Status of Vaccines for Porcine Epidemic Diarrhea Virus in the United States **and Canada**

Kimberly Crawford, Kelly M. Lager, Vikas Kulshreshtha, Laura C. Miller, and Kay S. Faaberg*

Virus and Prion Research Unit, National Animal Disease Center, USDA, Agricultural Research Service, Ames

Running title: US PEDV Vaccines

Abstract word count: 160

Text word count: 6010

*Corresponding author:

Kay S. Faaberg, Ph.D.
USDA, Agricultural Research Service, Virus and Prion Research Unit
Mailstop 2S-209
National Animal Disease Center
1920 Dayton Avenue
Ames, IA 50010
Phone: (515) 337-7250
Fax: (515) 337-7458
Email: kay.faaberg@ars.usda.gov

Disclaimer: Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

Abstract

In 2013, porcine epidemic diarrhea virus (PEDV) emerged in the United States as a rapidly spreading epidemic causing dramatic death losses in suckling piglets. Neonatal piglets are most vulnerable to clinical disease and their only protection is passive immunity from their dam. At the end of the third year of the PEDV outbreak, most US sow herds have been infected and many are entering into an endemic disease with much less, but still chronic losses. This endemic state and the occasional naïve herd that breaks with PEDV demonstrate a need to immunize sows to protect piglets. Stimulating PEDV immunity in the sow using safe and efficacious vaccines is the best course of action; however, conducting such studies to develop sow vaccines is very costly and logistically difficult. This manuscript reviews the status of PEDV vaccines available in the United States and describes an experiment evaluating the potential use of young pigs as a surrogate model to evaluate potential sow vaccines.

Introduction

Porcine epidemic diarrhea virus (PEDV) appeared suddenly in the United States for the first time in the spring of 2013 (Chen et al., 2014; Stevenson et al., 2013), spread rapidly throughout the country, and caused incredible mortality during the winter of 2013/2014. This turmoil led to regulation in June 2014 that new cases of PEDV infection were reportable events to the USDA, which provided the basis for building an extensive database. The latest statistics show that over 2900 swine premises have tested positive for PEDV, and about 10% of these have attained a negative status (APHIS, 2016). Incidence of new cases since 2014 show a cyclical pattern, peaking during winter with each winter having less cases than the previous year. Although the origin of the PEDV epidemic is not known, molecular epidemiology studies indicate the initial US PEDV isolate(s) were very closely related to each other and to Chinese PEDV isolates identified in Anhui Province in 2012. Once in the United States, the PEDV isolates have evolved into at least 4 lineages (Huang et al., 2013; Jung and Saif, 2015; Lee, 2015).

Neonatal piglets are most vulnerable to clinical disease and their only protection is passive immunity from their dam. Based on the APHIS PEDV data, most premises remain positive following the initial outbreak, which indicates a need to immunize sows to protect piglets. Ideally, stimulating PEDV immunity in the sow using safe and efficacious vaccines is the best course of action; however, conducting such studies to develop sow vaccines is very costly and logistically difficult. To reduce costs and difficulties associated with development of vaccines for sows, we propose the use of young pigs as a model to screen potential vaccines for future evaluation in a sow model. This review will address the status of vaccines that are available in the United States and describe an experiment evaluating the potential use of young pigs as a screening tool for sow vaccines.

Commercial Vaccines

The first conditionally USDA licensed vaccine (approved in June of 2014), *Porcine Epidemic Diarrhea Vaccine, RNA* (PED RNA), was developed at Harrisvaccines™ using the PEDV spike gene from a virus isolate in the SirraVaxsm RNA Particle Technology platform, which is a licensed method based on a replication deficient Venezuelan equine encephalitis virus packaging system that does not require live virus (Mogler et al., 2014; Vander Veen et al., 2012). The PEDV spike gene was chosen for expression as it has been shown to induce virus neutralization antibodies (Bosch et al., 2003). In manufacturer completed studies, first young pigs were immunized with replicon particles on days 0 and 21, and orally challenged three weeks later with homogenized gut tissue from a PEDV field case (Mogler et al., 2014). The pigs immunized with the replicon particles had less diarrhea compared to control animals, and had reduced incidence of viral shedding over the first 72 hours. In another study, sows were administered PED RNA at six and two weeks prior to farrowing. Piglets were then orally challenged at 10 days of age (trial 1), or within the first seven days (trials 2 and 3) with PEDV (Colorado/2013). The results showed that vaccinated dams had virus-neutralizing antibodies that were secreted in the milk for the duration of the studies. In addition, piglets that had suckled the vaccinated dams had detectable neutralizing antibodies. Reduction of piglet mortality was also seen (Mogler et al., 2015). In another study by the vaccine manufacturer, a sow herd that had experienced an outbreak was exposed to PEDV by feedback of the virus following initial diagnosis. Six weeks post-break, PEDV-RNA was administered intramuscularly into one of two paired sows, six pairs total, 7-10 days prior to farrowing. The other sow was vaccinated with diluent only to serve as the control. During the first 24 hours after farrowing, cross-fostering was

permitted. Although the preweaning mortality in the control group numerically increased over vaccine treatment animals, no statistical difference was seen between the two groups when compared on a weekly basis (Harris et al., 2015). Most recently, the company reported an additional study (Crawford et al., 2016). Both PEDV-exposed gilts and naïve sows were utilized. The naïve pregnant sows were vaccinated with PED RNA or placebo at about 8, 5 and 1 week pre-farrowing. The PEDV-exposed pregnant gilts, having been exposed to PEDV about 10 months before farrowing, were vaccinated with PED RNA approximately 4 and 1 weeks before giving birth. Control sows were also included for each group. After allowing natural farrowing but no piglet cross-fostering, piglets were challenged between 2-6 days of age, depending on farrowing date of the dam. All piglets from the naïve sow trial were orally challenged with 10^3 50% tissue culture infectious dose (TCID₅₀) PEDV/Colorado/2013. Piglets from the exposed gilt protocol were challenged orally with gut homogenate containing 10^8 genome copies by quantitative RT-PCR. After 14 days post challenge (DPC14), piglet mortality was evaluated as well as the presence of neutralizing antibodies in the colostrum (within 24 hours of farrowing) and milk (DPC7) of the sows. Piglet mortality was 69% and 91% in the naïve vaccinates and the sham controls, respectively, and serum and colostrum focus forming neutralization (FFN) antibodies were detected in the serum and colostrum as well as DPC7 milk in the naïve vaccinates only. From the previously-exposed gilts and their sham controls, piglet mortality was 45% and 59% (not statically significant due to small numbers), respectively, and both sham controls and vaccinates had significantly increased FFN antibodies over naïve sows in the serum and colostrum (Crawford et al., 2016).

A swine veterinary practice examined the use of PED RNA in a sow herd that had been naturally exposed to PEDV (Grenier et al., 2015). Eighty sows were identified for use in the

study and assigned to two groups. Three weeks pre-farrowing, one group was vaccinated with PEDV RNA, the other group was not vaccinated. Serum samples were collected from all sows at the start of the study as well as immediately prior to farrowing. Only sows that farrowed in the presence of a researcher were subsequently allowed to remain in the study. Colostrum samples were taken from those sows. Serum samples were also taken from piglets three days after birth to evaluate the anti-PEDV antibody transfer from the sow. Piglet mortality and diarrhea were also recorded until weaning. Antibody levels in sow serum, colostrum and piglet serum were assessed by ELISA to the S1 and S2 PEDV proteins. The study concluded that the use of PEDV RNA vaccine significantly increased the spike-specific IgG antibodies in the colostrum against both PEDV proteins, but piglet mortality was only reduced by a scant 3% (Grenier et al., 2015).

A conditional license was also approved in September of 2014 to Zoetis (Florham Park, New Jersey) for an inactivated whole-virus vaccine in the presence of an adjuvant (Study Report No. B826R-US-13-258, Zoetis Inc.). To demonstrate an expectation of efficacy, sows were vaccinated intramuscularly with one of three formulations or remained unvaccinated at 5 and 2 weeks pre-farrowing. The piglets born from vaccinated sows had 7.4 fold greater FFN antibody titers than piglets born from control sows. No information was given for piglet mortality. The sows from this study were revaccinated approximately 4 months after the initial vaccination and showed an average of 4-6 fold higher FFN titers than the titers after the initial 2 vaccinations. Using the same initial sow vaccination protocol in a commercial production facility experiencing a PEDV outbreak, field efficiency was then examined. Using gilts and sows of different parities, a 90.4% relative reduction in pre-weaning piglet mortality was seen (0.6% vaccinated compared 6.3% unvaccinated) and vaccinated sows had an additional 1.8 pigs survive. Yet the mortality rates due to PEDV for the sows in the study were only around 10% at most, a common

occurrence when a sow herd is pre-exposed to PEDV. The results in terms of farrowing data - including number of piglets born alive, of low viability, stillborn, or mummies - were similar when vaccinated sows and those receiving placebo were compared. However, when field efficacy economic data was analyzed, Zoetis stated that approximately \$100/litter could be realized by the use of their product.

In an independent study, investigators examined the effect of vaccination using the Zoetis killed virus preparation compared to no vaccination. This was accomplished using sows that had remained naïve to PEDV or had been previously exposed to the virus (Schwartz and Rademacher, 2015). Eight previously bred sows per group were placed into four Groups: 1. Previously Exposed, Zoetis PEDV vaccinated (PE Vac), 2. PE non-vaccinated receiving saline (PE Non-Vac), 3: Naïve, Zoetis PEDV vaccinated (Naïve Vac), and 4. Naïve non-vaccinated receiving saline (Naïve Non-Vac). The animals were tested to make sure they were negative for porcine reproductive and respiratory syndrome (PRRSV), a confounding factor that also causes premature abortions in the field. Sow Groups 1 and 2 were assigned so that an even number of sows with both low and high serum neutralization titers were present in both groups. After one week of acclimation, sows in Groups 1 and 3 received intramuscular vaccine injections at 5 and 2 weeks pre-farrowing, and sows in groups 2 and 4 received saline injections. All sows farrowed within a span of four days. On day 0 (farrowing), sow serum was collected for antibody testing (both IgA and IgG). Sow colostrum and milk was collected on days 0, 2 and 9. Piglet serum samples were collected for IgA and IgG analysis at 2 and 9 days of age. At five days of age, each piglet received an oral inoculation with 1 ml of fecal matter containing PEDV diluted to a qRT-PCR cycle threshold (Ct) of 19. Piglets were observed twice daily for 21 days. The study showed that sow serum IgG was induced by previous exposure to PEDV, but that naïve sow vaccination

can stimulate IgG antibody titers of a similar magnitude. PEDV vaccination of previously-exposed sows showed an increase in serum and colostrum IgG levels. The pattern of IgG response was not replicated in IgA titer data. Both PE Vac (Group 1) and PE Non-Vac (Group 2) had significant positive IgA titers in all serum and milk samples collected from both the sows and the piglets. Group 1 produced the highest IgA titers, Group 2 the next highest, with Group 3 (Naïve Vac) producing very low titers and Group 4 (Naïve Non-Vac) producing barely detectable titers. Lastly, piglet mortality was analyzed. Prior exposure of the sows with high IgA titers, whether revaccinated or not, prevented most clinical signs in their piglets after PEDV challenge, and no mortality was observed. Vaccination of naïve sows did induce a low IgA response in both milk and colostrum, but this response was unable to prevent 100% piglet mortality in 15 days. However, the mortality was delayed in Group 3 compared to Group 4, who displayed 100% mortality within 11 days after PEDV infection. The authors concluded that although the Zoetis vaccine stimulated IgG levels in colostrum and milk, the vaccine only marginally increased the levels of IgA in those sows that had been previously exposed to PEDV, and stimulated very low levels of IgA antibody in naïve sows. Furthermore, vaccination did not induce sufficient passive IgA antibody levels to decrease or prevent preweaning mortality in vaccinated naïve sows. The findings support the hypothesis that piglets must ingest fairly high levels of IgA to survive PEDV challenge (Schwartz and Rademacher, 2015).

Preparation of an autogenous vaccine from a PEDV field isolate was the focus of one other study (Collin et al., 2015). In this study, PEDV was isolated from a PEDV-positive pooled intestinal homogenate and passaged on Vero cells for two passages at Newport Laboratories (Worthington, MN). Viral cultures were then adapted to and scaled up in MARC145 cells, first in 25 cm² flasks and then 1700 cm² roller bottles to result in a large amount of virus for

autologous vaccine preparation (NPL PEDV 2013 P10.1). PEDV stock virus P10.1 was shown by quantitative RT-PCR to have a Ct value of 21.2 and a viral titer of $6.6 \log_{10}$ TCID₅₀/ml. The full-length genome was sequenced (GenBank Accession KM052365) and found to have greater than 99.9% identity with USA/Colorado/2013 (GenBank Accession KF272920). PEDV 2013

P10.1 was then inactivated by the addition of 0.1 M binaryethyleneimine (BEI; 5% final), neutralized with sodium thiosulfate and verified by passaging the inactivated fluids on cells three times with no increase in qRT-PCR titer and no evidence of CPE. The vaccine preparation was then concentrated 30X using a 10kD hollow fiber filter. To test the efficacy of the inactivated vaccine, 8 groups of pigs at about 4-weeks of age were divided into 5 to 9 animals per group and one unvaccinated control group of 5 animals. Table 1 lists the amount of virus pre-inactivation and the route of administration given to each group. All vaccines also contained 67% TS6, which is a proprietary oil and water adjuvant. The two different routes of vaccine intramuscular administration, neck and rear flank, was done to examine any potential changes in the immune response due to vaccination site. Pigs were vaccinated on days 0 and 21, and observed daily for signs of disease. Serum samples were collected on days 0, 21 and 35. Fecal swabs were collected 3 days after vaccination to confirm the absence of PEDV shedding by qRT-PCR targeting the spike gene. The study was terminated on Day 35. Serum samples from day 0 were analyzed by indirect immunofluorescence (IFA) and by the fluorescent foci neutralization assay (FFN) to confirm the absence of anti-PEDV antibody. Serology results (reflected as mean titer) showed that all groups (groups 1 =7.32 mean titer; 4=7.99; 5=6.99, 8=7.54) vaccinated with $8 \log_{10}$ TCID₅₀/ml inactivated virus, as well as the group (group 6=6.52) receiving $7 \log_{10}$ TCID₅₀/ml inactivated virus with Triton® X-100 (to assess treatment of antigen on immunogenicity), had statistically higher mean FFN neutralizing titers than the control group. The mean titers could not

be statistically differentiated from each other. The routes of administration as well as the presence or absence of detergent were also not statistically different. Those groups (2, 3 and 7) inoculated with less inactivated virus showed an FFN titer about 1 log lower than the other pig groups. Other tests were performed using recombinant PEDV nucleoprotein (NP) antigen, two ELISAs and a fluorescent microsphere immunoassay (FMIA), but these were less sensitive and thus the vaccine study results were not as robust. In addition, the data obtained from the study suggests that Triton® X-100 treatment of the antigen in the vaccine formulation (groups 5-8) altered the antigenicity or immunogenicity of the NP, leading to negative ELISA and FMIA results. The FFN, however, detects functional neutralizing antibody associated with epitopes on the PEDV S protein. In this trial, no protective titer could be determined as no animals were then challenged with wild-type PEDV (Collin et al., 2015).

Recently, three eukaryotic expression systems (mammalian, yeast and insect) were tested to define the optimum method to obtain the highest yield and most suitable for vaccine studies (Makadiya et al., 2016). The mammalian system, expressing the S1 protein of the USA/Colorado/2013 strain of PEDV (tagged with histidine at the carboxyl end) in HEK293T cells yielded the most protein per liter was appropriately N-glycosylated. In order to determine the immunogenicity of this preparation, two pregnant sows were immunized intramuscularly either with nickel column purified S1 protein (400 µg per dose) or saline mixed with TriAdj adjuvant [poly[di(sodium carboxylatoethylphenoxy)]-phosphazene (PCEP), a toll-like receptor (TLR) agonist, and an innate defense regulator (IDR) peptide; (Snider et al., 2014)] for a total of three times, days 0, 14 and 28. Serum samples were obtained on 0, 11, 28 and 35. After the last vaccination, farrowing was induced and colostrum samples were collected on the day of farrowing. Piglets were

allowed to suckle their respective dam, and were then each challenged orally with 3×10^2 TCID₅₀ PEDV on the 4th day of life. Piglet blood samples were collected at challenge. Daily rectal swabs were also obtained from each piglet. The results showed that the vaccine administration in a sow induced S1-specific IgG and IgA that were passively transferred to the piglets. High virus neutralization titers were also seen in the serum of both the S1-vaccinated sow and its piglets. Piglets (n=8) born to the vaccinated sow, when challenged, showed less severe signs of disease, but revealed no significant differences in diarrhea, body weight and virus shedding. The lower mortality, compared to the piglets of a control sow, seen within six (3 piglet died from the control sow versus one from the vaccinated sow) was promising.

Young Pig Model

Introduction

Although there must be differences between young and mature swine in the maturation of the immune system and their susceptibility to infection with PEDV, for the purposes of screening potential sow PEDV vaccines, we believe that these differences are negligible. The practical application of this logic is if a young pig responds to vaccine and develops protective immunity, then this may also occur in the sow. The converse is that if the pig develops an equivocal immune response to a vaccine, so will a sow. To test this logic, we conducted a PEDV vaccine trial using 8- and 20-week-old pigs and compared the results with what has been previously reported.

Materials and Methods

Virus. The US/Colorado/2013 strain of PEDV was grown on Vero cells as described previously (Crawford et al., 2015; Hofmann and Wyler, 1988).

Experimental Design. Two groups of different aged pigs were purchased as conventionally raised weaned pigs from a sow herd free of PEDV based on lack of clinical signs, specific antibody, and detection of PEDV nucleic acid in rectal swabs. Upon arrival at the National Animal Disease Center, pigs were randomly assigned to treatment groups and housed separately by age in isolation rooms until the beginning of the experiment, day 0 (Table 2). On day 0, Group A pigs (n=39) were about 8 weeks of age and Group B pigs (n=25) were about 20 weeks of age. For description of experimental design, both age groups that received the same treatment are considered one group. Treatment group 1 served as no-challenge control pigs and treatment group 2 pigs were wild-type (*wt*) USA/Colorado/2013 PEDV-challenge controls (Table 2). On day 7, group 3 received a single oral inoculation of *wt* PEDV (Table 2). Wild-type virus was given intranasally in a 5 ml dose with the pig physically restrained so the snout was elevated allowing the inoculum to enter the oropharyngeal cavity. On day 0 and 21, group 4 received an intramuscular 2 ml injection of a commercially available killed virus vaccine (Zoetis; Table 2). An experimental replication-deficient adenovirus-vectored PEDV vaccine was given intranasally to group 5 pigs on day 21 (Table 2). E1/E3-deleted adenovirus type 5 vaccine vector encoding the full-length and porcine codon-optimized S1 spike protein from the USA/Colorado/2013 strain of PEDV was manufactured in HEK293 cells, purified using isopycnic cesium chloride gradient centrifugation, formulated in a proprietary buffer and tested for bioburden and mycoplasma before use in the study. The volume given was 4 ml (4×10^{10} infectious units in 4 ml basal medium), split between each nostril. On day 43 for younger pigs and day 45 for older pigs, each pig in groups 2-5 were given a 5 ml intranasal challenge with the homologous *wt* PEDV. A timeline was developed (Figure 1).

Swine samples. Blood samples were collected at day -6 from all pigs, from pigs prior to any treatment, and at the conclusion of the experiment. Rectal swabs were collected from pigs in group 3 on day 7, the day of primary challenge, and then for 12 days-post-challenge (dpc). Beginning at day 43 for younger pigs and day 45 for older pigs, rectal swabs were collected daily for 12 days. Serum was harvested from blood samples and all serum and swab samples were stored at -80°C until antibody and qRT-PCR testing, respectively.

Diarrhea scoring. A clinical diarrhea score was developed to assess clinical disease following challenge. Scoring was done at the time of rectal swabbing using a scale from 0 to 4 with 0 being normal feces (a formed stool) to a 4, represented by voluminous watery diarrhea. Scores of 1, 2, 3 were based on appearance of feces with 1 being a “soft” stool that had a normal consistency, but it had lost its normal shape. A score of 2 and 3 were given to feces that was becoming consistently more fluid resulting in an appearance of a semi-liquid state, and a projectile liquid feces, respectively. In all treatment groups the individual diarrhea score was assessed when the pigs were handled at the time of rectal swabbing. If no evidence of abnormal feces was observed, then the pig was given a score of 0.

Serologic Assays. Serum samples were kindly tested for antibody by Tanja Opriessnig, et al., Iowa State University, USA, using an indirect ELISA based on the S1 portion of the PEDV spike protein (Gerber et al., 2014). After obtaining raw absorbance numbers for the samples as well as a known positive control, S/P ratios were determined by dividing the absorbance units of a test sample by the absorbance units of the positive control analyzed alongside the test sample. Virus neutralization (VN) assays were developed using Vero cells. Cell culture plates (96-well) were seeded with Vero cells (4×10^4 cells/well) in MEM supplemented with 10% fetal bovine serum. At 5-6 days after cell plating, serum samples were diluted in a two-fold series using basal

medium without trypsin in a separate 96-well plate. In a third 96-well plate, the series of diluted swine serum (50 µl/well; 4-8 wells/dilution) were mixed with 50 µl/well PEDV diluted to 4×10^3 – 6×10^3 50% tissue culture infectious dose (TCID₅₀)/ml in basal medium without trypsin. Positive and negative controls were also included. After 1h incubation at 37°C, all of the mixture (100 µl/well) of swine serum (potentially containing neutralizing antibodies to PEDV) and the virus were transferred to the 96-well plate of Vero cells from which the medium had been removed. The cells with virus:antibody complexes were incubated for 1h at 37°C to allow for virus attachment, and then washed 3 times with basal medium. Basal medium with 1.5 ml/L tryptose phosphate broth and 1.5 µg/ml trypsin was then added (200 µl/well), and the plates were incubated at 37°C and observed daily. The neutralization titer was read daily on days 3-5 post infection, and the endpoints were calculated by the Reed and Muench method (Reed and Muench, 1938).

Quantitative RT-PCR. Quantitative RT-PCR (qRT-PCR) was completed as described previously, targeting the PEDV nucleocapsid (N) gene (Miller et al., 2016).

Results

Clinical disease from day 0-43. No clinical disease was observed in groups 1 and 2 during this time. In group 3 pigs following *wt* virus challenge on day 7, mild-to-moderate clinical disease was observed beginning 2 dpc with 1 pig developing a diarrhea score of 1. By 3 dpc, a score of 1 and 4 were noted in two other pigs. Also observed at 3 dpc were piles of normal, soft, semi-solid, and watery diarrhea on the floor of the room. In addition, there were spatter marks on the wall of the room from pig(s) that had projectile diarrhea. On 4 dpc, 6 of 7 younger pigs were given a diarrhea score (1, 1, 2, 4, 4, 4) and the range of abnormal feces on the floor of the room was similar to 3 dpc. Also, several piles of vomitus were observed, and feed remained from the

previous day indicating a loss of appetite. The overall clinical picture was the same at 5 dpc and then began to reduce as the pigs recovered from the infection. By 9 dpc, no abnormal diarrhea score was recorded and pigs appeared normal when compared to the other groups. In the group 3 older pigs, no abnormal diarrhea scores for an individual animal was recorded, however from 2-3 dpc, several piles of soft or semi-solid feces were noted on the floor in addition to the presence of normal appearing stools. No clinical disease was observed following vaccine treatments in groups 4 and 5.

Clinical Disease from Day 43-70. Diarrhea scores after *wt* PEDV challenge were plotted by the group as a whole (Fig. 2A) or separately into younger (Fig. 2B) and older (Fig. 2C) pig groups. No clinical disease was seen in the sham-inoculated pigs (group 1). In group 2 pigs, clinical disease was recognized in the younger pigs at 2 dpc as a few soft and semi-solid fecal piles on the floor and one pig was given a score of 2. By 4 dpc, all 7 pigs had a score (1, 2, 2, 2, 2, 3, 4) resulting in the peak diarrhea score for this group with a return to normal scores by 7 dpc. During this time the character of the feces on the floor was mostly a liquid consistency and as a group, the pigs were lethargic and had a reduced appetite compared to before challenge. The overall diarrhea score for the older group 2 pigs was less in magnitude reflecting minimal diarrhea in the group, and that only 1-3 pigs received a score on any given day. When combined, group 2 pigs had the highest diarrhea score (Fig. 2A). Of the groups that were immunized and then received challenge virus, group 3 pigs (homologous challenge) had the least diarrhea score followed by group 4 (killed vaccine). The overall group 5 score was more similar to group 2 (Fig. 2A). In general, the diarrhea scores for each group were more elevated in the younger than older pigs except for group 5, the adenovirus vaccine group, which had the highest diarrhea score in the older pigs, and second highest score in young pigs compared to the challenge control group.

Other than diarrhea, all pigs appeared normal and maintained their appetite until conclusion of the experiment.

Seroconversion to PEDV S1. All animals inoculated (group 3) or vaccinated (groups 4 and 5) seroconverted to expressed S1 protein as detected by ELISA (Fig. 3). The killed virus (group 4) was able to readily induce an antibody response to the S1 protein in all animals for the duration of the experiment that was more robust than the *wt* PEDV (group 3) or the adenovirus construct (group 5) (Fig. 3A). The young pigs that were inoculated with *wt* PEDV produced a greater titer of anti-S1 antibodies than the older animals pre-challenge, an unexpected finding (Fig. 3B and C). The adenovirus expressing S1 produced an intermediate titer of antibodies in all animals (Fig. 3B and C).

Neutralizing antibody response. No VN antibody was detected in sera from group 1 pigs at any time and in any of the other groups prior to vaccination or challenge. Within 12 days of challenge, pigs in group 2 (challenge control) developed a detectable VN antibody (Fig. 4A). Primary challenge with *wt* PEDV (group 3) produced VN antibodies in all young pigs by time of secondary challenge (DPC 0)(Fig. 4B). Although there was a reduction in titer post challenge, no significant changes in titer were detected by DPC 12. In group 3 older pigs, only 2 pigs had developed a VN titer by time of secondary challenge. A significant increase in VN titer was found by 12 DPC in the older pigs even though one pig did not develop a VN titer (Fig. 4C). All group 4 pigs developed VN antibody following primary vaccination that increased after the second vaccination; however, the magnitude was less in the older pigs (Fig. 4). A significant increase was found in both 8 wk and 20 wk pigs following challenge. No VN antibodies were detected in the adenovirus vectored S1 pigs (group 5) 3 weeks after vaccination (DPC 0), but by 12 days after challenge, VN antibodies were detected.

Viral load. Viral RNA load was assessed using qRT-PCR after the initial infection with *wt* PEDV (group 3) and also post challenge. The initial inoculation of group 3 pigs showed the pigs were shedding virus RNA (data not shown). Post-challenge, when all animal virus loads were combined, group 3 pigs inoculated with *wt* virus prior to *wt* re-challenge showed a much greater reduction in viral RNA load than all other treatments combined (Fig. 5A). Group 4 pigs displayed a small delay in viral RNA detection when all animals were evaluated, but reached challenge virus load by 6 DPC. In younger pigs, this delay was more pronounced (Fig. 5B). Also note that the viral genome replicated to a higher copy number in younger pigs than in older animals (Fig. B and C). Finally, group 3 older pigs were seemingly not protected by previous exposure to *wt* PEDV, although a decrease in the length of time of PEDV shedding was noted, as in group 4 (killed virus) and group 5 (Adenovirus) vaccinates (Fig. 5C).

Summary of Young Pig Experiment

Most of the limited information available on the use of PEDV vaccines derived from US isolates describes field studies in sow herds. To date, there are no well-controlled sow studies that restrict potential exposure of animals to *wt* field virus. While such limitations do reflect reality, they also confound interpretations of results. There is a report of young pigs given a PEDV replicon particle vaccine under controlled conditions. Upon challenge, the pigs had reduced virus replication and diarrhea compared to non-vaccinated controls. Results from that pig experiment mimicked PEDV replicon particle vaccine studies in sows where incomplete clinical protection was found. Similar to the PEDV replicon particle vaccine observations, piglets in the present study developed an adaptive response to inactivated vaccine that provided incomplete protection. This response is similar to what has been reported in sow studies given the same vaccine that induced specific antibody and varying levels of clinical protection in their

suckling pigs. Although young pigs cannot be used to model PEDV vaccine effects on lactogenic immunity, based on what has been reported for the replicon particle vaccine and inactivated PEDV vaccine, it appears they could be used as a predictive model for the sow PEDV immune response. Accepting this premise implies the adenovirus vector which induced a humoral response and reduced replication of challenge virus, could be a vaccine prototype that merits additional study to determine if route, dose, and timing of vaccination might improve its efficacy.

One intriguing finding of this study was the fact that the group receiving 2 doses of killed vaccine was able to induce high levels of neutralizing antibodies, yet these animals were not well protected from *wt* PEDV challenge. At the same time, only modest levels of neutralizing antibodies were seen in the pigs that first had a *wt* PEDV challenge and then were re-challenged. Both findings suggest that other immune functions are necessary to adequately protect swine.

There is a trend for age-related differences between the young and old pigs. The most obvious differences are in group 3 in which the older pigs following primary *wt* PEDV challenge had less of a humoral response, and, compared to the younger pig response, replicated more virus and had a higher diarrhea score following secondary challenge with *wt* PEDV. In group 4 pigs, the older pigs had the maximum diarrhea score post challenge, and the onset and magnitude of fecal positive swabs were similar to group 2 pigs, the non-immunized pigs. In contrast, the younger pigs had less of a diarrhea score (although higher than groups 4 and 5), and the onset of fecal positive swabs was delayed by 2 days. We inoculated each group 3 young and old pig similarly with *wt* virus which included the same challenge dose. Based on VN and ELISA results, it appears some of the older pigs had a minimal or non-detectable humoral immune response to primary *wt* virus challenge despite being inoculated and exposed to penmates presumably shedding infectious virus following primary challenge (Fig. 4C). It is possible the methods used

were adequate to demonstrate treatment effects in young pigs, but may be inadequate for older pigs. However, additional studies investigating dose effect may be necessary to determine if young pigs can be an efficient model for sows, and if older pigs and sows may require an increased dosage of vaccines/antigenic mass.

Conclusion

The control of PEDV clinical disease is dependent on inducing a protective immune response in the sow to reduce transmission in the sow herd and provide quality passive immunity to piglets. Inducing a protective immune response in the sow herd is limited to vaccination and/or exposure to wild-type virus either through natural or forced exposure. The latter option is fraught with difficulties, which makes the use of a standardized PEDV vaccine desirable. Although a variety of vaccine technologies can potentially be used for PEDV vaccine development, such studies have a common logistical difficulty, the size of the target host which limits the potential for maintaining adequate experimental treatment group sizes in biocontainment. This is critical because without a controlled environment, the actual exposure history to PEDV is unknown, making it difficult to assess the efficacy of a vaccine. The goal of this study was to evaluate the potential of using a young pig model to screen PEDV vaccine candidates. This goal is based on the assumption that a vaccine that provides clinical protection in a young pig would provide clinical protection in a sow resulting in the development of passive immunity that would protect her piglets. With the clinical response of young pigs vaccinated with either the PEDV RNA replicon particles or PEDV inactivated virus similar to sows, i.e., that sows did develop specific antibody and varying levels of clinical protection, it seems possible that young pigs can work as a surrogate model for screening potential sow vaccines.

Given that pigs receiving a parenteral vaccination developed a specific immune response that was not fully protective, the route of immunization may be an important factor in stimulating an optimum mucosal immune response for protection from enteric disease and producing quality passive immunity for piglets.

Acknowledgements

The authors greatly appreciate our collaboration with M. Scot Roberts and Jianfeng Zhang of Altimune, Inc. in the young pig experiment. We also want to acknowledge the laboratory technicians Deb Adolphson and Sarah Anderson, as well as animal caretakers Jason Huegel, Keiko Sampson, Aaron Heibheisen and Justin Miller. This study was funded by the USDA Agricultural Research Service project number 3625-32000-108-00D.

References

- APHIS, 2016. Swine Enteric Coronavirus Disease (SECD) Situation Report – Mar 17, 2016, pp. 1-22.
- Bosch, B.J., van der Zee, R., de Haan, C.A., Rottier, P.J., 2003. The coronavirus spike protein is a class I virus fusion protein: structural and functional characterization of the fusion core complex. *J. Virol.* 77(16), 8801-8811.
- Chen, Q., Li, G., Stasko, J., Thomas, J.T., Stensland, W.R., Pillatzki, A.E., Gauger, P.C., Schwartz, K.J., Madson, D., Yoon, K.J., Stevenson, G.W., Burrough, E.R., Harmon, K.M., Main, R.G., Zhang, J., 2014. Isolation and characterization of porcine epidemic diarrhea viruses associated with the 2013 disease outbreak among swine in the United States. *J. Clin. Microbiol.* 52(1), 234-243.
- Collin, E.A., Anbalagan, S., Okda, F., Batman, R., Nelson, E., Hause, B.M., 2015. An inactivated vaccine made from a U.S. field isolate of porcine epidemic disease virus is immunogenic in pigs as demonstrated by a dose-titration. *BMC Vet Res* 11, 62.
- Crawford, K., Lager, K., Miller, L., Opriessnig, T., Gerber, P., Hesse, R., 2015. Evaluation of porcine epidemic diarrhea virus transmission and the immune response in growing pigs. *Vet. Res.* 46, 49.
- Crawford, K., Mogler, M., Hicks, J., Harris, D.L., 2016. Protective efficacy of a replicon particle vaccine in both naïve sows and previously exposed gilts against porcine epidemic diarrhea virus. *Proceedings of the Annual Meeting of the American Association of Swine Practitioners.* Vol. 47, 212-213.

- Gerber, P.F., Gong, Q., Huang, Y.W., Wang, C., Holtkamp, D., Opriessnig, T., 2014. Detection of antibodies against porcine epidemic diarrhea virus in serum and colostrum by indirect ELISA. *Vet. J.* 202(1), 33-36.
- Grenier, L., Connor, J., Graham, A., Mellor, J., Lowe, J., 2015. Evaluation of a PED vaccine on piglet mortality and sow immunity. *Proc Ann Mtg Am Assoc Swine Practitioners*. Vol. 46, 361.
- Harris, A.J., Wiseman, B., Harris, D.L., 2015. Evaluation of a porcine epidemic diarrhea vaccine to reduce preweaning mortality. *Proceedings of the Annual Meeting of the American Association of Swine Practitioners*. Vol. 46, 127.
- Hofmann, M., Wyler, R., 1988. Propagation of the virus of porcine epidemic diarrhea in cell culture. *J. Clin. Microbiol.* 26(11), 2235-2239.
- Huang, Y.W., Dickerman, A.W., Pineyro, P., Li, L., Fang, L., Kiehne, R., Opriessnig, T., Meng, X.J., 2013. Origin, evolution, and genotyping of emergent porcine epidemic diarrhea virus strains in the United States. *MBio* 4(5), e00737-00713.
- Jung, K., Saif, L.J., 2015. Porcine epidemic diarrhea virus infection: Etiology, epidemiology, pathogenesis and immunoprophylaxis. *Vet. J.* 204(2), 134-143.
- Lee, C., 2015. Porcine epidemic diarrhea virus: An emerging and re-emerging epizootic swine virus. *Virol J* 12(1), 193.
- Makadiya, N., Brownlie, R., van den Hurk, J., Berube, N., Allan, B., Gerdts, V., Zakhartchouk, A., 2016. S1 domain of the porcine epidemic diarrhea virus spike protein as a vaccine antigen. *Virol J* 13, 57.

- Miller, L.C., Crawford, K.K., Lager, K.M., Kellner, S.G., Brockmeier, S.L., 2016. Evaluation of two real-time polymerase chain reaction assays for Porcine epidemic diarrhea virus (PEDV) to assess PEDV transmission in growing pigs. *J. Vet. Diagn. Invest.* 28(1), 20-29.
- Mogler, M.A., Gander, J., Harris, D.L., 2014. Development of an alphavirus RNA particle-based vaccine against porcine epidemic diarrhea virus. *Proceedings of the Annual Meeting of the American Association of Swine Practitioners.* Vol. 45, 63-64.
- Mogler, M.A., Gander, J.F., Ray, D.D., Harris, D.L., 2015. Evaluation of PED RNA vaccine efficacy in dam vaccination, suckling piglet challenge model. *Proceedings of the Annual Meeting of the American Association of Swine Practitioners.* Vol. 46, 257-258.
- Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent end-points. *Amer. J. Hyg.* 27, 493–497.
- Schwartz, T.J., Rademacher, C.J., 2015. Evaluation of the effects of PEDv vaccine on PEDv naïve and previously PEDv-exposed sows in a challenge model comparing immune response and preweaning mortality. *Iowa State University James D McKean Swine Disease Conference Proceedings*, 36-40.
- Snider, M., Garg, R., Brownlie, R., van den Hurk, J.V., van Drunen Littel-van den Hurk, S., 2014. The bovine viral diarrhea virus E2 protein formulated with a novel adjuvant induces strong, balanced immune responses and provides protection from viral challenge in cattle. *Vaccine* 32(50), 6758-6764.
- Stevenson, G.W., Hoang, H., Schwartz, K.J., Burrough, E.R., Sun, D., Madson, D., Cooper, V.L., Pillatzki, A., Gauger, P., Schmitt, B.J., Koster, L.G., Killian, M.L., Yoon, K.J., 2013. Emergence of Porcine epidemic diarrhea virus in the United States: clinical signs, lesions, and viral genomic sequences. *J. Vet. Diagn. Invest.* 25(5), 649-654.

Vander Veen, R.L., Harris, D.L., Kamrud, K.I., 2012. Alphavirus replicon vaccines. *Anim Health Res Rev* 13(1), 1-9.

Table 1

Group	Vaccine*	No. of Pigs
0	Negative control	5
1	8.0 IM	8
2	7.0 IM	5
3	6.0 IM	5
4	8.0 RF	9
5	8.0 IM + Triton® X-100	9
6	7.0 IM + Triton® X-100	5
7	6.0 IM + Triton® X-100	5
8	8.0 RF + Triton® X-100	9

*PEDV titer in vaccine prior to inactivation (log₁₀ TCID₅₀/ml) and route of intramuscular administration (IM, neck; RF, rear flank)

Table 2

Group	Treatment	Challenge	Age of Pigs (weeks)	No. of Pigs
1	-	-	8	7
			20	5
2	-	<i>wt</i> PEDV	8	7
			20	5
3	<i>wt</i> PEDV - ON	<i>wt</i> PEDV	8	7
			20	5
4	Killed Vaccine* - IM	<i>wt</i> PEDV	8	9
			20	5
5	Adenovirus Vaccine - IN	<i>wt</i> PEDV	8	9
			20	5

*Administered on days 0 and 21; ON=oronasal, IM=intramuscular, IN=intranasal; *wt*=wild type

Figure Legends

Figure 1. Timeline of experiment. Abbreviations are as follows: Gr=group of pigs; *wt*=wildtype; KV=Killed Vaccine; Adeno=Adenovirus vectored PEDV S1; RS=Rectal Swab.

Figure 2. Diarrheic values (scored from 0-4 as described in the text) were evaluated at the time of rectal swabbing to assess clinical disease in the pigs. Mean and standard error of the mean $\pm$ (SEM) were calculated and plotted for each pig group. A. Whole group. B. 8 wk-old pigs. C. 20 wk-old swine.

Figure 3. ELISA results are shown. The S/P ratios were calculated [(Sample mean – negative control)/(mean positive control mean – negative control mean)] and plotted against the day of study. In this figure, the day of *wt* PEDV challenge is indicated, and pig samples were taken and assayed at the 12 dpc timepoint. A. All swine. B. 8 wk-old pigs. C. 20 wk-old swine.

Figure 4: Neutralization titers were assessed on prebleed serum samples of group 4 pigs (prior to first administration of the killed vaccine), day of vaccination (study day 21) for all pig serum samples except groups 1 and 2, and for all groups on DPC0 and DPC12. A. All swine. B. 8 wk-old pigs. C. 20 wk-old swine.

Figure 5: qRT-PCR assays were completed using a primer pair specific for the PEDV nucleocapsid gene (Miller et al., 2016). A. All swine. B. 8 wk-old pigs. C. 20 wk-old swine.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5