RESEARCH REPORT
SWINE HEALTH

Title: Development of swine influenza virus platform vector vaccines to control swine influenza virus and porcine epidemic diarrhea virus -NPB #15-013

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Industry Summary:
Influenza A virus (IAV) causes significant losses to the swine industry. Here we constructed a novel attenuated live IAV vaccine designed to elicit an immune response to conserved IAV proteins by removal of the highly variable hemagglutinin gene. A unique feature of this strategy was the ability of our engineered IAV vaccine to express protein derived from porcine epidemic diarrhea virus (PEDV). Laboratory characterization of the constructed IAV/PEDV vaccine strain found that the virus was capable of robust growth and expressed the PEDV S1 protein. Following two doses of vaccine delivered to pigs intranasally, pigs were challenged with PEDV or IAV to assess protection afforded by the vaccine. Analysis of sera collected from vaccinated pigs found that the vaccine failed to induce a measurable immune response. Likewise, analysis of IAV and PEDV shedding from pigs found no evidence for protection and suggested a possible enhancement of disease. Widespread severe clinical disease due to *Streptococcus suis* was manifest immediately following initial vaccination and may have adversely affected the immune system's ability to respond to the vaccine. Likewise, a number of viral co-infections were identified from the pigs during the experiment which may have adversely impacted vaccine performance.

Keywords: influenza, vaccine, respiratory disease, attenuated, porcine epidemic diarrhea virus**,** diarrhea

Scientific Abstract:
Influenza A virus (IAV) is a major cause of morbidity and economic loss in the swine industry. The newly discovered influenza D virus (IDV) is a seven-segmented virus that was initially isolated from swine but subsequently shown to exist in a bovine reservoir. Experimental inoculation of swine with IDV failed to produce any clinical disease and replication was limited to the upper respiratory tract. These results, in conjunction with a low seroprevalence in pigs (9.5%), suggest IDV is not a primary swine pathogen. A chimeric IAV was engineered by replacement of the IAV hemagglutinin (HA) gene with the hemagglutinin esterase fusion gene from IDV and replacement of the IAV neuraminidase gene with the S1 gene of porcine epidemic diarrhea virus (PEDV). This chimeric virus was further attenuated by mutating the HA trypsin-dependent cleavage site to an elastase-sensitive motif. Pigs were vaccinated twice intranasally with the chimeric viruses and challenged with a heterosubtypic IAV. No serological response to IAV or PEDV was

measured following vaccination and in contrast to our previous study, vaccine virus shedding was not detected following initial vaccination. Significantly higher IAV titers were measured in bronchoalveolar lavage fluids on day 3 post challenge for vaccinates as compared to controls however there were no differences between groups on day 5. Similarly, significantly higher PEDV titers were measured in feces from vaccinated pigs on days 2 and 4 post challenge as compared to controls. The shedding results for IAV and PEDV suggest a vaccine associated enhancement of infection however there were no differences between vaccine groups' histopathology scores. Of note, widespread severe clinical disease due to *Streptococcus suis* was manifest immediately following initial vaccination and may have adversely affected the immune system's ability to respond to the vaccine. Likewise, a number of viral co-infections were identified from the pigs during the experiment which may have adversely impacted vaccine performance.

Introduction:

Influenza viruses, members of the *Orthomyxoviridae* family, cause respiratory disease in a variety of species, including humans and swine (Tamura et al., 2005; Webster et al., 1992). Influenza A virus (IAV) and influenza B virus (IBV) are comprised of eight single-stranded negative sense RNA segments, whereas influenza C virus (ICV) and the newly proposed influenza D virus (IDV) consist of 7 segments (Tamura et al., 2005; Bouvier et al, 2008; Hause et al., 2013). ICV and IDV contain a hemagglutinin-esterase fusion (HEF) surface glycoprotein that performs the same receptor binding, fusion, and destruction functions as the IAV and IBV hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins (Matsuzaki et al., 1992; Suzuki et al., 2002; Rosenthal et al., 1998; Gao et al., 2008).

ICV and IDV do not cause serious illness in swine and a serosurvey of IDV in swine showed only 9.5% had measurable antibody titers (Hause et al., 2013; Guo et al., 1984). Virus replication was limited to the upper respiratory tract following experimental inoculation of swine. ICV and IDV differ from IAV and IBV such they utilize 9-O-acetyl-N-acetylneuraminic acid (Neu5,9Ac2) as receptors (Schultze et al., 1990). A lack of Neu5,9Ac2 in the swine lower respiratory tract may explain the inability of ICV/IDV to cause clinical disease in pigs and explain the low seroprevalence and infrequent detection in pigs.

Inactivated influenza vaccines afford protection by eliciting a humoral immune response primarily directed to the HA, which blocks receptor binding (Krammer et al., 2013). NA antibodies are also protective by limiting the spread of virus by blocking release from infected cells (Sylte et al., 2009). Recent studies in mice using an attenuated intranasal vaccine with a codon-deoptimized NS demonstrated heterologous protection despite a lack of protective hemagglutination inhibition assay antibody titers (Nogales et al., 2014). It was hypothesized that the codon-deoptimized NS virus was deficient in its ability to antagonize the innate immune system, thereby allowing for heterologous protection, possibly due to a cell-mediated response. A human adenovirus serotype 5 vectored vaccine expressing the NP of influenza was able to promote viral clearance and limit viral replication in pigs, facilitated via proposed T-helper 1 induction (Wesley et al., 2004). Together, these results suggest that influenza immunity is complex and represents a combination of a humoral immune response targeting immunodominant surface proteins and cell-mediated immunity focused on conserved T-cell epitopes.

While the aforementioned studies have shown the importance of the humoral immune response to the HA, as well as induction of a cell mediated response, another integral part of the immune response to influenza infection is the presence of IgA in the mucosal tissues of the respiratory tract (Hasegawa et al., 2014; Muramatsu et al., 2014). In contrast to IgG, IgA antibodies do not fix complement and are non-inflammatory in nature. Attenuated influenza virus vaccines given intranasally have been shown to induce higher levels of IgA than parenterally administered vaccines, as well as broader heterosubtypic immunity (Tamura et al., 2005; Muramatsu et al., 2014; Thomas et al., 2015). A method for attenuating influenza virus is by altering the HA cleavage site to an elastase-sensitive motif from a trypsin-sensitive motif (Gabriel et al., 2007; Masic et al., 2009). This previous work has shown that mutating the trypsin

specific arginine-glycine (Arg-Gly) cleavage site to the elastase-sensitive valine-glycine (Val-Gly) or alanine-glycine (Ala-Gly) yielded mutant viruses resistant to activation via trypsin-like proteases in a natural infection, but can be activated *in-vitro* with elastase supplementation. These mutant viruses were also attenuated in pigs (Masic et al., 2009).

PEDV was first detected in the U.S. in the spring of 2013 and rapidly spread throughout the country, causing the death of millions of pigs (Chen et al., 2014). Originally described in diarrheic pigs in Europe in the 1970's, PEDV has caused severe epidemics throughout Europe and especially Asia (Yang et al., 2013; Tian et al., 2013). PEDV is a member of the genus alphacoronavirus and is genetically closely related to another swine enteric pathogen, transmissible gastroenteritis virus (TGEV) (Song and Park, 2012). TGEV and PEDV are clinically difficult to distinguish. Both viruses are transmitted by the fecal-oral route and cause watery diarrhea in suckling pigs which commonly results in 30-100% mortality for a period of several weeks until immunity is established in the sow herd. Clinical severity is associated with pig age, with high mortality in nursing pigs and mild to unapparent disease in older animals.

PEDV is an enveloped virus with a large single-stranded RNA genome. The genome encodes four structural (spike, envelope, membrane and nucleocapsid) and three non-structural proteins (replicases 1a and 1b, and ORF3). The spike protein (S) is the main protein on the virion surface. The S protein is a glycoprotein that is essential for cell receptor binding and membrane fusion. The S protein likewise contains multiple neutralizing epitopes (Sun et al., 2007). Consequently, the S gene is the most variable region of the PEDV genome.

PEDV causes disease that can quickly kill young (<4 weeks of age) pigs and consequently lactogenic immunity is critical to protect piglets. Sows immunized by either natural exposure or vaccination produce antibodies which are secreted into the colostrum and milk. Both the nature and route of PEDV antigen exposure affect the immune response. While both IgG and IgA are important for passive immunity, IgA is more effective against orally transmitted pathogens as it persists longer in the gastrointestinal tract and has more potent virus neutralizing activity (Saif et al, 1972). IgA is most effectively stimulated when the immune system is presented antigen mucosally. The vaccines developed in this proposal will be designed to be given intranasally or orally to sows prior to farrowing, thus stimulating IgA secreting immunocytes that migrate to the mammary glands where they secrete IgA to the colostrum and milk. Passive antibodies can protect piglets as previous work has shown that piglets administered colostrum from cows hyperimmunized against PEDV afforded 100% protection to piglets challenged with PEDV (Shibata et al., 2001).

PEDV has proven difficult to control in areas where it has become endemic. In Asia, both inactivated and modified-live vaccines (MLV) are widely used. Inactivated vaccines have generally shown poor efficacy. Several MLV have shown efficacy to homologous challenge; however, genetic drift has resulted in circulating viruses with altered antigenicity, leading to loss of protection (Kweon et al., 1999; Song et al., 2006). In particular, a highly virulent PEDV was detected in China in 2011 that resulted in widespread severe epidemics despite the availability of numerous modified live vaccines (Pan et al., 2012). Subsequent genetic analysis found that highly virulent PEDV was genetically divergent from the MLV.

Another alphacoronavirus, TGEV, is genetically related to PEDV. Clinically and pathologically, TGEV is also similar to PEDV. TGEV was responsible for severe epidemics of gastrointestinal disease, including high morbidity and mortality, in the U.S. until the early 1990's and the emergence of porcine respiratory coronavirus (PRCV). PRCV was isolated from a porcine nasal swab and subsequently shown to be a variant of TGEV possessing a large deletion in the S gene (Wesley et al., 1991). Phenotypically, PRCV showed a change in tropism from enteric to respiratory and was non-pathogenic in naïve pigs. Immunization of pregnant sows with PRCV induced protective lactogenic immunity as evaluated in 4-day old pigs challenged with virulent TGEV (Wesley and Woods, 1993). PRCV was also shown to generate protective immunity after 6 days when administered to naïve pigs as young as 2 days

of age (Wesley and Woods, 1996). Additionally, PRCV could prime young gilts to induce a secondary immune response following exposure to virulent TGEV. Passive antibody transfer from PRCV-primed gilts gave increased survival rates in piglets challenged with TGEV as compared to piglets born to non-primed gilts (Wesley and Lager, 2003). This example illustrates the concept of the common mucosal immune system present in pigs where immunization via one mucosal site induces an immune response at distal mucosal surfaces and supports our vaccine strategy (Wilson et al., 2014).

Here, eight-segmented chimeric influenza viruses were generated containing six segments from IAV (A/California/04/2009 H1N1, segments M, NS, PB1, PB2, PA, NP), the HEF from IDV D/swine/OK/1334/2011 (D/OK), and the S1 gene from PEDV. A virus with elastase-dependent mutations in the HEF was also rescued. Protection to a heterosubtypic virus was evaluated in pigs vaccinated with the chimera vaccines.

Objectives:

Objective 1: Generate chimera influenza A/D viruses expressing porcine epidemic diarrhea virus (PEDV) spike (S) antigens. The complete S gene and a number of smaller S gene fragments will be cloned in our swine influenza virus (SIV) reverse genetics system and chimera influenza A/D viruses expressing PEDV S antigens will be rescued. Viruses will be characterized *in vitro* for growth and S antigen expression. The virus displaying the highest level of growth and S antigen expression will be identified. We hypothesize these viruses will replicate and be avirulent in pigs.

Objective 2: Generate single cycle infectious virus mutants for the lead vaccine candidate. The most promising vaccine candidate developed in Objective 1 will be attenuated by mutation of the hemagglutinin esterase fusion (HEF) trypsin cleavage site to that of elastase. Previous work has shown SIV with elastase dependent proteolytic cleavage of hemagglutinin (HA) are replication defective in pigs and induce superior immunity as compared to inactivated viruses while likewise being unable to cause disease.

Objective 3: Evaluate efficacy of the influenza A/D chimera virus and elastase mutant expressing the PEDV S gene in nursery pigs with maternally derived SIV antibodies to SIV and PEDV challenge. Three week old pigs will be vaccinated intransally with the chimera virus and subsequently challenged with either SIV or PEDV. Protection will be assessed using both immunoassays and clinical signs.

Materials & Methods:

Cells and viruses. Madin-Darby canine kidney (MDCK) cells and swine testicle (ST) cells were maintained in minimal essential media/Earl's balanced salt (MEM/HBSS), and 293T (human embryonic kidney) cells maintained in Opti-MEM, all supplemented with L-glutamine and 7.5% fetal bovine serum. Challenge virus A/swine/MN/2073/2008 (MN08) was obtained from South Dakota State University (SDSU), with viral titer determined by titration on ST cells. PEDV strain USA/Colorado/2013 (CO13) was obtained from NVSL and growth on Vero76 cells in the presence of trypsin.

Plasmids. A reverse genetics system for A/California/04/2009 was kindly gifted from Dr. Wenjun Ma (KSU). D/OK HEF pHW2000 plasmid was a kind gift of Dr. Feng Li. PCR was performed using primers to amplify D/OK HEF and add 5'-*NotI* and 3'-*SbfI* restriction sites. The IAV HA packaging

sequences were synthesized as a DNA fragment that contained *NotI* and *SbfI* restriction sites immediately downstream and upstream of the 5' and 3' packaging sequences, respectively, separated by an 18bp linker (Gao et al., 2008). The synthetic DNA was used as template for PCR using universal HA segment-specific primers that introduced *BsmBI* restriction sites at the product termini. The PCR product was purified using a Qiagen PCR cleanup column and then digested with *BsmBI* and ligated in to the pHW2000 plasmid to generate pHW-HA_{pack}. The D/OK HEF PCR product was digested with *NotI* and *SbfI* and ligated into a similarly digested pHW-HA_{pack} to yield pHW-HA:HEF. Additionally, the D/OK HEF segment was mutagenized using the QuikChange II SL Site-Directed Mutagenesis kit (Agilent Technologies) to introduce the elastase-dependent motifs as described by (Masic et al., 2009). The IAV NA packaging sequences were synthesized as a DNA fragment that contained *NotI* and *SbfI* restriction sites immediately downstream and upstream of the 5' and 3' packaging sequences, respectively, and cloned into pHW2000 to generate pHW-NA_{pack}. The PEDV spike S1 region (nt 1-2214) was amplified off CO13 using primers designed to introduce 5'-*NotI* and 3'-*SbfI* sites. The PCR product was digested with *NotI* and *SbfI* and cloned into a similarly digested pHW-NA_{pack} to generate pHW-NA:PEDV S1. Likewise, a smaller region of the PEDV S gene (region coding amino acids 636-789) was amplified from CO13 and cloned into pHW-NApack to yield pHW-NA:$PEDVS1_{636-789}$. A codon optimized complete PEDV S gene was synthesized by Genscript. Both the complete codon optimized S gene and codon optimized S1 genes were cloned into pHW-NApack to yield pHW-NA:S_{opt} and pHW-NA:$S1_{opt}$.

Generation of viruses by reverse genetics. Viruses were generated using an eight-plasmid reverse genetic system as previously described (Hoffman et al., 2000). Briefly, 293T and MDCK cells were co-cultured at equal density in a six-well plate in Opti-MEM (Invitrogen) with 5% fetal bovine sera and incubated overnight. Prior to transfection, the wells were rinsed once with phosphate buffered saline (PBS). 100ng/uL of each of the eight plasmid constructs were transfected using lipofectamine 2000 transfection reagent (Life Technologies). After a six hour incubation, the transfection mixture was replaced with 1mL of Opti-MEM plus penicillin/streptomycin solution (HyClone). At 24 hours post transfection, 1mL of MEM containing TPCK-treated trypsin was added to each well. At 48 hours post transfection, supernatants were harvested and passaged to ST cells in MEM. Viruses containing mutations conferring elastase sensitivity were supplemented with 1µg/mL of elastase from porcine pancreas (Sigma). The elastase mutations were confirmed by sequence analysis.

Vaccination/challenge pig experiment. Fifty-six 3-week-old pigs were obtained from a high health herd that was positive for maternally derived antibody (MDA) to IAV by IDEXX SIV ELISA were shipped to South Dakota State University and allowed one week to acclimate prior to vaccination. Pigs were divided into five rooms and seven treatment groups (Table 1).

Group	#Pigs	Vaccine	Challenge
A	8	HEF_{WT}/PEDV	SIV
B	8	HEF_{WT}/PEDV	PEDV
C	8	$HEF_{elastase}$/PEDV	SIV
D	8	$HEF_{elastase}$/PEDV	PEDV
E	8	None	SIV
F	8	None	PEDV
G	8	None	None

Table 1. Vaccination/challenge experiment experimental design

Vaccines were formulated at 640 HAU/mL. Sixteen pigs in groups A and B received 4.2 $\log_{10}$ $TCID_{50}$ of HA.HEF:NA.PEDVS1 intranasally, sixteen pigs in groups C and D received 4.7 $\log_{10}$ $TCID_{50}$ of HA.HEF-elast:NA.PEDVS1 intranasally, and groups E, F, and G consisted received mock vaccinations. All animals were re-vaccinated at 21 days, again formulated to 640 HAU/mL. Group A and B booster was 4.4 $\log_{10}$ $TCID_{50}$ and group C and D booster was 6.9 $\log_{10}$ $TCID_{50}$. Prior to the day 35 challenge, groups A and C were moved together and groups B and D were roomed together. At 35 days all animals, in groups A, C, and E were challenged intranasally with 4.0 $\log_{10}$ $TCID_{50}$ of MN08 and groups B, D, and F were challenged with 3.0 $\log_{10}$ $TCID_{50}$ of CO13. All animal experiments were conducted at South Dakota State University in accordance with the Institutional Animal Care and Use Committee guidelines.

Clinical observation and sampling. Pigs were bled on day 0 to determine serostatus for PEDV and SIV and fecal and nasal swabs were analyzed by PCR to verify absence of PEDV and SIV. Sera was also collected at day 21 and 35. Nasal swabs were collected from groups A, C, E, and G and fecal swabs from groups B, D, F, and G at day 0 (pre-vaccination) and day 1 post initial vaccination as well as day of booster, day 21, and one day post boost at day 22. Nasal swabs were collected from the same groups at day of challenge and days 1, 3, and 5 post SIV challenge (days 35, 36, 38, and 40) to monitor SIV shedding. Fecal swabs from groups B, D, F, and G were obtained at day of challenge and days 2, 4, and 6 post PEDV challenge (days 35, 37, 39, and 41). All swabs were placed in MEM and frozen at -80°C until ready for further testing. Animals were monitored daily for signs of illness. Three animals from groups A, C, E, and G were euthanized on days 3 and 5 post challenge and lungs and bronchial alveolar lavage fluids (BALF) samples collected and frozen. BALF was collected by adding 50mL of DMEM to the trachea from lungs collected *in toto* and gently palpating the lungs before pouring off fluid. Lung tissues were collected from the right cardiac lobe and analyzed by titration and histopathology. Two pigs in groups B, D, and F were euthanized on days 2, 4, and 6 post PEDV challenge and three samples from different sections of the intestine were collected and fixed in 10% buffered formalin.

Virus shedding from nasal swabs, fecal swab, and BALFs. Nasal and fecal swabs were tested by quantitative real-time reverse transcriptase PCR (qRT-PCR). RNA was extracted using the MagMax RNA isolation kit. qRT-PCR for SIV was performed according to the approved National Animal Health Laboratory Network universal IAV protocol employed at KSVDL. qRT-PCR for PEDV was performed by KSVDL. Nasal swabs from day 0, 1, 3, and 5 post challenge, as well as the BALF samples were titrated on ST cells. Samples were thawed and vortexed, then centrifuged at 2000 RPM for 5 minutes, supernatants were then serially diluted and plated (four replicates) onto confluent ST cells in 96-well plates. Plates were monitored for cytopathic effects (CPE) and confirmed via hemagglutination assay using turkey red blood cells. Titers were calculated by Spearman-Karber method.

IgA ELISA.

Enzyme-linked immunosorbent assay (ELISA) was performed on BALF samples, as previously described (Gauger et al., 2014). Briefly, vaccine virus HA.HEF:NA.PEDVS1 and challenge virus MN08 were ultracentrifuged at 27,000 RPM for three hours through a 20% sucrose cushion and coated overnight onto ELISA plates separately, at 75 HA units/50uL. Recombinant PEDV S1 was obtained from Dr. Ying Fang and coated at 2.5ug/mL (100uL/well). The plates were blocked for one hour at room temperature using starting block (Fisher). Plates were rinsed three times with phosphate buffered saline (PBS) with 0.05% Tween 20 (PBST). BALF samples were diluted 1:2 with 10mM dithiothreitol (DTT) to disrupt mucus, and incubated for one hour at 37°C before running the assay. Samples were then diluted 1:2 in PBS to a final dilution of 1:4. Fifty microliters of each DTT treated BALF sample was added to duplicate wells on each antigen-coated plate. Plates were incubated for one hour at room temperature then washed three times with PBST. Fifty microliters of goat anti-pig IgA HRP secondary

antibody (Abcam) was applied at 1:1500 (diluted in starting block) and incubated for one hour at room temperature. Plates were washed three times with PBST and 50uL of ABTS ELISA substrate was added and incubated at room temperature approximately eight minutes. The reaction was stopped by adding 50uL stop solution (KPL). The optical density (OD) was read at 405nm using an automated plate reader. OD values were averaged for each sample.

Hemagglutination inhibition assay

Hemagglutination inhibition (HI) assay was used to detect antibodies to vaccine virus HA.HEF:NA.PEDVS and challenge virus MN08. The test was performed using turkey red blood cells, according to the WHO manual. Samples assayed included serum from day 0 (pre-initial vaccination), day 21 (pre-second vaccination), and day 35 (pre-challenge).

PEDV Indirect Immunofluorescence Assay

Serum samples from days 0, 21, and 35 were assayed for antibody titers to PEDV at KSVDL by indirect immunofluorescence using CO13-infected Vero cells.

Histopathology and Immunohistochemistry

Gross lung lesion scores were determined as previously described (Henningson et al., 2015). The lung samples from the right cardiac lobe were submitted for microscopic lung lesion evaluation. Lungs were examined for airway epithelial necrosis and loss, airway inflammation, airway lymphocytic cuffing, and interstitial pneumonia. They were scored on a scale of 0 to 4 and reported as an average, as previously described (Henningson et al., 2015). Immunohistochemical (IHC) examination scored the lungs based on airway and interstitium IHC and reported as averages. The pathologist was blind to the identity of animals and groups the lung tissues originated from.

Statistical Analysis

Statistical analysis was performed using JMP Version 12, SAS Institute Inc.

Results:

Strain construction

We had the complete codon optimized S gene of PEDV synthesized. Both the full length S and S1 (nucleotides 1-2214, 738 amino acids) genes from the synthetic codon optimized S gene were cloned into pHW-NA_{pack}. The wild type S1 gene and a smaller region of S1 (amino acids 636-789) were amplified from CO13 and cloned into the plasmid pHW-NA_{pack}. Viruses were rescued using a contemporary triple reassortant internal gene cassette-bearing reverse genetics system (A/California/04/2009, from Dr. Wenjun Ma). For virus rescue, the hemagglutinin esterase fusion (HEF) gene from D/swine/Oklahoma/1334/2011 was inserted into the hemagglutinin (HA) segment of the genome (HA packaging sequences). The PEDV S genes were inserted into the NA segment of the genome (NA packaging sequences). The resulting viruses thus have influenza A virus internal genes (non-structural genes, matrix, nucleoprotein, polymerase acidic, polymerase basic 1 and polymerase basic 2) with the NA being replaced by PEDV S or S1 and the HA being replaced by the influenza D virus (IDV) HEF gene (Figure 1). Viruses were rescued by transfection of the eight plasmids into 293T cells followed by passaging virus to swine testicle (ST) cells followed by passage to eggs and ST cells. All viruses were rescued with the exception of the full length S gene. We suspect the S gene was too large (~4kb) to be packaged into the influenza genome.

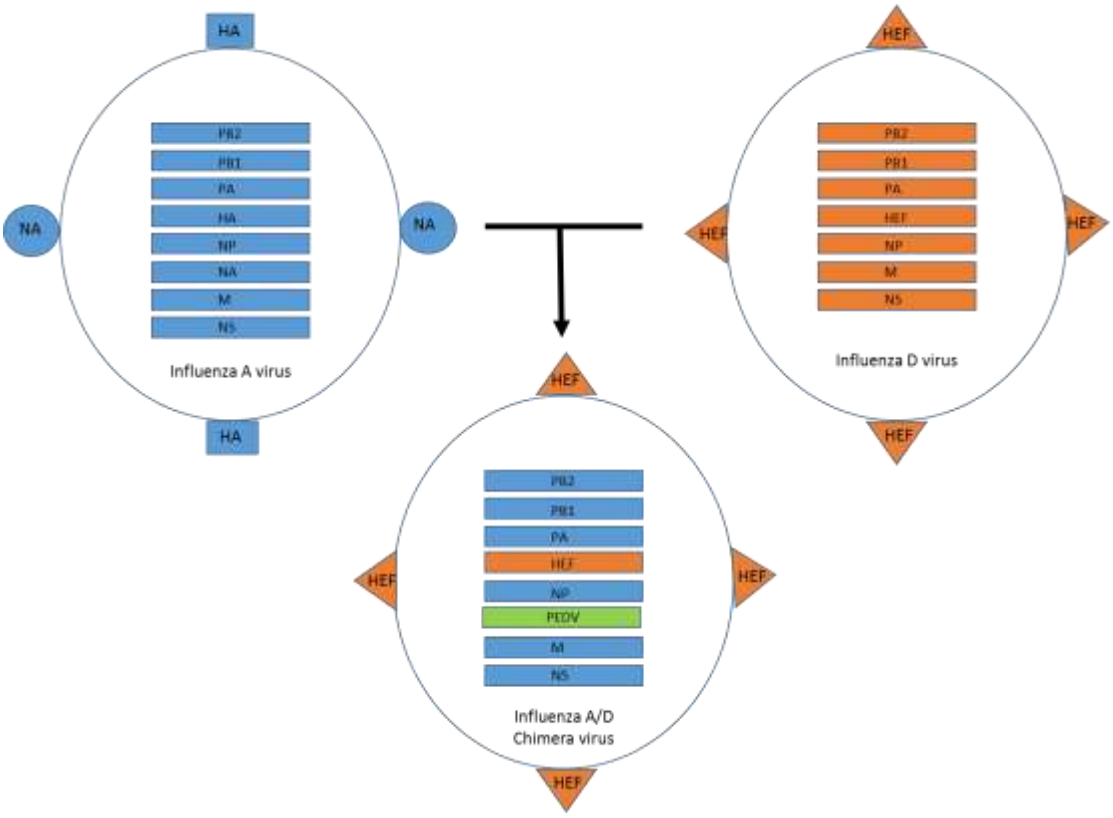

Figure 1. Diagram of strain construction. The HEF gene from IDV was cloned into the IAV genome using NA packaging sequences and the PEDV S1 gene was cloned into the IAV genome using HA packaging sequences.

Following virus rescue, full genome sequencing was performed to verify the proper genotype. Viruses were next passaged once on ST cells and IFA was performed using a monoclonal antibody generated against PEDV S1 (gift from Dr. Eric Nelson, South Dakota State University). The only viruses which stained positive were those expressing native S1 (both with wild type HEF and $HEF_{elastase}$, Figures 2a. and 2b., respectively). The elastase dependency for the virus carrying the $HEF_{elastase}$ bearing allele was confirmed *in vitro*.

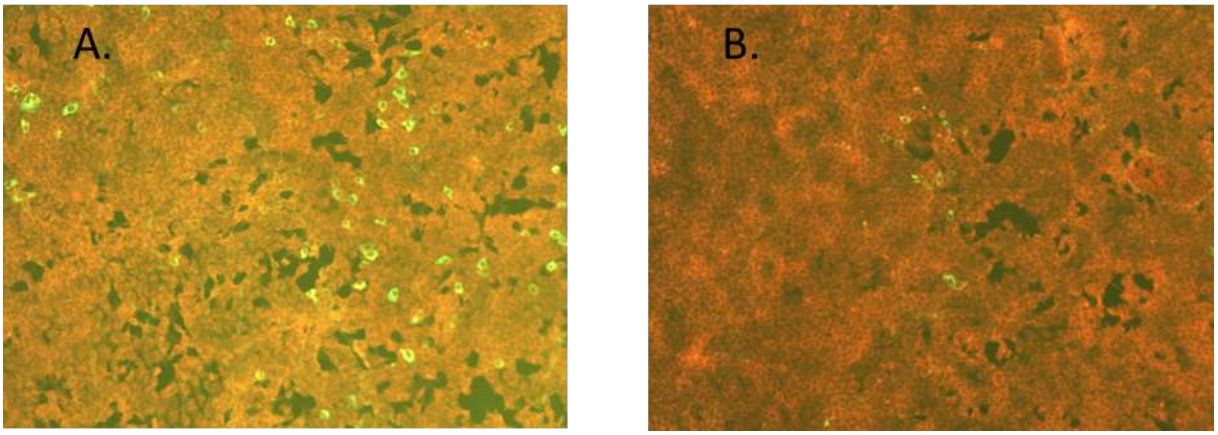

Figure 2. Chimeric influenza A/D viruses expressing native PEDV S1. Infected ST cells were fixed and stained with a monoclonal antibody generated against PEDV S1. A. wild type HEF, B. $HEF_{elastase}$

As only viruses carrying the native S1 expressed protein, these strains were selected for testing in pigs.

Vaccination

Litters were prescreened by ELISA to identify those with maternal antibodies to IAV. Average titers for pigs in all three treatment groups (A/B, C/D, and E/F/G) were positive (Figure 3, day 0; positive S/P<0.7). In contrast, sera had no measurable HI titer to IDV or measureable titer to PEDV at day 0.

Pigs were vaccinated intranasally on day 0 as listed in Table 1. Clinical illness was noted in a majority of the pigs beginning day 1 post vaccination. Clinical signs were noted in all rooms. Over the course of two weeks following initial vaccination, a total of six pigs died or were euthanized and they were submitted to the SDSU veterinary diagnostic laboratory for analysis. *Streptococcus suis* was identified as the causative agent. All pigs were treated with Excede periodically throughout the remainder of the study.

Nasal swabs collected on day 0 and day 1 post vaccination were all negative for IAV by PCR. Based upon our previous work, we had expected the wild type HEF bearing strains to replicate in the upper respiratory tract and be shed on day 1. All swabs collected on the day of booster vaccination (day 21) were also negative. One swab (out of 13) from a pig vaccinated with wild type HEF and 12/14 swabs from pigs vaccinated with $HEF_{elastase}$ were positive on day 22. All non-vaccinated control pigs were negative. These results were unexpected as our previous work found replication and shedding only for viruses carrying a wild type HEF.

Sera were collected on days 0, 21 and 35. While all groups were positive for IAV NP antibodies on day 0, antibody levels declined to negative levels by day 21 and remained negative at day 35 (Figure 3). There was no statistical difference in ELISA values between groups. All samples at all days were negative for antibodies to IDV on the HI assay. These results contrast our previous work where pigs vaccinated twice with viruses bearing a wild type HEF had mean HI titers of ~150 to IDV. All samples at all days were negative for antibodies to PEDV on the IFA assay.

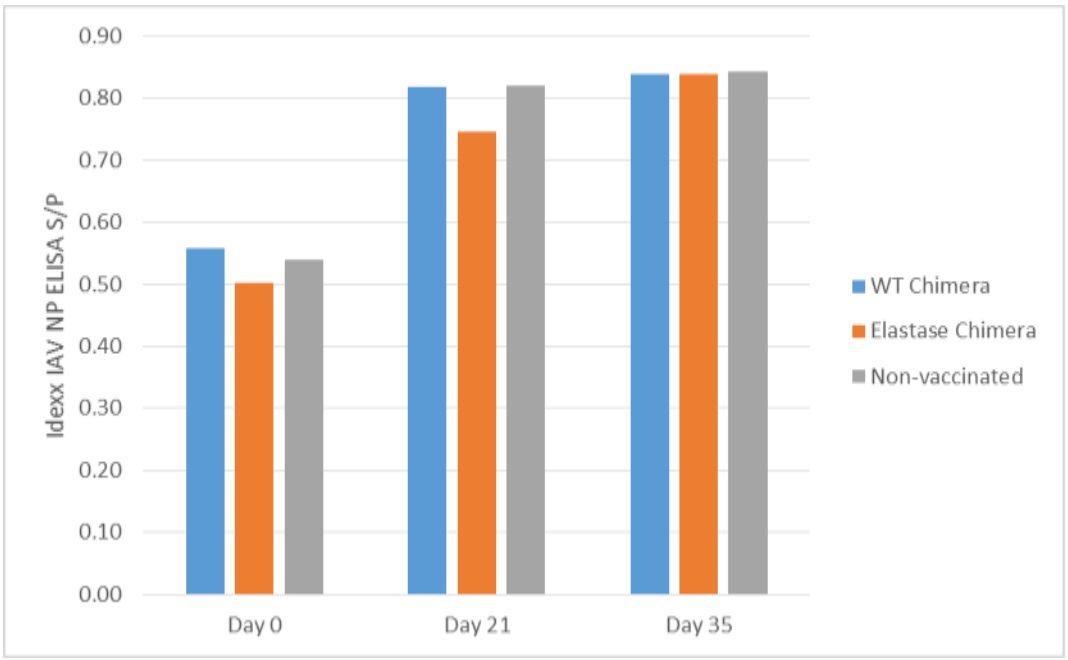

Figure 3. Mean IDEXX NP ELISA values on days 0, 21 and 35. Note: this is a competitive ELISA so lower values are increasingly positive. S/P<0.7 is positive.

Challenge

Nasal swabs collected on the day of challenge were negative for both IAV and PEDV by PCR. IAV titers were determined in nasal swabs on days 1, 3 and 5 post challenge by titration (Figure 4). All non-challenged pigs remained negative throughout the study. On day 1 post challenge, 3/7 non-vaccinated pigs had measureable IAV shedding (mean=1.1 $\log_{10}$ $TCID_{50}$/mL) while all vaccinated pigs were negative. On day 3 post challenge, pigs in both vaccinated and non-vaccinated groups shed approximately 1 $\log_{10}$ $TCID_{50}$/mL. On day 5, the viral titer in nasal swabs increased to approximately 2 $\log_{10}$ $TCID_{50}$/mL for both WT chimera and non-vaccinated control pigs however there was no statistically significant differences between groups at any time point.

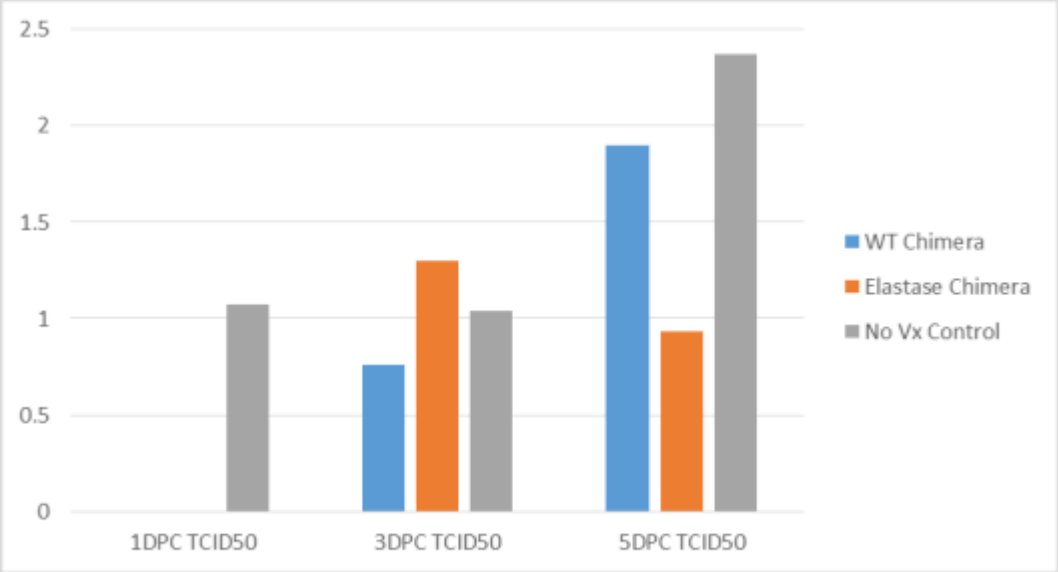

Figure 4. Mean influenza A virus titer in nasal swabs on days 1, 3 and 5 post-challenge. Titers reported are tissue culture infectious dose 50 per ml. No differences were statistically significant.

While titrating the nasal swabs, multiple different types of cytopathic effects (CPE) were evident. As IAV hemagglutinates turkey red blood cells, IAV titers were determined by adding TRBCs to the titration plates and titers were determined by HA activity. Swabs showing non-IAV CPE were analyzed by viral metagenomic sequencing. Complete genomes were assembled for porcine enterovirus G and porcine respiratory coronavirus.

Pigs were sequentially sacrificed on days 3 and 5 and BALF was collected and titrated. On day 3, IAV titers in BALF for both vaccine groups were ~3 $\log_{10}$ $TCID_{50}$/mL while the non-vaccinated challenge control group was negative (Figure 5). This difference was statistically significant. On day 5, all challenge groups had equivalent IAV titers in BALF.

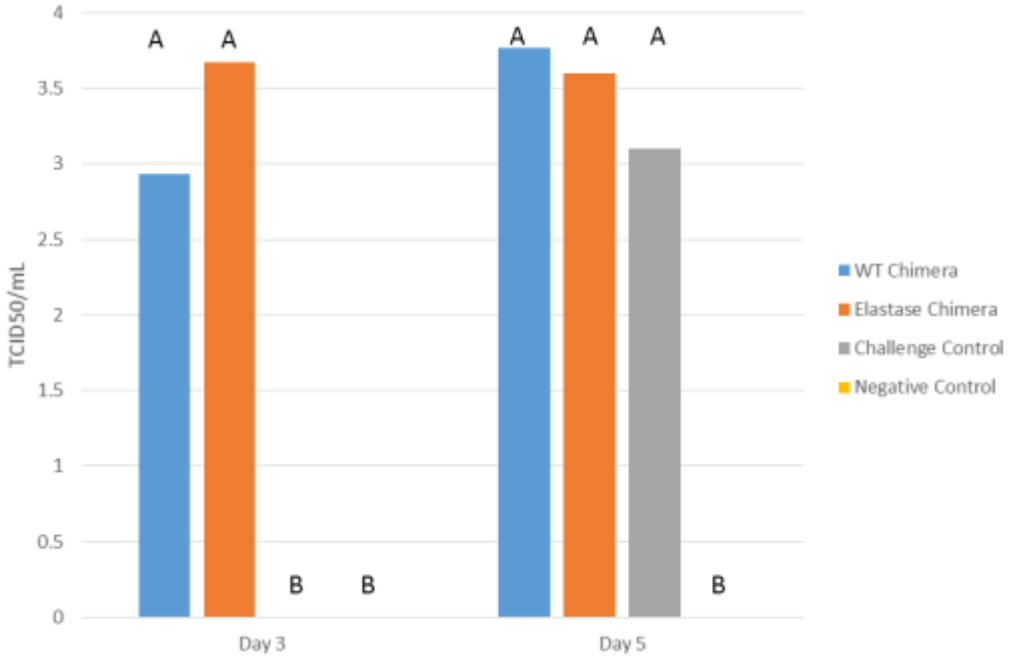

Figure 5. Mean influenza A virus titer in BALF on days 3 and 5 post-challenge. Titers reported are tissue culture infectious dose 50 per ml. Bars connected by different letters are statistically different with $P<0.05$.

BALF was also assayed for IgA that recognized the vaccine strain, the challenge virus and PEDV S1. There was no difference between the mean ELISA values for the different treatment groups (Figure 6).

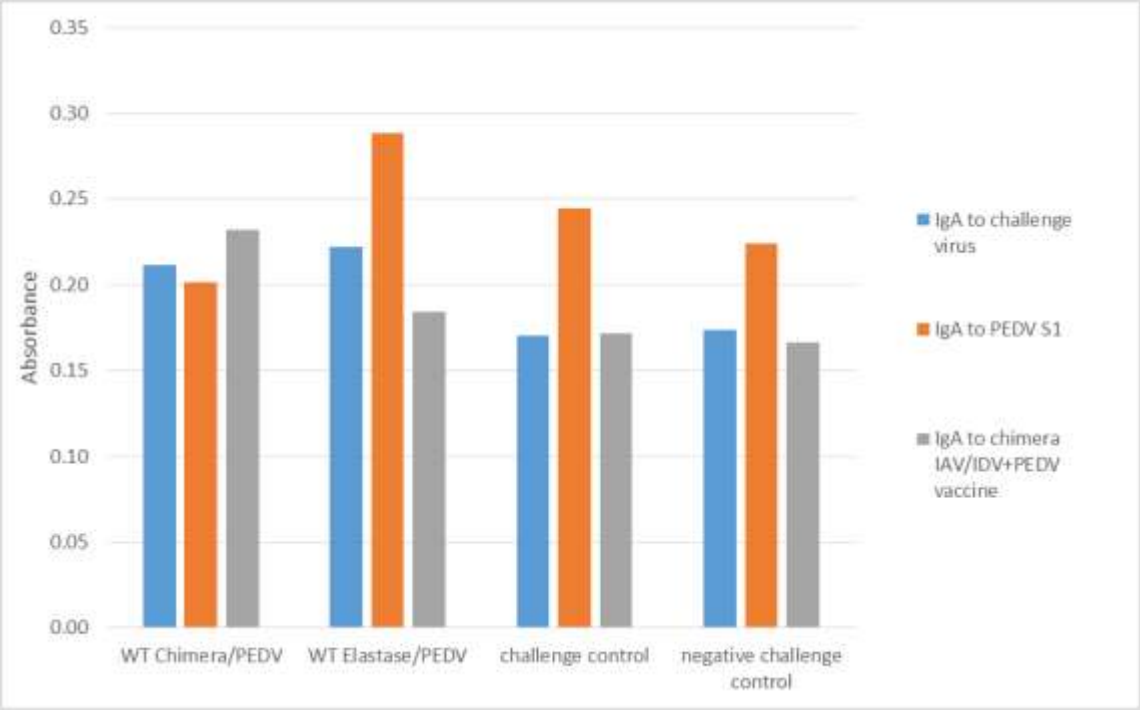

Figure 6. Mean IgA ELISA values in BALF to vaccine and challenge viruses and PEDV S1. No statistically significant differences were identified.

Lungs were submitted to KSVDL for histopathological examination using both H&E and IHC for IAV. There were no statistically-significant differences in microscopic lesions between treatment groups (Figure 7). Interestingly, lesions were noted by H&E staining of the negative control group. The lesions scores for this group were statistically indistinguishable from the other groups. As noted above, pigs were naturally infected with porcine enterovirus G and porcine respiratory coronavirus which may explain these results.

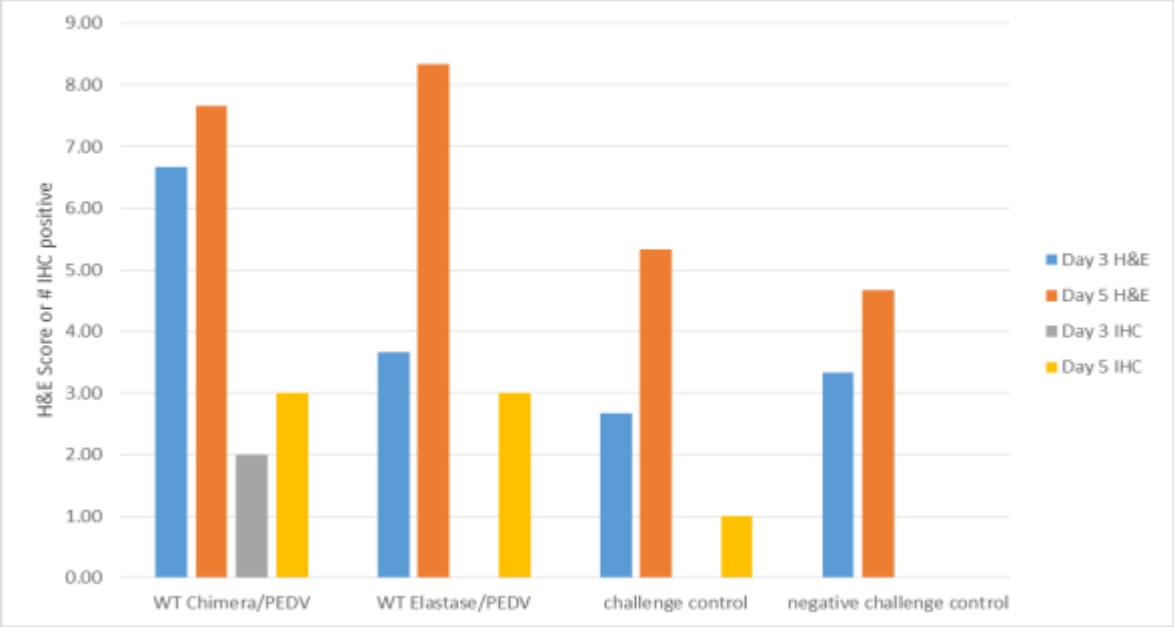

Figure 7. Mean microscopic lesion scores determined using H&E and IHC. Differences between groups were not statistically significant.

Finally, fecal swabs collected on days 0, 2, 4, and 6 were analyzed for PEDV by PCR. Pigs in the two vaccine groups had statistically higher titers of PEDV in fecal swabs than both the challenge and negative control (Figure 8).

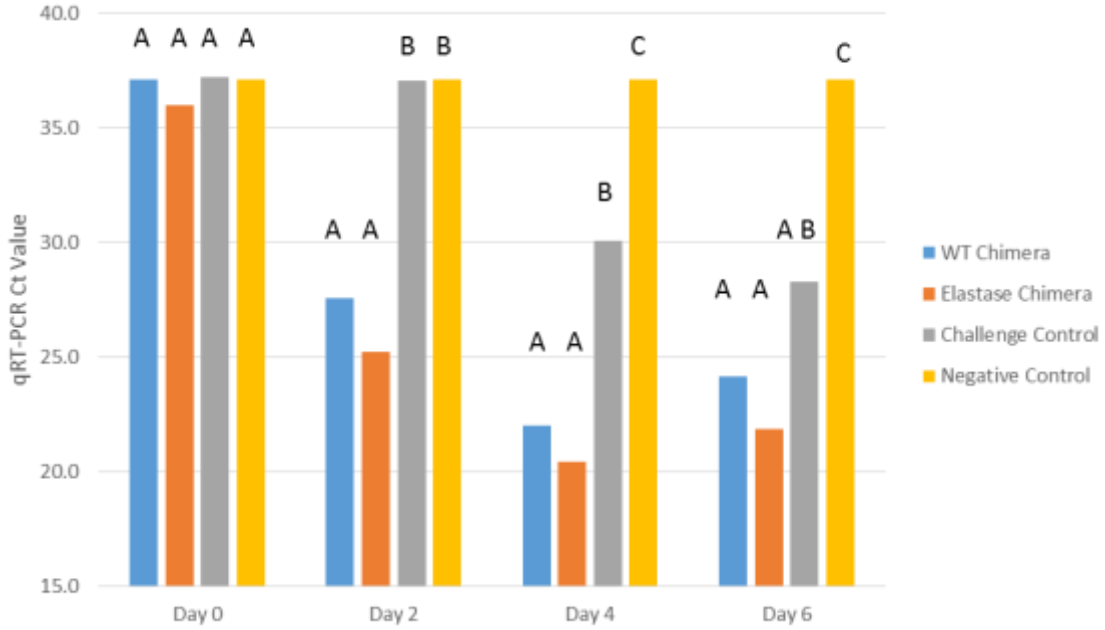

Figure 8. Mean PEDV qRT-PCR Ct value in fecal swabs collected on days 0, 2, 4 and 6. Note: a lower Ct value equates to higher viral load. Bars connected by different letters are statistically different with $P<0.05$.

Discussion

Influenza virus causes one of the most economically significant diseases affecting pigs worldwide. All currently commercially available IAV vaccines are inactivated and are widely perceived as lacking efficacy. Here we sought to develop a universal IAV vaccine by replacing the genetically highly variable HA gene with the HEF gene from IDV. Our hypothesis was that removal of the variable, immunodominant HA protein would lead to an increased immune response directed towards internal viral proteins. An additional anticipated benefit of this approach includes the ability to vaccinate young (~3 week old) pigs with maternal IAV antibodies as the chimera virus antigenically resembles IDV and pigs are largely seronegative to IDV. Also, as the HEF encodes receptor binding and destruction activities encoded by the IAV HA and NA proteins, inclusion of the NA gene in the IAV/IDV chimera virus is unnecessary for viral replication. We evaluated the chimera virus to serve as a vector to deliver PEDV S1 and protect pigs to PEDV in addition to IAV.

We attempted construction of a number of IAV/IDV chimera viruses encoding different forms of the PEDV S gene (full length, S1, $S_{636-789}$; both wild type and codon optimized). We were unable to rescue viruses encoding the complete S gene. We suspect the 4kb gene is too large to package into the virion (the largest influenza gene, PB2, is ~2.2kb). Viruses encoding the smaller S1 and $S_{636-789}$ were recoverable and were viable however S-antigen expression could only be confirmed for viruses carrying the wild-type (non-codon optimized) S1 gene. The codon optimized S gene was synthesized by Genscript. Gene synthesis took longer than anticipated and Genscript noted that the gene appeared unstable. We suspect that the codon optimized S gene was unstable and this is the underlying reason for the lack of detectable S and S1 protein expression by recombinant viruses carrying codon-optimized genes. The failure to detect protein $S_{636-789}$ could be due to the monoclonal antibody recognizing an epitope of S1 outside this region.

Vaccination with chimera viruses encoding WT and elastase-dependent HEF genes failed to elicit a detectable immune response. IDEXX NP ELISA values decreased during the course of the experiment, likely due to waning maternal antibodies. Based on a previous study, we expected the chimera with a WT HEF gene to replicate in the upper respiratory tract and be detectable in nasal swabs beginning day 1 post vaccination. Active chimera replication would be expected to result in an antibody response detectable by the IDEXX ELISA. PCR however failed to identify active replication of the vaccines post initial vaccination. Additionally, the pigs were seronegative to IDV throughout the study so maternal antibody interference is unlikely. We hypothesize that the active infection with *S. suis* triggered an immune response which neutralized the vaccines. During the two weeks following vaccination, six pigs died or were euthanized and all diagnostic testing suggested *S. suis* was the causative agent. Interestingly, vaccine viruses were detected in nasal swabs the day after booster vaccination. One out of 13 and 12/14 pigs receiving the vaccine with WT HEF and $HEF_{elastase}$, respectively, were positive by PCR. These results contrast with our previous work where only virus containing WT HEF was identified in nasal swabs post vaccination. Our previous study used vaccines formulated at 6.0 $\log_{10}$ $TCID_{50}$/mL. In this study we formulated all vaccines at 640 HA units/mL. Titration of vaccines found 4.2 and 4.4 $\log_{10}$ $TCID_{50}$/mL for the WT HEF vaccine at day 0 and 21, respectively, and 4.4 and 6.9 $\log_{10}$ $TCID_{50}$/mL for the $HEF_{elastase}$ vaccine. We hypothesize that either the lower live titer of the WT HEF vaccine used in this study or complicating co-infections with *S. suis*, porcine respiratory coronavirus and porcine enterovirus G, prohibited active replication of the WT HEF vaccine. The higher

titer of the $HEF_{elastase}$ vaccine at booster may also have been responsible for its detection the day following booster administration. It is unclear if the detected HEFelastase virus was replicating in vivo or merely still present in the nasal cavity following vaccination. Previously, we observed an IgA response against both vaccine and challenge virus in BALF both on days 3 and 5 post challenge for animals vaccinated twice with WT HEF. Here, no IgA response was directed against the vaccine, challenge or PEDV S1. The failure of the WT HEF vaccine to replicate in pigs is likely the underlying reason for the lack of a measurable mucosal immune response.

While we found no evidence for induction of an immune response following vaccination, IAV titers in BALF at day 3 post-challenge were significantly higher for vaccinated pigs as compared to non-vaccinated and challenged controls. Likewise, PEDV Ct values in fecal swabs collected from vaccinated pigs were significantly lower than those of non-vaccinated/challenged controls. Both of these results suggest vaccine-associated enhancement of disease. Vaccine-associated enhancement of respiratory disease has been observed for IAV when vaccine and challenge viruses are genetically distinct (Rajao et al., 2016).

In contrast to the results here, our previous study using a chimera IAV/IDV virus found good efficacy for an eight segmented IAV virus where the HA gene was replaced with the HEF of IDV. Following vaccination, the vaccine strain was detected in nasal swabs for seven days and a HI titer ~120 was evident by three weeks post vaccination to the vaccine strain. Despite no measurable HI titer to the heterosubtypic challenge virus, vaccinated pigs were able to clear the viral infection by day 5 post challenge where non-vaccinated controls still had >4 $\log_{10}$ $TCID_{50}$/mL at the same time point. Several differences between the two studies could explain these discrepancies: presence of the NA gene in the original study vaccine, co-infections of *S. suis*, porcine enterovirus G and porcine respiratory coronavirus interfering with vaccination and possibly causing immunosuppression, and lower vaccine titers in the current study as compared to the initial study. The finding of vaccine-associated enhanced disease as evident by significantly increased viral titers in BALF (IAV) and fecal swabs (PEDV) however suggests that the vaccine was immunogenic however the immune response elicited was not-protective.

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