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US feral swine were exposed to both avian and swine influenza A viruses

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Abstract

Influenza A viruses (IAVs) in swine can cause sporadic infections and pandemic outbreaks among humans, but how avian IAV emerges in swine is still unclear. Unlike domestic swine, feral swine are free ranging and have many opportunities for IAV exposure through contacts with various habitats and animals, including migratory waterfowl, a natural reservoir for IAVs. During 2010–2013, 8,239 serum samples were collected from feral swine across 35 US states and tested against 45 contemporary antigenic variants of avian, swine, and human IAVs; of these, 406 (4.9%) samples were IAV-antibody positive. Among 294 serum samples selected for antigenic characterization, 271 cross-reacted with ≥1 testing virus whereas the other 23 did not cross-react with any testing virus. Of the 271 IAV-positive samples, 236 cross-reacted with swine IAVs, 1 with avian IAVs, and 16 with avian and swine IAVs, indicating that feral swine were exposed to both swine and avian IAVs but predominantly to swine IAVs. Our findings suggest that feral swine could potentially be infected with both avian and swine IAVs, generating novel IAVs by hosting and reassorting IAVs from wild birds and domestic swine and facilitating adaptation of avian IAVs to other hosts, including humans, before their spillover. Continued surveillance to monitor the distribution and antigenic diversities of IAVs in feral swine is necessary to increase our understanding of the natural history of IAVs.

Keywords: feral swine, influenza A virus, influenza surveillance, mixing vessel, seroprevalence, swine influenza virus, United States

Importance

There are more than 5 million feral swine distributed across at least 35 states in the USA. In contrast to domestic swine, feral swine are free ranging and have unique opportunities for contact with wildlife, livestock and their habitats. Our serological results indicate that feral swine in the United States have been exposed to influenza A viruses (IAVs) consistent with those found in both domestic swine and wild birds, with the predominant infections consisting of swine adapted IAVs. Our findings suggest that feral swine having been infected with IAVs at low levels and could serve as hosts for the generation of novel IAVs at the interface of feral swine, wild birds, domestic swine, and humans.

Introduction

Influenza A virus (IAV), a negative-stranded RNA virus with 8 genomic segments, can infect a wide range of hosts, including humans, wild birds and domestic poultry, swine, canines, felines, equines, mink, ferrets, sea mammals, and bats. IAVs have been recovered from at least 105 wild bird species of 26 different families (1). Migratory waterfowl, such as *Anseriformes* spp. birds (e.g., ducks, geese, and swans) and *Charadriiformes* spp. birds (e.g., gulls, terns, and waders), are considered the major natural reservoirs of IAVs (2). Sixteen IAV HA (H1–H16) and nine NA (N1-N9) subtypes have been recovered from migratory waterfowl. The prevalence of IAV infection is up to 30% among wild birds (2), and virus transmission typically occurs via exposure to virus shed in the feces of infected animals (3, 4). It has been conceptually proposed that antigenic evolution in migratory waterfowl could be static (5); for example, this theory is supported by recent studies indicating a lack of antigenic diversity among H3 and H7 IAVs in migratory waterfowl in North America (6, 7).

IAVs in domestic swine are genetically and antigenically diverse. In the past decade, the predominantly circulating domestic swine strains in the United States were IAV subtypes H1N1, H1N2, and H3N2 (8, 9). Subtypes such as H4N6, H2N3, and H3N1 were also identified in North American domestic swine (10-12), but these viruses did not become endemic. The H1 subtypes circulating in domestic swine form four genetic clades: swH1α (classic H1N1), swH1ß (reassortant H1N1-like), swH1γ (H1N2-like), and swH1δ (human-like H1). Clade swH1γ is further divided into subclades swH1γ1 and swH1γ2, and clade swH1δ is subdivided into swH1δ1 (human-like H1N2) and swH1δ2 (human-like H1N1) (13). In addition, the 2009 H1N1 pandemic virus, A(H1N1)pdm09 emerged from a classic H1N1 virus and evolved into a distinct genetic and antigenic lineage (13). There are 4 genetic clusters (I-IV) of the H3N2 subtype of IAVs present in the US swine population (14-16). Cluster IV, currently the predominant IAV cluster circulating among domestic swine, can be further divided into at

least 2 antigenic clusters H3N2-α and H3N2-ß (17). Antigenic characterization suggests that these genetically diverse H1 and H3 viruses are antigenically distinct, showing different extents of cross-reactivity in serologic assays (17, 18). Influenza surveillance studies in domestic swine from 2009 through 2012 identified the co-circulation of H1N1, H1N2, and H3N2 IAVs, including 6 H1 genetic clades (H1α, H1ß, H1γ, H1δ1, H1δ2, A[H1N1]pdm09) and 2 H3 cluster IV antigenic clusters (H3SIVα and H3SIVß) (19, 20).

Avian- and human-origin IAVs typically preferentially bind to receptor saccharides containing terminal α2,3-linked sialic acid-galactose (SA2,3Gal) or α2,6-linked sialic acid-galactose (SA2,6Gal), respectively (21, 22). Swine tracheal epithelium expresses both SA2,3Gal and SA2,6Gal receptors (23), and swine are therefore proposed as the intermediate host for avian IAV adaptation and as a "mixing vessel" for generating novel viruses by reassortment between avian-origin and human-origin IAVs (24-26). In addition to avian-origin H2N3 and H4N6 IAVs, which were identified in domestic swine in North America, avian-origin H1N1(27, 28), H1N2 (29), H3N3 (30), H5N1 (31), H6N6 (32), and H9N2 (33, 34) IAVs were also identified in domestic swine in Eurasia. Among these avian-origin IAVs, only subtypes H1N1 and H3N2 have become endemic in domestic swine; the other avian-origin IAVs caused only low seroconversion rates and have been transient in domestic swine. Nevertheless, under laboratory conditions, avian-origin IAVs of subtypes H1-H13 can infect and replicate in swine at varying susceptibilities (26).

Feral swine in the US are domestic swine that escaped from commercial operations or were intentionally released, descendants of Eurasian wild boar introduced for hunting purposes, or hybrids of the two (35). In 2013, an estimated 5 million feral swine were found in at least 35 US states, with both numbers and geographic range increasing. H1N1 and H3N2 IAVs have been recovered from feral swine, and serologic surveillance conducted during 2011–2012 showed that 9.2% of 1,983 serum samples from

111 feral swine in 31 states were IAV-seropositive (17). Similar to domestic swine, feral swine can be
112 infected with IAVs under laboratory conditions (36). Feral swine have opportunities to encounter wild
113 waterfowl by frequenting the same bodies of water, feeding in the same areas, and preying or
114 scavenging on wild waterfowl, which can provide potential for IAV transmission from wild birds to
115 feral swine. Because feral swine are highly mobile, they can also have opportunities to come into contact
116 with IAVs from infected domestic swine, poultry, and even humans via contaminated fomites or aerosol
117 dispersal (37).

118 Our objective was to conduct a serological survey of feral swine for IAV exposure. Utilizing
119 8,239 serum samples collected from feral swine in 35 US states between 2010-2013, we explored
120 patterns of IAV seropositivity and further characterize seropositive samples' cross-reaction to 45
121 antigenically diverse prototype IAVs from avian, domestic swine and human hosts.

122

123 **Materials and Methods**

124 **Sample collection and serology testing**. From October 1, 2009–September 30, 2013, the United States
125 Department of Agriculture (USDA) Animal and Plant Health Inspection Service's Wildlife Services
126 collected postmortem serum samples from 8,239 individual feral swine across 35 US states. This
127 collection period included fiscal years (FY) 2010 (October 1, 2009–September 30, 2010; 1,818
128 samples), 2011 (October 1, 2010–September 30, 2011; 2,467 samples), 2012 (October 1, 2011–
129 September 30, 2012; 1,846 samples), and 2013 (October 1, 2012–September 30, 2013; 2,108 samples)
130 (Table 1). Serum samples from October 1, 2011–September 30, 2012, were previously reported as 1,989
131 totally, yet 143 serum samples had duplicate information and were ignored for this study. The date of
132 collection, geographic location, age (juvenile, subadult, adult, and unknown), and sex were recorded.

Antibody status was determined with the IDEXX AI MultiS-Screen Ab Test (IDEXX, Westbrook, Maine, USA). Serum samples with a sample-to-negative control ratio of ≤0.681 were determined to be IAV-positive (36). The ELISA results for a subset of feral swine samples (76 of 111) from FY2012 are reported elsewhere (17) in an assessment of the seroprevalence of subtype H3 IAV in feral swine. To ensure complete results, we included all feral swine serum samples collected for the study. Of the samples tested, 406 were identified as IAV-positive; from these 406 IAV positive samples, all of 294 IAV positive samples collected in the most recently three years (FY2011, FY2012, and FY2013) were selected for subtyping by HI assay.

Viruses and Reference Sera. A total of 45 IAVs were selected to represent the following antigenic groups of contemporary IAVs: endemic swine IAVs H1 (α, ß, γ1, γ2, δ1, δ2) and H3 cluster IV (α and ß); human influenza viruses (A[H1N1]pdm09, swine-origin influenza viruses A[H3N2]v, and seasonal H3N2); and avian influenza viruses H1–H14 (Table 2). Swine viruses were propapgated in Madin-Darby Canine Kidney (MDCK) epithelila cells and avian viruses were propagated in Specific Pathogen Free (SPF) embryotic eggs. These viruses were used in the serologic characterization. The reference swine, ferret, and chicken sera (Table S1) used to assess cross-reactivity among testing viruses were generated as described elsewhere (20, 38, 39).

Hemagglutination (HA) and Hemagglutination Inhibition (HI) Assays. HI assays were performed according to the World Health Organization Global Influenza Surveillance Network Manual for the laboratory diagnosis and virologic surveillance of influenza (40). In brief, we treated 1 volume of feral swine serum with 3 volumes of receptor-destroying enzyme (RDE; Denka Seiken Co., Japan) overnight at 37°C and then heat-inactivated the serum at 56°C for 30 minutes. After returning to room temperature, treated antisera were diluted with 6 volumes of 1× PBS (pH 7.4). To minimize nonspecific agglutination, we treated RDE-treated serum samples with 0.5% turkey red blood cells (RBCs; (41-43))

and then incubated them at 4°C for 1 hour, followed by centrifugation at 1,200 rpm for 10 minutes; we then collected the serum samples without disturbing the packed RBCs. RBC absorption was repeated until no nonspecific agglutination was associated with any serum sample. In the HI assay, 0.5% turkey RBCs were used for absorption; serum samples were determined to be positive against a specific virus if the HI titer was ≥1:40, as described previously (17, 44).

Virus neutralization assays. RDE-treated feral swine serum was serially diluted 1:2 in a microtiter plate, and 100 µL of 100 50% Tissue Culture Infectious Dose ($TCID_{50}$) virus was added to each well, and then the plate was incubated at 37°C for 1 hour. The sera–virus mixture was then incubated with Madin-Darby canine kidney (MDCK) cells for one hour in a 96-well tissue culture plate (USA Scientific; Ocala, FL, USA), washed twice with 200µL 1× PBS (pH =7.4), washed with 200 µL of Opti-MEM (Thermo Fisher; Waltham, MA, USA), and then incubated for 96 hours at 37°C in 5% CO_2. Detection of non-neutralizaed virus was conducted using HA assays with 0.5% turkey RBCs. Serum samples were determined to be positive against a specific virus if the neutralization titer was ≥1:40 (45).

Feral swine experiments. All work was registered and conducted under the supervision of the USDA, NWRC Institutional Animal Care and Use Committee using approved protocols to assure humane handling and use. A total of 16 juvenile feral swine (body weight, 16–22 kgs) were trapped in a rural area of Oktibbeha county, Mississippi, USA, transported to the research facilities, and housed as described elsewhere (36). The captured swine were quarantined for one week; before the experiments, all animals were tested to be seronegative to brucellosis, pseudorabies, and IAV by ELISA.

Eight animals were used to test susceptibility of A/mallard/Wisconsin/A00661712/2009 (H3N2); four animals were used to test susceptibility of feral swine to A/mallard/Ohio/648/2002 (H6N2); four additional feral swine were used as negative controls. For H3N2 virus, eight feral swine, housed in four

individual pens (2 per pen), were intranasally inoculated with a 10^6 50% tissue culture infectious dose ($TCID_{50}$) of A/mallard/Wisconsin/A00661712/2009 (H3N2). Nasal wash samples were collected from all eight feral swine daily from 1-10 days post infection (DPI) and titrated in SPF embryotic eggs; and serum was collected on 0, 7, 14, and 21 DPI to determine seroconversion. To detect pathogenesis of virus to swine, two infected and one control swine were necropsied at 5 and 7 DPI, respectively; turbinate, trachea and lung of each feral swine were collected and virus titer in these tissues were detected in SPF eggs. For H6N2 virus, four feral swine were intranasally inoculated with a 10^6 $TCID_{50}$ of influenza A/mallard/Ohio/648/2002 (H6N2) in a 1-mL volume. Serum was collected on 0, 5, 7, and 21 DPI to determine seroconversion. The animals used as the negative control were inoculcated with sterile PBS. Swine were monitored daily for subjective signs of influenza infection (e.g., lethargy, nasal discharge, coughing, and dyspnea) and objective signs (e.g., body temperature) until 14 DPI.

Data analyses. To understand the risk factors (i.e. seasonality and host factors) affecting IAV seroprevalence in feral swine, Chi-square tests were used to assess the differences of IAV seroprevalence between sex, age group, and month, year, and state of sample collection. We developed a multilevel multivariable logistic regression model to test potential risk factors associated with IAV seroprevalence in feral swine, focusing on the population sizes of domestic swine and poultry. The logistic regression model was developed using generalized estimation equations with binomial distribution and logit link function and accounted for clustering of pigs samples on the same date and location. Variables were manually selected if they contributed significance to the likelihood ratio statistic for Type 3 analysis at an alpha level of <0.05. IAV seroprevalence for sex and age group of feral swine, month, fiscal year, and state of sample collection were analyzed as individuals and feral swine groups and best fit was assigned based on QIC (Quasilikelihood under the Independence model Criterion). Feral swine groups were defined to eliminate confounding variables; we grouped samples if

collected on the same date at the same location; in theory, swine from the same group would be exposed to the same virus. In addition, an odds ratio (OR) was estimated using the GLIMMIX procedure for generalized linear mixed models with binomial distribution and logit link function. Observations from states with less than 100 samples were excluded from the analysis. We obtained population data for domestic swine and poultry per state from the 2012 USDA Census of Agriculture (https://www.agcensus.usda.gov/Publications/2012/). Small domestic swine farms were considered as those which had less than or equal to a total of 1,000 swine. All statistical analyses were conducted in SAS 9.5 (SAS Institute Inc., Cary, NC, USA).

Results

IAVs exposure in feral swine. To evaluate the overall seroprevalence of IAVs among feral swine, we used 8,239 serum samples collected across 35 US states during October 1, 2009–September 30, 2013; this collection period included FY2010–FY2013 (Fig. 1; Table 1). Serologic testing by the IDEXX AI MultiS-Screen Ab test suggested that 4.9% (406) of the samples were IAV-positive.

An association was identified between fiscal years and IAV seroprevalence and months and IAV seroprevalence for individual feral swine ($p = 0.0002$ and $p < 0.0001$ respectively) but not for groups ($p = 0.1717$ and $p = 0.1184$ respectively). An analysis of seroprevalence by month determined that during FY2010 (October 1, 2009–September 30, 2010), the highest and lowest seroprevalence rates were detected among samples collected in April (12.3%, 20 of 162 swine) and September (1.8%, 1 of 57 swine), respectively. This temporal pattern, with a relatively higher IAV seroprevalence in spring and winter, was similar to the patterns seen in FY2011 and FY2012 and to those seen in FY2013 in our previous report (17) (Fig. 2A).

To understand variables that are associated with IAV seropositivity among feral swine, we analyzed our results by swine age group and sex. IAV seroprevalence was highest among the adult swine (5.8%, 350/5,984) and lowest among juvenile swine (1.6%, 3/190); this pattern was consistent for all years; 2010 had the largest percent positives only due to have the smallest sampling of juveniles (14.29%; 1/7 samples), (Table S2). We identified an association between swine age group and IAV seroprevalence ($p < 0.0001$) for both individual swine and groups. In addition, IAV seroprevalence was higher among female (5.4%, 233/4,333) than male (4.5%, 173/3,871) swine; this pattern was consistent for all fiscal years except FY2011 (Table S2), in which seroprevalence was higher among male (4.1%; 48/1,170) than female (3.7%; 47/1,287) swine. However, no association was identified between sex and IAV-positive samples for individual swine ($p = 0.0583$) and groups ($p = 0.0665$). Overall, most IAV-percent positive samples were from adult (86.2%) and female (57.4%) feral swine (Fig. 2B).

Although our data set is comprised of samples collected from 35 states, sample sizes were not evenly distributed because feral swine populations vary widely between states. Consequently only 23 states had samples which tested IAV-positive. The seroprevalence of IAV was highest in North Carolina (16.1%, 34/211 samples) and Texas (10.5%, 164/1,561 samples) (Table S3).

Of 438 counties, 112 (25.56%) were IAV-positive (Fig. 1). Texas had the most IAV-positive samples (40.4%; 164/406 total). Of 31 counties sampled in Texas, the highest seroprevalence rates were in Dickens County (36.2%; 42/116 total), Hall County (42.3%; 22/52 total), and Freestone County (35.7%; 5/14 total). The number of IAV-positive samples varied by year, Hall County for example: in 2010, 0 of 1 samples were positive; in 2011, 0 of 12 were positive; in 2012, 6 (30.0%) of 20 were positive; and in 2013, 16 (84.2%) of 19 were positive.

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Distinction between swine and avian IAVs. HI assays were performed on 294 ELISA positive samples in the most recent three fiscal years (FY 2011, FY2012, and FY2013) and tested against 45 IAVs, including HA subtypes H1–H14 and diverse antigenic clusters of contemporary avian-, swine-, and human-origin IAVs (Table 2). Of note, among the testing strains, there were different extents of cross-reactivity against the reference sera against these viruses although most of these viruses are antigenically different with ≥4-fold loss in HI activity relative to homologous titer (Table S1).

Of the 294 feral swine serum samples tested, 271 from 71 counties within 21 states tested positive by HI assay for at least one virus in the reference panel, and 23 samples from 17 counties within 13 states tested negative to all viruses in the reference panel. Of the feral swine samples tested 38.4% were positive against H1 swine IAVs (113 out of 294), and 53.7% were positive against H3 swine IAVs (158 out of 294). Totally, 52 (17.7%) were positive to both H1 and H3 swine IAVs; among these H1- and H3-positive samples, 106 (36.1%) and 233 (79.3%) were also positive against H1 and H3 human IAVs, respectively (Fig. 3). The serologic characterization suggests that the swine-origin IAVs in H3α and H3ß clusters cross-reacted with the ferret reference sera against H3 seasonal and A(H3N2)v human IAVs, and vice versa (Table S4).

Although 271 of the 294 feral swine serum samples tested HI positive to swine and human IAV, only 16 (5.4%) cross-reacted to one of the four avian IAVs included in the reference panel: 13 (4.4%) samples cross-reacted to subtype H1 virus, one (0.3%) crossreacted to H3 virus, and one (0.3%) each cross-reacted to H6 or H7 virus (Table 2). HI with reference sera showed H1 avian IAVs cross-reacted with ferret reference sera against H1N1 human IAVs and that avian subtypes H3N2, H6N2, and H7N3 did not cross-react with the reference sera against the testing human and swine IAVs. To confirm the HI results, we performed neutralization assays, which also showed that the 16 feral swine serum samples were indeed cross-reactive against avian IAVs (Table S4).

Distribution of avian IAVs positive feral swine sera. Sixteen feral swine serum samples from 10 counties in six US states were positive for avian IAV (Fig. 3). The states with the highest number of positive samples were Texas (5), California (4), and Hawaii (4); the remaining samples were from Iowa, Kansas, and Ohio. The surveillance year with the highest number of avian IAV–positive samples was 2013 (7 samples), followed by 2011 (6 samples) and 2012 (3 samples). Thirteen samples were positive for avian subtype H1. Linn County, Iowa, was the only location with an avian H3 subtype. Colusa County, California, was the only location with an avian H6 subtype, and Jefferson County, Oklahoma, was the only place where an avian H7 subtype was identified.

Factors associated with IAV seroprevalance in feral swine. Using manual forward variable selection to determine factors that affect the likelihood for IAV exposure in feral swine, we first tested four variables using a multivariable model: the number of domestic swine farms, small domestic swine farms, poultry farms, and human population per state. We considered farms with 1,000 or fewer domestic swine to be small farms. Small farms were of interest due to less biosecurity and increased chance for contact with feral swine. Individually, the number of domestic swine farms, small domestic swine farms, and poultry farms were significant ($p < 0.05$) but the number of small domestic swine farms had a better fit based on QIC (2.29). The number of domestic swine farms was highly correlated with the other variables and were confounded, making each not significant. There were more IAV seropositive samples from states with more small domestic swine farms.

Feral swine are susceptible to H3 and H6 avian IAVs. For the eight feral swine that were inoculated with A/mallard/Wisconsin/A00661712/2009 (H3N2), viral shedding was detected from 1-6 DPI from 5 of 8 pigs with viral titer of 0.625 to 2.5 $\log_{10}(EID_{50})$ (Table S5). The results from HI assays demonstrated that one feral swine seroconverted with HI titers of 1:80 on 21 DPI (Table S6). The two treatment swine that were necropsied on 5 DPI both had viral titers of 1.333 to 3.5 $\log^{10}EID_{50}$/gram/mL

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in all tissues collected, except turbinate of one feral swine (Table S7). Neither of the control swine had any viral titers in the tissues collected.

For the four feral swine inoculated with H6N2 avian IAV, all pigs seroconverted at 21 DPI with titers ranging from 1:20 to 1:80 (Table S8). All control pigs remained seronegative against IAVs during this experiment. Clinical signs were not observed in any of the experimental feral swine.

Discussion

Feral swine are a potential reservoir for infectious pathogens of domestic swine, including IAVs, because bidirectional transmission of pathogens occurs through direct and indirect contact between feral and domestic swine, primarily through backyard farming operations with poor biosecurity (46). A previous study suggested that IAV circulating among feral swine are antigenically and genetically similar to those circulating among domestic swine (17). Laboratory experiments have demonstrated that swine IAV can infect feral swine and transmit efficiently among them (36). In addition, feral swine may be exposed to avian IAVs through direct and indirect contact with wild birds via scavenging or preying and by using common sources of water and forage. Our study findings confirm that although feral swine may be exposed to swine or avian IAVs, exposure to swine IAVs is much more common, especially with subtypes H1 and H3. Exposure to avian IAV was rare from our finding reported here; yet, there is concern that feral swine could have a mixed IAV infection, and generate reassortants between swine and avian IAVs that could ultimately be transmitted to domestic swine or humans.

Our analyses only focused on those serum samples in the most recent three years (FY2011, FY2012, and FY2013), where H3N2v emerged in domestic swine and caused outbreaks in humans (17, 47, 48). Our prior studies have demonstrated H3N2ß-like viruses were predominant in feral swine in

FY2012. This study suggested that, even though there is no apparent temporal pattern, a relatively higher IAV seroprevalence in the summer could be due to an increased change of contact between domestic and feral swine, e.g., through an increase of pasture time for noncommercial swine. Additionally, the highest seroprevalence in adults can be explained by the more opportunities for IAV exposure as compared to that of younger swine. Because there was no significant difference in IAV seroprevalence for sex, we can assume there was equal opportunity for IAV exposure.

Based on serological evidence, our findings suggest that IAV-positive feral swine in the United States were predominantly exposed to subtypes H1 and H3. H3N2 and H1N1 IAVs have been isolated from feral swine and are genetically close to endemic domestic swine IAVs(17, 49). It is unclear if there is an epidemiological link; however, this finding is consistent with a scenario where domestic swine IAV's occasionally spill over into feral swine populations. Feral swine may be more likely to have contact with domestic swine in backyards or small farming operations that have less biosecurity than large swine operations and it is possible that direct or indirect (i.e. through fomites) transmission occurs between feral swine and domestic swine. Another possible source of domestic-like IAVs in feral swine could be from escaped infected domestic swine, however, the recruitment rate of domestic swine into the feral swine population is not clear. Additionally, some feral swine serum samples cross-reacted with both H1 and H3 subtypes of IAVs which suggests these swine could have been exposed to more than one IAV and is consistent with previously reported findings (17). These possibilities need to be investigated by isolating IAVs currently circulating in feral swine and comparing their genetics to those of nearby IAVs circulating in the domestic swine populations.

A significant portion of the tested feral swine serum samples (78.57%; 231/294) cross-reacted with human-origin IAVs, including H1N1 and H3N2 viruses. The source of feral swine exposure to human H1N1 and H3N2 viruses is unknown. In 1934, Elkeles demonstrated the susceptibility of swine

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334 to human influenza virus strains (50). In the past five decades, genomic analyses suggested at least 20
335 introductions of IAVs from humans to swine, the majority being human seasonal subtype H3N2 viruses
336 (51). Human-to-swine transmission of influenza A(H1N1)pdm09 virus was detected in domestic swine
337 approximately one month after the virus was detected in humans (52). After this "reverse zoonosis"
338 event, A(H1N1)pdm09 virus co-circulated with endemic swine influenza virus, including triple-
339 reassortant H3N2, human-origin H1N2 (H1 δ1), and classical H1N1 (H1 γ) swine influenza viruses (19),
340 resulting in reassortment events (53-56). Additionally, in our reference sera panel, H3N2 seasonal
341 viruses did not cross-react with any reference sera against any contemporary H3 swine IAVs, this is
342 consistent with the H3 human-like viruses found in domestic swine (57). As early as 2010, within the
343 domestic swine population, novel HAs of H3 viruses emerged and were most genetically similar to
344 human H3N2 strains from the 2010-2011 season; this spillover event of human H3N2 into swine is
345 currently being sustained within the domestic swine population (57).

346 Recently, through evaluating dynamics of serological responses in feral swine inoculated with
347 influenza A viruses, we optimized the S/N cutoff for using IDEXX AI MultiS-Screen Ab Test to
348 determine the seropositivity for serum samples from feral swine, and a cutoff of S/N ≥ 0.681 was
349 determined (36). In this study, we adapted this cutoff of S/N ≥ 0.681 for all feral swine serum samples
350 collected in this study. Because this cutoff was more stringent than the one used in another study (17), a
351 lower seroprevalence was obtained. For example, for those samples collected in FY2012, a
352 seroprevalence of 6.0%, was obtained from this study, compared with the seroprevalence of 9.2%
353 reported in the prior study (17).

354 The serological data present in this study could be affected by limitations of serological assays.
355 We could have false-negative results because the avian IAVs usually induce a low serological titer, that
356 may decrease with time resulting in serum samples below the threshold of detection. In addition, we

could not test all possible antigenic variants in contemporary avian IAVs. Furthermore, for those samples that cross react with the IAVs of both avian and swine hosts, it will be difficult to conclude the IAVs exposure were from avian or swine hosts.

In summary, feral swine were predominantly exposed to H1 and H3 swine IAV, but 5.4% of IAV seropositive samples cross-reacted with avian IAV. Thus, there is potential for feral swine to generate novel IAVs by hosting and reassorting IAVs from wild birds with those from domestic swine facilitating adaptation of avian IAVs before their spillover to other hosts, including humans. Continued surveillance is warranted to monitor the distribution and genomic/antigenic diversity of IAVs in feral swine to assess their risk to human health and commercial livestock producers.

Acknowledgments

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References

1. Olsen B, Munster VJ, Wallensten A, Waldenstrom J, Osterhaus AD, Fouchier RA. 2006. Global patterns of influenza a virus in wild birds. Science 312:384-8.
2. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. 1992. Evolution and ecology of influenza A viruses. Microbiol Rev 56:152-79.
3. Stallknecht DE, Kearney MT, Shane SM, Zwank PJ. 1990. Effects of pH, temperature, and salinity on persistence of avian influenza viruses in water. Avian Dis 34:412-8.
4. Ito T, Okazaki K, Kawaoka Y, Takada A, Webster RG, Kida H. 1995. Perpetuation of influenza A viruses in Alaskan waterfowl reservoirs. Arch Virol 140:1163-72.
5. Sturm-Ramirez KM, Hulse-Post DJ, Govorkova EA, Humberd J, Seiler P, Puthavathana P, Buranathai C, Nguyen TD, Chaisingh A, Long HT, Naipospos TS, Chen H, Ellis TM, Guan Y, Peiris JS, Webster RG. 2005. Are ducks contributing to the endemicity of highly pathogenic H5N1 influenza virus in Asia? J Virol 79:11269-79.
6. Bailey EL, LP Zhao, N. Hall, JS Baroch, JA Nolting, J. Senter, L. Cunningham, FL Pharr, GT Hanson, L. Slemons, RD DeLiberto, TJ Wan, XF. 2016. Antigenic characterization of H3 subtypes of avian influenza a viruses from North America. Avian Dis.
7. Xu YB, E. Spackman, E. Li, T. Wang, H. Long, LP. Baroch, JA Cunningham, FL Lin, X. Jarman, RG DeLiberto, TJ Wan, XF. 2016. Limited antigenic diversity in contemporary H7 avian-origin influenza a viruses from North America. Sci Rep 9:20688.
8. Vincent AL, Ma W, Lager KM, Janke BH, Richt JA. 2008. Swine influenza viruses a North American perspective. Adv Virus Res 72:127-54.
9. Kaplan BS, DeBeauchamp J, Stigger-Rosser E, Franks J, Crumpton JC, Turner J, Darnell D, Jeevan T, Kayali G, Harding A, Webby RJ, Lowe JF. 2015. Influenza Virus Surveillance in Coordinated Swine Production Systems, United States. Emerg Infect Dis 21:1834-6.
10. Karasin AI, Brown IH, Carman S, Olsen CW. 2000. Isolation and characterization of H4N6 avian influenza viruses from pigs with pneumonia in Canada. J Virol 74:9322-7.
11. Webby RJ, Rossow K, Erickson G, Sims Y, Webster R. 2004. Multiple lineages of antigenically and genetically diverse influenza A virus co-circulate in the United States swine population. Virus Res 103:67-73.
12. Ma W, Gramer M, Rossow K, Yoon KJ. 2006. Isolation and genetic characterization of new reassortant H3N1 swine influenza virus from pigs in the midwestern United States. J Virol 80:5092-6.

13. Lorusso A, Vincent AL, Harland ML, Alt D, Bayles DO, Swenson SL, Gramer MR, Russell CA, Smith DJ, Lager KM, Lewis NS. 2011. Genetic and antigenic characterization of H1 influenza viruses from United States swine from 2008. J Gen Virol 92:919-30.
14. Hause BM, Oleson TA, Bey RF, Stine DL, Simonson RR. 2010. Antigenic categorization of contemporary H3N2 Swine influenza virus isolates using a high-throughput serum neutralization assay. J Vet Diagn Invest 22:352-9.
15. Olsen CW, Karasin AI, Carman S, Li Y, Bastien N, Ojkic D, Alves D, Charbonneau G, Henning BM, Low DE, Burton L, Broukhanski G. 2006. Triple reassortant H3N2 influenza A viruses, Canada, 2005. Emerg Infect Dis 12:1132-5.
16. Richt JA, Lager KM, Janke BH, Woods RD, Webster RG, Webby RJ. 2003. Pathogenic and antigenic properties of phylogenetically distinct reassortant H3N2 swine influenza viruses cocirculating in the United States. J Clin Microbiol 41:3198-205.
17. Feng Z, Baroch JA, Long LP, Xu Y, Cunningham FL, Pedersen K, Lutman MW, Schmit BS, Bowman AS, Deliberto TJ, Wan XF. 2014. Influenza A subtype H3 viruses in feral swine, United States, 2011-2012. Emerg Infect Dis 20:843-6.
18. Lewis NS, Russell CA, Langat P, Anderson TK, Berger K, Bielejec F, Burke DF, Dudas G, Fonville JM, Fouchier RA, Kellam P, Koel BF, Lemey P, Nguyen T, Nuansrichy B, Peiris JM, Saito T, Simon G, Skepner E, Takemae N, consortium E, Webby RJ, Van Reeth K, Brookes SM, Larsen L, Watson SJ, Brown IH, Vincent AL. 2016. The global antigenic diversity of swine influenza A viruses. Elife 5.
19. Anderson TK, Nelson MI, Kitikoon P, Swenson SL, Korslund JA, Vincent AL. 2013. Population dynamics of cocirculating swine influenza A viruses in the United States from 2009 to 2012. Influenza Other Respir Viruses 7 Suppl 4:42-51.
20. Feng Z, Gomez J, Bowman AS, Ye J, Long LP, Nelson SW, Yang J, Martin B, Jia K, Nolting J, Cunningham F, Cardona C, Zhang J, Yoon KJ, Slemons R, Wan X-F. 2013. Antigenic characterization of H3N2 influenza A viruses from Ohio agricultural fairs. Journal of Virology 87:7655-7667.
21. Rogers GN, Paulson JC. 1983. Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. Virology 127:361-73.
22. Rogers GN, D'Souza BL. 1989. Receptor binding properties of human and animal H1 influenza virus isolates. Virology 173:317-22.
23. Ito T, Couceiro JN, Kelm S, Baum LG, Krauss S, Castrucci MR, Donatelli I, Kida H, Paulson JC, Webster RG, Kawaoka Y. 1998. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. J Virol 72:7367-73.
24. Ma W, Kahn RE, Richt JA. 2008. The pig as a mixing vessel for influenza viruses: Human and veterinary implications. J Mol Genet Med 3:158-66.
25. Scholtissek C. 1990. Pigs as 'mixing vessels' for the creation of new pandemic influenza a viruses. Medical Principles and Practice 2:64-71.
26. Kida H, Ito T, Yasuda J, Shimizu Y, Itakura C, Shortridge KF, Kawaoka Y, Webster RG. 1994. Potential for transmission of avian influenza viruses to pigs. J Gen Virol 75 (Pt 9):2183-8.
27. Pensaert M, Ottis K, Vandeputte J, Kaplan MM, Bachmann PA. 1981. Evidence for the natural transmission of influenza A virus from wild ducts to swine and its potential importance for man. Bull World Health Organ 59:75-8.
28. Guan Y, Shortridge KF, Krauss S, Li PH, Kawaoka Y, Webster RG. 1996. Emergence of avian H1N1 influenza viruses in pigs in China. J Virol 70:8041-6.
29. Karasin AI, Olsen CW, Anderson GA. 2000. Genetic characterization of an H1N2 influenza virus isolated from a pig in Indiana. J Clin Microbiol 38:2453-6.

30. Karasin AI, West K, Carman S, Olsen CW. 2004. Characterization of avian H3N3 and H1N1 influenza A viruses isolated from pigs in Canada. J Clin Microbiol 42:4349-54.
31. Choi YK, Nguyen TD, Ozaki H, Webby RJ, Puthavathana P, Buranathal C, Chaisingh A, Auewarakul P, Hanh NT, Ma SK, Hui PY, Guan Y, Peiris JS, Webster RG. 2005. Studies of H5N1 influenza virus infection of pigs by using viruses isolated in Vietnam and Thailand in 2004. J Virol 79:10821-5.
32. Zhang G, Kong W, Qi W, Long LP, Cao Z, Huang L, Qi H, Cao N, Wang W, Zhao F, Ning Z, Liao M, Wan XF. 2011. Identification of an H6N6 swine influenza virus in southern China. Infect Genet Evol 11:1174-7.
33. Peiris JS, Guan Y, Markwell D, Ghose P, Webster RG, Shortridge KF. 2001. Cocirculation of avian H9N2 and contemporary "human" H3N2 influenza A viruses in pigs in southeastern China: potential for genetic reassortment? J Virol 75:9679-86.
34. Xu C, Fan W, Wei R, Zhao H. 2004. Isolation and identification of swine influenza recombinant A/Swine/Shandong/1/2003(H9N2) virus. Microbes Infect 6:919-25.
35. Gipson PS, B. Hlavachick, T. Berger, C. D. Lee. Explanations for recent range expansions by wild hogs into midwestern states, p 148-150. *In* (ed),
36. Sun H, Cunningham FL, Harris J, Xu Y, Long LP, Hanson-Dorr K, Baroch JA, Fioranelli P, Lutman MW, Li T, Pedersen K, Schmit BS, Cooley J, Lin X, Jarman RG, DeLiberto TJ, Wan XF. 2015. Dynamics of virus shedding and antibody responses in influenza A virus-infected feral swine. J Gen Virol 96:2569-78.
37. Kristensen CS, Botner A, Takai H, Nielsen JP, Jorsal SE. 2004. Experimental airborne transmission of PRRS virus. Vet Microbiol 99:197-202.
38. Xu Y, Bailey E, Spackman E, Li T, Wang H, Long LP, Baroch JA, Cunningham FL, Lin X, Jarman RG, DeLiberto TJ, Wan XF. 2016. Limited Antigenic Diversity in Contemporary H7 Avian-Origin Influenza A Viruses from North America. Sci Rep 6:20688.
39. Balzli C, Lager K, Vincent A, Gauger P, Brockmeier S, Miller L, Richt JA, Ma W, Suarez D, Swayne DE. 2016. Susceptibility of swine to H5 and H7 low pathogenic avian influenza viruses. Influenza Other Respir Viruses 10:346-52.
40. WHO. 2011. Manual for the laboratory diagnosis and virological surveillance of influenza. Geneva : World Health Organization,
41. WHO. 2013. The serological detection of avian influenza A(H7N9) virus infections by turkey haemagglutination-inhibition assay. World Health Organization, Geneva.
42. Pawar SD, Parkhi SS, Koratkar SS, Mishra AC. 2012. Receptor specificity and erythrocyte binding preferences of avian influenza viruses isolated from India. Virology Journal 9:251.
43. Killian ML. 2008. Hemagglutination Assay for the Avian Influenza Virus, p 47-52. *In* Spackman E (ed), Avian Influenza Virus doi:10.1007/978-1-59745-279-3_7. Humana Press, Totowa, NJ.
44. Hobson D, Curry RL, Beare AS, Ward-Gardner A. 1972. The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. J Hyg (Lond) 70:767-77.
45. Potter CW, Oxford JS. 1979. Determinants of immunity to influenza infection in man. Br Med Bull 35:69-75.
46. Wyckoff AC, Henke SE, Campbell TA, Hewitt DG, VerCauteren KC. 2009. Feral swine contact with domestic swine: a serologic survey and assessment of potential for disease transmission. J Wildl Dis 45:422-9.
47. Centers for Disease C, Prevention. 2011. Swine-origin influenza A (H3N2) virus infection in two children--Indiana and Pennsylvania, July-August 2011. MMWR Morb Mortal Wkly Rep 60:1213-5.
48. Biggerstaff M, Reed C, Epperson S, Jhung MA, Gambhir M, Bresee JS, Jernigan DB, Swerdlow DL, Finelli L. 2013. Estimates of the number of human infections with influenza A(H3N2) variant virus, United States, August 2011-April 2012. Clin Infect Dis 57 Suppl 1:S12-5.

49. Clavijo A, Nikooienejad A, Esfahani MS, Metz RP, Schwartz S, Atashpaz-Gargari E, Deliberto TJ, Lutman MW, Pedersen K, Bazan LR, Koster LG, Jenkins-Moore M, Swenson SL, Zhang M, Beckham T, Johnson CD, Bounpheng M. 2013. Identification and analysis of the first 2009 pandemic H1N1 influenza virus from U.S. feral swine. Zoonoses Public Health 60:327-35.

50. Elkeles G. 1934. Experimentelle Untersuchungen zur Aetiologie der Influenza (in German). Mededeelingen uit het Instituut vor Praeventieve Geneeskunde:60-79.

51. Nelson MI, Wentworth DE, Culhane MR, Vincent AL, Viboud C, LaPointe MP, Lin X, Holmes EC, Detmer SE. 2014. Introductions and evolution of human-origin seasonal influenza a viruses in multinational swine populations. J Virol 88:10110-9.

52. Howden KJ, Brockhoff EJ, Caya FD, McLeod LJ, Lavoie M, Ing JD, Bystrom JM, Alexandersen S, Pasick JM, Berhane Y, Morrison ME, Keenliside JM, Laurendeau S, Rohonczy EB. 2009. An investigation into human pandemic influenza virus (H1N1) 2009 on an Alberta swine farm. Can Vet J 50:1153-61.

53. Ducatez MF, Hause B, Stigger-Rosser E, Darnell D, Corzo C, Juleen K, Simonson R, Brockwell-Staats C, Rubrum A, Wang D, Webb A, Crumpton JC, Lowe J, Gramer M, Webby RJ. 2011. Multiple reassortment between pandemic (H1N1) 2009 and endemic influenza viruses in pigs, United States. Emerg Infect Dis 17:1624-9.

54. Lam TT, Zhu H, Wang J, Smith DK, Holmes EC, Webster RG, Webby R, Peiris JM, Guan Y. 2011. Reassortment events among swine influenza A viruses in China: implications for the origin of the 2009 influenza pandemic. J Virol 85:10279-85.

55. Vijaykrishna D, Poon LL, Zhu HC, Ma SK, Li OT, Cheung CL, Smith GJ, Peiris JS, Guan Y. 2010. Reassortment of pandemic H1N1/2009 influenza A virus in swine. Science 328:1529.

56. Starick E, Lange E, Fereidouni S, Bunzenthal C, Hoveler R, Kuczka A, grosse Beilage E, Hamann HP, Klingelhofer I, Steinhauer D, Vahlenkamp T, Beer M, Harder T. 2011. Reassorted pandemic (H1N1) 2009 influenza A virus discovered from pigs in Germany. J Gen Virol 92:1184-8.

57. Rajao DS, Gauger PC, Anderson TK, Lewis NS, Abente EJ, Killian ML, Perez DR, Sutton TC, Zhang J, Vincent AL. 2015. 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. J Virol 89:11213-22.

524 **TABLES**

525 **Table 1.** Summary of feral swine serum samples used to determine the predominant source of feral
526 swine exposure to endemic influenza A virus (IAV) in the United States.

Fiscal year samples collected[a]	No. samples collected	No. IAV-positive samples (% positive)[b]	No. positive samples selected for testing by HI assay[c]
2010	1,818	112 (6.2%)	0 [d]
2011	2,467	95 (3.9%)	95
2012	1,846	111 (6.0%)	111
2013	2,108	88 (4.2%)	88
Total	8,239	406 (4.9%)	294

527 [a]Fiscal years run from October 1st of one year through September 30th of the next year; the FY is
528 named according to the second year.
529 [b]Serum samples were considered IAV-positive if the sample-to-negative control ratio was ≥ 0.681by
530 ELISA (IDEXX AI MultiS-Screen Ab Test; IDEXX, Westbrook, Maine, USA) (36).
531 [c]HI, hemagglutinin inhibition.
532 [d] Not analysed to make more cost effective.
533

534

Table 2. Cross-reactivities of feral swine serum samples against testing influenza A viruses in hemagglutinin inhibition assays

Virus	Antigenic group	Source of virus[a]	No. seropositive samples (%)[b]	GMT (LB-HB)[c]
A/swine/Minnesota/02093/2008	H1N1-α	domestic swine	44 (14.67)	138.85 (40-1280)
A/swine/Minnesota/A01394082/2013	H1N2-α	domestic swine	20 (6.67)	80 (40-320)
A/swine/Nebraska/A01399642/2013	H1N1-ß	domestic swine	58 (19.33)	124.49 (40-1280)
A/swine/Nebraska/A01240348/2011	H1N1-ß	domestic swine	57 (19.00)	146.94 (40-1280)
A/swine/Indiana/13TOSU0832/2013	H1N1-γ	domestic swine	54 (18.00)	204.19 (40-1280)
A/swine/Indiana/13TOSU1154/2013	H1N1- γ	domestic swine	53 (17.67)	187.19 (40-1280)
A/swine/Illinois/A01076767/2010	H1N1- γ2	domestic swine	5 (1.67)	45.95 (40-80)
A/swine/South Dakota/A01349306/2013	H1N1- γ2	domestic swine	63 (21.00)	271.32 (40-1280)
A/swine/Iowa/15/2013	H1 δ1	domestic swine	12 (4.00)	75.51 (40-160)
A/swine/Iowa/18/2013	H1 δ2	domestic swine	22 (7.33)	600.92 (40-1280)
A/swine/Iowa/19/2013	H1 δ2	domestic swine	12 (4.00)	59.93 (40-320)
A/swine/Iowa/7/2013	H1 2009p	domestic swine	91 (30.33)	264.51 (40-1280)
A/swine/Iowa/8/2013	H1 2009p	domestic swine	81 (27.00)	301.39 (40-1280)
A/California/04/2009	H1 2009p	human	61 (20.33)	212.57 (40-1280)
A/mallard/Wisconsin/A00751454/2009	H1N1	avian	13 (4.33)	75.85 (40-640)
A/mallard/Oregan/A0030758/2007	H2N3	avian	0	0
A/swine/Ohio/09SW96/2009	H3N2 α	domestic swine	84 (28.00)	105.04 (40-1280)
A/swine/Ohio/10SW215/2010	H3N2 ß	domestic swine	117 (39.00)	140.45 (40-1280)
A/swine/Ohio/11SW347/2011	H3N2 ß	domestic swine	93 (31.00)	146.31 (40-1280)
A/swine/Texas/A01104013/2012	H3N2 ß	feral swine	123 (41.00)	64.58 (40-1280)
A/Perth/16/2009	H3N2	human	177 (59.00)	109.86 (40-1280)
A/Wisconsin/112/2010	H3N2v	human (spillover from domestic swine)	119 (39.67)	153.61 (40-1280)
A/Pennsylvania/14/2010	H3N2v	human (spillover from domestic swine)	89 (29.67)	89.91 (40-640)
A/Minnesota/10/2011	H3N2v	human (spillover from domestic swine)	105 (35.00)	106.96 (40-640)
A/Iowa/07/2011	H3N2v	human (spillover from domestic swine)	118 (39.33)	148.24 (40-1280)
A/Victoria/361/2011	H3N2	human	195 (65.00)	154.18 (40-1280)
A/mallard/Wisconsin/A00661712/2009	H3N2	avian	1 (0.33)	80 (±0.00)
A/blue-winged teal/Colorado/A00170379/2006	H3N8	avian	0	0
A/mallard/Washington/A00714770/2009	H4N6	avian	0	0
A/mallard/Wisconsin/10os3845/2010	H5N2	avian	0	0
A/mallard/Oregon/A00571208/2007	H6N1	avian	0	0
A/mallard/Ohio/648/2002	H6N2	avian	1 (0.33)	40 (±0.00)
A/bufflehead/Virginia/A00120022/2008	H7N2	avian	1 (0.33)	40 (±0.00)
A/American black duck/Delaware/A00870108/2010	H7N3	avian	0	0
A/northern shoveler/Illinois/10os3632/2010	H8N4	avian	0	0
A/mallard/Minnesota/10os4670/2010	H9N2	avian	0	0
A/northern shoveler/Arkansas/11os386/2011	H9N2	avian	0	0
A/mallard/South Dakota/A00536114/2007	H10N7	avian	0	0
A/mallard/Illinois/10OS3249/2010	H11N2	avian	0	0
A/mallard/Wisconsin/10OS2889/2010	H11N9	avian	0	0
A/American green-winged teal/Missouri/10OS4622/2010	H12N4	avian	0	0
A/bufflehead/Wisconsin/10OS3204/2010	H12N5	avian	0	0
A/hooded merganser/New Brunswick/03750/2009	H13N6	avian	0	0
A/white-winged scooter/Wisconsin/10OS3922/2010	H14	avian	0	0
A/long-tailed duck/Wisoconsin/10OS3912/2010	H14N6	avian	0	0

[a]The host from which the virus was isolated;

[b]Serum samples were determined to be positive against a testing virus if the associated HI titer was ≥1:40;

[c]The geometric mean titer (GMT) was calculated for each group of positive samples. LB, high boundary of HI titer; LB, low boundary of HI titer.

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FIGURE LEGENDS

Fig. 1. Geographic distribution of swine and of influenza A virus (IAV)–positive and IAV-negative serum samples collected from feral swine across the United States during fiscal years (FY) 2010–2013. A) Distribution of feral and domestic swine, and the density unit was 1,000,000. B–D) Distributions of IAV ELISA–negative and –positive feral swine serum samples collected in FY2011 (October 1, 2010–September 30, 2011) (B), FY2012 (October 1, 2011–September 30, 2012) (C), and FY2013 (October 1, 2012–September 30, 2013) (D).

Fig. 2. Epidemiologic analyses of the percentage of influenza A virus–positive feral swine serum samples collected across the United States during fiscal years (FY) 2010–2013. Samples were determined to be positive by ELISA. A) Temporal distribution of positive serum samples. B) Age distribution of feral swine with positive serum samples. C) Sex distribution of feral swine with positive serum samples. FYs run from October 1st of one year through September 30th of the next year; the FY is named according to the second year.

Fig. 3. Geographic distribution of domestic and feral swine across the United States and distribution of influenza A virus (IAV)–positive serum samples (by antigenic characterization) collected from feral swine during fiscal years (FYs) 2011–2013. A) Distribution of feral and domestic swine, and the density unit was 1,000,000. Antigenic characterization was determined by hemagglutination inhibition assay for FY2011 (B), FY2012 (C), and FY2013 (D). FYs run from October 1st of one year through September 30th of the next year; the FY is named

565 according to the second year. Dots (pie charts) indicate US counties where samples positive for

566 different IAV subtypes were collected.

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