In North American swine, there are numerous antigenically distinct H1 influenza A virus (IAV) variants currently circulating, making vaccine development difficult due to the inability to formulate a vaccine that provides broad cross-protection. Experimentally, live-attenuated influenza virus (LAIV) vaccines demonstrate increased cross-protection compared to inactivated vaccines. However, there is no standardized assay to predict cross-protection following LAIV vaccination. Hemagglutination-inhibiting (HI) antibody in serum is the gold standard correlate of protection following IAV vaccination. LAIV vaccination does not induce a robust serum HI antibody titer; however, a local mucosal antibody response is elicited. Thus, a live-animal sample source that could be used to evaluate LAIV immunogenicity and cross-protection is needed. Here, we evaluated the use of oral fluids (OF) and nasal wash (NW) collected after IAV inoculation as a live-animal sample source in an enzyme-linked immunosorbent assay (ELISA) to predict cross-protection in comparison to traditional serology. Both live-virus exposure and LAIV vaccination provided heterologous protection, though protection was greatest against more closely phylogenetically related viruses. IAV-specific IgA was detected in NW and OF samples and was cross-reactive to representative IAV from each H1 cluster. Endpoint titers of cross-reactive IgA in OF from pigs exposed to live virus was associated with heterologous protection. While LAIV vaccination provided significant protection, LAIV immunogenicity was reduced compared to live-virus exposure. These data suggest that OF from pigs inoculated with wild-type IAV, with surface genes that match the LAIV seed strain, could be used in an ELISA to assess cross-protection and the antigenic relatedness of circulating and emerging IAV in swine.

Influenza A virus (IAV), of the family Orthomyxoviridae, infects many species, including humans, pigs, horses, sea mammals, and birds. The structural proteins hemagglutinin (HA), neuraminidase (NA), and matrix 2 (M2), along with the cellular lipid bilayer, form the envelope of IAV. The HA and NA proteins play essential roles in virus entry and release of progeny, respectively, and are primary immunogens involved in a protective antibody response (reviewed in reference 1), while internal genes such as nucleoprotein (NP), polymerase subunit PB1, and matrix protein (M)1 are also important for a protective immune response (2).

Pigs are possible “mixing vessels” for the emergence of antigenically distinct IAV isolates since the respiratory tracts of pigs contain receptors for both avian and mammalian IAV (3, 4). Interspecies transmission of IAV is a real concern and has been well documented, with transmission between pigs and humans (5–7) as well as between pigs and birds or poultry (8–10). The relationship between human IAV and swine IAV is an animal and public health concern because of noted spillover events. Routine IAV vaccination is an important tool in the management of animal health to reduce economic loss associated with IAV infection (11) and for pigs displayed at agricultural fairs (12) in terms of both public and animal health.

One of the main roadblocks for swine IAV vaccine development is the presence of multiple antigenic variants within H1 and H3 subtypes, which results in the need for vaccines that provide cross-protection. Classical H1N1 (cH1N1) was the predominant IAV in U.S. swine until the introduction of the novel H3N2 strain in 1998 (13). H3N2 quickly became endemic in U.S. swine and reassorted with extant cH1N1, resulting in genes from human, avian, and swine IAV combining into a single IAV strain (13, 14). Through introductions of human seasonal IAV into swine and genetic evolution of existing strains, there are currently six phylogenetic clusters of H1 IAV circulating in U.S. herds that are designated beta (β), gamma (γ), γ-2, delta-1 (δ1), delta-2 (δ2), and pandemic (15–19). There is limited hemagglutination-inhibiting (HI) cross-reactivity between H1 clusters when using pig hyperimmune serum (derived through vaccination with adjuvanted,
monovalent whole-inactivated virus [WIV] vaccine) to evaluate antigenic relatedness (17, 18).

The majority of currently licensed IAV vaccines for swine are adjuvanted, WIV vaccines given by the intramuscular (i.m.) route. This vaccine formulation induces IAV-specific antibody in serum as measured by the HI assay or a whole-virus enzyme-linked immunosorbent assay (ELISA) (20). WIV vaccines primarily provide protection against homologous or antigenically related strains but provide limited cross-protection against heterologous strains (i.e., strains with the same subtype but limited antigenic cross-reactivity) (21, 22). Notably, differences in the immune response following vaccination through the i.m. route compared to natural infection include minimal mucosal IAV-specific IgA and cell-mediated immune responses (21). To overcome limitations associated with WIV vaccines, several next-generation IAV vaccines, including live-attenuated influenza virus (LAIV) vaccines and replication-defective adenovirus vectors that encode IAV genes, have been experimentally tested for use in pigs (23). LAIV and adenovirus-vectored vaccines have been shown experimentally to provide better cross-protection than WIV vaccines (24–28) and to elicit cellular immunity (24, 29), and IAV-specific maternal immunity does not interfere with adenovirus-vectored or LAIV vaccine efficacy in piglets (29–31).

Nonstructural protein 1 (NS1) of IAV is a virulence factor as a consequence of its type I interferon (IFN) antagonist activity (32). Introducing nucleotide mutations into the NS1 gene causes the loss of type I IFN inhibition; thus, the host IFN response inhibits growth of the virus (33). Through truncation of the last 93 amino acids of the NS1 IAV protein, an LAIV was generated that was shown to induce a robust IFN response and to be attenuated in pigs (34). This truncated NS1 LAIV with H3 surface genes was further shown to elicit protection against homologous and heterologous H3 challenge viruses, as well as partial heterosubtypic protection when vaccinated pigs were challenged with H1 virus (35).

Human replication-defective adenovirus serotype 5 encoding HA (Ad5-HA) has been shown experimentally to provide homologous protection (24, 31, 36) when administered by either the intramuscular or intranasal (i.n.) route and to overcome the problem of interference by maternal-derived immunity (31). Additionally, Ad5-HA IAV vaccines in swine were shown to provide partial heterologous protection when administered intranasally (24).

Despite advantages of LAIV- and Ad5-based vaccines, these platforms have yet to come to market. A live-animal sample and assay to predict vaccine efficacy and cross-protection, as well as additional cross-protection studies using contemporary viruses, will aid in the continued development of these platforms. Serum HI titer is the established gold-standard method to predict IAV cross-reactivity and, subsequently, the cross-protective efficacy of a particular vaccine. A reciprocal serum HI titer of >40 is usually considered protective (correlated with a 50% reduction in the risk of IAV infection [37]), and a greater than 4-fold reduction in HI titer between viruses is considered to represent significant antigenic drift with a predicted loss in cross-protection (37). LAIV vaccination elicits modest serum HI antibody responses to homologous antigens, but LAIV also provides protection against heterologous IAV in which LAIV antibodies did not cross-react (25, 38). Thus, HI titers following LAIV vaccination may be useful for evaluating vaccine immunogenicity and predicting homologous protection, but predictions of cross-protection using this gold standard method are unreliable.

Since intranasal LAIV vaccination elicits IAV-specific antibody at mucosal surfaces (29, 39), it is possible that a live-animal mucosal sample would be better for evaluating intranasal IAV vaccine immunogenicity and cross-protective efficacy. To test this hypothesis, a study was completed in which groups of pigs were exposed to wild-type (WT) IAV, LAIV, or Ad5-HA, all with a pandemic lineage surface gene(s), for collection of mucosal and serum samples postexposure. Vaccinated pigs were then challenged with either a heterologous β-cluster or γ-cluster IAV to evaluate the cross-protective efficacy of the vaccines and to evaluate IAV-specific antibody levels in prechallenge samples in association with protective efficacy. Taking the results together, WT IAV inoculation or LAIV or Ad5-HA vaccination provided some level of cross-protection (depending on the challenge strain), and immunogenicity likely impacted the extent of cross-protective efficacy. IAV-specific IgA in oral fluids was found to be associated with cross-protective efficacy; the data may serve as predictive indices, and additional work is warranted to further validate these findings.

MATERIALS AND METHODS

Ethics statement. Animal experiments were approved by the Institutional Animal Care and Use Committee of the National Animal Disease Center in Ames, IA. Animals were housed in animal biosafety level 2 (ABSL-2) conditions for the entirety of the study.

Vaccines and viruses. The LAIV vaccine expressed the 2009 pandemic H1N1 (H1N1pdm09) (pdm) surface genes from A/New York/18/2009 (NY/09) with the A/turkey/Ohio/313053/2004 (H3N2) internal genes, in which a truncated NS1 gene (34) was carried (see Fig. S1 in the supplemental material). The reverse-engineered, genetically matched parent virus was derived by rescuing the HA and NA genes from A/New York/18/2009 with the A/turkey/Ohio/313053/2004 internal genes (no attenuation mutations introduced) as previously described (40, 41) and is referred to as RG NY/09. The replication-defective adenovirus type 5 vector HA (Ad5-HA) vaccine was derived using an AdEasy system (Agilent, Santa Clara, CA) by cloning the A/California/04/2009 HA gene into the pShuttle-cytomegalovirus (CMV) vector, and adenovirus rescue was performed as previously described (42). Heterologous challenge viruses were H1N1 β-cluster A/swine/Minnesota/03012/2010 virus (MN/10) and H1N2 γ-cluster A/swine/Illinois/3134/2010 IAV (IL/10) obtained through submission of clinical samples to the University of Minnesota Veterinary Diagnostic Laboratory (kindly provided by Marie Culhane). All vaccines and viruses were propagated in Madin-Darby canine kidney (MDCK) cells in serum-free Opti-MEM media (Gibco, Grand Island, NY) supplemented with tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Sigma, St. Louis, MO), t-glutamine, and antibiotics, with the exception of Ad5-HA, which was grown in AD-HEK293 cells with high-glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum. Ad5-HA was purified using a double discontinuous cesium chloride gradient per the manufacturer’s instructions and dialyzed as previously described (42). All vaccines and viruses were diluted in phosphate-buffered saline (PBS) to the desired concentration immediately prior to administration.

Experimental design. Three-week-old pigs were obtained from a high-health-status herd free from IAV and porcine reproductive and respiratory syndrome virus. Pigs were administered enrofloxacin (Baytril; Bayer, Pittsburgh, PA) and cefitoxor crystalline free acid (Excede; Zoetis, Florham Park, NJ) upon arrival according to the recommendations of the manufacturers. IAV antibody status was confirmed to be negative using the IDEXX Multi-Screen Ab test (IDEXX, Westbrook, ME). In part 1 of the experiment (attenuation), piglets were randomly assigned to groups...
of eight and immunized i.n. at 4 weeks of age. Groups received LAIV, WT NY/09, or RG NY/09 parent virus or were not vaccinated (NV). Pigs were humanely euthanized at 3 days postvaccination (dpv) to assess the attenuation and pathogenicity of the LAIV compared to the wild-type parent virus. In part II of the experiment (immune measures of cross-protection), piglets were randomly assigned to groups of eight, immunized i.n. at 4 weeks of age, and boosted 3 weeks later by the same route with the same dose (Table 1). LAIV vaccine and WT NY/09 virus were targeted at 10^6 50% tissue culture infective doses (TCID50)/ml and at 2 ml per dose. The back titer of the inoculum after administration indicated that the WT NY/09 dose was 10^5 TCID50 per pig, whereas the LAIV dose was 10^2 TCID50 per pig. Pigs in the Ad5-HA group received 10^6 TCID50 in 2 ml i.n. Nonvaccinated (NV) controls received 2 ml PBS i.n. Three weeks following the boost, all pigs except for the nonvaccinated/nonchallenged (NV/NC) controls were challenged i.n. with 10^6 TCID50 MN/10(β) or IL/10(γ). Pigs were humanely euthanized at 5 days postinfection (dpi) for collection of samples to evaluate vaccine efficacy.

**Sample collection.** For postvaccination sample collection, nasal swabs (NS) were taken at 0 to 3 dpv for the subset of pigs necropsied on day 3 postpriming. Serum, nasal washes (NW), and oral fluids (OF) were collected from all remaining pigs every 7 days following primary immunization. Blood was collected by venous puncture and stored in BD Vacutainer serum separator tubes (BD, Franklin Lakes, NJ). Serum was collected by centrifugation at 800 x g for 20 min, divided into aliquots, and frozen at −80°C. NW were collected by instilling 5 ml PBS into a nares and collecting effluent as previously described (43), divided into aliquots, and frozen at −80°C. Typically, 0.5 to 2 ml of NW was collected. OF were obtained following previously described methods (44) with few modifications. Briefly, cotton ropes were hung in each room for approximately 30 min for the pigs to chew. Ropes from each treatment group were placed into separate zip lock bags. The rope was manually squeezed inside the bag and liquid sample decanted into a 30-ml conical tube. The tubes were centrifuged at 800 x g for 20 min, and the supernatant was filtered through 0.45-μm-pore-size syringe filters and immediately frozen at −80°C. For postchallenge sample collection, nasal swabs were collected daily beginning on the day of challenge (0 dpi) through necropsy (5 dpi) using polyester-tipped swabs (Puritan, Guilford, ME) pretwisted in 2 ml minimal essential media (MEM) and stored frozen. At necropsy, trachea wash was obtained by removing the trachea prior to lung lavage. The trachea, from 1 cm below the larynx to 2 cm above the bifurcation, was removed and submerged in 3 ml MEM and vigorously agitated for 15 s. The trachea was then removed, and the medium was aliquoted and frozen at −80°C. Lung lavage samples were obtained by lavage performed with 30 ml MEM as previously described (38). An aliquot of lung lavage fluid was plated on blood agar and Casman’s agar plates containing 0.01% (wt/vol) NAD and 5% horse serum for routine aerobic culture to rule out bacterial infection. All postchallenge samples were stored on ice until they were divided into aliquots and frozen within 2 h of collection.

### Pathological examination
At necropsy, the percentage of lung affected with the purple-red consolidation typical of IAV infection was evaluated (45). The total percentage of pneumonia was calculated on the basis of the weighted proportions of each lobe with respect to the total lung volume (46). A portion of the right middle 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 using parameters previously described (47).

### Antibody evaluation
The HI assay was performed as recommended in the WHO animal influenza training manual using turkey red blood cells and WT NY/09 virus or H1 challenge virus as the target antigen as previously described (41). The HI assay using OF and NW was performed following the same protocol as the serum HI assay, beginning with an initial dilution of 1:10. Data are expressed as the reciprocal titer. The serum neutralization assay was performed by generating serial 2-fold dilutions of heat-inactivated sera in MEM supplemented with 5% bovine serum albumin and antibiotics and by incubating with 100 TCID50 before inoculation of confluent MDCK monolayers in 96-well plates as previously described (48). Cells were incubated for 48 h, fixed, and stained for viral nucleoprotein (NP) antigen by immunocytochemistry (49). OF and NW neutralization assays were performed similarly except that the NW samples were not heat inactivated prior to use in the neutralization assay. Reciprocal titers were Log2 transformed and used for statistical analysis of HI and neutralization titer data. IAV-specific IgA and IgG levels in the OF and the NW were recorded as the optical density (OD) value using an indirect whole-virus ELISA and the viruses listed in Table 2 as previously described (38), with the exception that the NW and OF samples were diluted 1:2 in PBS for use in the assay. Antibody levels were reported as the mean OD at 405 nm for each vaccine group. Endpoint antibody titers were obtained by initially diluting OF and NW samples (pooled by treatment group) 1:2 in PBS and titrating 2-fold in duplicate before performing the ELISA. The resulting OD data were modeled as a nonlinear function of the Log2 dilution using the GraphPad Prism (GraphPad software Inc., La Jolla, CA) Log (agonist) versus response-variable slope four-parameter logistic model. Endpoints were interpolated by using 2 x the average OD of the nonvaccinated control as the cutoff. Endpoints for each virus were organized by cluster, and average cluster endpoint dilutions are expressed for both OF and NW. Total IA and IgG ELISAs were performed using a pig IgA or IgG quantification kit (Bethyl Laboratories, Montgomery, TX) following the manufacturer’s protocol.

### Virus titration
To determine viral loads, nasal swab, trachea wash, and lung lavage samples were titrated on MDCK cells to determine the TCID50/ml as previously described (38). Briefly, frozen samples were thawed and filtered through 0.45-μm-pore-size syringe filters, and 10-fold serial dilutions were made in triplicate in serum-free media containing TPCK-trypsin (Sigma, St. Louis, MO) (1 μg/ml) and added to confluent MDCK monolayers in 96-well plates. Cells were incubated for 48 h, fixed, and stained for NP by immunocytochemistry (49). For each titre-

### Table 1 Experimental design for evaluation of IAV vaccine immunogenicity and cross-protective efficacy

| Group (n = 8) | Vaccine | Challenge virus |
|--------------|---------|-----------------|
| NV/NC        | None    | None            |
| NV/CH        | None    | MN/10(β)        |
| WT           | NY/09   | MN/10(β)        |
| LAIV         | LAIV    | MN/10(β)        |
| Ad5-HA       | Ad5-HA  | MN/10(β)        |
| NV/CH        | None    | IL/10(γ)        |
| WT           | NY/09   | IL/10(γ)        |
| LAIV         | LAIV    | IL/10(γ)        |
| Ad5-HA       | Ad5-HA  | IL/10(γ)        |

### Table 2 IAV isolates used to evaluate antibody responses following intranasal immunization

| Cluster | Virus name | Abbreviation | Subtype |
|---------|------------|--------------|---------|
| Pandemic | A/NY/18/2009 | NY/09 | H1N1 |
| Pandemic | A/CA/04/2009 | CA/09 | H1N1 |
| Pandemic | A/swine/IL/3134/2010 | IL/10 | H1N1 |
| γ       | A/swine/IN/3134/2010 | IL/10 | H1N2 |
| γ       | A/swine/MN/30012/2010 | MN/10 | H1N1 |
| β       | A/swine/MN/30012/2010 | MN/10 | H1N1 |
| δ-2      | A/swine/MO/30013/2010 | MO/10 | H1N2 |
| δ-1      | A/swine/MN/02011/2008 | MN/08 | H1N2 |
| H3      | A/turkey/OH/313053/2004 | OH/04 | H3N2 |
tion, the Log_{10} transformed TCID_{50}/ml was calculated for each sample using the method of Reed and Muench (50).

**Statistical analysis.** Reciprocal HI and neutralization titers were Log_{2} transformed and viral titers were Log_{10} transformed for analysis and expressed as reciprocal titer values. ELISA data are presented as the average optical density at 405 nm. Statistical analysis was completed with Graph Pad prism version 6 using the Kruskal-Wallis test followed by Dunn’s posttest or using two-way analysis of variance (ANOVA) and Tukey’s multiple comparison where appropriate.

**RESULTS**

**Attenuation characteristics of H1 LAIV vaccine.** Pigs were i.n. inoculated with WT NY/09, H1 LAIV, or the reverse-genetics-rescued isogenic parent virus (RG NY/09) to assess the attenuation characteristics of the H1 LAIV vaccine. Macroscopic lung lesions (pneumonia) at 3 dpv were significantly reduced in pigs inoculated with LAIV compared to those in pigs inoculated with RG NY/09 (Fig. 1A). Amounts of WT NY/09 virus were detected in nasal swabs by 1 dpv, peaked at 2 dpv, and began to decline by 3 dpv. The amount of virus detected in nasal swabs from pigs inoculated with LAIV was significantly reduced at each time point tested compared to the amount of virus detected in nasal swabs from pigs exposed to WT NY/09 and was significantly reduced at 3 dpv compared to the amount detected in pigs exposed to RG NY/09 (average, 0.03 versus 1.8 Log_{10} TCID_{50}/ml) (Fig. 1B). Pigs inoculated with LAIV had significantly reduced mean viral titers in the trachea (0.2 Log_{10} TCID_{50}/ml) compared to animals exposed to WT NY/09 (P = 0.01) (Fig. 1D). The back titer of the challenge indicated that each pig in the RG NY/09 groups received 10^{5.9} TCID_{50} each pig in the WT NY/09 group received 10^{5.3} TCID_{50} and each pig in the LAIV group received 10^{4.2} TCID_{50}.

**IAV-specific antibody in serum, nasal wash, and oral fluids.** Serum, NW, and OF samples were collected weekly following primary exposure (to WT NY/09, LAIV, and Ad5-HA) for evaluation of IAV-specific antibodies. At 42 dpv, serum HI antibodies against WT NY/09 virus were detected for all immunization groups, though there were differences in titers between treatment groups (Table 3). Average serum HI titers against homologous NY/09 virus were 180, 55, and 31 in animals immunized with WT NY/09, LAIV, and Ad5-HA, respectively. Average serum HI titers against the challenge viruses were below the limit of detection, with the exception of the WT NY/09 group, which had a low but detectable (12 ± 2) serum HI titer against the β-cluster (MN/10) virus. NW and OF samples collected at 42 dpv were also evaluated as a sample source in the HI assay, but there was no measureable HI activity using these samples, even at a dilution of 1:10. All samples from nonvaccinated animals (NV) had no detectable HI titers.

Functional IAV-specific antibody levels were also measured using a virus neutralization assay (Table 3). WT NY/09 and LAIV exposure induced production of serum neutralizing antibody against NY/09 virus (titers, 168 ± 27 and 74 ± 27, respectively), and titers against IL/10(β) virus were similar. However, serum neutralization titers against the MN/10(β) challenge virus compared to neutralization of NY/09 virus were significantly reduced (54 ± 6.7 [P = 0.005] for WT NY/09-immunized animals and

**FIG 1** Attenuation characteristics of H1 NS1-truncated LAIV vaccine. Groups of pigs were inoculated with WT NY/09 virus, LAIV, or the reverse-genetics (RG)-rescued NY/09 parent virus by the intranasal route or were left nonvaccinated (NV) and samples collected at 3 days postvaccination to evaluate attenuation. (A) Percent macroscopic lung lesions. (B) Viral shedding in nasal swabs collected 0 to 3 days postimmunization. (C and D) Viral load in trachea wash (C) and lung lavage (D) fluids. Each dot represents a single animal in that respective treatment group, with the means ± standard errors of the means (SEM) for n = 8 shown for the group indicated. Data were analyzed with the Kruskal-Wallis test and Dunn’s posttest. P values of <0.05 were considered significant. *, P < 0.05; ***, P < 0.01.
TABLE 3 Serum hemagglutination inhibition and virus neutralization titers measured at 42 days postvaccination

| Vaccine | Serum titer of indicated target virus | Neutralization |
|---------|--------------------------------------|---------------|
|         | HI NY/09 (pdm) | IL/10(γ) | MN/10(β) | NY/09 (pdm) | IL/10(γ) | MN/10(β) |
| WT NY/09 | 180 ± 32.9 | <10 | 12.5 ± 1.6 | 168 ± 26.8 | 152 ± 24 | 54 ± 6.7** |
| LAIV | 55 ± 7.3 | <10 | <10 | 74 ± 26.8 | 72 ± 17 | 25 ± 6.3* |
| Ad5-HA | 31 ± 10 | <10 | <10 | <2 | <2 | <2 |
| NV | <10 | <10 | <10 | <2 | <2 | <2 |

***(P = 0.03; **, P = 0.005 (compared to NY/09 neutralization). Statistical significance was measured by the Kruskal-Wallis test (n = 8).**

25 ± 6.3 [P = 0.03] for LAIV-immunized animals. Ad5-HA immunization did not result in detectable serum neutralization antibody. Moreover, OF samples from animals exposed to WT NY/09 were able to neutralize WT NY/09 virus (titer 8 ± 1) but not the heterologous challenge viruses, while NW samples were unable to neutralize any virus used in the assay.

NW and OF samples were used in an indirect whole-virus ELISA to detect antibody specific to H1N1pdm09 lineage virus throughout the course of the vaccine regimen (Fig. 2). By 14 dpv, significant levels of IAV-specific IgA were measured in the NW (Fig. 2A) and OF (Fig. 2B) from pigs in the WT NY/09 (NW and OF, P < 0.0001) and LAIV (NW, P < 0.001; OF, P < 0.0001) groups and were maintained throughout the experiment. H1N1pdm09 IAV-specific IgG was detected only in OF samples beginning at 28 dpv (1 week postboost) and was maintained above background levels (NV group) only in samples from pigs in the WT NY/09 group (Fig. 2C). The total IgA and IgG antibody levels in each sample were determined, and amounts never varied more than 2-fold between time points (data not shown), suggesting that sample collection was consistent throughout the experiment. The Ad5-HA vaccine did not elicit detectable levels of H1N1pdm09-specific IgA or IgG in NW or OF samples (see Fig. S2A in the supplemental material). Due to an inability to measure H1N1pdm09-specific IgA in NW or OF collected from Ad5-HA-vaccinated animals, additional heterologous viruses were not evaluated using samples from Ad5-HA vaccines.

**Macrosopic and microscopic lung pathology.** Macrosopic and microscopic lung lesions were evaluated at 5 dpi following challenge with heterologous H1 β-cluster MN/10 or γ-cluster IL/10 virus (Fig. 3). Pigs in the nonvaccinated, nonchallenged (NV/NC) control group had negligible macroscopic pneumonia (score, 0.16% ± 0.07). Pigs in the nonvaccinated challenged (NV/CH) groups challenged with IL/10(γ) or MN/10(β) had minimal macroscopic pneumonia (3.5% ± 1.1 and 2.1% ± 0.3, respectively). Pigs with prior exposure to WT NY/09 virus, challenged with MN/10(β), did not have reduced pneumonia compared to NV/CH pigs due to overall low scores at 5 dpi (Fig. 3A); however, these animals did not have significantly increased pneumonia scores compared to the NV/NC controls (P = 0.63). Prior WT NY/09 inoculation did reduce macroscopic lung lesions following IL/10(γ) challenge (0.63%, P = 0.001) (Fig. 3B). While LAIV vaccination did not statistically significantly reduce pneumonia following challenge with either virus, there was a trend for reduced macroscopic pneumonia following challenge and the percentage of pneumonia for the LAIV groups was not significantly increased over that seen with NV/NC pigs (P = 0.41). While LAIV-vaccinated pigs challenged with MN/10(β) had microscopic pathology scores greater than those seen with NV/NC controls (1.1 and 1.3 versus 0.3, respectively), these scores were not significantly increased over NV/CH pig scores (0.79) (Fig. 3C). Pigs challenged with IL/10(γ) demonstrated mild microscopic lesions and did not have scores greater than those determined for the NV/NC controls (Fig. 3D). Ad5-HA vaccination did not protect against the development of lung lesions following heterologous IL/10(γ) or MN/10(β) challenge (see Fig. S2B and C in the supplemental material).

**Viral load in the respiratory tract.** Following challenge, viral titers in nasal swabs (NS), trachea washes, and lung lavage fluid were measured to evaluate protection against virus replication. NS were collected daily from 0 dpi through necropsy at 5 dpi (Fig. 4A and D). Pigs exposed to WT NY/09 virus and subsequently challenged with MN/10(β) had reduced average viral titers in NS on dpi 1 through 4 (P < 0.0001), but the average viral titers in NS

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**FIG 2** Kinetics of IAV-specific mucosal IgA and IgG levels following immunization. Groups of pigs were intranasally inoculated with WT NY/09 or LAIV on day 0 and boosted on day 21. NW and OF were collected weekly to day 42 to evaluate IAV H1N1pdm09 (CA/09)-specific mucosal antibody by whole-virus ELISA compared to nonvaccinated (NV) animals. (A and B) IAV-specific IgA detected in nasal wash (A) and oral (B) fluids. (C) IAV-specific IgG detected in OF. Data are expressed as means ± SEM of OD determined for n = 8 for the indicated group.
collected on dpi 5 were similar to the titers measured in NS collected from NV/CH animals (Fig. 4A). Virus was not recovered from any NS collected from pigs exposed to WT NY/09 and challenged with IL/10(γ) (Fig. 4D). LAIV vaccination limited the amount of MN/10(β) challenge virus in NS only at dpi 1 (P < 0.0001), and NS mean viral titers were similar to the titers measured in NS from NV/CH pigs on dpi 2 to 5. However, LAIV vaccination significantly limited the amount of IL/10(γ) challenge virus shed and the length of time during which the virus was shed in nasal passages (P = 0.005) (Fig. 4D). Pigs vaccinated with Ad5-HA also had reduced amounts of challenge virus in NS on dpi 1 to 2, but the average titers were the same as those in the respective NV/CH groups on dpi 3 to 5 (see Fig. S2D in the supplemental material).

Mean titers of the MN/10(β) challenge virus were significantly reduced in trachea washes from pigs previously exposed to WT NY/09 virus compared to NV/CH pigs (1.5 Log_{10} TCID_{50}/ml and 4.6 Log_{10} TCID_{50}/ml, respectively) (Fig. 4B). LAIV-vaccinated animals had mean titers of MN/10(β) virus in the trachea on dpi 5 similar to those measured from NV/CH pigs (Fig. 4B). Additionally, mean viral titers in the lung lavage fluid of all immunization groups challenged with MN/10(β) pigs did not differ from the levels seen with NV/CH pigs (Fig. 4C). Pigs exposed to WT NY/09 or LAIV and challenged with IL/10(γ) did not have any virus recovered from trachea wash or lung lavage fluid (Fig. 4E and F) on dpi 5. Ad5-HA-vaccinated animals also exhibited increased cross-protection against IL/10(γ) (see Fig. S2E and F in the supplemental material).

**Measures of IAV-specific cross-reactive immunity.** Since both live-virus exposure and LAIV vaccination provided increased cross-protection against the γ-cluster H1 variant and less cross-protection against the β-cluster H1 variant, we evaluated whether levels of IAV-specific IgA in a live-animal sample source were predictive of cross-protection, given that the standard HI assay was unable to measure cross-reactivity to either challenge virus (Table 3). Using NW and OF samples collected from all pigs at 42 dpv, the cross-reactivity of IgA to eight H1 viruses from different phylogenetic clusters (listed in Table 2) and one H3 virus (Fig. 5) was determined. Mucosal IAV-specific IgA reactive to homologous, H1N1pdm09 lineage viruses as well as IgA cross-reactive to representative viruses from each H1 cluster and an H3 virus was detected in NW from WT NY/09 virus-immunized animals (P = 0.05), while LAIV-immunized animals did not have detectable IgA reactive to all representative clusters (Fig. 5A). OF samples from WT NY/09- and LAIV-immunized animals had significantly higher levels of IAV-specific IgA than those from the NV controls (P < 0.0001 for all clusters and vaccine groups) (Fig. 5B). IgA in NW from LAIV-vaccinated animals had lower OD values across all phylogenetic clusters compared to homologous NY/09 antigen. However, IgA in OF from LAIV-vaccinated animals had lower OD values across all phylogenetic clusters compared to homologous NY/09 antigen.
heterologous viruses compared to those seen with viruses of the pandemic cluster (P < 0.001).

Since reductions in OD values compared to those of homologous IAV antigen were similar across tested heterologous H1 variants, average endpoint titers of IgA specific to each H1 cluster were determined to further elucidate differences in cross-reactive IgA. IAV-specific IgA endpoint titers in NW and OF against heterologous H1 cluster viruses were reduced compared to the homologous H1 endpoint titers (Fig. 5C and D). Differences between the OF IgA endpoint titer against the NY/09 vaccine virus and mean IgA endpoint titers in OF from WT NY/09-exposed pigs were an average of 1.8-fold for similar H1N1pdm09 lineage viruses, 4-fold for /H9253-cluster viruses, 4.8-fold for /H9252-cluster viruses, 6.7-fold for /H9254-cluster viruses, and 8.4-fold for H3 viruses.

**DISCUSSION**

In this study, we evaluated the ability of H1N1pdm09 cluster H1 LAIV and a replication-defective viral vector encoding IAV H1N1pdm09 HA to provide cross-protection against representative heterologous γ- and β-cluster H1 IAV infections compared to live virus infections and investigated predictive immune measures of cross-protection in samples collected from a live animal. In evaluations of viral loads, vaccination with LAIV or exposure to wild-type virus resulted in significant cross-protection against the γ-cluster challenge IL/10 IAV; however, protection against β-cluster MN/10 was more limited. The H1N1pdm09 cluster and /H9253-cluster viruses are more closely phylogenetically and antigenically related than the β-cluster and H1N1pdm09 cluster viruses (17, 18); thus, the increased protection against IL/10(γ) compared to MN/10(β) was somewhat anticipated. Together, these data suggest that a multivalent next-generation swine IAV vaccine may be necessary to protect against the diverse H1 viruses cocirculating in U.S. swine.

Although cold-adapted, temperature-sensitive LAIV has been approved for use in humans (51) and in horses (52), LAIV vaccines have not been approved for use in pigs. Original work on the LAIV vaccine focused on H3N2 surface genes (25, 34, 35). Here we describe the first use of an H1N1 NS1-truncated virus as an LAIV vaccine and compared the levels of immunogenicity and efficacy following exposure to WT NY/09 virus, which shares the same HA and NA as the LAIV. Similarly to previous reports of H3 NS1-truncated LAIV vaccination in pigs (35) and H1 NS1-truncated LAIV vaccination in mice (33), the H1 NS1-truncated LAIV in pigs was attenuated, with significantly less virus detected in the nose, trachea, and lungs as well as reduced levels of macroscopic pneumonia (Fig. 1). A previous study used a lower dose of challenge WT virus (3 Log10 TCID50 less than was used in the current study) (27), and it was as pathogenic as the typical dose of WT virus used in the current study. This suggests that, regardless of dose, WT IAV induces significant pathology, and although LAIV was administered at a level 1.8 Log10 TCID50 lower than that of the WT virus, the LAIV was attenuated. In addition, there are signif-
icant reports that truncating the NS1 gene of IAV leads to attenuation (33, 34, 53, 54). Despite the dose of LAIV vaccine administered and the minimal evidence of LAIV replication in vivo, the LAIV elicited a measureable IAV-specific antibody response, similarly to previous studies (33, 35). Administering a higher dose of LAIV may increase the degree of cross-protection, similarly to the cross-protection observed with live virus; however, this may not translate into a substantial increase in immune responses. In a follow-up study using a 1.5-log10-higher dose of LAIV administered i.n. to 21-day-old pigs, minimal LAIV was recovered from NS and IAV-specific mucosal IgA endpoint titers were not increased over the titers observed in the current study (data not shown).

Direct and indirect transmission of IAV is a major animal health as well as public health concern. Direct pig-to-pig transmission has been shown with the 2009 pandemic H1N1 virus (55), as well as indirect aerosol transmission with emerging H3N2 variant viruses (38). Agricultural fairs present a unique interface for interspecies transmission of IAV between pigs and people (6, 7, 12). WIV vaccines for IAV have been shown to limit disease but do not always limit virus shedding (21, 38). An IAV vaccine that limits nasal shedding of virus would likely have the ability to limit further transmission; therefore, we evaluated nasal shedding as a measure of cross-protection following challenge. All animals challenged with MN/10(β) had detectable virus in NS as early as 1 dpi. All immunized animals had reduced viral shedding of MN/10(β) in nasal swabs compared to NV/CH controls at 1 dpi, though all animals had similar levels of shedding by the end of the study (5 dpi). In contrast, LAIV-vaccinated pigs challenged with IL/10(γ) demonstrated reduced virus replication in the nose that was cleared by 4 dpi. These reductions in nasal shedding in vaccinated, challenged animals suggests that LAIV vaccination could limit the transmission of heterologous virus to naive or vaccinated contact animals (38, 56, 57), thus slowing or breaking the transmission cycle. Studies are under way to investigate this hypothesis.

A common parameter used to evaluate IAV vaccine efficacy is the prevention or significant reduction of lung lesions following challenge compared to nonvaccinated challenged animal results (45). LAIV-vaccinated animals challenged with IL/10(γ) had pneumonia scores that were not significantly different from the scores calculated for NV/CH controls. However, pneumonia scores following challenge were relatively low across all treatment groups, with NV/CH pigs averaging 3.5%, making significant reductions in pneumonia difficult to assess. Our observation of low pneumonia scores following challenge is consistent with previous studies of H1 IAV intranasal challenge in pigs and is not uncommon in 10-week-old pigs (24, 58). Despite the low pneumonia scores, virus was recovered from the lungs of all NV/CH pigs, and IL/10(γ) was not recovered from lung lavage fluid or tracheal washes of LAIV-vaccinated pigs, indicating protection against virus replication. The observation of lung pathology in the absence of virus in the respiratory tract is consistent with previous work evaluating LAIV or Ad5-HA efficacy in pigs (24, 29, 53). Whereas Ad5-HA-vaccinated animals had increased macroscopic lung le-
sions following MN/10(β) challenge compared to the NV/CH controls (see Fig. S2B in the supplemental material), the microscopic lesion characteristics were not consistent with previous descriptions of lesions associated with vaccine-associated enhanced respiratory disease (47). Collectively, these observations suggest that intranasal LAIV and/or Ad5-HA vaccination primes a mucosal cell-mediated immune response that is rapidly activated upon heterologous challenge and that this activation may control viral burden but result in immunopathology (59). Future work evaluating the mucosal IAV-specific cell-mediated immune response following intranasal IAV vaccination may provide insight into the mechanism associated with pneumonia following heterologous challenge; however, such an evaluation is unlikely to provide a useful live-animal sample that could be readily used for evaluating LAIV immunogenicity and cross-protection. While lung lesions are often used to evaluate IAV vaccine efficacy, together, these data indicate that some vaccines may significantly limit virus replication but that lung lesions may not be reduced.

The HI assay is commonly used to evaluate WIV vaccine immunogenicity and efficacy, and hyperimmune sera (generated through WIV vaccination) are often used to evaluate antigen relatedness between swine IAV strains (17, 60). Previous studies have shown that LAIV or Ad5-HA vaccination results in lower serum HI titers to homologous IAV and undetectable cross-reactive HI titers to IAV (24–26, 38). Consistent with previous reports, LAIV and Ad5-HA vaccination resulted in low HI titers to homologous IAV and did not elicit cross-reactive HI titers to heterologous IAV in the current study. These data reemphasize that neither LAIV vaccination nor Ad5-HA vaccination induces a robust peripheral HI antibody response and that the commonly used serum HI assay may not be suitable for predicting LAIV or Ad5-HA vaccine efficacy. In contrast, serum virus neutralization antibody was detected following LAIV vaccination and neutralization titers may be a useful measure of LAIV immunogenicity and efficacy. The greater sensitivity of the neutralization assay than the HI assay has made it an important tool in the development of IAV vaccines that are poorly immunogenic (37, 61, 62). Serum from LAIV vaccinates neutralized homologous H1N1 pdm09 virus and IL/10(γ) virus equally. Pigs exposed to WT NY/09 or LAIV had significant reductions in serum neutralization against MN/10(β) compared to homologous NY/09 virus neutralization. Higher serum neutralization titers measured against IL/10(γ) virus than against MN/10(β) virus were associated with control of virus replication in the lungs and trachea. These data suggest that the serum neutralization assay may be an effective tool to predict cross-protection of LAIV vaccines in pigs, though further work is warranted to evaluate cross-reactive titers against the panel of H1 viruses, particularly as it relates to cross-protection.

While serum can be an easy sample source for evaluating vaccine immunogenicity, compartmentalization of immune responses may limit the utility of peripheral blood and antibody assays to predict immunogenicity and efficacy associated with intranasally delivered vaccines. Previous studies have shown that IAV antigen-specific antibody can be detected by ELISA in NW and OF samples collected following vaccination or infection (24, 38, 39, 63). Experimental IAV infection elicits an IAV-specific antibody response detectable in OF (63), and OF have become a rapidly evolving diagnostic sample source for several human and animal pathogens (63, 64). We detected IAV-specific antibody in both NW and OF following WT NY/09 exposure and LAIV immunization using whole-virus ELISA. OF samples from immunized animals had increased IAV-specific antibody compared to NW samples as determined by a standard OD reading performed with a single dilution of sample or by sample endpoint titration. Previous studies have shown that intranasal LAIV vaccination induces a local IAV-specific IgA response measurable by ELISA and that the response was associated with protection from homologous challenge in humans (65) as well as in pigs (21, 38). Although we did not evaluate protection from homologous challenge, the higher antibody response observed in OF than in NW following vaccination would suggest that OF could serve as a sample source to evaluate LAIV immunogenicity and homologous protective efficacy as well as provide a measure of herd immune status (63).

Cross-reactive IAV-specific IgA in NW following LAIV vaccination has been shown to correlate with improved heterologous cross-protection in humans and in animal models of human IAV infection (26, 65, 66). Given the reductions in heterologous virus nasal shedding and in measurable IAV-specific IgA levels in mucosal samples prechallenge (42 dpv), we evaluated the association of cross-reactive mucosal IgA in immunized pigs with the level of cross-protection observed. Both NW and OF had measurable cross-reactive IgA; however, using ELISA OD levels as an association value, a broadly cross-reactive response to all H1 cluster viruses as well as to an H3 virus was observed, with OD values nearly the same across heterologous phylogenetic clusters. Using this association alone, we would have predicted complete protection against both challenge viruses, since mucosal IAV-specific IgA was cross-reactive with both MN/10(β) and IL/10(γ) antigens at similar OD values; however, this was not the case. Endpoint titrations of IAV-specific IgA in NW and OF from WT NY/09-exposed pigs separated the data showing cross-reactivity between virus clusters, and a general trend for decreasing IgA endpoint titers was evident, as phylogenetically distant viruses were used as the test antigen (pdm > γ-cluster > β-cluster > δ-cluster) (19), and these levels were associated with cross-protection. Due to the reduced immunogenicity of the LAIV vaccine, there was no clear association between IgA endpoint titers and cross-protection. Performing this analysis with OF samples from WT NY/09-inoculated animals, which had higher IAV-specific IgA titers, did show an association with antigenic relatedness, cross-reactive IgA titers, and cross-protection (Fig. 4 and 5B). Collectively, these data suggest that IAV-specific IgA in OF could be used to assess antigenic relatedness and cross-protection of circulating and emerging IAVs but that it may require that pigs be inoculated with wild-type viruses to elicit a response such that sufficient levels of mucosal IAV-specific IgA are induced. Vaccine or surveillance researchers would be able to curate a collection of OF samples from IAV-infected pigs and measure the antigenic relatedness of the IAV immunogen to currently circulating IAV strains and update and modify vaccine strains as necessary. This approach is somewhat similar to that of human seasonal IAV vaccine selection, where ferrets are infected with live IAV to elicit a high serum HI titer in order to assess the antigenic relatedness of circulating IAV isolates (67, 68).

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