RESEARCH REPORT
SWINE HEALTH

Title: Live-animal assay for identifying correlates of cross-protection for swine influenza virus vaccines when vaccinated in the presence of maternal antibody. **NPB#14-093**

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Industry Summary: Experimental live-attenuated influenza virus (LAIV) vaccines, delivered into the nose, have been shown protect pigs against influenza infection, more so than traditional inactivated commercial vaccines. In addition, LAIV vaccines have been shown to provide protection in piglets that have suckled from sows with influenza-specific immunity. Currently, inactivated vaccines are administered to sows thus, if a LAIV were to be used in piglets it is likely going to be administered in the presence of maternal-derived immunity (MDI). In addition, LAIV may be administered to sows in the future and it is important to understand the impact of MDI on LAIV vaccine efficacy in piglets. Data from a separate study suggests measuring antibody from LAIV vaccinated piglets with MDI may not be an effective measure of immunogenicity and cross-protection. The work in this proposal aimed to validate a live-animal assay that could be used as a predictor of vaccine-induced immunity and protection following LAIV vaccination in both MDI-positive and MDI-negative piglets. To these ends, sows were purchased and bred on-site, and vaccinated three times with either LAIV (4 sows) or WIV (4 sows), and naïve, non-vaccinated sows were purchased separately with similar farrowing dates. At three days of age, groups of piglets from sows were vaccinated intranasally with LAIV or non-vaccinated. At 42-days post vaccination blood, nasal wash, and oral fluids were collected from animals to evaluate the presence of IAV-specific immunity in piglets. Piglets were then challenged with influenza mismatched to the vaccine strain and vaccine efficacy was evaluated by measuring lung pathology and virus shedding in the nose, trachea, and lungs. Our results indicate that piglets were protected against influenza even when vaccinated in the presence of maternal immunity, but the ability to evaluate vaccination status was impeded by transferred maternal immunity.

Key words: influenza virus, live-attenuated influenza virus vaccine, maternal-derived immunity, cross-protection, immunity

Scientific Abstract: Live-attenuated influenza virus (LAIV) vaccines have been previously shown to confer protection against heterologous H3N2 IAV challenge in piglets with maternally-derived immunity (MDI) while whole-inactivated virus (WIV) vaccines were associated with enhanced disease. Protection in MDI positive piglets was associated with T cell priming measured in the periphery. This study was aimed at identifying a live-animal assay to predict cross-protection in piglets when vaccinated in the presence of WIV- or LAIV-induced MDI. At three days of age, piglets from each sow were vaccinated intranasally with LAIV or left non-vaccinated. Unexpectedly, LAIV vaccine transmitted from vaccinated litter mates to non-vaccinated litter mates (this did not occur in a preliminary test of vaccination in 3-week old pigs) resulting in all piglets being IAV antibody positive by 42 days post vaccination. Nonetheless, the presence of both WIV and LAIV MDI inhibited the development of IAV-specific IgA in the nasal wash compared to piglets vaccinated without MDI. At 42 days post vaccination, interferon-gamma (IFNγ) producing T cells in the periphery were not inhibited by MDI, however

overall levels of IFNγ producing T cells were low. IFNγ production in the periphery significantly increased post-challenge in piglets without MDI or with WIV MDI, but not in piglets with LAIV MDI. This data suggests that piglets vaccinated with WIV MDI primed a T cell response that was measurable following challenge, while LAIV had a trend for a primed T cell response following challenge. Despite the unmeasurable immune response to the LAIV in pre-challenge samples from piglets with LAIV MDI, these vaccinates had significantly reduced lung lesions compared to piglets vaccinated without MDI or with WIV MDI suggesting these piglets were protected from lung pathology. Overall, LAIV vaccination in the presence of MDI provided protection against heterologous challenge, but a live-animal assay for evaluating vaccine immunogenicity in MDI-positive piglets has not been clearly identified.

Introduction: 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 (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).

One of the main roadblocks for swine IAV vaccine development include 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 US swine until the introduction of novel H3N2 in 1998 (3). H3N2 quickly became endemic in US swine and reassorted with extant cH1N1, resulting in genes from human, avian, and swine IAV into a single IAV (3, 4). 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 US herds that are designated beta (β), gamma (γ), γ -2, delta-1 (δ1), delta-2 (δ2), and pandemic (5-9).

The majority of vaccines currently available for the prevention of influenza A virus (IAV) in swine are formulated as whole-inactivated virus(es) (WIV) with adjuvant, and delivered by the intramuscular route. WIV vaccines primarily provide protection against homologous or antigenically related strains, but provide limited cross-protection against heterologous strains (i.e., same subtype but limited antigenic cross-reactivity) (10, 11). There are a large number of different strains of IAV currently circulating in US swine and therefore, cross-protection against more distantly related viruses is important.

Several next-generation IAV vaccines have been experimentally tested for use in pigs, including live-attenuated influenza virus (LAIV) vaccines (12). LAIV vaccines have been shown experimentally to provide better cross-protection than WIV vaccines (13-16), elicit cellular immunity (17), and IAV-specific maternal immunity does not interfere with LAIV vaccine efficacy in piglets (17, 18).

While LAIV vaccines proved significant cross-protection and are a likely candidate for future commercial use, one obstacle to their introduction is an assay that predicts subsequent cross-protection from infection. While our research group has made significant progress towards developing such an assay (19), recent work by our research group also suggests mucosal antibody will not be predictive when LAIV is administered in the presence of IAV-specific MDI (17), but cell mediated immunity may be a better predictor of cross-protection.

Objectives: The **primary objective** of this research was to optimize and validate a live-animal assay that could provide a predictive measure of protection to influenza A virus (IAV) infection following intranasal vaccination with live-attenuated influenza virus (LAIV) vaccine, regardless of the immune status of the piglet at the time of vaccination. Prior work from our work has shown that IAV-specific immunoglobulin in oral fluids may be useful in evaluating LAIV vaccine immunogenicity and efficacy. Thus, we sought to identify an assay would be predictive even when administered in the presence of maternal-derived antibody (MDA). Inactivated IAV vaccines do not provide significant cross-protection against the large and diverse pool of IAV currently circulating in the North American pig population. Published research indicates that LAIV vaccines delivered by the intranasal route provide protection against diverse IAV, a key feature of their anticipated market availability and applicability. In addition, they provide protection when delivered in the presence of MDA, unlike WIV vaccines. However, what's lacking in this approach is a way to measure the immune

response in a sample collected from a live animal to predict protection following LAIV vaccination – regardless of piglet immune status.

Objective 1: Evaluate cross-reactive IAV cellular and humoral immune responses elicited following intranasal LAIV vaccination administered in the presence and absence of maternally-derived antibody (MDA) using samples from live animals.

Objective 2: Determine if the quantitative measure of cross-reactive immunity (antibody and/or cellular IFN-γ production) correlates with cross-protection in a vaccination/challenge experiment.

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.

Vaccines and Viruses. The LAIV vaccine expressed the 2009 pandemic H1N1 (H1N1pdm09) 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 (20) was encoded. Heterologous challenge virus H1N2 γ-cluster A/swine/Illinois/3134/2010 (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 OptiMEM media (Gibco) supplemented with TPCK-trypsin (Sigma), L-glutamine and antibiotics. All vaccines and viruses were diluted in phosphate-buffered saline (PBS) to the desired concentration immediately prior to administration.

Experimental Design: Eight sows were obtained from a high health herd and confirmed as IAV antibody negative by the AI Multi-Screen Antibody Test (Idexx). Four sows received 2 ml of 10^6 $TCID_{50}$/ml of LAIV intranasally, four sows received 2 ml of 10^6 $TCID_{50}$/ml of LAIV which had been UV inactivated for 120 s in a stratalinker system (Stratagene), mixed 1:5 v/v with Emulsigen-D (MVP Technologies) and administered intramuscularly. Sows were vaccinated once prior to breeding, and boosted at 6- and 3-weeks prior to the anticipated farrowing date. Six naïve sows with similar farrowing dates were purchased from the same herd and confirmed as IAV antibody negative. At three days of age, half of each litter was vaccinated intranasally with 2 ml of 10^6 $TCID_{50}$/ml of LAIV, and placed in an isolation pen for 20 min before returning to the sow. Piglets were weaned at 21 days of age and placed in isolation rooms based upon vaccine status and MDI status. At 42 days post vaccination (dpv), piglets were challenged with 10^6 $TCID_{50}$ of heterologous γ-cluster IL/10. Pigs were humanely euthanized 5 days post infection (dpi) for the collection of samples to assess vaccine efficacy.

Sampling: Pre-challenge: Blood was collected into BD SST for sera. Nasal wash (NW) was collected by instilling 5 ml PBS into a nare and collecting effluent as previously described (21), aliquoted and frozen at -80 °C. Typically 0.5-2 ml of NW was collected. Oral fluids (OF) were collected on the day of challenge by hanging cotton rope in each isolation pen and allowing the pigs to chew on the rope for 30 min. Oral fluids were rung from the ropes, centrifuged to pellet debris, supernatant filtered, and frozen as previously described (19). Nasal swabs (NS) were collected daily from all pigs beginning on the day of challenge (0 dpi) through necropsy (5 dpi) using pre-wet FLOQswabs (Copan Diagnositcs Inc.), which were stored frozen at -80°C in 2 ml MEM media. **Post-challenge:** At necropsy, trachea wash (19) and bronchioalveolar lavage fluid (BALF) was obtained as previously described (22). An aliquot of lung lavage was plated on blood agar and Casman's agar containing 0.01% (w/v) NAD and 5 % horse serum plates for routine aerobic culture to rule out secondary bacterial pneumonia. Lung lavage and trachea wash were aliquoted and frozen at -80 °C. Virus isolation of BALF from pigs was performed by inoculating 0.2 ml filtered BALF samples on MDCK monolayers in 24-well plates. Inoculated plates were incubated for 48 h at 37 °C, fixed with 10% phosphate buffered formalin, and evaluated by immunocytochemistry for IAV nucleoprotein antigen (23). Macroscopic lung lesions were scored as previously described (24) and percent pneumonia was calculated (25).

Antibody evaluation: The HI assay was performed as recommended in the WHO animal influenza-training manual using turkey red blood cells and NY/09 virus or H1 challenge virus as the target antigen as previously described (26). IAV-specific IgA levels in the NW were recorded as the optical density (OD) value using an indirect whole-virus ELISA with LAIV vaccine virus and IL/10 virus as previously described (22). Briefly, 0.75 µg per well of purified virus was adsorbed in

96-well microtiter plates (NUNC) in carbonate buffer overnight at room temperature. Plates were blocked with Starting Block (Pierce) for 30 min at room temperature, washed three times with PBS/0.05% tween-20. NW diluted 1:2 in PBS was added and incubated at room temperature for 1 h, washed three times, and goat anti-pig IgA horseradish peroxidase (Bethyl Laboratories) was incubated for 1 h at room temp. Plates were washed developed with 2-step ABTS (KPL) and stopped with ABTS stop solution (KPL). Antibody levels were reported as the mean OD at 405 nm for each vaccine group. IAV antibody in serum was determined using the AI Multi-Screen Antibody Test (Idexx) following manufacturer's protocol.

IFNγ ELISpot: Blood was collected in BD CPT with sodium citrate for collection of peripheral blood mononuclear cells (PBMCs) according to manufacturer's recommendations (Becton Dickinson). PBMCs were washed with RPMI-1640, passed through a 40 µm screen, washed and enumerated for the ELISpot assay. The ELISpot assay for enumerating IFNγ secreting cells (IFNγ SC) was performed as previously described (27) using porcine IFN-gamma ELISpot kit, following manufacturer's protocol (R&D systems). Briefly, antibody coated membrane plates were seeded with $2.5x10^5$ PBMCs per well and stimulated in triplicate with $2.5x10^5$ $TCID_{50}$ (MOI=1) LAIV, IL/10 virus, or non-infected MDCK culture supernatant (sham) as antigen, incubated 18 h and developed according to manufacturer's protocol. Spot counts for the plates were determined with a CTL-ImmunoSpot S5UV analyzer using ImmunoSpot software (Cellular Technology Ltd).

Statistical Analysis. ELISA data is 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 multiple comparison, or Two-way ANOVA and Tukey's multiple comparison where appropriate.

Results:

Objective 1: Evaluate cross-reactive IAV cellular and humoral immune responses elicited following intranasal LAIV vaccination administered in the presence and absence of maternally-derived antibody (MDA) using samples from live animals.

Table 1. Transfer of maternal immunity from sows of varying influenza immune status to piglets.

Maternal vaccine[a]	HI[b] titer
Naïve	>10
WIV	185
LAIV	87

[a] Influenza naïve sow (Naïve), Whole-virus inactivated (WIV) vaccinated sow, Live-attenuated influenza virus (LAIV) vaccinated sow

[b] Serum Hemagglutination Inhibition geometric mean titer to homologous virus of piglets at three days of age prior to vaccination

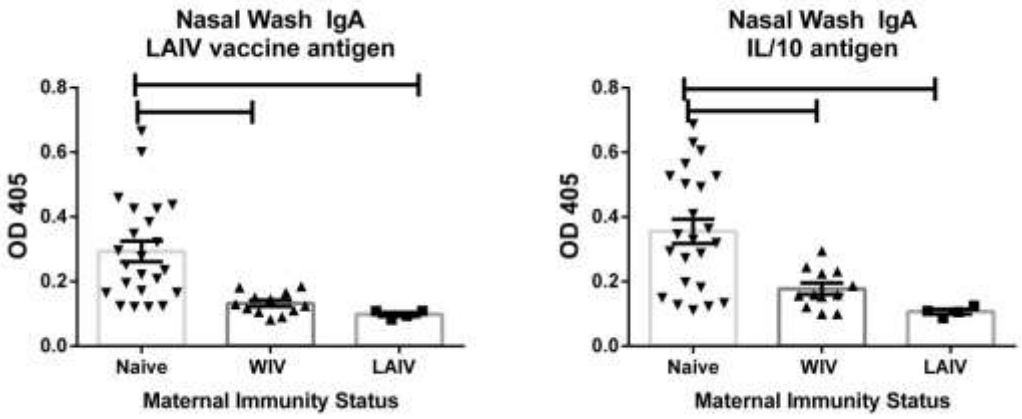

Figure 1. IAV-specific maternal immunity inhibited the production of IAV-specific IgA in nasal wash. Sows were influenza naïve or vaccinated with whole-virus inactivated (WIV) vaccine or live-attenuated influenza virus (LAIV) vaccine. Piglets of different maternal immunity status were vaccinated at three days old. Nasal wash was collected at 42 days post vaccination and tested by whole-virus ELISA for cross-reactive IgA to LAIV vaccine antigen and heterologous challenge IL/10 antigen. Each symbol represents an individual within the respective group with the mean ± s.e.m. indicated for each group. Data was analyzed with a Kruskal-Wallis test and Dunn's post test. Significant differences ($p<0.05$) are indicated with bars.

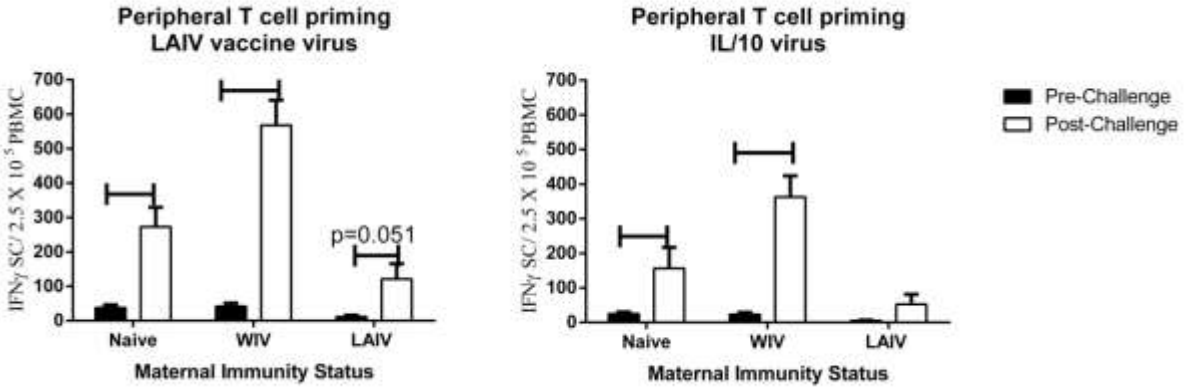

Figure 2. LAIV vaccination of piglets at three days old, primes an anamnestic T cell response detectable in the periphery post challenge. Sows were influenza naïve or vaccinated with whole-virus inactivated (WIV) vaccine or live-attenuated influenza virus (LAIV) vaccine. Piglets of different maternal immunity status were vaccinated at three days old. Peripheral blood mononuclear cells (PBMCs) were isolated pre-challenge (black bars) and post-challenge (white bars) and tested for IAV-specific IFNγ secreting cells (SC) by ELISpot. Data is represented as the mean ± s.e.m. for the indicated group and analyzed with a two-way ANOVA and Tukey's post test. Significant differences ($p<0.05$) between pre-challenge and post-challenge are indicated with bars.

Table 2. IAV-specific antibody in serum at 42 days post vaccination from piglets of different maternal immune status.

Maternal immunity[a]	Vaccinated[b]	IAV NP[c] antibody Positive/total
Naïve	NV/NC[d]	8/8
Naïve	Yes	8/8
Naïve	No	7/7
WIV	Yes	7/7
WIV	No	6/6
LAIV	Yes	4/4
LAIV	No	4/4

[a] Influenza naïve sow (Naïve), Whole-virus inactivated (WIV) vaccinated sow, Live-attenuated influenza virus (LAIV) vaccinated sow

[b] Piglets vaccinated at three days old with LAIV (Yes) or non-vaccinated (No)

[c] Nucleoprotein antibody positive

[d] Non-vaccinated, non-challenged (NV/NC)

Objective 2: Determine if the quantitative measure of cross-reactive immunity (antibody and/or cellular IFN-γ production) correlates with cross-protection in a vaccination/challenge experiment.

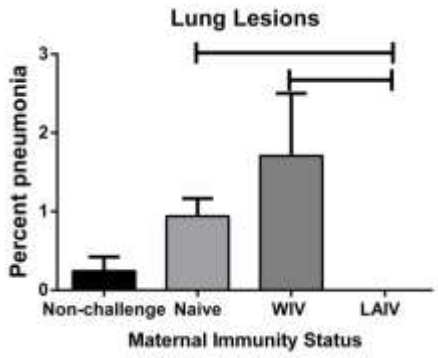

Figure 3. Lung lesions of vaccinated piglets of varying maternal immune status following challenge with heterologous IAV. Sows were influenza naïve or vaccinated with whole-virus inactivated (WIV) vaccine or live-attenuated influenza virus (LAIV) vaccine. Piglets were vaccinated at three days of age intranasally with LAIV and challenged 42 days post vaccination intranasally with heterologous IL/10 IAV or left non-challenged. Lung lesions were evaluated at 5 days post infection. Data is represented as percent pneumonia mean ± s.e.m. and analyzed with Kruskal-Wallis and Dunn's post test. Significant differences ($p<0.05$) are indicated by bars.

Table 3. Virus isolation in bronchioalveolar lavage fluid (BALF) in the lungs of vaccinated pigs 5 days post challenge.

Maternal immunity[a]	Vaccinated[b]	Positive/Total
Naïve	NV/NC[c]	0/8
Naïve	Yes	0/8
Naïve	No	0/7
WIV	Yes	1/7
WIV	No	0/6
LAIV	Yes	0/4
LAIV	No	0/4

[a] Influenza naïve sow (Naïve), Whole-virus inactivated (WIV) vaccinated sow, Live-attenuated influenza virus (LAIV) vaccinated sow

[b] Piglets vaccinated at three days old with LAIV (Yes) or non-vaccinated (No)

[c] Non-vaccinated, non-challenged (NV/NC)

Discussion:

In this study we evaluated the ability of pandemic lineage H1N1 LAIV vaccine to protect piglets vaccinated at three days of age with varying maternal immune status against heterologous γ-cluster H1N2 IAV challenge. In addition, we investigated possible predictive measures of immunogenicity and cross-protection, which included mucosal IgA and peripheral cell-mediated immunity. Vaccination of piglets at three days of age was chosen to coincide with current industry processing of newborn piglets (tail docking, iron shots, etc). Vaccination of 3-day old piglets from naïve sows induced IAV-specific IgA detectable in the nasal wash, and a primed anamnestic T cell response that was detectable only after challenge. In addition, LAIV vaccination of 3-day old piglets provided against virus replication in the lungs following heterologous IAV challenge, and pneumonia scores were not statistically different when compared to non-challenged controls. These data suggest that one dose of LAIV vaccine to 3-day old piglets derived from IAV-negative sows is immunogenic and protective against heterologous IAV challenge.

Vaccination of sows with IAV vaccines is routinely practiced to induce passive transfer of immunity to piglets through colostrum and milk and subsequently protect young piglets from clinical influenza. Passively acquired immunity has been shown to reduce infection and disease against strains similar to that in the IAV vaccine until maternal antibody titers wane below a protective threshold (28). In addition, maternal immunity can interfere with piglets' immune response to vaccination or infection (23). In this study we evaluated LAIV vaccination immunogenicity and efficacy in 3-day old piglets that had suckled from either WIV- or LAIV-vaccinated sows. Maternal immunity was successfully transferred to piglets prior to vaccination as piglets from naïve sows did not have measurable serum HI titers while piglets from both WIV- and LAIV-vaccinated sows had serum HI titers to vaccine virus (Table 1). The presence of either WIV or LAIV MDI interfered with the ability of the LAIV vaccine to induce a measurable mucosal IgA response in piglets 42 days post vaccination (Figure 1). IAV-specific IgA in nasal wash from LAIV vaccinated piglets with WIV or LAIV MDI was significantly lower than LAIV vaccinated piglets with naïve MDI. This data is consistent with previous studies by our research group that have shown maternal immunity interferes with LAIV vaccination's ability to elicit measurable antibody in serum, nasal wash, or BALF (17, 18). Taken together these studies suggest that mucosal antibody is not an effective live-animal sample source for evaluating LAIV vaccine efficacy when administered in the presence of IAV-specific MDI.

LAIV vaccines delivered intranasally have been shown to induce IAV-specific T cell responses in pigs (16, 29, 30). Peripheral T cell responses to LAIV vaccination were low to both homologous vaccine virus and heterologous challenge virus IL/10 (Figure 2, black bars). However, following challenge a rapid anamnestic peripheral IFN-γ T cell response was appreciated in LAIV vaccinated piglets derived from naïve and WIV vaccinated sows, while piglets given LAIV in the presence LAIV MDI had a trend for increased T cell IFN-γ responses post challenge. These data suggest MDI does not interfere with the priming of peripheral IAV-specific T cell immunity by LAIV vaccination, although heterologous IAV challenge was required to expand the response to a detectable level. Previous studies have shown LAIV vaccination of piglets with WIV MDI induced a detectable peripheral T cell response prior to challenge (17), however pigs in that study received a second vaccination at 21 days post priming (LAIV administered at 21 and 42 days of age), and pigs that received a single dose of LAIV at 21 days of age only did not have a measureable peripheral IFN-γ T cell recall response. Collectively, these data suggest one dose of LAIV vaccine to piglets with influenza specific MDI does not elicit a peripheral T cell response that is measureable in the IFN-γ ELISpot assay, but further testing may be required to identify the optimal assay for evaluating T cells in a recall assay to determine if peripheral cell-mediated immunity could be used a predictive measure of cross-protection.

Although LAIV vaccination of piglets with IAV-specific MDI did not elicit measureable peripheral IAV-specific antibody or IFN-g producing T cells, LAIV vaccination did limit lung lesions following heterologous challenge. Piglets given LAIV in the presence of LAIV MDI did not have any measureable lung consolidation/pneumonia (Figure 3) following challenge, and macroscopic pneumonia in this group was significantly lower than piglets vaccinated with LAIV in the presence of naïve MDI or WIV MDI. This suggests LAIV vaccination, reduces influenza-associated pneumonia.

Vaccine efficacy was also measured by the presence or absence of virus in the lungs following challenge (Table 3). Virus was not recovered from the lung lavage collected on day 5 post-challenge from any vaccinated or non-vaccinated pigs

post challenge. These results were somewhat unexpected, as we anticipated isolating virus from the lungs on non-vaccinated/challenged piglets derived from naïve sows. Prior work from our group has shown that LAIV vaccination does not induce a robust serum HI titer (13, 19, 22), thus we evaluated the immune status of piglets in this study using the Multispecies AI ELISA test, which measures antibody against the IAV nucleoprotein (NP). Using serum collected on the day of challenge (day 42 post-vaccination) we found that all of the piglets were IAV NP antibody positive regardless of assigned vaccination status, suggesting that the LAIV transmitted amongst piglets. We had performed a small pilot study to evaluate transmission of the LAIV, where groups of 3-week old piglets were vaccinated and non-vaccinated contacts introduced immediately following vaccination. In that study, vaccine virus was not recovered from the nose of any naïve piglet, nor did they seroconvert using HI or Multispecies ELISA (data not shown). Data from the current study suggests LAIV vaccine transmitted between 3-day-old vaccinated and non-vaccinated litter mates. Therefore, while our data suggests LAIV vaccination of three day old piglets with IAV-specific MDI is efficacious (particularly when compared to piglets given LAIV with WIV-derived MDI), the experiment remains somewhat incomplete without the proper non-vaccinated challenged controls.

Overall, the detection of LAIV-specific immunity following administration of a single dose of LAIV vaccine in the presence of IAV-specific MDI was limited, and our data suggest that mucosal antibody nor IFN-g ELISpot is the proper assay to measure LAIV immunogenicity in piglets that suckle IAV-specific MDI. However, our data suggest that cell-mediated immunity may be a good measure, as responses following challenge we detectable, suggesting these cells were primed by LAIV administration. Work is ongoing to repeat the experiment, with sow penning in separate isolation rooms to allow derivation of naïve piglets (i.e. one room is entirely vaccinated, while a second isolation room houses non-vaccinated sow and piglet controls). A limitation of this design is that piglets will not be randomized into groups to control for litter/genetic variables however this design will ensure vaccine is not transmitted between litters and true controls are obtained. We ask that modifications to the original budget be allowed, we are not asking for additional funds, but to move expenses across categories. Specifically, we did not use funds for salary on this project, and we ask that these be allowed to expend against consumables and additional animals.

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