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## Quantifying the effect of lactogenic antibody on porcine epidemic diarrhea virus infection in neonatal piglets



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### ABSTRACT

The contribution of lactogenic antibody to the protection of piglets against porcine epidemic diarrhea virus (PEDV) was evaluated. Pregnant multiparous sows and their litters were allocated to one of 3 treatment groups: Group 1–6 serum antibody-negative sows and a subset ( $n = 11$ ) of their piglets. Group 2–8 serum antibody-positive sows and their 91 piglets. Piglets were orally inoculated with PEDV at 4 (Group 1) or 2 (Group 2) days of age. Group 3–2 PEDV serum antibody-negative sows and 22 piglets, provided a baseline for piglet survivability and growth rate. Piglets were monitored daily for clinical signs, body weight, and body temperature through day post-inoculation (DPI) 12 (Groups 2 and 3) or 14 (Group 1). Serum and mammary secretions were tested for PEDV IgG, IgA, and virus-neutralizing antibody. Feces were tested by PEDV real-time, reverse transcriptase PCR (rRT-PCR). Piglets on sows without (Group 1) or with (Group 2) anti-PEDV antibody showed significantly different responses to PEDV infection in virus shedding ( $p < 0.05$ ), thermoregulation ( $p < 0.05$ ), growth rate ( $p < 0.05$ ), and survivability ( $p < 0.0001$ ). Specifically, Group 1 piglets shed more virus on DPIs 1 to 5, were hypothermic at all sampling points except DPIs 9, 11, and 12, gained weight more slowly, and exhibited lower survivability than Group 2 piglets. Within Group 2 litters, significant differences were found in virus shedding ( $p < 0.05$ ), and body temperature ( $p < 0.05$ ), but not in piglet survival rate. The number of sows and litters in Group 2 was insufficient to derive the relationship between specific levels of lactogenic antibody (FFN, IgA, and IgG) and the amelioration of clinical effects. However, when combined with previous PEDV literature, it can be concluded that the optimal protection to piglets will be provided by dams able to deliver sufficient lactogenic immunity, both humoral and cellular, to their offspring.

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### 1. Introduction

Porcine epidemic diarrhea virus (PEDV), a member of family *Coronaviridae*, is a cause of diarrhea and vomiting in pigs of any age (Pensaert and de Bouck, 1978; Pospischil et al., 2002; Wood, 1977). In neonatal piglets, PEDV can cause severe diarrhea and vomiting, thereby leading to dehydration, metabolic acidosis, and death (Pospischil et al., 2002; Pensaert, 1999). Clinical outbreaks of porcine epidemic diarrhea were first reported in 1971 in England,

with its viral etiology determined in 1978 (Pensaert and de Bouck, 1978; Wood, 1977). Previously free of the virus, outbreaks of PEDV in North America were first reported in April 2013 (Alvarez et al., 2016; Stevenson et al., 2013). Spreading rapidly across the continent, PEDV caused a 6–7% decline in U.S. pork production in 2014 (Alvarez et al., 2015).

The impact of PEDV infection is age-dependent. In piglets, the greater severity of PEDV is the result of slower villus-epithelial repopulation and the immaturity of the neonatal immune system (Annamalai et al., 2015; Jung et al., 2015; Jung and Saif, 2015; Madson et al., 2014; Moon, 1971; Shibata et al., 2000). Thus, the introduction of PEDV into a naïve swine farm can result in  $\geq 90\%$  mortality in piglets less than 2 weeks of age and up to 40% mortality in 2- to 4-week-old pigs (Stevenson et al., 2013). PEDV

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has also been reported to impact reproductive performance in pregnant gilts and sows. [Olanratmanee et al. \(2010\)](#) reported a 12.6% decrease in the farrowing rate, 5.7% increase in the rate returns, a 1.3% increase in the abortion rate, and a 2.0% increase in mummified fetuses per litter following an outbreak of PEDV in a large production system.

In the field, it has been observed that prior exposure of the sow herd to PEDV ameliorates the impact of PEDV infection in neonates, presumably via maternal immune mechanisms ([Goede and Morrison, 2016](#)). Based on these observations, it has been postulated that PEDV-specific IgG and IgA in colostrum and milk are critical for the protection of neonatal piglets against PEDV ([Chattha et al., 2015](#); [Song et al., 2015](#)). Using TGEV as a model, the assumption is that lactogenic immunity against PEDV is induced by enteric infection following oral exposure ([Song et al., 2007](#)). Thereafter, IgA plasmablasts stimulated in the sow's gut migrate to the intestinal lamina propria and then to the mammary glands where they produce PEDV-specific IgA ([Bohl et al., 1972a](#); [Saif et al., 1972](#)). Lactogenic IgA is effective within the intestinal tract of the neonatal pig because it has high affinity and is resistant to proteolysis ([Song et al., 2015](#)). However, as discussed by [Langel et al. \(2016\)](#), there are no published reports on the role of lactogenic antibody in the protection of piglets against PEDV. Therefore, the objective of this experiment was to quantify the effect of lactogenic immunity in PEDV-inoculated piglets by comparing viral shedding, piglet growth, thermoregulation, and survival in litters with PEDV-immune vs naïve dams.

## 2. Materials and methods

### 2.1. Experimental design

The study was conducted under the approval of the Iowa State University Office for Responsible Research. Pregnant multi-parous sows ( $n = 16$ ), 8 PEDV serum antibody-negative and 8 PEDV serum antibody-positive, were farrowed in biosafety level 2 (BSL-2) research facilities. Sows and their litters were allocated to one of 3 treatment groups: Group 1 (positive controls) consisted of 6 PEDV serum antibody-negative sows and 11 of their 74 piglets. The remaining piglets from these litters were used in an experiment described elsewhere ([Poonsuk et al., 2016](#)). Group 1 piglets provided a baseline response for PEDV infection in the absence of maternal immunity. Group 2 consisted of 8 PEDV serum antibody-positive sows and 91 piglets. Group 2 provided a measure of the effect of maternal immunity. Group 3 consisted of 2 PEDV serum antibody-negative sows and 22 piglets. This group provided a baseline for piglet survivability and growth rate.

Piglets were orally inoculated with PEDV at 4 (Group 1) or 2 (Group 2) days of age; sows were not inoculated, but had contact with PEDV-contaminated piglet feces. All piglets were closely observed for clinical signs through day post-inoculation (DPI) 12 (Groups 2 and 3) or 14 (Group 1) or until humane euthanasia was necessary. Body weight and body temperature measurements were taken daily. Sow mammary secretions and piglet fecal samples were also collected daily. Serum samples were collected from sows prior to farrowing and at the termination of the experiment, i.e., DPI 12 (Groups 2 and 3) or 14 (Group 1). Serum samples were collected from piglets at DPI 0 and either at the time of euthanasia or the termination of the experiment. Serum, colostrum, and milk were tested for PEDV IgG, IgA, and virus-neutralizing antibody. Feces were tested by PEDV real-time, reverse transcriptase PCR (rRT-PCR). Data were analyzed for the effect of maternal PEDV antibody levels in colostrum and milk on piglet PEDV serum antibody levels, PEDV fecal shedding, body temperature, weight gain, and mortality.

### 2.2. Animals and animal care

Sixteen clinically healthy, multiparous sows were acquired from 2 commercial sow farms between 84 and 110 days of gestation. To verify their PEDV status, serum samples collected on DPI -7 were tested for PEDV antibody by PEDV fluorescent focus neutralization (FFN) assay and a PEDV whole-virus indirect ELISA. To verify the absence of acute coronavirus infection, fecal swabs were collected immediately prior to receipt of animals and tested for PEDV, transmissible gastroenteritis virus (TGEV), and porcine delta coronavirus (PDCoV) using agent-specific qRT-PCRs. Animals were housed throughout the experiment in the Iowa State University Livestock Infectious Disease Isolation Facility (LIDIF), a BSL-2 research facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAA-LAC). The facility is equipped with a single-pass non-recirculating ventilation system that provided directional flow from low contamination areas to high contamination areas and zones of negative pressure to prevent airborne contamination from area-to-area or room-to-room. Each room was ventilated separately and humidity and temperature was strictly controlled. Animals were closely observed (multiple times daily) from the time they entered LIDIF to the end of the observation period by researchers, animal caretakers, and veterinary staff.

Sows were housed two per room in Danish-style free stall farrowing crates (Thorp Equipment Inc., Thorp, WI) and supplemental heat was provided for piglets. To induce parturition, all sows were administered 10 mg of dinoprost tromethamine (Lutalyse<sup>®</sup>, Zoetis Inc., Florham Park, NJ) 24 h prior to the expected farrowing date, i.e., on day 113 of gestation. The piglets included in the study ( $n = 124$ ) were ear-tagged and administered 1 ml iron hydrogenated dextran (VetOne<sup>®</sup>, Boise, ID) and 5 mg (0.1 ml) ceftiofur sodium (Excenel<sup>®</sup>, Zoetis). Piglets remained on the sow continuously throughout the observation period.

### 2.3. Porcine epidemic diarrhea virus (PEDV) inoculum

The PEDV isolate used in the study (USA/IN/2013/19338E) was isolated in 2013 at the Iowa State University Veterinary Diagnostic Laboratory from piglet small intestine submitted from an Indiana swine farm ([Chen et al., 2014](#)). The inoculum used in this study was the 7th passage of the virus on cell culture. For this study, the virus was serially propagated on Vero cells (African green monkey kidney) in flasks using methods described elsewhere ([Hofmann and Wyler, 1988](#); [Chen et al., 2014](#)). In brief, Vero cells (ATCC<sup>®</sup> CCL-81<sup>™</sup>, American type culture collection, Manassas, VA) were cultured in 25 cm<sup>2</sup> flasks (Corning<sup>®</sup>, Corning, NY) using maintenance medium (minimum essential medium (MEM) (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (Life Technologies), 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO), 0.05 mg/ml gentamicin (Life Technologies), 10 units/ml penicillin (Life Technologies), 10 µg/ml streptomycin (Sigma-Aldrich) and 0.25 µg/ml amphotericin (Sigma-Aldrich). Vero cells were inoculated with virus 24 h after reaching 100% confluency. Thereafter, maintenance medium was decanted from contiguous cell monolayers, the monolayer was washed twice with maintenance medium, and the flask was inoculated with 0.5 ml of a mixture of PEDV and post-inoculation medium (MEM supplemented with tryptose phosphate broth (0.3%) (Sigma-Aldrich), yeast extract (0.02%) (Sigma-Aldrich) and trypsin 250 (5 µg/ml) (Sigma-Aldrich). Flasks were then incubated at 37 °C with 5% CO<sub>2</sub> for 2 h to allow virus adsorption, after which 5 ml of post-inoculation medium was added to each flask without removing viral inoculum. Flasks were incubated at 37 °C with 5% CO<sub>2</sub> until cytopathic effect (CPE) was observed and then subjected to one freeze-thaw cycle (−80 °C). The contents were harvested,

centrifuged at 3000 for 10 min at 4 °C to remove cell debris, aliquoted into 2.0 ml microcentrifuge tubes, and stored in –80 °C until used.

PEDV titration was performed on confluent Vero cells monolayers grown in 96-well plates (CoStar™, Corning®). Eight 10-fold dilutions of virus stock solution were made using post-inoculation medium. Five wells were inoculated with 100 µl at each dilution, plates were incubated at 37 °C with 5% CO<sub>2</sub> for 1 h, and then 100 µl post-inoculation medium was added. Plates were incubated at 37 °C with 5% CO<sub>2</sub> for 5 days, after which wells were subjected to IFA staining and evaluated for the presence of virus. Wells with specific staining were classified PEDV-positive. Based on the titration results, the 50% endpoint was calculated as  $1 \times 10^5$  TCID<sub>50</sub>/ml using the Reed-Muench method (Reed and Muench, 1938).

#### 2.4. PEDV inoculation

On DPI 0, the PEDV stock solution ( $1 \times 10^5$  TCID<sub>50</sub>/ml) was diluted with PBS (1X, pH 7.4, Gibco®, Thermo Fisher Scientific, Grand Island, NY) to an estimated concentration of  $1 \times 10^3$  TCID<sub>50</sub>/ml, mixed 1:4 with milk replacer (Esbilac®, PetAg Inc., Hampshire, IL), and administered orally (5 ml) to all piglets of the PEDV antibody-positive sows, thereby delivering a total PEDV exposure dose of  $1 \times 10^3$  TCID<sub>50</sub> to each piglet. Thereafter, sows were monitored daily for diarrhea, milking ability, anorexia, and alertness. Piglets were monitored daily for diarrhea, rectal body temperature, dehydration, and ability to stand, walk, and suckle. Piglets unable to suckle, reluctant to stand, or demonstrating  $\geq 10\%$  dehydration based on skin tenting were humanely euthanized by intravenous administration of pentobarbital sodium (Fatal-Plus®, Vortech Pharmaceuticals, MI) at a dose of 100 mg/kg.

#### 2.5. Biological sample collection

##### 2.5.1. Serum

A total of 210 piglet serum samples and 32 sow serum samples for antibody testing were collected on DPI 0 and DPI 12 (Groups 1 and 3) or 14 (Group 2). Blood samples were drawn from the jugular vein or cranial vena cava using a single-use blood collection system (Becton Dickinson, Franklin Lakes, NJ) and serum separation tubes (Kendall, Mansfield, MA). Blood samples were processed by centrifugation at  $1500 \times g$  for 15 min, aliquoted into 2 ml cryogenic tubes (BD Falcon™, Franklin Lakes, NJ), and stored at –20 °C until tested.

##### 2.5.2. Mammary secretions

A total of 248 colostrum and milk samples for antibody testing were collected from sows between DPI 1 to 12 (Groups 1 and 3) or 14 (Group 2). Sows were administered 20 USP units of oxytocin (VetOne®) to facilitate collection of mammary secretions. Samples were processed by centrifugation at  $13,000 \times g$  for 15 min at 4 °C to remove fat and debris. The defatted samples were then aliquoted into 2 ml cryogenic tubes (BD Falcon™) and stored at –20 °C until tested.

##### 2.5.3. Feces

Samples for porcine coronavirus RT-PCR testing included fecal swab samples collected from individual sows immediately prior to receipt of the animals and placed in transport medium (BD BBL™ CultureSwab™ Collection/Transport system, Thermo-Fisher Scientific) and 1204 individual piglet fecal samples collected between DPI 0 and 12. Approximately 1 g of feces was collected from each piglet using a disposable fecal loop (VetOne®). The sample was mixed with 1 ml PBS (1X pH 7.4, Gibco®) immediately after collection, placed in a 2 ml cryogenic tube (BD Falcon™). Fecal

samples of Group 1 and 3 were pooled by litter i.e., 100 µl of feces of each piglet were pooled together in 2 ml cryogenic tube (BD Falcon™) for rRT-PCR testing. Fecal samples of Group 2 were tested individually.

#### 2.6. Coronavirus reverse-transcriptase polymerase chain reactions (rRT-PCR)

##### 2.6.1. RNA extraction

In brief, 90 µl of viral RNA was eluted from sow fecal swab samples or 50 µl of piglet fecal:PBS sample (2.5.3 above) using the Ambion® MagMAX™ viral RNA isolation kit (Life Technologies) and a KingFisher® 96 magnetic particle processor (Thermo-Fisher Scientific) following the procedures provided by the manufacturers.

##### 2.6.2. Porcine coronavirus real time RT-PCRs (rRT-PCR)

Sow fecal swab samples and piglet fecal samples were tested for PEDV using a PEDV N gene-based rRT-PCR described by Madson et al. (2014) and performed routinely at the Iowa State University-Veterinary Diagnostic Laboratory (ISU-VDL SOP 9.5263).

Sow fecal swab samples were tested for TGEV using a spike (S) gene-based procedure described by Kim et al. (2007) and performed routinely at the ISU-VDL (ISU-VDL SOP 9.5575). Primers and probes targeting conserved regions of the TGEV S gene were designed to match 9 TGEV strains, including Purdue 46-MAD (GenBank NC00236), TO14 (GenBank AF302264), TS (GenBank DQ201447), SC-Y (GenBank DQ443743), Miller M6 (GenBank DQ811785), TH-98 (GenBank AY676604), HN2002 (GenBank AY587884), 96-1993 (GenBank AF104420), and FS772/70 (GenBank Y00542) (Kim et al., 2007).

Sow fecal swab samples were tested for PDCoV using a membrane (M) gene-based rRT-PCR described by Chen et al. (2014) and performed routinely at the ISU-VDL (ISU-VDL SOP 9.5478). The protocol included positive control standards of known infectivity titers (TCID<sub>50</sub>).

##### 2.6.3. Real time RT-PCR

The eluted RNA, primers, and probe were mixed with commercial reagents (Path-ID® Multiplex One-Step RT-PCR kit, Life Technologies) and the RT-PCR reactions were conducted on an ABI 7500 Fast instrument (Life Technologies) as follows: 48 °C for 10 min, 95 °C for 10 min, 95 °C for 15 s (45 cycles), and 60 °C for 45 s. The results were analyzed using an automatic baseline setting with a threshold at 0.1. Quantification cycle (Cq) values <35 were considered positive for the PDCoV and Cq values <40 were considered positive for the PEDV and TGEV. Data were reported as “adjusted Cq”:

$$\text{Adjusted Cq} = (35 - \text{sample Cq}) \quad (1)$$

#### 2.7. Coronavirus antibody assays

##### 2.7.1. PEDV indirect immunofluorescence assay (IFA)

IFA plates were prepared by inoculating confluent monolayers of Vero cells (ATCC® CCL-81™) in 96-well plates (CoStar™, Corning®) with 100 µl/well of PEDV (US/Iowa/18984/2013) at  $1 \times 10^3$  plaque-forming units/ml. The plates were then incubated for 18–24 h, after which the inoculum was removed and the cell monolayers fixed with cold acetone:alcohol (70:30) solution (Sigma-Aldrich). Plates were then air-dried, sealed, and stored at –20 °C. To perform the test, serum samples were two-fold diluted (1:40–1:320) in PBS (1X pH 7.4) and then 100 µl of each dilution was transferred to IFA plates and incubated at 37 °C for 1 h. After incubation, the diluted serum samples were removed from test



plates, the plates rinsed 3 times with PBS (1X pH 7.4), and 50  $\mu$ l of 1:50 diluted goat anti-swine IgG antibody conjugated with FITC (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added to each well. After a 30 min incubation at 37 °C, the plates were rinsed again with PBS (1X pH 7.4) and the cells were observed under an inverted fluorescent microscope for PEDV-specific cytoplasmic staining.

#### 2.7.2. PEDV whole virus antibody ELISA

PEDV (USA/IN/2013/19338E) was used in the PEDV antibody ELISA. In brief, virus was propagated on Vero cells, the flasks subjected to one freeze-thaw, and the harvested material centrifuged at 4000  $\times$  g for 15 min to remove cell debris. The virus was then pelleted by ultracentrifugation at 140,992  $\times$  g for 3 h, after which the virus pellet was washed once with sterile PBS (1X pH 7.4). The purified virus was resuspended in PBS (1X pH 7.4) at a dilution of 1:100 of the original supernatant volume and stored at –80 °C. Following titration and optimal dilution, polystyrene 96-well microtitration plates (Nunc, Thermo Fisher Scientific) were manually coated (100  $\mu$ l/well) with the viral antigen solution and incubated at 4 °C overnight. After incubation, plates were washed 5 times, blocked with 300  $\mu$ l per well of a solution containing 1% bovine serum albumin (Jackson ImmunoResearch Inc., West Grove, PA), and incubated at 25 °C for 2 h. Plates were then dried at 37 °C for 4 h and stored at 4 °C in a sealed bag with desiccant packs. Plate lots with a coefficient of variation  $\geq$  10% were rejected.

ELISA conditions for the detection of anti-PEDV IgA and IgG antibodies in serum and colostrum/milk (defatted) specimens, including coating and blocking conditions, reagent concentrations, incubation times, and buffers, were identical. Positive and negative plate controls, i.e., antibody-positive and -negative experimental serum or milk samples, were run in duplicate on each ELISA plate. All samples were diluted 1:50, after which plates were loaded with 100  $\mu$ l of the diluted sample per well. Plates were incubated at 25 °C for 1 h and then washed 5 times with PBS (1X pH 7.4).

To perform the assay, 100  $\mu$ l of peroxidase-conjugated goat anti-pig IgG (Fc) antibody (Bethyl Laboratories Inc., Montgomery, TX) diluted 1:20,000 for serum and colostrum/milk samples or goat anti-pig IgA (Bethyl Laboratories Inc.) diluted 1:3000 for serum and 1:45,000 for colostrum/milk samples was added to each well and the plates incubated at 25 °C for 1 h. After a washing step, the reaction was visualized by adding 100  $\mu$ l of tetramethylbenzidine-hydrogen peroxide (TMB, Dako North America, Inc., Carpinteria, CA) substrate solution to each well. After a 5 min incubation at room temperature, the reaction was stopped by the addition of 50  $\mu$ l of stop solution (1 M sulfuric acid) to each well. Reactions were measured as optical density (OD) at 450 nm using an ELISA plate reader (Biotek<sup>®</sup> Instruments Inc., Winooski, VT) operated with commercial software (GEN5<sup>™</sup>, Biotek<sup>®</sup> Instruments Inc.). The antibody response in serum and colostrum/milk samples was represented as sample-to-positive (S/P) ratios calculated as:

$$S/P \text{ ratio} = \frac{(\text{sample OD} - \text{blank well control mean OD})}{(\text{positive control mean OD} - \text{blank well control mean OD})} \quad (2)$$

Analysis of the PEDV serum IgG WV ELISA data showed that a S/P cutoff of  $\geq$  0.40 provided a diagnostic sensitivity of 0.99 (95% CI: 0.96, 1.00) and specificity of 0.98 (95% CI: 0.97, 0.99) (Bjstrom-Kraft et al., 2016). No cutoffs have been established for mammary secretion samples.

#### 2.7.3. PEDV fluorescent focus neutralization (FFN) assay

Colostrum, milk, and serum samples were tested for neutralizing antibody. Prior to FFN testing, milk and colostrum samples were defatted by treatment with Rennet (Rennet from *Mucor miehei*, Sigma-Aldrich). In brief, 5  $\mu$ l Rennet was added to 1 ml of defatted milk or colostrum and briefly vortexed. The mixture was then incubated at 37 °C for 30 min, vortexed, and then centrifuged at 2000  $\times$  g for 15 min. The supernatant was then harvested and tested for neutralizing antibody.

To perform the FFN, test samples, antibody-positive control serum, and antibody-negative control serum were heat inactivated at 56 °C for 30 min and then 2-fold serially diluted (1:4–1:512) in 96-well dilution plates (Axygen<sup>®</sup>, Corning<sup>®</sup>) using post-inoculation medium to give a final volume of 100  $\mu$ l. Then, 75  $\mu$ l of each dilution was transferred to new dilution plate (Axygen<sup>®</sup>, Corning<sup>®</sup>), mixed with 75  $\mu$ l of PEDV ( $1 \times 10^{3.6}$  TCID<sub>50</sub>/ml) to give final serum dilutions of 1:8–1:1024, and incubated at 37 °C with 5% CO<sub>2</sub> for 1 h. Vero cell confluent monolayers in 96-well plates (CoStar<sup>™</sup>, Corning<sup>®</sup>) were washed twice with post-inoculation medium, inoculated with 100  $\mu$ l of the sample-virus mixture, incubated at 37 °C with 5% CO<sub>2</sub> for 1 h, and washed twice. 100  $\mu$ l of post-inoculation medium was then added to each well and the plates incubated at 37 °C with 5% CO<sub>2</sub> for 48 h. Finally, cells were fixed with 80% cold acetone:alcohol (80:20), stained with FITC-conjugated monoclonal antibody (SD6-29, Medgene Labs, Brookings, SD) for 1 h, and observed under an inverted fluorescent microscope for PEDV-specific cytoplasmic staining. Positive neutralizing endpoints were determined as the highest dilution resulting in a  $\geq$ 90% visual reduction in fluorescing foci relative to the antibody-negative serum control. Plates in which the positive control deviated more than 2-fold from its expected antibody titer were considered invalid.

#### 2.8. Data analysis

Area under the curve (AUC) analysis was used to calculate total PEDV mammary secretion antibody for each sow using FFN, WV-ELISA IgA, and WV ELISA IgG testing results for DPI 1 through 12 (MedCalc<sup>®</sup> version 16.4.3, Ostend, Belgium). Sow 5 was not included in this analysis because all piglets succumbed to PEDV on DPI 5 and mammary secretions were not collected thereafter. Thereafter, the association between DPI -7 serum antibody levels and total PEDV mammary secretion antibody were analyzed by regression analysis (SAS<sup>®</sup> 9.4, SAS<sup>®</sup> Institute Inc., Cary NC, USA).

Means and standard errors (SE) for percent body weight change, body temperature, PEDV fecal shedding (adjusted Cq), FFN antibody titer, WV-ELISA IgA and IgG S/P were calculated for each day using SAS<sup>®</sup> 9.4 (SAS<sup>®</sup> Institute Inc., Cary NC, USA). For individual piglets, the percent change in body weight was calculated relative to the weight of the piglet at 1 day of age:

$$\text{Percent change} = \frac{(\text{weight} - \text{weight at 1 day of age})}{(\text{weight at 1 day of age})} \times 100 \quad (3)$$

Prior to analysis, FFN data were transformed by dividing the reciprocal by 10 and taking the log<sub>2</sub>. Results were back-transformed and expressed as antibody titers. One-way analysis of variance (ANOVA) with repeated measures (DPI) was used to assess differences in body weight change, body temperature, PEDV fecal shedding (adjusted Cq), and antibody responses (FFN, IgA, and IgG) among groups over time. When differences were detected, compound symmetry covariance parameter estimates were used in the repeated measures analysis to detect differences among groups by DPI. Differences among groups in the time to

death were analyzed using proportional hazard regression analysis with a robust sandwich covariance matrix estimate.

### 3. Results

#### 3.1. Sows

All sows were clinically normal throughout the study. Sows exhibited normal maternal behavior and litters were kept intact and with their dam throughout the study. All sow fecal samples collected prior to inoculation on DPI 0 were PEDV, TGEV, and PDCoV qRT-PCR-negative.

Group 1 ( $n=6$  sows) and Group 3 ( $n=2$ ) consisted of animals without prior exposure to PEDV. All serum samples collected at DPI -7 were negative by FFN (antibody titer  $<1:8$ ) and PEDV WV IgG ELISA ( $S/P < 0.4$ ). All Group 1 serum samples collected on DPI 14 were negative by FFN and positive on the WV IgG ELISA (mean  $S/P$  1.8, SE 0.4). In Group 3, serum samples collected at DPI 14 were negative by both FFN ( $<1:8$ ) and PEDV WV IgG ELISA ( $S/P < 0.4$ ).

Group 2 ( $n=8$  sows) consisted of animals previously exposed to PEDV. All serum samples collected at DPI -4 were positive by FFN (mean antibody titer 1:8, SE 0) and PEDV WV IgG ELISA (mean  $S/P$  1.6, SE 0.2). Serum samples collected at 12 DPI showed a strong response to PEDV exposure compatible with an anamnestic response, i.e., FFN (mean antibody titer 1:26, SE 1) and WV IgG ELISA (mean  $S/P$  2.2, SE 0.2).

As shown in Table 1, total mammary secretion antibody levels were relatively uniform among sows within Groups 1 and 2 (samples were not collected from Group 3). Day-by-day comparisons showed higher antibody levels (FFN, IgA, IgG) in Group 2 (ANOVA,  $p < 0.0001$ ) at all sampling points for all tests, with the exception of the WV IgG ELISA on DPIs  $\geq 4$  (Fig. 1). Correlation analysis did not detect an association between Group 2 sow serum antibody levels (FFN, IgG) on DPI -4 and FFN, IgG, or IgA levels in mammary secretion samples collected on DPI 0 ( $p > 0.05$ ).

#### 3.2. Piglets

All piglets were clinically normal in appearance and behavior prior to PEDV inoculation. As shown in Table 2, testing of serum samples collected on DPI 0 showed that Group 1 and 3 piglets were antibody negative by FFN, PEDV WV IgA ELISA, and PEDV WV IgG ELISA. In contrast, Group 2 piglets were positive on all three assays

due to maternal antibody. All piglet fecal samples collected prior to inoculation on DPI 0 were PEDV qRT-PCR-negative.

Piglets were 4 days of age (Group 1) or 2 days of age (Group 2) at the time of PEDV inoculation. Diarrhea was observed in 1–11 Group 1 piglets on DPI 1, increasing to 6–8 piglets on DPI 4 and declining thereafter such that no diarrheic piglets were observed in the group on DPI 7. In Group 2, diarrhea was observed in 2 of 91 piglets on DPI 1. This increased to 26 of 87 piglets on DPI 4 and decreased thereafter. On DPI 11, 1 diarrheic piglet was observed among the 70 pigs remaining in the group. No diarrhea was observed in Group 3 piglets (uninoculated controls) at any time in the study.

PEDV RT-PCR positive fecal samples were detected in Groups 1 and 2 from DPI 1 through 12 (Fig. 2A). Compared to Group 1, Group 2 piglets shed significantly less virus on DPIs 1–5 (ANOVA,  $p < 0.05$ ). Thereafter, no significant difference was found between the two groups in the level of fecal viral shedding, although numerically lower adjusted Cqs were suggestive of less virus shedding in Group 2.

Body temperature data collected on DPIs -1 and 0 was used to calculate mean piglet body temperature ( $38.9^\circ\text{C}$ ) and 95% confidence intervals ( $38.3^\circ\text{C}$ ,  $39.1^\circ\text{C}$ ). Using these values as the “normal range”, the mean body temperature of Group 1 piglets was below the lower bound of the normal range on DPIs 2 through 8 and again on DPI 10 (Fig. 2B). The mean body temperature of Group 2 piglets was within the normal range throughout the study. A significant difference in body temperature was detected between Group 1 and 2 piglets at all sampling points (ANOVA,  $p < 0.05$ ) except DPIs 9, 11, and 12. Within Group 2, repeated measures ANOVA analysis detected a significant difference in body temperature response ( $p < 0.05$ ) among litters, but this could not be explained by differences in mammary secretion antibody levels among Group 2 sows.

The piglet rate of growth was expressed as percent weight change relative to piglet weight on the first day post farrowing. Statistical analysis found differences in the rate of growth between Group 1 and 2 piglets at all sampling points (ANOVA,  $p < 0.05$ ) except DPIs 2–4 (Fig. 2C). Thus, the two groups were different from the beginning of the observation period, with a significantly lower rate of growth in Group 2 piglets. After DPI 5 and through the end of the trial, Group 2 piglets performed better than Group 1 piglets ( $p < 0.05$ ). Further analysis based on repeated measures ANOVA found no significant difference in percent weight change among litters within Group 2 ( $p > 0.05$ ).

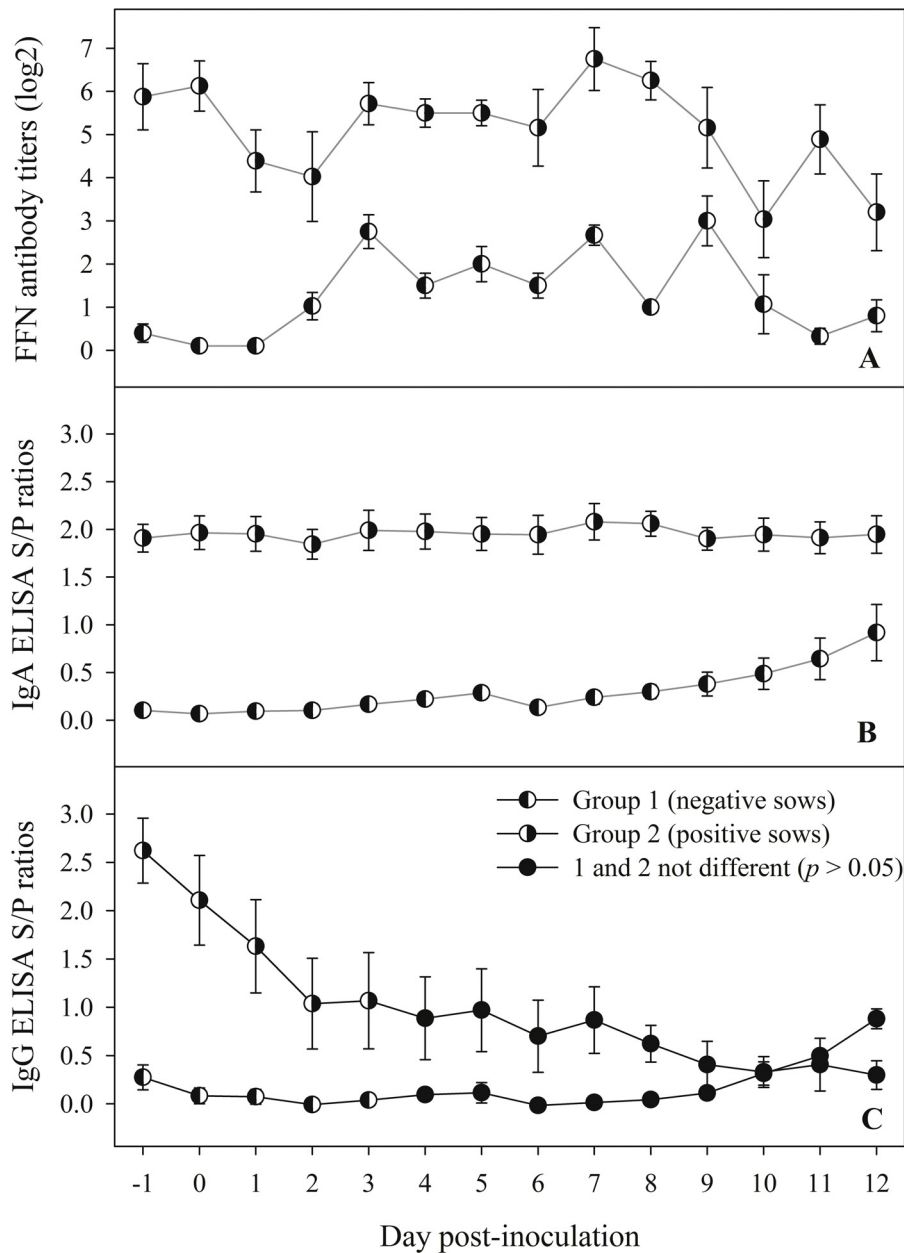
**Table 1**

Total PEDV mammary secretion antibody by group and sow based on area under the curve (AUC) analysis.<sup>a</sup>

Group	Sow	FFN		IgA ELISA		IgG ELISA	
		AUC	Maximum FFN titer	AUC	Maximum IgA S/P	AUC	Maximum IgG S/P
1 <sup>b</sup>	1	11.3	1:4	5.2	1.2	1.7	0.9
	2	12.5	1:16	2.7	0.2	1.2	0.6
	3	9.0	1:8	3.1	0.5	4.2	1.2
	4	12.5	1:4	2.9	0.6	1.9	0.7
	6	13.2	1:16	5.8	2.0	1.3	0.9
	$\bar{x}$ (SE)	11.7 (0.7)	1:8 (0.4)	3.9 (0.7)	0.9 (0.3)	2.1 (0.5)	0.9 (0.1)
	2	7	51.0	1:256	24.7	2.3	13.3
8		76.6	1:1024	23.7	2.1	15.1	3.3
9		47.3	1:64	21.2	2.6	13.1	1.9
10		75.1	1:512	24.1	2.2	14.1	2.3
11		68.6	1:256	33.2	2.7	28.8	3.5
12		68.5	1:128	26.8	2.4	19.4	2.9
13		80.0	1:256	32.6	2.7	23.2	4.4
14		41.0	1:64	26.4	1.7	16.3	3.3
$\bar{x}$ (SE)		63.5 (5.3)	1:223 (0.5)	26.6 (1.5)	2.3 (0.1)	18.0 (2.0)	3.1 (0.3)

<sup>a</sup> Area under the curve (AUC) for each sow and assay was calculated using mammary secretion antibody data for samples collected DPI -1 to 12 (MedCalc<sup>®</sup> version 16.4.3, Ostend, Belgium).

<sup>b</sup> Sow 5 not included in this analysis because all piglets succumbed to PEDV on DPI 5 and mammary secretions were not collected thereafter.

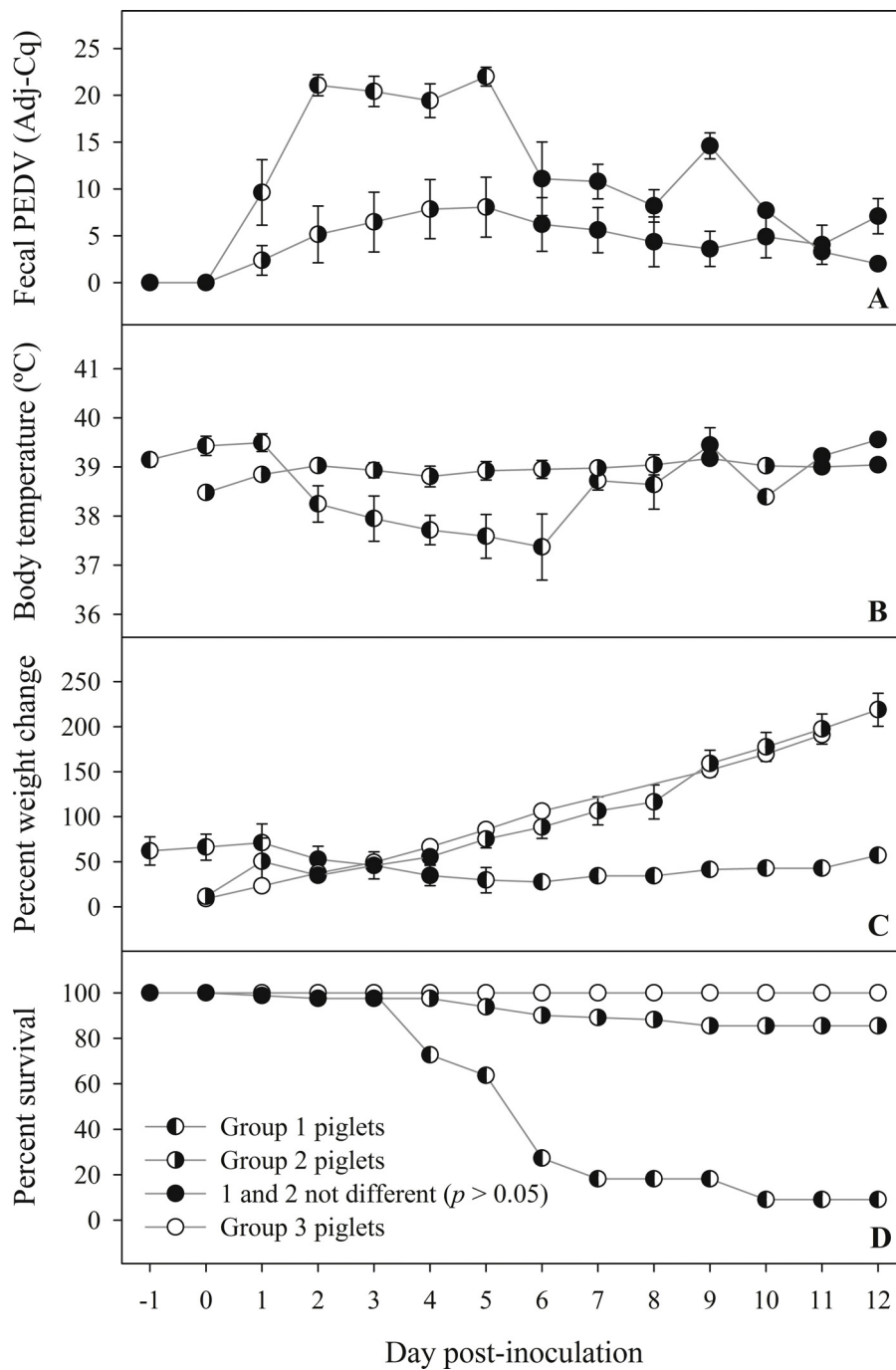


**Fig. 1.** PEDV antibody in mammary secretions tested by (A) fluorescent focus neutralization (FFN) assay, (B) PEDV indirect IgA ELISA, and (C) PEDV indirect IgG ELISA. Group 1 sows ( $n = 6$ ) were PEDV serum antibody negative and Group 2 sows ( $n = 8$ ) were PEDV serum antibody positive. Piglets in both groups were orally inoculated with PEDV (USA/IN/2013/19338E) on DPI 0.

**Table 2**  
Piglet serum antibody levels by group and day post inoculation (DPI).

Assay	Group	DPI 0	No. of piglets	DPI 12 or 14 <sup>a</sup>	No. of piglets
FFN antibody titer arithmetic mean (SE)	1	<1:8 (0)	11	1:16	1
	2	1:39 (1.1)	91	1:13 (1.1)	78
	3	<1:8 (0)	22	<1:8 (0)	22
PEDV IgA ELISA least square mean S/P (SE)	1	0.6 (0.1)	11	1.7	1
	2	2.5 (0.06)	91	1.9 (0.07)	78
	3	0.6 (0.01)	22	0.3 (0.009)	22
PEDV IgG ELISA least square mean S/P (SE)	1	0.2 (0.05)	11	2.1	1
	2	5.4 (0.06)	91	2.1 (0.1)	78
	3	0.3 (0.01)	22	0.02 (0.004)	22

<sup>a</sup> Observation period ended on DPI 12 for Groups 1 and 3 and DPI 14 for Group 2.



**Fig. 2.** Clinical data from piglets orally inoculated with PEDV (USA/IN/2013/19338E): (A) PEDV fecal shedding (adjusted Cq = 35 - sample Cq); (B) mean body temperature (°C); (C) percent weight change; and (D) percent survival within the group. Group 1 piglets (n = 11) were from serum antibody negative sows and Group 2 piglets (n = 91) were from serum antibody positive sows.

All uninoculated control piglets (Group 3, n = 22) survived to the end of the observation period. In contrast, 10 piglets (91%) in Group 1 died between DPIs 4 and 10 and 14 piglets (15%) in Group 2 died between DPIs 1 and 9 (Fig. 2D). Among these piglets, 79% (n = 11) were humanely euthanized because they met welfare thresholds (unable to suckle, reluctant to stand, or  $\geq 10\%$  dehydration). Proportional hazard regression analysis comparing Groups 1 and 2 found a significant difference in piglet survivability ( $p < 0.0001$ ).

Proportional hazard regression comparing litters within Group 2 found no difference in the rate of survival ( $p > 0.05$ ).

#### 4. Discussion

Neonatal pigs are born agammaglobulinemic, possess limited peripheral lymphoid cells, undeveloped lymphoid tissues, and no effector and memory T-lymphocytes (Sinkora and Butler, 2009). To



provide some level of immunological protection, neonatal piglets receive maternal cell-mediated (CMI) and humoral immune components through the ingestion of colostrum and milk (Saif and Jackwood, 1990). In this discussion, we review and discuss the elements of this process in the context of neonatal protection against PEDV and other swine enteric coronaviruses.

Sow mammary secretions contain a significant number of immune cells ( $\sim 1 \times 10^7$  per ml) (Evans et al., 1982). Cell numbers and types reflect both the developmental stage of the mammary gland and the physiological and/or immunological conditions of the sow (Magnusson et al., 1991). After ingestion by piglets, maternally-derived lymphocytes are able to cross the duodenal and jejunal epithelium by intercellular migration for at least one week after birth (Bianchi et al., 1999; Tuboly et al., 1988; Williams, 1993). Once absorbed, these cells migrate to mesenteric lymph nodes and a variety of other tissues, including liver, lung, spleen, the lamina propria, and submucosal spaces of the duodenum and jejunum (Tuboly et al., 1988; Tuboly and Bernath, 2002; Williams, 1993). Thus, peripheral blood mononuclear T-lymphocytes and peripheral B-lymphocytes in piglets that received colostrum cells showed higher responses to mitogens compared to controls (Williams, 1993). Research on the role of colostrum immune cells in protecting piglets against coronaviruses is scant, but the transfer of peripheral blood mononuclear cells from adult pigs into neonates was shown to delay the onset of TGEV enteritis (Cepica and Derbyshire, 1984). The effects of colostrum immune cells were not accounted for in this study, but maternal CMI may have been contributed to the protection against PEDV infection observed in Group 2 suckling pigs.

In addition to maternal CMI, antibody provided in colostrum and milk protects the piglet in the interval between birth and the development of a functional immune system. IgG is the major antibody isotype in colostrum whereas IgA predominates in milk (Curtis and Bourne, 1971). The enhancement of colostrum immunity depends on the stimulation of systemic immunity, whereas the enhancement of lactogenic immunity depends on appropriate and “local” stimulation at induction sites (i.e., gut and/or upper respiratory track) (Salmon et al., 2009). Bourne and Curtis (1973) showed that nearly 100% of IgG, 40% of IgA, and 85% of IgM in colostrum was derived from serum; whereas, 70% of IgG, and  $\geq 90\%$  of IgA and IgM in milk was produced locally in the mammary glands.

In this study, correlation analysis did not find an association between serum antibody levels (FFN, IgG) and FFN, IgG, or IgA levels in mammary secretions in sows previously exposed to PEDV (group 2). Furthermore, the lack of correlation between FFN and ELISA observed within some specimens may be due to the fact that FFN detects functional antibodies capable of neutralizing PEDV infectivity, whereas the WV ELISA detects PEDV isotype-specific (IgG or IgA) antibodies.

In late pregnancy, coinciding with high progesterone and low estrogen levels, binding of the IgG1 heavy chain by Fc $\gamma$  receptors on mammary epithelial cells promotes the selective transport of IgG from serum into mammary secretions (Butler, 1998; Chamley et al., 1973; Hammer and Mossmann, 1978; Killian et al., 1973; Porter et al., 1988; Schnulle and Hurley, 2003; Watson, 1980).

Postpartum, lower serum progesterone and higher serum corticoid and prolactin levels induce milk production and suppress colostrum production (Banchemo et al., 2006; Delouis, 1978; Hartmann and Prosser, 1984). This switch coincides with lower IgG and higher IgA concentrations in mammary secretions (Ash and Heap, 1975; Devillers et al., 2004a, 2004b; Foisnet et al., 2010). After parturition, lactogenic immunity predominantly involves secretory IgA (S-IgA) produced from plasma cells in mammary gland tissues. Beginning shortly before parturition and continuing throughout lactation, IgA plasma cells preferentially migrate from

mesenteric lymph nodes to the mammary glands (Roux et al., 1977). Mammary gland lymphocyte accumulation differs between gilts and sows, i.e. multiparous sows have more mammary tissue and are able to accumulate more plasma cells than primiparous females (Bischof et al., 1994; Sordillo et al., 1997; Sordillo and Streicher, 2002). Therefore, sows produce higher concentrations of S-IgA in mammary secretions (Cabrera et al., 2012; Klobasa et al., 1986). Like S-IgA, secretory IgM (S-IgM) is produced from IgM plasma cells in mammary gland tissues (Sordillo et al., 1997), but the mechanisms that regulates S-IgM plasma cell localization postpartum are not clear. (Note that multiparous sows were used in this study).

The enhancement of lactogenic immunity against enteric coronaviruses requires “productive” enteric viral replication to stimulate the development of specific IgA plasmablasts, to increase in cell trafficking from the gut to the mammary gland, and to enhance the immunoglobulin production and secretion in milk (Langel et al., 2016). Thus, parenteral immunization of sows with modified-live TGEV vaccines increased IgG in colostrum, but not IgA (Saif and Jackwood, 1990; Bohl et al., 1972a; Saif et al., 1972). Following enteric infection, IgA plasmablasts migrate to the mammary glands where they contribute S-IgA to milk and colostrum (Bohl et al., 1972a; Roux et al., 1977). Bohl et al. (1972b) found that sows orally inoculated with virulent TGEV produced high titers of S-IgA in mammary secretions and protected their piglets against the clinical effects of TGEV, whereas highly attenuated oral TGEV vaccines resulted in lower S-IgA antibody titers in mammary secretions and inadequate lactogenic immunity in suckling piglets (Jackwood et al., 1995; Moxley and Olson, 1989; Saif, 1999a, 1999b). Thus, amongst the PEDV immune sows (group 2) used in this study, sows were previously exposed to PEDV orally through feed-back. Notably, significant differences were found among litters from PEDV-immune dams in the quantity of virus shedding ( $p < 0.05$ ) and return to normal body temperature ( $p < 0.05$ ), but not in piglet survival rate. Although these differences were detected, it was not possible to assign a causal role to antibody isotype or concentration in mammary secretions because of sample size. In addition, some potentially contributing factors were not accounted for, e.g., the amount of colostrum or milk consumed by each pig and the role of CMI. Given that the ultimate goal of this line of research is to provide specific guidelines for monitoring and maintaining lactogenic immunity against PEDV infection in sow herds, these remain important topics for future research.

Within the mammary gland, polymeric immunoglobulins (dimeric S-IgA or pentameric S-IgM) secreted by local plasma cells bind to polymeric immunoglobulin receptors (pIgR) on the basolateral pole of the secretory epithelial cell (Johansen et al., 2000) and are then transported through the endosomal compartment to the luminal surface of the epithelial cell (Rojas and Apodaca, 2002). Upon reaching the luminal side of the epithelial cell, the pIgR molecule is enzymatically cleaved; a process that leaves a receptor fragment (“secretory component”) attached to the immunoglobulin molecule (Hunziker and Kraehenbuhl, 1998). The secretory component functions to protect the hinge region of S-IgA and S-IgM from protease cleavage, thereby extending their stability on mucosal surfaces (Hurley and Theil, 2011). The concentration of S-IgA and S-IgM in milk is dependent upon the rate of transportation across epithelial cells, but the rate of transport is dependent upon the expression of pIgR on mammary epithelial cells, which is under the control of hormones responsible for the initiation of lactation (Rosato et al., 1995; Scicchitano et al., 1986; Sheldrake et al., 1984). In sows, the hormonal regulation of the expression of pIgR on mammary epithelial cells has not been described, although it is known that pIgR density is highest from 0 to 3 days postpartum (Schnulle and Hurley, 2003).

In addition to endogenous hormones, exogenous hormones can also affect colostrum yield and composition (Dawe et al., 1982; Field et al., 1989). It has been reported that sows chemically induced to farrow trended toward the production of less colostrum and that the colostrum contained less fat (Farmer and Quesnel, 2009; Jackson et al., 1995; Milton et al., 1983). This is relevant because the sows in this study were induced to farrow using dinoprost tromethamine (Lutalyse®), a tromethamine salt of prostaglandin F2 alpha, on day 113 of gestation. It is possible that this procedure may have impacted the concentration and total quantity of colostrum antibody. Although the results of this study showed that induction of sows did not affect the overall capacity of lactogenic humoral immunity to protect piglets against PEDV, this may be a consideration in the field when optimization of herd immunity against PEDV is an objective.

Within the neonatal digestive tract, immunoglobulins remain intact because proteolytic activity in the digestive tract is low and is further reduced by “sow colostrum trypsin inhibitor (SCTI)” (Jensen, 1978; Chamberlain et al., 1965; Kunitz, 1947). Before gut closure, enterocytes of the immature neonatal intestinal epithelium retain the ability to pinocytose immunoglobulins in the lumen of the intestine. Immunoglobulins are released in the lamina propria, and then diffuse to the intestinal lymphatic system, from where they enter the circulatory system (Moog, 1979; Murata and Namioka, 1977; Bush and Staley, 1980).

Some absorbed maternal IgM, IgA, and IgG undergo transudation through the epithelial cells to reach the intestinal mucosal surfaces, where they play a major role in protecting neonates against enteric pathogens, e.g., TGEV and rotavirus (Bohl et al., 1972b; Saif et al., 1972; Saif, 1999a; Ward, 1996). In addition, IgG appears in the neonatal circulatory system within 48 h after birth (Curtis and Bourne, 1973; Porter et al., 1988; Watson, 1980). Depending on the sow's antibody profile, circulating maternal IgG can ameliorate the clinical effects of systemic infections, e.g., porcine parvovirus, porcine circovirus, and (Langel et al., 2016; Ostanello et al., 2005). Using a passive antibody model, Poonsuk et al. (2016) found that PEDV-inoculated neonates with circulating anti-PEDV antibodies returned to normal body temperature earlier and exhibited higher survivability compared to piglets without PEDV antibody. Although circulating antibody did not neutralize the negative effects of PEDV on growth nor reduce shedding of PEDV in feces, the results implied that colostrum IgG contributed to the protection of neonates against PEDV.

After gut closure, the majority of macromolecules are degraded by digestive enzymes, but some immunoglobulins remain intact and are transported across intestinal epithelium via antibody-specific FcRn receptors on gut epithelial cells (Stirling et al., 2005). By this mechanism, IgG, IgM, and IgA in mammary secretions may undergo selective transcytosis into enterocytes. In addition, S-IgA and S-IgM are stabilized by binding to secretory component and can be transcytosed by Fc receptor of enterocytes without degradation (Brandtzaeg, 1981).

The objective of the experiment was to quantify the protective effects of lactogenic immunity by comparing the course of PEDV infection in neonatal piglets farrowed by immune vs naïve dams. Overall, piglets that received lactogenic antibody shed less PEDV and exhibited better thermoregulation, higher growth rates, and higher survivability. These results are compatible with observations for other swine coronaviruses. Bohl et al. (1972a,b) found that sows previously infected with TGEV delivered levels of lactogenic immunity that reduced TGEV shedding, morbidity, and mortality in their piglets. Wesley and Lager (2003) reported that TGEV inoculated sows provided strong lactogenic antibody responses resulted in effectively protection against TGEV in neonates on basis of reduced clinical illness, and increased litter survival rates (Wesley and Lager, 2003).

When combined with previous PEDV literature, it can be concluded that the optimal protection to piglets will be provided by dams able to deliver sufficient lactogenic immunity to their offspring.

### Declaration of conflicting interests

The authors declare no conflicting interests with respect to their authorship or the publication of this article.

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