Active Influenza A Virus Surveillance in Swine at Agricultural Fairs

DISSERTATION

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Abstract

Pigs are recognized to play a key role in the ecology and epidemiology of influenza A virus infections and in the emergence of novel viruses which may progress to pandemics. Retrospective epidemiological investigations have demonstrated multi-directional interspecies transmission of influenza A viruses can occur between humans and swine at agricultural fairs. Following the emergence of the influenza A(H1N1)pdm09 virus, we hypothesized that an active, influenza A virus surveillance program at agriculture exhibitions could be used as an active, cost-effective, and efficient, approach for the early detection of zoonotic transmission of influenza A virus between swine and humans.

The second chapter of this dissertation describes the results of a study investigating the effectiveness of two virus isolation methods for the recovery of contemporary influenza A virus strains circulating in swine at agricultural exhibitions. Results demonstrated MDCK cells adapted to serum free medium were better than embryonating chicken eggs for the recovery of contemporary swine-origin influenza A viruses circulating in swine at agriculture exhibits.

The third chapter examines influenza A virus activity at selected agricultural fairs in Ohio over three years, 2009-2011. The results demonstrated that subclinical influenza
A virus infections were common among exhibition swine in Ohio and these infections were going unreported in current national swine influenza virus surveillance programs. These subclinical infections at the swine-human interface were hypothesized to be playing a role in variant influenza A virus infections in humans.

The fourth chapter expanded upon the previous chapters to conduct active virus surveillance at Ohio agriculture exhibitions during 2012. This study used nucleotide sequencing of isolates concurrently recovered from humans and pigs at the same location to confirm that H3N2pM influenza A virus infections in pigs were the source of H3N2v influenza A viruses infecting humans.

In chapter five, further analyses of isolates from pigs and humans from across Ohio during 2012 provided evidence that interspecies transmission was not limited to one rare event but the occurred at six additional agriculture exhibits over a three week period. Nucleotide identity of the H3N2 isolates from humans and swine at all seven fairs was greater than 99%, indicating that this H3N2pM swine-origin influenza A virus became widely disseminated among exhibition swine across Ohio during the 2012 fair season and was transmitted to humans at each of the seven fairs.

In chapter six, the final study seeked to examine fair-level risk factors potentially contributing to influenza A virus infections in the pigs at agricultural fairs. These data collected during 2012 indicate that larger swine shows have an increased risk for the presence of influenza A virus among the swine at the exhibition.
Overall, this project has established an active surveillance program to study influenza A virus activity at the swine-human interface. The first three years of the program provide an invaluable set of baseline data to which future results can be compared. The outcomes documented in 2012 highlight the need for effective mitigation strategies to control intra- and inter-species spread of influenza A virus at swine exhibitions.
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Introduction

Influenza A virus is a common cause of respiratory infections in swine and has recently had an increasingly negative economic impact on the swine industry. While production losses are an ongoing concern, they have paled in comparison to the public health, public relation, and trade issues associated with recent and repeated influenza A virus transmission from pigs to people and people to pigs, including the emergence of the 2009 pandemic H1N1 virus. Accumulating point mutations and genomic reassortment facilitate the appearance of new influenza A virus strains that can pose a significant threat to swine and public health. Actively monitoring influenza A viruses circulating in swine allows for early detection and identification of novel strains. This knowledge allows animal and public health officials to update diagnostic tests and reagents, modify vaccines, and develop appropriate precautions to protect public and animal health.

Surveillance is an essential component to all prevention, control, and eradication efforts but publicly available data from active surveillance for influenza A virus among swine has been limited to date. Currently, influenza A virus surveillance in the U.S. swine herd is almost entirely passive, which usually requires recognition of influenza-like-illness in pigs to initiate testing. For this reason, additional active surveillance for type A influenza viruses in the U.S. swine herd is necessary. However, the large number of samples required, additional labor, and extra time associated with traditional active
surveillance is often cost-prohibitive. Ultimately, surveillance systems that are not practical, user-friendly, and low cost are usually implemented in an unproductive manner and are eventually abandoned. This project utilizes active, practical, systematic, mutually beneficial, sustainable influenza A virus surveillance efforts targeted at enchaining current influenza A virus surveillance in swine.

Pigs infected with swine-, human-, and avian-origin influenza A viruses potentiate the emergence of novel influenza A viruses. If these novel strains are able to move between human and swine populations, efficiently infect humans and transmit from person to person, they can present a pandemic threat. The zoonotic transmission of these novel viruses between swine and humans usually requires close contact as occurs at the swine-human interface on farms, in packing plants and at agricultural exhibitions. People in close contact with swine could play multiple roles in inter-species transmission of influenza A viruses: they might transmit human-origin influenza virus to swine resulting in novel reassortant viruses; they may be the first to become infected with a new reassortant influenza A viruses thus serving as early sentinels; and they may disseminate swine-origin influenza A viruses to other members of their community. The outcome is highly dependent on the strain of virus involved, which emphasizes the need for a continued effort to understand zoonotic influenza virus transmission.

Agricultural exhibitions serve as the face of agriculture to the nearly 150 million people who attend fairs in North America each year. These annual celebrations of agricultural heritage and achievement allow for many people to come into direct contact with pigs. Many of these attendees have limited exposure to swine. This prolonged
The commingling of multi-source pigs with a large number of diverse people, many with limited prior exposures to swine-origin influenza A viruses, creates an environment ripe for intra- and inter-species influenza virus infections. Several recent variant influenza A viruses infections in humans have been associated with pig contact and attendance at fairs. Agricultural exhibitions are unique sites, which have an increased potential for zoonotic disease transmission and have not been substantially investigated. Sampling pigs at fairs provides access to a unique human-swine interface and an increased probability of examining a relatively rare event, the bi-directional transmission of type A influenza viruses between pigs and people.

The specific aims for this project were to:

1) Compare virus isolation methodologies for contemporaneous influenza A viruses present in swine nasal swabs using embryonating chicken eggs and MDCK cell cultures.

2) Examine type A influenza virus activity in pigs at the swine-human interface at agricultural fairs and assess the implications for public health.

3) Monitor the genomic properties of influenza A viruses circulating in swine being exhibited at the selected Ohio agricultural fairs.

4) Evaluate risk factors contributing to the occurrence of influenza A virus infections in pig at agricultural fairs in Ohio.
This dissertation is organized in the journal publication format that includes a literature review followed by five manuscripts that have been previously published, or in preparation for publication.
Chapter 1 - Literature review

1.1 Influenza A Virus

Orthomyxoviruses are medium sized (80-120nm), pleomorphic, enveloped viruses containing a single-stranded, negative-sense, segmented RNA genome.\(^1\) This family is divided into five genera *Thogotovirus, Isavirus, Influenzavirus A, Influenzavirus B*, and *Influenzavirus C*.\(^1\) Type A influenza viruses have been recovered from many host species and are the predominant orthomyxovirus infecting swine.\(^1\)-\(^3\) Type B influenza viruses have never been isolated from pigs, but on rare occasion, influenza B virus antibodies have been detected in Chinese and British swine.\(^4\), \(^5\) Type C influenza viruses are relatively common among children\(^6\) but are believed to be uncommon in swine\(^4\), \(^7\), \(^8\) with the only recovery occurring in China in 1981.\(^9\) Interestingly, a type C “like” influenza virus was recently recovered from pigs in the Untied States suggesting there may be more influenza C virus activity in swine than previously documented.\(^10\) Nonetheless the term swine influenza virus still refers to influenza A viruses (IAV) infecting pigs.\(^2\)

The 13,588 base genome of IAV is made up of eight RNA gene segments that are named for the protein(s) for which they encode, of which there are at least 12.\(^1\) Hemagglutinin (HA) and neuraminidase (NA) are the two primary surface glycoproteins
projecting from the surface of the viral envelope making them primarily responsible for the antigenic properties of the virus and are the major driver of immune response in respective hosts. These two proteins are also the basis for subtyping IAVs into 17 recognized HA subtypes and 10 separate NA subtypes which in combination are used to catalog IAVs into HA and NA combinations (e.g. H3N2). This HA and NA combination is used in conjunction with the type specific antigen and other host and laboratory data to form a complete isolate name (e.g. A/swine/Ohio/12TOSU268/2012(H3N2)). Within the virion, viral RNA is wrapped around nucleoproteins (NP) in packages referred to as ribonucleoprotein complexes. Additionally, because host cells cannot replicate negative sense RNA, IAVs must bring along their own polymerase complex which is comprised of three proteins (polymerase acidic protein (PA), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2)).

The active state of the homotrimer protein hemagglutinin, the product of a post-translational cleavage of HA0 into subunits HA1 and HA2, initiates viral entry into the host cell. The HA1 subunit contains the site for binding to specific sialic acid-containing oligosaccharides on the surface of susceptible host cells making it a major determinant of host range. Most avian-origin IAV strains preferentially bind to α-2,3 linked sialic acid receptors whereas many mammalian-origin strains, especially those infecting human beings, prefer α-2,6 linked sialic acid receptors. Once the virus attaches to the host cell surface, receptor-mediated endocytosis transports the entire virion and cell receptor complex into the cell. The decreasing pH within the endosome
activates M2 protein, an ion channel, allowing protons to enter the virion and release the viral ribonucleoproteins from the M1 protein which leads to expression of the nuclear localization signal. Additionally, the acidic endosomal environment causes a configurational change in HA2 subunit allowing for fusion of the viral lipid envelope with the endosomal membrane; this results in the subsequent release the viral ribonucleoproteins into the host cell cytoplasm.

Influenza A viral RNA must enter the nucleus of the host cell in order replicate making influenza viruses some of the few RNA viruses to be transcribed in the host cell nucleus. The ribonucleoprotein complexes containing the negative sense RNA, along with the PA, PB1, PB2, and NP proteins are imported into the nucleus of the host cell. The ribonucleoprotein complexes are then copied into viral mRNA using the virus’s RNA polymerase. In a process called “cap snatching,” viral mRNAs are capped with 10-13 nucleotides from the 5’ end of host cell transcripts that have been cleaved by influenza viral protein endonuclease. The viral mRNAs are transported out of the nucleus and new proteins are synthesized from viral mRNA using the host cells ribosomes. The HA, NA, and M2 enter the host cell’s secretory pathway and are transported to the cell surface to begin formation of new virions.

The ribonucleoprotein complexes are also used to copy the genomic RNA segment into complementary negative sense RNA which is subsequently packaged into new viral ribonucleoprotein complexes by the NP protein. Newly synthesized M1 and NS2 proteins are imported into the nucleus where they bind just created viral ribonucleoprotein complexes. This binding to M1 halts mRNA synthesis and allows
binding of NS2, causing the progeny ribonucleoprotein complexes to be trafficked out of the nucleus into the cytoplasm.\textsuperscript{25} Newly created ribonucleoprotein complexes and proteins are transported through the cytoplasm to the host cell membrane where they are assembled into progeny virus particles.\textsuperscript{26} Virus particles bud from the apical surface of the host cell and neuraminidase facilitates release of progeny virus by catalyzing the cleavage of terminal sialic acid residues on the cell surface.\textsuperscript{27}

Evolution of influenza A viruses happens through two primary mechanisms: accumulating mutations resulting in antigenic drift and genomic reassortment causing antigenic shift.\textsuperscript{28} The RNA polymerases of RNA viruses such as influenza lack proofreading ability\textsuperscript{1} and therefore are prone to making errors such as substitutions, insertions and deletions. Accumulated nucleotide mutations will cause the nucleotide sequence to drift leading to alterations in the translated amino acids, which in turn may change the structure and function of the resulting protein.\textsuperscript{26} The resulting gradual change in antigenic features is called “antigenic drift.” Nucleotide mutations that do not change the amino acid sequence are said to be synonymous substitutions because they have little or no affect on viral function. Conversely, non-synonymous substitutions in the nucleotide sequence do result in altered amino acid sequences and likely modify the antigenicity and/or structure of the viral proteins.

Unlike the slow evolutionary process of accumulating mutations in the nucleotide sequences, rapid changes in antigenic and functional properties of the viral proteins occur due to exchanging gene segments from multiple IAVs co-infecting a host cell at the same time. Swapping of gene segments, know as reassortment, not only changes the HA and
NA subtype but can result in numerous genomic constellations in the next generation of viruses.\textsuperscript{26} Genomic reassortment is commonly detected when exchanges alter the hemagglutinin and/or neuraminidase combination of a viral strain. These sudden and major antigenic changes are referred to as “antigenic shift.” When a reassortant virus has a competitive advantage, it can result in the emergence of a novel virus that can become dominant and dramatically affect the health of a host population. Swine have been called ‘mixing vessels’ for the development of new reassortant IAVs because pigs have both $\alpha$-2,3 and $\alpha$-2,6 linked sialic acid receptors in their respiratory tract allowing them be infected with swine-, human-, and avian-origin IAVs and thus allowing for simultaneous co-infection and the potential for genetic reassortment and the formation of novel IAVs.\textsuperscript{29,30}

\subsection*{1.2 Human Influenza}

In the United States, endemic strains of IAV commonly cause seasonal epidemics of acute respiratory disease resulting in approximately 200,000 hospitalizations and an estimated 3,000 to 49,000 deaths annually.\textsuperscript{31,32} On the other hand, pandemic IAVs infect a significant proportion of humans, spread rapidly over multiple contents, and the number of fatalities can climb into the millions around the world.\textsuperscript{33} Descriptions of influenza-like pandemics date back to ancient times and clear references to influenza can be found as early as 1650, but these cannot be confirmed because signs and symptoms of influenza are similar to other diseases.\textsuperscript{34} While four major and one minor pandemic have occurred over the last 100 years, there is descriptive demographic data suggesting there
have been several pandemics in earlier centuries. Each of the modern major pandemics starting with the 1918 Spanish flu pandemic was the result of a novel strain of influenza A virus being introduced to an immunologically naïve human population.

The 1918 influenza pandemic, known as the Spanish Flu, was caused by an H1N1 influenza A virus of which all eight segments have been retrospectively identified as having ancestral origins in the avian influenza A viruses. This pandemic is the deadliest documented influenza pandemic in history. This H1N1 virus was unusually lethal in humans with mortality estimates for the United States exceeding 675,000 and an estimated 50-100 million people succumbing to the virus worldwide. Unlike seasonal influenza, which typically more severely affects the young and elderly, the 1918 H1N1 influenza virus disproportionately caused mortality in previously healthy young adults. This virus, which was reported to kill within hours, is believed to cause severe disease and high mortality by inducing a “cytokine storm” following infection.

Following the 1918 pandemic, this H1N1 lineage demonstrated antigenic drift as they circulated in humans until 1957, when they were replace by a pandemic H2N2 IAV. This pandemic was referred to as the "Asian Flu" and was much less severe than the 1918 pandemic, leading to the death of approximately 70,000 people in the United States and 1-2 million people around the globe. This H2N2 IAV was a reassortant virus comprised of avian lineage HA, NA and PB2 genes and five genes retained from the H1N1 Spanish Flu pandemic virus lineage.

In 1968 another major pandemic occurred when a reassortant H3N2 IAV emerged. This pandemic was even milder than the 1957 Asian Flu pandemic with
approximately 34,000 deaths in the United States and nearly 700,000 worldwide. This pandemic was called “Hong Kong Influenza” and caused by a virus formed when avian-origin H3 and PB1 genes reassorted with the persisting lineage of the 1957 pandemic H2N2 Asian Flu virus. Following this pandemic, H3N2 viruses, as expected, became established in the human population where they continue to circulate widely today.

In 1977, a "pandemic-like" event occurred after the “Russian Flu” virus was detected in northeastern China. This H1N1 virus was a reintroduction of the 1955 H1N1 strain and as such, initially the cases were age-restricted to primarily younger people born after the emergence of 1957 pandemic virus who had no pre-existing immunity to H2N2 IAV. The origin of this H1N1 “Russian flu virus” is the topic of speculation. This H1N1 virus did not replace the previously circulating H3N2 subtype but rather H1N1 and H3N2 subtypes continued to co-circulated in humans into the 21st century.

The first influenza pandemic of the new millennium started in 2009 and was caused by a triple reassorant H1N1 IAV virus. This virus contained PB2 and PA genes similar to avian-origin IAVs found in North America; a PB1 gene from human-origin IAV; HA, NP, and NS genes of North American swine influenza; and NA and M genes from Eurasian swine-origin IAVs. Because of its many swine lineage segments, it was initially labeled as swine flu, a name that is estimated to have cost the U.S. agriculture sector millions of dollars. The estimated number of human deaths worldwide was between 151,700 and 575,400. Much like the 1918 pandemic, the mortality rate was higher for younger people rather than those older than 65 years-of-age. This H1N1 virus, called A(H1N1)pdm09 virus, has now replaced the previously
circulating H1N1 virus in the human population and now co-circulates with H3N2 viruses to cause seasonal influenza.\textsuperscript{55}

1.3 Swine Influenza

\textit{Evolution of IAVs in swine}

The first description of swine influenza occurred in the US during the 1918 influenza pandemic in humans\textsuperscript{56} and the etiologic agent was isolated in 1930 by Dr. Richard Shope.\textsuperscript{57} Three years later the first human-origin H1N1 influenza A virus was isolated.\textsuperscript{58} Once the hemagglutination inhibition test was developed and standardized\textsuperscript{59} the antigenic relationship between the human-origin and swine-origin H1N1 IAVs supported early speculation that the viruses causing human and swine influenza were similar. Subsequently, phylogenetic analyses confirmed the genetic relationship between the 1930’s swine and human H1N1 IAVs and it is widely held position that the swine IAV is a descendent of the 1918 H1N1 pandemic IAV.\textsuperscript{60-62} This lineage of H1N1 virus remained rather genetically and antigenically stable and was the predominant subtype circulating in U.S. swine and for more than 70 years.\textsuperscript{63-66} This lineage of swine-origin H1N1 IAV is now referred to as "classical" swine influenza virus.\textsuperscript{67}

While human-origin H3N2 IAVs were previously detected among swine in Taiwan (1968)\textsuperscript{68}, Canada (1990 and 1991)\textsuperscript{69-72}, Japan (1993)\textsuperscript{73}, the 1998 introductions of multiple reassortant H3N2 IAVs into North American swine dramatically changed the relatively stable landscape of IAVs in swine. The 1998 H3N2 viruses contained human lineage HA and NA genes, and were concurrently detected after pigs in North Carolina,
Texas, Minnesota and Iowa developed severe influenza-like-illness. Genetic analyses of the isolates from these cases revealed two distinct viral genotypes, a double reassortant and a triple reassortant. The double reassortant was recovered from swine in North Carolina and contained PA, PB2, NP, M, and NS genes from the classical H1N1 swine IAV lineage, along with PB1, HA, and NA genes from H3N2 human lineage IAV. The H3N2 IAV isolates recovered from pigs in Iowa, Minnesota and Texas were triple reassortants containing classical swine IAV lineage NP, M, and NS gene segments, PB2 and PA gene segments of avian lineage, and PB1, HA, and NA gene segments from human lineage IAVs. The six internal genomic segments of the triple reassortant viruses are referred to as the triple reassortant internal gene constellation, or “TRIG” cassette. The double reassortant H3N2 virus never became established in swine and disappeared unlike the triple reassortant H3N2 IAVs which quickly became endemic in the U.S. swine herd.

Phylogenetic analyses conducted on contemporary H1 IAVs endemic in North American swine reveals at least four discrete H1 genetic clusters referred to as α-, β-, and γ-δ- clusters. The viruses in α-cluster represent the classical swine influenza lineage of H1 IAVs. The β-cluster consists of HA genes derived from reassortments between the triple reassortant swine H3N2 and classical H1N1 swine-origin IAVs which resulted in swine H1N1 viruses containing the TRIG cassette. A reassortment event between classical swine influenza H1N1 with a triple-reassortant swine H3N2 IAVs led to the γ-cluster H1 gene segments which used to be called “H1N2-like.” The δ-cluster is the most genetically and antigenically distinct group of H1s in North American swine and it
contains two subclusters, designated δ1 and δ2, representing two introductions of human seasonal influenza viruses (H1N2 and H1N1) into North American swine.\textsuperscript{77, 78, 84}

The A(H1N1)pdm09 virus is another triple reassortant virus. It contains six gene segments closely related to the North American swine-origin triple-reassortant IAVs along with Eurasian lineage M and NA genes.\textsuperscript{49, 50, 85} The H1 of this pandemic virus is highly similar to the North American γ-cluster swine H1 IAVs yet it readily forms a subclade within this cluster.\textsuperscript{77, 86} Although similar to various swine-origin IAVs, no IAVs with this gene combination had been identified in swine or any other host species prior to its 2009 emergence in humans. The A(H1N1)pdm09 virus is now endemic in North American swine populations following introduction from humans and it continues to circulate and reassort in swine with the previously introduced H1 and H3 strains.\textsuperscript{87, 88}

The hemagglutinin genes of H3 IAVs in swine are classified with a different naming system which is linked to three distinct introductions of human H3 IAVs into North American swine populations.\textsuperscript{69, 89-91} Cluster I H3s are descendants of the seasonal H3N2 infecting humans in 1995. The introduction of the 1997-1998 human seasonal H3N2 into swine populations resulted in Cluster II. Human H3N2 IAVs circulating in humans during 1996 are most closely related Cluster III H3s which became established in pigs and subsequently evolved into the Cluster IV H3s. Presently cluster IV HA genes predominate the H3 IAV scene in North American swine populations with contemporary isolates rarely classified into the three other clusters.\textsuperscript{88}

All of these diverse H1 and H3 subtypes are currently co-circulating in North American swine demonstrating the speed with which IAVs are able to drift and/or shift.
within modern swine production. For nearly ten years between the introduction of the triple reassortant H3N2 and the emergence of A(H1N1)pdm09, the vast majority of IAV isolated from North American swine contained the TRIG cassette regardless of HA or NA subtype.\(^{75}\) Soon after the introduction of A(H1N1)pdm09 into North American swine, reassortant swine IAVs possessing the Eurasain M gene descended from A(H1N1)pdm09 virus instead of the TRIG (classical swine influenza) M gene were reported.\(^{87}\) Continued reassortments between IAVs circulating in swine populations have led to numerous internal and external gene constellations being reported worldwide.\(^{92-96}\)

**Disease in Swine**

Worldwide, IAV is a significant cause of respiratory disease in swine and is one of the primary components of the porcine respiratory disease complex (PRDC).\(^3, \textit{97-99}\) Depending on the operation, swine farms may be affected by sporadic or endemic infections of IAV costing North American pork producers estimated $3.23-$10.31 per pig.\(^{100, 101}\) Transmission of IAV in swine occurs via inhalation of infectious virus into the respiratory tract.\(^{102, 103}\) IAV infections in swine are usually mild and uncomplicated infections and typically result in high morbidity but very low mortality with recovery usually occurring within 4-8 days after infection.\(^2, \textit{104}\) However, IAV infections commonly disrupt mucociliary clearance leading to secondary bacterial or viral infections which complicate the disease process.\(^{99, 105, 106}\) *Haemophilus parasuis* and Porcine Reproductive and Respiratory Syndrome virus (PRRSv) are both known to result in severe clinical disease when they infect pigs already infected with IAV.\(^{107, 108}\)
Conversely, pigs co-infected with IAV and Mycoplasma hyopneumoniae show little or no change in the course of disease.\textsuperscript{109}

Clinical signs of IAV infection in pigs are strain dependent and can range from mild to severe with typical clinical signs of IAV infection in pigs including fever, anorexia, lethargy, coughing, labored breathing, sneezing, nasal discharge and poor weight gain.\textsuperscript{90,110,111} Fever, sometimes higher 40°C, is a consistent clinical sign which can peak as early 1 day post-infection and usually persist up to 5 days.\textsuperscript{112-114} Other clinical signs such as coughing and nasal discharge typically develop 2-3 days post-infection and can persist for 2-3 days. IAV can be particularly costly to sow farm owners and operators where reproductive failure due to IAV induced fever and weak-born piglets have been documented.\textsuperscript{74,82,115,116} Viral shedding in pigs has been documented to start as early a 24 hours post exposure and last from 1-4 weeks depending upon the strain and the pre-existing level of immunity.\textsuperscript{114,117,118}

1.4 Zoonotic Transmission

Beginning in the 1970s several new and re-emerging infectious agents have caused significant human disease.\textsuperscript{119,120} Recent evaluations estimate that 73\% of emerging or re-emerging diseases are zoonotic in origin.\textsuperscript{121} Type A influenza viruses are zoonotic and a worldwide threat to both human and animal health. Sporadic reports of swine-origin IAV infections in humans can be found in domestic and international medical literature.\textsuperscript{122-126} Since it is not possible to clinically differentiate variant influenza
in humans from strains endemic in the human population, the true incidence of zoonotic transmission is unknown.

In 1918, veterinarian Dr. J. S. Koen described the bidirectional zoonotic transmission of IAV after observing frequent outbreaks of influenza in families and their swine herds.\textsuperscript{127} Swine influenza virus was recovered from human lung during an autopsy in 1974.\textsuperscript{128} Two years later in Wisconsin, Drs. Pawlisch and Easterday documented transmission of swine influenza virus between pigs and their caretakers.\textsuperscript{129} Also in 1976, Ft. Dix, New Jersey was the site of an outbreak of swine influenza virus in humans which was detected when one soldier died and 12 others became severely ill with a respiratory illness.\textsuperscript{130} A follow-up investigation indicated nearly 230 soldiers were likely infected with the swine-origin virus even though none of them had any documented swine exposure.\textsuperscript{131-133}

In 1988 a pregnant woman in Wisconsin died after infection with an H1N1 swine influenza virus.\textsuperscript{134} Four days prior to onset, this woman visited an agricultural fair where there was prevalent respiratory disease among the pigs. Human-to-human transmission was documented in association with this case when several health care workers developed an influenza-like illness after caring for the patient.\textsuperscript{135}

More recently in August 2007, more than two thirds of the pigs being shown at the Huron County (Ohio) Fair were observed with influenza-like-illnes and approximately two dozen people exposed to the pigs at the fair developed influenza like illness simultaneously.\textsuperscript{136} Swine-origin triple reassortant H1N1 IAV was recovered from both humans and the pigs and the sequence analysis of the isolates revealed 100%
homology demonstrating that the IAV was transmitted from the pigs to the humans during the agricultural fair.\textsuperscript{79}

Serologic surveys of humans that work with swine demonstrated those persons have elevated antibodies to swine-origin influenza viruses. In a 1981 study, 11\% of persons exposed to pigs as part of their job were seropositive to swine H1N1, with swine veterinarians and swine farmers having the highest prevalence.\textsuperscript{137} A study in Wisconsin showed the risk for having antibodies to swine-origin H1N1 virus was elevated for veterinarians and farmers.\textsuperscript{138} More recent studies show that swine farmers, swine veterinarians and veterinary technicians along with meat processing workers have higher odd of exposure to swine-origin IAVs than people with no work-related exposure to swine.\textsuperscript{139, 140}

Human-to-swine transmission can result in severe disease, economic losses, and accounts for most of the IAV diversity current seen in North American swine populations. H3 and δ- cluster H1 IAVs were clearly introduced into the swine population via human-to-swine transmission. Soon after the A(H1N1)pdm09 virus began spreading humans and it was transmitted to pigs in the Canada and the United States.\textsuperscript{141, 142} These interspecies transmissions significantly impact the ecology of IAVs in swine populations.
1.5 Direct Detection Methods

Viral isolation

Viral isolation is the method of choice for influenza virus detection. It is highly specific and results in the propagation of live virus that can be cryogenically preserved and further used for virus characterization or vaccine development. Nasal swabs, oropharyngeal swabs, broncho-alveolar lavage fluid, or lung tissue provide the best samples for viral isolation.²,¹⁴³ The preferred hosts for influenza virus are embryonating chicken eggs (ECE) and cell cultures such as Madin-Darby canine kidney (MDCK) cells.¹⁴⁴-¹⁴⁶ Egg inoculation (EI) using 9-to-11-day-old ECEs is considered the gold standard for isolation and propagation of avian-origin IAVs and has been used for mammalian IAVs¹⁴⁷-¹⁴⁹ However, antigenic differences resulting from amino acid changes in the HA gene are known to occur in human influenza viruses propagated by this method.¹⁵⁰-¹⁵² These same viruses show little to no genetic or antigenic variation when propagated in mammalian cell lines including Vero, MRC-5, BHK-21, and fetal porcine kidney cells.¹⁵⁰-¹⁵⁴ Today, MDCK cells are most commonly used for research and diagnostic purposes as they have high sensitivity.¹¹¹,¹⁵⁵,¹⁵⁶ However, the genetic drift and frequent genetic reassortment occurring among IAVs in swine populations makes inoculation of ECEs and/or another cell line in addition to culture in MDCK cells a recommended approach for maximum sensitivity.

In cell cultures viral growth induces the production of cell lysis or cytopathic effects (CPE) which is visually identifiable. For certain strains a second blind passage is often necessary to elicit CPE. Culture supernatant is used to confirm viral identity after
the virus has grown in cell cultures. Hemagglutination (HA) of erythrocytes can be interpreted as a presumptive diagnosis of IAV and used to approximate the amount of virus present in the supernatant (1 HA unit approximates 5-6 log₁₀ of virus), but is not a definitive assay. For definitive viral identification, RT-PCR or a commercial influenza antigen test kit based on NP or M antigens in the culture supernatant can be used. Since viral culture amplifies viral concentration higher than that of the original sample, sensitivity issues incurred with clinical samples are usually not problematic when using commercially available antigen test kits. Viral titration by inoculating a set of serial dilutions in cell culture is a common quantitative method.

Viral isolation is time consuming (taking up to 5-14 days), the virus may be inactivated during shipping and preparation, and host preferences for various strains may be unknown, complicating viral culture.¹⁴⁴, ¹⁵⁷-¹⁵⁹ Despite these potential pitfalls and its reliance maintaining cell cultures and/or ECEs, viral isolation is a standard procedure available in most veterinary diagnostic laboratories.

**Antigen Detection**

Compared to viral isolation, the fluorescent antibody test (FA) provides a shorter detection time for influenza viruses, giving results after only 48 hours of incubation.¹⁵⁷, ¹⁶⁰ In this method, monoclonal antibodies specific for influenza virus proteins are applied to frozen sections of lung or cytological smears of nasal swabs.¹⁶¹ These antibodies are usually type-specific influenza A or B, NP, or M1 proteins. Besides providing a short turnaround time, FA also requires less sample preparation time and has the added
advantages of high specificity, the ability to detect live or inactivated virus, and localization of the virus to the lesions.\textsuperscript{157, 160, 162} However, this method also incurs variable sensitivity depending on the quantity and quality of samples tested, with higher sensitivity obtained as the sample number and quality increase.\textsuperscript{161} Because FA requires highly skilled technicians and laboratories with fluorescent microscope capabilities, other diagnostic tests which are even more rapid, such as immunoassays or RT-PCR, are becoming preferable and replacing FA.

Commercially available rapid immunoassays, such as enzyme-linked immunosorbent assay (ELISA)-based tests kits, are useful for detecting influenza virus antigen in clinical samples.\textsuperscript{160, 163-165} Most have been developed to detect viral protein NP using specific monoclonal antibodies; results are indicated by the presence or absence of a color change. While immunoassay testing is rapid (producing results in <15 minutes) and highly specific, the sensitivity of the test varies by strain, and it is fairly expensive for individual animal use.\textsuperscript{161, 166, 167} Rapid tests also does not allow for viral subtyping, detecting only the presence or absence of IAV.

Immunohistochemistry (IHC) chromogen-labeled polyclonal or monoclonal antibodies can be used to detect antigens in formalin-fixed paraffinembedded or frozen tissues.\textsuperscript{168} While the benefits of IHC are comparable to those described for FA, this method has the additional safety advantage of using fixed tissue in which the virus has been inactivated.\textsuperscript{169} IHC is frequently used to study viral pathogenesis because pathologists are able to localize the viral antigen to the sites of viral replication within the respiratory track.\textsuperscript{169} False negative results can occur in autolyzed samples or samples that
have undergone harsh chemical treatments. Other disadvantages include a slower turnaround time (at least 3 days from collection of tissue to results) and increased cost. As with ELISA testing, influenza IHC does not allow for subtyping, detecting only the presence or absence of viral antigens.

**Nucleic Acids**

Reverse transcription-polymerase chain reaction (RT-PCR). First described in 1985, polymerase chain reaction (PCR) has been used to sequence and analyze genes and diagnose infectious and genetic diseases. The production of complementary DNA (cDNA) from RNA was made possible by the development of RT-PCR. With the availability of real-time PCR (RRT-PCR), PCR became an even more powerful tool. Several RRT-PCR testing protocols have been developed for the detection and quantification of IAVs, with those developed for avian species birds being adapted to swine.

RNA extraction and purification methods can vary according to the type of sample being tested. Direct extraction of RNA is possible from infected amnioallantoic fluids, cell culture supernatants, bronchoalveolar lavage fluids, and oral fluids, though additional processing (i.e. homogenizing tissue samples with viral culture medium) may be needed for certain clinical diagnostic samples. Commercial kits that use magnetic beads or solid-phase adsorption are highly sensitive, easier to use, and provide consistent results with magnetic bead extraction kits proving very useful for liquid samples that
have low virus concentrations or contain PCR inhibitors, including oral fluids, blood, urine, feces, and semen.  

Primers for RRT-PCR are designed to target the conserved M or NP genes allowing detection of all IAV subtypes. RT-PCR assays are nearly 100% specific with properly designed primers and probes and are known to be highly sensitive with detection of 0.01 TCID$_{50}$/sample test volume, 0.1 TCID$_{50}$/ml, 10 TCID$_{50}$/ml, 1000 copies/600 ng of RNA, or 1–10 viral genomes per PCR reaction reported from multiple sources. An avian influenza RRT-PCR for the M gene, validated by the USDA, is now widely used for the detection of IAV in swine samples but it does not differentiate HA or NA subtype. Depending on the strain, the minimum detectable concentration of the virus for this procedure ranges from 10$^{-1}$ to 10$^{1}$ TCID$_{50}$/ml.  

RT-PCR is a sensitive and specific technique that can be used to rapidly screen a large number of samples making it the test of choice for influenza surveillance. Additionally beneficial, the technology also allows for genomic sequencing and does not require live virus for detection. However, RT-PCR involves high cost technology investment, skilled labor, and does not produce live virus for further studies or technology development. These disadvantages lead to testing schemes using both RT-PCR and viral isolation either in series or parallel. The continuous evolution of IAVs dictates frequent re-validation of RT-PCR protocols with contemporaneous strains.
Virus subtyping and sequencing

The 17 HA and 10 NA IAV gene variations are used for subtyping influenza viruses and determining host range, antigenicity and pathogenesis of various strains. Historically, antigenic subtyping using HA and NA inhibition assays (HI, NI) performed on cultured viruses were the gold standard methods for subtyping IAVs. Because both HI and NI assays are time-consuming and require standardized NA and HA antisera which are difficult to obtain, genomic subtyping using specific RT-PCRs targeting any of the eight genes is now more commonly used for subtyping IAVs. Multiplex formats consisting of several primer sets permit simultaneous amplification and detection of several genes.

After the 1998 emergence of triple-reassortant H3N2 IAVs in North American swine there has been ongoing rapid genetic and antigenic change among swine-origin IAVs. Analyzing these viruses requires molecular characterization which was initially accomplished by sequencing individual gene segments using capillary electrophoresis technology. Cell culture supernatants and amnioallantoic fluid containing a large concentration of whole virus are recommended for sequencing but direct sequencing from clinical samples can be accomplished. Universal primers amplifying the 3' end of the influenza genomic RNA and full genome sequencing using next generation sequencing platforms is now common and being used to conduct comprehensive analyses of IAVs. Using molecular analysis tools, the sequences can then be compared with others, revealing the evolutionary and geographic relationships of influenza viruses.
1.6 Indirect detection methods

Detection of influenza-specific serum antibodies is a useful surveillance measure for IAV especially when IAV is no longer directly detectable at the time of testing. Because IAV antibodies can form in response to both vaccination and exposure, determining such status is crucial when interpreting test results. Common serologic tests used to detect and measure influenza antibodies include hemagglutination inhibition (HI), serum neutralization (SN), and enzyme-linked immunosorbent assays (ELISA).\textsuperscript{2, 200, 201} In swine, immunoglobulins (predominantly IgG) rise to detectable levels within 1-2 weeks post-infection and peak at 4-7 weeks.\textsuperscript{148}

The HI test is a standard method for detecting IAV antibodies, although it is somewhat prone to operator subjectivity, and results can vary upon repeated sample testing.\textsuperscript{160} Agglutination of red blood cells occurs in the presence of HA protein on the viral surface and can be specifically inhibited by IAV antibodies.\textsuperscript{59} This inhibition can be measured by the HI assay which is inexpensive and simple to perform. Turkey or chicken red blood cells (RBCs) are commonly used for HI reactions for IAV in swine. Swine serum require removing non-specific inhibitors of viral hemagglutination and naturally occurring non-specific agglutinins which is commonly achieved by treatment with receptor destroying enzyme (RDE) from \textit{Vibrio cholera} or heat inactivation at 56°C.\textsuperscript{201} Titers are reported as the highest dilution of serum that prevents hemagglutination, with titers of 1:40 and higher typically considered specific and positive.\textsuperscript{202} Disadvantages of HI include the requirement of standardized live viral references; low repeatability
between runs, technicians, laboratories; and heterologous cross reactions may provide false positives.\textsuperscript{201, 203, 204}

Virus-specific neutralizing antibodies in serum samples are capable of blocking viral infection in cell culture, and can be detected by the serum neutralization test. In contrast, if these antibodies are absent, the virus is able to infect cells and cause CPE in the culture.\textsuperscript{201} A known quantity of pre-incubated IAV is added to MDCK cells and the highest dilution of serum that can neutralize cell infection and produce CPE is determined; the SN titer of the sample is calculated as the reciprocal of that dilution.\textsuperscript{200} This test is often called microneutralization because it uses minute volumes of serum in cell monolayers contained in 96-well microtiter plates. SN has the advantage of demonstrating biological activity of serum antibodies, rather than just their presence. However, it requires virus culture, and results can take up to 72 hours to obtain. As with the HI assay, SN titers may vary upon repeated testing.

The ELISA method for detecting IAV antibodies provides an automated, high-throughput, low labor-intensive method of detection. Antigen-antibody reactions are the basis for the test and the addition of an enzyme substrate causes a color-changing reaction that is measured by a spectrophotometer as an optical density (O.D.), which is inversely proportional to the quantity of anti-influenza antibodies present in the sample.\textsuperscript{205} A commercially available ELISA kit developed for avian use relies on the detection of a highly conserved epitope of IAV nucleoprotein and appears to detect antibodies against IAV subtypes commonly found in swine.\textsuperscript{206, 207} There are H1N1 and H3N2 antibody detection test kits commercially available, but because the commercial ELISAs only use
antigens derived from an α-cluster H1N1 IAV and a Cluster I H3N2 IAV they detects a limited range of swine subtypes.\textsuperscript{208}
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Chapter 2 - Comparative effectiveness of isolation techniques for contemporary Influenza A virus strains circulating in exhibition swine


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2.1 Abstract

The current study sought to compare the effectiveness of 2 virus isolation methods for the recovery of contemporary Influenza A virus (FLUAV) strains circulating in swine at agricultural exhibitions. Following the emergence of the influenza A (H1N1)pdm09 virus, increased surveillance of FLUAV strains among swine was recommended for early detection of emerging strains that threaten animal and human health. The increase in genetic drift and genomic reassortment among FLUAV strains infecting swine since 1998 necessitates that detection protocols be periodically validated for contemporary FLUAV strains. During 2009, nasal swabs were collected from 221 clinically healthy pigs at 12 agricultural exhibitions in Ohio. Nasal swabs were tested in parallel for the presence of FLUAV strains using 3 methodologies: 2 passages through Madin–Darby canine kidney (MDCK) cells adapted to serum-free medium (SFM), 2 passages through embryonated chicken eggs (ECEs), and real-time reverse transcription polymerase chain reaction (real-time RT-PCR). Of the 221 samples, 40 (18.1%) were positive for FLUAV recovery in MDCK cell culture and 13 (5.9%) were positive in ECEs (P = 0.015). All samples positive in ECEs were also positive in MDCK cell culture. MDCK cell culture virus isolation results were in perfect agreement with results of the real-time RT-PCR. Hemagglutinin and neuraminidase combinations of the recovered isolates were H1N2 and H3N2, which were consistent with FLUAV strains circulating in U.S. pigs. Effectiveness and cost savings justify the use of SFM-adapted MDCK cell culture over ECEs for the recovery of contemporary FLUAV strains from exhibition swine.


2.2 Introduction

The emergence of the influenza A (H1N1)pdm09 virus and the subsequent detection of numerous genomic variant strains of Influenza A virus (FLUAV) infecting swine and human beings have resulted in a wide call to increase FLUAV surveillance in swine.\textsuperscript{16,17,35} This request is being met in human and veterinary diagnostic laboratories using several established and widely accepted virus detection techniques for FLUAV strains. However, with the apparent increase in the frequency in genetic drift and genomic reassortment among multispecies-origin FLUAV strains infecting swine, especially since 1998, there is a need to continually evaluate the effectiveness of assays to ensure accurate results from virus surveillance efforts in swine.

Pigs are often called “mixing vessels” for FLUAV strains because the receptors present in the porcine respiratory tract allow pigs to become infected with swine-, human-, and avian-origin FLUAV strains.\textsuperscript{21} The pig’s susceptibility to FLUAV strains originating from multiple host species facilitates interspecies virus transmission and genomic reassortment in pigs thus making swine a source of novel FLUAV strains.\textsuperscript{26,31} Consequently, comprehensive surveillance programs targeted at the FLUAV strains circulating in swine populations could aid in the detection of emerging viruses that may threaten animal and human health. The effectiveness of these virus surveillance programs is reliant upon the effectiveness of virus isolation protocols to detect and characterize all FLUAV strains in the swine population.
Nasal swabs are routinely used for the antemortem detection of FLUAV strains in swine.\textsuperscript{33} Isolation of such strains from nasal swabs collected from pigs has been performed using a variety of substrates,\textsuperscript{10,12,14,39} with embryonated chicken eggs (ECEs) and Madin–Darby canine kidney (MDCK) cells being most common.\textsuperscript{8,25,45} The preferential growth of FLUAV strains in various culture substrates has been repeatedly documented and is strain dependent.\textsuperscript{6,29,36,45} Embryonated chicken eggs have historically been the gold standard for isolating FLUAV strains.\textsuperscript{7,33,51} More recently, MDCK cells have been shown to be sensitive to a wide range of FLUAV strains, especially those circulating in mammals, and thus, are now routinely used for the isolation and propagation of mammalian-origin FLUAV strains.\textsuperscript{10,13}

The continuous genetic drift and frequent genetic reassortment occurring among swine-origin FLUAV strains since the emergence of the triple-reassortant H3N2 virus in 1998\textsuperscript{23,34,52} requires virus isolation protocols to be periodically validated with contemporary viral strains. The purpose of the present study was to compare the effectiveness of MDCK cells adapted to serum-free culture and ECEs for the recovery of contemporary FLUAV strains circulating in swine at agricultural exhibitions.

\textbf{2.3 Materials and Methods}

Study sites for the investigation included 12 agricultural exhibitions in Ohio during July, August, and September of 2009. Nasal swabs were collected from 221 clinically healthy, market-weight pigs according to the World Organization for Animal Health and the Food and Agriculture Organization of the United Nations joint network of expertise on animal influenza guidelines (OFFLU: 2009, Collection of specimens for
detection of influenza from swine. Available at http://www.offlu.net/index.php?id=184. Accessed on November 12, 2012). Samples were collected on the last or next to last day of the swine exhibition period at each fair. Protocol number 2009A0134 for animal use was approved by the Institutional Animal Care and Use Committee of The Ohio State University. A single nasal swab collected from each pig was placed in an individual vial containing 1.8 ml of brain heart infusion (BHI) broth supplemented with penicillin (10,000 U/ml) and streptomycin (10 mg/ml). The vials were kept in a cooler with cool packs during sample collection, transported directly to the laboratory on dry ice, and then stored at −70°C until testing was initiated.

At the time of testing, vials were quickly thawed in a 37°C water bath. Amphotericin B (20 μg/ml), gentamicin sulfate (1,000 μg/ml), and kanamycin sulfate (325 μg/ml) were added to each vial as previously described to control bacterial and fungal contamination. Each vial was then vigorously agitated, after which, the vial was centrifuged at 1,200 × g for 30 min at 4°C. The BHI broth supernatant was then tested in parallel for the presence of infectious FLUAV in MDCK cell culture and ECEs, and for the FLUAV matrix (M) gene using targeted real-time reverse transcription polymerase chain reaction (real-time RT-PCR).

*Virus isolation in MDCK cell culture*

The methods used for FLUAV isolation in MDCK cell culture were adapted from previously described protocols to utilize a serum-free culture system. To summarize, MDCK cells were seeded in tissue culture flasks and grown in minimum essential
medium with Earle salts and L-glutamine\textsuperscript{b} supplemented with 1.0 mM sodium pyruvate,\textsuperscript{c} 0.1 mM nonessential amino acids,\textsuperscript{d} and 10% fetal bovine serum (FBS).\textsuperscript{e} Cells were maintained at 37°C in a 5% CO2 incubator. Once a productive cell line was established, MDCK cells were transitioned to serum-free medium (SFM)\textsuperscript{f} by decreasing the concentration of FBS over 4 passages in a stepwise fashion according to the manufacturer’s guidelines.\textsuperscript{f} Less than passage-25 MDCK cells fully adapted to SFM were used for all virus isolation attempts. The SFM-adapted MDCK cells were seeded into every other vertical column of a 24-well cell culture plate. One of the 4 wells in each empty column of wells was also seeded with SFM-adapted MDCK cells to serve as a negative control. This allowed for 3 samples to be tested in quadruplicate on one 24-well plate, with each sample having a negative control. Immediately prior to inoculation, the SFM from confluent cell monolayers in 24-well tissue culture plates was removed, and the monolayers were washed once with viral growth medium (VGM), which was removed immediately prior to inoculation. Viral growth medium was prepared by supplementing SFM with antimicrobials\textsuperscript{g} (1,200 U/ml of penicillin, 1.2 mg/ml of streptomycin, and 3 µg/ml of amphotericin B) and tolylsulfonyl phenylalanyl chloromethyl ketone–treated trypsin (0.5 µg/ml).

For each sample, 150 µl of BHI broth supernatant was inoculated into each of 4 wells of monolayered MDCK cells prepared as described above. Inoculum was allowed to adsorb for 60 min at 37°C before being removed and replaced with 0.5 ml of VGM. Plates were incubated at 37°C in 5% CO2 for 72 hr. Wells were observed daily for cytopathic effects (CPE) by inverted light microscopy, and the results were recorded.
After 72 hr, supernatant from each well was tested for hemagglutination activity using 0.5% turkey red blood cells in V-bottom plates. Hemagglutinating agents were screened for FLUAV strains using a commercially available diagnostic kit to identify FLUAV antigen. If CPE and hemagglutination were not present, media from the 4 wells representing 1 original sample was aseptically harvested and pooled. Pooled first-pass media was stored at −70°C until a second passage was performed. Second passage was carried out by inoculating the pooled first-passage media in quadruplicate onto monolayers of MDCK cells as described above. If no hemagglutinating activity was detected after 2 passages, the media was harvested and pooled in the same manner used for the first-pass media, and subsequently tested with a FLUAV M gene–targeted real-time RT-PCR (described below) to verify the negative result.

Virus isolation in embryonated chicken eggs

All samples were tested with standard methods previously described. Briefly, 150 μl of clarified viral transport media was inoculated, in quadruplicate, via the allantoic sac into 10 day-old, specific pathogen–free embryonated chicken eggs. Eggs were incubated at 37°C with 45% relative humidity and candled daily with dead eggs removed and placed in refrigeration at 4°C. Embryos alive after 60 hr were chilled at 4°C overnight. Chorioallantoic fluids from all eggs were tested for hemagglutinating agents and FLUAV strains as described above. If hemagglutination was not detected, the chorioallantoic fluids from the 4 eggs representing 1 original sample were aseptically harvested, pooled, and stored at −70°C until a second passage was performed. Pooled
first-passage chorioallantoic fluids were inoculated into 4 eggs to perform a second passage. If no hemagglutinating activity was detected after 2 passages, chorioallantoic fluids were harvested and pooled in the same manner as the first passage and then tested for the FLUAV M gene with real-time RT-PCR.

Real-time reverse transcription polymerase chain reaction

All original clarified samples and samples found negative after 2 passages in either virus isolation method were tested using a modified version of previously described FLUAV M gene–targeted real-time RT-PCR procedures.\textsuperscript{41,42} Briefly, RNA was purified using an RNA extraction kit according to the manufacturer’s protocol. Following extraction, the RNA was subjected to real-time RT-PCR using a one-step quantitative (q)RT-PCR kit\textsuperscript{i} in a 20-μl reaction mixture containing 10 μl of 2× qRT-PCR master mix, 8 pmol of each primer, 2.4 μM probe, 0.3 μl of diluted reference dye (5-carboxy-X-rhodamine, 1:500), 0.2 μl of 100 mM of dithiothreitol, 1 μl of RT/RNase block enzyme mixture, and 5 μl of extracted RNA. The reactions were performed on a qPCR system\textsuperscript{k} under the following thermocycling conditions: stage 1, 50°C for 10 min; stage 2, 95°C for 3 min; and stage 3, 40 cycles of 95°C for 1 sec and 60°C for 20 sec. Threshold cycle (Ct) values were calculated for each sample automatically by the qPCR system’s software using the background-based method. Samples with a Ct of ≤40 were considered positive.
**Subtyping**

The genomic hemagglutinin and neuraminidase subtypes of the recovered FLUAV isolates were determined by real-time RT-PCR with primers and probes developed for swine-origin FLUAV strains. RNA was extracted as described above and subjected to real-time RT-PCR using a one-step multiplex RT-PCR kit in a 25-μl reaction mixture containing 12.5 μl of 2× multiplex RT-PCR master mix, 10 pmol of each primer, 0.2 μM each probe (except N2 probe, which was increased to 0.4 μM), 0.25 μl of RT mixture, and 8 μl of extracted RNA. Hemagglutinin H1 and H3 probes were labeled with hexachlorofluorescein and 5-carboxyfluorescein, respectively, and run as a duplex reaction. The neuraminidase N1 and N2 probes labeled with hexachlorofluorescein and 5-carboxyfluorescein, respectively, were run as a separate duplex reaction. The reactions were performed on a qPCR system under the following thermocycling conditions: stage 1, 50°C for 20 min; stage 2, 95°C for 5 min; and stage 3, 40 cycles of 95°C for 15 sec and 54°C for 30 sec. Threshold cycle values were calculated for each sample automatically by the qPCR system’s software using the adaptive algorithm. Samples with a Ct of ≤40 were considered positive.

**Statistical analysis**

The results from virus isolation with MDCK cell culture and ECEs were cross-tabulated and analyzed using the McNemar chi-square statistic with a standard statistical program. Because the nasal swabs were collected from groups of commingled pigs, the underlying assumption of independent samples was not valid, thus the clustering effect was adjusted.
for using the Eliasziw–Donner procedure. Samples from the fairs where FLUAV strains were not detected by any method were excluded from the statistical analysis because these results did not contribute to the number of discordant pairs and severely skewed the within-cluster correlation.

Detection of α-2,3– and α-2,6–linked sialic acid residues by flow cytometry. A previously described protocol using flow cytometry15 was modified to quantify the abundance of α-2,3 sialic acid (SA 2,3) and α-2,6 sialic acid (SA 2,6) linkages to galactose expressed by the SFM-adapted MDCK cells. Briefly, freshly harvested passage-20 SFM-adapted MDCK cells (1 × 10^6 cells/sample) were washed with 5% FBS in phosphate buffered saline with sodium azide and incubated in the dark at 4°C for 30 min in the presence of biotinylated Sambucus nigra (SNA) lectin and fluorescein isothiocyanate–conjugated Maackia amurensis (MMA) lectin. After washing, cells were stained with streptavidin–phycoerythin, rewarshed, and then fixed for later analysis. Samples were analyzed on a flow cytometer. A minimum of 50,000 events were recorded per sample. Data was processed using commercial software. Side scatter and forward scatter gating isolated the SFM-adapted MDCK cells, and fluorescence-minus-one controls along with negative controls without lectins were used to define the gating strategy (Fig. 1). Purified mouse alveolar epithelial type II cells were used as a positive control for MMA staining.
2.4 Results

Of the 221 samples, FLUAV was detected in 40 samples (18.1%) with real-time RT-PCR, 40 samples (18.1%) with virus isolation with MDCK cells, and 13 samples (5.9%) with virus isolation with ECEs. The positive nasal swabs were collected from pigs from 3 of the 12 separate agricultural exhibitions. Virus isolation results with SFM-adapted MDCK cells and real-time RT-PCR analysis of original samples showed perfect agreement. All samples that were positive by ECEs were also positive in MDCK cells, but there were 27 specimens positive in MDCK cells and by real-time RT-PCR that failed to grow in ECEs (Table 1). The adjusted McNemar chi-square value comparing virus isolation in ECEs with virus isolation in MDCK cells was 5.95 (P = 0.015).

All CPE-positive cell culture wells were also positive for hemagglutinating activity, and all hemagglutinating agents were identified as FLUAV strains. No FLUAV strains were identified in CPE-negative MDCK monolayers or hemagglutination-negative cell culture fluids. Subtypes H1N2 and H3N2 were recovered from both MDCK cell culture and ECE virus isolation systems. For the 13 isolates recovered in ECEs, 8 (61.5%) were H1N2 and 5 (38.5%) were H3N2. Examining the 40 isolates recovered using SFM-adapted MDCK cells, 12 (30%) were H1N2 and 28 (70%) were H3N2. These hemagglutinin–neuraminidase subtype combinations were consistent with FLUAV strains contemporaneously circulating in U.S. pigs.30

All FLUAV isolates recovered with MDCK cell culture were recovered during first passage. No additional strains were recovered from second passage in MDCK cell culture (Table 2) or detected by real-time RT-PCR on second-passage cell culture
supernatant. In contrast, 8 FLUAV isolates were detected in first ECE passage and 5 additional FLUAV isolates were recovered from second passage in ECEs that were not detected in first passage. Real-time RT-PCR analyses confirmed all other second-passage chorioallantoic fluids as negative for FLUAV strains.

Two-color staining using biotinylated (SNA) lectin and fluorescein isothiocyanate–conjugated MMA lectin demonstrated that more than 98% of SFM-adapted MDCK cells reacted with SNA, indicating dominant SA 2,6 residues in SFM-adapted MDCK cells (Fig. 2). Of those, less than 2.25% of the cells were double-positive, also reacting with the MMA lectin, indicating the presence of SA 2,3 residues. The SFM-adapted MDCK cells with only SA 2,3 residues were lacking, as demonstrated by the failure to detect cells reacting with only the MMA lectin and not the SNA lectin. The finding was further validated by demonstrating that the MMA lectin bound as expected to the positive controls (mouse alveolar epithelial type II cells, which express high levels of SA 2,320; Fig. 3).

2.5 Discussion

Veterinary and public health officials are increasing surveillance of FLUAV strains in swine in response to numerous calls for earlier detection of emerging strains that may threaten animal and human health.\textsuperscript{16,17,35} Depending on the objective, virus isolation and/or real-time RT-PCR are currently the primary methods used to detect FLUAV strains among swine. The results of the current study show excellent agreement between real-time RT-PCR and virus isolation in SFM-adapted MDCK cell culture.
However, caution must be exercised when interpreting the agreement between real-time RT-PCR and MDCK found in the study because the sampled pigs were in a noncommercial setting, and were likely exposed to FLUAV 5–7 days earlier upon entry into their respective exhibitions via a point-source or near point-source introduction of virus. Previous studies have shown pigs infected with FLUAV begin shedding virus 48 hr postexposure and can continue to shed for up to greater than 10 days. In the present study, nasal swabs were collected at the end of the exhibition, which likely coincided with peak viral shedding in the swine population. Consequently, real-time RT-PCR and virus isolation in SFM-adapted MDCK cell culture performed equally well in this setting. Under different circumstances it would not be surprising to find more real-time RT-PCR–positive samples than virus isolation–positive samples.

In order to amplify FLUAV strains to a detectable level during virus isolation procedures, the use of multiple passages in cell culture and ECE has been widely recommended. The results of the present study show that second passage failed to yield any additional virus isolates in the SFM-adapted MDCK cell culture system. While additional viruses were recovered with a second passage in the ECE system, virus isolation with MDCK cell culture still greatly outperformed virus isolation with ECEs. The additional time and costs associated with performing a second pass in SFM-adapted MDCK cell culture were not necessary under the circumstances of the current investigation. Moreover, even with good laboratory procedures, each additional handling of potentially infectious material significantly increases the likelihood of cross-contamination and thus spurious results. The need to perform multiple passages in either
system would be warranted when an original sample or first-passage cell culture fluid is real-time RT-PCR positive but FLUAV was not detected after a single passage.

In the present study, the use of SFM-adapted MDCK cell culture was highly sensitive for the recovery of H1N2 and H3N2 FLUAV strains circulating among swine at the selected agricultural exhibitions. The use of SFM-adapted MDCK cells to cultivate human-origin FLUAV strains has been previously described and is currently being investigated as a substrate to produce live attenuated FLUAV vaccines for human use. Human-origin strains have been reported to grow to higher titers in MDCK cells adapted to SFM than in traditional MDCK culture systems. The use of SFM culture maintains physiological consistency and eliminates the problems associated with the lot-to-lot variability of FBS. At the time of writing, the cost of SFM and traditional culture media were comparable.

Binding to cell surface receptors with specific sialic acid groups is needed for FLUAV strains to enter a host cell and thus a substrate with an appropriate receptor is required for successful virus isolation. Most avian-origin FLUAV strains preferentially bind to SA 2,3 whereas mammalian-origin strains, especially those infecting human beings, prefer SA 2,6. Conventional MDCK cells express both SA 2,3 and SA 2,6 linkages in similar proportions. The SFM-adapted MDCK cells used in the present study clearly have higher SA 2,6 and lower SA 2,3 receptor levels than traditional MDCK cells. In contrast to the SFM-adapted MDCK cells, the allantoic cells of ECE are known to be rich in SA 2,3 but low in SA 2,6, which likely contributes to the improved recovery of swine-origin FLUAV strains with SFM-adapted MDCK cells. Cells
predominantly expressing SA 2,6 could improve the recovery of FLUAV strains threatening swine and human health while selecting against avian-origin strains. Given the lack of SFM-adapted MDCK cells expressing SA 2,3, the protocol described above may be of limited value to investigations of FLUAV strains at the avian–swine interface.

Presently, there are several different lineages of MDCK cells commercially available,\textsuperscript{9} which makes poorly defined diagnostic protocols subject to unintentional variability. Additionally, the parental lines of MDCK cells, like those used in the present study, are a heterogeneous population of epithelial cells, which allows for preferential selection of a particular subpopulation of cells.\textsuperscript{19} The long-term effects of maintaining MDCK cells in SFM culture have not been thoroughly investigated. Although, only low passage number cells (≤ passage 25) were used in the present study in an attempt to minimize the impact of selection pressure on the MDCK cells, the use of SFM may alter the virus susceptibility of MDCK cells. Traditional MDCK cell culture protocols using FBS \textsuperscript{44,49} were not used in the present study due to difficulty maintaining consistently adhered monolayers of MDCK cells when FBS containing cell growth medium was removed and replaced with VGM without FBS following inoculation. By adapting to the protocols to utilize SFM, the MDCK cells were no longer subjected to the sudden removal of FBS, and the resulting monolayers remained adhered to the plate surface, thus aiding in the visual detection of CPE. Ultimately, further studies are needed to compare the use SFM-adapted MDCK cell culture to traditional MDCK cell culture with FBS for the isolation of FLUAV strains from swine.
Under the circumstances of the present study, virus isolation with SFM-adapted MDCK cell culture was significantly superior to virus isolation with ECEs for the recovery of the contemporary FLUAV strains circulating in selected swine populations at the time of sampling. While the strains isolated in the study preferentially grew in MDCK cell culture over ECE, this finding may not hold true for all contemporary strains in the U.S. swine herd. Indeed, it has been shown that differences in growth of swine-origin FLUAV strains in ECE and cell culture can be associated with viral strain. A previous study compared ECE to MDCK cell culture for the isolation of FLUAV strains and found ECE superior to MDCK cell culture. Although the current results contradict those previous findings, it is important to note that the previous study used one H1N1 egg adapted strain, not field strains from original samples. Additionally, all 3 previous studies utilized traditional MDCK cell culture methods rather than the SFM-adapted MDCK cell culture utilized in the present study. Nevertheless, the previous findings led to the suggestion that ECE and cell culture should be used in tandem for the detection of swine-origin FLUAV strains. However, the financial cost and labor required to conduct virus isolation in ECE is significantly higher than that of MDCK cell culture, which makes conducting blind passage in parallel virus isolation systems unfeasible for large-scale surveillance. The results of the current study, as well as one previous study, show that MDCK cell culture provides better results for primary isolation of swine-origin FLUAV strains than ECE. However, the use of ECE and/or other cell lines should be considered when real-time RT-PCR–positive samples do not yield recoverable FLUAV in MDCK cell culture.
The methods chosen for the detection and/or characterization of FLUAV strains from swine will vary based on viral strains, animal population dynamics, laboratory resources, and goal of the project. The results of the present study support the use of MDCK cells adapted to serum-free culture as the primary isolation method for FLUAV surveillance among swine at agricultural exhibitions. Regardless of what methodology is used, the genetic drift and genomic reassortment of FLUAV strains requires continuous validation of any protocol with contemporary viruses to ensure highest sensitivity and accurate results.

2.6 Acknowledgments

The authors wish to acknowledge the support of the personnel at the fairs for participating in this study. Additionally, the authors acknowledge assistance of Dr. Jillian Yarnell.

2.7 Article Notes

Declaration of conflicting interests

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Surveillance (CEIRS), National Institute of Allergy and Infectious Diseases, National
Institutes of Health, Department of Health and Human Services, under contract no.
HHSN266200700007C.

2.8 Notes

a. MDCK cell line canine (catalog no. 84121903), Sigma-Aldrich, St. Louis, MO.
b. MEM/EBSS, HyClone Laboratories Inc., Logan, UT.
c. Gibco MEM sodium pyruvate solution (100 mM), Invitrogen Corp., Carlsbad, CA.
d. Gibco MEM non-essential amino acids solution (10 mM), Invitrogen Corp., Carlsbad, CA.
e. Gibco fetal bovine serum (certified, heat inactivated), Invitrogen Corp., Carlsbad, CA.
f. BioWhittaker UltraMDCK serum-free medium (document no. P-SFC 11/09), Lonza
Walkersville Inc., Walkersville, MD.
g. Gibco antibiotic-antimycotic (100×), Invitrogen Corp., Carlsbad, CA.
h. Flu DETECT, Synbiotics Corp., Kansas City, MO.
i. PrepEase RNA Spin kit, Affymetrix Inc., Cleveland, OH.
k. Mx3000P QPCR system, Agilent Technologies Inc., Santa Clara, CA.
l. QuantiFast Multiplex RT-PCR kit, Qiagen Inc., Valencia, CA.
m. Vector Laboratories Inc., Burlingame, CA.
n. EY Laboratories Inc., San Mateo, CA.

o. R&D Systems Inc., Minneapolis, MN.

p. CytoFix, BD, San Jose, CA.

q. FACS Calibur flow cytometer, BD, Franklin Lakes, NJ.
r. FlowJo, Treestar Inc., Ashland, OR.
2.9 References


Table 2.1: Isolation of Influenza A virus from clinically healthy swine at 3 county fairs in embryonated chicken eggs and in Madin–Darby canine kidney (MDCK) cell culture using serum-free medium.*

<table>
<thead>
<tr>
<th></th>
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<td>Total</td>
</tr>
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<tr>
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* Adjusted McNemar $\chi^2 = 5.95$ ($P = 0.0015$).
Table 2.2: Recovery of Influenza A virus from 2 passages through Madin–Darby canine kidney (MDCK) cell culture and embryonated chicken eggs (ECEs).

<table>
<thead>
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</tr>
<tr>
<td>ECEs</td>
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</table>
Figure 2.1: Isolated cells and negative controls to define the flow cytometry gating strategy

Side scatter (SSC) and forward scatter (FSC) analysis were used to isolate serum-free medium (SFM)-adapted Madin–Darby canine kidney (MDCK) cells (A). Negative controls without lectins (B, C) and fluorescence-minus-one (FMO) controls (D) were used to define the gating strategy. SNA = Sambucus nigra lectin; FITC = fluorescein isothiocyanate; MMA = Maackia amurensis lectin.
Flow cytometric analysis of serum-free medium (SFM)-adapted Madin–Darby canine kidney cells

Flow cytometric analysis of serum-free medium (SFM)-adapted Madin–Darby canine kidney (MDCK) cells for the detection of α-2,3– and α-2,6–linked sialic acid (SA 2,3 and SA 2,6, respectively) residues. Greater than 98% of SFM-adapted MDCK cells expressed SA 2,6 residues, and of that population less than 2.25% of the cells also expressed SA 2,3 residues. Cells expressing only SA 2,3 residues were not detected. SNA = Sambucus nigra lectin; FITC = fluorescein isothiocyanate; MMA = Maackia amurensis lectin.
Figure 2.3: Positive controls for the detection of α-2,3–linked sialic acid residues

Flow cytometric analysis of mouse alveolar epithelial type II (ATII) cells used as positive controls for the detection of α-2,3–linked sialic acid (SA 2,3) residues. Greater than 98% of mouse ATII cells expressed SA 2,3 as expected. FITC = fluorescein isothiocyanate; MMA = Maackia amurensis lectin.
Chapter 3 - Subclinical Influenza Virus A Infections in Pigs Exhibited at Agricultural Fairs, Ohio, USA, 2009–2011

Andrew S. Bowman, Jacqueline M. Nolting, Sarah W. Nelson, and Richard D. Slemons

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3.1 Abstract

Agricultural fairs are associated with bidirectional, interspecies transmission of influenza virus A between humans and pigs. We examined pigs exhibited at agricultural fairs in Ohio during 2009–2011 for signs of influenza-like illness and collected nasal swab specimens from a representative subset of these animals. Influenza virus A was recovered from pigs at 12/53 (22.6%) fairs during the 3-year sampling period. Pigs at 10/12 (83.3%) fairs from which influenza virus A was recovered did not show signs of influenza-like illness. Hemagglutinin, neuraminidase, and matrix gene combinations of the isolates were consistent with influenza virus A concurrently circulating among swine herds in the United States. Subclinical influenza virus A infections in pigs at agricultural fairs may pose a risk to human health and create challenges for passive surveillance programs for influenza virus A in swine herds.

3.2 Introduction

Awareness of bidirectional zoonotic transmission of influenza virus A between pigs and humans was heightened by the emergence of the influenza A(H1N1)pdm09 virus, which resulted in an influenza pandemic among humans starting in 2009. Interspecies transmission of influenza virus A is believed to be a principal mechanism contributing to the emergence of novel influenza virus A strains that pose a threat to human and swine health (1,2). Pig respiratory tracts have receptors for swine-, human-,
and avian-origin influenza virus A, which facilitates genomic reassortment among viruses from multiple host species. As a result, swine have been identified as mixing vessels for influenza virus A and a source of emergence for novel viruses (3).

For >60 years after its identification as a swine pathogen, influenza virus A circulating among North American swine was predominantly the H1N1 subtype (4). In 1998, triple-reassortant influenza virus A (H3N2), containing genes originating from swine-, human-, and avian-origin influenza virus A, was identified among swine in the United States (5). This lineage quickly became established among North American swine (6), and the 6 gene segments coding for internal proteins, including the matrix (M) gene, subsequently served as a common backbone for many new reassortant viruses appearing among pigs (7). Various subtype H1N1, H1N2, and H3N2 influenza virus A lineages continue to cocirculate and evolve among North American swine (6–9).

Swine are a source of novel and existing influenza virus A strains that infect humans (10–13). These strains pose a pandemic threat if they become capable of being transmitted efficiently from person to person and if limited protective immunity exists in the human population. Bidirectional zoonotic transmission of influenza virus A strains usually involves close contact between humans and swine. The United States has 3 major swine–human interfaces: commercial swine production, abattoirs, and agricultural fairs. Agricultural fairs are unique because they facilitate prolonged commingling of pigs from numerous sources raised under varied management programs with millions of persons who have widely disparate histories of exposure to various influenza viruses. This situation creates an environment conducive to zoonotic transmission of influenza virus A.
More persons come in contact with live swine at agricultural fairs than in any other setting in the United States, and several human cases of influenza A have been linked to swine exposure occurring at fairs. In 1988, a woman died of infection with a variant influenza virus A (H1N1) that she acquired while attending a Wisconsin fair where numerous pigs showed signs of influenza-like illness (ILI); a follow-up investigation identified more human infections (14). In Ohio, human infections with variant influenza virus A after exposure to pigs with ILI were detected at the 1988 Ohio State Fair, 2 weeks before the Wisconsin case was reported (R.D. Slemons, unpub. data), and more recently at the 2007 Huron County Fair (15).

Because of dynamic human and swine populations at fairs and the number of human influenza A cases associated with swine exposure that occurs at fairs (13–15), we hypothesized that influenza virus A infections in swine occur undetected at agricultural fairs. This study was initiated after the emergence of influenza A(H1N1)pdm09 to actively monitor the antigenic and genomic properties of influenza virus A among pigs at agricultural fairs in Ohio, with a goal of protecting the health of swine and the public.

### 3.3 Materials and Methods

#### Study Sites and Samples

During each year of this 3-year study, 2009–2011, agricultural fairs in Ohio were strategically recruited to participate on the basis of the size of the county’s commercial swine industry, the number of 4-H/FFA swine exhibitors, the number of pigs previously exhibited, or the geographic proximity to study sites used for influenza virus A
surveillance in wild birds. Selection criteria were chosen to provide a diverse representation of Ohio’s exhibition swine and the influenza virus A strains they might harbor. Before visiting the fair, the study team provided the leaders of each participating agricultural fair with an educational fact sheet on swine influenza.

The agricultural fair season in Ohio begins in June and continues into October; the fairs participating in this study occurred throughout the fair season (Figure). Fairs were visited at the end of the swine exhibition period, at which time pigs were visually examined for signs of ILI, and nasal swab specimens were collected from 20 selected pigs representing all areas of the exhibit, without consideration for individual pig health status (healthy or ill). Each nasal swab was placed in an individual vial containing brain–heart infusion broth supplemented with penicillin and streptomycin (16). The samples were frozen at −70°C until testing was initiated. The Institutional Animal Care and Use Committee of The Ohio State University approved protocol no. 2009A0134 for the use of animals in this study.

*Virus Isolation from Swine Nasal Swab Specimens*

Samples were thawed and treated with amphotericin B, gentamicin sulfate, and kanamycin sulfate (17); they then underwent centrifugation at 1,200 × g for 30 min at 4°C. The brain–heart infusion broth supernatant was added to 24-well plates containing monolayers of Madin-Darby canine kidney (MDCK; catalog no. 84121903, Sigma-Aldrich Co., St. Louis, MO, USA), adapted to and maintained in serum-free medium (A.S. Bowman et al., unpub. data). MDCK monolayers were examined for cytopathic
effect (CPE) daily for 3 days after the supernatant was added, at which time the cell culture supernatant was tested for hemagglutination activity by using 0.5% turkey erythrocytes (18). All hemagglutinating agents in cell culture supernatant and all MDCK cell cultures showing CPE were tested for the presence of influenza virus A by using Flu DETECT (Synbiotics Corporation, Kansas City, MO, USA). Each cell culture supernatant that had positive test results with Flu DETECT was identified as an influenza A viral isolate. Influenza virus A isolates were further characterized by using real-time reverse transcription PCR (rRT-PCR) assays.

RNA Extraction and rRT-PCR

RNA was extracted from original samples and influenza virus A isolates by using the PrepEase RNA Spin Kit (Affymetrix, Inc. Cleveland, OH, USA) according to the manufacturer’s instructions. Pan–influenza virus A rRT-PCR (19,20) was used to screen all original samples for influenza virus A. Hemagglutinin (HA) and neuraminidase (NA) subtypes of the influenza isolates were determined by using rRT-PCR assays specific for swine-origin influenza A virus H1 and H3 HA genes and N1 and N2 NA genes by using either a previously published protocol (21) modified to laboratory conditions (A.S. Bowman et al., unpub. data) or a commercially available swine influenza virus subtyping assay (Applied Biosystems, Foster City, CA, USA).

The M gene of the influenza A virus isolates was further characterized by differentiating between the North American swine triple-reassortant and the influenza A(H1N1)pdm09 virus M genes by using an rRT-PCR protocol (22) adapted to laboratory
conditions. The reactions were carried out by using the QuantiFast Multiplex RT-PCR +R Kit (QIAGEN, Valencia, CA, USA) in a 20-μL reaction mixture containing 10 μL 2× quantitative RT-PCR master mix, 7.5 pmol of forward primer, 2.5 pmol of each reverse primer, 0.125 μmol/L EA minor groove binder probe, 0.0625 μmol/L of each NA minor groove binder probe, 0.4 μL 50× ROX reference dye, 0.2 μL of reverse transcription product, and 5 μL of extracted RNA. The reactions were performed on an Mx3000P QPCR System (Agilent Technologies, Inc., Santa Clara, CA, USA) under these thermocycling conditions: 50°C for 20 min, then 95°C for 5 min, followed by 50 cycles of 97°C for 2 s and 60°C for 40 s. Cycle threshold values were calculated for each sample automatically by the QPCR System’s software by using the background-based method. Samples with cycle threshold <40 were considered positive.

3.4 Results

Fifty-three fair events were included in this study: 15 fairs during 2009, 16 fairs during 2010, and 22 fairs during 2011(Figure). Influenza virus A was recovered from >1 pigs at 12/53 (22.6%) fair events (Table 1). Results of the pan–influenza virus A rRT-PCR performed on original samples and virus isolation were completely concordant. Pigs with signs of ILI were observed and sampled at 2/53 (3.7%) fair events, and influenza A virus isolates were recovered from pigs at both fairs; pigs without signs of ILI but with positive test results for influenza A virus were found at 10/53 (18.9%) fair events. Therefore, pigs at 10/12 (83.3%) fairs at which pigs had influenza virus A infection did not exhibit signs of ILI.
A total of 1,073 pigs were tested during the 3-year study; influenza virus A was recovered from 155 (14.4%). The frequencies of virus isolation by year were 40/299 (13.4%) during 2009, 34/315 (10.8%) during 2010, and 81/459 (17.7%) during 2011. For the 12 fairs with >1 pigs testing positive for influenza A virus, the average frequency of virus isolation from nasal swab specimens was 62.9% (range 5%–100%; Table 2). Influenza virus A subtypes recovered were H1N2 and H3N2 during 2009, H3N2 during 2010, and H1N2 and H3N2 during 2011 (Table 3). The North American swine triple-reassortant M gene was found in all isolates recovered during 2009 and 2010, whereas the M gene from the influenza A(H1N1)pdm09 virus was found in all of the 2011 H3N2 and H1N2 isolates (Table 3).

3.5 Discussion

Our findings highlight the limitations of relying on visual examination for ILI to identify pigs infected with influenza virus A at agricultural fairs. Subclinical influenza virus A infections predominated among the pigs we tested, with subclinical infections detected among pigs at 10/53 (18.9%) participating fairs during 2009–2011. These findings may explain the frequency of variant influenza virus A infections among humans who have only been exposed to apparently healthy swine at fairs.

Agricultural fairs are often the face of agriculture to the general public. The International Association of Fairs and Expositions estimates annual attendance at fairs in North America to be 150 million persons (The Association, pers. comm.). Agricultural fairs have been occurring in the United States since 1811 (23) and are special community
events with a strong tradition and history of celebrating agricultural heritage and
achievement (24). As the agricultural workforce has decreased to <2% of the US
population (25), fairs have added educational programs to showcase advancements in
food production systems in an effort to maintain attendance (26) and meet societal needs.
These much-needed educational efforts often provide an opportunity for attendees to
have direct contact with all facets of agriculture, including pork production. Many of
these persons would not otherwise have any exposure to swine and the pathogens they
harbor, so their close contact with pigs at fairs may play multiple roles in the transmission
of influenza A viruses: they may pass human-origin influenza virus A to swine, leading
to novel reassortant viruses; they may serve as early sentinels by becoming infected first
with a novel swine-origin influenza A virus; or they may disseminate a novel swine-
origin influenza virus A in their local communities (27).

The long duration of many agricultural fairs (3–10 days) is distinctly different
than other swine concentration points or commingling events (i.e., abattoirs, markets,
auctions, or shows), where interactions are limited to hours. In addition to their long
duration, agricultural fairs also enable the comingling of pigs from multiple locations and
various production systems (backyard to intensive commercial) at 1 site. Exhibition
swine are commonly a unique population of noncommercial swine, reared by the use of
management practices that differ greatly from standard commercial swine production
practices (28). These pigs likely have varying levels of immunity to influenza virus A and
may bring a variety of influenza virus A strains with them to the fair, where the viruses
can spread to other pigs, possibly reassort, and potentially transmit to humans.
Swine-to-human transmission of influenza virus A has been sporadically reported worldwide (11), but the true incidence of this transmission is unknown. The Centers for Disease Control and Prevention reported that 36 humans were infected with variant influenza virus A in the United States during December 2005–April 2012 (29). Of these cases, 15 occurred after July 2011, and 6 cases, all involving infection with influenza A (H3N2) viruses containing the M gene from the influenza A(H1N1)pdm09 virus (H3N2v), were associated with exposure to swine at agricultural fairs. However, none of the implicated fairs reported signs of ILI in the pigs, and influenza virus A could not be isolated from the pigs that were suspected to be the sources because of delays and lack of the availability of the pigs. Nonetheless, it is possible that subclinical influenza infections in pigs at these swine–human interfaces played a key role in zoonotic infections.

The increased swine–human exposure occurring at agricultural fairs may also facilitate human-origin influenza A virus transmission to swine. The earliest reports of introductions of the influenza A(H1N1)pdm09 virus into the US swine herd occurred at the state fairs in Minnesota and South Dakota (30,31). Human-to-swine transmission is credited as a primary source of the genetic diversity seen in currently circulating swine influenza virus strains (32–34). Human-to-swine transmission of influenza virus A can be economically devastating for the pork industry because of decreased domestic sales, restrictions imposed by export partners, and production losses due to disease. Agricultural fairs may provide a conduit to introduce human-origin influenza virus A into the US swine herd.
No human cases of variant influenza A associated with any of the agricultural fairs included in this study were reported, even though influenza A (H3N2) viruses containing the M gene from the influenza A(H1N1)pdm09 virus were recovered from pigs at 6 of the participating fairs in 2011. However, the number of confirmed H3N2v cases dramatically increased during the summer of 2012, with most cases epidemiologically linked to swine exposure occurring at agricultural fairs (35,36).

The HA, NA, and M gene combinations of the influenza virus A isolates recovered from 155/1,073 (14.4%) sampled pigs were consistent with influenza virus A concurrently circulating in the US swine population (37,38). The high frequency of virus isolation from the pigs at the 12 fairs at which influenza virus A was found is likely due to sample collection occurring at the end of the exhibition period, ≈5–7 days after arrival, which probably coincided with peak viral shedding in the swine population.

A limitation of the study is that extrapolating the findings to other Ohio fairs and fairs in other states may not be possible because of the selection bias and inherent variability among agricultural fairs. Although the fairs where influenza virus A was recovered were diverse regarding the predetermined selection criteria (data not shown), the participating fairs were included in the study because they were ranked relatively high among Ohio fairs within >1 selection category. Expanded surveillance efforts for agricultural fairs are underway to more accurately estimate the true prevalence of influenza virus A infections among swine at agricultural fairs in Ohio. Recognized risk factors and accurate prevalence estimates are needed to lay the foundation for studies
investigating potential interventions to decrease the probability of swine-to-human and human-to-swine transmission of influenza virus A at agricultural fairs.

The subclinical influenza virus A infections identified in this study would not be detected by the current national swine influenza virus surveillance program (39), which is passive and focuses on swine showing signs of ILI and on reacting to reports of variant influenza A cases in humans (39). Thus, subclinical influenza virus A infections among pigs are likely underreported. This passive surveillance strategy does not adequately describe the breadth of influenza virus A circulating in swine because it does not identify less virulent strains of influenza virus A (40) and does not collect metadata on host, environmental, and agent factors that affect severity of illness. Therefore, to accurately capture the risk influenza virus A in swine populations presents to swine and public health, surveillance efforts should include healthy and clinically ill pigs.

Reducing bidirectional zoonotic transmission of influenza virus A between pigs and humans is crucial to agriculture and biomedical science. Unfortunately, little scientific evidence exists on which to base changes in policies and management practices to reduce the risk for interspecies transmission of influenza virus A between pigs and humans. This investigation highlights the need for additional studies to quantify the risk for interspecies influenza A virus transmission at fairs and to evaluate interventions to mitigate the risk.

Potential strategies to mitigate the risk for intra- and interspecies transmission of influenza virus A at fairs on the swine side of the human-swine interface include shortening the swine exhibition period, preventing interfair movement of pigs, and
vaccinating exhibition swine for appropriate influenza A viruses. Recommendations have previously been made for mitigating risk on the human side of the human–swine interface (www.cdc.gov/flu/swineflu). Expanded risk assessments at agricultural fairs will provide animal and public health officials with scientific data that will enable them to make appropriate decisions to protect animal and public health while still furthering appreciation and understanding of agriculture and ensuring our future food security.

3.6 Biography

Dr Bowman is a graduate research associate at The Ohio State University. His research interests include the ecology and epidemiology influenza virus A at the swine-human interface along with developing preventive strategies to protect animal and public health.

3.7 Acknowledgments

We thank leaders at the agricultural fairs participating in this study for their support; Jillian Yarnell, Justin Dickey, Kelsi Wittum, Charles Martin, Carina Vitullo, and Lori Bowman for their assistance collecting samples; and Jody Edwards for laboratory support.

This work has been funded in part by the Minnesota Center of Excellence for Influenza Research and Surveillance (MCEIRS) with federal funds from the Centers of Excellence for Influenza Research and Surveillance (CEIRS), National Institute of
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Table 3.1: Clinical signs of ILI and influenza virus A recovery from pigs at agricultural fairs, Ohio, USA, 2009–2011*

<table>
<thead>
<tr>
<th>Year</th>
<th>No. participating fairs</th>
<th>No. fairs with pigs with ILI</th>
<th>No. (%) fairs with ≥1 pig testing positive for influenza virus A</th>
<th>No. (%) fairs with subclinical influenza virus A infection in pigs</th>
</tr>
</thead>
<tbody>
<tr>
<td>2009</td>
<td>15</td>
<td>0</td>
<td>3 (20.0)</td>
<td>3 (20.0)</td>
</tr>
<tr>
<td>2010</td>
<td>16</td>
<td>1</td>
<td>3 (18.8)</td>
<td>2 (12.5)</td>
</tr>
<tr>
<td>2011</td>
<td>22</td>
<td>1</td>
<td>6 (27.3)</td>
<td>5 (22.7)</td>
</tr>
</tbody>
</table>

*Influenza A virus was recovered from pigs at both fairs where there were pigs with ILI. ILI, influenza-like illness.
Table 3.2: Frequency of influenza virus A isolation from individual pigs exhibited at agricultural fairs with >1 pig testing positive for influenza virus A, Ohio, USA, 2009–2011*

<table>
<thead>
<tr>
<th>Fair</th>
<th>2009</th>
<th>2010</th>
<th>2011</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>18/20 (80)</td>
<td>0/20</td>
<td>0/20</td>
</tr>
<tr>
<td>B</td>
<td>10/20 (50)</td>
<td>0/20</td>
<td>20/20 (100)</td>
</tr>
<tr>
<td>C</td>
<td>19/20 (90)</td>
<td>15/20 (75)</td>
<td>20/20 (100)†</td>
</tr>
<tr>
<td>D</td>
<td>0/20</td>
<td>1/20 (5)†</td>
<td>19/20 (95)</td>
</tr>
<tr>
<td>E</td>
<td>0/20</td>
<td>18/20 (90)</td>
<td>0/20</td>
</tr>
<tr>
<td>F</td>
<td>0/20</td>
<td>0/20</td>
<td>3/20 (15)</td>
</tr>
<tr>
<td>G</td>
<td>0/20</td>
<td>0/20</td>
<td>3/20 (15)</td>
</tr>
<tr>
<td>H</td>
<td>16/40 (40)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Fair G did not participate in 2009; fair H did not participate in 2009 or 2010.
†Fairs where there were pigs with influenza-like illness.
<table>
<thead>
<tr>
<th>Fair</th>
<th>2009</th>
<th>2010</th>
<th>2011</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>H1N2</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>B</td>
<td>H3N2</td>
<td>Negative</td>
<td>H3N2</td>
</tr>
<tr>
<td>C</td>
<td>H3N2</td>
<td>H3N2</td>
<td>H1N2, H3N2</td>
</tr>
<tr>
<td>D</td>
<td>Negative</td>
<td>H3N2</td>
<td>H1N2, H3N2</td>
</tr>
<tr>
<td>E</td>
<td>Negative</td>
<td>H3N2</td>
<td>Negative</td>
</tr>
<tr>
<td>F</td>
<td>Negative</td>
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<td>H3N2</td>
</tr>
<tr>
<td>G</td>
<td>Negative</td>
<td>Negative</td>
<td>H3N2</td>
</tr>
<tr>
<td>H</td>
<td></td>
<td></td>
<td>H1N2, H3N2</td>
</tr>
</tbody>
</table>

*Fair G did not participate in 2009; fair H did not participate in 2009 or 2010. **Boldface** indicates North American triple-reassortant swine-origin influenza A virus M gene segment; **underlining** indicates influenza A(H1N1)pdm09 virus M gene segment. HA, hemagglutinin; NA, neuraminidase; M, matrix.

Table 3.3: Characterization of HA, NA, and M gene segments of influenza virus A from agricultural fairs with >1 pig testing positive for influenza virus A, Ohio, USA, 2009–2011*
Figure 3.1: Frequency distribution of agricultural fairs, by week of the state fair season, Ohio, June–October 2009–2011. Black bar sections, fairs with pigs positive for influenza virus A; gray bar sections, fairs with no pigs positive for influenza virus A; white bar sections, fairs not enrolled in this study.
Chapter 4 - Molecular evidence for interspecies transmission of H3N2pM/H3N2v influenza A viruses at an Ohio agricultural fair, July 2012

Andrew S. Bowman, Srinand Sreevatsan, Mary L. Killian, Shannon L. Page, Sarah W. Nelson, Jacqueline M. Nolting, Carol Cardona, Richard D. Slemons

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4.1 Abstract

Evidence accumulating in 2011–2012 indicates that there is significant intra- and inter-species transmission of influenza A viruses at agricultural fairs, which has renewed interest in this unique human/swine interface. Six human cases of influenza A (H3N2) variant (H3N2v) virus infections were epidemiologically linked to swine exposure at fairs in the United States in 2011. In 2012, the number of H3N2v cases in the Midwest had exceeded 300 from early July to September, 2012. Prospective influenza A virus surveillance among pigs at Ohio fairs resulted in the detection of H3N2pM (H3N2 influenza A viruses containing the matrix (M) gene from the influenza A (H1N1) pdm09 virus). These H3N2pM viruses were temporally and spatially linked to several human H3N2v cases. Complete genomic analyses of these H3N2pM isolates demonstrated >99% nucleotide similarity to the H3N2v isolates recovered from human cases. Actions to mitigate the bidirectional interspecies transmission of influenza A virus between people and animals at agricultural fairs may be warranted.

4.2 Introduction

The commingling of pigs and people at agricultural fairs represents a potentially important and inadequately evaluated interface in the evolution and transmission of zoonotic influenza A viruses. This interface may be a critical point for the movement of influenza A viruses and/or genes between human and swine populations.1,2,3,4,5,6,7,8,9,10,11,12
Interspecies transmission of influenza A viruses has been sporadically confirmed at agricultural fairs.\textsuperscript{7,13,14,15} Recent cases of influenza A (H3N2) variant virus (H3N2v) infection in humans have been epidemiologically associated with exposures to swine at agricultural fairs in North America.\textsuperscript{16,17} Since 2009, we have conducted a prospective influenza A virus surveillance study among pigs exhibited at county fairs in Ohio. In July 2012, as part of routine sampling, H3N2pM viruses (H3N2 influenza A viruses containing the matrix (M) gene from the influenza A (H1N1) pdm09 virus) were recovered from pigs at a county fair associated with concurrent cases of human H3N2v infections among its participants.\textsuperscript{17} Here we report that next generation sequencing of the complete H3N2pM virus genomes, isolated from pigs at the fair, exhibited >99\% similarity at the nucleotide level to H3N2v isolates concurrently recovered from the human cases.

### 4.3 Materials and Methods

As part of our ongoing surveillance project, nasal swabs were collected from 34 randomly selected pigs (out of a total of 348 pigs) exhibited at an Ohio county fair on the last day of the fair, 28 July 2012, approximately 7 days after the pigs arrived at the fair. Visual examination of all pigs in the barns immediately prior to sample collection did not reveal any pigs with overt clinical signs consistent with influenza-like illness (ILI) although swine exhibitors reported pigs at the fair had a variety of maladies (diarrhea, vomiting and fever) 3–5 days prior to the sampling day, which resulted in the early dismissal of a few pigs from the fair. Swabs were placed singly into vials containing viral
transport media and stored at −80 °C until they were tested. Real-time reverse transcriptase polymerase chain reaction (RRT-PCR) was used to screen the original samples for the presence of influenza A virus, to determine hemagglutinin (HA) and neuraminidase subtypes, and to characterize the M gene lineage. Viral transport media supernatant was also inoculated onto Madin–Darby canine kidney cells for virus isolation. Representative influenza A virus isolates were sent to the United States Department of Agriculture National Veterinary Services Laboratories for confirmational testing and complete genome sequencing using integrated semiconductor sequencing (Ion Torrent).

All eight segments of two isolates were amplified using gene-specific and universal primers for each segment. The cDNA was purified and cDNA libraries were prepared for the Ion Torrent using the IonXpress Plus Fragment Library Kit (Life Technologies Corp., Grand Island, NY, USA) with Ion Xpress barcode adapters (Life Technologies Corp.). Prepared libraries were quantitated by qPCR using the Ion Library Quantitation Kit (Life Technologies Corp.). Quantitated libraries were diluted and pooled for library amplification using the Ion One Touch and ES systems (Life Technologies Corp.). Following enrichment, DNA was loaded onto an Ion 314 chip (Life Technologies Corp.) and sequenced using the Ion PGM 200 Sequencing Kit (Life Technologies Corp.).

Sequences were assembled using Lasergene DNASTar SeqMan NGen 4.0 software (DNASTAR, Inc., Madison, WI, USA). Reference based assemblies were performed using H3N2pM reference sequences generated at National Veterinary Services Laboratories (full open reading frames). Single nucleotide polymorphisms and short
insertions and deletions were evaluated as potential errors. The number of bases used for reference-based assembly was equal to the open reading frame length, i.e. 2280 bp for the PB2 gene, 1701 bp for the HA gene, etc. The output of the assemblies generated a full-length contiguous sequence for each gene segment spanning the complete open reading frame. Full-length sequences from the two swine-origin influenza A virus isolates reported here, A/swine/Ohio/12TOSU268/2012(H3N2) and A/swine/Ohio/12TOSU293/2012(H3N2), have been deposited in GenBank (accession numbers JX534958–JX534973).

On 27 July 2012, the Ohio Department of Health was notified by a county health department of two individuals who presented with ILI to local medical facilities and had significant direct swine contact over a number of days at their local county fair. Specimens were submitted for preliminary testing to the Ohio Department of Health laboratory and then forwarded to the Centers for Disease Control and Prevention for final confirmation. Genetic sequence results confirmed H3N2v infection in both patients. Over the next 3 weeks, an additional 20 cases of human illness associated with this county fair were confirmed to be caused by H3N2v. Most of the cases had direct exposure to swine. Sequences from the influenza A virus isolates recovered from the swine were compared to the human-origin influenza A H3N2v virus sequences (partial and full length) of A/Ohio/13/2012(H3N2), A/Ohio/14/2012(H3N2), A/Ohio/15/2012(H3N2), A/Ohio/16/2012(H3N2) and/or A/Ohio/20/2012(H3N2) recovered from people with ILI following exposure to swine at the fair (Global Initiative on Sharing All Influenza Data and the Influenza Research Database).
All segments were analyzed separately, and their evolutionary history was inferred using the Maximum Parsimony or Maximum Likelihood methods. A bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The matrix protein tree was obtained using the Close-Neighbor-Interchange algorithm with search level 1 in which the initial trees were obtained with the random addition of sequences (10 replicates). The trees are drawn to scale, with branch lengths calculated using the average pathway method and are in the units of the number of changes over the whole sequence. Evolutionary analyses were conducted in Molecular Evolutionary Genetics Analysis software version 5.0.

4.4 Results

Influenza A (H3N2) viruses containing the M gene from the influenza A (H1N1) pdm09 virus (H3N2pM) were detected in 31 of the 34 swine nasal swabs using RRT-PCR and H3N2pM viral isolates were recovered from 29 of the 31 RRT-PCR-positive samples. Analysis of the HA gene placed the concurrently recovered H3N2v and H3N2pM isolates tightly within the contemporary Cluster IV H3 viruses circulating in the US swine herd (Figure 1). The clustering of all HA genes of the human- and swine-origin viruses from this fair suggests common source dissemination. Furthermore, the phylogenetic structure of the HA gene suggests that H3 from the new variant viruses are evolving away from the classical Cluster IV H3. Neuraminidase (Figure 2), the polymerase complex (Figure 3), nucleoprotein (Figure 4) and non-structural (Figure 5)
genes all show a similar trend with near 99% nucleotide similarity between the swine-origin H3N2pM and human-origin H3N2v viruses (Table 1). The M genes of both the H3N2pM and the analyzed H3N2v influenza A viruses clustered into a single clade with H1N1pdm segments from 2009 and 2012 and were separated from M genes from older, as well as more recent classical H3N2 viruses (Figure 6). For some segments, isolates from pigs and people from Illinois (2011) and Nebraska clustered with the H3N2v genes.

4.5 Discussion

This study presents clear molecular evidence that pigs and humans were concurrently infected with the same strain of influenza A virus at an Ohio county fair in July 2012. The most recent ancestors of all the genetic segments of the H3N2v/H3N2pM viruses can be found in various triple reassortant H3N2, H1N2 and H1N1 influenza A viruses which have been detected in pigs in North America. The cocirculation of the genetic elements found in the H3N2v/H3N2pM viruses makes reassortment events occurring in swine the most likely path of emergence. Although similar influenza A H3N2pM viruses have been previously reported in pigs and in commercial swine herds, they had not been reported in exhibition pigs temporally and spatially linked to human cases of H3N2v until July 2012. Twelve pigs at the LaPorte County Fair in Northwestern Indiana tested positive for H3N2pM in early July 2012, but to date, there has been no documented comparison of genomic sequences of the viruses recovered from people and pigs. The temporal and spatial proximity of the human cases and swine reported here, the frequency of virus isolation from the pigs, and the >99% sequence identity of the swine-
and human-origin viruses originating from this fair clearly demonstrates inter- and intra-
species transmission of H3N2pM/H3N2v viruses (Figures 1–6). However, the route by
which the virus was introduced into the swine and human populations at the fair has not
been resolved.

Pigs are unique in their ability to serve as hosts of avian-, swine- and human-
origin influenza A viruses and thus have been identified as a source of novel reassortant
strains with zoonotic potential.\textsuperscript{29,30} The remarkable nucleotide sequence similarity
between the swine-origin and human-origin H3N2 viruses reported here provides
evidence that there are few or no adaptive genetic changes needed for the virus to
replicate in either host. None of the nucleotide differences between the H3N2pM and
H3N2v isolates resulted in a change in the predicted amino acid sequence, although the
ambiguous base call at position 221 in the M genes of the H3N2pM isolates makes it
unable to predict the resulting amino acid in that position (Table 1). Historically, as an
influenza A virus emerges in a new host, there is commonly a series of genotypic changes
that occur as the virus adapts to grow and transmit more efficiently in the new host. The
lack of difference between the genotypes of these isolates suggests that there are virtually
no innate species barriers preventing bidirectional interspecies transmission of
H3N2pM/H3N2v viruses between humans and pigs. Furthermore, this close phylogenetic
relationship between the swine and human viruses is consistent with a recent interspecies
transmission. Fortunately, the symptoms exhibited among most human cases of H3N2v
in 2012 have been relatively mild and sustained human-to-human transmission of these
viruses has not been observed. However, with the growing number of documented human cases during recent months, the continuing evolution H3N2v is a concern.

Agricultural fairs are unique settings where pigs and people from multiple sources and immunological status are concentrated at one location, creating an interface where new or existing influenza A viruses and/or genes can move within or between human and swine populations. The repeated swine–swine and human–swine interactions occurring at agricultural fairs are distinctly different from those occurring in commercial swine production settings. These unique interactions undoubtedly facilitate bidirectional zoonotic transmission of influenza A viruses at fairs. This statement is supported by the increasing number of human H3N2v cases in the United States in late summer 2012 and previously documented influenza A virus transmission from humans-to-swine and swine-to-humans at agricultural fairs. Appropriate steps to prevent and/or reduce intra- and inter-species transmission of influenza A viruses at fairs should be undertaken to protect animal and human health while considering short- and long-term implications for the cultural value of livestock exhibitions. Potential strategies to mitigate the transmission of influenza A viruses between pigs and people are available from several sources including The Ohio State University’s Animal Influenza Ecology and Epidemiology Research Program website (http://vet.osu.edu/preventive-medicine/AIEERP), Centers for Disease Control and Prevention and United States Department of Agriculture information publications.
4.6 Acknowledgements

The authors wish to acknowledge the assistance of Mary DiOrio, Brian Fowler, Kathy Smith (Ohio Department of Health, USA), Tony Forshey (Ohio Department of Agriculture, USA) and Sabrina Swenson (United States Department of Agriculture, USA) for support during the investigation. This work has been funded by the Minnesota Center of Excellence for Influenza Research and Surveillance with federal funds from the Centers of Excellence for Influenza Research and Surveillance, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under contract NO. HHSN266200700007C and the USDA/APHIS NVSL. Any mention of trade names or commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

4.7 References


Table 4.1: Variations in nucleotide sequences of cDNA from human-origin H3N2v and swine-origin H3N2pM influenza A viruses Abbreviations: M, matrix; NA, neuraminidase; NP, nucleocapsid protein; NS, non-structural protein.

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Gene segment (nucleotide position)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NP (666)</td>
</tr>
<tr>
<td>A/Ohio/14/2012(H3N2)</td>
<td>G</td>
</tr>
<tr>
<td>A/Ohio/16/2012(H3N2)</td>
<td>G</td>
</tr>
<tr>
<td>A/swine/Ohio/12TOSU268/2012(H3N2)</td>
<td>G</td>
</tr>
<tr>
<td>A/swine/Ohio/12TOSU293/2012(H3N2)</td>
<td>A</td>
</tr>
</tbody>
</table>
Figure 4.1: H3 Phylogeny

Phylogeny of HA gene segment from swine lineages of H3N2 viruses. The percentage of replicate trees in which the associated HA gene segments clustered together in the bootstrap test using the Maximum Parsimony method are shown next to the branches. The analysis involved 45 nucleotide sequences and there were a total of 863 positions in the final dataset. The cluster-based classification is derived from previously established nomenclature. The four major clusters of H3 that have been circulating in the swine populations are shown. All H3N2v/H3N2pM HA segments (identified by a dot alongside the isolate) clustered together as a sublineage of previously described Cluster IV viruses.
Figure 4.2: N2 Phylogeny

Maximum Parsimony analysis of NA gene from classical swine H3N2 and H3N2pM/H3N2v viruses. The NA genes of all the isolates (identified by a dot alongside the isolates) clustered together in a single lineage suggesting a clonal pattern. NA, neuraminidase.
Figure 4.3: Phylogenies of the polymerase complex (PA, PB1, PB2) genes.

Phylogenies of the polymerase complex (PA, PB1, PB2) genes. All sequences of the respective genes from the H3N2v/H3N2pM viruses clustered together. PA, polymerase A; PB1, polymerase B1; PB2, polymerase B2.
Figure 4.4: NP Phylogeny

Molecular phylogenetic analysis for NP gene. Shown is the evolutionary history of NP segment from swine-origin H3N2pM and human-origin H3N2v isolates inferred by using the Maximum Likelihood method based on the Hasegawa–Kishino–Yano model. All NP sequences from the H3N2pM/H3N2v (Ohio and other geographical locations) share high levels of identity. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was <100 or less than one-fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. A discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories (+G, parameter=0.3714)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 22 nucleotide sequences. There were a total of 1497 positions in the final dataset. BIONJ, BIO neighbor joining; MCL, Markov cluster; NP, nucleocapsid protein.
Figure 4.4
Figure 4.5: NS Phylogeny

Phylogenetic analysis of the NS gene. The bootstrap consensus tree inferred using the Maximum Parsimony method is taken to represent the evolutionary history of the NS segments analyzed. The analysis involved 16 nucleotide sequences with a total of 838 positions in the final dataset. All NS genes of H3N2v/H3N2pM influenza A viruses clustered in a single clade suggesting clonality. NS, non-structural protein.
Figure 4.6: MP Phylogeny

Phylogenetic analysis of gene coding for the MP from the (H1N1) pdm09, H3N2v/H3N2pM, North American swine-origin H3N2 and human-origin seasonal H3N2 influenza A viruses. Shown is a bootstrap consensus tree inferred using the Maximum Parsimony method. The analysis involved 22 nucleotide sequences of matrix genes from human, swine and recent pandemic influenza A viruses. All positions containing gaps and missing data were eliminated. There were a total of 978 positions in the final dataset.

Matrix of all H3N2v/H3N2pM isolates clustered with the pandemic H1N1 segment sequence (boxed sequences in the tree). There is an indication of minor microscale evolution away from influenza A (H1N1) pdm09 virus within this segment with specific polymorphisms that favor human infection being maintained. MP, matrix protein.
Figure 4.6
Chapter 5 - Molecular confirmation of inter- and intra-species transmission swine-origin

H3N2 influenza A virus at agricultural fairs, Ohio, 2012

This chapter has been prepared for submission to a peer reviewed journal.
5.1 Abstract

Agricultural fairs are the primary setting in the United States in which swine from various sources and humans comingle, providing opportunity for bi-directional zoonotic transmission of influenza A viruses. We conducted active influenza A virus surveillance in pigs at 40 selected Ohio agricultural fairs during 2012. Influenza A (H3N2) virus was recovered from pigs at 10 (25%) fairs. Seven of the fairs were epidemiologically linked to human influenza cases caused by influenza A (H3N2) variant virus. Nucleotide identities of the H3N2 isolates from humans and swine at all fairs was greater than 99%, providing molecular evidence to confirm transmission of influenza A(H3N2) virus between pigs and people during at least those seven fairs. When viewed in conjunction with the dramatic increase in the number of reported variant influenza cases during 2012, these results highlight the need to reduce inter- and intra-species transmission of influenza A viruses at swine exhibitions.

5.2 Introduction

More than 300 human cases of infection with influenza A (H3N2) variant virus, called H3N2v, were reported in the United States during 2012, resulting in 16 hospitalizations and one death.\(^1\) Interspecies transmission of influenza A viruses plays a significant role in the evolution of influenza A viruses infecting humans.\(^2,3\) Genomic reassortment resulting in novel influenza A viruses can occur in swine because they are susceptible hosts for avian and human as well as endemic swine strains of these viruses.\(^4\)\(^5\) Thus, swine are critically important in the ecology and emergence of influenza A
viruses threatening human health, as illustrated by the emergence of the H1N1(pdm09) virus, a reassortant virus with origins traced to influenza A viruses circulating among North American and European swine.6-8

Bidirectional zoonotic transmission of influenza A viruses between swine and humans requires a swine-human interface. Agricultural fairs are a unique interface allowing for comingling of pigs from multiple sources with humans (exhibitors and visitors),9 making them key settings for zoonotic influenza A virus transmission. Prior to 2012, outbreaks of variant influenza A were sporadically reported,10-12 and these cases were frequently linked to swine exposure at agricultural fairs.13-15 Epidemiological investigations into the H3N2v cases occurring during 2012 implicated swine exposure at agricultural fairs as the primary source of the viruses.16,17

During 2012, public health officials in Ohio documented 107 H3N2v cases, second only to the number of cases reported from Indiana. Epidemiologic investigations in Ohio concluded that human-to-human transmission was limited, linking the majority of these cases to direct or indirect swine exposure occurring at agricultural fairs across the state. Interspecies transmission of swine-origin H3N2 influenza A viruses containing the M gene from the influenza A (H1N1) pdm09 virus, termed H3N2pM viruses, from swine to humans was previously confirmed for one Ohio agricultural fair in July of 2012.18 This report details the results of active influenza A virus surveillance in pigs at Ohio agricultural fairs during 2012. Comparisons of the H3N2 influenza A viruses recovered from pigs and humans provides molecular confirmation that zoonotic transmission was not isolated to the one previously reported fair.
5.3 Materials and Methods

In 2012, 18 additional fairs were randomly selected to augment the 22 fairs previously enrolled in the study, bringing the total to 40 fairs distributed across Ohio. As in previous years, study team members visited at the end of each fair to visually examine the pigs for signs of respiratory disease and collect nasal swab specimens from at least 20 animals, regardless of the health status (healthy or ill) of each individual pig. Nasal swabs were placed in individual vials containing viral transport medium, and frozen at −70°C until the time of testing. The Ohio State University’s Institutional Animal Care and Use Committee approved the use of animals in this study under protocol no. 2009A0134.

Sample testing was performed as previously described. Briefly, samples were screened by real-time reverse transcriptase polymerase chain reaction (rRT-PCR) for the presence of influenza A virus (VetMAX-Gold SIV Detection Kit, Applied Biosystems, Austin, TX). Serum-free medium-adapted Madin-Darby canine kidney cells were inoculated with viral transport medium supernatant for viral isolation. Hemagglutinin (HA) and neuraminidase (NA) subtype determination, along with M gene lineage characterization, were performed with rRT-PCR using a commercially available swine influenza viral subtyping kit (Applied Biosystems, Austin, TX) and/or formerly described protocols.

Previously described procedures were used to sequence two representative swine-origin H3N2pM isolates from each fair. Two isolates from Fair D were previously sequenced and reported. Briefly, all segments of 18 remaining swine-origin isolates
were amplified using PCR, followed by purification of the cDNA and preparation of cDNA libraries. Quantitated libraries were diluted and pooled for library amplification. After enrichment, DNA was sequenced and the sequences were assembled using standard procedures. Sequences from the swine-origin influenza A virus isolates reported here have been deposited in GenBank (Technical Appendix).

Specimens collected from suspect human H3N2v cases were submitted to the Ohio Department of Health (ODH) laboratory for influenza testing. Samples with preliminary test results consistent with H3N2v infection were forwarded to the Centers for Disease Control and Prevention (CDC) for confirmatory testing and sequencing. Sequences of the human-origin H3N2v isolates are available in the EpiFlu database, which is available on the Global Initiative on Sharing All Influenza Data website (http://www.gisaid.org).

Genomic analyses were conducted using the full-length sequences of one human-origin and two swine-origin H3N2 isolates per fair, for a total of 27 influenza A virus isolates (Table 5.2). These were combined with all unique complete gene segments sequences of swine-origin influenza A virus available on the Influenza Resource Database. The nucleotide sequences of each segment were aligned individually using Geneious 6.0.5 [Biomatters Ltd. Auckland, New Zealand]; phylogenetic trees were generated using Maximum Likelihood methods, and the resulting trees were edited with MEGA 5.1.
5.4 Results

Influenza virus A was recovered from ≥1 pig at 10/40 (25%) fairs. As displayed in the Table 5.1, a total of 834 pigs were sampled; influenza A virus was recovered from 161 (19.3%). While H1N1 and H3N2 influenza A viruses were recovered during 2012, the vast majority of the isolates, 158/161 (98.1%), were H3N2pM. All isolates, including the three H1N1 isolates, contained an M gene derived from the A(H1N1)pdm09 virus.

A total of 107 confirmed human H3Nv cases were identified, and ODH identified 14 separate agricultural fairs in Ohio as sites of likely swine-to-human transmission of H3N2 influenza A. Of the 14 fairs epidemiologically linked to H3N2v cases, seven (fairs D through J) had participated in our swine surveillance project described above during 2012 (Table 5.1). These seven fairs occurred during four consecutive weeks of Ohio’s 18 week agricultural fair season, which runs from June through October (Figure 5.1).

Overall nucleotide identity of the H3N2v and H3N2pM isolates was greater than 99%. Phylogenetic analysis of each gene segment of the H3N2v and H3N2pM isolates demonstrated tight clustering with each other (Figure 5.2 - 5.9). Interestingly, the NA segments of the H3N2pM and H3N2v isolates described here clustered tightly together as a sublineage of the 2002 lineage of North American swine N2 (Figure 5.3A). The only other virus included in this NA sublineage recovered prior to 2012 was the NA segment from West Virginia H3N2v cases occurring in 2011, represented by the A/West Virginia/06/2011(H3N2) isolate (Fig 5.3B).
5.5 Discussion

The results of the present study provide clear molecular evidence that influenza A (H3N2)pM viruses were common among exhibition swine in Ohio during the 2012 agricultural fair season and zoonotic transmission occurred during at least seven fairs resulting in H3N2v cases. A previous publication documented that a swine-origin H3N2 influenza A virus infected both pigs and humans at one Ohio fair in July 2012. The current molecular investigation demonstrates that the previously confirmed transmission of swine-origin H3N2 influenza A virus from pigs to humans at an Ohio agricultural fair in 2012 was not an isolated event, but rather that H3N2pM isolates recovered from pigs at six additional agricultural fairs in Ohio are molecularly linked to human H3N2v infections contracted at each of those respective fairs.

Recently, there has been increased emphasis placed on educating fair organizers and exhibitors about implementing appropriate precautions when exhibition swine become ill. While swine showing clinical signs of influenza-like-illness at agricultural fairs are typically removed from public display and/or excused from the exhibition, a previously reported high prevalence of subclinical infection in swine at agricultural fairs suggests that many exhibitors and visitors are unknowingly being exposed to pigs infected with influenza A virus. In this 2012 investigation, 6/10 (60%) agricultural fairs with influenza A infected pigs did not report any influenza-like illness among the exhibition swine (Table 5.1), further demonstrating the public health risk posed by pigs with subclinical infections.
Swine-to-human and human-to-swine\textsuperscript{25} influenza A virus transmission is known to occur at fairs, highlighting the fact that pigs in this setting are potentially exposed to multiple lineages of influenza A viruses simultaneously, making fairs ideal locations for genomic reassortment and novel virus formation. The swine exhibited at agricultural fairs and livestock exhibitions are a small but distinct subset of the United States swine herd, frequently reared in very small herds as part of youth educational programs,\textsuperscript{26} and are generally segregated from swine reared for commercial pork production. H3N2pM influenza A viruses are not unique to fairs, and viruses similar to those described in the present study have also been detected in commercial swine populations,\textsuperscript{27} indicating that commercial swine and exhibition swine populations are not completely separated. Therefore, influenza A virus infected exhibition swine not only threaten public health, but also serve as a potential pathway for the spread of novel influenza A viruses and might allow for novel viruses to become established in the larger commercial swine population. The rapid dissemination of highly similar H3N2pM viruses among pigs at ten fairs across the state highlights the need for studying the transmission dynamics of influenza A viruses within exhibition swine populations. However, while there is certainly fair-to-fair movement of exhibition swine, the role of infected humans spreading these variant viruses has not been investigated.

The results of this study support previous calls for enhancing surveillance of influenza A viruses in swine, especially at high-risk swine human interfaces.\textsuperscript{28} Across the United States, 309 human H3N2v cases were reported in 2012 (http://www.cdc.gov/flu/swineflu/h3n2v-case-count.htm), with 306 cases occurring in the
summer. Investigations seeking to identify infected pigs were frequently hampered by the limitations of retrospectively tracing suspect pigs after exhibition. While the seasonal pattern in other states was similar to that seen in Ohio, more robust influenza A virus surveillance in swine at agricultural exhibitions in other states is needed to extrapolate the findings of this beyond Ohio. The failure to detect the sublineage of N2 associated with the variant influenza cases of 2012 in swine prior to this year further illustrates the need for better influenza A virus surveillance in swine populations.

Evaluation of the swine side of the swine-human interface at fairs appeared similar to previous years in which no human cases of variant influenza were reported in Ohio. We detected influenza A virus infected pigs at 25% of the fairs tested in 2012, which corresponds well with our previous work showing infection at 22.6% of Ohio fairs in 2009-2011. The temporal pattern was also similar to previous years, with positive fairs popping up sporadically during the early summer, a peak of fairs with infected pigs in the middle of the season, and no influenza A virus detected among the pigs at the autumn fairs. Of interest is the lack of H3N2v cases associated with fairs A, B, and C when similar H3N2pM viruses were circulating among the swine at those fairs earlier in the fair season (Table 5.1). One potential explanation is that increased awareness and surveillance led to a surveillance artifact caused by previous under-diagnosis and/or under-reporting. No H3N2v cases were reported in Ohio until after the initial reports of H3N2v cases in Indiana during July 2012 became public. Yet, almost immediately following that publication in the MMWR, Ohio local public health jurisdictions began
alerting the ODH that individuals with exposure to swine at agricultural fairs were seeking medical care for influenza-like illness.

Whatever the reason for the increased incidence of H3N2v cases during 2012, mitigation strategies must be undertaken to decrease the risk of influenza A virus transmission across the swine-human interface at fairs. Influenza A virus infections in exhibition swine represent an unquantified public health risk. Rigorous efficacy evaluations and expanded risk assessments of adopted mitigation strategies to protect public health are needed to assist animal and public health experts in making evidence-based recommendations for reducing intra- and inter-species transmission of influenza A virus in this setting. Fair organizers, animal health officials, and public health agencies should take additional steps to decrease the threat to human and animal health. Recently the National Assembly of State Animal Health Officials and the National Association of State Public Health Veterinarians jointly released some potential measures for fair organizers and exhibitors to consider when hosting and participating in swine exhibitions.24 Active communication and partnership between human and animal health agencies is needed to develop and implement appropriate prevention and control plans, and local health care providers should be alerted to variant influenza A viruses, especially when agricultural fairs or exhibitions occur in the community.

5.6 Acknowledgements

We thank our collaborators from the participating agricultural fairs. We also thank Jeffery Workman, Wendell Bliss, Mary DiOrio, Brian Fowler, Kathy Smith, Celia Quinn,
Tony Forshey, Susan Skorupski and Sabrina Swenson for their assistance and support of these investigations. This work has been funded by the Minnesota Center of Excellence for Influenza Research and Surveillance with federal funds from the Centers of Excellence for Influenza Research and Surveillance, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under contract NO. HHSN266200700007C and the USDA/APHIS NVSL. Any mention of trade names or commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.
References


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Table 5.1: Temporal, clinical observations, and test results for influenza A virus from pigs at agricultural fairs, Ohio, USA, 2012.

The table is limited to agricultural fairs with >1 pig testing positive for influenza A virus.

Pigs at 30 additional fairs tested negative for influenza A virus.
Figure 5.1: Epidemic Curve

Distribution of agricultural fairs and human influenza A (H3N2)v cases, by week of the Ohio fair season, June–October 2012. Agricultural fair data references the left axis with: black bar sections, fairs with pigs positive for influenza A virus; gray bar sections, fairs with no pigs positive for influenza A virus; white bar sections, fairs not enrolled in this study. Reported human H3N2v cases are represented by black triangles and are measured on the right axis.
Table 5.2: Isolate name, associated agricultural fair, and GenBank or Epiflu accession numbers for the gene segments of the influenza A virus isolates used in this study.
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* FAir D was previously described.18
Figure 5.2: HA Phylogeny

Panel A. Phylogenetic relationships of the HA sequences of swine origin H3 influenza A viruses. Swine and human isolates recovered from the same fair are identified with the same color and symbol (Fair A: teal open diamond, Fair B: black downward pointing open triangle, Fair C: pink upward pointing open triangle, Fair D: Red closed circle, Fair E: Green upward pointing closed triangle Fair F: Blue closed square, Fair G: gold downward pointing closed triangle, Fair H: orange open circle Fair I: purple closed diamond Fair J: Brown open square) Panel B. Expanded view of isolates from this study.
Figure 5.2
Figure 5.3: NA Phylogeny

Panel A. Phylogenetic relationships of the NA sequences of swine origin N2 influenza A viruses. Swine and human isolates recovered from the same fair are identified with the same color and symbol (Fair A: teal open diamond, Fair B: black downward pointing open triangle, Fair C: pink upward pointing open triangle, Fair D: Red closed circle, Fair E: Green upward pointing closed triangle Fair F: Blue closed square, Fair G: gold downward pointing closed triangle, Fair H: orange open circle Fair I: purple closed diamond Fair J: Brown open square) Panel B. Expanded view of isolates from this study.
Figure 5.3
Figure 5.4: MP Phylogeny

Phylogenetic relationships of the MP sequences. Swine and human isolates recovered from the same fair are identified with the same color and symbol (Fair A: teal open diamond, Fair B: black downward pointing open triangle, Fair C: pink upward pointing open triangle, Fair D: Red closed circle, Fair E: Green upward pointing closed triangle, Fair F: Blue closed square, Fair G: gold downward pointing closed triangle, Fair H: orange open circle, Fair I: purple closed diamond, Fair J: Brown open square) Panel B. Expanded view of isolates from this study which clustered together with other influenza A viruses containing the MP gene from A(H1N1)pdm09 virus.
Figure 5.5: NP Phylogeny

Phylogenetic relationships of the NP sequences. Swine and human isolates recovered from the same fair are identified with the same color and symbol (Fair A: teal open diamond, Fair B: black downward pointing open triangle, Fair C: pink upward pointing open triangle, Fair D: Red closed circle, Fair E: Green upward pointing closed triangle, Fair F: Blue closed square, Fair G: gold downward pointing closed triangle, Fair H: orange open circle, Fair I: purple closed diamond, Fair J: Brown open square) Panel B. Expanded view of isolates from this study.
Figure 5.5
Figure 5.6: NS Phylogeny

Phylogenetic relationships of the NS sequences. Swine and human isolates recovered from the same fair are identified with the same color and symbol (Fair A: teal open diamond, Fair B: black downward pointing open triangle, Fair C: pink upward pointing open triangle, Fair D: Red closed circle, Fair E: Green upward pointing closed triangle Fair F: Blue closed square, Fair G: gold downward pointing closed triangle, Fair H: orange open circle  Fair I: purple closed diamond Fair J: Brown open square) Panel B. Expanded view of isolates from this study.
Figure 5.7: PA Phylogeny

Phylogenetic relationships of the PA sequences. Swine and human isolates recovered from the same fair are identified with the same color and symbol (Fair A: teal open diamond, Fair B: black downward pointing open triangle, Fair C: pink upward pointing open triangle, Fair D: Red closed circle, Fair E: Green upward pointing closed triangle, Fair F: Blue closed square, Fair G: gold downward pointing closed triangle, Fair H: orange open circle, Fair I: purple closed diamond, Fair J: Brown open square) Panel B. Expanded view of isolates from this study.
Figure 5.7
Figure 5.8: PB1 Phylogeny

Phylogenetic relationships of the PB1 sequences. Swine and human isolates recovered from the same fair are identified with the same color and symbol (Fair A: teal open diamond, Fair B: black downward pointing open triangle, Fair C: pink upward pointing open triangle, Fair D: Red closed circle, Fair E: Green upward pointing closed triangle, Fair F: Blue closed square, Fair G: gold downward pointing closed triangle, Fair H: orange open circle, Fair I: purple closed diamond, Fair J: Brown open square) Panel B. Expanded view of isolates from this study.
Figure 5.9: PB2 Phylogeny

Phylogenetic relationships of the PB2 sequences. Swine and human isolates recovered from the same fair are identified with the same color and symbol (Fair A: teal open diamond, Fair B: black downward pointing open triangle, Fair C: pink upward pointing open triangle, Fair D: Red closed circle, Fair E: Green upward pointing closed triangle, Fair F: Blue closed square, Fair G: gold downward pointing closed triangle, Fair H: orange open circle, Fair I: purple closed diamond, Fair J: Brown open square) Panel B. Expanded view of isolates from this study.
Chapter 6 - Preliminary investigation of risk factors contributing to the presence of influenza A virus in swine at agricultural fairs

This chapter has been prepared for submission to a peer reviewed journal.
6.1 Abstract

Influenza A virus infections occurring in exhibition swine populations at agricultural fairs during 2012 served as the source of H3N2pM influenza A viruses transmitted to humans resulting in more than 300 documented cases of variant influenza A. This investigation was initiated to identify potential risk factors contributing to influenza A virus infections in the pigs at agricultural fairs. Swine nasal swabs and associated data were collected from pigs at 40 junior market swine shows held at fairs in Ohio during the 2012 fair season as part of an ongoing active surveillance program. Results show that adjusted odds of having influenza A virus infected pigs at a fair were 1.27 (95% CI: 1.04-1.66) higher for every 20 pig increase in the size of the swine show. Four of the 5 fairs which hosted breeding swine shows in addition to their junior market swine shows had pigs test positive for influenza A virus. While limited in size, the findings of the current study will be helpful to veterinary and public health officials evaluating mitigation strategies to decrease the intra- and inter-species transmission of influenza A virus at fairs. The findings of the current study highlight the need to gather more robust estimates of risk factors associated with zoonotic transmission of influenza A virus in these settings.

6.2 Introduction

The comingling of swine from numerous premises with varied management practices and their interaction with large numbers of exhibitors and visitors naïve to animal origin-influenza A viruses make agricultural fairs an ideal setting for the intra-
and inter-species transmission of influenza A viruses (IAV) between swine and human populations.\textsuperscript{1,2} Swine are a host species in which reassortment events may lead to emergent novel strains, since they are susceptible to infection from swine, human, and avian influenza A viruses.\textsuperscript{3,4} For this reason, limiting the bi-directional zoonotic transmission of these viruses at agricultural fairs is important for public and animal health.

The association between human and swine influenza A viruses was reported after respiratory disease in pigs was noted during the 1918 human Spanish flu pandemic;\textsuperscript{5} H1N1 IAV subsequently became established in swine populations worldwide. For nearly 80 years, classical swine influenza H1N1 virus was the dominant endemic IAV strain in the North America swine population.\textsuperscript{6} In 1998 triple reassortant H3N2 viruses containing PB1, HA, and NA gene segments from human IAV lineages, PB2 and PA genes from avian lineages, and NP, M, and NS gene segments from swine lineages, emerged in North American swine.\textsuperscript{7} Subsequently this lineage became established in the U.S. and Canadian swine herds and has resulted in an increased rate of genetic and antigenic change among swine-origin IAVs.\textsuperscript{8-10}

Reported cases of humans contracting influenza A virus directly or indirectly from pigs has been historically sporadic and variant IAVs frequently show limited capability for sustained human-to-human transmission.\textsuperscript{11-13} However, the emergence of the influenza A(H1N1)pdm09 virus, a strain containing gene segments from North American and European swine lineages,\textsuperscript{14} illustrated the pandemic potential of swine lineage IAVs crossing the species barrier to humans. While A(H1N1)pdm09 rapidly
spread worldwide and became endemic in the human population,\textsuperscript{15} sequencing of this virus has to date failed to elucidate any virulence or adaptation markers that would explain its human-to-human transmission efficiency, highlighting our inability to predict IAVs with pandemic potential. While the origin of the A(H1N1)pdm09 virus remains unknown, the virus was introduced into the North American swine population in 2009 and has since reaassorted with other swine-origin IAVs.\textsuperscript{16,17}

Zoonotic transmission of IAV to humans has been documented at unprecedented levels in recent years. Since 2011, more than 320 human cases of infection with variant influenza A viruses were reported to the Centers for Disease Control. These zoonotic viruses contained 7 genes from contemporary North American swine lineage IAV and one gene (M) derived from the H1N1pdm09 virus.\textsuperscript{18} The majority of the cases were epidemiologically linked to swine exposure occurring agricultural fairs across several states.\textsuperscript{19-21} Within Ohio, 107 H3N2v cases documented during 2012 resulted in eleven hospitalizations and one fatality.\textsuperscript{22} Genomic analyses of H3N2 IAV isolates recovered from pigs at agricultural fairs in the state during 2012 demonstrated >99% nucleotide similarity to H3N2v isolates recovered from concurrent human cases, providing molecular confirmation of zoonotic IAV transmission during at least 7 fairs.\textsuperscript{2} This record number of variant influenza A cases has resulted in a “one health” effort to minimize intra- and bi-directional inter-species IAV transmission at swine exhibitions.\textsuperscript{23} However, the paucity of scientific evidence makes it difficult for veterinary and public health officials to make evidence based recommendations to protect public and animal health. We previously reported the recovery of IAV from exhibition swine at 10 of 40 sampled
Ohio fairs in 2012. In the present study we investigate risk factors potentially contributing to the emergence of influenza A virus in swine at these fairs.

6.3 Material and Methods

As part of an ongoing active IAV surveillance project, swine nasal swabs and associated metadata about management practices were collected at 40 Ohio fairs in 2012. Molecular and microbiological assays for IAV were performed on the swabs and previously reported. Sample size was selected to provide a 95% probability of detecting IAV infection if greater than 15% of the pigs at each fair were infected. All swine exhibitions enrolled in this study were junior fair market swine shows occurring at agricultural fairs. For the outcome of interest, a fair was considered positive if viable influenza A virus was recovered from one or more pigs at the fair.

Data collection focused on fair level variables possibly contributing to the presence or absence of influenza A virus in the pigs during each exhibition. Junior fair shows are limited to exhibitors approximately nine years of age through nineteen years of age participating in 4-H, FFA, or another youth organization whereas open class shows are generally open to all participants regardless of age or affiliation. Classification of swine included market swine (pigs bred, raised, and intended for food purposes) and breeding swine (gilts, sows, and/or boars being raised for breeding purposes). Terminal swine shows are those in which all participating livestock are consigned to harvest immediately following the exhibition and partial terminal shows usually require the
champion animals to be harvested following the exhibition and other pig may or may not go to harvest.

The length of the swine exhibition was calculated as the number of days between the required arrival of the pigs and the time the pigs were sampled. Study team members calculated the area per pig (ft.²/pig) from the recorded size of the pens and the number of pigs per pen. While on the fairgrounds, study team members also documented if there was an easily identifiable and operational hand-wash and/or hand-sanitizer station within close proximity to the swine barn(s). These sanitation stations were used by study team members to determine if they were functional.

Additional variables included the number of pigs at the fair, number of swine exhibitors, fair attendance (number of people), and vaccine requirements, all of which were reported to the study team by the fair organizers. Fair officials also reported if there was a commingling event, such as a tag-in or weigh-in, during the weeks or months prior to the fair. Exhibition directors also reported if there were other pigs besides the exhibition swine on the fairgrounds (i.e. petting zoo, pig races, education displays).

The commercial swine inventory was retrieved from United States Department of Agriculture’s 2007 Census of Agriculture. The county human population was defined as the value reported in the 2010 U.S. census report. Weather data was collected from the National Weather Service’s weather station nearest to each fair.

Data were analyzed using STATA Version 11.1 (StataCorp, College Station, TX). Fisher exact test was used to assess differences in proportions and the Kruskal-Wallis equality-of-populations rank test was used for continuous variables. Univariate analysis
was performed to calculate unadjusted odd ratios to identify factors contributing to the presence of IAV in pigs at fairs. Exact logistic regression was used for multivariable modeling using a forward stepwise model building approach. A cut-off of $P \leq 0.05$ was used for inclusion in the model.

6.4 Results and Discussion

The results of the current study provide the first look at fair-level risk-factors associated with IAV infections in swine at agricultural fairs. While it is likely impossible to completely prevent IAV transmission at swine exhibitions, these data can be used to evaluate mitigation strategies to reduce the impact of intra- and inter-species IAV infections at swine exhibitions. Mitigation strategies which are practical, user-friendly, low cost, and do not dramatically alter the fair experience for exhibitors and visitors are most likely to be considered, implemented, and maintained.

Influenza A virus was recovered from pigs at 10 of the 40 fairs included in the investigation. The presence or absence of IAV infection among the pigs at the fairs could not be associated with county population, county swine inventory, and number of people attending the fair (Table 1). All IAV positive fairs and 27 of 30 (90%) of negative fairs were mixed sex (barrows and gilts) market swine exhibitions. Properly functioning hand-wash and/or hand-sanitizer stations were available at 25 of 40 (62.5%) fairs. While hand hygiene stations were not linked to presence or absence of IAV among the pigs at the respective fairs they may be critically important in controlling other zoonotic diseases at these venues.\textsuperscript{27}
Previous research has shown that environmental stressors (heat, lack of space, noise) on pigs can affect the course of various diseases in commercial swine operations.\textsuperscript{28} The average space per pig at the studied fairs was 12.8 sq.ft./pig, well above 6-8 sq.ft./finishing pig common through the swine industry.\textsuperscript{29} Average daily mean temperature was almost 4°C higher for fairs with IAV positive pigs suggesting that heat stress may be a contributing factor to IAV infections in exhibition swine. Caution must be used when interpreting this result because the vast majority of the fairs with IAV positive pigs occurred in a four week period during the middle of summer. This trend of mid-summer IAV activity in Ohio’s exhibition swine was also observed in the previous four years\textsuperscript{1} and may be more related to animal and/or people movement between these fairs than the weather. Environmental temperature failed to meet the selection criteria for inclusion the final multivariable model.

Not surprisingly, larger pig shows appear to be more likely to have IAV infected pigs than smaller swine exhibitions (Table 2). For every increase of 20 pigs at a fair, the risk of IAV infection in the pigs went up 1.27 times. Also, larger swine exhibitions also tend to have open class and breeding swine shows in addition to junior market shows. While open class shows were common among our studied fairs (16 of the 40), only 5 of the 40 (12.5%) fairs in this study had a breeding show; 4 of those 5 (80%) fairs had IAV infected pigs at the fair. The small number of fairs with breeding shows in this study makes analysis of this risk factor problematic but the finding is enough to warrant concern given that breeding swine are intended to leave the exhibition and enter a herd for progeny production. This fair-to-farm movement of pigs is a virus introduction risk
for the receiving herd and a potential method to disseminate IAV strains across a large
geographic area.

One potential mitigation strategy is to shorten the exhibition period.\textsuperscript{30} This would limit the time for IAV to spread among susceptible pigs and decrease the time humans are exposed to IAV infected pigs. The length of the exhibitions in the current study was similar between IAV positive and negative fairs. Active recruitment of fairs with more diverse management practice is needed to study the impact of a shortened swine exhibition. No matter the length of the exhibition, the disposition of the pigs following the show must be considered. The majority of fairs in this study (65\%) had terminal junior market shows. As expected, the practice of having a terminal show was not associated with decreased odds, but sending all the pigs to harvest at the of each fair is expected to help protect subsequent fairs by decreasing fair-to-fair spread of IAV.

Mandated vaccinations were almost non-existent with only one fair in the current study requiring any vaccinations for the swine coming to the fair (\textit{Erysipelothrix rhusiopathiae}); no fairs required IAV vaccination. Use of IAV vaccine in exhibition swine has been one of the most debated topics following the H3N2v outbreak of 2012. Commercially available swine influenza vaccines are universally indicated to reduce clinical signs in pigs associated with disease. Although their impact on transmission dynamics and zoonotic transmission remains unclear,\textsuperscript{31} it is expected that IAV vaccine use will impart at least partial immunity to circulating strains of IAV which should decrease viral shedding during a fair. An unintended consequence IAV vaccine use may be vaccine-associated enhanced respiratory disease which has been reported in swine
vaccinated with swine influenza virus vaccines that are mismatched to circulating strains. Furthermore, a decrease clinical signs without reducing shedding will only prevent recognition and response to active IAV infections in exhibition pigs.

Some of the major pitfalls of mandated IAV vaccination lay with the practical application of vaccines in this setting. The distribution of vaccines to swine exhibitors prior to the fair becomes difficult because most exhibition swine are raised by youth exhibitors in small herds (<10 pigs per herd). Commercial vaccines are usually sold in >50 doses per bottle making the cost per vaccinated pig in these small herds quite excessive. Additionally problematic is that most IAV vaccines labeled for swine require two doses to provide protection which can be difficult for youth exhibitors and their family members to accomplish. Even in properly vaccinated pigs, the immunity stimulated by current IAV vaccines is limited in scope and duration.\textsuperscript{33, 34} The strains used for commercial swine influenza vaccines are irregularly updated and the constant genetic and antigenic change occurring in contemporary swine-origin IAVs makes viral antigens unpredictable and difficult vaccine targets.

Pre-fair tag-in and/or weigh-in events were rather common with 23/40 (57.5\%) of the enrolled fairs holding one of these events. Tagging or weighing events are frequently used as a way for exhibitors to declare ownership of the pigs prior to the fair. These pre-fair events may provide an opportunity for mass vaccination of pigs prior to the fair but the application of such events may facilitate disease spread between animals.

The results presented herein are from one year of surveillance in a limited geographic area of the United States.
Additional assessments of swine exhibitions in multiple states across several years are needed to provide more comprehensive evaluations risk factors contributing to the problem. However, these data provide a critical first step toward developing effective IAV mitigation strategies that benefit fairs, exhibitors, visitors and the swine industry.

6.5 Acknowledgements

We thank our collaborators from the participating agricultural fairs. We also thank Jeffery Workman, Wendell Bliss, Gary Bowman, Dimitria Mathys, and Tony Forshey for their assistance and support with this project. This work has been funded by the Minnesota Center of Excellence for Influenza Research and Surveillance with federal funds from the Centers of Excellence for Influenza Research and Surveillance, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under contract no. HHSN266200700007C.
6.6 References


<table>
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<tr>
<th></th>
<th>Flu + ( \bar{x} )</th>
<th>Flu - ( \bar{x} )</th>
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<th>Odds Ratio</th>
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<td>70,490</td>
<td>.71</td>
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<td>Number of exhibitors</td>
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<td>146,446</td>
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<td>1.01</td>
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<td>217,5667</td>
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<td>Length of Exhibition</td>
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<td>5.33</td>
<td>.64</td>
<td>0.89</td>
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<td>80,175.79</td>
<td>.52</td>
<td>1.00</td>
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<td>Pig space (sq. ft./pig)</td>
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<td>12.56</td>
<td>.15</td>
<td>1.13</td>
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<td>Average daily mean temp</td>
<td>24.22</td>
<td>20.67</td>
<td>.03</td>
<td>1.32</td>
<td>1.01-1.75</td>
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<th>n</th>
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<td>40</td>
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<td>6</td>
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<td>29</td>
<td>97.7</td>
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<td>6</td>
<td>60</td>
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<tr>
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<td>40</td>
<td>11</td>
<td>36.7</td>
</tr>
<tr>
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<td>6</td>
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<td>8</td>
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<td>Is the junior market swine show terminal?</td>
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<td>9</td>
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<td>8</td>
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<td>21</td>
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<td>7</td>
<td>70</td>
<td>9</td>
<td>30.0</td>
</tr>
<tr>
<td>No</td>
<td>3</td>
<td>30</td>
<td>21</td>
<td>70.0</td>
</tr>
<tr>
<td>Sex Requirements of junior market swine</td>
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<td>0</td>
<td>3</td>
<td>10.0</td>
</tr>
<tr>
<td>Barrows only</td>
<td>10</td>
<td>100</td>
<td>27</td>
<td>90.0</td>
</tr>
<tr>
<td>Barrows and gilts</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>10.0</td>
</tr>
<tr>
<td>Showmanship occurs separate from the junior market show?</td>
<td>4</td>
<td>40</td>
<td>19</td>
<td>63.3</td>
</tr>
<tr>
<td>Yes</td>
<td>6</td>
<td>60</td>
<td>11</td>
<td>36.7</td>
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<tr>
<td>Pre-fair tag-in/weigh-in</td>
<td>0</td>
<td>0</td>
<td>1</td>
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<td>Are any swine vaccines required?</td>
<td>10</td>
<td>100</td>
<td>29</td>
<td>97.7</td>
</tr>
<tr>
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<td>0</td>
<td>0</td>
<td>1</td>
<td>3.3</td>
</tr>
<tr>
<td>No</td>
<td>10</td>
<td>100</td>
<td>29</td>
<td>97.7</td>
</tr>
<tr>
<td>Are there other pigs on the fairgrounds?</td>
<td>6</td>
<td>60</td>
<td>12</td>
<td>40</td>
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<tr>
<td>Yes</td>
<td>4</td>
<td>40</td>
<td>18</td>
<td>60</td>
</tr>
<tr>
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<td>10</td>
<td>100</td>
<td>29</td>
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Table 6.1: Summary statistics and crude odds ratios for presence of influenza A virus in pigs at fairs.
<table>
<thead>
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<th></th>
<th>Adjusted Odds Ratio</th>
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<tr>
<td>Breeding Show</td>
<td>21.676</td>
<td>.005</td>
<td>2.417-∞</td>
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<td>Number of pigs</td>
<td>1.012</td>
<td>.012</td>
<td>1.002-1.026</td>
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Table 6.2: Adjusted odds ratios from multivariable exact logistic regression model.
Chapter 7 – General Conclusions and Future Directions

Transmission of influenza A viruses between host species are threats to both animal and public health. This zoonotic transmission of type A influenza viruses and/or their gene segments contribute to the formation and emergence of novel influenza A viruses. Around the world there are unique niche settings conducive to intra- and inter-species influenza A virus transmission which include: agricultural fairs, live animal markets, and zoos. These locations represent an increased potential for interspecies transmission of influenza A viruses because they are concentration points and create animal-animal or human-animal interfaces. The data presented in Chapters 3, 4 and 5 demonstrate that intra-species transmission of influenza A virus in exhibition swine at agricultural fairs in Ohio is common and inter-species transmission represent significant animal and public health threats. Agricultural fairs allow animals with diverse histories of influenza A virus infections to mix with fair attendees creating the most dense swine-human interface in the United States. Investigations of the human cases of H3N2v and H1N2v influenza A in 2011 and 2012 linked most of the cases to human-swine exposure occurring at fairs and the data in chapters 4 and 5 provide the molecular confirmation that swine-to-human transmission is occurring.
The prospective virus surveillance data from 93 fairs during four years (2009-2012) indicates there were pigs infected with influenza A virus at approximately 25% of the fairs in Ohio. Furthermore, greater than 80% of the influenza A virus infected pigs at these fairs did not exhibit typical clinical signs of respiratory disease thereby presenting an undetected public health threat. The observed number of human variant influenza A cases in 2012 raised questions about the actual frequency of zoonotic influenza A virus transmission in the settings. Prospective surveillance of humans as well as animals will provide insight into this question and provide valuable information about the human side of the animal-human interface. Conducting active surveillance in humans to detect zoonotic transmission, a relatively rare event, will be difficult to accomplish on a large scale in the general public. Agricultural fairs provide a setting where zoonotic influenza A virus transmission is more common and thus makes them idea places to study the otherwise uncommon happening. An animal model system seems to be a viable option to further assess the risk of interspecies transmission of influenza A viruses at fairs and to evaluate interventions to mitigate the risk.

Unfortunately, there is little scientific evidence upon which to base changes in policies and management practices to mitigate the risk of the zoonotic transmission of influenza A viruses between pigs and people at fairs. The preliminary data presented in Chapter 6 provided an initial model for evaluation of risk factors that could be modified to decrease the risk of influenza A virus at agricultural fairs or other swine-human interfaces. In order to make more meaningful recommendations, more data about influenza A virus activity in pigs at fairs inside and outside Ohio is needed. A
collaborative project utilizing the talents, resources, and skills of The Ohio State University, Department of Veterinary Preventive Medicine (OSU-VPM); Minnesota Center for Influenza Research and Surveillance (MCEIRS), Centers for Disease Control and Prevention (CDC); and the United States Department of Agriculture, Animal and Plant Health Inspection Service (USDA-APHIS) is being organized to conduct active influenza A virus surveillance in pigs at fairs in five state during 2013. This expanded surveillance project is aimed at refining the understanding of fair-level risk factors influencing the prevalence of influenza A virus infections among swine at agricultural exhibitions and make accurate assessment of the effectiveness of mitigation strategies to minimize zoonotic transmission of influenza A viruses at agricultural exhibitions.

In depth investigations of influenza A virus infection status of swine at fairs will be needed to elucidate the dynamics of influenza A virus infections in these settings. Longitudinal sampling of pigs during fairs will provide information about the introduction of the viruses to the fairs and will shed light on how the length of exhibition impacts the number of infected animals. This information will be vital for designing effective mitigation strategies but will require developing alternate and practical sampling strategies to overcome the obstacle of sampling pigs prior to the show.

Additional future research plans to examine influenza A virus activity at animal-human interfaces include expanding surveillance efforts at fairs to include other host species at key interface locations. Influenza A virus activity in domesticated avian host species (ducks, chickens, turkeys, and geese) being exhibited at agricultural fairs has not been examined, thus gaps in knowledge about animal-avian-human interfaces exists.
Identifying and understanding these interface environments where bidirectional transmission can potentially occur is imperative to identifying emerging novel viruses and protecting public health.

Finally, it is believed that commercial swine serve as the major source of the influenza A viruses infecting exhibition swine. Exhibition swine are a unique population of noncommercial swine, reared using management practices that differ greatly from standard commercial swine production practices. While these exhibition swine are not typical of the U.S. swine herd, they are the pigs on public display at fairs and shows and represent the largest human-swine interface in the U.S. We know that influenza A viruses in the exhibition swine population are ultimately transmitted to humans at agricultural fairs. What remains unknown is how influenza A viruses become established in the exhibition swine population. Clarifying the genomic linkages between influenza A viruses maintained in commercial pigs and exhibition swine is crucial to understanding the intra-species transmission of influenza A viruses and ultimately protecting public health.

The United States is a leader in swine influenza surveillance but the majority of that surveillance is passive, depending upon recognition of influenza-like-illness in pigs to initiate testing. This passive surveillance strategy provides a high level view of influenza A virus activity in the U.S. swine herd but it fundamentally fails to detect subclinical infections in swine creating a gap in knowledge of viral genomic diversity in swine. Targeted investigations of influenza A virus using active surveillance of both ill and healthy pigs in commercial swine are needed to supplement the passive and more
traditional virus surveillance methods. Actively monitoring influenza A viruses circulating in swine populations allows for more timely detection of novel strains which will allow the scientific community time to update diagnostic tests and reagents, modify vaccines, and develop appropriate precautions to protect public health.
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