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Vaccines provide a primary means to limit disease but may not be effective at blocking infection and pathogen transmission. The objective of the present study was to evaluate the efficacy of commercial inactivated swine influenza A virus (IAV) vaccines and experimental live attenuated influenza virus (LAIV) vaccines against infection with H3N2 virus and subsequent indirect transmission to naive pigs. The H3N2 virus evaluated was similar to the H3N2v detected in humans during 2011-2012, which was associated with swine contact at agricultural fairs. One commercial vaccine provided partial protection measured by reduced nasal shedding; however, indirect contacts became infected, indicating that the reduction in nasal shedding did not prevent aerosol transmission. One LAIV vaccine provided complete protection, and none of the indirect-contact pigs became infected. Clinical disease was not observed in any group, including nonvaccinated animals, a consistent observation in pigs infected with contemporary reassortant H3N2 swine viruses. Serum hemagglutination inhibition antibody titers against the challenge virus were not predictive of efficacy; titers following vaccination with a LAIV that provided sterilizing immunity were below the level considered protective, yet titers in a commercial vaccine group that was not protected were above that level. While vaccination with currently approved commercial inactivated products did not fully prevent transmission, certain vaccines may provide a benefit by limitating shedding, transmission, and zoonotic spillover of antigenically similar H3N2 viruses at agriculture fairs when administered appropriately and used in conjunction with additional control measures.

In 2007, human infection with novel influenza A virus (IAV), including swine origin IAV, became a nationally notifiable event in the United States. Until 2011, reported spillover of IAV from pigs to people occurred sporadically (three to five reports per year) and was most often associated with pig exposure (1). In the fall of 2011, approximately 12 cases of variant H3N2 IAV (here H3N2v) were described, and from July to September of 2012, more than 300 cases were reported, the majority occurring at agricultural fairs in the summer. This particular H3N2v virus is novel, as it contains seven gene segments from swine lineage triple-reassortant (tr) H3N2 viruses and the M gene from the 2009 H1N1 pandemic (pH1N1) virus (rH3N2p). However, the H3N2v of 2011-2012 is not the only rH3N2p identified in swine (2–4) in recent years. In the 1990s in North America, introductions of human seasonal H3N2 IAV into swine resulted in three genetic variants of H3 viruses circulating in swine (clusters I, II, and III) (5, 6). The H3N2 viruses continued to circulate and evolve, with the disappearance of clusters I, II, and III and a cluster IV evolving from cluster III. Cluster IV viruses remain one of the dominant IAV subtypes identified in the U.S. swine population (7).

Given the frequent spillover events associated with this particular rH3N2p virus, there is an urgent need to identify methods to mitigate transmission from pigs to people, as well as among pigs. IAV vaccines are commonly used in the U.S. swine industry and provide a likely means to prevent IAV-associated disease. However, the rapidly evolving diversity of IAV currently circulating in U.S. swine (8–11) has made controlling IAV with vaccines very difficult, even with multivalent formulations. While live attenuated influenza virus (LAIV) vaccines have been approved for use in people and horses, a LAIV for swine has yet to make it to market. This is despite a number of LAIV vaccines that have been developed and demonstrated to provide significant cross-protection in experimentally infected pigs (12–15).

Most commercial IAV vaccines for pigs are inactivated, multivalent formulations using field-sourced IAV as the seed virus, with each strain reflecting a genetically and antigenically distinct hemagglutinin (HA) lineage with possible combinations of H1α, H1β, H1γ, H1δ1, H1δ2, H1N1pdm09, and/or H3 clusters I to IV. The present study was designed to evaluate the efficacy of three different commercially available swine IAV vaccines and two experimental LAIV vaccines against an rH3N2p virus that is genetically similar to the H3N2v that spilled over to people in the summer of 2012. In addition, the ability of vaccine immunity to decrease shedding and limit indirect transmission to naive pigs was evaluated. As noted in a prior pig experiment (16) and again observed in the present study, rH3N2p virus infection did not cause significant clinical disease or lung pathology, even in naive pigs. Thus, clinical presentation is unlikely to be useful for identifying infected pigs. One commercial vaccine provided significant protection from nasal shedding of the challenge virus; however, it did not

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completely prevent indirect transmission to naive pigs. One LAIV provided complete protection against the challenge, as the challenge virus was not recovered from any principal or contact pig and the contact pigs did not seroconvert. Taken together, the results indicate that immunization with a commercial vaccine may not be sufficient to prevent the transmission of this particular rH3N2p virus in settings where the population has pigs with mixed immune statuses. When used appropriately and paired with other control measures, vaccination may be useful in decreasing the amplification of IAV at points of animal concentration associated with events at which humans and animals interface, ultimately reducing the frequency of spillover.

MATERIALS AND METHODS

Phylogenetic analysis. Representative H3N2 influenza A virus HA sequences from contemporary swine field strains and putative vaccine strains, as well as human H3N2v sequences, were downloaded from the NCBI GenBank and GISAID EpiFlu databases. Sequences were aligned by using default settings in MUSCLE v.3.8.31 (17) with subsequent manual correction in Mesquite, v. 2.75 (http://mesquiteproject.org/mesquite/mesquite.html). A maximum-likelihood (ML) tree was inferred by using the RAxML v7.3.4 program (18), employing a general time-reversible (GTR) model of nucleotide substitution with Γ distributed rate variation among sites. Statistical support for individual nodes within the best-scoring tree was estimated by using the rapid bootstrap algorithm (1,000 replications) in RAxML v7.3.4.

Vaccines and viruses. Commercial vaccines were procured from veterinary suppliers and administered according to label instructions. KV-1 (FluSure XP; Zoetis) is a fully licensed quadrivalent commercial product containing the cluster IV H3N2, γ-cluster H1N1, δ1-cluster H1N2, and δ2-cluster H1N1 IAVs as vaccine seed viruses. KV-2 (PneumoStar SIV; Novartis Animal Health, Basel, Switzerland) is a fully licensed bivalent commercial product containing the cluster I H3N2 and α-cluster H1N1 IAVs as vaccine seed viruses. KV-3 (MaxiVac Excell 5.0; Intervet/Schering-Plough Animal Health, Boxmeer, The Netherlands) is a fully licensed pentavalent commercial product containing the cluster I and IV H3N2 and β-, γ-, and δ-cluster H1N1 IAVs as vaccine seed viruses. Both LAIV-1 and LAIV-2 are experimental monovalent vaccines previously described by our group (12, 13, 19–21). LAIV-1 was derived from a triple-reassortant cluster I H3 virus (A/swine/Texas/4199-2/98 [TX98]) and was attenuated by engineering a series of stop codons that truncate the nonstructural (ns1) protein, which in turn attenuates the virus's ability to antagonize the effects of the host's type I interferon antiviral response (22). LAIV-2 was derived from a swine-like triple-reassortant cluster IV H3 virus (A/turkey/OH/313053/04 [OH04]) and attenuated by introducing mutations into the polymerase genes, impairing polymerase activity and restricting virus growth at elevated temperatures (20). A swine isolate of rH3N2p (A/swine/Indiana/A00968373/2012 [IN12]) that was isolated from a pig exhibited at a fair and associated with human H3N2v cases in the summer of 2012 was used as the challenge virus. The virus isolated from the pigs was genetically similar to the H3N2v viruses isolated from human cases from the same fair. Wild-type parent virus of each LAIV strain was used as the antigen in serological assays and representative cluster I (TX98) and IV (OH04) viruses. LAIV-2, challenge virus, and viruses used for serological assays were propagated in Madin-Darby canine kidney (MDCK) cells. LAIV-1 was propagated in 7-day-old embryonated chicken eggs incubated for 48 h at 35°C (kindly provided by Matthew Sandbulte, Iowa State University).

Experimental outline. Three-week-old pigs were procured from a high-health status herd free of influenza and porcine reproductive and respiratory syndrome viruses and treated with ceftiofur crystalline antibiotic (Excede; Zoetis) upon arrival at the National Animal Disease Center. All pigs were free of IAV and IAV antibodies at the start of the experiment. At 5 weeks of age, pigs were vaccinated according to the manufacturer's recommendations for all of the commercial vaccines—two doses of FluSure XP and MaxiVac Excell were administered 21 days apart; one dose of PneumoStar was administered. For experimental LAIV vaccines, pigs were vaccinated with 2 ml of 10^6 50% tissue culture infective doses ($TCID_{50}$)/ml by the intranasal route twice, 21 days apart. Each principal vaccinated or nonvaccinated (NV) group included six pigs, and each group was housed in a separate room. Six weeks following primary vaccination, principal pigs were challenged (Ch) by the intranasal route with 2 ml of 10^6 $TCID_{50}$/ml IN12 virus. A group of NV-nonchallenged (NCh) controls was included. Rectal temperatures were measured daily from the day before challenge through the day of necropsy for principal pigs. Five naive, age-matched pigs were placed in a neighboring pen (indirect contact) in each principal room at 2 days postinfection (dpi). Challenged and indirect-contact pigs were housed in separate raised decks approximately 0.5 m apart. Each deck had solid sides 0.4 m tall and topped with a wire mesh that prevented the pigs from jumping out of the deck, thus preventing direct contact. During the 3 days principal and indirect-contact pigs were housed in the same room, pigs in the indirect-contact pen were processed and chores involving them (sample collection, feeding, any handling) were performed first to minimize fomite transfer as much as possible. Principal pigs were humanely euthanized 3 days after indirect-contact pigs were placed in the same room at 5 dpi for macroscopic pneumonia evaluation, collection of bronchoalveolar lavage fluid (BALF), and collection of lung tissue for evaluation of microscopic lesions. Nasal swabs (NS) were collected from principal pigs at 0, 2, 4, and 5 dpi and from naive indirect-contact pigs at 0 to 5, 7, and 9 days postcontact (dpc). Blood was collected for serum by venipuncture from the principal pigs at the start of the study (prevaccination), at days 21 and 42 postvaccination, and at necropsy (dpi 5). Nasal wash (NW) samples were collected from all principal pigs on the day of the challenge. Serum samples were collected from the indirect-contact pigs on the day they were placed in the room with principal pigs and at necropsy (dpc 0 and 16). Animal studies were conducted in accordance with the National Animal Disease Center's Institutional Animal Care and Use Committee.

Pathological examination of lungs. At necropsy, the lungs of principal pigs were removed and the percentage of the lung affected with purple-red consolidation typical of IAV infection was evaluated. The total percentage of the entire lung affected (percent pneumonia) was calculated on the basis of weighted portions of each lobe with respect to the total lung volume as previously described (23). A portion of the right middle lung lobe was fixed in 10% buffered formalin for 48 h, processed by routine histopathologic procedures, and stained with hematoxylin and eosin. Microscopic lesions were evaluated and scored by a veterinary pathologist blinded to the treatment groups by using parameters as previously described (24).

Viral isolation and titration. NS samples were collected and placed into 2 ml minimal essential medium (MEM), and BALF samples were collected by lung lavage with 50 ml of MEM. All samples were stored at −80°C until viral titers were measured at the completion of the animal study. NS samples were thawed, vortexed, and centrifuged at 300 × *g* for 5 min to pellet debris. Each NS sample was filtered through a 0.45-μm syringe filter, and the fluid was subsequently used for virus isolation and titration. For virus isolation, 0.2 ml of NS or BALF sample was inoculated onto MDCK monolayers in a 24-well plate, and for virus titration, 10-fold serial dilutions of sample were inoculated onto MDCK monolayers in triplicate in a 96-well plate. Samples were incubated for 48 h at 37°C in 5% CO_2. Cells were fixed with 4% phosphate-buffered formalin and stained by immunocytochemistry with an anti-influenza A virus nucleoprotein monoclonal antibody (Hb56) as previously described (25). For virus titration analysis, the ($\log_{10}$-transformed) number of $TCID_{50}$/ml of each sample was calculated by the method of Reed and Muench (26). Samples that were negative on virus titration but positive on virus isolation were assigned a value of 0.75 ($\log_{10}$) $TCID_{50}$/ml. Samples that were negative for both virus isolation and virus titration were assigned a value of 0.

Antibody evaluation. For hemagglutination inhibition (HI) antibody assays, all serum samples were heat inactivated at 56°C for 30 min, subsequently treated with a 20% kaolin (Sigma-Aldrich, St. Louis, MO) suspension, and adsorbed with 0.5% turkey red blood cells to remove nonspecific agglutinins. HI antibody titers were determined with 0.5% turkey red blood cells by using the TX98, OH04, and IN12 viruses as the antigens. Reciprocal HI antibody titers were divided by 10 and $\log_2$ transformed, analyzed, and reported as the average geometric mean reciprocal titer for each group.

For evaluation of levels of IgA and IgG antibodies to the rH3N2p challenge virus, an enzyme-linked immunosorbent assay (ELISA) was performed. IN12 virus was concentrated over a 20% sucrose cushion and subsequently used to coat Immulon-2 plates (Nunc) at 50 hemagglutination units per well. NW and BALF samples were incubated at 1:2 (vol/vol) with 10 mM dithiothreitol for 1 h at 37°C to dissociate mucus before use in the ELISA. Serum was diluted 1:2,000 for IgG evaluation and 1:4 for IgA evaluation. Horseradish peroxidase-labeled anti-swine IgG (Kirkegaard and Perry) and anti-swine IgA (Bethyl Laboratories) antibodies were diluted 1:1,500 and used as detection antibodies. After substrate addition, the optical density (OD) at 405 nm was measured with an automated plate reader. Antibody levels are reported as the mean OD of each treatment group for each antibody isotype (IgA or IgG).

Data analysis. Macroscopic pneumonia scores, microscopic lung scores, $\log_{10}$-transformed BALF virus titers, and ODs for IgG and IgA assays were subjected to analysis of variance with $P \leq 0.05$ considered significant and a Tukey posttest performed for pairwise comparisons (GraphPad Prism; GraphPad Software, La Jolla, CA). Log_{10}-transformed NS virus titers for each time point (dpc) were analyzed by using a mixed linear model for repeated measures (Proc Mixed, SAS 9.2 for Windows; SAS Institute, Cary, NC). Linear combinations of the least-squares mean estimates of NS virus titers were used in *a priori* contrasts after testing for a significant ($P < 0.05$) effect of the vaccine treatment group on NS virus titers. Comparisons were made between groups at each time point by using a 5% level of significance ($P < 0.05$) to assess statistically significant differences. The correlation between the time (dpc) when a single naive indirect-contact pig became positive for virus isolation by NS and the mean viral load of the principal pigs during the time they were cohoused was determined by calculating the Pearson product-moment correlation (Proc Corr, SAS 9.2 for Windows; SAS Institute, Cary, NC).

RESULTS

Vaccine strain and challenge strain relatedness. Swine H3 viruses isolated from 1998 to 2013 form four distinct phylogenetic clusters, designated I through IV (Fig. 1). As the virus seed strains used in each of the commercial vaccines are proprietary, phylogenetic analysis including the exact vaccine HA genes could not be performed. However, since vaccine manufacturers indicate the phylogenetic cluster of the vaccine seed virus, inferences were made on the basis of representative published sequences from each cluster and year of isolation. The LAIV-1 and LAIV-2 vaccines are cluster I and IV viruses, respectively, and are identified by solid squares in Fig. 1. The challenge strain is a cluster IV virus identified by a solid diamond in Fig. 1. Although the LAIV-2 and IN12 challenge viruses are both within the monophyletic cluster IV clade, they are highly divergent within that clade (96.3% amino acid identity). The LAIV-1 and IN12 challenge virus HAs show 89.9% amino acid sequence identity.

HI cross-reactivity following vaccination. Serum was collected from all vaccinated pigs on the day of challenge (day 42 following primary immunization) to evaluate titers of HI antibodies to representative cluster I (TX98) and cluster IV (OH04) H3 viruses and the rH3N2p challenge virus (IN12) (Table 1). Vaccination with KV-1 and KV-3 resulted in significant titers of HI antibodies to IN12 challenge virus. KV-2 vaccination induced seroconversion, to the cluster I TX98 virus, but the average titer of antibody to IN12 was less than 20. LAIV vaccination did not induce titers of HI antibodies to IN12 of greater than 40, but the titer of HI antibody to each respective homologous antigen was greater than 40. The KV-3 vaccine, which includes a cluster I and a cluster IV H3N2 virus, induced significant titers of HI antibodies (>120) to all three of the antigens tested, although the titers of antibody to the cluster I virus (TX98) were highest. None of the NV pigs seroconverted (data not shown).

Protection against rH3N2p challenge following vaccination. Six weeks following primary immunization, pigs in the principal groups were challenged intranasally with IN12 and necropsied 5 days later to evaluate lung viral titers and pathology. A group of NV pigs were included as controls. Four of the five vaccines tested provided significant protection against viral replication in the lungs, although the amount of virus recovered varied between the groups (Fig. 2A). LAIV-2 provided complete protection, as virus was not recovered from the lungs of any pig. LAIV-1 and KV-1 provided significant protection, as virus was recovered from the lungs of only two of six pigs in each group and the average virus titers were less than 1.75 TCID_{50} ($\log_{10}$). While there was a significant reduction in the average lung viral titer in KV-3-vaccinated pigs, there was a wide range of amounts of virus recovered within the group, from no virus isolated to a titer of 5.25 TCID_{50}/ml ($\log_{10}$). One commercial vaccine, KV-2, did not provide protection from viral replication in the lungs, as mean titers were not significantly different from the mean titer of the NV group (4.7 ± 0.7 versus 5.3 ± 0.4 TCID_{50} [$\log_{10}$], respectively).

Challenge with the rH3N2p virus, even in NV pigs, did not induce any obvious clinical disease, such as anorexia or fever (data not shown). In addition, there were no significant differences in macroscopic or microscopic pneumonia at 5 dpi between the NV-Ch and NV-NCh groups (Fig. 2B and C). Thus, while the viral load in the lung was significantly different between vaccine groups at 5 dpi, this was not correlated with significant pathological changes in the lung (Fig. 2).

Shedding dynamics following challenge of vaccinated principal pigs and indirect contact with naive pigs. To test whether vaccination could prevent or limit transmission, this study included an evaluation of viral titers in NS from principal pigs, as well as naive pigs placed in the same room (indirect contact) as vaccinated-challenged pigs. When naive, indirect-contact pigs were placed in the room at 2 dpi, there were significant differences in the NS viral titers of principal pigs in the different vaccine groups (Fig. 3A). Vaccination with neither KV-2 nor KV-3 reduced virus replication in the nose, as there was no significant difference in NS titers between the NV group and the KV-2 or KV-3 group at dpi 2 or dpi 4 or 5. Vaccination with LAIV-1 did not significantly reduce NS titers on dpi 2 or 4, but by dpi 5, there was a significant reduction in NS virus titers compared to those of the NV group. Vaccination with KV-1 significantly reduced viral replication in the nose, as the average titer in NS samples was significantly reduced by 4 to 5 $\log_{10}$ TCID_{50} (Fig. 2A) and virus was isolated from only three of the six pigs in that group (Table 2). Virus was not isolated from the NS of any pig vaccinated with LAIV-2, indicating complete protection against the challenge.

The time (dpc) at which naive, indirect-contact pigs had a positive NS virus isolation was inversely correlated with the average NS titer (dpi 2, 4, and 5 combined) of the principal pigs (Fig. 3 and

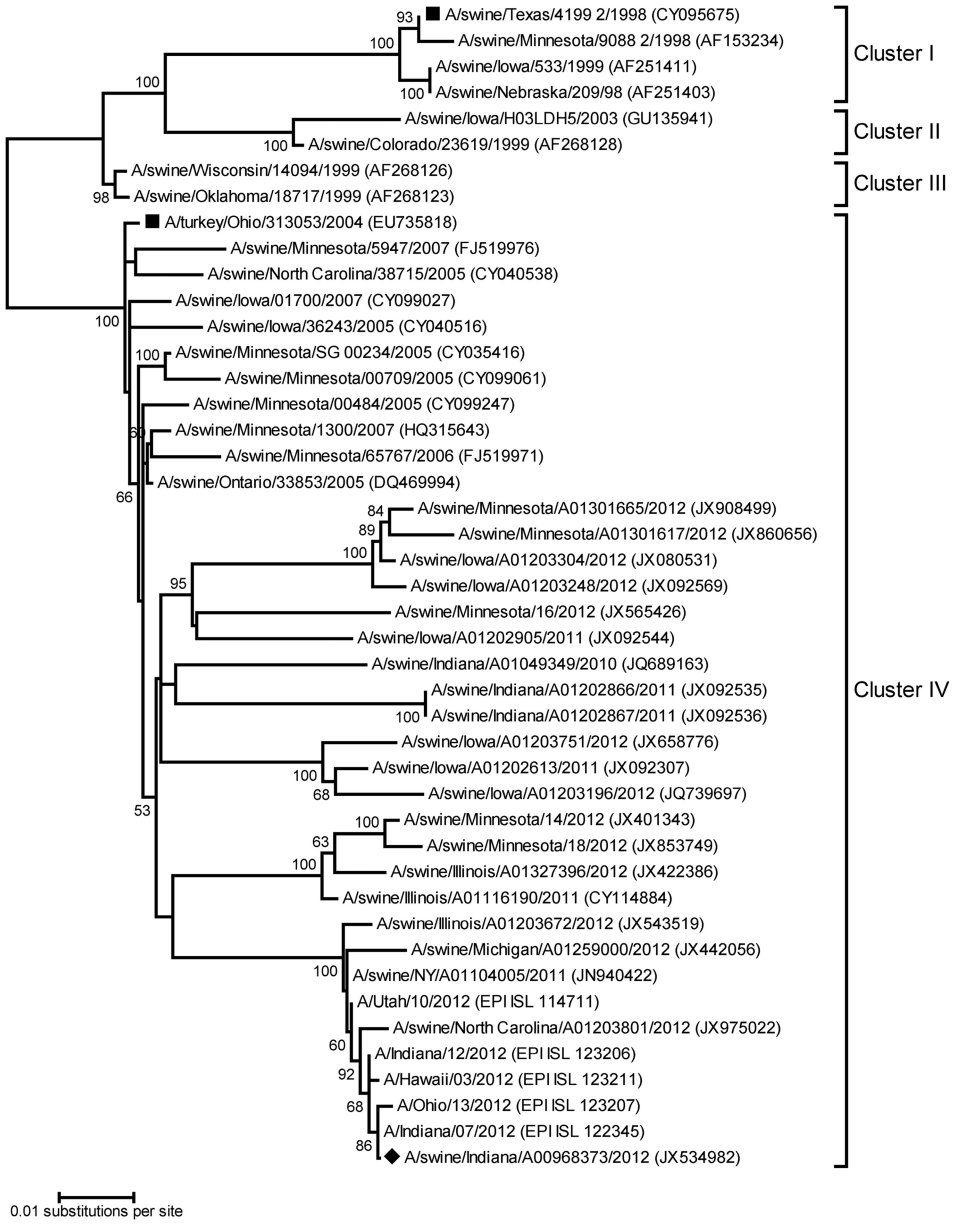

FIG 1 Phylogeny of representative H3N2 influenza A virus isolates on the basis of the HA-encoding gene. The tree shown was constructed by the ML method and a GTR+Γ substitution model implemented in RAxML v.7.3.4. Values above or below branches indicate bootstrap support (percent) estimated from 1,000 resamplings of the sequence data; bootstrap values of ≤50% are not shown. H3N2 HA sublineages are indicated by the brackets on the right (clusters I, II, II, and IV). Taxon names indicate viral isolates, followed by GenBank or GISAID EpiFlu accession numbers in parentheses. LAIV vaccine isolates are marked with solid squares, and the challenge virus is marked with a solid diamond. The scale bar indicates the number of nucleotide substitutions per site.

TABLE 1 Geometric mean reciprocal titers of HI antibodies to different viral antigens in sera collected 6 weeks following priming

Vaccine group[a]	Geometric mean reciprocal titer of HI antibody to viral antigen:[b]		
	IN12 (IV)	OH04 (IV)	TX98 (I)
KV-1	453	226	28
KV-2	18	28	71
KV-3	143	127	320
LAIV-1	10	13	71
LAIV-2	36	113	10

[a] See Materials and Methods for full descriptions of the vaccines used.
[b] The virus used as the antigen in the assay is shown. The H3 phylogenetic cluster is shown in parentheses.

Table 3). Three of five naive pigs in indirect contact with NV-Ch pigs were positive for virus in the NS by dpc 1, and all five pigs were positive by dpc 2. As noted above, vaccination with KV-1 significantly reduced NS titers in principal pigs following a challenge and virus was not recovered from any pigs with indirect contact with KV-1–Ch pigs until 4 dpc. In contrast, there was no significant difference in NS virus titers between KV-3 and NV principal pigs, and by 1 dpc, one indirect naive contact in the KV-3 group was shedding virus. Virus was not isolated from any pig in the group with indirect contact with the LAIV-2-vaccinated pigs.

Once a single pig in the indirect-contact pen became infected and began shedding virus, direct transmission between naive pen-

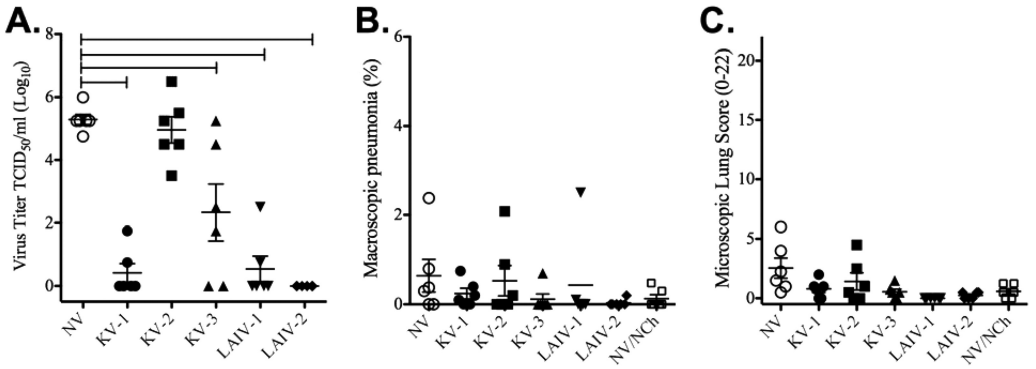

FIG 2 Lung viral titers and pathology following primary challenge of vaccinated pigs. Groups of pigs were vaccinated with commercial killed swine IAV vaccine (KV) or experimental LAIV vaccine or sham vaccinated (NV) as described in Materials and Methods. Six weeks following primary immunization, pigs were intranasally challenged with rH3N2p virus and their lungs were collected at 5 dpi for evaluation of viral titers in BALF (A) and macroscopic (B) and microscopic (C) lung lesions. Each data point represents an individual animal in the vaccine group indicated.

mates in the contact group occurred. In the KV-1 indirect-contact group, the first contact pig was positive on dpc 4 and that same pig was negative for virus by dpc 9. In the NV-Ch group, the first naive indirect contact was positive by dpc 1 and negative on dpc 9, indicating a longer duration of infection. In addition, the average peak NS titer of the KV-1 indirect-contact group was 4.55 ± 0.4 $TCID_{50}$/ml (log_{10}) on dpc 7; which was significantly less than the peak titer of the NV indirect-contact group, which was 5.75 ± 0.8 $TCID_{50}$/ml (log_{10}) on dpc 4 ($P < 0.01$). Together, these data indicate that the level of exposure to shedding principal pigs had an impact on the kinetics of viral replication and subsequent shedding in naive, indirect-contact pigs.

Antibody responses following vaccination and challenge. To further understand the cross-reactive immune response elicited following vaccination, as well as gain insight into the mechanism of protection, IgA and IgG in serum and NW cross-reactive to the challenge virus were evaluated. On the day of challenge, 6 weeks following the primary vaccination, serum and NW were collected from all of the vaccinated principal pigs. Serum levels of IgG to the rH3N2p IN12 challenge virus were significantly higher in KV-1- and KV-3-vaccinated pigs than in NV pigs (Fig. 4A). There was not a significant increase in IgG to IN12 in LAIV-vaccinated pigs compared to the levels in NV pigs, although a trend toward increased IgG was appreciated. In contrast, IgA levels to IN12 in the NW were highest in LAIV-vaccinated pigs, as both LAIV-1- and LAIV-2-vaccinated pigs had significantly elevated levels of IgA cross-reactive to IN12 virus (Fig. 4B). However, IgA to IN12 in the NW was not detected in any pig that received one of the commercial killed vaccines (KV). Neither IN12-specific serum IgA nor NW IgG was detected in any group (data not shown).

Additionally, antibody levels in the BALF collected on dpi 5 were evaluated (Fig. 5). Levels of IgA in the BALF reactive to the IN12 challenge virus mirrored the IgA levels in the NW, as IgA was detected only in the BALF and NW of LAIV-vaccinated pigs (Fig. 5A). While there was a trend toward increased IgA levels in the BALF of KV-1-vaccinated pigs, the levels were not significantly higher than those of NV-Ch or NV-NCh pigs. The IN12-reactive IgG level in the BALF of LAIV-vaccinated pigs was significantly higher than that in NV-Ch pigs. The IgG levels in the BALF of KV-1 pigs were significantly higher than the levels in NV-Ch pigs, but the levels were not significantly increased in the BALF of KV-2- or KV-3-vaccinated pigs (Fig. 5B).

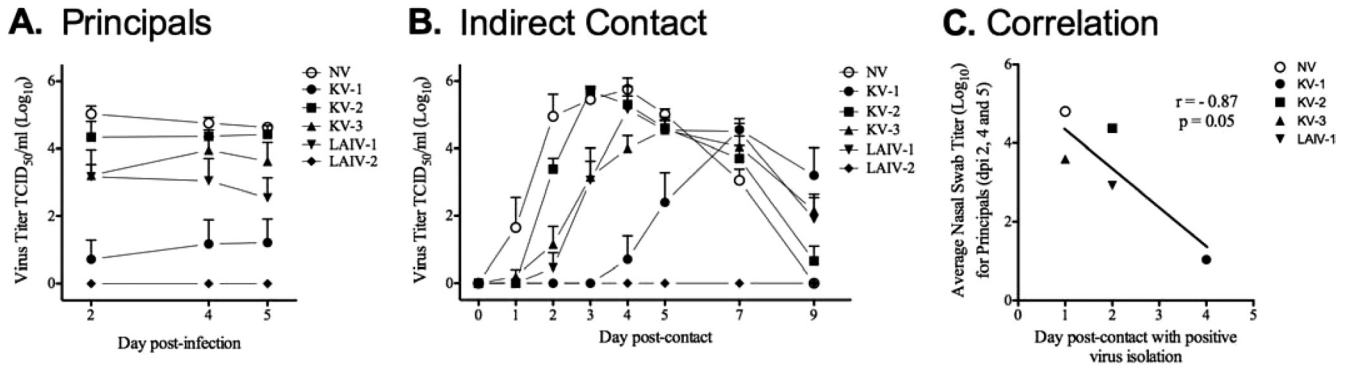

FIG 3 Shedding dynamics following primary inoculation or natural acquisition of rH3N2p virus. Groups of pigs were vaccinated with commercial killed swine IAV vaccine (KV) or experimental LAIV vaccine or sham vaccinated (NV) as described in Materials and Methods. Six weeks following primary immunization, pigs in the principal group (A) were challenged intranasally with rH3N2p virus and NS was collected at 2, 4, and 5 dpi for quantification of viral titers. (B) At 2 dpi of principal pigs, naive pigs were placed in the same room as the principal pigs (indirect contact) in each respective vaccine group and NS was collected at the indicated time (dpc) for quantification of viral titers. Data are expressed as the mean + the standard error of the mean of each group. (C) Indirect correlation between the average viral titer in the NS of principal pigs (dpi 2, 4, and 5 averaged) and the day on which an NS from an indirect, naive contact was positive for virus.

TABLE 2 Numbers of principal pigs per group positive for virus in the nose at different times postinfection

	No. of pigs positive/total in vaccine group:[a]					
No. of dpi	KV-1	KV-2	KV-3	LAIV-1	LAIV-2	NV
0	0/6	0/6	0/6	0/6	0/6	0/6
2	2/6	6/6	5/6	6/6	0/6	6/6
4	3/6	6/6	6/6	5/6	0/6	6/6
5	3/6	6/6	6/6	5/6	0/6	6/6

[a] See Materials and Methods for full descriptions of the vaccines used.

DISCUSSION

Situations in which pigs from mixed sources, and thus with mixed infection statuses and swine IAV immunological profiles, come into close contact with humans are unique settings for the potential interspecies transmission of IAV. Agricultural fairs are one such venue where pigs and people from multiple sources and locations concentrate in relatively close proximity, and combined with the propensity for prolonged exposure, this may facilitate interspecies transmission. The transmission of IAV is bidirectional, with several reported human-to-swine and swine-to-human transmission events associated with agricultural fairs (11, 27). In the fall of 2011, there were a limited number of reports of human IAV infection with a swine origin H3N2 virus, all of which were epidemiologically linked to exposure to pigs at county fairs. Through the summer of 2012, the number of cases increased to more than 300 in North America. The H3N2v virus was characterized as a reassortant (r) H3N2 in which seven genes originated from swine triple-reassortant IAV and the matrix (M) gene of pandemic (p) H1N1 origin (16). While numerous rH3N2p genome constellations have been identified in IAV isolated from pigs, this particular gene constellation is the only rH3N2p virus that has also been associated with zoonotic transmission to people to date (28).

Serologic studies conducted with the H3N2v viruses indicated that certain human age groups have purported seroprotection (HI antibody titers of >40) whereas others do not (29). The presence of cross-reacting antibodies in those born after 1990 is not surprising, since the H3N2 originally introduced into swine was most closely related to human seasonal H3N2 isolated in the mid-1990s (30, 31). However, current human seasonal trivalent influenza vaccine (TIV) did not boost antibody levels (HI antibody titers) in individuals with pre-existing antibody to H3N2v virus (32). In addition, seasonal TIV did not protect again H3N2v infection or disease in ferrets (33). Thus, if H3N2v were to establish itself in the human population by gaining the ability to be transmitted between people, a new specific vaccine would be needed (32).

Methods to potentially reduce the exposure of people to swine IAV at agricultural exhibits include veterinary inspections of swine stock upon entry and/or during the exhibit, strict policies for the removal of sick pigs, limitation of the time pigs spend at the exhibit, limitation of movement between exhibits, and improved hygiene of the stock and human caretakers. Another additional method to limit the potential for zoonotic transmission is to control the virus in the swine stock through vaccination. However, the vaccine must significantly decrease, if not eliminate, the viral burden to decrease shedding and transmission, in addition to protection from clinical disease and/or lung pathology. Oftentimes, vaccination limits disease but does not prevent shedding; thus, the transmission cycle is not broken. A previous report indicated that the rH3N2p viruses do not cause overt clinical disease or lung pathology in pigs (16), and that was true in the present study as well. In addition, pigs were not reported to display overt signs of clinical disease when NS at a county fair were collected for an ongoing surveillance project, despite positive virus isolation (27). Taken together, these data indicate that clinical presentation is not a reliable indicator of infection for all IAV strains.

In North America, there are a number of commercial swine IAV vaccines available, the majority of which contain inactivated or killed virus (34). Although manufacturers disclose the phylogenetic cluster of the seed virus, the strain name and genetic sequence are proprietary. In the present study, the commercial inactivated vaccine with a cluster I H3 (KV-2) failed to protect, as expected. However, neither of the cluster IV H3 vaccines (KV-1 and KV-3) was fully protective either. A LAIV vaccine encoding a cluster IV H3 (LAIV-2) and matched 2002 lineage N2 provided sterilizing immunity against infection, even though the H3 in the LAIV-2 is highly divergent (96.3% amino acid identity) from the cluster IV challenge virus (Fig. 1). As the HA sequences of the cluster IV H3 viruses in KV-1 and KV-3 are proprietary, the genetic relationship to the LAIV-2 or challenge virus HA could not be determined; thus, it is difficult to determine if genetic differences could be related to the lack of protection. Although neither the LAIV-1 nor the KV-2 vaccine containing cluster I H3 viruses provided sterilizing immunity, the LAIV-1 vaccine was more efficacious than the KV-2 vaccine. Hence, while the antigen in the vaccine is important to consider for cross-protection, it appears that the vaccine platform significantly impacts vaccine efficacy. Our results support previous studies that show better heterologous immunity with LAIV vaccines than with intramuscular delivery of inactivated products (13, 21, 35).

To control IAV in the swine population and potentially reduce zoonotic events, a vaccine that reduces shedding of the virus is necessary. We found a correlation demonstrating that the greater the amount of virus detected in principal pigs during the time of exposure, the more quickly a single naive indirect-contact pig was found to be positive for the virus (Fig. 3C). KV-1 provided significant protection against nasal shedding in principal pigs, and virus was not isolated from the NS of an indirect-contact pig until 4 dpc, compared to 1 to 2 dpc with other vaccine groups. We interpret these data to indicate that while a vaccine may not provide sterilizing immunity, if it is able to significantly control nasal shedding,

TABLE 3 Numbers of indirect-contact pigs per group positive for virus in the nose at different times postcontact with challenged pigs in different vaccine groups

	No. of indirect-contact pigs positive for virus/total in vaccine group:[b]					
No. of dpc[a]	KV-1	KV-2	KV-3	LAIV-1	LAIV-2	NV
0	0/5	0/5	0/5	0/5	0/5	0/5
1	0/5	0/5	1/5	0/5	0/5	3/5
2	0/5	5/5	3/5	1/5	0/5	5/5
3	0/5	5/5	5/5	5/5	0/5	5/5
4	1/5	5/5	5/5	5/5	0/5	5/5
5	5/5	5/5	5/5	5/5	0/5	5/5
7	5/5	5/5	5/5	5/5	0/5	5/5
9	4/5	2/5	5/5	4/5	0/5	0/5

[a] Numbers of days after indirect contact are shown.
[b] See Materials and Methods for full descriptions of the vaccines used.

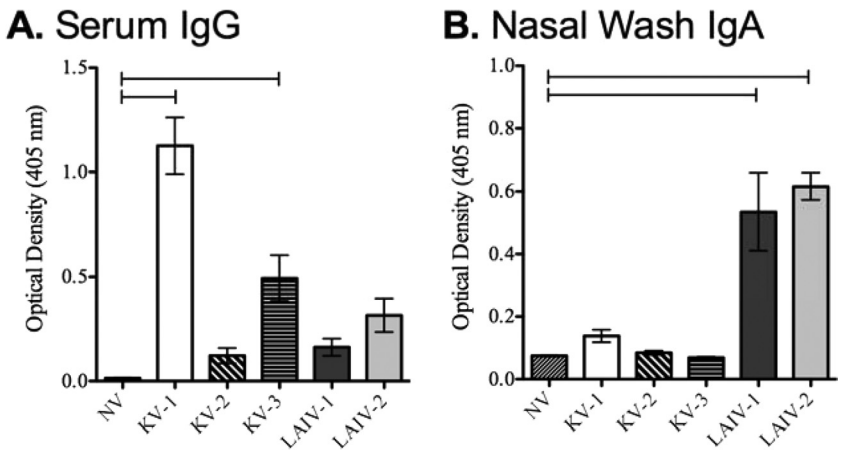

FIG 4 Levels of antibodies specific to the rH3N2p challenge virus in the serum and nasal cavities of pigs following vaccination. Groups of pigs were vaccinated with commercial killed swine IAV vaccine (KV), experimental LAIV vaccine or sham vaccinated (NV) as described in Materials and Methods. Six weeks following primary immunization, samples were collected for evaluation of serum IgG (A) and NW IgA (B) levels. Data are expressed as the mean OD of each group ± the standard error of the mean.

it may decrease the likelihood of indirect transmission to naive pigs. However, this benefit may be further reduced in naive pigs with nose-to-nose contact. It is likely that once IAV infects a single naive indirect-contact pig, direct transmission among the naive pigs in that pen occurred. Work has shown that vaccination does prevent susceptibility to IAV infection upon exposure to naive, infected pigs (36). Further work is needed to determine if vaccination of both the principal group and the indirect-contact group would further limit direct or indirect transmission between pigs. However, this study shows that vaccination affording partial protection of a subset of pigs that share air space with naive pigs may not control IAV shedding and transmission.

HI antibody titer of serum is the typical immune parameter used as a correlate of protection from influenza, and reciprocal titers of greater than 40 are usually considered protective. The homologous H3N2 vaccine antigen for the commercial vaccines was not available to be used in the HI assay to determine the antigenic relationship to the challenge virus. Although cross-reactivity with the challenge virus was evident in the HI assay, the *n*-fold reduction compared to the homologous reaction would be the best indicator of antigenic divergence between the vaccine strain and the challenge strain. The average reciprocal serum HI antibody titer for the most efficacious vaccine (LAIV-2) was 36. However, the KV-3 commercial vaccine average reciprocal serum HI antibody titer was 143 but was only partially protective (Table 1 and Fig. 2). Thus, using serum HI antibody titers as a measure of efficacy and cross-protection was misleading. Serum IgG specific to IN12 challenge virus mirrored serum HI antibody titers, with levels highest in the KV-1 group, followed by the KV-3 group, and both of these vaccines contained H3 cluster IV viruses. Virus-specific IgA in the NW of LAIV-vaccinated children has been shown to be correlated with protection (reviewed in reference 37). NW IgA specific to IN12 challenge virus was significantly elevated in both LAIV-vaccinated groups prior to a challenge (Fig. 4B);

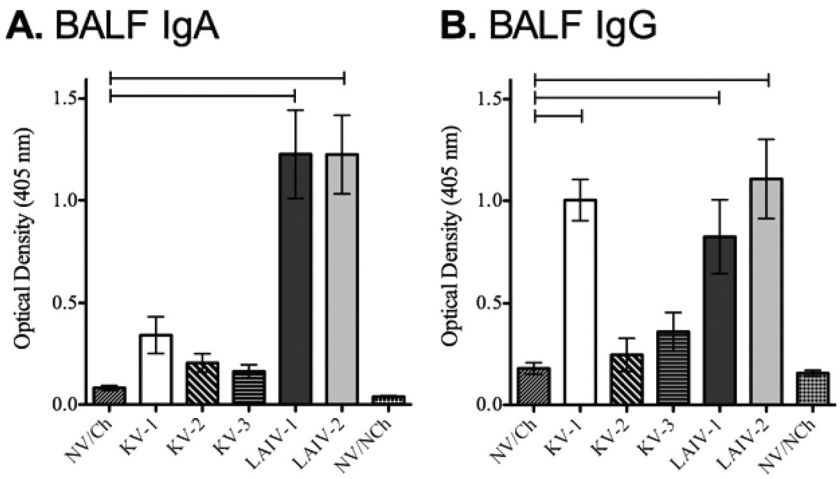

FIG 5 Levels of antibodies specific to the rH3N2p challenge virus in BALF 5 days after a challenge. Groups of pigs were vaccinated with commercial killed swine IAV vaccine (KV) or experimental LAIV vaccine or sham vaccinated (NV), and 6 weeks following primary immunization, the pigs were challenged by the intranasal route with rH3N2p IAV. Five days following the challenge (Ch), BALF samples were collected and levels of IgG (A) and IgA (B) antibodies to the challenge virus were evaluated by ELISA as described in Materials in Methods. Data are expressed as the mean OD of each group ± the standard error of the mean.

however, a difference between levels in LAIV-1- and LAIV-2-vaccinated pigs was not observed, though there was a difference in the protection afforded by these two vaccines (Fig. 2 and 4B). It is likely that the IgA detected in the ELISA was cross-reactive to conserved regions of the IN12 challenge virus. Although the ELISA evaluates virus-specific antibody levels, it does not evaluate functional antibody and is less likely to be used to predict protection.

Overall, our results provide pig owners and agricultural personnel experimental data for determining the value of commercial vaccines in providing protection against this particular rH3N2p virus. None of the commercial vaccines provided complete protection against nasal shedding, though one significantly decreased the amount of virus being shed over time. LAIV vaccination of children has been shown to induce a weak HI antibody response, and cell-mediated immunity is believed to contribute to LAIV vaccine efficacy against phylogenetically distant viruses (38, 39). While antigen matters in a vaccine regardless of the platform, the LAIV vaccines provided broader heterologous protection and induced a robust mucosal immune response that likely plays a significant role in protection from virus replication in the upper respiratory tract. Further work is needed to identify an assay that can be used to predict LAIV vaccine efficacy. One concern often raised with LAIV vaccines is the potential for reversion; however, LAIV vaccine has been used for nearly a decade in humans and reversion has not been documented (40).

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