RESEARCH REPORT

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SWINE HEALTH

Title: Defining PEDV maternal humoral immunity and correlates of neonatal protection – **NPB #13-264**

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INDUSTRY SUMMARY

Through the proposed approach we tried to the answers PEDV serology questions from the field regarding the association between maternal anti-PEDV antibody and protection of neonates against PEDV. It would allow producers to better assure the sows have been effectively exposed and thereby their pigs effectively protected through colostrum and milk.

The primary objective of this project was to determine correlates between maternal anti-PEDV antibody and protective immunity against PEDV in neonates. The objective was accomplished through two phases of experimental research:

Phase 1. Evaluation of PEDV protection in the context of maternal immunity against PEDV challenge in piglets from immune sows.

- Newborn piglets from 8 PEDV-immune sows were allowed to suckle colostrum for 2 days before they were inoculated with 10^3 TCID50 PEDV. No challenged piglets from 2 PEDV negative sows were included as negative control group. Piglets were monitored for 14 days to observe clinical outcomes, including PEDV real time reverse transcriptase PCR (rRT-PCR) of piglets feces (analyzed as converted CT), survival (%), daily weight, (analyze as weight changes), and daily temperature.
- Specific anti-PEDV antibodies (IgG, IgA, and virus neutralizing) were found in the colostrum and milk of immune sows but not in those from naïve negative control sows. Subsequently, significant levels of specific PEDV antibodies (IgG, IgA, and VN) were found in serum from piglets born to immune sows. Thus, serological results for neonatal piglets that have received colostrum/milk provide a crude means of assessing of transfer and decline of maternal antibodies.

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- Results showed that antibody-based detection methods are applicable to monitoring sows for PEDV exposure and evaluate their immune status. The determination of antibody isotypes (IgG, IgA and VN) in serum and colostrum/milk specimens provided valuable information regarding dynamic, timing, sensitivity and source of humoral response against PEDV. Particularly remarkable is the specific role of mucosal IgA and VN antibodies as first and principal line of defense against PEDV infection.
- Following inoculation with PEDV, virus shedding was significantly reduced in piglets that had received passively transferred immunity. Specifically, a linear relationship between colostrum/milk IgA and virus shedding from piglets were observed where piglets receiving milk with higher IgA titers had lower PEDV shedding in feces, suggesting that IgA local gut (milk) antibodies play a crucial role in the clearance of the virus.

Phase 2. Evaluation of PEDV protection in naïve piglets administered specific levels of anti-PEDV antibody.

- Each litter of piglets from 7 PEDV negative sows were administered (intraperitoneally) with 6 specific levels of anti-PEDV antibody [0 (saline solution), 1:80; 1:160; 1:320; 1:640; 1:1280] harvested from phase 1, and subsequently inoculated with 10^3 TCID50 PEDV. Piglets were monitored as in Phase 1.
- PEDV-specific antibody (IgG, IgA, and VN) levels in sera of pigs receiving immune serum intraperitoneally were significantly higher than those that received control saline solution (PBS).
- The proportion of pigs shedding PEDV after inoculation was also lower in pigs receiving any of the 5 levels of immune serum compared to those receiving the control saline solution. However, it was difficult to establish a quantitative correlation between specific antibody levels and protection in terms on reduction of the virus shedding.
- Mortality data reported in that experiment indicate that systemic (serum) antibody titers do not correlate with protection of piglets against PEDV.

KEYWORDS – Porcine epidemic diarrhea virus, maternal immunity, neonatal, antibody, serum, colostrum, milk, feces.

SCIENTIFIC ABSTRACT

The level and isotype (IgG, IgA, and neutralizing antibodies) of anti-PEDV antibody necessary to protect neonatal pigs against clinical PEDV infection was investigated using laboratory measurements. As a first step, all tools [virus isolation, PCR (feces), IFA (IgG in serum), FNN (neutralizing antibodies in serum and colostrum/milk), ELISA (IgG and IgA antibodies in serum and colostrum/milk)] required for this study were developed and validated. The experiment was conducted in two phases.

In the first phase, we evaluated protection (passive maternal immunity) against PEDV in piglets from sows previously exposed to PEDV (at ~100 days of gestation). Piglets from 2 negative control and 8 PEDV-immune sows were inoculated with 10^3 TCID50 PEDV at 3 days of age and monitored for 14 days to observe clinical profiles and PEDV shedding. Serum and colostrum/milk samples were tested for PEDV-specific VN, IgG and IgA antibodies. Fecal samples were tested individually pooled by litter by PEDV rRT-PCR. No clinical sign were observed in control sow litters, with a piglet mortality <7%. In litters from PEDV immune sows, diarrhea was observed in 27.3 to 100% of piglets and mortality ranged from 0 to 40%. IgG antibody levels in colostrum from PEDV immune sows significantly decrease after 48-72 h post-partum (milk). A linear relationship between milk IgA levels and virus shedding from piglets were observed where piglets receiving milk with higher IgA titers had lower PEDV shedding in feces (p value = 0.04).

In the second phase, we investigated the role of systemic antibody in the protection of PEDV infection in naïve piglets. At 2 days of age, new born piglets from 7 PEDV naïve sows were intraperitoneally administered 1 of 6 levels of PEDV antibody harvested from PEDV immunized sows. Piglets were orally inoculated with 10^3 TCID50 PEDV at 5 days of age. Observation, sampling, and testing were performed as in Phase 1. Fecal samples were tested individually by PEDV rRT-PCR. Correlation between clinical profiles, PEDV shedding from piglet feces and immunity levels administered to each pig will be analyzed to determine the systemic antibody level necessary to provide piglet protective immunity against PEDV infection. All piglets (100%) develop diarrhea within 48h post-inoculation and mortality by treatment ranged from 50 to 100%. Efficient uptake of the injected antibodies into the circulation was evidenced by the correlation between the level of antibodies injected (1 of 6 levels) and the titers of VN, IgG, and IgA in serum from piglets grouped for specific treatment.). A linear relationship (log2) between treatment levels and virus shedding in piglets were observed. However, comparative mortality rates showed that piglets received milk from PEDV immune sows (Phase 1) had higher survival rates to piglets from PEDV naive sows (Phase 2) after experimental PEDV inoculation. Thus, lactogenic antibodies may play more important role than systemic antibodies in protecting new born piglets against PEDV infection.

INTRODUCTION

Porcine epidemic diarrhea virus (PEDV) is the causative agent of porcine epidemic diarrhea (PED) that was first recorded in England in the early 1970s (Oldham et al., 1972). Since then, PED had been reported in European countries and several Asian countries such as Japan, South Korea, China, Thailand, and Vietnam (Chasey et al., 1978; Nagy et al., 1996; Puranaveja et al., 2009; Song et al., 2012; Van Reeth & Pensaert., 1994; Vui et al., 2014). In North America, PEDV was detected for the first time in the U.S. in April 2013 (Stevenson et al., 2013) and subsequently PEDV was reported in Canada (Pasick et al., 2014) and Mexico (Vlasova et al., 2014). Currently PEDV remains a challenge for the global swine industry.

PEDV is an enteropathogenic coronavirus that infects the villous enterocytes, resulting in villous atrophy and malabsorptive diarrhea. PEDV infection can affect pigs of all ages but with more severe form in neonates. While waiting for various vaccines to become widely available, the stimulation of sow (maternal) immunity by offering her 'feedback' combined with a decrease of the viral load in the environment (cleaning and disinfection process) has been a widely applied strategy for producers and veterinarians.

Maternal antibody in the lumen of the piglet intestine can provide piglet protection. Maternal antibodies in newborn piglets come from colostrum and milk (lactogenic immunity) of dams. The immunoglobulins in colostrum are mainly IgG and the antibodies in milk are mainly IgA. The absorbed colostral IgG by piglets eventually enters the neonatal blood circulation while the milk IgA acquired by piglets stays on the mucosal surfaces of intestines and provides local protective immunity. Questions regarding the association between maternal anti-PEDV antibody and protection of neonates against PEDV are still in doubt and have not been addressed by recent research.

Because of this, the objective of our experiment was to provide a broader knowledge of PEDV-specific immunity and immune-protection against PEDV, in an experimental study for further application in field PEDV management.

OBJECTIVES

The goal of the present study was to stablish the association between maternal anti-PEDV antibody and protection of neonates against PEDV. Two independent animal experiments, conducted under the approval of the Iowa State University Office for Responsible Research (ISU #2-14-7736-S), were performed to analyze the role of both maternal and systemic antibodies in protection against infection with PEDV.

MATERIALS AND METHODS

Using laboratory measurements, the level and isotype of anti-PEDV antibody necessary to protect neonatal pigs against clinical PEDV infection were determined.

Experimental design phase 1. To evaluate the association of antibody levels in colostrum/milk with the protection of neonatal piglets against clinical PEDV. Ten multi-parous sows, 2 PEDV serum antibody-negative and 8 PEDV serum antibody-positive, were acquired from two commercial farms at ~100 days of gestation, placed into biosafety level 2 (BSL-2) research facilities, and farrowed 113 viable piglets. All sows were clinically healthy post-farrowing and lactated normally throughout the study. Piglets from antibody-positive sows (n = 91) were orally inoculated with PEDV 2 days after birth. Piglets from antibody-negative sows (n = 22) served as environmental sentinels and assay negative controls.

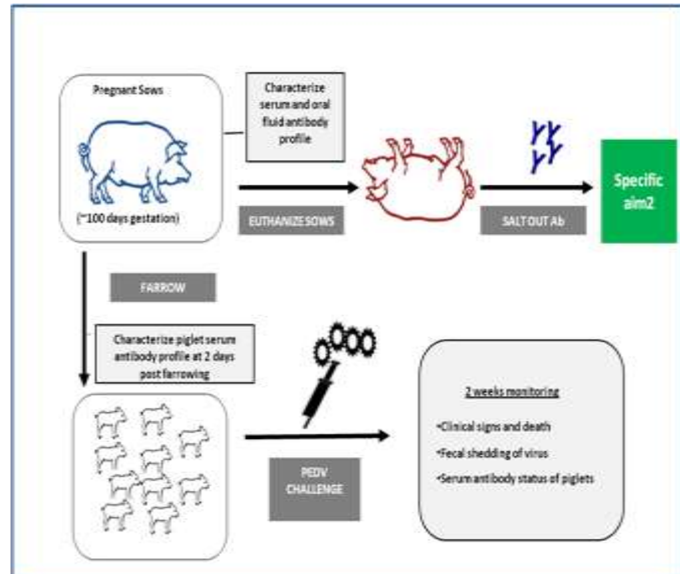


Fig 1. Evaluation of humoral response of PEDV-immune sows and their piglets before and after PEDV experimental infection. Comparative antibody profile, neutralizing antibodies production and correlation with protected immunity.

All piglets were closely observed for clinical signs through day post inoculation (DPI) 12 or until humane euthanasia was necessary. Daily observations included measurement of body weight and body temperature. Samples collected for analysis included daily sow milk and piglet fecal samples, serum samples from sows and piglets at 0 and 12 DPI or at the time of euthanasia. Serum, colostrum, and milk were tested for PEDV IgG, IgA, and VN antibody. Feces were pooled by litter and tested by PEDV rRT-PCR. Data were analyzed for the effect of maternal PEDV antibody levels in colostrum and milk on piglet PEDV systemic antibody levels, fecal shedding (converted PEDV rRT-PCR CTs values), body temperature, weight gain, and mortality (% survival). At the end of the 14 day observation period, all sows were euthanized and exsanguinated. Total serum antibody from each sow was collected and antibodies purified for use in phase 2.

Experimental design phase 2.

To evaluate the ability of systemic PEDV antibody to protect neonatal piglets against PEDV, 7 clinically healthy sows were acquired from one commercial sow farm at ~110 of their second gestation. To verify their negative status, sow fecal swabs were tested for PEDV, TGEV, and PDCoV using agent-specific rRT-PCRs and serum samples were tested for PEDV antibody using a whole-virus indirect ELISA. Piglets (n = 73) from 7 PEDV-naïve sows were intraperitoneally (IP) administered concentrated PEDV antibody sufficient to achieve one of 6 targeted levels of circulating antibody. 24 h later, all piglets were inoculated with PEDV and then observed until DPI 14 or until humane euthanasia was necessary.

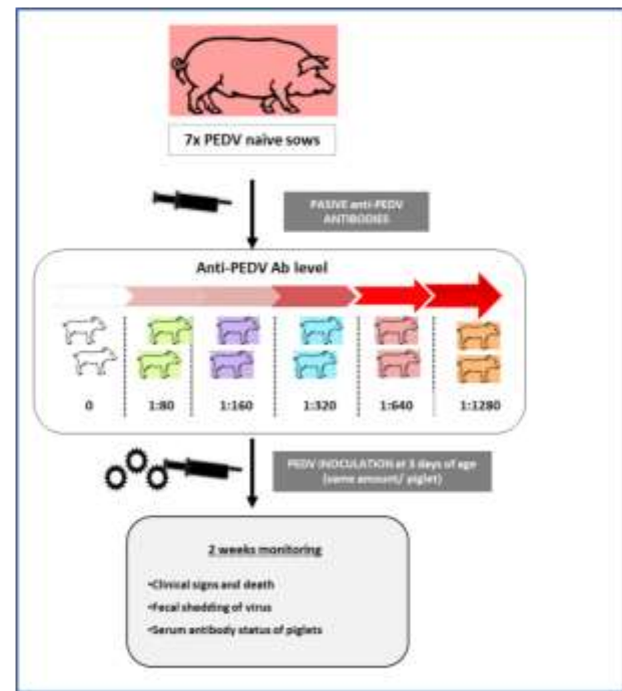


Fig 2. Evaluation PEDV protection in naïve piglets administered specific levels of anti-PEDV antibody.

On a daily basis, sow milk and piglet fecal samples and data on piglet clinical signs, body weight, and body temperature were collected. Serum samples were collected from sows at DPI -7 and 14 and from piglets at DPI -1, 0, and 14 or at the time of humane euthanasia. Fecal samples were tested by PEDV rRT-PCR. Serum, colostrum, and milk were tested for PEDV IgG, IgA, and virus-neutralizing antibody. The data were evaluated for the effects of systemic PEDV antibody levels on the outcomes measured. The experiment was implemented as follow:

Concentrated PEDV antibody: The procedure used to precipitate swine serum proteins and antibodies was a modification based on previous publications (Hebert et al., 1973; Grodzki and Berenstein, 2010). Specifically, swine serum proteins and serum antibody were precipitated by single fractional precipitation using 30% and then 40% ammonium sulfate, respectively. The entire process was conducted in an environmental chamber (Caron®, Marietta, OH) maintained at 4°C. Initially, whole blood was collected from humanely euthanized and exsanguinated PEDV antibody-positive sows (ISU #2-14-7736-S). Serum was harvested and stored in 1 L bottles (BiotainerTM, Nalge Nunc Corp., Rochester, NY) at -20°C. For the first precipitation, the serum was thawed for 24 h at 4°C, the volume of saturated ammonium sulfate (Sigma-Aldrich) calculated to achieve 30% concentration was added in a drop-by-drop fashion while stirring continuously, and then the mixture was incubated at 4°C for 16 h with continued stirring. Following incubation, the mixture was centrifuged (4°C) at 4,000 x g for 10 min to remove protein aggregates and less soluble proteins. For the second precipitation, the volume of the supernatant (antibody fraction) was measured, additional saturated ammonium sulfate was added as before to achieve a final concentration of 40%, and then the mixture was incubated for 16 h at 4°C with stirring. Thereafter, the mixture was centrifuged (4°C) at 4,000 x g for 10 min to recover the antibody fraction. The pelleted antibody was gently resuspended with PBS (1X pH 7.4) at a 1:5 (pellet:PBS) volume ratio. To remove salts, the solution was dialyzed in 250 ml dialysis flasks (Pierce®, Thermo-Fisher Scientific, Waltham, CA) floating vertically in ~15 liters of 4°C, continuously stirred PBS (1X pH 7.4). The entire volume of PBS was replaced every 4 h for 5 times and then the antibody solution was concentrated by polyacrylamide gel dialysis (Spectra/Gel®

Absorbent, Spectrum Laboratories, Inc. Rancho Dominguez, CA). The concentrated antibody solution was then aliquoted into 50 ml centrifuge tubes and stored at -20°C until used.

Treatments: The concentrated PEDV antibody solution was thawed at 22°C for 2 h and then 2-fold diluted with PBS (1X pH 7.4) to create 6 treatments consisting of 5 different concentrations of PEDV antibody and an antibody-negative control (PBS 1X pH 7.4). Piglets were blocked by sow and randomized to treatments using commercial statistical software (SAS/STAT® 9.2). Treatments were administered by intraperitoneal inoculation at the rate of 1.35 ml of the solution per kg of piglet bodyweight. Allocation of treatments by litter and piglet age are given in Table 1.

Table 1. Assignment of piglets to treatments

Litter	No. of piglets	Treatments (no. piglets within treatments)[a]					
		1 (Negative)	2 (1:80)	3 (1:160)	4 (1:320)	5 (1:640)	6 (1:1280)
1	13	2	3	2	2	2	2
2	11	2	1	2	2	2	2
3	9	1	2	1	2	2	1
4	10	2	1	2	1	1	3
5	10	2	2	1	2	2	1
6	9	2	2	2	1	1	1
7[b]	12	1	1	3	3	2	2
Totals	74	12	12	13	13	12	12

[a] Treatments consisted of 5 different concentrations of PEDV antibody and an antibody (Ab)-negative control (PBS 1X pH 7.4).

b Data from litter 7 were excluded from the analysis because the sow exhibited clinical signs of

Animals and animal care. Animals used in both phases of the study were housed throughout the experiment in the Iowa State University Livestock Infectious Disease Isolation Facility (LIDIF). LIDIF is a biosafety level 2 research facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The facility is equipped with a single-pass non-recirculating ventilation system that provides directional flow from low contamination areas to high contamination areas and zones of negative pressure to prevent control airborne contamination from area-to-area or room-to-room. Each room is ventilated separately and humidity and temperature is 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.

All sows within each phase had been bred on the same day. To induce parturition, all sows were administered 10 mg of dinoprost tromethamine (Lutalyse®, Zoetis Inc., Florham Park, NJ) 24 hours prior to the expected farrowing date, i.e., day 113 of gestation. All viable piglets were ear-tagged and administered 1 ml iron hydrogenated dextran (VetOne®, Boise, ID) and 5 mg (0.1 ml) ceftiofur sodium (Excenel®, Zoetis). Piglets remained on the sow continuously throughout the 2-week observation period.

PEDV inoculation. On DPI 0, the PEDV stock solution (1 x 105 TCID50/ml) was diluted to an estimated concentration of 1 x 103 TCID50/ml, mixed 1:4 with milk replacer (Esbilac®, PetAg Inc., Hampshire, IL) and administered orally (5 ml) to each piglet. This provided a total dose of 1 x 103 TCID50 PEDV 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. Animals unable to suckle, reluctant to stand, or demonstrating ≥ 10% dehydration based on skin tenting were euthanized by intravenous administration of pentobarbital sodium (Fatal-Plus®, Vortech Pharmaceuticals, MI) at a dose of 100 mg/kg.

Biological sample collection

Serum: Serum samples for antibody testing were collected from sows (DPI -7, 14) and piglets (DPI -1, 0, 14). Blood samples were drawn from the jugular vein or cranial vena cava using a single-use blood collection

system (Becton Dickson, Franklin Lakes, NJ) and serum separation tubes (Kendall, Mansfield, MA). Blood samples were processed by centrifugation at 1,500 x g for 15 min, aliquoted into 2 ml cryogenic tubes (BD Falcon™, Franklin Lakes, NJ), and stored at -20°C until tested.

Mammary secretions: Colostrum and milk samples for antibody testing were collected from sows between DPI -3 to 14. Sows were administered 20 USP units of oxytocin (VetOne®) to facilitate collection of mammary secretions. Samples were processed by centrifugation at 13,000 x 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.

Fecal samples: Fecal samples for porcine coronavirus RT-PCR testing included fecal swab (BD BBLTM CultureSwabTM Collection/Transport system, Thermo-Fisher Scientific) samples collected from individual sows immediately prior to receipt of the animals and individual piglet fecal samples collected between DPI 0 and 14. Approximately 1 gram of feces was collected from each piglet using a disposable fecal loop (VetOne®), mixed with 1 ml PBS (1X pH 7.4, Sigma-Aldrich) immediately after collection, placed in a 2 ml cryogenic tube (BD Falcon™), and stored at -80°C.

Coronavirus reverse-transcriptase polymerase chain reactions (rRT-PCR)

RNA extraction: In brief, 90 µl of viral RNA was eluted from sow fecal swab samples or 50 µl of piglet fecal:PBS sample (2.6.3 above) using the Ambion® MagMAXTM viral RNA isolation kit (Life Technologies) and a KingFisher® 96 magnetic particle processor (Thermo-Fisher Scientific) following the procedures provided by the manufacturers.

Coronavirus primers and probes: Sow fecal swab samples and piglet fecal samples were tested for PEDV using a PEDV N gene-based rRT-PCR described in Madson et al. (2014) and performed routinely at the Iowa State University-Veterinary Diagnostic Laboratory (ISU-VDL SOP 9.5263). Primers and probes targeting conserved regions of the PEDV nucleocapsid gene were designed to match a U.S. PEDV nucleotide sequences published in GenBank (accession no. KF272920) (Madson et al., 2014).

Sow fecal swab samples were tested for TGEV using a using a spike (S) gene-based procedure described in 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 in 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 (TCID50). In brief, the forward primer, reverse primer, and probe were designed to match the membrane protein of global and U.S. PDCoV isolates. The probe was labeled with FAM/ZEN/3'Iowa Black Detector (Integrated DNA Technologies, Coralville, IA).

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 corresponding coronavirus.

Coronavirus antibody assays

PEDV indirect immunofluorescence assay (IFA): IFA plates were prepared by inoculating confluent monolayers of Vero cells (ATCC® CCL-81™) in 96-well plates (CoStarTM, Corning®) with 100 µl/well of PEDV (US/IN/2013/19338E) at 1 x 103 plaque-forming units/ml. The plates were then incubated for 18 to 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 serially two-fold diluted (1:40 to 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 µl of 1:50 diluted goat anti-swine IgG antibody conjugated with FITC (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added to each well. After 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.

PEDV whole virus antibody ELISA: PEDV (USA/Iowa/18984/2013) 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 4,000 x g for 15 min to remove cell debris. The virus was then pelleted by ultracentrifugation at 140,992 x 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 (Nalge Nunc Corp.) were manually coated (100 µl/well) with the viral antigen solution and incubated at 4°C overnight. After incubation, plates were washed 5 times, blocked with 300 µl 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 ≥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 µl 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 µl 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 1:3,000 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 µl 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 µl 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® Instruments Inc., Winooski, VT) operated with commercial software (GEN5TM, Biotek® Instruments Inc.). The antibody response in serum and colostrum/milk samples was represented as sample-to-positive (S/P) ratios calculated as:

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

PEDV fluorescent focus neutralization (FFN) assay: Colostrum, milk, and serum samples were tested for neutralizing antibody. Prior to FFN testing, defatted milk and colostrum samples were treated with Rennet (Rennet from Mucor miehei, Sigma-Aldrich). In brief, 5 µl 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 2,000 x 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 to 1:512) in 96-well dilution plates (Axygen®, Corning®) using post-inoculation medium to give a final volume of 100 µl. Then, 75 µl of each dilution was transferred to new dilution plate (Axygen®, Corning®), mixed with 75 µl of PEDV (1 x 103.6 TCID50/ml) to give final serum dilutions of 1:8 to 1:1024, and incubated at 37°C with 5% CO2 for 1 h. Vero cell confluent monolayers in 96-well plates (CoStarTM, Corning®) were washed twice with post-inoculation medium, inoculated with 100 µl of the sample-virus mixture, incubated at 37°C with 5% CO2 for 1 h, and washed twice. 100 µl of post-inoculation medium was then added to each well and the plates incubated at 37°C with 5% CO2 for 48 h. Finally, cells were fixed with 80% cold acetone:alcohol (80:20), stained with FITC-conjugated monoclonal antibody (SD6-29, Medgene Labs, Brooking, 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 ≥ 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.

Data analysis

To determine correlations between colostrum/milk PEDV-specific IgG, IgA or VN antibody levels of each sow and outcomes, area under the curve (AUC) of antibodies (IgG, IgA, VN) measured by ELISA (S/P ratio) or FNN (titer) from day of inoculation to end of experiment were calculated from each sow. Similarly, AUC of adjusted CT in pooled feces, ADG, and litter survival rates were calculated from each litter. Linear correlation analyses were evaluated by SigmaPlot (SigmaPlot 12.5©, Systat Software Inc.) based on AUC of IgA compared to AUC of adjusted CT (adjusted CT= reported CT-35) and AUC of survival rate of each litter. Two-tailed p-value less than 0.05 was used as statistically significant.

RESULTS

PHASE I

Passive transference of maternal antibodies

The kinetics and levels of maternal antibodies (IgG, IgA and VN) secreted on colostrum/milk from PEDV naïve sows (n=2; sows 1-2) and PEDV immune sows (n=8; sows 3-10) collected daily from farrowing to 14 days post-partum is showed in Figure 1. PEDV specific IgG levels (Fig. 3-A) but not the IgA (Fig. 3-B) and VN (Fig. 3-C) found in colostrum from immune sows decrease significantly after 3-5 days post farrowing (colostrum→milk).

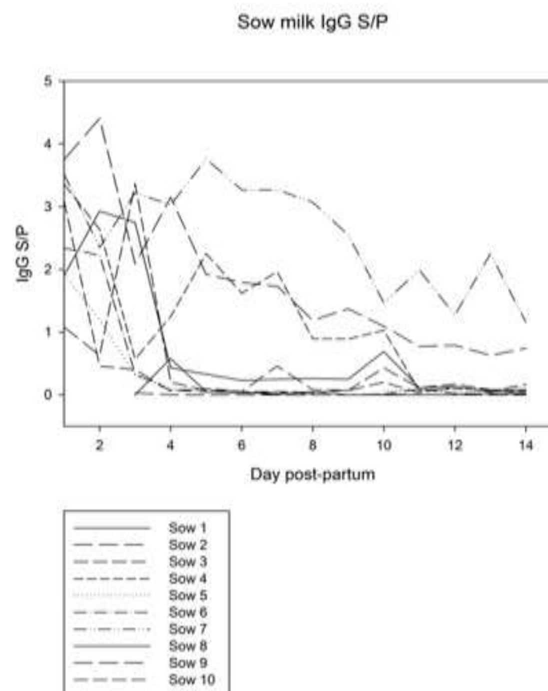


Fig 3-A. PEDV WV IgG ELISA on colostrum/milk

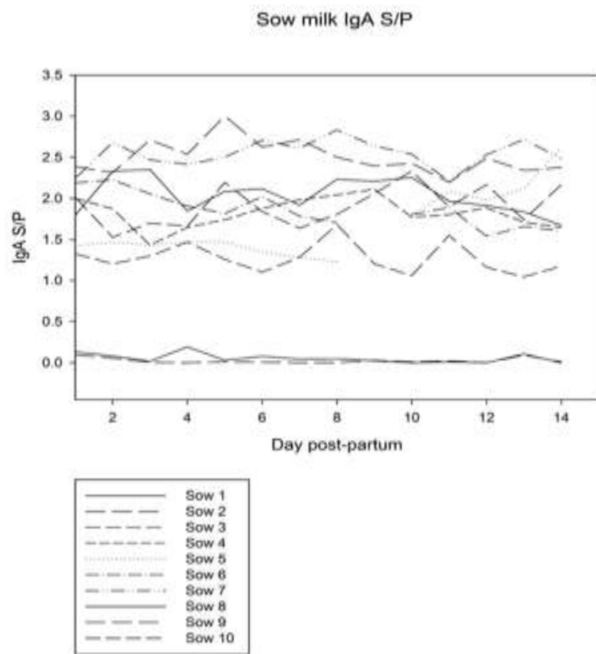


Fig 3-B. PEDV WV IgA ELISA on colostrum/milk.

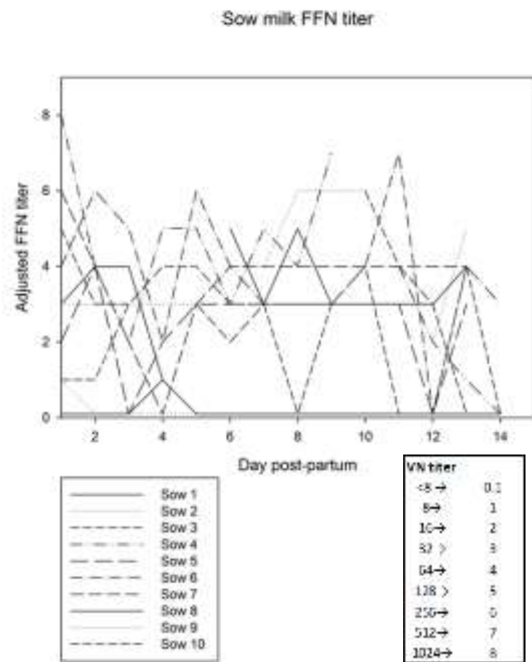


Fig 3-C. PEDV FFN test on colostrum/milk.

Passive protection after PEDV inoculation

The area under the curve (AUC) of adjusted CTs of PEDV rRT-PCR in litter pooled feces (Fig. 4) and litter survival rate (Fig. 5) were plotted by day post-inoculation to demonstrate PEDV shedding in feces from litter exposed to PEDV (litter 3-10) and survivals of piglets in each litter (litter 1-10), exposed or not to PEDV during the experiment.

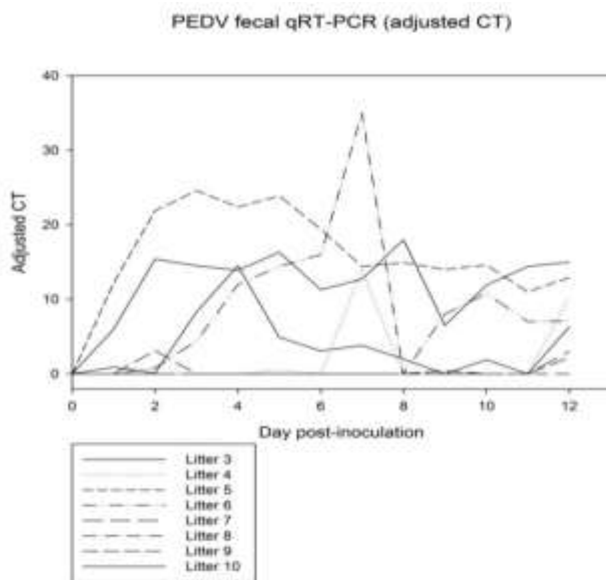


Fig 4. Fecal PEDV shedding in challenged pigs by DPI. Data are presented as AUC of adjusted PEDV rRT-PCR CT values in pooled feces (litter).

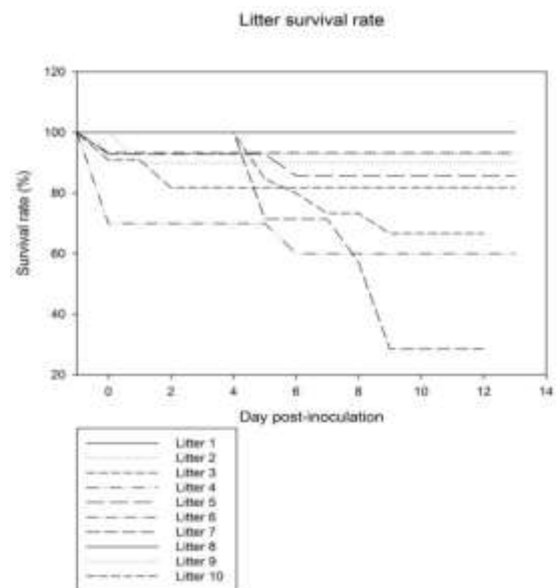


Fig 5. Litter survival rate (%). Data are presented as AUC of survival rate.

No clinical sign were observed in control sow litters, with a piglet mortality <7%, and piglet average daily gain (ADG) was 0.19 and 0.2 kg/day. In litters from PEDV immune sows, diarrhea was observed in 27.3 to 100% of piglets; mortality ranged from 0 to 40%; and piglet ADG ranged from 0.02 to 0.19 kg/day.

Correlation analysis

Correlations between milk PEDV-specific antibody levels of each sow from day of inoculation (DPI0) to end of experiment (DPI12) and virus shedding in pooled feces from each litter were calculated. A linear relationship between milk IgA (Fig. 6) levels and virus shedding from piglets were observed, where piglets receiving milk from sows that had higher milk IgA or VN titer had lower PEDV shedding in feces (p value = 0.04). No linear relationship between milk IgA and survival of the piglets was proved (data not showed).

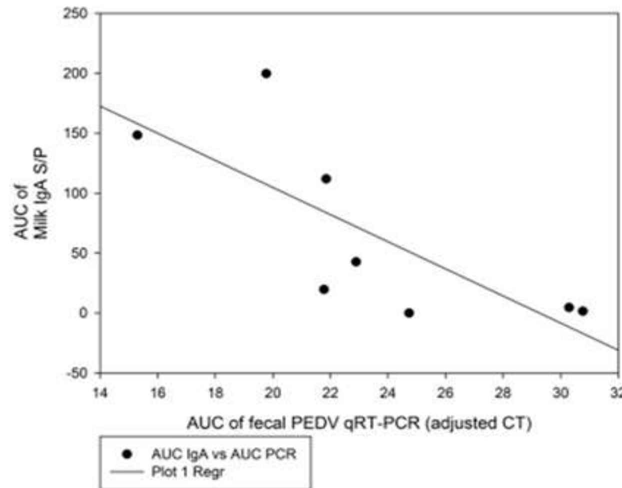


Fig 6. Correlation between AUC of S/P values of milk IgA ELISA and AUC of converted CT of PEDV rRT-PCR in piglet feces.

PHASE II

Passive transference of intraperitoneal injected antibodies

The PEDV-specific levels of systemic antibodies in piglets after intraperitoneal injection of different treatment is presented in Figure 7. Efficient uptake of the injected antibodies into the circulation was evidenced by the increased titers of IgG and IgA antibodies measured by a PEDV WV ELISA, and VN antibodies measured by a FFN test, in serum of piglets injected with treatment 2 to 6 of given immune serum. Those levels were higher than those of control group (treatment 1).

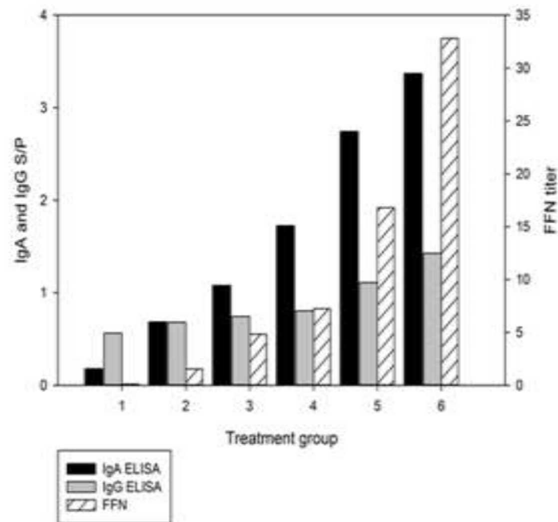


Fig 7. PEDV-specific levels of systemic antibodies (IgA, IgG and VN) in piglets after intraperitoneal injection of different treatments

Passive protection after PEDV inoculation

The fecal PEDV shedding and survival rate of piglets grouped by treatment after experimental inoculation of PEDV is presented in Figures 8 and 9, respectively. All piglets (100%) developed diarrhea within 48h post-inoculation. Mortality by treatment ranged from 50 to 91%, where piglets receiving control saline solution (treatment 1) showed the lowest survival rate. Piglet ADG ranged from 0 to 0.105 kg/day without correlation

with treatment received.

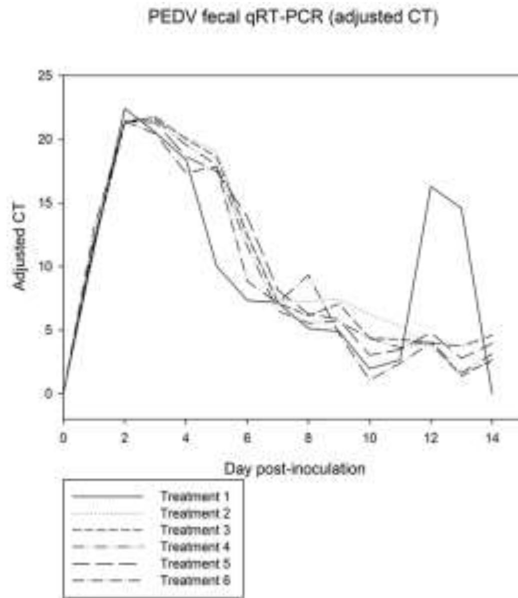


Fig 8. Fecal PEDV shedding of piglets by treatment and after PEDV inoculation by DPI.

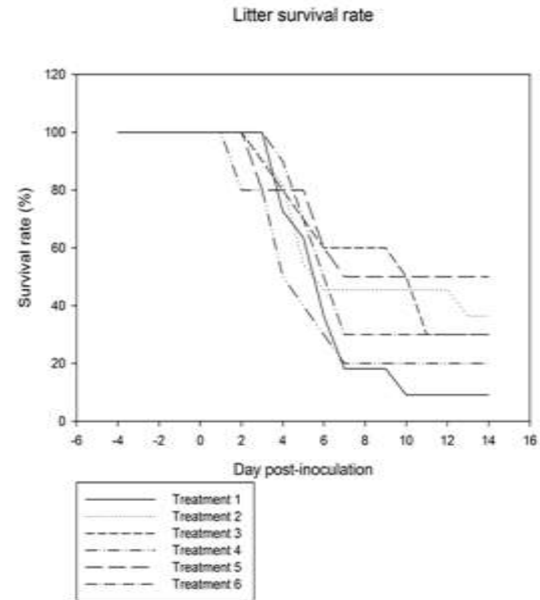
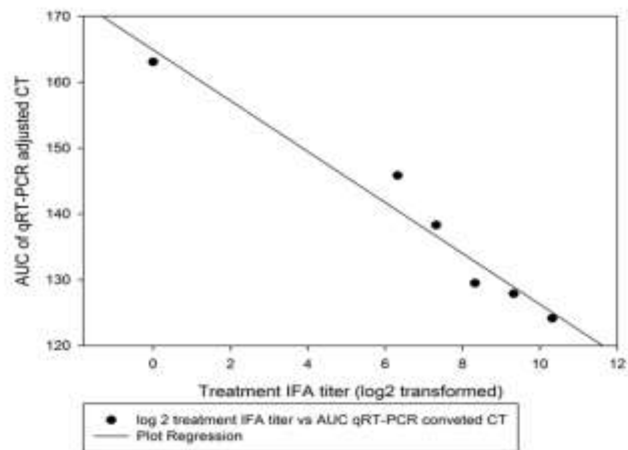


Fig 9. Piglet survival rate (%) of piglets by treatment.

Correlation analysis

We found a correlation between treatment dose received by log 2 of intraperitoneal injection and the PEDV shedding in piglet feces (p-value = 0.0008) (Fig. 10). However, there was a discrepancy between viral shedding data and clinical signs. Specifically, we found a reduced viral shedding by treatment but a significant incidence of diarrhea and mortality levels in piglets receiving high levels of antibodies intraperitoneally.



DISCUSSION

We investigate the effects on maternal and systemic passive antibody responses against PEDV infection in two different experimental studies. Correlation between clinical profiles and immunity levels of each pig was analyzed to determine the maternal or systemic antibody profile necessary to provide piglet protective immunity against PEDV infection in commercial herds.

We found evidences of the contribution of maternal (lactogenic) immunity but not of the systemic immunity in providing protection against severity of PED disease by reducing the virus shedding and mortality in new born piglets after experimental inoculation of a PEDV U.S. prototype strain. Thus, the present work provides preliminary evidence of a protective effect of local (maternal immunity) antibodies beyond that of circulating

antibodies (systemic immunity) alone. Although we have not addressed the role of cell-mediated immunity, it is know that a combination of both humoral and cell-mediated immunity is required for optimal disease protection.

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PUBLICATIONS RELATED TO THE PROJECT: PROCEEDINGS AND ABSTRACTS

Poonsuk K, Giménez-Lirola LG, Gonzalez W, Zhang J, Chen Q, Correa da Silva Carrion L, Olsen C, Magtoto R, Johnson J, Wang C, Madson D, Main R, Zimmerman J, Yoon KJ. March 2015. Defining PEDV maternal immunity and correlates of neonatal protection. 46th Annual Meeting of the American Association of Swine Veterinarians. Orlando, Florida. Proc Seminar #3, pp. 26-27.

Poonsuk K, Giménez-Lirola LG, Gonzalez W, Zhang J, Chen Q, Correa da Silva Carrion L, Olsen C, Magtoto R, Johnson J, Wang C, Madson D, Main R, Zimmerman J, Yoon KY. December 2014. Relationship between maternal immune status and neonatal protection against PEDV infection. Proc 95th Ann Meet Conference of Research Workers in Animal Diseases. Chicago, Illinois, p. 74, No 97.

Giménez-Lirola LG, Baum D, Bower L, Chen Q, Sun D, Johnson JK, Madson D, Magtoto R, Poonsuk K, Sarmento L, Yoon KJ, Zhang JQ, Zimmerman JJ, Main R. February 2015. Serology: Overview of PED antibody response. Proc 46th Annual Meeting of the American Association of Swine Veterinarians. Orlando, Florida. Proc Seminar #2, pp. 7-9.

ORAL PRESENTATIONS

Poonsuk K, Giménez-Lirola LG, Gonzalez W, Zhang J, Chen Q, Correa da Silva Carrion L, Olsen C, Magtoto R, Johnson J, Wang C, Madson D, Main R, Zimmerman J, Yoon KJ. March 1, 2015. Defining PEDV maternal immunity and correlates of neonatal protection. 46th Annual Meeting of the American Association of Swine Veterinarians. Orlando, Florida.

Poonsuk K, Giménez-Lirola LG, Gonzalez W, Zhang J, Chen Q, Correa da Silva Carrion L, Olsen C, Magtoto R, Johnson J, Wang C, Madson D, Main R, Zimmerman J, Yoon KY. December 8, 2014. Relationship between maternal immune status and neonatal protection against PEDV infection. 95th Ann Meet Conference of Research Workers in Animal Diseases. Chicago, Illinois.

Giménez-Lirola LG, Baum D, Bower L, Chen Q, Sun D, Johnson JK, Madson D, Magtoto R, Poonsuk K, Sarmento L, Yoon KJ, Zhang JQ, Zimmerman JJ, Main R. February 28, 2015. Serology: Overview of PED antibody response. 46th Annual Meeting of the American Association of Swine Veterinarians. Orlando, Florida.