# CHARACTERIZATION OF H1N2 VARIANT INFLUENZA VIRUSES IN PIGS

by

MICHAEL ALAN DUFF

B.S., University of Arizona, 2009

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2014

Approved by:

Major Professor
Wenjun Ma

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# Abstract

With introduction of the 2009 pandemic H1N1 virus (pH1N1) into swine herds, reassortment between the pH1N1 and endemic swine influenza viruses (SIVs) has been reported worldwide. Recently, reassortant H3N2 and H1N2 variant SIVs that contain the M gene from pH1N1 virus and the remaining seven genes from North American triple-reassortant (TR) SIVs have emerged. These variant viruses have caused more than 300 cases of human infections and one death in the USA, creating a major public health concern. To date, the pathogenicity and transmissibility of H1N2 variant viruses in pigs has not been investigated. Through passive surveillance, we have isolated two genotypes of reassortant H1N2 viruses with pH1N1 genes from diseased pigs in Kansas. Full genome sequence and phylogenetic analysis showed that one is a swine H1N2 variant virus (swH1N2v) with the M gene from pH1N1; the other is a reassortant H1N2 virus (2+6 rH1N2) with six internal genes from pH1N1 and the two surface genes from endemic North American TR H1N2 SIVs. Furthermore, we determined the pathogenicity and transmissibility of the swH1N2v, a human H1N2 variant (huH1N2v), and the 2+6 rH1N2 in pigs using an endemic TR H1N2 SIV (eH1N2) isolated in 2011 as a control. All four viruses were able to infect pigs and replicate in the lungs. Both H1N2 variant viruses caused more severe lung lesions in infected pigs when compared to the eH1N2 and 2+6 rH1N2 viruses. Although all four viruses are transmissible in pigs and were detected in the lungs of contact animals, the swH1N2v shed more efficiently than the other three viruses in the respective sentinel animals. The huH1N2v displayed delayed and inefficient nasal shedding in sentinel animals. Taken together, the human and swine

H1N2 variant viruses are more pathogenic and the swH1N2v more transmissible in pigs and could pose a threat to public and animal health.

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# Acknowledgements

I would first like to thank my parents, Dennis and Isabel Duff, for inspiring me to aim high and to dream big. They have stood by me through my successes and failures and it is an honor to follow in their footsteps. I only hope that I can live up to their expectations.

I am ever indebted to my major professor, Dr. Wenjun Ma, for accepting me into his laboratory. At the time, I was a relatively "research-inexperienced" veterinary student with an improbable dream of becoming a DVM/PhD. And now, thanks to Dr. Ma's continued support and guidance, that dream is on its way to becoming a reality.

I am very grateful to my committee members, Drs. Juergen Richt and Bob Rowland, for their guidance and support in my research and my pursuit of a doctorate of veterinary medicine. I must also recognize Drs. Michael Kenney, Elizabeth Davis, and Annelise Nguyen for opening the doors to my research career through the Veterinary Research Scholars and NIH-BRITE Programs.

My sincere appreciation goes to all of the members of the Ma and Richt laboratories for their help with this project, especially Jingjiao Ma, for her dedication in training me. Thank you to my friends and family for their well wishes. And I owe a great many thanks to my best friend, Alexa Ukena, for her never-ending patience, support, and motivation to keep going strong.

This project would not have been possible without financial support from the National Institutes of Health, the National Pork Board, and the Kansas State University College of Veterinary Medicine Department of Diagnostic Medicine/Pathobiology.

# Dedication

In loving memory of my Tata, Alfredo Quen Martinez, and my Nana, Dolores Martinez:

‘Gone yet not forgotten,
Although we are apart,
Your spirit lives within me,
Forever in my heart.’
~ Unknown ~

# Chapter 1 - Influenza Virus Literature Review

## Introduction

Influenza, commonly known as flu, is an infectious zoonotic disease caused by negative sense, single-stranded RNA viruses of the *Orthomyxoviridae* family which are able to infect a variety of species including birds, ferrets, cats, pigs, and humans [1]. In water fowl, the disease is largely asymptomatic and primarily affects the gastrointestinal tract while poultry can develop disease of variable severity. In swine, the virus can cause high morbidity resulting in substantial economic losses for the pork industry and even food shortages for local and national municipalities. In humans, the virus causes a contagious respiratory disease of variable severity that can lead to death [2].

Although every person is at risk for the flu, the CDC identifies persons at high-risk for flu-related complications as children under the age of five, adults 65 years and older, pregnant women, and people with underlying medical conditions such as asthma or heart disease. Despite the development and wide use of vaccines, it is estimated that 500,000 persons die annually from the flu during non-pandemic years [3]. In pandemic years, the death toll can climb to millions like the 1918 "Spanish Flu" which killed upwards of 50 million people worldwide [4].

## *Orthomyxoviridae* Family of Viruses

There are five RNA viruses in the *Orthomyxoviridae* family of viruses: Influenza A Virus, Influenza B Virus, Influenza C Virus, Isavirus, and Thogotovirus. As the names suggest, Influenza A, Influenza B, and Influenza C viruses cause influenza [2]; however, their host range and severity of infection varies widely between genera. Influenza A viruses (IAVs) are the most predominant of the three genera, infecting both birds and

mammals, and consists of numerous subtypes based on the combination of its surface proteins, hemagglutinin (HA) and neuraminidase (NA) [5]. Additionally, IAVs change more rapidly than influenza B and C viruses which is why they are responsible for all pandemics to date including the 1918 Spanish flu, 1957 Asian flu, 1968 Hong Kong flu, and the 2009 swine flu [6,7].

Influenza B viruses cause severe disease and are responsible for annual epidemics in humans which is why they are included in the annually produced trivalent seasonal influenza vaccine. However, they change much slower than IAVs and their host range is largely restricted to humans with only occasional infections in seals [8]. On the other hand, influenza C viruses are only found in pigs and humans where they cause a mild upper respiratory illness without pandemic potential and are therefore not included in vaccine therapy [9].

## Influenza A Virus

### *Morphology*

The IAV can vary greatly in size and shape, from small spherical morphologies of only 80-120 nanometers (nm) in length to long, filamentous particles measuring several micrometers (μm) in length [10,11]. It is thought that smaller droplets (less than 1 μm in length) travel further in aerosols and are therefore more transmissible than larger droplets (larger than 6 μm) which travel only a few meters. Although most human IAVs are filamentous in structure, continual passages in eggs or tissue cultures leads to a more spherical formation [12,13] which results in higher titers and possibly more efficient transmission [14,15]. Host cell type and actin cytoskeleton have also been shown to play a

role in influenza morphology with the formation of filamentous morphologies best seen in polarized epithelial cells [16].

### *Virus Genome and Structure*

The IAV is a negative-sense, single-stranded RNA virus consisting of eight RNA segments that encode for 12-16 proteins [17]. These segments are labeled according to size with segment one being largest and segment eight being smallest. Segments one, two, and three encode for the polymerase basic 2 (PB2), polymerase basic 1 (PB1), and polymerase acidic (PA) proteins, respectively. Collectively, these three proteins comprise the polymerase complex. Segment two also encodes two additional proteins in some viruses: the pro-apoptotic virulence factor named PB1-F2 which is translated via an alternative open reading frame (ORF) [18] and the N40 protein for which there is no well-defined role via an in-frame downstream initiation site [19]. Segment three also encodes for an additional protein named PA-X that is involved in shutoff of host protein synthesis via a ribosomal frame-shift during translation. Segment four encodes the HA protein responsible for cellular receptor binding. Segment five encodes the nucleoprotein (NP) which is bound to the viral RNAs (vRNAs) and carries nuclear localization signals (NLSs). Segment six encodes the sialic-acid cleaving transmembrane NA protein. Segment seven encodes both the multifunctional matrix one (M1) protein which supports the structure of the virus and the matrix two (M2) protein that functions as an ion channel. Segment eight encodes both the host antiviral response antagonist non-structural protein 1 (NS1) and the nuclear export protein (NEP), formerly known as non-structural protein 2 (NS2).

The viral lipid envelope is derived from the host plasma membrane and contains a lipid bilayer comprised of transmembrane proteins HA and NA as well as the ion channel M2 [20]. HA and NA are similar in that they both protrude from the surface of the influenza virion as "spikes," are major targets for the host humoral response, and are bound to the viral envelope by a short sequence of hydrophobic amino acids; however, they are structurally different. The HA protein is rod-shaped and much more prevalent than NA which is mushroom-shaped [21,22].

The ion channel M2 is also present in the membrane and is involved with un-coating the virus during entry [23] as well as dissociating the vRNPs from M1 during the early phase of the infectious cycle [20]. The remaining proteins are enclosed within the M1 protein which supports the morphology of the virion [24]. Enclosed within the M1 proteins are the NEPs and the RNP complex. The RNP complex, also known as the virion core, is required for viral replication and transcription [25] and consists of the viral RNA bound by NP and the RNA-dependent RNA polymerase complex [23,26,27].

## *Viral Proteins*

### *Surface Proteins Hemagglutinin and Neuraminidase*

The HA protein is critical for virus entry and fusion within the endosome. Infectivity is dependent on cleavage of the HA0 precursor by furin into the mature HA1/HA2 form. The HA1 contains the sialic acid binding domain and HA2 contains the fusion peptide. Cleavage of HA0 is a prerequisite for the conformational change to the low pH after which the fusion peptide is hidden preventing new virions from aggregating and losing infectivity [28].

It is believed that HA evades antibody recognition by antigenic shift [29] with mutations in the N-linked glycosylation thought to alter the oligosaccharide layer surrounding HA [30]. Truncation of the HA protein can lead to a change in virion morphology which can affect transmissibility [31-33]. Several antigenic and species-specific phylogenetically important regions (PIRs) have been identified in HA. PIRs for human-like H1 viruses include 96A, 133S, and 183P [34]. Additionally, putative antigenic sites have been identified including 91Y, 134A, 180H, and 225G [34,35] with mutation G225D/E playing a role in altering viral receptor specificity during adaptation of avian influenza viruses (AIVs) to pigs [36-39].

The NA is found as a tetramer on the surface of the host-derived lipid envelope and functions to cleave the sialic acid containing receptors to allow for the release of progeny virions from infected cell surfaces [23,40]. NA contains a large number of highly conserved, charged residues in the pocket enclosing the sialic acid binding sites and around its rim including 118R, 119E, 151N, 152R, 198N, 224R, 227E, 243N, 274H, 276E, 277E, 292R, 330N, 350K, and 425E. It is thought that these amino acids are in locations where they are either 1) inaccessible to antibodies, 2) accessible to antibodies but only in such a way that non-essential amino acids also form the antigen-antibody complex, or 3) are not antigenic [41]. Three human-like PIRs have been detected as 51M, 331S, and 403R [34] with mutations M51I, S331R, and R403M suspected to play a role in adaptation of viruses containing human-like N2s to swine [42].

It is important for the HA and NA activities to be balanced in order for productive rounds of infection to be maintained [43]. Although NA is not required by the virus to infect

cells, antibodies to NA do mitigate the disease resulting in lower pulmonary viral titers and less severe lung lesions [41,44].

***Matrix and Non-structural Proteins***

The M1 protein forms the majority of the virion and has several known functions associated with viral assembly and budding [23]. In fact, expression of M1 alone can lead to budding of viral vesicles from the plasma membrane [45,46]. It accumulates at the plasma membrane interacting with the cytoplasmic tails of HA and NA and is thought to initiate bud formation by inducing membrane curvature [14,32,47,48]. This was demonstrated by inducing mutations in the cytoplasmic tails of HA and NA and deletions in M2 which exhibited reduced incorporation of M1 and vRNPs and reduced the stability of progeny virions [14,32,49,50]. Additionally, increased viral-like particle (VLP) formation was observed by association of membrane targeting peptide to M1 suggesting that the plasma membrane-M1 association is critical for VLP formation [51].

M1 also plays a role in virion morphology [24] and assembly through interactions with HA, NA, M2, and vRNPs [52]. It is believed that a long filamentous virion may have the potential for increased cell-to-cell transmission in the respiratory mucous by infecting neighboring cells prior to even being released [16]. On the other hand, small spherical cells are likely to be more easily incorporated into aerosols and thus, to transmit more efficiently [10]. Bourmakina et al first demonstrated the importance of M1 in determining virion morphology by replacing the spherical forming M1 of A/WSN/33 with the filamentous forming M1 of A/Udorn/72 which resulted in a filamentous formation. They found that mutations R95K in the amino-terminal and E204N in the carboy-terminal regions of M1 resulted in a significant reduction in filamentous formation. This suggested

that these regions play an important role in determining filamentous or spherical viral morphology [10].

While some studies have shown that mutation A41V in M1 resulted in loss of filamentous particle formation [53-55], another study showed that V41A had no effect on formation but that mutation K95R abrogated filamentous particle formation [10]. Position 95 is important because it is on the surface of the molecule and amino acid changes can affect the charge which in turn can affect its interactions with other cellular components [10,56-58]. Additional sites suspected to play a role in virion morphology include amino acids 30, 102, 204, 207, 209, and 218 [10,14,31,54]. M1-M1 interaction has also been shown to be a determinant of virion morphology because M1 forms a helical net under the viral membrane and the pitch of the helical turn of the M1 protein differs between spherical and filamentous virion formation [59].

The NS1 protein has been shown to inhibit host IFN-α and IFN-β synthesis [60]. In fact, the NS1 of the 1918 H1N1 IAV increased the pathogenicity of the virus by inducing numerous pro-inflammatory chemokine and cytokine genes which in turn attracted pro-inflammatory innate immune cells. It also down-regulated the expression of IFN-stimulated genes decreasing the host's immune response [61]. It also appears to play a role but does not seem to be required for host protein shutoff [62]. Moreover, it is involved with nuclear export and stability of cellular mRNA and enhances the efficiency of viral mRNA translation [63-65].

The NEP protein was originally named non-structural 2 protein because it was thought to have no function in the virion. However, studies have proven that it is involved in exporting vRNPs from the nucleus for packaging into new virions. It is also

thought to support viral budding and to regulate the accumulation of viral mRNA, cRNA, and vRNA[66].

***RNP Complex Proteins***

The RNP complex consists of the PB2, PB1, PA and NP proteins and has long been known to be important in determining host specificity [67]. For instance, amino acid changes at position 627 have been identified as important for AIV replication competence in mammalian cells. It has been shown that avian 627E restricts viral growth in humans and monkeys but that a mutation to lysine can restore viral replication [68]. However, it was recently discovered that 627K is not required for efficient infection or induction of disease in mammals. This is demonstrated by the fact that equine and swine IAVs possess amino acid 627E, the same as is found in HPAI H5N1 [69-72]. Additionally, one study showed that residues 627E in conjunction with 701N function similarly to the E627K mutation [73]. Other amino acid changes in the PB2 protein have been shown to be important in adapting AIVs to mammals. Amino acids residues 271 and 588 have been shown to improve polymerase activity [74] while amino acid 701 has been shown to increase polymerase activity of HPAI in mammals [75]. Additionally, phenylalanine at position 636 has been shown to improve polymerase activity in mammals [69].

The PB1 protein has been shown to be important for pathogenicity. For example, other than HA and NA it was the only gene segment to be exchanged in the 1957 and 1968 pandemics [76,77]. Additionally, this protein from the 1918 H1N1 virus was shown to be required for full pathogenicity of the virus in mice [78].

The PB1-F2 protein is a 99 amino acid protein translated from alternative initiation of PB1 and is thought to initiate host cell apoptosis by targeting mitochondria

[18]. It initiates apoptosis by permeating the mitochondria resulting in the dissipation of the mitochondrial membrane and causing the release of cytochrome c [79,80]. It is also thought to suppress the adaptive immune response by decreasing antigen presentation [76]. This has been shown by the decreased presence of lymphocytes and impaired immune response seen in humans infected with HPAIs [76,81]. It also acts to delay viral clearance and prolong viral replication by impairing $CD8^+$ T cells (those responsible for viral clearance) leading to the development of irreversible pulmonary immunopathology [82,83]. Moreover, studies have shown that HPAIs in which PB1-F2 was knocked out resulted in decreased pathogenicity in mice [84].

The c-terminal domain of PB1-F2 is significant for mitochondrial targeting and contains five species-specific signatures [85,86]. Specifically, position 66 is in the α-helical domain and mutation N66S is thought to have a pro-apoptotic function [76]. This is because N66S causes a significant increase in IFN-γ and TNF-α causing a cytokine dysregulation similar to that of 1918 H1N1 and HPAI H5N1 [81,87]. N66S is also thought to increase apoptosis by affecting interactions with ANT3 and VDAC1 [80].

The PA protein is believed to be a multi-functional protein though its exact roles have not been well defined. Studies have shown that it is involved with both replication and transcription. Mutation T97I has been shown to play a role in adapting AIVs to mammals and increasing virulence in a mouse model [88].

Compared to other ORFs, NP displays distinct boundaries between human and avian species-specific amino acids. Several of these are located in regions that interact with cellular proteins and play a role in antiviral mechanisms of host cells. Notably, amino acid 313 is highly conserved between species with human viruses possessing

tyrosine (Y), avian and swine viruses possessing phenylalanine (F), and 2009 pandemic H1N1 viruses possessing valine (V) [89,90].

## *Replication*

### *Virus Attachment*

The HA protein is the major envelope protein of IAV and forms a projection-like spike on the surface of the virion that elicits neutralizing antibodies [91]. It enables the virus to attach to host cells by binding the N-acetylneuraminic (sialic) acid expressed on the surface of many host cells in many species. This sialic acid terminal binds to either carbon-3 or carbon-6 galactose through its carbon-2 resulting in two conformations: α-2, 3 and α-2, 6 linkages [92]. Accordingly, the HA of different strains of IAVs will preferentially bind either the α-2, 3 or α-2, 6 linkages.

Most avian and equine IAVs bind the sialic acid α-2, 3 linkage while human and swine IAVs preferentially bind the sialic acid α-2, 6 linkages [93]. In poultry, the α-2, 3 linkages are predominantly expressed in the gastrointestinal tract. In humans, the α-2, 6 linkages are predominantly expressed in the upper respiratory tract while some α-2, 3 linkages are also be expressed in the lower respiratory tract. This may explain why humans can be infected with AIVs, albeit rarely. It may also explain the high pathogenicity seen in humans infected with AIVs.

Swine also possess both α-2, 3 and α -2, 6 receptors in their respiratory tract, similar to that in humans, [94] which allows them to be infected with human, swine, and avian influenza viruses and is why they are considered the "mixing vessels" for IAVs [95]. Further supporting the "mixing vessel" hypothesis is the fact that reassortant SIVs containing genes from swine, avian, and human IAVs have been isolated from pigs which

can then transmit to humans [96]. Furthermore, at least 1-13 HA subtypes of AIVs are able to infect and replicate in pigs [97]. This has led to the belief that many AIVs gain adaptation to mammals via swine.

### *Virus Entry*

After the virus attaches to host cells, it enters the cell via receptor-mediated endocytosis. Once encapsulated, the virus is translocated toward the nucleus. Concurrently, the hydrogen ions within the endosome drive the pH down to approximately five which aids in un-coating the virus and the subsequent release of vRNPs into the cytoplasm [98]. The exact mechanism for this acidification and release of vRNPs occurs in a few of steps. First, the low pH in the endosome creates a conformational change in the HA0 which exposes a fusion peptide (HA2) that mediates the fusion of the viral envelope with the host membrane. This in turn opens a pore in the endosome that allows the vRNPs to be released into the cytoplasm. However, prior to the membrane fusion, the hydrogen ions from the endosome enter the virus via the M2 ion channel and disrupt the M1 matrix proteins polymerization and interaction with the viral RNPs. This then allows the vRNPs to be released into the cytoplasm [99]. Once released from the virion, vRNPs and viral proteins are imported into the nucleus via NLSs in the NP [99].

### *Synthesis of Viral RNA*

Once inside the nucleus, the viral RNA-dependent RNA polymerase is responsible for unprimed replication of vRNAs and initiates viral mRNA synthesis by "cap snatching" in which the PB2 subunit binds the 5' cap of host pre-mRNAs. The RNA-dependent RNA polymerase uses the negative-sense vRNA as a template to

synthesize positive-sense mRNAs and cRNAs. The mRNAs serve as a template for viral proteins synthesis while the cRNA is transcribed into more copies of negative-sense vRNA. The NPs are required for these steps of replication and are deposited on the cRNA and vRNA during RNA synthesis. This is unique to influenza viruses because most other single-stranded RNA viruses are not replicated or transcribed in the nucleus [100]. After transcription, the endonuclease domain of the PA subunit then cleaves the cap. From there, viral mRNAs are transported to the cytoplasm for translation into viral proteins mediated by an M1-NS2 complex that is bound to the vRNPs. The NS2 interacts with the human chromosome maintenance region 1 protein that exports the vRNPs from the nucleus [100].

***Synthesis of Viral Proteins and Assembly***

In the early stage of infection, the polymerase subunits PB2, PB1, and PA are translated in the cytoplasm using vRNA as a template and are then transported back to the nucleus via NLSs. Some of the M1 proteins are transported back to the nucleus while the NEP proteins re-enter the nucleus via free diffusion [99]. During the late stage of infection, surface proteins HA, NA, and M2 are synthesized on membrane-bound proteins in the endoplasmic reticulum and glycosylated in the Golgi apparatus before being transported to the cell membrane. The remaining proteins are transported back into the nucleus for formation of more vRNPs for the new virus particle [100].

Once sufficient viral proteins have been produced, the virus begins the assembly process. Although the process is not fully understood, there are two proposed mechanisms for viral assembly: 1) the random packaging model and 2) the specific packaging model. The random packaging model suggests that the viral segments will be

randomly packaged into the virions while the specific packaging model suggests that viral segments will be selectively packed into virions by signals from each RNA segment [101].

### *Virus Budding and Release*

The vRNPs translocate to the cell membrane where they bind to the terminal sialic acids of the HA and NA proteins and clump together. The NA of the newly formed viruses then cleaves the sialic acid residues from cell surface glycoproteins and gangliosides, allowing for the release of the progeny virus from the infected cell [100]. The new virus can then infect neighboring cells or be transmitted to another host.

### *Antigenic Drift and Antigenic Shift*

IAVs can undergo a number of changes in their genomes via two mechanisms: antigenic drift and antigenic shift. Antigenic drift is the result of gradual accumulations in point mutations caused by lack of proofreading of the RNA-dependent RNA polymerase during viral replication. Antigenic drift is responsible for currently circulating seasonal influenza viruses which cause epidemics in humans. Since 1968, most severe epidemics have been the result of the H3N2 IAVs [2].

Antigenic shift is an abrupt change in the subtypes of a host species and occurs via one of three mechanisms: 1) genetic reassortment, 2) direct transfer of an entire virus from one host species into another species, and 3) the re-emergence of a virus that was previously seen in a species but is no longer endemic. Genetic reassortment occurs when two or more different influenza viruses (parent strains) simultaneously infect a cell and yield new viruses (progeny viruses) with genes from different parent strains. Genetic reassortment between different strains can result in the emergence of a new strain and

new subtype. Additionally, genetic reassortment between avian, equine, canine, swine, and human IAVs can lead to the emergence of hybrid viruses [2]. The end result of antigenic shifts is often a novel influenza virus capable of evading the immune systems thereby leading to an epidemic or a pandemic as was seen in 1918, 1957, 1968, and 2009 [2].

## Influenza A Virus Ecology

Given the relative frequency of IAV epidemics and pandemics, much research has been done to investigate the pathogenicity of virus and host factors in the transmissibility and pathogenicity of IAVs. The use of reverse genetics has helped to study the role of each viral protein while the use of different animal models including mice, ferrets, pigs, and rhesus macaque monkeys has led to an understanding of the roles of these viruses in different animals [17,102]. Recent pandemics and the advances in detection have placed more and more emphasis on the continual surveillance and reporting of IAVs to authorities [2].

### *Avian Influenza A Viruses*

While waterfowl and shorebirds are the natural reservoirs for most IAVs, infections in these birds are largely asymptomatic. However, infections of poultry with IAVs can cause avian influenza. AIVs are divided into low pathogenic avian influenza (LPAI) viruses and high pathogenic avian influenza (HPAI) viruses based on the genetic features of the virus and the severity of disease in birds. LPAI viruses begin shedding within 1-2 days of infection and continue shedding for 1-2 weeks. In contrast, HPAI viruses can be shed for up to 30 days. To date, only the H5 and H7 subtypes have caused

HPAI while all other HA subtypes have caused LPAI [2]. However, the H1-H16 subtypes have been isolated from waterfowl [103].

In wild birds, a large amount of virus is shed in the feces and the virus is primarily spread via the fecal-oral route. Adding to the transmissibility is the fact that AIVs are relatively stable in the environment and have been detected in waters where ducks swim. In poultry, the virus can quickly spread through the flock via both the fecal-oral route in addition to aerosols transmission due to the proximity of the birds. Recently, isolates of HPAI H5N1 have been detected in higher quantities from tracheal samples than from fecal samples of chickens suggesting that the fecal-oral route may no longer be the primary route of infection. AIVs can also spread via vectors such as flies, fomites, migratory birds, and even cracked eggs infected with the virus [2].

### *Human Influenza A Viruses*

In humans, H1N1 viruses related to the 1918 H1N1 pandemic circulated alone until the 1957 pandemic introduced H2N2 viruses which circulated until 1968. These viruses continued with antigenic drift away from the 1918 H1N1 [104,105]. In 1977, the H1N1 viruses from the 1950s reemerged [91] and led to more reassortment which led to substantial further antigenic evolution of the human H1N1 viruses [106]. There are currently three endemic subtypes of viruses: H1N1, H1N2, and H3N2. The H1N2 virus was first detected in humans in 2001 and is most likely the result of genetic reassortment between H1N1 and H3N2 human IAVs.

Humans infected with IAVs usually begin shedding the virus 3-4 days after infection and about 1-2 days before the onset of symptoms. The virus is transmitted in two ways: 1) aerosols produced by sneezing and coughing and 2) direct contact with

nasal discharge or fomites exposed to nasal discharge. In general, close contact and closed environments enhance transmission. Unlike AIVs, mammalian IAVs are relatively unstable and do not survive well in the environment outside of dried mucus [2].

In humans, the seasonal influenza virus transmits very efficiently and causes mostly mild cases of flu. In contrast, the HPAI H5N1 virus has demonstrated very limited human-to-human transmission but causes severe pneumonia that can result in systematic infection and multiple organ failure. Other IAVs that infect humans cause a wide range of disease, ranging from mild to severe, often depending on host factors including age and health conditions [2].

### *Swine Influenza A Viruses*

SIVs cause an acute respiratory disease in pigs and play an important role in porcine respiratory disease complex. It has also been associated with outbreaks of abortions on swine farms [107-109]. Although mortality is usually low, less than 1%, morbidity can be as high as 100%. Additionally, SIVs can transmit within a herd quickly causing pigs to become ill and experience clinical signs of lethargy, fever, dyspnea, cough, and oculonasal discharge for 5-10 days [110-112]. Most pigs begin shedding within 24 hours of infection and continue to shed for 7-10 days [2]. Additionally, there is evidence to suggest that piglets play a more important role than sows in maintaining influenza infections, transmission, and persistence [112].

Classical H1N1 SIV (cH1N1) was first isolated from swine in the US in 1930 [112] and is antigenically similar to a reconstructed 1918 H1N1 virus [104]. The cH1N1 remained antigenically stable and was exclusively prevalent in North American swine populations until 1998 when it reassorted with both a human H3N2 virus and an American-lineage

AIV of unknown origin which resulted in a triple-reassortant (TR) H3N2 SIV [108-110,113-115]. This TR H3N2 virus contains genes of human- (PB1, HA, NA), swine- (NP, M, NS), and avian-lineage (PB2, PA) influenza viruses. The prevalence of this constellation of internal genes (human-lineage PB1, swine-lineage NP, M, and NS, and avian-lineage PB2 and PA) has warranted the name "triple-reassortant internal gene" (TRIG) cassette [110]. Interestingly, TRIG readily accepts different HA and NA combinations leading to an increase in the generation of reassortant IAVs [42,96]. As a result, several reassortant viruses including H1N1, H1N2, H3N1, and H3N2 viruses been detected in swine herds in the US [116,117].

Three types of H1 viruses have been detected in US swine populations: classical H1N1, TR H1N1 which contains HA and NA from cH1N1 and TRIG from H3N2, and human-like H1N1 and H1N2 viruses that contain human-like HA and NA and TRIG from TR SIVs [42]. Based on phylogenetic analysis, there are four H1 phylogenetic clusters detected in swine in the US: α (classical swine), β (reassortant H1N1-like), γ (H1N2-like), and δ (human-like H1). In addition to the clades above, there are two sub-clusters of the H1 δ clade that are from independent introductions of human seasonal HA into SIVs: δ-1 and δ-2 [35]. Human-like H1 SIVs were first detected in Canada in 2004 [118] and in the US in 2005 [42]. Until 1998 there was much cross-reactivity between H1; however, there is little H1 cross-reactivity between these four clades [42,117].

The H1N2 SIV is one of the major subtypes of IAVs circulating in swine herds worldwide. Different H1N2 SIVs have become established and maintained in North American and Eurasian swine populations [119]. The H1N2 viruses isolated from pigs in North America are typically triple-reassortant viruses containing human-, swine- and

avian-origin IAVs [119]. In the United States, the H1N2 virus is a reassortant virus containing genes from cH1N1, human-like H1N1, and TR H3N2 viruses and has increased in prevalence ever since.

The Eurasian swine H1N2 viruses are double-reassortant viruses between avian-like H1N1 and human H3N2 viruses [120]. H1N2 SIVs were first isolated from pigs Sweden in the winter of 2008-2009 [121]. In Japan and France, H1N2 SIVs were the result of reassortment between cH1N1 and human H3N2 viruses in pigs [107]. In the United Kingdom, multiple reassortment events are suspected between human H1N1 and H3N2 which generated a novel reassortant H1N2 virus that in turn acquired all six internal genes from a wholly avian H1N1 virus [95,107].

Although the H1N2 virus has not caused disease any more severe in pigs than that of other subtypes of IAVs, a TR H1N2 SIV has been detected in mink on a 15,000-head mink farm in the Midwest United States [122], showing that the swine H1N2 virus is able to cross the species barriers.

## 2009 Pandemic H1N1 "Swine Flu"

The 2009 pandemic H1N1 (pH1N1) emerged in April 2009 and spread to more than 70 countries in less than two months. Shortly thereafter the virus was detected in more than 200 countries causing the World Health Organization (WHO) to declare it a pandemic [123]. According to WHO, there were between 43 and 89 million cases of infection with pH1N1 from April 2009 to April 2010 resulting in 195,000 – 403,000 hospitalizations and 8,870 – 18,300 confirmed deaths worldwide, though these numbers are thought to be conservative. The virus has since been detected from a variety of other species including swine, turkeys, ferrets, and cats [1]. The virus was dubbed "Swine Flu"

because it is antigenically most related to cH1N1 and North American TR H1N1 SIVs that have circulated in the US for more than 10 years and have sporadically caused human infections [114,124]. However, there are many genetic and antigenic distinctions between pH1N1 and SIVs [125].

Sequence analysis revealed that pH1N1 was the result of multiple reassortments and contains a unique constellation of genes [114]. The HA, NP, and NS genes are from cH1N1 that entered swine around 1918 and continued to circulate in cH1N1 and TR SIVs [126]. The NA and M genes are of Eurasian swine-lineage that entered the Eurasian swine population from avian source in 1979 [127] and continued to circulate exclusively in Eurasia [128]. The PB1 gene is from TR swine-lineage and was transmitted from humans around 1998, though it was originally transmitted from an avian source to humans in 1968 [77]. Finally, the PB2 and PA genes are of TR swine-lineage that first entered North American swine from avian in 1998 [109]. Interestingly, six of the eight genes in pH1N1 are found in North American TR SIVs and despite their longtime establishment in swine, have shown limited human-to-human transmission, rarely causing human infections [14,114,129,130].

The Eurasian-lineage NA and M genes are thought to enhance the spread of pH1N1 and has been demonstrated in ferret studies [131]. Specifically, the Eurasian-lineage NA is thought to exhibit higher neuraminidase activity in vivo and may increase enzymatic activity causing the release of more viral particles resulting in enhanced transmission [14]. Susceptibility of pigs to pH1N1 was shown experimentally [36,132,133] after outbreaks of pH1N1 in pigs were detected in 2009 in which direct human-to-pig transmission was suspected [34,134,135]. Since then, pH1N1 has become established in North

American swine herds based on genetic analysis of more than 1,500 SIVs isolated between 2009 and 2010 [136].

### *Reassortant SIVs with Genes from pH1N1 Detected Worldwide*

The first reassortment of endemic SIVs and pH1N1 was detected in Hong Kong in 2010. In this case the virus possessed pH1N1-lineage NA, Eurasian-lineage HA, and TRIG [86]. Since then, reassortant viruses between SIVs and pH1N1 have been detected worldwide. In England, a reassortant virus with European H1N2 SIV-derived HA and NA and the other six genes from pH1N1 was detected [137]. In Italy, a reassortant virus was isolated that possessed all pH1N1-derived genes except for an H1N2 SIV-derived NA [138]. In Thailand, a reassortant H1N1 virus was detected with all pH1N1-derived genes except for an endemic H1N1 SIV-derived NA [139]. In Argentina, an outbreak of pH1N1 in swine led to the detection of two reassortant H1N1 SIVs that possessed human-like SIV-derived HA and NA and all remaining genes from pH1N1 [134].

### *Reassortant SIVs with Genes from pH1N1 Detected in the United States*

In the US, reassortant SIVs representing several distinct genotypes of pH1N1 and SIVs have been identified including TR H1N2 and H3N2 subtype SIVs [86]. Most of these viruses possess HA and NA genes of currently circulating SIVs and all contain the M gene from pH1N1 suggesting that this is a favorable constellation [106]. Viruses that have been reported to cross the species barrier to infect humans and are known as "variant" viruses [137]. However, testing of both human and swine isolates of H3N2 variant (H3N2v) viruses in ferret and swine models showed no increased pathogenicity or transmissibility compared to the endemic SIVs [122,140]. Other reassortant viruses between pH1N1 and H3N2 SIVs containing 3-5 internal genes from pH1N1 have been identified [1].

Novel reassortant H1N2 viruses with genes from pH1N1 virus have also been isolated from swine in Europe [122,137] and North America [140]. Recently, the novel TR H1N2 variant (H1N2v) viruses with only M gene from pH1N1 have been reported to infect humans in Minnesota (www.WHO.int). In fact, there were two cases of children infected with a swine variant IAV after being exposed to pigs at a state fair although no further human-to-human transmission was reported.

### *Zoonosis*

Although waterfowl are the natural reservoirs for most IAVs [91], interspecies transmission of IAVs has been well documented despite the detection of host-range restrictions [134,141-146]. Evidence of zoonotic transmission of IAVs from pigs to humans has been especially well documented [107,147-150]. According to the CDC, from December 2005 – November 2010, there were more than 300 human infections with variant SIVs in the US including H1N1v, H1N2v, and H3N2v viruses [151]. Then, in 2011 an H3N2v virus with pH1N1 M gene and the remaining genes from endemic H3N2 SIVs emerged and caused 12 human infections (mostly in children) in Indiana, Maine, Pennsylvania, Iowa, Utah and West Virginia. Also in 2011, an H1N2v virus with pH1N1 M gene infected a child at a state fair. There were 12 more confirmed cases of humans infected with H3N2v viruses in 2011 and an alarming 309 cases in 2012 [152,153]. This has raised public health concerns that the introduction of human IAVs into swine could generate novel reassortant viruses that spill back into the human population [96].

## Purpose of Research

The H1N2v virus has been reported to infect humans in the US though no further human-to-human transmission has been reported. To our knowledge, H1N2 viruses with

genes from pH1N1 virus isolated from swine have not been characterized in animal models. This demonstrates the importance of understanding the virulence and transmissibility of these novel reassortant H1N2v viruses in the event that a more virulent and transmissible reassortant virus emerges, which might pose significant economic and socio-economic threats to the swine industry and public health. In this study we characterized H1N2v viruses isolated from human and swine *in vitro* and *in vivo* compared with a reassortant H1N2 virus containing genes from pH1N1 using an endemic H1N2 virus as a control.

# Chapter 2 - Materials and Methods

## Cells

Madin-Darby canine kidney (MDCK) cells were cultured in Eagle's minimum essential medium (MEM) containing 5% fetal bovine serum (FBS) (Fischer Scientific, Houston, TX), 1% antibiotics (Invitrogen, Carlsbad, CA), 1% L-glutamine (Invitrogen, Carlsbad, CA), and 1% 1x MEM vitamins (Invitrogen, Carlsbad, CA). Human alveolar basal epithelial (A549) cells and porcine kidney (PK-15) cells were grown in Dulbecco's modified Eagle medium (DMEM) with 10% FBS, 1% antibiotics, 1% L-glutamine, and 1% 1x MEM vitamins. Cells were inoculated with viruses using infecting MEM prepared by adding 0.3% BSA (Sigma), 1 μg tosyl phenylalanyl chloromethyl ketone-treated trypsin (TPCK Trypsin) (Sigma, St. Louis, MO), 1% antibiotics, L-glutamine, and 1x MEM vitamins to MEM. All cells were incubated at 37ºC with 5% CO2.

## Viruses

One novel reassortant H1N2 SIV with six internal genes from pH1N1 and two H1N2v viruses, one swine and one human variant with M gene from pH1N1, were characterized in this study using an endemic TR H1N2 SIV isolated in 2011 as a control. The reassortant A/swine/Kansas/12-117893/2012 (2+6 rH1N2) with PB2, PB1, PA, NP, NS, and M genes from pH1N1 was isolated from nasal swabs of a four-week old weaned pig with fever, coughing, and depression in a nursery with high morbidity and approximately 5% mortality. The swine variant A/swine/Kansas/12-156064/2012 (swH1N2v) with M gene from pH1N1 was isolated from nasal swabs of a gilt with depression, dyspnea, and nasal discharge. The endemic A/swine/Kansas/11-104259/2011 (eH1N2) with all genes from North American TR SIVs was isolated from a 28-day old

pig with a history of streptococcal infections but no physical symptoms of illness and served as a control in this study. These samples were submitted for diagnostic testing to the Kansas State Virology Diagnostic Laboratory (KSVDL) in Manhattan, KS where they were initially identified as IAVs by reverse-transcription (RT) polymerase chain reaction (PCR) before being transferred to Dr. Wenjun Ma's laboratory for further characterization. The human variant virus A/Minnesota/14/2012 (huH1N2v) was isolated from a human and kindly provided by the Centers for Disease Control and Prevention (CDC).

## Hemagglutinin Inhibition Assay

Hemagglutinin inhibition (HI) assays were completed using sera of all direct-infection pigs prior to infection [42]. For this, sera was first heat-inactivated at 56°C, then treated with a 20% suspension of kaolin (Sigma Aldrich, St. Louis, MO) in order to eliminate non-specific inhibitors, and lastly absorbed with 0.5% chicken red blood cells. To confirm that sera was negative for SIVs, the HI assay was performed to test antibodies against a panel of reference SIV strains including A/swine/Iowa/1973 H1N1, A/swine/Texas/1998 H3N2, and A/swine/North Carolina/2001 H1N1v.

## Molecular Characterization

Each virus was first amplified in MDCK cells to create sufficient working stocks for further characterization. Viral RNA was extracted from MDCK cell culture supernatants using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). Viral cDNAs of each gene segment were obtained by RT using vRNA as templates and a universal influenza (Uni12) primer. Each gene was amplified by PCR using a respective segment-specific primer. The amplified full-length gene products were separated onto

agarose gel and purified using the Qiaquick Gel Extraction Kit (Qiagen Inc., Valencia, CA, USA). The RT-PCR products were sequenced using specific sequencing primers and full genomes were sequenced (sequence primers are available upon request). Sequences were analyzed using the Lasergene sequencing analysis software (DNASTAR, Madison, WI) and BLAST (http://blast.ncbi.nlm.nih.gov) to determine virus subtype. Sequences were aligned using CLUSTAL W and phylogenetic tree analyses were generated with 1,000 bootstrap replicates using MegAlign software (version 4.1). Amino acid mutations were identified using MEGA software (version 5.1).

## Titrations

Viral titrations of cell culture supernatants, bronchoalveolar lavage fluid (BALF), and nasal swab samples were performed in quadruplicate using 96-well plates of confluent MDCK cells maintained as previously described. Serial dilutions for each sample were completed from $10^{-1}$ to $10^{-8}$ using infecting MEM and then 100 µl of each dilution was added to MEM-washed confluent MDCK cells in each well. Cells were incubated at 37°C with 5.0% $CO_2$ and evaluated for cytopathogenic effect (CPE) at 24- and 48-hours post-infection (hpi). At 72-hpi, plates were fixed in methanol and immunocytochemically stained with a monoclonal antibody specific to influenza NP.

For this, plates previously fixed for 10 minutes with methanol were washed three times with washing solution consistent of 0.05% Tween in PBS. Then, 50 µl of MAb-anti Influenza A virus nucleoprotein (ATCC #HB-65; H16-L10-4R5) diluted 1:500 in diluting solution consistent of 0.05% Tween in PBS plus 1.0% BSA was added to each well and allowed to incubate at room temperature for 30 minutes. Plates were then washed three times in washing solution. Next, 50 µl of rabbit anti-mouse IgG conjugated HRP (DAKO

catalog #P0260) diluted 1:300 in diluting solution was added to each well and allowed to incubate at room temperature for 30 minutes. Plates were again washed three times in washing solution. Next, 50 µl of AEC substrate prepared by adding 19 mL of AEC diluent (0.1M acetic acid plus 5.75 ml glacial acetic acid plus 1 liter of ddH20) plus 1 ml AEC solution (400 mg AEC plus 100 ml dimethylformamide mixed in a chemical fume hood) plus 20 µl $H_2O_2$ was added to each well and allowed to incubate for 30 minutes until a red color change was evident. Finally, plates were rinsed thoroughly with water and allowed to air dry before reading under a microscope.

## Real-time RT-PCR for M gene of pH1N1

A previously published real-time RT-PCR assay was employed in this study to detect the presence of the pH1N1 M gene from nasal swabs of contact-exposure pigs in the swH1N2v and huH1N2v groups[154]. In this assay, the primers and probes were designed specifically for targeting the pH1N1 M gene (Table 2-1). RNA was extracted from nasal swab samples and a known pH1N1 virus (positive control) using the QIAamp viral RNA kit (Qiagen, Valencia, CA, USA). This assay was performed on a Smart Cycler using a Qiagen One-Step RT-PCR master mix prepared as follows: 5 µl of 5X buffer, 2µl of dNTP (10mm), 0.625µl of forward primer (20µm), 0.625 µl of reverse primer (20µm), 0.25 µl of probe (10 µm), 0.2 µl of BSA (25 µg/µl), 2.5 µl of $MgCl_2$ (50mm), 0.25 µl of RNAse inhibitor (10 U/µl), 1 µl of enzyme mix, 5.55 µl of RNAse-free water, and 8 µl of sample template. A total of 8 µl sample from negative-control pigs was used as a negative control. The Smart Cycler thermocycling conditions was programmed as follows: 50ºC for 30 minutes, 95ºC for 15 minutes, then 40 cycles of

94ºC for 15 seconds and 60ºC for one minute. Cutoff for this assay was set at 35 threshold cycles (Ct).

**Table 2-1 Primers and probe used for the one-step real-time RT-PCR assay.**

| | Primers/Probe | Location of Primers* | Amplicon (bp) |
|---|---|---|---|
| Real-time RT-PCR | Pan-M-Probe: 5'- TTG CATGGG CCT CAT ATA CAA C-3' | --------- | 92 |
| pH1N1 specific | Pan-M-F: 5'- GGT GTC ACT AAG CTA TTC AA-3' | 342-361 | --------- |
| | Pan-M-R: 5'- CAA AAG CAG CTT CTG TGG TC-3' | 414-433 | --------- |

Table 2-1 Location of primers was calculated based on the ORF of the Matrix 1 gene. This table used with permission of the original authors.

## Plaque Assay

Viral dose and the size of plaques formed by each virus was measured by traditional plaque assay. For this, 10-fold serial dilutions were made for each virus using infecting MEM. One milliliter of each dilution was inoculated onto a confluent monolayer of MDCK cells in six-well plates and allowed to incubate for 60 minutes at 37$^{o}$C with 5% CO2. Medium was removed from each well and cells were washed with fresh (unaltered) MEM. After removing the fresh MEM, 3mL of an MEM, 1% agarose, and 1µg/mL TPCK Trypsin mixture was added to each well and allowed to set at 4$^{o}$C for 10 minutes until gel solidified. Plates were then incubated in an inverted position for 72 hours at 37$^{o}$C with 5% CO2. Gels were carefully removed from each well and cells fixed with methanol for 10 minutes. Cells were then stained with 0.5% crystal violet dye in PBS for 15 minutes at room temperature before gently rinsing with a slow stream of cool water and allowed to air-dry.

## Replication Kinetics

Viral replication kinetics were performed for all viruses on monolayers of MDCK (MOI 0.001), A549 (MOI 0.01) and PK-15 (MOI 0.01) cells in 12-well plates at the indicated multiplicity of infection (MOI). Viral samples were collected at 0-, 12-, 24-, 36-, and 48- hpi, centrifuged at 1,200 rpm for five minutes to remove cellular contents, and stored at -20$^{o}$C. Samples were titrated in quadruplicate onto confluent MDCK cells in 96-well plates. CPE was visualized at 48-hpi and cells were fixed with methanol for 10 minutes at 72-hpi. Immunocytochemistry test (ICC) was performed using the monoclonal NP antibody to confirm CPE. Viral titer ($TCID_{50}$/mL) was calculated for each sample by the method of Reed and Meunch.

## Animal Ethics Statement

All animal studies were conducted at the Large Animal Research Center (LARC) at Kansas State University (KSU), a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and performed under the guidance of two experienced large animal research veterinarians.

## Pig Study

A total of 45 healthy four- to five-week old outbred pigs from an SIV-negative and porcine reproductive and respiratory syndrome virus-negative farm in Texas were randomly divided into direct-infection, contact-exposure, and negative-control groups: four direct-infection groups consistent of six pigs each, four contact-exposure groups consistent of four pigs each, and one negative-control group consistent of five pigs.

Direct-infection pigs were anesthetized on day zero using telazol and xylazine and inoculated intra-tracheally with 1.5mL of $10^5$ $TCID_{50}$/mL of each virus. Four naïve contact animals were added to each direct-infection group at 2-days post-infection (dpi) and allowed to comingle freely to assess virus transmission. Pigs were observed for clinical signs of infection and body temperatures were recorded daily. Three pigs from each direct-infection group and two negative-control pigs were euthanized at 5-dpi by administering FATAL-PLUS intravenously at a dosage of 1mL/10 lbs. of body weight. The remaining direct-infection and negative-control pigs as well as all contact-exposure pigs were euthanized at 7-dpi and 5-days post-contact (dpc).

Nasal swabs were collected from all direct-infection pigs at 0-, 3-, 5-, and 7-dpi and from contact pigs at 0-, 1-, 3-, and 5-dpc. Blood samples were collected from each

pig at day 0 and on necropsy days. Macroscopic lung lesions were evaluated by a single, experienced research veterinarian and scored based as a percentage of each lung lobe with lesions. BALF samples were collected from all lungs by flushing lungs with 50 mL of MEM. BALF and nasal swab samples were titrated onto MDCK cells to determine viral titers as previously described [116]. Lung lobes were collected and fixed in 10% formalin during necropsy before staining with haemotoxylin and eosin for histopathological examination. Anti-influenza A mAb against NP was used for immunohistochemistry (IHC) staining. Lung samples were examined blindly by a veterinary pathologist and scored on a scale of 0-3 based on severity of bronchial epithelial injury [155].

## Statistical Analysis

Statistics are shown as the mean and the standard error for each mean. Viral titers, macroscopic lung lesions, and microscopic lung lesions were analyzed by student's t-test. A p-value $\leq 0.05$ was considered significant.

# Chapter 3 - Results

## Subtyping and Molecular Analysis of eH1N2, 2+6 rH1N2, swH1N2v, and huH1N2v

The three Kansas swine isolates were identified as H1N2 viruses by nucleotide sequencing and a BLAST search of the National Library of Medicine (http://blast.ncbi.nlm.nih.gov). Full genome sequences for each virus were deposited into Genbank and can be found using the following accession numbers: eH1N2 (KJ528280-KJ528287), 2+6 rH1N2 (KJ528288-KJ528295), swH1N2v (KJ528296-KJ528303), and huH1N2v (KJ620412-KJ620419).

### *Molecular Analysis of the Hemagglutinin and Neuraminidase Molecules*

The HA molecules of all viruses clustered into the δ-1 sub-cluster (human-like H1 clade) based on phylogenetic analysis (Figure 3-1) and contain a total of 566 amino acids. Molecular comparison of the HA molecules revealed that eH1N2, 2+6 rH1N2, and huH1N2v all share 99% identity at the nucleotide level and 98-99% identity at the amino acid level. In contrast, swH1N2v was different sharing only 96% identity at the nucleotide level and 95-96% identity at the amino acid level with the other three viruses.

Amino acid differences were identified between the HA molecules of all viruses, especially between swH1N2v and the other three viruses (Table 3-1). The receptor binding sites of all viruses are similar and contain human seasonal H1N1/pH1N1 amino acid residues 155G, 158N, 190T, 219K, 223Q, 224E, 225G, and 230I [156]. Similarly, the cleavage sites of these viruses are highly homologous with human seasonal and swine H1N1 viruses. The PIRs of all viruses are similar including residues 96A, 133S, and 183P [183S in swH1N2v], all of which are common to North American TR H1N2

viruses, human seasonal viruses, and pH1N1 viruses[34,42,157,158]. Additionally, the putative antigenic sites of all viruses contain the human-like amino acid residues 91Y, 134A, 180H, 187N (187D in swH1N2v), and 225G which support preference for α-2, 6 sialic acid binding [156,159].

The NA molecules of all viruses clustered into the American TR, recent-human clade (Figure 3-2) and consist of 470 amino acids. The NA molecules of eH1N2, 2+6 rH1N2, and huH1N2v were very similar sharing 99% identity at the nucleotide level and 98-99% identity at the amino acid level. However, the swH1N2v was different, sharing only 95% homology at both the nucleotide and amino acid levels with the other three viruses. Notably, eH1N2, 2+6 rH1N2, and huH1N2v all differed by five or fewer amino acids while swH1N2v differed by 22 amino acids (Table 3-1). Interestingly, the PIRs of all viruses contain the human-like amino acids 51M, 331S, and 403R instead of the proposed M51I, S331R, and R403M mutations thought to support introduction into swine [42,157].

**Figure 3-1 Phylogenetic tree of HA molecules.**

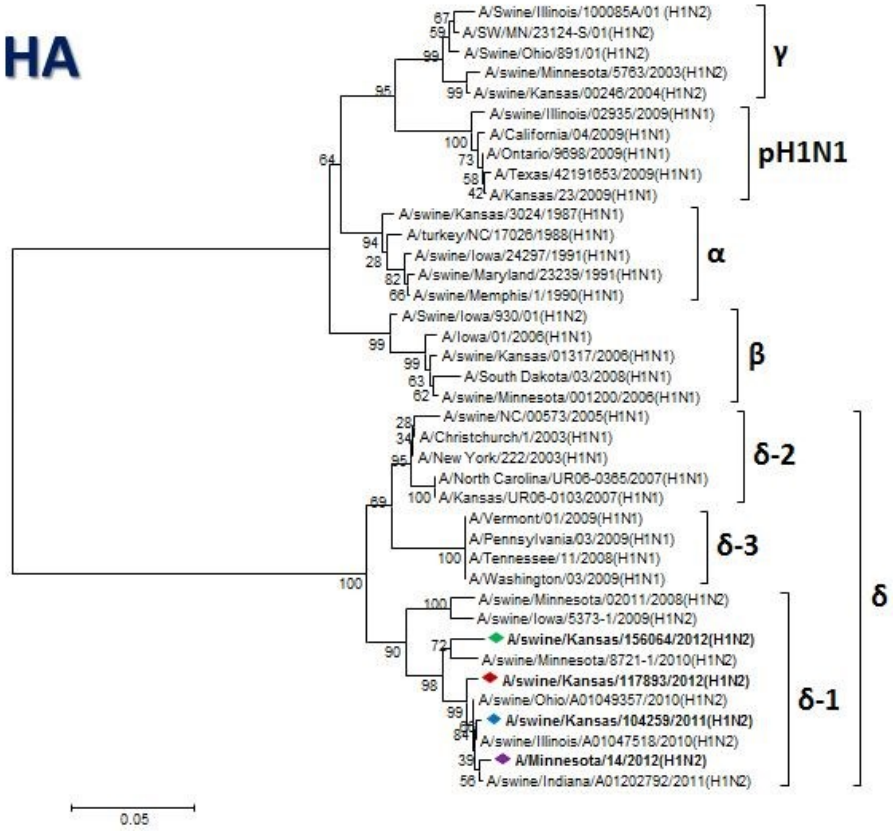


Figure 3-1 Phylogenetic tree of the HA molecules based on nucleotide sequences from eH1N2 (blue diamond), 2+6 rH1N2 (red diamond), swH1N2v (green diamond), and huH1N2v (purple diamond) and other sequences from GenBank. The phylogenetic tree shows the relationship of viruses in five clades: the α clade has closest homology to classical swine viruses, the β clade has closest homology to reassortant H1N1-like viruses, the γ clade has closest homology to H1N2-like viruses, the pH1N1 clade has closest homology to pH1N1 viruses, and the δ clade has closest homology to human-like H1 viruses as indicated by the bars on the right of the tree. Phylogenetic trees were generated with 1,000 bootstrap replicates using MegAlign software (version 4.1).

**Figure 3-2 Phylogenetic tree of NA molecules.**

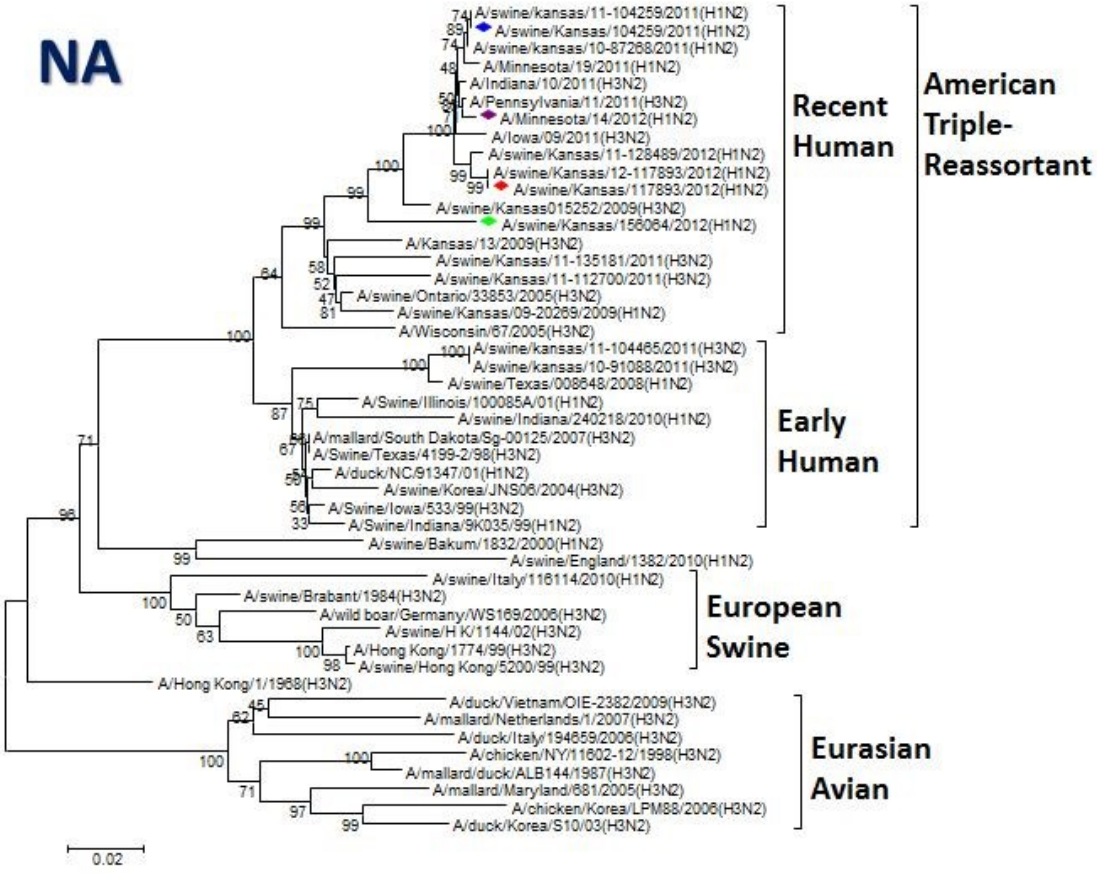


Figure 3-2 Phylogenetic tree of the NA molecules based on nucleotide sequences from eH1N2 (blue diamond), 2+6 rH1N2 (red diamond), swH1N2v (green diamond), and huH1N2v (purple diamond) and other sequences from GenBank. The phylogenetic tree shows the relationship of viruses in three clades: the American triple-reassortant clade, the European swine clade, and the Eurasian Avian clade as indicated by the bars on the right of the tree. Phylogenetic trees were generated with 1,000 bootstrap replicates using MegAlign software (version 4.1).

**Table 3-1 Amino acid differences between the HA and NA molecules of the swH1N2v and huH1N2v viruses.**

| Protein | Position huH1N2v | swH1N2v | huH1N2v | Protein | Position | swH1N2v | |
|---|---|---|---|---|---|---|---|
| HA | 24 | I | T | NA | 41 | D | N |
| | 26 | L | F | | 50 | V | A |
| | 32 | A | T | | 60 | K | R |
| | 33 | H | Y | | 71 | I | T |
| | 108 | E | K | | 126 | P | T |
| | 119 | S | P | | 147 | D | N |
| | 120 | D | E | | 149 | I | V |
| | 127 | Q | E | | 176 | I | M |
| | 161 | V | A | | 236 | T | A |
| | 164 | V | E | | 257 | I | V |
| | 174 | G | K | | 262 | V | I |
| | 202 | G | E | | 267 | T | K |
| | 215 | S | P | | 284 | S | F |
| | 217 | G | E | | 309 | D | N |
| | 218 | D | N | | 310 | H | Y |
| | 240 | R | G | | 312 | V | I |
| | 276 | T | A | | 329 | N | S |
| | 309 | A | S | | 332 | F | V |
| | 342 | T | A | | 336 | H | Y |
| | 483 | N | S | | 344 | E | K |
| | 512 | R | K | | 367 | D | N |
| | 522 | S | Y | | 369 | D | T |
| | 552 | A | V | | 370 | S | L |
| | - | - | - | | 386 | P | A |
| | - | - | - | | 390 | S | L |
| | - | - | - | | 402 | N | D |

Table 3-1 List of amino acid differences between the HA and NA molecules for swH1N2v and huH1N2v. The HA protein is numbered according to the H1 numbering system. There are 23 unique amino acid differences between the HA molecules accounting for approximately 5% of the total amino acids. There are 26 unique amino acid differences between the NA proteins accounting for approximately 5.5% of the total amino acids.

### *Molecular Analysis of Matrix and Non-Structural Molecules*

The M molecules of the 2+6 rH1N2, swH1N2v, and huH1N2v viruses clustered into the pH1N1 clade while eH1N2 clustered into the North American TR swine clade (Figure 3-3). The M1 molecules of all four viruses contain 238 amino acids. Molecular comparison of the M1 molecules shows that the 2+6 rH1N2, swH1N2v, and huH1N2v molecules share 99% homology at the nucleotide and amino acid levels. In contrast, the eH1N2 shared 86-87% homology at the nucleotide level and 94% homology at the amino acid level.

The M1 molecules of all viruses contain amino acids residues 41A, 102K, and 204E [54] which contribute to a filamentous virion morphology; however, viruses with pH1N1 M gene contain unique amino acid differences that contribute to a more spherical virion morphology. These amino acid residues include 30S (30N in huH1N2v) (Table 3-2), 31A, 95R, 142A, 207N, 209T, and 218T which are thought to increase transmission of pH1N1 [10,14,31]. Additionally, the avian/swine signature 137T was found in all viruses. Viruses with pH1N1 M gene contain avian signatures 115V and 121T while eH1N2 contains human signatures 115I and 121A [89].

The M2 molecules of all viruses contain 98 amino acids. Similar to the M1 molecules, the M2 molecules of 2+6 rH1N2, swH1N2v, and huH1N2v all shared 99% homology at the nucleotide and amino acid levels. However, the eH1N2 differed, sharing only 91-92% homology at the nucleotide level and 86-87% homology at the amino acid level. Notably, only four substitutes were identified between viruses with the pH1N1 M gene and A/California/04/2009 (pH1N1) (Table 3-2): S13N in 2+6 rH1N2, D24N in swH1N2v, P25S in swH1N2v, and V27A (associated with amantadine resistance) in both variant viruses. Another mutation associated with amantadine resistance, S31N, was also

found in all viruses with the pH1N1 M gene. Avian/swine signatures were identified in all viruses at residues 57Y, 86V, and 93N as well as in viruses with pH1N1 M gene at residues 11T and 20S. In contrast, eH1N2 contained two human signatures, 11I and 20N [89].

The NS molecules of eH1N2, swH1N2v, and huH1N2v all clustered into the North American TR swine clade while 2+6 rH1N2 clustered in the pH1N1 clade (Figure 3-4). The NS1 molecules of all viruses contain 220 amino acids with considerable variation. The NS1 molecules of eH1N2 and swH1N2v were highly homologous sharing 99% identity at the nucleotide level and 98% identity at the amino acid level. The huH1N2 shared 96% identity at the nucleotide level and 95% identity at the amino acid level with both the eH1N2 and swH1N2v viruses. Predictably, identity between the 2+6 rH1N2 and the other three viruses was considerably lower, sharing only 92-94% identity at the nucleotide level and 91-92% at the amino acid level. Notably, both variant viruses differed by 10 amino acids (Table 3-2).

The NS2 molecules of all viruses contain 122 amino acids. As with the NS1 molecules, the eH1N2 and swH1N2v viruses were most similar, sharing 99% identity at the nucleotide level and 98% identity at the amino acid level. The huH1N2 shared 97% identity at the nucleotide level and 95-97% identity at the amino acid level with both the eH1N2 and swH1N2v viruses. Predictably, identity between the 2+6 rH1N2 and the other three viruses was considerably lower, sharing only 93-94% identity at the nucleotide level and 93-95% identity at the amino acid level. All viruses possess the avian/swine signature 107L and amino acid residue 70G, both of which are present in pH1N1 viruses

[70,89]. Only five amino acid differences were identified between swH1N2v and huH1N2v (Table 3-2).

**Figure 3-3 Phylogenetic tree of M molecules.**

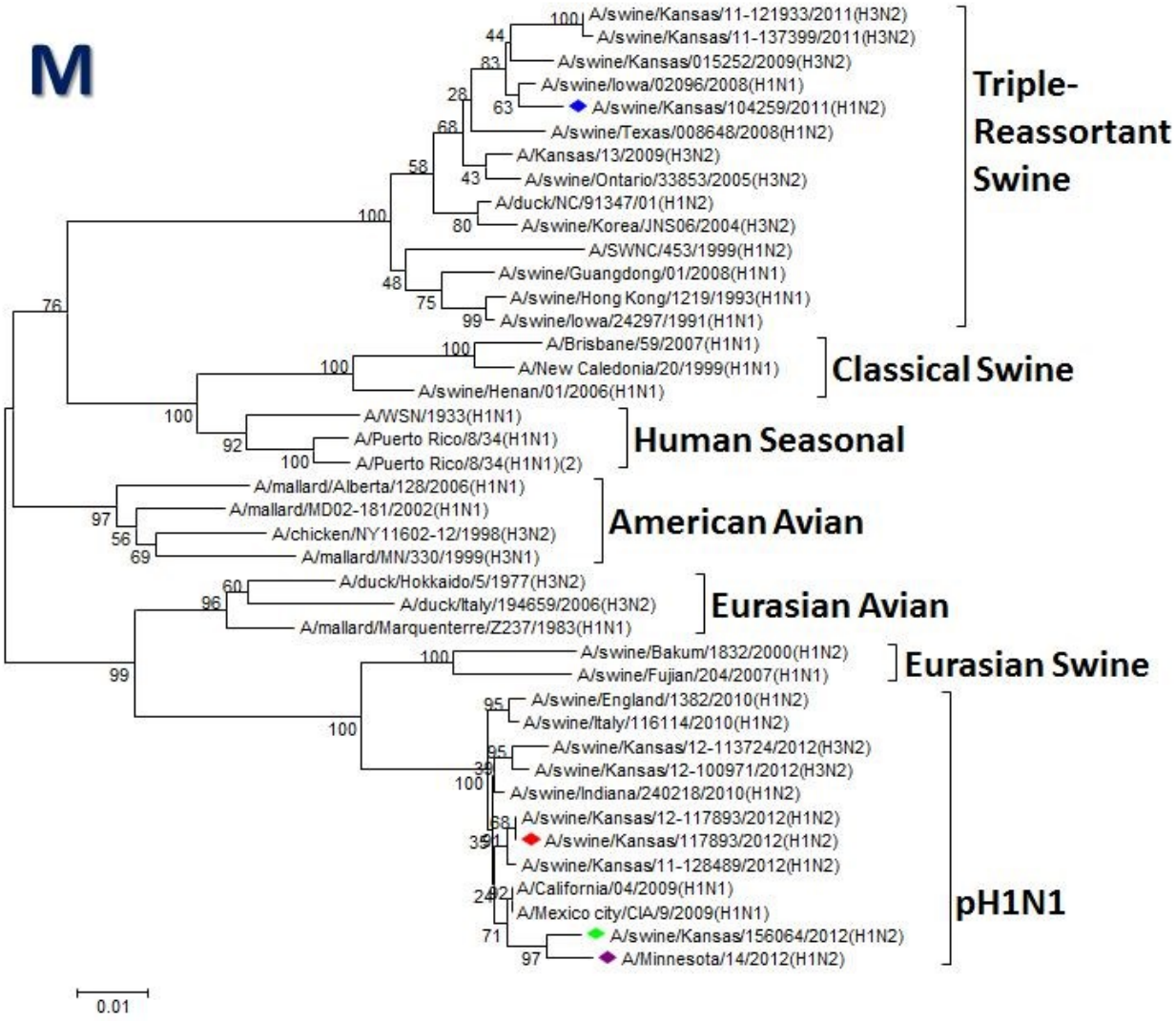


Figure 3-3 Phylogenetic tree of the M molecules based on nucleotide sequences from eH1N2 (blue diamond), 2+6 rH1N2 (red diamond), swH1N2v (green diamond), and huH1N2v (purple diamond) and other sequences from GenBank. The phylogenetic tree shows the relationship of viruses in seven clades: the triple-reassortant swine clade, the classical swine clade, the human seasonal clade, the American avian clade, the Eurasian Avian clade, the Eurasian swine clade, and the pH1N1 clade as indicated by the bars on the right of the tree. Phylogenetic trees were generated with 1,000 bootstrap replicates using MegAlign software (version 4.1).

**Figure 3-4 Phylogenetic tree of NS molecules.**

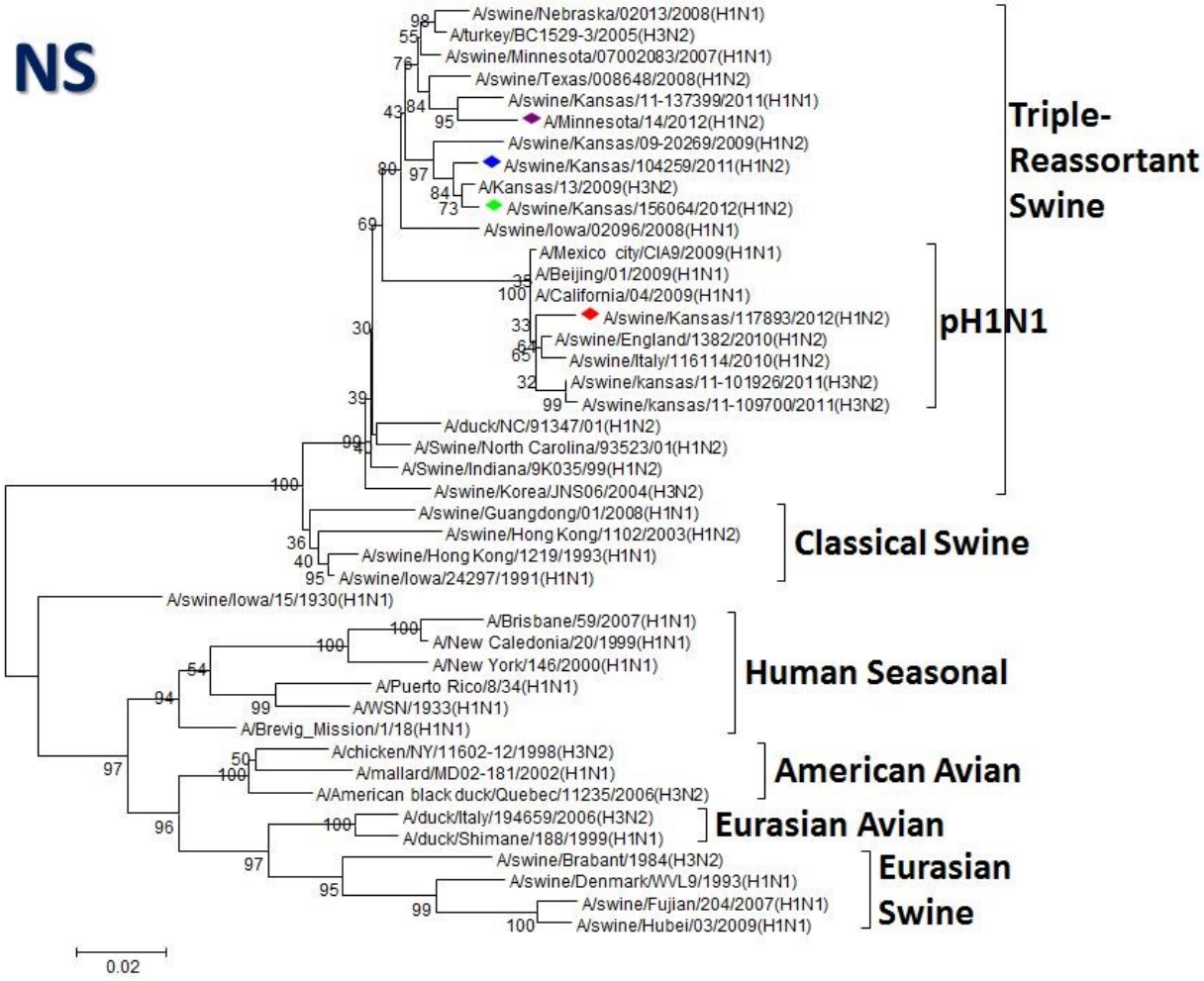


Figure 3-4 Phylogenetic tree of the NS molecules based on nucleotide sequences from eH1N2 (blue diamond), 2+6 rH1N2 (red diamond), swH1N2v (green diamond), and huH1N2v (purple diamond) and other sequences from GenBank. The phylogenetic tree shows the relationship of viruses in seven clades: the triple-reassortant swine clade, the classical swine clade, the human seasonal clade, the American avian clade, the Eurasian Avian clade, the Eurasian swine clade, and the pH1N1 clade as indicated by the bars on the right of the tree. Phylogenetic trees were generated with 1,000 bootstrap replicates using MegAlign software (version 4.1).

**Table 3-2 Amino acid differences between the M1, M2, NS1, and NS2 molecules of the swH1N2v and huH1N2v viruses.**

| Protein | Position | swH1N2v | huH1N2v | Protein | Position | swH1N2v | huH1N2v |
|---|---|---|---|---|---|---|---|
| M1 | 30 | S | N | NS1 | 6 | M | V |
| M2 | 24 | N | D | | 45 | G | R |
| | 25 | P | S | | 51 | G | S |
| NS2 | 6 | M | V | | 70 | R | K |
| | 11 | D | G | | 84 | V | I |
| | 15 | R | K | | 90 | I | L |
| | 16 | Q | R | | 119 | M | I |
| | 19 | I | T | | 124 | M | I |
| | - | - | - | | 139 | S | N |
| | - | - | - | | 178 | V | I |

Table 3-2 List of amino acid differences between the M1, M2, NS1, and NS2 molecules of swH1N2v and huH1N2v. There is only one unique amino acid difference between the M1 molecules accounting for 0.42% of the total amino acids. There are two unique amino acid differences between the M2 molecules accounting for approximately 2.0% of the total amino acids. There are 10 unique amino acid differences between the NS1 molecules accounting for approximately 4.5% of the total amino acids. There are five unique amino acids between the NS2 molecules accounting for 3.3% of the total amino acids.

### *Molecular Analysis of the Polymerase Complex (PB2, PB1 [PB1-F2], PA [PA-X]) and NP Molecules*

The polymerase complex molecules PB2, PB1, PA, and NP of eH1N2, swH1N2v, and huH1N2v clustered into the North American TR swine clade while 2+6 rH1N2 clustered into the pH1N1 clade (Figure 3-5, Figure 3-6, Figure 3-7, Figure 3-8).

The PB2 molecules of all viruses contain 760 amino acids. The eH1N2, swH1N2v, and huH1N2v viruses shared 98-99% identity at the nucleotide level and 99% homology at the amino acid level. In contrast, the 2+6 rH1N2 virus differed from the other three viruses, sharing only 93-94% identity at the nucleotide level and 97% at the amino acid level. The PB2 molecules of all viruses possess the same host-range-specific residues 271A, 588T, 627E, 636L, and 701D [69]. Avian/swine signatures were identified in all viruses at residues 44A, 199A, 475L, 567D, 588T, 613V, 627E, 674A, and 702K while a single human signature was identified at 271A [89,160]. Although the viruses in this study possess avian-adapted residue 627E instead of mammalian-adapted 627K, it has been reported that mutations found in these viruses including L89V, T271A, G309D, T339K, R477G, I495V, G590S, Q591R, and 676T act in lieu of the E627K mutation to support adaptation to replication in mammals by changing the electrostatic charge on the surface of residue 627 from negative to positive [69,160,161]. A total of seven distinct amino acids were identified between swH1N2v and huH1N2v (Table 3-3).

The PB1 molecules of all viruses contain 758 amino acids and showed considerable variation. The eH1N2 and swH1N2v viruses were most similar, sharing 97% identity at the nucleotide level and 99% at the amino acid level while 2+6 rH1N2 and swH1N2v were least similar, sharing only 93% identity at the nucleotide level and 97% identity at the amino acid level. Comparison of the other viruses showed 94-95%

identity at the nucleotide level and 97-98% identity at the amino acid level. Two genetic signatures were identified in viruses with pH1N1 M gene: avian/swine signature 327R and human signature 336I, both of which are present in pH1N1 viruses [89]. A total of 14 amino acid differences were identified between swH1N2v and huH1N2v (Table 3-3).

Analysis of the PB1-F2 molecules revealed that the eH1N2, swH1N2v, and huH1N2v viruses possess a full-length PB1-F2 ORF encoding 80 amino acids while 2+6 rH1N2 is truncated and possesses only 12 amino acids, consistent with pH1N1 viruses. The eH1N2 and swH1N2v viruses share 99% identity at the nucleotide level and 97% identity at the amino acid level. In contrast, the huH1N2v virus shares 95% and 96% identity at the nucleotide level with the eH1N2 and swH1N2v viruses, respectively. Moreover, it shares only 89% and 86% identity at the amino acid level with the eH1N2 and swH1N2v viruses. There are 11 unique amino acid differences between swH1N2v and huH1N2v (Table 3-3). While these viruses lack the N66S mutation found in the Hong Kong 1997 H5N1 [78] and 1918 pandemic viruses which is thought to increase virulence, they do possess mutations E6D, R53K, and R75H which are thought to increase pathogenicity [77].

The PA molecules of all viruses contain 717 amino acids. The eH1N2, swH1N2v, and huH1N2v viruses all shared 98-99% identity at the nucleotide level and 99% identity at the amino acid level. However, the 2+6 rH1N2 virus differed from the other three viruses, sharing only 93-94% identity at the nucleotide level and 96-97% identity at the amino acid level. Avian/swine genomic signatures (also present in pH1N1 viruses) were found in all viruses at residues 55D, 225S, 268L, 404A, 409N, and 552T [89]. Human genomic signatures were identified at 57Q in swH1N2v and 356R in 2+6 rH1N2 while a

single human genomic signature (also present in pH1N1 viruses) was identified in all viruses at 409N. Interestingly, all viruses retained amino acid residue 97T instead of 97I which is thought to play a role in the adaptation of influenza viruses to mammalian hosts [88]. A total of six amino acid differences were identified between swH1N2v and huH1N2v (Table 3-3).

The PA-X molecules of all viruses contain 233 amino acids. The eH1N2, swH1N2v, and huH1N2v viruses all share 99% identity at both the nucleotide and amino acid levels. In contrast, the 2+6 rH1N2 virus differed from the other three viruses, sharing only 92% identity at the nucleotide level and 93-94% identity at the amino acid level. The swH1N2v virus possesses only two unique amino acid, E209G and A212E, while the huH1N2v virus possesses only one unique amino acid difference, V100I.

The NP proteins of all viruses contain 499 amino acids. The eH1N2, swH1N2v and huH1N2v viruses shared 98-99% identity at the nucleotide level and 99% identity at the amino acid level. However, the 2+6 rH1N2 virus differed significantly, sharing only 92-93% identity at the nucleotide level and 94% identity at the amino acid level with the other three viruses. Avian/swine genomic signatures (also present in pH1N1 viruses) were identified in all viruses at 16G, 61I, 109I (eH1N2, swH1N2v, huH1N2v), 214R, 283L, 293R, 305K, 372E, 422R, 442T, and 445D. Human genomic signatures (also present in pH1N1 viruses) were found in all viruses at 100V (eH1N2, swH1N2v, huH1N2v), 33I, 305K, and 357K[89]. Additionally, all viruses contain amino acid residue 53E while 2+6 rH1N2 contained amino acid mutations G34A and I109T, all of which are thought to help incorporate the pH1N1 M gene into the TRIG cassette [34,162]. Only four amino acid differences were identified between swH1N2v and huH1N2v (Table 3-3).

**Figure 3-5 Phylogenetic tree of PB2 molecules.**

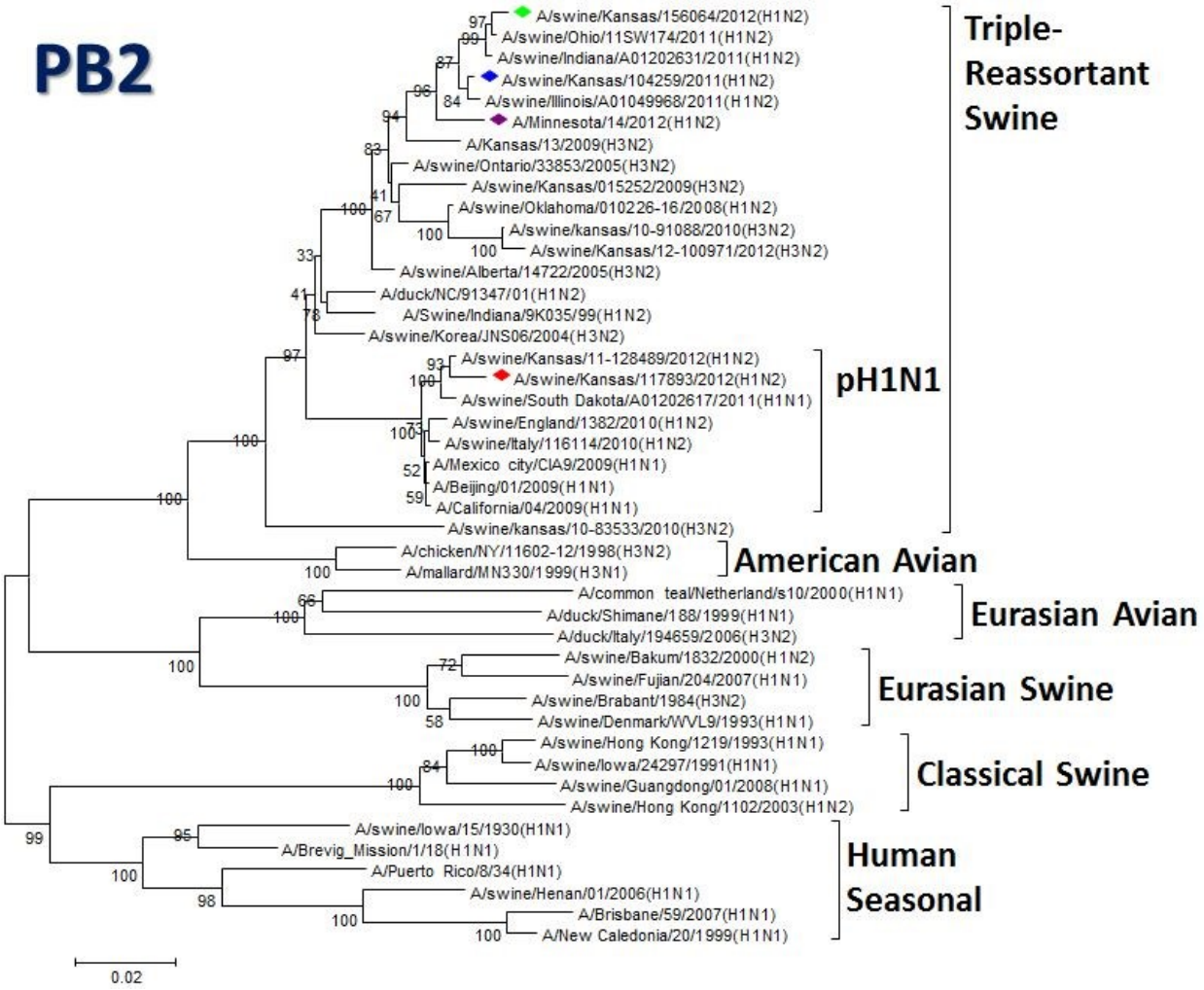


Figure 3-5 Phylogenetic tree of the PB2 molecules based on nucleotide sequences from eH1N2 (blue diamond), 2+6 rH1N2 (red diamond), swH1N2v (green diamond), and huH1N2v (purple diamond) and other sequences from GenBank. The phylogenetic tree shows the relationship of viruses in seven clades: the triple-reassortant swine clade, the classical swine clade, the human seasonal clade, the American avian clade, the Eurasian Avian clade, the Eurasian swine clade, and the pH1N1 clade as indicated by the bars on the right of the tree. Phylogenetic trees were generated with 1,000 bootstrap replicates using MegAlign software (version 4.1).

**Figure 3-6 Phylogenetic tree of PB1 molecules.**

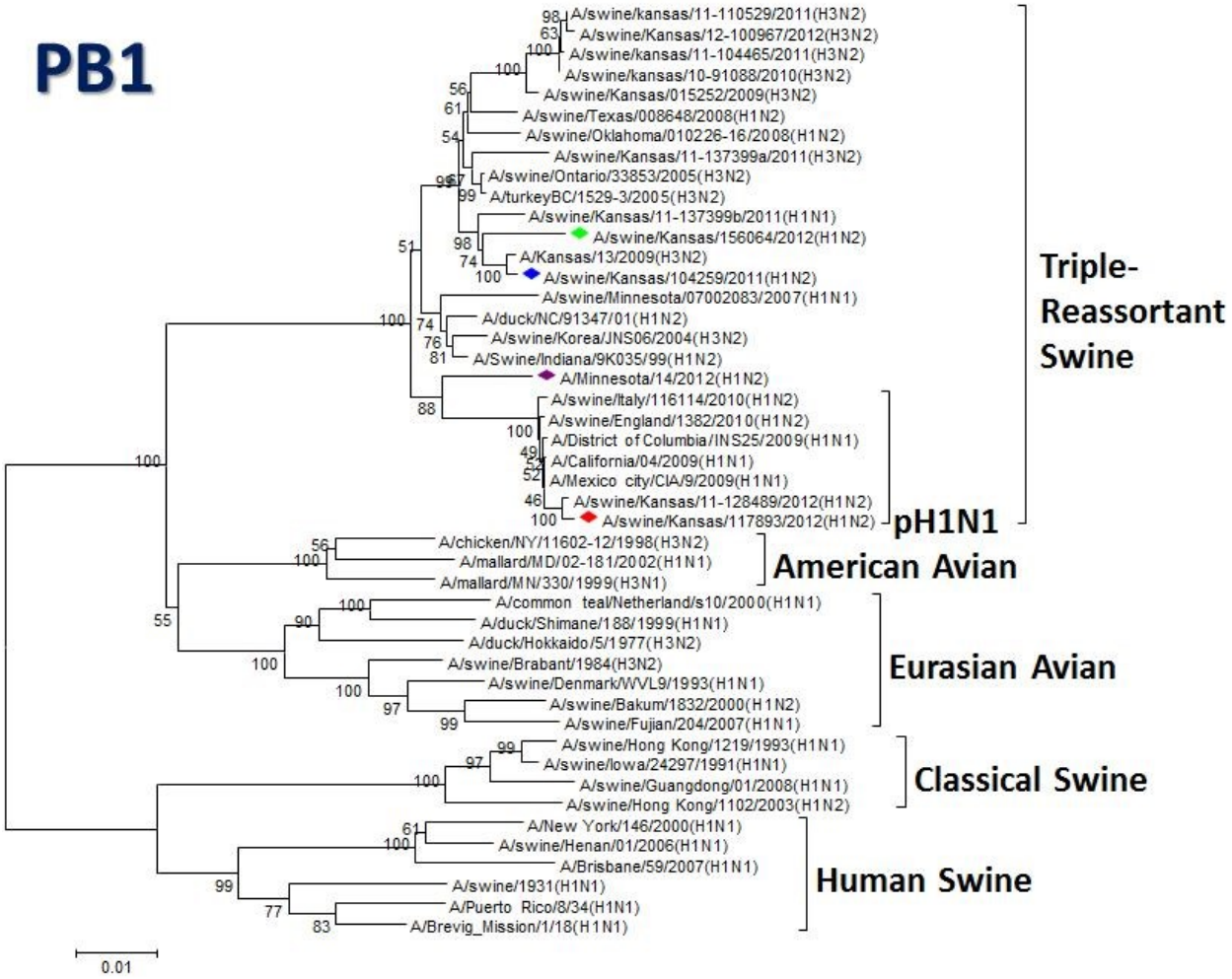


Figure 3-6 Phylogenetic tree of the PB1 molecules based on nucleotide sequences from eH1N2 (blue diamond), 2+6 rH1N2 (red diamond), swH1N2v (green diamond), and huH1N2v (purple diamond) and other sequences from GenBank. The phylogenetic tree shows the relationship of viruses in seven clades: the triple-reassortant swine clade, the classical swine clade, the human seasonal clade, the American avian clade, the Eurasian Avian clade, the Eurasian swine clade, and the pH1N1 clade as indicated by the bars on the right of the tree. Phylogenetic trees were generated with 1,000 bootstrap replicates using MegAlign software (version 4.1).

**Figure 3-7 Phylogenetic tree of PA molecules.**

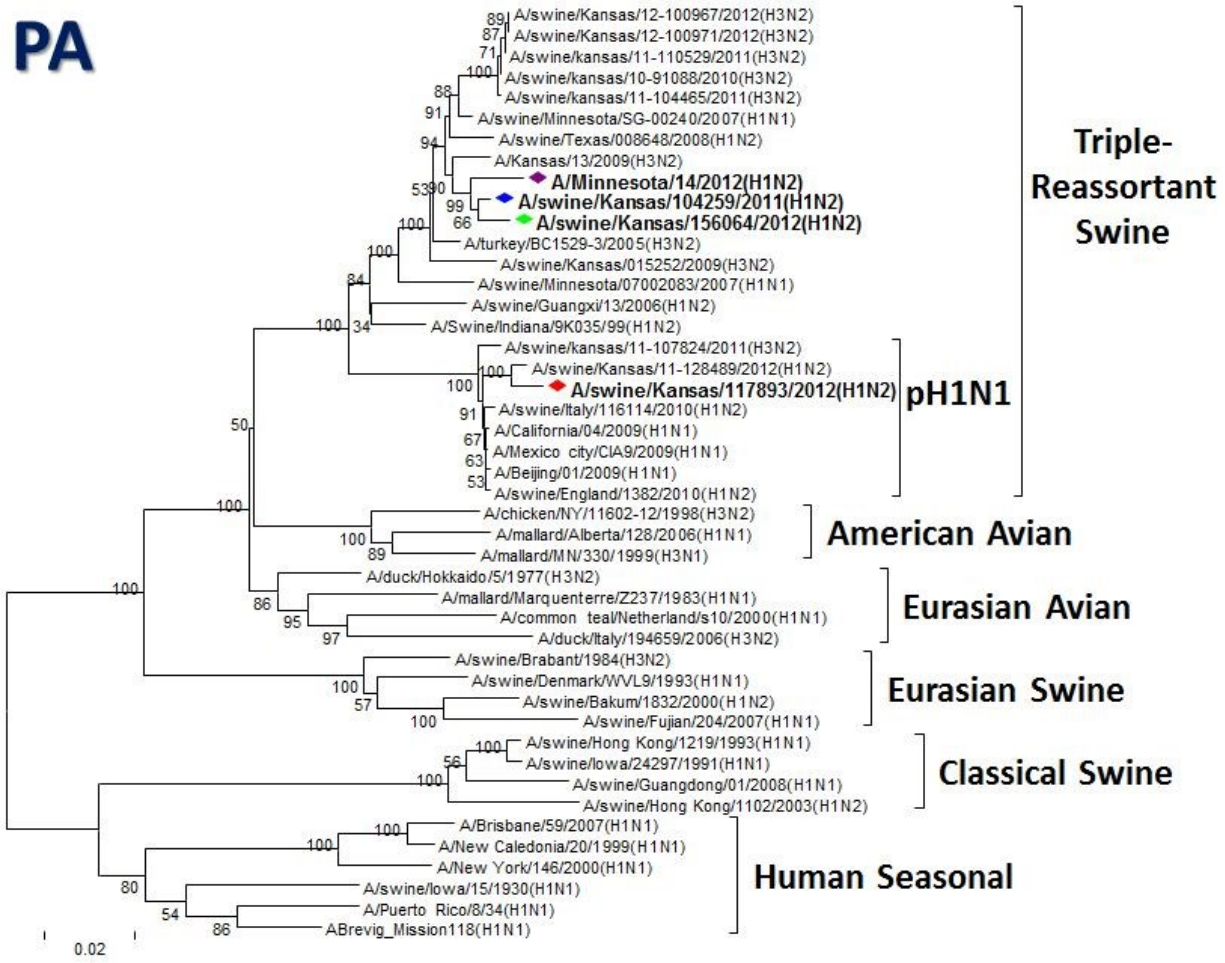


Figure 3-7 Phylogenetic tree of the PA clades based on nucleotide sequences from eH1N2 (blue diamond), 2+6 rH1N2 (red diamond), swH1N2v (green diamond), and huH1N2v (purple diamond) and other sequences from GenBank. The phylogenetic tree shows the relationship of viruses in seven clades: the triple-reassortant swine clade, the classical swine clade, the human seasonal clade, the American avian clade, the Eurasian Avian clade, the Eurasian swine clade, and the pH1N1 clade as indicated by the bars on the right of the tree. Phylogenetic trees were generated with 1,000 bootstrap replicates using MegAlign software (version 4.1).

**Figure 3-8 Phylogenetic tree of NP molecules.**

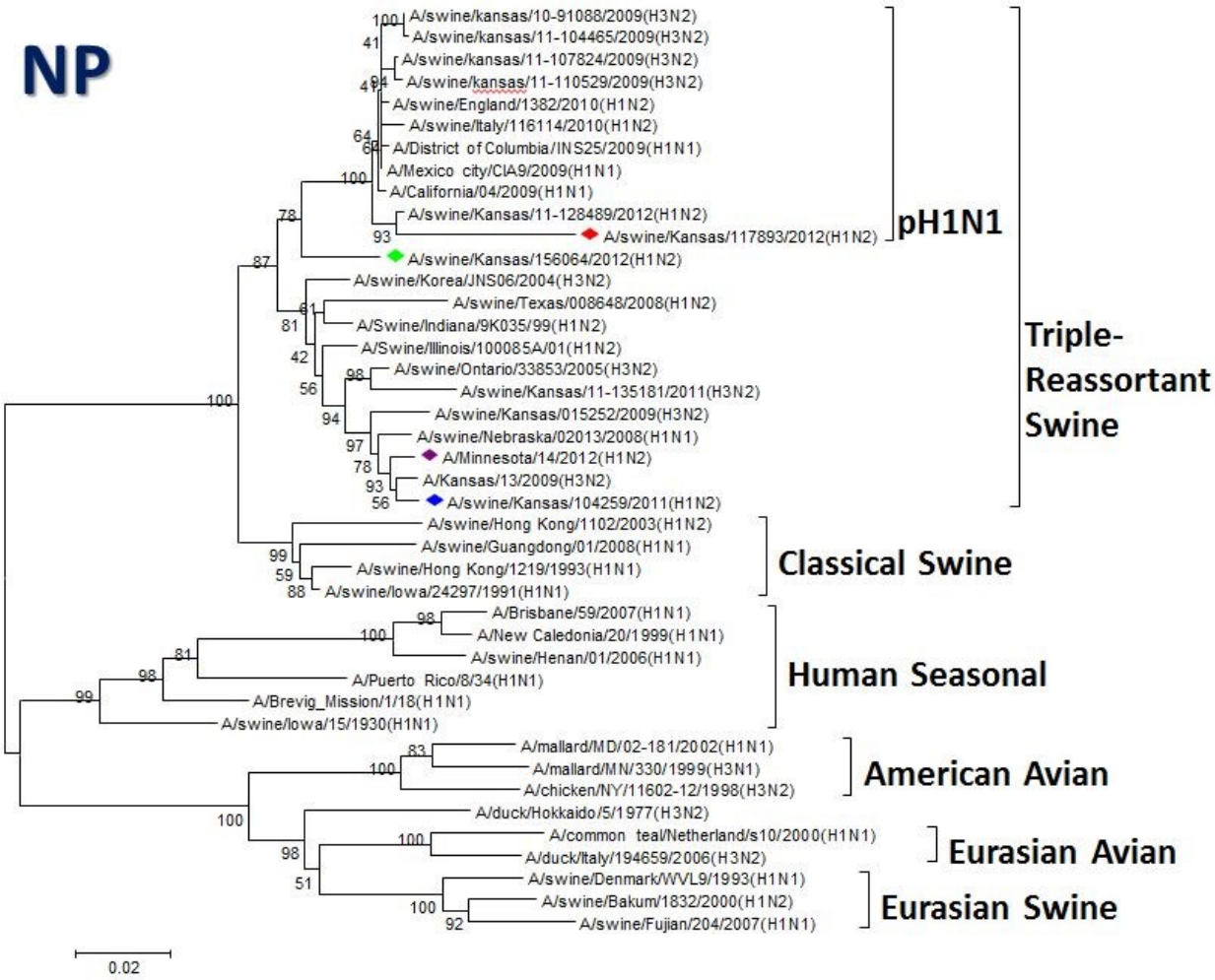


Figure 3-8 Phylogenetic tree of the NP molecules based on nucleotide sequences from eH1N2 (blue diamond), 2+6 rH1N2 (red diamond), swH1N2v (green diamond), and huH1N2v (purple diamond) and other sequences from GenBank. The phylogenetic tree shows the relationship of viruses in seven clades: the triple-reassortant swine clade, the classical swine clade, the human seasonal clade, the American avian clade, the Eurasian Avian clade, the Eurasian swine clade, and the pH1N1 clade as indicated by the bars on the right of the tree. Phylogenetic trees were generated with 1,000 bootstrap replicates using MegAlign software (version 4.1).

**Table 3-3 Amino acid differences between the PB2, PB1, PB1-F2, PA, PA-X, and NP molecules of the swH1N2v and huH1N2v viruses.**

| Protein | Position | swH1N2v | huH1N2v | Protein | Position | swH1N2 | huH1N2v |
|---|---|---|---|---|---|---|---|
| PB2 | 456 | S | N | PB1-F2 | 4 | E | G |
| | 480 | V | I | | 27 | I | T |
| | 543 | E | K | | 36 | I | T |
| | 556 | N | H | | 37 | L | R |
| | 559 | S | T | | 40 | D | G |
| | 560 | L | V | | 43 | P | L |
| | 607 | L | F | | 50 | G | D |
| PB1 | 4 | N | H | | 56 | V | A |
| | 111 | M | H | | 67 | P | L |
| | 140 | A | S | | 68 | T | I |
| | 171 | M | I | | 79 | Q | R |
| | 212 | V | M | | 80 | STOP | STOP |
| | 223 | A | T | PA | 100 | V | I |
| | 374 | S | A | | 469 | F | L |
| | 433 | R | K | | 547 | E | D |
| | 435 | T | V | | 614 | N | D |
| | 517 | I | V | | 626 | K | R |
| | 581 | N | D | | 628 | M | V |
| | 587 | A | V | NP | 125 | N | S |
| | 618 | E | D | | 217 | V | I |
| | 642 | S | N | | 353 | L | I |
| PA-X | 100 | V | I | | 400 | R | K |
| | 209 | E | G | | - | - | - |
| | 212 | A | E | | - | - | - |

Table 3-3 List of amino acid differences between swH1N2v and huH1N2v. There are seven unique amino acid differences between the PB2 molecules accounting for less than 1.0% of the total amino acids. There are 14 unique amino acid differences between the PB1 molecules accounting for approximately 1.8% of the total amino acids. There are six unique amino acid differences between the PA molecules accounting for less than 1.0% of the total amino acids. There are four unique amino acids between the NP molecules accounting less than 1.0% of the total amino acids. There are three unique amino acid differences between the PA-X molecules accounting for 1.2% of the total amino acids. There are 11 unique amino acid differences between the PB1-F2 molecules accounting for 18.75% of those amino acids.

### *Origin of Each Gene Segment*

Molecular analysis of each virus revealed that all gene segments from eH1N2 have closest homology to endemic North American TR SIVs. Similarly, all genes from both variant viruses share highest homology to North American TR SIVs with exception of the matrix gene which has highest homology to pH1N1 viruses. Dissimilarly, all internal gene segments from the 2+6 rH1N2 virus share highest homology with pH1N1 viruses while only its surface proteins, HA and NA, have highest homology with North American TR SIVs (Table 3-4).

**Table 3-4 Virus subtype and origin of each gene from eH1N2, 2+6 rH1N2, swH1N2v, and huH1N2v.**

| Virus | Subtype | PB2 | PB1 | PA | HA | NP | NA | M | NS |
|---|---|---|---|---|---|---|---|---|---|
| **eH1N2** | **H1N2** | **T** | **T** | **T** | **T** | **T** | **T** | **T** | **T** |
| **2+6 rH1N2** | **H1N2** | **P** | **P** | **P** | **T** | **P** | **T** | **P** | **P** |
| **swH1N2v** | **H1N2** | **T** | **T** | **T** | **T** | **T** | **T** | **P** | **T** |
| **huH1N2v** | **H1N2** | **T** | **T** | **T** | **T** | **T** | **T** | **P** | **T** |

Table 3-4 Genes denoted with 'T' have closest homology with North American triple-reassortant swine influenza viruses. Genes denoted with 'P' have closest homology with the 2009 pandemic H1N1 virus.

## In Vitro Studies

### *Plaque Assay*

Plaque assays showed that swH1N2v and huH1N2v formed large, similar-sized plaques compared to eH1N2 and 2+6 rH1N2 which formed small, similar-sized plaques at 72-hpi in MDCK cells (Figure 3-9).

**Figure 3-9 Viral plaque assay on MDCK cells.**

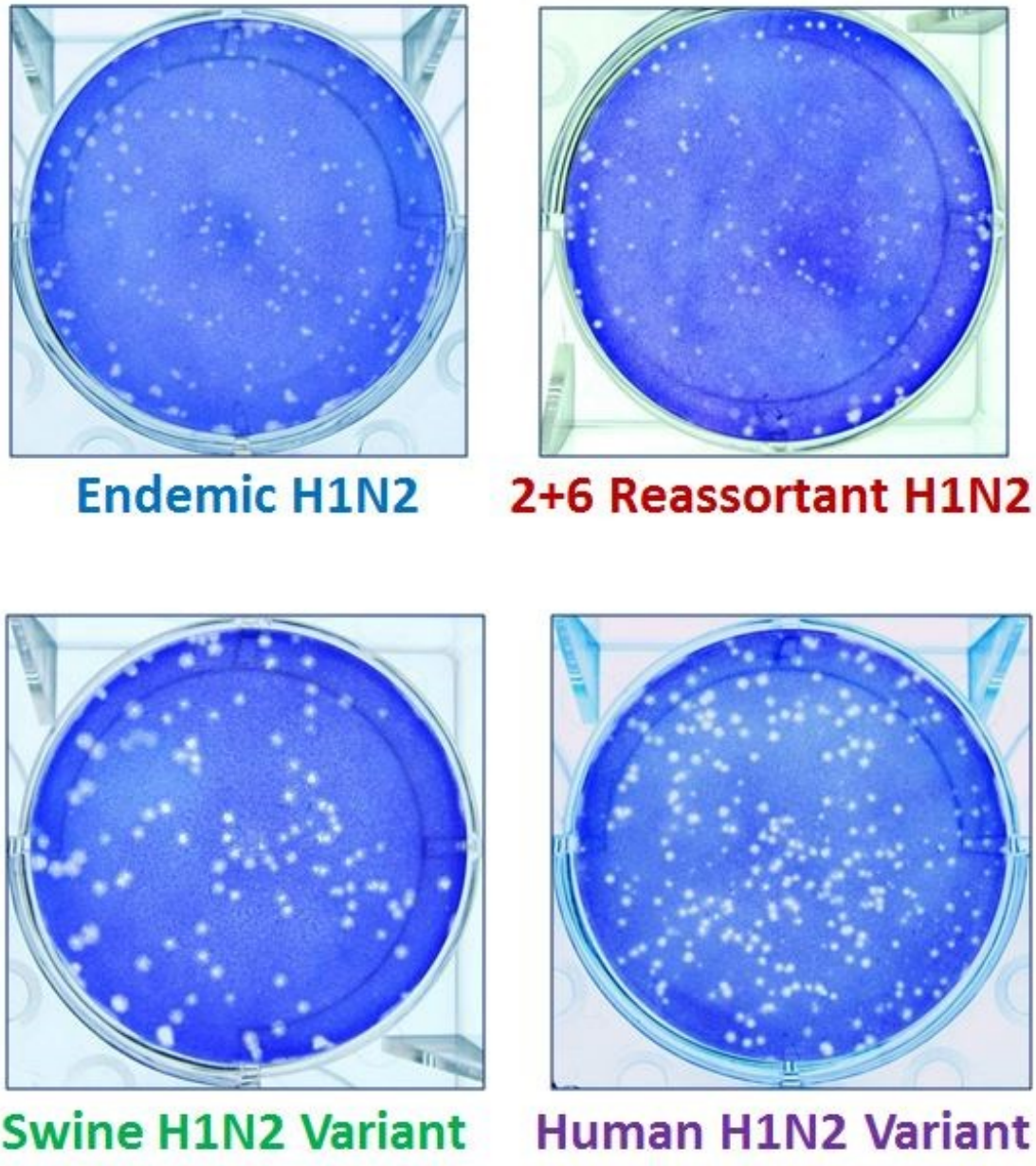


Figure 3-9 Plaque assays for each virus on MDCK cells at 72-hpi. MDCK cells infected with each virus at MOI 0.001. All viruses formed plaques; however, both variant viruses formed significantly larger plaques than the eH1N2 and 2+6 rH1N2 viurses.

### *Viral Replication Kinetics*

Growth studies showed that all viruses replicated efficiently in PK-15, A549, and MDCK cells. In PK-15 cells, the huH1N2v virus grew to significantly higher titers than the other three viruses at 24-, 36- and 48-hpi (Figure 3-10). In A549 cells, the huH1N2v virus grew to significantly higher titers than the other three viruses at 24-hpi. By 36-hpi, the 2+6 rH1N2 and huH1N2v viruses grew to significantly higher titers than the swH1N2v virus which grew to significantly higher titers than the eH1N2 virus. By 48-hpi, the 2+6 rH1N2, swH1N2v, and huH1N2v viruses all grew to similar titers which were significantly higher than the eH1N2 virus (Figure 3-11). In MDCK cells, the 2+6 rH1N2 virus grew to significantly higher titers than the other three viruses at 24- and 36-hpi while all viruses grew to similar titers by 48-hpi (Figure 3-12).

**Figure 3-10 Viral replication kinetics in PK-15 cells.**

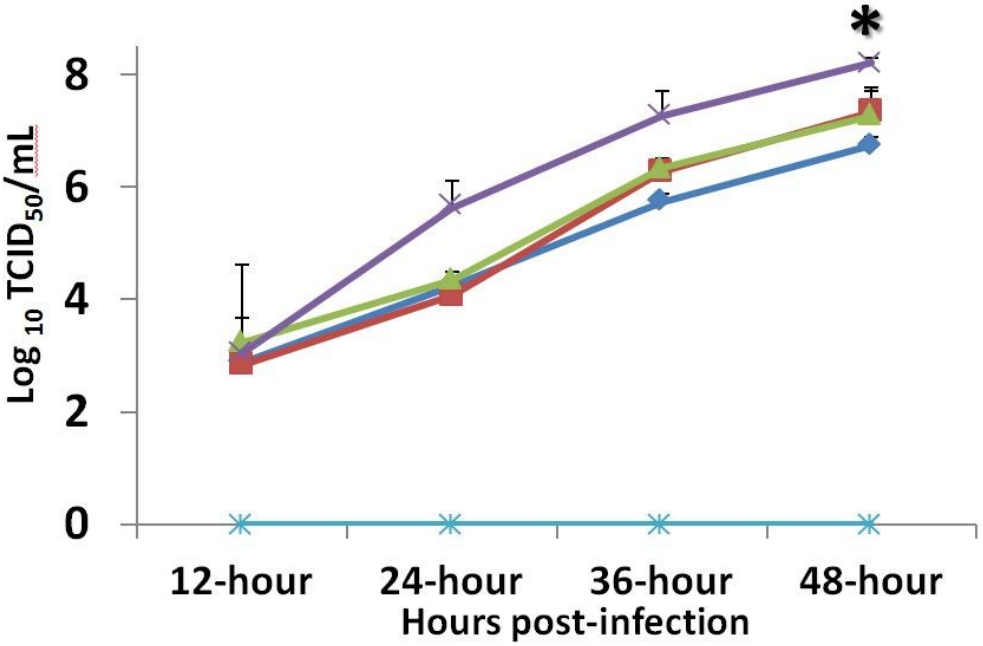


Figure 3-10 Viral replication kinetics for eH1N2 (blue), 2+6 rH1N2 (red), swH1N2v (green), and huH1N2v (purple) viruses in PK-15 (porcine kidney) cells. * represents p-value of $< 0.05$.

**Figure 3-11 Viral replication kinetics in A549 cells.**

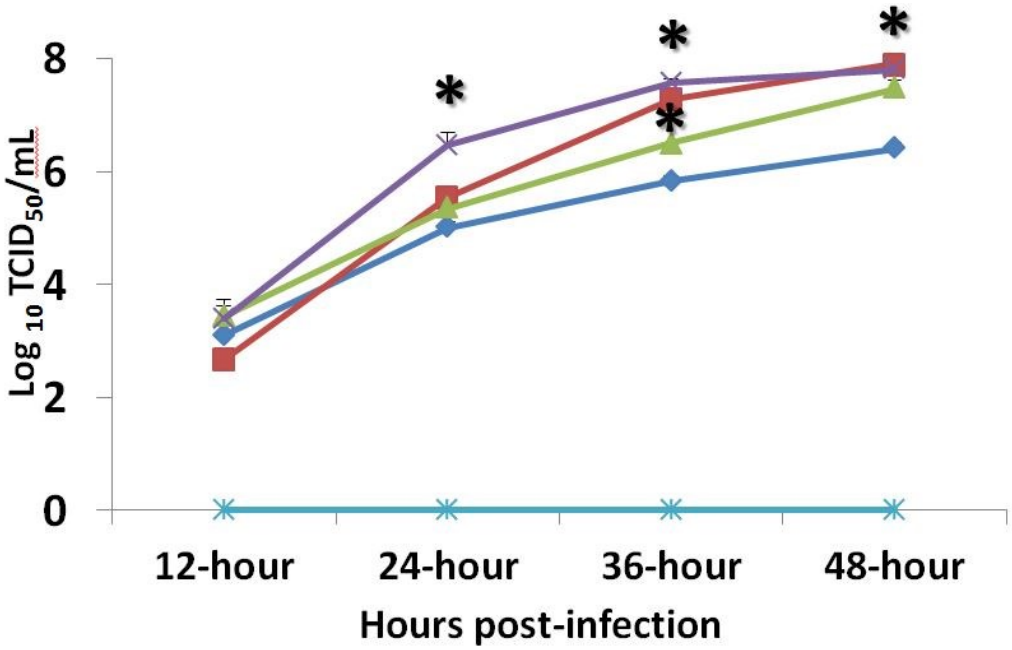


Figure 3-11 Viral replication kinetics for eH1N2 (blue), 2+6 rH1N2 (red), swH1N2v (green), and huH1N2v (purple) viruses in A549 (human adenocarcinomic basal epithelial) cells. * represents p-value of $< 0.05$.

**Figure 3-12 Viral replication kinetics in MDCK cells.**

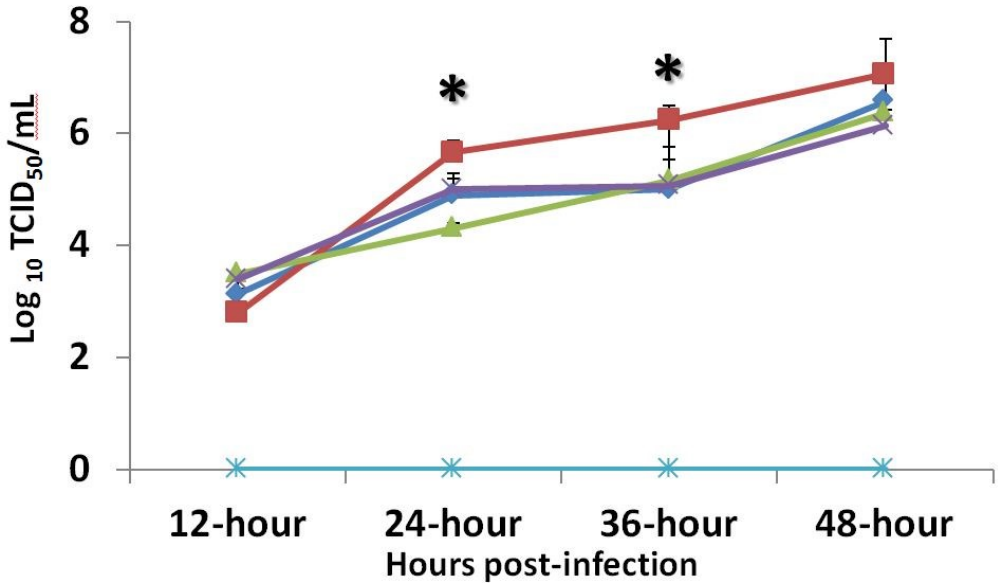


Figure 3-12 Viral replication kinetics for eH1N2 (blue), 2+6 rH1N2 (red), swH1N2v (green), and huH1N2v (purple) viruses in MDCK (Madin-Darby canine kidney) cells. * represents p-value of $< 0.05$.

## Viral Infection and Replication in Direct-Infection Pigs

### *Clinical Signs*

All pigs were observed daily for clinical signs of disease. Although pigs did not show obvious signs of disease (respiratory distress, lethargy, anorexia), several pigs showed fever defined as temperature of 104°C and higher. This fever lasted 1-3 days between 2- and 5-dpi and included four pigs from the eH1N2 group, five pigs from the 2+6 rH1N2 group, one pig from the swH1N2v group, and two pigs from the huH1N2v group.

### *Macroscopic Lung Lesions*

All direct-infection pigs showed macroscopic lung lesions indicative of swine influenza (Figure 3-13) and lungs were assigned scores as a "percent of the lung with lesions". Pigs inoculated with eH1N2 showed lesions on 13.46% ± 3.58% of lungs at 5-dpi and 6.57% ± 1.40% of lungs at 7-dpi. Pigs inoculated with 2+6 rH1N2 showed lesions on 11.92% ± 2.28% of lungs at 5-dpi and 8.13% ± 4.16% of lungs at 7-dpi. Pigs inoculated with swH1N2v showed lesions on 30.76% ± 6.39% of lungs at 5-dpi and 34.10% ± 5.47% of lungs at 7-dpi. Pigs inoculated with 2+6 rH1N2 showed lesions on 26.05% ± 5.16% of lungs at 5-dpi and 33.95% ± 5.51% of lungs at 7-dpi. Control pigs showed no macroscopic lung lesions and were assigned scores of zero. Overall, the swH1N2v and huH1N2v viruses caused significantly more lung lesions in all direct-infection pigs compared to the eH1N2 and 2+6 rH1N2 viruses (Figure 3-14).

**Figure 3-13 Macroscopic lung lesions from direct-infection pigs at 5-dpi.**

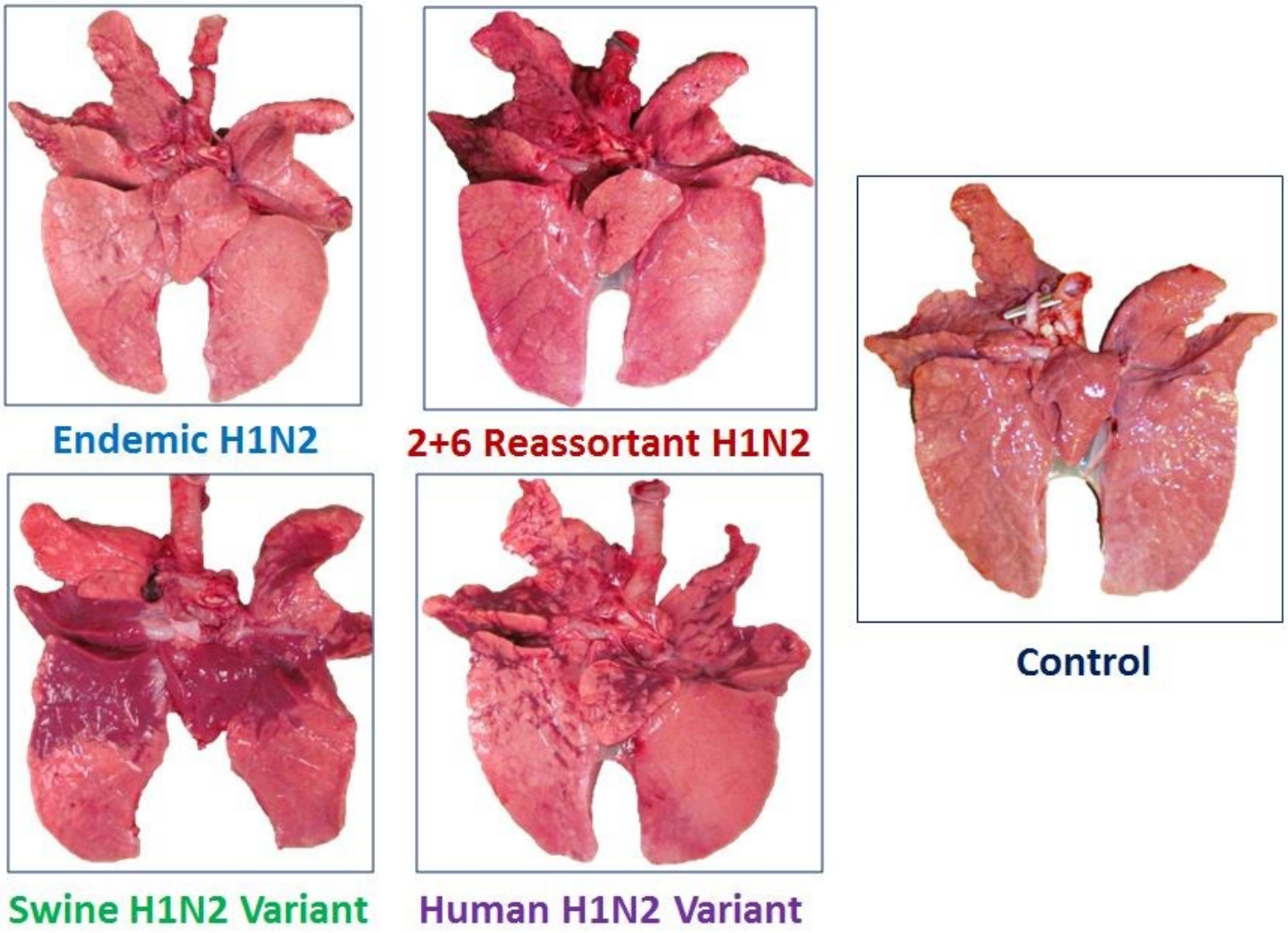


Figure 3-13 Macroscopic lung lesions of a single direct-infection pig from each direct-infection group at 5-dpi.

**Figure 3-14 Macroscopic lung lesion scores from direct-infection pigs at 5- and 7-dpi.**

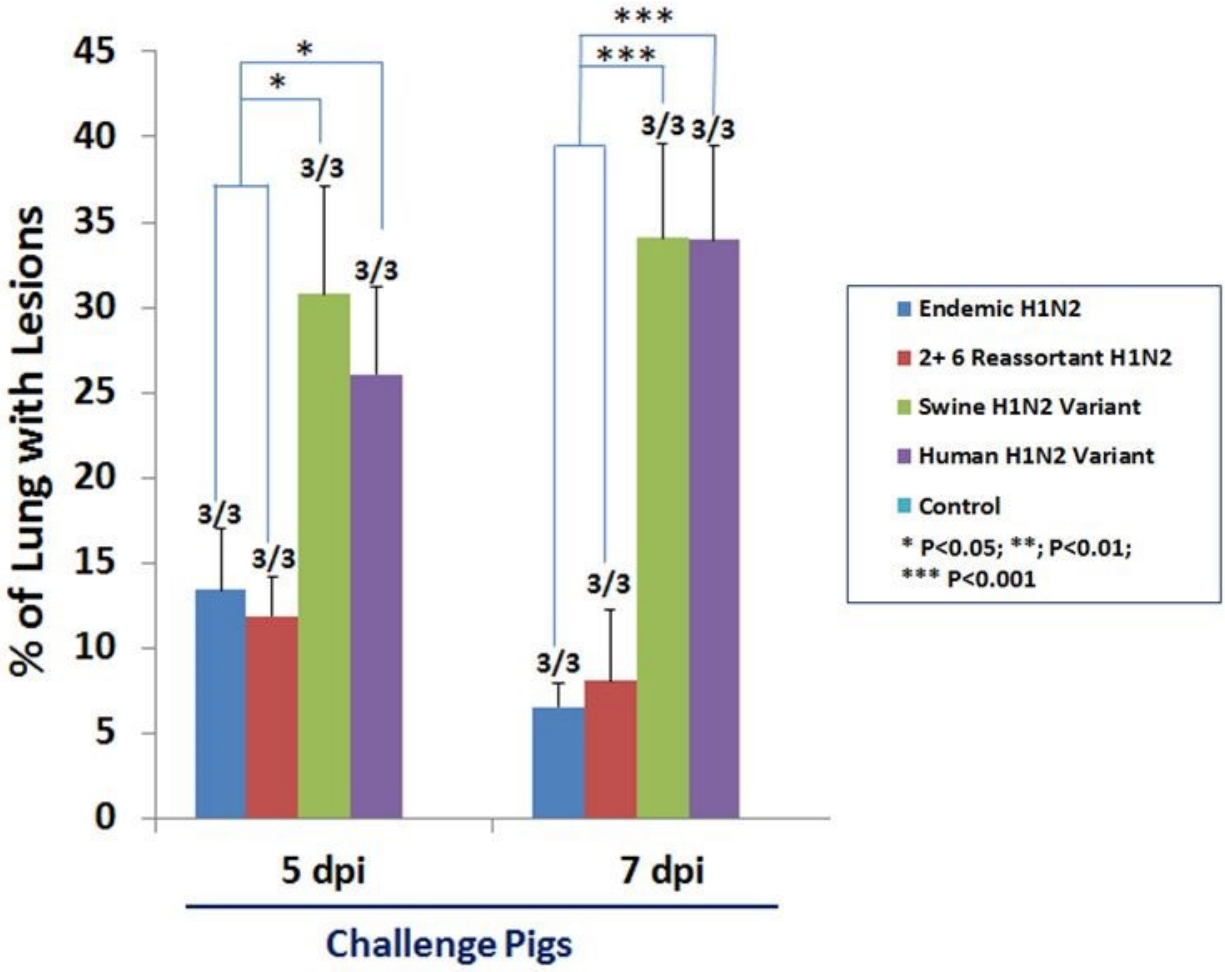


Figure 3-14 Macroscopic lung lesion scores from direct infection pigs at 5- and 7-dpi. Lungs were assigned scores by a single, experienced research veterinarian as a "percent of lungs with lesions." A p-value < 0.05 was considered statistically significant.

### *Microscopic Lung Lesions*

All direct-infection pigs showed microscopic lung lesions indicative of swine influenza as described by a veterinary pathologist using histopathology (Figure 3-15). For histopathology, scores were assigned to the lung tissues on a scale of 0-3 with 0 being no lesion, 1 being mild lesions, 2 being moderate lesions, and 3 being severe lesions (Table 3-5). Pigs inoculated with eH1N2 showed minimal to complete lack of bronchiolar epithelial degeneration and necrosis. A mild alveolar epithelial degeneration and necrosis was observed with plugging of alveolar lumen with cellular debris. Lung lesions for this group were scored as 0.67 ± 0.29 at 5-dpi and 1.17 ± 0.44 at 7-dpi. Pigs inoculated with 2+6 rH1N2 showed minimal to mild bronchiolar and alveolar epithelial degeneration and necrosis. There was mild interstitial pneumonia characterized by small numbers of lymphocytes infiltrating the alveolar septa and perivascular areas. Lung lesions for this group were scored as 1.00 ± 0.44 at 5-dpi and 0.67 ± 0.17 at 7-dpi. Pigs inoculated with swH1N2v showed severe bronchiolar epithelial necrosis and moderate numbers of neutrophils were present in the bronchiolar and alveolar lumen. Lung lesions for this group were scored as 2.17 ± 0.44 at 5-dpi and 2.17 ± 0.44 at 7-dpi. Pigs inoculated with huH1N2v showed moderate to severe bronchiolar epithelial degeneration and necrosis with infiltration of neutrophils into the alveolar septa and alveolar and bronchiolar lumen. Lung lesions for this group were scored as 2.50 ± 0.29 at 5-dpi and 2.18 ± 0.44 at 7-dpi. Overall, both the swH1N2v and huH1N2v viruses caused significantly more severe lesions than the eH1N2 and 2+6 rH1N2 viruses (Figure 3-16).

**Figure 3-15 Microscopic lung lesions from direct-infection pigs at 5-dpi.**

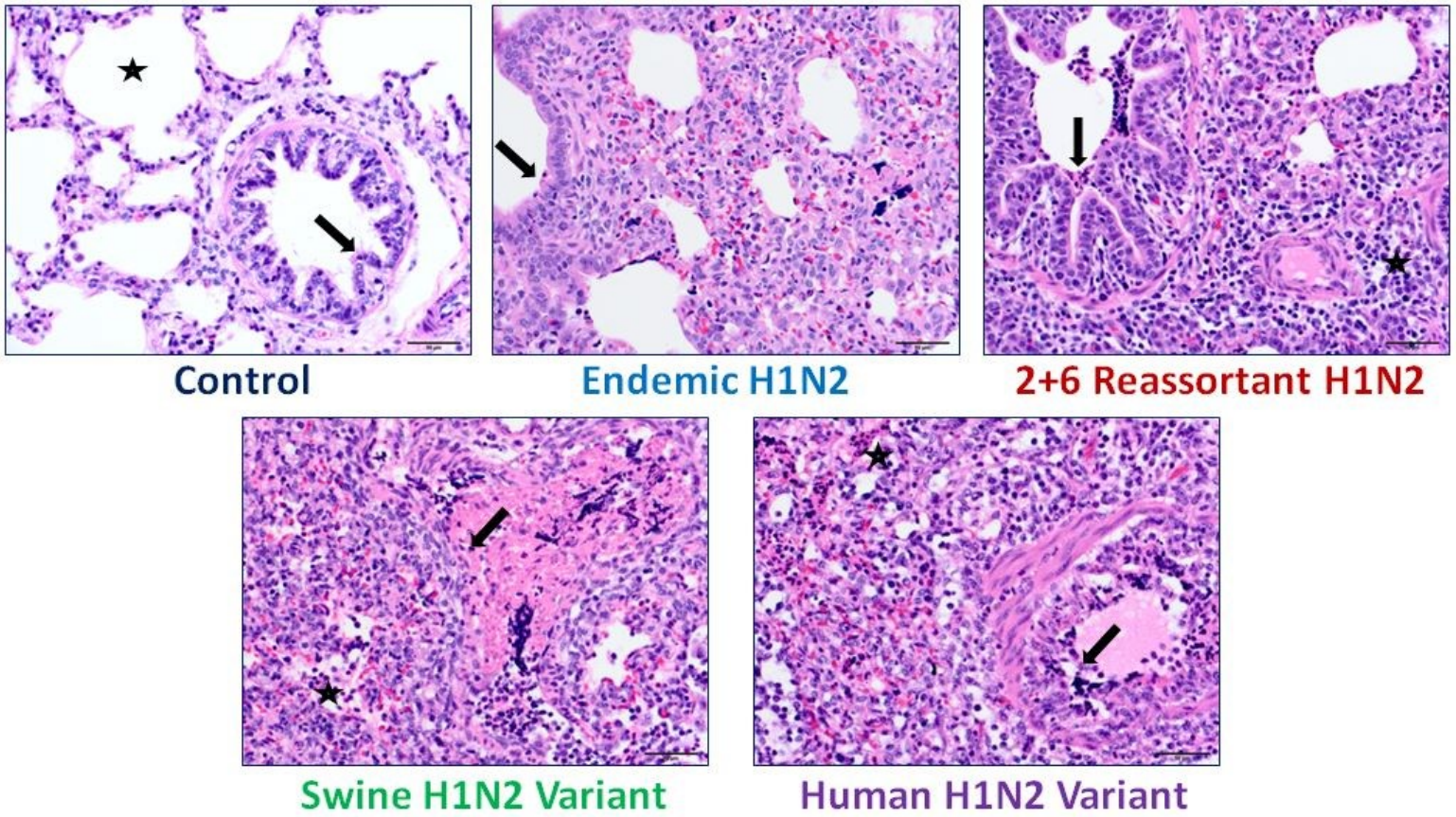


Figure 3-15. Microscopic lung sections from direct-infection pigs infected at 5-dpi. The bronchioles are lined by normal cuboidal epithelium (arrow) and the alveoli are clear (asterisk) in the control group. In eH1N2, there was minimal to complete lack of bronchiolar epithelial degeneration and necrosis. A mild alveolar epithelial degeneration and necrosis was observed with plugging of alveolar lumen with cellular debris (asterisk). In 2+6 rH1N2, there was minimal to mild bronchiolar and alveolar epithelial degeneration and necrosis (arrows). There was mild interstitial pneumonia characterized by small numbers of lymphocytes infiltrating the alveolar septa and perivascular areas (asterisk). In contrast, swH1N2v caused severe bronchiolar epithelial necrosis (arrow). Moderate numbers of neutrophils were present in the bronchiolar and alveolar lumen (asterisk). ThehuH1N2v also caused moderate to severe bronchiolar epithelial degeneration and necrosis (arrow) with infiltration of neutrophils into the alveolar septa and alveolar and bronchiolar lumen (asterisk). Scale bar is 50 μm in all the photographs.

**Figure 3-16 Microscopic lung lesion scores from direct-infection pigs at 5- and 7-dpi.**

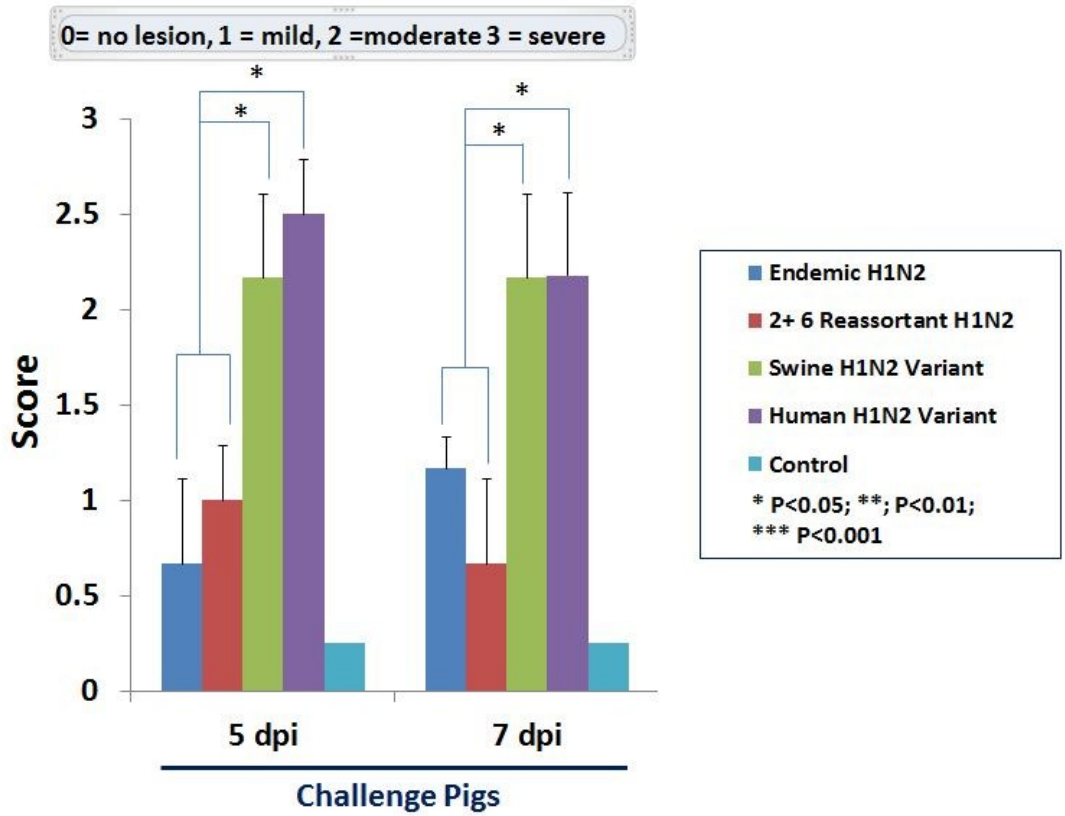


Figure 3-16 Microscopic lung lesion scores from direct-infection pigs at 5- and 7-days post infection. Lung lobes were collected and fixed in 10% formalin during necropsy before staining with Haemotoxylin and eosin for histopathological examination. Anti-influenza A monoclonal antibody against NP was used for immunohistochemistry staining. Lung samples were examined blindly by a veterinary pathologist and scored on a scale of 0-3 based on severity of bronchial epithelial injury.

**Table 3-5 Histopathology scoring guide.**

| Lesions | 0=no lesion | 1= mild lesion | 2=moderate lesion | 3=severe lesion |
|---|---|---|---|---|
| **Bronchiolar epithelial necrosis** | | Segmental epithelial necrosis | Diffuse epithelial necrosis in 2-4 bronchioles in a slide | Diffuse epithelial necrosis in more than 5 bronchioles |
| **Neutrophilic bronchiolitis/alveolitis** | | Mild inflammation | Moderate inflammation | Severe inflammation, completely obliterating the bronchiole |
| **Bronchiolar submucosal inflammation** | | Mild infiltration of lymphocytes/ neutrophils | Moderate inflammation | Severe inflammation |
| **Peri- Bronchiolar lymphoid inflammation** | | Mild infiltration | Moderate infiltration | Severe infiltration |
| **Interstitial pneumonia** | | Mild thickening of alveolar septa | Moderate thickening of septa | Marked thickening of septa |
| **Bronchiolar epithelial atypia/hyperplasia** | | Segmental epithelial atypia and mild hyperplasia | Diffuse and moderate epithelial hyperplasia and moderate atypia | Diffuse and marked epithelial hyperplasia with atypia |
| **Interlobular edema** | | Mild | Moderate | Severe |
| **Hemorrhage** | | Mild | Moderate | Severe |

Table 3-5 Scoring guide used for histopathological examination of pigs' lungs.

### *Viral Replication in Pigs' Lungs*

All viruses replicated efficiently in direct-infection pigs' lungs as measured by viral titers from BALF samples. Pigs inoculated with eH1N2 had an average viral titer of 4.50 ± 1.22 detected from pigs necropsied at 5-dpi while no virus was detected at 7-dpi. Pigs inoculated with 2+6 rH1N2 had an average viral titer of 4.97 ± 0.37 detected from pigs necropsied at 5-dpi while no virus was detected at 7-dpi. Pigs inoculated with swH1N2v had an average viral titer of 6.23 ± 0.15 detected from pigs necropsied at 5-dpi while no virus was detected at 7-dpi. Pigs inoculated with huH1N2v had an average viral titer of 4.70 ± 0.00 detected from 2/3 pigs necropsied at 5-dpi while no virus was detected at 7-dpi. Overall, viral titers from BALF samples of pigs inoculated with the swH1N2v virus were significantly higher than those from pigs inoculated with the 2+6 rH1N2 and huH1N2v viruses (Figure 3-17).

**Figure 3-17 Viral titers from BALF samples of direct-infection pigs at 5- and 7-dpi.**

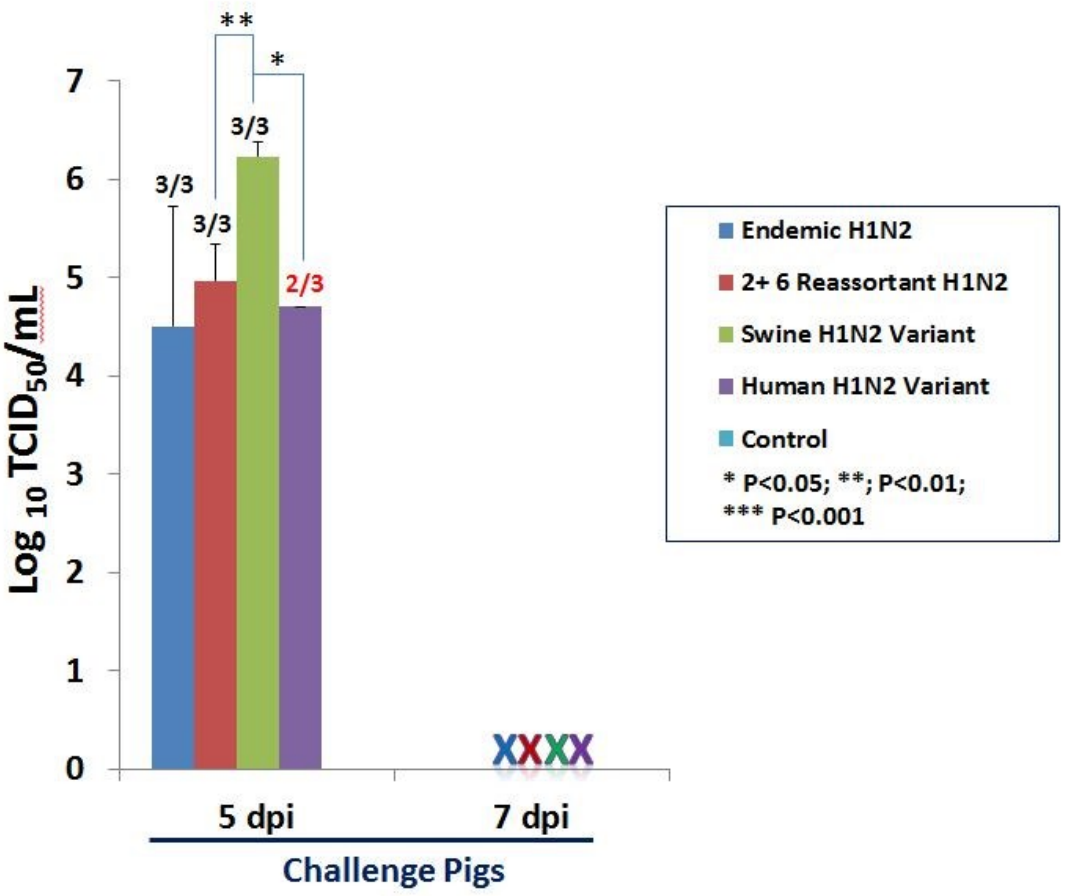


Figure 3-17 Viral titers from BALF samples of direct-infection pigs. Samples were titrated in quadruplicate using 96-well plates of confluent MDCK cells. Serial dilutions for each sample were completed from $10^{-1}$ to $10^{-8}$ using infecting MEM and then 100 µl of each dilution was added to MEM-washed confluent MDCK cells in each well. Cells were incubated at 37ºC with 5.0% $CO_2$ and evaluated for CPE at 24- and 48-hpi. At 72-hpi, plates were fixed in methanol and immunocytochemically stained with a monoclonal antibody specific to influenza NP. A p-value of $< 0.05$ was considered statistically significant.

### *Viral Shedding from Pigs*

All viruses shed efficiently from nasal cavities of direct-infection pigs as detected by viral titers in nasal swab samples. In pigs inoculated with eH1N2, an average viral titer of 3.93 ± 0.14 was detected in 4/6 pigs at 3-dpi. By 5-dpi, an average viral titer of 3.82 ± 0.30 was detected in 6/6 pigs; however, no virus was detected at 7-dpi. In pigs inoculated with 2+6 rH1N2, an average viral titer of 4.20 ± 0.33 was detected in 5/6 pigs at 3-dpi. By 5-dpi, an average viral titer of 4.80 ± 0.35 was detected in 6/6 pigs. At 7-dpi, an average viral titer of 2.95 ± 0.25 was detected in 2/3 pigs. In pigs inoculated with swH1N2v, an average viral titer of 5.27 ± 0.29 was detected in 6/6 pigs at 3 dpi. By 5-dpi, an average viral titers of 4.73 ± 0.37 was detected in 6/6 pigs; however, no virus was detected at 7-dpi. In pigs inoculated with huH1N2v, an average viral titer of 3.24 ± 0.15 was detected in 5/6 pigs at 3-dpi. By 5-dpi, an average viral titer of 4.03 ± 0.22 was detected in 6/6 pigs; however, no virus was detected at 7-dpi (Figure 3-18).

**Figure 3-18 Viral titers from nasal swab samples of direct-infection pigs at 3-, 5-, and 7-dpi.**

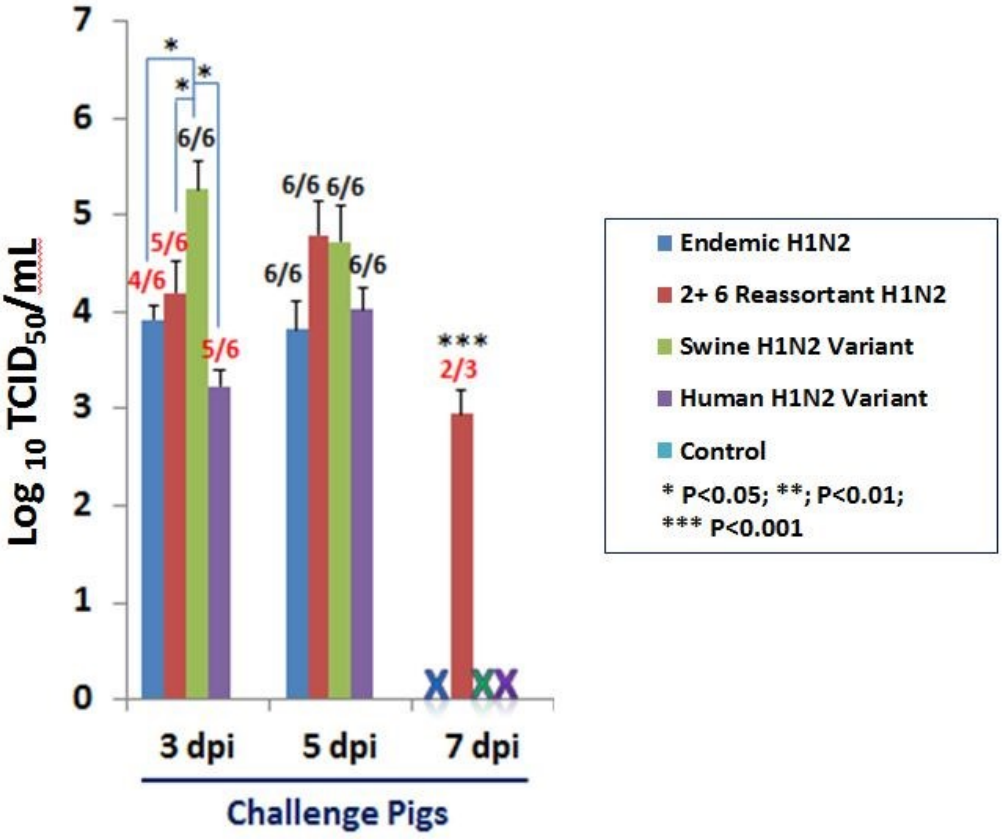


Figure 3-18 Viral titers from nasal swab samples of direct-infection pigs at 3-, 5-, and 7-dpi. Samples were titrated in quadruplicate using 96-well plates of confluent MDCK cells. Serial dilutions for each sample were completed from $10^{-1}$ to $10^{-8}$ using infecting MEM and then 100 µl of each dilution was added to MEM-washed confluent MDCK cells in each well. Cells were incubated at 37°C with 5.0% $CO_2$ and evaluated for CPE at 24- and 48-hpi. At 72-hpi, plates were fixed in methanol and immunocytochemically stained with a monoclonal antibody specific to influenza NP. A p-value of $< 0.05$ was considered statistically significant.

## Viral Transmission to Contact-Exposure Pigs

### *Clinical Signs*

All pigs were observed daily for clinical signs of disease. Although pigs did not show obvious signs of disease (respiratory distress, lethargy, anorexia), several pigs showed fever defined as a temperatures of 104°C and higher. This fever lasted 1-2 days between 2- and 5-dpc and included two pigs from the eH1N2 group, three pigs from the 2+6 rH1N2 group, one pig from the swH1N2v group, and two pigs from the huH1N2v group.

### *Macroscopic Lung Lesions*

Several contact-exposure pigs showed macroscopic lung lesions indicative of swine influenza (Figure 3-19) and lungs were assigned scores as a "percent of the lung with lesions" by a single, experienced research veterinarian. Only 2/4 pigs comingled with the eH1N2 direct-infection pigs showed lesions on 4.69% ± 0.82% of lungs at 5-dpc. Also, only 3/4 pigs comingled with 2+6 rH1N2 direct-infection pigs showed lesions on 3.22% ± 0.36% of lungs at 5-dpc. In contrast, 4/4 animals pigs comingled with swH1N2v direct-infection pigs showed lesions on 7.44% ± 1.98% of lungs at 5-dpc. Similarly, 4/4 pigs comingled with huH1N2v direct-infection pigs showed lesions on 4.29% ± 0.457% of lungs at 5-dpc. Overall, 100% of pigs exposed to the swH1N2v and huH1N2v direct-infection pigs showed lesions compared with 50% and 75% of pigs exposed to the eH1N2 and 2+6 rH1N2 direct-infection pigs, respectively.

**Figure 3-19 Macroscopic lung lesions from contact-exposure pigs at 5-dpc.**

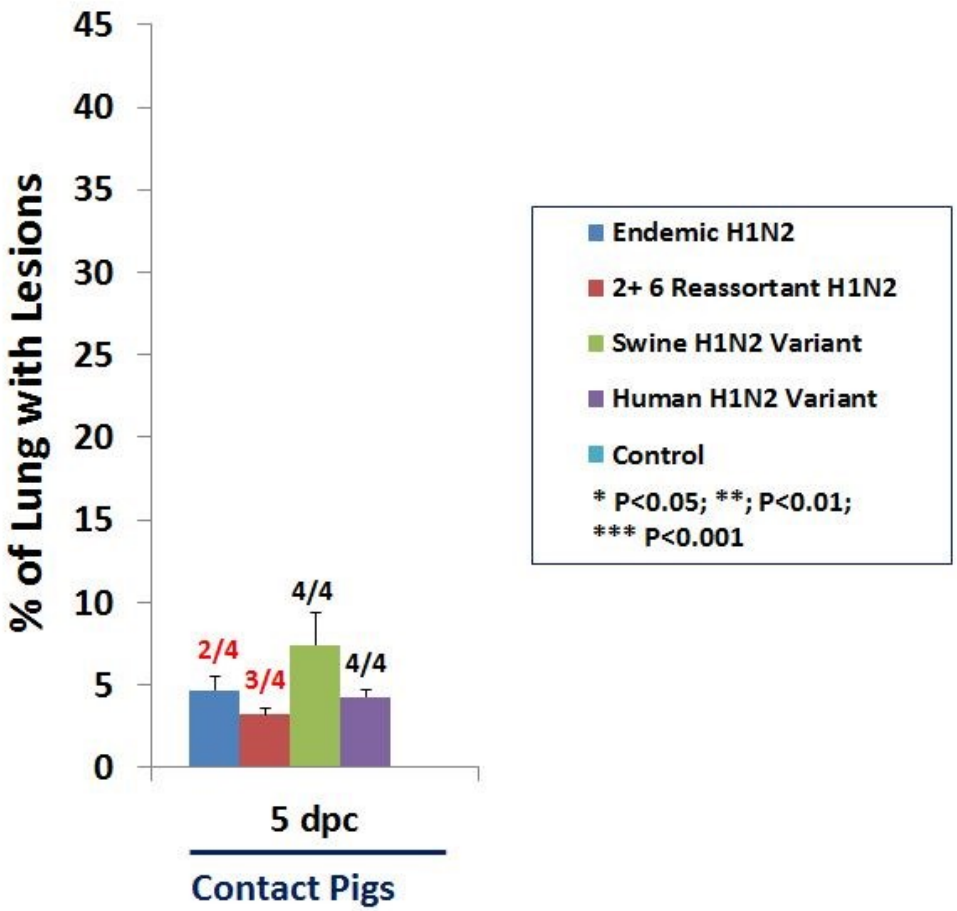


Figure 3-19 Macroscopic lung lesions from contact-exposure pigs at 5-dpc. Lungs were assigned scores by a single, experienced research veterinarian as a "percent of lungs with lesions." A p-value < 0.05 was considered statistically significant.

### *Microscopic Lung Lesions*

All contact-exposure pigs showed microscopic lung lesions indicative of swine influenza as described by a veterinary pathologist using histopathology. For histopathology, scores were assigned to the lung tissues on a scale of 0-3 with 0 being no lesion, 1 being mild lesions, 2 being moderate lesions, and 3 being severe lesion. Pigs comingled with eH1N2 direct-infection pigs showed minimal to complete lack of bronchiolar epithelial degeneration and necrosis. Lung lesions for this group were scored as 0.05 ± 0.14 at 5-dpc. Pigs comingled with 2+6 rH1N2 direct-infection pigs showed minimal to complete lack of bronchiolar epithelial degeneration and necrosis. Lung lesions for this group were scored as 0.25 ± 0.20 at 5-dpc. Pigs comingled with swH1N2v direct-infection pigs showed mild to moderate bronchiolar epithelial necrosis and moderate numbers of neutrophils were present in the bronchiolar and alveolar lumen. Lung lesions for this group were scored as 1.15 ± 0.13 at 5-dpc. Pigs comingled with huH1N2v direct-infection pigs showed mild to moderate bronchiolar epithelial degeneration and necrosis. Lung lesions for this group were scored as 0.89 ± 0.13 at 5-dpc. Overall, pigs exposed to the swH1N2v and huH1N2v direct-infection pigs showed significantly more severe lesions when compared to the pigs exposed to the 2+6 rH1N2 direct-infection pigs (Figure 3-20).

**Figure 3-20 Microscopic lung lesions from contact-exposure pigs at 5-dpc.**

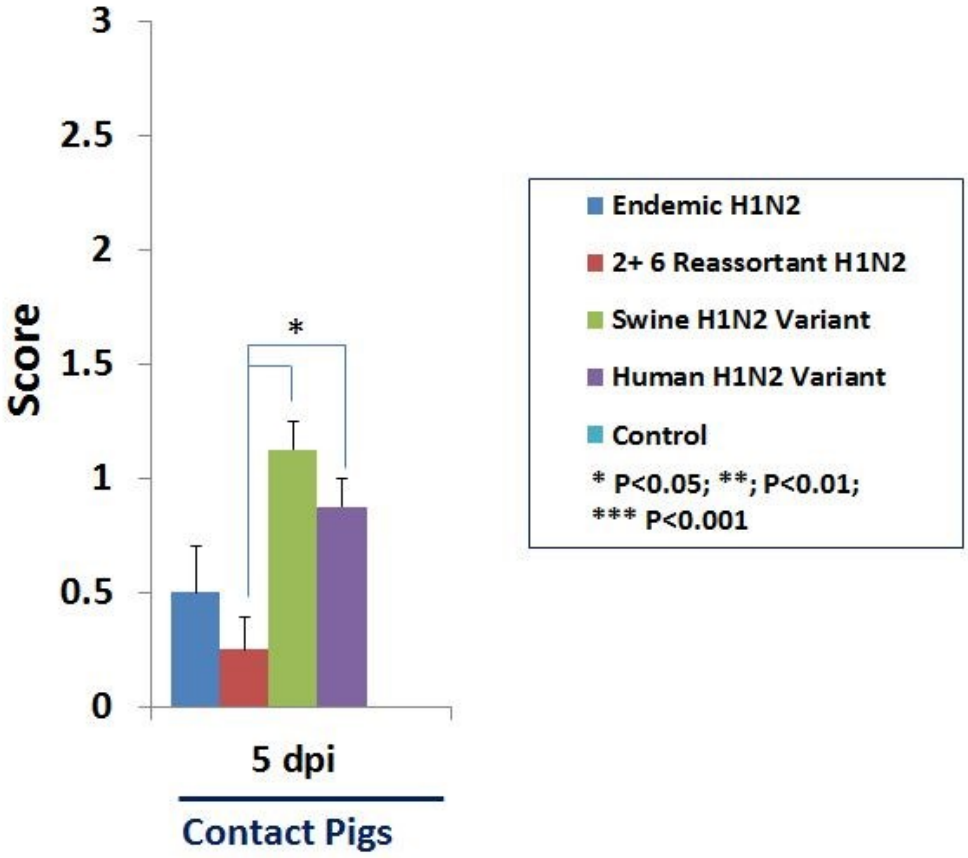


Figure 3-20 Microscopic lung lesions from contact-exposure pigs at 5-dpc. Lung lobes were collected and fixed in 10% formalin during necropsy before staining with Haemotoxylin and eosin for histopathological examination. Anti-influenza A monoclonal antibody against NP was used for immunohistochemistry staining. Lung samples were examined blindly by a veterinary pathologist and scored on a scale of 0-3 based on severity of bronchial epithelial injury.

### *Viral Replication in Pigs' Lungs*

All viruses replicated efficiently in contact-exposure pigs' lungs as measured by viral titers from BALF samples. Pigs comingled with eH1N2 direct-infection pigs had an average viral titer of 4.35 ± 0.27 detected from pigs necropsied at 5-dpc. Pigs comingled with 2+6 rH1N2 direct-infection pigs had an average viral titer of 4.83 ± 0.38 detected from 3/4 pigs necropsied at 5-dpc. Pigs comingled with swH1N2vdirect-infection pigs had an average viral titer of 5.90 ± 0.06 detected from pigs necropsied at 5-dpc. Pigs comingled with huH1N2v direct-infection pigs had an average viral titer of 4.13 ± 0.56 detected from pigs necropsied at 5-dpc. Overall, pigs exposed to the swH1N2v direct-infection pigs had significantly higher viral titers in BALF compared with pigs exposed to the eH1N2 and huH1N2v direct-infection pigs (Figure 3-21).

**Figure 3-21 Viral titers from BALF samples of contact-exposure pigs at 5-dpc.**

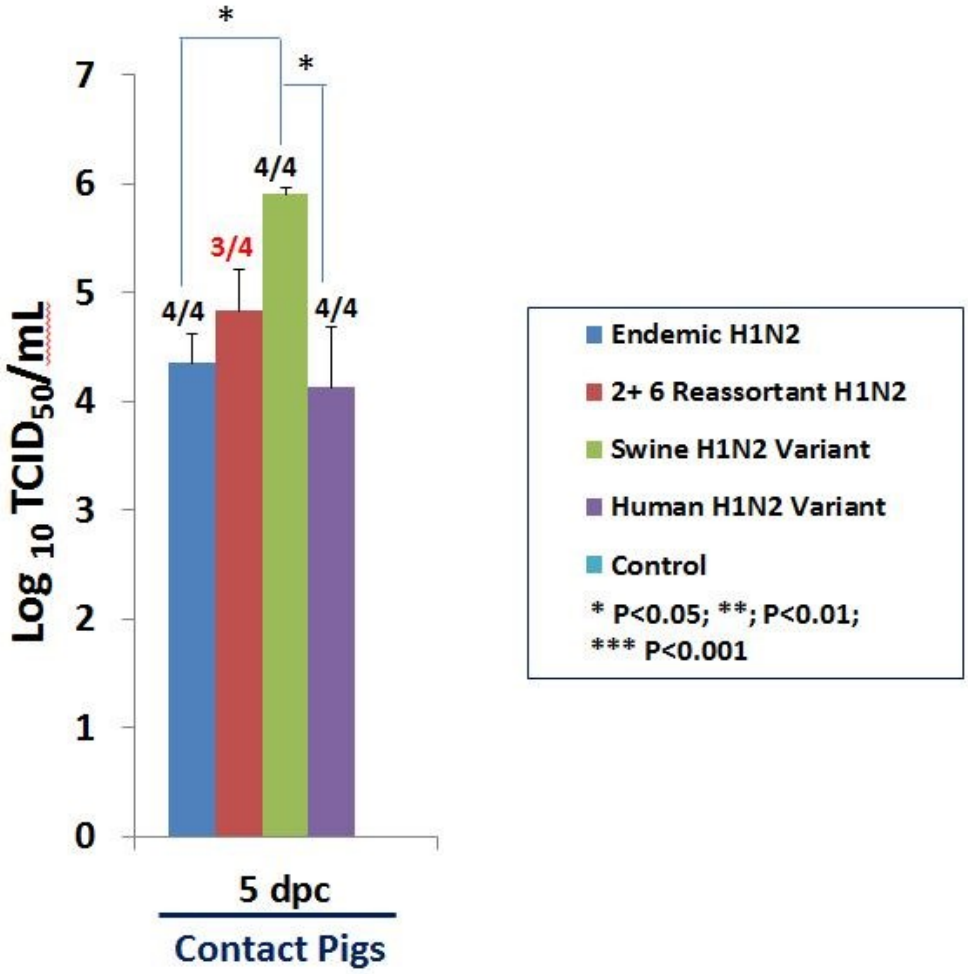


Figure 3-21 Viral titers from BALF samples of contact-exposure pigs at 5-dpc. Samples were titrated in quadruplicate using 96-well plates of confluent MDCK cells. Serial dilutions for each sample were completed from $10^{-1}$ to $10^{-8}$ using infecting MEM and then 100 µl of each dilution was added to MEM-washed confluent MDCK cells in each well. Cells were incubated at 37°C with 5.0% $CO_2$ and evaluated for CPE at 24- and 48-hpi. At 72-hpi, plates were fixed in methanol and immunocytochemically stained with a monoclonal antibody specific to influenza NP. A p-value of $< 0.05$ was considered statistically significant.

### *Viral Shedding from Pigs*

Viruses shed efficiently from nasal cavities of contact-exposure pigs comingled with eH1N2-, 2+6 rH1N2-, and swH1N2v-direct-infection pigs as detected by viral titers in nasal swab samples. In pigs comingled with eH1N2 direct-infection pigs, viral titers of 5.98 ± 0.42 was detected in 4/4 pigs at 3-dpc while viral titers of 3.00 ± 0.24 was detected in 4/4 pigs at 5-dpc. In pigs comingled with 2+6 rH1N2 direct-infection pigs, viral titers of 4.58 ± 0.77 was detected in 4/4 pigs at 3-dpc while viral titers of 3.08 ± 0.22 was detected in 4/4 pigs at 5-dpc. In pigs comingled with swH1N2v direct-infection pigs, a viral titer of 3.70 was detected from 1/4 pigs as early as 1-dpc. An average viral titer of 5.75 ± 0.17 was detected in 4/4 pigs at 3-dpc while an average viral titer of 3.88 ± 0.55 was detected in 4/4 pigs at 5-dpc. However, no virus was detected in nasal swab samples of pigs comingled with huH1N2v direct-infection pigs (Figure 3-22).

**Figure 3-22 Viral titers from nasal swab samples of contact-exposure pigs at 1-, 3-, and 5-dpc.**

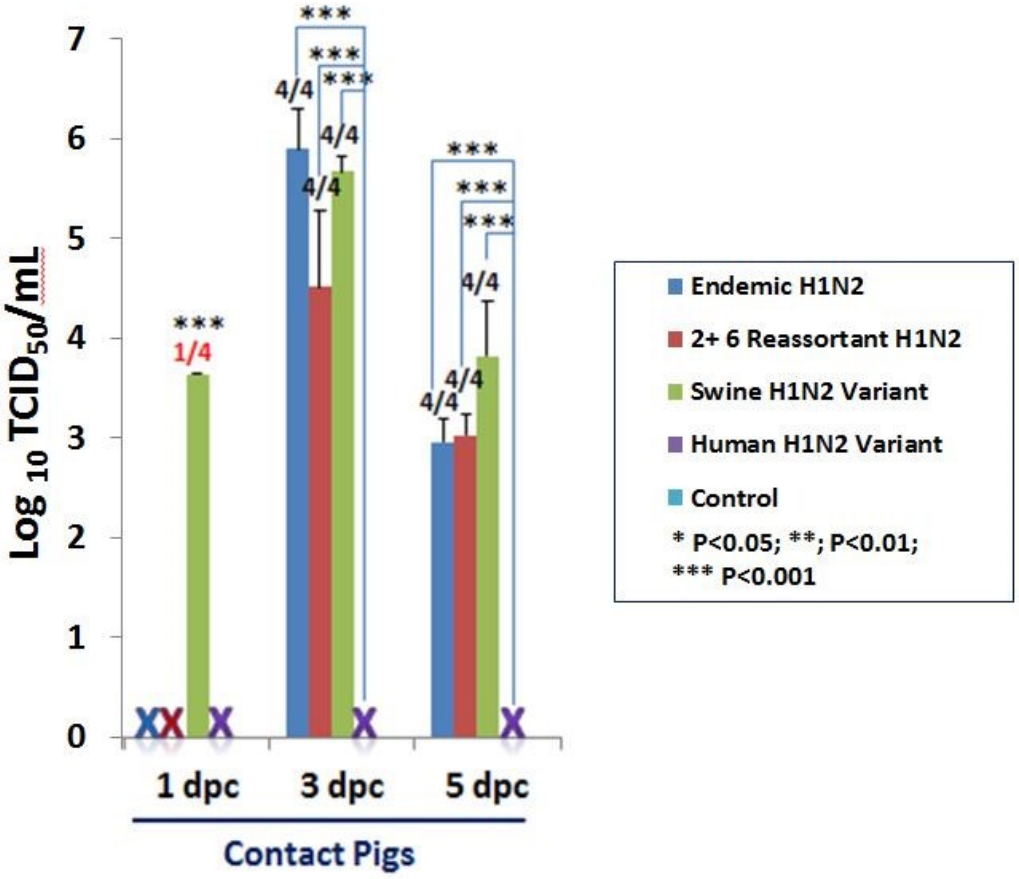


Figure 3-22 Viral titers from nasal swab samples of contact-exposure pigs at 3- and 5-dpc. Samples were titrated in quadruplicate using 96-well plates of confluent MDCK cells. Serial dilutions for each sample were completed from $10^{-1}$ to $10^{-8}$ using infecting MEM and then 100 µl of each dilution was added to MEM-washed confluent MDCK cells in each well. Cells were incubated at 37°C with 5.0% $CO_2$ and evaluated for CPE at 24- and 48-hpi. At 72-hpi, plates were fixed in methanol and immunocytochemically stained with a monoclonal antibody specific to influenza NP. A p-value of $< 0.05$ was considered statistically significant.

### *One-step real-time RT-PCR for pH1N1 M gene in swH1N2v and huH1N2v contact-exposure pigs*

Nasal swabs collected from contact-exposure pigs in the swH1N2v and huH1N2v groups at 1-dpc were all found to be negative with Ct values greater than the cutoff value of '35'. At 5-dpc, 4/4 (100%) of the pigs exposed to the swH1N2v virus were positive with Ct values ranging from 26.45-34.59 ($\bar{x}$ = 31.67) while only 3/4 (75%) of the pigs exposed to the huH1N2v virus were positive with Ct values ranging from 31.56-33.15 9 ($\bar{x}$ = 32.26, excluding the negative sample). By 5-dpc, 4/4 (100%) of the pigs exposed to the swH1N2v virus were positive with Ct values ranging from 22.34-26.29 ($\bar{x}$ = 25.44). Similarly, 4/4 (100%) of the pigs exposed to the huH1N2v virus were positive, although with significantly higher Ct values ranging from 31.72-33.50 ($\bar{x}$ = 33.01) (Table 3-6).

**Table 3-6 Ct values for one-step real-time RT-PCR of nasal swab samples from swH1N2v and huH1N2v contact-exposure pigs.**

| Sample ID | 1-dpc | 3-dpc | 5-dpc |
|---|---|---|---|
| **Pig #33 – swH1N2v** | 35.07 | 26.45 | 25.90 |
| **Pig #34 – swH1N2v** | 37.88 | 32.68 | 24.94 |
| **Pig #35 – swH1N2v** | 36.71 | 32.96 | 24.62 |
| **Pig #36 – swH1N2v** | 38.54 | 34.59 | 26.29 |
| **Pig #37 – huH1N2v** | 36.22 | 31.56 | 31.72 |
| **Pig #38 – huH1N2v** | 37.07 | 33.15 | 33.31 |
| **Pig #39 – huH1N2v** | 35.86 | 32.09 | 33.50 |
| **Pig #40 – huH1N2v** | 38.04 | 0.00 | 33.60 |
| **Positive control** | 22.39 | 22.56 | 22.34 |
| **Positive control** | 21.99 | 23.06 | 21.09 |
| **Negative-control Pig** | 37.75 | 36.87 | 35.73 |
| **Negative-control Pig** | 39.16 | 37.59 | 35.01 |

Table 3-6 Ct values for each nasal swab sample from pigs exposed to swH1N2v and huH1N2v at 1-, 3-, and 5-dpc. Cutoff for this assay was set at 35 threshold cycles (Ct = 35). A total of 8 µl from a known pH1N1 virus was used as a positive control and 8 µl of sample template from a negative-control pig was used as a negative control.

# Chapter 4 - Discussion and Conclusions

The ecology of North American SIVs has become increasingly diversified in recent years. Prior to 1998, most cases of swine influenza in the US was caused by an H1N1 virus known as "classical swine" [122]. In 1998 a novel TR H3N2 virus emerged in North American swine herds. Reassortment of this TR H3N2 virus with H1N1 viruses resulted in the generation of another subtype, the H1N2 virus [116]. Introduction of the 2009 pH1N1 into swine herds has further diversified the ecology and led to the generation of numerous reassortant SIVs, all of which contain the pH1N1 M gene based on surveillance.

From December 2005 – November 2010, there were more than 300 human infections with SIVs in the US including H1N1v, H1N2v, and H3N2v viruses [151]. According to the CDC, in 2011 an H3N2v virus with pH1N1 M gene and the remaining genes from endemic H3N2 SIVs emerged and caused 12 human infections (mostly in children) in Indiana, Maine, Pennsylvania, Iowa, Utah and West Virginia. Also in 2011, an H1N2v virus with pH1N1 M gene infected a child at a state fair. There were 12 more confirmed cases of humans infected with H3N2v viruses in 2011 and an alarming 309 cases in 2012 [152,153]. Fortunately, to date these human infections with variant viruses have shown little to no human-to-human transmission.

Based on our own passive surveillance of Kansas swine herds from 2010-2013, we have identified 18 H1N1 SIVs, 22 H3N2 SIVs, and 25 H1N2 SIVs. Preliminary sequencing of the HA, NA, and M genes of all viruses revealed a number of reassortant viruses with genes from pH1N1 including three H1N1 viruses, 11 H3N2 viruses, and 16 H1N2 viruses. Full genome sequencing revealed that a number of these reassortant

viruses possess only the M gene from pH1N1 including 2/3 of the H1N1 viruses and 12/16 of the H1N2 viruses. This suggests that this may be a favorable constellation and may become established in US swine herds.

In this study, three swine isolates and one human isolate were characterized *in vitro* and identified as H1N2 SIVs: two variant viruses (one swine and one human) with only M gene from pH1N1, one reassortant virus with PB2, PB1, PA, NP, M, and NS genes from pH1N1, and one endemic virus with no genes from pH1N1. The results show that all viruses replicated efficiently *in vitro*, growing to significant titers in porcine, human, and canine cell lines. Notably, the reassortant and variant viruses with genes from pH1N1 grew to significantly higher titers than the endemic virus at various time points in all three cell lines. Additionally, both variant viruses formed significantly larger plaques than the reassortant and endemic viruses. Taken together, one should expect the viruses with pH1N1 M gene to replicate more efficiently in pigs and the variant viruses to be more pathogenic.

Although no obvious signs of influenza were observed in pigs, all viruses caused a similar degree of fever in both direct-infection and contact-exposure pigs. Both variant viruses caused significantly more severe macroscopic and microscopic lung lesions in direct-infection animals than the other two viruses. This trend was also observed in the microscopic lung lesions of contact-exposure animals. While the severity of macroscopic lung lesions was less pronounced in contact-exposure animals, it should be noted that both variant viruses caused lesions in 100% of these animals compared with the endemic and reassortant viruses which caused lesions in only 50 – 75% of these animals, respectively.

The swine variant virus showed enhanced replication and pig-to-pig transmissibility compared to the other three viruses. BALF samples show that all viruses are able to replicate efficiently in the lower respiratory tract where alpha 2, 3 N-linked sialic acids are present; however, the swine variant grew to higher titers in BALF samples of both direct-infection and contact-exposure pigs. Nasal swab samples of direct-infection pigs show that all viruses are capable of replicating in the upper respiratory tracts of pigs where alpha 2, 6 N-linked sialic acids are present. The swine variant virus seems able to replicate more efficiently than the other three viruses as it was shed in highest titers and was the only group in which 100% of direct-infection animals were shedding at 3-dpi. In contrast, the human variant was only detected in BALF samples from 67% of direct-infection pigs at 5-dpi. Additionally, it was only detected at low titers from the nasal swabs of contact-exposure pigs using one-step real-time RT-PCR specific for the pH1N1 M gene suggesting that it may be incapable of sustained pig-to-pig transmission.

The data from this pig study highlights differences from a previous study in pigs involving H3N2v viruses published by Kim, Seong-Hee et. al. [163]. In that study, fever was accompanied by nasal discharge from all pigs which progressed from mucoserous to mucopurulent while no nasal discharge was observed in the current study. They also found higher copies of RNA in contact-exposure pigs compared with direct-infection pigs while no significant difference was observed in this study. Moreover, their study found high RNA copies in BALF samples for as long as 14 dpi (our study concluded at 7 dpi) suggesting that the virus may not have been cleared; however, in the current study, all virus appeared to have been cleared from the lungs by 7 dpi. Moreover, the viruses in that

study only caused pathogenesis similar to that of typical SIVs while the variant viruses in the current study were far more pathogenic than typical SIVs and caused significantly more severe lung lesions than the other two viruses. This demonstrates differences in behavior between the H3N2v and H1N2v viruses, further supporting the importance of studying the H1N2v viruses.

The apparent increased transmissibility and pathogenicity of the swH1N2v is most likely due to a number of factors. Given the roles of HA and NA with virus attachment, the immune response, and viral budding, it is interesting that the HA and NA molecules of swH1N2v varied significantly from the other three viruses. As aforementioned, there are 23 unique amino acids in the HA molecule of swH1N2v compared with the other three viruses. Although the PIRs of the HA molecules are highly similar between the viruses, swH1N2v possessed one difference from the other three viruses, P187S. Similarly, the putative antigenic sites of the HA molecules were similar with one exception in swH1N2v, N183D. It is possible that either of these differences enhances the virus's ability to attach to host cells. In contrast, the HA molecule of huH1N2v possessed only two unique amino acid differences from the other three viruses: E164V and K184R. The NA molecule of swH1N2v possesses 21 unique amino acids compared to the other three viruses while huH1N2v possesses only three: D42N, P126T, and T267K. To our knowledge, changes in these amino acids of the HA and NA molecules have not been previously published and may be targets for transmissibility studies.

As expected, few amino acid differences were identified between the M1 and M2 molecules of the viruses. The huH1N2v possessed one unique amino acid in its M1

molecule, S30N, while none were identified in swH1N2v. Interestingly, amino acid residue 30 is thought to play a role in determining morphology of the virus and its possible that a change in this amino acid can alter the morphology from spherical to more filamentous, affecting its transmissibility. Analysis of the M2 molecules revealed only one unique amino acid difference for each variant virus: D24N in swH1N2v and P25S in huH1N2v. These sites have not been investigated and may serve a functional importance in the transmissibility and replication of these viruses.

Analysis of the NS1 molecules revealed only one unique amino acid to swH1N2v, N139S, while huH1N2v possesses five unique amino acids: G45R, G51S, V84I, M124I, and V178I. Given the role of NS1 as a host antiviral antagonist, it is possible that these changes in huH1N2v affect the virus's ability to overcome the host antiviral response. This might explain the decreased titers observed in BALF and nasal swab samples collected. It might also explain why BALF samples from only 2/3 pigs collected at 5-dpi and nasal swab samples from only 5/6 animals collected at 3-dpi were positive for virus. In contrast, only one unique amino acid is present in the NS2 of huH1N2v compared with the other three viruses: I19T. The NP molecules of both variant viruses were highly homologous with only one unique amino acid residue identified in each: K400R in swH1N2v and N125S in huH1N2v.

Our results show that the swH1N2v virus is more pathogenic and transmissible in pigs when compared to the endemic and novel reassortant H1N2 SIVs. Furthermore, our surveillance data revealed that the H1N2v is the predominant genotype of H1N2 SIVs circulating in Kansas swine herds suggesting that they might become established in pigs causing more challenges for the pig industry. Taken together, these results warrant the

continued surveillance and analysis of SIVs. In Kansas alone, there are more than 1,500 hog farms with 3.1 million hogs that account for 95% of the state's pork supply and 2.7% of the nation's pork supply (www.nationalhogfarmer.com). Should a more virulent variant virus emerge it could result in disaster for local hog farmers and the nation's food supply in addition to posing a major public health concern.

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