RESEARCH REPORT
SWINE HEALTH

Title: Heterologous prime-boost vaccination protocols using whole inactivated influenza A virus vaccines to drive improved heterologous cross-protection**, NPB# 16-111.**

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Industry Summary

Influenza A virus (IAV) is a highly diverse and contagious swine pathogen that causes financial losses to the US swine industry through decreased production, increased vaccine and treatment costs, and often leads to elevated mortality rates due to an increase in secondary bacterial pneumonia. Additionally, IAV in swine (IAV-S) is a zoonotic pathogen with public health concerns. Therefore, veterinarians and pork producers have a major stake in finding alternative methods that can be implemented to reduce the negative impact of IAV in their herds and decrease the diverse ecology of influenza viruses circulating in swine. Whole inactivated virus vaccines (WIV) played an important role in the prevention and control of IAV-S in previous years. However, repeated failures of current WIV products and the overall lack of efficacious IAV vaccines have become extremely frustrating for producers, and the choices are limited in the types of vaccines currently licensed for use in swine, although custom made WIV vaccines are available. A major cause of vaccine failure on farms is presumed to be the diversity of strains that co-circulate in swine. Not only are there multiple subtypes and lineages of IAV co-circulating, but each lineage is prone to rapid mutation and evolution, which creates mismatches to strains used in the vaccines. Although protection against infection with identical or very similar IAV are adequate under ideal conditions, WIV vaccines do not prevent infection with mismatched, antigenically diverse strains of IAV. The goal of our proposal was to evaluate novel vaccination protocols to improve cross-protection induced by WIV vaccines by alternating the administration of relevant IAV antigens from the H1 subtype (heterologous prime-boost), in comparison to giving multiple viruses together in one vaccine. A similar strategy was reported to be effective against H3 subtype viruses in Europe. We selected 3 commonly identified H1 IAV, viruses 1, 2, and 3, and tested 6 different vaccination scenarios, with vaccines containing either one or two viruses. However, the heterologous prime-boost strategy was not successful for the H1 viruses evaluated in our study. In fact, all groups vaccinated with only WIV exhibited greater respiratory disease than the unvaccinated challenge control pigs and were not protected from infection. The only group included in our study that had protection from disease and infection were the pigs that were infected with the live mismatched Virus 1, allowed to recover, vaccinated with a WIV with the mismatched Virus 2, followed by challenge with Virus 3. These results demonstrate the extreme difficulty in controlling H1 viruses from the U.S. with WIV and the potential advantage for modified live virus vaccines against IAV in swine.

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Keywords
Influenza A virus, swine, vaccine, heterologous

Scientific Abstract
Influenza A virus (IAV) is a highly diverse and contagious swine pathogen, which causes financial losses to the US swine industry through decreased production, increased vaccine and treatment costs and in many cases elevated mortality due to an increase in secondary bacterial pneumonia. Additionally, IAV-S is a zoonotic pathogen with public health concerns for swine workers, attendees at swine exhibitions or fairs and the emergence of IAV with pandemic potential. Whole inactivated virus vaccines (WIV) played an important role in the prevention and control of IAV-S in previous years. However, repeated failures of current WIV products and the overall lack of efficacious IAV vaccines have become extremely frustrating for producers in the swine industry, and the choices are limited in the types of vaccines currently licensed for use in swine, even though custom made WIV vaccines are available. A major cause of vaccine failure on farms is presumed to be the diversity of strains that co-circulate in North American swine. Although protection against homologous infection is adequate under ideal conditions, WIV vaccines do not prevent infection with heterologous or antigenically diverse strains of IAV. We evaluated different vaccination protocols by alternating the administration of relevant IAV antigens from the H1 subtype, in comparison to giving antigens as multivalent vaccines. A similar strategy was reported to be effective against H3 subtype viruses in Europe. We tested 6 different vaccination scenarios, with vaccines containing either one or two viruses, and selected 3 commonly identified H1 IAV, Viruses 1, 2, and 3. The heterologous prime-boost strategy was not successful for the H1 viruses selected for our study. In fact, all groups that received only WIV exhibited greater respiratory disease than the unvaccinated challenge control pigs and were not protected from infection. The only group included in our study that demonstrated protection from disease and infection were pigs that were infected with live mismatched strain 1, allowed to recover, vaccinated with a WIV with mismatched strain 2, followed by challenge with Virus 3. This vaccination group was also the only group that had a robust immune response to the strains contained in the vaccine before challenge. These results demonstrate the extreme difficulty in controlling H1 viruses from the U.S. with WIV and a potential advantage for modified live virus vaccines against IAV in swine.

Introduction
Influenza A virus (IAV) remains an important cause of respiratory disease in swine and humans around the world. Influenza A viruses in swine (IAV-S) are members of the *Orthomyxoviridae* family with segmented, single-stranded, negative-sense RNA genomes (1). Clinical signs of influenza illness in pigs can display a range of severity, but often occurs as moderate to severe respiratory disease with high morbidity and rapid recovery (2). Influenza infections associated with other respiratory pathogens, as part of the porcine respiratory disease complex, can lead to complicated pneumonia and severe clinical signs. Mortality is typically low but nontrivial: wean to finish death due to influenza has been estimated as high as 2.9% and finisher mortality estimated at 2.5% in different field studies (3-5). Respiratory disease due to IAV has substantial economic burden as a result of weight loss, reduced weight gain and, in some cases, reproductive failure in affected sows due to high fevers (6). One production system estimated the cost of influenza at $10.31 per market pig with 64% attributed to lost production and 36% due to increased veterinary-related expenses and treatment (3).

USDA surveillance has revealed considerable genetic diversity that has led to phylogenetic clade terminology supported by antigenic data. Influenza viruses with hemagglutinin (HA) genes most similar to human seasonal H1 viruses were termed δ-cluster, and those more similar to the classical H1N1 viruses were termed α-, β-, or γ-clade (7-9). The δ-clade was further divided into δ-1 and δ-2

subclusters. Within the δ-1 H1 viruses in particular, this genetic clade has shown a high degree of expansion in the last 5 years, both in number of detections as well as in genetic diversity. Many phylogenetically distinct HA genetic clades co-circulate in North American swine populations and include: H1α, H1β, H1γ, H1γ-2, H1δ1 and H1δ2; the H1pdm09; and H3 cluster IV viruses that subdivided into emerging clusters H3 A–F (7, 10, 11), along with a recently introduced human seasonal H3 (12). The most frequently detected H1 viruses are γ and δ-1.

The complexity and genetic diversity demonstrated among the multiple IAV-S clusters bring additional challenges in diagnosis and control of this important swine pathogen. Vaccination against influenza is routinely used by U.S. swine producers and veterinarians to reduce clinical disease; however, infection, transmission and virus evolution may still occur. Traditional commercial swine influenza vaccines licensed in the U.S. are oil-in-water adjuvanted, bivalent or multivalent vaccines composed of inactivated whole viruses. Vaccination usually consists of two intramuscular injections, 2 to 4 weeks apart with all antigens administered in the same, repetitive doses (2). Intramuscular vaccination with killed vaccines can induce high levels of serum antibody to the IAV HA, but limited mucosal antibodies (13). Whole inactivated virus (WIV) vaccine protection depends on the priming antigen and challenge virus being closely related or antigenically matched. Vaccination with commercial WIV vaccines has been shown to partially protect against clinical signs and reduce nasal viral excretion when pigs are challenged with closely related viruses (14-18). However, when infected with antigenically distinct viruses, these vaccines have reduced efficacy (19-21). Intramuscular vaccination with killed vaccines can induce high levels of serum antibody to the IAV HA, but limited mucosal antibodies (12). The continual co-circulation of antigenically diverse swine IAV greatly complicates control by traditional vaccines. As a result, the increased use of herd-specific autogenous killed vaccines once represented half of the vaccine doses produced for swine in the U.S. (21). Unfortunately, autogenous vaccines also offer suboptimal cross-protection and doses produced of traditional WIV autogenous vaccine dramatically decreased in 2012 (personal communication, Byron Rippke, CVB). Collectively, veterinarians and swine producers have become extremely frustrated with the suboptimal performance of traditional WIV influenza vaccines used in swine and alternative vaccine platforms that offer broad cross-protection are needed.

Although WIV vaccines have demonstrated suboptimal performance against antigenically distinct IAV, a different approach to how inactivated vaccine antigens are administered has been explored with some success. Recent research in mice has demonstrated increased cross-protection against diverse H5N1 clades using a WIV heterologous prime-boost vaccine regimen (23). Compared to a homologous prime-boost protocol where the same antigen was administered in repetitive doses, a heterologous prime-boost approach by alternating H5N1 clade 1 and clade 2.1 (antigenically related but distinct clades) WIV vaccines demonstrated more robust hemagglutination inhibition (HI) titers against the vaccine strains and increased cross-protection against clades 2.2 and 2.3 that was not apparent in the mice that received only homologous doses of vaccine. A similar approach was recently described by Qiu et al., 2015 (3rd International Symposium on Neglected Influenza Viruses) where antigenically distinct European and North American H3N2 WIV vaccines were used in a successful heterologous prime-boost protocol (24). We will investigate the hypothesis that consecutive vaccinations with antigenically distinct influenza viruses of the H1 subtype will elicit a more broadly cross-protective antibody response using North American strains of IAV-S. If successful, a heterologous prime-boost vaccination protocol could be used as a gilt acclimation tool and tailored to the specific IAV-S circulating in certain breeding farms well as used to control IAV in downstream production flows. Similar vaccination approaches could be used in the entire breeding herd as well.

The cross-protective immune response observed when alternating antigenically diverse IAV in a WIV vaccine protocol may be due to a phenomenon called “back-boost” where the primary immune

response to the initial vaccine antigen, or natural exposure, is boosted by administration of an antigenically different IAV of the same HA subtype. Rather than resulting from the production of novel antibodies with extensive cross-reactivity, the back-boost appears most consistent with memory-cell stimulation and antibody recall (25). Repeated use of the same monovalent, or multivalent, WIV vaccines may lead to a phenomenon known as “antigen trapping” where binding of antigen by pre-existing cross-reactive antibodies and memory-cells decreases the antigenic load available for priming naïve B-cells and leads to a diminished novel antibody response to subsequent vaccine antigens. Alternatively, boosting with antigenically distinct heterologous IAV of the same subtype may allow conserved epitopes on the surface of the HA to create a “back-boost” response in concert with robust primary immune responses to the novel boost antigen.

Here, we investigate the heterologous prime-boost vaccination hypothesis using the two most predominant and contemporary IAV-S H1 clades (γ and δ-1) currently circulating in swine. Alternating IAV-S antigens in vaccine protocols specific to the IAV-S circulating on the farm or production system would be a rapid and convenient method to help swine producers control influenza infections on the farm. New protocols or different IAV vaccine antigens could be implemented quickly if the lateral introduction of a new IAV occurs, particularly in replacement gilts during acclimation periods prior to entry into the breeding herd. A heterologous prime-boost approach with WIV has the potential to increase the efficacy of a vaccination program.

Objectives

Objective 1. Evaluate protection against infection, shedding and lung lesions with a novel WIV heterologous prime-boost vaccination protocol using different H1 cluster IAV antigens compared to protection induced by a traditional bivalent WIV homologous prime-boost approach followed-by challenge with a contemporary heterologous IAV.

Objective 2. Evaluate the systemic and mucosal humoral cross-reactive immune response induced by a novel WIV heterologous prime-boost vaccination protocol using different H1 cluster viruses compared to a traditional WIV homologous prime-boost protocol.

Materials and Methods

Experimental Design. IAV were selected from the USDA IAV-S surveillance repository based on HA and NA phylogenetic characterization: Virus 1 was A/Swine/Minnesota/A01567490/2014 γ H1N1; Virus 2 was A/Swine/Nebraska/A01492366/2014 δ-1a H1N2; and Virus 3 was A/Swine/Missouri/A01444664/2013 δ-1b H1N2. Viruses were grown in Madin Darby Canine Kidney (MDCK) cells. Seventy-five, three-week-old crossbred IAV and PRRSV seronegative pigs were divided into eight groups: non-vaccinated/non-challenged (NC/NV), non-vaccinated/challenged (NV/Ch), and the five different vaccination protocol groups (Mon-Hom, γ-δMon-Het, δ-γMon-Repeat, Biv-Switch, Biv-Het) according to the experimental design in Table 1. Group 8 is similar to Group 4, but this group was exposed to live γ-cluster virus 1 prior to administering the δ1 Virus 2 Mon-Het WIV to mimic field scenarios where vaccines are administered to gilts with prior natural exposure. Pigs were administered a 2 ml intramuscular dose of their respective WIV vaccine twice at 4 and 7 weeks of age.

Table 1. Experimental design

Group	Vaccine Group	Vaccination	Boost	Challenge
1	NV/NC	None	None	None
2	NV/Ch	None	None	δ1 (Virus 3)
3	Mon-Hom	WIV δ1 (Virus 3)	WIV δ1 (Virus 3)	δ1 (Virus 3)
4	γ-δ Mon-Het	WIV γ (Virus 1)	WIV δ1 (Virus 2)	δ1 (Virus 3)
5	δ-γ Mon-Het	WIV δ1 (Virus 2)	WIV γ (Virus 1)	δ1 (Virus 3)

6	Biv-Repeat	WIV γ/δ1 (Virus 1 & 2)	WIV γ/δ1 (Virus 1 & 2)	δ1 (Virus 3)
7	Biv-Switch	WIV γ/δ1 (Virus 1 & 3)	WIV δ1 (Virus 2)	δ1 (Virus 3)
8	Live-γ/Mon-Het	Live γ (Virus 1)	WIV δ1 (Virus 2)	δ1 (Virus 3)

Pigs were challenged with 2 ml intratracheally and 1 ml intranasally with 1 x 10^5 $TCID_{50}$/ml of δ1 Virus 3 H1N2 at 10 weeks of age, three weeks post-boost vaccination. Inoculation was performed under injectable anesthesia. Nasal swab samples were collected pre-vaccination, pre-challenge (0 days post infection [dpi]) 1, 3, and 5 dpi. Serum samples were collected at pre-vaccination, post-boost vaccination, pre-challenge and 5 dpi. Pigs were humanely euthanized with a lethal dose of pentobarbital and necropsied at 5 dpi. Postmortem samples collected included bronchoalveolar lavage fluid (BALF), trachea and right cardiac or the affected lung lobe.

Viral Replication and Shedding. Nasal swab (NS) specimens were subjected to virus isolation. BALF and virus isolation-positive NS were subjected to virus titration in MDCK cells. Virus titer was calculated for each sample to evaluate the level of virus replication (26).

Pathology. At necropsy, lungs were evaluated and the percent of the surface affected with pneumonia calculated (27). Trachea and lung tissue samples were fixed in 10% buffered formalin, routinely processed and stained with hematoxylin and eosin. Microscopic lesions will be evaluated by a veterinary pathologist blinded to treatment groups, and scored according to previously described parameters. An individual composite score for each pig will be computed for lung and trachea microscopic lesions. Immunohistochemical (IHC) was used to confirm influenza A virus-specific antigen in lung tissues (28). IHC scores will be subjectively evaluated for each lung and trachea.

IAV Antibody Assays. Sera samples were heat inactivated at 56°C for 30 min and treated to remove nonspecific hemagglutinin inhibitors and natural serum agglutinins. HI assays were performed with each virus (γ Virus 1, δ1 Virus 2, and δ1 Virus 3) and turkey red blood cells using standard techniques (29). Enzyme-linked immunosorbent isotype specific assays (ELISA) were performed to detect NP antibodies to indicate exposure to IAV using a commercial kit (IDEXX). Additional custom ELISAs (30) were also performed using the γ and δ1 clade viruses (Virus 2 & 3).

Statistical Analysis. Analysis of variance (ANOVA) with a p-value ≤ 0.05 considered significant (GraphPad Prism, GraphPad Software, La Jolla, CA) was performed to analyze log10 transformed BALF and nasal swab virus titers, log2 transformations of HI reciprocal titers, mean ODs for ELISA assays and macroscopic and microscopic pneumonia scores. Kruskal–Wallis tests were used to analyze microscopic pneumonia and IHC scores. Response variables shown to have a significant effect by treatment group was subjected to pair-wise comparisons using the Tukey– Kramer test or the Dunn's test with Bonferroni correction.

Results

Objective 1: *Macroscopic and microscopic lung lesions.* The average macroscopic lung lesions by group are shown in Figure 1. Groups 3-7 had mean macroscopic pneumonia significantly greater than the non-vaccinated challenge control group. Mean microscopic lung and trachea lesions are reported in table 2. Group 8 lung histopathologic lesion scores were similar to the non-vaccinated, non-challenged group 1. In contrast, groups 2-7 lung lesions scores were significantly higher compared to group 1 and 8, and all WIV vaccinated groups except Group 8, the group first primed by live virus, had significantly higher lung lesion scores compared to the non-vaccinated, challenged pigs. Non-vaccinated and non-challenged group microscopic trachea scores are similar in group 1, 2 and 8 where pigs were non-vaccinated and non-challenged, non-vaccinated and challenged and pigs that were primed with live IAV, respectively. Vaccine groups 3, 4, 5, and 7 had significantly higher trachea scores compared to groups 1, 2 and 8.

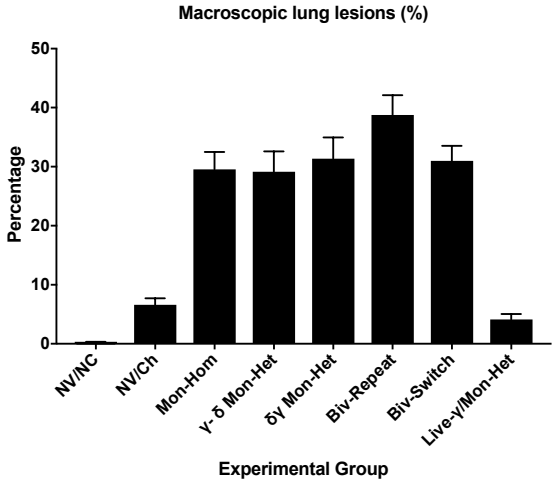

Figure 1. Mean percentage of macroscopic lung lesions. A visual estimate of the surface area of the lung consistent with influenza pneumonia was assessed for each pig and then averaged for each group. Means with standard error of the mean are shown for each group.

Immunohistochemistry (IHC) scores for lung and trachea are reported in table 2. Challenge virus was not detected by IHC in groups 1, 5 and 8 although differences in IHC lung scores were minimal and similar between groups 1, 2, 3, 4, 5, 7, and 8. Group 6 pigs vaccinated with the WIV γ/δ1 (Virus 1 & 2) twice and challenged with the δ1 (Virus 3) had the highest IHC lung score that was significantly higher compared to the pigs administered the live IAV vaccine. Trachea IHC scores were not significantly different across all groups.

Table 2: Composite microscopic lung and trachea scores and composite immunohistochemistry lung and trachea scores.

Group	Lung Composite Score* (0-22)	Trachea Composite Score (0-8)	IHC: Lung Score (0-8)	IHC: Trachea Score (0-4)
1	1.4 (0.43)^{a}	0.0 (0.26)^{a}	0.0 (0.06)^{ab}	0.0 (0.06)^{a}
2	8.0 (0.30)^{b}	2.3 (0.26)^{ab}	0.4 (0.06)^{ab}	0.4 (0.06)^{a}
3	15.8 (0.66)^{cd}	5.4 (0.17)^{cd}	0.1 (0.04)^{ab}	0.0 (0.00)^{a}
4	19.4 (0.28)^{cd}	6.1 (0.12)^{d}	0.3 (0.06)^{ab}	0.1 (0.04)^{a}
5	19.8 (0.16)^{d}	6.5 (0.17)^{d}	0.0 (0.00)^{a}	0.6 (0.09)^{a}
6	17.8 (0.26)^{cd}	4.1 (0.27)^{bc}	0.6 (0.08)^{b}	0.2 (0.06)^{a}
7	15.2 (0.63)^{c}	4.8 (0.15)^{cd}	0.2 (0.05)^{ab}	0.5 (0.09)^{a}
8	2.5 (0.32)^{a}	0.4 (0.08)^{a}	0.0 (0.00)^{a}	0.0 (0.00)^{a}

*Means with standard error of the mean. Rows within the same column with different superscripts are significantly different (P<0.5).

Virus replication. The vaccine regimen given to groups 2-7 did not protect from virus replication in the lungs. Likewise, significant levels of virus were detected in nasal swabs on at least one of the days sampled. Consistent with the observed protection from lung lesions, Group 8 primed with the mismatched live Virus 1 and boosted with Virus 2 WIV did not have significant levels of virus detected in the lungs or nose.

Table 3. Virus titers in bronchoalveolar lavage fluid (BALF) and nasal swabs.

	Virus titers (log10/ml)		
	BALF	Nasal Swab	
Group	5 DPI	3 DPI	5 DPI
NV/NC	0[a]	0	0
NV/Ch	6.6±0.32[b]	2.67±1.74	0.97±1.65
Mon-Hom	2.9±2.65[c]	1.43±2.04	0.20±0.43
γ - δ Mon-Het	3.4±2.67[c]	2.00±1.96	0.76±1.11
δ - γ Mon-Het	4.0±2.19[c]	2.45±1.92	1.05±1.60
Biv-Repeat	2.9±2.85[c]	2.88±2.00	1.52±1.72
Biv-Switch	6.0±1.70[b]	1.27±1.83	2.20±1.71
Live-γ/Mon-Het	0.6±1.73[a]	0	0

Objective 2. *Antibody response.* Homologous HI titers were modest (<40) in the WIV groups and there was no advantage seen in HI response following dose 2 of WIV with heterologous virus (data not shown), although all groups seroconverted to WIV vaccines and live exposure based on the NP ELISA (Figure 2A). All vaccinated groups also had antibodies that bound to Virus 3 whole virus in the IgG ELISA (Figure 2B). Further detailed analysis of HI data and whole virus ELISA with Virus 2 and 3 are on-going.

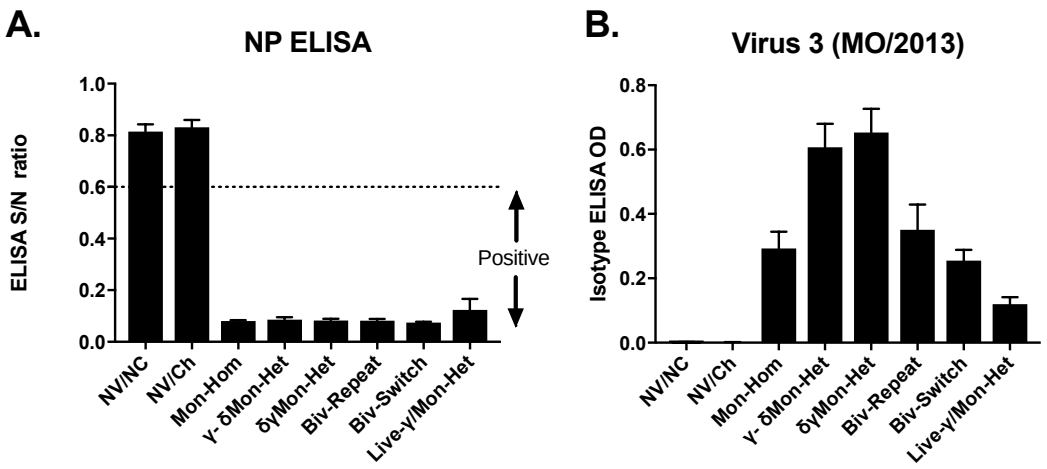

Figure 2. Antibody responses measured by NP ELISA and Virus 3 whole virus ELISA. All vaccinated groups were positive by NP ELISA and showed antibody binding to Virus 3. Data are reported as group means with standard error of the mean.

Discussion

In a short term immunity model, we tested to see if heterologous prime and boost with different combinations of monovalent and bivalent WIV would increase the breadth of protection against δ-1b H1N2 challenge. The HA proteins from the γH1N1 and the δ-1 lineage H1N2 viruses are antigenically distinct with minimal cross-reactivity. And, although from the same genetic ancestral lineage, the δ-1a and δ-1b H1N2 viruses also show loss in antigenic cross-reactivity. Due to the dominance of viruses with these HA clades found in the USDA surveillance, the chance of exposure to these combinations of viruses is a very possible scenario on pig farms.

The viruses used in WIV formulations, either given once or twice, did not induce levels of reciprocal HI titers predicted to be correlated with homologous or heterologous protection (≥40) prior to challenge. Each of the three viruses were incorporated into the WIV with the same quantity of HA

units and with the same adjuvant, so the minimal HI response highlights that some IAV strains are poor inducers of HI antibody in WIV. This is in contrast to the robust homologous HI response to the live challenge with the γH1N1. Although even homologous HI titers were modest in the WIV groups, all groups seroconverted to the vaccines and live exposure based on positive NP and Virus 3 IgG ELISAs. Further evidence of priming by the WIV was a rapid increase in HI titers in the WIV group at 5 dpi compared to 0 dpi. The absence of neutralizing antibody to the challenge Virus 3 is likely involved in the presence of the increased lung pathology, known as vaccine associated enhanced respiratory disease (VAERD). VAERD has been described previously with WIV in pigs under various conditions (27-32).

Based on macroscopic lung lesions and virus titers in the lungs, none of the WIV vaccine strategies provided complete protection from infection with the δ1 (Virus 3) challenge virus, and lung lesions were increased in the WIV vaccinated groups. Only group 8 pigs (live-γ/Mon-Het) primed with live virus followed by heterologous WIV were significantly protected from lung lesions and virus replication. These data support the conclusion that the WIV vaccine protocols did not protect pigs from infection and lung lesions and had more severe clinical disease compared to pigs that did not receive vaccine, but were challenged with the same IAV strain.

These results demonstrate the extreme difficulty in controlling H1 viruses from the U.S. with WIV and a potential advantage for modified live virus vaccines against IAV in swine. Implementing autogenous WIV in a herd without checking for post-vaccination increase in HI titers to the vaccine strains runs a risk of inducing VAERD. Further study into other combinations of viruses or different order of application of the two doses of WIV is warranted, as is investigation of the mechanism of the failure to induce robust HI response with these H1 viruses and the correlation of the non-neutralizing antibody response with VAERD.

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