RESEARCH REPORT
SWINE HEALTH

Title "Live-animal assay for identifying correlates of protection/cross-protection for intranasal swine influenza virus vaccines" – **NPB #13-122**

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Industry Summary. 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. While WIV vaccines may provide protection against infection with strains closely related to those in the vaccine, they provide limited cross-protection. 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. Live-attenuated influenza virus (LAIV) vaccines, delivered by the intranasal route have been shown to provide increased cross-protection when compared to WIV vaccines. In addition, replication-defective virus (RDV) vectors have been shown to provide cross-protection. However, the immune response after LAIV or RDV vaccination is different than that following WIV vaccination and immune assays typically used to predict cross-protection are inaccurate when applied to LAIV vaccines. Specifically, measurement of IAV-specific antibody in the serum using the hemagglutination inhibition (HI) assay does not accurately predict cross-protection. Because LAIV vaccines are delivered by the intranasal route (and some RDV vaccines are also) we evaluated the ability of mucosal antibody to predict cross-protective efficacy of LAIV and RDV vaccines. Groups of pigs were given either LAIV or RDV and then nasal wash and oral fluids were collected weekly for 6 weeks. Groups of pigs were then challenged with heterologous IAV and efficacy evaluated by reduction in nasal shedding of virus, virus load in the respiratory tract (trachea and lung), and protection against lung pathology. Our data indicate that oral fluids can serve as a useful sample source for evaluating the ability of LAIV to induce an immune response to IAV. In addition, IAV-specific IgA (measured in in oral fluids) were associated with protection against heterologous challenge.

Keywords: Influenza virus, live-attenuated influenza virus vaccine, immunity, correlate of protection, vaccine

Scientific Abstract:

Introduction

Stated Objectives from original proposal

The **primary objective** of this research is to optimize and validate a live-animal assay that provides a predictive measure of protection to heterologous influenza A virus (IAV) infection following intranasal vaccination with live-attenuated influenza virus (LAIV) or replication-defective virus (RDV) encoding IAV genes. Inactivated IAV vaccines do not provide adequate cross-protection against the large and diverse pool of IAV currently circulating in the North American pig population. Published research indicates that LAIV and RDV vaccines delivered by the intranasal route provide protection against diverse IAV, a key feature of their anticipated market availability and applicability. 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 cross-protection.:

- **Objective 1**: Evaluate cross-reactive cellular and mucosal antibody elicited following intranasal LAIV and RDV vaccination using samples from live animals.

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

Materials and Methods:

Vaccines and Viruses. NS1LAIV vaccine expressed the HA and NA surface genes from A/New York/18/2009 with the A/turkey/Ohio/313053/2004 internal genes. NS1LAIV was derived from the A/turkey/Ohio/313053/2004 internal genes, in which a truncated NS1 gene (1) was encoded. The replication-defective Adenovirus type 5 vector HA (Ad5-HA) vaccine was derived using the AdEasy system (Agilent, Santa Clara, CA) by cloning the A/California/04/2009 HA gene into pShuttle-CMV vector as previously described (2). Heterologous challenge viruses were H1N1 β-cluster A/Swine/Minnesota/03012/2010 (MN/10), and H1N2 γ-cluster A/Swine/Illinois/3134/2010 (IL/10) obtained through submission of clinical samples to the Diagnostic Virology Laboratory (USDA, Ames, IA). All vaccines and viruses were propagated in Madin-Darby Canine Kidney (MDCK) cells in serum-free OptiMEM media (Gibco, Grand Island NY) supplemented with TPCK-trypsin (Sigma, St. Louis, MO), L-glutamine and antibiotics, with the exception of the Ad5-HA which was grown in AD-HEK-293 cells with high glucose DMEM (Gibco) supplemented with FBS. Ad5-HA was purified using double discontinuous cesium chloride gradient as per manufacturer's instructions, and dialyzed as previously described (2). 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 (PRRSV). Pigs were administered Baytril (Bayer, Pittsburgh, PA) and Excede (Zoetis, Florham Park, NJ) upon arrival according to manufacturer's recommendations. IAV antibody status was confirmed as negative using the IDEXX Multi-Screen Ab test (Westbrook, ME). Piglets were randomly assigned to 8 pigs per group and immunized intranasally (IN) at 4 weeks of age and boosted 3 weeks later by the same route and dose. LAIV vaccines and WT virus were targeted at 10^6 $TCID_{50}$/ml at 2 ml per dose. Back titer of vaccines after administration indicated NY/09 WT immunized pigs received $10^{5.5}$ $TCID_{50}$, and NS1LAIV vaccinated pigs were given $10^{4.2}$ $TCID_{50}$. Ad5-HA pigs received 10^{10} $TCID_{50}$ in 2 ml. Non-vaccinated, challenged (NV/CH) controls received 2mL PBS IN. A subset of pigs in the NS1LAIV group, NY/09 WT group, and NV group (N=8 per group) were humanely euthanized 3 days post-primary immunization to assess viral attenuation. Three weeks following the boost, all pigs except for the non-vaccinated/non-challenged controls (NV/NC) were challenged IN with $10^{5.8}$ $TCID_{50}$ MN/10 (β), or IL/10 (γ). Pigs were humanely euthanized on 5 days post infection (dpi) for collection of samples to evaluate vaccine efficacy.

Sample collection. Pre-challenge. Nasal swabs were taken at 0-3 days post vaccination (dpv) for the subset of pigs necropsied on day 3 post-priming. Three dpv 8 pigs per group were euthanized for evaluation of attenuation. Serum, nasal wash (NW), and oral fluids (OF) were collected from all remaining pigs every seven days following primary immunization. Blood was collected by venous puncture into BD Vacutainer serum separator tubes (BD, Franklin Lakes, NJ). Serum was collected by centrifugation at 800 x g for 20 minutes, aliquoted and frozen at -80°C. NW was collected by instilling 5ml PBS into a nare and collecting effluent as previously described (3), aliquoted and frozen at -80 °C. On average 0.5-2 ml of nasal wash was collected. OF were obtained following previously described methods with few modifications (4). Briefly, cotton ropes were hung in each room for approximately 30 minutes. Ropes from each group were placed into separate ziplock bags. The rope was manually squeezed inside the bag and liquid sample decanted into a 50 mL conical tube. The tubes were centrifuged at 800 x g for 20 minutes, supernatant filtered through 0.45 µm syringe filters and immediately frozen at -80 °C. **Post Challenge.** Nasal swabs were collected daily beginning on the day of challenge [0 days post-infection (dpi)] through necropsy (5 dpi) using pre-wet polyester tipped swabs (Puritan, Guilford, ME) and stored frozen in 2 ml minimal essential media (MEM). At necropsy, trachea wash was obtained by removing the trachea prior to lung lavage. The trachea, one cm below the larynx to 2 cm above the bifurcation was removed and submerged in 3 mL MEM and vigorously agitated for 15 sec. The trachea was removed and media was aliquoted and frozen at -80 °C. Lung lavage samples were obtained by lavaging with 50mL MEM as previously described (5). An aliquot of lung lavage was plated on blood agar and Casman's agar plates for routine aerobic culture to confirm status of bacterial infection. All post challenge samples stored on ice until they were aliquoted and frozen within 2 hours of collection.

Pathological examination. At necropsy, the percent of lung affected with purple-red consolidation typical of IAV infection was evaluated (6). The total percent of pneumonia was calculated on the basis of weighted proportions of each lobe with respect to the total lung volume (7). A portion of the right middle lobe was fixed in 10% buffered formalin for 48 hours, processed by routine histopathologic procedures and stained with hematoxylin and eosin. Microscopic lesions were evaluated and scored by a veterinary pathologist blinded to treatment groups using parameters previously described (8).

Antibody evaluation. The hemagglutination inhibition (HI) assay was performed as recommended in the WHO animal influenza-training manual using turkey red blood cells and NY/09 WT virus or challenge virus H1 strains as the target antigen as previously described (9). Serum neutralization assay was performed by heat-inactivating serum samples and subsequent serial 2-fold dilutions of sera in MEM supplemented with 5% bovine serum albumin and antibiotics, and incubation with 100 $TCID_{50}$ before inoculation of confluent 96-well MDCK monolayers as previously described (10). Cells were incubated for 48 hours, fixed and stained for NP antigen by immunocytochemistry (11). OF and NW neutralization was performed similarly except NW samples were not heat-inactivated prior to use in the neutralization assay. Log_2 transformations of reciprocal titers were 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 as previously described (5) using the viruses listed in Table 1, with the exception that NW and OF samples were diluted 1:2 in PBS. Antibody levels are reported as the mean OD at 405 nm for each vaccine group. Endpoint antibody titers

were obtained by initially diluting OF and NW samples 1:2 in PBS and titrating two-fold in duplicate. The resulting OD data were modeled as a nonlinear function of the Log_{10} dilution using Graph Pad Prism (La Jolla, CA) log (agonist) vs. response-variable slope four-parameter logistic model. Endpoints were interpolated by using 2X the average OD of the non-vaccinated control as the cutoff. Total IgA and IgG ELISAs were performed using pig IgA or IgG quantification kit (Bethyl Laboratories, Montgomery, TX) following the manufacturer's protocol.

Virus titration. To determine viral loads, nasal swabs, trachea wash and lung lavage were titrated on MDCK cells to determine the $TCID_{50}$/mL as previously described (5). Briefly, frozen samples were thawed, filtered through 0.45 µm filters, and ten-fold serial titrations were made in triplicate in serum-free media containing TPCK-trypsin (Sigma, St. Louis, MO) and added to confluent MDCK monolayers in 96-well plates. Cells were incubated for 48 hours, fixed and stained for NP protein by immunocytochemistry (11). For each titration the Log_{10} transformed number of $TCID_{50}$/mL of each sample was calculated using the method of Reed and Muench (12).

Statistical Analysis. HI and neutralization titers were Log_2 transformed and viral titers were Log_{10} transformed for analysis and expressed as reciprocal titer. ELISA data is expressed as the average optical density at 405 nm. Statistical analysis was done in Graph Pad prism 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 cellular and mucosal antibody elicited following intranasal LAIV and RDV vaccination using samples from live animals.

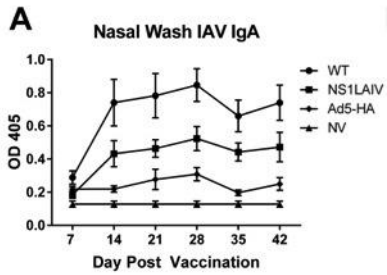

Figure 1. IAV-specific kinetics of mucosal IgA and IgG following immunization. IAV H1 pandemic cluster specific mucosal antibody from immunized animals measured by whole-virus ELISA compared to non-vaccinated (NV) animals. IAV-specific IgA detected in (A) nasal wash and (B) oral fluids. (C) IAV specific IgG detected in OF. Data expressed as mean ± s.e.m. OD405 of N=8 for indicated group

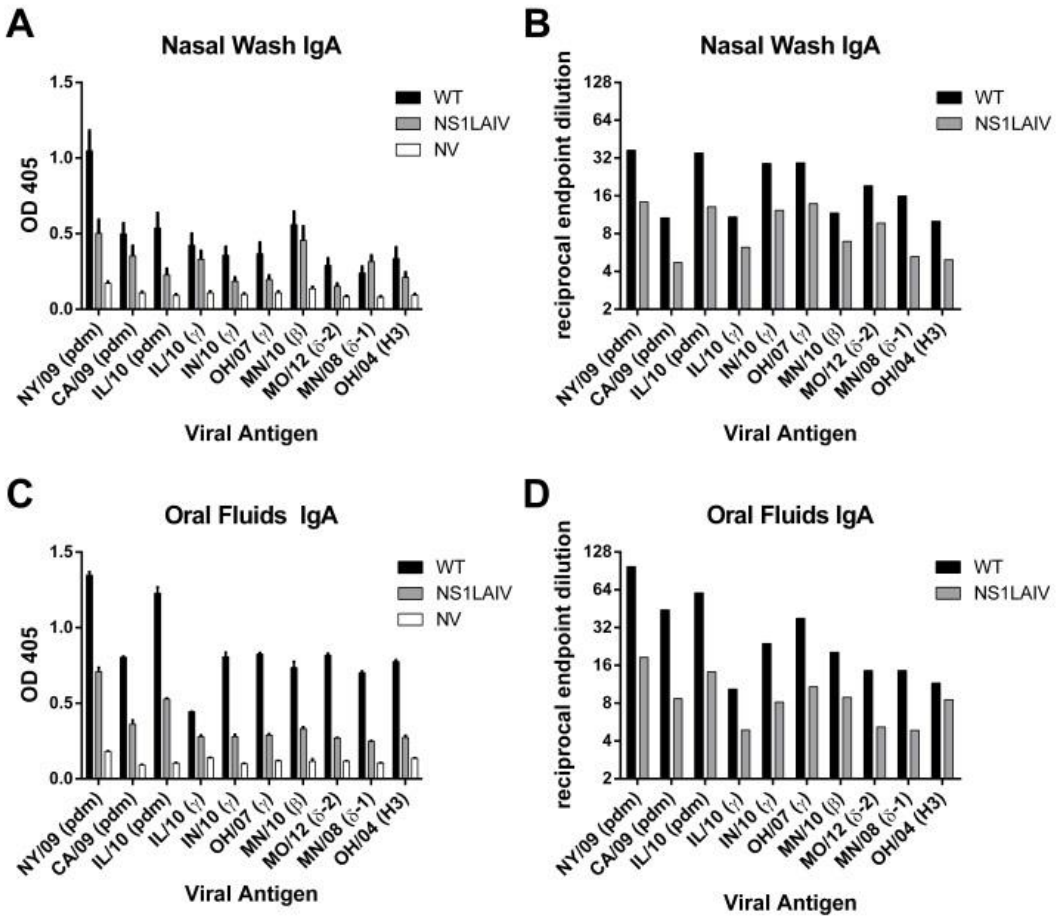

Figure 2. NS1LAIV vaccine elicits IgA cross-reactive to heterologous H1 and H3 IAV. Nasal wash and oral fluid samples were collected at 42 dpv and used to measure IAV-specific IgA by whole virus ELISA. Samples were tested against representative viruses from each H1 cluster and a H3 virus. Cross-reactive IgA data expressed as mean ± s.e.m. optical density (OD) 405 in (A) nasal wash and (C) oral fluids. Reciprocal endpoint titers of IAV specific IgA in (B) nasal wash samples pooled by respective group and (D) group oral fluids

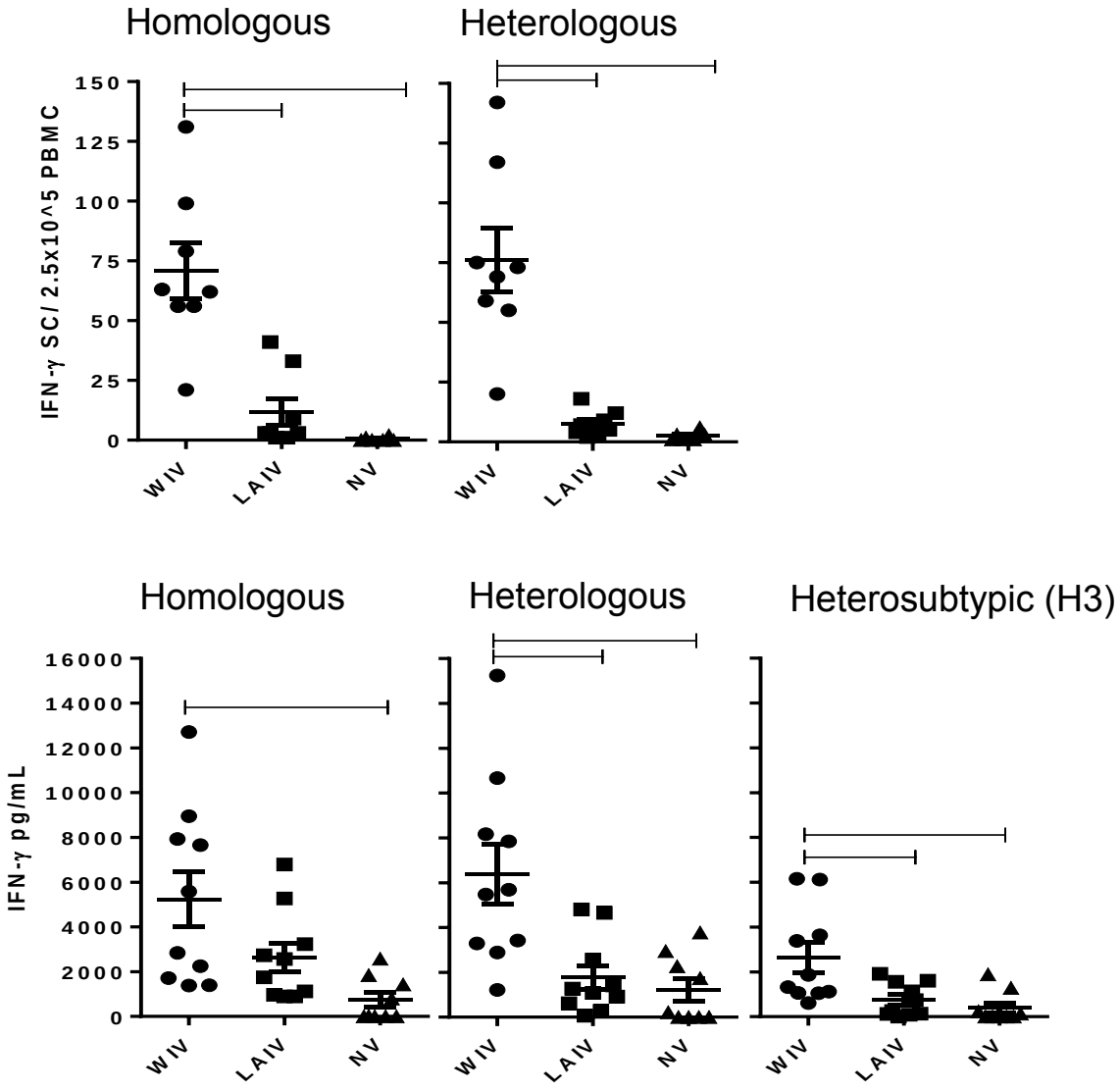

Figure 3. WIV vaccination induced a stronger peripheral cell-mediated immune response when compared to LAIV vaccine. Peripheral blood mononuclear cells were isolated 3 weeks post-boost for evaluation of IFN-g production by A)ELISpot and B) ELISA following restimulation with homologous, heterologous, or heterosubtypic IAV.

Table 1. Hemagglutination Inhibition titers measured at 42 days post vaccination

	Serum HI titer			Oral Fluid HI titer			Nasal Wash HI titer		
	Target virus			Target Virus			Target Virus		
Vaccine	NY/09 (pdm)	IL/10 (γ)	MN/10 (β)	NY/09 (pdm)	IL/10 (γ)	MN/10 (β)	NY/09 (pdm)	IL/10 (γ)	MN/10 (β)
NY/09 WT	180 ± 32.9	<10	12.5 ± 1.6	<10	<10	<10	<10	<10	<10
NS1LAIV	55 ± 7.3	<10	<10	<10	<10	<10	<10	<10	<10
Ad5-HA	31 ± 10	<10	<10	<10	<10	<10	<10	<10	<10
NV	<10	<10	<10	<10	<10	<10	<10	<10	<10

Table 2. Virus neutralization titers measured in serum, oral fluids, and nasal wash collected 42 days post vaccination.

	Serum Neutralization titer			Oral Fluid Neutralization titer			Nasal Wash Neutralization titer		
	Target virus			Target Virus			Target Virus		
Vaccine	NY/09 (pdm)	IL/10 (γ)	MN/10 (β)	NY/09 (pdm)	IL/10 (γ)	MN/10 (β)	NY/09 (pdm)	IL/10 (γ)	MN/10 (β)
NY/09 WT	168± 26.8	152 ± 24	54 ± 6.7**	8 ± 1	<2	<2	<2	<2	<2
NS1LAIV	74 ± 26.8	72 ± 17	25 ± 6.3*	<2	<2	<2	<2	<2	<2
Ad5-HA	<2	<2	<2	<2	<2	<2	<2	<2	<2
NV	<2	<2	<2	<2	<2	<2	<2	<2	<2

**p=0.005 compared to neutralization by NY/09
*p=0.03 compared to neutralization by NY/09
Statistical significance was measured by Kruskal-Wallis test N=8

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

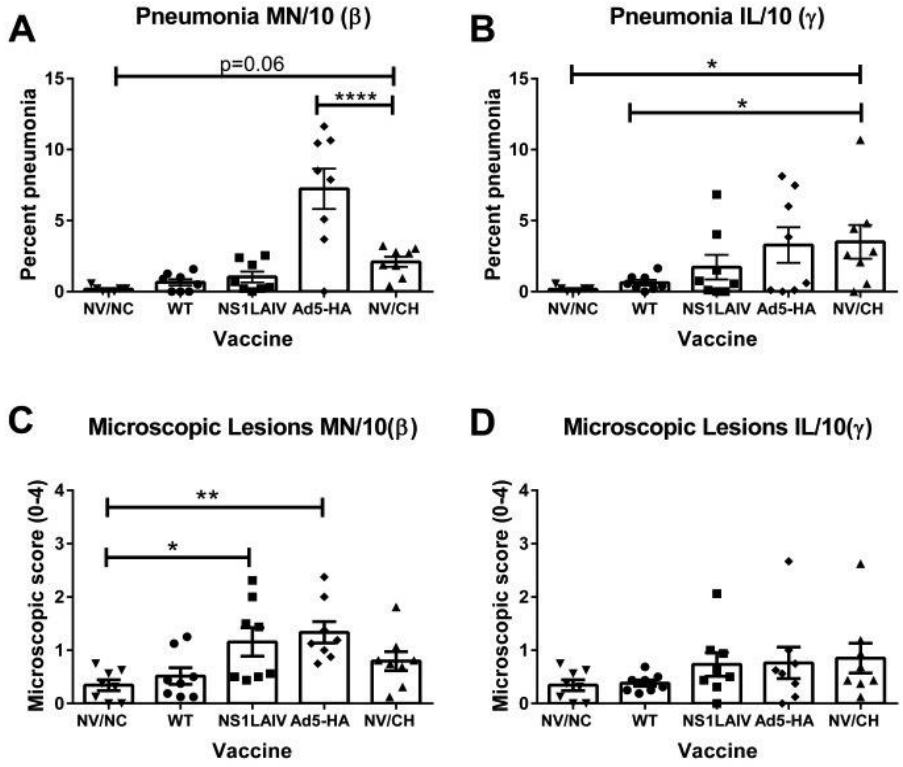

Figure 4. NS1LAIV or Ad5-HA vaccination does not reduce pneumonia following heterologous IAV challenge. Macroscopic lesion scores of animals following challenged with (A) β cluster H1N1 MN/10 or (B) γ cluster H1N2 IL/10 IAV compared to non-vaccinated, challenged (NV/CH) or non-vaccinated, non-challenged (NV/NC) animals. Microscopic lesion scores following challenge (C) β cluster H1N1 MN/10 or (D) γ cluster H1N2 IL/10 IAV. Data are expressed as the mean ± s.e.m. for N=8 and analyzed with Kruskal-Wallis test and Dunn's post test. $P<0.05$ were considered significant. One asterisk $p<0.05$, two asterisks $p<0.01$, four asterisks $p<0.0001$.

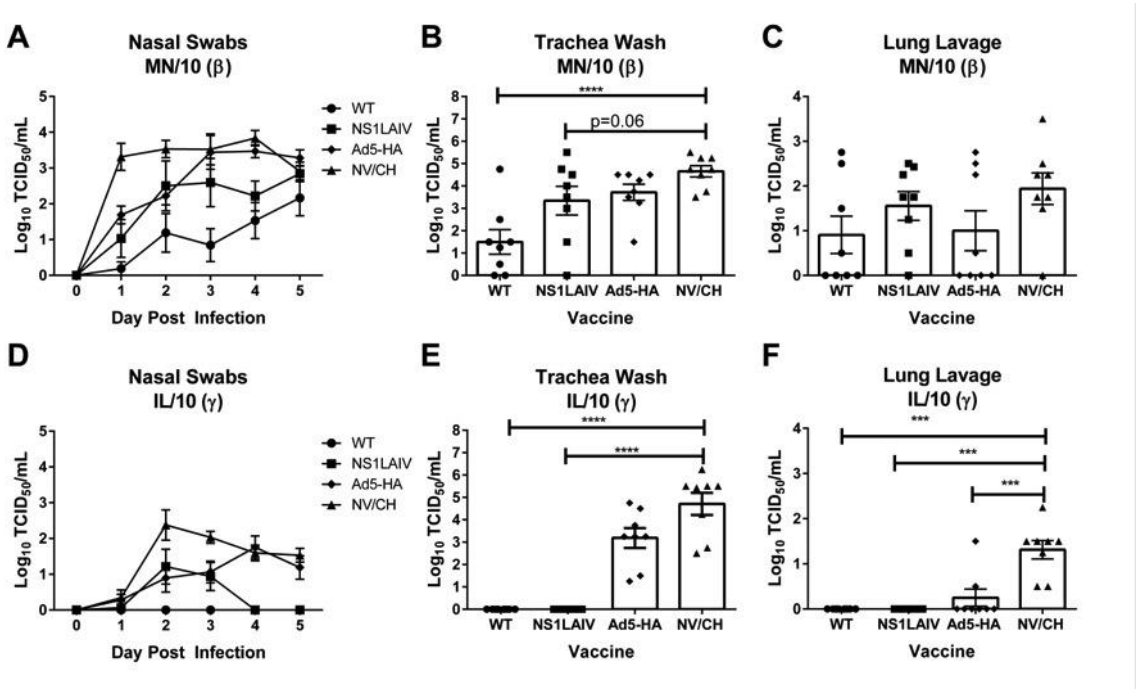

Figure 5. NS1LAIV and Ad5-HA vaccines reduce virus load and shedding following heterologous γ cluster challenge. Viral titers from animals challenged with MN/10 (β) (A-C) or IL/10 (γ) (D-F). Viral titers recovered from (A and D) nasal swabs 0-5 dpi, (B and E) trachea wash, or (C and F) lung lavage. Data are expressed as the mean ± s.e.m. for N=8. Kruskal-Wallis and Dunn's multiple comparison test were used for analysis. P<0.05 were considered significant. Three asterisks p<0.001, four asterisks p<0.0001.

Discussion

In this study we evaluated the ability of pandemic HA (H1) encoded in a LAIV or a replication-defective viral vector to provide cross-protection against representative heterologous β- and γ-cluster H1 IAV viruses compared to wild-type virus exposure. In addition, possible predictive immune measures of cross-protection were investigated, which included serum antibody and cell-mediated immune responses, as well as mucosal antibody responses. Vaccination with NS1LAIV resulted in significant cross-protection against the IL/10 γ-cluster IAV; however, protection against MN/10 β-cluster was reduced in comparison. Cross-protection against the MN/10 (β) virus was limited to a reduction in nasal shedding the first few days post-infection, with no detectable reduction in titers in the lower respiratory tract. Exposure to NY/09 WT virus protected against subsequent IL/10 (γ) virus challenge, but offered minimal protection against MN/10 (β) virus challenge. The pandemic cluster and γ-cluster viruses are more phylogenetically and antigenically related than the β-cluster and pandemic cluster viruses (13, 14); thus, the increased protection against IL/10 (γ) compared to MN/10 (β) was somewhat anticipated. Together, these data suggest a multivalent IAV vaccine may be necessary to afford protection against antigenically distinct H1 viruses.

Lung lesions are a common parameter measured following IAV challenge to assess IAV vaccine efficacy. WIV vaccines have been shown to provide protection against clinical disease and lung pathology, but not always limit virus replication and shedding. On the contrary, LAIV vaccination provided protection against virus replication in the lower respiratory tract and nasal shedding, but did not limit the induction of lung pathology. There was minimal induction of peripheral cell-mediated immunity following LAIV vaccination, which is in contrast to the response elicited following WIV vaccination (Figure 3). However, LAIV vaccination has been shown to induce mucosal cell-mediated immunity, which upon heterologous challenge, may be activated and control virus replication, but also result in immunopathology. This was also observed in pigs challenged with IAV following Ad5-HA vaccination, as lung lesions were not reduced when compared to NV/CH controls, though in some cases, there was a reduction in viral titers.

In addition to evaluating mucosal antibody responses, peripheral antibody responses specific to IAV were also evaluated because they are the traditional standard for evaluating IAV immunogenicity and predicting cross-protection. The HI assay is commonly used to evaluate WIV vaccine immunogenicity, and hyperimmune antisera has been used to evaluate antigenic relatedness between H1 viruses (REF). Serum HI antibody titers following WT infection, NS1LAIV and Ad5-HA vaccination were measured to assess peripheral, cross-reactive antibody responses elicited by the different

vaccine platforms. NS1LAIV vaccination elicited a serum HI antibody response, but it was limited to reactivity with homologous vaccine virus, which is in agreement with previous LAIV vaccine reports (5, 15-17). Average reciprocal HI titers for NY/09 WT and NS1LAIV immunized animals were greater than 40, which is considered protective. However, serum HI titers against heterologous challenge virus were below the limit of detection (<10) although partial (MN/10) or full (IL/10) protection was observed following challenge (Figure 4). These data reemphasize that LAIV vaccination does not induce a robust peripheral antibody response and the commonly used serum HI assay may not be suitable for predicting LAIV vaccine efficacy. Ad5-HA vaccination elicited a very low serum HI antibody against homologous virus (pandemic HA) at 42 dpv. This is in contrast to previous reports by our group which did not measure any serum antibody responses, including HI antibody, following IN Ad5-HA administration (15); however, this difference is likely due to differences in the vaccination regimen used as a single dose of Ad5-HA was used previously while two doses were used in this current study.

Oral fluids (OF) serve as an easy sample source to collect for evaluating porcine immunity, and thus, we evaluated OF as a sample for evaluating LAIV immunogenicity and predicting cross-protection. Nasal wash (NW) samples were also collected for comparison because of the intranasal delivery of vaccine. Our results indicate that OF contain IAV-specific IgA, and a greater amount than NW samples. In addition, IgA cross-reactive to a panel of H1 IAV strains could be detected in OF, and the decrease in titers was associated with phylogenetic relatedness of the H1 viruses. Cross-reactive IAV-specific IgA in NW following LAIV vaccination has been shown to correlate with improved heterologous cross-protection in humans and animal models of human IAV infection (17-19). Given the reductions in heterologous virus nasal shedding, and measureable IAV-specific IgA in mucosal samples pre-challenge (42dpv), we evaluated the association of cross-reactive mucosal IgA in immunized pigs to the level of cross-protection observed. Both NW and OF had measurable cross-reactive IgA (Figure 5A and C), however when using ELISA OD values as an association value, a broadly cross-reactive response was observed to all H1 cluster viruses as well as a H3 virus, with OD's 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 to similar OD values; however, this was not the case. Endpoint titrations of IAV-specific IgA in NW and OF separated the cross-reactivity between virus clusters (Figure 2B and D) and a general trend for decreasing IgA endpoint titers was evident as phylogenetically distant viruses were used as test antigen (pdm>γ-cluster>β-cluster>δ-cluster). However within a cluster certain viruses [i.e. IL/10(γ)] had significantly reduced endpoint titrations to the extent that we would not have predicted protection. However, strong cross-protection was observed as IL/10(γ) virus replication was significantly limited in LAIV vaccinated pigs. Moreover, the generally lower antibody response detected in the NS1LAIV vaccinated animals and the lack of detectable mucosal antibody response in Ad5-HA vaccinated animals, made this analysis most appropriate for the NY/09 WT exposed animals, which had higher IAV-specific IgA. These data suggest the use of OF IAV-specific IgA to predict cross-protection may require samples be collected from animals exposed to (or hyperimmunized) non-attenuated wild-type viruses to elicit a response for sufficient levels of mucosal IgA to be detected. This approach is currently used for evaluation of cross-reactive immunity against drifted human seasonal IAV viruses in which antisera from ferrets exposed to wild-type IAV is used in immune assays (20, 21).

Collectively, these results reiterate prior findings that show that LAIV vaccines provide cross-protection against antigenically distinct viruses, and that peripheral immune responses (serum antibody or peripheral cellular IFN-g production) cannot be used to accurately predict cross-protection. Mucosal antibody levels (IAV-specific IgA in NW and OF) measured in a whole-virus ELISA may serve as a better sample source and assay for evaluating protective efficacy of IN delivered LAIV vaccines. However, exposure to wild-type virus (encoding the same surface genes as LAIV) may be necessary for eliciting antibody levels to a high enough titer such that inferences of cross-reactivity can be made. Additional work is warranted to determine a threshold for protection and/or cross-protection.

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