Transmission and control of influenza virus in pig populations

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Dedication

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General introduction

Influenza A virus (IAV) is a common cause of respiratory disease in pigs and has been detected in pigs in most pig producing regions of the world, including recent reports from countries previously deemed free of common IAV subtypes in pigs (Hofshagen et al., 2009; Deng et al., 2012). Influenza A viruses are able to infect many different animal species, including pigs, humans, and birds. In addition, influenza A viruses may be transmitted between pigs and other species, as highlighted by the pandemic 2009 H1N1 virus and H3N2 variant viruses (Myers et al., 2007; Forgie et al., 2011; Bowman et al., 2012).

Following the first clinical description of influenza virus infection in pigs in the United States in 1918 (Koen. 1919), research targeting influenza A viruses in pigs and other animal species has intensified quite rapidly. At the same time, control of influenza in swine farms has become increasingly challenging as there are many diverse influenza virus lineages present in the United States pig population (Ducatez et al., 2011; Corzo et al., 2013). Furthermore, current vaccine based control strategies may be effective under specific conditions (Romagosa et al., 2011a; Corzo et al., 2012), but less effective in other situations (Gauger et al., 2012; Vincent et al., 2012). Influenza control is further hindered by the relatively small amount of information assessing influenza virus transmission and within herd infection dynamics as compared to other research disciplines.

A major focus for control for other common swine pathogens, including porcine reproductive and respiratory syndrome virus (PRRSv), has been virus elimination from the herd. This has been accomplished using several different methods and based on a thorough understanding of infection patterns within herds (Corzo et al., 2010). While procedures used to eliminate IAV from a three-site pig herd have been described, additional knowledge regarding influenza virus transmission and infection dynamics may aid elimination and control measures in a variety of settings (Torremorell et al., 2009).

Due to the narrow window of influenza virus detection from individual animals (i.e. days), describing infection patterns within swine herds can be difficult and costly. Limited information describing the temporal patterns of influenza virus infection in specific subpopulations exists, specifically at the breeding herd level. Studies are also needed to assess the resultant downstream impact on grow-finish herds following infection of pigs at the breeding herd. Herd level risk factors for influenza virus infection have been described, however; little information regarding virus circulation within and across herds has been described.

Vaccination for influenza is a widely used control measure in pigs and has been adopted by approximately 70% of large sow farms in the United States (USDA. 2007; Beaudoin et al., 2011). The impact of vaccine use on sow farms may vary from controlling the expression of influenza associated disease to reducing influenza virus transmission. The use of influenza virus vaccines in sow herds is common, however; the impact of vaccine use on influenza virus transmission has not been described at the sow herd level. This knowledge would better inform vaccine-based control measures and their impact on virus transmission and ultimately virus elimination.

While vaccination has been the major control measure for influenza in swine populations, knowledge concerning other potential transmission routes and risk factors for infection may also aid in influenza control. Indirect transmission routes, including transmission by fomites, have not been studied in detail to assess the plausibility and commonality of these events in swine populations. This knowledge may help reduce the incidence of influenza virus infections and in combination with other control measures may also help eliminate influenza virus from swine herds.

In conclusion, there is a wealth of information available to help address influenza virus infections in the United States pig population. However, influenza virus continues to be a major health challenge for swine producers, veterinarians, and public health officials. Limited information exists regarding influenza virus epidemiology,

transmission, and infection dynamics within herds. A more thorough understanding of these specific topics will allow for better implementation of control strategies and mitigation of influenza virus infections in pig populations. This thesis aims to 1) describe within herd influenza virus infection dynamics and temporal patterns of infection in breeding and grow-finish herds, 2) assess the prevalence and temporal patterns of influenza virus infection in weaning-age pigs on commercial swine breeding herds, 3) evaluate influenza virus transmission via indirect routes, and 4) determine the impact of vaccination and maternally derived immunity on influenza virus transmission in weaning-age pigs.

Chapter 1: Literature review

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Influenza virus characteristics

Influenza viruses are members of the family *Orthomyxoviridae*, with influenza type A viruses comprising the important zoonotic viruses and the viruses affecting pigs. Influenza A viruses are enveloped RNA viruses with a segmented genome. The proteins encoded by the 8 RNA genomic segments include the hemagglutinin (HA) and neuraminidase (NA) glycoproteins into which influenza A viruses are further subtyped. The following proteins are also encoded by the genomic segments: matrix protein 1 (M1), matrix protein 2 (M2), polymerase basic 1 (PB1), polymerase basic 2 (PB2), polymerase acid (PA), nucleoprotein (NP), nonstructural protein 1 (NS1), nuclear export protein/nonstructural protein (NEP/NS2), and polymerase basic 1-F2 (PB1-F2) (Knipe et al., 2007).

The HA surface protein is important for virus-cell interaction, virus entry into host cells, and it is a major surface antigen (Wiley and Skehel, 1987). Influenza viruses are able to evolve over time due to genetic changes, termed antigenic drift and shift. Antigenic drift is the accumulation of small changes in genes over time due to mutations that result in immunologically distinct IAVs. Antigenic shift results when gene segments are exchanged between viruses and novel IAVs emerge. These important mechanisms allow for influenza viruses to evade existing control strategies and lead to the complex epidemiology of influenza viruses.

History of influenza virus infection of pigs in the United States

Influenza virus was first isolated from pigs in 1930 (Shope. 1931). However, the clinical disease was described earlier in 1918, at the same time as the 1918 human influenza pandemic (Koen. 1919). This causative IAV was later termed classical swine influenza virus and was of the subtype H1N1. The classical H1N1 influenza viruses then remained the dominant viruses in the United States pig population until 1998. In the United States the seroprevalence to classical H1 influenza viruses was 20-47% in 1976-1977 and 51% in 1988-89 (Hinshaw et al., 1978; Chambers et al., 1991). These studies both reported lower seroprevalence to H3 influenza viruses at 1.4% in 1976-1977 and 1.1% in 1988-

1989 (Hinshaw et al., 1978; Chambers et al., 1991). The epidemiology of influenza virus in the United States changed following the introduction of H3N2 viruses in 1998 (Zhou et al., 1999).

A severe respiratory disease outbreak was observed in a pig farm in North Carolina in 1998, followed by outbreaks in Texas (Zhou et al., 1999). It was later confirmed that these outbreaks were caused by H3N2 influenza viruses derived from genetic reassortments of avian, swine, and human influenza A viruses (double and triple reassortant viruses) (Zhou et al., 1999). Triple reassortant viruses then became widespread in the United States pig population (Webby et al., 2000). Today, there are many distinct viral lineages present in the United States including: classical H1N1, H3N2, reassortant H1N1, H1N2, human-like H1N1, and human-like H1N2 (Vincent et al., 2008). More recently, the viral lineages present in the United States have been expanded to include the pandemic 2009 H1N1 influenza virus, first reported in pigs on an Alberta, Canada swine farm (Howden et al., 2009), and H3N2 variant or H3N2v viruses (Bowman et al., 2012).

2009 pandemic H1N1 influenza virus

The 2009 pandemic H1N1 influenza virus contained a combination of gene segments from both North American and Eurasian swine lineages that had never been detected previously (Garten et al., 2009; Smith et al., 2009). Further analyses concerning the 2009 H1N1 pandemic virus and the evolution of influenza viruses in general have provided important information regarding the potential origin of this novel virus. It has been suggested that the virus may have been circulating in swine, albeit undetected, for some time prior to its initial identification (Garten et al., 2009; Smith et al., 2009). Southern China received much focus due to the presence of both North American and Eurasian swine lineage viruses in Chinese pigs. Reassortment events between North American and Eurasian influenza viruses have led to the establishment of stable virus lineages in swine and these reassortment events were shown to occur in China before and after the

pandemic. However, a direct 2009 pandemic H1N1 precursor virus has not been recognized (Lam et al., 2011; Vijaykrishna et al., 2011).

Infection of a commercial swine farm with the 2009 pandemic H1N1 virus was first confirmed in May 2009 in Alberta, Canada (Howden et al., 2009). Following the description in Canada, the 2009 pandemic H1N1 virus was identified in many other areas around the world (Hofshagen et al., 2009; Moreno et al., 2010; Pasma and Joseph, 2010; Pereda et al., 2010; Song et al., 2010; Sreta et al., 2010; Welsh et al., 2010; Forgie et al., 2011). These reports describe some of the initial 2009 pandemic H1N1 infections in swine herds and provide details including clinical signs, pathogenesis, source of virus and the course of infection within herds. Gray and Baker (2011) summarized the reports to the world organization for animal health (OIE) of pandemic H1N1 virus infections in pigs, and indicated that the virus was present worldwide (Gray and Baker, 2011).

Human-to-swine transmission of 2009 pandemic H1N1 influenza virus was suspected in a majority of these case descriptions. Forgie et al. (2011) provided detailed epidemiologic data from a Canadian swine research farm in which humans were infected before swine and swine became infected following contact with the human index case (Forgie et al., 2011). Hofshagen et al. (2009) described the spread of 2009 pandemic H1N1 influenza virus in Norway, previously free of classical swine influenza, in which humans infected with 2009 pandemic H1N1 influenza virus were the most likely source of virus (Hofshagen et al., 2009). However, in many of these cases, definitive evidence of human-to-swine transmission was lacking, and human-to-swine transmission was suspected after other potential sources were ruled out. The 2009 pandemic H1N1 virus is also known to transmit efficiently between pigs, making the introduction of infected pigs into a population another likely route of virus entry into populations (Brookes et al., 2009). The detailed description of 2009 pandemic H1N1 virus infections within herds also pointed to the fact that this virus behaved similarly to endemic swine viruses and all susceptible swine populations were likely able to become infected and transmit the virus.

The worldwide presence of the 2009 pandemic H1N1 virus provided the opportunity for reassortment events between this virus and endemic or other viruses. A novel reassortant virus (A/swine/Hong Kong/201/2010) was identified in swine through systematic virologic surveillance in Hong Kong in January 2010 (Vijaykrishna et al., 2010). Additional reassortant viruses derived from the 2009 pandemic H1N1 virus were then discovered including novel H1N2 viruses in Italy and Argentina, and novel H1N1 reassortant viruses in Germany, Southern China, Thailand, and Argentina (Kitikoon et al., 2011; Moreno et al., 2011; Pereda et al., 2011; Starick et al., 2011; Zhu et al., 2011). Similarly, nine pandemic/endemic reassortant influenza viruses were described and isolated during syndromic and active surveillance from across the United States (Ducatez et al., 2011).

Epidemiology of influenza virus in pigs in the United States

Prior to the emergence of H3N2 viruses in 1998, seroprevalence data indicated that influenza viruses were widespread in the United States pig population. Although seroprevalence data has been quite useful historically to describe the epidemiology of influenza virus in the United States pig population, virologic surveillance can provide additional information even in the presence of routine influenza virus vaccination. A recent virologic surveillance study in the United States identified 91% of enrolled growing pig farms as influenza virus positive during a 12 to 24 month period (Corzo et al., 2013). Several farms in the aforementioned study had different viruses detected throughout the study period (Corzo et al., 2013). This study not only described the ubiquitous nature of influenza virus infections, but it also showed that there is a wide variety of influenza virus subtypes and genotypes present across the United States, including the 2009 pH1N1 and H3N2v viruses.

Epidemiology of influenza virus within pig farms

Herd estimates indicate that influenza viruses are common with a seroprevalence of 83% in sow herds in Ontario, Canada and over 90% in sow herds in Belgium, Germany, and Spain (Poljak et al., 2008a; Van Reeth et al., 2008). The ubiquitous nature of influenza

viruses and the continual circulation of the virus in pig populations have brought forth several suggestions regarding maintenance of endemic influenza virus infections in pig populations. Historically, recurring influenza virus infections were thought to be due to long-term carrier pigs and intermediate hosts (Shope. 1941; Blaskovic et al., 1970a). While influenza virus replicates almost exclusively in epithelial cells of the respiratory tract of pigs, infectious virus has been detected in brain and this finding could also have implications on virus transmission (De Vleeschauwer et al., 2009; Loeffen et al., 2011). In both studies, the transference of virus from the nasal turbinates, nasopharynx or tonsils into the brain tissue during the dissection process was not ruled out conclusively. Further study concerning the duration of infection and infectiousness in individual pigs has shown that the duration is relatively short and raises serious doubts regarding the potential existence of carrier pigs (Vannier et al., 1985; Clavijo et al., 2002; Romagosa et al., 2011a).

Swine breeding herds are a very dynamic population as within swine breeding herds there is considerable variation in terms of pig age, level of immunity, animal turnover, movement within farm, and previous exposure to certain pathogens. All of these factors are likely to play a critical role in the epidemiology of IAV. The maintenance of influenza viruses in breeding herds can be explained via the continual infection of susceptible pigs introduced or produced in herds (Brown. 2000). The maintenance of influenza virus within herds over time has also been suggested following observational studies in pig populations over extended periods (de Jong et al., 2001; Poljak et al., 2008b). These temporal observations are further strengthened by work identifying influenza virus positive neonatal pigs in the absence of positive sows in breeding herds, with susceptible neonatal pigs potentially serving as the maintenance host population over time (Larsen et al., 2010). Virus circulation among weaned pigs, which was evident in up to one half of Dutch breeding herds (Loeffen et al., 2003), could serve to maintain infections within herds and as a source of virus dissemination.

Understanding the dynamics of influenza viruses in swine populations can help lead to effective strategies to eliminate influenza virus from herds. An H3N2 influenza virus was eliminated from a three-site pig herd by altering the timing of gilt introductions and the handling of suckling pigs in combination with nursery and finish depopulation (Torremorell et al., 2009). Strict all-in/all-out procedures by nursery room did not eliminate influenza virus in the nursery of the three-site herd mentioned above, likely due to continued indirect exposures within the site. These findings indicate that herd management factors and farm structure can impact the population dynamics of infection. This was shown when the incidence of influenza virus infection was found to be higher at the beginning of the finishing period in farrow-to-finish herds, whereas in finishing herds, the incidence of infection was highest at the end of the finishing period (Loeffen et al., 2009). Clearly, the structure of the pig herd must be taken into account when assessing influenza virus infection dynamics and control at the population level. Within growing pig populations, different infection patterns have been described, including an endemic (wave) form and an epidemic form (Simon-Grife et al., 2012). In addition, several pigs were shown to be influenza virus positive in non-consecutive weeks within a defined population (Simon-Grife et al., 2012). These observations provide insight into the epidemiology of influenza virus within populations and resultant impact on influenza virus prevalence and incidence.

Various risk factors for influenza virus infection at the herd level have been established. In Ontario, multiple risk factors for influenza virus seropositivity including herd size, pig density, farm type, and source herd infection status were identified (Poljak et al., 2008a). Pig density was also a significant risk factor for infection in Belgium (Maes et al., 1999). Herd seropositivity in Malaysia was associated with farm size, the importation of pigs, proximity of the farm to other pig farms, and the presence of mammalian pets on the farm (Suriya et al., 2008). Three risk factors for influenza virus seropositivity were identified in Spain, including replacement rate, open partitions between pens, and uncontrolled entrance to farms (Simon-Grife et al., 2011). Most recently, an active virologic surveillance study in the United States identified season of

the year as a predictor of influenza virus positivity at the group level and observed a high level of subclinical influenza virus infections (Corzo et al., 2013).

Influenza virus transmission

While transmission experiments and models exist for influenza virus in multiple mammalian and avian hosts, few have been conducted in pigs. Transmission experiments allow one to assess the spread of infection within a population via estimation of the reproduction ratio (R) and allow one to assess the effect of control measures in a population (Velthuis et al., 2007). The basic reproduction ratio (R0) is defined as the expected number of secondary cases in a completely susceptible population because of a typical infectious individual during its entire infectious period (Diekmann et al., 1990). In general, when R0 is >1, an infection will spread in a population, and when R0 is <1, the infection will not spread in a population. Therefore, the impact of control measures on R0 will highlight the ability to control the spread of influenza virus within a population.

One tool that may impact the reproduction ratio is vaccination. Several studies have estimated the reproduction ratio for avian influenza virus and have shown that vaccination may reduce transmission (van der Goot et al., 2005; van der Goot et al., 2007; Bos et al., 2008; Bouma et al., 2009). However, vaccination does not always reduce transmission of avian influenza virus and the impact of vaccination may vary across species (van der Goot et al., 2007; Poetri et al., 2011). Therefore, it is important to assess virus transmission and the impact of control measures in each specific host species.

Influenza virus has been traditionally characterized as a pathogen that moves rapidly in swine herds, resulting in high morbidity (Olsen et al., 2006). However, experimental quantification of virus transmission and the impact of control measures in pig populations are limited. Multiple examples of transmission from infected to susceptible pigs exist, as well as transmission from infected to seropositive contact pigs (Choi et al., 2004; Brookes et al., 2009; Lange et al., 2009). An ‘avian-like’ H1N1

influenza virus was shown to transmit through four pairs of vaccinated pigs at antibody levels thought to protect against infection (Lloyd et al., 2011). Influenza virus transmission was quantified in non-vaccinated and vaccinated pig populations with a reproduction ratio estimate of 10.66 in non-vaccinated pigs and reproduction ratio estimates of 1 and 0 for pigs vaccinated with heterologous and homologous inactivated vaccines (Romagosa et al., 2011a). Further study concerning influenza virus transmission in pigs may elucidate measures that can mitigate transmission and reduce the burden of influenza infections in pig populations.

Transmission between pigs and people

Following the first published description of clinical disease in pigs in 1918 and the isolation from pigs in 1930, influenza viruses have continued to evolve and impact animal and human health across the world (Koen. 1919; Shope. 1931). During the initial description of disease in pigs, it was noted that an outbreak in a family would occur concurrent with an outbreak in pigs (Koen. 1919). Serological evidence of influenza virus transmission from people to pigs under field conditions was later described (1938) in the United States and a human seasonal influenza virus isolate was first obtained from pigs in Taiwan (1969) (Shope. 1938; Kundin. 1970). Influenza virus transmission between people and pigs has been examined extensively and has been shown to occur sporadically over the last century (Nelson et al., 2012).

The transmission of influenza virus between people and pigs focuses on type A influenza virus strains, as types B and C are thought to primarily infect humans. Transmission of influenza viruses between species occurs, but this is an uncommon event. There are many barriers that limit interspecies transmission including influenza virus receptor specificity; however, all barriers are not completely known and understood. Influenza virus replicates primarily in the respiratory tract of pigs and humans, and therefore, similar modes of transmission apply to both pigs and humans. Recent work with the pandemic H1N1 virus showed that viable virus was only detected in the respiratory tract of infected pigs, other samples including muscle were negative

(Vincent et al., 2010). Not only did this study prove that the respiratory tract of pigs is the single most important site of replication, it also demonstrated that IAV transmission via consumption of pork is considered negligible.

In the United States, the classical H1N1 virus was the predominant viral lineage circulating in pigs until 1998. Several serologic studies in pigs before 1998 revealed that pigs were infected with H3 viruses, but prevalence of infection was quite low (Hinshaw et al., 1978; Chambers et al., 1991). A study in 1997/1998 indicated that pig exposure to human H3 viruses was greater than previously reported (Olsen et al., 2000). The diversity of influenza viruses then changed in the US in 1998 when a genetic reassortment of human H3N2, classical swine H1N1, and avian influenza virus genes was observed (Zhou et al., 1999). This reassortant virus became established in the North American pig population along with other diverse viral lineages including H1 viruses with a human origin HA gene (Zhou et al., 1999; Webby et al., 2004; Karasin et al., 2006; Vincent et al., 2009). Clearly, transmission of influenza viruses between people and pigs shaped the current epidemiology of influenza virus in North America (Nelson et al., 2012).

In contrast to the observed low level of H3 influenza virus in North America prior to 1998, H3N2 influenza virus related to human seasonal viruses were circulating in Europe and Asia at seemingly higher levels since the 1970s (Kundin. 1970; Shortridge et al., 1977; Ottis et al., 1982; Haesebrouck et al., 1985). This is one example of how influenza virus epidemiology in pigs differs by geographic region. However, similar to North America, the epidemiology of influenza virus is quite complex with multiple subtypes and variants circulating in Europe and Asia. One common observation regarding the transmission of influenza virus between people and pigs is the appearance of the 2009 pandemic H1N1 virus in pig populations around the world as this specific virus was easily transmitted between people and pigs.

Influenza virus transmission between pigs and people has played a large role in the complex epidemiology and evolution of influenza virus in pig populations. In cases

where a particular influenza virus can be transmitted efficiently in pig and human populations, it can be difficult to discern the direction of the transmission event, pigs to people versus people to pigs. Transmission of influenza viruses from pigs (swine-origin viruses) to people has been documented on sporadic occasions. The sporadic nature of the transmission is in spite of the fact that during routine animal husbandry practices, there is actually quite close contact between people and pigs occurring on a frequent basis. However, a swine influenza virus was first isolated from a man in 1974 (Smith et al., 1976). A swine-origin influenza virus of H1N1 subtype (A/New Jersey/76) was then isolated from soldiers at Fort Dix, NJ, USA in 1976 following a respiratory disease outbreak (Gaydos et al., 1977; Gaydos et al., 2006). However, the definitive source of the virus at Fort Dix is unknown and the virus did not spread outside of Fort Dix (Gaydos et al., 2006). Following the initial description of swine-origin influenza viruses in humans and the Fort Dix outbreak, additional swine-origin influenza virus infections of humans were observed.

One of these events includes probable human-to-human transmission following exposure to pigs at an agricultural fair in Wisconsin, USA (Wells et al., 1991). A literature review by Myers et al.(2007) revealed 50 cases of apparent zoonotic swine influenza virus infection and a review by Van Reeth (2007) displayed all documented human infections with swine-origin influenza viruses, including cases in Europe, Asia and North America (Myers et al., 2007; Van Reeth. 2007). A recent summary concerning human infections with triple reassortant swine-origin influenza viruses (H1) in the United States revealed that 11 human cases were reported between 2005 and 2009, most having exposure to pigs (Shinde et al., 2009). The most recent example of this event was the detection of H3N2v viruses in pigs and people at agricultural fairs (Bowman et al., 2012). These cases over time illustrate the sporadic nature of interspecies transmission and the ultimate role of surveillance to identify these events in pigs and humans. The clinical manifestations following swine influenza virus infection in humans do not seem to differ from those of typical influenza virus infections; however, many report previous contact

with pigs (Myers et al., 2007). Due to similar clinical manifestations, swine influenza virus infections in people are likely underreported.

In addition to documented cases of swine-origin influenza virus infections in humans, serological evidence has identified exposure to pigs as a risk of swine-origin influenza virus infection (Olsen et al., 2002; Myers et al., 2006; Terebuh et al., 2010a; Gerloff et al., 2011). For example, farmers, veterinarians and meat processing workers with swine exposure were shown to be at higher odds of exposure to H1N1 and H1N2 swine influenza viruses versus controls (Myers et al., 2006). Additional studies have also shown that swine workers and their non-exposed spouses are at increased risk of swine influenza virus infection (Gray et al., 2007). More recently, swine workers in Europe were shown to have more frequent and higher titers to swine influenza viruses, including the pandemic H1N1 virus compared to controls (Gerloff et al., 2011).

Even though occupational exposure to swine has been shown to increase the risk of swine influenza virus infection, there is more involved in transmission events than just exposure and contact with infected pigs. This was highlighted by an H2N3 infection of swine in which transmission from ill pigs to employees was not evident (Beaudoin et al., 2010). A prospective cohort study which assessed the transmission of influenza virus between pigs and swine workers found serologic evidence for infection with human and swine-origin influenza viruses in swine workers (Terebuh et al., 2010b). Additionally, this study identified a low rate of symptomatic infection and virus isolation among participant swine workers. Certain employee behaviors and attributes may impact the risk of human and swine-origin influenza virus infection, including the use of personal protective equipment, smoking and pre-existing immunity (Ramirez et al., 2006; Terebuh et al., 2010b; Beaudoin et al., 2011).

Influenza virus transmission via indirect routes

Transmission of infectious agents can occur via direct contact with infected hosts or via indirect contact. Potential indirect routes include both aerosols and fomites. However, the

importance of indirect routes of transmission such as fomites and aerosols has not been studied in detail regarding influenza virus in swine. These indirect routes of transmission have been suggested following influenza virus outbreaks in swine herds with no obvious link to infection via the introduction of infected pigs (Tofts. 1986; Desrosiers et al., 2004). Also, local spread of influenza virus has also been documented with indirect routes of transmission among the possible explanations (Poljak et al., 2008b).

Aerosol transmission of influenza virus has been documented in many hosts including humans, ferrets, guinea pigs, and birds (Alford et al., 1966; Mubareka et al., 2009; Yee et al., 2009a; Yee et al., 2009b). Aerosol transmission is thus a likely and important route of influenza virus transmission (Tellier. 2006). Influenza virus has also been detected in the air of rooms with experimentally infected pigs; however, aerosol transmission in pigs has not been demonstrated (Loeffen et al., 2011; Corzo et al., 2012; Corzo et al., 2013).

Fomites or inanimate objects can also play a role in influenza virus transmission. Influenza virus has been shown to contaminate inanimate surfaces including banknotes and various household surfaces (Boone and Gerba, 2005; Thomas et al., 2008). Human hands can also be contaminated with influenza virus and serve as a source of infection (Weber and Stilianakis, 2008; Mukherjee et al., 2012). Fomite transmission has also been observed in avian and guinea pig models (Mubareka et al., 2009; Yee et al., 2009b). However, the role of fomites in the transmission of influenza virus within and between swine farms has not been elucidated.

Factors that influence transmission

Even if the specific routes of transmission for an infectious agent are known, there will be certain factors that influence transmission events. Many factors can influence whether direct contact will lead to a successful transmission event including host susceptibility. For example, the presence of homologous maternal immunity has been shown to prevent transmission of influenza virus in pig populations (Romagosa et al., 2011a).

Indirect transmission routes can be influenced by environmental conditions. In a guinea pig model, aerosol transmission was dependent upon temperature and relative humidity. Cold and dry environmental conditions were shown to favor aerosol transmission events (Lowen et al., 2007). The survival of influenza virus in the environment is also heavily influenced by certain conditions. Hard, non-porous surfaces have been shown to extend the survival of influenza virus in the environment compared to other surfaces including cloth and paper (Bean et al., 1982). The inactivation of influenza virus on non-porous surfaces is also dependent upon relative humidity (Weber and Stilianakis, 2008). Temperature, salinity, and pH are also significant predictors of influenza virus survival in the environment (Irwin et al., 2011).

Influenza virus control and prevention measures

Vaccination has been the main focus of influenza control and prevention in swine. Two common vaccination approaches include sow vaccination and growing pig vaccination. According to the USDA, 70% of large sow farms and 29.3% of all sow farms vaccinated breeding females in 2006 for influenza virus (USDA. 2007). In contrast, 10.8% and 4.3% of nursery pig sites and grower/finisher sites vaccinated for influenza virus, respectively (USDA. 2007). Sow farm vaccination provides a benefit to the sows and gilts vaccinated, as well as neonatal pigs through passive immunity via colostrum. While maternal immunity can last for a long period of time depending on the initial titer, ultimately this immunity will wane and likely leave grow/finish pigs with low levels or undetectable levels of immunity (Loeffen et al., 2003; Markowska-Daniel et al., 2011). Biosecurity measures are also vital for the prevention and control of influenza virus. Pig to pig contact is the primary mode of influenza virus transmission (Olsen et al., 2006). Therefore, controlled pig movement may help prevent influenza virus infections.

Influenza virus vaccination

Influenza virus vaccination is a common practice on swine farms in the United States. The impact of vaccination is largely studied in experimental challenge models. Since the

introduction of H3N2 influenza virus in 1998, vaccination for influenza virus has become more complex. Influenza virus vaccines are typically adjuvanted and inactivated (Thacker and Janke, 2008). Killed influenza virus vaccines are commercially available and autogenous vaccines are also used. In 2006, autogenous vaccines accounted for around 20% of influenza virus vaccine use in US swine breeding females (USDA. 2007).

A multitude of influenza virus vaccine studies have been performed in pigs. A majority of these studies assess the impact of vaccine on serological, virological, clinical, and pathological parameters following challenge with either homologous or heterologous influenza viruses in an experimental setting. Complete protection following homologous (identical vaccine virus and challenge virus) influenza virus experimental challenge has been demonstrated via the use of inactivated influenza virus vaccines (Bikour et al., 1996; Lee et al., 2007; Romagosa et al., 2011a). However, partial protection is commonly observed when the challenge virus and the priming vaccine antigen are not identical or heterologous (Heinen et al., 2001; Lee et al., 2007; Vincent et al., 2008). Enhanced pneumonia in vaccinated pigs following infection with a heterologous virus (genetically and antigenically distinct) has also been observed (Vincent et al., 2008; Gauger et al., 2012).

In contrast to vaccination, live exposure and recovery from influenza virus infection has been shown to provide complete protection to homologous and/or heterologous challenge viruses (Van Reeth et al., 2003; Vincent et al., 2008). It appears that live virus exposure activates the cell mediated (CMI) and humoral components of the immune system, thus providing greater cross-protection (Vincent et al., 2008). This has prompted the search for modified-live vaccines and other vaccines in swine that offer greater cross-reactivity potential. However, inactivated influenza virus vaccines are currently the only available option for swine.

Influenza virus maternally derived immunity (MDI)

Maternally derived immunity (MDI) is obtained in neonatal pigs through ingestion of colostrum rich in antibodies, cells, and other factors (Salmon et al., 2009). Colostrum is of vital importance as the epitheliochorial placentation of swine prevents the transfer of antibodies and cells in utero (Kim. 1975). Therefore, MDI is necessary in order to prevent and reduce clinical disease from a variety of causes in the first weeks of life in pigs.

Specifically, the impact of maternally derived immunity (MDI) on influenza virus infection in swine has been studied. Complete protection following homologous influenza virus challenge in pigs with MDI has been shown (Blaskovic et al., 1970b). However, further study has shown that MDI is able to reduce clinical signs associated with influenza virus infection, but protection is not complete (Mensik et al., 1971; Loeffen et al., 2003; Choi et al., 2004; Kitikoon et al., 2006). In fact, pigs with MDI have been shown to excrete virus for a longer period of time than pigs without MDI, and MDI can potentially enhance lesions associated with a heterologous influenza virus challenge (Loeffen et al., 2003; Kitikoon et al., 2006; Vincent et al., 2012). It is also generally believed that a negative correlation exists between HI titers at vaccination and/or at exposure to influenza virus and the development of active immunity (Renshaw. 1975; Loeffen et al., 2003; Kitikoon et al., 2006; Markowska-Daniel et al., 2011). Therefore, the major impact of influenza virus MDI is related to its ability to reduce the clinical effects of disease without a major impact on influenza virus infection.

It must be noted that a majority of studies regarding influenza virus infection and MDI have been individual pig challenge studies. Although this type of study can evaluate the typical infectious disease study parameters including clinical signs, virus shedding, and respiratory tract lesions, these studies do not allow for the evaluation of virus transmission in a population setting. Also, pigs can be exposed to influenza virus at an early age in which the levels of MDI are likely at their peak (Larsen et al., 2010). Influenza virus challenge studies are often performed in older pigs in which the level of MDI may be lower (Loeffen et al., 2003). Knowledge concerning the transmission of

influenza virus in the presence of MDI would provide additional important knowledge in regards to control of influenza virus.

Chapter 2: Infection dynamics of pandemic 2009 H1N1 influenza virus in a two-site swine herd

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Introduction

Following the initial description of clinical influenza in pigs in 1918 and the first viral isolation from pigs in 1930, influenza viruses have continued to evolve and impact animal and human health (Koen. 1919; Shope. 1931). Intermittent reports over the past 35 years indicate that influenza virus infections are common in swine dense areas of the United States (Hinshaw et al., 1978; Chambers et al., 1991; Olsen et al., 2000; Choi et al., 2002). Influenza viruses are also widespread in other swine-producing regions with herd prevalence estimates as high as 83% in sow herds in Ontario, Canada, and over 90% in sow herds in Belgium, Germany and Spain (Poljak et al., 2008a; Van Reeth et al., 2008).

The emergence of the pandemic 2009 H1N1 influenza virus in multiple host species highlighted the evolving nature of influenza viruses and the potential for inter-species transmission. The pandemic 2009 H1N1 virus spread rapidly across the world, and transmission from humans to pigs has been described (Forgie et al., 2011). In addition, the sporadic identification of uncommon influenza virus subtypes in pigs including H4N6, H9N2, H2N3, H5N2 and H7N2 also demonstrates the complexity of influenza virus epidemiology (Karasin et al., 2000; Peiris et al., 2001; Ma et al., 2007; Lee et al., 2009; Kwon et al., 2011).

Several risk factors for influenza virus infection at the herd level have been identified including high pig density in an area, increased replacement rates, open partitions between pens and uncontrolled entrances to farms (Maes et al., 1999; Poljak et al., 2008a; Poljak et al., 2008b; Suriya et al., 2008; Simon-Grife et al., 2011). These risk factors suggest potential areas of focus for influenza virus control and prevention. While experimental studies have provided considerable understanding of influenza pathogenesis and infection dynamics in individual hosts, there is sparse information in commercial pig populations. Continuous circulation or maintenance of influenza viruses within populations over time, such as swine breeding herds, has been suggested (Easterday and Couch, 1975; Brown. 2000; de Jong et al., 2001; Poljak et al., 2008b). However, the expected period of persistence of influenza viruses following introduction into swine

populations and factors that may influence it are unknown. The objectives of this study were to compare the occurrence of influenza virus infection in defined subpopulations of a swine breeding herd and to describe temporal patterns of infection in a growing pig population partially sourced from that breeding herd.

Materials and Methods

Study herd

A swine breeding herd (Site 1) and one of its associated wean-to-finish sites (Site 2) were enrolled in this study in July 2010. Site 2 received weaned pigs from Site 1 and one other swine breeding herd. All sites were located in Southern Minnesota, USA. Site 1 operated with strict biosecurity measures including shower-in/shower-out procedures and controlled movement of pigs, people and materials into the site. Site 2 also practiced controlled movement of pigs, people and materials into the site. Both sites were free of porcine reproductive and respiratory syndrome virus (PRRSv) during the course of the study according to regular veterinary inspection and diagnostic testing. Animals at Site 1 and Site 2 were not vaccinated for influenza virus during the course of this study. Specific site characteristics of Site 1 and Site 2 are described below. All animals were under the direct supervision of the respective site caretakers, and all animal handling by study personnel was approved according to University of Minnesota IACUC protocols 0912A75417 and 0912A75418.

Site 1

Site 1 consisted of four interconnected buildings housing approximately 3000 sows/gilts and 4000 neonatal pigs (from birth until an average weaning age of 21 days). Two buildings housed open and pregnant sows/gilts in individual stalls, and two buildings housed periparturient and lactating sows and neonatal pigs from birth until an average weaning age of 21 days in individual farrowing crates. Mature gilts (6–7 months of age) were introduced to the herd on a weekly basis from a continuous flow gilt development unit (GDU), which was located offsite. The GDU received maternal line weaned pigs from Site 1 on a monthly basis. Just prior to enrolment of this herd into the study,

influenza virus genome was detected at Site 1 based on routine oral fluid testing of weaned pigs conducted by the herd veterinarian. The original timing and source of influenza virus infection at Site 1 was unknown; however, influenza virus infection was previously suspected based on downstream pig performance and diagnostic testing.

Site 2

Site 2 (Figure 2.1) was a tunnel-ventilated wean to finish barn housing approximately 5000 pigs and managed in an all-in/all-out manner by site (one barn on the site). All pigs at this site were housed in one building with 2 rooms separated by a solid wall. There were 24 open partition pens of approximately 210 pigs each. Pigs at Site 2 were weaned from two different breeding herds, with 2175 pigs originating from Site 1, which was described above. Influenza virus genome was not detected in a previous sampling of weaned pigs from the second breeding herd source approximately 2 months earlier. At this previous sampling, 60 nasal swab and 10 oral fluid samples were influenza virus genome negative via matrix gene-based real-time reverse transcription-PCR (RRT-PCR) assay.

The barn was fully populated over a period of 11 days. Initially, pigs were systematically placed within pens by breeding herd source based on the date of arrival to Site 2. However, mixing of pigs by breeding herd source within pen did occur within sort pens and to balance the number of pigs within pens. Pigs from each breeding herd source were also represented in each room of the building. Therefore, pens and rooms could not be assigned by breeding herd source. In addition, the open partition pens allowed for direct contact across pens immediately following placement. Forty days following the initial introduction of pigs, 2160 pigs were transferred to a separate grow–finish site to maintain adequate space allowances. Following the transfer event, initial pen integrity was lost as mixing of pigs occurred across pens. Pigs were not sampled at the second grow–finish site following movement. A timeline of salient management events at Site 2 and the sampling protocol at Site 1 are displayed in Figure 2.2.

Sampling

Site 1

Approximately sixty bilateral nasal swab samples (BBL CultureSwab™, Stuart single plastic applicator, Becton, Dickinson and Co., Sparks, MD, USA) were collected at two sampling events (27 days apart) from the following four animal subpopulations: sows, gilts, neonatal pigs 3–10 days of age and neonatal pigs >11 days of age (range: 11–26 days of age). Sixty samples were collected from each subpopulation to achieve the power to detect at least one influenza virus–positive animal with 95% confidence assuming a prevalence of at least 5% at each sampling event. With the aim of increasing the likelihood of detection in each group, animals in each subpopulation were screened clinically by study personnel for the presence of clinical signs suggestive of respiratory disease including: anorexia, coughing, sneezing, nasal and ocular discharge, lethargy and dyspnea, and affected animals selected for targeted sampling. When <60 clinically suspect animals were identified in a group, systematic random sampling of clinically healthy animals was performed to achieve the target sample size. To conduct systematic random sampling, the number of sows, gilts or litters present on the farm was estimated and divided by the required sample size to calculate the sampling interval based on space (e.g. every 4th stall or crate). Following influenza virus–positive results from neonatal pigs >11 days of age at the first 2 sampling events, a third sampling event (29 days after the second sampling) was added to collect 60 nasal swab samples from this subpopulation. At each sampling event, blood samples were also collected from animals in each subpopulation, and a random subset of 15 samples were tested per subpopulation per sampling event.

Additionally, trachea and lung tissue samples from neonatal pigs that died or were euthanized between the first and second sampling events were collected by farm personnel under instruction of the study personnel. Trachea and lung samples were stored at -20°C at the farm until collected by study personnel at the subsequent farm visit.

Site 2

Sixty nasal swab samples were collected via systematic and convenience sampling of pigs at each of 5 sampling events at 7- to 10-day intervals. These sampling events were continued until two consecutive negative sampling events occurred. Twelve oral fluid samples were collected at each of 9 sampling events at 7- to 17-day intervals until two consecutive negative sampling events occurred. Oral fluid samples were collected using 3-strand twisted cotton rope following methods published previously (Prickett et al., 2008). Ropes were suspended on open partition metal gates so that pigs in two adjacent pens had access to each rope, ensuring pigs in all 24 pens in the barn had access to a rope. At six of the sampling events, blood samples were also collected and tested from randomly selected subsets of 15 animals.

Diagnostic testing

Nasal swab, trachea and lung tissue samples

Following collection and transport, each nasal swab was suspended in 2 ml of minimum essential medium supplemented with 2% bovine serum albumin, trypsin and antibiotics. The suspension was vortexed and centrifuged, and the supernatant of each of three samples was pooled and tested by a matrix gene-based real-time reverse transcription-PCR (RRT-PCR) assay (Slomka et al., 2010) at the University of Minnesota Veterinary Diagnostic Laboratory. A positive result was defined by a cycle threshold (Ct) value of <35. If a pool of three samples tested negative, each individual sample was considered negative. When a pooled sample was positive, all 3 individual samples were tested via RRT-PCR. A subset of RRT-PCR-positive nasal swab samples from Sites 1 and 2 were cultured on Madin–Darby canine kidney (MDCK) cells (Meguro et al., 1979) to obtain virus isolates for genetic sequencing and long-term storage. Hemagglutinin (HA) and neuraminidase (NA) gene sequences were also obtained using previously described specific primers for HA and NA (Hoffmann et al., 2001) at the University of Minnesota Veterinary Diagnostic Laboratory. Gene sequences were obtained from extracted viral RNA of nasal swabs, virus isolates and oral fluid samples. For all individual pig tissue samples collected, sections of the trachea and the right middle lung lobe were homogenized and tested via matrix RRT-PCR.

Oral fluid samples

Oral fluid samples were also tested by matrix RRT-PCR with modifications (Detmer et al., 2011). A subset of RRTPCR- positive oral fluid samples were cultured on MDCK cells. All matrix RRT-PCR-positive oral fluid samples were subtyped according to their respective hemagglutinin and neuraminidase genes (Richt et al., 2004). Differential pandemic 2009 H1N1 matrix and differential pandemic 2009 H1N1 neuraminidase RT-PCR assays were also conducted on all positive matrix RRT-PCR oral fluid samples (Lorusso et al., 2010; Sponseller et al., 2010). Cut-off Ct values for the differential assays at the University of Minnesota Veterinary Diagnostic Laboratory were <35 positive, >35 and <40 suspect and >40 negative.

Serology

Blood samples were collected via jugular venipuncture. After collection, serum was separated and stored at -20°C. Sera were subsequently analyzed for antibodies to influenza virus by ELISA (FlockChek® Avian Influenza MultiS-Screen Antibody Test Kit, IDEXX Laboratories Inc., Westbrook, ME, USA) following manufacturer's protocols (Ciacci-Zanella et al., 2010). This assay measures antibody directed against the nucleoprotein (NP) of influenza A viruses, and samples with an S/N ratio ≤0.673 are considered positive.

Analysis

Statistical analyses were performed using SAS (SAS System, v 9.2; SAS Inst., Cary, NC, USA) and R (R Foundation for Statistical Computing, Vienna, Austria). The prevalence of influenza virus-infected pigs and influenza virus-infected pens and 95% confidence intervals based on the exact method were calculated over time based on RRT-PCR testing of nasal swabs and oral fluids. Odds ratios were calculated via logistic regression methods. Site 1 median ELISA S/N ratios were calculated by subpopulation, and Site 2 ELISA S/N ratios were displayed via boxplot. To compare ELISA S/N ratios between the first and last sampling event at Site 2, the means and 95% bootstrap percentile confidence

intervals were calculated and compared for the respective sampling events. Briefly, bootstrap distributions for ELISA S/N ratio means were created via resampling with replacement from the original sample of 15 within each group with 1,000 replications. Hemagglutinin and NA gene sequences were aligned and compared using the ClustalW algorithm using MegAlign™ software (DNASTAR, Inc., Madison, WI, USA).

Results

Site 1

Clinical signs

Clinical signs of respiratory disease were infrequent at Site 1 and were limited to mild coughing, sneezing, nasal discharge and dyspnea. Clinical signs of respiratory disease were not observed in pigs of 3–13 days of age, but in pigs >14 days of age 28% (29 of 104) of animals sampled at sampling events 1 and 2 displayed at least one of the following clinical signs: coughing, sneezing, nasal discharge and/ or dyspnea. Coughing was evident in just one sow and one gilt across sampling events 1 and 2. In addition to clinical signs, influenza virus genome was also detected in pigs >14 days of age. The odds of prevalent influenza virus infection in pigs >14 days of age were significantly higher in those pigs with at least one clinical sign versus those without clinical signs at the time of sampling (odds ratio: 4.63; 95% confidence interval: 1.20–17.9; P value 0.03).

Nasal swabs

Prior to the initiation of the study, influenza virus genome was detected in the production system via routine oral fluid sampling of weaned pigs. At no time in the study was influenza virus genome detected in sows or gilts at Site 1 (Table 2.1). Influenza virus genome was detected at a low prevalence in neonatal pigs at sampling events 1 and 2 (Figure 2.3). The prevalence of infection in pigs 3–10 days of age was 2% at sampling event 1. In pigs ≥11 days of age, the prevalence of infection was 10% at sampling event 1 and 7% at sampling event 2. At sampling event 3, influenza virus genome was not detected in pigs ≥11 days of age. Influenza virus was also isolated from 2 of 6 nasal swab samples cultured in MDCK cells.

Hemagglutinin and NA gene sequencing of a subset of matrix RRT-PCR-positive samples revealed that 7 (three at sampling event 1, three at sampling event 2 and one from pig lung and trachea samples between sampling events 1 and 2) shared >99.2% nucleotide similarity among them and clustered tightly with the pandemic 2009 H1N1 virus, A/CA/4/2009 H1N1. All sequenced viruses shared at least 98.9% HA nucleotide similarity and 99.4% NA nucleotide similarity to the pandemic 2009 H1N1 virus, A/CA/4/2009 H1N1.

Trachea and lung tissue

Between sampling events 1 and 2, 46 trachea and lung samples were collected from individual piglets. Real-time RT-PCR-positive samples were obtained from 4% (1 of 25) of tissue samples collected from pigs ≤10 days of age and 29% (6 of 21) of tissue samples collected from pigs ≥11 days of age.

Serology

Ninety-five per cent of animals sampled and tested via ELISA were seropositive at Site 1. All pigs 3–10 days of age were seropositive with a median ELISA S/N ratio of 0.15 (Table 2.2). One sow, two gilts and three pigs ≥11 days of age were either suspect or negative, and the subpopulation median ELISA S/N ratios were 0.17, 0.23 and 0.26, respectively.

Site 2

Nasal swabs and oral fluids

Thirteen per cent (8 of 60) of nasal swabs collected at sampling event 1 (day 0) at Site 2 were RRT-PCR positive for influenza virus genome (Table 2.3). By the second sampling event, when the site had been fully populated, 47% of nasal swabs and 100% of oral fluid samples tested positive. The prevalence of infection was reduced (10%) by the third sampling event, after which influenza virus genome was not detected in nasal swabs. As per the study design, nasal swab sampling was discontinued after two consecutive

negative sampling events. Based on the nasal swab results, influenza virus genome was detected for at least 16 days post-weaning in this population. Influenza virus was also isolated from 5 of 11 nasal swab samples cultured in MDCK cells.

Oral fluids were not collected at sampling event 1; however, influenza virus genome was detected in 100% of oral fluid samples at the second sampling event and in 58%, 25% and 33% of oral fluid samples at sampling events 3, 4 and 5. There was then a numerical increase in the percentage of positive oral fluid samples at sampling event 6 (83%), followed by a numerical decline at sampling events 7 (8%) and 8 (17%). Influenza virus genome was not detected at sampling events 9 and 10. Influenza virus genome was consistently detected via matrix RRT-PCR of oral fluid samples until 69 days following the first detection of influenza virus at this site.

All nine oral fluid samples cultured in MDCK cells were virus isolation negative. The viruses detected at day 0 at Site 2 shared 99.2% HA and NA nucleotide similarity or higher to the viruses previously detected at Site 1. Hemagglutinin gene sequencing from samples collected at sampling events 1, 2 and 3 and NA gene sequences from sampling events 1, 2 and 6 also indicated that the viruses detected shared at least 99.2% nucleotide similarity to each other. All sequenced viruses from Sites 1 and 2 also clustered tightly with the pandemic 2009 H1N1 virus, A/CA/4/2009 H1N1, and shared at least 98.9% HA nucleotide similarity and 99.4% NA nucleotide similarity to A/CA/4/2009 H1N1.

Matrix RRT-PCR identified 39 positive oral fluid samples from sampling events 2 through 8. All matrix RRTPCR- positive oral fluid samples were subtype H1N1. Site 2 differential pandemic 2009 matrix RT-PCR and differential pandemic 2009 N1 RT-PCR results from the 39 matrix RRT-PCR-positive oral fluid samples are displayed in Table 2.4. Ninety-two per cent (36 of 39) of the oral fluid samples were positive or suspect via the differential matrix RT-PCR, and 100% (39 of 39) of the oral fluid samples were positive or suspect via the differential N1 RT-PCR. Negative and/or suspect results on the differential tests only occurred when the matrix RRT-PCR Ct values were >30.

Serology

Eighty-four per cent of animals sampled and tested via ELISA from sampling events 1 through 5 were seropositive (Figure 2.4). At sampling event 10, 40% (6 of 15) of animals sampled and tested via ELISA were seropositive, and the bootstrap mean ELISA S/N ratios were significantly lower at sampling event 1 (0.29, 95% bootstrap percentile confidence interval 0.20–0.40) compared with this final sampling event (0.63, 95% bootstrap percentile confidence interval 0.52–0.72), which was 96 days after sampling event 1.

Discussion

Limited research exists concerning the population infection dynamics and the factors that may affect influenza virus maintenance and transmission in pig populations. This study was designed to assess the epidemiology of influenza virus in a two-site swine herd infected with the pandemic 2009 H1N1 virus, and it provided novel insight into the infection dynamics within pig populations under field conditions typical of current commercial production practices.

Based on results from Site 1, it appeared that neonatal pigs were important for the maintenance of influenza virus in the breeding herd between sampling events 1 and 2, highlighting the potential importance of this subpopulation in the persistence of influenza virus in breeding farms. Genetically similar influenza viruses were identified in neonatal pigs over a 27-day period via nasal swab and tissue samples, while influenza virus genome was never detected in sows or gilts at these sampling events. It has been hypothesized that influenza virus persistence on swine breeding farms depends on the ongoing availability of susceptible animals (Brown. 2000). The repeated absence of positive results in gilts and sows in this herd indicates that the continuous supply of susceptible neonatal pigs may have provided the primary mechanism for virus maintenance between sampling events 1 and 2. While influenza virus genome was detected in neonatal pigs at and between sampling events 1 and 2 at Site 1, influenza

virus genome was not detected at sampling event 3. This could indicate that influenza virus circulation ceases over time or continues to circulate at levels below that of our detection threshold. It is plausible the importance of neonatal pigs as a reservoir of virus may fluctuate over time and be affected by fluctuations in the immune status of the sow herd.

At Site 1, clinical signs were relatively prevalent in pigs ≥14 days of age but were minimal in neonatal pigs <14 days of age, sows and gilts. Virus prevalence also displayed a similar pattern as 10 of the 11 positive nasal swab samples were from pigs ≥14 days of age. Additionally, the odds of prevalent influenza virus infection in pigs ≥14 days of age were significantly higher in those pigs with at least one clinical sign versus those without clinical signs at the time of sampling. Therefore, the presence of clinical signs at the subpopulation and individual animal level were predictive of influenza virus positivity. Because the sampling in this study was partly based on targeted sampling of clinically ill pigs versus random sampling, influenza virus prevalence in pigs ≥11 days of age (where targeted sampling took place) may be slightly overestimated compared with the other subpopulations in this study. However, influenza virus genome was also detected in pigs without clinical signs of respiratory disease. The sow and gilt populations cannot be excluded in terms of virus maintenance as our sampling protocol was designed to detect a prevalence of at least 5% with 95% confidence. Therefore, these subpopulations cannot be regarded as truly influenza virus negative, but we are 95% confident that prevalence in these groups was <5%.

The findings regarding clinical signs and virus prevalence have several implications. First of all, in veterinary medicine, diagnostic testing is often conducted to assess whether a specific herd is infected with a pathogen of interest (e.g. influenza virus). The identification of a specific subpopulation (e.g. weaning-age pigs) with higher prevalence of infection within a herd can aid in detecting pathogens of interest by increasing herd sensitivity at a constant sample size. Secondly, influenza virus can be

detected in pigs without apparent clinical signs. Therefore, the absence of clinical signs at the individual animal level cannot be used to rule out influenza virus infection.

In addition to the potential role neonatal pigs may serve in terms of virus maintenance, this study identified that transport of weaned pigs is a potentially important means of dissemination of influenza viruses among farms. The transport event in this case resulted in the introduction of virus from Site 1 to Site 2, leading to variable and prolonged detection of influenza virus genome over time. This event not only impacted animal health at Site 2, but it created a reservoir of influenza virus in the growing pig population. Multisite pig production systems (Alexander et al., 1980; Harris. 1988) can effectively reduce infection pressure on swine breeding farms by moving growing pigs to a distant site, however; this also may lead to the dissemination of pathogens in weaned pigs to other sites as shown in this study.

Although weaned pigs from the second breeding herd source for Site 2 previously tested negative for influenza virus genome, the impact of this source on influenza virus prevalence and herd immunity at Site 2 was unknown. Because the two sources could not be treated as separate populations once at Site 2, the results of this study illustrate the infection dynamics in a comingled source of weaned pigs. However, Fig. 4 does illustrate that most pigs were seropositive via ELISA in the first few samplings post-placement, indicating the presence of maternal immunity or previous exposure. Prevalence and risk factor studies in Ontario, Canada, have indicated that exposure to endemic influenza virus may be a result of continuous virus circulation in source herds (Poljak et al., 2008a; Poljak et al., 2008b). The virus transport event observed from Site 1 to Site 2 in this study is supported by the aforementioned risk factor studies. In addition, the transport event preceded prolonged detection of virus over time. This finding has implications for influenza virus transmission knowing that regional spread can be observed due to risk factors such as high pig density and uncontrolled entrance to farms (Maes et al., 1999; Poljak et al., 2008b; Simon-Grife et al., 2011). Swine movement has also been implicated in the spatial dissemination of influenza viruses in the United States (Nelson et al., 2011).

The prolonged infection of a population shown in this study may also have implications for public health and increased risk of exposure to farm workers.

Although it appears that neonatal pigs were important for the maintenance of influenza virus over time in this swine breeding herd and the transport of influenza virus to Site 2, the transmission dynamics are likely impacted by many factors that vary within and among swine farms. These factors may include contact rates, animal housing structure, the presence of pre-existing immunity and the rate of animal turnover and introduction. For example, a numerical increase in the percentage positive oral fluid samples at sampling event 6 was observed. This was the first sampling event following disruption of pen integrity due to the movement of pigs offsite. While this was only one observation, altered contact rates and patterns within barn may have impacted the infection dynamics. Therefore, more extensive longitudinal studies across farms are needed to further the understanding of the epidemiological drivers of influenza virus persistence in swine herds.

Serologic assessment at Site 1 and Site 2 provided additional information that influenza virus can circulate in seropositive populations. Although animals at Site 1 and Site 2 were not vaccinated for influenza virus during the course of this study, most animals were seropositive via the NP ELISA assay indicating previous exposure. Adult animals were likely seropositive due to previous vaccination or infection, while the universal seropositivity of neonatal pigs ≤10 days of age likely indicates passive transfer of maternal immunity from their respective dams. While complete protection following homologous influenza virus challenge in pigs with maternally derived antibody (MDA) has been shown (Blaskovic et al., 1970b), additional studies indicate that MDA may reduce clinical signs associated with influenza virus infection, but protection is not complete (Mensik et al., 1971; Loeffen et al., 2003; Choi et al., 2004; Kitikoon et al., 2006). This scenario is likely at Site 1 where clinical signs were mild, but pigs were still infected at sampling events 1 and 2 and were able to transport influenza virus to Site 2.

Additionally, influenza virus genome was continually detected at Site 2 via oral fluids and nasal swabs, while most pigs were seropositive between sampling events 1 and 5. Figure 4 also depicts the wide range in antibody titers in the population, which could presage temporal variability in age susceptibility to influenza infection and delayed but extended propagation of infection through the population as animals become susceptible. Figure 4 also reveals that the majority of pigs sampled at the final sampling event were seronegative via ELISA, while the majority of pigs sampled previously were seropositive. Although paired serum samples were not collected to allow for individual pig assessment, the population serum profile likely changed over time due to declining levels of maternal and naturally induced antibodies. In addition, seroconversion following infection may have been inhibited due to the presence of maternally derived immunity.

A limitation of this study was the inability to isolate and genetically sequence influenza virus from oral fluid samples. While oral fluids were superior to nasal swabs for detecting influenza virus genome at sampling events 4 and 5, virus isolation was unsuccessful and subsequent genetic characterization was limited. Oral fluids have been shown to be a sensitive method to monitor populations despite the decreased success in virus isolation (Detmer et al., 2011; Romagosa et al., 2011b). Therefore, HA and NA sequences were not available at the later sampling events to clearly distinguish whether a genetically similar virus was maintained for the duration of the study at Site 2. However, genetic subtyping revealed that all positive oral fluid samples were subtype H1N1, indicating that a virus of the same subtype was maintained during the study period. In addition, RT-PCR assays specific for the matrix and NA genes of the pandemic 2009 H1N1 virus (the virus detected in this study) identified each positive sample as at least suspect in one of the assays (Table 2.4). These additional results, along with the general decline in prevalence following peaks at sampling events 2 and 6, further point towards the maintenance of a similar virus in the pig population over time.

Influenza virus prevalence is assumed to be high in most swine-producing regions of the world (Hinshaw et al., 1978; Chambers et al., 1991; Olsen et al., 2000; Choi et al., 2002; Poljak et al., 2008a; Van Reeth et al., 2008). Prevalence of influenza virus in swine herds is dictated by the number of new influenza virus infections at the herd level and the duration of infection at the herd level. To have an impact on or to describe virus prevalence at the herd level, knowledge concerning virus maintenance and transmission must be obtained. This study provides a unique and comprehensive description of influenza virus infection dynamics in a two-site swine herd. Neonatal pigs appeared to be important in terms of virus maintenance at the herd level and transport of the virus to another site. In addition, prolonged influenza infections in populations need to be taken in consideration in order to assess risk of influenza exposure to other populations including people.

Table 2.1. Site 1 proportion ( ) and 95% C.I. [ ] of matrix RRT-PCR positive nasal swab samples.

Day of sampling	Sampling event	Sows	Gilts	Pigs 3-10 days of age	Pigs 11-26 days of age
0	1	0/60 (0)	0/59 (0)	1/60 (0.02)	6/60 (0.10)
		[0-0.06]	[0-0.06]	[0-0.09]	[0.04-0.21]
27	2	0/60 (0)	0/60 (0)	0/60 (0)	4/58 (0.07)
		[0-0.06]	[0-0.06]	[0-0.06]	[0.02-0.17]
56	3	NT (Not tested)	NT	NT	0/58 (0)
					[0-0.06]
Overall		0/120 (0)	0/119 (0)	1/120 (0.01)	10/176 (0.06)
		[0-0.03]	[0-0.03]	[0-0.05]	[0.03-0.10]

Table 2.2. Median ELISA S/N ratios for each subpopulation at Site 1.

Subpopulation	N	Median (Interquartile range)[a]
Sows	30	0.17 (0.14-0.25)
Gilts	30	0.23 (0.17-0.40)
Pigs 3-10 days of age	30	0.15 (0.13-0.22)
Pigs 11-26 days of age	30	0.26 (0.19-0.47)

[a]S/N ratios $\leq$0.673 are considered positive

Table 2.3. Site 2 proportion ( ) and 95% C.I. [ ] of matrix RRT-PCR positive nasal swab and oral fluid samples.

Day of study	Sampling event	Nasal swabs	Oral fluids
0	1	8/60 (0.13) [0.06-0.25]	Not tested (NT)
9	2	28/60 (0.47) [0.34-0.60]	12/12 (1.0) [0.74-1.0]
16	3	6/60 (0.10) [0.04-0.21]	7/12 (0.58) [0.28-0.85]
26	4	0/60 (0) [0-0.06]	3/12 (0.25) [0.05-0.57]
35	5	0/60 (0) [0-0.06]	4/12 (0.33) [0.10-0.65]
47	6	NT	10/12 (0.83) [0.52-0.98]
58	7	NT	1/12 (0.08) [0-0.38]
69	8	NT	2/12 (0.17) [0.02-0.48]
79	9	NT	0/12 (0) [0-0.26]
96	10	NT	0/12 (0) [0-0.26]
Overall		42/300 (0.14) [0.10-0.18]	39/108 (0.36) [0.27-0.46]

Table 2.4. Site 2 oral fluid results.

Sample	Sampling event	M RRT-PCR Ct value[a]	Differential M RT-PCR[b]	Differential N1 RT-PCR[b]
1	2	**30**	**+**	**S**
2	2	**26**	**+**	**+**
3	2	**26**	**+**	**+**
4	2	**28**	**+**	**+**
5	2	**26**	**+**	**+**
6	2	**27**	**+**	**+**
7	2	**26**	**+**	**+**
8	2	**31**	**S**	**S**
9	2	**31**	**+**	**+**
10	2	**27**	**+**	**+**
11	2	**28**	**+**	**+**
12	2	**30**	**+**	**+**
13	3	**26**	**+**	**+**
14	3	**24**	**+**	**+**
15	3	**35**	**S**	**S**
16	3	**26**	**+**	**+**
17	3	**29**	**+**	**+**
18	3	**28**	**+**	**+**
19	3	**31**	**+**	**+**
20	4	**34**	**S**	**S**
21	4	**34**	**-**	**S**
22	4	**30**	**+**	**+**
23	5	**29**	**+**	**+**
24	5	**34**	**-**	**S**
25	5	**30**	**+**	**+**
26	5	**29**	**+**	**+**
27	6	**31**	**S**	**S**
28	6	**34**	**S**	**S**
29	6	**23**	**+**	**+**
30	6	**33**	**S**	**S**
31	6	**29**	**+**	**+**
32	6	**23**	**+**	**+**
33	6	**31**	**S**	**S**
34	6	**31**	**S**	**+**
35	6	**28**	**+**	**+**
36	6	**24**	**+**	**+**
37	7	**34**	**S**	**S**
38	8	**33**	**-**	**S**
39	8	**34**	**S**	**S**

[a]Positive samples (Ct ≤35); [b](+) positive; (-) negative; (S) suspect

Figure 2.1. Site 2 barn layout (numbers 1-24 represent separate pens).

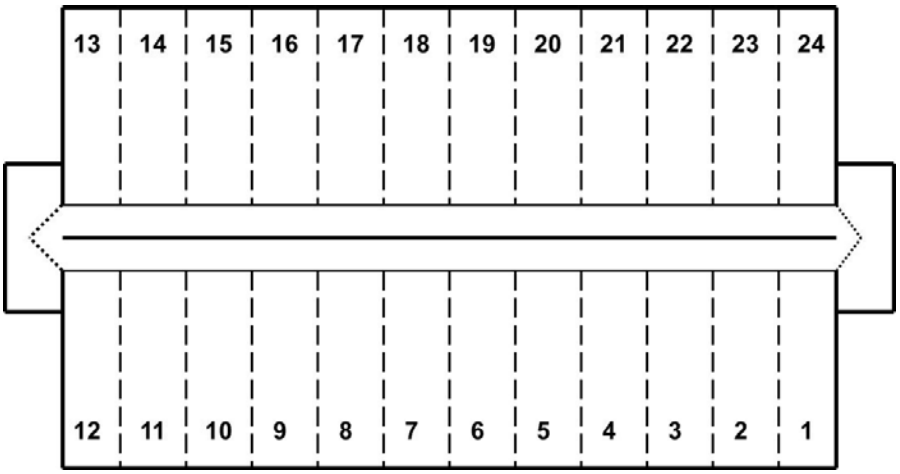
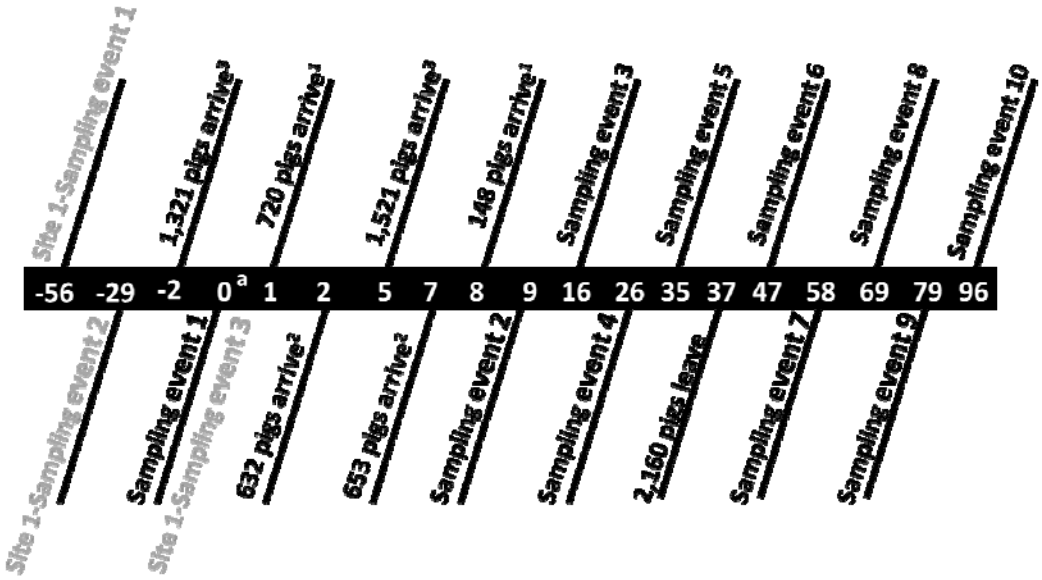


Figure 2.2. Timeline (days) of management events at Sites 1 and 2.



[a]Days in relation to Site 2 sampling event 1 (day 0 in the timeline)

[1]Pigs from Site 1

[2]Pigs from the second breeding herd source

[3]Approximately equal number of pigs from each breeding herd source

Grey color = Site 1 event, Black color = Site 2 event.

Figure 2.3. Distribution and boxplot of pig age by matrix RRT-PCR result.

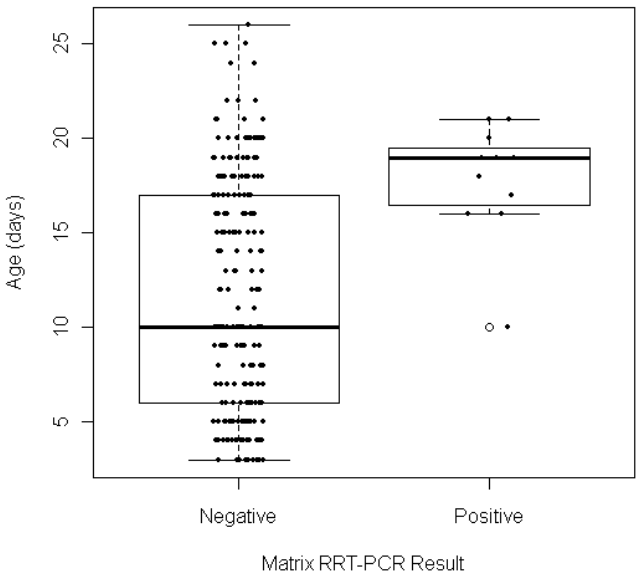
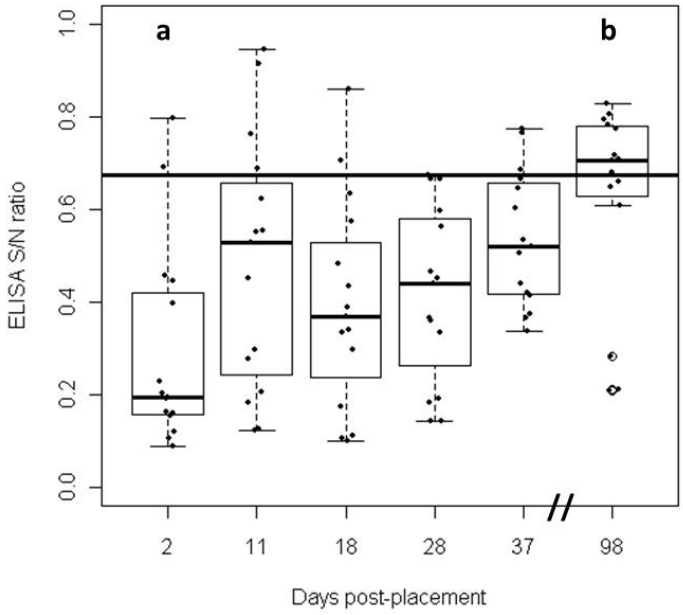


Figure 2.4. Distribution and boxplot of ELISA S/N ratios for each sampling event at Site 2.



The black horizontal line represents the cutoff (≤0.673 is considered positive).

[a,b] Statistically significant differences based on non-overlapping 95% bootstrap percentile confidence intervals of means.

Chapter 3: Influenza A virus surveillance of weaning age pigs on farrow to wean sow herds in the United States, 2013

Introduction

The prevalence of influenza virus infection at the herd level is assumed to be high in most swine producing regions of the world. Historical surveillance information in the United States, based mainly on serological results, indicated that influenza virus infections were common in the 1970's-1990's (Hinshaw et al., 1978; Chambers et al., 1991; Olsen et al., 2000). Limited active surveillance data based on virologic surveillance existed before a recent study in the United States described that 91% of enrolled growing pig sites tested influenza virus positive at least once based on monthly testing over a 1 to 2 year period (Corzo et al., 2013). The aforementioned study highlights the commonality of influenza virus infection in growing pigs, but limited information exists regarding influenza virus prevalence and temporal patterns in sow herds.

Several risk factor studies have been conducted to elucidate key factors associated with influenza virus infection and the resultant high prevalence in pig populations. One common finding has been the association of influenza virus positivity and pig density or proximity to other pigs (Maes et al., 1999; Poljak et al., 2008a; Suriya et al., 2008). This finding highlights the potential external risk or lateral introduction of influenza virus into a herd that will influence influenza virus prevalence. Internal factors may also impact influenza virus prevalence, such as the maintenance of IAV within a herd over time.

Swine breeding herds or sow herds are comprised of very dynamic pig populations. Young pigs are generally born on a daily basis and replacement gilts are introduced on a regular basis due to sow attrition. These populations have been hypothesized to contribute to influenza virus maintenance over time in sow herds (Brown. 2000). Additionally, young pigs may be moved to distant sites at weaning age which allows for influenza virus to be transported to distant geographic areas. Swine movement has been associated with influenza virus dissemination across the United States (Nelson et al., 2011); however, the role of weaning age pigs at sow farms has not been assessed specifically. The objectives of this study were to assess the prevalence and

temporal patterns of influenza virus infection in weaning age pigs from a selected cohort of sow farms and to characterize the viruses obtained within these selected farms.

Materials and Methods

Sow farm selection

Veterinarians and swine producers were invited to participate in this study by convenience selection. Veterinarians and swine producers that agreed to participate in the study identified farrow to wean sow farms that met several inclusion criteria and also provided consent for nasal swab samples to be collected by farm personnel and tested for IAV. Inclusion criteria included: 1) farrow to wean sow herd 2) history of influenza virus infection at the sow herd within the previous year 3) replacement gilts introduced to the sow herd from a source outside of the sow herd premises. All pigs were under the direct supervision of the respective site caretakers and this study was approved according to University of Minnesota IACUC protocol 1207B17281 and IBC protocol 1208H18341. Fifty-two sow herds were enrolled from 6 different states across the United States and from 8 different swine production systems/ownerships.

Nasal swab collection

Each sow herd enrolled in this study had 30 individual bilateral nasal swab samples (liquid Stuart transport media) collected from weaning-age pigs at approximately monthly intervals for a 3 to 6 month period. A nasal swab sampling guide was sent to all participants to standardize the collection procedure. Personnel at each farm identified the oldest suckling pigs present on the premises and sampled pigs no more than 3 days younger than the oldest suckling pigs. In addition, the 30 samples were evenly distributed between litters in the eligible rooms and only one swab was collected per litter via convenience sampling. The age of the pigs sampled was recorded for each sampling event. For classification and analysis purposes, each monthly sample collection event of 30 nasal swab samples was termed a sampling event. Thirty samples were collected to achieve the power to detect at least one influenza virus positive animal with 95% confidence assuming a prevalence of at least 10% at each sampling event. All nasal swab

samples were shipped to the University of Minnesota. Samples were collected from January 2013 to August 2013.

Diagnostic testing

Nasal swab samples were placed in 1.8 mL of MEM supplemented with 2% bovine serum albumin, trypsin, amphotericin B, and antibiotics. The suspension was vortexed and the supernatant was tested for IAV in pools of 3 via matrix gene RRT-PCR (Slomka et al., 2010). A positive result was defined by a cycle threshold (Ct) value of <35. The RRT-PCR-positive pool with the lowest Ct value was cultured on Madin–Darby canine kidney (MDCK) cells (Meguro et al., 1979) and hemagglutinin (HA) gene sequences were obtained using previously described specific primers for HA (Hoffmann et al., 2001). All testing was performed at the University of Minnesota Veterinary Diagnostic Laboratory. Hemagglutinin gene sequences were aligned and compared using the ClustalW algorithm using MegAlign™ software (DNASTAR, Inc., Madison, WI, USA).

Results

Nasal swab results

From January 2013 to August 2013, a total of 7,560 nasal swab samples were collected and tested in 2,520 pools of 3 samples for IAV RNA. These 7,560 samples were from 252 sampling events across 52 farrow to wean sow herds (Table 3.1). The average age of pigs from which samples were collected was 20 days (range 13-30 days). Of the 2,520 pools tested, 2,132 (85%) were negative or suspect and 388 (15%) were positive for IAV by RRT-PCR (Figures 3.1 and 3.2). The average Ct value of IAV positive pools was 27.4 (range 19.2-34.9). Of the 252 sampling events, 187 (74%) were classified as IAV negative and 65 (26%) were classified as IAV positive by RRT-PCR. Of the 52 sow herds, 23 (44%) tested IAV positive for at least one sampling event.

Of the 33 sow herds that tested IAV negative at the first sampling event, 29 (88%) continued to test negative throughout the study period. Of the 19 sow herds that tested influenza virus positive at the first sampling event, 16 (84%) tested positive for at least

one additional sampling event. Three sow herds tested positive for 6 consecutive sampling events over durations of 156, 165, and 165 days.

Genetic sequencing (HA)

An HA gene sequence was obtained from 58 of 65 positive sampling events and two HA gene sequences were obtained from one sampling event, for a total of 59 HA gene sequences. HA gene sequences from at least two separate sampling events were obtained and compared within farm from seventeen farms. The comparison revealed that all HA gene sequences within farm shared greater than 98.8% nucleotide similarity (Table 3.2), with the exception of one farm in which an H1 and an H3 sequence were obtained at the same sampling event. In contrast, HA sequences were generally different across farms.

Discussion

Weaning age pigs have been a sampling target for many swine pathogens, including porcine reproductive and respiratory syndrome virus (Holtkamp et al., 2011). This first large scale assessment of influenza A virus in this specific subpopulation identified influenza virus positive weaning age pigs in at least one sampling event at 23 of the 52 (44%) enrolled sow herds based on monthly sampling over 3 to 6 sampling periods. Additionally, 26% of sampling events contained at least one influenza virus positive sample and 15% of pools tested influenza virus positive.

The herd level estimate above is lower than the herd level estimate recently obtained via an active surveillance study in growing pig herds (Corzo et al., 2013); however, the sampling duration in this study was significantly shorter and could partially explain the lower herd level prevalence estimate. Regardless, the estimate obtained in this study illustrates the commonality of influenza virus in pig populations, including sow herds. Interestingly, the sampling event results at the herd level were quite repeatable. Of the 33 sow herds that tested IAV negative at the first sampling event, 29 (88%) continued to test negative throughout the study period. Of the 19 sow herds that tested influenza virus positive at the first sampling event, 16 (84%) tested positive for at least one

additional sampling event. This included 3 sow herds which tested positive for 6 consecutive sampling events over durations of 156, 165, and 165 days. All farms were farrow to wean sow herds; therefore, these results were obtained even though a new cohort of pigs was sampled at each sampling event.

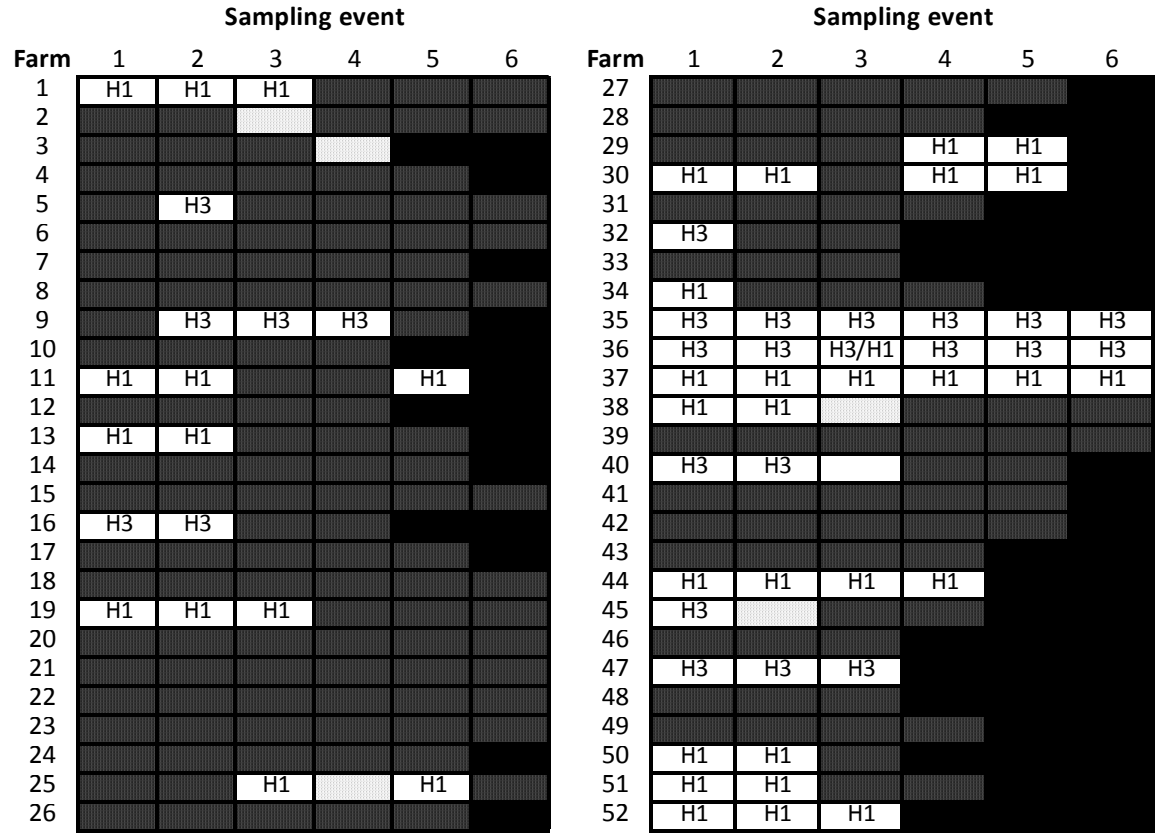
Not only were sampling event results repeatable over the study period, but HA gene sequences were also similar over positive sampling events within herds. A strength of this study is that multiple HA gene sequences were obtained from 17 sow herds that tested positive on at least 2 separate sampling events. HA gene sequences were therefore compared within farm and found to share greater than 98.8% nucleotide similarity, with one exception. Two HA gene sequences (1 H3 and 1 H1) were obtained from one sampling event at one sow farm. Although the H3 and H1 virus were not similar, the H3 virus shared greater than 98.8% nucleotide similarity with H3 viruses obtained at five other sampling events in this specific farm. Although HA sequences within farm were similar, HA sequences between herds were quite variable. This is important as weaned pig populations can serve as a reservoir of diverse influenza virus lineages.

The repeatability of sampling event positivity and HA gene similarity within farm over time suggest that a similar virus may have been maintained in weaning age pigs within these specific sow herds. One limitation of this study is that virus similarity is based only on HA gene sequencing. The hemagglutinin surface protein is important for virus-cell interaction, is a common vaccine target, and is a major surface antigen (Wiley and Skehel, 1987). However, changes in other gene segments or reassortment events may have taken place throughout this study which would not be captured with the methods employed in this study. Additionally, major antigenic changes could have occurred even though minor HA genetic change was observed. Another limitation of this study is that sampling only occurred in weaning age pigs. Therefore, pigs of other ages or production stages may have been influenza virus positive within these sow herds while weaning age pigs tested negative. The results of this study only reflect influenza virus prevalence in weaning age pigs, not sow herds in general.

The results of this study have several implications regarding influenza virus epidemiology and control. First of all, influenza virus prevalence may be high in swine herds due to a longer duration of infection within the weaning age pigs at the herd than previously suspected. Influenza virus prevalence will be high if the duration of infection at the herd level is extended. Control strategies at the herd level then need to not only focus on preventing influenza virus introductions (reducing incidence and prevalence) but also on reducing the duration of infection at the herd level (reducing prevalence). The primary means for influenza virus control and prevention are vaccination and biosecurity measures. Vaccination of breeding females is a common practice as 70% of large sow farms and 29% of all sow farms vaccinated breeding females in 2006 for influenza virus (USDA. 2007). While weaning age pigs may not be vaccinated directly, sow vaccination may result in protective levels of maternal immunity in weaning age pigs. Future research is needed to explore the impact of maternally derived immunity and other control measures on influenza virus transmission within sow herds. Weaning age pigs from several sow herds within this study consistently tested influenza virus negative. A thorough assessment of factors associated with these specific herds may also lead to measures that can reduce influenza virus prevalence.

Multi-site pig production systems commonly utilized in the United States have allowed for the segregation of differing production stages. These systems also necessitate the movement of weaning age pigs to many different sites distant from the sow herd source. Therefore, weaning age pigs are an important subpopulation to consider when assessing influenza virus epidemiology in pigs. This large scale study of weaning age pigs has provided evidence to support the commonality of influenza virus in this specific subpopulation. Additionally, it appears that this population is able to maintain influenza virus infections in sow herds for an extended period of time. Weaning age pigs need to be considered in comprehensive influenza virus control strategies and measures to reduce the prevalence of influenza virus in this population need to be explored further to minimize the impact of influenza virus in pig populations.

Table 3.1. Sampling event results and HA subtype for each of 52 sow farms



Positive (at least one pool RRT-PCR positive)

Negative (all pools RRT-PCR negative)

Suspect (at least one pool RRT-PCR suspect, the remaining pools negative)

Testing not conducted

Table 3.2. HA gene nucleotide sequence comparison from 17 sow herds with 2 or more HA gene sequences from separate sampling events

Farm ID	Count of HA gene sequences compared	Subtype	Lowest % nucleotide identity between all sequences
1	3	H1	99.8
9	3	H3	99.7
11	2	H1	99.8
13	2	H1	99.4
19	3	H1	99.5
25	2	H1	99.8
30	2	H1	99.9
35	6	H3	98.8
36	6*	H3	98.8
37	6	H1	99.4
38	2	H1	99.9
40	2	H3	100
44	4	H1	99.8
47	2	H3	99.8
50	2	H1	99.7
51	2	H1	99.9
52	3	H1	99.8

* Two HA gene sequences (1 H3 and 1 H1) were obtained from one sampling event. The % identity in this table is based on the 6 H3 sequences obtained over time.

Figure 3.1. Percentage of IAV positive sow farms, sampling events, and nasal swab pools

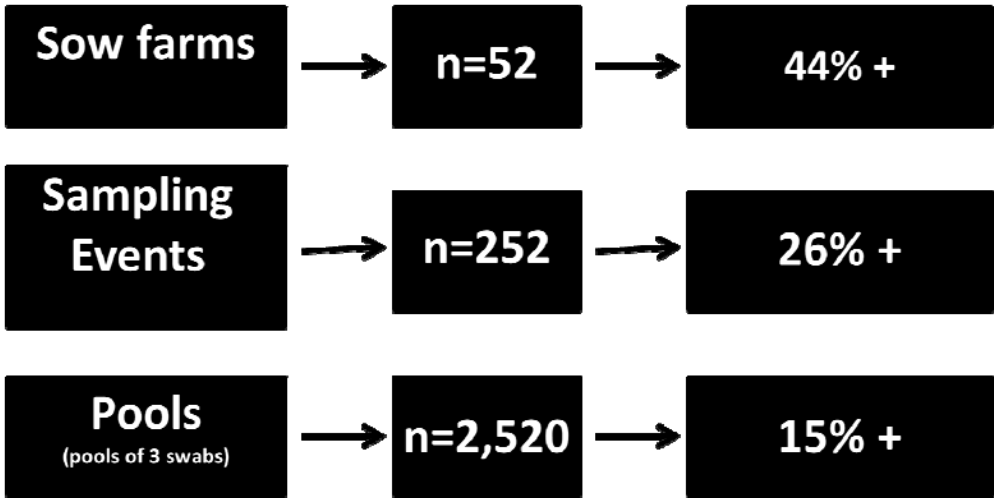
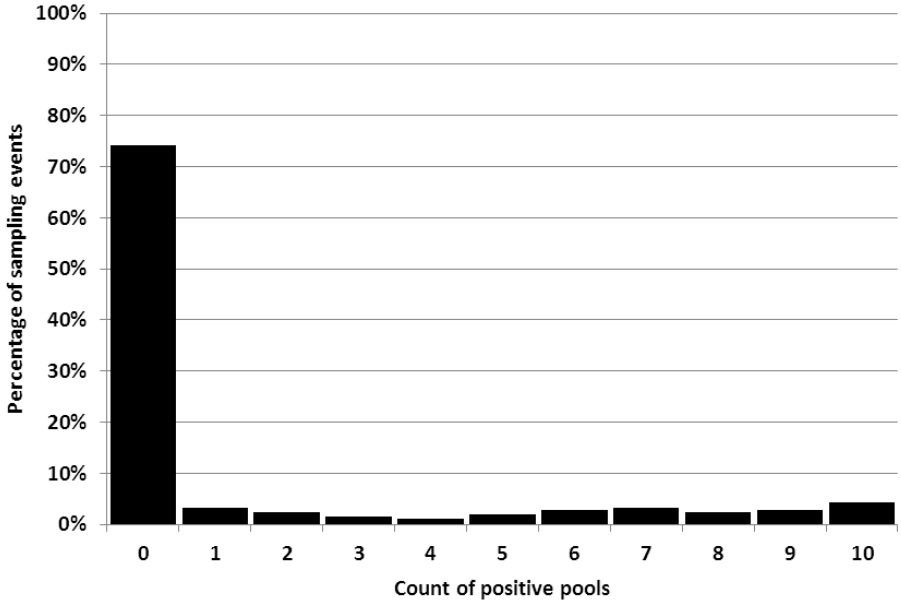


Figure 3.2. Number of positive pools at each sampling event



Chapter 4: Indirect transmission of influenza A virus between pig populations under two different biosecurity settings

This work has been published in:
Allerson, M.W., Cardona, C.J., Torremorell, M., 2013. Indirect Transmission of Influenza A Virus between Pig Populations Under Two Different Biosecurity Settings. PLoS One 8, e67293.

Introduction

During the period of 1976-2007, 1.4 to 16.7 deaths per 100,000 persons were influenza-associated in the United States each year (Centers for Disease Control and Prevention (CDC). 2010). In addition to the significant mortality and morbidity associated with influenza virus in the human population, influenza A virus (IAV) is a common pathogen in many animal species, including pigs. Influenza virus has been considered widespread in the United States pig population since first described clinically in 1918 (Koen. 1919). Classical H1N1 viruses were the dominant circulating influenza viruses in pigs in the United States until the appearance and subsequent circulation of triple reassortant H3N2 viruses in 1998, leading to a more complex epidemiologic picture (Zhou et al., 1999).

While many different IAV subtypes and genotypes have been described in pigs and circulate today, common transmission routes exist and transmission of IAV can occur via several different routes. In addition to direct contact with infected hosts, aerosols and fomites may serve as transmission routes for IAV (Tellier. 2006). These transmission routes are not only applicable for within species transmission, but they are also important for interspecies transmission. In 2009, an H1N1 influenza virus with gene segments of swine lineage and a gene combination not previously reported was detected in humans in North America (Garten et al., 2009). This virus ultimately infected humans across the globe and became widespread in animal populations, including pigs. The 2009 pandemic H1N1 and H3N2 variant (H3N2v) viruses (Bowman et al., 2012; Centers for Disease Control and Prevention (CDC). 2012) have recently highlighted the role of interspecies transmission in IAV epidemiology.

While it is known that direct pig to pig transmission of IAV occurs, other transmission routes have not been fully elucidated. Indirect transmission routes, such as contaminated personnel or fomites, have been shown to transmit other pathogens from infected to susceptible pigs (Otake et al., 2002; Amass et al., 2003). Influenza viruses have been recovered from fomites and the hands of people (Bean et al., 1982; Boone and Gerba, 2005; Mukherjee et al., 2012); however, limited information exists regarding the

subsequent infection of susceptible hosts from contaminated fomites. Furthermore, biosecurity measures (e.g. hand washing and wearing clean outerwear and boots) are commonly utilized by animal and public health personnel between different populations or visits to prevent IAV transmission, but there is limited information on the effectiveness of these measures. The objective of this study was to evaluate the role of fomites in IAV transmission between pig populations separated by two different biosecurity settings and this was accomplished using a pig challenge and exposure model.

Materials and methods

Ethics statement

All pigs were monitored daily and cared for according to the University of Minnesota Animal Care and Use Protocol number 1109A05201. This study and the Animal Care and Use Protocol were specifically approved by the University of Minnesota Institutional Animal Care and Use Committee (IACUC). Due to the use of an infectious agent (IAV), this study was also approved by the University of Minnesota Institutional Biosafety Committee (IBC), protocol number 1109H04982. Personnel wore the following clothing and personal protective equipment (PPE) when in contact with pigs: coveralls, boots, bouffant cap, protective eyewear, N-95 respirator, and gloves. Fomite sample collection was from clothing and PPE, not from study personnel directly. In addition, fomite samples were collected to assess whether IAV could be detected from the specific fomites and results were not linked to a specifically named person. The interaction with pigs simulated contact normally encountered in pig facilities. Human subject approval was therefore not applicable and was not obtained for this study; however, personnel provided verbal consent to participate in the study with the process documented as part of regular planning meetings prior to the start of the study. Personnel were also approved to participate as part of the aforementioned approved IACUC and IBC protocols.

Animals and animal housing

Thirty-five pigs (average age of 6 weeks) were assigned to one of four experimental groups. Ten pigs (1 replicate) were assigned to the infected group (I), 10 pigs (2 replicates of 5 pigs) were assigned to the low biosecurity sentinel group (LB), 10 pigs (2 replicates of 5 pigs) were assigned to the medium biosecurity sentinel group (MB), and 5 pigs (1 replicate) were assigned to the negative control (NC) group (Table 4.1). All pigs used in this study tested negative for IAV antibodies at the source herd and 5 days prior to the start of the study after movement to the University of Minnesota animal isolation facility. Serum samples were tested via enzyme linked immunosorbent assay (ELISA) (FlockChek® Avian Influenza MultiS-Screen Antibody Test Kit, IDEXX Laboratories Inc., Westbrook, ME, USA) as described previously (Ciacci-Zanella et al., 2010). Nasal swabs collected from all pigs 5 days prior to the start of the study also tested negative for IAV RNA via a matrix gene based real-time reverse transcription PCR (RRT-PCR) (Slomka et al., 2010).

A convenience selection of 9 serum samples from pigs used in this study were determined to be negative for porcine reproductive and respiratory syndrome virus (PRRSv) (HerdChek® PRRS Antibody X3 Test Kit, IDEXX Laboratories Inc., Westbrook, ME, USA) and Mycoplasma hyopneumoniae (HerdChek® M. hyopneumoniae Antibody Test Kit, IDEXX Laboratories Inc., Westbrook, ME, USA) antibodies. In addition, the source herd was determined to be free of PRRSv and Mycoplasma hyopneumoniae by historical antibody testing.

All pigs were housed at the University of Minnesota animal isolation facility (St. Paul, MN, USA) with each replicate placed in a separate isolation room (Figure 4.1). The isolation barn had 11 individual animal isolation rooms (6 used for this study) with a shared hallway. Each animal isolation room had an anteroom with footbaths, a sink for hand washing, and a storage area for equipment. In addition, each animal isolation room had a storage area of 2.08 m2 and one animal housing area of 7.28 m2 (1.5 m2/pig in sentinel pig rooms). The floor of the animal housing area was solid concrete and the housing area had one water line with two water nipples. All rooms had negative pressure

ventilation systems with one air inlet and one air outlet and the total airspace volume of each room was 35.1 m3. The incoming air to each room was filtered with a 3 ply panel filter (TRI-DEK® 15/40, TRI-DIM Filter Corp., Louisa, VA, USA) and exhaust air was filtered with a HEPA filter (XH Absolute HEPA filter, Camfil Farr, Inc., Stockholm, Sweden).

Experimental groups

Infected group (I)

Eight pigs were challenged intra-tracheally and intra-nasally with 1 mL of viral inoculum in each location, containing 4.6×10^6 tissue culture infective dose (TCID50/mL) of a delta cluster H1N1 IAV (A/Sw/MN/07002083/07). The virus was originally isolated at the University of Minnesota Veterinary Diagnostic Laboratory from an outbreak of respiratory disease in pigs and has been used previously (Vincent et al., 2009). The viral isolate was grown in bulk quantities using Madin-Darby canine kidney (MDCK) cells (Meguro et al., 1979). Before the challenge inoculation, all pigs were sedated by an intramuscular injection of Telazol® (6 mg/kg, Telazol®, Fort Dodge Animal Health, Fort Dodge, IA, USA). Two pigs in this group (direct contact sentinels) were not inoculated and were moved to a separate isolation room prior to the challenge virus inoculation. Twenty-four hours later, the direct contact sentinel pigs were moved back with the 8 experimentally inoculated pigs.

Low biosecurity sentinel group (LB)

Ten IAV negative pigs were placed in two separate isolation rooms (5 pigs/room, 2 replicates). Two study personnel (personnel A and B) each moved directly from group I to one of the LB replicates. The same person moved to the same replicate for all nine movement events with the exception of a replacement for person A at movement event 1.

Medium biosecurity sentinel group (MB)

Ten IAV negative pigs were placed in two separate isolation rooms (5 pigs/room, 2 replicates). Two study personnel (personnel C and D) each performed a series of

biosecurity measures before moving to one of the MB replicates. The same person moved to the same replicate for all nine movement events.

Negative control group (NC)

Five IAV negative pigs were placed in an isolation room. All personnel caring for this group did not have contact with the I, LB, or MB groups during the entire movement period.

Study personnel

Study personnel had no other direct pig contact for the duration of this study. Personnel A and B had direct contact with pigs in groups I and LB and personnel C and D had direct contact with pigs in groups I and MB during the course of the movement period. Isolation facility personnel and other personnel that did not have contact with pigs in groups I, LB, or MB cared for pigs in group NC. Personnel A, B, C, and D performed all necessary procedures (e.g. pig nasal swab collection, feeding of pigs, cleaning rooms) in their respective sentinel rooms in order to prevent entry of other personnel into rooms during the movement period and the sampling dates thereafter.

Clothing and personal protective equipment (PPE)

All personnel wore the same clothing and PPE, except person A wore cloth coveralls at all times in contrast to Tyvek® coveralls worn by personnel B, C, and D (Table 4.2). Person A wore cloth coveralls so that both types of coveralls could be assessed in the LB setting where coveralls were not changed prior to movement to sentinel rooms. Due to the potential for interspecies transmission and to comply with approved University of Minnesota Institutional Biosafety Committee protocol 1109H04982, personnel were required to wear PPE in addition to boots, coveralls, and gloves commonly worn by study and farm personnel. The PPE included a bouffant cap, protective eyewear, and N-95 respirator.

Before entry into the group I room, personnel showered in the isolation facility. Following the shower procedure, personnel placed on an undershirt and pants and a pair of disposable plastic boots and entered the animal isolation hallway after stepping in an iodine footbath. In the animal isolation hallway, personnel placed on coveralls, latex gloves, a bouffant cap, and transported a pair of disposable plastic boots to the group I room. Upon entry to the group I anteroom, personnel stepped in an iodine footbath and placed on a N-95 respirator, room specific rubber boots, and room specific protective eyewear. Before entering the animal housing area, personnel placed on a pair of disposable plastic boots over their rubber boots.

Movement between experimental groups

A movement event was defined as the movement of personnel from group I to groups LB and MB. There were a total of 9 movement events over a 5 day period for each LB and MB replicate. The first movement event took place in the afternoon, approximately 36 hours following the experimental inoculation of pigs in group I (Figure 4.2). Movement events then took place during the morning (am) and afternoon (pm) of 4 consecutive days. Therefore, the second movement event (am) followed the first movement event (pm) by approximately 16 hours, the third movement event (pm) followed the second movement event by approximately 8 hours. Movement event duration was timed according to the estimated infectious period (1 to 5 days post-inoculation) of pigs in group I, and confirmed by sampling pigs in group I daily.

Exposure of personnel to infected and sentinel pigs

For each of the 9 movement events, 4 study personnel (A, B, C, and D) were exposed to pigs in group I at the same time for a period of 45 minutes after following procedures outlined above in the clothing and PPE section. Pigs were allowed to have direct contact with clothing and PPE for the 45 minute period. Personnel spent the 45 minute period in the infected pig room standing and sitting on the floor while handling pigs as they moved throughout the room. Therefore, pigs had access to all clothing and PPE worn by personnel. This period allowed for thorough exposure to potentially infectious secretions.

All pigs in group I interacted with all study personnel during the exposure period and all personnel interacted in a similar manner with the infected pigs. Following movement of personnel to sentinel groups LB and MB, the same exposure period of 45 minutes was repeated to allow for sentinel pigs in groups LB and MB to contact potentially infectious clothing and PPE.

Movement of personnel A and B to LB rooms

Following the 45 minute interaction period of personnel A and B with group I pigs, personnel A and B placed their used coveralls, disposable plastic boots, latex gloves, and bouffant cap in a clean plastic bag in the group I storage area. Personnel A and B exited the group I room via the outside door and entered their respective LB sentinel group storage area through the outside door and placed on the used coveralls, disposable plastic boots, gloves, and bouffant cap. Each person collected four separate fomite swab samples from (1) coveralls, (2) disposable plastic boots, (3) latex gloves, and (4) the bouffant cap and outer surface of the N-95 respirator in the group LB storage area. Following the fomite swab sample collection, personnel interacted with sentinel group LB pigs (Table 4.3). Personnel A and B did not wash their hands or face during this process.

Movement of personnel C and D to MB rooms

Following the 45 minute interaction period of personnel C and D with group I pigs, each person collected four separate swab samples from (1) coveralls, (2) disposable plastic boots, (3) latex gloves, and (4) the bouffant cap and outer surface of the N-95 respirator in the group I storage area. Personnel C and D entered the group I anteroom and disposed of their used coveralls, disposable plastic boots, latex gloves, bouffant cap, and N-95 respirator. Personnel washed their hands and face with soap and water for approximately 40-60 seconds according to World Health Organization (WHO) guidelines and moved towards the MB sentinel rooms through the animal isolation hallway. In the anteroom of the MB sentinel rooms, personnel C and D washed their hands and face again and placed on new coveralls, a new pair of latex gloves, a new bouffant cap, room specific rubber boots, a new pair of disposable plastic boots, a new N-95 respirator, and room specific

protective eyewear (Table 4.3). Each person collected four separate swab samples from (1) coveralls, (2) disposable plastic boots, (3) latex gloves, and (4) the bouffant cap and outer surface of the N-95 respirator in order to ensure that the new materials were negative for IAV following the biosecurity procedures. Following the fomite swab sample collection, personnel C and D interacted with sentinel group MB pigs.

Sample collection, processing, and testing

Fomite swabs

For all fomite and nasal swab samples, a sterile rayon-tipped swab was used (BD BBL™ CultureSwab™, liquid Stuart medium, single plastic applicator, Becton, Dickinson and Co., Sparks, MD, USA). The chest area, front and back of each leg, and front and back of each arm were swabbed in a zigzag pattern for each coverall sample. For the boot sample, the entire surface of each disposable plastic boot was swabbed via a zigzag pattern in addition to visibly contaminated areas of the boot surface. The entire surface of each latex glove was swabbed and the outer surfaces of the bouffant cap and N-95 respirator were swabbed. Following collection and transport, each swab was suspended in 1.8 mL of brain–heart infusion (BHI) medium. Samples were tested for IAV via a matrix gene based real-time reverse transcription PCR (RRT-PCR) (Slomka et al., 2010). Cutoff Ct values for the RRT-PCR assay used in this study were: ≤35 positive, >35 and ≤40 low level positive or suspect, and >40 negative. In addition, all fomite samples in which IAV RNA was detected were tested by virus isolation on MDCK cell monolayers.

Nasal swabs

Nasal swabs were collected from all pigs prior to the start of the study and following inoculation of pigs in group I (Figure 4.2). Bilateral nasal swabs were collected using sterile rayon-tipped swabs and placed in liquid Stuart medium. Following collection and transport, each nasal swab was suspended in 1.8 mL of brain–heart infusion (BHI) medium. Samples were tested for IAV via matrix gene RRT-PCR. In addition, one nasal swab sample from each pig in the infected group (I) was tested by virus isolation and virus titration on MDCK cell monolayers with TCID50/mL calculated by the method of

Spearman–Karber. The nasal swab samples tested by virus isolation and virus titration were from 2 days post inoculation (DPI) from the inoculated pigs (n = 8) and 5 DPI from the direct contact sentinels (n = 2). Hemagglutinin (HA) gene sequences were obtained from positive nasal swab samples from each infected pig in the LB and MB groups (n = 10), the inoculum (n = 1), and two positive samples from two different pigs in group I using previously described specific primers for HA (Hoffmann et al., 2001) at the University of Minnesota Veterinary Diagnostic Laboratory. HA1 gene sequences obtained from all groups were compared to ensure that there were no new virus introductions.

Blood samples

Blood samples were collected via jugular venipuncture and serum was separated and stored at −20°C until testing. Samples were collected from all pigs prior to the start of the study at -5 DPI. Samples were also collected from pigs prior to euthanasia (15 DPI for group I and 21–28 DPI for the remainder of the pigs). Samples were tested for IAV antibodies via enzyme linked immunosorbent assay (ELISA) (FlockChek® Avian Influenza MultiS-Screen Antibody Test Kit, IDEXX Laboratories Inc., Westbrook, ME, USA) as described previously with an S/N ratio ≤ 0.673 considered positive and an S/N ratio > 0.673 considered negative (Ciacci-Zanella et al., 2010). The Influenza A Multiscreen ELISA measures antibodies directed against the nucleoprotein (NP) of influenza A viruses.

Statistical Analyses

Statistical analyses were performed using SAS (SAS System, SAS Inst., Cary, North Carolina, v 9.2) and R (R Foundation for Statistical Computing, Vienna, Austria). ELISA antibody titers at -5 DPI and prior to euthanasia were compared via Student's paired t-test. Hemagglutinin gene sequences were aligned and compared using the ClustalW algorithm using MegAlign™ software (DNASTAR, Inc., Madison, WI, USA).

Results

Fomite swabs

Of the 144 samples collected following contact with infected pigs but prior to biosecurity procedures, 11 (8%) were low level positives via RRT-PCR (Table 4.4). All samples collected following contact with infected pigs but prior to biosecurity procedures from gloves and bouffant cap/respirator were negative, while 7/36 (19%) and 4/36 (11%) samples collected from boots and coveralls were low level positives, respectively. All RRT-PCR low level positive fomite swabs were virus isolation negative. All four personnel had at least one low level positive fomite sample following contact with infected pigs during the movement period with 1, 4, 3, and 3 samples low level positive from personnel A, B, C, and D respectively. Following biosecurity procedures practiced by personnel C and D in the MB replicates, all fomite samples (n = 72) were negative via RRT-PCR.

Nasal swabs

Infected group (I)

Influenza virus RNA was detected via nasal swab sampling from all pigs in group I at least once (Table 4.5). Both direct contact sentinel pigs were infected 1 to 2 days after the eight inoculated pigs first tested positive. The average detection period (number of days between the first and last detection of IAV RNA via RRT-PCR from nasal swabs) for animals in group I was 7.3 days (range 6–9 days). During the 5 days in which the 9 movement events took place, 5, 8, 9, 10, and 10 pigs were IAV positive or low level positive via RRT-PCR in the infected group. The subset of nasal swab samples (n = 10) tested by virus isolation were virus isolation positive and titers ranged from 1.47×10^{3} to 6.81×10^{4} TCID50/mL.

Low biosecurity sentinel group (LB)

Influenza virus was not detected via nasal swabs from pigs in LB replicate 1; however, all 5 pigs in LB replicate 2 were infected with IAV. The average detection period for animals in LB replicate 2 was 6.4 days (range 5–8 days).

Medium biosecurity sentinel group (MB)

Influenza virus was not detected via nasal swabs from pigs in MB replicate 1; however, all 5 pigs in MB replicate 2 were infected with IAV. The average detection period for animals in MB replicate 2 was 5.6 days (range 5–7 days).

Negative control group (NC)

Influenza virus was not detected via nasal swab samples from pigs in the negative control group.

Serology

All pigs were seronegative via ELISA (S/N ratio > 0.673) at the beginning (-5 DPI) of the study (Figure 4.3). Pigs in LB replicate 1, MB replicate 1, and the NC group were seronegative via ELISA prior to euthanasia, while ELISA S/N ratios were significantly lower (positive) in pigs from LB replicate 2 ($P = 0.002$) and MB replicate 2 ($P = 0.001$) prior to euthanasia compared to paired ELISA S/N ratios at -5 DPI. In addition, ELISA S/N ratios were significantly lower (positive) in pigs from group I prior to euthanasia compared to paired ELISA S/N ratios at -5 DPI ($P < 0.0001$).

Genetic sequencing (HA1)

HA1 gene sequencing of one positive matrix RRT-PCR positive sample from each infected pig in the LB and MB groups (n = 10), the inoculum (n = 1), and two RRT-PCR positive samples from two different pigs in group I revealed that all HA1 gene sequences shared greater than 99.7% nucleotide similarity.

Discussion

Influenza virus transmission routes within and between pig populations have not been fully elucidated. In addition, experimental studies evaluating the entire indirect (fomite and/or contaminated personnel) transmission chain from influenza infected to susceptible hosts are scarce across all species, including humans. This experimental study provides evidence that fomites can be contaminated with IAV following interaction with infected

pigs, IAV can be transported via fomites to non-contiguous groups of sentinel pigs, and that sentinel pigs can become infected with IAV following the contamination and transport of fomites by personnel. Furthermore, additional biosecurity measures did not prevent transmission in one of two replicates.

The first necessary step in an indirect (fomite) transmission chain is an infected population capable of contaminating fomites, such as coveralls and boots. A strength of this study was the presence of many acutely infected pigs during the nine movement events. This allowed personnel to have extended, close contact with known infected pigs for the duration of the study. At 2 DPI (movement events 2 and 3), all nasal swab samples from inoculated pigs were RRT-PCR and virus isolation positive. In addition, at 5 DPI (movement events 8 and 9), nasal swab samples from the direct contact sentinels were RRT-PCR and virus isolation positive. This confirms that not only were pigs in the infected group influenza virus positive, but they were also shedding infectious virus.

Contact with infected pigs resulted in 7/36 (19%) and 4/36 (11%) swab samples collected from boots and coveralls respectively as low level positives for IAV via RRT-PCR. Virus isolation was attempted from RRT-PCR low level positive fomite samples; however, all samples were virus isolation negative. While the sensitivity of the sampling method is unknown and difficult to quantify in this setting, IAV RNA was present on boots and coveralls of personnel shortly after contact with infected pigs. The swab based sampling method used in this study may have impacted the recovery rate of IAV from certain materials, such as those that are more absorbent (e.g. N-95 mask and bouffant cap). Enhanced detection methods have been used to successfully recover influenza virus from absorbent surfaces (Mukherjee et al., 2012) and use of such methods may have resulted in greater recovery of IAV from clothing and PPE used in this study.

The number of positive fomite samples was also likely dictated by the interaction preference of pigs as boots and coveralls were more accessible to pigs during this study. A unique aspect of this study was the subsequent exposure of sentinel pigs to these

materials as the ultimate measure of IAV infectivity and transmission. The contamination of hands, oral mucosa, and nasal mucosa of personnel following contact with infected pigs could not be assessed in this study as necessary personnel protective equipment (gloves and N-95 respirator) was worn by personnel at all times to prevent interspecies transmission.

While the role of fomites concerning IAV transmission has been studied previously, often the transmission chain is ended following the contamination of fomites or assessing the transmission chain further is not possible and inferences on the likelihood of transmission are made based on the duration or frequency of IAV presence on specific materials (Boone and Gerba, 2005; Greatorex et al., 2011; Mukherjee et al., 2012). This study was able to continue the transmission chain by exposing sentinel pigs to recently contaminated fomites. With all other known transmission routes outside of fomites controlled for and minimized (e.g. direct pig contact, infected personnel, aerosol) the role of fomites via movement of personnel could be assessed specifically.

Following the first detection of IAV via daily sampling from one pig in the LB and MB replicates, all 5 pigs in each replicate became infected over time. Based on the timing of IAV detection in the LB and MB groups and the 1–2 day latent period observed in group I following experimental inoculation, it appears that a small subset of pigs may have been infected due to IAV exposure via fomites and the remainder was likely due to pig to pig transmission. Differences in pig behavior and interaction with the study personnel and exposure dose may explain why all pigs did not become infected at the same time.

Movement events from group I to groups LB and MB did not occur after DPI 5. Therefore, it is unlikely that new infections $\geq$ 8 DPI were due to exposure to contaminated fomites from group I. This observation along with the low frequency of virus isolation negative, but RRT-PCR low level positive fomite samples following contact with infected pigs may indicate that the amount of infectious IAV on fomites was

low. In addition, one of the LB replicates remained IAV negative following nine personnel movement events over a five day period from a room with 5, 8, 9, 10, and 10 infected pigs each day and evidence of IAV RNA on coveralls and boots transported to the respective room. The LB replicate that did become infected was visited by person A who wore cloth coveralls during the nine movement events in contrast to person B who wore Tyvek® coveralls. Influenza virus RNA was detected from coverall swab samples of personnel A and B, while only the sentinel pig room visited by person A became infected. The coverall material may have impacted the survival of IAV and the resultant transmission results; however, all other equipment worn by study personnel was the same and IAV RNA was also detected from the boot swab samples of person B. Additional research would be required to assess the potential impact of different coverall types on transmission.

The frequency at which infection events would take place in this setting under the specific biosecurity procedures cannot be assessed due to limited replication. Of particular interest was the infection of pigs in one of the medium biosecurity replicates. Due to the limited replication in this study, we cannot conclude that the biosecurity measures practiced reduced or increased the frequency of IAV infection. In this experimental setting, the medium biosecurity groups were placed either between or in an adjacent room to the low biosecurity groups. While it is unlikely that a higher containment population would be placed directly adjacent to or between lower containment populations in a field setting, the procedures practiced in this study and the experimental layout avoided the potential transmission from LB to MB replicates. For example, the shared animal isolation hallway was only entered after personal hygiene measures were performed and contaminated outer clothing and PPE were removed in the anteroom. In addition, all rooms were separately ventilated as described in the methods section to prevent aerosol transmission between rooms.

It has been well documented in experimental and observational studies that certain biosecurity measures may prevent or limit the spread of pathogens in pigs such as

PRRSv, Escherichia coli, and Mycoplasma hyopneumoniae (Otake et al., 2002; Amass et al., 2003; Batista et al., 2004; Pitkin et al., 2011). In humans, a review of physical measures including hand washing and mask use to reduce respiratory virus transmission indicated that these measures were also effective and should be implemented and assessed further (Jefferson et al., 2009). Knowing that biosecurity and hygiene measures can be effective in preventing or limiting spread of pathogens, the results of this study do not significantly alter current recommendations. However, this study does highlight the potential for IAV transmission by fomites in the presence of biosecurity and hygiene measures.

Personnel did not shower or change clothes worn underneath coveralls between movement events from group I to the MB replicates. Therefore, it is possible that contamination of clothes worn underneath coveralls or areas of the skin that were not washed could have been contaminated with IAV over the 9 movement events. While assessing the mechanical transmission of enterotoxigenic Escherichia coli, transmission was prevented when people showered and wore clean outerwear after interacting with infected pigs; however, transmission was not fully prevented via hand washing and wearing clean outerwear (Amass et al., 2003). Most importantly, our study indicates that IAV can be transmitted via fomites and result in the infection of previously negative pigs.

Influenza virus is a common respiratory pathogen in pigs and this study illustrates that fomites can transmit IAV from infected to susceptible populations. While the frequency of this event cannot be determined, this transmission route should be taken into account under existing comprehensive biosecurity protocols in the field. In addition to the fomites assessed in this study, influenza viruses have been detected and shown to remain viable on various other fomites. For example, viable influenza virus has been recovered from stainless steel and plastic surfaces, paper tissue, and banknotes following contamination and has been detected on fomites in human settings, such as day care centers (Bean et al., 1982; Boone and Gerba, 2005; Thomas et al., 2008). While fomites can be contaminated, recent work has shown that influenza virus may not survive for a

long period following natural contamination (Mukherjee et al., 2012). Movement to sentinel rooms following contact with infected pigs was immediate in this study, and thus long term survival of IAV on fomites was not necessary for transmission. Short time intervals between contact with infected individuals and susceptible individuals may occur in many settings, including within day care centers and pig barns, indicating that transmission can take place even though the survival of IAV may be short.

Influenza virus infections are common in both pig and human populations and the pandemic 2009 H1N1 and H3N2 variant (H3N2v) viruses have recently highlighted the interspecies transmission potential of IAV (Forgie et al., 2011; Bowman et al., 2012). A thorough understanding of critical transmission routes is needed in order to mitigate IAV transmission both within species and between species. This study has confirmed the transmission of IAV by fomites between pig populations in an experimental setting under differing biosecurity measures. Biosecurity and hygiene measures aimed at indirect routes of transmission, including fomites, should be incorporated and further assessed as part of comprehensive biosecurity protocols to prevent IAV transmission.

Table 4.1. Experimental groups.

Group	Code	N	Replicates	Isolation room	Person in contact
Infected	I	10	1	1	A, B, C, D
Low biosecurity	LB	10	2 (n=5)	2 and 4	A, B
Medium biosecurity	MB	10	2 (n=5)	3 and 5	C, D
Negative control	NC	5	1	6	Other

Table 4.2. Clothing and personal protective equipment (PPE).

Clothing or PPE	**Manufacturer**
Undershirt and pants	Various manufacturers (55% cotton, 45% polyester)
Tyvek® coverall	DuPont™ Tyvek® Wilmington, DE, USA
Cloth coverall	Various manufacturers (65% polyester, 35% cotton)
Rubber boots	Tingley Rubber Corp. South Plainfield, NJ, USA
Disposable plastic boots	KNOT-a-BOOT™, Continental Plastic Corp. Delavan, WI, USA
Polypropylene bouffant cap	Medline Industries Inc. Mundelein, IL, USA
Protective eyewear	MSA, Safety Works® Cranberry Township, PA, USA
N-95 respirator	3M™ (9210/37021) St. Paul, MN, USA
Powder-free latex gloves	Microflex® Evolution One® Reno, NV, USA

Table 4.3. Clothing and PPE changed after contact with group I.

Clothing or PPE	**Movement to LB group**	**Movement to MB group**
Undershirt and pants	No	No
Coverall	No	Yes
Rubber boots	Yes (room specific)	Yes (room specific)
Disposable plastic boots	No	Yes
Polypropylene bouffant cap	No	Yes
Protective eyewear	Yes (room specific)	Yes (room specific)
N-95 respirator	No	Yes
Powder-free latex gloves	No	Yes

Table 4.4. Fomite swab results following contact with infected pigs and prior to biosecurity measures.

	Personnel*			
Move	**A (LB-2)**	**B (LB-1)**	**C (MB-2)**	**D (MB-1)**
1				
2			Boots (39)	Boots (39)
3				
4			Boots (39)	
5		Coveralls (39)		
6	Coveralls (38)**	Coveralls (39)	Boots (39)	Boots (37)
7		Boots (38), Coveralls (39)		Boots (40)
8				
9				

*All samples not listed were negative via RRT-PCR
**( ) RRT-PCR Ct value

Table 4.5. Pig RRT-PCR results by day from nasal swab samples.

		Study Day																	
Group	**Pig**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
I	I-1	-	+	+	+	+	+	S	S	-	+	-	-	N	N	N	N	N	N
	I-2	-	-	+	-	+	+	-	S	-	-	-	-	N	N	N	N	N	N
	I-3	-	-	+	+	+	+	S	+	+	-	-	-	N	N	N	N	N	N
	I-4	-	+	+	+	+	+	S	S	-	S	-	-	N	N	N	N	N	N
	I-5	-	-	+	+	+	+	-	S	-	-	-	-	N	N	N	N	N	N
	I-6	-	S	+	+	+	+	S	+	-	-	-	-	N	N	N	N	N	N
	I-7	-	+	+	+	+	+	+	S	-	-	-	-	N	N	N	N	N	N
	I-8	-	+	+	+	+	+	S	S	-	-	-	-	N	N	N	N	N	N
	I-9*	-	-	-	+	+	+	S	+	+	S	-	-	N	N	N	N	N	N
	I-10*	-	-	-	+	+	+	+	+	+	+	S	-	N	N	N	N	N	N
MB-1	MB-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	N	-
	MB-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	N	-
	MB-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	N	-
	MB-4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	N	-
	MB-5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	N	-
MB-2	MB-6	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	S	-	N
	MB-7	-	-	-	-	-	-	-	-	-	-	+	+	+	+	S	-	-	N
	MB-8	-	-	-	-	-	-	-	-	-	-	S	+	+	+	S	-	-	N
	MB-9	-	-	-	-	-	-	-	-	-	S	+	+	+	+	+	S	-	N
	MB-10	-	-	-	-	-	-	-	+	+	+	+	+	S	-	-	-	-	N
LB-1	LB-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	N	-
	LB-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	N	-
	LB-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	N	-
	LB-4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	N	-
	LB-5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	N	-
LB-2	LB-6	-	-	-	-	-	-	-	-	S	-	+	+	+	S	+	-	-	N
	LB-7	-	-	-	-	-	-	-	S	+	+	+	+	+	-	-	-	-	N
	LB-8	-	-	-	-	-	-	-	-	-	+	+	+	+	S	-	-	-	N
	LB-9	-	-	-	-	-	-	-	+	+	+	+	+	S	-	-	-	-	N
	LB-10	-	-	-	-	-	S	+	+	+	+	+	S	S	-	-	-	-	N
NC	NC-1	-	N	-	N	-	N	N	N	N	-	N	N	N	N	-	N	N	-
	NC-2	-	N	-	N	-	N	N	N	N	-	N	N	N	N	-	N	N	-
	NC-3	-	N	-	N	-	N	N	N	N	-	N	N	N	N	-	N	N	-
	NC-4	-	N	-	N	-	N	N	N	N	-	N	N	N	N	-	N	N	-
	NC-5	-	N	-	N	-	N	N	N	N	-	N	N	N	N	-	N	N	-
Group	**Pig**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
		Study Day																	

*Direct contact sentinel
(-) = Negative (Ct value >40)
(+) = Positive (Ct value ≤35)
S = Low level positive or suspect (Ct value >35 and ≤40)
N = Not tested

Figure 4.1. Isolation facility layout.

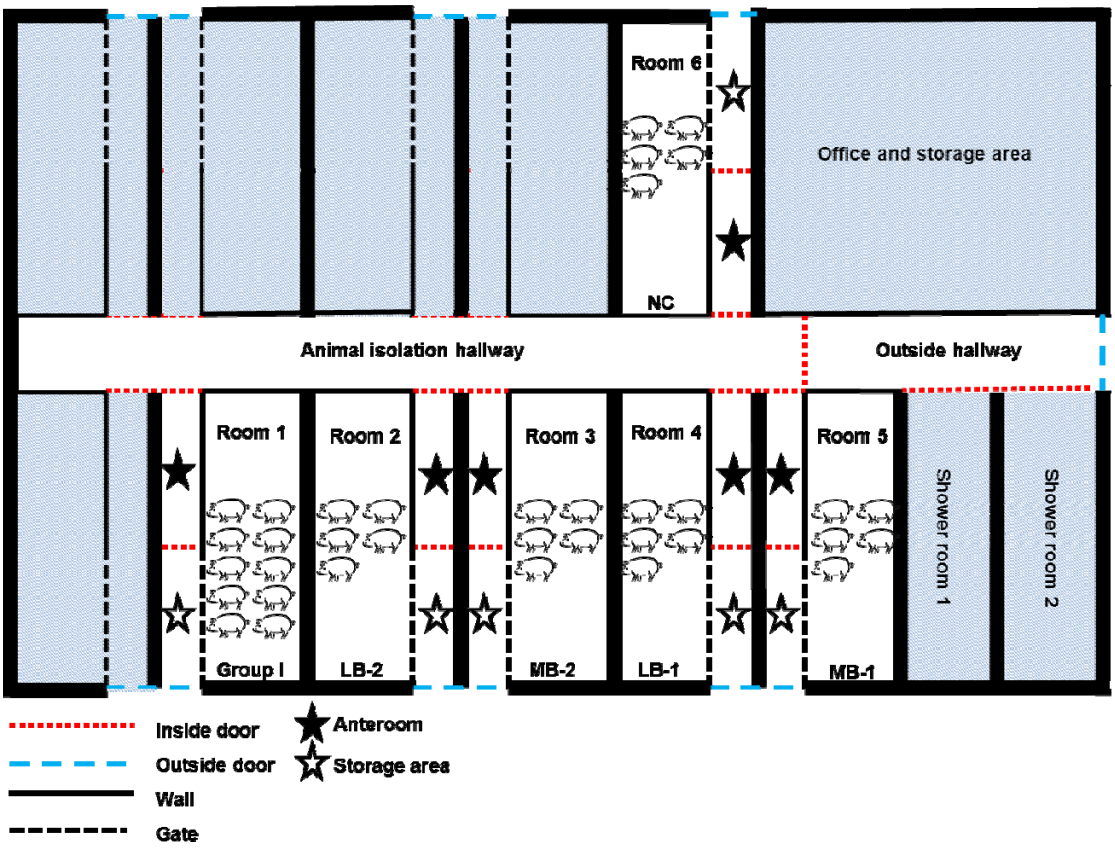
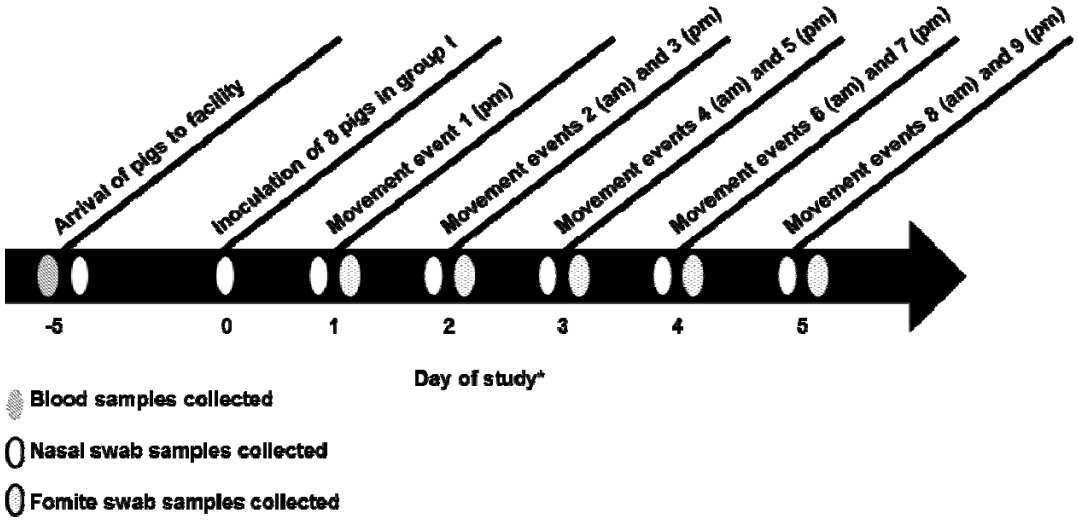
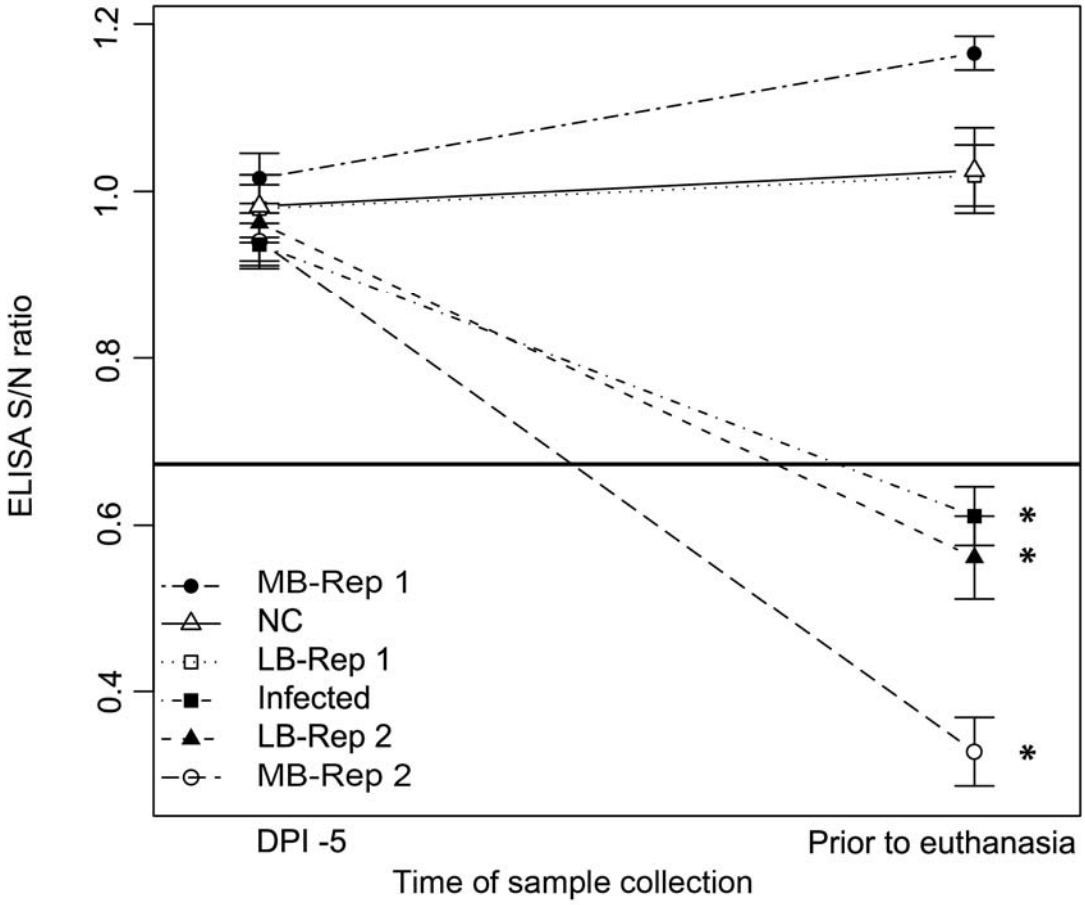


Figure 4.2. Timeline following arrival and during movement events.



*In relation to day of inoculation.

Figure 4.3. Influenza A Multiscreen ELISA S/N ratios (±SE) by experimental group and replicate.



The black horizontal line represents the cutoff value (≤0.673 is considered positive).

*Significantly lower ELISA S/N ratios prior to euthanasia compared to -5 DPI ($P < 0.05$).

Chapter 5: The impact of maternally derived immunity on influenza A virus transmission in neonatal pig populations

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Introduction

Influenza A viruses (IAV) have been a significant cause of respiratory disease in pigs for nearly a century. Prevalence estimates indicate that IAV are common and widespread in pig populations across the United States (Hinshaw et al., 1978; Chambers et al., 1991; Olsen et al., 2000). In addition, transmission of IAV between humans and pigs has been documented including the pandemic 2009 H1N1 virus (Myers et al., 2007; Howden et al., 2009; Forgie et al., 2011) and H3N2v viruses (Centers for Disease Control and Prevention (CDC). 2012). Therefore, vaccination has been adopted as a control measure in pigs with approximately 70% of large sow farms in the United States vaccinating breeding females (USDA. 2007; Beaudoin et al., 2011). Vaccination of breeding females for IAV not only provides immunity to the swine breeding herd, but also passive or maternal immunity through colostrum to the progeny.

Maternally derived immunity has been shown to have several impacts following IAV exposure. The ability to recover IAV from exposed pigs has been shown to be inversely related to the level of specific passive antibodies at the time of exposure (Renshaw. 1975). However, additional research has shown that maternally derived antibodies do not protect pigs from IAV infection but rather reduce clinical disease (Mensik et al., 1971; Loeffen et al., 2003; Choi et al., 2004; Kitikoon et al., 2006). While existing research provides a wealth of information regarding maternally derived immunity and individual animal outcomes following IAV challenge, the impact of maternally derived immunity on IAV transmission has not been assessed in detail.

The information needed to define transmission characteristics of IAV and other pathogens in pigs can be obtained experimentally (Velthuis et al., 2007). These studies not only describe the infection status of individuals under various treatments or immune statuses, but also allow one to quantify specific transmission parameters such as the transmission rate, infectious period, and ultimately the basic reproduction ratio (Ro). Concerning influenza virus, the basic reproduction ratio would be defined as the expected number of secondary influenza virus infections in a susceptible population due to a

typical infectious individual during the duration of that individual's infectious period (Diekmann et al., 1990). Therefore, when Ro is >1 each infectious individual will infect on average at least one other susceptible individual and transmission will occur, whereas, when Ro is <1 transmission will eventually cease. In certain situations, the goal of an intervention measure such as vaccination is to reduce Ro to <1. Transmission of IAV has been observed in the presence of passively acquired immunity (Choi et al., 2004), but transmission parameters have not been calculated with varying immune statuses as expected to occur in field situations. Therefore, the main objective of this study was to assess the impact of maternally derived immunity on IAV transmission by estimating and comparing the transmission rates, infectious periods, and reproduction ratios between groups of neonatal pigs with varying maternal immunity. In addition, the number of sentinel pigs infected per day and serum antibody titers were compared between treatment groups.

Materials and methods

Animals

Sows from a swine breeding farm previously determined to be free of IAV infection were randomly assigned to one of three treatment groups (Table 5.1) at 4–5 weeks pre-farrow and vaccinated intra-muscularly with inactivated experimental IAV vaccines. Prior to the initial vaccination, all sows in the heterologous (PASSV-HET) and homologous (PASSV-HOM) groups were confirmed to be seronegative for IAV by an enzyme linked immunosorbent assay (ELISA – FlockChek® Avian Influenza MultiS-Screen Antibody Test Kit, IDEXX Laboratories Inc., Westbrook, ME, U.S.A.). All sows farrowed at the breeding farm of origin and following parturition, neonatal pigs were individually identified, allowed to suckle colostrum from their respective dams, and not cross-fostered throughout the study. Sows in the PASSV-HET group and PASSV-HOM group were initially vaccinated on average at 32 and 33 days pre-farrow and the booster vaccine dose was administered on average at 18 and 19 days pre-farrow, respectively. Prior to transport to the University of Minnesota, nasal swab and blood samples were collected from all neonatal pigs. Nasal swabs were confirmed to be IAV negative via a matrix

gene-based real-time reverse transcription-PCR (RRT-PCR) and sera were tested by ELISA and hemagglutination inhibition (HI) assay with the respective vaccine viruses used as antigens in the HI assay. Pigs in the CTRL group were confirmed to be negative by ELISA and pigs in the PASSV-HOM and PASSV-HET groups were confirmed positive via ELISA and HI assay. All pigs were seronegative for porcine reproductive and respiratory syndrome virus (PRRSV) and Mycoplasma hyopneumoniae.

Animal housing

All selected pigs were transported to the University of Minnesota animal isolation facility (St. Paul, MN, U.S.A.) at 2–3 weeks of age. Pig selection was not random in the PASSV-HOM and PASSV-HET groups as pigs with the highest homologous HI titers were purposely selected within replicates. Selected pigs were placed in 8 groups of 11 pigs each in separate isolation rooms. Each separately ventilated isolation room had one animal housing pen of 7.28 m2 (0.66 m2/pig). Pigs were cared for according to the approved University of Minnesota Institutional Animal Care and Use Committee (IACUC) protocol 1010A91533.

Experimental design

The study was conducted with 3 replicates (11 pigs/replicate) for each of the PASSV-HET and CTRL treatment groups and 2 replicates for the PASSV-HOM group. Each replicate of 11 pigs consisted of 1 seeder pig (IAV naïve prior to infection) and 10 sentinel pigs (ELISA negative in the CTRL group and seropositive in the PASSV-HET and PASSV-HOM groups). The 10 sentinel pigs/replicate were selected from 2 to 3 different sows in each PASSV-HET and CTRL replicate and from 3 sows in the PASSV-HOM replicates. Twenty-four to forty-eight hours post-arrival to the isolation facility, nasal swabs were collected from all pigs and confirmed to be negative via IAV RRT-PCR. Pigs were also injected once with an antibiotic (Ceftiofur crystalline free acid, 5.0 mg/kg body weight Excede®, Pfizer Animal Health, New York, NY, U.S.A.) in order to reduce bacterial contaminants prior to the start of the study. Four to five days post-arrival, one naïve pig from each room (designated as the seeder pig) was moved to a separate

isolation room and inoculated with the challenge virus A/Sw/IA/00239/04 (IA/04). Forty-eight to seventy-two hours following inoculation, seeder pigs were moved back into their original rooms with the sentinel pigs. Nasal swabs were collected daily from all pigs for a period of 13 or 14 days following the movement of infected seeder pigs into the respective rooms. Thirteen to fourteen days following the introduction of the infected seeder pig, all pigs were humanely euthanized with an intravenous lethal dose of pentobarbital at 100 mg/kg.

Challenge virus and preparation of vaccines

A β cluster H1N1 triple reassortant IAV strain A/Sw/IA/00239/04 (IA/04) was used as the challenge virus in this study. This virus was isolated from a field sample and has been used in previous studies (Vincent et al., 2006; Vincent et al., 2007; Vincent et al., 2009; Romagosa et al., 2011a). The same virus (IA/04) was used to prepare the homologous vaccine (PASSV-HOM). The heterologous vaccine (PASSV-HET) was created using an α cluster H1N1 IAV strain A/Sw/IL/02450/08 (IL/08) (Detmer et al., 2012). Both viruses were grown in bulk quantities using Madin–Darby canine kidney (MDCK) cells (Meguro et al., 1979), adjusted to an HA titer of 1:128/0.1 ml and inactivated by the addition of formalin at a final concentration of 0.1%. The formalized virus was mixed with an adjuvant at 12% (v/v) (Emulsigen®-D, MVP Technologies, Omaha, NE, U.S.A.). Based on HA gene sequencing, the IL/08 virus shared 86% nucleotide similarity with the IA/04 virus. To reiterate, the terms homologous (PASSV-HOM) and heterologous (PASSV-HET) used in this study describe immunity in neonatal pigs based on the vaccines and challenge strains used.

Virus inoculation/seeder pigs

Seeder pigs were challenged intra-tracheally and intra-nasally with 0.5 ml of virus inoculum in each location, containing 1×10^7 tissue culture infective dose (TCID50)/ml of the IA/04 virus. Before the challenge inoculation, all pigs were sedated by an intramuscular injection of Telazol® (6 mg/kg, Telazol®, Fort Dodge Animal Health, Fort Dodge, IA, U.S.A.).

Sample processing and diagnostic tests

Blood samples

Blood samples were collected via jugular venipuncture and serum was separated and stored at −20 °C until testing. Samples were collected at the sow farm of origin, 24–48 h prior to mixing of sentinel and seeder pigs following inoculation (pre-contact), and on the day of euthanasia (13 or 14 days post-contact). Samples were tested for IAV antibodies via ELISA and HI assay. Samples were tested by HI using the IA/04 and IL/08 viruses as separate antigens using a standard procedure (Direksin et al., 2002). Serial 2-fold dilutions of treated sera were tested beginning at a 1:20 dilution and ending at a 1:640 dilution. Samples were also tested via ELISA assay as described previously with an S/N ratio ≤0.673 considered positive and an S/N ratio >0.673 considered negative (Ciacci-Zanella et al., 2010). The Influenza A Multiscreen ELISA measures antibodies directed against the nucleoprotein (NP) of influenza A viruses.

Nasal swabs

Nasal swabs were collected daily from all animals for 13–14 days using sterile rayon-tipped swabs (BD BBL™ CultureSwab™, liquid Stuart medium, single plastic applicator, Becton, Dickinson and Co., Sparks, MD, U.S.A.). Following collection, each nasal swab was suspended in 1 ml of MEM supplemented with 2% bovine serum albumin, trypsin, and antibiotics. Samples were tested for IAV via matrix gene RRT-PCR at the University of Minnesota Veterinary Diagnostic Laboratory (Slomka et al., 2010). In addition, nasal swab samples collected from seeder pigs on the day of movement back to their respective rooms following inoculation were cultured on MDCK cell monolayers with virus titers obtained via serial dilution and calculated by the method of Spearman–Karber.

Transmission parameters

Pigs were characterized on a daily basis according to a SIR model as S (susceptible), I (infectious), or R (recovered) as previously described (Romagosa et al., 2011a). Briefly,

on day 0, each respective room consisted of one infectious pig (seeder) and 10 susceptible (sentinel) pigs. The status of each pig was confirmed on a daily basis via the collection of a nasal swab which was tested for IAV RNA via matrix RRT-PCR. A pig was considered infectious (I) if positive for IAV via RRT-PCR from a nasal swab. A pig was considered recovered (R) if the pig was positive for IAV and then became negative. The transmission rate parameter (β) was estimated by day ($\Delta t = 1$) for each treatment group using a generalized linear model (GLM) with a complementary log–log link function and an offset variable of log $I\Delta t/N$ (number of infectious pigs per day/total number of pigs) as described previously (Velthuis et al., 2003; Velthuis et al., 2007; Romagosa et al., 2011a). The reproduction ratio (R) was then estimated for each treatment group via the product of the transmission rate parameter per day (β) and the infectious period of infected sentinel pigs. In the CTRL group, the reproduction ratio estimate is by definition the basic reproduction ratio estimate as this population was completely susceptible to the challenge virus in contrast to the PASSV-HET and PASSV-HOM groups with maternal immunity.

For statistical analyses, replicates were combined for each treatment group. Individual pig infectious periods (IP) were defined as the number of days between the first and last detection of IAV via RRT-PCR from nasal swabs. Mean infectious periods and 95% percentile confidence intervals were calculated and compared between the PASSV-HET and CTRL groups via bootstrap methods with 1000 replications. Briefly, bootstrap distributions for infectious period means and their difference were created via resampling with replacement from the original sample of 30 pigs within each group with 1000 replications. Differences between β values were compared using contrast comparisons and differences considered statistically significant at $p < 0.05$. The statistical comparison between R estimates was based on non-overlapping 95% confidence intervals. Statistical analyses were performed using SAS (SAS System, SAS Inst., Cary, NC, U.S.A. v 9.2) and R (R Foundation for Statistical Computing, Vienna, Austria).

Additional statistical methods

Survival curves comparing time to IAV infection were created and compared via Kaplan–Meier methods and the log-rank test. Pigs remaining IAV negative at the end of the study in the PASSV-HOM group were right-censored at 14 days post-exposure. Log2 transformed HI reciprocal antibody titers and ELISA S/N ratios were compared by analysis of variance (ANOVA) by treatment group with pair-wise comparisons conducted using the Tukey–Kramer method. Hemagglutination inhibition antibody titers <1:20 (first dilution tested) were given the value of 1:10 in the analyses. Antibody titers 24–48 h pre-contact and 13–14 days post-contact were analyzed via Student's paired t-test.

Stochastic SIR model

The direct method of Gillespie was used to model the random events of transmission and recovery (Gillespie. 1976) as previously described in a similar experimental setting (Romagosa et al., 2011a). For each simulation the total population size was 11, with initial values of S = 10, I = 1, and R = 0. The proportion of 10,000 simulations by the number of new cases (IAV infections) for each group was displayed in graphical format.

Results

Serology

The serologic status of sentinel pigs pre-contact and post-contact with seeder pigs are summarized and displayed in Table 5.2 and Figure 5.1. All sentinel pigs in the CTRL group were seronegative by ELISA prior to contact with seeder pigs and all sentinel pigs in the PASSV-HET and PASSV-HOM groups were seropositive based on both ELISA and HI assays with the respective vaccine antigens. The homologous reciprocal geometric mean HI titers in the PASSV-HET and PASSV-HOM groups were 143 and 331, respectively.

Transmission

Nasal swab matrix RRT-PCR

All seeder pigs were RRT-PCR and virus isolation positive at 48–72 h post inoculation when placed with sentinel pigs and virus titers ranged from 3.2×10^3 to 1×10^5

TCID50/mL. In addition, seeder pigs were positive for at least 3 days following contact with the sentinel pigs. All sentinel pigs in the PASSV-HET (30/30) and CTRL (30/30) groups were RRT-PCR positive for at least one day (infectious) following the introduction of seeder pigs; whereas just one pig in the PASSV-HOM group (1/20) with a pre-contact homologous HI titer of 1:320 was positive for 6 days. The proportion of IAV negative pigs by day following contact with each respective seeder pig differed between groups over the study period ($p < 0.001$, Figure 5.2).

Infectious period

The mean length of the infectious period was longer at 4.78 days in the CTRL group compared to the PASSV-HET group at 4.06 days (mean difference 0.70, 95% percentile bootstrap CI 0.10–1.27, $p = 0.03$, Table 5.3). Only one pig was infected in the PASSV-HOM group and had an infectious period of 6 days.

Transmission rate parameter (β) and reproduction ratio (R) estimates

The transmission rate parameter (β) and the reproduction ratio estimate were significantly lower in the PASSV-HOM group compared to the PASSV-HET and CTRL groups (Table 5.3). There were no statistically significant differences between the transmission parameters and the reproduction ratio estimates for the PASSV-HET and CTRL groups.

Stochastic modeling

Stochastic modeling based on the transmission parameters generated from the experimental study showed that 80% and 89% of simulations of the PASSV-HET and CTRL groups, respectively resulted in all susceptible pigs ($n = 10$) becoming infected with IAV (Figure 5.3). In contrast, 0.3% of simulations of the PASSV-HOM group resulted in all susceptible pigs ($n = 10$) becoming infected.

Discussion

To address the lack of information regarding IAV transmission in pigs with maternal immunity, an experimental transmission study was conducted to estimate the

reproduction ratio (R) of IAV in pigs with varying levels of maternal immunity. In the experimental model, IAV was transmitted to all sentinel pigs that were seronegative to the challenge virus and sentinel pigs with heterologous maternal immunity, and the R estimates did not differ significantly between these groups. In contrast, transmission was a low probability event in the presence of homologous maternal immunity. While pigs were infected in all groups, clinical signs were mild in all infected pigs throughout the study (results not shown).

The reproduction ratio estimates obtained from the experimental model provide useful insights regarding IAV transmission in populations. As expected, all sentinel pigs became infected in the CTRL group with a basic reproduction ratio (Ro) estimate (95% confidence interval) of 10.4 (6.6–15.8). This is very similar to a previously determined Ro of 10.7 in an older population of seronegative sentinel pigs (Romagosa et al., 2011a). The stochastic models for the CTRL group demonstrate that major IAV outbreaks are likely to occur in small naïve populations following the introduction of an infected pig.

Similar to what was observed in the CTRL group, all sentinel pigs with heterologous maternal immunity were infected with IAV and the estimated value of R was 7.1 (4.2–11.3). While the transmission parameter (β) was numerically lower in the PASSV-HET group, the estimates were not statistically different between the CTRL and PASSV-HET groups. In contrast, the infectious period was slightly shorter in the PASSV-HET group compared to the CTRL group although the biological significance of this slightly shorter infectious period may be minimal. The resultant stochastic PASSV-HET group model showed this numerical difference with a slightly lower proportion of simulations with all pigs becoming infected and a slightly higher proportion of simulations with no new cases compared to the CTRL group. In contrast, the reproduction ratio estimate was significantly lower in the PASSV-HOM group at 0.8 (0.1–3.7) compared to the PASSV-HET and CTRL groups. Based on stochastic modeling, 57% of simulations resulted in no new cases in the PASSV-HOM group.

This study confirmed that IAV infection and transmission can take place in the presence of maternal immunity. The PASSV-HET group reinforced previous reports of IAV infection in the presence of maternal immunity and showed that the R estimate was similar to that of the CTRL group. This study also showed that infection was prevented in most pigs and transmission reduced in the presence of homologous maternal immunity. In addition, stochastic modeling showed that over half of the time an infected pig was introduced in the PASSV-HOM population described in our experimental setting, transmission was prevented. Optimization of passive or maternally derived immunity through sow vaccination is a widely practiced control measure for influenza transmission. The results from this experiment involving pigs in the PASSV-HOM group appear to justify that practice. The PASSV-HOM group demonstrates, in a "best case scenario," that when pigs are challenged with IAV when maternal antibody titers to the challenge virus are high and with the same virus as contained in the sow vaccine preparation, then indeed transmission is decreased. Although transmission was decreased in the PASSV-HOM group one pig with a pre-contact homologous HI titer of 1:320 was infected, but secondary transmission from this pig was not observed.

While this study shows IAV transmission may be reduced given the specific settings of this study, field conditions may alter this impact. Pigs with homologous maternal immunity in this study had high and uniform titers, whereas pigs in a field setting may have more variable levels of maternally derived immunity as the concentration at weaning age depends on many factors including the initial IgG level in the colostrum, quantity of colostrum ingested, and gut closure timing (Rooke and Bland, 2002). In addition, the main purpose of the PASSV-HET group in this study was to create a population of pigs with high levels of maternally derived immunity with limited cross-reactivity to the challenge virus. This situation may occur in field settings, but the level of cross-reactivity will differ. The terms homologous and heterologous were used in this study to describe immunity in neonatal pigs based on the vaccines and challenge strain used, but there is likely great variation within these descriptions in field settings. In this

study, 4 ml of vaccine was administered as a booster dose 2–3 weeks pre-farrow to sows in the PASSV-HOM group (Table 5.1), while 2 ml was administered to sows in the PASSV-HET group. In addition to the factors mentioned above, this difference may have impacted the results observed in this study. However, the ultimate measure of interest regarding immunity in this study was HI antibody titer to the challenge virus (IA/04). In this study, a 4 ml vaccine dose was needed as a booster in order to achieve desirable HI titers in the PASSV-HOM group. The resultant HI titers in neonatal pigs to the challenge virus (Table 5.2) need to be taken in account when interpreting results of this study. Animal housing types and the initial number of IAV infected pigs will also differ between farms, which could alter contact patterns between infected and susceptible pigs.

The level of specific passive antibody at the time of exposure has been regarded as an indicator of immune protection to IAV in young pigs (Renshaw. 1975). This study provides further evidence regarding this point in a transmission experiment. The majority of pigs that suckled colostrum from sows vaccinated with a homologous killed vaccine and then challenged via direct contact with an experimentally infected pig were completely protected. One pig did become infected even with a high HI titer to the challenge virus. However, the reproduction ratio was lower compared to pigs with high levels of heterologous maternal immunity and pigs seronegative to the challenge virus. These results suggest that while homologous immunity may not completely prevent transmission, it is still beneficial to decrease transmission and prevent disease.

In order to reduce the prevalence of IAV in swine, transmission routes and the impact of common control measures on transmission must be understood. Influenza virus transmission was reduced but not prevented in pigs with homologous maternal immunity compared to pigs with heterologous maternal immunity and pigs seronegative to the challenge virus. Furthermore, there was no difference in IAV transmission between pigs that were seronegative to the challenge virus and pigs with heterologous maternally derived immunity. This study provides important information regarding a commonly used control measure for IAV and its impact on virus transmission, and highlights the

role of pigs with passive immunity as potential disseminators of IAV despite a potential reduction of clinical disease.

Table 5.1. Treatment groups and experimental vaccines

Treatment group (Abbreviation)	Sow vaccine virus (Subtype/H1 cluster)	Initial vaccine (Dose volume)	Booster vaccine (Dose volume)
Heterologous (PASSV-HET)	A/Sw/IL/02450/08 [IL/08] H1N1/α cluster	4-5 wks pre-farrow (2 ml)	2-3 wks pre-farrow (2 ml)
Homologous (PASSV-HOM)	A/Sw/IA/00239/04 [IA/04] H1N1/β cluster	4-5 wks pre-farrow (2 ml)	2-3 wks pre-farrow (4 ml)
Control (CTRL)	Not vaccinated	Not vaccinated	Not vaccinated

Table 5.2. HI titers (reciprocal geometric means) against IA/04 virus (challenge virus and PASSV-HOM group vaccine virus) and IL/08 virus (PASSV-HET group vaccine)

IA/04 virus			IL/08 virus		
Group	Pre-contact	Post-contact	Group	Pre	Post
PASSV-HET	17[b]	70[c*]	PASSV-HET	**143[a]**	**111[a]**
PASSV-HOM	**331[a]**	**139[b*]**	PASSV-HOM	61[b]	39[b*]
CTRL	10[c]	**393[a*]**	CTRL	21[c]	124[a*]

[a,b,c]Statistically significant differences between groups at each time period (columns) ($p < 0.05$)

[*]Statistically significant differences between pre-contact and post-contact paired samples (rows) ($p < 0.05$)

Titers in bold are homologous titers, e.g., the virus in the assay was homologous to the virus to which the pigs or the pigs' dam were exposed via vaccine or contact with a seeder pig

Table 5.3. Infectious period, transmission rate parameter, and reproduction ratio estimates with 95% confidence intervals (95% CI) by treatment group

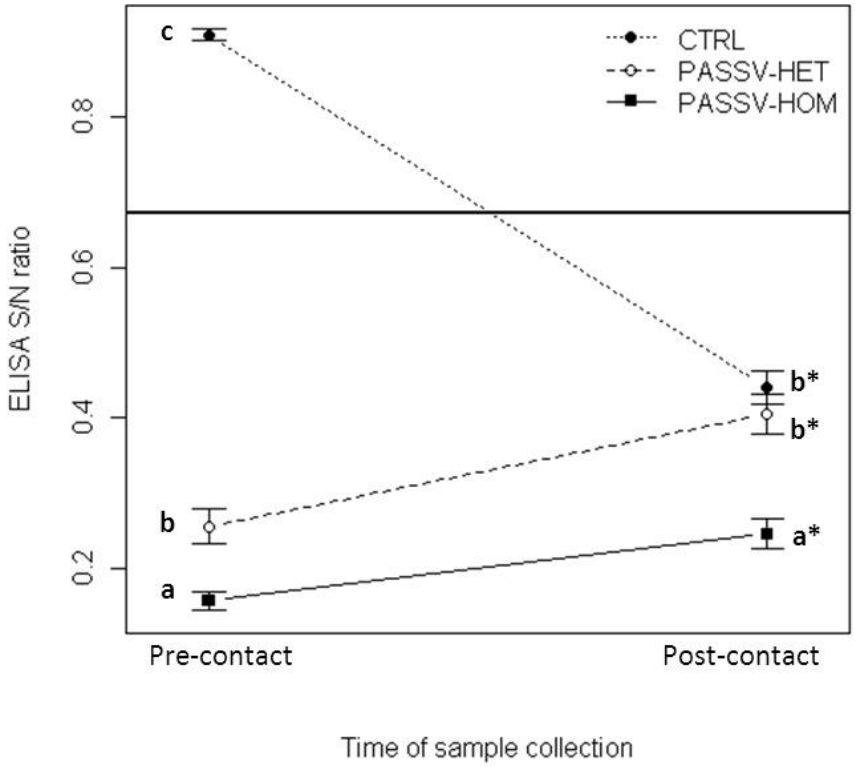
Group	Infectious period (days) (95% CI)	Transmission rate parameter (95% CI)	Reproduction ratio estimate (95% CI)
PASSV-HET	4.06 (3.57-4.60)[a]	1.74 (1.18-2.46)[b]	7.1 (4.2-11.3)[y]
PASSV-HOM	6[*]	0.14 (0.01-0.61)[a]	0.8 (0.1-3.7)[x]
CTRL	4.78 (4.47-5.10)[b]	2.18 (1.47-3.10)[b]	10.4 (6.6-15.8)[y]

[a,b]Statistically significant differences ($p < 0.05$).

[x,y]Statistically significant differences based on non-overlapping 95% CI's

[*]Infectious period of one infected sentinel pig

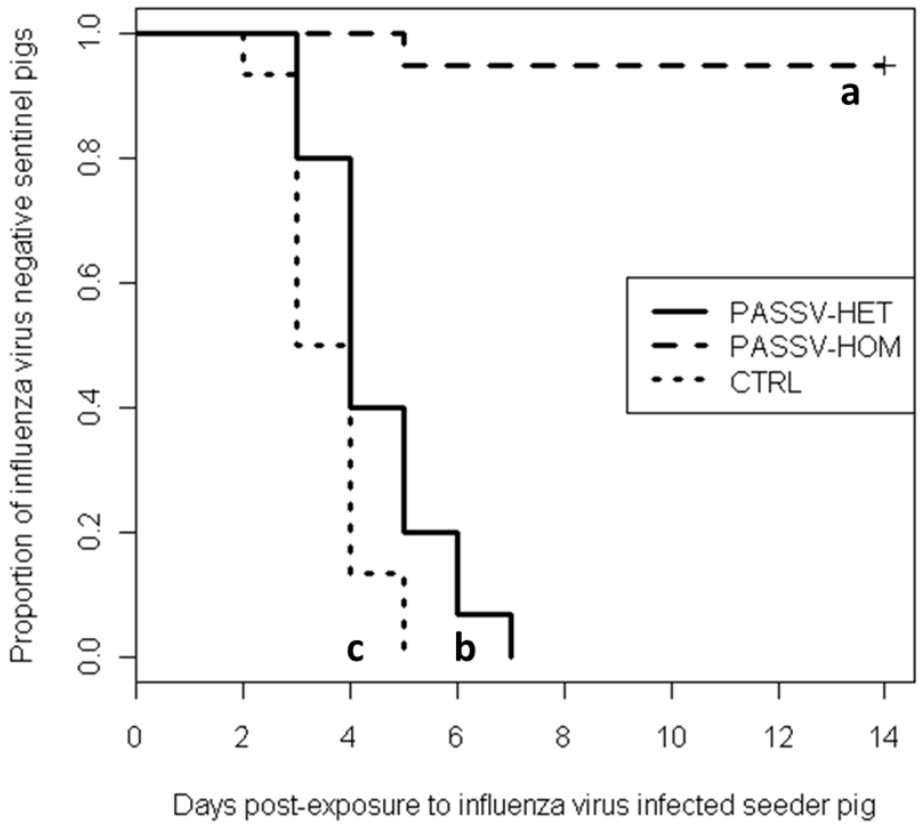
Figure 5.1. Influenza A Multiscreen ELISA S/N ratios (±SE) pre-contact and post-contact by treatment group. The black horizontal line represents the cutoff (≤0.673 is considered positive).



[a,b,c]Statistically significant differences between groups at each time period ($p < 0.05$)

*Statistically significant differences between pre-contact and post-contact paired samples ($p < 0.05$)

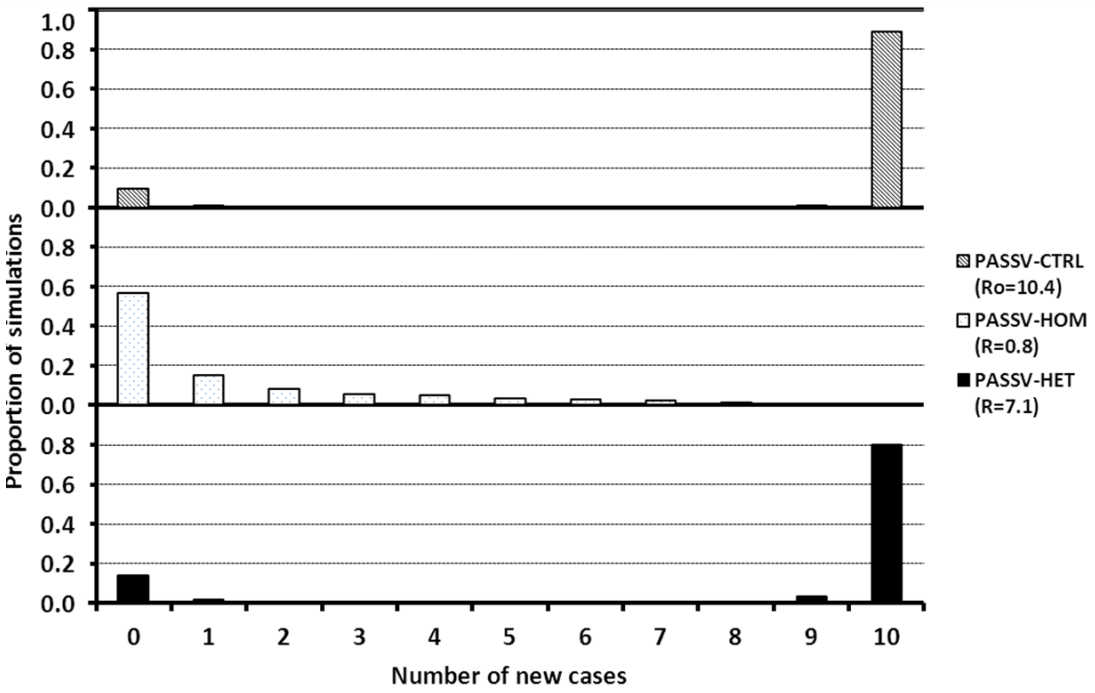
Figure 5.2. Survival curve from influenza virus infection by treatment group



+ Censored data

[a,b,c]Statistically significant differences ($p < 0.001$), log-rank test

Figure 5.3. Number of new cases (influenza virus infection) represented as the proportion of 10,000 simulations from the stochastic SIR model with initial values of (S=10, I=1, R=0) for each treatment group



Chapter 6: General discussion and conclusions

General discussion and conclusions

Influenza A virus has been a significant pathogen in pig populations for almost 100 years. The ability of this virus to maintain itself and adapt over this period of time has frustrated researchers, veterinarians, and pig producers. Additionally, influenza virus is a common cause of disease in humans and other animal species. The pandemic 2009 H1N1 virus and H3N2 variant viruses have illustrated the close connection of humans and pigs and the exchange of influenza viruses between species. While the literature contains much information assessing influenza virus epidemiology and control in humans, the knowledge base surrounding influenza virus epidemiology, control, and transmission in pig populations is more limited. Therefore, the objectives of this thesis were to assess influenza virus epidemiology and transmission within pig herds and to assess currently practiced control measures and their ultimate impact of influenza virus transmission and prevalence.

The first chapter provided a thorough review of relevant literature that led to the questions addressed in this thesis. There is a wealth of information available assessing influenza virus in pigs and other animal species. However, there is a gap in the literature regarding influenza virus transmission within herds. This knowledge could better inform control measures and allow for more efficient, targeted surveillance of influenza virus in pig populations. The second chapter of this thesis narrowed the gap in understanding of influenza virus transmission and infection dynamics within herds. This chapter summarized the infection dynamics of influenza virus in a two-site swine herd following infection with pandemic 2009 H1N1 influenza virus. This study demonstrated that young pigs in sow herds can be infected with influenza virus, even in the absence of infected sows and gilts. This population was also able to maintain the infection over time as genetically similar influenza viruses were identified over a 27-day period via nasal swab and tissue samples. Weaning age pigs were then able to transport this virus from the sow herd to a distant wean to finish site. Based on repeated sampling at the wean to finish site, influenza virus genome was consistently detected in oral fluid samples until 69 days

following the first detection of influenza virus at the site. This study provided an in depth understanding of influenza virus infection dynamics within a sow herd and an associated wean to finish site. Others had previously hypothesized that influenza viruses could be maintained for extended periods of time within herds and this study provided further confirmation regarding this epidemiologic feature of influenza viruses.

To further describe the infection dynamics and prevalence of influenza virus in sow herds, chapter 3 assessed the prevalence and temporal patterns of influenza virus infection in weaning age pigs from a selected cohort of sow farms and characterized the viruses obtained within these selected farms. This study further strengthened the findings of chapter 2 as 44% of the 52 enrolled sow herds tested IAV positive for at least one sampling event. This further confirms the importance of weaning age pig regarding influenza virus epidemiology. Additionally, 84% of sow herds that tested influenza virus positive at the first sampling event tested positive for at least one additional sampling event and three sow herds tested positive for 6 consecutive sampling events over durations of 156, 165, and 165 days. The comparison of HA gene sequences within farm also showed that viruses obtained within farm over the study period shared greater than 98.8% nucleotide similarity, with the exception of one farm infected with 2 different subtypes. In many sow herds, weaning age pigs were able to maintain influenza virus infections over extended periods of time, up to 165 days. This large scale assessment within sow herds greatly contributes to the understanding of influenza virus epidemiology within sow herds.

While Chapters 2 and 3 provided additional explanations regarding the high prevalence of influenza virus across pig herds, the ultimate cause for influenza virus maintenance at the herd level remained unknown. Chapters 4 and 5 explored the transmission of influenza virus and the impact of commonly practiced control measures on influenza virus transmission. Direct pig to pig transmission of IAV is known to occur; however, other transmission routes have not been studied in detail in pigs. Chapters 2 and 3 of this thesis described the continual infection of weaning age and weaned pigs in sow

and grow finish farms without assessing transmission routes within these farms. Indirect transmission routes, such as contaminated personnel or fomites, were thought to be an important transmission route within sow farm and grow finish sites, but indirect transmission routes had not been previously assessed for IAV in pig populations. Chapter 4 evaluated the role of fomites in IAV transmission between pig populations that were separated by two different biosecurity settings. This was accomplished by using a pig challenge and exposure model so that the entire infection chain could be monitored in a pig model. Interestingly, fomites were contaminated with IAV following interaction with infected pigs, IAV was transported via fomites to sentinel pigs, and sentinel pigs were infected with IAV. Furthermore, additional biosecurity measures did not prevent transmission in one of two replicates. This chapter provided evidence for an additional transmission route for IAV within pig populations. Indirect transmission routes must also be taken into account when investigating influenza virus infections in pig herds. Measures to prevent influenza virus transmission via fomites should be included in comprehensive biosecurity plans.

Chapter 4 described transmission of influenza virus by fomites, which could be contributing to the infection dynamics described in chapters 2 and 3. One control measure that may have an impact on influenza virus transmission within sow farms is vaccination. Sow vaccination is a common practice in sow herds that results in neonatal pigs obtaining maternally derived immunity via colostrum; however, the impact of sow vaccination on influenza virus transmission in weaning age pigs had not been described previously. Chapter 5 assessed the impact of maternally derived immunity on IAV transmission in weaning age pigs. To represent possible scenarios that may occur in a field setting, influenza virus negative sows were either not vaccinated or vaccinated pre-farrow with a heterologous or homologous vaccine (as compared to the challenge virus). Neonatal pigs then suckled colostrum and obtained their respective maternal immunity. Weaning age pigs were then challenged via a seeder pig model and transmission parameters were estimated following daily sampling of seeder and sentinel pigs. Influenza virus was transmitted to all sentinel pigs that were seronegative to the challenge virus and sentinel

pigs with heterologous maternal immunity. In contrast, transmission was a low probability event in the presence of homologous maternal immunity and the reproduction ratio was significantly lower. Therefore, homologous maternal immunity may reduce influenza virus transmission, specifically in sow herds, but may not prevent transmission. This finding is important and this control measure may be utilized alone or in combination with other measures to reduce influenza virus transmission in sow herds. Moreover, this finding could account for the differences in infection dynamics seen in sow farms in Chapter 3 of this thesis. The impact of maternally derived immunity may differ across herds and different virus types; however, the findings of this study describe the impact of a commonly used control measure for influenza virus in pigs.

Future influenza virus research may focus on controlled, prospective studies that are able to test hypotheses related to influenza virus control and potentially elimination of influenza virus from commercial swine herds. This thesis has identified weaning age pigs as an important subpopulation to which control measures may be targeted. However, there is limited research assessing control measures such as vaccination on influenza virus prevalence and transmission in commercial swine herds. Additionally, improved diagnostic testing strategies and better defined subpopulations for targeted surveillance (such as weaning age pigs) will allow for more accurate influenza virus prevalence and incidence data in commercial herds. This will allow veterinarians and producers to more accurately identify important influenza virus transmission routes and risk factors for infection.

In summary, the findings of this thesis have advanced the understanding of influenza virus transmission and epidemiology in swine. Researchers, veterinarians and swine producers may utilize this information to investigate influenza virus infections within herds and to help mitigate influenza virus infections. This thesis provides science based explanations that help describe the complex epidemiology of influenza virus and generate a path for future research in commercial settings to minimize the impact of influenza virus in pigs and other animal species.

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