Impact of dietary spray-dried bovine plasma addition on pigs infected with porcine epidemic diarrhea virus

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ABSTRACT: Experimental data suggest that the addition of spray-dried plasma (SDP) to pig feed may enhance antibody responses against certain pathogens and negatively impact virus survival. The benefit of SDP on Escherichia coli infection is well documented. The aim of this study was to determine the effect of bovine SDP (BovSDP) in the pig diet on acute porcine epidemic diarrhea virus (PEDV) infection. A total of 16 3-wk-old conventional crossbred pigs were used and divided into three groups. Treatments included 1) a negative control group fed a commercial diet and sham inoculated with commercial liquid porcine plasma (n = 3), 2) a positive control group fed a commercial diet and inoculated with PEDV-spiked porcine plasma (PEDV; n = 8), and 3) a third group of pigs fed the commercial diet with inclusion of 5% spray-dried bovine plasma and inoculated with PEDV-spiked porcine plasma (BovSDP; n = 5).

Although clinical signs associated with PEDV infection were mild in the BovSDP group, two of eight pigs in the PEDV group developed moderate clinical disease and had to be euthanized. The PEDV IgG and IgA antibody levels and prevalence rates were significantly (P < 0.05) higher in the PEDV–BovSDP group compared with the PEDV group at 7 d postinoculation. The average fecal PEDV RNA shedding time was 7.2 ± 1.0 d for the PEDV–BovSDP group and 9.3 ± 1.1 d for the PEDV group with an overall time to clearance of PEDV shedding of 11 d for PEDV–BovSDP pigs and at least 14 d for PEDV pigs, which was not different (P = 0.215). The results indicate that addition of BovSDP induced an earlier anti-PEDV antibody response in pigs experimentally infected with PEDV thereby reducing clinical disease and the amount and duration of viral shedding during acute PEDV infection.

Key words: bovine plasma, feed, pig model, porcine epidemic and diarrhea virus

INTRODUCTION

Porcine epidemic diarrhea virus (PEDV) is a large enveloped RNA virus belonging to the Alphacoronavirus genus in the family Coronaviridae. PEDV isolates can be divided into two genogroups: G1, which encompasses a majority of strains isolated before 2010, and G2, which contains more recently identified strains (Huang et al., 2013). PEDV was first discovered in North America in April 2013 and since its emergence has caused substantial losses to the U.S. pork industry. The clinical disease associated with PEDV is characterized by watery diarrhea and vomiting in ...
all ages of pigs and high mortality rates in young pigs (Pensaert and Debouck, 1978).

An intact intestinal mucosa, which prevents the entrance of agents across the epithelium, is a first line of protection with great importance in weaned pigs that, due to separation from their dam and comingling with other pigs, are under high stress and exposed to numerous pathogens they have not encountered before (Pitman and Blumberg, 2000; Boudry et al., 2004; Pie et al., 2004; Bailey et al., 2005; Moretó and Pérez-Bosque, 2009). Producers commonly add spray-dried plasma (SDP) of porcine or bovine origin to weaned pig diets as it has been shown to promote growth (Grinstead et al., 2000) and aids in combating postweaning pathogens such as Escherichia coli (Torrallardona et al., 2003; Bosi et al., 2004). It is well recognized that the addition of SDP to pig feed enhances the immune response and decreases pathogen loads compared with pigs without plasma access through feed. For instance, plasma protein supplements modulated the mucosal immune response in organized and diffuse gut-associated lymphoid tissue, which is accompanied by a reduction of proinflammatory cytokine production (Bosi et al., 2004; Nofrarias et al., 2006; Pérez-Bosque et al., 2008).

A Canadian case–control study investigating factors associated with mortality due to porcine circovirus type 2 (PCV2) found that nursery rations were more likely to contain SDP in control herds compared with clinically affected case herds (Dewey et al., 2006); however, alternatively the observed differences may be completely unrelated to the SDP inclusion in the diets. In addition, the biological neutralization activity of antibodies against common pig pathogens present in SDP was conserved and may contribute to the biosafety of commercially available SDP (Polo et al., 2013). Previously, it has also been shown that SDP has an intrinsic effect reducing survival of PEDV under in vitro conditions (Quist-Rybachuk et al., 2015). After the introduction of PEDV into the United States, concerns on possible contribution of porcine-based SDP in rapid farm-to-farm transmission were raised (Pasick et al., 2014) despite studies clearly demonstrating that PEDV is inactivated during the spray-drying process (Opriessnig et al., 2014; Pujols and Segales, 2014; Gerber et al., 2014b). To eliminate any possible risk of pig pathogen spread via SDP while retaining its benefits as part of a pig diet, pork producers may switch from porcine-origin SDP toward bovine-origin SDP (BovSDP). The purpose of this study was to determine whether there is any benefit of adding BovSDP to a diet of pigs during acute PEDV infection.

**MATERIALS AND METHODS**

**Ethical Statement**

The experiment was approved by the Iowa State University Institutional Animal Care and Use Committee (IACUC approval number 2-14-7742-S).

**Animals, Housing, and Experimental Design**

Sixteen, 2-wk-old, crossbred, colostrum-fed pigs were selected from a commercial PEDV-free herd. The farm of origin was tested on a monthly basis for PEDV RNA on representative fecal samples and for PEDV antibodies on selected serum samples and had no history of clinical diarrhea. The farm of origin was also free of porcine reproductive and respiratory syndrome virus (PRRSV), influenza A virus (IAV), and Mycoplasma hyopneumoniae. Before shipment to the research facility, the pigs received a one dose commercial PCV2 vaccine (Merck Animal Health, Inc.). The pigs were transported to the research facility at Iowa State University, randomly assigned to one of three groups of three to eight pigs, and housed in separate rooms on solid concrete floors (Table 1). Upon arrival of the pigs in the research facility, they were tested negative for PEDV antibodies in serum and PEDV RNA in fecal samples. The experimental design is summarized in Figure 1. Briefly, the pigs were inoculated with PEDV at 3 wk of age (day postinoculation or dpi 0), and all pigs were necropsied at dpi 14.

**Feed**

Starting with arrival in the research facility and for the duration of the study, all pigs were

| Group designations | Number of pigs per group | Percentage of BovSDP in the diet | Inoculation | ADG in grams |
|--------------------|--------------------------|----------------------------------|-------------|--------------|
| NEG                | 3                        | 0                                | Saline      | 224.5 ± 14.6 |
| PEDV               | 8                        | 0                                | PEDV        | 110.6 ± 40.0 |
| PEDV-BovSDP        | 5                        | 5                                | PEDV        | 128.4 ± 33.1 |
fed the same standard commercial corn-soybean meal-dried whey-based diet (Table 2) except for the diet of the PEDV–BovSDP group, which was supplemented with 5% spray-dried commercial bovine plasma replacing soy protein concentrate on an equal total lysine basis (Figure 1). The commercial spray-dried bovine plasma (Lot #S510011001) was produced in a manufacturing plant located in Kansas, United States, and submitted to commercial spray-drying conditions including a minimum outlet temperature of 80 °C throughout substance (throughout the entire particle's mass). The control diet did not contain any BovSDP.

**Inoculation**

For the PEDV inoculation, the passage 7 PEDV G2b isolate 13-19338E (Chen et al., 2014) at viral concentration of $10^{3.0}$ 50% tissue culture infectious dose per milliliter was used. In brief, PEDV was propagated on Vero cells (ATCC CCL-81) with minimal essential medium supplemented with tryptose phosphate broth (0.3%), yeast extract (0.02%), trypsin 250 (5 µg/mL), gentamicin (0.05 mg/mL), penicillin (100 unit/mL), streptomycin (100 µg/mL), and amphotericin (0.25 µg/mL) as previously described (Chen et al., 2014). Commercial liquid porcine plasma negative for PEDV RNA was used as diluent to adjust the PEDV inoculum (PEDV and PEDV–BovSDP groups) or saline (negative control [NEG] group) to a total volume of 10 mL for each pig. Inoculation was performed orally by the same team for all pigs. The pigs were held in vertical recumbence between the legs of the holder while the second person administered the material by slowly dripping the inoculum into the mouth of the pig using a syringe on dpi 0 when the pigs were 3 wk old.

**Clinical Evaluation**

After PEDV inoculation, all pigs were monitored daily for signs of illness such as lethargy, vomiting, or diarrhea. Rectal temperatures were taken every other day on each pig, and the fecal consistency score ranging from 0 = normal, 1 = semisolid, 2 = pasty, and 3 = liquid was determined (Gerber et al., 2016). All pigs were weighed at dpi −6 and again at dpi 14, and the ADG was calculated.

**Serology**

To verify the presence of PEDV IgA and IgG antibodies, blood samples were collected in serum separator tubes on dpi 0, 7, and 14 and centrifuged at 4,000 rpm for 10 min at 4 °C. All serum samples were tested for the presence of anti-PEDV IgG or IgA by an “in-house” indirect ELISA based on the spike 1 gene of a prototype U.S. PEDV strain similar to the one used as challenge virus in this study (Gerber et al., 2014a; Gerber and Opriessnig, 2015). A sample with a sample-to-positive (S/P) ratio equal to or greater than 0.2 was considered positive for PEDV IgG, and a sample with an S/P ratio equal to or greater than 0.13 was considered positive for PEDV IgA.

**PEDV Real-Time Reverse Transcription PCR (Real-Time RT PCR) on Rectal Swabs**

To determine fecal PEDV shedding, rectal swabs were collected from all pigs on dpi 6 and from dpi 1 to 14 using polyester swabs and stored in 5-mL plastic tubes containing 1-mL sterile saline. Total nucleic acids were extracted from fecal swab suspensions using the MagMax Pathogen RNA/DNA Kit and an automated nucleic acid extraction system (Thermo Scientific Kingfisher Flex, Thermo Fisher Scientific, Pittsburgh, PA) according to the instructions of the manufacturer.
...Presence of PEDV RNA was determined by using a quantitative real-time RT-PCR that was set up using the Path-ID Multiplex One-Step RT-PCR Kit (Thermo Fisher Scientific), and amplifications were performed on the Applied Biosystems 7500 Fast Real-Time PCR System thermocycler and the accompanying software (Opriessnig et al., 2014). Necropsy, Histopathology, and Immunohistochemistry

On dpi 14, all pigs were killed by intravenous pentobarbital overdose (FATAL-PLUS, Vortech Pharmaceuticals Ltd, Dearborn, MI) and necropsied. Tissues collected from pigs at necropsy included eight sections of small intestines, three sections of large intestine, and one section of mesenteric lymph node. Tissues were immediately put in 10% buffered formalin and routinely processed for histopathology and assessed for lesions by a veterinary pathologist blinded to the treatment status. Presence and degree of atrophic enteritis were scored ranging from 0 = normal to 3 = severe. To determine presence and amount of PEDV antigen in tissue sections, a PEDV immunohistochemical stain was performed on intestines from all pigs as described (Stevenson et al., 2013). The amount of PEDV antigen was scored by a veterinary pathologist blinded to the treatment status with 0 = no signal, 1 = 1% to 10% staining, 2 = 11% to 50% staining, and 3 = greater than 50% staining.

Statistical Analysis

The statistical software used was JMP Pro 11. Analysis of variance (ANOVA) was used for cross-sectional assessment of the average daily weight gain and continuous measures including viral shedding, IgA, and IgG antibody levels. The PEDV genomic copy numbers per milliliter of fecal suspension were log transformed before statistical analysis. If a significant (P < 0.05) difference was detected, pairwise testing using the Tukey–Kramer adjustment was performed to determine which groups were different. Daily rectal temperature data were analyzed with multivariate ANOVA. Non-repeated measures of necropsy and histopathology data were assessed using non-parametric Kruskal–Wallis ANOVA. If a non-parametric ANOVA test was significant (P < 0.05), then Wilcoxon tests were used to assess the differences between pairs of groups. Differences in incidence of clinical scores were evaluated by using the Fisher’s exact test. For fecal shedding of PEDV RNA, an area under the curve (AUC) was calculated for each animal individually and differences between groups were assessed by unpaired t-test (data passed Shapiro–Wilks normality test).

RESULTS

Clinical Signs

There was no difference in rectal temperatures among the groups over time, and none of the pigs developed elevated rectal temperatures during the experiment. Diarrhea was not observed in the NEG...
group while individual pigs in the PEDV group and PEDV–BovSDP group had fluid-to-pasty-to-semi-solid feces between dpi 2 and 6 with no significant differences among groups. In addition, vomiting was occasionally observed in single pigs during this time. By dpi 9, two PEDV pigs became lethargic and had reduced appetite. One of these pigs developed mild pasty diarrhea. Due to welfare concerns, these pigs were euthanized between dpi 11 and 13. The ADG is summarized in Table 1. Overall, there were numerical differences among groups for the ADG: NEG pigs had the highest ADG, and PEDV pigs had the lowest ADG; however, the differences were not significant.

**Anti-PEDV IgA and IgG Antibody Responses**

All pigs were negative for PEDV IgA and IgG antibodies at arrival, and the NEG pigs remained seronegative for the duration of the study (Figures 2 and 3). Three of five PEDV-BovSDP pigs had detectable anti-PEDV IgA antibodies in serum by dpi 7, and all pigs in this group and five per eight PEDV pigs were positive by dpi 14. Group mean IgA levels were higher (P < 0.011) for PEDV–BovSDP pigs compared with PEDV pigs at dpi 7 (Figure 2). Group mean IgG levels were also higher (P < 0.021) in PEDV–BovSDP pigs at dpi 7 compared with the PEDV pigs (Figure 3). All PEDV-infected pigs in both groups had anti-IgG antibodies by dpi 14 (Figure 3).

**Fecal Shedding of PEDV**

All pigs were PEDV RNA negative at dpi −6, and PEDV RNA was not detected in any of the NEG pigs throughout the study. Fecal shedding of PEDV RNA was first detected in a PEDV pig at dpi 1 and shedding in this group lasted until termination of the study at dpi 14 (Figures 4 and 5). Pigs in the PEDV–BovSDP group shed PEDV from dpi 2 to 11 (Figures 4 and 5). The prevalence of PCR positive PEDV–BovSDP pigs compared with the PEDV group was higher (P = 0.021) at dpi 2, while it was lower (P = 0.028) at dpi 12. The average shedding period was 9.3 ± 1.1 for PEDV pigs and 7.2 ± 1.0 for PEDV–BovSDP pigs, which was not different (P = 0.215). Similarly, the cumulative fecal viral RNA shedding was not different (P = 0.384) between PEDV pigs and PEDV–BovSDP pigs.

**Microscopic Lesions and PEDV Antigen in Tissues**

In the two pigs that were euthanized due to welfare reasons, in addition to the standard set of enteric tissues, sections of liver, lung, spleen, tonsil, heart, and kidney were also collected and assessed to rule out any concurrent systemic infection. One of these two pigs had moderate diffuse atrophic enteritis associated with PEDV antigen as determined by immunohistochemical stains (score 2). This pig also had the highest amount of PEDV RNA measured (log_{10} 9.5 genomic copies per fecal swab at dpi 6 and

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**Figure 2.** Mean group anti-PEDV IgA ELISA S/P ratios ± SEM at days 0, 7, and 14 after PEDV inoculation (dpi) in the different treatment groups and number of positive pigs/total number of pigs in the group for each day. The results of the two pigs that were killed on dpi 11 and 13 were included at dpi 14. An S/P ratio greater than 0.13 was considered positive. Different superscripts (A,B) indicate significantly different means at a given day. Statistical analysis was performed by one-way ANOVA followed by pairwise testing using the Tukey–Kramer adjustment if P < 0.05. The statistical software used was JMP Pro 11. The number of ELISA positive pigs per total number of pigs per group is listed next to each group mean.
log_{10} 8.9 at dpi 11) when it was killed. The other pig had its peak PEDV shedding at dpi 6 (log_{10} 6.3 PEDV genomic copies), but its shedding decreased to log_{10} 5.6 genomic copies by dpi 13. No other lesions were seen in these two pigs. The remaining pigs were necropsied at the scheduled time at dpi 14, and lesions or PEDV antigen were not seen in any of these pigs suggesting that the PEDV infection and associated lesions had resolved.

**DISCUSSION**

PEDV has become a major economic concern for North American pig producers since it was first

Figure 3. Mean group anti-PEDV IgG ELISA S/P ratios ± SEM at days 0, 7, and 14 after PEDV inoculation in the different treatment groups and number of positive pigs per total number of pigs in the group for each day. The results of the two pigs that were killed on dpi 11 and 13 were included at dpi 14. An S/P ratio greater than 0.2 was considered positive. Different superscripts (A, B) indicate significantly different means at a given day. The number of ELISA positive pigs per total number of pigs per group is listed next to each group mean.

Figure 4. Mean group log_{10} amount of PEDV RNA fecal samples ± SEM at days 0, 7, and 14 after PEDV infection and number of positive pigs per total number of pigs in the group for each day. An ‘A’ on a given day indicates significant (P < 0.05) different group means. Statistical analysis was performed by one-way ANOVA followed by pairwise testing using the Tukey–Kramer adjustment if P < 0.05. The statistical software used was JMP Pro 11. 443.
identified in the United States in 2013 (Stevenson et al., 2013; Chen et al., 2014). The lack of highly effective vaccines and the relative ineffectiveness of common treatment methods have led to a search for alternative methods of treating and preventing outbreaks. The inclusion of SDP into the diets of young pigs has been shown to have health benefits for other diseases. This study was designed to determine whether there is any benefit to adding BovSDP to the diet during PEDV infection.

Supplementing feed rations of pigs, fish, poultry, cats, and dogs with SDP as protein source is performed on a regular basis. SDP is a protein-rich product obtained from blood from healthy animals (cattle or pigs) at slaughter (Torrallardona, 2010; Pérez-Bosque et al., 2016). In pet food, SDP is a preferred binder in canned food products due to its high-protein content and its physicochemical properties (Polo et al., 2005; Rodriguez et al., 2016). Porcine SDP was first introduced as protein source for pigs during the early 1990s (Cole and Sprent, 2001) and since has been used widely in the diet of weaned pigs (Torrallardona, 2010). Benefits of adding porcine SDP include improvement of weight gain mainly due to increased feed intake and reduction of incidence and severity of diarrhea after weaning (Adewole et al., 2016). Comprehensive information on the effect of SDP obtained in 75 trials involving over 12,000 pigs has been summarized (Torrallardona, 2010).

In this study, the PEDV challenge was performed after an acclimation period of 1 wk to minimize stress from weaning and transport to the new facility. Successful PEDV challenge was confirmed by detecting PEDV RNA in fecal swabs. All pigs (100%) from the BovSDP–PEDV group shed PEDV in fecal samples from dpi 2 to 6, whereas between 25% and approximately 87% of the animals in PEDV group shed the virus during that time. These results highlight that a higher number of animals excreted PEDV during the early stages of infection in BovSDP–PEDV group. Differences in the challenge dose or challenge process can be ruled out. The PEDV inoculum was prepared in a similar manner and at the same time for both groups by a single person and stored on ice until challenge of each group. The challenge was conducted by the same personnel for both groups with approximately 20 min between the two groups. The obtained results could have been by chance due to the group sizes. Alternatively, the BovSDP–PEDV animals could be more prone to excrete PEDV in the early stages in the infection. Unlike in suckling pigs that are very susceptible to PEDV infection, only 2 per 13 PEDV-infected pigs (both from the PEDV challenged groups) had to be killed due to the severity of clinical signs. This is expected and comparable with other trials infecting 3-wk-old pigs (Crawford et al., 2015).

In the current study, the PEDV IgG and IgA antibody responses were more rapid in the BovSDP group compared with the PEDV group. Specifically, by dpi 7, 60% of the BovSDP pigs were anti-IgG and
IgA positive compared with 12.5% of the PEDV pigs. Of note, while systemic IgA antibodies were measured, mucosal IgA levels were not determined, and it is therefore unknown how the addition of the BovSDP affected the gut immunity. In a previous study, a good correlation of IgA levels in serum and feces was found (Gerber and Opriessnig, 2015), and as fecal samples and gut mucosa are more difficult to process during routine lab work, serum IgA levels are commonly tested. Explanations for the earlier humoral immune response in the BovSDP group may include acceleration of the clinical course by the dietary supplement; however, a more rapid immune response due to earlier replication of the virus in more pigs unrelated to the diet modification is also possible. Serum anti-PEDV Ig in pig serum has been demonstrated to neutralize infectivity of PEDV (Hofmann and Wyler, 1989), and bovine plasma could have a similar neutralizing activity, which was not further assessed in this study. Besides the presence of possible neutralizing antibodies in SDP, other plasma compounds such as peptides (Anderson and Anderson, 2002) could contribute to the benefits seen with SDP addition to a diet.

Virus shedding in the BovSDP group was 2.1 d shorter than in the PEDV group. The fecal PEDV RNA shedding in this group was terminated by dpi 11. A previous study has shown that PEDV-infected pigs shed infectious PEDV capable of horizontal transmission for 14 to 16 d after infection (Crawford et al., 2015). This is similar to what was observed in the PEDV-infected pigs without BovSDP in the diet in this study.

The data from this trial indicate a beneficial effect of BovSDP on acute PEDV infection, which is similar to previous reports using the PRRSV infection model (Pujols et al., 2011). Specifically, pigs fed BovSDP were able to clear virus shedding 2.1 d sooner than non-BovSDP pigs. The addition of BovSDP to the diet resulted in faster and stronger PEDV antibody responses and reduced PEDV shedding time compared with pigs with no BovSDP in the diet. Limitations of this study include the usage of one single virus titer and the low numbers of pigs tested. In addition, two PEDV pigs had to be removed early from the study, which could have impacted the outcomes. A larger study with higher numbers of pigs per group comparing porcine and bovine-derived SDP with multiple necropsy days should be conducted to further confirm the possible benefits of SDP in PEDV-infected pigs.

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