Evolution of Influenza A Viruses in Exhibition Swine and Transmission to Humans, 2013-2015

Thesis

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Abstract

Influenza A virus is a zoonotic pathogen whose introduction to humans from animals could potentially cause a pandemic. Animal-linage influenza A viruses (IAVs) that infect humans are referred to as variant IAVs, which are designated with a 'v' after the subtype. To better understand the epidemiology of IAV in exhibition swine and resulting H3N2v in humans, we performed a phylogenetic analysis using full genome sequences from 279 IAV isolates collected from exhibition swine in 5 states from 2013-2015 and 23 of the 25 H3N2v cases reported during those same years. Sixty-six fairs (23.7%) had at least one sample that was positive for IAV and 20 of those fairs (30.3%) had more than one IAV genotype circulating in the pigs. An overall 3-year prevalence of 9.7% (95% CI: 9.1-10.3) was observed. However, the prevalence of IAV in swine significantly decreased from 2014 to 2015 when the proportion of fairs with IAV infected pigs decreased from 30.14% (95% CI: 19.6-40.1) in 2014 to 13.5% (95% CI: 6.9-20.1) in 2015. We found 19 IAV genotypes infecting swine and 6 IAV genotypes in humans, with 5 genotypes in both host species. There was a positive correlation between the number of fairs at which a genotype was present among the pigs and the number of human cases of that same genotype. Additionally, we showed that H3N2v isolates clustered tightly with exhibition swine isolates that were prevalent in the same year. Our data indicate that there are multiple genotypes of swine-lineage IAV that can infect humans, and highly prevalent IAV genotypes during a given year are the strains most likely to infect humans.

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Nolting JM, Szablewski CM, Edwards JL, Nelson SW, Bowman AS. "Nasal wipes for influenza A virus detection and isolation from swine." Journal of Visualized Experiments. Vol. 106, e. 53313. (4 Dec 2015).

Szablewski CM, Hendricks K, Bower WA, Shadomy SV, Hupert N. Anthrax Cases Associated with Animal-Hair Shaving Brushes. *Emerging Infectious Diseases*. 2017;23(5):806-808. doi:10.3201/eid2305.161554.

Fields of Study

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Chapter 1. Literature Review

1.1 Influenza History

Influenza is a contagious respiratory illness that has affected humans since ancient times. There are recorded cases of highly contagious, acute respiratory illnesses that date as early as 412BC. However, the actual term "influenza" wasn't coined until the 15th century. At that time the name denoted the belief that the disease was caused by "the influence of the stars". (1) For centuries, man had searched for the cause of these epidemics. Many sources were implicated including the weather and poisonous gases from swamps. At the end of the 19th century, the germ theory was becoming widely accepted and microbiology of infectious diseases had become firmly rooted. In 1889 a bacillus, *haemophilus influenza*, was found in the throat swabbing of influenza patients. This lead to the belief that *haemophilus influenza* was the causative agent of influenza. (1) During the 1918-1919 human H1N1 pandemic, a veterinarian discovered that a respiratory illness seen in pigs was strikingly similar to the one seen in humans at the same time. He concluded they were caused by the same agent. (2) However, it wasn't until the influenza A virus (IAV) was isolated from a pig in 1930 that the true cause of the illness was discovered. (3) Even after that, it took three more years before the first strain of IAV was isolated in humans in 1933. (4) In that same year, researchers at the

National Institute for Medical Research in London were able to successfully inoculate ferrets with influenza from human throat washings. (1)

Anecdotal evidence indicates that animals have suffered from influenza-like illnesses for as long as humans. (5) There are numerous historical accounts of respiratory illness in animals, especially horses, either before or after human epidemics. (1) However, the correlation wasn't officially established until the fall of 1918, when the first official cases of influenza in animals were seen in pigs. (6)

1.2 Etiology of Influenza

Influenza A virus is a member of the family *Orthomyxovirddae*, which is a family of 7 different genera. Members of this family include influenza A, B, C, and D. (7) Although A, B, and C influenza genera can cause respiratory disease in humans, IAV is the most burdensome. (8) IAV is the type of influenza that is of routine clinical significance in swine. IAV is an enveloped, single-stranded, negative-sense RNA virus. It is polymorphic, about 80-120nm in diameter, and encodes 10 to 11 viral proteins on eight separate RNA segments. The eight viral RNA segments are named for the proteins that they encode: polymerase basic 1 and 2 (PB1 and PB2), polymerase acidic (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M), and non-structural protein (NS). (9) PB2 acts as a cap binding transcriptase, PB1 acts as an elongation transcriptase, PA is thought to act as a protease activity transcriptase, HA is a viral surface projection that binds the virus to the cell it is infecting, NP binds RNA and transports vRNA , NA is a viral surface projection and acts to relase virus from the cell, M is a matrix protein, and NS functions for RNA transport, translation, and splicing. (10)

These segments are folded into rod-shaped ribonucleotide complexes (RNP). Each of these complexes contains the viral RNA, a polymerase, and copies of the viral encoded nucleoprotein. (11) These eight distinct RNPs are packaged into an enveloped virion which is then complete enough to infect an animal or human. When a host is infected with an IAV, hemagglutinin will attach to sailic acid receptors on the surface of the host cell and will enter the cell by endocytosis. Once in the cell, the virus will release the RNPs into the cytosol. From there, the RNPs move into the nucleus where it will use the host cell's machinery to make viral proteins and new copies of the RNPs. Finally, these new RNPs will be gathered along the edge of the cell membrane, where they will be encapsulated into a new budding virion. (11) Since IAV is an RNA virus, it lacks proofreading among its RNA polymerases leading to replication errors. (12) The standard rate of error for the influenza virus is 1 in 10^4 bases. (13) The evolutionary rate for the NS gene in influenza is 2×10^{-3} substitutions per site per a year. This is about 10^6 times the evolutionary rate of germlines in mammals. (14)

1.3 Epidemiology of Influenza

In the present day, IAV is regularly found in nature. Many species of animal suffer from strains of influenza virus including humans, ducks, chickens, pigs, horses, seals, cats, dogs, and mink. (13, 15) Influenza occurs seasonally in humans with significant influenza epidemics in humans occurring about every 3 years, typically during the winter season. (16) Although H3N2 and H1N1 viruses are both associated with these epidemics, H3N2 viruses are more frequently associated due in part to their more rapid antigenic drift. (16) It has been speculated that over the last 250 years there have been 10

to 20 influenza pandemics. (1) (17) One of the most deadly influenza pandemics is the great pandemic of 1918-1919. It is estimated that one third of the world's population or about 500 million people were infected and showed clinical signs of illness. Although it is hard to know the exact number, it is estimated that as many as 20-100 million people died as a result of infection. (1, 18) This makes the case fatality rate (CFR) > 2.5%, which is very high considering other influenza pandemic CFR's were <0.1%. All influenza pandemics since that time have been caused by descendants or reassortants of this virus. This includes all H1N1 viruses as well as reassorted H3N2 and H2N2. (18)

Depending on the vaccination rate and success, anywhere from 15 to 61 million people suffer from influenza A, B, or C associated illness (influenza illness) each year. This comes out to about 5-20% of the population. (19, 20) There is an average of about 24.7 million cases of influenza illness each year in the United States with around 41,000 deaths. (21) Many of these deaths are in older patients and are attributed to co-morbidities. This amounts to about 610,656 life years lost per a year. (22) It is estimated medical care and indirect costs associated with influenza total over 16 billion dollars a year. (23) In 2003, it was estimated that the total economic burden of influenza across all age groups in the US was $87.1 billion dollars. (22)

Although a clear majority of influenza cases in humans are endemic human strains, in recent years there has been an increase in the number of zoonotic cases of influenza. When a human is infected with an IAV strain that normally circulates in animals, not humans, it is called a variant virus. This is denoted with a lower case "v" after the subtype, i.e. H3N2v. Human infections with H3N2v, H1N1v, and H1N2v have

all been detected in the United States. (24) There have been a total 421 cases of variant influenza in humans since 2005. A majority of these cases (390) were due to H3N2v, but there were also 20 cases of H1N1v and 11 cases of H1N2v. (25)

1.4 Human Pandemics

Pandemics arise when an IAV contains 3 properties. It must be antigenically unique enough to be able to avoid the population's immunity, it must be sufficiently adapted enough to easily spread from person to person, and it must cause severe disease. (26) Due to the quick turn-around time for raising swine, there is always a large population of naïve animals. This allows for less severe immunologic pressure compared to human populations. (27) Consistent with this practice is the fact that current European strains of H1N1 show cross reactivity with IAV from the 1980's meaning that antigenic drift is much slower than in human influenza viruses. (16) In comparison, the human population has had several shifts in the H1N1. From 1977 to 2009 there has been enough shift to warrant 8 updates of the H1 component of the influenza vaccine. (28) The fact that the human seasonal H1 has drifted much faster than the swine H1 has led to a significant antigenic gap. This makes swine a reservoir for H1 viruses that could cause significant illness in humans and could possibly even cause a pandemic. (29)

1.4.1 Spanish, Asian, and Hong Kong Influenza

In the last century, there have been 4 major pandemics. The 1918 Spanish flu (H1N1), the 1957 Asian flu (H2N2), the 1968 Hong Kong flu (H3N2), and the 2009 Swine flu (H1N1). Analysis of the 3 viruses from the 20th century suggested that the strains may have been generated through a series of reassortment events and that these

happened over a period of several years prior to the pandemic occurrences. It is believed that all 3 viruses were reassorted with at least one virus of animal origin. The origin of the animal segments from the 1957 and 1968 pandemics appear to be of avian origin but the origin of the 1918 pandemic remains ambiguous. (30-32) It is believed that these reassortments may have occurred in swine, but there is no conclusive proof. (16) (33) We do know that both the Spanish flu and the Hong Kong influenza A viruses were passed from humans to pigs regularly under natural conditions. (30, 34) This is in contrast to the Asian flu which was found to not readily infect swine. Therefore, swine are not believed to have played a significant role in the epidemiology of the Asian flu. (35)

1.4.2 2009 H1N1

The 2009 H1N1 pandemic was the first influenza pandemic of the 21st century. Genomic analysis showed that all the segments were from well-established swine influenza lineages that had been circulating in swine populations for at least 10 years. Most of the genes were from North American H3N2 and H1N2 viruses but the NA and M genes were closely related to an H1N1 avian-like swine virus found in European swine in 1979. This strain was one that had not previously been reported in North American swine. (32) This pandemic serves as an example of why surveillance in swine populations is important. The H1N1 pandemic circulated in swine populations for 10 years before making the jump to humans. (29)

1.5 Human Influenza

Classic influenza syndrome is signaled by sudden onset of fever, headache, cough, sore throat, myalgia, nasal congestion, weakness, and/or loss of appetite. This

typically occurs between the months of December and April. Transmission between humans is mostly from direct person to person contact with respiratory secretions. (16) Influenza in humans can be treated with anti-viral drugs such as Zanamivir and oseltamivir. For antivirals to be maximally effective, they must be instituted at symptom onset. (36) This would be before laboratory testing. However, laboratory testing in very important for characterizing the strain of influenza. Viral collection for human influenza virus is similar to swine collection. Optimum upper respiratory tract samples include nasal swabs, nasopharyngeal aspirates, nasal washes, and throat swabs. More invasive procedures for lower respiratory tract infections such as transtracheal aspirates, bronchoalveolar lavage, and lung or tracheal tissue are also acceptable. Specimens that are taken within 3 days of symptom onset are preferable, especially for viral isolation. Samples need to be stored in a viral transport medium and refrigerated (26-46 °F). If samples cannot be processed within 2 to 3 days of sampling, they should be kept at -70 °C. (37) (38)

1.5.2 Influenza Surveillance

Domestically, the CDC reports cases of variant influenza in Fluview, its weekly national influenza surveillance report. (24) (39) As part of the International Health Regulations set forth by the World Health Organization in 2007, the CDC reports all cases of novel influenza viruses including variant cases to the WHO. (40) (41) In addition, influenza associated deaths in children less than 18 years of age is a nationally notifiable condition. (42)

The influenza division within the Centers for Disease Control and Prevention (CDC) collects, compiles, and analyzes influenza activity in the United States, Puerto Rico, and the Virgin Islands. This surveillance system is a combination of several voluntary reporting sources: virologic surveillance, out-patient illness surveillance, hospitalization surveillance, and mortality surveillance. Virologic surveillance is conducted through either the National Respiratory and Enteric Virus Surveillance System (NREVSS) or the U.S. World Health Organization Collaborating Laboratories System. (43) Through these tools, the CDC is informed of testing results from clinical and public health laboratories. Clinical test information includes numbers of positive samples and age groups of persons infected. Information from public health laboratories include virus type, subtype, and lineage. The second source of data is the U.S Outpatient Influenza-like Illness Surveillance Network. (44) This network is comprised of more than 2,800 outpatient healthcare facilities. Each week these facilities report the number of patients they saw that week as well as the number of patients that presented for an influenza-like illness (ILI). (5) This is defined as a fever of ≥100 °F and a cough and/or sore throat without a known cause other than influenza. The percentage of ILI cases are then compared to the national baseline of 2.2%. Hospitalization rates for influenza are recorded through the Influenza Hospitalization Surveillance Network. (45) Cases are identified by a positive influenza test. This can be a viral culture, direct/indirect fluorescent antibody assay, rapid influenza diagnostic test, or molecular assay like reverse transcription -polymerase chain reaction. Mortality surveillance data is procured through the National Center for Health Statistics mortality surveillance data. (46) Death

identified from pneumonia and influenza on the death certificate are compared against a seasonal baseline. This baseline changes based on a periodic regression model. Deaths of children under 18 years of age from influenza are recorded in the Influenza-Associated Pediatric Mortality Surveillance System. (42)

1.6 Influenza A Virus in Swine

Influenza in swine has an incubation period of about 1 to 3 days. After which time there is a sudden onset of clinical signs. Labored breathing and dyspnea are the most typical signs of IAV infection. They can also be characterized by high fever (105-106.7 °F), anorexia, inactivity, huddling, reluctance to rise, and tachypnea. Coughing begins a few days later. The clinical signs only last about 5-7 days with rapid recovery. Acute, clinical outbreaks of IAV typically occur in pigs that are seronegative and fully susceptible to the strain of influenza. This could occur in all ages. (26) The primary route of infection in swine is pig to pig contact via nasopharyngeal exposure. (16) This is because viral replication is seen in the epithelial cells of the upper and lower respiratory tracts in pigs. This includes the lungs, nasal mucosa, and trachea. (26) A heavy viral load in the lungs is required to produce clinical signs. This is due to the production of cytokines that are in turn induce lung inflammation. (26)

Influenza virus can go undetected in swine. (47) (48) (49) (50) Infection with influenza virus can often be subclinical with only 25 to 30% of the herd showing influenza-like illness. (51) One experiment found that pigs intranasally infected with IAV can maintain the virus in their lung tissue for at least 3 months without clinical signs during which time the virus can be spread to other susceptible animals. (52)

1.6.1 Influenza A Virus in Commercial Swine

There are 3 different subtypes of influenza virus that are currently circulating in swine worldwide. These are H1N1, H3N2, and H1N2. Because of the ease of spread of the infection, it is hard to prevent infection especially in densely populated swine regions where the virus is enzootic. (16) Studies looking at influenza strains in the commercial population have found similar IAVs across the country. One study sampled swine from multiple midwestern states. It was found that they had similar genomic diversity and it was believed that they came from the same progenitor. This is consistent with the knowledge that swine are frequently transported through the country. (53) Geographical segregation can lead to differences in viruses circulating in swine populations. Animal movement patterns can lead to genomic diversity in viral populations. Producers in the USA commonly move swine around the country at different ages after weaning compared to the EU which very rarely move swine. This leads the Midwest US to have a greater source of genomic diversity than would be seen with isolated raising regions. (54) (55)

1.6.2 Influenza A virus in Exhibition Swine

In the United States, there are 3 major swine-human interfaces: commercial swine production, abattoirs, and agricultural exhibitions. The commercial swine industry is mostly made up of large, high population facilities with stringent biosecurity protocols and separation of populations based on age and origination site. The USDA conducts year-round surveillance of influenza A viruses in swine but exhibition swine are distanced from the commercial population. Most commercial swine will not come into direct contact with swine used in exhibitions. (56) Exhibition swine only make up

approximately 1.5% of the US swine heard. Most are raised in backyards or small-farm settings. They are primarily raised as agricultural projects for education or competition. (57) Nevertheless, phylogenetic mapping of influenza viruses in exhibition swine show that there is cross over between commercial swine and exhibition swine populations. (9)

In commercial swine facilities and abattoirs, access is limited to a few individuals with similar animal exposure histories. In contrast, agricultural fairs facilitate the exposure of millions of individuals with varying exposure histories to swine. Swine at agricultural exhibitions come from differing management practices and co-mingle for extended periods of time. (58) Exhibition pigs can attend multiple agricultural exhibitions in multiple states each year which provides opportunity for intrastate and interstate spread of viruses. (9, 56) Since more people come into contact with live swine at agricultural exhibitions than any other setting in the United States, it is no surprise that we see cases of influenza in humans from this exposure. (58)

Studies have shown that IAV has been circulating in the exhibition swine population for decades. In 2016, one study found the prevalence of influenza virus at studied fairs was 77.5%, indicating that influenza virus spreads quickly in fair populations. Swine at only 2 out of the 6 fairs sampled displayed wide-spread influenza-like illness. This demonstrates that many pigs can have subclinical infections of IAV. (59) One study that sampled pigs at county fairs recovered influenza A virus circulating in exhibition swine populations without influenza-like Illness. A study from 2009 to 2011 showed that 12/53 (22.6%) of fairs sampled were positive for influenza virus. Of those 12 fairs, only 2 (3.7%) of the fairs had pigs that looked sick with influenza-like illness.

Therefore, the prevalence of subclinical influenza at the fair level was 18.9%. (58) Risk factors for fairs to have Influenza A virus in their pigs include whether the fair has a breeding show in addition to a junior fair market show and a higher number of pigs at the fair. The odds of a fair having IAV infected pigs are 1.27 times higher for every 20-pig increase in swine show size. (60)

Genomic analysis of influenza in exhibition swine from 2009-2013 demonstarted that influenza viruses in exhibition swine did not form discrete clades separate from commercial swine. This suggests that new viral diversity in exhibition swine is introduced from commercial pigs. There was also little association between geographical distance and genomic distance between US exhibition swine, which is also consistent with the theory that commercial swine introduced genomic diversity into the exhibition swine population. (9) When influenza viruses from 2013 were further analyzed it was discovered that there were 12 different genotypes. There were nine different H1 genotypes and three different H3 genotypes. (9) During 2013, H3-G1 was found to have emerged in exhibition swine at least 7 independent times. Further analysis indicates that H3-G1 genotypes emerged by direct introduction from commercial swine as well as in-situ reassortment events between non-H3-G1 viruses already circulating in exhibition swine. (9) Analysis of influenza genotypes from 2009 to 2013 concluded that 5 of the 11 genotypes found had only one segment different from H3-G1. This means that a single segment substitution through reassortment would produce the H3-G1 genotype. (61)

1.6.3 Basic Swine Influenza Surveillance

Influenza A virus in swine is not a reported or regulated disease in the United States. The virus is considered endemic in swine populations in North and South America, Asia, and Europe. The United States Department of Agriculture cooperates with state and industry partners to conduct voluntary IAV surveillance. This surveillance is used to determine what subtypes are currently circulating in the swine population. Between October 2010 and July 2012, the USDA's IAV Surveillance Program tested 12,662 samples from 3,766 lab submissions. Over that time-period, they found 1,488 case submissions that were positive for IAV. Acceptable samples for this program are nasal swabs or lung tissues. (62) Virus can be isolated from day 1 to day 7 post-inoculation. However, it is less likely to be detected after day 7. (26) Virus can be isolated from upper and lower respiratory tract epithelial cells as well as the tonsils, respiratory lymph nodes, bronchoalveolar lavage (BAL) fluids, oral fluids, and nasal, tonsil, or oropharyngeal swabs. (26, 63, 64)

1.6.3.1 Sampling Materials

Over the past few years there have been many publications describing the various sampling materials and methods for the detection of influenza A virus in non-human mammalian species. These papers compare several nasal swab materials as well as new, innovative ways of sampling including Swiffer cloths, cotton gauze, and oral fluid collecting ropes. Swabs are considered the gold standard ante-mortem specimen for diagnosis of IAV in swine by the WHO. (41) Although nasal, throat, and tracheal swabs are all listed as potential sample methods, nasal swabs are the least invasive swab and

quickest to obtain. Many swab materials have been researched including nylon flocked, rayon budded, urethane foam, and Dacron budded swabs. (65, 66) Several research papers looking at IAV in avian and human hosts agree that flocked nylon and rayon budded swabs have equal efficacy in IAV detection for both PCR and virus isolation. Limited data indicates that urethane foam swabs have lower detection rates than nylon flocked swabs but possibly better than Dacron budded swabs for PCR detection of IAV from avian hosts. (67)

Several studies compared oral fluid collection with cotton ropes to the nasal swab. Data show that oral fluids are a viable alternative to nasal swabbing individual pigs for the PCR detection of IAV in a swine population. Proposed probabilities of IAV detection ranged from 80% - 100% oral fluid consensus for PCR testing. (63, 64) Oral fluids may be more useful for detecting IAV for a prolonged period of time following initial infection. However, the major limitation to the use of oral fluids for IAV surveillance is poor ability of viable virus recovery from these samples. The only IAV positive virus isolation results yielded just a 50% recovery. (68)

Other new surveillance methods that seem promising but need further research include the method of nasal swiping. (69) Swiffer cloths and cotton gauze have been used to collect IAV and other organisms in the environment and from the snout of pigs with variable results. (70-72)

1.6.3.2 Testing Methods

A definite diagnosis of IAV is only possible by isolation of the virus, detection of viral proteins or nucleic acid, or discovery of virus-specific antibodies. (26) The methods

currently used for diagnosis include virus isolation in embryonated chicken eggs and cell culture, antigen detection via antibody and ELISA testing, reverse transcriptase polymerase chain reaction (RT-PCR) assay, and serologic analyses. (73) Testing techniques are described in full detail by the OIE in their Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. (74)

Real-time RT-PCR assays are highly specific and sensitive. Studies have shown that RT-PCR can have 100% specificity and greater sensitivity than viral isolation. (75) (73, 76) However, due to increased sensitivity, a PCR weak positive sample may actually contain degraded or dead virus rather than infectious virus. (26) PCR can be used as a screening tool to determine if a sample is IAV positive. Two of the most common viral isolation (VI) mediums are embryonated chicken eggs (ECE) or Madin-Darby Canine Kidney cells. Although ECE is the gold standard VI medium for avian influenza, swine influenza virus is not readily isolated in ECE. (77) (78, 79) Additionally, ECE are an expensive testing method and recent studies have shown that MDCK cell lines are a better alternative to ECE. (80) (81) Original samples or viral isolated propogated samples can be sequenced.

1.7 Genomic Variability

There are many mechanisms that can cause genomic variability in IAV. These mechanisms can be divided into those that cause a new subtype and those that cause variation within an existing subtype. Those that cause a new subtype include reassortment of surface protein genes between different strains and re-emergence of strains which had previously circulated and subsided. The term for a reassortment of the

HA and NA gene is antigenic shift. This occurs when a new HA gene, typically from another species, is gained possibly leading to increased transmissibility. (82) Causes that lead to variation within a subtype include point mutations in genes, short deletions and insertions in genes coding for the HA and NA proteins, and reassortment of internal genes. (83) Genetic drift is the term used to refer to the continuous accumulation of these nucleotide substitutions over time. (84) Most base substitutions don't cause a change in the amino acid sequence or cause a change in the fitness of the virus. A majority of the amino acids encoded in the viral genome are negatively selected. Therefore, a gene that doesn't benefit the virus will soon disappear. However, if the mutated amino acid has a positive effect on the virus it will be positively selected allowing future generations of the virus to contain that nutation. This leads to diversity of the gene pool. One example of this selection is the gradual change of HA genes which allowed immune escape. This is known as antigenic drift. (84) Antigenic drift within influenza viral lineages is usually slower in swine than in humans. (26, 85) (16)

1.7.1 Influenza A Virus Reassortment in Swine

Swine have long been considered a perfect mixing vat for the next novel pandemic virus. Although avian influenza strains have been seen in humans and it is believed that some human seasonal strains are from avian sources, differences in HA receptors prohibit avian influenza viruses from readily infecting humans. (86) Cells in the swine respiratory tract contain both α2-6-galactose linked (Human preferred) and α2,3-galactose linked (avian preferred) sialic acid receptors. (87, 88) Therefore, a pig can be infected with swine, human, and avian influenza viruses at the same time. Human

influenza virus strains have repeatedly been isolated in swine. This includes H3N2 in pigs in Asia, Europe, and North America. (51) (89) (53). A study published in 2000, found an isolate from a pig in Colorado in 1977 and a piglet from Ontario in 1997 that were wholly human influenza viruses. Fortunately, the 1997 virus from Ontario was not able to spread from pig to pig and lacked 11 of the 12 HA mutation found in other swine H3N2 viruses circulating in the same population at that time. (53) However, the risk of a pig being infected with a human and/or avian influenza strain in always present. If this occurs, reassortment between the viruses can cause a novel virus to be created. This novel virus could now have the capability to infect human cells and elude the immune system. (13) Pigs are presumed to be the species where a swine, avian, and human influenza reassortment occurs, however, this hasn't been proven. Initially, this was thought to be because swine had large amounts of both human and avian receptors. However, it has recently been found that human type receptors are more abundant than avian receptors on the tracheal epithelium of pigs. (90) One recent paper looked at the replication and transmission of H5N1, H5N2, and H5N8 avian strains in swine and found that the avian virus is poorly adapted for replication and transmission in swine. (91) Another reason that swine are implicated is because they are the only domesticated mammalian species reared in abundance that is susceptible to, and allows productive replication of, avian and human influenza viruses. (51) Reassortment, when it does happen, can take years before it is found. Genomic analysis of the H1N1pdm09 virus demonstrated that this H1N1 reassortment circulated in pigs for 10 to 17 years before it infected humans. (92) Although we cannot prove that the 2009 H1N1 pandemic virus came from swine, it is

speculated that pigs were the source since it was a reassortment of swine, avian, and human influenza viruses. (92)

1.7.2 Influenza A Virus Lineages in Swine

Influenza virus is categorized by type, subtype, and genotype. The subtype is defined by the hemagglutinin and neuraminidase glycoproteins found on the outside of the viral envelope. Although there are 16 different HA forms and 9 different NA forms, the most common ones seen in swine are H1N1, H1N2, and H3N2. (93) The genotype is found by sequencing the RNA segments and analyzing them independently on a phylogenetic tree. This will show the evolutionary lineage of each strain based on host species and geographic region of the world. (26)

The diversification of H1 in North American swine in the last century is due to the establishment of non-swine lineage IAV in swine populations. These strains have subsequently evolved through antigenic shift and drift. Prior to 1998, North American swine were exclusively infected with classical H1N1 lineage. This strain was the one discovered in swine in 1930 and was believed to be introduced to swine during the 1918 "Spanish flu" pandemic. In 1998, there was a series of outbreaks of influenza-like illness in swine in the south and midwest. The causative agent of these outbreaks was discovered to be H3N2. Further analysis of this outbreak demonstrated that there were 2 separate genotypes of H3N2 present, most likely introduced from human sources. One genotype, found in North Carolina, was a double assortment with human HA, NA and PB1 and swine NS, NP, MP, PB2, and PA segments. The other genotype, found in Minnesota, Iowa, and Texas, proved to be a triple reassortment with human HA, NA, and PB1, swine

NS, NP, and MP, and avian PB2 and PA segments. (6, 94) By the end of 1999, viruses related to the triple reassortment were widespread in the US. However, the double assortment did not spread well in swine. (94) Co-circulation of the triple reassorted or TRIG strain of H3N2 with classical swine lineages subsequently generated other strains of H1N1 and H1N2. (92, 95) Subsequent to the 2009 human H1N1 pandemic, an H1 was seen in swine that contained 6 gene segments of North American TRIG with a MP and NA of Eurasian lineage. (96) (29)

Today, there are 6 antigenically distinct H1 clusters, the H1α, H1β, H1γ, H1δ1, H1δ2, and H1N1pdm09. (97) Viruses from the classical H1N1 lineage which acquired the TRIG cassette around 1998 have evolved to form the α-, β-, and γ- clusters based on the genetic makeup of the HA gene. Starting in 2005, H1 subtypes that had HA strains most similar to human seasonal H1 viruses form the δ- cluster. (96) The HA from the δ-cluster appears to have emerged from 2 separate introductions of human seasonal HA in 2003 and 2005. (98) These two introductions, from H1N2 and H1N1 viruses, led to two distinct subclusters, δ1 and δ2, respectively. (99) The α-, β-clusters have not been isolated as often in US swine now, having been overshadowed by the more dominant γ- and δ-clusters. (98) The presence of "human-like" residues in the receptor binding pocket of HA on some δ- cluster viruses isolated in 2008 demonstrate that although these viruses have been circulating in pigs for years, they can still retain human-adapted phenotypes. This adaption allows the possibility of novel reassortment viruses to spill back into the human population. (98, 99)

From 1930 to the mid 1990's North American pigs were almost exclusively infected by "classical" H1N1. Seroprevalence reports for over half of that time estimated positive rates from 28-51%. There was evidence that H3 from human seasonal influenza periodically infected swine during that time as well, but it failed to establish a sustained transmission pathway, and therefore, was only seen in about 1% of pigs. (100) (98) However, a dramatic shift occurred in 1997 where seropositivity to H3 jumped to 8%. (6) Now seropositivity for H3N2 is as high as 28%. (101) Genetic evaluation of H3N2 subtypes in swine since 1998 show at least three separate introductions of human H3 have become established in swine. These introductions have led to the phylogenetic clusters I, II, and III. Cluster III became dominant in North America and has continued to evolve into cluster IV. (98)

The genotypes of IAV are decided based on the sequences of each of the 8 segments. Plotting each of the individual segments on a phylogenetic tree can help to show how each strain evolved from previous strains. When North American internal segments (PB2, PB1, PA, NP, MP, and NS) are plotted on a tree they break out into two major clades; these clades are categorized into TRIG or pdm09 lineage. The neuraminidase protein can be broken down into 2 commonly occurring classifications in swine, N1 and N2. All N1 circulating in the US swine population is of a classical origin. There are two N2 lineages from separate introductions in 1998 and 2002. (97) (101)

1.7.2.1 Common Swine Influenza A virus genotypes

Since swine carry many different strains of IAV, and because they can serve as a mixing vat for influenza viruses, it is important that we understand the patterns of genetic

and antigenic diversity in swine to assess potential risks to both human and swine populations. Presently, seven distinct HA clusters co-circulate in the US swine population H1α, H1β, H1γ, H1δ1, H1δ2, H1N1pdm09, and H3 cluster IV. (98) However, recently there has been an increase in novel human-like H3's which cluster with human seasonal H3's from 2010-2011. (102) By 2011, at least 49 incidences of H1N1pdm09 and at least 23 incidences of H3 and H1 seasonal virus transmission from humans to swine have been identified. (103) These introductions have led to reassortment events. The reassortment of H3N2-TRIG viruses with H1N1pdm09 viruses (rH3N2p) are of particular concern. (104) In 2010, phylogenetic analysis revealed at least 10 different genotypes of rH3N2p circulating in US swine. (97) By 2016, this number had increased to 44. (88) Genotype 1, (H3-G1) was detected more frequently in swine than any other genotype at that time. (97, 101) It was also the most spatially diverse having been found in 6 different states. (97) H3-G4 was the second most frequent genotype in 2010. In that same study, H3-G6 and H3-G9 were the only other genotypes that exhibited sustained transmission in swine populations. All the other genotypes did not have sustained transmission and appeared to have evolved through multiple independent reassortment events. (97) Presently, studies still show H3-G1 as the predominant genotype with a prevalence of around 32%. (88)

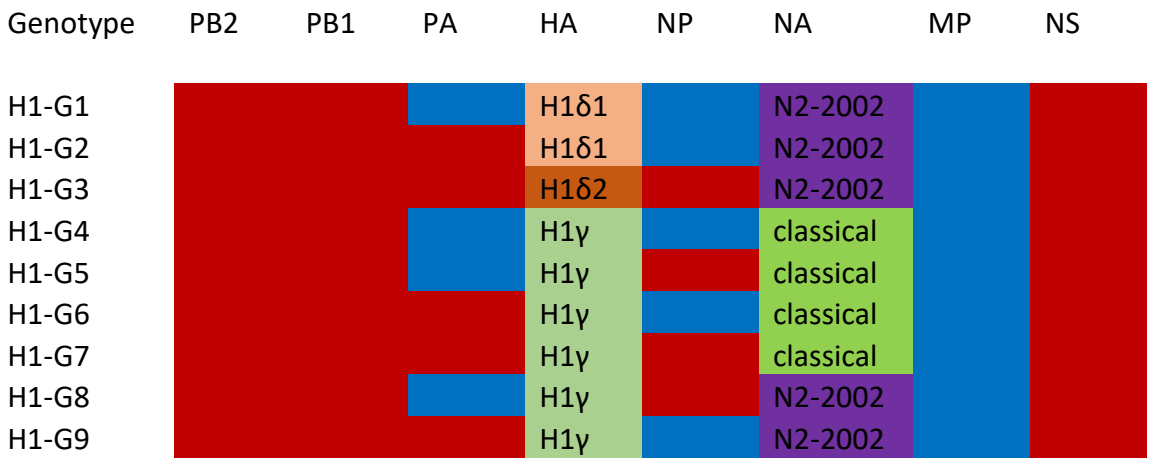
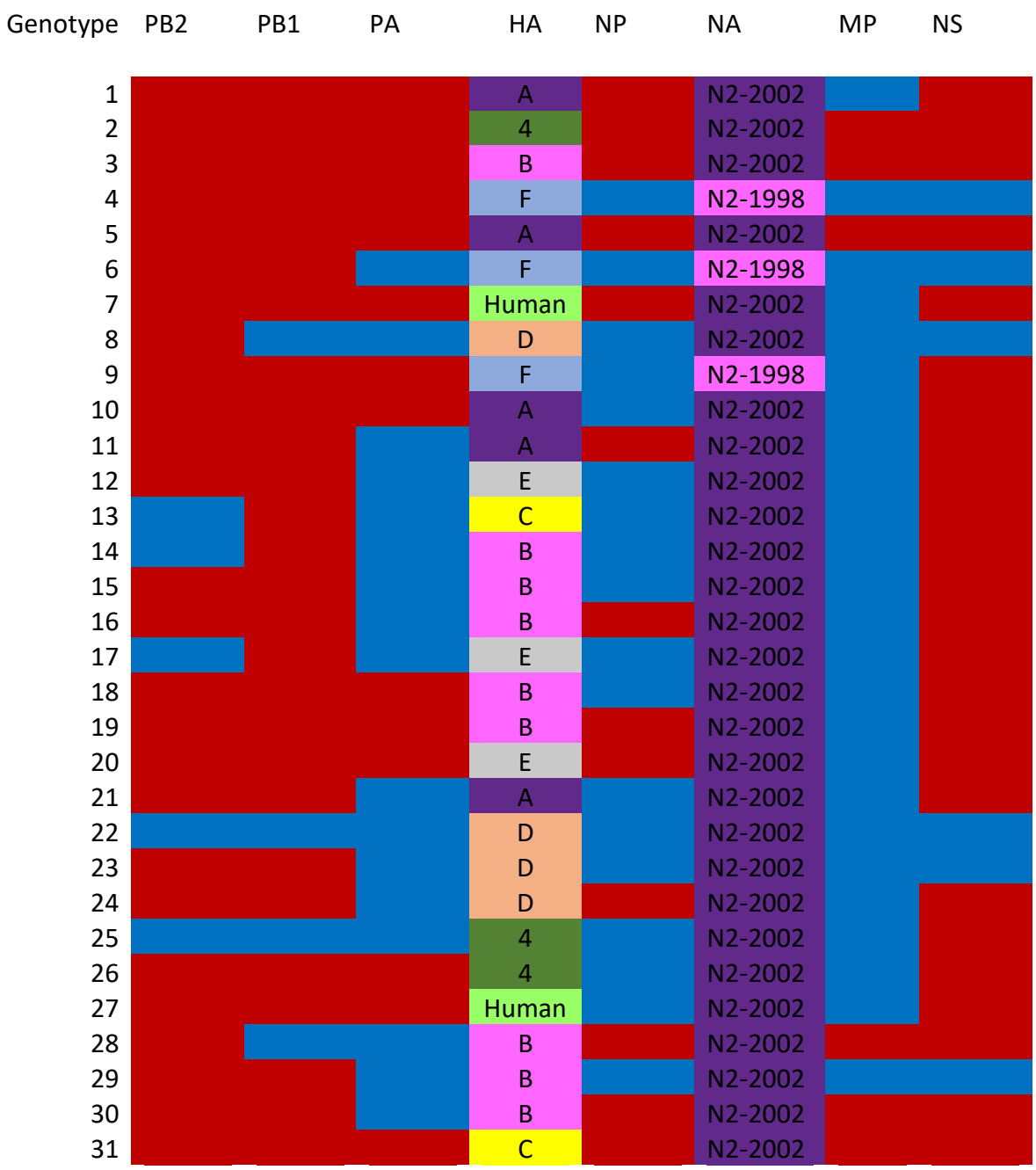


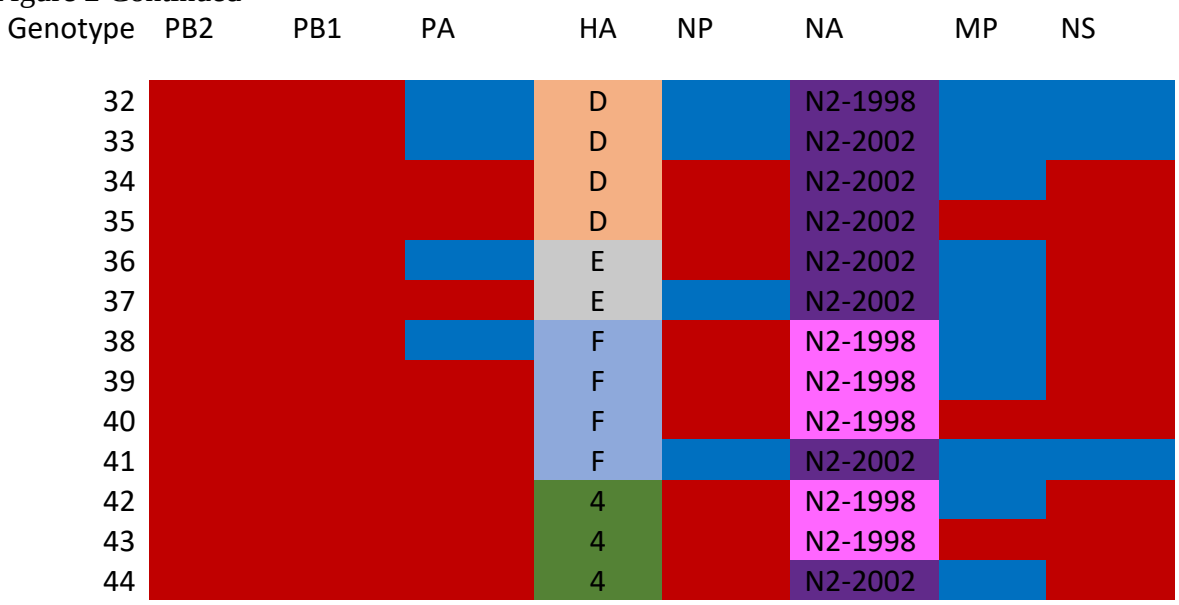
Figure 1: H1 influenza A virus genotypes identified in exhibition swine in 2013. The internal segments (PB2, PB1, PA, NP, MP, and NS) of H1N1pdm09 origin are indicated in blue and TRIG origin segments are indicated in red. The H1 segments are classified as H1δ1 (peach), H1δ2 (orange), and H1γ (green). The neuraminidase (NA) segments are classified as classical N1 (green) or N2-2002 (purple). (9)



Continued

Figure 2: H3N2 influenza A virus genotypes identified in US swine in 2016. The internal segments (PB2, PB1, PA, NP, MP, and NS) of H1N1pdm09 origin are indicated in blue and TRIG origin segments are indicated in red. The H3 segments are classified as cluster IV (green), cluster IV sub-cluster A-F (A: purple; B: pink; C: yellow; D: peach; E: grey; F: stone), and human origin (neon green). The neuraminidase (NA) segments are classified as N2-1998 lineage (pink) or N2-2002 lineage (purple). (87)

Figure 2 Continued



1.8 Past Cases of Variant Influenza

During the early years of influenza studies, the transmission of swine influenza to humans was not seriously considered. No extensive studies were done to look at influenza in persons with swine contact. Since a large percentage of the population had antibodies to the 1918 swine influenza strain, there wouldn't be a generation that was free of 1918 antibodies until the 1950's. (105)

A literature review of human cases of swine influenza from 1958 to 2005 produced 37 civilian cases. Of the civilian cases, 61% of the patients had swine exposure including living or working on a swine farm, working with sick laboratory pigs, county fair visitors or attendants, and abattoir workers. The median age of the patients was 24.5 years. Twenty of the civilian cases were described as healthy. Seven cases were known to have an underlying medical condition. All six of the fatal cases died of pneumonia and 2 of the fatal cases were reported as previously healthy. H1N1 was the most common subtype found but H3N2v was found in 4 cases. (106)

1.8.1 H1N1v

In 1961, the WHO had an unpublished report of 5 swine strains of influenza being isolated from humans However, there was no epidemiologic links to swine. (105) In 1974, a 13-year old boy with Hodgkin's disease died of respiratory failure due to pneumonia and underlying Hodgkin's disease. Postmortem viral isolation discovered that the boy was infected with an influenza A virus that was antigenically identical to a swine isolate. Epidemiologic investigation found that the boy lived on a farm with swine. Influenza A virus was found in some of the pigs on the farm. (107) This was the first-

time swine influenza was isolated from a human in the United States. In October 1975, an 8-year old boy living on a swine farm was infected with swine influenza. Investigations proved that the in addition to the boy, 5 family members who had close contact to swine on the farm, had antibodies to swine influenza virus. The swine on their farm were also infected with swine influenza virus. It was concluded that the boy's illness and the seropositivity of the family members resulted from direct contact with swine influenza shed from sick pigs. (108)

In 1976, 13 military trainees at Fort Dix were hospitalized with acute respiratory disease associated with swine influenza and there was 1 death. Although it is believed around 230 soldiers were infected, the outbreak only lasted 3 weeks. There was no epidemiologic evidence of swine exposure and it was believed that the virus was brought into the camp by a recent recruit. (109, 110)

The first confirmed case of swine influenza in 1976 outside of Fort Dix occurred in Wisconsin. The patient was a 23- year old male who worked on a pig farm. Surveillance of the pigs at the farm where he worked showed that there were pigs infected with influenza at the same time as the patient. A second case in Wisconsin occurred in a 13-year old boy who lived on a swine farm with sick pigs. (111) Serum from contacts of the child found antibodies to swine influenza in a classmate without swine contact. This is the first strong evidence of person-to-person transmission outside of Fort Dix. (105) In 1979, Dr. Easterday conclusively showed that swine influenza had been transmitted to farm personnel. Farm workers as well as other occupations that had

close contact with swine were found to have a higher antibody titer level to swine influenza. (112)

The first cases of swine influenza associated with exhibition swine occurred in 1979. In February of that year, a 20-year old college student was hospitalized with fever and influenza-like illness. It was found that the week before illness onset, he served as a swine barn attendant at a major livestock show. He slept in the barn and had contact with over 2000 pigs, one of which died of unknown causes. No other pigs appeared sick. Comparison of his viral proteins indicated that it was of swine origin. In 1980, a 6-year old boy tested positive for swine origin influenza. Although he did not have direct contact with swine, he did visit the swine area of a regional livestock show 2 days before symptom onset. (113) In 1988, a 32- year old women who was 36 weeks pregnant was admitted to the hospital for pneumonia. She died 7 days later. Post-mortem testing discovered that she was infected with influenza A virus. RNA fingerprinting and partial RNA sequencing of 7 of the 8 segments indicated that the genome of her isolate was similar to that of enzootic swine influenza. Epidemiologic investigation of her exposures discovered that she had attended a pig barn at a county fair four days before the onset of symptoms. Veterinarians at the fair indicated that there was ILI present in some pigs at the fair. (114) (115) In the last decade we have seen a decrease in H1N1v case with only 20 being reported since 2005. (25)

1.8.2 H3N2v

The first human case of H3N2v was reported in The Netherlands in 1993 but H3N2v wasn't significant in the United States until 2011. (116) Since 2005, there have

been 431 H3N2v and 11 H1N2v cases in the United States. (25) Ninety-eight percent of the H3N2v cases were reported after 2011. (117) In 2011, there were 12 cases of H3N2v. Six of those cases had exposure to swine. (118) In August of 2011, 2 children under the age of 5, from 2 different states, were diagnosed with H3N2v virus. One child had direct contact with swine at a county fair and the other child's caregiver had direct contact with swine. Both patients had respiratory specimens tested and they showed a reassortment of swine H3N2 currently circulating in the swine population and the 2009 influenza A (H1N1) virus. (119) In 2012 there were 306 cases of H3N2v in 10 states. The median age of patients was 7 years old. There were 16 patients hospitalized and 1 patient died. Ninety-three percent of patients reported attendance at an agricultural fair and 95% reported swine contact. (120) All 320 human cases in 2011-2012 were found to caused by the H3N2-TRIG/H1N1pdm09 (genotype H3-G1). H3-G1 viruses were known to have been circulating in swine for at least 8 months before they were isolated in humans. It's proliferation in swine coincided with the increased human cases. However, it was found that although all the human cases were within the H3-G1 genotype, the human cases clustered differently on the N2 phylogeny. This suggests that these human transmission events were independent. Human to human transmission was very limited with transmission cases making up less than 5 isolates in the study. (97)

Between 2013 and 2017, there have been 102 cases of H3N2v influenza. Of these cases 100 had direct swine exposure at a county fair or agricultural exhibition within a week of symptom onset. (121) (122) (123-126) Phylogenetic analysis of the 18 H3N2v strains from 2016 found that the variant strains were nearly identical to the strains found

in the exhibition swine. The variant strains were also found to come from 2 different clades showing that they emerged separately from each other. (59)

Chapter 2. The Study

This chapter was prepared as a manuscript for submission to Emerging Infectious Diseases journal.

2.1 Introduction

As swine can be infected with avian-lineage and human-lineage influenza A viruses (IAV), in addition to endemic swine-lineage IAVs, they serve as a potential source of reassorted novel IAVs that are capable of infecting humans. (87, 88) This risk became apparent with the 2009 H1N1 influenza pandemic. Subsequently, in 2011-2012 there was an outbreak of H3N2 variant cases in humans associated with swine exposures. Animal-linage IAVs that infect humans are referred to as variant IAV, which are designated with a 'v' after the subtype. (127). Since 2005, there have been 431 variant H3N2 (H3N2v) cases reported in the United States. (117) Of the 423 cases reported during or after 2011, 397 (94%) reported swine exposure, and a majority of those exposures were to exhibition swine at agricultural fairs. (118, 120, 121) While the number of swine to which humans can be exposed in commercial swine production is much higher than exhibition swine, access to commercial swine is limited to relatively few individuals. In contrast, agricultural fairs facilitate swine exposure to millions of individuals with varying influenza exposure histories. Since more people come into contact with live swine at agricultural exhibitions than any other setting in the United States, there is a risk of IAV transfer from infected swine to humans with varying degrees of protective immunity. (58) Understanding the patterns of IAV genomic diversity in exhibition swine populations, and how to control IAV spread within these populations, can help to protect the health of swine and the public.

Exhibition swine only make up approximately 1.5% of the United States' swine herd. Most are raised in backyards or small-farm settings and are primarily raised as agricultural projects for education or competition. (57) A unique aspect of livestock exhibitions is that animals from many source farms comingle for extended periods of time; this comingling allows for a mixing of diverse IAVs. (58) Furthermore, exhibition pigs can attend numerous agricultural exhibitions across multiple states each year, which provides repeated opportunities for intrastate and interstate spread of IAVs. (9, 56)

Influenza A virus has been found in the exhibition swine population since active surveillance began in 2009. (58) Genomic analyses of IAVs in exhibition swine from 2009-2013 indicated that new viral diversity in exhibition swine is primarily introduced from commercial swine. (9) To date, forty-four different genotypes of H3N2 have been described in the US swine population, 37 of which contain H1N1pdm09 gene segments. (88) One of the predominant genotypes, H3-G1, a H3N2 reassorted with the pdm09 M gene is the genotype that caused all 320 H3N2v cases in 2011-2012 and had a prevalence of around 32% in US swine from 2009-2016. (88, 97)

The large number of H3N2v cases seen in agricultural fair visitors since 2011 serves as a reminder that exhibition swine are a potential source of zoonotic IAV. Since IAV can be shed from apparently healthy animals, pigs who are shedding the virus may not be tested. (51) Furthermore, since many exhibition swine go to market at the conclusion of the fair, swine may not be available for sampling after human cases are discovered. In response to the increased H3N2v cases in 2012, we expanded our swine IAV surveillance program. During the course of our active surveillance from 2013-2015,

H3N2v cases were reported in or near states where we sampled. We sought to use our active surveillance data to demonstrate that there are multiple genotypes of IAV circulating in the exhibition swine population and that new genotypes are continuously emerging. Furthermore, combining our surveillance data with public health epidemiologic investigations provided proof that multiple IAV genotypes were transmitted from swine to humans at agricultural fairs over that time span. To investigate this, we performed a phylogenetic analysis using full genome sequences from 279 IAVs collected from exhibition swine in 5 states via active surveillance from 2013-2015 and whole genome sequences from 24 of the 25 H3N2v cases described during those same years.

2.2 Materials and Methods

Surveillance for IAV in exhibition swine was conducted by collecting nasal swab or nasal wipe samples from selected swine at participating agricultural fairs in Ohio, Indiana, Michigan, Iowa, Texas, Colorado, West Virginia, and Kentucky from 2013-2015. Fairs were selected based on their willingness to participate, the size of their swine shows, and their geographical and temporal locations. Samples were taken from swine regardless of whether they showed outward signs of illness. (58) These samples were placed in viral transport medium and stored at -80 °C until they were tested. The samples were screened for IAV using real-time reverse transcriptase polymerase chain reaction. (VetMAX-Gold SIV Detection Kit; Applied Biosystems, Austin, TX, USA) Those samples that were RRT-PCR positive were then inoculated onto Madin-Darby kidney

cells for viral isolation. The complete genome of representative isolates were sequenced using previously described protocols. (128)

Whole genome sequences (WGS) of all sequenced strains collected as part of the exhibition surveillance program from 2013-2015 along with WGS for contemporary swine-lineage IAVs were downloaded from Genbank. (9) The sequences from our isolates were combined with the background sequences and sequences for each gene segment were aligned separately, with independent alignments made for H1, H3, N1, and N2. Maximum likelihood trees were built using Influenza Research Database's phylogenetic tree generator. (129) We inferred maximum-likelihood trees under a general time reversible evolutionary model and assessed branch support using a bootstrap approximation with 1,000 replicates. (130) Trees were visualized and edited using MEGA7 and Geneious 9.1.8. (131, 132) Internal gene segments (PB2, PB1, PA, NP, M, and NS) were categorized as triple reassortant internal genes (Trig) or A(H1N1)pdm09 lineage (pdm). The H1 trees were assigned a clade based on the Swine H1 Clade Classification tool on the Influenza Research Database website. (133, 134) These clades were then categorized into H1δ1, H1δ2, or H1γ. (134) The H3 segments all fell within the CIV clade but were further categorized into subclades of A-F. All N1 segments were classified as classical origin. N2 segments were classified as N2-1998 or N2-2002.

Nucleotide sequences from all H3N2v cases reported during 2013-2015 were downloaded from GISAID. One Michigan case from 2013 was excluded from the analyses because it only had partial genomic sequences. The 2015 New Jersey case was missing 2 segment sequences, therefore, it was included in the trees but was not

designated a genotype. County level exposure data was obtained from the Centers for Disease Control and Prevention.

2.3 Results

Diverse reassorted genotypes were seen in exhibition swine from 2013-2015. Influenza A virus surveillance among exhibition swine occurred from 2013-2015 in 277 fair/exhibition events across the eight states. Influenza A virus was found at 66 (23.8%) of the fair/exhibition events. There were 15 exhibitions that were IAV positive more than one year. In total, there were 47 different exhibitions that were positive in 5 different states: Ohio, Indiana, Iowa, Texas, and Kentucky. Human cases of H3N2v were reported in Illinois, Indiana, Iowa, Michigan, Ohio, New Jersey, and Minnesota. There was a statistically significant decrease in the proportion of fairs with at least 1 IAV positive pig per a fair between 2014 and 2015 ; 31% (31/100; 95% CI: 22.0-40.0) in 2013, 30.14% (22/73; 95% CI: 19.6-40.1) in 2014, and 13.5% (14/104; 95% CI: 6.9-20.1) in 2015. Prevalence of IAV within fairs that had at least 1 IAV positive pig was 41.2% in 2013 (95% CI: 31.6-50.8), 34% in 2014 (95% CI: 23.1-44.9), and 26.0% in 2015 (95% CI: 17.6-34.4). There was a statistically significant decrease in the prevalence of IAV in exhibition swine every year with 14.9% (487/3276; 95% CI: 13.7-16.1) in 2013, 8.9% (209/2349; 95% CI: 7.7-10.1) in 2014 and 4.6% (134/2918; 95% CI: 3.8-5.4) in 2015. The overall prevalence for the three years was 9.7% (95% CI: 9.1-10.3).

There were 19 different genotypes found over the span of 2013-2015 (Figure 3). These genotypes included 5 antigenically distinct HA segment lineages (H1δ1, H1δ2, H1γ, H3-C4A, and H3-C4B), 3 NA lineages (Classical N1, N2-1998, and N2-2002), and

2 types of internal gene segments (TRIG and pdm09). In all years, the PB2, PB1, and NS gene segments were TRIG origin and the M segment was pdm09 origin. The fifteen previously described genotypes were assigned categories based on published nomenclature. (9, 88, 97) There was a variety of distinct genotypes seen each year ranging from 13 genotypes seen in 2013 to 7 seen in 2015. We documented four new genotypes, all of which were of the H1N2 subtype. The number of genotypes per a fair varied from 1 to 4 (Table 2). H3-G1 was the most prevalent H3N2 virus seen in 2013 (19.2%) and 2015 (57.1%) (Table 1 and Figure 4). Although there were no positive H3-G1 isolates in 2014, there were 4 other H3N2 genotypes with varying prevalences seen in the swine over the 3 years.

Transmission of swine H3N2 to humans. There were 5 different genotypes of H3N2v infecting humans. All 5 of the H3 genotypes seen in swine were associated with at least one human case over the 3-year time span. Based on epidemiologic exposure data for human cases, we sampled swine at 4 fairs where a total of 10 human cases were exposed. Three of these exhibitions had at least one positive pig and the same genotype was described in swine and humans at 2 of the positive exhibitions.

In all 3 years, the H3 genotypes that were isolated from the highest number of fairs were also genotypes that were seen in humans (Figure 3). Spearman's Rank-Order Correlation demonstrated a positive correlation between the number of fairs a genotype was found at and the number of human cases of the same genotype (spearmen $\rho = 0.6711$; p-value= 0.0023). (135) Phylogenetic analysis of H3N2v isolates from humans and IAV isolates from exhibition swine the same year demonstrated tight clustering (supplemental

figures 1-10). The hemagglutinin segments of all H3N2v isolates fell within the cluster IV in the A and B subclusters (figure 5). All H3N2v neuraminidase segments, except for a New Jersey isolate from December 2015, clustered together with swine isolates within the N2-2002 lineage.

In 2013, seventy-two percent of human cases were caused by H3-G1, which was the most prevalent H3 genotype in swine that year (table 1). Interestingly, in 2014 we did not see H3-G1 in swine. Instead we saw a predominance of H3-G18 which was unique in that it carried an HA gene from the C4B clade. The following year, there was a human case associated with another genotype (H3-G19) that contained a HA from the C4B clade.

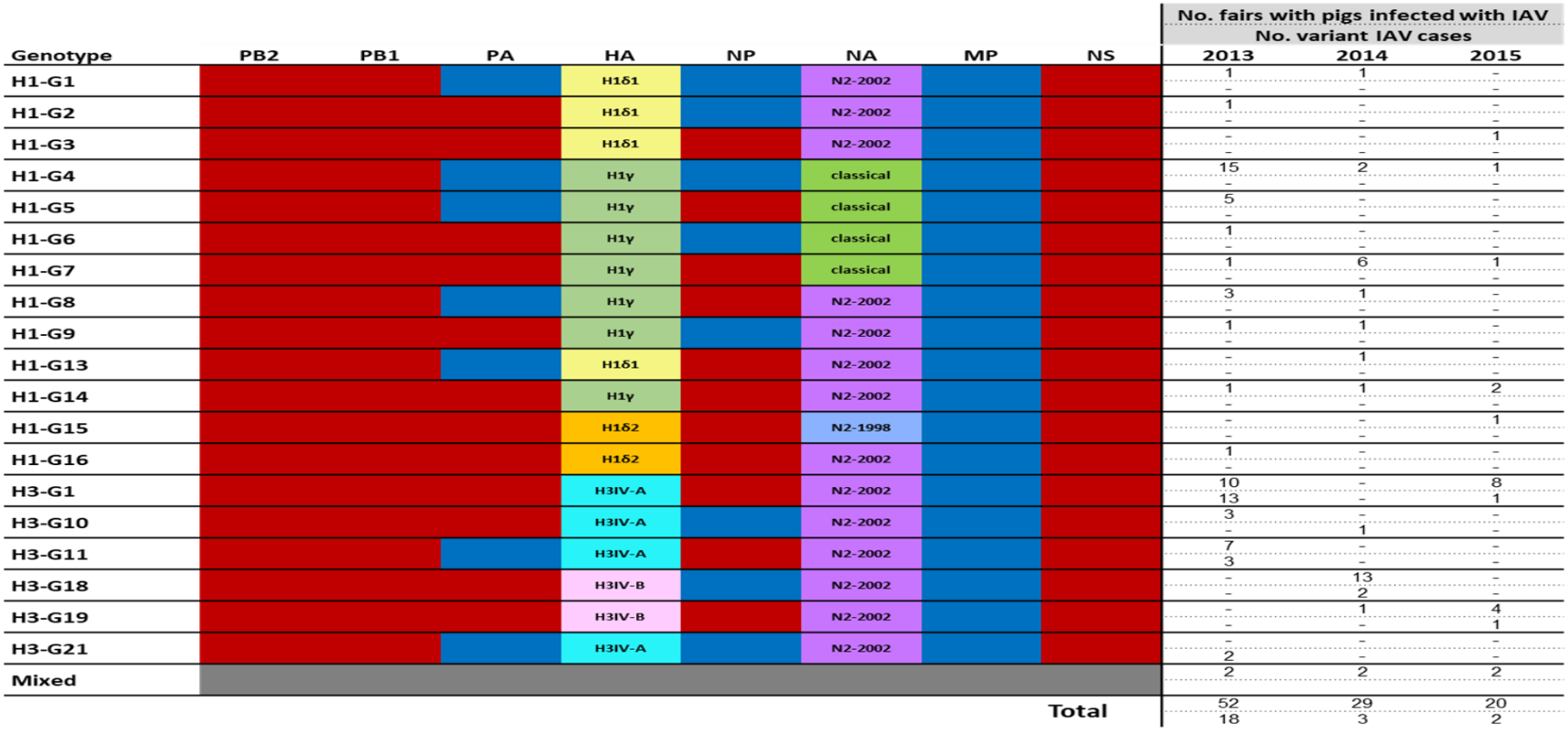


Figure 3: Nineteen influenza A virus genotypes found in exhibition swine or humans from 2013-2015. The internal segments (PB2, PB1, PA, NP, M, and NS) of H1N1pdm09 origin are indicated in blue and TRIG origin segments are indicated in red. The H1 segments are classified as H1δ1 (yellow), H1δ2 (orange), and H1γ (green). The H3 segments are classified as H3IV-A (cyan) and H3IV-B (lavender). The neuraminidase (NA) segments are classified as classical N1 (green), N2-1998 (light blue) or N2-2002 (purple). The A(H1N1)/A(H1N2) genotypes are numbered H1-G1 to H1-G16. The A(H3N2) genotypes are numbered H3-G1 to H3-G21, consistent with genotype nomenclature used previously. (9, 88)

	Positive Fairs by Year		
	2013	2014	2015
Genotype	# of fairs/31 (%)	# of fairs/22 (%)	# of fairs/14 (%)
H1-G1	1 (3.2)	1 (4.5)	--
H1-G2	1 (3.2)	--	--
H1-G3	--	--	1 (7.1)
H1-G4	15 (48.4)	2 (9.1)	1 (7.1)
H1-G5	5 (16.1)	--	--
H1-G6	1 (3.2)	--	--
H1-G7	1 (3.2)	6 (27.3)	1 (7.1)
H1-G8	3 (9.7)	1 (4.5)	--
H1-G9	1 (3.2)	1 (4.5)	--
H1-G13	--	1 (4.5)	--
H1-G14	1 (3.2)	1 (4.5)	2 (14.3)
H1-G15	--	--	1 (7.1)
H1-G16	1 (3.2)	--	--
H3-G1	10 (32.3)	--	8 (57.1)
H3-G10	3 (9.7)	--	--
H3-G11	7 (22.6)	--	--
H3-G18	--	13 (59.1)	--
H3-G19	--	1 (4.5)	4 (28.6)
H3-G21	--	--	--
mixed	2 (6.5)	2 (9.1)	2 (14.3)

Table 1: Prevalence of genotypes by fair from 2013-2014. Total number of fairs where a genotype was found divided by total number of positive fairs each year.

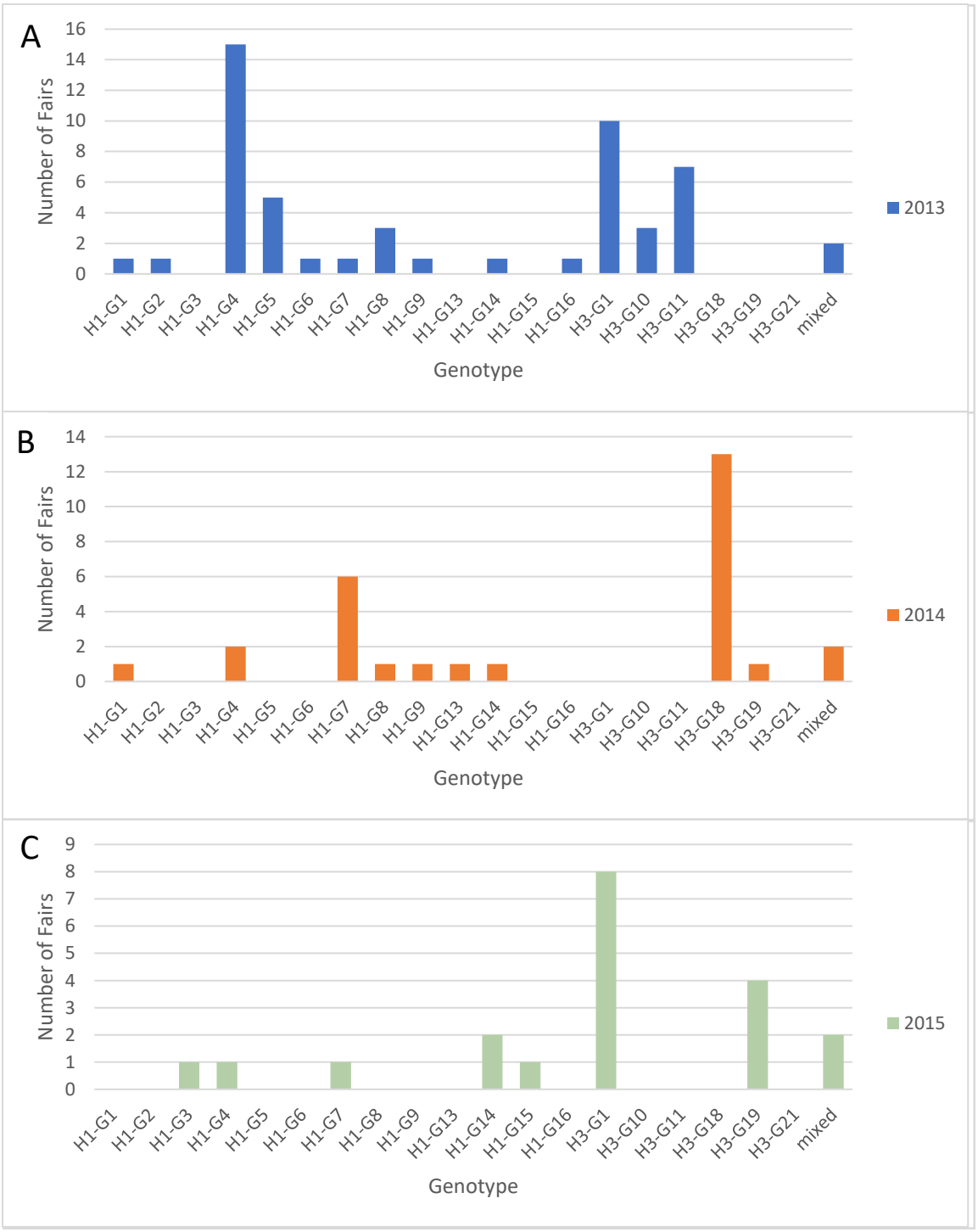


Figure 4: Count of the number of fairs where each genotype was found by year.

Exhibition ID	No. Genotypes per fair		
	2013	2014	2015
Iowa A		1	
Iowa B	1		
Indiana A	1	2	1 + mixed
Indiana B	1	1	
Indiana C	1		
Indiana D	1	1	
Indiana E			1
Indiana F		1	
Indiana G	2		
Indiana H	1		
Indiana I	5	2	
Indiana J	1	1	
Indiana K	4	2 +mixed	1
Indiana L			1
Indiana M	2 + mixed		
Indiana N	3	1	
Indiana O			1
Indiana P			2
Indiana Q		1	
Indiana R	2	1	
Indiana S			1
Indiana T			1
Indiana U		1	
Indiana V			3
Indiana W	1	1	
Indiana X		1	
Indiana Y			1
Indiana Z	1		
Indiana AA	1	1	

Exhibition ID	No. Genotypes per fair		
	2013	2014	2015
Kentucky A			1
Kentucky B		2	2 + mixed
Ohio A	1		
Ohio B	2		
Ohio C	1		
Ohio D	2	1	
Ohio E		1	
Ohio F		1	
Ohio G	1		
Ohio H			1
Ohio I	1		
Ohio J	1	1	
Ohio K	1		
Ohio L		2	
Ohio M	3 + mixed	1 +mixed	
Ohio N	1		
Ohio O	1		
Ohio P	1		
Ohio Q			1
Ohio R	1		
Texas A	4		
Texas B	1		

Table 2: Number of influenza A virus genotypes seen by exhibition each year

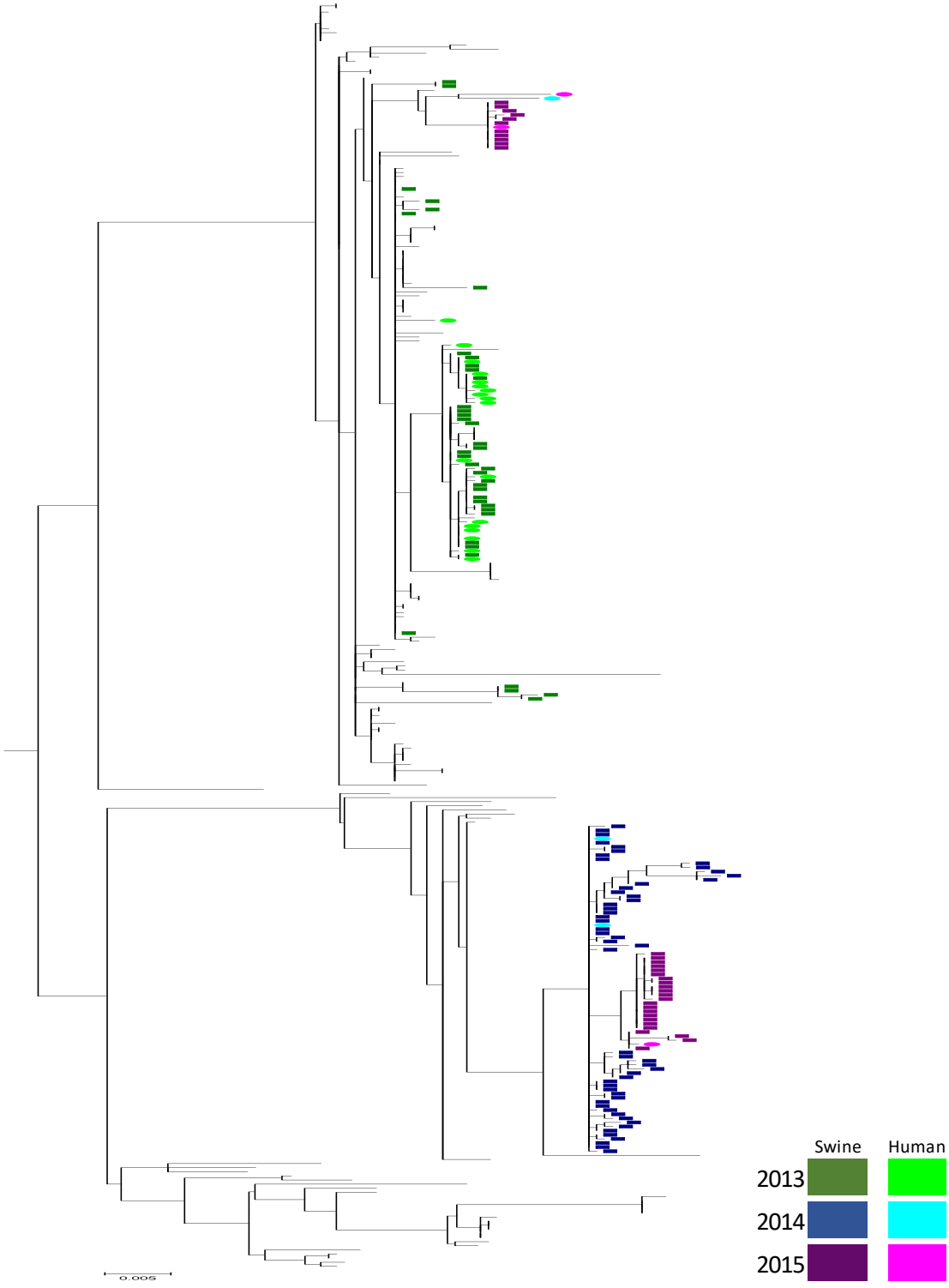


Figure 5: Expanded view of phylogenetic relationships of the HA sequences of exhibition swine-origin H3N2 and human H3N2v isolates from 2013-2015

2.4 Discussion

Identification of variant IAVs in humans with exposure to exhibition swine is a recent topic that has garnered much attention from both the scientific and mainstream press. (57, 103, 117, 119, 130, 131) Although the instances of human H3N2v cases decreased from 2013-2015, we have seen another increase in cases over the last 2 years. There is concern that the next H3N2v strain will infect a larger group of individuals or acquire the ability to transmit between humans. Since the exhibition swine population is a source of new viral diversity, studying the genotypes of IAV circulating in swine populations can help us better understand the potential risks to swine and human health from reassorted viruses. (61) Genomic analysis over a three-year time-period demonstrates that there are multiple different genotypes circulating in exhibition swine each year and these are continuously evolving from year to year. (figure 3). Pandemics arise when an influenza virus contains 2 properties. It must be antigenically unique enough to be able to avoid the population's immunity and it must be sufficiently adapted enough to easily spread from person to person. (26) The emergence of new genotypes provides a continuous source of novel IAV's to which swine and human populations might lack immunity. If a newly reassorted genotype also gains the ability to transmit between humans it could pose a pandemic threat.

Sometimes, when there is a high prevalence of an H3N2 genotype in pigs, we will see that genotype affect human populations. (96) Fortunately, most H3N2v cases in humans are not well adapted for sustained human-to-human transmission. (23) In our study, there were 6 different genotypes that zoonotically infected humans affecting

anywhere for 1 to 13 people. Although the H3-G1 genotype has affected more humans than any other genotype, there has been an increase of variant IAV from other genotypes in humans over the last several years (table 1). We found 5 different H3N2 genotypes in exhibition swine from 2013-2015 and all 5 were found in at least 1 human case. This demonstrates that reassortments of the H3-G1 genotype are also able to infect humans. Although it remains unclear why H3-G1 surged in humans in 2011-2012 and decreased the following years, explanations include immunity being carried over to the following year, implementation of prevention strategies, or lower exposure levels due to a decrease in prevalence within pig populations. (9)

Two of the new genotypes we described only differed from previously established genotypes by one segment. Although the other two new genotypes, H1-G15 and H1-G16, varied from the more established genotypes, they only differed from each other by the NA segment. The increase in genotypes supports previous studies describing continuous reassortment of H3N2 viruses with the H1N1pdm09 viruses in the swine population. (9, 60, 96) Although the H1N1pdm09 strain was introduced to the commercial swine population, it did not thrive in the population. Instead, H3N2 viruses reassorted with H1N1pdm09 segments appeared. (61) The H3-G1 genotype emerged with a pdm09 M gene, which has displayed increased replication and transmission characteristics in animal models. (136) All genotypes that we found contained the pandemic M, which is the dominant matrix protein in the commercial swine population. Additionally, as the prevalence of H3-G1 has ebbed and flowed in the exhibition swine population we have seen the emergence of other genotypes that contain other pdm09 internal genes (figure 3).

(9, 61) Their proliferation increases the probability that a novel virus will emerge with the potential to transmit among humans or cause severe infection.

The size of the exhibitions we sampled could have some bearing on how many genotypes were recovered. The extent of our sampling ranged from large national livestock expositions to small, local county fairs. The larger, regional exhibitions tended to have an average of 2.7 genotypes compared to the local fairs that only had an average of 1.5 genotypes. This proves that not only are individual animals a source of reassortment but that large regional exhibitions foster greater reassortment. Expositions that comingle multiple animals from various management backgrounds and geographical regions can be seeding genetic diversity by bringing together IAV's from different regions into one central location that might not otherwise be united. In recent history, we have seen novel zoonotic reassorted viruses coming out of live animal markets especially in Asia. (4, 132) However, we can see the same potential at swine exhibitions right here is the United States. Although we have been fortunate that the H3N2v cases have not been highly virulent, the risk that a new reassortment could have this ability is ever present.

Since multiple genotypes of swine-lineage IAV are able to infect humans, the best strategies to prevent inter-species spread is to use a multi-prong approach to decrease prevalence in swine and to halt risky behaviors in humans that encourage disease transmission. Factors that can contribute to the increased prevalence of IAV in exhibition swine populations include participation in multiple shows and allowing pigs to comingle for multiple days. (133) Since reassortment of IAV happens readily in swine populations,

the best practice is produce blanket recommendations that can help to decrease the prevalence of IAV in swine coming into the fair and decrease the intra-species spread of influenza during the exhibitions. Some of these methods have been outlined by the National Association of State Public Health Veterinarians. (http://nasphv.org/Documents/Influenza_Transmission_at_Swine_Exhibitions_2016.pdf)

Although IAV can infect any person, those individuals with compromised immune systems such as those under the age of 5, over the age of 65, pregnant, or immunosuppressed are at an increased risk of complications from infection. (134) Limiting swine contact with these individuals can help to prevent severe IAV infections. Supplying handwashing or hand sanitizer stations along with signage suggesting washing hands, could increase hand hygiene compliance, a recommendation to decrease IAV spread. Discouraging sleeping in the swine barns is another recommended control strategy. Since a large portion of those affected by H3N2v are youth exhibitors, educating youth on zoonotic disease and disease prevention is a crucial component to control.

This study was not without its limitations. Most of our samples were obtained from exhibitions within the Midwest. Expanding sampling in the other regions of the US might discover different genotypes. Additionally, representative samples taken from each fair ranged from 20 to 200 samples. It is possible that there are other genotypes that are circulating at low levels that were not picked up by our surveillance program. Finally, increasing sampling to accommodate more exhibitions within each state will help ensure more accurate prevalence data.

With an ever-increasing percentage of our population unfamiliar with food production, county fairs and agricultural exhibitions are an important component of public agricultural education. However, this education should not come at the risk of public health. Therefore, surveillance and control strategies need to be present to protect the health of the public. As we have seen an increase in H3N2v cases the last 2 years, there is growing concern that the next variant strain might be more virulent. Therefore, more research is needed to help understand and limit zoonotic transmission of IAV. Understanding how these viruses evolve and reassort can help predict and potentially prevent occurrences. Ideally, active surveillance of influenza dynamics within the exhibition swine population would inform annual predictions of the threat of zoonotic IAV. Ultimately, controlling IAV in exhibition swine should help to decrease the risk of inter-species IAV spread and help to ensure a better public health outcome.

2.5 Conclusions

Although there has been a decrease in the prevalence of IAV in exhibition swine populations from 2013 to 2015, there are still cases of H3N2v in humans attending swine exhibitions. All five H3 genotypes found in swine during those years were also found in humans. Even though the fair level prevalence of IAV significantly decreased from 2013-2015, a positive correlation was discovered between the number of fairs a genotype was found at and the number of human cases of that genotype. Additionally, large regional exhibitions were more likely to have multiple genotypes present in their pigs. Strategies to decrease the incidences of H3N2v include decreasing fair and pig IAV prevalences as well as halting risky human behaviors.

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Bibliography

1. Kaplan MM, Webster RG. The epidemiology of influenza. Scientific American. 1977 Dec;237(6):88-106.
2. Koen J. A practical method for field diagnosis of swine diseases. Am J Vet Med. 1919;14(9):468-70.
3. Shope RE. THE ETIOLOGY OF SWINE INFLUENZA. Science (New York, NY). 1931 Feb 20;73(1886):214-5.
4. Smith W, Andrewes CH, Laidlaw PP. A VIRUS OBTAINED FROM INFLUENZA PATIENTS. The Lancet. 1933 1933/07/08/;222(5732):66-8.
5. Wan X-F, Dong L, Lan Y, Long L-P, Xu C, Zou S, et al. Indications that Live Poultry Markets Are a Major Source of Human H5N1 Influenza Virus Infection in China. Journal of virology. 2011 05/31/received

09/22/accepted;85(24):13432-8.
6. Zhou NN, Senne DA, Landgraf JS, Swenson SL, Erickson G, Rossow K, et al. Genetic reassortment of avian, swine, and human influenza A viruses in American pigs. Journal of virology. 1999 Oct;73(10):8851-6.
7. Viruses ICoTo. Orthomyxoviridae. 2016 [cited; Available from: https://talk.ictvonline.org/ictv-reports/ictv_9th_report/negative-sense-rna-viruses-2011/w/negrna_viruses/209/orthomyxoviridae
8. McDonald SM, Nelson MI, Turner PE, Patton JT. Reassortment in segmented RNA viruses: mechanisms and outcomes. Nature reviews Microbiology. 2016 05/23;14(7):448-60.
9. Nelson MI, Wentworth DE, Das SR, Sreevatsan S, Killian ML, Nolting JM, et al. Evolutionary Dynamics of Influenza A Viruses in US Exhibition Swine. The Journal of infectious diseases. 2016 Jan 15;213(2):173-82.
10. Calisher CH. Swine flu. Croatian medical journal. 2009 Aug;50(4):412-5.
11. Zheng W, Tao YJ. Structure and assembly of the influenza A virus ribonucleoprotein complex. FEBS Letters. 2013;587(8):1206-14.
12. Holland J, Spindler K, Horodyski F, Grabau E, Nichol S, VandePol S. Rapid Evolution of RNA Genomes. Science (New York, NY). 1982;215(4540):1577-85.
13. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. Microbiological reviews. 1992 Mar;56(1):152-79.
14. Buonagurio DA, Nakada S, Parvin JD, Krystal M, Palese P, Fitch WM. Evolution of human influenza A viruses over 50 years: rapid, uniform rate of change in NS gene. Science (New York, NY). 1986 May 23;232(4753):980-2.

15. Parrish CR, Murcia PR, Holmes EC. Influenza virus reservoirs and intermediate hosts: dogs, horses, and new possibilities for influenza virus exposure of humans. Journal of virology. 2015 Mar;89(6):2990-4.
16. Van Reeth K. Avian and swine influenza viruses: our current understanding of the zoonotic risk. Veterinary research. 2007 Mar-Apr;38(2):243-60.
17. Potter CW. A history of influenza. Journal of applied microbiology. 2001 Oct;91(4):572-9.
18. Taubenberger JK, Morens DM. 1918 Influenza: the Mother of All Pandemics. Emerging infectious diseases. 2006;12(1):15-22.
19. Klepser ME. Socioeconomic Impact of Seasonal (Epidemic) Influenza and the Role of Over-the-Counter Medicines. Drugs. 2014 08/23;74(13):1467-79.
20. Prevention CfDCa. Estimated Influenza Illnesses, Medical Visits, Hospitalizations, and Deaths Averted by Vaccination in the United States. 2017 [cited; Available from: https://www.cdc.gov/flu/about/disease/2015-16.htm
21. Dushoff J, Plotkin JB, Viboud C, Earn DJ, Simonsen L. Mortality due to influenza in the United States--an annualized regression approach using multiple-cause mortality data. American journal of epidemiology. 2006 Jan 15;163(2):181-7.
22. Molinari NA, Ortega-Sanchez IR, Messonnier ML, Thompson WW, Wortley PM, Weintraub E, et al. The annual impact of seasonal influenza in the US: measuring disease burden and costs. Vaccine. 2007 Jun 28;25(27):5086-96.
23. McLaughlin JM, McGinnis JJ, Tan L, Mercatante A, Fortuna J. Estimated Human and Economic Burden of Four Major Adult Vaccine-Preventable Diseases in the United States, 2013. The Journal of Primary Prevention. 2015 06/02;36(4):259-73.
24. Centers for Disease Control and Prevention NCfIaRDN. Variant Influenza Viruses: Background and CDC Risk Assessment and Reporting. September 12, 2016 [cited; Available from: https://www.cdc.gov/flu/swineflu/variant.htm
25. Centers for Disease Control and Prevention NCfIaRDN. Reported Infections with Variant Influenza Viruses in the United States since 2005. September 6, 2017 [cited 2017; Available from: https://www.cdc.gov/flu/swineflu/variant-cases-us.htm#table-infections
26. Zimmerman J, Karriker L, Ramirez A, Schwartz K, Stevenson G. Diseases of Swine. Hoboken, UNITED STATES: John Wiley & Sons, Incorporated; 2012.
27. Webby RJ, Swenson SL, Krauss SL, Gerrish PJ, Goyal SM, Webster RG. Evolution of swine H3N2 influenza viruses in the United States. Journal of virology. 2000 Sep;74(18):8243-51.
28. Hay AJ, Gregory V, Douglas AR, Lin YP. The evolution of human influenza viruses. Philosophical transactions of the Royal Society of London Series B, Biological sciences. 2001 Dec 29;356(1416):1861-70.
29. Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, et al. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science (New York, NY). 2009 Jul 10;325(5937):197-201.
30. Kilbourne ED. Influenza pandemics of the 20th century. Emerging infectious diseases. 2006 Jan;12(1):9-14.

31. Smith GJ, Bahl J, Vijaykrishna D, Zhang J, Poon LL, Chen H, et al. Dating the emergence of pandemic influenza viruses. Proceedings of the National Academy of Sciences of the United States of America. 2009 Jul 14;106(28):11709-12.
32. Guan Y, Vijaykrishna D, Bahl J, Zhu H, Wang J, Smith GJ. The emergence of pandemic influenza viruses. Protein & cell. 2010 Jan;1(1):9-13.
33. Kawaoka Y, Krauss S, Webster RG. Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. Journal of virology. 1989 Nov;63(11):4603-8.
34. Kundin WD. Hong Kong A-2 influenza virus infection among swine during a human epidemic in Taiwan. Nature. 1970 Nov 28;228(5274):857.
35. Wallace GD, Kissling RE. Studies in swine of Asian influenza virus. Bulletin of the World Health Organization. 1959;20(2-3):455-63.
36. Monto AS, Gravenstein S, Elliott M, Colopy M, Schweinle J. Clinical signs and symptoms predicting influenza infection. Archives of Internal Medicine. 2000;160(21):3243-7.
37. Organization WH. WHO guidelines for the collection of human specimens for laboratory diagnosis of avian influenza infection. January 12, 2005 [cited; Available from: http://www.who.int/influenza/human_animal_interface/virology_laboratories_and_vaccines/guidelines_collection_h5n1_humans/en/
38. Services USDoHaH. Influenza Specimen Collection. In: Prevention CfDCa, editor.
39. Prevention CfDCa. Flu Activity & Surveillance. October 21, 2015 [cited October 13, 2017]; Available from: Flu Activity & Surveillance
40. Organization WH. International Health Regulations. Third Edition ed; 2016. p. 84.
41. WHO. WHO manual on animal influenza diagnosis and surveillance. 2002.
42. Centers for Disease Control and Prevention NCfIaRDN. Overview of Influenza Surveillance in the United States. October 13, 2017 [cited; Available from: https://www.cdc.gov/flu/weekly/overview.htm
43. Prevention CfDCa. The National Respiratory and Enteric Virus Surveillance System (NREVSS). October 5, 2017 [cited; Available from: https://www.cdc.gov/surveillance/nrevss/index.html
44. Yih WK, Cocoros NM, Crockett M, Klompas M, Kruskal BA, Kulldorff M, et al. Automated Influenza-Like Illness Reporting—An Efficient Adjunct to Traditional Sentinel Surveillance. Public Health Reports. 2014 Jan-Feb;129(1):55-63.
45. Chaves SS, Lynfield R, Lindegren ML, Bresee J, Finelli L. The US Influenza Hospitalization Surveillance Network. Emerging infectious diseases. 2015 Sep;21(9):1543-50.
46. Centers for Disease Control and Prevention NCfIaRDN. National Center for Health Statistics Mortality Surveillance Data. October 7, 2016 [cited; Available from: https://www.cdc.gov/flu/weekly/nchs.htm

47. Kothalawala H, Toussaint MJ, Gruys E. An overview of swine influenza. The Veterinary quarterly. 2006 Jun;28(2):46-53.
48. Pomorska-Mol M, Kwit K, Pejsak Z, Markowska-Daniel I. Analysis of the acute-phase protein response in pigs to clinical and subclinical infection with H3N2 swine influenza virus. Influenza and other respiratory viruses. 2014 Mar;8(2):228-34.
49. Pomorska-Mol M, Markowska-Daniel I, Pejsak Z. Acute phase protein response during subclinical infection of pigs with H1N1 swine influenza virus. Veterinary microbiology. 2012 Oct 12;159(3-4):499-503.
50. Busquets N, Segales J, Cordoba L, Mussa T, Crisci E, Martin-Valls GE, et al. Experimental infection with H1N1 European swine influenza virus protects pigs from an infection with the 2009 pandemic H1N1 human influenza virus. Veterinary research. 2010 Sep-Oct;41(5):74.
51. Brown IH. The epidemiology and evolution of influenza viruses in pigs. Veterinary microbiology. 2000 5/22/;74(1–2):29-46.
52. Blaskovic D, Jamrichova O, Rathova V, Kaplan MM, Kociskova D. Experimental infection of weanling pigs with A-swine influenza virus. 2. The shedding of virus by infected animals. Bulletin of the World Health Organization. 1970;42(5):767-70.
53. Karasin AI, Schutten MM, Cooper LA, Smith CB, Subbarao K, Anderson GA, et al. Genetic characterization of H3N2 influenza viruses isolated from pigs in North America, 1977-1999: evidence for wholly human and reassortant virus genotypes. Virus research. 2000 Jun;68(1):71-85.
54. Nelson MI, Lemey P, Tan Y, Vincent A, Lam TT, Detmer S, et al. Spatial dynamics of human-origin H1 influenza A virus in North American swine. PLoS pathogens. 2011 Jun;7(6):e1002077.
55. Lewis NS, Russell CA, Langat P, Anderson TK, Berger K, Bielejec F, et al. The global antigenic diversity of swine influenza A viruses. eLife. 2016 Apr 15;5:e12217.
56. Bliss N, Stull JW, Moeller SJ, Rajala-Schultz PJ, Bowman AS. Movement patterns of exhibition swine and associations of influenza A virus infection with swine management practices. Journal of the American Veterinary Medical Association. 2017 Sep 15;251(6):706-13.
57. Nelson MI, Wentworth DE, Das SR, Sreevatsan S, Killian ML, Nolting JM, et al. Evolutionary Dynamics of Influenza A Viruses in US Exhibition Swine. The Journal of infectious diseases. 2016 08/04

03/25/received

06/15/accepted;213(2):173-82.
58. Bowman AS, Nolting JM, Nelson SW, Slemons RD. Subclinical influenza virus A infections in pigs exhibited at agricultural fairs, Ohio, USA, 2009-2011. Emerging infectious diseases. 2012 Dec;18(12):1945-50.
59. Bowman AS, Walia RR, Nolting JM, Vincent AL, Killian ML, Zentkovich MM, et al. Influenza A(H3N2) Virus in Swine at Agricultural Fairs and Transmission to Humans, Michigan and Ohio, USA, 2016. Emerging infectious diseases. 2017 Sep;23(9):1551-5.

60. Bowman AS, Workman JD, Nolting JM, Nelson SW, Slemons RD. Exploration of risk factors contributing to the presence of influenza A virus in swine at agricultural fairs. Emerging microbes & infections. 2014 Jan;3(1):e5.
61. Nelson MI, Stucker KM, Schobel SA, Trovão NS, Das SR, Dugan VG, et al. Introduction, Evolution, and Dissemination of Influenza A Viruses in Exhibition Swine in the United States during 2009 to 2013. Journal of virology. 2016 11/14

07/21/received

09/21/accepted;90(23):10963-71.
62. Service USDoAAaPHI. USDA Swine Influenza Surveillance Update. July 31, 2015 [cited October 13, 2017]; Available from: https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/animal-disease-information/swine-disease-information/CT_Siv_surv_manual
63. Detmer SE, Patnayak DP, Jiang Y, Gramer MR, Goyal SM. Detection of Influenza A virus in porcine oral fluid samples. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc. 2011 Mar;23(2):241-7.
64. Goodell CK, Prickett J, Kittawornrat A, Zhou F, Rauh R, Nelson W, et al. Probability of detecting influenza A virus subtypes H1N1 and H3N2 in individual pig nasal swabs and pen-based oral fluid specimens over time. Veterinary microbiology. 2013 Oct 25;166(3-4):450-60.
65. Esposito S, Molteni CG, Daleno C, Valzano A, Cesati L, Gualtieri L, et al. Comparison of nasopharyngeal nylon flocked swabs with universal transport medium and rayon-bud swabs with a sponge reservoir of viral transport medium in the diagnosis of paediatric influenza. Journal of medical microbiology. 2010 Jan;59(Pt 1):96-9.
66. Fereidouni SR, Globig A, Starick E, Harder TC. Effect of swab matrix, storage time, and temperature on detection of avian influenza virus RNA in swab samples. Avian diseases. 2012 Dec;56(4 Suppl):955-8.
67. Spackman E, Pedersen JC, McKinley ET, Gelb J, Jr. Optimal specimen collection and transport methods for the detection of avian influenza virus and Newcastle disease virus. BMC veterinary research. 2013;9:35.
68. Romagosa A, Gramer M, Joo HS, Torremorell M. Sensitivity of oral fluids for detecting influenza A virus in populations of vaccinated and non-vaccinated pigs. Influenza and other respiratory viruses. 2012 Mar;6(2):110-8.
69. Dobesh K, Waddell J, Polson D. Use of buffered Swiffer cloth for collection of PRRSv from the snout and face of growing pigs. 2012 AASV Annual Meeting Proceedings. 2012.
70. Edwards JL, Nelson SW, Workman JD, Slemons RD, Szablewski CM, Nolting JM, et al. Utility of snout wipe samples for influenza A virus surveillance in exhibition swine populations. Influenza and other respiratory viruses. 2014 Sep;8(5):574-9.
71. Kenney K, Polson D. Validation of Swiffer cloth-origin neutralizing broth samples for detection of PRRS virus in the environment. 2011 AASV Annual Meeting. 2011.

72. Zewde BM, Robbins R, Abley MJ, House B, Morrow WE, Gebreyes WA. Comparison of Swiffer wipes and conventional drag swab methods for the recovery of Salmonella in swine production systems. Journal of food protection. 2009 Jan;72(1):142-6.
73. Landolt GA, Karasin AI, Hofer C, Mahaney J, Svaren J, Olsen CW. Use of real-time reverse transcriptase polymerase chain reaction assay and cell culture methods for detection of swine influenza A viruses. American journal of veterinary research. 2005 Jan;66(1):119-24.
74. Health WOfA. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. 2017.
75. Watzinger F, Ebner K, Lion T. Detection and monitoring of virus infections by real-time PCR. Molecular aspects of medicine. 2006 Apr-Jun;27(2-3):254-98.
76. Choi YK, Goyal SM, Kang SW, Farnham MW, Joo HS. Detection and subtyping of swine influenza H1N1, H1N2 and H3N2 viruses in clinical samples using two multiplex RT-PCR assays. J Virol Methods. 2002 Apr;102(1-2):53-9.
77. Swenson SL, Vincent LL, Lute BM, Janke BH, Lechtenberg KE, Landgraf JG, et al. A comparison of diagnostic assays for the detection of type A swine influenza virus from nasal swabs and lungs. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc. 2001 Jan;13(1):36-42.
78. Clavijo A, Tresnan DB, Jolie R, Zhou EM. Comparison of embryonated chicken eggs with MDCK cell culture for the isolation of swine influenza virus. Canadian journal of veterinary research = Revue canadienne de recherche veterinaire. 2002 Apr;66(2):117-21.
79. Zhang J, Gauger PC. Isolation of swine influenza virus in cell cultures and embryonated chicken eggs. Methods in molecular biology (Clifton, NJ). 2014;1161:265-76.
80. Gaush CR, Smith TF. Replication and plaque assay of influenza virus in an established line of canine kidney cells. Applied microbiology. 1968 Apr;16(4):588-94.
81. Bowman AS, Nelson SW, Edwards JL, Hofer CC, Nolting JM, Davis IC, et al. Comparative effectiveness of isolation techniques for contemporary Influenza A virus strains circulating in exhibition swine. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc. 2013 Jan;25(1):82-90.
82. Hampson AW, Mackenzie JS. The influenza viruses. The Medical journal of Australia. 2006 Nov 20;185(10 Suppl):S39-43.
83. Palese P, Young JF. Variation of Influenza A, B, and C Viruses. Science (New York, NY). 1982;215(4539):1468-74.
84. Zell R, Scholtissek C, Ludwig S. Genetics, evolution, and the zoonotic capacity of European Swine influenza viruses. Current topics in microbiology and immunology. 2013;370:29-55.
85. Sheerar MG, Easterday BC, Hinshaw VS. Antigenic conservation of H1N1 swine influenza viruses. The Journal of general virology. 1989 Dec;70 (Pt 12):3297-303.

86. Fang R, Min Jou W, Huylebroeck D, Devos R, Fiers W. Complete structure of A/duck/Ukraine/63 influenza hemagglutinin gene: animal virus as progenitor of human H3 Hong Kong 1968 influenza hemagglutinin. Cell. 1981 Aug;25(2):315-23.
87. Ito T, Couceiro JN, Kelm S, Baum LG, Krauss S, Castrucci MR, et al. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. Journal of virology. 1998 Sep;72(9):7367-73.
88. Rajao DS, Walia RR, Campbell B, Gauger PC, Janas-Martindale A, Killian ML, et al. Reassortment between Swine H3N2 and 2009 Pandemic H1N1 in the United States Resulted in Influenza A Viruses with Diverse Genetic Constellations with Variable Virulence in Pigs. Journal of virology. 2017 Feb 15;91(4).
89. Hinshaw V, Webster R. The natural history of influenza A viruses. CRC Press, Boca Raton, Florida; 1982. p. 79-104.
90. Imai M, Kawaoka Y. The role of receptor binding specificity in interspecies transmission of influenza viruses. Current Opinion in Virology. 2012 2012/04/01/;2(2):160-7.
91. Kaplan BS, Torchetti MK, Lager KM, Webby RJ, Vincent AL. Absence of clinical disease and contact transmission of HPAI H5NX clade 2.3.4.4 from North America in experimentally infected pigs. Influenza and other respiratory viruses. 2017 Jul 08.
92. Smith GJ, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, et al. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature. 2009 Jun 25;459(7250):1122-5.
93. Vincent AL, Lager KM, Ma W, Lekcharoensuk P, Gramer MR, Loiacono C, et al. Evaluation of hemagglutinin subtype 1 swine influenza viruses from the United States. Veterinary microbiology. 2006 Dec 20;118(3-4):212-22.
94. Vincent AL, Ma W, Lager KM, Janke BH, Richt JA. Swine influenza viruses a North American perspective. Advances in virus research. 2008;72:127-54.
95. Karasin AI, Landgraf J, Swenson S, Erickson G, Goyal S, Woodruff M, et al. Genetic characterization of H1N2 influenza A viruses isolated from pigs throughout the United States. Journal of clinical microbiology. 2002 Mar;40(3):1073-9.
96. Vincent AL, Ma W, Lager KM, Gramer MR, Richt JA, Janke BH. Characterization of a newly emerged genetic cluster of H1N1 and H1N2 swine influenza virus in the United States. Virus Genes. 2009 July 14;39(2):176.
97. Kitikoon P, Nelson MI, Killian ML, Anderson TK, Koster L, Culhane MR, et al. Genotype patterns of contemporary reassorted H3N2 virus in US swine. The Journal of general virology. 2013 Jun;94(Pt 6):1236-41.
98. Lorusso A, Vincent AL, Gramer ME, Lager KM, Ciacci-Zanella JR. Contemporary epidemiology of North American lineage triple reassortant influenza A viruses in pigs. Current topics in microbiology and immunology. 2013;370:113-32.
99. Lorusso A, Vincent AL, Harland ML, Alt D, Bayles DO, Swenson SL, et al. Genetic and antigenic characterization of H1 influenza viruses from United States swine from 2008. The Journal of general virology. 2011 Apr;92(Pt 4):919-30.

100. Olsen CW. The emergence of novel swine influenza viruses in North America. Virus research. 2002 May 10;85(2):199-210.
101. Nelson MI, Vincent AL, Kitikoon P, Holmes EC, Gramer MR. Evolution of Novel Reassortant A/H3N2 Influenza Viruses in North American Swine and Humans, 2009–2011. Journal of virology. 2012 02/01/received

06/06/accepted;86(16):8872-8.
102. Rajão DS, Gauger PC, Anderson TK, Lewis NS, Abente EJ, Killian ML, et al. Novel Reassortant Human-Like H3N2 and H3N1 Influenza A Viruses Detected in Pigs Are Virulent and Antigenically Distinct from Swine Viruses Endemic to the United States. Journal of virology. 2015 08/26

06/30/received

08/19/accepted;89(22):11213-22.
103. Nelson MI, Gramer MR, Vincent AL, Holmes EC. Global transmission of influenza viruses from humans to swine. The Journal of general virology. 2012 Oct;93(Pt 10):2195-203.
104. Lindstrom S, Garten R, Balish A, Shu B, Emery S, Berman L, et al. Human infections with novel reassortant influenza A(H3N2)v viruses, United States, 2011. Emerging infectious diseases. 2012 May;18(5):834-7.
105. Dowdle WR, Hattwick MAW. Swine Influenza Virus Infections in Humans. The Journal of infectious diseases. 1977;136:S386-S9.
106. Myers KP, Olsen CW, Gray GC. Cases of Swine Influenza in Humans: A Review of the Literature. Clinical Infectious Diseases. 2007 April 15, 2007;44(8):1084-8.
107. Smith TF, Burgert EO, Jr., Dowdle WR, Noble GR, Campbell RJ, Van Scoy RE. Isolation of swine influenza virus from autopsy lung tissue of man. The New England journal of medicine. 1976 Mar 25;294(13):708-10.
108. O'Brien RJ, Noble GR, Easterday BC, Kendal AP, Shasby DM, Nelson DB, et al. Swine-like influenza virus infection in a Wisconsin farm family. The Journal of infectious diseases. 1977 Dec;136 Suppl:S390-6.
109. Gaydos JC, Top FH, Jr., Hodder RA, Russell PK. Swine influenza a outbreak, Fort Dix, New Jersey, 1976. Emerging infectious diseases. 2006 Jan;12(1):23-8.
110. Top FH, Jr., Russell PK. Swine influenza A at Fort Dix, New Jersey (January-February 1976). IV. Summary and speculation. The Journal of infectious diseases. 1977 Dec;136 Suppl:S376-80.
111. Control CfD. Influenza-Wisconsin. Morbidity and Mortality Weekly Report. 1976;25:402.
112. Easterday BC. The epidemiology and ecology of swine influenza as a zoonotic disease. Comparative Immunology, Microbiology and Infectious Diseases. 1980 1980/01/01;3(1):105-9.
113. Dacso CC, Couch RB, Six HR, Young JF, Quarles JM, Kasel JA. Sporadic occurrence of zoonotic swine influenza virus infections. Journal of clinical microbiology. 1984 Oct;20(4):833-5.

114. Human infection with swine influenza virus--Wisconsin. MMWR Morbidity and mortality weekly report. 1988 Nov 04;37(43):661-3.
115. Rota PA, Rocha EP, Harmon MW, Hinshaw VS, Sheerar MG, Kawaoka Y, et al. Laboratory characterization of a swine influenza virus isolated from a fatal case of human influenza. Journal of clinical microbiology. 1989 Jun;27(6):1413-6.
116. Claas EC, Kawaoka Y, de Jong JC, Masurel N, Webster RG. Infection of children with avian-human reassortant influenza virus from pigs in Europe. Virology. 1994 Oct;204(1):453-7.
117. Prevention CfDCa. Case Count: Detected U.S. Human Infections with H3N2v by State since August 2011. 2017 [cited 2017 october 21, 2017]; Available from: https://www.cdc.gov/flu/swineflu/h3n2v-case-count.htm
118. Evaluation of rapid influenza diagnostic tests for influenza A (H3N2)v virus and updated case count--United States, 2012. MMWR Morbidity and mortality weekly report. 2012 Aug 17;61(32):619-21.
119. Swine-origin influenza A (H3N2) virus infection in two children--Indiana and Pennsylvania, July-August 2011. MMWR Morbidity and mortality weekly report. 2011 Sep 09;60(35):1213-5.
120. Jhung MA, Epperson S, Biggerstaff M, Allen D, Balish A, Barnes N, et al. Outbreak of Variant Influenza A(H3N2) Virus in the United States. Clinical Infectious Diseases. 2013 December 15, 2013;57(12):1703-12.
121. Minnesota Uo. CDC reports H3N2v case, 1st pediatric flu death of season. News Scan for October 24, 2014 2014 October 24, 2014 [cited 2017; Available from: http://www.cidrap.umn.edu/news-perspective/2014/10/news-scan-oct-24-2014
122. Blanton L, Brammer L, Smith S, Mustaquim D, Steffens C, Abd Elal AI, et al. Update: influenza activity -- United States and worldwide, May 18-September 20, 2014. MMWR Morbidity and mortality weekly report. 2014 Oct 03;63(39):861-4.
123. prevention Cfdca. 2012-2013 Influenza Season Summary. Fluview 2013 [cited; Available from: https://www.cdc.gov/flu/weekly/pdf/12-13%20Season%20Summary.pdf
124. Prevention CfDCa. 2013-2014 Influenza Season Week 39 ending September 27, 2014. Fluview 2014 [cited; Available from: https://www.cdc.gov/flu/weekly/weeklyarchives2013-2014/weekly39.html
125. Prevention CfDCa. 2015-2016 Influenza Season Week 37 ending September 17, 2016. Fluview 2016 [cited; Available from: https://www.cdc.gov/flu/weekly/weeklyarchives2015-2016/Week37.htm
126. Prevention cfDCa. 2016-2017 Influenza Season Week 39 ending September 30, 2017. Fluview 2017 [cited; Available from: https://www.cdc.gov/flu/weekly/weeklyarchives2016-2017/Week39.htm
127. Organization WH. Standardization of terminology for the influenza virus variants infecting humans: Update. Joint announcement of FAO, OIE and WHO 2014 [cited 2017; Available from: http://www.who.int/influenza/gisrs_laboratory/terminology_variant/en/
128. Bowman AS, Sreevatsan S, Killian ML, Page SL, Nelson SW, Nolting JM, et al. Molecular evidence for interspecies transmission of H3N2pM/H3N2v influenza A

viruses at an Ohio agricultural fair, July 2012. Emerging microbes & infections. 2012 Oct;1(10):e33.
129. Database IR. Generate Phylogenetic Tree. 2017.
130. Stamatakis A, Ludwig T, Meier H. RAxML-III: a fast program for maximum likelihood-based inference of large phylogenetic trees. Bioinformatics. 2005 Feb 15;21(4):456-63.
131. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Molecular biology and evolution. 2016 Jul;33(7):1870-4.
132. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, et al. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. 2012 04/27

01/09/received

03/25/revised

04/16/accepted;28(12):1647-9.
133. database IR. Swine H1 Clade Classification Tool. 2017 [cited; Available from: https://www.fludb.org/brc/tree.spg?method=ShowCleanInputPage&decorator=influenza
134. Anderson TK, Macken CA, Lewis NS, Scheuermann RH, Van Reeth K, Brown IH, et al. A Phylogeny-Based Global Nomenclature System and Automated Annotation Tool for H1 Hemagglutinin Genes from Swine Influenza A Viruses. mSphere. 2016 Nov-Dec;1(6).
135. Inc. SI. JMP®, Version 13.; 1989-2007.
136. Pearce MB, Jayaraman A, Pappas C, Belser JA, Zeng H, Gustin KM, et al. Pathogenesis and transmission of swine origin A(H3N2)v influenza viruses in ferrets. Proceedings of the National Academy of Sciences of the United States of America. 2012 02/21;109(10):3944-9.

Appendix A. Phylogenetic Trees

See Supplemental Document