The spray-drying process is sufficient to inactivate infectious porcine epidemic diarrhea virus in plasma

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ABSTRACT

Porcine epidemic diarrhea virus (PEDV) is considered an emergent pathogen associated with high economic losses in many pig rearing areas. Recently it has been suggested that PEDV could be transmitted to naïve pig populations through inclusion of spray-dried porcine plasma (SDPP) into the nursery diet which led to a ban of SDPP in several areas in North America and Europe. To determine the effect of spray-drying on PEDV infectivity, 3-week-old pigs were intragastrically inoculated with (1) raw porcine plasma spiked with PEDV (RAW-PEDV-CONTROL), (2) porcine plasma spiked with PEDV and then spray dried (SD-PEDV-CONTROL), (3) raw plasma from PEDV infected pigs (RAW-SICK), (4) spray-dried plasma from PEDV infected pigs (SD-SICK), or (5) spray-dried plasma from PEDV negative pigs (SD-NEG-CONTROL). For the spray-drying process, a tabletop spray-dryer with industry-like settings for inlet and outlet temperatures was used. In the RAW-PEDV-CONTROL group, PEDV RNA was present in feces at day post infection (dpi) 3 and the pigs seroconverted by dpi 14. In contrast, PEDV RNA in feces was not detected in any of the pigs in the other groups including the SD-PEDV-CONTROL group and none of the pigs had seroconverted by termination of the project at dpi 28. This work provides direct evidence that the experimental spray-drying process used in this study was effective in inactivating infectious PEDV in the plasma. Additionally, plasma collected from PEDV infected pigs at peak disease did not contain infectious PEDV. These findings suggest that the risk for PEDV transmission through commercially produced SDPP is minimal.

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1. Introduction

Spray-dried porcine plasma (SDPP) is used widely in diets for weanling pigs to improve their growth rate and feed intake, and to reduce diarrhea (Peace et al., 2011; Pettigrew, 2006; Van Dijk et al., 2001; Zhao et al., 2007). The plasma utilized for production of SDPP is collected at veterinary-inspected abattoirs from animals designated as fit for human consumption. Specifically, blood is collected into containers with anticoagulant and the erythrocytes are removed by centrifugation. The plasma obtained is subsequently spray-dried and used for the production of food, feed and for industrial applications. It has been shown that specific spray-drying conditions substantially reduce the number of viable microorganisms (Ananta et al., 2005; Polo et al., 2005; Pujols et al., 2007). The blood is

pooled from 6000 to 10,000 animals slaughtered on the same day, resulting in a mixture of antibodies against various pathogens that have a neutralizing effect on potential pathogens present in the plasma and can be considered as an effective biosafety step in the manufacturing process of SDPP (Polo et al., 2013).

Porcine epidemic diarrhea virus (PEDV), an enveloped single-stranded, positive-sense RNA virus which belongs to the family Coronaviridae, was first reported in feeder and fattening pigs in the UK in 1971 (Wood, 1977). PEDV causes an acute, highly contagious diarrhea in pigs of all ages with up to 90–95% mortality in suckling pigs in naïve breeding herds (Stevenson et al., 2013; Sun et al., 2012; Temeeyasen et al., 2013; Wang et al., 2013). Outbreaks of PEDV have occurred in all pig producing areas in Europe (Song and Park, 2012) followed by introduction into Asia in the early 1990s (Kusanagi et al., 1992). More recently, PEDV was introduced to North America and rapidly spread across farms and states causing large-scale out-breaks with high rates of morbidity and mortality (Huang et al., 2013; Mole, 2013; Stevenson et al., 2013). Thus far, there is no effective vaccine or specific treatment available, and the only measures to control the disease are those directed at preventing the introduction of the virus to farms (Park et al., 2011; Song and Park, 2012; Temeeyasen et al., 2013).

The source of introduction and routes of transmission of PEDV in North America are being investigated. Potential routes of PEDV transmission include direct and indirect contact with feces of infected animals (Jung et al., 2014; Saif et al., 2012), contaminated transport vehicles (Lowe et al., 2014), and milk from infected sows (Sun et al., 2012). Recently, SDPP products were implicated to transmit PEDV in the USA and Canada (Anonymous, 2014). Analytical results released by the Ontario Ministry of Agriculture and Food indicated the presence of PEDV RNA in commercial SDPP; bioassay tests confirmed infectious PEDV in the SDPP but not in the associated feed in which it was used as an ingredient (Pasick et al., 2014).

To investigate the potential of plasma obtained from PEDV infected pigs to contain infectious PEDV and to investigate the infectivity of PEDV after the spray-drying process, plasma samples derived from PEDV positive piglets or plasma samples spiked with cell culture propagated PEDV were tested in a swine bioassay, either untreated (raw) or after spray-drying. The objectives of this study were to (1) evaluate whether plasma from PEDV infected pigs at the peak of disease contains infectious PEDV and (2) if the spray-drying process is efficient to inactivate PEDV.

2. Material and methods

2.1. Ethical statement

The experimental protocol in this study was approved by the Iowa State University Institutional Animal Care and Use Committee (Approval no. 2-14-7742-S; approved on 5-Mar-14).

2.2. Animals and housing

Colostrum-fed, crossbred, specific-pathogen-free, 2-week-old pigs were purchased from a PEDV-free herd. Sixteen pigs obtained from six litters were transported to biosafety level 2 research facilities at Iowa State University, Ames, IA, USA. Upon arrival, the pigs were ear-tagged, randomly divided into groups of two-to-four pigs and housed in five separate rooms. Each room had $18\,m^2$ of solid concrete floor space, separate ventilation systems, and one nipple drinker. All groups were fed ad libitum a balanced, pelleted, complete feed ration free of animal proteins (Heartland Coop, Prairie City, IA, USA).

2.3. Experimental design and treatments

The experimental design is summarized in Table 1. Treatments included spray-dried plasma from PEDV negative pigs (SD-NEG-CONTROL), spray-dried plasma from pigs experimentally infected with PEDV (SD-SICK), PEDV negative plasma spiked with PEDV and then spray dried (SD-PEDV-CONTROL), liquid (raw) PEDV negative plasma spiked with PEDV (RAW-PEDV-CON-TROL), and liquid (raw) plasma from pigs experimentally infected with PEDV (RAW-SICK). Experimental inoculation was done after a one week acclimation period when the pigs were 3 weeks old. Fecal swabs were collected using polyester swabs two days prior to inoculation, and at days post-inoculation (dpi) 3, 5, 7, 11, 14, 21 and 28 and stored in 5 ml plastic tubes containing 1 ml of sterile saline solution (Fisher Scientific, Inc.). Blood samples were collected at dpi 2, and at dpi 7, 14, 21 and 28. The blood was collected in 8.5 ml serum separator tubes (Fisher Scientific, Inc.), immediately centrifuged at $3000 \times g$ for 10 min at $4\,°C$, separated, and stored at $-80\,°C$ until use. Following challenge, the pigs were individually monitored daily. Pigs were weighed at dpi 0 and 28.

2.4. Plasma sources and processing

Plasma was obtained by collecting blood from pigs euthanized with an overdose of pentobarbital (Fatal Plus, Vortech Pharmaceutical, Dearborn, MI, USA) in jars containing 12,000 USP heparin units (Hospira Inc., Lake Forest, IL, USA) per liter of blood. The plasma was immediately centrifuged at $2000 \times g$ for 10 min at $4\,°C$ in 50 ml centrifuge tubes and stored at $4\,°C$ until use.

2.4.1. PEDV-negative plasma
PEDV RNA and antibody negative plasma was obtained from five 15-week-old crossbred PEDV naïve pigs as part of another study (T. Opriessnig, unpublished data). One liter of the PEDV-negative plasma was spray-dried two hours after plasma collection and served as sham-inoculum for the NEG-CONTROL group.

2.4.2. PEDV-spiked plasma
Ten milliliter of the PEDV-negative plasma described under "PEDV-negative plasma" was spiked with a cell culture propagated PEDV strain ISU13-19338E (Chen et al., 2014) to a final concentration of 5×10^2 50% tissue culture

Table 1
Experimental design.

Group designation	n	PEDV status of the pig utilized as plasma source	Plasma treatment		Inoculation dose per pig	Log$_{10}$ PEDV genomic equivalent copies/ml[b]
			PEDV spiking (PEDV titer)	Spray-drying		
SD-NEG-CONTROL	4	Negative	No	Yes	5 ml	0
SD-SICK	4	Positive[a]	No	Yes	0.9 g	0
SD-PEDV-CONTROL	4	Negative	Yes (1.8×10^4 TCID$_{50}$/ml)	Yes	0.9 g	3.56
RAW-PEDV-CONTROL	2	Negative	Yes (5.0×10^2 TCID$_{50}$/ml)	No	5 ml	4.28
RAW-SICK	2	Positive[a]	No	No	5 ml	0

[a] Experimentally infected with PEDV 3 days previously.
[b] Final product used for inoculation of the pigs (raw plasma or reconstituted spray-dried plasma powder).

infectious dose (TCID$_{50}$) per ml (total dose of 5×10^3 TCID$_{50}$) and served as inoculum for the RAW-PEDV-CONTROL group. In addition, 1 l of the PEDV-negative plasma was spiked with PEDV to a final concentration of 1.8×10^4 TCID$_{50}$ per ml, stored at 4 °C, and subsequently spray-dried 5 h after plasma collection, and served as inoculum for the SD-PEDV-CONTROL group.

2.4.3. Plasma from PEDV sick pigs

Plasma was obtained from three 3-week-old crossbred pigs from another study where they were orally infected with 5×10^3 TCID$_{50}$ of PEDV ISU13-19338E and euthanized at dpi 3 when the pigs had developed diarrhea (Opriessnig et al., 2014). Specifically, PEDV RNA was demonstrated in serum samples by RT-PCR in two of three individual pigs from the PEDV control group ranging from 2.2 to 3.2 log$_{10}$ genomic equivalent copies per ml (Opriessnig et al., 2014). However, PEDV RNA was not detected in the pooled plasma by real-time RT-PCR. Assessment of the *in vitro* infectivity of the pooled plasma was not performed. A total of 15 ml of the plasma were used in its liquid form and served as inoculum for the RAW-SICK group and 110 ml of the plasma were spray-dried and served as inoculum for the SD-SICK group.

2.5. Spray-drying process

To ensure that the spray dryer was not contaminated with PEDV, it was disinfected with an oxidizing disinfectant (Virkon® S, DuPont) according to the manufacturers' recommendations and subsequently subjected to UV light for 30 min from all sides before the initiation of each run. Seven swabs were then taken from various components of the apparatus, placed in sterile saline, and confirmed to be PEDV-negative by real-time RT-PCR.

The collected plasma was spray-dried using a benchtop spray-dryer (Yamato Model ADL310, Yamato Scientific Co. Ltd., Tokyo, Japan) as previously described (Patterson et al., 2010). Briefly, a 0.4-mm nozzle was used with the following parameters: inlet air temperature of 166 °C, aspiration rate of 0.6 m^3/min, outlet temperature of 80 °C, and an 820 ml/h sample flow rate under 0.1 MPa of pressure. The resulting spray-dried plasma powder was stored at 4 °C for seven days, at which time it was reconstituted in sterile saline to a concentration of 0.18 g/ml.

2.6. Inoculation

Inoculation was done by intragastric administration of 5 ml of each of the final treatments as outlined in Table 1 using a stomach tube. Pigs in the SD-NEG-CONTROL, SD-PEDV-CONTROL and SD-SICK groups were inoculated with the respective reconstituted spray-dried plasma. Pigs in the RAW-PEDV-CONTROL and the RAW-SICK groups were inoculated intragastrically with the liquid plasma samples.

2.7. Anti-PEDV-IgG antibodies

Serum samples were tested by a spike (S) 1-based PEDV IgG ELISA described elsewhere (Gerber et al., 2014) and an indirect immunofluorescence assay (IFA) according to routinely performed standard protocols at the Veterinary Diagnostic Laboratory at Iowa State University (VDL-ISU). Briefly, an immunogenic fragment spanning amino acids 1 through 718 of the S1 domain of the PEDV IA1 strain (Huang et al., 2013) expressed in an eukaryotic expression vector was used as antigen for the S1-ELISA. Microtiter plates were coated with the S1 polypeptide diluted in carbonate coating buffer and incubated overnight at 4 °C. Plates were then blocked with 1% bovine serum albumin for 2 h at 22 °C and incubated with samples diluted 1:100 in PBS containing 10% goat serum for 30 min at 37 °C. After a washing step, a 1:20,000 diluted peroxidase-conjugated goat anti-swine IgG (Jackson ImmunoResearch) was added and incubated at 37 °C for 30 min. The peroxidase reaction was visualized by using tetramethylbenzidine-hydrogen peroxide solution as the substrate for 10 min at room temperature and stopped by adding 50 μl of 2.5 M sulfuric acid to each well. Optical densities (OD) were measured at 450 nm using an ELISA plate reader and were considered positive if the OD value was 0.3 or greater.

2.8. PEDV RNA detection and quantification

Total nucleic acids was extracted from the fecal swab suspensions, serum, raw plasma samples and spray-dried plasma samples using the MagMax^{TM} viral isolation kit (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) on the KingFisher Flex System (ThermoFisher Scientific, Pittsburgh, PA, USA). Spray-dried plasma was diluted 1:20 in saline prior to extraction. The extracts were tested for presence and quantity of PEDV genomic equivalent copy

numbers by a real-time RT-PCR targeting the conserved ORF1b gene as described (Kim et al., 2007).

2.9. Sequencing

A PEDV real-time RT-PCR positive fecal sample collected at dpi 7 from a pig in the RAW-PEDV-CONTROL group was sequenced and compared with the sequence of the PEDV strain used for inoculation. In brief, a RT-PCR was used to amplify the S1 and nucleocapsid genes as described (Chen et al., 2014). The PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen) according to the manufacturers' directions and sequenced at the Iowa State University DNA facility. The sequences were aligned with published data using BLAST at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) and compiled using Lasergene software and the Clustal W alignment algorithm (DNASTar, Madison, WI, USA).

2.10. Necropsy, histopathology and immunohistochemistry

All pigs were necropsied at dpi 28. Severity of macroscopic lesions in the small and large intestines were estimated by a pathologist blinded to the treatment status. Six sections of small intestines, three sections of large intestines, and one section of lung were collected at necropsy, fixed in 10% neutral-buffered formalin, and routinely processed for histological examination. Microscopic lesions were evaluated by a pathologist blinded to treatment groups as described (Stevenson et al., 2013). Detection of PEDV-specific antigen in selected formalin-fixed and paraffin-embedded intestinal sections was performed by using immunohistochemistry and a monoclonal antibody specific for PEDV (BioNote, Hwaseong-si, Gyeonggi-do, Korea) as described (Kim et al., 1999; Stevenson et al., 2013).

2.11. Statistical analysis

For data analysis, JMP® Pro software version 10.0.0 (SAS Institute, Cary, NC, USA) was used. Statistical analysis of the data was performed by one-way analysis of variance (ANOVA) for the average daily weight gain. A p-value of less than 0.05 was set as the significant level.

3. Results

3.1. Clinical presentation, average daily weight gain, gross and microscopic lesions

The pigs in the RAW-PEDV group developed semisolid-to-fluid diarrhea around dpi 3 which lasted until dpi 7–10. Clinical disease was not observed in any of other groups for the duration of the study. From the time of challenge to the termination of the study, there was no difference in the average daily weight gain ($p = 0.58$) among groups (data not shown). At dpi 28, macroscopic or microscopic lesions were not observed and PEDV antigen was not detected by immunohistochemistry in any of the pigs.

3.2. Anti-PEDV-IgG antibody detection

There was no difference between the detection rates using the S1-ELISA and IFA and only S1-ELISA results are presented in Fig. 1. Pigs in the SD-NEG-CONTROL, SD-PEDV-CONTROL, RAW-SICK and SD-SICK groups remained seronegative throughout the duration of the study. In the RAW-PEDV-CONTROL group, pigs seroconverted by dpi 14 (Fig. 1).

3.3. PEDV RNA detection, quantification and sequencing

Log$_{10}$ transformed mean group PEDV genomic equivalent copy numbers per fecal swab ($\pm$SEM) are presented in Fig. 2. Pigs within the SD-NEG-CONTROL, SD-PEDV-CONTROL, SD-SICK, and RAW-SICK groups remained PEDV negative throughout the duration of the trial. In the RAW-PEDV-CONTROL group, PEDV RNA in fecal swabs was detected from dpi 3 until termination of the study at dpi 28 (Fig. 2). PEDV RNA was not detected in serum samples at any collection point. Sequence analysis of the PEDV recovered from the RAW-PEDV-CONTROL revealed 100% similarity at the nucleotide level with the PEDV strain used for inoculation (data not shown).

4. Discussion

After initial identification of PEDV in the U.S., the virus spread rapidly across all major swine production regions resulting in high mortality and great economic losses (Mole, 2013; Stevenson et al., 2013). It has been suggested that secondary contamination of transport vehicles at collection points (Lowe et al., 2014) and SDPP products fed to young piglets (Anonymous, 2014) played a role in the PEDV dissemination in North America. Detection of PEDV RNA positive spray-dried plasma lots further raised awareness among pork producers leading to ban of SDPP in certain areas (Anonymous, 2014).

As a member of the *Coronavirus* family PEDV is an enveloped RNA virus and as such is sensitive to heat inactivation (Hofmann and Wyler, 1989). It has been confirmed that PEDV can be rapidly inactivated when heated at 60 °C for 30 min (Hofmann and Wyler, 1989). Other enveloped viruses with similar heat resistance such as porcine reproductive and respiratory syndrome virus and pseudorabies virus, and even more stable non-enveloped RNA viruses such as swine vesicular disease virus (Turner and Williams, 1999) have been successfully inactivated by spray-drying (Polo et al., 2005; Pujols et al., 2007). In contrast, porcine circovirus type 2, a non-enveloped DNA virus, is considered a model for heat resistant viruses (Martin et al., 2008; O'Dea et al., 2008) and the spray drying process using laboratory spray dryer models is not fully capable of inactivation (Patterson et al., 2010).

The present study was designed to test the degree of inactivation of PEDV during the spray-drying process. To make sure that an appropriate positive control was utilized, plasma was spiked with a known amount of infectious PEDV. In addition, the plasma was free of PEDV antibodies to avoid potential virus neutralization prior to

□SD-NEG-CONTROL ■SD-SICK ▤SD-PEDV-CONTROL ▨RAW-PEDV-CONTROL ▩RAW-SICK
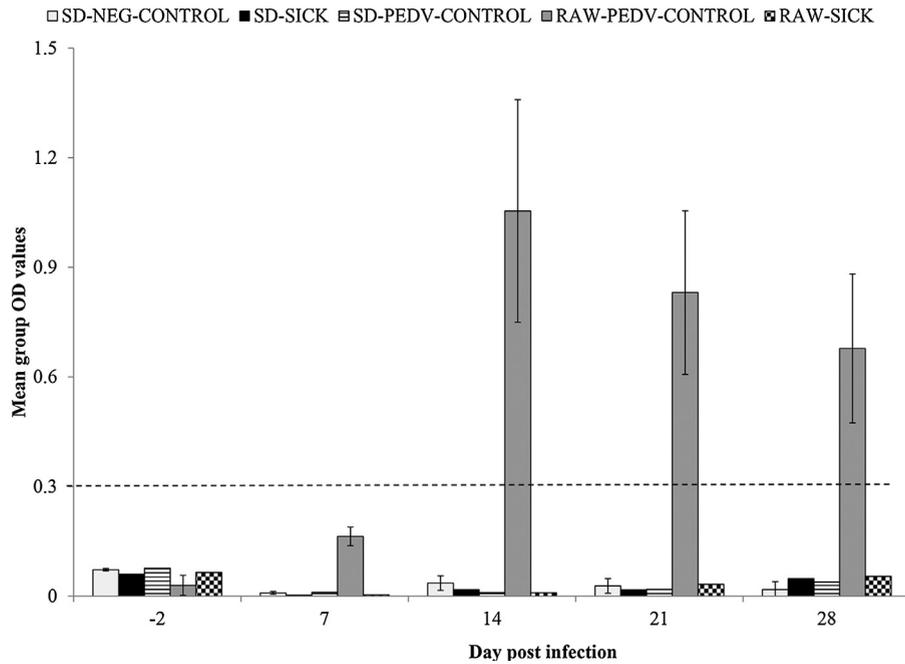
Fig. 1. Group mean anti-PEDV-IgG optical density (OD) values. Group means (±SEM) were calculated using serum samples from all animals in each group at each time point. Any OD value equal or greater than 0.3 was considered positive.

□SD-NEG-CONTROL ■SD-SICK ▤SD-PEDV-CONTROL ▨RAW-PEDV-CONTROL ▩RAW-SICK
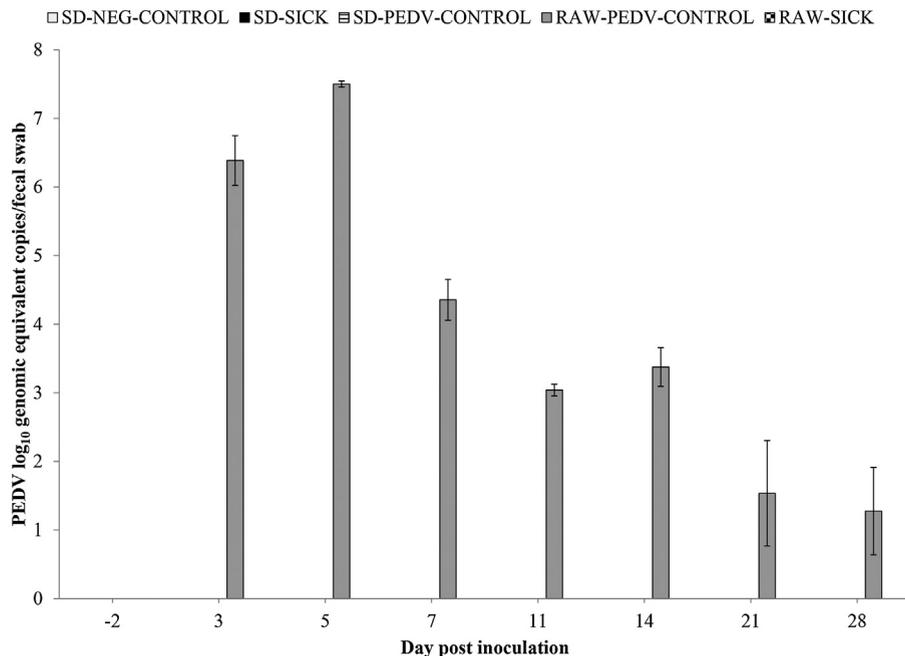
Fig. 2. Group mean PEDV RNA levels in fecal samples over time. Group means (±SEM) were calculated using the $\log_{10}$ PEDV genomic equivalent copy number per fecal swab from all pigs in each treatment group at each time point.

spray drying. When administered directly in untreated liquid form, as expected, infectious PEDV-spiked into plasma retained its infectivity and was capable of infecting the PEDV naïve pigs in the RAW-PEDV-CONTROL group as determined by viral shedding in feces and seroconversion to PEDV. To further test if PEDV is capable of surviving the

spray-drying process, plasma containing infectious PEDV (SD-PEDV-CONTROL) was spray-dried using a laboratory bench spray-dryer. Although PEDV RNA could be detected in the plasma powder after spray drying, none of the SD-PEDV-CONTROL pigs became PEDV infected, as evidenced by the lack of PEDV RNA in feces and no evidence of seroconversion.

The PEDV-spiked spray-dried plasma powder used in this study was stored at 4 °C for seven days. A recent report on the biosafety of spray-dried plasma (Biosafety of spray dried plasma relative to porcine epidemic diarrhea virus; http://www.functionalproteins.com/documents/news/21.pdf; Accessed on May 1, 2014) has shown that PEDV survived up to 14 days in contaminated spray-dried bovine plasma stored at 4 °C using an *in-vitro* model. Therefore, the storage conditions used in the present study were not detrimental to the virus viability indicating that the viral inactivation in the present study was due to the spray-drying process itself. It is worth noting that the concentration of PEDV in the plasma that was spray-dried and used for the SD-PEDV-CONTROL group was higher than the concentration in the liquid plasma used in the RAW-PEDV-CONTROL group. Together with the data obtained in the RAW-PEDV-CONTROL group, this indicates that the spray drying process inactivated the virus.

The design and configuration of the particular spray-dryer utilized and the specific spray-drying process have been shown to affect the efficacy of virus inactivation. Therefore, it has been suggested that it may not be appropriate to fully extrapolate data obtained through use of the bench-top laboratory spray dryer to the commercial spray drying processes (Thybo et al., 2008). In general, laboratory spray dryers have less capability of viral inactivation compared with commercial spray-driers (Shen et al., 2011). Therefore the results from the present study are encouraging as they indicate complete inactivation of infectious PEDV even under less stringent conditions supporting an overall low risk that infectious PEDV, if present, may survive the commercial spray-drying process. In the present study, the outlet temperature used was the same as suggested for spray-dried plasma production under commercial processes, as this parameter has been shown to have the single major effect on microorganism viability (Ananta et al., 2005). Usage of different spray-drying conditions or lapses in the consistency of the processes could potentially lead to an incomplete viral inactivation and further work needs to be done to understand and perhaps improve the quality assurance of the processes utilized by the SDPP industry. Alternatively, contamination of PEDV in SDPP and by-products may occur post-processing as suggested by recovery of infectious PEDV from feed without inclusion of animal by-products (Dee et al., 2014). Additionally, PEDV has been demonstrated to become aerosolized and transported up to 10 miles from infected sites (Alonso et al., 2014) thereby showing a great potential for wide spread and environmental contamination.

Although it was initially thought that enteric coronaviruses including PEDV are not associated with viremia (Saif and Heckert, 1990), viral loads of 4.0–7.6 $\log_{10}$ PEDV genome equivalent copy numbers per ml of serum have been described recently in experimentally and naturally PEDV-infected pigs (Jung et al., 2014). In contrast, the outcomes of another study using an experimental PEDV model in young pigs suggest that detection of PEDV RNA in blood occurs sporadically and at low levels (Opriessnig et al., 2014). Similarly, no PEDV RNA could be detected in serum samples of the RAW-PEDV-CONTROL pigs in the present study. Differences in the ability of the PEDV to reach the vascular system may be associated with the viral strain (Park and Shin, 2014). Sporadic detection of viral RNA of another enteric coronavirus, feline enteric coronavirus (FECV), in blood seems to be associated with mononuclear cells that engulf the virus in the gut and subsequently gain access to the vascular system (Vogel et al., 2010). The susceptibility of mononuclear cells to FECV is however poor and viral spread is inefficient (Rottier et al., 2005). Rather than direct PEDV viremia, secondary contamination of blood and blood products with PEDV RNA at the slaughterhouse level, for example with fecal material, seems to be a more likely source for the observed PEDV contaminations of pooled plasma samples. This should be further investigated to improve the overall production and manufacturing processes.

Similar to the FECV infection in cats, PEDV infection in pigs appears to be restricted to intestinal cells (Jung et al., 2014; Kim et al., 1999; Stevenson et al., 2013). PEDV antigen has also been demonstrated in macrophages infiltrating the gut lamina propria (between 18 and 110 h post inoculation) (Lee et al., 2000) and more recently, in alveolar macrophages (Park and Shin, 2014). Transient detection of PEDV RNA in serum samples could be partially explained by association of PEDV with macrophages in acutely infected pigs. In the present study, there was no evidence of active PEDV infection when plasma obtained from previously PEDV infected pigs was given to the RAW-SICK group. Plasma PEDV RNA level in the pooled samples utilized for the present study was below real-time RT-PCR detection limit. Further studies are necessary to fully understand the extent and significance of PEDV RNA detection in serum of infected animals and whether the virus in the blood stream retains infectivity.

In summary, the data from this study indicate that the spray-drying process was highly effective in PEDV inactivation as determined by using plasma spiked with infectious PEDV. Therefore it can be concluded that commercial SDPP does not represent a significant risk factor for the transmission of PEDV infection in commercial swine production. PEDV viremia levels in pigs experimentally infected with PEDV are low to undetectable and consumption of plasma obtained from PEDV-infected piglets did not result in transmission of PEDV under the conditions of this study.

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