Epidemiology of Influenza A viruses of Swine: Surveillance, Airborne Detection and Dissemination

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Dedication

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Research Abstracts

Chapter 2: Swine Influenza Active Surveillance in the United States

Influenza A virus (IAV) in swine continues to be an important swine respiratory agent along with being a source of concern to public health authorities. While veterinary diagnostic laboratories are a valuable source of information with regards to the identification and genetic characterization of newly emerged virus through passive surveillance, there is still a need for additional surveillance programs that can aid in detecting new viruses in a timely manner. An active surveillance program was performed in 32 pig farms throughout the Midwestern United States between June 2009 and December 2011. Thirty nasal swabs were collected from growing pigs on a monthly basis and tested for IAV by RRT-PCR. During sample collection, data on sample collection date, pig age, pig group respiratory signs, clinical status and vaccination history were recorded. A total of 16,170 nasal swabs from 540 groups of growing pigs were collected from which 746 (4.6 %) nasal swabs and 117 (21.7 %) groups tested positive for IAV, respectively. Throughout the study, IAV was consistently detected in at least one farm except in two months. From the positive groups of pigs, H1N1, H1N2, H3N2, 2009 pandemic H1N1were detected in 18%, 16%, 7.6% and 14.5% of the groups, respectively. In seven groups, H1N2 or H3N2 reassortants containing genes from 2009 pandemic H1N1 were found. There were eight groups in which an H1N2 and the 2009 pandemic H1N1 were identified simultaneously. Groups of pigs were more likely to test positive for IAV during the spring and summer seasons compared to the fall. Age and group respiratory clinical signs were not predictors of group IAV status. This active IAV surveillance program provided quality data and increased the understanding of the current situation of circulating viruses in the U.S. pig population. Further studies in swine should be conducted to increase our knowledge regarding the characteristics of IAV.

Chapter 3: Swine influenza virus risk factors in growing pigs

Influenza A virus (IAV) is an important cause of respiratory disease in swine. Understanding the epidemiology of the disease is in its early stages and is needed to develop effective control and prevention strategies. A study was conducted to assess the relationship between the presence of IAV in growing pig farms and farm level risk factors. Twenty-six pig farms participated in the study from which 30 nasal swabs from growing pigs were collected on a monthly basis for 12 or 24 consecutive months between 2009 and 2011. Nasal swabs were tested for IAV by RRT-PCR. Weather stations were located at every participating farm for monitoring temperature, relative humidity, light intensity, wind speed and wind gusts. Farm level data was obtained through a questionnaire to assess the relationship between the presence of IAV and farm level characteristics. At the individual level, 4.6% of the nasal swabs from growing pigs tested positive for IAV. Of the monthly groups of pigs from which nasal swabs were collected, 20.8% had at least one positive nasal swab. Positive nasal swabs originated from 23 of the 26 participating farms. Farm type, pig flow and gilt source were associated with the presence of IAV. Environmental temperature and wind speed were associated with the presence of IAV. Overall, this study provides insights into the ecology of IAV which can aid in the development of control and prevention strategies.

Chapter 4: Prevalence and risk factors for H1N1 and H3N2 influenza A virus infections in Minnesota turkey premises

Influenza virus infections can cause respiratory and systemic disease of variable severity and also result in economic losses for the turkey industry. Several subtypes of influenza can infect turkeys causing diverse clinical signs. Influenza subtypes of swine origin have been diagnosed in turkey premises. However, it is not known how common these infections are nor the likely routes of transmission. We conducted a cross-sectional study to estimate the seroprevalence of influenza viruses in turkeys and examine factors associated with infection on Minnesota turkey premises. Results for influenza diagnostic tests and turkey and pig premises location data were obtained from the Minnesota Poultry Testing Laboratory (MPTL) and the Minnesota Board of Animal Health (MBAH)

respectively from January 2007 to September 2008. Diagnostic data from 356 premises were obtained, of which 17 premises tested positive for antibodies to influenza A virus by agar gel immunodiffusion (AGID) assay and were confirmed as either H1N1 or H3N2 influenza viruses by hemagglutination and neuraminidase inhibition assays. Influenza infection status was associated with proximity to pig premises and flock size. The latter had a sparing effect on influenza status. This study suggests that H1N1 and H3N2 influenza virus infections of turkey premises in Minnesota are an uncommon event. The route of influenza virus transmission could not be determined, however, the findings suggest that airborne transmission should be considered in future studies.

Chapter 5: Characterization of the temporal dynamics of airborne influenza A virus detection in acutely infected pigs

Influenza A viruses infect many species including avians, mammals and humans. Aerosol transmission is one route that enables the virus to infect populations. This study explored the relationship between number of infected pigs and the probability of detecting influenza virus RNA in bioaerosols through the course of an acute infection. Bioaerosols were collected using a cyclonic collector in two groups of seven week-old pigs that were experimentally infected upon exposure with a contact infected pig (seeder pig). After contact exposure, individual pig nasal swab samples were collected daily and air samples were collected three times per day for eight days. All samples were tested for influenza by RRT-PCR targeting the influenza virus matrix gene. All pigs' nasal swabs became influenza virus RRT-PCR positive upon exposure to the infected seeder pig. Airborne influenza was detected in 58% (25/43) of the air samples collected. Temporal dynamics of influenza virus detection in air samples were in close agreement with the nasal shedding pattern in the infected pigs. First detection of positive bioaerosols occurred 2 days post contact (DPC). Positive bioaerosols were consistently detected between 3 and 6 DPC, a time when most pigs were also shedding virus in nasal secretions. Overall, the odds of detecting a positive air sample increased 2.2 times with every additional nasal swab positive pig in the group. In summary, there was a strong relationship between the number of pigs shedding influenza virus in nasal secretions and

the detection of bioaerosols during the course of an acute infection in non-immune population.

Chapter 6: Detection of airborne influenza A virus in experimentally infected pigs with maternally derived antibodies

This study assessed whether recently weaned piglets with maternally derived antibodies were able to generate infectious influenza aerosols. Three groups of piglets were assembled based on the vaccination status of the dam. Sows were either non vaccinated (CTRL) or vaccinated with the same (VAC-HOM) strain or a different (VAC-HET) strain than the one used for challenge. Piglets acquired the maternally derived antibodies by directly suckling colostrum from their respective dams. At weaning, pigs were challenged with influenza virus by direct contact with an infected pig (seeder pig) and clinical signs were evaluated. Air samples, collected using a liquid cyclonic air collector, and individual nasal swabs were collected daily for 10 days from each group and tested by matrix real-time reverse transcriptase polymerase chain reaction (RRT-PCR) assay. Virus isolation and titration were attempted for air samples on Madin-Darby canine kidney (MDCK) cells. All individual pigs from both VAC-HET and CTRL groups tested positive during the study but only one pig in the VAC-HOM group was positive by nasal swab RRT-PCR. Influenza virus could not be detected or isolated from air samples from the VAC-HOM group. Influenza A virus was isolated from 3.2% and 6.4% air samples from both the VAC-HET and CTRL groups, respectively. Positive RRT-PCR air samples were only detected in VAC-HET and CTRL groups on day 7 post-exposure. Overall, this study provides evidence that recently weaned pigs with maternally derived immunity without obvious clinical signs of influenza infection can generate influenza infectious aerosols which is relevant to the transmission and the ecology of influenza virus in pigs.

Chapter 7: Detection of airborne swine influenza A virus in air samples collected inside, outside and downwind from swine barns

Airborne transmission of influenza A virus (IAV) in swine is speculated to be an important route of virus dissemination, but data are scarce. This study attempted to detect airborne IAV by virus isolation and RRT-PCR in air samples under field conditions. This was accomplished by collecting air samples from four acutely infected pig farms and locating air samplers inside the barns, at the external exhaust fans and downwind from the farms and at distances up to 2.1 km. Weather data was also collected to explore the relationship between detection of IAV and temperature, relative humidity and sunlight intensity. IAV was detected in air samples collected in all the farms included in the study. On average, 96% and 85% of the air samples collected inside and at the exhaust fans from positive farms tested positive through RRT-PCR, respectively. Isolation of IAV was possible from air samples collected inside the barn at two of the farms and in one farm from the exhausted air. Influenza virus RNA was detected in air samples collected between 1.5 and 2.1 Km away from the farms. The odds of detecting IAV decreased with distance from the farm and greater levels of sunlight intensity. The results from this study prove evidence of the risk of aerosol transmission in pigs under field conditions and perhaps to other species as well.

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Chapter 1: Literature Review

Influenza Virus Characteristics

Influenza virus belongs to the *Orthomyxoviridae* family of viruses. Within the same family of viruses there are five different genera: influenza virus types A, B and C, *Thogotovirus* and *Isavirus*. Influenza A virus (IAV) is a 100 nm enveloped spherical particle that has a negative-sense single stranded RNA is single-stranded genome. The IAV structure is particular in that it possesses projections on its surface membrane. The virus has a segmented genome with a total of eight genes encoding for 11 different proteins. The encoded proteins are: polymerase B2 (PB2), polymerase B1 (PB1), PB1-F2, polymerase A (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix 1 (M1) and matrix 2 (M2), non-structural 1 (NS1) and non-structural 2 (NS2). The HA and NA surface glycoproteins play a key role during infection since they are the antigenic portions of the virus and at the same time facilitate entry into and exit from the hosts respiratory epithelial cells. These two glycoproteins are used to subtype IAV into 16 HA types and 9 NA types. Viruses are then classified by species from which the isolate originates, location, arbitrary numerical designation, year and subtype, e.g., A/Swine/Iowa/15/1930 H1N1 (Palese and Shaw, 2007).

Due to its phospholipid layer, IAV are labile to lipid solvents and also susceptible to heat inactivation (56°C for 30 minutes) and acidic (pH = 3) compounds as mentioned in one report (Murphy et al., 2008). Successful inactivation of IAV has been reported with several disinfectants including those classified as phenolic, quaternary ammonia, chlorine and peroxigen compounds (Suarez et al., 2003). Furthermore, IAV can be inactivated through the exposure to ultraviolet light which damages its proteins thus hindering the replication process (Jensen, 1964; McDevitt et al., 2012).

Viral evolution

The virus has developed two main mechanisms for genetic evolution by which it can maintain the infection chain within and among populations. These are known as antigenic drift and shift. The former mechanism involves minor mutations in the HA gene

which may allow the virus to escape from the hosts' immune system. Such types of mutations have long been described in the literature (Brown et al., 1997; Olsen et al., 2002). Antigenic shift causes major changes in the genome of the virus and can occur when a single permissive host cell is infected by two different influenza A viruses. These dual infections give the viruses the opportunity to replicate in the same cell. Upon replication and assembly but before budding gene segments from two different viruses, if present in the cell, can be mixed and assembled into a single virion creating a new combination of genes in a process called reassortment. These newly reassorted IAV have commonly been found circulating within pig populations and add a great degree of viral genetic diversity (Ma et al., 2010; Webby et al., 2004). Reassortment is purported to occur readily in swine due to the presence of both α2-3 and α 2-6 receptors in the respiratory tract, thus enabling binding of avian and mammal influenza A viruses, respectively (Ito et al., 1998; Ma et al., 2009). However, the respiratory tract of humans and birds such as quail have both receptor types as well (Shinya and Kawaoka, 2006; Wan and Perez, 2006; Kuchipudi et al., 2009). The presence of both receptor types in certain species gives viruses from different hosts the ability to infect a single species, resulting in an opportunity to reassort, but it also facilitates the movement of a virus across the species barrier (Webster et al., 1992).

Epidemiology

Prevalence

Prevalence studies of IAV around the world have been related to research projects trying to estimate the presence of this pathogen in pig populations. This estimation can be accomplished through the detection of specific anti-influenza antibodies or through the detection of the virus by either isolation or molecular methods. Within each technique there is variation with regards to laboratory methods which have an influence on detection of either antibodies or virus. It is also important to mention that these two methods have different time frames limiting our ability to make objective comparisons, thus, interpretation of prevalence estimations should be done with caution. Because

serologic methods are more common and perhaps less expensive together with the short window of time in which the virus persists in the respiratory system, most studies assessing prevalence have been serology-based.

Antibodies against IAV in swine have been found in many countries around the world highlighting the widespread nature of these organisms. In Europe, a Spanish seroprevalence study that included a total of 98 commercial farms demonstrated 92.9%, 64.3% and 92.9% of farms tested positive for antibodies against influenza A virus subtypes H1N1, H1N2 and H3N2 respectively by hemagglutination inhibition (HI) assays. Interestingly, the proportion of positive sows was higher than growing pigs which could have been due to age acting as a confounder. Furthermore, there were samples in which antibodies to more than one subtype were detected which could suggest either cross reactivity or that pigs were infected by more than one viral subtype (Simon-Grife et al., 2010). Another serological study conducted in Great Britain included 2000 culled sows. Samples from these animals were tested through HI and 59.7% of the sows tested had anti-influenza antibodies against either H1N1 or H3N2 influenza. A total of 277 pigs had antibodies to both H1N1 and H3N2 but more importantly, antibodies to influenza B and C viruses were also found in 8 and 198 sows respectively which is not a common finding (Brown et al., 1995). Recently, another study conducted in 146 English farrow-to-finish farms from which 20 samples were collected and tested through HI identified anti-influenza antibodies in all farms. Similar to the previous study, antibodies to different subtypes were found within the same farm samples suggesting concurrent circulation of viruses (Mastin et al., 2011). Van Reeth, et al. (2008) conducted a seroprevalence study in seven European countries including Belgium, the Czech Republic, Germany, Italy, Ireland, Poland and Spain. A total of 4190 sow serum samples originating from 651 farms with at least 100 sows which had never been vaccinated for influenza were tested by HI. All seven countries tested positive for influenza antibodies. However, the Czech Republic, Ireland and Poland had lower seroprevalence compared to the other participating countries. Overall, the H1N1 subtype was the most widespread followed by

H1N2 and H3N2 subtypes (Van Reeth et al., 2008).

Asian countries have also reported influenza antibodies in pigs. A Chinese study using pooled data analysis from 19 publications estimated that the seroprevalence of H1 and H3 antibodies was 31.1% and 28.6% respectively (Liu et al., 2009). Another Chinese study conducted in Fujian province detected antibodies to H1, H3 and H9 subtypes. In 2007, a total of 25 farms were surveyed and 45.5%, 2.9% and 2.6% of the samples tested positive for H1, H3 and H9 subtypes respectively (Song et al., 2010). In Korea, 14 finishing pig samples per farm were collected from 53 different farms. Antibodies to H1N1, H3N2 and to both viruses were found in 51.2%, 43.7% and 25.3% of the samples, respectively (Jung et al., 2007). A different study was conducted by Pascua et al. in which serum samples submitted for other diagnostics at a university laboratory were tested through HI. Overall, a total of 6418 growing-finishing pig sera were used in this study from which 2959 (46.1%) had antibodies to IAV. It was estimated that 41.5% of the samples had antibodies for H1, whereas only 3.7% had antibodies for H3. However, 0.9% of the samples tested positive for both subtypes (Pascua et al., 2008). Studies in Malaysia (Suriya et al., 2008) and India (Chatterjee et al., 1995) also detected antibodies to both H1N1 and H3N2 viruses in pig populations.

In North America, several influenza seroprevalence studies conducted in the 1970's, 1980's and 1990's in the United States detected antibodies to all three major subtypes (Chambers et al., 1991; Hinshaw et al., 1978; Olsen et al., 2000). The latest prevalence study in the United States was conducted by Choi et al. in which 111,418 samples were submitted to the University of Minnesota Veterinary Diagnostic Laboratory and screened for IAV antibodies by HI. Out of the total number of sera tested, 22.8% had antibodies to H1N1 or H3N2. Sixty six percent of the total number of positive samples accounted for H1N1 infections, whereas the remaining positive samples were due to H3N2. However, authors stressed that detected antibodies could be the result of either natural infection or vaccination (Choi et al., 2002a). In Ontario Canada, 83.1% and

40.3% of the sows and finishing pigs tested, respectively, were positive to H1N1 in a sample of herds. In 2003, only sows were tested for H3N2 antibodies using two strains, A/Swine/Colorado/1/77 and A/Swine/Texas/4199-2/98. Sera to both strains reacted from the study population in which a total of 9.2% and 7.9% of the sows tested positive to the Colorado and Texas strain respectively (Poljak et al., 2008). A similar study in Ontario was conducted in which only finisher pig sera was tested. The study yielded similar results in that antibodies to both viruses were detected. In 2004 the prevalence for H1N1 and H3N2 was 13.4% and 2.7% respectively. The following year, the prevalence for H1N1 increased to 14.9% and for H3N2 the increase was more dramatic since the estimate was 25.9% (Poljak et al., 2008).

In South America, an Argentinean study reported findings from a small scale seroprevalence study involving 13 farrow-to-finish farms. Antibodies to H1N1 and H3N2 were detected in 89% and 73% of the samples respectively. Additionally, 62% of the sera tested positive for both subtypes by HI (Pineyro et al., 2010). A Brazilian study detected antibodies to influenza A H3N2 in 46% of the samples tested by HI from a total of 675 samples (Caron et al., 2010), and in another study conducted in the state of Sao Paulo, 97% of the serum samples had detectable levels of anti-influenza antibodies. Interestingly, 91% of the samples had antibodies to influenza B virus (Mancini et al., 2006). In Colombia, a seroprevalence study conducted in 2008 concluded that 0.8% and 12.9% of the samples tested through ELISA tested positive for IAV H1N1 and H3N2, respectively (Correa et al., 2011).

Besides commercial farms, wild pig populations have serological evidence of IAV infections. A multi-state project was conducted in the United States in which blood samples from the feral swine population were collected in six states. Seropositivity against H3N2 subtype was the most common with all positive samples reacting against this subtype. No positive samples were detected in samples derived from Florida, Missouri and Oklahoma wild pigs and 1% of the samples were positive in Mississippi,

5.3% in California and 14.4% in Texas (Hall et al., 2008). Another study focusing on investigating the prevalence of influenza A in feral swine located in dense commercial pig farm regions in North and South Carolina resulted in higher prevalence levels. Antibodies to three different antigenic variants of H1N1 (human-like H1N1 in 73%), reassortant avian/swine-like H1N1 (7% of the samples), classical-like H1N1 (14% of the samples) and in 47% of the samples H3N2 was detected (Corn et al., 2009). A recent study tested 50 serum samples from wild pigs originating from Arkansas, Louisiana, Oklahoma and Texas generated similar results in that 2% and 40% of the samples were positive for H1 and H3 influenza antibodies, respectively (Baker et al., 2011). In Europe, a study conducted in specific German regions found that 5.2% of wild pigs had antibodies to both H1N1 and H3N2. Two viruses were isolated from samples collected in the same study (Kaden et al., 2008). Overall, these serologic studies demonstrate that IAV circulates among swine populations around the world at different levels.

Besides detection of anti-influenza antibodies, the presence of IAV can also be determined through molecular methods that directly detect the antigen. Nasal swabs are commonly collected for this purpose either for diagnostic or surveillance purposes. These studies may not be directly comparable to the serologic studies mentioned above due to the fact that detection of antibodies in serum can be accomplished for many weeks (Desrosiers et al., 2004) whereas the duration of the virus in respiratory nasal secretions is quite short (Brown, 2000). Data on the prevalence of IAV in swine nasal swabs is scarce and the few reports that are in the literature are related to research studies related to surveillance programs in different areas of the world. In North America, more specifically in the United States, two different studies have been conducted by collecting nasal swabs from market age pigs at the slaughter plants. The first study was conducted in two different slaughter plants located in Tennessee and Wisconsin that received pigs for slaughter from seven different states. Two-hundred randomly selected pigs were selected for nasal swab sampling on a weekly basis for a total of 9,400 samples from which 478 (5.1%) nasal swabs tested positive for IAV (Wright et al., 1992). The second

study followed similar methods in that nasal swabs were collected at a slaughter plant from 1,200 pigs for virus isolation. A total of 26 (2.1%) H1N1 viruses were isolated from these samples (Olsen et al., 2000). Similar studies have been conducted in other parts of the world, for instance, in Hong Kong, tracheal swabs from pigs at a slaughter plant were collected on a monthly basis for a period of over two years. A total of 184 (3.7%) viruses out of 4,957 samples were isolated from pigs without respiratory clinical signs (Peiris et al., 2001). A Taiwanese study conducted a serological surveillance study in 1974 farms from which nasal swabs were only collected in farms where there was evidence of IAV antibodies. A total of 881 nasal swabs were collected for RRT-PCR and virus isolation finding eight RRT-PCR positive swabs and isolating two viruses (Shieh et al., 2008). In South Korea, lung samples were collected in the slaughter house and sent to a diagnostic laboratory for virus isolation. From 532 lung samples, 5 (0.93%) tested positive for IAV through virus isolation. Interestingly, one sample was subtyped as H7N2 which is not a common subtype found in pigs (Kwon et al., 2011).

Overall these studies demonstrate that IAV do circulate ubiquitously in pig populations increasing the probability of maintaining the transmission chain. Additionally, the detection of anti-influenza antibodies may be more practical and cost effective than molecular methods or virus isolation. However, the information provided by serological studies is not as powerful as the information provided by detecting antigen since the complete genetic characterization is not performed.

Risk Factors

There have been several groups attempting to determine the possible farm-level risk factors associated with influenza infections especially with avian influenza in poultry. However, risk factor studies involving swine influenza are scarce.

A study performed in Canada found an association between H1N1 influenza virus seropositive finishing pigs and herd size measured in 1000 finishing pig increments (OR = 4.44; CI 1.9-13.07) and pig farm density (OR = 1.41; CI 1.0-2.04). In the same study,

the presence of finishing pigs only in the farm was a sparing factor (OR = 0.11; CI 0.01-0.62) (Poljak et al., 2008). An association between replacement rate and seropositivity to H1N2 and H3N2 in sows in Spanish herds has also been reported. Furthermore, uncontrolled access to farms was associated with seropositivity to H1N1 and H1N2 in finishing pigs (Simon-Grife et al., 2010). Another risk factor study was conducted in Malaysia where farm size, purchase of pigs and proximity to other pig farms were associated to seropositivity to influenza viruses (Suriya et al., 2008). More recently, a study conducted in England found a higher likelihood of seropositivity between the number of pigs per water space and pigs housed indoors versus outdoors. Pigs housed in straw yards and sampling time (i.e. July-September) were found to be factors that reduced the likelihood of testing seropositive (Mastin et al., 2011).

Loeffen et al. (2009) reported that farrow-to-finish herds had a different infection dynamic than finishing units. Apparently transmission of the virus differs in these two type of units since antibody prevalence was highest at the beginning of the finishing phase in farrow-to-finish farms whereas the highest seroprevalence in finishing units was highest at the end of the finishing phase suggesting that farm type could also be a risk factor (Loeffen et al., 2009).

Transmission

Transmission of influenza virus in pigs is mainly through nose-to-nose contact, exposure to nasal secretions from infected individuals and aerosolized droplets (Brown, 2000). Pigs shed virus in nasal secretions one day after infection and the viral load in these secretions gradually increases and then decreases until shedding stops approximately 5 to 7 days post infection (Van Reeth et al., 2003). Direct contact transmission in pigs was first demonstrated in the 1930's (Shope, 1931). However, there is an increasing body of scientific literature highlighting the importance of influenza aerosol transmission in influenza infections (Tellier, 2009). It has been suggested in epidemiological studies that farm density plays a role in the airborne transmission IAV

(Maes et al., 2000; Poljak et al., 2008).

Aerosol transmission has been suspected (Desrosiers et al., 2004; Mohan et al., 1981) but has not been proven. Recently, airborne detection of IAV from pigs has been reported in a vaccine efficacy study (Loeffen et al., 2011) supporting aerosol or airborne as a possible route for virus dissemination. Airborne transmission of IAV has also been reported in humans, (Blachere et al., 2009; Brankston et al., 2007; Tellier, 2009) mice, guinea pigs, ferrets and chickens (Mubareka et al., 2009; Munster et al., 2009; Schulman, 1967; Yao et al., 2011; Yee et al., 2009). Recently, two studies provided insights into an outbreak of equine IAV in which the airborne route seemed to play an important role on virus dissemination and there was a relationship with specific environmental conditions (Davis et al., 2009; Firestone et al., 2012). Furthermore, the transmission of influenza in a guinea pig model provided evidence that airborne transmission may depend on climatic conditions. Transmission occurred when the relative humidity (RH) was 20 % and 35 % and the temperature was 20°C. When the temperature was 5°C and the RH was 35 % and 50 % transmission occurred efficiently but at RH of 65 % and 80% transmission was less efficient. In the same report, researchers found that guinea pigs housed at low temperatures shed virus for a longer period of time compared to those housed at warmer temperatures (Lowen et al., 2007). Recently, another study using the guinea pig model provided evidence that aerosol transmission will not occur when the temperature is 30°C compared to temperatures of 20°C suggesting that at high temperatures transmission may only occur through the direct contact route (Lowen et al., 2008). Additionally, aerosol transmission seems to be influenced by genetic components of the virus in that there are reports showing that viruses with different genetic compositions in specific sites in the PB2 gene are more likely to transmit through the airborne route than others (Steel et al., 2009).

Another indirect route of transmission that has been suspected is through exposure to contaminated water (Karasin et al., 2000; Karasin et al., 2004). Migratory birds are

known to carry the virus in their intestines and when landing in ponds or lakes they can discharge great quantities of viral particles in the water becoming a source of virus (Hinshaw et al., 1979).

Transmission through vectors such as insects has been reported. Avian influenza H5N1 has been isolated from insects in and around poultry farms after an epidemic of disease (Barbazan et al., 2008; Sawabe et al., 2006; Sawabe et al., 2009). In pigs, IAV does not seem to enter the bloodstream which may reduce the likelihood of this occurring despite the fact that virus can occasionally be detected in blood samples (Romijn et al., 1989).

Environmental Survival

Information about the survival of IAV outside the host is crucial for the understanding of the transmission of this agent. Presently there is scarce data on the survival of the virus outside a host and the available data has been generated through diverse experimental studies. The persistence of avian influenza viruses has been studied and has been limited to avian IAV based on the fact that waterfowl are the reservoir for IAV (Stallknecht et al., 1990; Webster et al., 1992). Survival of IAV in water increased with decreasing water temperature. The virus survived for 207 days when the water temperature was 17°C compared to 102 days when water temperature was 28°C (Stallknecht et al., 1990b). Water characteristics (pH and salinity) together with temperature were also evaluated and it was concluded that the virus survived for longer periods at low temperature and salinity and high pH (Stallknecht et al., 1990a; Brown et al., 2007; Brown et al., 2009).

Survival of the virus on different surfaces has been studied and provides a guide for fomite transmission of IAV at the farm level. One study concluded that the virus survives more efficiently on non-porous (plastic, stainless steel) compared to porous materials (pajamas, tissues, handkerchiefs, magazines) (Bean et al., 1982; Tiwari et al., 2006). A

similar IAV survival study was conducted using banknotes (i.e. money bills) in which different concentrations of human influenza viruses including type A and B were placed on banknotes. The study concluded that survival of the virus depended on the concentration and the environment (presence or absence of mucus) (Thomas et al., 2008) which provides key information for understanding the indirect transmission of the virus. Additionally, there has been data suggesting that the survival of the virus on the aerosols is related to exposure to ultra-violet light which can also play a role when the virus is placed on different surfaces that are exposed to this type of light (McDevitt et al., 2012).

Even though the importance of the survival of IAV in slurry is well understood in the control of avian IAV (Webster et al., 1992), it is not fully understood in pigs since the pig does not shed the virus in feces. However, spread of manure is a common practice in the United States during the fall season and has been suspected as one mechanism that could have helped the virus spread (Desrosiers et al., 2004). Additionally, Haas, et al. reported that influenza virus can survive in slurry for as long as 9 weeks if maintained at 5°C and 2 weeks if maintained at 20°C (Haas et al., 1995).

Clinical signs

Clinical signs due to IAV infections in swine appear suddenly and can be variable. Normally, coughing, sneezing, nasal discharge, anorexia, pyrexia and lethargy are the major signs in infected pigs. The virus can infect most of the pigs in a group but mortality is generally low and may also depend on the presence of other pathogens in the herd that can act as secondary / opportunistic invaders. It has been reported that clinical signs can also be related to virus-related pathogenicity which can vary among strains within the same subtype (Vincent et al., 2006; Vincent et al., 2008). Experimental infections with IAV have resulted in the absence of clinical signs (Van Reeth, 2007) suggesting great variability in viral pathogenicity. In addition to the major respiratory signs typically described, reduced reproductive performance, agalactia, diarrhea in piglets (Moreno et al., 2010) edematous eyelids, and conjunctivitis (Howden et al., 2009; Sreta et al., 2010)

have also been reported in herds infected with the 2009 pandemic strain H1N1 (Moreno et al., 2010).

When IAV is part of a co-infection, clinical signs are evident (Thacker et al., 2001; Van Reeth et al., 1996) which may reflect the health status of the groups of pigs used in this study. Abortions have been occasionally reported after the introduction of a virus into a breeding herd (Karasin et al., 2000). However, absence of clinical signs in IAV infected pigs has been reported which has important public health implications. The roots of this phenomenon are not fully understood. However, this can be the result of a combination of events such as the presence of antibodies conferred by colostrum (e.g. maternal immunity), vaccination or previous exposure and low viral exposure which may preclude pigs from exhibiting clinical signs (Thacker and Janke, 2008; Van Reeth et al., 1999; Van Reeth, 2000; Van Reeth, 2000; Van Reeth, 2007).

Pathogenesis

Once influenza virus enters the swine respiratory system, it binds to the epithelium of the bronchi and bronchioles, is engulfed and starts to replicate, stimulating inflammation and eventually causing respiratory epithelial cell death (Olsen et al., 2006). Microscopically, the damaged epithelium is accompanied by infiltration of inflammatory cells in both bronchi and bronchioles. Such infiltration of inflammatory cells increases and peaks at days 5 and 7 post infection (Jo et al., 2007). Due to the inflammatory process and cell debris, there is obstruction of the airway which causes labored breathing (Jung et al., 2005). All these processes are related to a cascade caused by pro-inflammatory cytokines which can be detected in broncho-alveolar lavage fluids (BALF). Lung viral titers have been positively correlated with interferon alpha (IFN-α), interleukin (IL)-1, IL-6, IL-12 and tumor necrosis factor alpha (TNF-α) (Barbe et al., 2011; Van Reeth et al., 1999; Van Reeth et al., 2002a; Van Reeth et al., 2002b). Besides the inflammatory response, these cytokines will have an indirect metabolic impact by accelerating muscle protein degradation, inhibiting anabolic effects of insulin, increase

lipolysis together with pyrexia and anorexia (Johnson, 1997). It has been reported that not all strains of IAV have the same pathogenic effect in pigs and there is strain variability within subtype suggesting that some strains may be more pathogenic than others (Vincent et al., 2006).

Diagnosis

Diagnosis of swine influenza can be performed by either a presumptive clinical diagnosis or through diagnostic tests. Clinical diagnosis may not be accurate since respiratory clinical signs are non-specific and especially when there are subclinical infections. However, the clinical picture can aid a definitive diagnosis once samples and diagnostic tests are performed.

Diagnostic tests can be used in a direct (antigen) or indirect (antibodies) manner. The former can be performed ante or post-mortem by collecting the appropriate samples. Ante-mortem diagnosis of IAV can be performed by collection of either nasal secretions or oral fluids through the use of nasal swabs from the nasal cavity or cotton ropes, respectively. Once the sample is collected, either detection of virus RNA by reverse transcription polymerase chain reaction (RT-PCR) or virus isolation (VI) can be performed.

The commonly used RRT-PCR for the detection of influenza A virus in mammals targets the highly conserved matrix gene. The advantage of this technique is that it can be performed within hours and at a relatively lower cost than VI. Additionally, RRT-PCR tests have been developed to subtype different HA and NA genes and have proven to be more sensitive than VI. This technique does not require the presence of viable virus to yield positive results and can detect as few as 10^{-1} tissue culture infectious dose 50 per ml ($TCID_{50/ml}$) (Choi et al., 2002b; Landolt et al., 2005; Richt et al., 2004). Recently, a new technique has allowed samples collected in different areas of the world to be shipped internationally without any risk of disease introduction. This consists of a cotton-based

membrane on which the sample is placed and reagents inactivate the microorganisms in the sample. The technology is known as Flinders Technology Associates filter paper (FTA® card). Such technology has been used for the detection of avian IAV (Abdelwhab et al., 2011; Kraus et al., 2011).

Virus isolation has been routinely performed in embryonated chicken egg (ECE) and cell cultures such as Madin-Darby Canine Kidney cells (MDCK). Comparisons between these two methods for VI have concluded that ECE has higher sensitivity than the MDCK method for swine influenza viruses (Clavijo et al., 2002; Swenson et al., 2001). However both methods are still being used widely. Furthermore, the literature reports that not all viruses are isolated in a certain cell line, suggesting there may exist strain-cell line growth viral phenotypes which indicate why more than one virus isolation method should be used (Landolt et al., 2005).

The second way by which influenza infection can be diagnosed is through serology. Among the serologic tests available, indirect fluorescent antibody test (IFAT), serum neutralization (SN), hemagglutination inhibition (HI) and enzyme-linked immunosorbent assay (ELISA) are currently being used for swine. These tests (HI and ELISA) can detect immunoglobulin G (IgG) and M (IgM) (Skibbe et al., 2004) and are reference tests for influenza in swine. Comparisons between these two tests have been able to prove a slight superiority from a sensitivity standpoint of HI over ELISA (Leuwerke et al., 2008; Skibbe et al., 2004; Yoon et al., 2004).

Histopathology would be the third way in which a diagnosis can be performed. This technique may be used as a confirmatory test after serology and molecular techniques have been used. Immunohistochemistry (IHC) detects the virus in the respiratory epithelium and has been proven to be a reliable technique for the diagnosis of influenza infections (Haines et al., 1993; Vincent et al., 1997) but fixation conditions may affect the specificity and sensitivity of the IHC test.

Prevention and control

Preventive and control measures for IAV infections are based on biosecurity and vaccination practices. Due to the fact that the epidemiology of IAV is in its early stages perhaps not all measures are being adopted to reduce or avoid transmission. The introduction of animals into breeding herds has become an important topic since new pathogen introductions can come through this route. Replacement breeding stock is placed in areas known as gilts developer units for disease monitoring and to meet the quarantine requirements of the receiving farm. Once these animals have been isolated and their vaccination program completed, they are allowed to enter the farm.

There are no reported anti-viral treatments for IAV infection in swine. Most of the scientific literature regarding prevention is related to vaccination. Presently, there are only inactivated vaccines that are commercially available for swine and which are aimed at preventing disease but not infection (Olsen et al., 2006). Such vaccines have been licensed for use in sows and pigs three to five weeks or older depending on the manufacturer. Usually IAV vaccines will contain more than one to stimulate cross-protection. Inactivated whole virus vaccines stimulate the production of antibodies against the HA surface protein. These anti-HA antibodies are aimed at blocking the attachment of the virion to respiratory epithelial cells (Ma and Richt, 2010). The concentration of the antigen per dose, the adjuvant and the percent homology between the strain in the vaccine and the challenge strain partly determine the efficacy of vaccines (Kyriakis et al., 2010). Vaccinated pigs subsequently challenged with a strain homologous to the vaccine can be fully protected. However, heterologous challenge may induce some cross-protection and reduced viral shedding as has been reported in the literature (Kyriakis et al., 2010; Van Reeth et al., 2003; Vincent et al., 2010). Recently, the impact of vaccination on the transmission of IAV in pigs was assessed in an experimental setting. Vaccinated pigs were able to reduce the speed by which the virus spread throughout the population (Romagosa et al., 2011a).

Influenza vaccination practices have been most popular in swine breeding herds based on data generated through a United States Department of Agriculture (USDA) - National Animal Health Monitoring System (NAHMS) survey (NAHMS, 2006). Such immunization practices are aimed at increasing the immunity in the breeding herd with the secondary aim of providing maternally derived antibodies (MDA) to their offspring. A report in the literature concluded that MDA may have a negative impact on piglet vaccination for influenza virus since the antigen in the vaccine will not be processed by the piglets' immune system causing interference (Kitikoon et al., 2006). Another study found certain protection of MDA against major clinical features of the disease and at the same time stated that such circulation of antibodies can be the reason why there are sub-clinical infections (Loeffen et al., 2003).

Vaccination of growing pigs is not a common practice in the North American swine industry (NAHMS, 2006). Information on influenza vaccination efficacy field trials is scarce but needed. In 2010, a study evaluated the effect of an inactivated bi-valent vaccine on growth performance in two cohorts in a multi-site farm with a clinical history of respiratory disease due to influenza. Unfortunately, the study was confounded by an outbreak of porcine circovirus associated diseases (PCVAD) that occurred during the study. Vaccinated pigs grew more quickly than non-vaccinates in one of the two cohorts leading to inconclusive results (Poljak et al., 2010). A similar situation was assessed and somewhat agreed with the previous report in which the efficacy of an influenza vaccine in pigs infected with porcine reproductive and respiratory syndrome virus had poor protection suggesting that concomitant diseases may have an impact on influenza vaccine efficacy (Kitikoon et al., 2009). There have been reports with other types of vaccines such as live-attenuated, baculovirus-derived, vectored, virus-like particle and plasmid DNA vaccines which are still on the developmental stages (Ma and Richt, 2010; Macklin et al., 1998).

Elimination of influenza viruses from pig farms has been recently reported which provides promising data for control strategies. Torremorell et al. (2009) eliminated an H3N2 from a Mexican multi-site pig farm through herd closure in the breeding herd and partial depopulation of the nursery site. A positive impact was observed in performance since a decrease in nursery mortality was seen together with increased growth, decrease in feed conversion and reduced medication costs (Torremorell et al., 2009).

Surveillance

Infectious disease surveillance is defined as: " …an active, ongoing, formal, and systematic process aimed at early detection of a specific disease or agent in a population or early prediction of elevated risk of a population acquiring an infectious disease, with a pre-specified action that would follow the detection of disease"(Thurmond, 2003). Surveillance is a key component especially when emerging zoonotic diseases are taken into account. However, efforts to integrate surveillance in both humans and animals should be addressed to be able to act in a timely manner (Kuiken et al., 2005) so that measures to manage risks are in place for preventing health related disasters.

Surveillance can be divided in two different categories depending on how samples are collected; passive and active (Pfeiffer, 2010). Passive surveillance describes the method by which diagnostic laboratory data is analyzed for increase in diagnosis of a certain pathogen. This type of surveillance depends on the willingness and frequency by which veterinarians or owners submit their cases for investigation. It is an inexpensive procedure but at the same time it only takes into account cases with clinical presentation leaving subclinical cases underdiagnosed. On the other hand, active surveillance describes the method by which a systematic approach is created for sample collection and diagnosis regardless of clinical manifestations. The disadvantage with this surveillance system is that is costly since creating a sampling network will demand important economic resources (Salman, 2003).

With the objective of allocating resources efficiently, a similar approach to active surveillance has emerged recently in which high risk sub-populations for a certain condition are sampled. The theory behind this new approach is based on the high probability that these sub-populations have for acquiring such condition. This new approach has been referred to as risk based surveillance (Stark et al., 2006).

In pigs, surveillance can be conducted by collection of different samples such as blood, nasal or oral fluid samples. The latter sample type has become an important tool for animal disease surveillance in that it is a fast and non-invasive way of assessing the disease status of a pig population (Ramirez et al., 2012).

In summary, there is a great deal of information regarding IAV in the literature that applies mostly to the diagnostic properties and pathogenesis of the virus in swine. Such data has enabled researchers to continue the process of understanding how this virus behaves in the pig. However, there is still a significant gap on the understanding of the epidemiology of this disease especially in the field and research should focus on this area to start generating hypotheses and management procedures that can aid in the reduction of disease transmission within the herd and between herds. Therefore, studies focused on the ecology and transmission of this virus not only from a herd standpoint but from a regional standpoint will provide insight into the control and prevention of IAV in swine populations.

Chapter 2: Influenza active surveillance in Swine in the United States

Introduction

During the last decades, influenza A virus (IAV) has become an important pathogen due to epidemics of respiratory disease causing not only an increase in mortality but also raising public health organizations alarms regarding the need for further understanding and control of this organism (Salomon and Webster, 2009). Additionally, the ability of the virus to cross species barriers has raised further concern in that the probability of reassortment and generation of highly transmissible viruses are a constant threat to mankind (Donatelli et al., 2003; Webster et al., 1992; Yassine et al., 2010).

Despite evidence in other species, swine have been labeled as the "mixing vessel" since both avian and mammalian type receptors for IAV have been found in the pigs' trachea making it a potential source of new viruses through reassortment (Ito et al., 1998; Ma et al., 2009). Humans can become infected with influenza viruses originating from swine which represents a constant threat to public health (Myers et al., 2006; Myers et al., 2007). Three major subtypes (H1N1, H1N2, H3N2) of IAV circulate in the pig population causing widespread respiratory disease characterized by dry coughing, sneezing, fever, anorexia, rhinorrhea and lethargy (Brown, 2000; Olsen et al., 2006) .

In the United States (US), the disease in swine has been present for almost a century (Koen, 1919). Monitoring of influenza viruses has been conducted through seroprevalence studies in the 70's (Hinshaw et al., 1978) and 80's (Chambers et al., 1991). These studies revealed that H1N1 viruses were the predominant circulating subtype in the swine population. In 1997-1998, another large scale seroprevalence study was performed in which H1N1 viruses continued to predominate. However, the proportion of pigs with antibodies to H3N2 increased from 1.1% to 8.0% (Olsen et al., 2000).

At the end of 1998, new H3N2 viruses emerged in the swine population in North

Carolina, Texas, Iowa and Minnesota as a result of either a double or triple reassortment between human, avian and swine viruses (Zhou et al., 1999). A US serological survey was being conducted approximately at the same time this new virus emerged. The survey found that the US swine population from the 23 participating states had been exposed to these newly emerged H3N2 viruses since 20.5% tested positive for H3N2. Additionally, 28.3% of the samples tested positive for H1N1, but most interesting was the fact that 9.8% of the samples had antibodies to both H1N1 and H3N2 suggesting that both subtypes may have been circulating simultaneously or sequentially in the pig population (Webby et al., 2000). Between 1999 and 2001, a new influenza virus subtype (H1N2) was detected in the US swine population after outbreaks of respiratory disease in different states were reported (Karasin et al., 2000; Karasin et al., 2002). Ever since, circulating influenza viruses in pigs have been able to change due to mutations and reassortment further adding more diversity (Webby et al., 2004). All these studies provided valuable information into the understanding of the disease but still the epidemiology of the disease was not fully understood.

Detection of newly emerged pathogens can be performed through passive or active surveillance. The former is driven by laboratory submission of samples after outbreaks of respiratory disease whereas the active surveillance method is based on purposely collecting and screening field samples regardless of the clinical status. There are reports of active surveillance for influenza through collection of nasal swabs at slaughter plants in Asia detecting uncommon (i.e. H3N1, H7N2, H9N2) circulating viruses in the local pig population (Kwon et al., 2011; Peiris et al., 2001; Shieh et al., 2008). In the US, similar studies following the same sample collection methodology have been reported during the early and late 90's (Olsen et al., 2000; Wright et al., 1992). However, there is still need for a surveillance program that will identify newly emerged viruses (Webby et al., 2004).

Overall, these studies have elucidated epidemiologic traits of the virus in swine

such as the constant circulation of IAV in the swine population and the sporadic infections with rare subtypes. However, there has not been a proactive approach for influenza leaving a gap that needs to be filled (Butler, 2012; Guan et al., 2012). Therefore, the objectives of this study were to 1) conduct an active surveillance program to better characterize the presence of influenza viruses in the swine population and, 2) make available live viruses for genetic characterization, potential vaccine and diagnostic use.

Methods

Farm selection

Thirty-three mid-western US pig farms were conveniently selected by contacting swine practitioners. Veterinarians that agreed to participate in the study were asked to enroll growing pig farms that were representative of modern swine production systems and whose owners were interested in participating in the study. Farms were visited once the producer agreed to participate in the study. The producer was given the option to withdraw from the project at any point throughout the study.

Farm visit frequency, pig selection and sample collection

Participating farms were visited every month for 12 to 24 four consecutive months. Upon arrival at the farm, the investigator would meet with the farm manager/owner to decide which group of pigs was to be sampled. If there was only one age group, samples were collected from such group whereas if there were more than one age group, samples would be collected from the age group that would be closest to 10-weeks of age based on previous reports (Beaudoin et al., 2012).

A total of 30 nasal swabs were collected allowing us to be 95% confident of detecting at least one positive sample when the prevalence of disease was at least 10%. Pigs were restrained by a snare and a nasal swab (Starswab II, Starplex Scientific Inc. Etiobiocke, Ontario, Canada) was inserted two to three inches into the back of each

nostril with rotating movements. Nasal swabs were labeled with a specific code containing the farm identification number, month of the year, two letter state abbreviations and the nasal swab number to maintain confidentiality.

During the visit, the age of the pigs and group respiratory clinical signs (absence or presence of sneezing, coughing and nasal secretion) were recorded. Both nasal swabs and submission sheet were placed into a Styrofoam container with ice-packs and shipped overnight to the laboratory for testing.

Nasal swab testing, subtyping

All nasal swabs collected during the project were tested at the virology department laboratory of St. Jude Children's research hospital (Memphis, TN, USA). Nasal swabs were initially screened for influenza A virus by real-time reverse transcription (RRT-PCR) targeting the matrix gene. Positive RRT-PCR samples underwent further diagnostics for subtype determination including the 2009 A H1N1 pandemic virus and swine H1 and H3 viruses (Ducatez et al., 2011; Richt et al., 2004; World Health Organization, 2009).

Statistical analyses

A group of pigs was defined as the 30 pigs that were sampled in a given month and would be considered positive if at least one positive swab was detected. Descriptive statistics were performed on measured variables. Data were analyzed by repeated measures logistic regression. Model building was performed by first screening independent variables through univariate analysis. Variables with a *P*-value below 0.25 were retained for the multivariable model. All selected variables were forced in the model including two-way interactions and were sequentially removed if the P-value was greater than 0.05.

Two different models were built. The first model assessed the relationships between

the group status (positive vs. negative) for influenza virus and age, year, clinical signs and season. The second model assessed the relationships between the presence of respiratory clinical signs (presence vs. absence) with subtype and season. Season was included in the models by categorizing the four seasons by including the three full months in each season as follows: Winter (January, February, March), Spring (April, May, June), Summer (July, August, September), Fall (October, November, December). Clustering of groups by farm was accounted for by including farm as a random effect and month was included as the repeated measure. Statistical procedures were performed in SAS 9.2 (SAS Institute Inc., Cary, NC, USA).

Results

Producers who agreed to participate were located throughout the mid-western US including the states of Illinois, Indiana, Iowa and Minnesota. Farms were enrolled in the program as veterinarians and producers agreed to participate, thus, the program started in June 2009 and concluded in December 2011.

Thirty-three farms were initially enrolled in the project from which 17 were located in Iowa, 4 in Illinois, 8 in Indiana and 4 in Minnesota. All farms except one were visited at least one time during the project. On August 2009, a group of 7 farms in Iowa withdrew from the program due to financial constraints following the 2009 pandemic H1N1 (2009pH1N1) related swine industry crisis.

Nasal swab results

A total of 16,170 nasal swabs from 540 groups of growing pigs originating from 32 farms were collected. From the total number of nasal swabs collected, 746 (4.6 %) nasal swabs tested IAV RRT-PCR positive and 178 viruses were isolated out of these samples. At least one positive swab was detected in 116 (21.7%) groups of pigs and thus, these groups were classified as positive. Twenty-nine (90.6%) farms out of the 32 participating farms had at least one positive group throughout the study.

Attempts to characterize the viruses either by subtyping or sequencing were done on an ad hoc basis and dependent on limited available resources. Therefore detailed characterization for all viruses detected is not available, yet all viruses have been archived for future studies. From the 117 groups that tested RRT-PCR IAV positive between June 2009 and December 2011, complete or partial subtype details were obtained for 99 (84.6%) pig groups. Single subtype IAV infection with H1N1, H1N2, H3N2 or 2009pH1N1 was detected in 21, 19, 9 and 17 groups, respectively. In seven groups, reassortant viruses were found; e.g. the matrix gene from the 2009pH1N1 strain was detected in five H1N2 and two H3N2 viruses. Dual infections were detected in ten groups in which eight were concurrently infected with H1N2 and 2009pH1N1 viruses, one group harbored an H1N1 plus an H3N-untypable virus and the remaining group was dually infected with an H1N1 and an H1N-untypable virus. Partial subtyping information was obtained for 16 pig groups from which 11, 4 and 1 were infected with an H1N-untypable, H3N-untypable and H1N-untypable with pandemic matrix gene, respectively. In 18 groups, a subtype could not be defined either through RRT-PCR or sequencing. Viruses were isolated from 178 swabs that originated from 62 pig groups.

The mean number of IAV RRT-PCR positive swabs per positive pig group was 6.4 and the median was 2. The number of positive swabs ranged from 1 to 30, with most groups (n = 48) having 1 positive swab. There were 15, 10, 4 and 2 groups that had 2, 3, 4, and 5 positive swabs. There were 13 groups that had more than 20 positive swabs with two having 29 and once having 30 (Figure 2.1).

The number of positive groups per farm ranged from 1 to 18. In average, 31 % of the groups tested by farm throughout the program were classified as positive (Figure 2.2). Farms that did not test positive for IAV were monitored for approximately one year but lacked consistency in the testing frequency and time intervals between tests.

The average age of the pigs in the 540 groups was 13.7 weeks with a SD of 5.7 weeks. Influenza virus was detected in pigs as young as 4 weeks and as old as 30-32 weeks of age. Positive groups had a mean age of 12.4 weeks with a SD of 5.2 and negative groups had a mean age of 13.9 weeks with a SD of 5.8 (Figure 2.3).

Clinical signs

Respiratory clinical signs were observed in 187 (34.6 %) out of 540 groups. From the groups that were reported to exhibit clinical signs, 43 (22.9 %) tested positive for influenza A virus. On the other hand, from the groups of pigs that had no clinical signs (n=353), 74 (20.6 %) tested positive for influenza (Table 2.1).

Season

The number (proportion) of groups sampled in each season throughout the study was similar with winter, spring, summer and fall having 124 (22.9 %), 137 (25.4 %), 150 (27.8 %) and 129 (23.9 %) respectively (Table 2.2).

Logistic regression

In the first model, all variables (age, season and year) except clinical signs had a P-value below 0.25 in the univariate analysis (Table 2.3). Two variables were retained in the multivariable model: season and age. Groups of pigs tested during the spring and summer had 2 (95% CI 1.1, 3.8) and 1.9 (95% CI 1.0, 3.5) times higher odds of testing positive for IAV, respectively, when compared to pigs tested during the fall. There was no association between the winter season and influenza virus detection. Age was not significantly (P = 0.09) associated with IAV group status. However, it was left in the model due to confounding.

In the second model, a total of 81 groups of pigs that had complete subtype information were used for model building. Neither subtype nor season had a significant relationship with the presence of clinical signs.

Discussion

Surveillance will continue to be an important tool for infectious disease epidemiology since it will provide data that will aid in the understanding of the determinants of disease allowing scientists and practitioners to work towards generation of disease prevention and control strategies (Salman, 2003). The contribution made by surveillance studies is imperative when not only zoonotic but also emerging pathogens are a threat for humans (Kuiken et al., 2005) . This holds true for IAV in swine since through surveillance programs, emerging viruses have been identified circulating in pig populations (Fouchier et al., 2003; Kwon et al., 2011; Peiris et al., 2001).

Results from this study agree with what has been previously published (Kwon et al., 2011; Olsen et al., 2000; Peiris et al., 2001; Shieh et al., 2008; Wright et al., 1992) in that IAV detection rate either through virus isolation or RRT-PCR on individual nasal swabs was low (e.g. $\leq 5\%$), presenting a challenge for surveillance programs. However, new sample collection techniques in swine such as the collection of pen-based oral fluids (e.g. saliva) for antibody and antigen detection are gaining importance due to practicality and lower testing costs (Prickett et al., 2008; Prickett and Zimmerman, 2010). Oral fluids are collected by hanging cotton ropes and allowing pigs to chew on them for 10 to 30 minutes saturating the cotton with oral fluids which are then transferred to test tubes and shipped to the diagnostic laboratory for testing. This sample, originates from an important number of pigs in the pen increasing the probability of detection making it a suitable and essential tool for population surveillance (Ramirez et al., 2012; Romagosa et al., 2011b). Through oral fluid sampling of clinically healthy populations, asymptomatic carriers of IAV can be detected more efficiently than through nasal swab sampling.

The absence of respiratory clinical signs in groups of pigs harboring IAV in our study is notable. This can be the result of a combination of events such as the presence of antibodies conferred by colostrum ingestion (e.g. maternal immunity), vaccination or

previous exposure, and low viral exposure which may preclude pigs from exhibiting clinical signs (Thacker and Janke, 2008; Van Reeth et al., 1999; Van Reeth, 2000; Van Reeth, 2007). Subclinical infections in pigs have important public health implications due to the fact that humans can come in contact with apparently healthy pigs and thus become infected (Gray et al., 2007; Killian et al., 2012; Myers et al., 2006; Myers et al., 2007; Newman et al., 2008; Olsen et al., 2002). Subclinical infections are perhaps one of the most common routes for IAV to enter a pig farm since replacement breeding stock or recently weaned animals are constantly moved within and between states and countries. In fact, there is evidence that movement of pigs may have been the cause of dissemination of certain virus lineages of viruses within the US and subclinical infections may have played a role (Nelson et al., 2011).

It has been reported that pathogenicity can vary among strains within the same subtype (Vincent et al., 2006; Vincent et al., 2008) Certain IAV experimental infections have resulted in the absence of clinical signs (Van Reeth, 2007) suggesting great variability. When IAV is part of a co-infection, clinical signs are evident (Thacker et al., 2001; Van Reeth et al., 1996) which may reflect the health status situation of the groups of pigs used in this study since the likelihood of co-infections in the field was high. However, the lack of association between subtype and clinical signs in the present study is difficult to explain. More studies are needed to further understand the role of subtype on the presence of respiratory signs.

Our study demonstrated that IAV is present in growing pigs throughout the year and that groups of pigs were more likely to test positive during the spring and summer compared to the fall season. This finding is contrary to what has been suggested in the past (Brown, 2000). It is important to mention that the suggested seasonal trend could have been based on presence of clinical signs that appear during a time of the year where there are other factors present (e.g. cold weather, bad air quality inside barns, co-infections) (Brown, 2000; Olsen et al., 2006; Straub, 1994) which made veterinarians

submit samples to diagnostic laboratories for the detection of IAV. However, as mentioned earlier, subclinical infections may have played a role in that the presence of disease may have been overlooked during warm months misleading the interpretation of the information available at that time. Another possible explanation for finding more IAV positive groups in the spring and summer is the increase in the finishers of pigs born to gilts during these seasons. This increase in potentially more susceptible growing pigs is a result of increased breeding of gilts during the previous four months in an attempt to achieve breeding targets. The breeding target concept is a management tool used to increase the productivity of breeding herds in that for a constant weekly production of weanling pigs, producers have to breed a certain number of females including replacement gilts and sows that have recently weaned their litters. During the summer months, there is a phenomenon known as seasonal infertility in which female breeding pigs fail to come into estrus. This phenomenon may be managed by breeding more replacement gilts than usual to meet their production targets (Leman, 1992). The window of time between conception and weaning is approximately 137 days (gestation - 116 days and lactation - 21 days), thus, more pigs from gilt litters which are thought to have lower levels of antibodies will be weaned during the winter and will be growing throughout the spring and summer seasons which may provide a source of susceptible individuals for viruses to circulate during these two seasons. However, this needs to be further investigated.

The results from the present study contribute to a better understanding of the epidemiology of IAV in swine by highlighting three different aspects: 1) different influenza viruses circulate simultaneously within pig populations; 2) influenza is present in different pig age populations at a rather low prevalence throughout the year; and 3) subclinical infections are frequent among groups of pigs. More studies are needed to continue to understand IAV.

Table 2.1. Contingency table summarizing swine influenza virus group status and presence or absence of respiratory clinical signs for each of the 540 groups of growing pigs tested between June 2009 and December 2011.

	Influenza Positive	Influenza Negative	Total
Presence of Clinical Signs	43	144	187
Absence of Clinical Signs	74	279	353
Total	116	424	540

Table 2.2. Summary of the number of pig groups tested and status for influenza virus in each season between June 2009 and December 2011.

	Winter	Spring	Summer	Fall	Total
Positive	19	37	40	21	116
Negative	105	100	110	108	425
Total	124	137	150	129	540

Table 2.3. Logistic regression, repeated measures univariate analysis for the relationship between group influenza status with age, season and year in pigs tested for influenza between June 2009 and December 2011.

Variable	Odds Ratio (95% CI)	*P*- value
Season		
- Fall (Referent)	- -	- -
- Spring	1.9 (1.0, 3.5)	0.02
- Summer	1.8 (1.0, 3.4)	0.04
- Winter	0.9 (0.4, 1.7)	0.76
Age	0.9 (0.9, 1.0)	0.10
Year		
- 2011 (Referent)	- -	- -
- 2010	0.6 (0.4, 1.0)	0.09
- 2009	0.9 (0.4, 1.8)	0.91

Figure 2.1. Frequency distribution of number of positive nasal swabs per group for influenza A virus in 540 groups of pigs tested between June 2009 and December 2011.

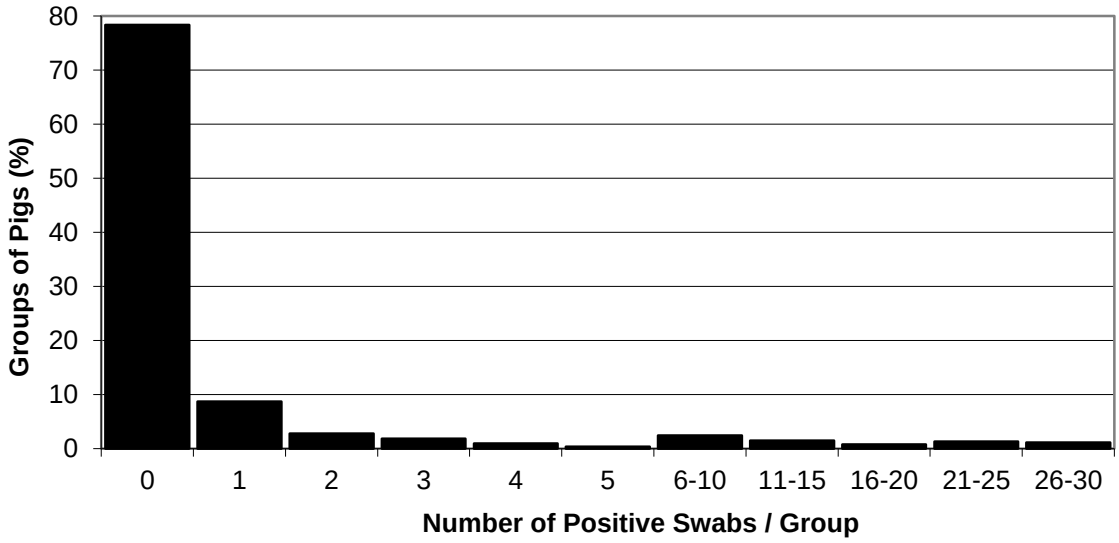


Figure 2.2. Number of influenza A virus positive and negative groups by farm between June 2009 and December 2011.

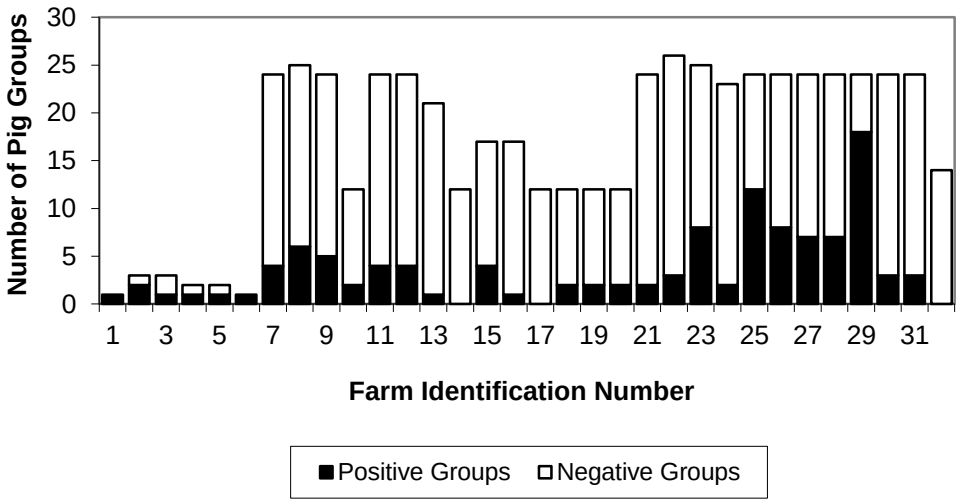
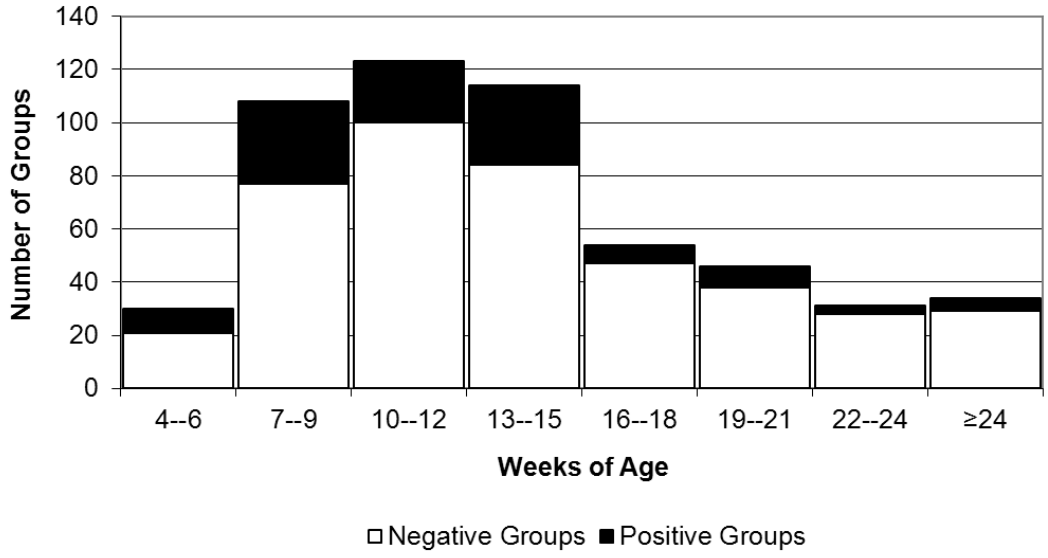


Figure 2.3. Age distribution of RRT-PCR influenza A positive and negative groups between June 2009 and December 2011.



Chapter 3: Influenza A virus risk factors in growing pigs

Introduction

Influenza A virus (IAV) is an RNA single-stranded negative sense virus belonging to the *Orthomyxovirodae* family. The virus can infect humans and certain domestic and wild animal species including avian, porcine, equine, canine, feline and marine mammals. In swine, the virus is considered to play a primary role in polymicrobial respiratory disease events (Opriessnig et al., 2011). Swine have been recognized as an important host species for IAV since they may be potential sources for both zoonotic infections and novel viruses through reassortment (Ma et al., 2009; Van Reeth, 2007; Webster et al., 1992).

Among the different IAV subtypes, three (H1N1, H1N2, H3N2) have been circulating in the swine population during recent decades (Brown, 2000; Vincent et al., 2008; Webby et al., 2004). Infections in swine are characterized by high morbidity and low mortality. Infected pigs may exhibit sneezing, coughing, lethargy, fever, anorexia, and rhinorrhea (Olsen et al., 2006). Infected pigs start shedding infectious viral particles through their nasal secretions 1 day after infection and can continue to shed for 7 days. Introduction of new viruses into pig farms may be preceded by introducing infected replacement animals (Brown, 2000; Mohan et al., 1981). IAV infected pigs can generate infectious aerosols which may play a role in the regional dissemination (Corzo et al, 2011). However, there are many unanswered questions with regards to the airborne transmission route.

Risk factor studies based on serologic data have generated valuable information regarding the epidemiology of influenza in swine. The presence of anti-influenza antibodies has been associated with high farm density, farm type, herd size, female replacement rates, pen density, uncontrolled access of individuals and indoor housing (Ewald et al., 1994; Maes et al., 2000; Mastin et al., 2011; Poljak et al., 2008; Simon-Grife et al., 2010; Suriya et al., 2008). However, there are no risk factor studies associating the presence of IAV with farm level characteristics. Therefore, the objective

of this study was to investigate whether certain farm level risk factors were associated with the presence of IAV in growing pigs.

Methods

Procedures performed in this study were in accordance with the University of Minnesota Institutional Care and use Committee (IACUC) guidelines.

Study population

This study was part of a larger IAV active surveillance study conducted in the Midwestern United States which started in June 2009 and ended in December 2011. A total of 32 conveniently selected farms were enrolled for which the primary objective was to actively monitor IAV in growing pigs over time.

Six farms withdrew from the surveillance study leaving 26 farms located in Illinois (n=4), Indiana (n=8), Iowa (n=10) and Minnesota (n=4) remaining enrolled.

Sample collection and testing

Sample collection began once producers' agreed to participate and continued for 12 or 24 months. On each farm, nasal swabs were collected monthly from a convenience sample of 30 (95% confident of detecting at least one positive swab when virus prevalence is at least 10%) growing pigs at approximately 10 weeks of age (Starswab II, Starplex Scientific Inc. Etiobiocke, Ontario, Canada). On pig farms where there were multiple age groups, the investigator selected the age group of pigs that was closest to 10-weeks of age. Nasal swab samples collected during the monthly visit belonged to pigs from the same age-group cohort. Nasal swabs were individually labeled with the farm identification number, state, month and sample number. Samples were placed in an Styrofoam container with ice-packs and shipped overnight to the virology department of St. Jude Children's research hospital (Memphis, TN, USA) for influenza A virus testing by RRT-PCR (Ducatez et al., 2011; Richt et al., 2004).

Data collection

A questionnaire containing close ended questions was designed to capture data on farm characteristics. Specifically the survey assessed farm type, regional farm density, topography surrounding the site, entrance biosecurity measures for employees and visitors, pig flow, origin of pigs, source of gilts , vaccination history, water treatment protocols and number of people working at the farm (Table 3.1). The person collecting the monthly nasal swabs administered the survey to the owner or farm manager.

Meteorological data collection

A weather station (HOBO®, Onset Computer Corporation, Bourne, MA, USA) was placed 20 – 30 m away from the pig barns on each of the participating farms. The weather stations were set up to log data every hour. Meteorological data recorded included temperature (°C), relative humidity (%), sunlight intensity (watts/m^2), wind direction (degrees), wind speed (kph) and gust speed (kph). Data were downloaded into a portable computer from the weather station monthly on the day nasal swabs were collected.

Data analysis

For the purpose of this study, a group of pigs was defined as the 30 growing pigs selected for monthly monitoring. A group was considered positive when at least one RRT-PCR tested positive for IAV.

Unviariate analysis was performed as a screening tool and variables with a *P*-value below 0.25 were considered for inclusion in a repeated measures logistic regression model. One logistic model examined the relationship between IAV status of the group and farm level characteristics and a second model assessed the weather data.

The multivariable model was built by forcing all variables that met the screening

criteria plus two-way interaction terms. A stepwise backward elimination procedure was employed for model simplification by removing variables with a $P \geq 0.05$. Farm was included in the model as a random effect to account for clustering of the groups of pigs per farm. Month was included in the model as the repeated measure under an autoregressive correlation structure matrix. SAS 9.2 (SAS Institute Inc., Cary, NC, USA) were used for all statistical procedures.

Results

A total of 522 groups with a mean and median age of 13.5 and 13 weeks, respectively were screened for IAV. Age ranged from 3.5 to 31 weeks of age. Eight farms were finishing farms, 8 were wean-to-finish farms, 4 were farrow-to-finish farms, 4 were nursery-finisher farms, 1 was a nursery and 1 was a gilt developer unit.

The number of visits per farm was not constant due to absence of pigs, time constraints or farms withdrawing from the study. Of the 26 farms enrolled in the study, one was visited 25 times, 14 were visited 24 times, one was visited 23 times, one was visited 21 times, 2 were visited 17 times, 6 were visited 12 times and one was visited 11 times between June 2009 and December 2011. Thus, 32.1%, 24.7%, 18.3%, 15.5%, 4.6% and 4.6% of the samples originated from groups of pigs in finisher, wean-to-finish, farrow-to-finish, nursery-finisher, nursery and gilt developer unit farms respectively.

At the individual level, 730 (4.7%) of 15,630 nasal swabs tested positive for IAV whereas at the group level, 110 (21.1%) of 522 groups of pigs had at least one RRT-PCR positive nasal swab. All but three farms had at least one IAV positive group. These three farms that had no positive IAV groups were monitored for 11 or 12 months.

Farm level factors

The odds of testing positive for IAV increased 3.05 and 13.55 times for farrow-to-finish and nursery farms respectively, when compared to finishing farms (Table 3.2). Pig

farms that managed their pigs under an all-in all-out (AIAO) by barn or by site compared to farms that managed pigs in a continuous flow had lower odds of testing positive for IAV. Growing pigs born in sow farms that raised their own gilts had higher odds of testing positive for IAV compared to pigs that were born in sow farms in which replacement gilts originated from an external multiplier system.

Four variables were included in the multivariable model (e.g. farm type, pig flow, gilt source and presence of other animals). One variable (farm type) remained in the multivariable model.

Meteorological factors

All measures met the multivariable model inclusion criteria (Table 3.3). Two variables (temperature and wind speed) remained in the final multivariable model after backwards stepwise elimination. Each degree increase in temperature increased the likelihood of a group of pigs testing positive for IAV by 1.04 (CI 1.01-1.07). Similarly, the likelihood of testing positive for IAV increased 1.31 times with every kilometer per hour (kph) increase in wind speed (CI 1.03-1.23).

Discussion

To the authors' knowledge, this is the first study in which the presence of IAV in pig farms has been associated with farm level and meteorological risk factors in swine.

Finding farm type as a significant risk factor mirrors findings reported in a serological risk factor study in that finisher pigs were more likely to be seropositive for IAV when sows were on-site compared to finisher pigs raised on farms without the presence of the sow herd (Poljak et al., 2008). It has been reported that IAV infection dynamics in swine farms is farm-type dependent since pigs become infected earlier in lifetime in farrow-to-finish farms compared to finisher herds (Loeffen et al., 2009). This difference may be explained by the population structure in these farms since farrow-to-

finish farms contain all the different age groups on the same site allowing the virus to perpetuate due to the constant presence of susceptible individuals in which the presence of maternal antibodies is waning. The existence of susceptible individuals of varying ages is absent in finishing farms that contain pigs only in the later stages of growth. Nursery farms follow the same trend in that the constant influx of recently weaned pigs provide the necessary conditions for an infectious agent to maintain transmission of the virus between older pigs and the incoming pigs. Additionally, it has been reported that recently weaned pigs can be a source of virus by introducing new viruses into recipient barns (Allerson et al., 2011; Brown et al., 2012). Furthermore, personnel and equipment used between these different age groups may play a role on IAV transmission since it is known that the virus can survive outside the host (Bean et al., 1982; Haas et al., 1995; Stallknecht et al., 1990b; Thomas et al., 2008; Tiwari et al., 2006).

Pig flow describes the way animals flow through the buildings. There are two main animal flows, AIAO and continuous flow. AIAO corresponds to filling and emptying the building at once having then one age-group of pigs in the building. Conversely, continuous flow means that pigs are constantly entering and exiting the building having pigs of different age-groups in the same building. AIAO pig flow has been reported to have a significant impact on growth rate since pigs are not being challenged with infectious agents from older pigs (Diekman et al., 1999; Ice et al., 1999; Scheidt et al., 1995). Pigs were less likely to test positive for AIV if they were raised under an AIAO by barn (OR = 0.31; CI=0.14-0.66) or by site (OR=0.36; CI=0.15-0.88) flow compared to pigs raised under a continuous flow manner. AIAO flow prevents having different age-groups of pigs together which precludes horizontal transmission of infectious agents from older pigs to younger pigs, a known mechanism for IAV maintenance in pig populations (Brown, 2000).

Groups of pigs that were born in farms where the source of the gilts was either a multiplier farm or gilts that had been born in the sow farm then taken to an off-site

facility and returned had lower likelihood of testing positive for IAV compared to groups of pigs born in sow farms where gilts were born and raised on-site. This finding disagrees with what has been published in that farms introducing replacement animals are at higher risk of being seropositive (Brown, 2000; Poljak et al., 2008; Simon-Grife et al., 2010; Suriya et al., 2008) and at the same time supports IAV maintenance (Brown, 2000). In today's swine farms, veterinarians are aware of the importance of gilt introduction and perhaps these incoming gilts either come from a high health multiplier system or even from AIAO gilt developer units decreasing the likelihood of viral introductions.

In today's swine farms, weather conditions influence the environment of the pig inside the barn (for example, low temperatures trigger the need to provide the pig with a heat source increasing barn temperature). Data on the relationship between environmental conditions and the presence of IAV in pigs is scarce. Meteorological conditions have been associated with regional dissemination of equine IAV in Australia (Firestone et al., 2012) and porcine reproductive and respiratory syndrome virus and *Mycoplasma hyopneumoniae* in pigs (Dee et al., 2009; Otake et al., 2010). Our study detected an association between two meteorological variables and the presence of IAV in groups of growing pigs. As outside temperature and wind speed increased, the likelihood of a group of pigs being infected with IAV increased. One reasonable interpretation for the relationship between temperature increase and presence of IAV may be that as environmental temperature increases, the temperature of the barn will increase as well, creating the need for increased air movement to reduce ambient temperature. A higher rate of air exchange in the building can be accomplished either by increasing exhaust fan speed or by lowering the curtains increasing the entry of external air particles that may include airborne pathogens. However, it has been reported that higher air exchange in pig units have a protective effect on pneumonia lesions at slaughter (Flesja et al., 1982) suggesting that the concentration of housing unit particles decreases and thus respiratory lesions decrease. On the other hand, increasing wind speed may reduce temperature but it is not clear what impact would this have on pig barn environmental conditions. These

associations require further investigation to deepen our understanding of the impact of meteorological conditions on disease within the barn.

Farm level risk factors identified in this study provide insights into the understanding of the epidemiology of the disease. This study emphasizes the importance of population dynamics in that certain farm type facilities and pig flow play a role in the epidemiology of the disease due to the constant entry of animals into a population, providing the necessary susceptible hosts for virus maintenance. Therefore, efforts into managing closed populations should be performed to decrease virus transmission and persistence which will benefit not only pigs but more importantly humans. It is important to mention that there is still need for more studies to fully understand the involvement of farm level factors in the presence of influenza virus in pig farms.

Table 3.1. Variables for the assessment of the presence of IAV in growing pig farms.

Variable	Type of variable	Categories
Farm type	Categorical	Gilt developer unit; Nursery; Nursery-Finisher; Wean-to-finish; Farrow-to-finisher vs. Finisher
Number of finisher barns on site	Continuous	
Barn ventilation	Categorical	Mechanical; Natural vs. Combination
Topography around the site	Binary	Gentle rolling hills vs. flat
Forestation around the site	Binary	No vs. Yes
Number of pig farms within 1 mile	Continuous	
Distance to closest pig farm	Continuous	
Distance to closest road	Continuous	
Drinking water originates from well	Binary	No vs. Yes
Drinking water chlorinated	Binary	No vs. Yes
Drinking water acidified	Binary	No vs. Yes
Recycled lagoon water for flush or recharge	Binary	No vs. Yes
Number of employees at the site	Continuous	
Entrance sanitation procedure	Categorical	No measures; Boot wash and disinfection; Coverall and boot change, hands washed vs. Shower in and clothes changed
Employee restrictions on visits to other pig farms	Binary	No vs. Yes
Downtime required for employees after visiting other pig farms	Continuous	
Downtime required for visitors	Continuous	
Frequency of veterinary visits	Continuous	
Pig flow	Categorical	All-in All-out by site; All-in All-out by bar vs. Continuous flow
Number of sow herds supplying pigs	Continuous	
Sow herd-size	Continuous	
Number of workers at the sow herd	Continuous	

Gilt source	Categorical	Multiplier; Born at breeding site and moved to another site and later returned vs. Born at breeding site and never moved from that site
Sows vaccinated for influenza	Binary	No vs. Yes
Growing pigs vaccinated for influenza	Binary	No vs. Yes
Presence of migratory birds within 1 mile radius of the site	Categorical	No; Rarely (less than once every 6 months); Occasionally (every 3 to 6 months) vs. Frequently (once per month)
Presence of feral pigs near the site	Binary	No vs. Yes
Presence of birds inside buildings	Binary	No vs. Yes
Use of insecticides on building exterior	Binary	No vs. Yes
Presence of other animals in the farm	Binary	No vs. Yes

Table 3.2. Farm level factors associated with the presence of swine influenza in farms in the Midwestern United States.

Risk Factor	Odds Ratio	95% CI	*P*
Farm type			
Finisher (Referent)	-	-	-
Farrow-to-finish	3.05	1.56-5.95	<0.001
Wean-to-finish	0.89	0.44-1.80	0.764
Nursery	16.69	5.34-52.18	<0.001
Gilt developer unit	1.11	0.31-3.94	0.864
Nursery-finisher	0.78	0.33-1.82	0.575
Sow vaccination for influenza	1.09	0.31-3.75	0.887
Number of barns on site	1.036	0.89- 1.20	0.642
Barn ventilation			
Natural and mechanical (Referent)	-	-	-
Natural ventilation	0.559	0.17-1.77	0.323
Mechanical ventilation	1.331	0.52-3.40	0.550
Topography - Gentle rolling hills	0.769	0.30-1.94	0.557
Absence of trees surrounding the site	0.724	0.24-2.13	0.558
Number of pig farms within 1 mile	1.015	0.70- 1.46	0.935
Distance to closest pig farm	0.863	0.62-1.19	0.369
Distance to closest road	0.891	0.71-1.10	0.296
Drinking water chlorinated	1.566	0.52-4.70	0.423
Drinking water acidified	1.566	0.52-4.70	0.423
Recycled lagoon water for flush or recharge	1.080	0.11-9.88	0.945
Number of employees at the site	1.042	0.88-1.22	0.621
Entrance sanitation procedure			
Shower in and clothes changed (Referent)	-	-	-
No measures	0.399	0.10-1.56	0.187
Boot wash and disinfection	0.544	0.11-2.67	0.452
Coverall and boot change, hands washed	0.440	0.16-1.19	0.107
Employee restrictions on visits to other pig farms	0.877	0.37-2.03	0.759
Downtime required for employees after visiting other pig farms	1.199	0.74-1.93	0.456
Downtime required for visitors	1.126	0.67-1.86	0.644
Frequency of veterinary visits	0.977	0.93-1.02	0.341
Pig flow			
Continuous flow (Referent)	-	-	-
All-in All-out by barn	0.309	0.14-0.66	0.002
All-in All-out by site	0.359	0.14-0.88	0.027
Number of sow herds supplying pigs	1.513	0.59-3.82	0.379
Sow herd size	1.0	1.0-1.0	0.306

Number of workers at the sow herd	0.974	0.90-1.04	0.460
Growing pigs vaccinated for influenza	1.143	0.27-4.78	0.854
Gilt source			
Born at breeding site and never moved from that site (Referent)	-	-	-
Born at breeding site and moved to another site and later returned	0.175	0.04-0.63	0.008
Multiplier	0.256	0.09-0.68	0.007
Presence of migratory birds within 1 mile radius of the site			
Frequently (once per month)(Referent)	-	-	-
No	1.489	0.38-5.81	0.565
Rarely (less than once every 6 months)	2.122	0.70-6.37	0.179
Occasionally (every 3 to 6 months)	1.708	0.53-5.50	0.368
Presence of feral pigs near the site	1.590	0.20-12.14	0.654
Presence of birds inside buildings	0.767	0.33-1.74	0.525
Use of insecticides on building exterior	1.740	0.55-5.49	0.344
Presence of other animals in the farm	1.586	0.72-3.45	0.244

Table 3.3. Meteorological factors associated with the presence of influenza virus in growing pigs.

Variable	Odds Ratio	95% CI	*P*
Temperature (°C)	1.027	0.999, 1.056	0.0550
Relative humidity (%)	0.965	0.937, 0.995	0.0223
Light intensity (watts/m^2)	1.006	1.001, 1.010	0.0205
Wind direction (Ø degrees)	0.993	0.984, 1.002	0.1054
Wind speed (kph)	1.175	1.026, 1.346	0.0201
Wind gusts (kph)	1.132	1.024, 1.250	0.0150

Chapter 4: Prevalence and risk factors for H1N1 and H3N2 influenza A virus infections in Minnesota turkey premises

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Introduction

Influenza virus infections of turkeys can be asymptomatic or cause clinical disease depending on the virulence of the strains and presence of co-infections (Anonymus, 2009; Suarez et al., 2002). Clinical signs can range in severity from none to mild to severe and may include ruffled feathers, diarrhea, anorexia, incoordination, opisthotonos, paddling movements, spastic paralysis, somnolence and death (Narayan et al., 1972; Saif et al., 2003). In 1992, Halvorson et al. (Halvorson et al., 2003) reviewed the occurrence of influenza in turkeys raised in the United States, and reported that influenza viruses had been detected in 25 states. Eleven hemagglutinin subtypes (H1, H2, H3, H4, H5, H6, H7, H8, H9, H10 and H13) had been identified up until 1991. Influenza viruses H1 and H9 were detected frequently from 1978 through 1991. Wild birds were considered the most likely source of these viruses.

In Minnesota, influenza infections with several subtypes have been reported in turkeys, especially during the cold months of the year (Halvorson et al., 1985), and all viruses have been classified as low pathogenicity (Poss and Halvorson, 2003). In September 1978 influenza outbreaks involving three subtypes (H6N1, H4N2, H1N1) occurred in turkey flocks and an egg producing chicken premises in Minnesota. It was hypothesized that the origin of the H6N1 virus outbreak in the chicken premises was a nearby turkey premises. However, possible origins of the other two subtypes were not identified (Moulthrop and Langston, 1980). In August 1995, an H9N2 influenza outbreak in commercial turkeys was reported in central Minnesota. Interestingly the virus continued spreading and infected multiple turkey premises until March 1996. Factors such as presence of wild birds on turkey ranges, cross contamination between farms, contaminated trucks and poor cleaning and disinfection protocols were identified as potential contributors to the epidemic (Halvorson et al., 1998).

Infections of turkeys with swine influenza viruses (i.e. H1N1, H3N2) have also been reported. In 1980, a farm in Ohio housed breeder flocks and 1000 pigs (farrow-to-

finish) on the same premises but in separate barns separated by just 12 meters (Mohan et al., 1981). Two separate outbreaks were reported in consecutive years. The first outbreak in turkeys followed an outbreak of respiratory disease that affected all age groups of pigs following the introduction of recently purchased boars infected with influenza A virus. Immediately, decreased egg production and an increase in culled eggs were noticed in the breeder flock. The second outbreak followed a similar pattern. The introduction of boars with respiratory signs into the pig farm was followed by a respiratory disease outbreak in the growing pigs and subsequently a drop in egg production from the turkeys was recorded. Laboratory diagnostics confirmed that an identical H1 swine influenza virus had infected both pigs and turkeys but the mechanism of transmission of the virus from pigs to the turkeys was not elucidated (Mohan et al., 1981). Subsequently, there have been numerous reports (Andral et al., 1985; Choi et al., 2004; Ficken et al., 1989; Suarez et al., 2002; Tang et al., 2005) of swine influenza viruses infecting turkey flocks where no pigs were kept at the same premises.

With approximately 600 turkey premises, Minnesota is the leading turkey producing state in the US and it is also the third largest swine producing state (Minnesota Turkey Factsheet 2009a; Quick Facts 2009b). In certain areas of the state, production of both turkeys and pigs occurs in close proximity. Estimates of the economic impact of influenza in turkeys in the US have ranged from $200,000 up to five million dollars during influenza outbreaks between 1978 and 1991. In the same report, authors estimated a loss of US$ 0.5 to 2 per turkey marketed (Halvorson et al., 2003). It is estimated that economic losses to Minnesota turkey growers due to the 1995 H9N2 influenza outbreak, were US$ 6.2 million, with 87% of the losses attributable to mortalities, condemnation and depopulation (Halvorson et al., 1998).

Due to the potential for interspecies transmission of influenza viruses and the presence of co-localized premises of turkeys and pigs in agricultural regions such as Minnesota, understanding the disease ecology of virus infections is key for control.

Additionally, epidemiologic information on the prevalence and risk factors for interspecies transmission between pigs and turkeys is scarce. Therefore, the objectives of this study were to estimate the prevalence of H1N1 and H3N2 influenza virus infections in turkey flocks in Minnesota and examine some factors that may be associated with infection.

Methods

For the purpose of this cross-sectional study, turkey premises are defined as barns on the same location where single-age or multiple-age groups of turkeys are raised. Flocks are identified as those individual groups at a premises and comprising a specific age and/or gender of turkey. Pig premises are defined as locations housing any number of pigs for breeding, exhibition or market. Infection is defined as presence of detectable antibodies against influenza A viruses in any bird in the flock.

Study population and design.

The study was based on an avian influenza surveillance program in Minnesota conducted under the supervision of the Minnesota Board of Animal Health (MBAH) at the Minnesota Poultry Testing Laboratory (MPTL). Under this program, blood samples from 20 birds from every flock to be marketed were collected within three weeks prior to processing at the abattoir. This sample size is adequate to provide 95% confidence for detecting at least one positive bird if the prevalence of infection were at least 15%. Blood samples were screened by an influenza A virus antibody detection test, specifically the agar gel immunodiffusion (AGID) test as performed at the Minnesota Poultry Testing Laboratory in Willmar, Minnesota using reagents provided by the United States Department of Agriculture (USDA) National Veterinary Services Laboratory (NVSL) in Ames, Iowa. The protocol contained in the National Poultry Improvement Plan and Auxiliary Provisions (NPIP) was followed for all AGID testing conducted (NPIP, 2011) in which positive samples were further characterized by subtyping through hemagglutination inhibition (HI) for subtypes H1 through H16 and neuraminidase

inhibition (NI) for subtypes N1 through N9 at the NVSL per the testing guidelines set forth in the OIE Terrestrial Animal Health Code (OIE, 2009). For the purpose of this study, a premises was classified as positive when at least one flock tested positive for influenza A virus antibodies by AGID.

Specific antigens used by the National Veterinary Service Laboratories for HI were H1 - A/Equine/NJ/8/76 H1N7, H3 - A/Duck/Ukraine/1/63 H3N8, H3 - A/Swine/NC/35922/98 H3N2 and for NI, N1- Equine-Swine/TN/3/76 H7N1, N2 - A/Turkey/MA/65 H6N2, N2 - A/Swine/TX/1/98 H3N2. Infection is presumed to be swine-origin H3N2 when positive H3N2 antibodies are detected in HI tests using A/Swine/NC/98 H3N2 and A/Duck/Ukraine/1/63 H3N8 as the H3 antigens and A/Swine/TX/98 H3N2 and A/Turkey/MA/65 H6N2 as N2 antigen. Infection in turkeys is presumed to be swine-origin H1N1 when positive H1N1 antibodies are detected in HI tests using A/Equine/NJ/8/76 H1N7 as the H1 antigen and A/Equine-Swine/TN/3/76 H7N1 as N1 antigen.

All influenza testing data for this study was obtained following the approval from Minnesota agencies. The data set contained all testing information between February 2007 and September 2008, and included information on turkey premises location (longitude, latitude), flock size (number of turkeys), gender (hen or tom), number of blood samples collected, test results and number of flocks tested per premises. Data from a total of 356 premises uniquely identified with a number were obtained. A database of Minnesota pig premises locations (longitude, latitude) was obtained from MBAH.

Data management and analysis

Both turkey and pig premises databases were provided as Excel spreadsheets (Microsoft, Excel 2003, Microsoft Corporation, Seattle, USA). Both turkey and pig farm locations were imported into a geographic information system (ArcGIS 9.3, ESRI, Redlands, California. 2008) and mapped. Information (shape files) regarding major roads

and hydrography (lakes and rivers) in Minnesota were obtained from the Minnesota Department of Natural Resources and imported into ArcGIS 9.3.

Distances between each turkey premises and the nearest pig farm as well as to the nearest major road were calculated using the near tool in ArcGIS 9.3. Two, four and six kilometer (Km) buffers were created around each turkey premises. The number of pig premises within each buffer was recorded for influenza-negative and positive turkey premises. Another 1 Km buffer was created around each turkey premises and the presence or absence of either a river or lake was recorded.

Data were analyzed using logistic regression where the outcome variable was seropositivity to influenza A virus by AGID assay. Variables were initially screened by univariate analysis and variables with a *P*-value less than 0.25 were retained for the final multivariable logistic regression model. For the final multivariable model, all selected variables plus two-way interaction terms were forced in the model and subsequent backward elimination was performed by sequentially removing variables with a p-value greater than 0.05 until all variables in the model contain a p-value equal or smaller than 0.05.

Results

Between February 2007 and September 2008, turkeys on 17 (4.7 %) out of 356 turkey premises tested positive for influenza A virus antibodies by AGID (Table 4.1). In all 17 cases a subtype was identified through HI/NI assays. In 13 cases, antibodies were subtyped as H3N2 whereas in 3 cases they were H1N1. Interestingly there was one of the 17 premises with antibodies to both H1N1 and H3N2 subtypes.

Positive turkey premises were located in 11 different counties (Figure 4.1). Four, three and two positive premises were located in Brown, Olmsted and Waseca Counties respectively. The remaining positive premises were singly located in Chippewa,

Cottonwood, Dodge, Kandiyohi, Le Sueur, Redwood, Steele, Stearns and Winona Counties (Table 4.1). During the fall (September, October, and November) and winter (December, January and February) months there were 3 and 9 positive premises, respectively. The remaining 5 positive premises were detected in April (three premises), June and August.

Eight variables (presence of pig premises, gender, distance to nearest major road, presence of water sources (in particular, lakes) within 1 kilometer, number of all water sources (lakes and rivers) within 1 kilometer, number of lakes within 1 kilometer, flock size, distance to nearest pig premises) were identified to have a p-value less than 0.25 through univariate analysis (Table 4.2). The remaining variable (number of flocks tested) was not statistically associated with H1N1 or H3N2 influenza virus infection in turkeys ($P>0.25$).

Of these eight variables, two remained in the multivariable logistic regression model after a backward stepwise elimination process was performed (Table 4.3), including pig premises proximity and flock size. Turkey premises with pigs located within 2 kilometers had 8 times higher odds of testing influenza positive than those with the closest pigs located between 4 and 6 kilometers. Interestingly, increasing flock size (interpreted as 1000 birds) reduced the odds of testing positive for influenza virus.

Discussion

The results of this study indicate that influenza A virus antibodies to H1N1 and H3N2 (presumably swine-origin) viruses were found in turkey premises in Minnesota between February 2007 and September 2008. The overall estimated prevalence for influenza A antibodies for either H1N1 or H3N2 viruses was 4.7 % in Minnesota turkey premises. The data presented here suggest that indeed inter-species transmission did occur. Furthermore, although such transmission events may seem infrequent, it is important to mention that the AGID test is not a sensitive test (Peng et al., 2007; Yee et

al., 2008), perhaps yielding false negative results and thereby underestimating the frequency of inter-species transmission.

HI and NI tests have been a reliable tool for subtyping avian influenza antibody positive samples in different avian species, including turkeys (Brown et al., 2010; Halvorson et al., 1985). Our assumption that the detection of anti-influenza A virus antibodies in turkeys in Minnesota is a result of infection with swine-origin influenza A viruses is based on the fact that HI and NI assays were performed using not only swine origin viruses but also genetically similar avian viruses. For example, the viruses used in the NI assay (A/Turkey/MA/65 H6N2 and A/Swine/TX/1/98 H3N2) share 99% NA gene nucleotide similarity with each other, concluding that NI serological tests are adequate for detecting N2 antibodies against H3N2 strains of swine origin. While these antigens were adequate for subtype detection in all of our cases, they may not be able to determine the subtype in other cases due to lack of serological cross-reactivity. The antigens used at the USDA NVSL during the study period, while having sufficient genetic similarity to detect swine influenza A virus antibodies, are not the recently circulating subtypes of influenza A viruses of turkeys or swine in the US (Vincent et al., 2008).

Although no information, other than geographical was gathered with respect to any kind of managerial or physical relationships between the turkey and pig premises that could have suggested a transmission route, we cannot rule out the possibility of fomite or vector transmission. It has been reported that influenza viruses have better survivability in non-porous surfaces compared to porous surfaces (Bean et al., 1982) suggesting that any equipment shared between pig and turkey premises could potentially transmit the virus. Research on avian influenza has indicated that transmission of the virus through contaminated bedding, manure and carcasses can play an important role (Yee et al., 2008). However, it is unlikely that movement of these types of waste materials between pig and turkey premises occurs frequently. On the other hand, information on influenza transmission through vectors is scarce. There are two reports in which H5N1 influenza

virus was detected through RT-PCR and virus isolation in mosquitoes and blow flies caught in the vicinity of H5N1 infected poultry farms (Barbazan et al., 2008; Sawabe et al., 2006). Unlike turkeys, which can have both respiratory tract and systemic influenza virus infections, influenza infections in swine are restricted to only the respiratory tract with rare viremia (Romijn et al., 1989) suggesting that the blood borne route may be an unlikely route for transmission. The primary subtypes infecting pigs are H1N1, H1N2 and H3N2 and the most common clinical signs observed are pyrexia, anorexia, nasal discharge, sneezing and coughing. The virus spreads rapidly among the population with morbidity close to 100% but generally low mortality. Individual pigs recover within one week of infection (Olsen et al., 2006). Transmission between pigs is mainly by nose-to-nose contact and pigs usually shed virus in their nasal secretions for 5-7 days. However, there is potential for aerosol transmission (Brown, 2000). Influenza vaccination in swine has become more common over the last 15 years. Usually, sows are vaccinated with the objective of conferring extended maternal immunity to their litters that will protect the young pigs from clinical disease (Thacker and Janke, 2008). In the US, the 2006 National Animal Health Monitoring System (NAHMS) survey concluded that 26.4%, 9.2% and 3.8% breeding, nursery and growing-finishing pig herds respectively vaccinated against both H1N1 and H3N2 influenza viruses (NAHMS, 2006).

Most (12 out of 17) of the turkey premises infected with influenza in our study were detected during the cold months of the year suggesting that there may be a seasonal effect. There are different reports where low ambient temperature is highlighted as an important component for increased influenza virus survival either in water (Stallknecht et al., 1990b) or slurry (Haas et al., 1995), with both water and slurry possible sources of virus transmission via fomites or vectors. Additionally, experimental aerosol transmission studies in guinea pigs under different environmental conditions were able to demonstrate that aerosol transmission is most efficient when low temperature and humidity are present (Lowen et al., 2007) suggesting the possible role of airborne influenza in the spread of the virus from a regional standpoint.

In this study, proximity to pig farms was significantly associated with turkey seropositivity to H3N2 influenza viruses and H1N1 influenza viruses of probable swine-origin. Turkey premises that had pig farms within a 1.9 km radius had 8 times higher odds of testing serologically positive for H3N2 and H1N1 influenza viruses than turkey farms that had pig farms located within a 4 to 6 km radius. Our findings agree with anecdotal reports (Andral et al., 1985; Choi et al., 2004; Ficken et al., 1989; Suarez et al., 2002; Tang et al., 2005) in which proximity to pig farms may play a role in infection. It is difficult to conclude by which route infection occurred in the positive premises in our study. However, there is an increasing body of literature suggesting that aerosol transmission plays an important role in the spread of this virus (Lu et al., 2004; McQuiston et al., 2005; Tellier, 2009). In pigs, there have been epidemiologic studies (Maes et al., 2000; Poljak et al., 2008) in which pig farm density has been found to be a risk factor for infection in pig farms which again points to aerosol as a likely route of influenza transmission. Given that swine influenza is very common in pigs in the USA, it is feasible that aerosol transmission between swine and turkey farms may occur only when specific environmental conditions (temperature, humidity, wind direction) prevail.

Increasing flock-size by one thousand birds was found to have a protective trend (OR = 0.9, 95% CI 0.9, 1.0) in our multivariable logistic regression model. Although literature on flock size being a risk factor for the introduction of pathogens to poultry farms is scarce, there have been some reports examining this relationship. Increasing flock size has been found to be associated with increasing odds of H7 and H5 infections only in univariate models (Henning et al., 2009; Lu et al., 2004; McQuiston et al., 2005). Thomas et al. (Thomas et al., 2005) reported that increasing the number of houses and birds were significantly associated with the introduction of highly pathogenic avian influenza in the Netherlands. This study also suggested these factors may have been confounded in their analysis. It is difficult to conclude why an increase in flock-size would be associated with reduced risk in our study. We can speculate that compared to

small turkey premises, larger turkey premises may have stricter management and biosecurity measures in place such as restricting access to unwanted personnel, all-in all-out by site, shower-in shower-out, raising birds with a known influenza status and dedicated personnel that work exclusively at the farm.

It is important to mention that our study is based on premises locations which were recorded by different government agencies. These could not be practically validated, and likely included some positional errors (Wayne and Davies, 2007). If spatial errors in farm locations are assumed to have occurred at random, this would likely lead to an underestimation of the effect of proximity to pig farms. The alternative possibility of having the observed association between influenza risk and pig farms due primarily to spatial errors in farm location is considered highly unlikely, but cannot be eliminated.

In summary, we found that infection of turkey premises with influenza viruses of presumed swine origin is an uncommon event. The cessation of Minnesota range turkey production in 1998 decreased the total number of influenza positive flocks, but the number and percentage of flocks identified with H1N1 and/or H3N2 antibodies increased. With few if any clinical signs in commercial market turkeys, H1N1/H3N2 infections of turkey breeding flocks have become increasingly important to the Minnesota turkey industry. Additionally, seasonal patterns of infection have changed and in the past 5 to 6 years there have been more spring – early summer introductions for unexplained reasons. H1N1/H3N2 vaccination of turkey breeders is now routine in an effort to fend off infection from these swine influenza strains which has led to significant egg production drops in the past. Additionally, the significant association of pig farm proximity to turkey premises as a probable risk factor for infection sheds light on important epidemiologic information regarding the interspecies transmission of influenza in the field. More studies are needed to further understand the risks of influenza transmission between these two species and to define practical guidelines to mitigate the risk.

Table 4.1. Turkey premises test results for influenza A virus in Minnesota between February 2007 and September 2008.

County	Number of Positive Premises / Premises Tested (Subtypes)	County	Number of Positive Premises / Premises Tested (Subtypes)
Aitkin	0/1	Meeker	0/15
Anoka	0/2	Morrison	0/25
Becker	0/16	Murray	0/1
Big Stone	0/2	Nobles	0/1
Blue Earth	0/4	Norman	0/1
Brown	4/10 (H1N1, H3N2, H3N2, H1N1+H3N2)	Olmsted	3/5 (H3N2)
Cass	0/1	Ottertail	0/38
Chippewa	1/2 (H1N1)	Pipestone	0/1
Chisago	0/1	Pope	0/2
Clay	0/2	Redwood	1/7 (H3N2)
Cottonwood	1/4 (H3N2)	Renville	0/5
Dakota	0/4	Rice	0/8
Dodge	1/8 (H3N2)	Roseau	0/13
Douglas	0/3	Sibley	0/1
Filmore	0/7	Stearns	0/36
Freeborn	0/2	Steele	1/8 (H3N2)
Goodhue	0/7	Swift	0/12
Houston	0/1	Todd	0/21
Isanti	0/1	Wadena	0/3
Kandiyohi	1/42 (H3N2)	Waseca	2/6 (H1N1, H3N2)
Lac Qui Parle	0/1	Watonwan	0/1
Le Seur	1/4 (H3N2)	Wilkin	0/2
Lyon	0/4	Winona	1/4 (H3N2)
Marshall	0/1	Wright	0/4
Martin	0/3	Yellow Medicine	0/3

Total Number of Positive Premises/Premises Tested (subtypes)

17/356

(3 of H1N1; 13 of H3N2, 1 of H1N1+H3N2 mixed infection)

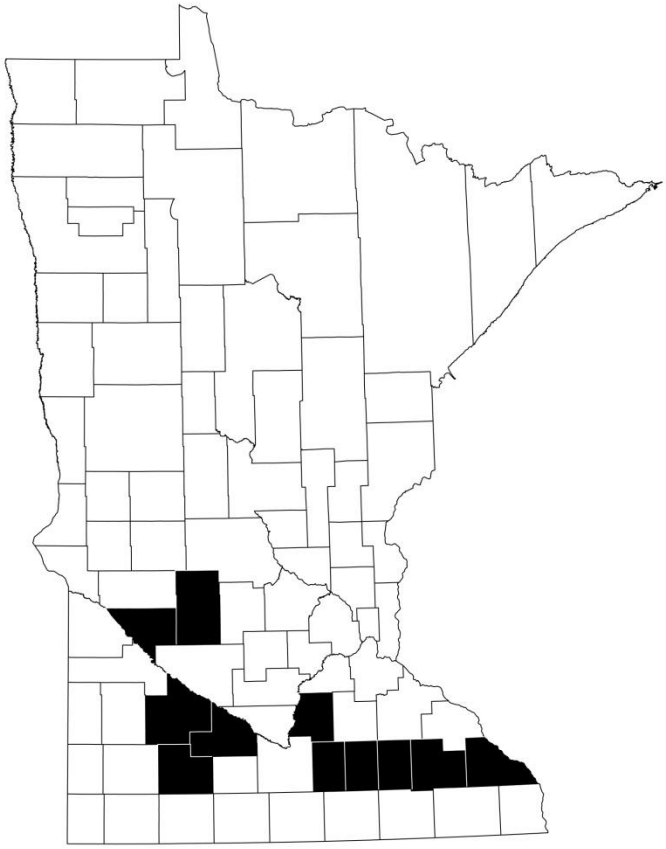
Table 4.2. Logistic regression univariate analysis for risk factors associated with swine influenza viruses infection in turkey premises in Minnesota.

Variable	**OR (95% CI)**	***P*- value**
Presence of pig farms		
- Between 4 and 5.9 km (Referent)	- -	
- Between 2 and 3.9 km	2.4 (0.4, 12.8)	0.80
- Between 0 and 1.9 km	8.0 (1.7, 37.4)	<0.01
Number of flocks tested per premise	1.02 (0.9, 1.0)	0.33
Gender	0.31 (0.1, 0.8)	0.02
Distance to nearest major road (m)	1.00 (1.0, 1.0)	0.10
Presence of water resources within 1 kilometer radius	3.57 (1.0, 12.6)	0.04
Number of water resources within 1 kilometer radius	0.67 (0.3, 1.2)	0.23
Number of lakes within 1 kilometer radius	0.68 (0.3, 1.2)	0.24
Increase in number of birds by 1000	0.94 (0.8, 0.9)	0.03
Distance to nearest pig farm (km)	1.00 (0.9, 1.0)	0.01

Table 4.3. Final logistic regression model for risk factors associated with swine influenza viruses infection in turkey premises in Minnesota.

Variable	OR (95% CI)	*P*- value
Presence of pig farms		
- Between 4 and 5.9 km (Referent)	-	-
- Between 2 and 3.9 km	2.7 (0.5, 14)	0.955
- Between 0 and 1.9 km	8 (1.7, 37)	0.003
Increase in number of birds by 1000	0.9 (0.9, 1.0)	0.053

Figure 4.1. Distribution of 13 influenza A seropositive (black colored) counties in Minnesota between February 2007 and September 2008 based on turkey premises serology.



Chapter 5: Characterization of the temporal dynamics of airborne influenza A virus detection in acutely infected pigs

Introduction

Influenza A virus (influenza) infections in swine cause respiratory disease and decreased performance. A recent survey in the United States concluded that at least 22% of the herds, regardless of the farm type (e.g. breeding, nursery or finisher), reported having problems attributed to influenza (NAHMS, 2006). Influenza is considered endemic and widespread in many countries and regions around the world (Brown, 2000). Because influenza has no species barrier and continues to be a significant zoonotic infection (Myers et al., 2007), understanding how the virus spreads within and between populations is crucial.

Influenza transmission among pigs is mainly through nose-to-nose contact and aerosolized droplets (Brown, 2000). Risk factor studies in Canada (Poljak et al., 2008) and Belgium (Maes et al., 2000) found that the likelihood of a pig farm being positive for influenza was significantly associated with pig farm density. In addition, recent data from studies performed with different species (Brankston et al., 2007; Mubareka et al., 2009; Munster et al., 2009; Tellier, 2009; Yee et al., 2009) suggests that airborne transmission of influenza may play an important role in the regional ecology of influenza, although the relative importance of this transmission route remains contentious.

Airborne spread of swine pathogens has been a topic of discussion in the last decades in that there is scarce data supporting this route as one of the main routes of transmission. Reports on the detection of swine pathogens (bacteria and virus) in air are available (Bourgueil et al., 1992; Dee et al., 2009; Hermann et al., 2008; Stark et al., 1998; Torremorell et al., 1997; Verreault et al., 2010; Weesendorp et al., 2008). However, there is still a gap on the understanding of the dynamics of bioaerosol generation and transmission. In addition, recent studies conducted in pigs to understand the regional spread of porcine reproductive and respiratory syndrome virus (PRRSv) and *Mycoplasma hyopneumoniae* confirmed that viable pathogens could travel between 3.5 and 9.1 km from the presumed source of infection (Dee et al., 2009; Otake et al., 2010).

Detection of airborne influenza has been reported (Blachere et al., 2009; Chen et al., 2009; Fabian et al., 2009; Yang et al., 2011) demonstrating that the virus can become airborne under different settings. Although detection of influenza in air samples from pigs has been reported (Loeffen et al., 2011), there is very limited information on the characterization of influenza bioaerosols generated by acutely infected pigs. Given the scarce information available, the need to understand bioaerosol generation and the potential for influenza airborne spread between populations, the objective of this study was to characterize the temporal dynamics of bioaerosol detection in an acutely infected population of pigs. Overall, this study explored the relationship between number of infected pigs and probability of detecting influenza in bioaerosols through the course of an acute infection

Methods

All procedures were conducted in accordance with the University of Minnesota Institutional Biosafety Committee (IBC) and Institutional Animal Care and Use Committee (IACUC).

Study site

The study was performed at the University of Minnesota College of Veterinary Medicine animal BSL-2 research facilities (St. Paul, Minnesota). Each isolation room had a utility area of 2.08 m^2 where the feed bin and cleaning tools were stored and also had one housing pen of 7.28 m^2 with a total airspace volume of 35.1 m^3 per room.

For the purpose of this study, enhanced personnel biosecurity measures were followed which included the use of personal protective equipment including N-95 masks, goggles, gloves, hairnets and dedicated clothing and footwear.

Study animals

Twenty-two, 7-week-old pigs were purchased from an influenza negative herd. Pigs were also free of major swine respiratory pathogens including PRRSv and *M. hyopneumoniae*. Prior to the start of the study and upon arrival to the isolation facility, the influenza negative status of the pigs was confirmed by collecting blood samples and nasal swabs. Serum samples were tested for influenza via hemagglutination-inhibition (HI) (Pedersen, 2008) and enzyme linked immunosorbent assay (ELISA) (Ciacci-Zanella et al., 2010) tests and nasal swabs were tested using real-time reverse transcriptase PCR (RRT-PCR) targeting the matrix gene (Slomka MJ, Densham AL, Coward VJ, Essen S, Brookes SM, Irvine RM, Spackman E, Ridgeon J, Gardner R, Hanna A, Suarez DL, Brown IH, 2010).

Two groups of eleven pigs were used in two independent replicates. Space allowance was 0.58 m^2 per pig and pigs had *ad-libitum* access to feed and water throughout the study.

Experimental pig infection

One pig (seeder pig) from each replicate was taken to a separate isolation room for challenge inoculation. Pigs were sedated by an intramuscular injection with a rapid-acting anesthetic (Telazol, Fort Dodge Animal Health) at a dose of 6.6 mg/kg. Once pigs were sedated, they were briefly restrained and the oral cavity exposed. A catheter attached to a syringe was used to deliver 1mL of A/Sw/IA/00239/2004 H1N1 influenza A virus containing 1 x 10^6 $TCID_{50}$/mL into the trachea. A separate syringe was used to deliver 0.5 mL of the same inoculum into each of the nostrils. Nasal swabs of seeder pigs were collected at 24 and 48 h post inoculation and tested by RRT-PCR. Upon confirmation of nasal shedding, seeder pigs were returned to their original room and commingled with the contact pigs. Pigs were observed daily and individual nasal swabs were collected from all pigs in the room until the study ended at 8 days post contact (DPC) exposure. The seeder pig remained in the room with the contact pigs for the duration of the study.

Air sampling

Air samples were collected using a liquid cyclonic collector (Midwest Micro Tek) as described in prior studies (Dee et al., 2009; Otake et al., 2010; Pitkin et al., 2009). The cyclonic collector was placed in the approximated middle of the pig housing area, hung from an aluminum pole 80 cm away from the ground and 1.45 m away from the wall. The pigs did not have direct access or contact with the device.

For sample collection, the device, which is able to process 400L of air per min, was allowed to run for 30 min at each sampling point. Ten mL of minimum essential media (MEM) supplemented with 4% bovine albumin serum were used as the collection media. After collection, approximately 5 mL of MEM were recovered using a sterile syringe (Tyco-Healthcare, Kendall Monoject) and stored in sterile 15 mL polystyrene tubes (Sarstedt). The collector was then disinfected with alkyl dimethyl benzyl ammonium chloride spray (Lysol, Reckitt Benckiser), rinsed with water, and dried with paper towels (Kim wipes, Kimberly-Clark).

A total of 3 air samples were collected each day. Samples were collected every 8 h at approximately 6:00 AM, 2:00 PM and 10:00 PM. A negative control sample was collected before the seeder pig was commingled with the contact pigs. Samples were collected until day 8 and 6 post contact for replicates 1 and 2 respectively.

Liquid cyclonic collector sensitivity

To determine the sensitivity of the liquid cyclonic collector used in this study, the validation of the cyclonic collector was performed by artificially generating aerosols into a plastic film chamber (L 1.83 m x W 2.24 m x H 1.95 m) within a designated isolation room through a cold fog mister (Dyna-Fog Hurricane ULV/Mister, Curtis Dyna-Fog, Ltd.) used in previous studies (Dee et al., 2006). The mister was located in one corner of the chamber with the nozzle set at a 40° angle. A small opening was made in the plastic

film where the mister nozzle was introduced and taped, thus, forcing the output to go straight into the chamber airspace. The cyclonic collector was hung from a metal pole and located in the middle of the chamber area 1.45 m away from the wall and 0.8 m from the ground.

For the generation of artificial bioaerosols, five 900 mL solutions with different concentrations of influenza virus A/Sw/IA/00239/2004 were prepared by diluting a 100 mL of stock virus with an original concentration of 10^6 $TCID_{50}$/mL in 10-fold dilutions to produce solutions ranging from 10^1 $TCID_{50}$/mL to 10^5 $TCID_{50}$/mL. The validation started with the aerosolization of 900 mL virus free MEM which served as a negative control and then followed by aerosolization of the other solutions in an ascending manner. Upon aerosolization, the cyclonic collector was placed in the chamber and allowed to run for 30 min. Samples were collected as described above. Time between collections ranged from approximately 10 to 20 min.

Diagnostic Assays

Serum samples were tested by HI (Pedersen, 2008) and ELISA (IDEXX Laboratories, Westbrook, Maine, USA) (Ciacci-Zanella et al., 2010). Air samples, cyclonic collector swabs and nasal swabs were tested by RRT-PCR (Slomka MJ, Densham AL, Coward VJ, Essen S, Brookes SM, Irvine RM, Spackman E, Ridgeon J, Gardner R, Hanna A, Suarez DL, Brown IH, 2010). All assays were conducted at the University of Minnesota Veterinary Diagnostic Laboratory. Virus titration was attempted from the RRT-PCR air positive samples in Madin-Darby canine kidney (MDCK) cells. Supernatants from air samples were titrated using 10-fold serial dilutions and expressed as a log 10 $TCID_{50}$/mL calculated by the Spearman–Karber method.

Statistical Analyses

Multivariate logistic regression using repeated measures was used to analyze the dataset. The outcome variable was air sample influenza status. A correlation matrix with

a compound symmetry structure was used to account for dependency between consecutive air samples. There were only two predictors in the model: number of positive pigs and replicate. It was assumed that the number of positive pigs detected every day was constant throughout the sampling day. Statistical procedures were performed in SAS 9.2 (SAS Institute Inc.).

Results

Experimental infection

Serum and nasal swab samples collected from the study pigs at the farm of origin and upon arrival at the isolation facilities were negative for influenza (data not shown). After challenge, both seeder pigs shed influenza in their nasal secretions. At 48 h post inoculation and prior to exposure with the contact pigs, virus shedding in nasal secretions of the seeder pigs was $5x10^3$ $TCID_{50}$/mL in replicate one and $1x10^4$ $TCID_{50}$/mL in replicate two.

A total of 154 nasal swabs were collected during both replicates, of which 88 were collected during the first replicate. In both replicates, one positive pig was detected at 1 DPC which corresponded to the seeder pig. On 2 DPC, the number of positive pigs increased to four and then continued to increase gradually until all contact pigs became positive (Figure 5.1). Over the time course of the experiment, the average ct value in nasal swabs of the infected pig population decreased. Air sample mean ct values followed a similar pattern (Figure 5.2).

Air sampling

All air samples collected during the validation of the cyclonic collector were RRT-PCR positive and titrated except for the negative control (Table 5.1). Limit of detection for the cyclonic collector was estimated at 10^1 $TCID^{50}$/mL.

A total of 43 air samples were collected during both experimental infection replicates. The first positive air sample was detected at 2 days post-contact (DPC) when the seeder pig and 3 contact pigs were detected RRT-PCR positive. Detection of positive air samples increased to 100% by day 4 in replicates 1 and day 3 in replicate 2 (Figure 5.1). In replicate 1, 13 of 25 (52%) air samples were RRT-PCR positive with a mean and standard deviation cycle threshold (ct) value of 33.97 ± 0.82 (Figure 5.2). In replicate 2, 12 of 18 samples (66.6%) were positive with a mean and standard deviation ct value of 32.95 ± 1.41.

Positive bioaerosols were consistently detected between 3 DPC and 6 DPC when most pigs were also shedding virus in nasal secretions. There were 3 RRT-PCR suspect bioaerosol samples (ct values >35 and <40) which were classified as negative by virus isolation. Two of the suspects were observed on 1 DPC in each replicate, a time when only the seeder pig was positive for influenza in nasal secretions.

No virus was isolated from the air samples collected from experimentally infected pigs. All cyclonic collector post-disinfection swabs were RT-PCR negative for influenza.

Multivariate logistic regression

The odds of detecting a positive air sample increased 2.2 times (95% CI 1.29, 3.76) per additional positive pig detected. There was no significant effect attributed to replicate (OR= 0.28, 95% CI 0.01, 5.69). However, replicate was retained in the model to adjust for any differences between replicates that might have occurred, such as the use of different cyclonic collector units.

Discussion

Understanding transmission of influenza in pigs is crucial. Pigs play a central role in the ecology of influenza since they can serve as sources of viruses for swine, avian and human influenza viruses which can result in new strains of zoonotic and pandemic

potential (Brown, 2000; Ma et al., 2009). Aerosol transmission of influenza viruses is poorly understood but this route may be significant in large pig populations in areas of high pig density (Maes et al., 2000; Poljak et al., 2008). In addition, understanding the risk of airborne transmission is important since infected pigs may pose a risk to other populations, including humans (Van Reeth, 2007). In this study, we were able to temporally characterize the detection of airborne influenza production by pigs in relation to prevalence of infection in an experimentally infected population. To our knowledge, this is the first study where a temporal relationship between shedding patterns, onset of infection, and prevalence has been established.

Influenza RNA was detected in 58% of the air samples collected from experimentally infected pigs. Detection was constant from 3 DPC through 6 DPC and coincided with maximum nasal secretion virus shedding prevalence. The timing of detecting influenza in aerosols was dependent on the course of infection, the quantity of animals shedding at a given point in time, and the quantity of virus being shed. The first positive air sample was detected at DPC 2 in both replicates when 4 pigs had already tested positive by individual RRT-PCR on nasal secretions. The detection of influenza in the air is related to individual pig results in that positive air samples were detected when the quantity of influenza positive pigs was increasing. Detection prior to 2 DPC yielded only suspect RRT-PCR results, although the seeder pig tested positive at that time. These results indicate that saturation of the air with viral particles is dependent on a minimum threshold of infected pigs. That is, for bioaerosols to be detected in both replicates of this study, four pigs had to be shedding influenza in nasal secretions. The threshold required to detect airborne influenza may vary according to housing conditions, air sampling methods, environmental conditions and viral genotype and/or phenotype (Pearce et al., 2012). Additionally, our logistic regression model identified a relationship between number of positive pigs and influenza detection in the air. The odds of detecting an influenza positive air sample increased 2.2 times per additional nasal secretion positive pig. This suggests that the risk of aerosolization, and perhaps aerosol transmission,

increases as the number of acutely infected pigs increase. More studies are needed to determine whether this relationship is maintained in endemically infected pig populations in agricultural settings.

RRT-PCR ct values were used as a semi-quantitative approach to determine virus levels. Mean ct values in air samples were higher but paralleled mean ct values in individual pigs. These results indicated that the quantity of virus detected in air samples was related to the amount shed by the pigs but overall virus in the air was lower than the amount shed by the individual pigs. It is known that influenza infected pigs can start shedding virus on day 1 after infection, and as the infection progresses, viral concentration in nasal secretions increases until it reaches a maximum and then gradually decreases (Van Reeth et al., 2003).

The study reported here was conducted in a mechanically controlled ventilated building with minimal environmental temperature and relative humidity oscillations. Differences in environmental conditions may affect virus survival and therefore ability to detect the virus from the air. Therefore our results may not precisely correlate with detection under field conditions and further investigation is required. In this study a single H1N1 viral strain was used. Differences in aerosol transmission have been reported for different strains of influenza (Chou et al., 2011; Pearce et al., 2012) and for other viruses (Cho et al., 2007). Therefore more studies are needed to determine whether the relationship observed in this study is maintained for other strains.

Even though the evaluation of the diagnostic sensitivity of the air sampling device was beyond the scope of this study, it is important to mention that the cyclonic collector proved to be an analytically sensitive method for detecting airborne influenza virus since influenza could be detected at very low aerosolized concentrations of 10^1 $TCID_{50}$/mL. Similar sensitivity has been reported for the detection of other viruses via the cyclonic collector (Pitkin et al., 2009). Additionally, cell culture titration of air samples from the

experimentally generated aerosols also proved that the device may be a good candidate for collecting viable viral particles. However, in this study influenza virus was not isolated from air samples originating from experimentally infected pigs. The most likely explanation was a low concentration of viral particles in air samples given the high RRT-PCR ct values obtained from the air samples.

This study characterizes the temporal relationship between influenza nasal secretion shedding in pigs and the likelihood of detection of airborne influenza A virus as a first step to assess risk of airborne influenza transmission from acutely infected pigs. The results provide a deeper understanding of the temporal dynamics of influenza A virus presence in aerosols and indicate that the risk of influenza aerosols is directly related to the number of pigs shedding in a group. Further studies are needed to determine the risk of influenza aerosols under field conditions. Understanding the significance of aerosol transmission under field conditions is crucial to assess the transmission risk in pigs and from pigs to other species including people.

Table 5.1. Virus titration and RRT-PCR cycle threshold values from air samples collected from artificially generated aerosols.

Influenza aerosolized stock dilutions[a] $TCID_{50}$/mL	Influenza titres in air samples $TCID_{50}$/mL	RRT-PCR cycle threshold values[b]
10^1	10^1	34.4
10^2	10^1	30.19
10^3	10^1	27.91
10^4	10^2	23.57
10^5	10^3	20.19

[a]A total of 900 mL were aerosolized per solution

[b]Cycle threshold: positive Ct <35, suspect = Ct 35.00 – 40, negative = Ct >40

Figure 5.1. Proportion of positive pigs and air samples detected throughout the study for replicate 1 (top) and replicate 2 (bottom).

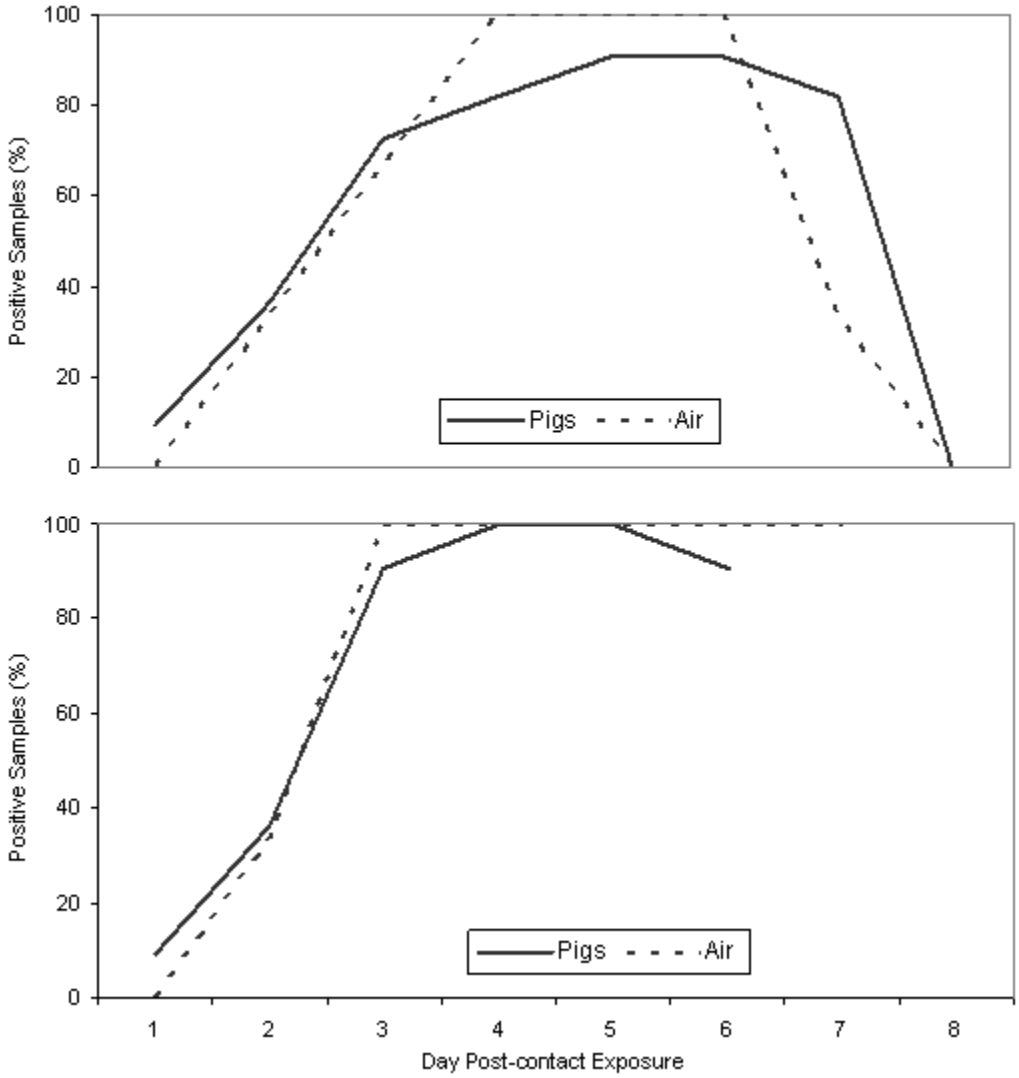
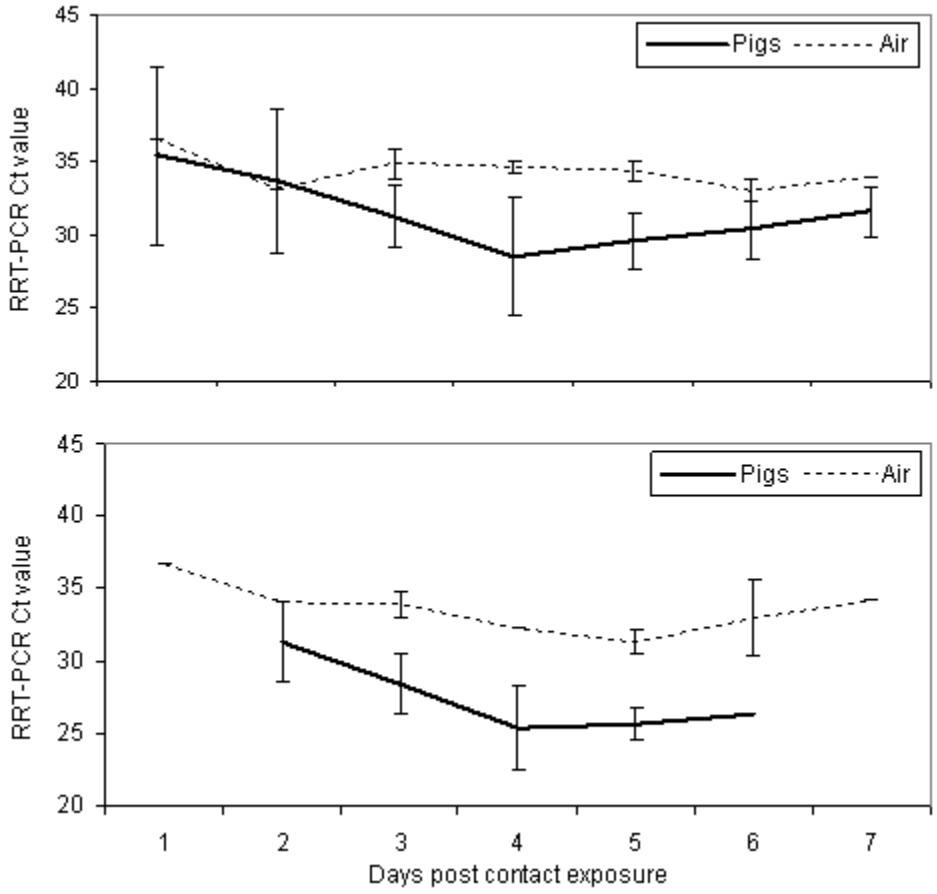


Figure 5.2. Mean and standard deviation RRT-PCR cycle threshold (ct) values for nasal swabs and air samples collected from experimentally infected pigs for replicate 1 (top) and replicate 2 (bottom).



Chapter 6: Detection of airborne influenza A virus in experimentally infected pigs with maternally derived antibodies

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Introduction

Influenza A virus infections (flu) in swine cause respiratory disease and decreased growth performance (Olsen et al., 2006). Different studies have demonstrated that flu is widespread in swine throughout the world and the constant risk of virus transmission, especially to humans (Hayden and Croisier, 2005; Jung et al., 2002; Maes et al., 2000; Maldonado et al., 2006; Myers et al., 2007; Poljak et al., 2008), calls for the need to have a better understanding of influenza transmission in pigs.

Influenza A virus can be transmitted through direct nose-to-nose contact and aerosols, however, information on flu transmission in pig populations is scarce (Brown, 2000). Neonatal pigs have been shown to play a key role in the transmission of influenza viruses within and between herds in that they represent a continual reservoir of infectious virus within breeding herds and a source for grow-finish sites (Allerson and Torremorell, 2011). Dam vaccination is a common approach for controlling influenza virus in swine breeding herds. In North America, pre-farrow dam vaccination is performed in 70% of the herds having 500 or more sows (NAHMS, 2006). Dam vaccination is directed at protecting breeding animals from reproductive losses such as abortions and returns to estrus (Karasin et al., 2000; Vannier, 1999). More importantly, dam vaccination aims at conferring maternal derived antibodies (MDA) to the newborn piglet (Thacker and Janke, 2008) to reduce clinical signs upon infection. Research suggests that pigs with MDA and challenged with homologous (Loeffen et al., 2003) and heterologous (Kitikoon et al., 2006) strains can be partially protected against influenza-like clinical signs although viral shedding might not appear to be influenced by the presence of MDA (Kitikoon et al., 2006; Loeffen et al., 2003). In contrast, other reports suggest that homologous maternal immunity achieved in neonatal pigs through dam vaccination and ingestion of colostrum can reduce the transmission of influenza virus in neonatal pig populations (Allerson et al., 2010). Overall, the degree that MDA impact the transmission of influenza viruses is not well understood and needs further evaluation.

Recent reports have also highlighted the importance of aerosol transmission, not only in humans (Tellier, 2009), but also in different species such as ferrets (Munster et al., 2009), guinea pigs (Lowen et al., 2006) and chickens (Yee et al., 2009). Airborne influenza transmission is considered important in pigs, although the data on swine influenza virus are scarce. There is anecdotal and epidemiological information that implicates pigs as the virus source for neighboring turkey flocks (Choi et al., 2004; Corzo et al., 2012c). Furthermore, epidemiological studies (Maes et al., 2000; Poljak et al., 2008) have also concluded that pig farm density is a risk factor for influenza seropositive pig herd status pointing at the airborne route as the likely route for regional virus spread. Recently, swine influenza virus could be detected in the air from experimentally infected pigs (Loeffen et al., 2011). Characterization of infectious aerosols was further evaluated in acutely infected pigs showing a strong correlation between presence of influenza virus in the air and the number of shedding pigs in a population (Corzo et al., 2011).

However, the impact of maternally derived immunity on influenza virus transmission, specifically aerosol transmission, in swine has not been assessed. Therefore the objective of this study was to determine if pigs with MDA are able to generate detectable levels of infectious influenza aerosols under experimental conditions.

Methods

All procedures were conducted in accordance with the University of Minnesota Institutional Biosafety Committee (IBC) and Institutional Animal Care and Use Committee (IACUC).

Study site

The study was performed at the University of Minnesota College of Veterinary Medicine animal research facilities (St. Paul, Minnesota). The facility is a BSL-2 research unit equipped with showers and a negative pressure ventilation system with dedicated materials and personnel. For the purpose of this study, enhanced personal

biosecurity measures were followed which included the use of room specific personal protective equipment including N-95 respirators, goggles, gloves, hairnets and dedicated clothing and footwear.

Each isolation room had a utility area of 2.08 m^2 where the feed bin and cleaning tools were stored and one housing pen of 7.28 m^2. The utility area and the pen were separated by a metal gate. The walls were made of brick and tile and the floor was solid concrete. Each pen had one water line with two water nipples. The distance between the floor and the ceiling was 3.75 m and the total airspace volume was 35.1 m^3 per room.

Study animals

Thirty-three individually identified pigs from an influenza virus free herd were used in this study. Prior to the start of the study the herd had been serologically tested and deemed negative for porcine reproductive and respiratory syndrome virus (PRRSv), *Mycoplasma hyopneumoniae* and influenza A virus. Pigs were derived from sows that had either been vaccinated or un-vaccinated (controls). Eight sows were divided into three groups in which three sows were vaccinated 38 and 17 days pre-farrow with a killed triple reassortant influenza virus, A/Swine/IA/00239/2004 (H1N1) belonging to the H1 beta cluster (VAC-HOM); three different sows were vaccinated at the same time with a killed triple reassortant influenza virus A/Swine/IL/02450/2008 (H1N1) belonging to the H1 alpha cluster (VAC-HET). The viruses used in the vaccines for the VAC-HOM and VAC-HET groups were genetically distinct and shared 86% hemagglutinin (HA) nucleotide gene similarity. The remaining sows were left unvaccinated and served as the source for control (CTRL) and seeder pigs. Upon farrowing, piglets were ear-tagged and remained with their biological mother until weaning. Piglets were handled following standard farm production practices. Blood samples were collected from piglets and tested for influenza antibodies via hemagglutination-inhibition (HI) (Pedersen, 2008) using the challenge virus (A/Swine/IA/00239/2004 H1N1) and enzyme linked immunosorbent assay (ELISA) (IDEXX Flock Chek™ AI MultiS-Screen, IDEXX Laboratories,

Westbrook, Maine, USA) (Ciacci-Zanella et al., 2010).

Each group consisted of 11 pigs total – 10 pigs born from sows vaccinated homologously, heterologously, or unvaccinated plus one "seeder pig" born from unvaccinated sows. Pigs within groups were selected based on HI titers. Pigs with the highest HI titers in each litter were included in the study. Upon weaning, pigs were transported to the isolation animal unit at the University of Minnesota and were housed in separate rooms based on their treatment allocation. Space allowance was 0.58 m^2 per pig and pigs had *ad-libitum* access to feed and water throughout the study. Nasal swabs from all piglets were collected on the same day and tested for influenza RNA using real-time reverse transcriptase PCR (RRT-PCR) targeting the matrix gene (Spackman et al., 2002; Spackman and Suarez, 2008) as described below.

Air sample collector

Air was sampled using a liquid cyclonic collector (Midwest Micro Tek, Brookings, South Dakota, USA) previously validated for the detection of airborne influenza A virus. Limit of detection was previously estimated at 10 $TCID_{50}$/ml (Corzo et al., 2011). Briefly, the sampling device is comprised of three components: a) an electric power source, b) a turbine powered by an electric motor which is attached to an aluminum four legged structure, and c) a removable aluminum-plastic collection vessel in which liquid media is placed for sample collection (Figure 6.1). When the collector is running, air is sucked in from underneath the collection vessel into the collection vessel containing liquid media. This turbulence creates the mixing effect between the air and the liquid media. Once the air hits the diagonal collection vessel wall, the air is forced to exit upwards through circular openings located on the aluminum four legged structure. This effect results in particles present in the air being washed with liquid media. This device is able to process 400L of air per minute. Three separate devices were used in our study and randomly assigned to a specific room.

Experimental inoculation and exposure

The seeder pig from each group was taken to a separate isolation room for challenge inoculation. Pigs were sedated by an intramuscular injection with a rapid-acting anesthetic (Telazol, Fort Dodge Animal Health, Fort Dodge, IA, USA) at a dose of 6.6 mg/kg. Once pigs were sedated, they were manually restrained and the oral cavity exposed. A catheter attached to a syringe was used to deliver 0.5 mL of A/Swine/IA/00239/2004 (H1N1) influenza A virus containing 1 x 10^7 $TCID_{50}$/ml into the trachea. Additionally, 0.25 mL of the same inoculum was delivered into each of the nostrils. Nasal swabs of seeder pigs were collected at 24 and 48 hours post inoculation and tested for influenza virus RNA by RRT-PCR. Upon confirmation of infection, seeder pigs were returned to their original room and commingled with the rest of the study (contact) pigs. Pigs were observed daily and individual nasal swabs were collected from all pigs in the room until the study ended at 10 days post contact exposure. The seeder pig remained in the room with the rest of the pigs throughout the duration of the study.

Clinical signs

Influenza like clinical signs consisting of cough, nasal discharge and sneezing were recorded daily throughout the study. Pigs were observed during 10 min by the same person and presence or absence of clinical signs was noted.

Personnel flow and air sampling process

A total of 3 air samples were collected each day in each group for a total of 10 days. Samples were collected every eight hours at approximately 6:00 AM, 2:00 PM and 10:00 PM. Personnel movement between rooms required the use of room specific coveralls, hairnets, gloves and boots. Personnel would start the sampling process by first entering the room housing the VAC-HOM, followed by the VAC-HET and finally the CTRL group.

Air sampling procedures were collected by pouring 10 mL of minimum essential

media (MEM) supplemented with 4% bovine serum albumin into the cyclonic collector collection vessel. The collector was hung from an aluminum pole 80 cm away from the ground and 1.45 m away from the wall and allowed to run for 30 minutes. The pigs did not have direct contact with the air sampling device. After collection, approximately 5 ml of MEM were removed from the collection vessel using a sterile syringe (Tyco-Healthcare, Kendall Monoject, Mansfield, MA, USA) and stored in sterile 15 ml polystyrene tubes (Sarstedt, Newton, NC, USA). The collector was then disinfected with alkyl dimethyl benzyl ammonium chloride spray (Lysol, Reckitt Benckiser, Wayne, NJ, USA), rinsed with water, and dried with paper towels (Kim wipes, Kimberly-Clark, Roswell, GA, USA). After disinfection, the collection vessel and turbine were swabbed for PCR testing.

Diagnostic Assays

All procedures were conducted at the University of Minnesota. Serum samples were tested by HI (Pedersen, 2008) and ELISA (IDEXX Flock Chek^{TM} AI MultiS-Screen, IDEXX Laboratories, Westbrook, Maine, USA) (Ciacci-Zanella et al., 2010). Air samples, nasal and cyclonic collector swabs were tested by RRT-PCR (Spackman et al., 2002; Spackman and Suarez, 2008). Virus isolation was attempted on air samples collected between days 3 and 9 post contact exposure on Madin-Darby canine kidney (MDCK) cells. Titration was attempted in VI positive air samples. Supernatants from air samples were titrated using ten-fold serial dilutions and expressed as a log 10 $TCID_{50}$/ml calculated by the Spearman–Karber method.

Statistical Analyses

All statistical procedures were conducted in SAS 9.2 (SAS Institute Inc., Cary, NC, USA). Differences were considered statistically significant at $P<0.05$. HI and ELISA antibody titers comparison between groups was performed through analysis of variance. Fisher's exact test was used to assess the relationship between treatment and detection of influenza A virus in air samples by either RRT-PCR or virus isolation. Logistic

regression was used to investigate whether there was a relationship between the numbers of RRT-PCR positive pigs and the ability to detect virus in the air. Relationship between the immune status of the pig and the presence of clinical signs was assessed by logistic regression repeated measures.

Results

Serum samples from pigs born to vaccinated sows collected before arrival to the isolation facilities were positive by HI and ELISA, and samples from pigs born to unvaccinated pigs were negative. HI titers against the challenge strain differed significantly between the VAC-HOM and the VAC-HET groups but not between the VAC-HET and the CTRL group (Table 6.1). A significant difference was detected on the ELISA titers between the pigs born to vaccinated sows and the pigs born to non-vaccinated sows. Nasal swabs from all three groups were RRT-PCR negative for influenza virus on arrival.

All three seeder pigs became infected with influenza A after challenge. A total of 330 nasal swabs were collected from all individual pigs from 0 days post contact (DPC) until the end of the study period. All seeder pigs tested positive for influenza during the first three DPC. Besides the seeder pigs, six positive swabs were detected in the control group and none in both the VAC-HOM and VAC-HET groups on three DPC. From four DPC onwards the number of positive swabs increased in both the CRTL and the VAC-HET group until 5 and 6, respectively, when they reached the maximum number of positive individual results (Figure 6.2). Clinical signs in all three groups were mild and there were no between groups significant differences ($P > 0.05$).

A total of 31 air samples were collected from each treatment group. In the VAC-HOM group, there were no positive air samples; However, one sample was classified as suspect at 6 DPC by RRT-PCR (Table 2). A total of 3.2% and 6.4% of the samples were positive in the VAC-HET and the CTRL groups, respectively. There were 19.3% samples

considered suspect in the VAC-HET group and 9.6% in the CTRL group. There were no statistically significant differences between influenza A virus detection in the air across treatment groups and the number of either positive RRT-PCR or virus isolation positive air samples.

Influenza virus was isolated and titrated from six air samples. Four of the samples originated from the VAC-HET group with a virus titer of 3.2×10^2 $TCID_{50}$/ml. Two of these samples were classified as suspect by RRT-PCR at 6 DPC, one was positive by RRT-PCR at 8 DPC and another was negative at 8 DPC by RRT-PCR. The other two titrated samples were from air samples from the CTRL group. The sample at 7 DPC which tested positive by RRT-PCR had a virus titer of 3.2×10^2 $TCID_{50}$/ml whereas the sample that was classified as suspect on 8 DPC had a virus titer of 1×10^2 $TCID_{50}$/ml. All cyclonic collector post-disinfection swabs were RRT-PCR negative for influenza.

Due to the low number of events (positive air samples) within groups, a logistic regression model to determine the relationship between positive pigs and detection of virus in the air could not generate estimates.

Discussion

Pre-farrow dam vaccination is widely used in swine to minimize the impact of influenza infections in breeding herds and neonatal pigs. The immune response generated in sows can be transferred to newborn piglets through ingestion of antibody rich colostrum. The transfer of MDA can potentially protect neonatal pigs against influenza challenge including the reduction of clinical signs. However, it is not well understood whether pigs with MDA can be a source of infectious influenza aerosols when they become infected with influenza virus. Therefore, we conducted this study to evaluate whether pigs with MDA can generate infectious aerosols upon infection with influenza virus.

Results from our study indicate that pigs can be a source of infectious aerosols for influenza since virus was isolated from air samples. Furthermore, infectious aerosols could be generated by pigs with MDA without obvious clinical signs of influenza infection. Generation of infectious aerosols was more relevant in pigs with partial immunity (heterologous MDA) compared to pigs from the homologous MDA group in that this situation somewhat reflects current field conditions. The VAC-HET group is most likely to reflect field situations given the diversity of influenza infections in pigs. The presence of both influenza RNA and viable viral particles in air samples collected from recently weaned pigs with and without maternal immunity highlight the risk of aerosol transmission in neonatal pigs. In this study, pigs became infected and were able to generate infectious aerosols suggesting that aerosol transmission can take place in partially immune breeding herds and weaned pig populations. Furthermore, pigs without obvious clinical signs of influenza infection could also generate infectious aerosols. Therefore, these results add one more piece of information to the complex ecology of influenza A virus in swine.

Individual pig results from the VAC-HOM group indicated that pigs were protected against influenza infection and virus transmission could only be detected in one contact pig. Protective immunity induced by vaccination can result in the reduction of nasal shedding and pneumonia (Bikour et al., 1996; Vincent et al., 2010) due to specific IgA and IgM limiting virus attachment and replication in the respiratory epithelial cells (Cox et al., 2004; Richt et al., 2006). In our study, pigs in the VAC-HOM group had significantly higher HI titers compared to the VAC-HET and CTRL groups. This may be the reason why there were no positive air samples identified in the VAC-HOM group. In contrast, there was active virus shedding in the CTRL and the VAC-HET group which paralleled the detection of the virus in the air. Detection of the virus in the air was positive at 6 and 7 DPC in the VAC-HET and CTRL groups, respectively, which also corresponded to the period of time when most (e.g. 80 – 100 %) of the pigs were positive. Cross-reactive HI titers in the VAC-HET and the CTRL were significantly lower

compared to the VAC-HOM group which most likely contributed to the susceptibility of influenza A virus infection and shedding of virus in aerosols in these groups.

The overall frequency of influenza RNA detection in air samples in this study is low compared to our previous experience (Corzo et al., 2011). One possible explanation is the bodyweight and age of the pigs used in each study. Pigs in this study were 3 week old with an average weight at infection of 5.17 kg (SD 2.32) versus the pigs used in the previous study which were 7 week old and weighed approximately 25 kg. Lung size is related to bodyweight and is considered a predictor of air exchange also known as tidal volume (TV). TV is the amount of air inspired and expired in one breath in a healthy animal (Tranquilli et al., 2007). The TV for swine has been estimated to be 11 ml/kg (Riebold et al., 1995) suggesting that the volume of air expired by the 3 week-old pigs would be five times smaller than the air expired by 7 week-old pigs. Based on this, and assuming that 3 and 7 week old pigs have similar virus titers in the lung (based on the same square area), the total amount of air containing viral particles aerosolized by 3 week-old pigs compared to 7 week-old pigs is predicted to be lower. Therefore, saturation of the air with influenza virus particles would be more efficient with larger pigs and therefore the likelihood of detection of viral particles in the air would also be higher in older pigs. Additionally, the level of virus replication in the lungs could also impact the level of virus found in aerosols. In this study we did not attempt to quantify the level of virus replication in the lungs. Whether younger pigs would have similar or distinct virus replication rates in the lung compared to older pigs and whether that would have a significant effect on virus aerosol generation needs to be further investigated.

Alternatively, the virus could have been present in the air but under the detection limit of the cyclonic collector. Although the limit of detection for the cyclonic collector for influenza is considered low at 10 $TCID_{50}$/mL (Corzo et al., 2011), there might not have been enough virus accumulated in the air during the first few days post contact to yield a positive result. This could be the result of specific housing conditions, rates of air

change (e.g. 12 to 15 per hour in this study) or environmental conditions that may have influenced our ability to detect the virus in the air. In addition, we were not able to explore the relationship between the number of pigs shedding virus with detection of virus in the air most likely due to the low number of positive results available to fit the statistical model (Hosmer and Lemeshow, 2000).

Results from this study point towards the importance of aerosol transmission under field conditions. However housing conditions in this study compared to field settings can be significantly different which may influence the direct applicability of these results. Pigs in this study were kept in small rooms with good air quality, constant ventilation rates and limited temperature oscillations that may not mimic field situations. Under field conditions weaned pigs of similar age to the ones used in this study are kept in nursery or wean-to-finished facilities and may have lower or variable ventilation rates and higher densities which may contribute to higher saturation rates of the air with infectious aerosols. In contrast, pigs in breeding herds prior to weaning are younger than the pigs used in this study and overall piglet density in farrowing rooms will be lower which may suggest that aerosol transmission might not be as relevant prior to weaning compared to post-weaning. Furthermore, under field conditions pigs will have more variable levels of MDA which will also influence the degree of influenza susceptibility, virus replication, virus transmission and therefore the level of infectious aerosols generated. Nevertheless further research is needed to assess the importance of aerosol transmission under field conditions and which factors contribute to it.

This study reports the detection of infectious airborne influenza A virus in air samples collected from weaned pigs with maternal immunity without obvious clinical signs. Despite the low number of positive air samples in pigs with partial immunity, this study highlights the potential for pigs with MDA to generate infectious aerosols. Airborne influenza A viral particles generated by weaned pigs or even suckling pigs can represent a risk for influenza transmission and further complicate the ecology of

influenza virus and complexity of influenza transmission. More studies are needed to further our understanding on the effect of immunity on the generation of infectious aerosols and their role on influenza transmission under field conditions.

Table 6.1. Geometric means of the reciprocal hemagglutination inhibition (HI) against the challenge virus and ELISA serologic results from pigs born to vaccinated and non-vaccinated sows before contact exposure.

Group	HI Geometric means		ELISA
	A/Swine/IA/00239/2004 H1N1	A/Swine/IL/02450/2008 H1N1	Mean ± SD
VAC-HOM	320 [a]	60.63 [a]	0.148 ± 0.048 [a]
VAC-HET	13.2 [b]	85.74 [a]	0.199 ± 0.134 [a]
CRTL	10 [b]	21.44 [b]	0.869 ± 0.024 [b]

Different superscripts within the column differ significantly $P<0.05$

Table 6.2. Number of positive and suspect RRT-PCR results and mean cycle threshold (Ct) values for nasal swabs and air samples, and number of virus isolation positive air samples and virus titers for each group throughout the study after introducing an influenza A virus infected pig (seeder pig) within each group.

	VAC-HOM						VAC-HET						CTRL					
	Nasal swabs		Air samples				Nasal swabs		Air samples				Nasal swabs		Air samples			
DPC[1]	No. Pos. RRT-PCR[2]	Mean Ct[3]	No. Pos. Susp. RRT-PCR[4]	Mean Ct[5]	No. V.I[6]	Virus titer[7]	No. Pos. RRT-PCR[2]	Mean Ct[3]	No. Pos. Susp. RRT-PCR[4]	Mean Ct[5]	No. V.I[6]	Virus titer[7]	No. Pos. RRT-PCR[2]	Mean Ct[3]	No. Pos. Susp. RRT-PCR[4]	Mean Ct[5]	No. V.I[6]	Virus titer[7]
0	1/11	28.36	0/3	>40	NT	NT	1/11	26.61	0/3	>40	NT	NT	1/11	29.19	0/3	>40	NT	NT
1	1/11	29.46	0/3	>40	NT	NT	1/11	29.52	0/3	>40	NT	NT	1/11	30.12	0/3	>40	NT	NT
2	1/11	31.57	0/3	>40	NT	NT	1/11	31.15	0/3	>40	NT	NT	2/11	33.5	0/3	>40	NT	NT
3	1/11	31.57	0/3	>40	NT	NT	1/11	33.15	0/3	>40	NT	NT	8/11	31.85	0/3	>40	NT	NT
4	1/11	35.07	0/3	>40	NT	NT	9/11	32.33	1/3	36.38	0/3	NT	10/11	30.24	0/3	>40	NT	NT
5	1/11	32.29	0/3	>40	NT	NT	9/11	31.67	1/3	36.85	0/3	NT	10/11	30.14	1/3	36.06	0/3	NT
6	1/11	32.48	1/3	39.71	0/3	NT	9/11	30.15	2/3	36.31	2/3	3.2	10/11	29.84	1/3	39.95	0/3	NT
7	1/11	30.48	0/3	>40	NT	NT	9/11	32.00	2/3	36.03	1/3	3.2	10/11	31.86	2/3	34.73	1/3	3.2
8	1/11	28.73	0/3	>40	NT	NT	8/11	34.15	1/3	36.92	1/3	3.2	9/11	34.53	1/3	38.4	1/3	1
9	1/11	32.62	0/3	>40	NT	NT	1/11	34.69	0/3	>40	NT	NT	1/11	39.21	0/3	>40	NT	NT
10	1/11	34.61	0/3	>40	NT	NT	1/11	34.06	0/3	>40	NT	NT	0/11	>40	0/3	>40	NT	NT

[1] Days post contact

[2] Number of RRT-PCR positive and suspect pigs / total number of pigs. Samples are considered RRT-PCR positive Ct <35; suspect Ct 35 – 40; and negative Ct >40.

[3] Mean RRT-PCR cycle threshold (Ct) value of positive and suspect nasal swab results

[4] Number of RRT-PCR positive and suspect air samples / total number of air samples

[5] Mean RRT-PCR Ct value of positive and suspect air samples

[6] Number of virus isolation/ Total. NT= Not tested.

[7] Virus titer in 10^2 $TCID_{50}$/ml. NT= Not tested.

Figure 6.1. Picture of liquid cyclonic air collector. Liquid cyclonic air collector assembled for air sample collection (left). Disassembled liquid cyclonic air collector where the propeller and collection vessel are shown (right).

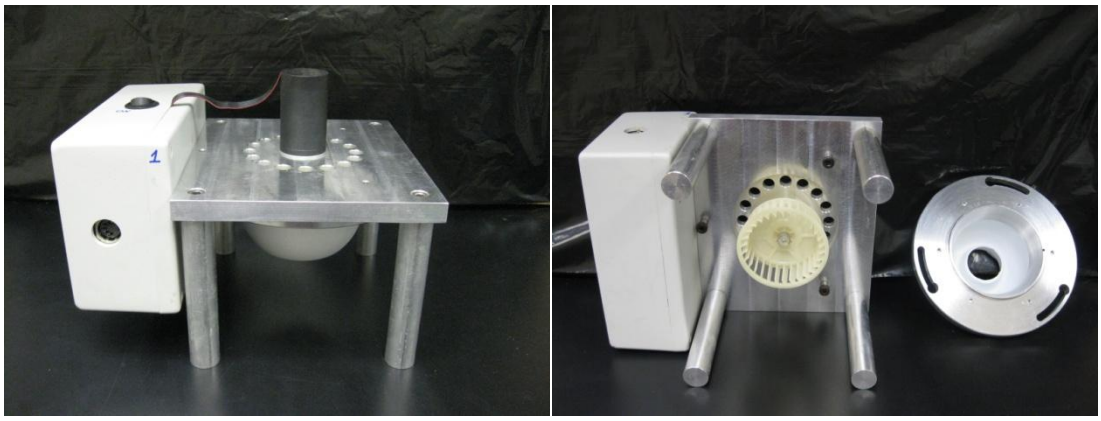
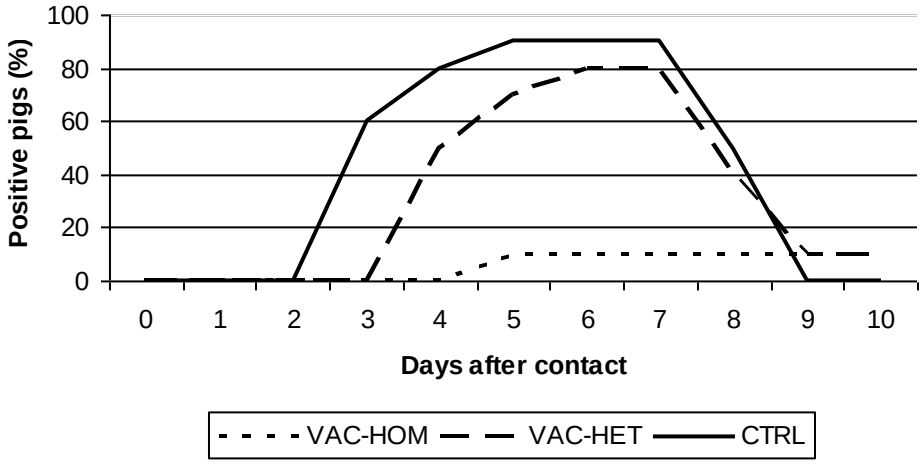


Figure 6.2. Percentage of influenza A virus RRT-PCR nasal swab positive pigs by day post contact-exposure in each group (seeder pigs are excluded from the graph).



Chapter 7: Detection of airborne swine influenza A virus in air samples collected inside, outside and downwind from swine barns

Introduction

Influenza A virus (IAV) is a negative sense single stranded RNA virus belonging to the *Orthomyxovirideae* family (Palese and Shaw, 2007). In swine, IAV causes respiratory disease characterized by anorexia, fever, sneezing, coughing, rhinorrhea and lethargy and the febrile state in pregnant animals can lead to abortions (Karasin et al., 2000; Olsen et al., 2006). The disease is characterized by low mortality but high morbidity, and decreased growth performance which results in increased pig weight variation. Besides the effects on animal health, IAV is an important zoonotic pathogen and pigs can be a source of novel reassortants (Ma et al., 2009), including viruses of pandemic potential. Therefore IAV has implications for both animal and public health, and understanding transmission of IAV in animal populations is crucial to prevent zoonotic infections.

Infected pigs can shed virus through nasal secretions for approximately 5 to 7 days allowing transmission to occur by direct nose-to-nose contact. The airborne route can also play a role in viral spread (Brown, 2000; Olsen et al., 2006). Commonly, sudden respiratory disease outbreaks follow the introduction of infected pigs originating from infected sources (Mohan et al., 1981) resulting in the introduction of new viruses. However, reports of respiratory illness may not always be related to pig introduction. Risk factor studies in Canada (Poljak et al., 2008) and Belgium (Maes et al., 2000) found that the likelihood of a pig farm being positive for influenza was significantly associated with pig farm density, suggesting that other routes, such as airborne, can play a role in between herd transmission. Recently, pig farm proximity to turkey flocks has been associated with turkey seropositivity to swine-origin IAV which suggest that the airborne route may have played a role in transmission (Corzo et al., 2012c). Aerosol transmission of IAV has also been reported in humans, (Blachere et al., 2009; Brankston et al., 2007; Tellier, 2009) mice, guinea pigs, ferrets and chickens (Mubareka et al., 2009; Munster et al., 2009; Schulman, 1967; Yao et al., 2011; Yee et al., 2009) suggesting that airborne transmission of IAV may play an important role in the ecology of influenza. However, the relative importance of this transmission route remains under debate.

In pigs, regional spread of infectious pathogens has been well documented.

Airborne spread has been documented for foot and mouth disease virus, pseudorabies virus, *Mycoplasma hyopneumoniae* and porcine reproductive and respiratory virus (PRRSV) (Christensen et al., 1990; Christensen et al., 1993; Dee et al., 2009; Gloster et al., 2003; Goodwin, 1977; Grant et al., 1994; Otake et al., 2010). Airborne transmission of IAV is poorly understood and thus needs further investigation.

Recently, IAV was detected in aerosols generated from infected pigs vaccinated for IAV and (Loeffen et al., 2011) and also in pigs with passive immunity (Corzo et al., 2012a). Corzo, et al. (Corzo et al., 2011) reported an association between detecting virus in nasal secretions and the likelihood of detecting virus in the air. In these studies, virus could be readily detected in air samples collected during the acute infection phase suggesting that acutely infected pigs can be a substantial source of infectious virus. However, the implications of these studies for field settings can only be speculated. To the authors' knowledge there is no data on the detection of IAV under field conditions. The objectives of this study were to determine if IAV can be detected in air samples collected in swine farms and downwind from them and to determine whether certain climatic conditions are associated with airborne detection.

Methods

Procedures and protocols used in this study were approved by the University of Minnesota Institutional Animal Care and Use Committee (IACUC).

Farm identification and selection

Four pig farms were selected for this study (farms 1 through 4). Farms were identified by contacting veterinarians that care for pig farms in Southern Minnesota and Northern Iowa. Veterinarians were asked to alert the investigators upon sudden onset of respiratory clinical signs in growing pig populations suggestive of acute influenza like illness (i.e. rapid onset of widespread dry hacking cough, sneezing, rhinorrhea, anorexia and lethargy). Farms were included in the study if the veterinarian had a presumptive diagnosis suggestive of influenza or was able to collect samples and confirm the presumptive diagnosis within 2 to 4 days from the onset of clinical signs, and was able to

communicate with the investigators within 2 to 3 days from the onset of disease.

Once the farms had been identified, the investigator traveled to the farm within 2 to 5 days from onset of clinical signs. The clinical history of the break was reviewed and recorded after discussing it with farm personnel. Attempts were made to collect a complete clinical history of the affected groups as well as assigning a clinical score based on severity of respiratory signs. Scores ranged from 1 to 3 where 1 = cough in less than 25% of the pens, 2 = cough and sneezing in 50% of the pens and 3 = cough, sneezing and lethargy in 75% or more of the pens. If there was more than one group of pigs affected in a farm, the group with the most recent onset of clinical signs was selected for testing.

Air sampling procedures

Air samples were collected using a liquid cyclonic collector (Midwest Micro-Tek, Brookings, SD, USA) capable of processing 400 L / min of air. This device has been previously validated for the collection of swine respiratory pathogens including PRRSV, *Mycoplasma hyopneumoniae* (Dee et al., 2009; Otake et al., 2010) and IAV (Corzo et al., 2011). Briefly, 10 mL of a minimum essential medium (MEM) solution supplemented with 4% bovine albumin serum (BAS) were added to the liquid cyclonic collector collection vessel. The cyclonic collector was run for 30 minutes allowing airborne particles to be mixed with the collection media solution. Once air sampling was completed, a sterile syringe (Tyco-Healthcare, Kendall Monoject, Mansfield, MA, USA) was used to recover and place the sample in a plastic vial (Thermo scientific capitol vial, Fisher Scientific, Waltham, MA, USA). Air samples were then stored in ice until they were transported to the laboratory for diagnostic procedures. The device would then be cleaned and disinfected according to a previously validated protocol by spraying alkyl dimethyl benzyl ammonium chloride (Lysol, Reckitt Benckiser, Wayne, NJ, USA) on the turbine and the collection vessel. These two surfaces were then sprayed with water to remove remaining disinfectant and dried with paper towels (Kim wipes, Kimberly-Clark, Roswell, GA, USA) (Corzo et al., 2011).

Air samples from inside swine barns were collected by simultaneously placing four

or five cyclonic collectors throughout the barn. Cyclonic collectors were placed 1.5 m above the floor and 1 m below the ceiling and secured to a feed line using rubber bungee cords. Pigs did not have direct access to the air collection devices. Power extensions were used to supply power to the air sampling devices.

Air samples were also collected from the exhaust fans located in the external barn wall. Air exiting the pig barn was sampled by placing the cyclonic collectors as close as possible to the draft of air exhausted from the pig barn. Cyclonic collectors were placed either on the ground when samples were collected from exhaust manure pit fans or were hung from a tripod when samples were collected from an external wall exhaust fan. Air sampling devices were run simultaneously.

In two of the farms (farms 3 and 4), additional air samples were collected 0.9 to 2.1 Km downwind from the infected pig population. Google Earth Map (Google, Mount View, CA, USA) was used to identify potential sampling locations based on wind direction obtained through www.weather.com. Potential sampling locations were identified as the closest two roads crossed by the downwind. Two collection locations were identified for farms 3 and 4. Upon arrival at these locations, a wind vane was used to identify and confirm the direction where the wind was blowing from. The cyclonic collectors were distributed along the side of the road covering a linear distance of 20 m and run simultaneously. The collectors were placed at distances ranging from 1 m to 1.85 m above the ground and connected through cord extensions to a power source located in the study vehicle.

Sample size and sampling scheme

Fifteen, 30 minute samples were collected from inside the barn and 15, 30 minute samples were collected at the exhaust fan at farms 1 and 2. The sample size was determined to detect IAV in the air in at least one sample when prevalence of detection was at least 10% given a 95% confidence. In farms 3 and 4, there were 15 air samples collected inside the barn and 30 air samples in each of the three exterior locations (exhaust fan, distance 1 and distance 2) for a total of 105 air samples per farm. A sample

size of 60 was chosen to detect at least one positive sample given a prevalence of detection of at least 5% with a 95% confidence. It was assumed that prevalence would be lower for samples collected outside the barn. Outside samples were collected for two consecutive days to increase the probability of detection.

Upon arrival at the farm and confirming observation of clinical respiratory disease, the first set of samples was collected inside the barn. Immediately after, exhaust air samples were collected. In farms 3 and 4, downwind samples were collected after exhaust samples were collected by starting at the location closest to the farm and ending at the farthest location. The following day, sampling collection protocol was reversed starting air sampling at the farthest location, followed by the closest location and ending sampling at the exhaust collection point. Exterior samples were collected first at dusk and into the night (first day) and dawn into the morning (second day) to increase the chances of virus detection (Dee et al., 2009).

Environmental conditions

Temperature (°C), relative humidity (%) and light intensity (watts/m^2) were recorded every minute using a weather station (HOBO, Onset Computer Corporation, Bourne, MA, USA) while air was being collected at the external locations at farms 3 and 4.

Pig population IAV status confirmation

To confirm that the population exhibiting respiratory clinical signs was undergoing an influenza epizootic 15 oral fluid samples (saliva) were collected (95% confident of detecting at least one positive sample given an estimated prevalence of disease of at least 20%). Oral fluids were collected as described previously (Detmer et al., 2011; Prickett et al., 2008; Prickett and Zimmerman, 2010; Ramirez et al., 2011; Romagosa et al., 2011b) by hanging 0.6 m of cotton rope from the pen division horizontal bars underneath where the cyclonic collectors were hung. Pigs were allowed to chew on the rope for approximately 30 minutes. At the end of sampling, oral fluids were obtained by placing the rope in a plastic bag (Ziploc bag, S.C. Johnson & Son, Inc. Racine, WI, USA) and

squeezing it until fluid would be deposited in the bottom of the bag. A 10 mL aliquot was transferred to a tube (Thermo scientific capitol vial, Fisher Scientific, Waltham, MA, USA) from each bag and refrigerated until it was transported to the laboratory.

Diagnostic testing

All air and oral fluid samples were tested at the University of Minnesota Veterinary Diagnostic laboratory for influenza A RNA by a RRT-PCR targeting the matrix gene (Slomka MJ, Densham AL, Coward VJ, Essen S, Brookes SM, Irvine RM, Spackman E, Ridgeon J, Gardner R, Hanna A, Suarez DL, Brown IH, 2010). Samples that yielded a cycle threshold (ct) value below 35 were considered positive whereas those that yielded a ct value between 35 and 40 or higher than 40 were considered low positive or negative respectively. If samples were RRT-PCR positive, they were further tested using virus subtyping, virus isolation using MDCK cells and sequencing (Detmer et al., 2012; Detmer et al., 2011).

Swine Bioassay

To determine whether viral particles contained in the downwind samples were infectious, samples were inoculated into serologically influenza negative pigs. A 2 mL aliquot per pig was used for intra-tracheal inoculation in anesthetized pigs housed at the University of Minnesota animal isolation facilities. Pigs were monitored through nasal swab sampling on days 3, 4, 5 and 6 post-inoculation by RRT-PCR. Pigs were humanely euthanized on day 7 post-inoculation. At necropsy, a tracheo-bronchial swab and lung tissues were collected for RRT-PCR testing. Trachea and lung sections were also examined for histopathological lesions.

Statistical analyses

Kruskall-Wallis test was used to compare the RRT-PCR cycle threshold (ct) values between oral fluids, air samples collected inside the barn and at the exhaust fan. Multivariable logistic regression was used to evaluate whether meteorological factors and sampling distance were associated with detection of IAV in outside air samples. Backward stepwise procedures were used for model building including variables that had

a $P<0.25$ in the univariate analysis and corresponding two-way interaction terms. Variables that were not normally distributed were log-transformed (distance, temperature, relative humidity, solar radiation). Differences were considered statistically significant when $P < 0.05$. All analyses were performed using SAS 9.2 (SAS Institute Inc., Cary, NC, USA).

Results

Detection of IAV by RT-PCR inside barns, at the exhaust fans and downwind

Table 7.1 summarizes the farms tested according to farm type, age, group size, air volume, clinical score and days between onset of clinical signs and investigator's visit.

All oral fluid samples in farms 1, 2 and 4 tested positive by RRT-PCR and 12 of 15 samples (80%) tested positive in farm 3 (Table 7.2). IAV was also detected in all air samples collected inside the barn in farms 1 and 4 whereas in farm 3, 13 of 15 air samples (87%) tested positive. Only two air samples were low positive in farm 2. The number of positive air samples collected at the exhaust fan were 15 of 15 (100%) in farm 1, 2 of 15 (13%) in farm 2, 20 of 30 (67%) in farm 3 and 26 of 30 (87%) in farm 4. H1N2 IAV subtype was detected in farm 1, H1N1 was detected in farms 2 and 3, and H3N2 subtype was detected in farm 4.

Average ct values differed significantly among oral fluids, inside air and exhaust air samples for farms 1, 3 and 4 ($P < 0.05$) with ct values being lowest in oral fluids and highest in outside air. In farm 2, significant differences were only observed between oral fluids and inside air samples ($P < 0.05$) but not between inside and outside air ct values ($P = 1.0$) (Table 7.3).

In regards to the downwind air sampling at farm 3, there was 1 (1.7%) RRT-PCR positive and 9 (15%) low positive air samples. The positive air sample was untypable and was collected 2.1 Km away, whereas the low positive samples were collected at 1.2, 1.8 and 2.1 Km. Partial subtyping in two of the low positive samples collected at 1.2 Km away from farm 3, yielded an H1N-untypable and an H-untypableN1 subtype. At farm 4,

4 (6.7%) and 25 (41.7%) air samples tested positive and low positive, respectively. Positive air samples were collected at 1.5 and 1.6 Km away from the farm whereas the low positive samples were collected at four different sampling points ranging from 0.8 to 1.9 Km. The two samples that tested positive at 1.5 Km yielded either a complete (e.g. H3N2) or a partial (e.g. H-untypableN2) subtype. As for the other two positive samples, both were detected 1.6 Km away from the farm. One of these samples yielded a partial subtype (e.g. H3N-untypable) whereas the second positive sample was untypable (Table 4).

Virus isolation and sequencing

In farm 1, virus was isolated from oral fluids (n=11), inside air (n=6) and exhaust air (n=1). In farm 4, virus was isolated from oral fluids (n=5) and inside air (n=1), but virus was not isolated from exhaust air samples. No viruses were isolated from samples in farms 2 and 3. Sequencing results from oral fluids and air samples was possible only in farms 1 and 4 and these shared ≥ 99% HA virus sequence similarity within each farm (Table 7.5). None of the downwind RRT-PCR positive air samples was virus isolation or swine bioassay positive.

Meteorological conditions and detection of influenza in air samples

Four variables (e.g. distance between sample collection point and the farm, relative humidity, light intensity and farm) were included in the multivariable model (Table 7.6). Two remained in the final multivariable logistic regression model: distance between sample collection point and source farm, and light intensity. Increasing the distance of sample collection from the farm had a sparing effect (OR = 0.15; CI = 0.08, 0.27) on detection and an increase in light intensity decreased the odds (OR = 0.73; CI = 0.54, 0.97) of detection of influenza in air samples.

Discussion

PRRSV, *Mycoplasma hyopneumoniae*, pseudorabies virus and FMDV have been documented to travel long distances and potentially infect other farms (Christensen et al., 1990; Christensen et al., 1993; Dee et al., 2009; Gloster et al., 2003; Goodwin, 1977;

Grant et al., 1994; Otake et al., 2010). Understanding the routes for IAV transmission is vital for designing appropriate IAV control strategies and prevention of zoonotic infections. In this study, we detected infectious IAV from air samples from the interior and at the exit point of swine farms. Three subtypes of IAV (H1N1, H1N2 and H3N2) were detected in barn air, air at the exhaust fans and at selected distances downwind from these farms. Furthermore, the IAV HA sequences identified in the swine oral fluids matched the sequences from IAV in aerosols providing evidence that the viruses detected in the aerosols originated from the pigs in the study.

Results from this study support previous epidemiological risk factor studies (Maes et al., 2000; Poljak et al., 2008) that concluded that swine density is a risk factor for IAV seropositivity. In those studies, the authors suggested that proximity may aid airborne spread of IAV due to generation of infectious particles that are transmitted between farms. Our results indicate that pigs can be a source for infectious aerosols. Although in our study we could not detect viable viral particles in the long distance sampling locations, we speculate that airborne transmission of IAV in pigs is possible, but its role in between herd transmission needs to be further evaluated. Exposure to infectious aerosols is also considered a significant route of transmission of IAV within swine populations (Brown, 2000).

The reasons for not isolating IAV from long distance samples in our study are not entirely clear. We speculate that infectious particles may have maintained their viability but our air collection methods were not able to preserve virus integrity. Isolation of infectious pathogens from air samples is in general poor due to the physical disruption of the pathogen (Fabian et al., 2009; Hermann et al., 2008; Torremorell et al., 1997). Additionally, the number of viable viral particles was probably diluted to very low levels which would most likely result in our inability to isolate the virus. Indeed, our PCR data suggest that there was low quantity of genetic material present in the distant samples given that many samples were low positive and partial subtyping was only successful in some of the samples. This observation is also supported by the direct relationship found between RRT-PCR ct values and sampling location in regards to proximity to the natural

environment of the pig. The closer the samples were taken to the pig, the higher the likelihood of finding positive samples in the air which should not come as a surprise. In addition, interior environments offer better conditions for particle saturation in the environment due to limited drafts whereas outside conditions favor the mixing with outside air drafts which dilutes the concentration of viral particles (Jiang et al., 2009; Moore et al., 1979; Tellier, 2009). Alternatively, the virus was not viable given that specific environmental conditions are required for the virus to be able to survive in the air or that it became inactivated due to residual disinfectant levels in the collection vessel. In our study, we found an association between IAV detection in the air and light intensity and distance from the farm but we could not establish such a relationship with virus viability. Spread of equine influenza virus to newly infected premises has been associated with wind direction, temperature and relative humidity (Davis et al., 2009; Firestone et al., 2012) supporting the hypothesis that regional transmission of infectious influenza particles can occur. However, further research is needed to understand the environmental conditions that support long distance IAV transmission in aerosols generated by pigs.

Exposure to people or other animal species to IAV infected aerosols of swine may be a risk factor for interspecies IAV transmission. Myers et al. reported increased IAV seroprevalence in personnel working with pigs (Myers et al., 2006) and our data suggest that in addition to other routes such as direct contact and fomites, zoonotic infections may also be the result of exposure to infectious aerosols, in particular exposure to indoor contaminated aerosols. However, additional research is needed to further assess the role of aerosols generated in pigs in the development of zoonotic infections.

In this study, temperature and relative humidity were not retained in the final statistical model evaluating the association between IAV detection and environmental conditions. Both of these parameters had low variation and perhaps this was the cause for the lack of association. However, they warrant further investigation since there are reports in which the stability of the particle and airborne transmission of IAV depends on these factors. (Lowen et al., 2007; Schaffer et al., 1976). In contrast, as mentioned above,

both sunlight intensity and distance from the farm had a sparing effect on virus detection supporting the concept of virus dilution or inactivation when exiting the facilities and the germicidal effects of ultraviolet light emissions (Jensen, 1964; McDevitt et al., 2012).

The populations selected for study were conveniently selected for presenting an acute outbreak of influenza infection. This was done to increase our chances to detect virus from the air. Rate of virus detection was low for the farm tested after the acute signs of the disease had disappeared (farm 2). Both time to onset of clinical disease and presence (or lack of) of immunity are associated with detection of virus in the air (Corzo et al., 2011; Corzo et al., 2012b). Whether detection of IAV in aerosols from endemically infected herds without obvious respiratory signs is relevant and whether those herds represent a risk needs to be further evaluated. Because endemically infected populations are common, the role that such populations have in IAV transmission to people or other species still needs to be further elucidated. Transport of IAV in the air might also be associated with co-infections which may increase the likelihood of generating infectious aerosols, but this was not assessed as part of this study and needs further investigation.

This study provides new information into the understanding of IAV aerosol generation in pigs and the role that infectious aerosols play on airborne transmission. Our study is the first to report that pigs acutely infected with IAV release viral particles into barn airspace that can also exit the building and be transported downwind. More importantly, some of these viral particles are infectious and may be a source for infections to other pigs and perhaps to other animal species and people. The distance that IAV can be transported through the air as well as whether viable virus can be isolated from long distance air samples remains to be resolved as it will depend on environmental conditions as well as pathogen load and diagnostic methods. The possibility that swine IAV can be a risk to people without direct pig contact, or people in close proximity with pigs or pig farms still needs to be determined. Data from this study suggest that the risk may be limited given the fact that only very low amounts of non-viable IAV could be detected downwind. Furthermore, the data from this study also emphasizes the need to generate biosecurity mechanisms by which airborne pathogens are prevented from exiting

livestock facilities.

Table 7.1. Summary of pig farms, air spaces and clinical signs from which air samples were collected.

Farm	Farm type	Age of pigs (wks)	Number of pigs per air space	Air volume (m^3) per air space	Clinical score*	Days between onset of clinical signs and air sampling
1	Nursery	7	1,456	1,082	3	2 – 4
2	Wean-to-finish	13	1,095	1,726	1	7 – 10
3	Wean-to-finish	7	2,198	1,726	2	4 – 6
4	Wean-to-finish	15	1,200	1,987	3	3 – 5

* 1 = cough and sneezing in less than 25% of the pens, 2 = cough and sneezing in 50% of the pens and 3 = cough and sneezing in 75% of the pens.

Table 7.2. Number of positive and percentage of influenza A virus (IAV) RRT-PCR and virus isolation results from oral fluid samples and air samples collected inside the barn and at the exhaust fan from acutely infected pig populations.

	Oral fluids		Inside air samples		Exhaust air samples		
Farm	RRT-PCR	Virus Isolation	RRT-PCR	Virus Isolation	RRT-PCR	Virus Isolation	IAV subtype
1	15/15* (100)	11/15 (73)	15/15 (100)	6/15 (40)	15/15 (100)	1/15 (7)	H1N2
2	15/15 (100)	NT	2/15 (13)	0/2 (0)	2/15 (13)	0/2 (0)	H1N1
3	12/15 (80)	0/5 (0)	13/15 (87)	0/5 (0)	20/30 (67)	0/5 (0)	H1N1
4	15/15 (100)	5/5 (100)	15/15 (100)	1/5 (0)	26/30 (87)	0/4 (0)	H3N2

* Number of positive/total number of samples (percentage)

NT= not tested

Table 7.3. Average influenza A virus RRT-PCR cycle threshold and standard deviation (SD) values for oral fluids and air samples collected from four acutely infected pig populations.

	Oral Fluids		Inside Air		Outside Air	
Farm	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
1	22.63 [a]	1.32	28.58 [b]	0.86	32.44 [c]	1.41
2	31.26 [a]	2.39	37.43 [b]	0.81	37.76 [b]	0.03
3	31.38 [a]	3.62	33.21 [b]	1.36	34.62 [c]	1.65
4	21.01 [a]	1.41	29.56 [b]	0.83	32.64 [c]	1.48

Means within row with different superscripts indicate statistically significant differences ($P \leq 0.05$).

Table 7.4. Summary of downwind RRT-PCR positive and low positive air samples for influenza A virus.

Farm	No. positives (%)[a]	Test Result[b]	Cycle threshold value	Distance (Km)	Subtype
3	1/60 (1.6)		37, 38	1.2	H1N?, H?N1
			37, 37.23, 37.67, 37.99	1.1	Untypable
		Low Positive	39.22	1.7	Untypable
			37.96	1.9	Untypable
			37.09	2.0	Untypable
		Positive	34.3	2.1	Untypable
4	4/60 (6.6)	Low Positive	36.24, 36.99, 37.46, 37.6, 38.32, 38.41	0.8	Untypable
			35.66, 35.73, 36.69, 37.36, 37.37, 37.38, 37.4, 38.37	1.5	Untypable
		Positive	34.24, 34.59	1.5	H3N2, H?N2
		Low Positive	35.95, 36.1, 36.95, 37.02, 37.05, 37.13, 37.98, 38.85	1.6	Untypable
		Positive	34.38, 34.48	1.6	H?N?, H3N?
		Low Positive	35.48, 37.02, 37.17	1.9	Untypable

[a] Number of positive/total number (percentage)

[b] Cycle threshold: positive Ct <35, low positive = Ct 35 – 40, negative = Ct >40

? = Untypable

Table 7.5. Percentage similarity matrix of hemagglutinin gene sequences obtained from oral fluid and air samples collected from two influenza A virus acutely infected pig farms.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	Sequence number	Sample Type and number
	100	100	100	100	99.9	99.9	100	100	100	100	100	100	100	54	53.6	54.2	54.1	1	Farm 1 Air 1
		100	100	100	99.9	99.9	100	100	100	100	100	100	100	54	53.6	54.2	54.1	2	Farm 1 Air 2
			100	100	99.9	99.9	100	100	100	100	100	100	100	54	53.6	54.2	54.1	3	Farm 1 Air 3
				100	99.9	99.9	100	100	100	100	100	100	100	54	53.6	54.2	54.1	4	Farm 1 Air 4
					99.9	99.9	100	100	100	100	100	100	100	54	53.6	54.2	54.1	5	Farm 1 Air 5
						100	99.9	99.9	99.9	99.9	99.9	99.9	99.9	54	53.6	54.2	54.1	6	Farm 1 Saliva 1
							99.9	99.9	99.9	99.9	99.9	99.9	99.9	54	54.1	54.2	54.1	7	Farm 1 Saliva 2
								100	100	100	100	100	100	54	54	54.2	54.1	8	Farm 1 Saliva 3
									100	100	100	100	100	53.1	53.1	53.1	53.1	9	Farm 1 Saliva 4
										100	100	100	100	54	54.1	54.2	54.1	10	Farm 1 Saliva 5
											100	100	100	54	53.9	54.2	54.1	11	Farm 1 Saliva 6
												100	100	54	54.1	54.2	54.1	12	Farm 1 Saliva 7
													100	53.1	53.1	53.1	53.1	13	Farm 1 Saliva 8
														54	54	54.2	54.1	14	Farm 1 Saliva 9
															99.9	100	100	15	Farm 4 Air 1
																99.9	99.9	16	Farm 4 Saliva 1
																	100	17	Farm 4 Saliva 2
																		18	Farm 4 Saliva 3

Table 7.6. Logistic regression univariate analysis for log-transformed risk factors (except farm) associated with detection of influenza virus in long distance air samples collected from two acutely infected pig populations.

Variable	OR (95% CI)	*P*- value
Distance between sample collection point and source farm	0.069 (0.033, 0.146)	<0.001
Temperature	0.967 (0.811, 1.154)	0.712
Relative humidity	0.193 (0.051, 0.732)	0.015
Light intensity	1.167 (1.038, 1.313)	0.010
Farm 3 versus 4	0.609 (0.316, 1.173)	0.138

Chapter 8: General Discussion and Conclusions

General Discussion and Conclusions

The history of influenza A virus (IAV) in swine started in 1918 when a veterinarian, Dr. J.S. Koen, an inspector for the Bureau of Animal Industry in charge of the hog cholera control program in the state of Iowa, described the disease for the first time. Koen's diagnosis and description in swine coincided with the respiratory syndrome physicians were observing in humans during the same period. This epizootic led Koen to suggest that what he was seeing in pigs was very similar to what physicians saw in humans and even suggested that humans could have infected pigs and vice versa (Koen, 1919; Taubenberger, 2006). That single event is perhaps the first and most important link between IAV, pigs and humans as evidenced by the volumes of reports in the literature which has led researchers to investigate the complex inter-species epidemiology of this disease. Ever since, research conducted on this pathogen has been focused mostly on the human-animal interface generating a great body of knowledge. However, the epidemiology of IAV in swine is still in its early stages. Therefore, the objective of this document was to further the understanding of the epidemiology of IAV in swine that could contribute to the generation of new hypotheses, prevention and control measures.

Through six chapters, this document provides insight into the complex epidemiology of IAV in pig farms. The first two chapters focused on describing the occurrence and herd-level determinants for the presence of IAV in commercial pig farms through the implementation of a surveillance program. The remaining four chapters described studies conducted to further understand one of the indirect routes of transmission of IAV, airborne transmission. These four chapters started with Chapter 4 in which the association between proximity of pig farms to turkey farms and the presence of anti-influenza antibodies in turkey flocks was investigated. This chapter is followed by three chapters (e.g. Chapters 5, 6 and 7) that focused on the development of protocols for the detection of IAV in air samples under different conditions from experimentally and naturally infected pigs under different settings.

The second chapter of this document summarized the findings of an IAV active

surveillance program conducted in Midwestern pig farms. Results from this study demonstrated that IAV is common among pig populations since the virus was found in at least one of the groups monitored in almost all (e.g. 90%) of the participating farms. Additionally, the virus was detected every month since the beginning of the program except for three months in which all groups monitored tested negative. However, is important to mention that IAV was in most of the groups at a low level since most of the groups had between 1 and 3 positive swabs. Furthermore, groups were more likely to test positive for IAV during the warm seasons of the year compared to the fall season. Overall, these findings provide insight into the epidemiology of IAV in swine in that the virus continues to circulate throughout the year in swine populations. Another interesting finding throughout this surveillance program is that pig farms are frequently having different IAV subtype introductions which perhaps help explain the continuous reassortment events occurring and thus the never ending diversity of the virus. This project opens a window of opportunity into the understanding of IAV dynamics in swine farms. It is unknown why most of the groups of pigs had a low (<10%) percentage of positive swabs and it allows us to speculate that this may have been due to either the starting or ending of an epidemic. Another possibility could be that due to immunity present in the group, transmission of the virus was allowed but at a low rate and thus this would fall into a subclinical scenario which needs to be further investigated.

Using the diagnostic data recorded in Chapter 2, we decided to assess farm level risk factors that could potentially be associated with the presence of IAV. Additionally, weather stations were located at these pig farms and recorded meteorological parameters (e.g. temperature, relative humidity, light intensity, wind speed and gusts speed) with the aim of exploring the association between weather and presence of IAV. At the univariate level, it was possible to demonstrate an association between farm type, pig flow and gilt replacement management and the presence of IAV. However, we were not able to find associations at the multivariable level. Similarly, temperature and wind speed were associated to IAV at the multivariable level. However, it is not well understood how these meteorological variables influence the occurrence of IAV inside the pig barn. It is clear

that environmental conditions outside the barn will influence care takers to act accordingly and provide optimum conditions inside the barn, but more research is needed to fully understand such relationships between weather and IAV in pig populations.

These two studies provided insight into the epidemiology of IAV in pig farms by highlighting that the virus circulates throughout the year in pig populations. These studies have added knowledge into the epidemiologic features of this virus. However, there is still need for more studies to fully understand how this virus behaves within and between pig populations. One area that needs full attention because of its importance, especially when dealing with an infectious disease that has zoonotic potential, is the means by which the etiologic agent is transmitted. In swine it is known that IAV is transmitted through nose-to-nose contact as its main route (Brown, 2000). However, data on the importance and understanding of airborne transmission of AIV in humans has only been generated in the last decade (Tellier, 2009). Thus, the same consideration should be given to airborne transmission in swine which has been thought to play a role since there have been reports that suggest that virus introductions into pig farms could have been through the airborne route (Andral et al., 1985; Choi et al., 2004; Desrosiers et al., 2004; Desrosiers et al., 2004) since these outbreaks were not preceded by pig introductions. Therefore, four chapters of this thesis were devoted into setting the foundation for the understanding of airborne transmission of IAV. The first of those four chapters, Chapter 3, focused on exploring the relationship between pig farm proximity and the likelihood of turkey flocks being seropositive for swine IAV. Minnesota, as the number 1 and 3 producer of turkeys and pigs in the United States, respectively, allowed us to investigate such hypotheses. Through geographic information systems (GIS) we were able to map turkey and pig farms in the state using databases provided by local agencies. Thanks to this software, distances between pig and turkey farms and number of pig farms within a specific radius of a turkey farm were obtained. This study demonstrated that the closer the pigs were to the turkeys, the higher likelihood of the turkeys being seropositive. Although we were able to find an association we could not prove causality. However, the low likelihood of the turkey and pig farms sharing employees or equipment which could

have been another way of transmission, the hypotheses of airborne transmission gains more weight especially when the antibodies found in turkeys were related to swine viruses. The literature has different examples of pig viruses being isolated from turkey flocks (Andral et al., 1985; Choi et al., 2004; Ficken et al., 1989; Mohan et al., 1981; Tang et al., 2005) which support furthermore that pigs can potentially be infectious aerosol generators.

Validation of air sampling protocols and detection of airborne IAV was investigated and described in Chapter 4. An air sample collection device (Liquid cyclonic collector, Midwest Micro-Tek, Brookings, SD) was validated for the collection and detection of IAV in air samples. Swine flu was detected in air samples through RRT-PCR and virus isolation when artificial aerosols were created. This finding allowed us to continue in our investigation process since we learned that the air collector is capable of capturing viral particles and maintain them in a viable state. The following step focused on the detection of IAV in air samples collected in an air space where experimentally infected pigs were housed. Detection of IAV was accomplished through RRT-PCR but it was not detected through virus isolation, most likely due to sample handling. Detection of the virus was possible once an aerosol generation minimum threshold was reached indicating that for the air sampling device to collect enough particles there needs to be a certain number of pigs actively shedding the virus. In this study, it was not until 4 pigs were shedding the virus that the machine captured enough airborne viral particles and thus yielded a positive RRT-PCR result. As expected, the virus was detected in air samples as long as pigs would be actively shedding. The odds of detecting a positive RRT-PCR air sample increased with every additional shedding pig. This study allowed us to understand that IAV can be detected in the air and that pigs can be a source of IAV aerosols for a short period of time. However, it is important to mention that this was the case for only one strain of an H1N1 virus and more research needs to be done to fully understand if aerosol generation in swine is strain or subtype dependent as has been suggested in the guinea pig model (Steel et al., 2009). Furthermore, the pigs in this study were completely naïve in that they originated from a herd with no history of influenza

based on several years of seronegative test results either through hemagglutination inhibition (HI) or ELISA.

The next step was conduct a similar study by attempting to emulate what is seen in the field by experimentally challenging recently weaned pigs with maternally derived immunity (MDI) since vaccination of sows is commonly used as means of prevention and control (Thacker and Janke, 2008). Additionally, it is known that immunity against influenza ameliorates clinical signs and decreases shedding (Van Reeth et al., 2003; Van Reeth, 2007). In this study, air samples from three groups of pigs (e.g. control-no MDI, homologous-MDI and heterologous-MDI) that had been exposed to an H1N1 virus were collected for 10 days. Both the control and heterologous groups generated infectious aerosols since samples yielded RRT-PCR and virus isolation positive results. From this study we were able to understand that indeed recently weaned pigs under the presence of MDI can generate infectious aerosols which may be transmitted onto pen mates or even humans. Additionally, the frequency of detection of airborne IAV was considerably lower compared to our earlier study which leads us to hypothesize that the size of the pig may have an important component in the dynamics of aerosol generation which needs to be determined. From this study we could corroborate that capturing the virus in the air with this air sampling device is possible and more importantly, it preserves the virus integrity. Both studies in chapters 5 and 6 provided us with confidence to take our next step into the understanding of IAV airborne, detection in the field.

The last chapter of this document (Chapter 7) focused on the detection of IAV under field conditions. For the purpose of this study, acutely infected pig populations were chosen as our target populations to increase the likelihood of detecting IAV. Detection of the virus by RRT-PCR in air samples was possible in all farms inside the barn and at the exhaust fan outside the barn. However, isolation of the virus from air samples was more challenging and was only possible in two of the farms. Furthermore, downwind detection of IAV was attempted in two of these farms and air samples yielded only RRT-PCR positive results between 1.5 and 2.1 Km away from the farm. Downwind

detection of IAV in air samples depended on distance and light intensity. Even though we were not able to prove that RRT-PCR air samples contained viable viruses, the data suggests that airborne transmission at the regional level can play a role and should be investigated. An important question that remains to be answered is what is the length of time an infected population can generate infectious aerosols that can travel to neighboring farms. The findings from this study provided an important piece of knowledge into the ecology and epidemiology of swine flu but more importantly, it provided methods for the understanding of regional transmission of IAV through the airborne route.

If one extrapolates the data from the chapters devoted to the understanding of airborne detection of IAV, it may be tempting to assume that airborne transmission occurs more frequently than expected but presently the role of transmission through the airborne route still needs to be further elucidated. It is known that other viruses (e.g. porcine reproductive and respiratory syndrome virus) do become airborne and thus farms are including air filtration technology for units housing breeding herds in an attempt to lower the risk of infection (Spronk et al., 2010).

This thesis has generated several grains of knowledge with regards to the epidemiology of IAV infection in swine. There are still many unanswered questions that need to be solved to further understand the mechanisms by which this virus maintains its circulation throughout the year. Thanks to the fast development of diagnostic technology and the interest of not only animal health authorities but also human health authorities, advancements into the understanding of influenza will occur at a faster pace which will reveal more characteristics of this threatening organism.

In conclusion, we are starting to learn how IAV behaves in swine. Based on its characteristics and due to the inter-species transmissibility of this virus, we can safely assume that IAV will continue to disseminate within and between populations. More efforts should be devoted to research of IAV in animal populations, especially swine, since the link between pigs and people and the implications this has on inter-species

transmission are likely considerable but still largely understudied.

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