CONTROL AND CHARACTERIZATION OF INFLUENZA A VIRUSES IN SWINE

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Susan Elisabeth Detmer

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Sagar Goyal, Co-advisor
Marie Gramer, Co-advisor

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DEDICATION

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GENERAL INTRODUCTION

Between 1958 and 2005, there were 37 human cases of zoonotic swine-origin influenza A virus (IAV) infection reported (Myers et al 2007; Van Reeth 2007). A majority of these infections were with classical swine H1N1 viruses and these 37 cases did not include the Fort Dix cases in 1976 that resulted in 1 death and up to 230 soldiers infected (Myers et al. 2007; Van Reeth 2007). However, a recent report of pig to human transmission was at an Ohio County Fair in 2007 (Vincent et al. 2009b). The sequence analysis of the HA gene segment of the IAVs isolated from the humans and pigs in this case revealed that it was a strain that was currently circulating in the U.S. pig population. The internal genes of this isolate were determined to be of the triple-reassortant swine influenza lineage, including a conserved avian PB2 gene sequence (Vincent et al. 2009b).

On June 11, 2009 the first influenza pandemic in 41 years was declared by the World Health Organization. This virus was like no virus previously seen in the human population with gene segments from both Eurasian and North American swine viruses. The 2009 pandemic H1N1 virus was called a "quadruple-reassortant" virus because it is composed of neuraminidase (NA) and matrix (M) gene segments from Eurasian swine influenza viruses combined with triple-reassortant proteins of North American swine influenza viruses (human-origin polymerase B1 (PB1), avian-origin polymerase B2 (PB2) and polymerase A (PA), and classical swine-origin hemagglutinin (HA), nucleoprotein (NP) and non-structural (NS) (Garten et al. 2009; Smith et al. 2009). The evolutionary analysis of the 2009 pandemic H1N1 shows that the generation of this strain was not likely a recent event. In fact, in order to facilitate human-to-human spread, it probably adapted to the human host through secondary reassortments in humans (Ding et al. 2009). However, the original source of this virus has not yet been determined.

The emergence of the 2009 pandemic H1N1 virus and scattered reports of human infections with swine-origin isolates underscores the importance of fully understanding the genetic, antigenic, and pathogenic characteristics of influenza A viruses so that we may limit the introduction of novel IAVs to the swine population and monitor for newly emerging and evolving viruses. In order to improve our understanding of IAVs in swine, the goal of this dissertation is to address the ability of genetic characterization to predict variations in virus phenotype, such as viral binding and antigenicity.

Understanding the genetic, antigenic and pathogenic features of viruses is important to prevent introduction of human and avian viruses into swine herds and the potential spillback of those viruses to the human population, as wells as preventing the sustained transmission of IAVs within an endemically infected herd. The control of influenza viruses in pig populations continues to be dynamic and complex, and is reliant on a number of factors. Two of these factors include appropriate selection and application of (1) diagnostic tests and (2) vaccines.

Routine surveillance for influenza viruses at the farm level, either syndromic or active surveillance, is often accomplished using real time RT-PCR tests on nasal swabs from live pigs and lung tissue samples from post-mortem examinations. Easily collected sample methods, such as oral fluids, could provide additional viruses for characterization of IAVs in swine. Oral fluids have been used extensively for diagnostic tests in human medicine and are now being applied in swine herds for detecting pathogens and antibodies against the pathogens (Prickett et al. 2010). As part of this dissertation, porcine oral fluids were validated as a viable sample collection method for routine RT-PCR and virus isolation tests (chapter 2). Another important control measure for influenza viruses in pigs continues to be vaccines. In order to assure continued efficacy of vaccines against currently circulating strains of virus, vaccine challenge trials are performed. In this dissertation, the efficacy of a commercial vaccine was examined against challenge with a contemporary field isolate (chapter 3).

To address the genetic and phenotypic characterization of influenza A viruses from swine, two sets of viruses were selected from the influenza database at the University of Minnesota, Veterinary Diagnostic Laboratory and sequenced. The first set was isolated from a group of endemically infected farms treated by the same veterinarian from 2005 to 2009. The selected viruses were either used to produce autogenous vaccines or they were the epizootic viruses found during outbreaks in the vaccinated herds (chapter 4). The second set of viruses were isolated from nursery pigs in one endemically infected multi-site swine production system (farm M) from 2007 to 2009 and either contained a distinct two amino acid insertion or were presumptive ancestral viruses without the insertion (chapter 5). The viruses from farm M were further characterized

along with representative viruses that have been previously studied *in vivo* using a new technique called virus histochemistry to examine the patterns of virus attachment in the respiratory tract (chapter 6).

For the purposes of this dissertation IAVs were classified as virulent increased virulence have some of the following clinical/case presentations: (a) morbidity approaching 90% and mortality approaching 10%, (b) sudden, unexpected deaths occurring early in the disease outbreak, (c) gross lesions that are not typical of swine influenza including profuse hemorrhage and/or edema, and (d) sufficient health and production records along with laboratory results that indicate that a highly virulent influenza virus is involved. The characteristics of highly virulent influenza viruses, such as A/swine/KS/77778/2007 H1N1 and A/swine/OH/511445/2007 H1N1, have been previously described in the literature (Ma et al. 2010; Vincent et al. 2009b). This classification was not related to the criteria for classification of avian viruses as having high or low pathogenicity.

CHAPTER 1

LITERATURE REVIEW

A portion of the material in this chapter has been submitted for publication:
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1.1 Influenza virus

Influenza in swine is an acute respiratory disease caused by influenza A (IAV) viruses within the *Orthomyxoviridae* (Palese and Shaw 2007). Orthomyxoviruses have a negative-sense, single-stranded, and segmented RNA genome and include five genera: the influenza viruses A, B, and C; *Thogotovirus*; and *Isavirus*. The orthomyxoviruses that infect pigs are predominantly IAV viruses. Antibodies to influenza B viruses have been detected in pigs in the United Kingdom and China, but influenza B virus has not been isolated from swine (Mu et al. 1988; Brown et al 1995). Although rare, influenza C viruses have been detected in swine (Brown et al. 1995; Youzbashi et al. 1996; Matsuzaki et al. 2002). The genus Thogotovirus has two species isolated from ticks: Dhori virus and Thogoto virus (Palese and Shaw 2007). The genus *Isavirus* has one species: infectious salmon anemia virus (Palese and Shaw 2007).

The eight genes of IAVs code for at least 10 proteins. The 2 major surface glycoproteins of IAVs, hemagglutinin (HA) and neuraminidase (NA), are important for host range, antigenicity, pathogenesis, and diagnostic detection. Two other proteins used for diagnostic detection are: nucleoprotein (NP) and matrix proteins (M1 and M2). The remaining proteins encoded by the viral RNA are: non-structural (NS1 and NS2 or nuclear export protein), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and polymerase acidic protein (PA). The pro-apoptotic protein PB1-F2 is the 11^{th} protein that often contains at least one premature stop codon within the coding sequence, preventing translation into the complete protein product.

Since the HA and NA proteins are large and located on the outside of the virion, these proteins are more accessible and the antigenic characteristics of these proteins have been well studied. One such technique using the antibody responses to HA and NA, is used for subtyping IAVs. This gives us the H*x*N*y* subtype, where x=1-16 and y=1-9. Beyond the H*x*N*y* subtyping of the influenza virus, the influenza virus isolates are given strain names composed of: influenza type (A, B or C) / species of infected animal/ location of infected animal / isolate number / year, followed by the H*x*N*y* subtype. For example: one of the first IAV isolated from swine by Richard Shope in 1930 was named A/swine/Iowa/15/1930 H1N1 (Shope 1931).

IAVs can also be divided into avian and mammalian groups based on whether they can infect both avian and mammalian species or only avian species. All of the IAVs can infect birds (H1-16 and N1-9), but only H1-3 and N1-2 have had broad infectivity in humans and pigs. The reason behind the species variation has a lot to do with how the cells are infected in humans compared to birds.

Infection with IAVs is initiated by the binding of HA to sialic acid (also called neuraminic acids) sugars on the surface of epithelial cells. The human influenza H1, H2 and H3 subtypes bind to the NeuAcα2-6Gal – linked sialic acid found in the human respiratory tract. The avian influenza viruses bind to the NeuAcα2-3Gal receptors in the intestines. Both of these receptors are found in the respiratory tract of swine, which is why pigs are considered susceptible to human, swine and avian IAVs. However, the lectin histochemistry technique used to qualitatively determine the location of these sialic acid receptors in the respiratory tract has led to discrepancies in the distribution of influenza receptors and specific cell types involved, and the actual cells that were infected in culture compared to those predicted by LH in the human airway (Matrosovich et al 2004; Nicholls et al. 2007; Nicholls et al. 2008; Shinya et al. 2006; Yao et al. 2008) and in the swine airway (Ito et al. 1998; Suzuki et al. 2000; Van Poucke et al. 2010). Additionally, how IAV enters and replicates in cells that have had these sialic acids removed from the surface, has not been determined (Stray et al. 2000).

After infection is initiated by HA binding to the sialic acid receptor, the virion is internalized as described in the replication cycle of influenza virus in 5th edition of Field's Virology (Palese and Shaw 2007):

> "On binding at the cell surface, the virus is internalized by receptor-mediated endocytosis. The low pH in the endosome triggers fusion of the viral and endosomal membranes, releasing the viral ribonucleoproteins (vRNPs) into the cytoplasm. vRNPs are imported into the nucleus, where they serve as the template for transcription. New proteins are synthesized from viral mRNA. The viral genome (vRNA) is replicated through a positive-sense intermediate (cRNA). Newly synthesized viral RNPs are exported from the nucleus to the assembly site at the apical plasma membrane, where virus particles bud and are released."

1.2 Clinicopathology

Most IAV infections in swine are mild with low mortality and recovery usually occurring within 7-10 days of infection (Van Reeth et al. 2007) or 5-7 days after the onset of clinical signs (Alexander et al. 2000). Recovery, in medical terms, is "the act of regaining or returning toward a normal or healthy state" and healthy is defined by the "absence of disease or another abnormal condition" (medical-dictionary.thefreedictionary .com). Therefore, for an animal to be recovered from IAV infection, the virus must be absent from the tissues that are known to be susceptible to the virus. In other words, no longer replicating and/or shedding the virus.

In the majority of experimental infections of swine with both human and swine isolates of IAV, the clinical signs can start as early as 1 day-post-infection (DPI) (Pospisil et al. 1973; Haesebrouck et al. 1985; Haesebrouck et al. 1986; Brown et al. 1993; Macklin et al. 1998; Heinen et al. 2001a; Heinen et al. 2001b; Heinen et al. 2002; Van Reeth et al. 2003; Lee et al. 2007; Sreta et al. 2009; Weingartl et al. 2009), 2 DPI (Blaskovic et al. 1970) and 3 DPI (Jo et al. 2007; Jung et al. 2005; Jung et al. 2006), which coincides with detection of virus in nasal secretions. IAV infection in pigs is similar to that in humans with clinical signs ranging from mild to severe from strain to strain. There can also be variation during an epizootic (outbreak) involving a single strain. The clinical signs of the acute respiratory disease caused by the virus in experimentally inoculated pigs include: fever, anorexia, coughing, labored breathing to "thumping", sneezing, nasal discharge and poor weight gain. Fever is the most consistent clinical sign and peaks within 24-48 hours of infection. The clinical signs ceased in experimentally infected animals between 4 and 8 DPI (Pospisil et al. 1973; Haesebrouck et al. 1985; Haesebrouck et al. 1986; Brown et al. 1993; Lee et al. 1995; Macklin et al. 1998; Heinen et al. 2001a; Heinen et al. 2001b; Heinen et al. 2002; Van Reeth et al. 2003; Jung et al 2006; Lee et al. 2007; Sreta et al. 2009; Weingartl et al. 2009). In one study, fevers persisted up to 14 dpi in 77-day-old weanlings (Blaskovic et al 1970; Lee 1995). In animals that have robust protection from a vaccine prior to challenge by an antigenically similar isolate, the viral shedding is reduced to a 48-72 hour period (Heinen et al. 2001b; Heinen et al. 2002; Van Reeth et al. 2001a; Van Reeth et al. 2003; Van

Reeth et al. 2006). In young pigs that have maternally derived antibodies (MDA) to a homologous influenza virus, viral shedding lasted 10 days in pigs with MDA compared to 6-8 days in the pigs without MDA (Loeffen et al. 2003).

During the first 24 to 48 hours of infection with IAV, changes are evident on the microscopic level. There is vacuolar degeneration and necrosis of the bronchial epithelial cells with loss of the apical cilia and an influx of moderate numbers of neutrophils, lymphocytes and macrophages. This leads to sloughing of the necrotic epithelial cells into the lumina of the bronchi and bronchioles, leaving an attenuated epithelium by 3-4 days after infection. Between days 4 and 5 after infection, the early signs of recovery include varying degrees of epithelial hyperplasia, mitotic figures within the epithelial cells and mild inflammation in the bronchi and bronchioles as the inflammation spreads outward, expanding the alveolar septae. By 7-10 dpi there is varying degrees of interstitial pneumonia, perivascular and peribronchiolar lymphoid proliferation and normal to hyperplastic bronchial epithelia. By 14-21 days after the initial infection with IAV, the damaged respiratory tissues should be fully recovered on the microscopic level (Nayak et al. 1965; Pospisil et al. 1973; Gourreau et al. 1980; Brown et al. 1993; Jung et al. 2005; Jo et al. 2007; Lee et al. 2007; Sreta et al. 2009).

In cases where the pig has a secondary infection with another virus or bacteria, results vary from transient to delayed recovery or decreased weight gain. Co-infection of influenza virus with *Haemophilus parasuis* or porcine reproductive and respiratory syndrome virus (PRRSv) are associated with more severe clinical disease in swine (Van Reeth et al. 1996; Van Reeth et al. 2001b). These results can be compared to co-infection of influenza virus with *Mycoplasma hyopneumoniae* which has been shown to have transient to no effect on the overall outcome (Richt et al. 2004; Yazawa et al. 2004). The animals used for experimental infections usually are “specific pathogen free” and of the highest health status prior to infection. Under field conditions, the disruption of the mucociliary clearance in the respiratory tract due to influenza infection can lead to more serious complications due to secondary viral or bacterial infections (Thacker et al. 2001; Jung et al. 2005; Weingartl et al. 2009).

1.3 Diagnostic sampling and detection methods

The clinical signs and characteristic macroscopic and microscopic lesions can be used to make a presumptive diagnosis, but to make a definitive diagnosis of IAV infection the virus needs to be detected either directly or indirectly within the infected cells. In order to accomplish this, it is critical to remember that viral replication and shedding will peak with or shortly after the fever peaks in the animal. Since viral shedding will decline rapidly after 48 hours, it is important to collect samples from animals in that are in the early stages of infection and still febrile.

Samples that can be collected ante-mortem include nasal swabs, tracheal swabs, tracheal fluid and oral fluids. Lung tissue can be collected post-mortem as part of a complete necropsy, providing further our understanding of the pathogenesis of IAV in swine through examination of the macroscopic and microscopic lesions. In addition to lung tissue, IAVs have been detected in other respiratory tissues (nasal turbinates and trachea) as well as in the tonsil and tracheobronchial lymph nodes (Vincent et al. 2009c; Yazawa et al. 2004). IAVs can also be measured and detected in the airways using bronchoalveolar lavage fluid (BALF) collected post-mortem. In order to decide on which sample to collect, it is important to understand how the tests directly or indirectly detect IAVs.

1.3.1 Direct detection methods

1.3.1.1 Detection of influenza virus antigen

Immunohistochemistry (IHC) and immunofluorescence (IFA) are used to detect influenza virus antigen in frozen or formalin-fixed tissues using different antibodies (Guarner et al. 2000; Haines et al. 1993; Larochelle et al. 1994; Onno et al. 1990; Vincent et al. 1997). The nucleoprotein (NP) is well conserved among IAVs; therefore, anti-NP antibodies can be used to detect all subtypes of IAV. However, the hemagglutinin (HA) protein is subtype specific and hence is used to detect specific subtypes of influenza virus. The NP antigen is located in the nucleus and cytoplasm of infected cells (Guarner

et al. 2000; Haines et al. 1993; Larochelle et al. 1994; Vincent et al. 1997) while the HA is located in the cytoplasm and along the cell surface (Guarner et al. 2000).

Direct immunostaining methods use antibodies that are labeled with biotin, fluorophore, enzyme or colloidal gold (Buchwalow and Bocker 2010). Although technically difficult and time consuming, indirect immunostaining methods have higher sensitivity and are more commonly used for diagnostic tests (Buchwalow and Bocker 2010). These methods use an unlabeled primary antibody followed by a labeled secondary antibody. The application of the substrate then results in amplification of the colorimetric signal produced by the enzyme attached to the secondary antibody (Buchwalow and Bocker 2010). Of the indirect methods, the standard avidin-biotin complex (ABC) method of IHC has been widely used for IAV detection (Haines et al. 1993; Vincent et al. 1997). However, with this method there can be background staining due to endogenous biotin in the tissues (Vosse et al. 2007). Therefore, these methods have been adapted to polymer-based IHC method (Richt et al. 2006) that uses a polymer backbone on the secondary antibody to attach to the enzyme instead of avidin-biotin complex (Sabattini et al. 1998).

A number of rapid immunoassays, most being enzyme-linked immunosorbent assay (ELISA)-based tests kits are commercially available that can detect influenza virus antigen in clinical samples. Most of these tests have been developed specifically for human and avian applications and the viral proteins that are detected by these kits are HA, neuraminidase (NA) or NP. Five of the kits licensed for human application were found to have sensitivity of 67-71% and specificity of 99-100% for IAV (Hurt et al. 2007). The sensitivity was higher for specimens containing more than 10^5 copies/ml of influenza virus RNA as determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (Cheng et al. 2009) or 10^3 to 10^5 $TCID_{50}$/ml of virus as determined by virus titration in cell cultures (Chan et al. 2009; Hurt et al. 2009). For avian samples, in which sensitivity of RT-PCR is known to be lower than that of virus isolation in embryonated chicken eggs, the sensitivity of antigen detection kits was comparable to that of RT-PCR (Cattoli et al. 2004); the minimum amount of virus needed was 5×10^4 $TCID_{50}$/ml (Fedorko et al. 2006).

1.3.1.2 Detection of nucleic acids

First described in 1985 (Saiki et al. 1985), the polymerase chain reaction (PCR) has been used to clone DNA, sequence and analyze genes, identify people by their unique genetic fingerprint and diagnose infectious and genetic diseases. The production of complementary DNA (cDNA) from RNA was made possible by the development of RT-PCR. In 1992, PCR was made even more powerful with the innovation of real-time PCR (RRT-PCR) (Higuchi et al. 1992). Although semi-quantitative in nature (Kubista et al. 2006), several RRT-PCR testing protocols have been developed for the detection and quantization of IAVs in birds and adapted to swine (Spackman et al. 2002; Spackman and Suarez 2008a).

The use of RNA extraction and purification methods varies by the type of sample being tested. For example, RNA can be extracted directly from infected amnioallantoic fluids, cell culture supernatants, bronchoalveolar lavage fluids (BALF) and oral fluids. However, for certain clinical diagnostic samples, prior processing is necessary. Tissue samples, such as lungs, are first made into a 10% w/v homogenate using a balanced salt solution or a viral culture medium while nasal swabs are usually suspended and vortexed in a test tube with 2 ml of the above media. Although labor intensive, standard organic extraction procedures produce high purity RNA from most any sample, including tissue homogenates, paraffin-embedded tissues, and body fluids (Sun 2010). However, commercial kits that use magnetic beads or solid-phase adsorption are more sensitive and easy to use with consistent results (Sun 2010). Commercial kits, such as RNeasy and QIAamp RNA kits (Qiagen, Valencia, CA) and PureLink™ RNA kit (Invitrogen, Carlsbad, CA) are based on solid-phase adsorption using silica-membrane spin columns. Commercial kits for magnetic bead extraction, such as MagMAX™ (Applied Biosystems, Foster City, CA) and EZ1 (Qiagen, Valencia, CA) are useful for liquid samples that have low virus concentration or contain PCR inhibitors, such as oral fluids, semen, urine, feces and blood (Chan and McNally 2008; Das et al. 2009).

To detect a broad range of IAV subtypes, primers for RRT-PCR are designed to target the conserved matrix (M) or nucleoprotein (NP) genes. The USDA-validated avian

influenza RRT-PCR for the M gene (Spackman et al. 2002; Spackman and Suarez 2008a) has been adapted for the detection of IAV in swine samples. The minimum detectable concentration of the virus for this procedure ranges from 10^{-1} to 10^{1} $TCID_{50}$/ml depending on the virus strain (Landolt et al. 2005; Richt et al. 2004). While virus isolation is still the gold standard test for IAVs, RT-PCR is an accurate, rapid and sensitive technique that can be used to screen a large number of samples in a short period of time. The main disadvantage of RT-PCR is that it detects only the viral RNA and does not determine whether virus is viable or not. Since virus isolation depends on sample inoculation in a live culture system and detects the presence of live virus, it is often used in conjunction with RT-PCR to verify the presence of viable virus.

1.3.1.3 Detection of whole virus

Egg inoculation (EI) using nine to eleven-day-old embryonated chicken eggs is considered the gold standard for isolation and propagation of avian influenza viruses and certain egg-adapted IAVs (Clavijo et al. 2002; Swenson et al. 2001). However, it has been demonstrated that human influenza viruses propagated in chicken embryos acquired amino acid changes in their HA gene resulting in antigenic variation of the virus (Katz et al. 1987; Katz and Webster 1992; Meyer et al. 1993; Robertson et al. 1995). Comparatively, there was little to no genetic or antigenic variation in the same viruses when propagated in mammalian cell lines (Katz et al. 1987; Katz et al. 1990; Katz and Webster 1992; Meyer et al. 1993; Robertson et al. 1995), including Vero, MRC-5, BHK-21, and fetal porcine kidney cells. Of these, the Madin-Darby canine kidney (MDCK) cells have the highest sensitivity and are most commonly used in research and diagnostic applications (Meguro et al. 1979). For maximum sensitivity, inoculation of chicken embryos and/or another cell line is recommended in addition to MDCK cells.

Sample preparation for virus culture is the same as described for RT-PCR (Meguro 1979). IAVs may replicate in cell cultures within 24-48 hours or may take up to 5-6 days if the initial virus concentration in the sample is low. Growth of virus in cell cultures induces the production of cell lysis or cytopathic effects (CPE). Often a second blind passage is necessary for certain strains to show CPE. Once the virus has grown in

cell cultures, tests can be performed on the culture supernatant to confirm viral identity. Although not a definitive assay, hemagglutination (HA) of chicken erythrocytes can be taken as a presumptive diagnosis of the virus and for approximation of the amount of virus present in the cell culture supernatant (1 HA unit approximates 5-6 $\log_{10}$ of virus). A more accurate method of quantifying virus is virus titration by inoculation of a set of serial dilutions in cell cultures (Villegas and Alvarado 2008). For definitive virus identification, the culture supernatant can be tested by RT-PCR or commercial influenza antigen test kits based on NP or M antigen. Since virus culture usually contains higher concentrations of virus than the original sample, sensitivity issue seen with clinical samples is usually not a problem when using antigen test kits.

Even though virus isolation requires specialized equipment and maintenance of cell cultures and/or embryonated eggs, it is a standardized procedure that is available in most diagnostic laboratories. The virus isolated in cell culture can be cryogenically preserved for years and used for further characterization and vaccine production.

1.3.2 Indirect detection methods

Although the clinical signs of influenza infection coincide with the presence of virus in nasal secretions, the isolation of virus by the gold standard method of virus culture or its detection by RT-PCR can be difficult when the period of virus shedding is brief. It has been found in vaccine challenge studies that shedding can be as transient as 24-72 hours (Heinen et al. 2001; Heinen et al. 2002; Van Reeth et al. 2001a; Van Reeth et al. 2003; Van Reeth et al. 2006).

In situations when IAV is suspected but no longer detectable at the time of testing, detection of specific immunoglobulins may be undertaken. Immunoglobulins (predominantly IgG) are formed in swine at detectable levels within 1 to 2 weeks post infection and peak at 4 to 7 weeks (Olsen et al. 2006). For this reason, it has been recommended that serum samples be collected from pigs at the time of infection and at 3-4 weeks after the onset of clinical signs to compare the acute vs. convalescent response (Rossow et al. 2003). Since IAV antibodies can be formed in response to both vaccination and exposure status, the interpretation of serologic assays will depend on

both the vaccination and exposure status of the animals being tested. The serologic tests used to detect and measure influenza antibodies include: hemagglutination inhibition (HI), serum neutralization (SN), and enzyme-linked immunosorbent assays (ELISA).

1.3.2.1 Hemagglutination inhibition

The agglutination of red blood cells (RBCs) is a natural reaction that occurs in the presence of HA protein on the surface of the virus. Hemagglutination can be specifically inhibited by IAV antibody, which can be measured in an HI assay. Optimum hemagglutination and HI reactions for IAV from swine occur with turkey or chicken RBCs, which are used in standardized tests (OIE 2008). Before conducting HI tests, it is imperative to remove non-specific inhibitors of viral hemagglutination and naturally occurring agglutinins from the serum samples to be tested. Inhibitors can be removed by treatment with receptor destroying enzyme (RDE) from *Vibrio cholerae,* heat inactivation, kaolin, or potassium periodate. Similarly, non-specific agglutinins can be removed by pretreatment of serum samples with chicken or turkey RBCs (Boliar et al. 2006; Pedersen 2008a; Regula et al. 2000; Ryan-Poirier and Kawaoka 1991; Springer 1958; Subbarao et al. 1992). RDE and heat inactivation at 56°C are the methods currently recommended to remove inhibitors (OIE 2008).

For the HI test, serial 2-fold dilutions of the test serum (starting at 1:10 and ending at 1:640 or 1:1280) are prepared in 96-well microtiter plates followed by the addition of 4-8 HA units of a single subtype of influenza virus in all wells containing serum dilutions. Following incubation for an hour at room temperature, 0.5% suspension of RBCs is added to each well. In the absence of specific antibody, the virus is uninhibited (unbound) and is free to bind to the RBCs resulting in hemagglutination. However, if anti-hemagglutinin antibodies are present in the serum, such as after exposure or vaccination, the antibodies will bind to the hemagglutinin protein on the surface of the influenza virus, thus *inhibiting* the virus' ability to agglutinate the RBCs. The reciprocal of the highest serum dilution that inhibits HA is considered to be the HI titer of that serum (Figure 1.1). HI titers greater than or equal to 1:40 are usually considered to be protective (Hancock et al. 2009).

The HI test is considered a standard test for the detection of IAV antibody (Villegas and Alvarado 2008) but is somewhat subjective in nature and the results may vary because of operator subjectivity and also upon repeating the test. Also, since there is broad cross-reactivity among the α, β, and γ clusters of the H1 subtype of IAVs, a positive HI titer may indicate a virus related to the virus of exposure, but does not definitively identify it. However, homologous virus reactions are typically stronger than heterologous virus reactions, resulting in higher HI titers. The advantages of this test are that it is a standardized procedure that is inexpensive and easy to perform and the results are comparable to more complicated tests, such as serum neutralization (Vincent et al. 2006; Leuwerke et al. 2008).

1.3.2.2 Serum neutralization or virus neutralization

The SN test detects virus-specific neutralizing antibody present in a serum sample. Serial 2-fold dilutions of the serum and a known amount of IAV are pre-incubated and then added to MDCK cells to determine the highest dilution of serum that can neutralize virus infection of cells and production of CPE (Figure 1.2). Neutralizing antibodies in serum sample block viral infection of cell culture and the virus is not available to produce CPE. However, if antibodies are not present, the virus is not blocked and is free to cause CPE in inoculated cell cultures. Reciprocal of the highest serum dilution that can neutralize virus infection is considered to be the SN titer of the serum. Since the test uses very small volumes of serum in cell monolayers contained in 96-well microtiter plates, it is often called microneutralization. One of the advantages of SN over HI and ELISA is that it demonstrates the biologic (neutralizing) activity of the antibodies present in the serum. Some of the disadvantages of this test are that it requires equipment and supplies used for virus cultures and the results can take up to 72 hours to obtain. Also, the SN titers may vary when the test is repeated.

1.3.2.3 Enzyme-linked immunosorbent assay

The ELISA test uses a 96-well plate that has been coated with influenza viral antigen. The serum sample is incubated in the coated wells for antibody attachment. After

the unbound material is washed away, an anti-influenza monoclonal antibody that is conjugated to an enzyme is bound to the antigen. The unbound conjugate is washed away and the enzyme substrate (that produces a color change in the presence of the enzyme) is added to the wells. The color-changing reaction is stopped after 15 minutes and the amount of color produced is read as an optical density (O.D.) in a spectrophotometer (figure 5.4). The O.D. is inversely proportional to the amount of anti-influenza antibodies present in the test sample. Commercially available ELISA test kits include separate ELISA tests for H1N1 and H3N2 subtypes of IAV. Another ELISA that detects antibodies to a range of IAV viruses is available and has been adapted for use in detecting swine anti-IAV antibodies (Ciacci-Zanella et al. 2010).

The commercial H1N1 ELISA uses an antigen prepared from a classical H1N1 IAV and, thus has a limited detection range of swine H1 subtypes. Although the H1N1 test is not designed to detect other IAV subtypes, it may sometimes cross react with H3N2 because of some common epitopes between H1N1 and H3N2 viruses. In addition, the H1N1 test has been found to miss recently infected animals (Yoon et al. 2004). The H3N2 ELISA test was developed from a cluster I virus leading to lower reactivity with cluster IV H3N2 viruses (Yoon et al. 2004). The MultiS-Screen ELISA (FlockChek™, Idexx, Westbrook, ME) uses a highly conserved epitope of IAV nucleoprotein (NP) (Ciacci-Zanella et al. 2010). Preliminary studies indicate that this kit, while originally designed for use in avian species, also detects antibodies against subtypes common to swine (Ciacci-Zanella et al. 2010).

1.3.3 Virus subtyping and sequencing

Important for host range, antigenicity and pathogenesis, the 16 HA and 9 NA genes are antigenically and genetically divergent and these variations are used for subtyping the influenza viruses. The cultured viruses were traditionally subtyped using HI and NA inhibition (NI) assays (Pedersen 2008a, Pedersen 2008b). The NI assay uses a dilution of the cultured virus between 1:4 and 1:32, depending on the virus concentration. There are several steps that include standardized NA antisera (N1-N9), fetuin, periodate, sodium arsenite and thiobarbituric acid which result in a dark color if there is no

inhibition and a light color if there is inhibition; The NA subtype has the light color result. Both of these assays are time-consuming and require standardized NA and HA antisera, which are often difficult to acquire. Therefore, RT-PCR is now regularly used for subtyping. Currently, HA and NA specific primers can be used for both detection and subtyping of IAVs. Additionally, a number of multiplex and nested RT-PCR have been developed for subtyping with and without simultaneous detection of IAV (Chander et al. 2010; Fereidouni et al. 2009; He et al. 2009; Lam et al. 2007; Li et al. 2001; Stockton et al. 1998; Yang et al. 2010).

In addition to subtyping, RT-PCR can also be used for sequencing all eight gene segments of influenza virus (Chander et al. 2010; Jindal et al. 2009). The sequences can be examined and compared to other sequences with molecular analysis tools; uncovering the evolutionary and geographic relationships of influenza viruses. However, the amount of RNA in clinical samples is usually low compared to the other cellular materials and contaminating bacteria (Spackman and Suarez 2008a). Therefore, cell culture supernatants and amnioallantoic fluid containing a large concentration of whole virus, are recommended for sequencing and other molecular analyses (Spackman and Suarez 2008a).

1.3.4 Limitations of diagnostic assays

The rapid evolution of IAVs over the last decade has led to genetic and antigenic variation of the virus in North American swine. This has led to limitations in cross-reactivity for the serologic assays. These changes need to be kept in mind when interpreting the results of these tests. Although there is some antigenic cross-reactivity among the classical and reassorted α, β and γ clusters of the swine H1 subtype, there is little to no cross-reactivity between these three clusters and the human-like δ cluster (Vincent et al. 2006). This variability in the antigenic cross-reactivity was demonstrated in 2009 pandemic H1N1 virus for both North American and European swine H1 subtypes using sera from experimentally infected and vaccinated pigs (Kyriakis et al. 2010a; Vincent et al. 2010a; Vincent et al. 2010b). The human-like viruses in the SwH1δ cluster were recently found to have two distinct antigenically divergent groups, which could

result in additional limitations for serologic assays (Vincent et al. 2009a). Similarly among the swine H3 viruses, there is little to no cross-reactivity between groups I and IV. There is also limited to no cross-reactivity between swine subtypes, which means that multiple viruses from each subtype need to be tested to determine the subtype of the virus that produced the antibodies. To overcome the limitations of cross-reactivity and broaden influenza surveillance, the samples may first be screened by the MultiS-Screen ELISA followed by more specific tests, such as SN and HI assays to determine the subtype of the virus of exposure.

As the influenza virus continues to evolve, the primers for RT-PCR for detection and subtyping need to be continually validated and updated. Current testing stratagems rely on conserved nucleotide sequences for the primers. However, the variability in the HA and NA genes in avian influenzas have resulted in the design of multiple *wobble primers* to detect one subtype of IAV without cross-reactivity with other HA and NA subtypes (Sidoti et al. 2010; Starick et al. 2000; Suarez et al. 2007). The avian influenza primers can be used for subtyping influenza viruses from swine or new subtyping primers can be designed using published sequences (He 2009; Huang et al. 2009; Lee et al. 2008; Nagarajan et al. 2010). New technologies, such as enzyme hybridization and microarray, are being used for subtyping of influenza viruses across species (avian, human and swine) and detection of specific influenza viruses like 2009 pandemic H1N1 (He 2009; Huang et al. 2009).

1.4 Evolution of influenza viruses in swine

In order to evade the immune response from natural infections or vaccinations, influenza viruses evolve by two mechanisms: antigenic drift and antigenic shift. Antigenic drift is the process by which the HA and NA gene segments change by mutations in the genetic code, through nucleotide substitutions, insertions and deletions. In general, most RNA viruses like influenza, have a high rate of change and mutations. These mutations can result in changes in the amino acid translated from the RNA, which in turn may or may not result in a change in the 3-dimensional structure of the protein. If the nucleic acid change does not result in an amino acid change, then it is a synonymous

substitution and there is no overall change in the antigenic properties of the virus. However, if the nucleic acid change does result in an amino acid change, then it is a non-synonymous substitution and there may be a change in the antigenic properties of the virus.

While antigenic drift is usually a random and slow process, antigenic shift is a rapid process involving the exchange of the hemagglutinin and neuraminidase gene segments between two or more influenza viruses infecting the same cell. This process is also called reassortment. When this process results in a completely unrelated or novel protein on the surface of the virus, there can be a dramatic effect on the infected population.

Antigenic shift and drift have resulted in regional variations in the predominant IAVs found in North America. The classic swine influenza (cH1N1) in North America was relatively stable since it was first isolated in 1930 (Shope 1930). However, the first appearance in North America for the H3N2 subtype was in Canada in 1988 and the first U.S. isolate was from North Carolina in 1998 (Zhou et al. 1999, Zhou et al. 2000). The 1998 U.S. isolate was a double-reassortant strain composed predominantly of cH1N1 proteins except for HA, NA and PB1 genes from human influenza virus (Zhou et al. 1999). The H3N2 swine isolates that followed were triple-reassortants composed of genes from avian, human and swine IAVs (Zhou et al. 2000). The HA, NA and PB1 genes were from human influenza virus, the matrix and nonstructural proteins and nucleoprotein genes were from cH1N1, and the PA and PB2 genes were from avian influenza virus.

Since the emergence of H3N2 subtypes in North American swine population, there has been another reassortment between the triple-reassortant H3N2 and the cH1N1 to produce H1N2 viruses. These continued reassortments have led to numerous variants to date of the reassortant H1N2 and H1N1 (rH1N1) variety in North America (Karasin et al 2006), as well as an H3N1 virus isolated in Minnesota (Ma et al. 2006). Since the H3N2 pandemic began in 1967-68, the Chinese have isolated human, avian and swine origin H1N1 strains from pigs, as well as the reassortant H3N2 and H1N2 strains of IAV from their pigs (Yu et al. 2007).

Among the North American swine H1 hemagglutinin genes, phylogenetic analysis has revealed 4 distinct genetic clusters which have been named alpha, beta, gamma, and delta (Vincent et al. 2009; Lorusso et al. 2011). The swH1α is most closely related to cH1N1. The swH1β cluster (also called rH1) resulted from H3N2 reassortments with cH1N1 that produced H1N1 and H1N2 viruses with hemagglutinin and neuraminidase genes from cH1N1 and all internal genes from the H3N2 (Gramer and Rossow 2004; Gramer et al. 2007). The SwH1γ cluster developed changes in the HA genes as a result of reassortments between the triple reassortant H3N2 and cH1N1 viruses (Webby et al. 2000), and includes both H1N1 and H1N2 viruses. The swH1δ clade is the most divergent and contains both H1N2 and H1N1 viruses that contain H1 segments derived from seasonal human IAVs that are paired with N1 and N2 genes (Vincent et al. 2009a, Nelson et al. 2011).

The internal genes from the North American reassortant swine IAVs possess what are referred to as a triple-reassortant internal gene (TRIG) cassette, which is found in all four clades of North American swine influenza viruses (Vincent et al. 2008). Additionally, all of the IAVs sequenced in the 21st century from swine that have been found to contain the HA gene of cH1N1 have been found to contain the TRIG cassette (Vincent AL, personal communication) creating a distinction between cH1N1 and SwH1α viruses as demonstrated in a phylogenetic tree of representative SwH1 viruses (Figure 4).

Molecular characterization of influenza viruses has become a benchmark for evaluating the evolutionary relationships and known antigenic coding regions. Molecular characterization is done using a number of techniques. For influenza viruses, the most common analysis is to use computer program such as MEGA 4 with Clustal W alignment (www.megasoftware.net) to align the full-length gene sequence of the viral gene segments along with IAV sequences found in GenBank and produce phylogenetic trees. Most often HA and NA gene segments are genetically characterized, but full genome sequencing is ideal for a comprehensive analysis of IAV evolution in swine.

1.5 Influenza virus vaccination and control for swine

While it is possible to eradicate IAV from a herd without using vaccines, vaccinations are usually an important part of a comprehensive plan to decrease influenza infection in a herd (Torremorell et al. 2009). IAV antibody titers (usually measured by HI) are one of the components that are necessary to limit clinical disease and virus shedding (Olsen et al. 2006). These protective antibodies need to arise from either natural infection or vaccination. Since it is not recommended to purposely inoculate pigs in an attempt to mimic natural infection due to human safety issues and the risks of secondary bacterial infections, decreased feed efficiency and increased nursery mortalities (Torremorell et al 2009), there is a range of vaccination strategies to consider for each farm.

The efficacy of the vaccine is determined by how well the genetic and antigenic properties of the vaccine virus match with the genetic and antigenic properties of the challenge virus. In addition: antigenic dose, adjuvants, timing of vaccine delivery, the presence of maternal antibody and co-infections also play a role in how well the vaccine may work.

One vaccine strategy is to continuously vaccinate the breeding animals in an attempt to give protection to the nursery pigs with maternally derived antibodies (MDA). MDA for IAV are found in the colostrum of naturally exposed or vaccinated sows (Blaskovic et al. 1970). The suckling piglet will get most of the MDA by drinking the colostrum in the first few hours of life and these antibodies may start to wane between 20 and 30 days of age (Blaskovic et al. 1970). MDA alone have been shown to help protect the piglets against disease (Kitikoon et al. 2006). However, the problem that MDA pose for vaccination of growing pigs is that it has also been shown that pigs with MDA develop a weaker immune response to vaccination (Loeffen et al. 2003) and may be at risk for enhanced IAV pneumonia (Kitikoon et al. 2006). Sow vaccination can be useful if you need to increase MDA levels in piglets to protect them prior to weaning and early into the nursery (Olsen et al. 2006). However, MDA levels by HI assay may not drop to below 1:40 until 16 weeks of age (Olsen et al. 2006). These antibodies can also interfere

with the piglet's ability to form its own active immune response at the time of vaccination and weaken overall protection (Blaskovic et al. 1970).

Another vaccine strategy for farms that have more losses in the growers and finishers is to vaccinate the pigs before they enter the finishing site. This model more closely matches what is done for most vaccine efficacy studies. These studies obtain 3-4 week old pigs from an influenza virus seronegative farm and vaccinate the pigs at two-week intervals. When the pigs are 9-12 weeks old (2-4 weeks after the second vaccination), the pigs are then challenged with either a homologous (same virus that is in the vaccine) or a heterologous (different virus from the one in the vaccine) influenza virus. In order to use this strategy in pigs with MDA, the pigs should not be vaccinated until after the MDA have disappeared for the best vaccine effect.

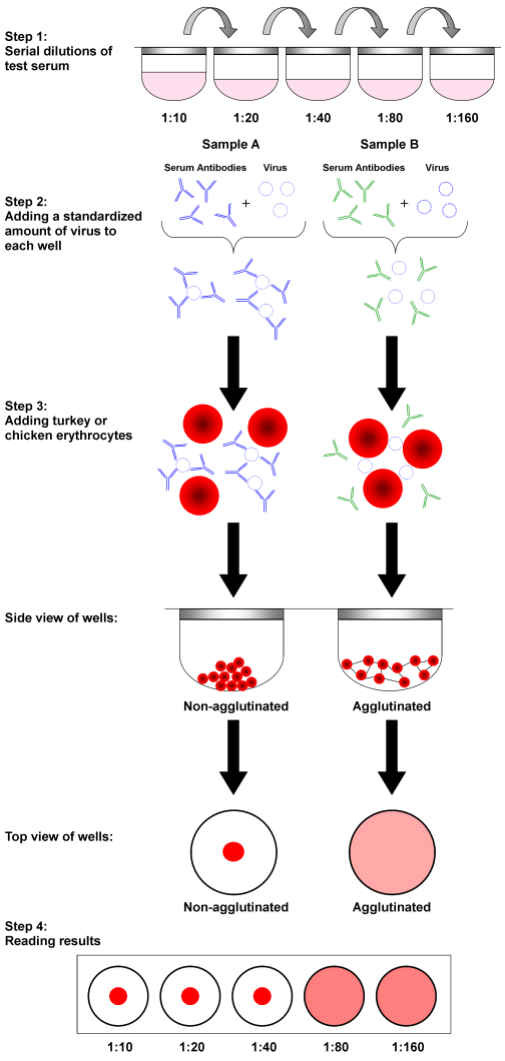
In the face of an IAV infection, the decision to vaccinate and which vaccine to use should be based on a scientific approach to the problem. This assessment might include antigenic and molecular characterization of the virus, then comparison of the field strains to both commercial and herd-specific influenza vaccines.

Vaccines that are sold in the U.S. for IAV are produced at establishments that are licensed through the U.S. Department of Agriculture; however, the vaccine itself falls into one of three licensure categories: fully approved, conditional licensure and autogenous. The vaccines that are usually referred to as "commercial" vaccines fall into the category of fully approved after the appropriate vaccine purity, potency, safety and efficacy studies have been conducted. The process for a vaccine to become fully approved usually takes 2-3 years and the viral isolates that are in these vaccines are owned by the company that manufactures them. Herd-specific vaccines or autogenous vaccines are also produced by companies under the regulation of the USDA. The regulations governing autogenous vaccines indicate their use when a fully approved commercial vaccine does not provide effective protection against a specific viral strain. The viral isolates that are in these vaccines are usually owned by the client who submits the tissue or virus to the company that makes the vaccine. A new area of vaccine technology includes recombinant viral subunit vaccines and vector particle vaccines. Some of the improvements over current vaccine technology are that these vaccines can be

produced in as little as 12 weeks and they may provide cross-protection against multiple strains.

There is no single best solution when it comes to vaccination strategies. It is important to consider the age groups affected and how the vaccine fits into the comprehensive influenza control program for the farm, as well as the overall health plan for the farm.

Figure 1.1 (left) Steps in a hemagglutination inhibition reaction. The antibodies on the left in sample A prevent the virus from agglutinating the erythrocytes. Whereas the antibodies on the right in sample B do not bind to the virus in step 2, which agglutinate the erythrocytes in step 3. The antibody titer shown in step 4 is read out as 1:40.
Figure 1.2 (right) Steps in a serum neutralization reaction. The antibodies in sample A on the left neutralized the virus in step 2. This resulted in no cytopathic effects (CPE) in step 4. Whereas the antibodies in sample B on the right did not neutralize the virus in step 2, resulting in infection of the MDCK cells and CPE in step 4.



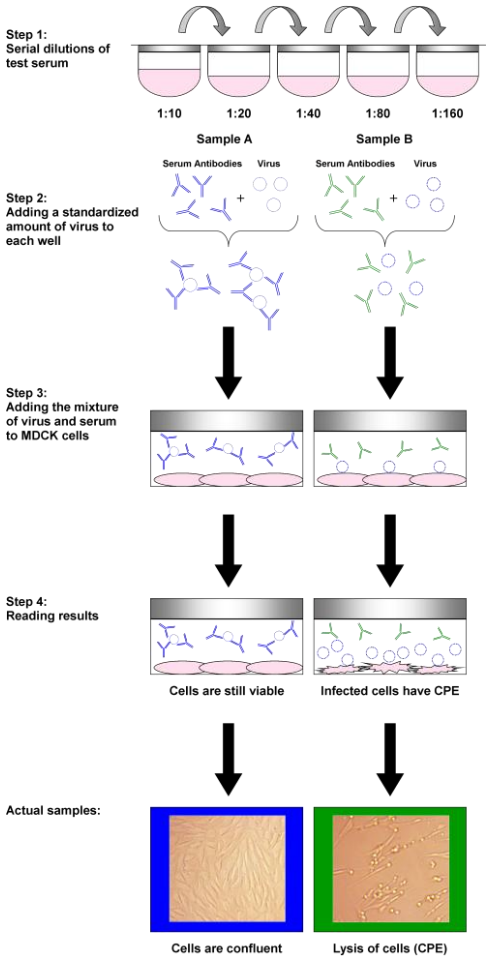


Figure 1.3 Steps in a blocking ELISA test. The optical density of sample A is lower than sample B because the IAV antibody in sample A bound to the antigen coated on the bottom of the well, partially blocking the binding of the enzyme bound conjugate. The antibodies in sample B did not bind to the antigen and were therefore washed out in step 2.
Figure adapted from http://www.idexx.com/pubwebresources/pdf/en_us/livestock-poultry/0965846.pdf

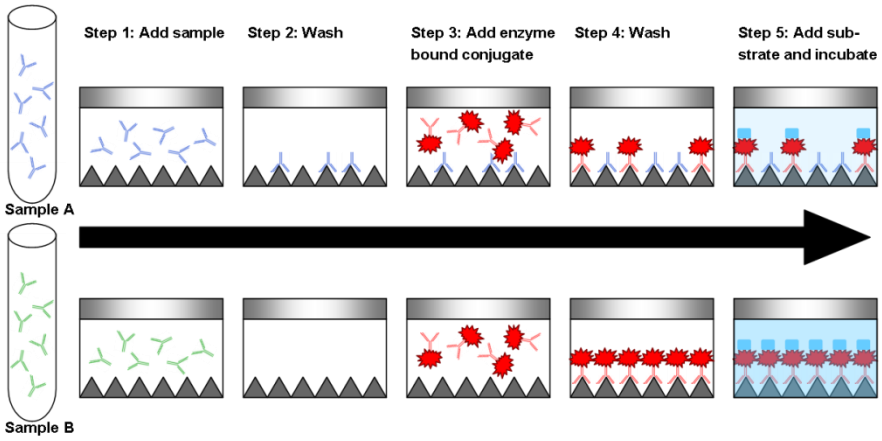
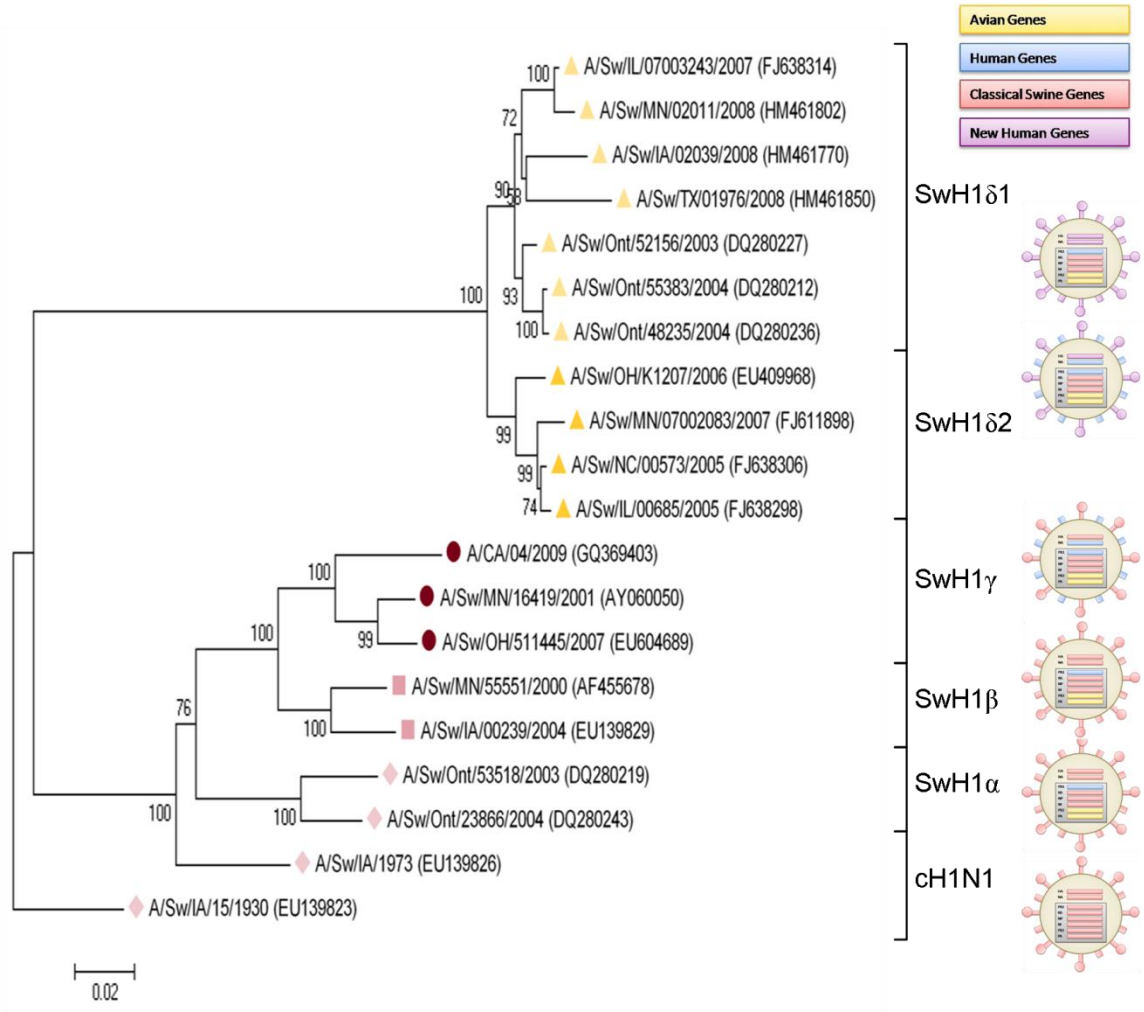


Figure 1.4 Phylogenetic tree containing classic swine influenza viruses (cH1N1) and representatives of the four swine H1 hemagglutinin clusters (SwH1α, β, γ, and δ). The internal genes are highlighted by a gray box and GenBank numbers follow the virus name in parentheses.



CHAPTER 2

DETECTION OF INFLUENZA A VIRUS IN PORCINE ORAL FLUID SAMPLES

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Porcine oral fluids have been used for the detection of *Porcine reproductive and respiratory syndrome virus* and *Porcine circovirus-2*. The objective of the present study was to determine if *Influenza A virus* (IAV) is present in porcine oral fluids at detectable levels and to validate a standard IAV molecular diagnostic test for porcine oral fluids. Pen-based oral fluid samples were collected on 3, 4, 5, and 6 days post-infection (DPI) from 4 groups of 6 pigs each that were inoculated intratracheally with A/Swine/Iowa/00239/2004 H1N1, and 2 untreated or mock-inoculated groups of 6 pigs each, which served as negative controls. Individual nasal swabs were also collected from these 36 pigs on 3 and 7 DPI. All oral fluid samples were examined for the presence of IAV by matrix gene real-time reverse transcription polymerase chain reaction (real-time RT-PCR) and virus isolation. Nasal swabs were tested initially by virus isolation followed by re-test of negative samples with real-time RT-PCR. No oral fluid sample from virus-inoculated pigs was positive by virus isolation but 15 out of 16 (94%) pigs were positive by real-time RT-PCR. In contrast, virus was isolated from 32 out of 48 (67%) nasal swabs collected from virus-inoculated pigs. In addition, 382 out of 910 porcine oral fluids collected from pigs in the field between August 1, 2009 and January 31, 2010 were positive by real-time RT-PCR. The results of the present study indicate that pen-based oral fluids provide an easy, effective, and safe collection method for the detection of swine influenza virus with rapid testing methods such as real-time RT-PCR.

2.1 Introduction

In human beings, oral fluids are predominantly composed of salivary gland secretions mixed with variable amounts of serum and inflammatory cells, bacteria, fungi, viruses, bronchial and nasal secretions, gingival epithelial cells, and food debris (Humphrey and Williamson 2001; Kaufman and Lamster 2002). As a diagnostic sample, human oral fluids are easily collected and have been used for virus and antibody detection and for drug (misuse and therapeutic) and hormone level monitoring (Kaufman and Lamster 2002; Robinson et al. 2008). For pigs, oral fluids have recently been used as a surveillance tool for Porcine circovirus-2 (PCV-2) and Porcine reproductive and respiratory syndrome virus (PRRSV) using quantitative reverse transcription polymerase chain reaction (qRT-PCR; Prickett et al. 2008a; Prickett et al. 2008a; Prickett et al. 2008c; Prickett et al. 2008d). Porcine oral fluids have also been used for the detection of PRRSV antibodies using enzyme-linked immunosorbent assay and indirect fluorescent antibody tests (Prickett et al. 2008a; Prickett et al. 2008a; Prickett et al. 2008d).

As influenza continues to evolve in North American swine, the genetic and antigenic variability of this virus and the presence of multiple co-circulating strains have resulted in the need for diagnostic tests that can be used for herd monitoring and surveillance, as well as for differentiation and characterization of influenza strains. The 2009 H1N1 influenza pandemic has also brought to light the need for better surveillance of influenza in animals and human beings. Influenza surveillance in pigs could be facilitated if easy, effective, and safe sample collection methods are available along with accurate and rapid diagnostic assays. The objective of the present study was to determine whether influenza A virus (IAV) could be detected in porcine oral fluids obtained from experimentally infected pigs and to validate the real-time reverse transcription polymerase chain reaction (real-time RT-PCR) and virus isolation assays for oral fluid samples.

2.2 Materials and methods

Virus

The influenza virus strain used was A/Swine/Iowa/00239/2004 H1N1 (IA/04 H1N1), which had previously been used in vaccine evaluation studies (Vincent et al. 2006; Vincent et al. 2007). The virus inoculum was prepared by propagation on Madin–Darby canine kidney (MDCK) cells (Meguro et al. 1979) and had a titer of 10^6 median tissue culture infective dose ($TCID_{50}$)/ml as calculated by the Spearman–Kärber method (Villegas and Alvarado 2008).

Animals

Thirty-six, 3-week-old pigs were purchased from a single, influenza-negative source and consisting of isolation rooms equipped with air filtration and negative pressure anterooms at the University of Minnesota (St. Paul, Minnesota). The animal phase of this study was conducted with the approval of the Institutional Care and Use Committee of the University of Minnesota. Upon arrival, the pigs were randomly assigned to 6 treatment groups of 6 pigs each and acclimatized for 1 week prior to the start of the experiment. On experimental day 0, all 24 animals in groups 1–4 received 2 ml of virus inoculum intratracheally. Groups 5 and 6 served as negative control groups. The animals in group 5 (n = 6) received 2 ml of minimal essential medium (MEM; Mediatech Inc., Manassas, VA) intratracheally to demonstrate that the inoculation procedure did not cause respiratory lesions or secondary infection. The animals in group 6 (n = 6) received no treatment. In order to monitor the progression of infection over a 2-week period, 2 pigs from each group were humanely euthanized on 7, 10, and 14 days post-infection (DPI) with a lethal dose of pentobarbital (Vortech Pharmaceuticals, Dearborn, MI), and necropsies were performed. The reason behind the need for 2 different negative control groups, 4 inoculated controls, and multiple euthanasia days was that these pigs were part of a larger, unrelated study.

Nasal swab sample collection and analysis

Nasal swabs were collected using a rayon-tipped swab (BBL™ CultureSwab™, BD Diagnostic Systems, Sparks, MD) from all pigs 1 day prior to virus inoculation (day 1) and at 3 and 7 DPI. The swabs were placed in a vial containing 2 ml of Eagle's MEM containing 4% bovine serum albumin, trypsin, and antibiotics (MEM+). The swab sample was vortexed for 30 sec and centrifuged for 30 min at 2,272 × *g*. For virus isolation, the

swab supernatant (in 400-µl amounts) was inoculated on monolayers of MDCK cells grown in 25-cm^2 flasks (Sarstedt Inc., Newton, NC) with 5 ml of MEM+ media (Meguro et al. 1979). All cultures were incubated at 37°C under a 5% CO_2 atmosphere. All flasks were examined daily for 7 days under an inverted light microscope to observe virus-induced cytopathic effects (CPE). Samples found negative by virus isolation were retested by a matrix real-time RT-PCR procedure as previously described (Spackman et al. 2002; Spackman and Suarez 2008a). Briefly, viral RNA was extracted from 50 µl of swab supernatant using a magnetic bead procedure (Ambion® MagMAX™ AM1835, Applied Biosystems, Foster City, CA). Following extraction, the RNA was subjected to real-time RT-PCR using a one-step RT-PCR kit[f] with reference dye (One-Step PCR kit, ROX™, QIAamp® viral RNA Mini kit, Qiagen Inc., Valencia, CA) and a matrix control provided by the United States Department of Agriculture (USDA)–National Animal Health Laboratory Network. A positive result was defined by a cycle threshold (Ct) value less than 35, the suspect range included Ct values greater than 35 and less than 40, and a negative result was greater than 40 (the Ct value is determined by the number of cycles needed to exceed the background signal).

Oral fluid sample collection and analysis

The pigs were acclimatized to the ropes used to collect samples 3 days prior to infection, and pen-based oral fluids were collected on 3, 4, 5, and 6 DPI from each of the 6 groups. A 70-cm length of 3-strand twisted rope (WebRiggingSupply.com, Barrington, IL) was untwisted and suspended at shoulder height from a metal bar in the pens. For convenience, the rope was left in the pens so that the pigs could chew on it for 12 hr on 3 and 4 DPI, and for 6 hr on 5 and 6 DPI. Pigs were observed chewing on the ropes from the time the ropes were put in the pens until the ropes were removed. As each rope was removed, it was placed inside a 3.7-liter plastic storage bag (SC Johnson & Son Inc., Racine WS), and the oral fluids were wrung out. The bag was sealed, and a bottom corner of the bag was cut off with scissors to pour the contents into a 50-ml centrifuge tube (Sarstedt Inc., Newton, NC). The tubes were centrifuged at 2,272 × *g* for 30 min to remove particulate debris. The samples were stored overnight at 4°C and assayed by virus isolation and real-time RT-PCR the following day.

All oral fluid samples were assayed using the same USDA-validated matrix real-time RT-PCR procedure (Spackman et al. 2002; Spackman and Suarez 2008a), with modifications: the sample was preprocessed using 175 µl of oral fluid supernatant and 235 µl of lysis/binding solution and the viral RNA was extracted using a magnetic bead procedure (Ambion® MagMAX™ AM1836, Applied Biosystems, Foster City, CA). All oral fluid samples were also assayed by virus isolation using 400 µl of the oral fluid supernatant passaged on MDCK cells in 25-cm^2 flasks (Sarstedt Inc., Newton, NC) with 5 ml of MEM+ media.

Evaluation of the IAV matrix real-time RT-PCR technique for oral fluids

To determine the lowest detectable quantity of IAV in an oral fluid sample and the effects of temperature and storage time on the detection of viral RNA in the sample, the following experiments were conducted. Fifty milliliters of oral fluid were collected from pigs in a *Mycoplasma hyopneumoniae,* IAV, PRRSV, and PCV-2–negative research herd and verified to be negative for IAV by real-time RT-PCR. Aliquots of virus-negative oral fluids were spiked with strain IA/04 H1N1 (initial titer = 3.16×10^5 $TCID_{50}$/ml). Five different dilutions (10^{-1} to 10^{-5}) with titers ranging from 3.16×10^5 to 3.16×10^1 $TCID_{50}$/ml were used. The spiked samples were stored at room temperature for 1, 2, 3, 4, and 5 hr prior to testing. Another aliquot of the dilutions was frozen at –80°C and tested after 12, 24, 48, and 72 hr of storage. All samples were also assayed using lysis/binding solution, magnetic bead extraction, and USDA-validated matrix real-time RT-PCR procedures as described above.

To evaluate the sensitivity of this real-time RT-PCR assay when 2 different influenza virus strains may be present in the saliva sample, this procedure was repeated with another two virus strains: A/Swine/Minnesota/01969/2007 H1N2 (MN/07 H1N2) and A/Swine/Kansas/02052/2008 H3N2 (KS/08 H3N2). The 2 virus strains were mixed together, calculated to have an original titer of $10^{7.2}$ $TCID_{50}$/ml, and used to make 6 serial 10-fold dilutions in virus-negative oral fluids (1.77×10^6 to 1.77×10^1 $TCID_{50}$/ml).

Evaluation of the virus isolation technique for oral fluids

To determine the effect of oral fluids on the survival of virus, the following experiment was performed. Oral fluid was collected from pigs in a *M. hyopneumoniae,*

IAV, PRRSV, and PCV-2–negative research herd and verified to be negative for IAV by real-time RT-PCR. The spiked oral fluids were made by mixing 10 ml of oral fluid with 10 ml of strain IA/04 H1N1 (initial titer = $10^{6.5}$ $TCID_{50}$/ml). The spiked oral fluids and the original undiluted virus (without oral fluid) were incubated at 4°C. At 0, 1, 2, 24, and 48 hr, 150 µl of samples were removed from both tubes for virus titration by serially diluting the samples 10-fold using 20 µl of sample and 180 µl of MEM. The serial dilutions were inoculated in 96-well plates containing monolayers of MDCK cells using 4 wells/dilution (Meguro et al. 1979). After 4 days of incubation, the plates were assessed for CPE, and virus titers were calculated by the Spearman–Kärber method (Villegas and Alvarado 2008).

Evaluation of oral fluid samples collected in the field

Using the recommended 20–30 min sampling time (Prickett et al. 2008a; Prickett et al. 2008a; Prickett et al. 2008c; Prickett et al. 2008d), pen-based and individual oral fluid samples were collected from pigs under field conditions by veterinarians and farm staff between August 1, 2009 and January 31, 2010. These samples were tested at the University of Minnesota Veterinary Diagnostic Laboratory using the IAV matrix real-time RT-PCR for oral fluids as described above. Virus isolation and viral sequencing were performed on influenza-positive samples if requested by the submitting party.

Necropsy findings

At necropsy, the lungs were removed, and the percent of lungs affected by lobular consolidation was determined (Halbur et al. 1995). To confirm the presence or absence of IAV at the time of necropsy, nasal swab and lung tissue samples were collected for virus isolation and real-time RT-PCR. The RNA extraction for lung tissue was performed using a viral RNA kit, as described above. Samples of nasal turbinates, trachea, lung with lesion (if present), and the middle right lung lobe were fixed in 10% neutral buffered formalin solution, paraffin-embedded (FFPE), thin-sectioned (5 µm), and then stained with hematoxylin and eosin by routine histological procedures. The lung sections were scored from 0 to 4 based on the severity of bronchial injury as detected by light microscopy (Richt et al. 2003). Using anti-influenza A nucleoprotein, monoclonal mouse antibody (Meridian Life Science Inc., Saco, ME), anti-mouse IgG polymeric peroxidase

conjugate (Dako, Glostrup, Denmark), and 3-amino-9-ethylcarbazole chromogen (Dako, Glostrup, Denmark), immunohistochemistry was performed on thin sectioned, FFPE respiratory tissues by standard techniques (Guarner et al. 2000; Sabattini et al. 2008).

Statistical analysis

For all statistical analyses, SAS version 9 (SAS Institute Inc., Cary, NC) was used. Macroscopic and microscopic lung scores were compared by one-way nonparametric analysis of variance with post hoc Tukey–Kramer and Kruskal–Wallis tests. The oral fluid and nasal swab results were compared by Chi-square tests. The minimum number of animals needed per group was determined to be 5 using a one-tailed *t*-test to compare means with unequal variance from 2 independent populations ($\alpha = 0.05$, $1 - \beta = 0.90$).

2.3 Results

Nasal swabs from all animals collected 1 day prior to the start of the experiment were influenza negative by real-time RT-PCR. IAV was detected by virus isolation in nasal swabs collected from 21 out of 24 inoculated pigs at 3 DPI. At 7 DPI, IAV was detected in nasal swabs collected from 11 out of 24 inoculated pigs; 3 of which were negative at 3 DPI. Therefore, although the overall detection of influenza in nasal swab samples was 67% (32/48) by virus isolation alone, each of the 24 inoculated pigs were positive at either 3 or 7 DPI, or on both days. Viral RNA was detected in 2 additional nasal swabs at 7 DPI making the overall detection of influenza 71% (34/48) for virus isolation and real-time RT-PCR combined (Table 2.1). In contrast, influenza was detected in 15 out of 16 (92%) oral fluid samples of infected pigs by real-time RT-PCR at 3–6 DPI (Table 2.2). The oral fluid samples collected from the 2 negative control groups were negative at all time points (0/8). All 24 oral fluid samples from both the infected and uninfected pigs were negative for virus isolation after 2 passages in MDCK cells (Table 2.2).

The minimal detectable limit of the real-time RT-PCR assay was 316 $TCID_{50}$/ml of influenza virus in oral fluids spiked with strain IA/04 H1N1 after storage at room temperature for 1 and 5 hr and after storage at –80°C for 24, 48, and 72 hr (Table 3). The

minimal detectable limit of the real-time RT-PCR assay was 17 $TCID_{50}$/ml of influenza virus in oral fluids spiked with strains MN/07 H1N2 and KS/08 H3N2 after storage at room temperature for 1 and 5 hr and after storage at –80°C for 24 and 48 hr (Table 2.4). For the validation of the virus isolation assay, the titer of undiluted virus inoculum (IA/04 H1N1) was $10^{6.7}$ $TCID_{50}$/ml for all time points at both storage temperatures. The virus titer in oral fluids spiked with strain IA/04 H1N1 and stored at 4°C was $10^{6.5}$ $TCID_{50}$/ml at 0, 1, 2, and 24 hr and $10^{5.5}$ $TCID_{50}$/ml at 48 hr.

Of the 910 field samples tested, 382 were positive, 83 were suspect (Ct values >35 and <40), and 444 were negative. One sample did not yield sufficient amount for testing. Additional procedures were requested by submitting parties: virus isolation was requested on 22 of the real-time RT-PCR–positive samples and virus was isolated from 11 of them. Subtyping was requested on 29 samples, and the results were H1N1 (7), H3N2 (3), and H1N2 (3) subtypes, and nontypeable (16). Hemagglutinin (HA) sequences were requested on 4 positive oral fluid samples, and sequences were obtained directly from 2 samples.

2.4 Discussion

Influenza A virus was detectable in porcine oral fluids by real-time RT-PCR. The results of the real-time RT-PCR analysis and virus isolation performed on the nasal swabs collected in the mornings of 3 and 7 DPI were compared to results of the real-time RT-PCR analysis and virus isolation performed on the oral fluids that were collected in the evening of 3 and 6 DPI, respectively. In making these comparisons, there was agreement between the test results of the oral fluids by real-time RT-PCR and nasal swabs by virus isolation. Similarly, there was also agreement between the expected and observed results when the test results were compared to the infection status of the pigs (Chi-square: $p < 0.0001$). The exception to these agreements was the pen-based oral fluid results from group 3 at 3 DPI, which were negative.

One nasal swab was positive by virus isolation and real-time RT-PCR at 7 DPI from a single mock-inoculated pig, ID 329. This nasal swab was collected during necropsy at 7 DPI and, other than this spurious result, there was no indication that this pig

was ever infected with IAV. In fact, the lung tissue collected at the same time as the nasal swab at necropsy was negative by real-time RT-PCR and virus isolation. Furthermore, there were no gross lesions in the lung, nasal turbinates, or trachea of this pig, nor was there any immunoreactivity to anti-IAV antibodies when immunohistochemistry was conducted on these tissues. Based on these findings, it has been determined that pig ID 329 was truly negative for IAV and that the false positive result was most likely due to a sampling error. Therefore, this nasal swab result was not used in the aforementioned comparisons.

In the current study, IAV was detected in oral fluids by real-time RT-PCR but not by virus isolation, even after 2 passages on MDCK cells. Several factors may have contributed to this result, such as inactivation by a naturally occurring enzyme or other component of oral fluids, high real-time RT-PCR Ct values (suggestive of low quantity and quality of viral RNA in the sample), and the prolonged oral fluid sample collection times (6 and 12 hr). The recommended oral fluid sample collection time is 30 min (Prickett et al. 2008a; Prickett et al. 2008a; Prickett et al. 2008c; Prickett et al. 2008d).

Salivary glycoprotein-340 (gp-340) and mucin (specifically, MUC5B; White et al. 2009) present in human oral fluids have significant inhibiting and neutralizing activities against IAV strains. Glycoprotein-340 is present in saliva and bronchoalveolar lavage fluid of human beings and has been shown to directly inhibit influenza viruses by binding the α(2,3)-linked sialic acid on the surface of gp-340 to HA on the surface of the virus (Hartshorn et a. 2003). It was also shown that the concentration of the α(2,3)- and α(2,6)-linked sialic acid on the surface of gp-340 varies between people (Harshorn et al. 2006). Such protein has not been examined in swine saliva, but human gp-340 has been shown to have inhibitory activity against strain A/Swine/Iowa/3421/1990 H1N1 (Harshorn et al. 2006). The protein MUC5B has similar effects on HA as gp-340, but does not have the same inhibitory effect that gp-340 has on neuraminidase (White et al. 2009). Inhibition of the virus by components found in normal oral fluids was addressed by the validation of the virus isolation procedure on oral fluids. Influenza A virus was detectable in experimentally spiked oral fluids, albeit at a lower titer after several days, which suggests that the components in swine oral fluids do not completely inhibit viral growth.

The real-time RT-PCR Ct value may be used as a semiquantitative indicator for the amount of viral RNA present in the sample (Kubista et al. 2006). The lung samples collected at necropsy at 3 and 7 DPI that were positive by both real-time RT-PCR and virus isolation had Ct values ranging from 21.94 to 33.56 whereas the lung samples that were positive by real-time RT-PCR but negative by virus isolation had Ct values ranging from 29.12 to 34.74. Of these samples, the highest Ct value (28.41) with a measureable virus titer ($10^{2.5}$ $TCID_{50}$/ml) for strain IA/04 H1N1 was from a lung sample at 3 DPI. The Ct values of the oral fluids ranged from 27.29 to 34.75, which overlaps the range for which the virus was inconsistently isolated from the lung tissue. Since real-time RT-PCR was not performed on the nasal swabs that were positive by virus isolation, there are no Ct values to compare to the nasal swab titers and the Ct values of the oral fluids. However, based on the lung results and the amount of RNA present in oral fluids estimated by the Ct values, the virus should have been isolated from at least one of the oral fluid samples if it were viable. This may indicate a lower sensitivity of virus isolation from oral fluids compared to lung samples.

It is possible that the environment played a role in decreasing virus viability. It had been determined in previous experiments that cotton ropes collected the most fluid. Although IAV has been shown to remain viable for 24–48 hr on hard, nonporous surfaces, it becomes inactivated in less than 12 hr on highly porous surfaces such as cloth (Bean et al. 1982). Due to non availability of personnel, the pigs were allowed to chew on the ropes for 6 or 12 hr instead of the recommended 30 min. It is possible that sample collection times of 6 and 12 hr using a porous cotton rope created an unfavorable environment for the virus to remain viable. Since there is potential in the field that a sample will be collected on a rope but not immediately extracted, further studies are needed to determine if the delay in extraction will affect virus viability. In the present study, shorter collection periods were examined, and it was determined that 20–30 min is adequate to obtain a sample of at least 5 ml from a group of 6–12 pigs weighing less than 25 kg (data not shown).

The real-time RT-PCR sensitivity assays for strain IA/04 H1N1 Ct values ranged from 23.16 for the first dilution (3.16×10^5 $TCID_{50}$/ml) in oral fluids to the highest

readable value of 39.43 for the fifth dilution (3.16×10^1 $TCID_{50}$/ml). Similarly, the real-time RT-PCR sensitivity assays for the combined strains MN/07 H1N2 and KS/08 H3N2 Ct values ranged from 26.44 for virus titer of 1.77×10^1 $TCID_{50}$/ml to 36.27 for the titer of 1.77×10^1 $TCID_{50}$/ml. While the IA/04 H1N1 assay had a range greater than 16 cycles, as would be expected for a dilution spanning 5 logs (Yang et al. 2002), the combined virus assay had a range of less than 10 cycles. This difference suggests that a potential inhibitor exists within the oral fluids that could be reducing the PCR efficiency (Kubista et al. 2006). Consequently, estimation of titers and the amount of RNA present in the sample using these Ct values may not be reliable. Further studies will need to be conducted to determine the cause of this variation in oral fluids.

Pen-based and individual oral fluid samples collected in the field using the recommended 20–30 min sampling time (Prickett et al. 2008a; Prickett et al. 2008a; Prickett et al. 2008c; Prickett et al. 2008d) were tested for PCV-2, PRRSV, and IAV strains. Viruses were detected in oral fluid samples collected in the field by veterinarians and farm staff demonstrating the practicality and use of these fluids for diagnostic tests. In summary, oral fluids provide a noninvasive sample that requires minimal training and fewer samples per group resulting in a lower overall cost when compared to individual animal samples. The results of the experimental and field samples showed that oral fluids can be used for testing groups of swine for the presence of IAV as has been shown for PRRSV and PCV-2 ((Prickett et al. 2008a; Prickett et al. 2008a; Prickett et al. 2008c; Prickett et al. 2008d). Additional procedures are available for testing oral fluids, such as IAV subtyping and sequencing of the virus strains using RT-PCR. However, the limited ability to isolate the virus from oral fluids could be a pitfall of the technique since viral isolates are particularly helpful when sequencing an influenza virus strain and are necessary for production of reagents for vaccines and diagnostic tests. Despite limited virus isolation, the ease of use and availability of PCR tests for multiple pathogens should be useful for disease surveillance projects and herd monitoring programs.

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Table 2.1 Detection of *Influenza A virus* using real-time RT-PCR* and virus isolation on Madin-Darby canine kidney cells for nasal swabs collected on study days 3 and 7 from pigs that were inoculated, mock-inoculated or noninoculated. †

Group no.	Treatment†	Virus isolation		Real-time RT-PCR‡		Cumulative result	
		Day 3	Day 7	Day 3	Day 7	Day 3	Day 7
1	Inoculated	6/6	2/6	NA	2/4	6/6	4/6
2	Inoculated	5/6	2/6	0/1	0/4	5/6	2/6
3	Inoculated	5/6	5/6	0/1	0/1	5/6	5/6
4	Inoculated	5/6	2/6	0/1	0/4	5/6	2/6
1–4	Inoculated	21/24	11/24	0/3	2/13	21/24	13/24
5	Mock inoculated	0/6	1/6§	0/6	1/6§	0/6	0/6
6	Noninoculated	0/6	0/6	0/6	0/6	0/6	0/6
5–6	Negative controls	0/12	0/12	0/12	0/12	0/12	0/12

* RT-PCR = reverse transcription polymerase chain reaction; NA = not applicable.
† Animals were inoculated with strain IA/04 H1N1 or minimal essential medium intratracheally, or not inoculated.
‡ Only the nasal swabs that were negative by virus isolation were retested by real-time RT-PCR.
§ It was concluded that this pig was truly negative by real-time RT-PCR and virus isolation results for the lung tissue collected at the same time as the nasal swab (at necropsy), the lack of gross or microscopic lesions in the lung, nasal turbinates and trachea, and the lack of immunoreactivity in the respiratory tissues to anti–*Influenza A virus* antibodies.

Table 2.2 Detection of *Influenza A virus* using real-time RT-PCR* and virus isolation on Madin-Darby canine kidney cells for in oral fluids collected on study days 3, 4, 5, and 6 from pigs that were inoculated, mock-inoculated or noninoculated. †

Group no.	Treatment†	Virus isolation‡				Real-time RT-PCR§			
		Day 3	Day 4	Day 5	Day 6	Day 3	Day 4	Day 5	Day 6
1	Inoculated	–	–	–	–	+(32.58)	+(33.82)	+(31.75)	+(29.62)
2	Inoculated	–	–	–	–	+(34.75)	+(33.16)	+(29.68)	+(31.11)
3	Inoculated	–	–	–	–	–(>40)	+(33.87)	+(30.15)	+(32.24)
4	Inoculated	–	–	–	–	+(33.60)	+(34.40)	+(27.58)	+(27.29)
5	Mock inoculated	–	–	–	–	–(>40)	–(>40)	–(>40)	–(>40)
6	Noninoculated	–	–	–	–	–(>40)	–(>40)	–(>40)	–(>40)

* RT-PCR = reverse transcription polymerase chain reaction.
† Animals were inoculated with strain IA/04 H1N1or minimum essential medium intratracheally, or not inoculated.
‡ Virus isolation was positive (+) or negative (–) for virus-induced cytopathic effects.
§ The detection of real-time RT-PCR product was positive (+), suspect (S), or negative (–). Numbers in parentheses are cycle threshold values.

Table 2.3 The sensitivity of the real-time reverse transcription polymerase chain reaction technique was determined for oral fluids spiked with *Influenza A virus* strain IA/04 H1N1.*

Virus titer ($TCID_{50}$/ml)	Time at room temperature (25°C)					Time between freezing (-80°C) and thawing		
	1 hr	2 hr	3 hr	4 hr	5 hr	24 hr	48 hr	72 hr
3.16×10^1	–(>40)	S(38.20)	S(39.43)	S(37.64)	S(38.55)	–(>40)	–(>40)	S(38.73)
3.16×10^2	S(35.18)	S(35.82)	S(35.48)	S(35.01)	+(34.40)	+(33.72)	+(33.78)	+(33.77)
3.16×10^3	+(32.29)	+(32.45)	+(31.99)	+(31.85)	+(30.96)	+(30.62)	+(30.76)	+(30.43)
3.16×10^4	+(28.82)	+(28.73)	+(28.23)	+(27.89)	+(27.43)	+(27.17)	+(26.93)	+(26.66)
3.16×10^5	+(25.10)	+(24.88)	+(24.52)	+(24.39)	+(23.84)	+(23.91)	+(23.31)	+(23.16)
3.16×10^6†	+(19.41)	+(19.29)	+(19.00)	+(18.87)	+(19.4)	+(17.78)	+(17.26)	+(17.39)

* $TCID_{50}$ = median tissue culture infective dose. The original titer was calculated to be $10^{6.5}$ $TCID_{50}$/ml (or 3.16×10^6 $TCID_{50}$/ml). The detection of real-time reverse transcription polymerase chain reaction product was positive (+), suspect (S), or negative (–).
† Undiluted virus.
Numbers in parentheses are cycle threshold values.

Table 2.4 The sensitivity of the real-time reverse transcription polymerase chain reaction technique was determined for oral fluids spiked with *Influenza A virus* strains MN/07 H1N2 and KS/08 H3N2.*

Virus titer ($TCID_{50}$/ml)	Time at room temperature (25°C)					Time between freezing (-80°C) and thawing		
	1 hr	2 hr	3 hr	4 hr	5 hr	24 hr	48 hr	72 hr
1.77×10^1	+(32.57)	S(35.02)	S(35.05)	S(36.27)	+(34.90)	+(34.28)	+(34.90)	S(35.88)
1.77×10^2	+(33.08)	+(33.58)	+(33.52)	+(33.89)	+(33.70)	S(35.49)	+(34.85)	S(36.24)
1.77×10^3	+(34.15)	+(32.56)	+(33.54)	+(33.57)	+(30.94)	+(34.49)	+(33.50)	+(33.21)
1.77×10^4	+(32.59)	+(32.07)	+(32.63)	+(31.99)	+(31.33)	+(30.02)	+(29.57)	+(30.02)
1.77×10^5	+(30.90)	+(30.47)	+(30.51)	+(31.16)	+(30.28)	+(26.44)	+(26.76)	+(27.72)
1.77×10^6	+(26.94)	+(27.27)	+(26.99)	+(27.47)	+(30.28)	+(25.93)	+(26.02)	+(26.49)

* $TCID_{50}$ = median tissue culture infective dose. The original titer was calculated to be $10^{7.2}$ $TCID_{50}$/ml (or 1.77×10^7 $TCID_{50}$/ml). The detection of real-time reverse transcription polymerase chain reaction product was positive (+), suspect (S), or negative (–).
Numbers in parentheses are cycle threshold values.

CHAPTER 3

IN VIVO EVALUATION OF VACCINE EFFICACY AGAINST CHALLENGE WITH A CONTEMPORARY FIELD ISOLATE FROM THE ALPHA CLUSTER OF H1N1 SWINE INFLUENZA VIRUS

The material in this chapter has been prepared for publication:
Detmer SE, Gramer MR, King VL, Mather S, Rapp-Gabrielson VJ (2011) *In vivo* evaluation of vaccine efficacy against challenge with a contemporary field isolate from the alpha cluster of H1N1 swine influenza virus. Can J Vet Res.

Influenza A virus vaccines currently contain a mixture of isolates that reflect the genetic and antigenic characteristics of the currently circulating strains. This study was conducted to evaluate the efficacy of an inactivated, trivalent, swine influenza virus vaccine (Flusure XP®, Canada) in pigs challenged with a contemporary alpha cluster H1N1 field isolate of Canadian swine origin. Pigs were allocated to two treatment groups that were either intramuscularly injected with the vaccine or an adjuvant placebo at two-week intervals and inoculated intratracheally and intranasally with the challenge virus. Four negative control pigs were neither vaccinated nor challenged. Following challenge, swine were monitored for clinical signs of respiratory disease and nasal swabs were taken for virus isolation daily until 5 days post-challenge when all pigs were euthanized for necropsy. On the challenge day and 5 days after challenge, the vaccinated pig sera had reciprocal HI titers ≥ 40 for all vaccine viruses, but ≤ 20 to the challenge virus. There were gross lesions present in the lungs of all pigs that were inoculated with the challenge virus but lung lesions did not differ significantly between the placebo and vaccinated pigs. However, there virus shedding was significantly reduced in nasal secretions, lungs and bronchoalveolar lavage fluid (BALF) in the vaccinated pigs compared to the placebo pigs. These results indicate that swine vaccinated with Flusure XP® (Canada) were partially protected against experimental challenge with a swine alpha cluster H1N1 virus that is genetically similar to those currently circulating in Canadian swine.

3.1 Introduction

Influenza A viruses (IAV) are a major cause of acute respiratory disease in swine that occurs year-round and has a significant economic impact on production (Torremorell et al. 2009). The predominant IAV subtypes found in North American swine are H1N1, H1N2, and H3N2. Within these subtypes, the viruses can be further divided into related groups by the genetic and antigenic properties of the hemagglutinin (HA) gene. Vaccines are one of the principle control measures used in order to mitigate the effects of IAV infection in pigs. The vaccines that are commercially available for use in swine in the United States and Canada contain a mixture of two or more inactivated viruses with genetic and antigenic characteristics of the currently circulating strains. Since the predominant antibody response to these inactivated vaccines is to the HA proteins of the viruses (Kitikoon et al. 2008), the cross-reactive properties of the antibodies to the HA proteins in vaccines and the genetic relationships of IAV in swine are used to evaluate the vaccine viruses for potential cross-reactivity and protection.

Within the North American swine HA subtype 1 viruses (SwH1), phylogenetic and antigenic analyses have revealed the presence of four distinct genetic clusters referred to as α, β, γ, and δ (Vincent et al. 2006; Vincent et al. 2009a). The SwH1 α, β, and γ cluster viruses share common ancestry with classical swine influenza virus isolates such as the prototypical A/swine/Iowa/15/1930 H1N1 (Vincent et al. 2006). The SwH1δ cluster viruses are most closely related to human seasonal influenza A viruses and upon further investigation, it was revealed that there are two distinct subclusters, δ1 and δ2, which can have substantial differences in all eight viral genes, likely the result of two separate introductions from humans to pigs (Vincent et al. 2009a). Due to their different ancestry, there is variable antigenic cross-reactivity between α, β, and γ clusters, but limited to no cross-reactivity between the δ viruses and the other 3 clusters (Vincent et al. 2006; Vincent et al. 2009a). The Canadian human-like swine viruses are in the δ1 cluster and American human-like swine viruses are in both δ1 and δ2 clusters (Karasin et al. 2006; Vincent et al. 2009a). The 2009 pandemic virus (pH1N1) possesses a HA gene similar to SwH1γ cluster and was the predominant virus circulating in humans and pigs in North America during the pandemic and immediate post-pandemic periods (MRG and

SED, unpublished data). Prior to the introduction of pH1N1 to pigs in Canada and the U.S., the predominant strains circulating in U.S. swine were SwH1β, γ and δ viruses (Lorusso et al. 2011), while SwH1α and δ predominated in Canadian swine (Nfon et al. 2011). For this reason, a SwH1α virus was selected for the challenge strain.

The vaccines that are commercially available in North America contain inactivated IAVs representing the subtypes and genetic clusters currently circulating in North America. Although hemagglutination inhibition (HI) cross-reactivity and analysis of HA similarity are used as predictors of vaccine cross-protection, challenge studies in pigs using heterologous contemporary field isolates are the gold standard to evaluate vaccine efficacy against new strains. The present study was conducted to evaluate the efficacy of a vaccine containing a SwH1γ virus (A/swine/Iowa/110600/2000 H1N1) in pigs challenged with a contemporary SwH1α field isolate of Canadian swine origin (A/Swine/Illinois/02450/2008 H1N1).

3.2 Materials and methods

Viruses and sequence analyses

The commercially available vaccine used for this experiment, FluSure XP® (Pfizer Animal Health, Madison, NJ) contained three influenza viruses: A/swine/Iowa/110600/2000 H1N1 (IA00 H1N1), A/swine/North Carolina/031/2005 H1N1 (NC05 H1N1), and A/swine/Missouri/069/2005 H3N2 (MO05 H3N2). This is the vaccine formulation available in Canada (the product available in the U.S. also contains A/swine/Oklahoma/0726H/2008). The challenge virus, influenza A/swine/Illinois/ 02450/2008 H1N1 (IL08 H1N1), was selected from an influenza A virus archive at the University of Minnesota Veterinary Diagnostic Laboratory (UMVDL). The virus was originally isolated from a postmortem lung tissue sample collected from an 11-week-old pig that had severe respiratory disease. Sequencing of the hemagglutinin (HA) gene was performed on the original sample submission to the UMVDL using a viral RNA extraction kit (Applied Biosystems, Foster City, California, USA), a combination of universal (Hoffman et al. 2001) and custom made primers (available upon request), and a genetic analyzer (Applied Biosystems, Foster City, California, USA). Phylogenetic

analyses, molecular evolutionary analyses and antigenic analyses were conducted using MEGA 4 (Tamura et al. 2007). IAV sequences available in GenBank and the MVDL database were used for the alignments using Clustal W and the phylogenetic analyses. The evolutionary history was inferred using the Neighbor-Joining method and evolutionary distances were computed using the Maximum Composite Likelihood method with Kimura 2-parameters (Kimura 1980; Felsenstein 1985; Saitou and Nei 1987; Tamura et al. 2007). Putative antigenic sites in the HA gene were identified by alignment to A/Puerto Rico/8/1934 (Caton et al. 1982; Qi and Lu 2006). The virus inoculum was prepared by propagation on Madin-Darby Canine Kidney (MDCK) cells (Meguro et al. 1979) and had a titer of $10^{5.5}$ $TCID_{50}$/ml as calculated by the Spearman-Karber method (Villegas and Alvarado 2008).

Serology

Serum samples were pretreated with receptor destroying enzyme (RDE) and turkey erythrocytes (Pedersen 2008a). Serial two-fold dilutions of the treated sera, from 1:10 to 1:640, were assayed for anti-influenza A antibodies by standard HI test with 4-8 hemagglutination units of virus and a 0.5% suspension of turkey erythrocytes (Pedersen 2008). The HI tests were performed against the challenge virus (IL08 H1N1) and the vaccine viruses (IA00 H1N1, NC05 H1N1, and MO05 H3N2).

Experimental design

Twenty-four 3-week-old pigs were purchased from Manthei Hog Farm, Elk River, Minnesota, USA and housed in an isolation facility at the University of Minnesota (St. Paul, Minnesota, USA). The animal phase of this study was conducted in accordance with the approval of the University of Minnesota Institutional Care and Use Committee. All pigs were clinically healthy upon arrival, free from infection with influenza A virus, *Mycoplasma hyopneumoniae* and porcine reproductive and respiratory syndrome (PRRS) virus, and had no serum antibodies against influenza A virus. All pigs were treated with a single intramuscular dose of ceftiofur crystalline-free acid (Excede®, Pfizer Animal Health, Kalamazoo, Michigan, USA) per label instructions prior to shipping to reduce bacterial contaminants prior to the start of the study. The animals were randomly assigned to treatments using a generalized block design that was based on body weight

and placed into assigned isolation rooms upon arrival to the facility (Table 3.1). The pigs were given free access to water and were fed an antibiotic-free, age appropriate diet ad libitum that met or exceeded NRC nutrient requirements.

On experimental day 0, the pigs in groups T01 and T02 received 2 ml of vaccine adjuvant alone or reconstituted vaccine, respectively, intramuscularly on the left side of the neck. The intramuscular injections for T01 and T02 were repeated 14 days later (day 14) on the right side of the neck. Pigs in the group NTX (n=4) were given neither treatment and served as the negative controls. Two of these pigs were housed in a separate room until the end of the study. Immediately prior to viral challenge, the two sentinel pigs in group NTX (*) which were housed in rooms 2 and 3 with the vaccinated pigs were removed for euthanasia and necropsy to demonstrate the health status of the pigs prior to challenge (Table 3.1). On experimental day 28 (two weeks following the second vaccination), all pigs in groups T01 and T02 were inoculated with 2 ml intratracheally and 2 ml intranasally of IL08 H1N1 while anesthetized with a single intramuscular dose (6.6 mg/kg) of tiletamine/zolazepam (Telazol®, Fort Dodge Animal Health, Fort Dodge, Iowa, USA). Two pigs were removed from the study due to a treatment allocation error, leaving nine pigs per treatment group.

Clinical observations, rectal temperatures, sampling and pathological examination

Personnel performing clinical and injection site observations, necropsy, and laboratory analysis of samples collected were blinded to the treatments given to the pigs. The pigs were examined daily throughout the study by a veterinarian for signs of lethargy, changes in respiration, appetite, injection site reactions and other changes in general health. The pigs were also examined for coughing, labored breathing, and nasal discharge from one day prior to challenge (day 27) until the end of the study (day 33). Rectal temperatures were measured and recorded starting two days prior to challenge (day 26) until the end of the study. Nasal swabs were collected from all animals one day prior to challenge to confirm the negative status of the pigs at challenge. Nasal swabs were collected every 24 hours after challenge (day 29-33). Serum was collected 12 and 2 days before the first vaccine (day -12 and -2), 1 day before the second vaccine (day 13), at challenge (day 28), and 5 days after challenge (day 33).

The two sentinel pigs in rooms 2 and 3 were removed prior to challenge and euthanized with a lethal dose of pentobarbital (Fatal-Plus Solution®, Vortech Pharmaceuticals, Dearborn, Michigan, USA) for necropsy. Five days post-challenge (day 33), all remaining pigs were euthanized with a lethal dose of pentobarbital for necropsy. All necropsies were performed immediately following euthanasia. The lungs were removed with the trachea attached and evaluated for the percentage of the lung affected with purple, lobular consolidation typical of influenza infection in swine. The percentage of consolidation for each lobe was calculated using weighted proportions of the total lung volume (Halbur et al 1995). The proportions were 10% each for the cranial, middle and accessory lobes, and 25% each for the caudal lobes for a total of 100%. The bronchoalveolar lavage fluid (BALF) sample was collected for virus isolation by clamping the cranial trachea, searing the outside of the caudal trachea with a hot spatula and using a sterile 14 gauge needle to inject 50 ml of Eagle's minimal essential medium (MEM) containing 4% bovine serum albumin, trypsin and antibiotics (MEM+) into the lungs. After gently massaging the lungs, approximately 25 ml of fluid was aspirated using the same needle and syringe. Two samples of the right medial lung lobe (or lesion, if present) were collected. One sample for influenza testing by RT-PCR and virus isolation and a second sample was fixed in 10% formalin for 48 hours and paraffin embedded for histopathology by standard techniques. The lung tissue was examined microscopically for bronchiolar epithelial changes and peribronchiolar inflammation in large, medium and small bronchioles. The following scoring stratagem was used: 0 = no airways affected, 1 = only a few isolated airways affected, 2 = localized cluster of affected airways (lobular), 3 = several airways affected throughout the section, and 4 = many airways affected, often severely (Richt et al. 2003). The scores were categorized for statistical analysis as 0-2 and 3-4.

Virus isolation and titration

Within 24 hours of collection, the nasal swabs were placed in a vial containing 2 ml MEM+, and vortexed for 30s, followed by centrifugation for 30 min at $2272 \times g$. Lung tissue was homogenized with MEM+ using a Stomacher® (Seward Laboratory Systems Inc., Bohemia, New York, USA) for 30s, followed by centrifugation for 60 min at

2272×*g* to collect the supernatant. Virus isolation was performed on all nasal swabs collected after challenge that were positive by matrix RT-PCR , as well as, all lung and BALF samples by inoculating the swab supernatant, lung homogenate or BALF on monolayers of MDCK cells grown in flasks with MEM+ media (Meguro et al. 1979). All cultures were incubated at 37°C under a 5% CO_2 atmosphere and examined daily for seven days under an inverted light microscope to observe cytopathic effects (CPE). Samples found positive by virus isolation were titered using 10-fold serial dilutions that were inoculated in 96-well plates containing monolayers of MDCK cells and using four 4 wells per dilution. After four days of incubation, the plates were assessed for cytopathic effects and virus titers calculated by the Spearman-Karber method (Villegas and Alvarado 2008).

The swab supernatant collected prior to the challenge (day 27) was tested by a matrix real-time RT-PCR procedure previously published by (Spackman et al. 2002; Spackman and Suarez 2008a) using magnetic bead RNA extraction (Ambion® MagMAX™, Applied Biosystems, Foster City, California, USA) and a real-time RT-PCR kit (Qiagen Inc., Valencia, California, USA). All swab supernatant, lung and BALF samples found negative by virus isolation were retested by the same matrix real-time RT-PCR procedure. Sequencing was performed on nasal swab viral isolates from one placebo pig and one vaccinated pig on day 32 (4 days post-inoculation) by extracting viral RNA from the MDCK cell fluids and the HA sequence was obtained using sequencing procedures as described above.

Statistical analysis

The rectal temperature, logarithm transformed virus isolation from nasal swabs and logarithm transformed antibody titer data was evaluated by a general linear repeated measures mixed model using SAS (SAS Institute, Cary, North Carolina, USA). The percentage of total lung with lesions, logarithm transformed area under the curve of virus isolation from nasal swabs, and logarithm transformed virus isolation from BALF and lungs were analyzed using a general linear mixed model. Microscopic lung lesions scores categorized as 0-2 or 3-4 were analyzed with a generalized linear mixed model with a logit link function and binomial distribution as well as an overdispersion parameter. A *P*-

value of <0.05 was considered to indicate a statistically significant difference between groups. All hypothesis tests were two-tailed.

3.3 Results

Viruses and sequence analyses

The inferred evolutionary history in the phylogenetic analysis of the HA gene sequences reveals that IL08 H1N1 clusters tightly within the swine hemagglutinin type 1 α-cluster (SwH1α) with isolates from Manitoba (Figure 3.1). In addition to the genetic similarity to Canadian swine origin viruses, there was an epidemiological link with the pigs in Illinois being geographically co-located with pigs imported from Canada. Pairwise distance analysis revealed that the challenge virus shares 99.7% nucleic acid similarity to A/swine/Manitoba/02414/2008 and 99.1% nucleic acid similarity to A/swine/Manitoba/01178/2006 and A/swine/Minnesota/01237/2006. Pairwise distance analysis of the challenge virus isolated on study day 32 from a vaccinated pig and placebo pig shows that there were no nucleotide changes in the hemagglutinin gene of the original virus isolate and after pig passage for both vaccinated and placebo pigs (Figure 3.1).

Phylogenetic analysis also reveals that the sequence of the SwH1α challenge virus has only 87.2% nucleic acid similarity to the SwH1γ vaccine virus (IA00 H1N1). This difference between the hemagglutinin gene of the challenge virus and the vaccine viruses is also demonstrated by comparing the number and positions of nucleotide changes within the putative antigenic sites (Table 3.2). This comparison reveals a total of 11 changes in antigenic sites between challenge virus and the SwH1γ vaccine virus (IA00 H1N1), and 24 changes between challenge virus and the SwH1δ vaccine virus (NC05 H1N1).

Antibody response to vaccination

The reciprocal HI titers for the three treatment groups and four viruses are shown in Figure 2. The reciprocal HI titers for all sera collected on day -12 and -2 were ≤ 10 (negative) against the vaccine and challenge viruses. The negative control and placebo pigs also had reciprocal HI titers ≤ 10 (negative) against the vaccine and challenge

viruses on day 13 and 28. The vaccinated swine had reciprocal HI titers ≥ 40 against the vaccine viruses and ≤ 20 against the challenge virus on Day 28 and 33 (0 and 5 DPC). For the SwH1γ vaccine virus, IA00 H1N1, there was no significant difference in HI titers between treatment groups on Days -2 and 13 ($p = 1.0$ and $p = 0.1002$), but there was a significant difference on Days 28 and 33 ($p < 0.0001$). For the SwH1δ vaccine virus, NC05 H1N1, there was no significant difference in HI titers between treatment groups on Day -2 ($p = 1.0$), but there was a significant difference on Days 13, 28 and 33 ($p = 0.0107$, $p < 0$ .0001 and $p < 0.0001$). For the SwH3 vaccine virus, MO05 H3N2, there was no significant difference in HI titers between treatment groups on Day -2 ($p = 1.0$), but there was a significant difference on Days 13, 28 and 33 ($p < 0.0001$). All swine had a reciprocal HI titer ≤ 20 against IL08 H1N1 on Day 33 (5 DPC) with no significant difference between treatment groups on Days -2 and 13 ($p = 1.0$), but there was a significant difference between treatment groups on Days 28 and 33 ($p = 0.0002$ and $p < 0.0001$).

Clinical signs and virus shedding after challenge

During the post-challenge period (day 29-33), mild clinical signs of respiratory disease were observed in five pigs (3 placebo and 2 vaccinated) for less than 24 hours; one of the vaccinated pigs that was observed coughing also had labored breathing. Fevers defined by a rectal temperature ≥ 40.1°C were observed for less than 48 hours in three pigs (33.3%) from the placebo group (T01). This was compared to no fevers in the vaccinated pigs (T02). Although the mean rectal temperature of the vaccinated pigs was consistently lower than the placebo group (Figure 3), there was no statistical difference between the two groups ($p > 0.05$).

Nasal swabs collected from all animals one day prior to challenge were negative by real time RT-PCR. The nasal swab virus titer for the vaccinated pigs (T02) was significantly lower than placebo (T01) at all time points except for Day 32 or 4 DPC (Figure 2). No virus was isolated from the nasal swabs of the vaccinated pigs (T02) on 5 DPC. All of the nasal swabs that were negative by virus isolation were also negative by real time RT-PCR. Geometric means of the area under the curve (AUC) of virus isolation from nasal swabs was significantly lower for the vaccinated pigs (T02) at 491.1

$TCID_{50}$/ml than it was for the placebo pigs (T01) at 13922.9 TCID50/ml ($p = 0.0055$). The BALF and lung tissue from the placebo pigs (T01) were all positive, whereas the BALF and lung tissue from the vaccinated pigs (T02) were all negative by virus isolation. In contrast, the geometric mean virus titer for the placebo pigs (T01) was 35535.9 $TCID_{50}$/ml in BALF and 1588.6 $TCID_{50}$/ml in lung, both significantly different from vaccinated pigs ($p < 0.0001$).

Gross lung lesions

There were gross lung lesions present in the lungs of all pigs that were inoculated with the challenge virus and there was no significant difference in gross lung lesions between treatment groups ($p = 0.4032$). Lung lesions consisting of multifocal, dense, purple areas of lobular consolidation, ranged from 2 to 34% of the total lung volume for both of the challenged groups. The right and left middle lung lobes were most often affected having on average more than half of the total lung lesions present. On average, more than half of the lung lesions observed were in the right and left middle lung lobes, making these the most often affected lobes. The back-transformed least square means of gross lung lesions for the placebo group (T01) was 12% with standard error of 5%. The back-transformed least square means of gross lung lesions for the vaccinated group (T02) was 9% with standard error of 4%. The four NTX pigs (two sentinels and two negative controls) had no macroscopic lung lesions.

Microscopic lung lesions

The acute lesions of purulent bronchiolitis that were observed in the epithelial lining of large, medium and small bronchioles were accompanied by loss of apical cilia, epithelial cell necrosis with intraluminal sloughing and attenuation, areas of epithelial hyperplasia in addition to transmigration of large numbers of neutrophils admixed with a few lymphocytes and macrophages. There was rare bronchiolitis obliterans in the most severely affected lung sections. Multifocally, there was peribronchiolar to lobular pneumonia characterized by moderate to large numbers of neutrophils, lymphocytes and macrophages within the alveolar spaces and occasionally expanding the alveolar septae. Five out of nine vaccinated pigs had a lesion score of 1 with only a few isolated airways affected compared to only one placebo pig. Three of the nine placebo pigs had the most

severely affected lung sections with a lesion score of 4. There was no significant difference between the categorized microscopic lung scores of the placebo (T01) group and the vaccinated (T02) group (p =0.1398). The NTX pigs (2 sentinels and 2 negative controls) had no microscopic lung lesions.

3.4 Discussion

Influenza vaccinations are an important part of a comprehensive herd plan to limit the effects of IAV infection by reducing clinical disease and virus shedding (Olsen et al. 2006; Torremorell 2009). Limiting the effects of infection in a swine herd will help mitigate the economic losses that are related to IAV infection. These losses can be significant, particularly in growing pigs, and are due to increased mortality, decreased feed efficiency, and treatment costs for secondary bacterial infections (Torremorell 2009).

The rapid evolution of influenza viruses over the last decade in North America, particularly of the SwH1 subtype, has resulted in remarkable genetic and antigenic variability. Since the efficacy of a vaccine is directly linked to genetic and antigenic matching of the vaccine virus to the challenge virus, the variability in circulating field strains is a major challenge for the North American swine industry. Cross-protection for this commercial vaccine, containing SwH1γ and SwH1δ viruses, has been demonstrated against challenge with SwH1γ virus A/swine/Minnesota/000581/2001 H1N1 and SwH1β virus PAH-NADC11 H1N1 demonstrating a 90.1% and 80.5% reduction in gross lung lesions, respectively (Rapp-Gabrielson et al. 2005). In other efficacy studies for this commercial vaccine, there were cross-reactive HI antibodies and significant reduction in gross lung lesions against challenge with classical swine H1N1 (cH1N1) virus A/swine/Iowa/40776/1992, and SwH1β virus A/swine/Minnesota/00040/2002 H1N1 demonstrated (Gramer and Rossow 2004; Kitikoon et al. 2006). In the most recent studies involving the 2009 pandemic virus (pH1N1) A/California/04/2009 H1N1, which contains a SwH1γ HA gene, the vaccine induced HI antibody titers of very low cross-reactivity yet vaccinated animals did have significant reductions in gross lung lesions and nasal swab virus titers (Vincent et al. 2010b).

The SwH1 β and γ-cluster challenge viruses in these published results were from early in the evolution of triple-reassortant swine viruses (1999-2002) and A/swine/Iowa/40776/1992, was a classical H1N1 virus from before the introduction of the triple reassortant internal gene (TRIG) cassette (Vincent et al. 2006). Since the viruses currently circulating in Canada with the cH1N1-like HA gene have the TRIG cassette, the present study was conducted to evaluate the efficacy of the commercial vaccine in pigs challenged with a contemporary SwH1α field isolate of Canadian swine origin, having classical-like swine H1 and N1 genes but with the triple reassortant internal gene (TRIG) cassette.

The origin of the challenge virus was determined by phylogenetic analysis of the HA gene sequence. The analysis revealed that IL08 H1N1 was indeed an α-cluster field isolate that was more genetically related (>99% nucleotide similarity) to isolates from Manitoba (MVDL influenza sequence database) than to influenza viruses previously isolated from Illinois. Further investigation revealed that the farm in Illinois where the isolate originated was surrounded by grower/finisher sites that had been recently populated with swine imported from Canada. Since none of the viruses isolated from this site or other sites within this multi-site production system are closely related to IL08 H1N1 by phylogenetic analysis, and although there was no virus available for comparison from the imported pigs, it is most likely that IL08 H1N1 is a virus of Canadian swine origin.

Vaccine-induced protection against influenza in the best case scenario should result in a reduction of gross and histopathologic lesions in the lungs, clinical signs and virus isolation in nasal swabs and BALF. The post-mortem examinations conducted on day 33 revealed that all challenged pigs, both vaccinated and placebo groups, had gross lesions present in the lungs and there was no significant difference between treatment groups ($p = 0.4032$). Two-thirds (6/9) placebo pigs had gross lesions present in 10% or more of the lung compared to only one-third (3/9) of the vaccinated pigs. There was also no significant difference between the categorized microscopic lung scores of the placebo pigs and the vaccinated pigs ($p = 0.1398$). Although the microscopic lesions were not significantly different between the placebo and vaccinated groups, it is important to note

that the moderate to severe lesions (scored 3-4) were found in the lungs of 77.8% (7/9) of the placebo pigs compared to only 22.2% of the vaccinated pigs.

The serology results indicate that all of the vaccinated pigs had a reciprocal HI titer ≥ 40 against the vaccine viruses, which is considered to be a protective titer (Hancock et al. 2009). However, the reciprocal HI titers to the challenge virus were ≤ 10 at the time of challenge, reflecting the HA sequence differences between the vaccine virus and this α-cluster SwH1N1 virus. Although virus was detected and lung lesions were present in almost all challenged pigs post-challenge, the infection induced only mild clinical signs that included fever of 24 to 48 hours duration in a few pigs and rare coughing. The virus titers of the nasal swabs taken during the challenge period (day 29-33) show that there was reduction in viral shedding for vaccinated swine compared to placebo pigs post-challenge and no virus was detected in the vaccinated pigs on day 5 post-challenge. There was also a significant difference between treatment groups for the geometric mean virus titer for both BALF and lung ($p < 0.0001$) with no virus being detected in the BALF or lung tissue collected from vaccinates on Day 33 (5 DPC). Thus, although the vaccine did not reduce lung lesions under the conditions of this study, it did reduce replication of the virus in the lung and nasal shedding.

In conclusion, the results indicate that vaccination with Flusure XP™ reduced virus shedding in nasal secretions, lungs and BALF in swine experimentally challenged with a SwH1α cluster virus that is genetically similar to those currently circulating in Canadian swine. This would indicate that the vaccine helped provide protection against this heterologous virus challenge.

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Table 3.1 Summary of the experimental design. The animals were allotted to three groups: no treatment and no challenge, vaccination and challenge, and vaccine adjuvant alone and challenge. Immediately prior to challenge, the no treatment pigs (*) in rooms 2 and 3 were removed for euthanasia and necropsy. Two weeks following the second vaccination, swine were inoculated 2 ml intratracheally and 2 ml intranasally with $10^{5.5}$ $TCID_{50}$/ml of influenza IL08 (H1N1), an alpha-cluster H1 of Canadian swine origin.

Room No.	Group	Treatment	No. swine	Challenge
1	NTX	No Treatment	2	No challenge
2	NTX	No Treatment	1	No challenge*
	T01	Vaccine	5	SwH1α
	T02	Adjuvant alone	5	SwH1α
3	NTX	No Treatment	1	No challenge*
	T01	Vaccine	4	SwH1α
	T02	Adjuvant alone	4	SwH1α

* removed for euthanasia and necropsy prior to challenge.

Table 3.2 Number and position of amino acid (AA) changes in putative antigenic sites for the HA1 of H1N1 vaccine strains and the challenge strain A/Sw/IL/02450/2008.

Antigenic site (total no. of AA residues)	A/Sw/IA/110600/2000		A/Sw/NC/031/2005	
	No. changes	Position(s)	No. changes	Position(s)
Sa (13)	4	125, 157, 161, 162	6	155, 157, 160, 161, 162, 164
Sb (9)	2	186, 190	8	153, 156, 186, 189, 190, 193, 194, 195
Ca (14)	4	137, 166, 170, 205	5	139, 142, 166, 170, 205
Cb (8)	1	73	5	69, 71, 72, 73, 74

Figure 3.1 Phylogenetic tree for the hemagglutinin gene segments based on nucleotide sequences of swine hemagglutinin subtype 1 viruses (SwH1) found in the UMVDL and GenBank databases, along with the sequences of the challenge virus (IL08 H1N1) before and after inoculation (◇), the SwH1γ vaccine virus (IA00 H1N1; ○), and the SwH1δ vaccine virus (NC05 H1N1; □). The four clusters of related viruses are indicated by brackets on the right of the tree as: SwH1α (cH1N1 or classical-like; ◆), SwH1β (reassortant H1N1-like; ■), SwH1γ (H1N2-like; ●), and SwH1δ (Human-like H1; ▲). There were 874 nucleotide positions in the final dataset, bootstrap test (1000 replicates) are shown next to the branches, and the reference viruses used are identified by GenBank accession numbers in parenthesis, when available.

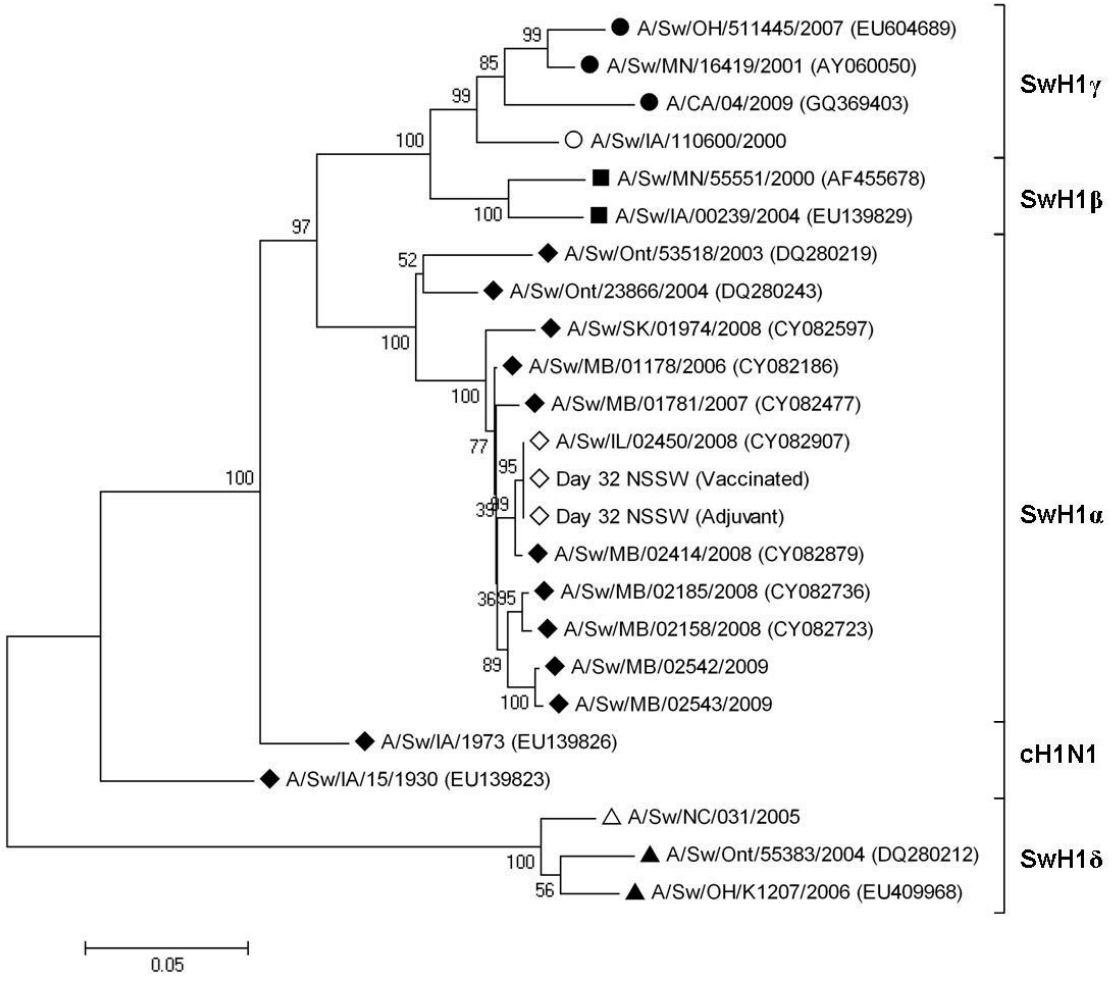


Figure 3.2 (a) The plot of back-transformed least-squares means of hemagglutination inhibition (HI) titers in HI units/50µl against A/Sw/IA/110600/2000 H1N1 from study day -12 to 33 for the negative control pigs (NTX, ●), placebo pigs (T01, ○), and vaccinated pigs ((T02, ▼). (b) The plot of back-transformed least-squares means of HI titers in HI units/50µl against A/Sw/NC/031/2005 H1N1 from study day -12 to 33 for the negative control pigs (NTX, ●), placebo pigs (T01, ○), and vaccinated pigs (T02, ▼). (c) The plot of back-transformed least-squares means of HI titers in HI units/50µl against A/Sw/MO/069/2005 H3N2 from study day -12 to 33 for the negative control pigs (NTX, ●), placebo pigs (T01, ○), and vaccinated pigs (T02, ▼). (d) The plot of back-transformed least-squares means of HI titers in HI units/50µl against A/Sw/IL/02450/2008 H1N1 from study day -12 to 33 for the negative control pigs (NTX, ●), placebo pigs (T01, ○), and vaccinated pigs (T02, ▼).

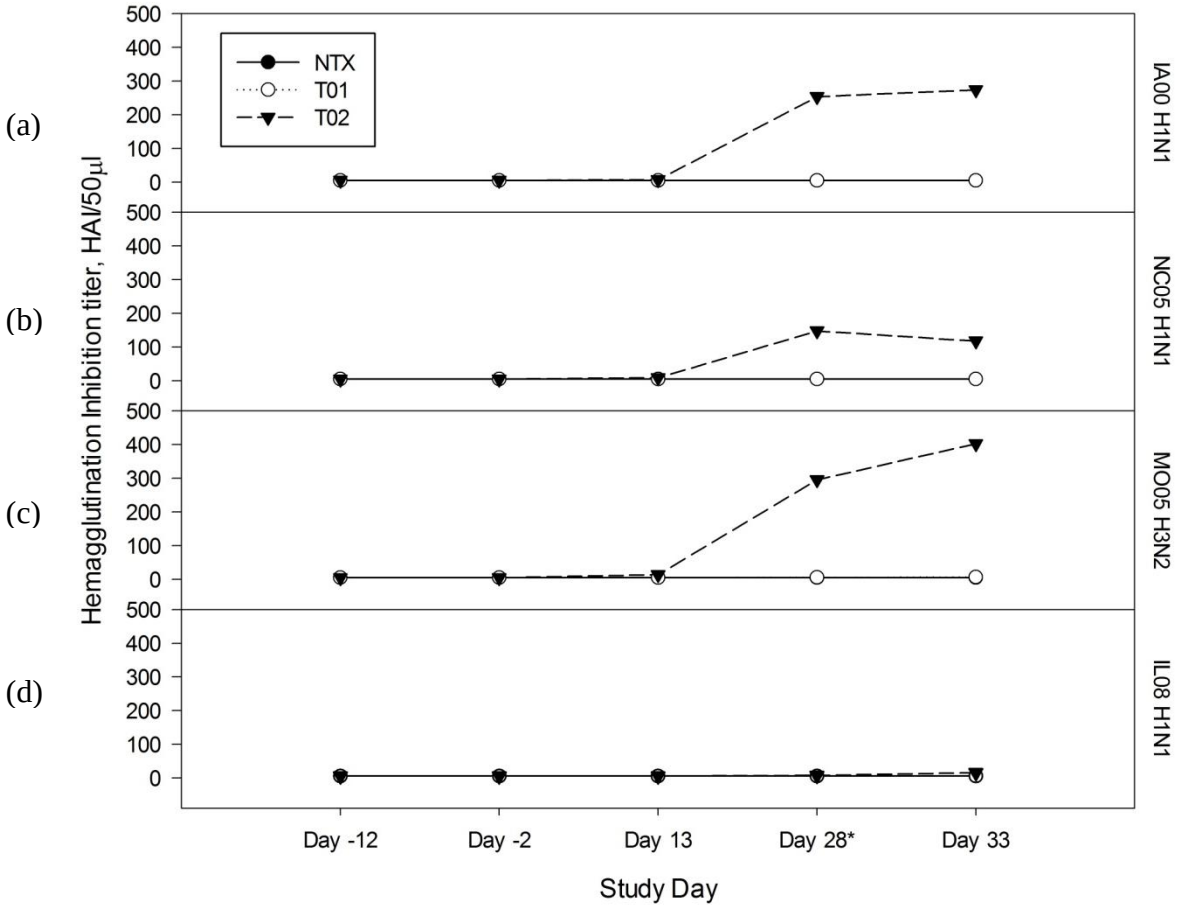
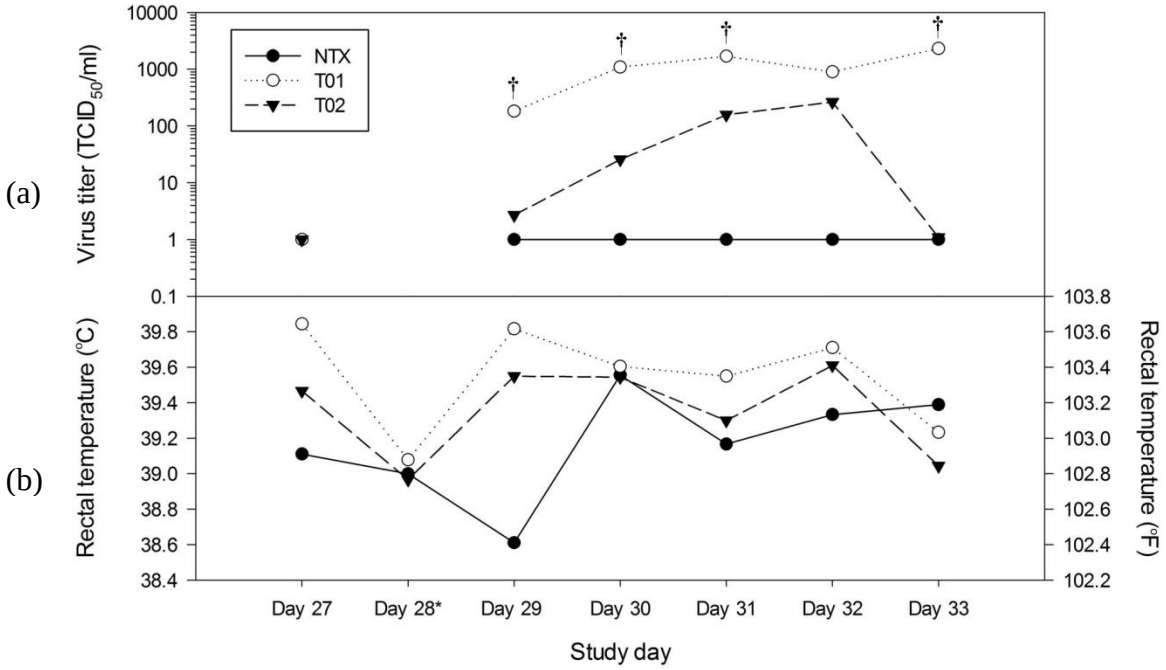


Figure 3.3 (a) The plot of back-transformed least square means of nasal swab virus titer in $TCID_{50}$/ml from study day 27 to 33 for the negative control pigs (NTX, ●), placebo pigs (T01, ○), and vaccinated pigs (T02, ▼). (b) The plot of least-squares means of rectal temperatures in degrees Celsius from study day 27 to 33 for the negative control pigs (NTX,●), placebo pigs (T01, ○), and vaccinated pigs (T02, ▼).



*Virus inoculation occurred on study day 28.
† Statistically significant difference in virus titer.

CHAPTER 4

MOLECULAR ANALYSIS OF VACCINE AND EPIZOOTIC VIRUS STRAINS OF INFLUENZA A VIRUSES IN SWINE

As influenza A viruses continue to emerge and evolve through reassortment and antigenic drift in the U.S. swine population, small genetic changes have resulted in viruses that evade the immune protection provided by highly similar autogenous vaccines. Here we present the molecular analyses of influenza A viruses isolated during an outbreak of respiratory disease in a group of nursery pigs where a herd-specific, inactivated, influenza virus vaccine had been used to immunize the dams of the infected piglets. To better understand how viruses evade vaccine protection, the vaccine (SG-238 and SG-239) and outbreak (SG-240) viruses were subjected to molecular characterization. The epizootic strain, SG-240, was most similar to SG-238 based on nucleotide sequences, in the hemagglutinin (98.8%), neuraminidase (98.7%), polymerase B2 (98.6%), polymerase B1 (99.0%), polymerase A (99.2%), and nonstructural (99.7%) gene segments. With the exception of nonstructural protein, which was identical between SG-240 and SG-239, these two viruses had significant differences at the nucleotide level for five other proteins. Although there was high nucleotide similarity between the vaccine virus SG-238 and the epizootic virus SG-240, most of the antibody induced by the inactivated vaccine virus would be primarily against the hemagglutinin protein where there was only one amino acid difference in an antigenic site between the two viruses. The molecular characterization tools used in this study provide benchmark for evaluating vaccine and epizootic viruses in swine populations that could be useful for screening viruses for genetic changes, comparing multiple viruses isolated from the same population over time and vaccine strain selection.

4.1 Introduction

The respiratory disease in a herd due to influenza A virus (IAV) infection causes increased susceptibility to secondary infections, spikes in morbidity and mortality and overall poor productivity, especially among the growing pigs. These can result in economic losses for a farm due to death losses, feed efficiency losses and increased treatment costs (Torremorell et al. 2009). Vaccination is one of the control measures that are used on swine farms to mitigate some of the losses due to IAV.

Vaccines that are sold in the United States for IAV are produced at establishments that are licensed through the United States Department of Agriculture (USDA); however, the vaccine itself falls into one of three licensure categories: fully approved, conditional licensure and autogenous. The vaccines that are usually referred to as "commercial" vaccines fall into the category of fully approved after the appropriate vaccine purity, potency, safety and efficacy studies have been conducted. The process for a vaccine to become fully approved usually takes 2-3 years and the viral isolates that are in these vaccines are owned by the company that manufactures them. Herd-specific or autogenous vaccines are also produced by companies under the regulation of the USDA. The regulations governing autogenous vaccines indicate their use when a fully approved commercial vaccine does not provide effective protection against a specific viral strain. The viral isolates that are in these vaccines are usually owned by the client who submits the tissue or virus to the company that makes the vaccine. Autogenous vaccines are to be made using one or more viral isolates from a farm or farm system and are solely for use on that farm or farm system.

IAV vaccines contain at least one virus and up to 5 viruses that have been inactivated. For commercial vaccines, the viruses represent strains in the North American swine population compared to the herd-specific strains found in autogenous vaccines. Since the viruses in these vaccines are inactivated, vaccines also contain an adjuvant to be delivered with the virus to enhance the immune response and a second injection or "booster" is performed in 2-4 weeks. These adjuvants are specifically developed by vaccine companies in order to obtain a prolonged immune response with minimal tissue

damage. Therefore, the adjuvant formulations vary among manufacturers and are usually considered to be proprietary information.

The efficacies of commercial vaccines are tested in vaccination-challenge studies. These studies are conducted on a regular basis by pharmaceutical companies on their own vaccines and by government entities when a novel strain becomes endemic in the swine population (Vincent et al. 2010b). Studies have also demonstrated that the genetic similarity between two strains of influenza virus does not guarantee cross-protective antibodies (Vincent et al. 2007; Vincent et al. 2008; Vincent et al. 2009a). However, since this option is expensive and requires special facilities, challenge studies are rarely performed on autogenous vaccines to verify efficacy of the vaccine. Therefore, the efficacy of most autogenous vaccines within a herd is usually unknown and even if the correct isolate was chosen for the vaccine, the changing nature of the virus and how the vaccination strategy is implemented on the farm present additional problems.

Whether commercial and/or autogenous vaccines are used on a farm, there are two main strategies for implementation of vaccination on a farm. The first strategy is to vaccinate the naïve young animals that do not have interfering maternal derived antibodies (MDA) around weaning time and two weeks later. The other strategy is to vaccinate the sows and protect their offspring via passive immunity through MDA in the colostrum. It has been demonstrated that during the first few weeks of life, MDA protect the young pigs from the clinical effects of an influenza infection (Blaskovic et al. 1970; Kitikoon et al. 2006). Unfortunately, while MDA can wane as early as 20-30 days (Blaskovic et al. 1970), MDA can also last up to 16 weeks (Olsen et al. 2006) and can interfere with vaccinations while present, resulting in a weaker immune response and incomplete protection (Loeffen et al. 2003).

This partial protection may actually provide pressure for the virus to change, but has not been closely examined in a population setting. Since antibodies are primarily produced against the surface protein hemagglutinin (HA), there is increased pressure on this protein to change in order to evade the immune system (Ferguson et al. 2003). This has been supported with phylogenetic analysis of selective pressure looking at positive selection of amino acid (AA) residues for HA (Shen et al. 2009). Changes in AAs occur

rapidly by antigenic shifts due to the acquisition of a novel surface protein through reassortment with another influenza virus. Gradual changes in the surface proteins occur by antigenic drift due to the accumulation of genetic mutations over time. Antigenic shift and drift are the sources of change that make influenza viruses a moving target for vaccine control strategies. These gradual changes can accumulate over time in HA and result in changes in antigenicity that can be detected by hemagglutination inhibition and serum neutralization (Vincent et al. 2006; Vincent et al. 2009a; Vincent et al. 2009b; Lorusso et al. 2011).

While antigenic and molecular characterization has been performed on IAVs circulating in North American swine (Vincent et al. 2006; Vincent et al. 2009a; Vincent et al. 2009b; Lorusso et al. 2011; Nfon et al. 2011) and passive immunity in pigs has been examined against homologous and heterologous IAV challenge (Blaskovic et al. 1970; Loeffen et al. 2003; Kitikoon et al. 2006), there is limited information on the molecular characteristics of the vaccine and epizootic viruses in the field. In the present study, an epizootic virus, A/Swine/Minnesota/001581/2007 H1N1 (SG-240), was isolated from a group of nursery pigs born to sows vaccinated with an autogenous vaccine containing isolates A/Swine/Minnesota/001200/2006 H1N1 (SG-238) and A/Swine/Minnesota/ 001482/2007 H1N2 (SG-239). The objective of this study was to perform molecular characterization of the epizootic and vaccine viruses in order to provide a benchmark for future evaluations of vaccine and epizootic viruses encountered in the field.

4.2 Materials and methods

Sample collection and virus selection

Nasal swab and lung tissue samples were collected for diagnostic purposes from the veterinarian on the farm and submitted to the University of Minnesota, Veterinary Diagnostic Laboratory (UMVDL) for IAV testing. Since these specimens were collected as part of routine veterinary care, approval was not sought from the University of Minnesota, Institutional Animal Care and Use Committee. The vaccine viruses, SG-238 and SG-239 were isolated at UMVDL from cases submitted by the veterinarian and provided to the autogenous vaccine manufacturer. The epizootic virus, SG-240, was

isolated from a 10-day-old pig born to a sow that had been vaccinated with SG-238 and SG-239 approximately one month prior to farrowing. Microscopic examination of the lung from this pig revealed purulent bronchopneumonia consistent with influenza infection. Although this pig was negative for porcine reproductive and respiratory syndrome virus, this pig was concurrently infected with porcine circovirus type 2 and aerobic culture of the lung yielded a light growth of *Streptococcus suis.*

Sample testing and sequencing

All samples were tested for IAV by a matrix real-time RT-PCR procedure (Spackman et al. 2002; Spackman and Suarez 2008a) using magnetic bead RNA extraction (Ambion® MagMAX™, Applied Biosystems, Foster City, California, USA) and a real-time RT-PCR kit (Qiagen Inc., Valencia, California, USA). Virus isolation was performed by inoculating the positive sample on monolayers of Darby Canine Kidney (MDCK) cells grown in flasks with Eagle's MEM containing 4% bovine serum albumin, trypsin, and antibiotics (Meguro et al. 1979). All cultures were incubated at 37°C under a 5% CO_2 atmosphere and examined daily for seven days under an inverted light microscope to observe cytopathic effects (CPE).

Sanger sequencing of the hemagglutinin (HA), neuraminidase (NA), polymerase B2 (PB2), polymerase B1 (PB1), polymerase A (PA), and nonstructural (NS) genes was performed using a viral RNA extraction kit (Applied Biosystems Inc., Foster City, California, USA), a combination of universal (Hoffman et al. 2001) and custom made primers (available upon request), using a genetic analyzer (Model 377; Applied Biosystems, Perkin-Elmer, Foster City, CA) and a Taq Dye Deoxy terminator cycle sequencing kit (Applied Biosystems, Perkin-Elmer, Foster City, CA) as previously described (Hoffman et al. 2001). Phylogenetic analyses, molecular evolutionary analyses and antigenic analyses were conducted using MEGA 4 (Tamura et al. 2007). Sequences were assembled with Lasergene SeqMan Pro (DNASTAR, Madison, WI) and visually checked. IAV sequences available in GenBank and the sequences obtained in this study were used for the alignments using Clustal W and the phylogenetic analyses. The evolutionary history was inferred using the Neighbor-Joining method and evolutionary distances were computed using the Maximum Composite Likelihood method (Felsenstein

1985; Saitou and Nei 1987; Tamura et al. 2007). Bootstrap values (1000 replications) were used. Putative antigenic sites in the HA gene were identified by alignment to A/Puerto Rico/8/1934 H1N1 (Caton et al. 1982; Qi and Lu 2006). A three-dimensional model of the hemagglutinin protein was examined in Pymol using PDB 1RUZ, the protein structure for A/South Carolina/1/1918 H1N1 (Gamblin et al. 2004).

4.3 Results

Full length gene sequences were obtained for the 3 polymerase genes, HA, NA and NS by the traditional Sanger method and used for molecular characterization. The HA nucleotide similarity of the epizootic virus SG-240 to the vaccine viruses was 99.1% for SG-238 with 3 AA differences and 68.2% for SG-239 with 104 AA differences. Sequence analysis of the HA protein sequence revealed that the AA difference between SG-238 and SG-240 were: T72A, A120V, and L337I with T72A being the only AA difference within an antigenic site (Table 4.1; Figure 4.1). Phylogenetic analysis of the HA gene revealed that the H1N2 vaccine virus, SG-239, clusters within the delta 1 cluster of swine H1 viruses, which originated from human seasonal viruses between 2003 and 2005 (Vincent et al. 2009a; Nelson et al. 2011). The H1N1 vaccine, SG-238, and the epizootic strain, SG-240 clustered tightly together in the beta cluster of the swine H1 viruses (Figure 4.2).

The NA gene segment analysis revealed that SG-240 and SG-238 had 98.7% nucleotide similarity with 8 AA differences in the translated proteins. SG-240 and SG-239 were 54.4% similar with 272 AA differences. The NA gene of SG-240 and SG-238 were of swine N1-lineage and the NA gene of SG-239 was of human N2-lineage, as revealed by phylogenetic analysis (Figure 4.2).

Phylogenetic analysis of the three polymerase genes revealed that PA and PB2 were of avian-origin and PB1 was of human-origin, as seen with swine viruses containing the triple reassortant gene cassette (Figure 4.3). The PB2 nucleotide sequence of SG-240 was 98.6% similar to SG-238 and 98.1% similar to SG-239 with 14 and 18 AA differences, respectively. One of the key changes in PB2 associated with virulence is at position 627. The human-lineage residue, lysine (K), at this position has been shown to

have the most effect on increasing IAV virulence for H5N1 and pH1N1 viruses compared to the avian-lineage glutamic acid (E) residue (Maines et al. 2011; Ozawa et al. 2011). It has also been shown that K591R and D701N can compensate for K627E (Yamada et al. 2010; Ozawa et al. 2011). All three viruses had the avian-lineage 627E, but did not have the compensatory 591R or 701D seen with virulent strains of triple-reassortant swine viruses (Lorusso et al. 2010).

The PB1 genes were human H3-lineage and the nucleotide sequence of SG-240 was 99.0% similar to SG-238 and 98.9% similar to SG-239 with 5 and 8 AA differences, respectively. All three viruses have a functional PB1-F2 gene with the human-lineage 66N seen with low pathogenicity H5N1 (Lorusso et al. 2011; Conenello et al. 2007). The PA nucleotide sequence of SG-240 was 99.2% similar to SG-238 and 98.9% similar to SG-239 with 6 AA differences for both comparisons. SG-238 and SG-239 had 241C found in human and Hong Kong pandemic-lineage viruses compared to the swine-lineage 241Y of SG-240 (Schultz et al. 1991), and SG-238 and SG-240 had 262K of the avian and human-lineage compared to the Eurasian avian-lineage 262R of SG239 (Dunham et al. 2009). All three viruses had the 627G associated with the non-virulent strains of H5N1 (Maines et al. 2011).

The NS nucleotide sequence of SG-240 was 100% similar to that of SG-239 and 99.7% similar to that of SG-238. These genes were within the classical swine cluster of NS genes (Figure 4.3). All three viruses had 103F and 106M AA residues that have been associated with increased viral replication (Tscherne and Garcia-Sastre 2011) and 128I associated with the less virulent strains of H5N1 (Maines et al. 2011).

4.4 Discussion

In this study, an IAV was isolated during an outbreak of respiratory disease where a herd-specific vaccine had been used to immunize the dams of the infected piglets. The epizootic virus isolated during the outbreak, SG-240, and the viruses in the vaccine, SG-238 and SG-239 were subjected to molecular characterization. The results of the molecular characterization showed that there was high nucleotide similarity between SG-238 and SG-240 and low similarity between SG-239 and SG-240.

Ideally, the complete genome of these viruses would be compared for more comprehensive analysis of IAV evolution of the selected viruses. However, this was not possible. The whole genome sequencing using the Sanger method was initially attempted, but the final sequence had numerous gaps that could not be filled. This sequencing was attempted using a low passage virus and the amount of virus in the sample was not measured either by RT-PCR or by hemagglutinating titer (HA titer). Additionally, the virus had been subjected to multiple freeze thaw cycles, which can damage the RNA. The viruses were regrown for the second attempt at sequencing using Next Generation Illumina® sequencing as passage 2 viruses that were frozen at -80°C in aliquots to prevent multiple freeze thaw cycles. The vaccine viruses were successfully sequenced using this method. However, since the epizootic virus, SG-240, had an HA titer of less than 1:64, sequencing was unsuccessful for this virus. A third and final attempt to sequence these viruses is currently underway at the J. Craig Venter Institute in Bethesda, Maryland. Passage 2 viruses were grown on MDCK cells at sufficient quantities verified by HA titer and RT-PCR Ct values for this attempt and results are currently pending.

Since full length gene sequences were obtained for the HA, NA, PB2, PB1, PA, and NS by the Sanger method, these sequences were used for molecular analysis of the three viruses with particular focus on the HA gene. The predominant antibody response to inactivated IAV vaccines is to the HA proteins of the viruses (Kitikoon et al. 2008). Therefore, the cross-reactive properties of the antibodies to the HA proteins in vaccines and the genetic relationships of IAV in swine are used to evaluate the vaccine viruses for potential cross-reactivity and protection. Serum samples from neither the vaccinated sows nor their affected offspring were available for evaluation of the cross-reactive properties of the antibodies using hemagglutination inhibition titers. However, to better understand how the epizootic virus evaded vaccine protection, the vaccine (SG-238 and SG-239) and outbreak (SG-240) viruses were subjected to molecular characterization that included assessment of the HA antigenic sites.

The antigenic sites Ca, Cb, Sa and Sb for the influenza A/Puerto Rico/8/1934 H1N1 virus were initially determined using hybridoma antibodies, radioimmunoassay, and x-ray crystallography (Gerhard et al. 1981; Caton et al. 1981). Since then, these

antigenic sites have been examined in classical swine viruses, triple reassortant swine viruses, vaccine challenge studies and the 2009 pandemic viruses (Arora et al. 1997; Qi and Lu 2006; Krause et al. 2010; Kyriakis et al. 2010b; Lorusso et al. 2011). Although these studies demonstrate that the genetic homology with the challenge virus is not the ultimate predictor for IAV vaccine performance in swine, they do provide a method of HA gene analysis that is targeted toward the sites. It has been demonstrated that sites adjacent to antigenic sites of a human HA protein are heavily glycosylated, protecting these sites from antibody recognition resulting in a higher frequency of variation in the areas that are not protected by glycosylation, the antigenic sites (Das et al. 2010).

In the present study, evaluation of the antigenic sites revealed that there was only one AA change within an antigenic site between SG-238 and SG-240. T72A is within the Cb antigenic site which lies within the vestigial esterase domain distal to the HA receptor binding pocket in the three-dimensional protein structure (Caton et al. 1982; Qi and Lu 2006). Since changes within Cb can alter neutralizing antibody binding to the HA protein, it is possible that this single amino acid change resulted in the decreased ability of the vaccine to reduce clinical signs of influenza in pigs. The other two AA changes (A120V and L337I) were not in antigenic sites, but it should be noted that position 120, next to the Sb antigenic site, has some variability in AA (T, A, V, S or E can be found at site 120 for swine isolates of IAV). One study found the directional mutation E120T for host adaptation from swine to human at this site (Hu 2010).

The effect that these three AA changes in HA would have on transmission, and replication efficacy of the virus, particularly in the presence of immune protection induced by a similar vaccine virus has not been studied. It is possible that the small variations between SG-238 and SG-240 could result in a reduction in the protection provided by the autogenous vaccine. In this study, antibody levels were not measured in the 10-day-old pig from which the epizootic strain was isolated. Since maternally derived antibodies (MDA) only start to wane between 20 and 30 days of age (Blaskovic et al. 1970), the affected 10 to 16-day-old pigs on this farm should have still had sufficiently protective titers. In order to determine if a single amino acid change resulted in the decreased ability of the vaccine to reduce clinical signs of influenza in pigs, these viruses

should be examined in a vaccine-challenge study which would examine both passive immune protection with MDA and traditional vaccination of naïve animals at 2-week intervals. The effectiveness of the strategy of using MDA to protect young pigs against IAV infection has been brought into question (Kitikoon et al. 2006) and the results of a the follow-up vaccine-challenge study would provide additional data to advance knowledge in this area. There is an additional possibility that in this case the MDA resulted in enhanced pneumonia, which has been described for both MDA and vaccine induced antibodies (Kitikoon et al. 2006; Gauger et al. 2011). Analysis of cytokines and memory T cells from the follow-up study would provide additional knowledge of this phenomenon.

Successful influenza control strategies for swine require appropriate timing, dose, and strain selection for vaccines, reduction of bacterial co-infections, and practical biosecurity measures. When these components are in place and a virus "escapes" the immune response, it is assumed that vaccine "failure" has occurred. Molecular characterization tools, such as those used here, have provided useful information regarding the continual changes that are found in viruses isolated from the U.S. swine population. In a broader sense, sequencing viruses that are endemic in pigs can be useful for screening the circulating viruses for significant genetic changes, comparing multiple viruses isolated from the same population over time, as well as influencing proper vaccine strain selection. The changes described in this situation will provide a benchmark for evaluating vaccine and epizootic viruses in swine populations.

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Figure 4.1 (a) Molecular model of the hemagglutinin gene with the Ca, Cb, Sa and Sb antigenic sites labeled along with the amino acid residue variations between SG-238 and SG-240 at positions 72 and 120.

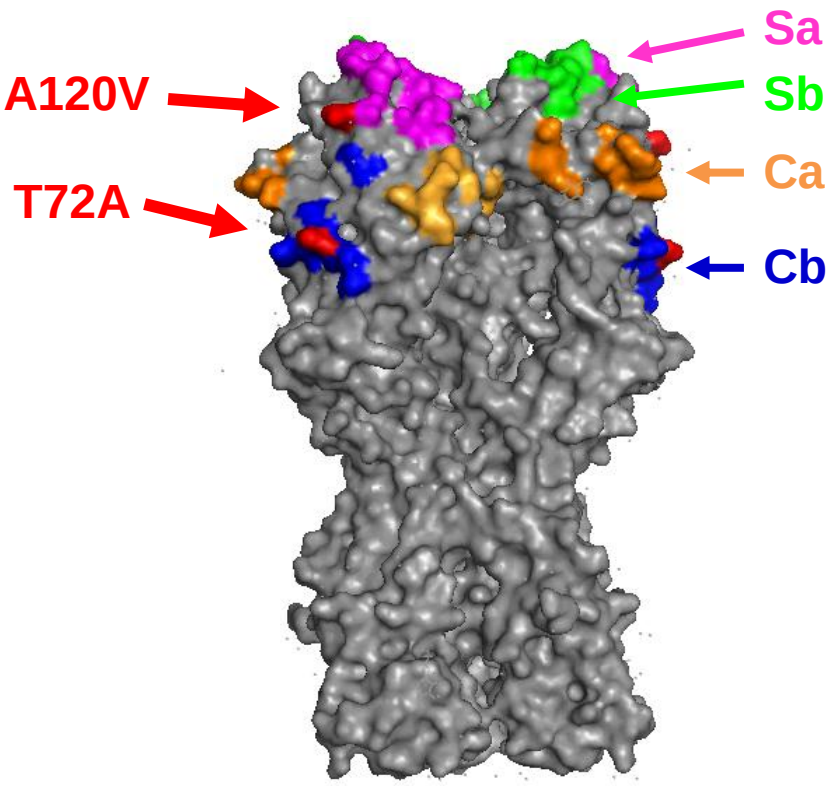


Table 4.1 Number and position of amino acid (AA) changes in putative antigenic sites for the HA1 of H1N1 vaccine strains SG-238 and SG-239, and the epizootic strain, SG-240.

Antigenic site (total no. of AA residues)	SG-238		SG-239	
	No. changes	Position(s)	No. changes	Position(s)
Sa (13)	0	n.a.	5	121, 155, 157, 160, 162
Sb (9)	0	n.a.	7	153, 156, 185, 189, 193, 194, 195
Ca (14)	0	n.a.	5	137, 138, 139, 142, 205
Cb (8)	1	72	5	69, 71, 72, 73, 74

n.a. = not applicable.

Figure 4.2 Phylogenetic trees for the swine H1 hemagglutinin (HA) clusters and human and swine neuraminidase (NA) proteins based on nucleotide sequences of SG-239 (▲), SG-238 (■) and SG-240 (□), along with reference sequences available in GenBank. The reference viruses used in the analysis have host abbreviations: *hu* human; *du* duck; *tu* turkey; *sw* swine and the GenBank accession number.

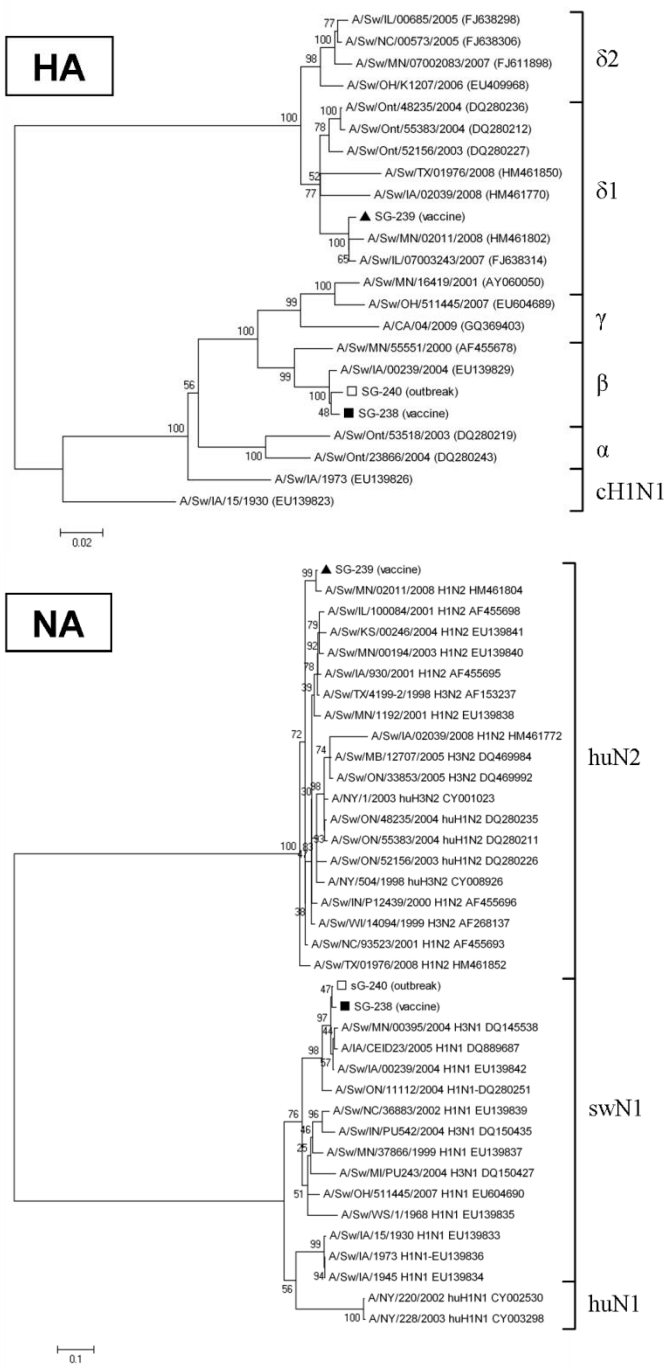
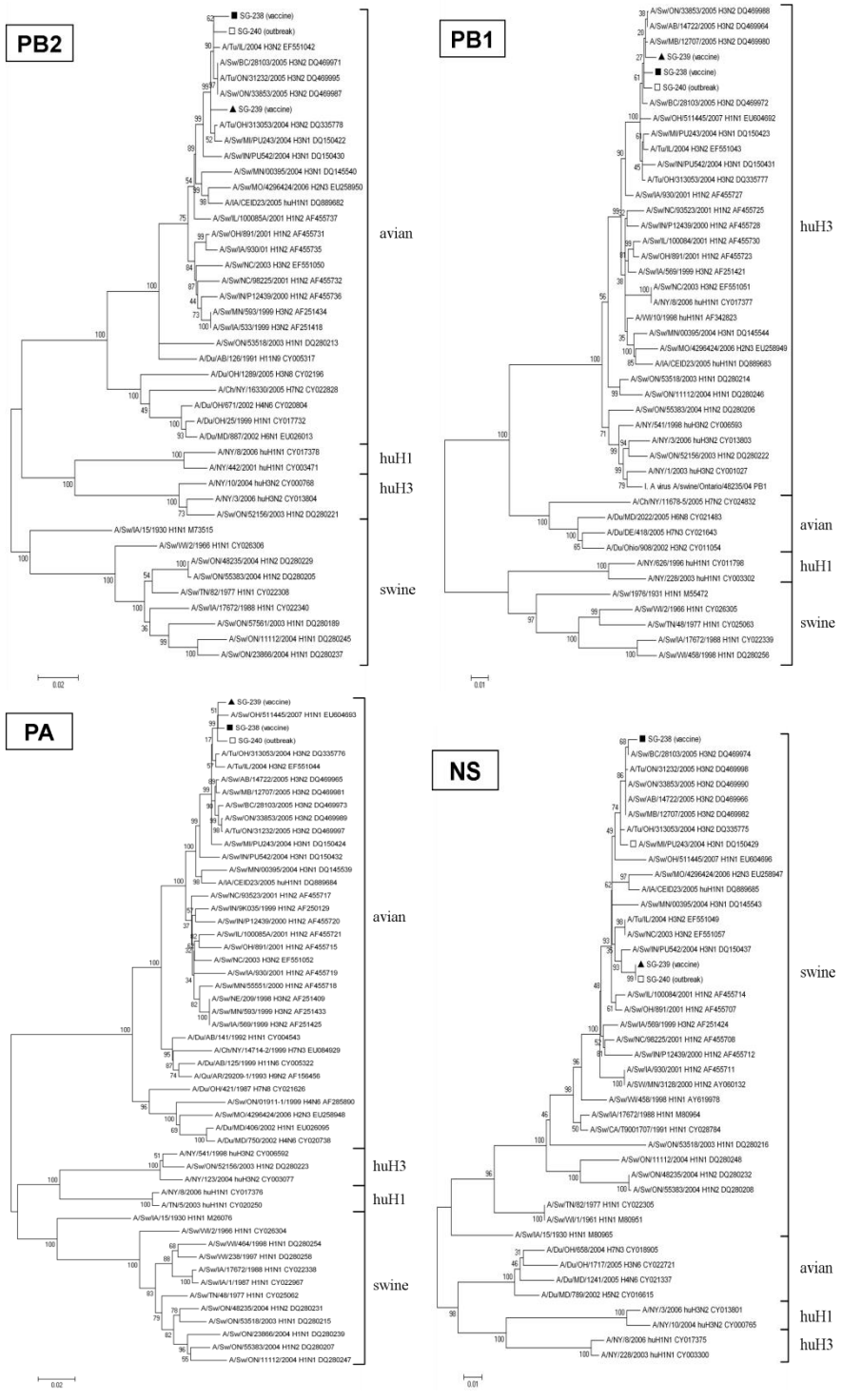


Figure 4.3 Phylogenetic trees for polymerase B2 (PB2), polymerase B1 (PB1) polymerase A (PA), and nonstructural (NS) proteins based on nucleotide sequences of SG-239 (▲), SG-238 (■) and SG-240 (□), along with reference sequences available in GenBank. The reference viruses used in the analysis have host abbreviations: *hu* human; *du* duck; *tu* turkey; *sw* swine and the GenBank accession number.



CHAPTER 5

MOLECULAR CHARACTERIZATION OF A VIRULENT INFLUENZA A VIRUS WITH A TWO AMINO ACID INSERTION IN HEMAGGLUTININ

The evolutionary trends of novel influenza A viruses (IAV) that have become endemic in North American swine have been studied at regional, national and global scales, but have not been well examined within a single swine production system or even a single farm. Thorough examination of endemic IAVs within a single production system at a molecular level would provide information critical for understanding the molecular epidemiology and evolution of IAVs in swine. In this study, eight viruses were selected from a single "swine flow" within a large multi-site production system that has conducted passive surveillance of IAVs since 2004. Five of the selected viruses contained a unique 2 amino acid insertion in the hemagglutinin protein within the Sb antigenic site and three of the selected viruses were potential ancestors to these insertion viruses. Molecular characterization of the selected viruses revealed up to five variations within the antigenic sites between the viruses. For two viruses the insertion was the only variation between ancestral and insertion virus. This was an interesting revelation for a virus that has demonstrated sustained transmissibility and fitness in swine over a three year period despite an alteration in hemagglutinin protein. The results of this study provide new understanding of IAV evolution in a single production system which can be used as a platform for future studies.

5.1 Introduction

Influenza A viruses (IAV) cause an acute respiratory disease in swine. Evolutionary analyses of IAV have demonstrated that the 1918 pandemic virus is the likely common ancestor for the classical swine and human-lineage viruses in North America and these classical H1N1 (cH1N1) swine viruses remained relatively stable in the swine population until 1998 (Vana and Westover 2008). However, in 1998 double-reassortant (human and swine-lineage) and triple-reassortant viruses (human, avian and swine-lineage) H3N2 viruses were first discovered in swine. Since then, triple reassortant IAVs have rapidly evolved through reassortment and antigenic drift, resulting in novel strains (Webby et al. 2004). These novel strains have resulted in changes in influenza epidemiology and persistence within endemically infected populations of pigs.

Most IAVs circulating in North American swine contain the triple-reassortant internal genes (TRIGs) comprised of human-origin polymerase B1 (PB1), avian-origin polymerase A (PA) and polymerase B2 (PB2), and classical swine-origin matrix (M), nonstructural (NS) and nucleoprotein (NP). The surface glycoproteins of IAVs, hemagglutinin (HA) and neuraminidase (NA) are important for virulence, host specificity, and the immune response (antigenicity). For the viruses that contain the TRIG constellation that became endemic within North American swine at the end of the 20th century (Webby et al. 2000), it has been shown that the match between HA and NA is an important factor in virulence and host specificity (Zhang et al. 2010). The predominant IAV subtypes of these swine viruses are H1N1, H1N2 and H3N2. However, within the H1 subtype alone, antigenic drift and shift have resulted in the formation of at least five antigenically and genetically distinct clusters known as α, β, γ, δ1, and δ2 (Vincent et al. 2009a; Lorusso et al. 2011; Nfon et al. 2011).

Using a defined population and by limiting both time and space parameters, it is possible to study the microevolution of IAVs in swine. In the present study, a novel IAV containing a unique two amino acid (AA) insertion in the HA gene was discovered within a single flow of a multi-site farm system. Genetic characterization of this virus over time will provide a benchmark to evaluate the past and future evolution of the virus in swine; critical information for understanding the epidemiology of the disease caused by the virus

within a farm or production system can be revealed when combined with clinical and post-mortem data.

5.2 Materials and methods

Virus selection

Nasal swab and lung tissue samples were collected for diagnostic purposes from swine on farm M and submitted to the University of Minnesota Veterinary Diagnostic Laboratory (UMVDL) for testing during the period of 2005 to 2009. Since these specimens were collected as part of routine veterinary care, approval was not sought from the University of Minnesota, Institutional Animal Care and Use Committee. All samples were tested for IAV by a matrix real-time RT-PCR procedure (Spackman et al. 2002; Spackman and Suarez 2008a) using magnetic bead RNA extraction (Ambion® MagMAX™, Applied Biosystems, Foster City, California, USA) and a real-time RT-PCR kit (Qiagen Inc., Valencia, California, USA). All samples that were positive by RT-PCR were submitted for sequencing and virus isolation. Virus isolation was performed by inoculating the positive sample on monolayers of Darby Canine Kidney (MDCK) cells grown in flasks with Eagle's MEM containing 4% bovine serum albumin, trypsin, and antibiotics (Meguro et al. 1979). All cultures were incubated at 37°C under a 5% CO_2 atmosphere and examined daily for seven days under an inverted light microscope to observe cytopathic effects (CPE). Sequencing of the HA gene was performed on the original sample submission to the UMVDL using a viral RNA extraction kit (Applied Biosystems Inc., Foster City, California, USA), a combination of universal (Hoffman et al. 2001) and custom made primers (available upon request), using a genetic analyzer (Model 377; Applied Biosystems, Perkin-Elmer, Foster City, CA) and a Taq Dye Deoxy terminator cycle sequencing kit (Applied BioSystems, Perkin-Elmer, Foster City, CA).

Clinicopathology

Based on initial HA sequences, eight viruses were selected from a single flow within the farm system for further analysis. Additional clinical information was obtained on the selected viruses and lung tissue was divided for aerobic bacterial culture and fixation in 10% formalin for 48 hours prior to being paraffin embedded for

histopathology by standard techniques. The lung tissue was examined microscopically for bronchiolar epithelial changes and peribronchiolar inflammation in large, medium and small bronchioles.

Sequencing and molecular analysis

The eight selected viruses were passaged on MDCK cells as described above (Table 5.1). A combination of Sanger and *de novo* Next Generation Illumina sequencing was performed using universal primers as described (Hoffman et al. 2001; Kuroda et al. 2010). Sanger sequences were assembled with SeqMan (DNASTAR, Madison, WI) and visually checked. Illumina sequences were assembled with Genomics Workbench (CLC bio, Cambridge, MA) and visually checked. Phylogenetic analyses, molecular evolutionary analyses and antigenic analyses were conducted using MEGA 4 (Tamura et al. 2007). IAV sequences available in GenBank and the sequences obtained in this study were used for the alignments using Clustal W and the phylogenetic analyses. The evolutionary history was inferred using the Neighbor-Joining method and evolutionary distances were computed using the Maximum Composite Likelihood method (Felsenstein 1985; Saitou and Nei 1987; Tamura et al. 2007). Bootstrap values (1000 replications) were used. Putative antigenic sites in the HA gene were identified by alignment to A/Puerto Rico/8/1934 (Caton et al. 1982; Qi and Lu 2006). A three-dimensional model of the hemagglutinin protein was examined in Pymol using PDB 1RUZ, the protein structure for A/South Carolina/1/1918 H1N1 (Gamblin et al. 2004).

5.3 Results

Virus selection

The HA gene sequence was obtained for 159 viruses of the H1 subtype from farm M between 2005 and 2009. In 13 of these viruses, a unique two amino acid (AA) insertion of lysine (K) and glutamic acid (E) was found at position 156 using the H1 numbering system. The insertion disrupted the Sb and Sa antigenic sites (Figure 5.1) which are proximal to the HA receptor binding pocket in the three-dimensional protein structure (Caton et al. 1982; Qi and Lu 2006). Positions 152 and 156 in the H1 numbering system have been associated with distinguishing avian and human influenza viruses from

those isolated from swine. Avian-like swine and classical swine viruses, usually contain valine (V) and asparagine (N), respectively, at positions 152 and 156 compared to avian viruses which contain threonine (T) at both sites (Fanning and Taubenberger 1999). The presumed ancestral viruses have V and N in these sites, but the insertion viruses have V and KEN at these sites (Figure 5.1).

In order to examine the gradual antigenic drift and to limit the potential of reassortment with viruses from outside the region, only barns within the same state receiving pigs from the same sow farm source were used. Eight of the 13 insertion viruses were from related farms within the same multi-site rearing system. Five of the related eight viruses were successfully grown on MDCK cells for full genome sequencing. Only two viruses were isolated within two weeks of each other in the same geographic area (SG-443 and SG-444). Additionally, three viruses that were highly similar to the insertion viruses, but did not have the two AA insertion were selected and grown for full genome sequencing. These viruses were isolated immediately prior to when the insertion first appeared and considered to be the likely ancestors to the insertion viruses (Table 5.1).

Clinicopathology

Additional clinical information was obtained on presumed ancestral and insertion viruses demonstrating that these viruses were associated with clinical signs of respiratory disease in weaned pigs that were 3-7 weeks old (average age 4.5 ± 1.3 weeks). The morbidity associated with these viruses was 15-85% and there was up to a 4% increase in mortality compared to the other groups of pigs placed in other nursery barns from the same sources at the same time. Lung tissue was available for assessment for all eight viruses. For both the insertion and ancestral viruses, microscopic examination of the lungs revealed moderate to severe purulent bronchopneumonia consistent with infection with IAV. Many of the lung samples had additional lesions consistent with secondary bacterial infections supported by isolation of bacteria. All lung samples were positive for bacterial growth by aerobic culture with *Haemophilus parasuis, Streptococcus suis* and *Pasteurella multocida* isolated most often. These viruses were classified as virulent based on the clinical and pathological assessment of these animals.

Genetic characterization

All eight segments were analyzed for molecular characteristics previously shown to carry virulence factors. Analysis of the antigenic sites in the HA protein revealed that the two AA insertion at position 156 was the only variation in the antigenic sites for two of the five viruses when compared to the ancestral viruses. One virus also had one AA change at position L70I. Two other viruses also had AA changes at S74G, A139D, K162M and I168T (positions 139, 162, and 168 are positions 138, 159 and 165 on virus A/Puerto Rico/8/1934 H1N1 used for H1 numbering alignment). Outside the antigenic sites, no AA variations were found in sites reported to be phylogenetically important regions and all eight viruses contained residues in the receptor-binding pocket that were typical of swine lineage viruses (Table 5.2). SG-445 and SG-1097b have the same HA sequence and these viruses are 99.8% similar to SG-1097a. The insertion viruses had 99.0-99.7% nucleotide similarity to SG-445.

The AA sequence of NA was identical for the presumed ancestral viruses and there were 2-6 AA differences between the ancestral and insertion viruses. The phylogenetically informative AA positions where these variations occurred were L15M, L85I, N221K, K222Q, K332E, I365N, and I389M (Table 5.2)(Fanning et al. 2000). The viruses were examined for the substitutions H274Y and N294S that have been described in association with oseltamivir resistance and found in 2008 swine viruses; the H274Y substitution was found in all of these viruses (Lorusso et al. 2011).

In PB1, the only variations were T257A and Q582K in SG-1150, and M317T in SG-1096. All of the viruses contained the full-length coding sequence for the pro-apoptotic PB1-F2 protein. At position 66 within the PB1-F2 protein, all viruses had N, compared to the S at this site that has been associated with increased virulence in the 1918 pandemic virus and H5N1 viruses (Conenello et al. 2007). In PB2, all insertion viruses had the A89V substitution, SG-444 had I30V, SG-1150 had T106A, SG-1096 and SG-1150 had V109I, SG-1096 had I461V, and SG-1094 had E677K. The basic amino acid at 591R has been found to compensate for 627E in the avian lineage PB2 gene found in triple-reassortant swine viruses (Yamada et al. 2010). The avian-lineage 701D, 627E and 591R were found in all viruses, with the exception of SG-443 having D701N. In PA,

all insertion viruses had the I322V substitution and SG-1096 had H437Y. With the exception of the 189D residue found in all of these viruses, the NS genes were similar to other viruses analyzed from 2008 (Lorusso et al. 2011).

Phylogenetic analysis

Phylogenetic analysis of the HA gene revealed that the insertion and ancestral viruses cluster tightly together with a well described pathogenic virus A/swine/Ohio/ 51145/2007 H1N1 in the SwH1 gamma cluster (Figure 5.2) (Ma et al. 2010). The NA, M, NS and NP genes are of swine-origin, the PA and PB2 are of avian-origin, and PB1 is of human-origin.

5.4 Discussion

Molecular characterization of influenza viruses has become one of the key tools used by researchers to examine the continual changes that are found in viruses isolated from swine populations. By sequencing the viruses that are endemic in pigs, we can screen the circulating viruses for genetic changes and compare multiple viruses isolated from the same population over time, as well as to viruses in available vaccines. This information is critical for understanding the epidemiology of the disease caused by the virus within a farm or production system, especially when the molecular data can be combined with clinical signs and post-mortem findings; providing a benchmark for evaluating IAV evolution in swine.

In this study, IAVs containing a unique two amino acid insertion at position 156 in the HA antigenic site Sb and the presumed ancestral viruses were studied for evolutionary changes over a three year period. There was minimal variability found in the AA sequences for all gene segments of these eight viruses with the most changes found in the last two viruses that were isolated (SG-1150 and SG-1094). The origin of the insertion is most likely a result of the error prone RNA polymerase, as the six inserted nucleotides are an exact repeat of the previous six nucleotides in this site. If this is the cause, there is no explanation why this insertion has not been seen before in any species, nor does it explain why this insertion has been maintained and the presumed ancestral virus has not been found since last isolated on January 16, 2008.

The evolutionary rate of HA has been estimated to be as low as 2×10^{-3} nucleotides/site/year for influenza B viruses and above 4×10^{-3} nucleotides/site/year for human H3N2 viruses (Hay et al. 2001). Since influenza viruses have a high rate of non-synonymous changes, the rate of change in H3N2 viruses translates to 5×10^{-3} AA/site/year (Hay et al. 2001). Although mutations in the HA gene resulting in AA insertion or deletion in the HA protein have been demonstrated in human and animal IAVs (McCullers et al. 1999; Webster et al. 2007), the rate of these events have not been determined. However, the insertion virus continues to be found every 2 to 3 months within this farm system, with the first isolation of this virus outside this system occurring on October 20, 2010. The virus isolated in October, 2010 was found in a geographic proximity to a farm within the original farm system with a nearly identical virus isolated four months earlier. The exact route that this virus traveled and was transmitted between the two farms has yet to be determined, and the undetected reservoir that harbors this virus between epizootics also remains to be determined.

The fact that the 2 AA insertion described in this study has been sustained in this virus and has resulted in a virus that continues to circulate in pigs after more than 3 years, is quite remarkable considering the location of this insertion. The functional HA protein product that studs the surface of the viral envelope is a trimer consisting of three identical HA proteins that form a cylindrical pocket in the distal protein surface where HA binds to sialic acid receptors on epithelial cells. Therefore, the 2 AA insertion that occurs at a site adjacent to the binding pocket would also be repeated three times in the three-dimensional (3-D) trimer (Figure 5.1b). The addition of a polar basic lysine (K) and a polar acidic glutamic acid (E) to an uncharged polar asparagine (N) in this site would result in a change in the overall 3-D conformation of the binding pocket. The exact change that this would cause would have to be confirmed by x-ray crystallography.

Changes in the 3-D shape of HA could result in increased or decreased binding of HA to sialic acid receptors and/or neutralizing antibodies. Receptor binding could be assessed by examining the ability of these viruses to agglutinate red blood cells from turkeys or chickens, by measuring the association constants (K_{ass}) using sialylglycopolymers conjugated with fetuin, and by glycan microarray. In order to

estimate the amount of virus present prior to sequencing, the hemagglutination (HA) titers and cycle threshold (Ct) values for the matrix gene RT-PCR test were measured. There did not appear to be a direct correlation between the HA and Ct values for these viruses (Figure 5.2). Antibody binding of HA can be assessed using cross-hemagglutination inhibition titers using these insertion and ancestral viruses and hyperimmune serum produced in either ferrets or pigs followed by antigenic cartography. Other functional studies would include measuring the replication kinetics of these viruses in MDCK cells and measuring protein expression with viral polymerase activity using ancestral and insertion viruses, as well as reverse genetics-derived syngenic strains. Additionally, the transmissibility, pathogenesis and fitness of the ancestral and insertion viruses, as well as reverse genetics-derived syngenic strains could be assessed *in vivo*.

Using molecular characterization, influenza researchers have started to examine the continually changing IAVs in swine populations. By sequencing the viruses that are endemic in pigs, we can screen the circulating viruses for significant genetic changes and compare multiple viruses isolated from the same population. For this reason, sequence analysis of HA has become routine practice for some swine farm systems that are endemically infected with influenza A viruses including the farm system where the unique 2 AA insertion described in this study was found. Retrospective analysis of the HA sequences from this farm revealed potential ancestral viruses that were isolated six months prior to when the viruses with the insertion were isolated. Additionally, it was found that all eight viruses characterized in this study were associated with elevated piglet mortality and severe pulmonary pathology. By limiting the scope of this study to a defined population and group of viruses, it was possible to study the microevolution of IAV in swine. The genetic characterization of these insertion and potential ancestral viruses over a three year period has provided new understanding the molecular epidemiology of IAV within a farm or production system. Future studies will add to this information and provide a benchmark to evaluate the past and future evolution of the virus in swine.

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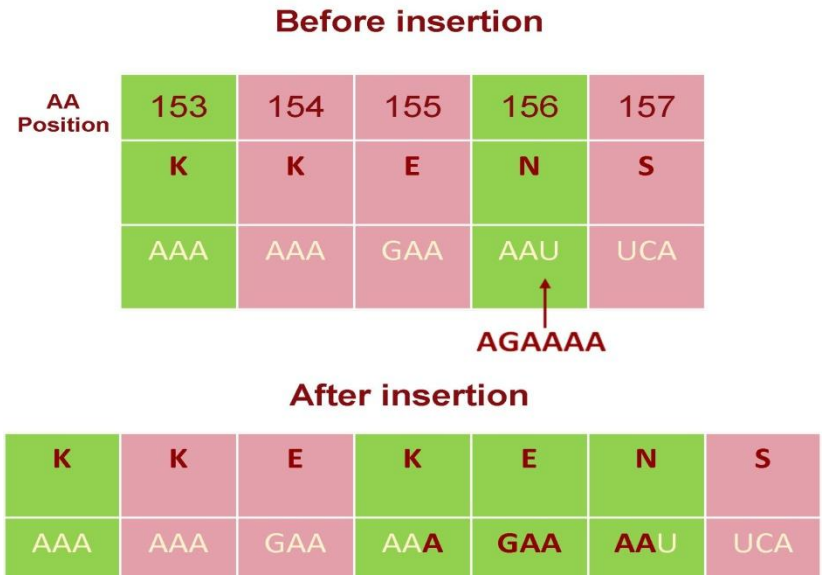
Figure 5.1 (a) Diagram of nucleotide and amino acid sequences before and after the 6 nucleic acid insertion. The Sa antigenic sites are 154, 155 and 157 and the Sb antigenic sites are 153 and 156. The insertion results in two additional amino acids within the Sb antigenic site. (b) Molecular model of the hemagglutinin gene with the Ca, Cb, Sa and Sb antigenic sites labeled along with the insertion site at position 156.

(a)



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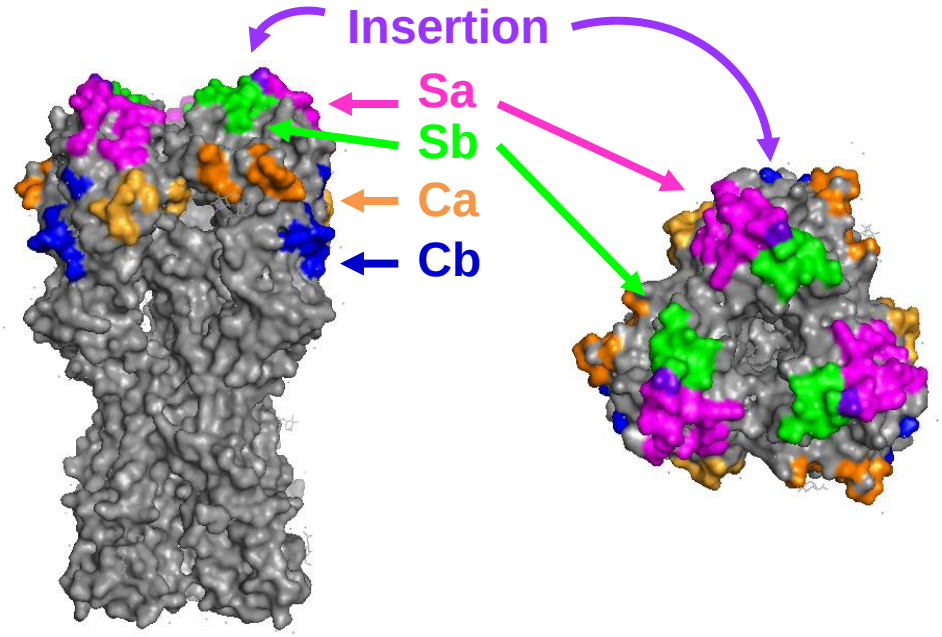


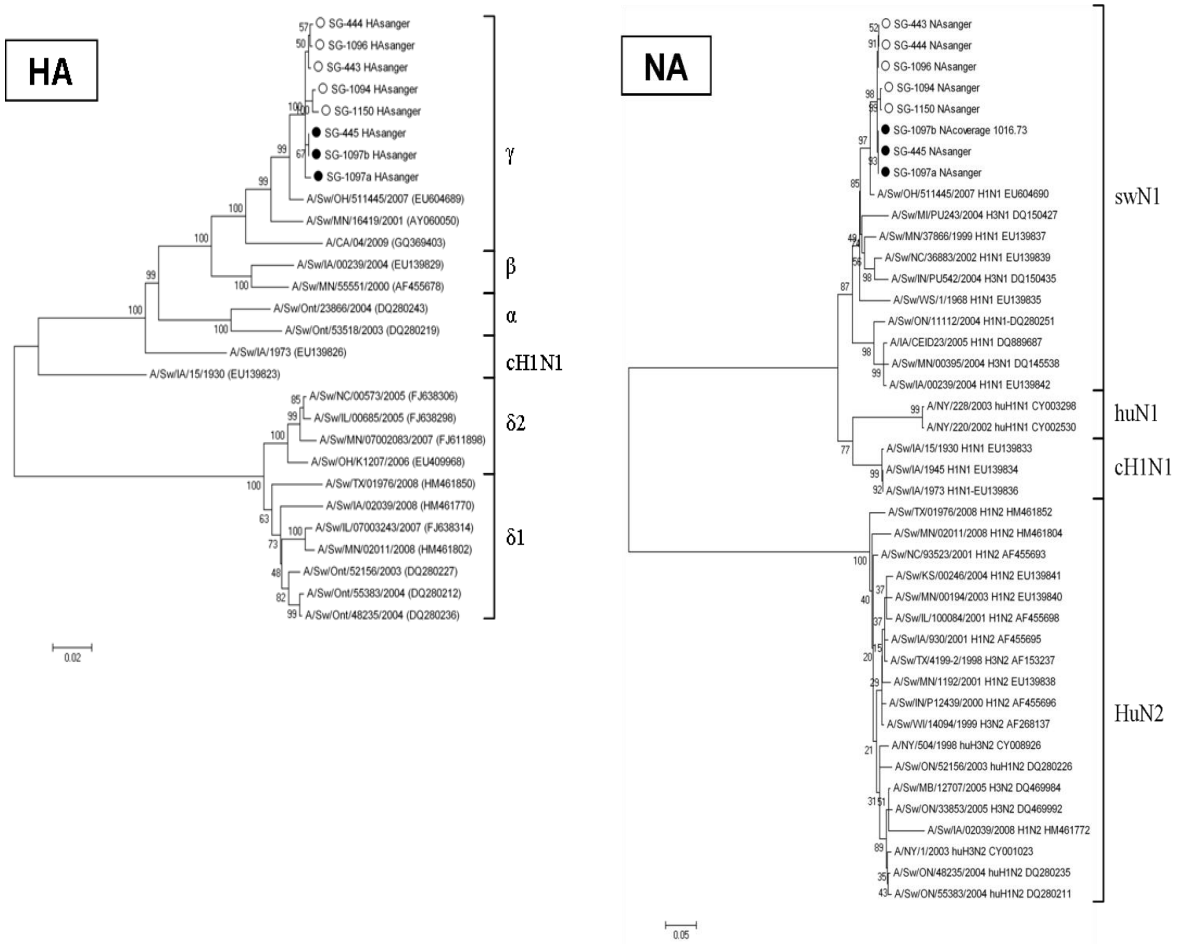
Table 5.1 Table of presumed ancestral and insertion viruses. The passage number, hemagglutination (HA) titers and cycle threshold (Ct) values are listed for the viruses grown for sequencing.

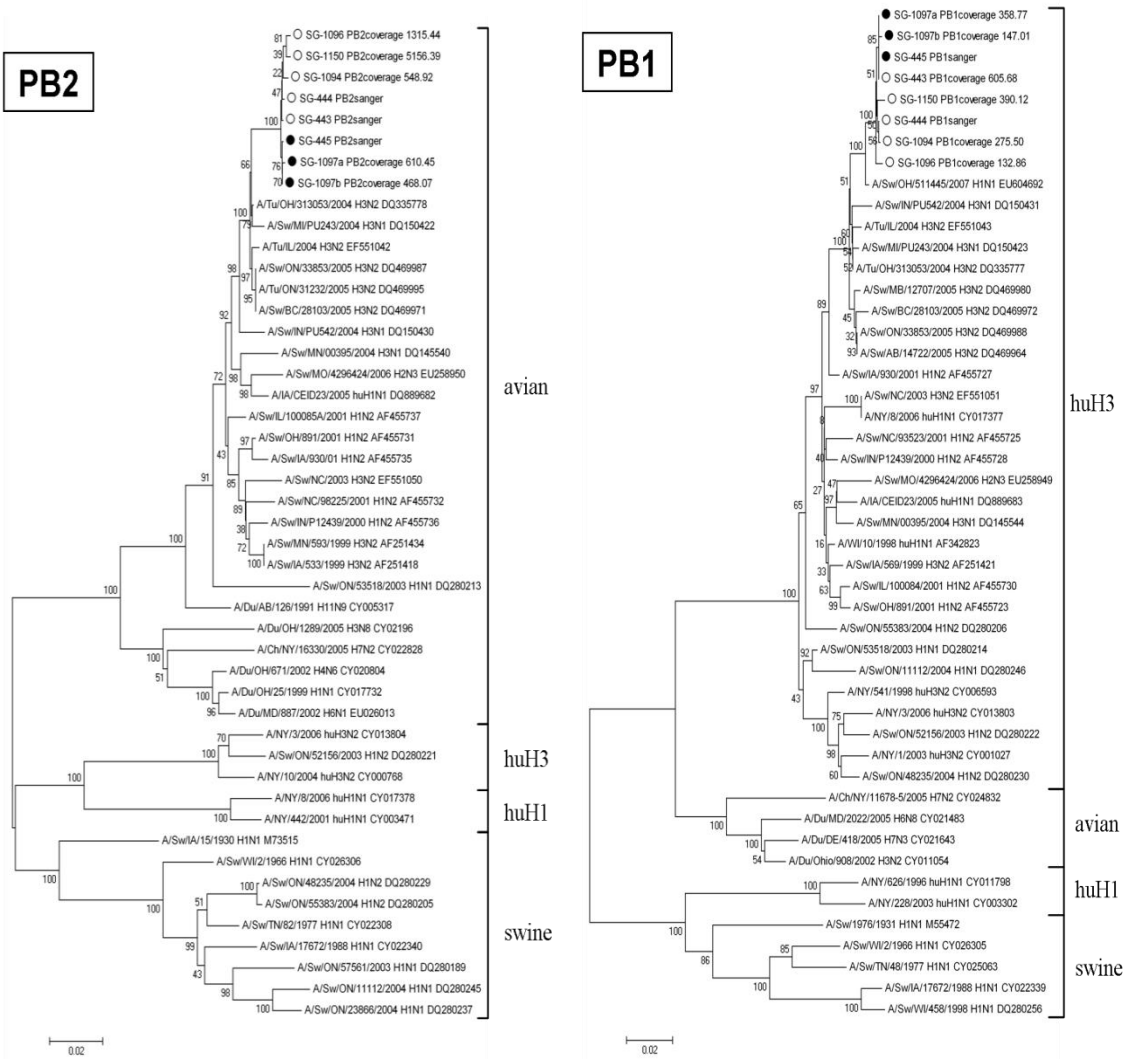
Virus	Insertion present?	Date virus isolated	Pig age (weeks)	Passage number	HA titer	Ct value
SG-1097a	No	12/07/2007	4	3	1:16	20.18
SG-1097b	No	12/07/2007	4	2	1:64	18.69
SG-445	No	01/16/2008	3	2	1:128	18.39
SG-443	Yes	07/16/2008	7	2	1:32	21.8
SG-444	Yes	07/31/2008	5	2	1:64-128	18.91
SG-1096	Yes	10/17/2008	5	2	1:32-64	20.3
SG-1150	Yes	01/12/2009	5	2	1:64-128	20.12
SG-1094	Yes	03/31/2009	3	2	1:64-128	20.25

Table 5.2 The number and position of amino acid changes in antigenic sites of the hemagglutinin protein of the viruses containing the insertion compared to the ancestral viruses and the AA substitutions in the phylogenetically important regions (PIR) of the neuraminidase gene.

	Residue	PIR	Ancestral	SG-443	SG-444	SG-1096	SG-1150	SG-1094
HA	70	Cb	L	L	I	L	L	L
	74	Cb	S	S	S	S	G	G
	139	Ca	A	A	A	A	D	D
	156	Sb	N	KEN	KEN	KEN	KEN	KEN
	162	Sa	K	K	K	K	M	M
	168	Ca	I	I	I	I	T	T
NA	15	A	L	L	L	L	M	L
	85	C	L	L	L	L	I	I
	221	E	N	N	N	K	N	N
	222	E	K	Q	Q	Q	Q	Q
	332	I	K	K	K	K	E	E
	365	L	I	N	N	N	N	N
	389	M	I	I	I	I	M	M

Figure 5.2 Phylogenetic trees for each of the eight gene segments based on nucleotide sequences from the eight viruses from farm M that contained the insertion (○) and the presumptive ancestral viruses (●) and sequences available in GenBank. The reference viruses used in the analysis have host abbreviations: *hu* human; *du* duck; *tu* turkey; *sw* swine and the GenBank accession number.

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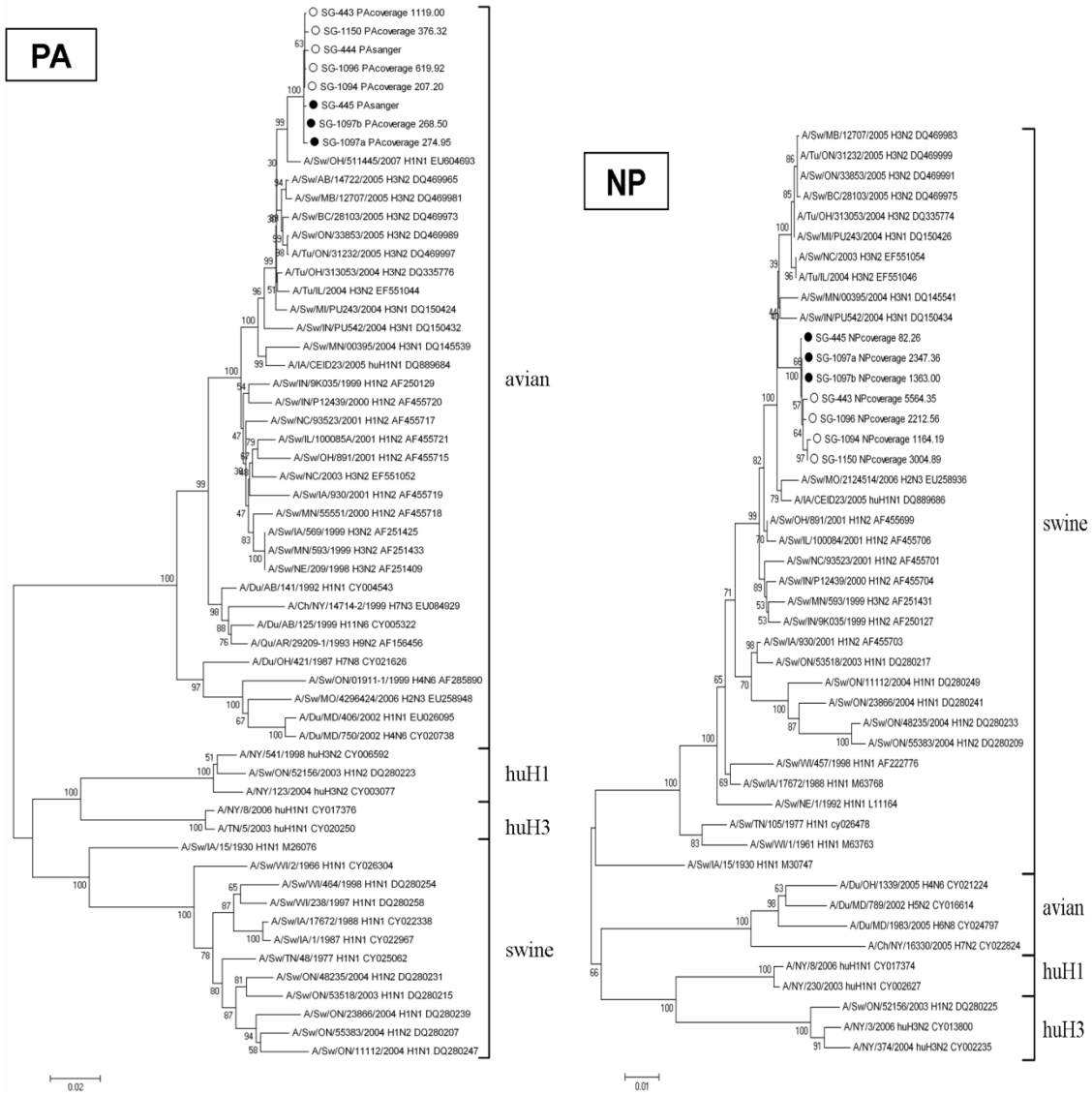
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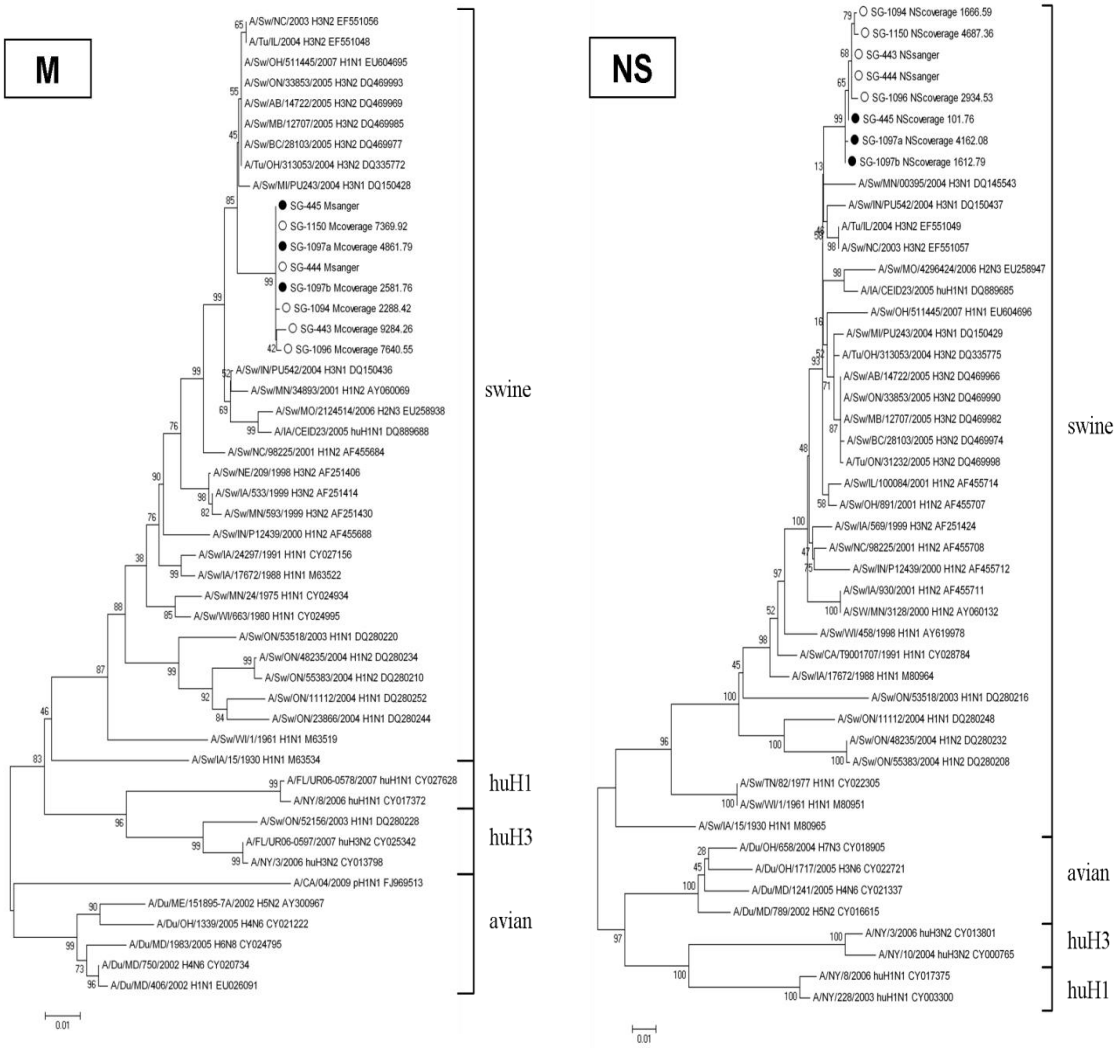
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A/Sw/NE/209/1998 H3N2 AF251406
A/Sw/IA/533/1999 H3N2 AF251414
A/Sw/MN/593/1999 H3N2 AF251430
A/Sw/IN/P12439/2000 H1N2 AF455688
A/Sw/IA/24297/1991 H1N1 CY027156
A/Sw/IA/17672/1988 H1N1 M63522
A/Sw/MN/24/1975 H1N1 CY024934
A/Sw/WI/663/1980 H1N1 CY024995
A/Sw/ON/53518/2003 H1N1 DQ280220
A/Sw/ON/48235/2004 H1N2 DQ280234
A/Sw/ON/55383/2004 H1N2 DQ280210
A/Sw/ON/11112/2004 H1N1 DQ280252
A/Sw/ON/23866/2004 H1N1 DQ280244
A/Sw/WI/1/1961 H1N1 M63519
A/Sw/IA/15/1930 H1N1 M63534
A/FL/UR06-0578/2007 huH1N1 CY027628
A/NY/8/2006 huH1N1 CY017372
A/Sw/ON/52156/2003 H1N1 DQ280228
A/FL/UR06-0597/2007 huH3N2 CY025342
A/NY/3/2006 huH3N2 CY013798
A/CA/04/2009 pH1N1 FJ969513
A/Du/ME/151895-7A/2002 H5N2 AY300967
A/Du/OH/1339/2005 H4N6 CY021222
A/Du/MD/1983/2005 H6N8 CY024795
A/Du/MD/750/2002 H4N6 CY020734
A/Du/MD/406/2002 H1N1 EU026091
0.01
swine
huH1
huH3
avian
NS
SG-1094 NScoverage 1666.59
SG-1150 NScoverage 4687.36
SG-443 NSsanger
SG-444 NSsanger
SG-1096 NScoverage 2934.53
SG-445 NScoverage 101.76
SG-1097a NScoverage 4162.08
SG-1097b NScoverage 1612.79
A/Sw/MN/00395/2004 H3N1 DQ145543
A/Sw/IN/PU542/2004 H3N1 DQ150437
A/Tu/IL/2004 H3N2 EF551049
A/Sw/NC/2003 H3N2 EF551057
A/Sw/MO/4296424/2006 H2N3 EU258947
A/IA/CEID23/2005 huH1N1 DQ889685
A/Sw/OH/511445/2007 H1N1 EU604696
A/Sw/MI/PU243/2004 H3N1 DQ150429
A/Tu/OH/313053/2004 H3N2 DQ335775
A/Sw/AB/14722/2005 H3N2 DQ469966
A/Sw/ON/33853/2005 H3N2 DQ469990
A/Sw/MB/12707/2005 H3N2 DQ469982
A/Sw/BC/28103/2005 H3N2 DQ469974
A/Tu/ON/31232/2005 H3N2 DQ469998
A/Sw/IL/100084/2001 H1N2 AF455714
A/Sw/OH/891/2001 H1N2 AF455707
A/Sw/IA/569/1999 H3N2 AF251424
A/Sw/NC/98225/2001 H1N2 AF455708
A/Sw/IN/P12439/2000 H1N2 AF455712
A/Sw/IA/930/2001 H1N2 AF455711
A/SW/MN/3128/2000 H1N2 AY060132
A/Sw/WI/458/1998 H1N1 AY619978
A/Sw/CA/T9001707/1991 H1N1 CY028784
A/Sw/IA/17672/1988 H1N1 M80964
A/Sw/ON/53518/2003 H1N1 DQ280216
A/Sw/ON/11112/2004 H1N1 DQ280248
A/Sw/ON/48235/2004 H1N2 DQ280232
A/Sw/ON/55383/2004 H1N2 DQ280208
A/Sw/TN/82/1977 H1N1 CY022305
A/Sw/WI/1/1961 H1N1 M80951
A/Sw/IA/15/1930 H1N1 M80965
A/Du/OH/658/2004 H7N3 CY018905
A/Du/OH/1717/2005 H3N6 CY022721
A/Du/MD/1241/2005 H4N6 CY021337
A/Du/MD/789/2002 H5N2 CY016615
A/NY/3/2006 huH3N2 CY013801
A/NY/10/2004 huH3N2 CY000765
A/NY/8/2006 huH1N1 CY017375
A/NY/228/2003 huH1N1 CY003300
0.01
swine
avian
huH3
huH1

CHAPTER 6

IN VITRO CHARACTERIZATION OF INFLUENZA A VIRUS ATTACHMENT IN THE UPPER AND LOWER RESPIRATORY TRACTS OF PIGS

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As influenza A viruses continue to emerge and evolve in the North American swine population, strains that are apparently associated with increased virulence have arisen. Virus binding to epithelial cells in the respiratory tract is a key step in the infection process. Therefore, direct assessment of virus-host cell interaction using virus histochemistry will enhance our understanding of the pathogenesis of these new viruses. For this study, we selected viruses that represented the four main genetic clusters of North American swine H1(SwH1) viruses, along with A/CA/04/2009 H1N1 and a vaccine strain for the positive controls, and the virus label, fluorescein isothiocyanate (FITC), for the negative control. A group of five viruses containing a two amino acid insertion in the binding site of the hemagglutinin gene and their presumed ancestral viruses were also examined for changes in binding patterns. Viruses were bound to formalin-fixed paraffin-embedded, 6-week-old (6w) and adult pig tissues. Virus histochemistry scores per respiratory zone ranged from + to +++, with bronchioles having the highest and most consistent scores, regardless of animal age. Virus attachment was quantified in digital images of five bronchioles per 6w tissue section using image analysis software. Significant differences in attachment were found among the SwH1 viruses ($p<0.0001$) and among the ancestral and insertion viruses ($p<0.0001$). The results of this study provide new insights regarding the virus binding to porcine respiratory epithelial cells. This information, combined with the information derived from the genetic analysis of these viruses will provide a benchmark for evaluating future changes in viral binding, as well as aid our understanding of influenza viral pathogenesis and virulence factors for pigs.

6.1 Introduction

Influenza is an acute respiratory disease that occurs year-round in swine and is caused by influenza A virus (IAV) subtypes H1N1, H1N2 and H3N2. While, most IAV infections in swine are mild with recovery usually occurring within 7-10 days of infection, the respiratory lesions can vary from mild tracheobronchitis to severe bronchoalveolar pneumonia (Van Reeth 2007). In order to further characterize the process of infection with different IAV strains, we need to examine the interaction of various viruses with cells that they infect.

It has been generally accepted that avian and equine IAV have a host cell receptor preference for α2,3-linked sialic acids (SAα2,3) and human and swine have a host cell receptor preference for α2,6-linked sialic acids (SAα2,6). The distribution of these receptors in the human and swine respiratory tract has been widely examined in a number of species using lectin histochemistry (LH) as an indirect measurement of IAV virus attachment (Matrosovich et al. 2004; Shinya et al. 2006; Nicholls et al. 2007; Yao et al. 2008). LH uses the SAα2,3 binding preference of *Maackia amurensis* agglutinin (MAA) and the SAα2,6 binding preference of *Sambucus nigra* agglutinin (SNA) to identify the IAV receptors in formalin-fixed or frozen tissues. In LH, a virus-free system, the amount of MAA binding is a proxy measurement of SA alpha 2-3 receptors and SNA binding indicates the presence of SA alpha 2-6 receptors.

Some of the limitations of LH technique are the binding of MAA-1 to non-sialic residues in contrast to poor recognition of alternative glycans by MAA-2, and cross-reactive binding of SNA to some SAα2-3 receptors (Nicholls et al. 2007; Nicholls et al. 2008). These limitations have led to several discrepancies in LH studies, including: the distribution of influenza receptors, the specific cell types involved, and the actual cells that were infected in culture compared to those predicted by LH in the human airway (Matrosovich et al 2004; Nicholls et al. 2007; Nicholls et al. 2008; Shinya et al. 2006; Yao et al. 2008) and in the swine airway (Ito et al. 1998; Suzuki et al. 2000; Van Poucke et al. 2010).

Another method that is used to characterize IAV virus interaction with host cells is virus histochemistry (VH). In contrast to the indirect methods of LH, VH allows for

direct examination of the pattern of virus attachment to tissues. VH has been used to visualize the pattern of viral attachment for avian-origin IAV H5N1, H5N9 and H6N1 and human-origin IAV H1N1 and H3N2 in paraffin embedded respiratory tissues of human, ferret, mouse, macaque, cat, dog and pig origin (van Riel et al. 2006; van Riel et al. 2007). This technique has yet to be applied to swine influenza viruses and respiratory tissues of pig origin.

In order to provide new insights regarding the pathogenesis of IAV in swine, the purpose of this study was to examine the binding patterns of swine-origin IAV to swine respiratory tissues using viruses that have been definitively characterized by prior genetic and antigenic analyses, and with known pathogenicity *in vivo*.

6.2 Materials and methods

Viruses

Well characterized viruses demonstrated to be virulent in previous studies were selected from the representative four genetic clusters (alpha, beta, gamma, and delta) of North American swine H1 viruses (Vincent et al. 2006; Vincent et al. 2009a; Vincent et al. 2009b; Ma et al. 2010; Lorusso et al. 2011). These included: SwH1α A/swine/Illinois/02450/2008 (IL08), SwH1β A/swine/Iowa/00239/2004 (IA04), SwH1γ A/swine/Ohio/511445/2007 (OH07), SwH1γ A/swine/Kansas/77778/2007(KS07), SwH1δ1 A/swine/Texas/01976/2008 (TX08), and SwH1δ1 A/swine/Illinois/07003243/2007 (IL07) (Table 6.1). Additionally, a group of H1N1 viruses isolated from pigs within a single farm system over a two year period that contained two amino acid (AA) insertion in the Sb antigenic site of the hemagglutinin gene (SG-443, SG-444, SG-1094, SG-1096, SG-1150) and their presumed ancestral viruses without the insertion (SG-445, SG-1097a) were selected. A/CA/04/2009 H1N1 (pH1N1) and A/swine/Minnesota/001482/2007 H1N2 (SG-239) were used as positive controls and the label, fluorescein isothiocyanate (FITC), was used as a negative control. OH07 and pH1N1 were kindly provided by United States Department of Agriculture, Agricultural Research Service, and KS07 was generously provided by Kansas State Veterinary Diagnostic Laboratory. All remaining viruses were

from the influenza virus archive at the University of Minnesota, Veterinary Diagnostic Laboratory (UMVDL).

Virus sequence analysis

HA gene sequences for IL08, IA04, OH07, KS07, TX08, IL07, pH1N1, SG-239 and the reference virus A/swine/Iowa/15/1930 H1N1 were available in GenBank (CY082907, EU139832, EU604689, GQ484355, HM461850, FJ638314, FJ966082, CY041903 and EU139823, respectively) and HA sequences for the remaining viruses were retrieved from the UMVDL database which is stored in the Influenza Sequencing Database, Los Alamos National Laboratories (Macken et al. 2001). For the UMVDL sequences, the HA gene was sequenced using the original material of lung or nasal swab collected from pigs with respiratory disease as part of diagnostic investigations, a viral RNA extraction kit (Applied Biosystems Inc., Foster City, CA), a combination of universal (Hoffman et al. 2001) and custom made primers (available upon request), and a genetic analyzer (Applied Biosystems Inc., Foster City, CA). Molecular evolutionary analyses were conducted using MEGA 4 with sequence alignments using Clustal W (Tamura et al. 2007). The evolutionary history was inferred using the Neighbor-Joining method and evolutionary distances were computed using the Maximum Composite Likelihood method with Kimura 2-parameters (Kimura 1980; Felsenstein 1985; Saitou and Nei 1987; Tamura et al. 2007).

Virus propagation and label attachment

Viruses were propagated on monolayers of Madin-Darby canine kidney (MDCK) cells grown in flasks with Eagle's minimum essential media containing 4% bovine serum albumin, trypsin and antibiotics (Meguro et al. 1979). All cultures were incubated at 37°C under a 5% CO_2 atmosphere and examined daily for up to 3 days under an inverted light microscope to observe cytopathic effects (CPE). After three freeze-thaw cycles, the supernatant was harvested and cleared by centrifugation for 10 min at $2272 \times g$. The cleared supernatant was then centrifuged at 4°C for 2 h at $85{,}000 \times g$ and the virus pellet was resuspended in 2 ml of phosphate buffered saline (PBS). The resuspended virus was inactivated by dialysis against 1% formalin overnight at 4°C followed by four cycles of dialysis against PBS for 2 h at 25°C. The virus was then inoculated in MDCK cells to

confirm inactivation. The label was prepared by mixing 0.1 mg/ml FITC in 0.5mol/L bicarbonate buffer at pH 9.6 (Sigma, St. Louis, MO). Equal volumes (1:1) of the inactivated virus and label solution were constantly stirred for 1 h at 25°C followed by four cycles of dialysis against PBS for 2 h at 25°C. The hemagglutination titers of all viruses were determined and the suspensions were diluted to contain 50 to 100 hemagglutinating units/50µl (Killian 2008).

Respiratory tract tissues of pigs

Formalin-fixed and paraffin-embedded (FFPE) respiratory tissues (nasal turbinate, trachea and lung) were selected from the UMVDL tissue block archive. All animals were from farms that have repeatedly tested negative for IAV, porcine respiratory and reproductive syndrome virus (PRRSv), porcine circovirus type 2 (PCV-2) and *Mycoplasma hyopneumoniae.* There was no macroscopic or microscopic evidence of respiratory disease and all animals tested negative for IAV, PRRSv, PCV-2 and *M. hyopneumoniae* at the time of necropsy. One 6-week-old pig (6w) and one 13-month-old sow (adult) were selected to test all of the viruses and three additional 6w pigs were selected to compare variations in binding among individual pigs using positive (SG-239) and negative controls. Additionally, since the slide incubation container could only hold 20 slides at a time, the variability between staining runs was compared for the positive control (SG-239) results.

Virus histochemistry

The FFPE respiratory tissues were microsectioned (4µm) and attached to adhesive glass slides. The tissues were deparaffinized with CitriSolv™ (Fisher Scientific, Waltham, MA) and hydrated using graded alcohols. After blocking with TNB buffer (Perkin Elmer, Waltham, MA), the FITC-labeled influenza viruses were incubated on the tissues overnight at 4°C. The virus label, FITC, was then detected using an anti-FITC antibody conjugated with horseradish peroxidase (Dako, Denmark) and this signal was then amplified using the tyramide signal amplification (TSA™) biotin system (Perkin Elmer, Waltham, MA). The amplified signal was then revealed using 3-amino-9-ethyl-carbazole (AEC) and the tissues were counterstained with Mayer's hematoxylin (Dako, Denmark) prior to cover slipping using glycergel mounting medium (Dako, Denmark).

The amount of virus attachment to the apical surface of the epithelial cells was scored as follows: (–) no attachment, (+) rare or few cells, (++) moderate number of cells, and (+++) abundant or many cells.

Image and statistical analyses

To quantify the level of virus attachment in the 6w bronchiole, 5 bronchioles approximately 250 μm in diameter were selected per tissue section. The images were captured at 40× magnification with an Olympus BX45 and an Olympus DP70 digital camera (Olympus America Inc., Center Valley, Pennsylvania). Digital images were analyzed with Image-Pro Plus 6.2 image analysis software (Media Cybernetics, Silver Springs, MD) as previously described (Belur et al. 2011). Thresholds (n = 3) were determined by using appropriate red color hues to identify positive staining for the red granular staining generated by the AEC chromogen. The total area (μm^2) and circumference of the area analyzed per bronchiole was recorded. The log transformed percentage of area containing granular red staining was calculated for the three threshold levels and evaluated by ANOVA using SAS (SAS Institute, Cary, North Carolina, USA) with post-hoc Tukey test. A *P*-value of <0.05 was considered to indicate a statistically significant difference between groups.

6.3 Results

Virus sequence analysis

The inferred evolutionary history of the selected viruses is shown in the phylogenetic tree of the HA sequences and the four SwH1genetic clusters are labeled (Figure 6.1). The ancestral and insertion viruses fall into the SwH1γ cluster with pH1N1, but are clearly divergent from pH1N1. BLAST search of the Influenza Research Database (www.fludb.org) revealed that the HA sequences of the ancestral and insertion viruses were most similar to OH07 and KS07. Using pairwise analysis between sequences of the first 901 nucleotides, the ancestral viruses were 98.2% similar and the insertion viruses were 96.8-97.9% similar to OH07 and KS07, respectively. The ancestral viruses were 96.6% similar and the insertion viruses were 93.6-95.9% similar to OH07 and KS07, respectively by pairwise analysis of the first 296 amino acids.

Virus histochemistry scores

The VH scores per respiratory zone (nasal turbinate, trachea, bronchus, bronchiole, and alveoli) ranged from + to +++ for all viruses. Virus attachment was most abundant in the bronchioles, regardless of virus or animal age, with respiratory binding scores ++ to +++ for all viruses at this location. Both positive control viruses had mild to moderate binding (+ to ++) throughout the upper and lower respiratory tract and there was no binding of the FITC alone for the negative controls (Table 6.2; Figure 6.2). The percentage of area containing granular red staining (AEC chromogen) was calculated for each of the five bronchioles using the cumulative area of the three thresholds divided by the total circular area analyzed. The results of the morphometric analysis are reported as the mean percent area of the five bronchioles ± standard deviation.

Variation between staining runs

Due to the space constraints of slide incubation chamber, the VH technique was performed in several different staining runs. The variation in the pattern of virus attachment for the positive control, SG-239, appeared to be minimal by the naked eye. However, the morphometric scores for SG-239 used on the lung tissue of the same 6w pig in four different slide runs had mean percent staining in the bronchioles of 2.61 ± 1.01%, 3.82 ± 1.28%, 5.43 ± 2.76%, and 1.38 ± 0.93%. A statistically significant difference was detected between the last two means by ANOVA analysis with post-hoc tukey test (p = 0.0038), indicating a limitation in comparing VH results from the different staining runs based on the analysis of 5 bronchioles per lung section. For the remaining comparisons, only results from the same staining run were compared.

Variation among individual animals

Initial assessment of the variation in the amount of viral binding using the control virus SG-239 was measured in four different 6w pigs with similar genetic background. The pattern of virus attachment in the upper and lower respiratory tract appeared to be the same for all four pigs (data not shown) and the morphometric scores were 2.61 ± 1.01%, 3.72 ± 1.62%, 3.70 ± 2.07%, and 2.27 ± 1.41%. No difference was detected between the means (p = 0.2789), indicating that VH results from these four 6w pigs in the same staining run were comparable.

Swine H1 cluster representatives

The SwH1α, β, and γ viruses had similar patterns of virus attachment with abundant binding in both the upper and lower respiratory tracts. The SwH1δ viruses had minimal binding throughout the respiratory tract, regardless of animal age. One of the SwH1γ viruses, KS07, had less binding compared to the genetically similar virus, OH07. One notable difference in the patterns of virus attachment for the two age groups was found in the nasal turbinates where there was more abundant binding to the 6w turbinate compared to the adult turbinate (Table 6.2; Figure 6.2). The computerized morphometric scores of the 6w bronchioles revealed more binding for the SwH1α, β, and γ viruses (>3%) compared to the SwH1δ viruses (<1%) that was demonstrated to be different in the post-hoc Tukey test ($p < 0.0001$).

Viruses with and without insertion

The patterns of attachment for viruses that had the 2 AA insertion were compared with those of their presumed ancestors (viruses without the insertion). The most abundant virus attachment to the bronchiolar epithelium of the 6w and adult pigs occurred with the ancestral viruses. Within the pulmonary parenchyma, there was abundant bronchiolar attachment of the ancestral virus for the 6w and adult pigs and there was moderate to abundant attachment to the alveoli. Comparatively, there was less abundant binding of the insertion viruses to the bronchioles and alveoli for the 6w and adult pigs. For both the ancestral and insertion viruses, there was less virus attachment in the upper respiratory tract than the lower respiratory tract. While there was only minimal (+) attachment to the 6w nasal turbinate and tracheal epithelium for both the insertion and ancestral viruses, there was moderate (++) attachment to the adult nasal turbinate and tracheal epithelium for the ancestral viruses and minimal to moderate attachment for the insertion viruses (Table 6.2; Figure 6.2). The morphometric scores of the ancestral viruses, SG-445 and SG-1097a, revealed more attachment to the bronchiolar epithelium (4.28 ± 1.81% and 6.19 ± 2.56%, respectively) than insertion viruses SG-443, SG-444, SG-1094, SG-1096 and SG-1150 (0.71 ± 0.46%, 0.40 ± 0.30%, 3.84 ± 2.18%, 3.62 ± 2.37%, and 2.52 ± 2.46%, respectively). According to the post-hoc Tukey test, the detectable difference ($p < 0.0001$) was between the two viruses with the lowest binding (SG-443 and SG-444) and

the other five viruses studied (SG-445, Sg-1097a, SG-1094, SG-1096 and SG-1150) with overlap between SG-444 and SG-1150.

6.4 Discussion

IAV had remained relatively stable in the North American swine population until the emergence of the triple reassortant H3N2 viruses in 1998 (Webby et al. 2000). The rapid evolution of North American swine lineage IAV over the last decade has resulted in remarkable genetic and antigenic variability and presented a major challenge to the swine industry. This has been particularly true for the HA gene of viruses in the SwH1 subtype. Although a number of studies have examined the genetic and antigenic characteristics of these emerging virus variants in swine (Vincent et al. 2006; Vincent et al. 2009a; Vincent et al. 2009b; Ma et al. 2010; Lorusso et al. 2011), there have been limited studies to determine if genetic and antigenic variation has led to changes in attachment of virus to the epithelial cells during infection. The purpose of this study was to examine the pattern of virus attachment to porcine respiratory tissues for IAV isolated from North American swine.

Although limited replication of influenza viruses can occur in cell cultures without SA receptors present (Stray et al. 2000), much of what is known about IAV attachment to epithelial cells in the respiratory tract focuses on α2-3 and α2-6 SA and has been determined by LH assays despite the discrepancies between studies and the limited specificity of SNA and MAA lectins (Matrosovich et al. 2004; Shinya et al. 2006; Nicholls et al. 2007; Yao et al. 2008). Recent studies have indicated that α2-3 SA and α2-6 SA are not the sole receptors for avian and mammalian IAV viruses (Stray et al. 2000; Kumari et al. 2007; Nicholls et al. 2007). Glycan microarrays allow for avidity comparisons due to modifications in the SA (e.g. total SA length and fucosylation, sulfation and sialylation at position 2 or 3) and microarray analysis has found influenza viruses bind to a wide spectrum of glycans including non-SA residues (Nicholls et al. 2007). As these newly identified glycans are identified in the host respiratory tract, these findings have led to the realization that the glycan structure can affect the ability of the

virus to bind to the host cell surface and that there may be other receptors that play a role in infection that are overlooked by LH assays (Nicholls et al. 2007).

In a recent glycan microarray study of human and swine viruses by Chen et al. in 2011, SwH1α, β and γ viruses were highly specific for the α2-6 SA and had limited affinity to α2-3 SA, but the human-like SwH1δ viruses had increased affinity to fucosylated and sulfated α2-3 SA, similar to human seasonal IAV. This study also demonstrated that AA substitutions D187N, D187V, D222G, and Q223R, were directly linked to increased α2-3 SA affinity and D127E was linked to increased α2-6 SA affinity in human isolates of pH1N1. One of the viruses analyzed by Chen, et al., A/Ohio/1/2007, demonstrated high affinity for α2-6 SA and limited affinity to α2-3 SA on microarray that was seen with all of the recently isolated SwH1α, β and γ viruses. Since A/Ohio/1/2007 was collected from the same zoonotic event as OH07 and the two are genetically related, it is likely that they would have similar binding affinity. Experimental infection of 4-week-old pigs with OH07 (n = 15) also demonstrated lung lesions typical of virulent strains of IAV in swine. Macroscopic lung lesions averaged 25% and microscopic lung lesions (0-3) were 2.4 on 5 days post-infection (DPI) (Vincent et al. 2009b). All eight gene segments of KS07 have at least 99.7% nucleotide similarity to OH07 and experimental infection with KS07 (n = 20) resulted in similar lesions with 21% macroscopic and 2.5 (0-3) microscopic scores 5 DPI (Ma et al. 2010). In the present study, OH07 and KS07 had similar patterns of virus attachment to the respiratory epithelium and morphometric scores (3.71 ± 2.29% and 3.13 ± 1.78%, respectively), consistent with the previously described gross and microscopic lesions attributed to OH07 and KS07 *in vivo* challenge (Table 6.3).

The SwH1β virus, IA04, had similar lesions *in vivo* (as studied in 15 pigs) compared to OH07 and KS07 with 24% gross pneumonia and a microscopic lung score of 2.8 (scale of 0-4) at 5 DPI (Vincent et al. 2009a). Similar findings were demonstrated *in vitro* using VH with IA04 having a similar pattern of virus attachment to the respiratory epithelium and morphometric score (4.03 ± 3.80) as the SwH1γ viruses. The SwH1α virus, IL08, also had a similar attachment pattern and morphometric score (3.37 ± 2.55), and experimental *in vivo* infection (n = 9) with IL08 resulted in a similar average

microscopic lung score of 3(0-4) 5 DPI, but macroscopic lung lesions averaged only 12% (see results in chapter 3). This may have been related to the D127E substitution that has been associated with increased α2-6 SA affinity described by Chen, et al. or virulence factors found in the other gene segments that do not play a role in attachment to the cell surface (e.g., polymerase basic 1 or 2 genes).

There was a stark contrast in virus attachment patterns between the strong binding of the SwH1α, β and γ viruses compared to the minimal binding of the SwH1δ1 viruses throughout the respiratory tract of both 6w and adult pigs. This difference was detected by the ANOVA for the morphometric scores of the SwH1 cluster viruses ($p < 0.001$) and the post-hoc Tukey test. The SwH1δ1viruses examined in the present study have the human-lineage T133S residue and 131G deletion in HA. TX08 had the D187N substitution which resulted in higher α2-3 and α2-6 SA affinity in the human isolates (Chen et al. 2011). Also, TX08 and SG-239 have the NS1 residues (108R, 125E and 189G) which have been associated with a loss of binding to the epithelial receptors (Lorusso et al. 2011). The SwH1δ1 viruses had minimal attachment to the respiratory epithelium, but the morphometric score for control virus (averaged for the four staining runs) was 3.31 ± 2.17% compared to 0.33 ± 0.20% for IL07 and 0.49 ± 0.33% for TX08. Experimental *in vivo* infection with TX08 (n = 5) resulted in 16.8% gross lung lesions and microscopic lung lesions averaged 2.1 (0-3) at 5 DPI (Ciacci-Zanella JR, unpublished data).

The patterns of virus attachment and the morphometric scores in the respiratory tissues of the 6w pig appear to be highly correlated to the macroscopic and microscopic lung lesions at 5 DPI for the SwH1α, β and γ viruses. However, the correlation between VH results and *in vivo* lung lesion did not hold true for the SwH1δ1 viruses which had minimal virus attachment throughout the respiratory tract and low morphometric scores in the bronchioles. This variation is likely due to other factors of virulence (e.g. viral polymerase activity) for these SwH1δ1 viruses. Although *in vivo* pathogenesis studies have not been performed on SG-239, it is important to note that this virus is also a SwH1δ1 virus and this virus had more binding in the 6w bronchioles and alveoli than IL07 and TX08 along with a higher morphometric score (3.31 ± 2.17%).

Clinical data were collected from the farm where the ancestral and insertion viruses originated. All of the viruses were isolated from pigs that were weaned and sent to multiple off-site nurseries. Different nursery sites within the farm system were infected with these viruses at different time points over a three year period, but SG-443 and SG-444 were collected at the same time from two different barns. The infected animals were 3 to 7-weeks-old (average age 4.6 ± 1.4 weeks). There was 15-85% morbidity associated with these viruses and up to a 4% increase in mortality compared to the other groups of pigs placed in other nursery sites at the same time. Histopathology of the lungs from which the farm M isolates were collected showed moderate to severe necropurulent bronchoalveolar pneumonia characteristic of IAV infection with secondary bacterial pneumonia (e.g. *Streptococcus suis*, *Haemophilus parasuis* and *Pasteurella multocida* were most often isolated from these lungs).

The pattern of virus attachment for the insertion and ancestral viruses showed strongest binding in the lower respiratory tract of both the 6w and adult pigs. Binding to the lower respiratory tract, particularly to the alveoli, and the subsequent diffuse alveolar damage, was the main pathologic abnormality in fatal cases of infection with pH1N1 in humans during the pandemic (Mukhopadhyay et al. 2010). Further studies will be needed to examine which specific mutations within HA or other genes are associated with the apparent lower respiratory tract binding predilection of the gamma cluster viruses examined in this study (Tables 6.2 and 6.3). Further studies will also be needed to determine if these changes translate directly to *in vivo* binding and replication models, or if these changes can be compensated for by synergistic effects of the virulence factors of the internal gene proteins.

While minimal variation was found in the viral binding to the bronchiole of the four 6w pigs, these four pigs were from 3 different litters on the same farm and therefore had a similar genetic background. The impact of swine genetics on the SA receptors in the respiratory tract has not been reported and further investigation is needed in order to determine if there is a genetic variation in swine SA receptors and patterns of virus attachment. Additionally, since a difference in patterns of virus attachment were found between the 6w and adult pigs, the dynamics of virus binding throughout the

developmental stages should be examined using the respiratory tracts of pigs from birth to slaughter (6 months). Another limitation that should be examined further is the difference that was found in the morphometric results between staining runs. Until repeatability of the technique is improved, comparisons between staining runs or even laboratories and other publications for this technique would be limited.

Since the VH technique has adapted some of its techniques and reagents from immunohistochemistry (IHC), it is likely that these techniques have similar limitations and sources of non-specific background staining. Some of the sources of increased background staining for IHC include: tissue drying out during incubation, antibodies produced in rabbits which can result in non-specific binding to cartilage and muscle, endogenous peroxidases found in red blood cells, granulocytes, monocytes, hepatocytes, muscle and kidney, endogenous biotin in tissues, and antibodies present within plasma cells (Wendelboe and Bisgaard 2006). Background produced by endogenous peroxidases can be limited by adding a 15 minute, 3% hydrogen peroxide blocking step before incubation with the detection antibody. Background produced by endogenous biotin in the tissues can be avoided by using a polymer-based IHC method, such as Dako's EnVision+ system (Sabatini et al. 1998; Vosse et al. 2007). Background produced by endogenous antibodies present in plasma cells can be limited by adding a 15 minute, 10% normal serum step after the hydrogen peroxide step and before the antibody incubation. Additionally, endogenous antibodies can be blocked by adding 2-4% normal swine serum to the rabbit EnVision+ solution. These modifications in the VH technique were tested using SG-239 in the adult respiratory tissues and although there was minimal background staining, there was strong non-specific immunoreactivity in the plasma cells. Further evaluations would be necessary to determine if these modifications would result in more reproducible VH results.

The temperature and humidity of the laboratory was recorded on each day that the VH procedure was performed. While the temperature remained consistent, ranging from 21 to 23°C, the humidity varied from 14% to 40%. This variation in ambient humidity could result in variations in binding of the virus, the antibody to the FITC attached to the virus, and the amplification of the antibody signal. Low humidity could also contribute to

drying out of the tissues during incubation. To limit the amount of variation in humidity within the incubation chamber, water was added to the wells below the slides in the chamber and a water bath was placed in the refrigerator for the virus incubation step. The amount of moisture present in the incubation chamber and refrigerator was not determined.

While there are limitations to the application of these results, the present study provides new insights on the patterns of virus attachment for influenza A viruses from swine to porcine respiratory epithelial cells. Additionally, the *in vitro* model demonstrated that viruses previously described in *in vivo* studies as causing severe lung lesions had strong binding to the bronchioles and alveoli. The computerized morphometric analysis used in this study also provided a quantitative measure to compare the binding results within the bronchiole. However, for this study, measurements were taken from only 5 bronchioles, resulting in a minimal detectable significant difference of 2.85%. In order to detect a difference of 2.02%, a sample size of 10 bronchioles would be needed and 40 bronchioles would be needed to detect a 1.01% difference. The preliminary information obtained in this study combined with the information derived from the genetic analysis of these viruses will provide a benchmark for evaluating interactions between IAV and the cells of the respiratory tract. The new insights into viral binding of swine viruses will lead to better understanding of the role of virus tropism and replication in IAV pathogenesis in pigs and may also provide new targets for more effective prevention and treatment measures.

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Table 6.1 Table of viruses selected for virus histochemistry.

Virus	Abbrev.[a]	Subtype	Cluster	Passage[b]	Citation
SwH1 cluster representatives:					
A/swine/Illinois/02450/2008	IL08	H1N1	SwH1α	P2	c
A/swine/Iowa/00239/2004	IA04	H1N1	SwH1β	P2	Vincent et al. 2006
A/swine/Ohio/511445/2007	OH07	H1N1	SwH1γ	P5	Vincent et al. 2009b
A/swine/Kansas/77778/2007	KS07	H1N1	SwH1γ	P3	Ma et al. 2010
A/swine/Illinois/07003243/2007	IL07	H1N2	SwH1δ1	P2	Vincent et al. 2009a
A/swine/Texas/01976/2008	TX08	H1N2	SwH1δ1	P2	Lorusso et al. 2011
Viruses with a 2 AA insertion:					
A/swine/Illinois/02238/2008	SG443	H1N1	SwH1γ	P3	d
A/swine/Illinois/02251/2008	SG444	H1N1	SwH1γ	P3	d
A/swine/Illinois/02695/2008	SG1094	H1N1	SwH1γ	P3	d
A/swine/Illinois/02385/2008	SG1096	H1N1	SwH1γ	P3	d
A/swine/Illinois/02510/2008	SG1150	H1N1	SwH1γ	P3	d
Presumed ancestral viruses:					
A/swine/Illinois/01981/2008	SG445	H1N1	SwH1γ	P2	d
A/swine/Illinois/01917/2008	SG1097a	H1N1	SwH1γ	P3	d
Positive controls:					
A/CA/04/2009	pH1N1	H1N1	SwH1γ	Unknown	Garten et al. 2009
A/swine/Minnesota/01482/2007	SG239	H1N2	SwH1δ1	P2	e

[a]Abbreviated name used.
[b]Final passage number of the virus used for virus histochemistry. Passage number of pH1N1 was unknown.
[c]See chapter 3.
[d]See chapter 5.
[e]See chapter 4.

Figure 6.1 Phylogenetic tree for the hemagglutinin gene segments based on nucleotide sequences of swine hemagglutinin subtype 1 viruses (SwH1) found in the UMVDL and GenBank databases was rooted using the reference virus A/swine/Iowa/15/1930. The four defined genetic clusters of related viruses are indicated by brackets on the right of the tree as: SwH1α (cH1N1 or classical-like; ◆), SwH1β (reassortant H1N1-like; ■), SwH1γ (H1N2-like; ●), and SwH1δ (Human-like H1; ▲). There were 901 nucleotide positions in the final dataset, bootstrap test (1000 replicates) are shown next to the branches, and the GenBank accession numbers are in parenthesis, when available.

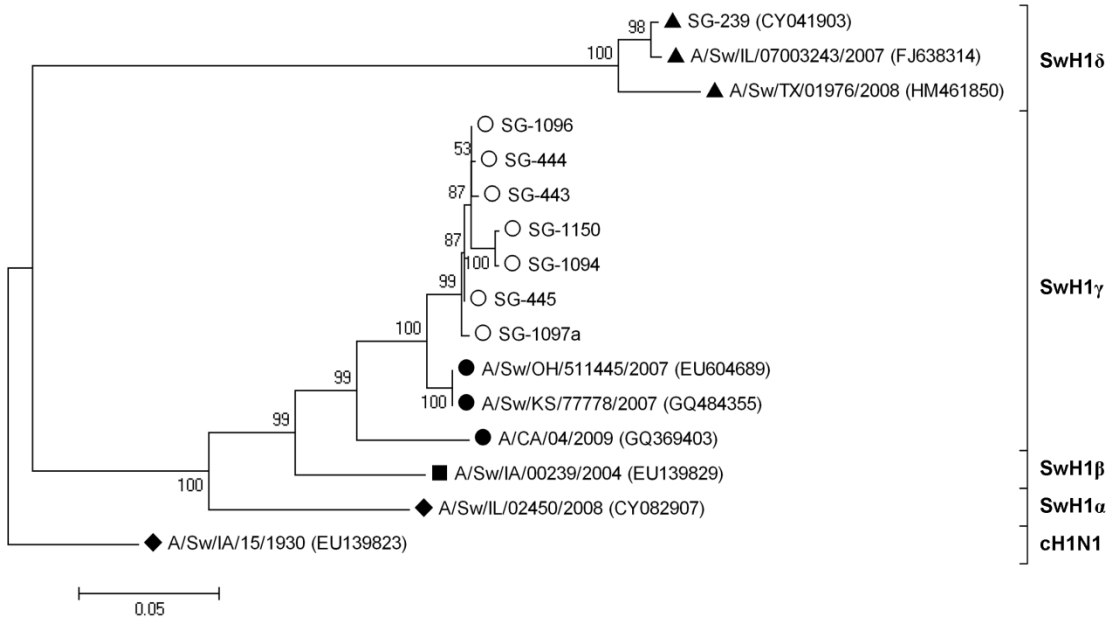

Table 6.2 Distribution and intensity of virus attachment to respiratory tract detected by the red chromogen signal. The amount of virus attachment to the apical surface of the epithelial cells was scored as follows: (-) no attachment, (+) rare or few cells, (++) moderate number of cells, and (+++) abundant or many cells.

		6-week-old				Adult			
Virus	Score[a]	Turbinate	Trachea	Bronchiole	Alveoli	Turbinate	Trachea	Bronchiole	Alveoli
SwH1 cluster representatives:									
IL08	3.37 ± 2.55	++	+++	+++	++	+	++	++	++
IA04	4.03 ± 3.80	+++	++	+++	++	++	+	++	++
OH07	3.71 ± 2.29	+++	++	+++	++	+	++	++	+++
KS07	3.13 ± 1.78	++	++	+++	++	+	+	++	++
IL07	0.33 ± 0.20	+	+	+	+	+	+	+	+
TX08	0.49 ± 0.33	+	+	+	+	+	+	+	+
Viruses with a 2 AA insertion:									
SG443	0.71 ± 0.46	+	+	++	+	+	++	++	+++
SG444	0.40 ± 0.30	+	+	++	+	+	+	+	+
SG1094	3.84 ± 2.18	+	+	+++	+	+	+	++	+
SG1096	3.62 ± 2.37	+	+	+++	++	++	++	++	++
SG1150	2.52 ± 2.46	+	+	+++	++	+	++	++	++
Presumed ancestral viruses:									
SG445	4.28 ± 1.81	++	+	+++	+++	++	++	+++	++
SG1097a	6.19 ± 2.56	++	+	+++	+++	++	++	+++	++
Positive controls:									
pH1N1	n.d.[b]	+	+	++	+	+	+	+	+
SG239	3.31 ± 2.17	+	+	++	++	+	+	+	+

[a]The mean morphometric scores for the 6w bronchioles are the percent area of staining ± standard deviation.
[b]n.d. = not done.

Figure 6.2 Attachment of virus A/swine/Illinois/02450/2008 to the nasal turbinate (2a), trachea (2b), bronchiole (2c) and alveoli (2d). AEC, hematoxylin counterstain. **Figure 6.3.** Attachment of virus A/swine/Iowa/00239/2004 H1N1 to the nasal turbinate (3a), trachea (3b), bronchiole (3c) and alveoli (3d). AEC, hematoxylin counterstain. **Figure 6.4.** Attachment of virus A/swine/Ohio/511445/2007 to the nasal turbinate (4a), trachea (4b), bronchiole (4c) and alveoli (4d). AEC, hematoxylin counterstain. **Figure 6.5.** Attachment of virus A/swine/Texas/01976/2008 to the nasal turbinate (5a), trachea (5b), bronchiole (5c) and alveoli (5d). AEC, hematoxylin counterstain. **Figure 6.6.** Attachment of ancestral virus SG445 to the nasal turbinate (Fig. 6a), trachea (Fig. 6b), bronchiole (Fig. 6c) and alveoli (Fig. 6d). AEC, hematoxylin counterstain. **Figure 6.7.** Attachment of insertion virus SG443 to the nasal turbinate (7a), trachea (7b), bronchiole (7c) and alveoli (7d). AEC, hematoxylin counterstain. **Figure 6.8.** Attachment of control virus SG239 to the nasal turbinate (8a), trachea (8b), bronchiole (8c) and alveoli (8d). AEC, hematoxylin counterstain. **Figure 6.9.** Attachment of negative control, FITC protein, to the nasal turbinate (9a), trachea (9b), bronchiole (9c) and alveoli (9d). AEC, hematoxylin counterstain.

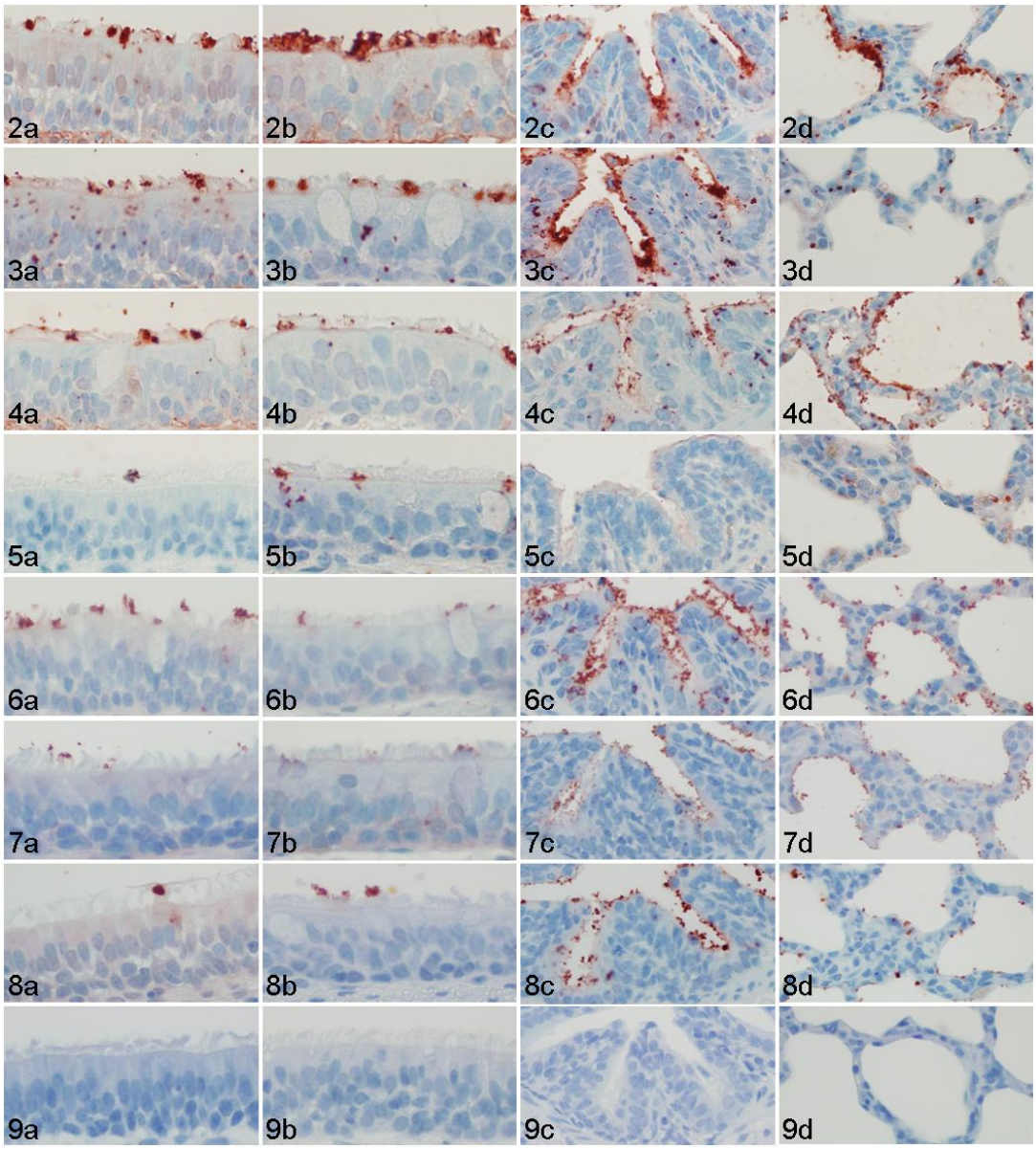

Table 6.3 Comparison of virus histochemistry score and morphometric area to macroscopic and microscopic lesions found on 5 days post-infection in the *in vivo* pathogenicity studies.

Virus	Cluster	Bronchiolar score	Bronchiolar area (%)	Macroscopic lesions (%)	Microscopic lesions (scale)	Citation
SwH1 cluster representatives:						
IL08	SwH1α	+++	3.37 ± 2.55	12	3.0 (0-4)	a
IA04	SwH1β	+++	4.03 ± 3.80	24	2.8 (0-4)	Vincent et al. 2006
OH07	SwH1γ	+++	3.71 ± 2.29	25	2.4 (0-3)	Vincent et al. 2009b
KS07	SwH1γ	+++	3.13 ± 1.78	21	2.5 (0-3)	Ma et al. 2010

[a]See chapter 3.

GENERAL DISCUSSION AND CONCLUSIONS

GENERAL DISCUSSION:

Over the last decade, influenza A viruses have rapidly evolved in the North American swine population with the identification of novel strains occurring at increased frequency (Karasin et al. 2000; Lekcharoensuk et al. 2006; Ma et al. 2006; Ma et al. 2007; Vincent et al. 2009a; Vincent et al. 2009b; Ma et al. 2010; Nelson et al. 2011). Many of these novel viruses have been shown to be capable of causing outbreaks in other species; the most notable example being the recent 2009 H1N1 pandemic virus which has caused interspecies transmission events between humans, pigs, turkeys, cats, dogs, and zoo animals throughout the world (http://www.usda.gov/documents/CHART-SAMPLES_FOR_CY2009_011910.pdf ; Berhane et al. 2010; Dundon et al. 2010; McCullers et al. 2011). In order to diagnose these novel strains, our diagnostic techniques have had to evolve as well.

Advances in molecular diagnostics have improved the sensitivity and specificity of PCR tests and made it possible to detect small amounts of pathogens in various samples. These advances have also brought about the development of new sampling strategies for testing swine pathogens within the environment or population. Alternative samples such as swabs of fomites or trucks, flies and air samples have been used to detect pathogens in the environment (Otake et al. 2002; Dee et al. 2004; Hermann et al. 2006; Pitkin et al. 2009; Otake et al. 2010). Pooled nasal swabs or ear notches and oral fluids have made it possible to test large numbers of animals using fewer samples (Landolt et al. 2005; Kennedy et al. 2006; Prickett et al. 2010). Oral fluid samples in particular, have become useful for surveillance and herd monitoring programs (Prickett et al. 2010). Oral fluids offer a collection method that is easy, safe and convenient to collect without handling the animals and provides sensitive and reliable results for swine pathogens such as: PRRSv, PCV-2 and IAV (Prickett et al. 2010). In chapter 2, we validated the influenza A Matrix real time RT-PCR and virus isolation tests, and tested samples from experimentally infected pigs and samples from the field submitted by veterinarians.

In addition to changes in sampling methods for molecular tests, there have been improvements in viral sequencing and changes in the applications of sequencing data. In the last five years, we went from getting 600-700 nucleotides per read from viral isolates

to over 900 nucleotides per read from original sample material using Sanger sequencing methods. For influenza viruses, these improvements have meant that instead of only having a partial HA1 sequence, we can now sequence whole gene segments in one reaction. Next Generation sequencing using Illumina and 454 sequencers are just starting to give promising results of deep sequencing from original samples to look for variability in influenza viruses within a sample (Ramakrishnan et al. 2009; Krause et al. 2011). As sequencing and analytical tools for sequencing improved, more sequences became available for analysis in both public and private databases. These newly available sequences were then used for molecular and antigenic characterization studies, providing new insights into the pathogenesis of influenza A viruses in swine (Vincent et al. 2006; Vincent et al. 2009a; Vincent et al. 2009b; Ma et al. 2010; Lorusso et al. 2011).

By applying molecular characterization to field and vaccine viruses, we can examine the changes within the challenge virus that are necessary to evade the immune system and the nuances of cross-reactivity in the antibody reaction. In chapter 3, we examined the efficacy of a commercial vaccine containing a SwH1γ virus against challenge with a contemporary SwH1α field isolate. The commercial vaccine provided partial protection with a reduction in viral shedding and minimal cross-reactivity detected in the hemagglutination inhibition titers (≤ 1:20). These results were consistent with the molecular assessment of antigenic sites within the HA gene having 11 differences between SwH1γ vaccine virus and SwH1α challenge virus. In chapter 4, we performed molecular characterization of an autogenous vaccine virus to examine the SwH1β virus that escaped immunity provided by a highly similar SwH1β vaccine virus. In this case, there was only 1 amino acid difference in an antigenic site of HA between the vaccine and epizootic viruses. Additionally, the NA, PB2, PB1, PA and NS genes of these viruses were compared and it was determined that these segments were at least 98.6% nucleotide similarity. However, since we did not have serologic results from the original case and we cannot confirm that these pigs were truly protected by maternally derived antibodies from the vaccinated sows, further evaluation of these viruses in a vaccine-challenge study would be necessary to assess the ability of the epizootic virus to escape immunity induced by these particular vaccine viruses.

To address the genetic and phenotypic characterization of influenza A viruses from swine, a set of viruses isolated from nursery pigs in one endemically infected multi-site swine production system (farm M) from 2007 to 2009 that either contained a distinct two amino acid insertion or were presumptive ancestral viruses without the insertion were selected. In chapter 5, the full genome of viruses from farm M were sequenced and characterized to examine the accumulation of amino acid changes between the viruses over time. These changes in the amino acid sequence of HA accumulated over time. The more recent isolates (SG-1094 and SG-1150) had the same changes as the earlier isolates plus new changes. The most recent isolates from 2010 (SG-1152 and SG-1156) had the same changes as SG-1094 and SG-1150, plus additional changes. However, while some of the changes in the NA and the internal genes also had a similar trend, some of the changes were random and did not show up in the other isolates.

In order to examine the effect of the genetic changes on the phenotype of these recently characterized viruses, a new technique called virus histochemistry was used to examine the patterns of virus attachment in the respiratory tract in chapter 6. In this study, the pattern of virus attachment and the morphometric score of bronchiolar attachment were examined for viruses containing the 2 amino acid insertion along with representative viruses of the swine H1 subtype that have been studied in-vivo and found to be virulent in swine. The morphometric score results showed a detectable difference in bronchiolar epithelial binding ($p < 0.0001$), where two of the insertion viruses had less binding than the other three insertion viruses and the two presumed ancestral viruses. A difference was also detected in the morphometric scores of the representative virulent strains ($p < 0.0001$), where the SwH1δ viruses had significantly less binding than the representative viruses for the SwH1α, β, and γ clusters.

In order to advance our understanding of influenza viruses and achieve applicable results from studies, it will remain important to translate the molecular and phenotypical characterization results derived from studies into clinically relevant information. To aid this, future pathogenicity, antigenicity and transmission studies comparing the ancestral viruses to insertion viruses, as well as viruses that have the insertion added to them via reverse genetics would need to be performed.

Controlling influenza viruses in swine will likely continue to be dynamic and difficult. With continued efforts of collaboration between agencies and institutions, surveillance and sequencing efforts can be improved by more open sharing of sequences between agencies/institutions, performing targeted surveillance in under-sampled populations or regions to fill in the gaps within the current data sets, and sequencing whole genomes of influenza viruses. Additionally, since influenza in swine not only causes economic losses for pork producers, but also poses a potential risk to public health, investigation into improved control and prevention strategies continues to be not only warranted but necessary as we move forward and advance our knowledge of influenza infections in swine.

GENERAL CONCLUSIONS:

In order to improve our understanding of IAVs in swine, the goal of this dissertation was to address the ability of genetic characterization to predict variations in virus phenotype, such as viral binding and antigenicity. The general conclusion for each of the five projects contained in chapters 2 through 6 of this dissertation supported this overall goal through methods that can be used to collect more viruses, by examining the genetic characteristics vaccine viruses, and by examining the genetic and phenotypical characteristics of novel and virulent viruses.

Chapter 2. Influenza A virus was detectable by real time RT-PCR and virus isolation in swine oral fluids collected from influenza negative pigs and spiked with influenza virus, and oral fluids collected from influenza infected pigs. The results this study demonstrated that pen-based oral fluids provide an easy, effective, and safe collection method for the detection of swine influenza virus with rapid testing methods such as real-time RT-PCR, which in turn will provide us with more viruses to add to our viral archives for molecular and phenotypical characterization.

Chapter 3. The results of the vaccine efficacy study indicated that the protection provided by the commercial vaccine, FluSure XP™, against challenge with a Canadian origin SwH1α cluster virus, resulted in a cumulative reduction in virus shedding in the nasal secretions over the five day challenge period and no detectable virus in the nasal secretions, lungs and BALF on day five post-challenge. Molecular characterization of the vaccine and challenge viruses can be used to explain why there was only reduction in virus shedding (partial protection) instead of complete prevention of virus shedding (complete protection). The hemagglutinin gene sequence of the challenge and vaccine viruses revealed that there were 11 and 24 amino acid differences in antigenic sites between the challenge virus and the SwH1γ and SwH1δ2 viruses, respectively. These variations could result in reduction in neutralizing effects of the antibodies produce by vaccination on the challenge virus.

Chapter 4. Outside of controlled vaccine efficacy experiments, influenza virus vaccines need to be examined for their effects in the field. In a clinical situation where an autogenous vaccine appeared to have failed to provide protection against challenge with a highly similar epizootic virus, molecular characterization revealed that there were 1 and 24 amino acid differences in antigenic sites of hemagglutinin between the SwH1β epizootic virus and the SwH1β and SwH1δ1 viruses, respectively. The changes described in this situation will provide a benchmark for evaluating vaccine and epizootic viruses in swine populations and molecular characterization will provide a useful tool for examining the continual changes that are found in viruses isolated from the U.S. swine population.

Chapter 5. Hemagglutinin gene sequencing has become routine practice for some swine farm systems that are endemically infected with influenza A viruses including the farm system where the unique two amino acid insertion described in this study was found. Retrospective analysis of the HA sequences from this farm provided potential ancestral viruses that were isolated six months prior to the viruses with the insertion were isolated. The viruses in this study had sustained transmissibility and fitness in swine over a three year period despite the two amino acids inserted in a key antigenic site. Additionally, the microevolution of these insertion and potential ancestral viruses has provided a benchmark to evaluate the past and future evolution of the virus in swine.

Chapter 6. In order to enhance our understanding of the pathogenesis of influenza viruses in swine, direct assessment of virus-host cell interaction using virus histochemistry was performed with formalin-fixed paraffin-embedded normal swine tissues. This study demonstrated that the patterns of virus attachment had strong virus binding within the bronchioles and alveoli for the insertion and ancestral viruses from chapter 5, and representative viruses previously described in in-vivo studies as causing severe lung lesions. By combining the results of this study with the results of molecular characterization of these viruses, we can bridge the gap between understanding genotype and understanding phenotypes of influenza A viruses in swine. The new insights into viral binding of swine viruses will lead to better understanding of the role of virus tropism and replication in IAV pathogenesis in pigs and may also provide new targets for more effective prevention and treatment measures.

LIMITATIONS AND FUTURE DIRECTIONS

The rapid evolution of influenza viruses in swine over the last decade has presented a challenge for both the detection and control of the virus in swine populations. While this dissertation addressed different aspects of control and characterization of influenza A viruses in swine, there were limitations within each study that need further investigation. In this section, some of the limitations of the experiments will be discussed along with the opportunities that these limitations present for future investigations.

Chapter 2: Detection of influenza A virus in porcine oral fluid samples.

While PCR based tests are sensitive enough to detect a small amount of RNA present in oral fluid samples, we also need to be able to isolate the virus from these samples in order to fully characterize viruses collected via influenza surveillance projects. The limited ability to isolate the influenza viruses in this study is a major pitfall of the technique and further studies need to be performed to determine what the underlying cause is and how this cause may be mitigated. In this study there was no virus isolated from samples using a 6 or 12 hour collection time, but there was virus isolated using the 30 minute time period, suggesting that collection time was a factor. Other factors that may be involved are enzymes within the swine oral fluids that could inactivate these viruses. While these enzymes have been studied in human saliva, they have not been examined in swine oral fluids. Another factor that could result in inactivation of influenza viruses is the use of cotton ropes for collection. Dacron swabs are used for nasal swabs because it was demonstrated that cotton provides an unfavorable environment for influenza viruses. Although nylon ropes may cause damage to the gingiva and may not provide sufficient sample for testing, it is possible that moisture wicking material used in exercise clothing may provide an alternative that is both absorbent and may keep the virus viable. Lastly, influenza antibodies (IgA, IgG and IgM) have not been well characterized in porcine oral fluids. These antibodies could have neutralizing effects on the virus and present an alternative source of antibodies for serologic tests. Further studies will need to be conducted to determine if the quantity of these antibodies in oral fluids reflects the quantity in serum and if they have an effect on virus isolation.

Chapter 3: In vivo evaluation of vaccine efficacy against challenge with a contemporary field isolate from the alpha cluster of H1N1 swine influenza virus.

In a typical study design, vaccinated animals are housed in one isolation room and non-vaccinated (placebo) animals are housed in another isolation room. In this vaccine efficacy study, the study design was different. Vaccinated and placebo pigs were mixed within two rooms. This reduced the effect that room would have on the statistical analysis and helped to maintain the blinded status of the person collecting clinical and post-mortem assessments. One of the pitfalls of this design is that the person performing the vaccinations and placebo injections needed to take extra care of which syringes contained which treatment and which animals received those treatments. Unfortunately, two pigs were removed from the study because they each received one vaccine and one placebo injection (they were only half vaccinated). Another limitation of this study was the number of pigs that were used. The number of pigs needed for the study was calculated using the minimal detectable difference in virus titer. However, taking into account that these animals were blocked by weight, if we wanted to assess the microscopic scores as 0-4 instead of 0-2 and 3-4, we would have needed four times as many pigs per experimental group. For future investigations, the mixed room study design should be implemented with care and random allotment instead of blocking by weight may make the statistical analysis less complicated.

The use of molecular characterization of the vaccine and challenge viruses may be useful for selecting viruses for future vaccine efficacy studies. In this study, molecular analysis was performed after the virus had been selected. In the future, use of molecular tools prior to selection may help with the selection process and help us better understand the link between genetic variation of influenza viruses and responses to vaccination in swine. This information could be critical in understanding vaccine efficacy and the formation of vaccination policies at farm, regional and national levels.

Chapter 4: Molecular analysis of vaccine and epizootic virus strains of influenza A viruses in swine.

The greatest challenge in this study was obtaining the full genome sequences for the molecular characterization of the vaccine and epizootic viruses. In the end, only 6 of 8 segments were sequenced for these viruses by the Sanger method. Despite the advances in sequencing techniques that have resulted in longer reads per trace file and more complete sequences for lower overall costs, there continue to be roadblocks to obtaining full genome sequences. Determining the proper balance of traditional and Next Generation sequencing that result in accurate sequences is an area that needs further exploration. As newer sequencing technologies and computer software are developed this balance will change and need to be adapted in order to produce results that best represent viral adaptations and changes within the host or population.

Another limitation of this study was the lack of serum samples from both the Dams and the 10 to 16 day old pigs that were infected with the epizootic strain of influenza. Theoretically, the sow responded to the vaccination prior to farrowing and produced antibodies that were transferred to the newborn pigs via colostrum, but we do not know what individual animal variations affected the sow's response to vaccination and formation of colostral antibodies, or the response in the offspring. However, for this study, we have no proof that the sows responded appropriately to the vaccine, produced antibodies in the colostrum and the antibody levels in the offspring. We also have no evidence that even if these pigs had passive antibody protection that it would have been effective against the epizootic strain. As a follow-up to this pilot study, further evaluation of these viruses in a vaccine-challenge study would be necessary to assess the ability of the epizootic virus to escape immunity induced by these particular vaccine viruses by both traditional vaccination of naïve animals at 3 and 5 weeks of age and through maternally derived antibodies. Potential challenge viruses would include the epizootic virus and a syngenic strain developed from the vaccine virus with the T72A variation within the hemagglutinin protein by reverse genetics. The results of this study could provide information critical to understanding vaccine efficacy and the formation of vaccination policies at farm, regional and national levels.

Chapter 5: Molecular characterization of a virulent influenza A virus with a two amino acid insertion in the hemagglutinin gene.

A unique two amino acid insertion was found within the Sb antigenic site of the hemagglutinin protein and this virus has demonstrated sustained transmissibility and fitness in swine over a three year period. While the insertion and ancestral viruses were genetically well characterized in this study, the potential effect that this insertion could have on the three-dimensional structure of hemagglutinin and protein function were not examined. For the three-dimensional structure, x-ray crystallography combined with homology modeling would provide us with a picture of how this insertion changes the receptor binding pocket and antibody binding site. The effects that these changes have on binding of HA to sialic acid receptors and/or neutralizing antibodies could be examined through functional assays in future studies.

Receptor binding could be assessed by examining the ability of these viruses to agglutinate red blood cells from turkeys or chickens, by measuring the association constants (K_{ass}) using sialylglycopolymers conjugated with fetuin, and by glycan microarray. Antibody binding of HA can be assessed using cross-hemagglutination inhibition titers using these insertion and ancestral viruses and hyperimmune serum produced in either ferrets or pigs followed by antigenic cartography. Other functional studies would include measuring the replication kinetics of these viruses in MDCK cells and measuring protein expression with viral polymerase activity using ancestral and insertion viruses, as well as reverse genetics-derived syngenic strains. Additionally, the transmissibility, pathogenesis and fitness of the ancestral and insertion viruses, as well as reverse genetics-derived syngenic strains could be assessed in-vivo.

In addition to studies that would characterize the effect of the insertion on the hemagglutinin protein function, the complete set of 20 insertion and ancestral viruses collected from this farm since 2007 provides an opportunity to examining the rate of evolution of a single virus strain in a multi-site production system using the Bayesian Markov Chain Monte Carlo approach available in the BEAST software package to estimate the rates of nucleotide substitution of each gene segment.

Chapter 6: In vitro characterization of influenza A virus attachment in the upper and lower respiratory tracts of pigs.

The technique used in this study, virus histochemistry, was established for use on human and avian influenza viruses at Erasmus University in the Netherlands. One of the goals of this pilot study was to get the protocol of this technique working at the University of Minnesota and this was accomplished. However, one of the limitations of this technique was found when the morphometric scores of the bronchioles were compared between staining runs for one of the control viruses. This finding indicates a limitation in the repeatability of this technique using this protocol. An alternative protocol using Dako's EnVision+ system to amplify the signal and adding 3% hydrogen peroxide and 10% normal serum blocking steps to block endogenous peroxides and biotins, respectively, was tested. It is possible that these changes in the protocol could result in improved repeatability of the technique, but this will need to be tested using the morphometric scoring. Another technical challenge was maintaining temperature and humidity within the laboratory, incubation chamber and refrigerator. Low humidity causes the tissue on the slide to dry out, which results in increased background and affects interpretation. Further studies are needed to determine the role of humidity on the variation between slide runs.

Since the pandemic strain A/CA/04/2009 had very limited binding throughout the respiratory tract, it may be useful to examine the pattern of virus attachment for another pandemic strain that has been demonstrated to be more pathogenic in swine, such as A/Mexico/4108/2009. Another limitation that should be addressed is the role of the host in the process of infection and the complexity of host variation. In this pilot study, only four 6-week-old pigs from 3 different litters on the same farm were compared. In order to investigate the effect that genetic background has on the pattern of virus attachment, respiratory tissues of pigs from diverse backgrounds would need to be collected for a future study examining this effect. Additionally, to investigate the effect that age (growth development) has on the pattern of virus attachment, respiratory tissues of pigs of different ages would need to be examined in future studies.

Exhaustive work has been done in order to advance our understanding of influenza viruses, yet it still remains important to translate the molecular and phenotypical characterization results derived from these studies into clinically relevant information. Therefore, future pathogenicity, antigenicity and transmission studies of influenza A viruses may need to employ very defined and precise reverse genetics techniques to elucidate genetic changes of importance. It is my hope that global influenza surveillance will lead to the sharing of viruses and techniques in order to more fully understand this ever-changing, ever-challenging pathogen of both humans and animals.

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