RESEARCH REPORT
SWINE HEALTH

Title: Evaluation of mucosal B cell response to PEDV in infected sows - **NPB # 14-187**

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Industry Summary:
Porcine epidemic diarrhea virus (PEDV) has rapidly become one of the most economically devastating enteric diseases in the US swine industry. The virus is most severe in neonatal pigs with mortality up to 100%. Thus, induction of strong maternal immunity is critical in reducing the production losses caused by PEDV. Therefore, research on understanding insights of PEDV specific memory B cell responses in the intestines and lymphoid tissues of infected sows, and its association with antibody response in clinical samples (plasma, fecal and oral swabs) is important. We used 36 sows resourced from multiple commercial swineherds in Mid-Eastern part of the US, with the clear history of approximately 1 and 6 months post-exposure to PEDV, and included both primiparous and multiparous and uninfected control age-matched sows. Our results suggested that analysis of PEDV specific IgA antibody response in the plasma and oral fluid is an ideal strategy for diagnosis, and plasma for immune status evaluation until six months post-infection that we tested. But fecal samples from sows are not suitable for diagnosis purpose in infected recovered sows. At the mucosal and systemic sites of pigs, PEDV specific memory B cell response was higher at one month compared to six months post PEDV-infection at both intestines and spleen; and though their levels at six months decline but still their levels and VN titers in plasma appear to be sufficient to respond rapidly to reinfection.

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Keywords: Porcine epidemic diarrhea virus (PEDV); B cell response; intestines and lymph nodes; IgA and IgG in clinical samples

Scientific Abstract:
Porcine epidemic diarrhea virus (PEDV) is the economically important enteric disease in the swine industry. Since the virus infects naïve and neonatal pigs, mortality is very high which reaches up to 100%. Currently, feedback methods utilizing PEDV infected material of piglets has demonstrated variable success in preventing reinfection of sows and their offspring. Thus, it is challenging for the swine veterinarians to understand the herd immune status against PEDV. Therefore, information on the duration of memory IgA and IgG antibody secreting B cell response in the intestines and lymphoid tissues of PEDV-infected sows, and its association with specific antibody levels in clinical samples such as plasma, oral, and fecal samples is important. Our goal of this study was to quantify PEDV specific IgA and IgG positive memory B cell response in sows with a clear history of infection of approximately 1 and 6 months post-exposure to virus. Further, due to expected differences in the immune status of primiparous vs. multiparous sows against PEDV, we included them in our study and compared the data with background values of uninfected age-matched sows. For this study, a total of 36 sows (n=6 sows per group), were resourced from multiple commercial swineherds in Mid-Eastern part of the US. Our results suggested that PEDV specific IgA response in the plasma and oral fluid (but not fecal samples) is ideal for diagnosis; and plasma for immune status evaluation in terms of virus neutralizing antibody titers until six months post-infection in sows. Data on PEDV specific IgA and IgG secreting B cell response in sows at mucosal and systemic sites indicated that, though their levels decline by 6 months at both ileum and spleen, but their levels and strong VN titers in plasma appears to be sufficient to react rapidly to reinfection in sows. In summary, PEDV specific B cell response at the intestines and spleen of infected sow associates strongly with the antibody response in plasma and oral fluid until six months post-infection that we evaluated, and such levels in sows might be sufficient to combat reinfection and protect offsprings.

Introduction:
Porcine epidemic diarrhea virus (PEDV) is the causative agent of porcine epidemic diarrhea (PED) that clinically manifests as severe watery diarrhea with subsequent dehydration in all ages of swine, but more severe in sucking pigs [1]. Other clinical signs of PED include vomiting and anorexia. PED is characterized by high mortality that may range from 30% to 100% in neonates, and high morbidity but low mortality in weaned pigs [2]. Economic losses due to elevated mortality and decreased production are significant in US swine herds that have been affected by the disease. PEDV was detected on multiple US swine farms in April of 2013 [1], and the virus has continued to spread through swine producing states at an alarming rate until mid part of 2014.

Over 45,779 PEDV tests have been conducted in the US between May 2013 and March 2014, and there have been 4,757 cases of PEDV in the US affected 27 states (http://www.aasv.org/pedv). PEDV continues to infect naive swine farms breaching strict biosecurity protocols for unknown reasons or has reinfected breeding farms after implementing feedback strategies. Piglets are expected to be protected from PED clinical disease through colostral immunity received from immune dams [3]; however, protection from infection and shedding has been variable with occasional failure of feedback regimens (personal communication). Control and prevention of PEDV is one of the major hurdles to the swine industry in the US. In spite of feedback methods to control PEDV outbreaks in subsequent litters, the results are not satisfactory. This could also be attributed to non-availability of reliable and cost-effective diagnostic tool to monitor protective herd immune status against PEDV. Standardization of antibody based assays and associating the same with B cell responses to identify PEDV immune levels in infected recovered sows in the breeding farms will immensely help swine farmers and veterinarians to take up appropriate control measures.

Objectives:
To evaluate B cell responses in mammary tissues and intestines of PEDV infected sows and associate the results with antibody responses

Materials & Methods:

Cells: Vero cells used in this study was provided by Dr. Jianqiang Zhang, Iowa State University. Cells were cultured in Minimum Essential Media (Gibco, CA) supplemented with 10% heat inactivated fetal bovine serum (Atlanta Biologicals, GA), 2mM L-glutamine (Gibco, CA) and antibiotic/antimycotic solution (HyClone, UT) at 37℃ in a humidified atmosphere with 5% CO_2.

Virus: PEDV strain (provided by Dr. Ying Fang, Kansas State University and Dr. Jianqiang Zhang, Iowa State University) was propagated in Vero cells. Confluent cell monolayer was flushed with sterile phosphate-buffered saline (PBS) twice before infecting with the virus, and after one hour of adsorption at 37℃, serum free MEM containing tocylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (1μg/ml) (Sigma, MO), 0.3% tryptose phosphate broth (TPB, sigma), 0.02% yeast extract) and antibiotic/antimycotic solution (HyClone, UT) were added without removing the inoculum. The virus induced cytopathogenic effect was close to 90% by about 2-3 days, and we collected the virus culture supernatant after freeze-thawing two times and clarified followed by ultracentrifugation through a 20% (wt/vol) sucrose cushion. Viral antigen was collected and the protein concentration was measured by micro BCA protein assay kit (Thermo Scientific, IL) and both the stock virus and viral antigen were stored at -80℃.

Animals: Three swine breeding farms located in the Middle Eastern part of the US, with the two farms having a clear history of 100% PEDV infection at approximately one month and 6 months before, and the third farm with no such infection were chosen for this study. All the three farms had also confirmed the PEDV infection or no infection status in the laboratory. Six sows each from primiparous and multiparous group with a total of 36 sows were used in this study. Animals were euthanized in a slaughter plant as per the standard procedures with necessary efforts to minimize suffering of animals. All the procedures of the animal use protocol were approved by the Committee on the Ethics of Animal Experiments of The Ohio State University. On the day of necropsy, plasma, oral swab and fecal swab samples were collected for virus specific antibody titration; and ileum, mesenteric lymph nodes (MLN), and spleen were collected in MEM containing antibiotics and antifungal.

Isolation of mononuclear cells (MNCs): MNCs from ileum, MLN, and spleen were isolated as previously described with a few modifications [4-7]. Briefly, ileum tissue was cut in to tiny pieces and treated with Type Ⅱ collagenase after treating with EDTA to remove epithelial cells. Cell suspension was obtained after passing the digested tissues of ileum, spleen, and MLN through stainless steel Cellector fitted with a 80 μM mesh screen (Cellector, FL). The harvested MNCs were centrifuged in the interface of 43% and 70% Percoll and the cells in the interface were collected and filtered through 40μm cell strainer (BD Falcon, MA) and resuspended in enriched-RPMI (E-RPMI, RPMI containing 10% FBS, 200μm HEPES, 1mM sodium pyruvate, 25μm 2-ME, 1x Non-Essential Amino Acid, and 1x antibiotic and antifungal). The viability of cells was confirmed by trypan blue dye exclusion method, and counted using a hemocytometer.

***In vitro* stimulation of MNCs with PEDV antigen:** MNCs isolated from ileum, MLN, and spleen were plated in 24-well cell culture plate (25×10^6 cells/well) in 2 ml E-RPMI in the presence of semi-purified PEDV viral antigen (25μg/ml), and as controls with medium alone or lipopolysaccharide (25μg/ml). Cells were cultured for 6 days at 39℃ with 5% CO_2, and 0.5 ml of E-RPMI was added to each well on every second day. Supernatants were collected to measure PEDV specific IgA and IgG antibody by ELISA. Cells were harvested, washed using PBS, resuspended in E-RPMI, counted and used for detecting the population of PEDV-specific IgA and IgG antibody secreting cells (ASC) by ELISPOT assay, and frequency of IgA^+ and IgG^+ B cells by flow cytometry.

Antibody isotype ELISA: PEDV-specific IgA and IgG antibody levels were determined as described previously [5, 6]. Briefly, pretitrated amounts of PEDV antigen (5μg/ml), recombinant PEDV S protein (5μg/ml) or M protein (10μg/ml) (provided by Dr. Ying Fang, Kansas State University) diluted in 50mM

carbonate buffer (pH 9.6) were coated overnight at 4℃ in the 96-well ELISA plate (Corning, MA). Plates were blocked using 10% nonfat milk in PBS containing 0.05% Tween 20. Plasma sample was diluted 1:200, 1:800, 1:3200, and 1:12800, and oral swab and fecal swab samples were diluted 1:8, 1:32, 1:128 and 1:512. The supernatants harvested from restimulated MNCs were diluted 1:2, and 50μl of each sample was applied into duplicate wells and incubated for 1 hr at room temperature (RT). Plates were washed and horseradish peroxidase-conjugated goat anti-pig IgA (Bethyl laboratories) or IgG antibody (KPL) (1:5000) were added to the plate and incubated for 1 hr. The reaction was developed using TMB peroxidase substrate and stopped using 1M phosphoric acid, and plates were read at OD_{450}.

Virus titration and virus neutralizing (VN) antibody assays: PEDV titer in stocks and VN antibody titer in clinical samples were analyzed by indirect immunofluorescence assay (IFA) as described previously [8, 9] with a few modifications. Briefly, confluent Vero cell monolayers in 96-well plates were washed once using PBS. For virus titration, 10-fold serial diluted virus fluid was added to cells plate in quadruplicate; for VN titration, clinical samples were UV (254 nm for 45 min) and heat (56℃ for 30 min) inactivated and 2-fold serially diluted samples were incubated with equal volume of PEDV (50 $TCID_{50}$ per well) for 1.5 hrs at 37 ℃, and the mixture was transferred into Vero cells grown in microtiter plates in duplicate. Plates were incubated for 1 hr at 37 ℃, infection medium was added after the wells were washed once using PBS and incubated for 24 hrs at 37 ℃. Plates were washed and fixed using an acetone-Milli-Q water (8:2) mixture for 10 min at RT, and dried completely before immunostained. Cells were treated with anti-PEDV N protein specific monoclonal antibody (SD6-29) (Medgene labs., SD) (1:1,000) for 2 hrs at 37 °C, followed by treatment with Alexa Fluor 488 conjugated goat anti-mouse IgG (H+L) (Invitrogen, CA) secondary antibody (1:4000). The plate was examined under a fluorescent microscope after mounting with glycerol-phosphate-buffered saline (PBS) (6:4). The virus induced cytopathic effect was examined under a fluorescent microscope, and the titer was calculated using the Reed and Muench method. The viral titer was expressed in 50% tissue culture infective dose ($TCID_{50}$) per ml. VN titer was determined to be the reciprocal dilution ratio of the sample which caused >90% reduction in virus induced fluorescence foci units compared to virus control wells.

Enzyme-Linked ImmunoSpot (ELISPOT) assay for quantifying PEDV-specific antibody secreting cells (ASCs): ELISPOT assay was performed to analyze the population of PEDV specific IgA and IgG antibody secreting B cells as described previously [4-7]. Briefly, two kinds PEDV antigen coated plates were used in this study. (i) Vero cells infected and fixed cell culture plates: PEDV (10^4 $TCID_{50}$/ml) containing 1μg/ml of TPCK-trypsin was inoculated to Vero cell monolayers in 96-well tissue culture plate. Cells were fixed with 80% acetone at 16 hrs post-infection and stored at -20℃. At that stage about 80% of cells were infected as tested by indirect fluorescence assay, and the cell monolayer was still intact. Mock-infected cells treated exactly the same way were used as control. (2) PEDV antigen (25μg/ml) immunocaptured plates: semi-purified PEDV antigen (25μg/ml) diluted in 50mM carbonate buffer (pH 9.6) was coated overnight at 4℃ in nitrocellulose-based 96-well microtiter plates (Millipore, MA). Plates were washed with PBS and blocked with E-RPMI for 1 hr at RT and MNCs of ileum, MLN, and spleen were plated at three 10-fold dilutions in duplicate wells, starting from 5×10^5 cells/well. All plates were incubated for 16 hrs at 39℃ with 5% CO_2, and then washed with PBS containing 0.05% Tween 20 (PBST). The antibody production was detected by using horseradish peroxidase-labeled affinity-purified goat anti-pig IgA (Bethyl laboratories) or IgG (KPL) both diluted 1:2000 in PBST and incubated for 2 hrs at 37℃.The color was developed using 3-amino-9-ethylcarbazole substrate (Sigma Chemical Co) and spots were counted using the AID ELISPot Reader System (Autoimun Diagnostika GmbH Strassberg, Germany). Data were expressed as the mean numbers of antibody-secreting cells (ASC) per 5×10^5 MNCs.

Flow cytometric analyses: The frequencies of IgA^+ or IgG^+ B cells from 100,000 acquired events of immunostained MNCs were determined by flow cytometry as described previously [10]. Briefly, MNCs were restimulated with PEDV antigen as described above and immunostained with mouse anti-pig IgA antibody

(Clone K60 1F1, AbD Serotec) followed by goat anti-mouse IgG1 conjugated to APC/CY7 and rabbit anti-pig IgG conjugated to Texas red. Subsequently, cells were fixed, permeabilized and then intracellular stained using FITC conjugated rat anti-mouse CD79β antibody (Clone AT107-2, AbD Serotec), which was shown to cross-react with pig B cells. Cells were acquired using BD Aria ΙΙ flow cytometer and analyzed using the FlowJo software.

Statistical analysis: All data were expressed as the mean value ± the standard error of mean (SEM) of six sows. Statistical analyses were performed by one-way ANOVA followed by Tukey's post hoc test using GraphPad InStat 5.0 prism software.

Results:

Antibody response in PEDV infected sows

Details of sow groups used in this study are provided in the Table 1. Clinically, sows were normal before moved to slaughter plant and at the time of necropsy. PEDV specific IgA and IgG antibody responses were determined in plasma, oral swab and fecal swab samples using PEDV antigen, recombinant PEDV S or M protein coated plates. Our results indicated that, PEDV specific IgA antibody response in the samples of plasma (Fig.1a) and oral swab (Fig.1b) from either one month or six months post PEDV infected sows was significantly higher than uninfected sow groups, and a similar trend was present in S protein coated plates (Fig.1d), but not in M protein plate (Fig.1e). Surprisingly, in fecal samples the specific IgA antibody against PEDV antigen (Fig.1c), S protein (Fig.1d), and M protein (Fig.1e) was absent, and the optical density values were significantly lesser than the uninfected sow samples (Fig.1, c, d, e).

Although PEDV specific IgG antibody levels in the plasma of infected sows were significantly higher than uninfected sows (Fig.1 f, i, j), the background optical density values of IgG from uninfected sows was very high compared to IgA levels (Fig. 1 a, d, e). In oral swab samples of only six months post PEDV-infected primiparous sows, specific IgG antibody levels against viral antigen, S protein, and M protein were significantly higher than uninfected and other infected sow groups (Fig. 1 g, i, j). Again PEDV IgG antibody in fecal samples was absent in infected sows (Fig. 1 h, i, j). Antibody data suggested that antibody analysis against PEDV in plasma and oral swabs but not fecal samples of infected sows are reliable for diagnosis purpose.

Further, virus neutralizing antibody (VN) titers against PEDV in infected sow samples were quantified, our data revealed that in plasma of all the four infected sow groups the titer was $\geq$ 512 (Fig. 2a), suggesting that even after 6 months the sows might have the ability to combat reinfection. However, the VN titers in oral and fecal swab samples were $\leq$ 8 in all the infected sows (Fig. 2 b, c), suggesting that these clinical samples are not ideal for monitoring the immune status in sows against PEDV.

Flow cytometry analysis of PEDV secreting IgA and IgG B cells in infected sows

Flow cytometry analysis allows simultaneous multiparametric analysis of the physical and chemical characteristics of immune cells. MNCs from ileum, MLN, and spleen were stimulated *ex vivo* using PEDV antigen for six days to analyze the frequency of memory IgA^+ and IgG^+ B cells by flow cytometry. In ileum MNCs, significantly higher frequency of $CD79^+IgG^+$ B cells (but not $CD79^+IgA^+$ B cells) were present in the infected sows even in the mock stimulated compared to uninfected sows (Fig. 3 a, g). When MNCs were stimulated *ex vivo* with PEDV antigen, the frequencies of $CD79^+IgA^+$ and $CD79^+IgG^+$ B cells of infected sows were significantly increased compared to uninfected sows (Fig. 2 b, h). Especially, antigen specific B cells of one month post PEDV-infected multiparous sows were significantly higher than their six months counterpart (Fig. 2 b, h). However, there was no significant increase in the frequency of both $CD79^+IgA^+$ and $CD79^+IgG^+$ B cells of MLN MNCs of PEDV infected sows, either stimulated with viral antigen (Fig. 3 d, j) or medium control (Fig. 3 c, i).

As expected, in the spleen of all the four post-PEDV-infected sow groups the frequency of $CD79^+IgA^+$ B cells were significantly higher compared to their uninfected age-matched counterparts, either unstimulated or stimulated with PEDV antigen (Fig. 3 e, f). But in both primiparous and multiparous one month post PEDV-infected sows, a significantly higher frequency of $CD79^+IgG^+$ B cell was observed compared to both uninfected and six months post-infected age-matched counterparts, either unstimulated or stimulated with PEDV antigen (Fig. 3 k, l). Further, in one month post PEDV-infected spleen of multiparous sows, increased frequency of $CD79^+IgA^+$ B cells in unstimulated MNCs was significantly higher than its six month post- infected counterpart (Fig. 3e). In one month post PEDV-infected spleen of primiparous sows, antigen specific $CD79^+IgA^+$ memory B cell population was significantly reduced compared to its six months post-infected counterpart (Fig. 3f). Interestingly, within the six month post PEDV-infected sows, the multiparous had significantly higher frequency of $CD79^+IgA^+$ memory B cell population compared to its primiparous counterpart (Fig. 3f). This data suggested that, in the spleen of PEDV-infected sows $CD79^+IgA^+$ memory B cell population persists for long period.

The culture supernatants harvested on the day six cultures of MNCs used above in Flow cytometry, were analyzed for the presence of secreted PEDV specific IgA and IgG antibodies to the whole virus and recombinant proteins S and M by ELISA (Fig. 4). Our results indicated that, corresponding to the Flow cytometry data, MNCs of ileum and spleen of group 3 and 4 sows (one and six month post PEDV-infected primiparous and multiparous) had secreted either significantly higher or at least increased trend in virus and S protein specific IgA and IgG antibodies (Fig. 4 a-c, e-g), but the levels of secreted antibodies specific to M protein was low (Fig. 4 d, h).

Quantification of PEDV secreting IgA and IgG antibody secreting cells in infected sows

ELISPOT assay allows visualization of the secretory product(s) of individual activated cells, and thus provides both qualitative and quantitative information on T and B cell responses. Population of PEDV specific IgA and IgG secreting cells (ASCs) present in every 0.5 million MNCs of ileum, MLN and spleen were enumerated by ELISPOT assay (Fig. 5). To standardize this assay, we used both PEDV antigen containing fixed Vero cells plate and PEDV antigen immunocaptured plate. Our results indicated similar trend in the frequencies of both IgA and IgG ASCs, but 5-10 fold higher numbers of ASCs were detected in fixed Vero cell antigen plates compared to the other, therefore fixed cell antigen plate data is shown here (Fig. 5). As expected we did not detect PEDV specific IgA and IgG ASCs in uninfected both primiparous and multiparous sows (Fig. 5). Overall the population of PEDV specific IgA ASCs was greater than IgG ASCs in the three tissues MNCs (Fig. 5).

In ileum, the number of PEDV-specific IgA ASCs (75-200) in one month post PEDV-infected multiparous sows was significantly greater than uninfected and six months post PEDV-infected (~10 ASCs) age-matched counterparts in unstimulated cells (Fig. 5a); and a similar trend was present in stimulated cells (Fig. 5b). In contrast, the number of virus-specific IgG ASCs were too low (<5) in the ileum MNCs of sows (Fig. 5 g, h). In MLN MNCs, the number of PEDV-specific IgA ASCs in one and six months post PEDV-infected multiparous sows were greater than uninfected age-matched sows, which were unstimulated and stimulated *ex vivo* with PEDV antigen, respectively (Fig. 5 c, d). In the unstimulated MNCs of spleen, frequency of PEDV-specific IgA ASCs (~400) in all the four PEDV-infected sow groups were significantly greater than uninfected sows (Fig. 5e); and only in PEDV-infected multiparous sows a significant increase in IgA ASCs in MNCs stimulated with virus antigen was observed compared to uninfected animals (Fig. 5 f). But significantly greater numbers of PEDV-specific IgG ASCs (~300) were present only in the PEDV antigen stimulated one month post PEDV-infected multiparous sows compared to uninfected sows (Fig. 5l).

Discussion

We evaluated PEDV specific B cell response in 24 infected sows at approximate post-infection time 1 and 6 months, comprising of both primiparous and multiparous sows, and as a control age-matched uninfected 12 sows. Earlier PEDV whole virus based ELISA was standardized to detect swine antibodies in serum samples

against PEDV at the swine herd level [11]. But similar studies on detecting antibodies in oral fluid and fecal samples are not available. In our studies, similar PEDV viral antigen based ELISA was standardized and satisfactory results were obtained in both plasma and oral fluid samples, but not in fecal samples. PEDV specific IgA and IgG response in clinical samples were compared with the antibody secreting B cell population in the ileum, mesenteric lymph nodes and spleen of sows using both Flow cytometry and ELISPOT assays. Our results suggested that PEDV specific IgA response in the plasma and oral swabs should be considered for diagnosis purpose, and it was detectable even after six months in infected sows irrespective of their primiparous and multiparous status, while the corresponding IgG response was low. Surprisingly, in fecal samples the specific response was masked by the background reactivity, and unlike in piglets it may not be an ideal clinical sample for diagnosis of PEDV in sows.

In an earlier study, 11 day old conventional pigs were infected with PEDV and specific IgA and IgG ASCs were detected in the intestines at higher levels than in systemic sites (spleen and blood) [6]. But in sows naturally infected with PEDV, we observed higher levels of IgA and IgG ASCs in spleen than in intestines. Like in our study, in that study too PEDV specific IgA and IgG antibodies were detected in the serum of infected pigs [6]. Studies conducted in sucking and gnotobiotic pigs infected with TGEV and rotavirus, respectively, had detected higher numbers of specific IgG ASCs compared to IgA ASCs in the intestines [4, 6, 12, 13]. But in our study, both in one and 6 months post PEDV-infected primiparous and multiparous sows; the numbers of specific IgA ASCs were greater than IgG ASC in the intestines, mesenteric lymph nodes and spleen. This suggests that the severity of infection in the gut of neonatal pigs would have induced greater levels of IgG ASC compared to low to milder grade of PEDV-infection in sows.

Since PEDV has only been located in intestinal mucosa and mesenteric lymph nodes, systemic stimulation of immune cells is very low [14, 15], but our study in sows suggested that systemic response in spleen is predominant over the response in intestines, the differences might be due to time frame of sampling and age of the animals. Immunologically, the possible reason could be migration of some percent of activated PEDV antigen bearing dendritic cells to spleen, resulting in activation of naïve B and T cells. Alternatively, the memory lymphocytes at the mucosa- association lymphoid tissues could migrate to spleen and bone marrow [16].

Consistent with results of others [4, 13, 17], we did not detect PEDV specific stimulation of intestinal MNCs of PEDV uninfected sows in ELISOPT assay, suggesting the need of *in vivo* priming to observe the recall response. We observed detection of significant population of virus specific IgA and IgG positive B cells by both flow cytometry and ASCs in ELISPOT in the MNCs of ileum and spleen of sows primarily infected by PEDV and without any *ex vivo* stimulation with PEDV antigen. Consistent with our findings, a similar detection of specific ASCs after primary infection of sucking pig with PEDV and without *ex vivo* stimulation in the intestines and spleen was reported [6].

In summary, PEDV specific IgA and IgG B cells in infected sows were detectable better in ileum and spleen lymphocyte population compared to mesenteric lymph nodes using both ELISPOT and Flow cytometry assays, with the later assay more robust than the former in detecting antigen specific B cells**. In summary, our results suggested that, detection of PEDV specific IgA in plasma and oral fluid samples are reliable for diagnosis in sows, and plasma could be used to determine the immune status. PEDV specific B cell response though declines by 6 months at both ileum and spleen of sows, its presence in those tissues and the high VN titers in plasma even after six months might protect piglets from the disease.**

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Table.1

Groups	No. of Sows	PEDV infection	Status	Abbreviation
1	6	mock	Primiparous	Mock-PP
2	6	mock	Multiparous	Mock-MP
3	6	1 month post-infection	Primiparous	1m PI-PP
4	6	1 month post-infection	Multiparous	1m PI-MP
5	6	6 months post-infection	Primiparous	6m PI-PP
6	6	6 months post-infection	Multiparous	6m PI-MP

Table 1. Grouping of sows at different stages of PEDV infection. A total of 36 sows from three different swine breeding farms located in the Mid-Eastern part of the US, having a clear PEDV infection history (uninfected, one month or six months post- infected) were selected and 6 sows each from primiparous and multiparous groups were transported to a slaughter plant in Ohio. Clinical samples such as blood (plasma), oral swab, and fecal swab, and tissues of ileum, mesenteric lymph nodes and spleen were collected on the day of necropsy.

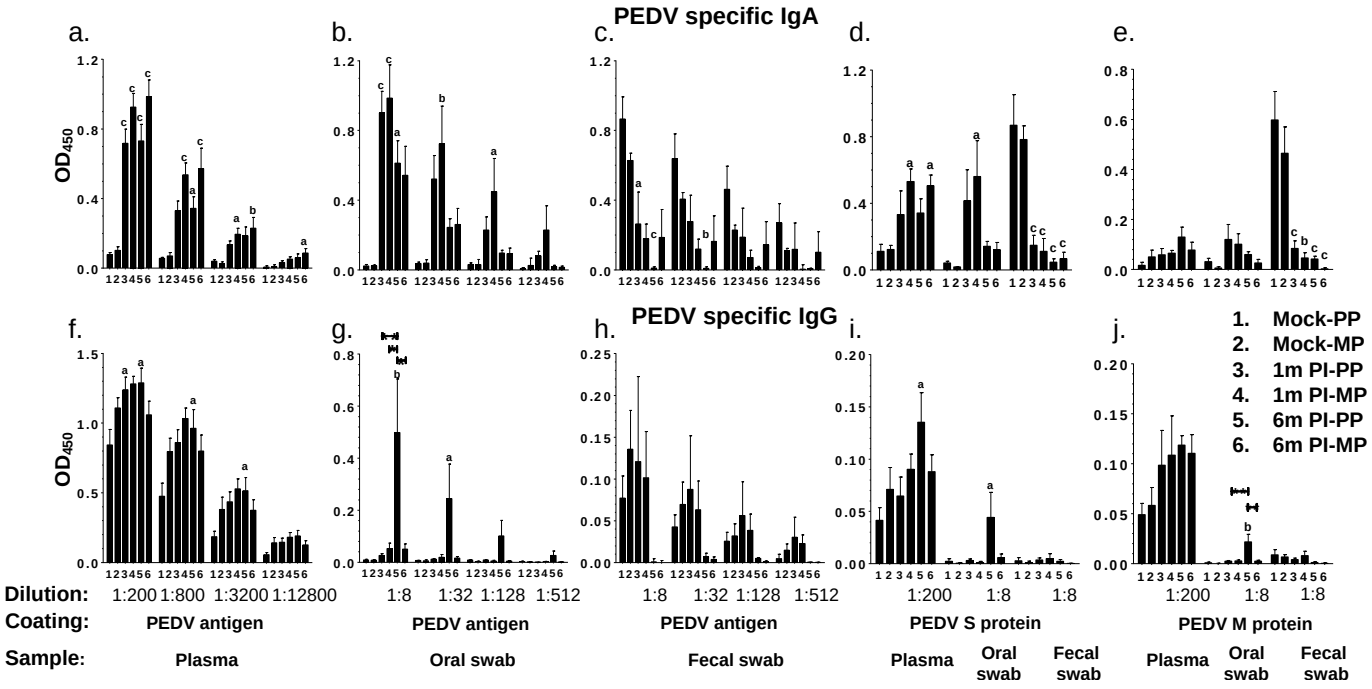

Fig. 1. Quantification of PEDV-specific IgA and IgG antibody titers in clinical samples of infected sows. Samples were collected from the uninfected and post PEDV infected sows as indicated in the Table 1. PEDV specific IgA (a-e) and IgG (f-j) antibody titers were quantified by ELISA, with plates coated either using PEDV antigen (a-c and f-h), S protein (d, i) or M protein (e, j); in plasma (a, f, d, e, i, j), oral swab (b, g, d, e, i, j) and fecal swab (c, h, d, e, i, j) samples. Each bar represents mean OD_{450} value ± SEM from 6 sows. Lowercase alphabet and asterisk indicates a statistically significant difference ('a' or * $P<0.05$, 'b' or ** $P<0.01$ and 'c' or * $P<0.001$) between mock-uninfected and PEDV-infected corresponding primiparous and multiparous sow groups or between the two indicated PEDV-infected sow groups.

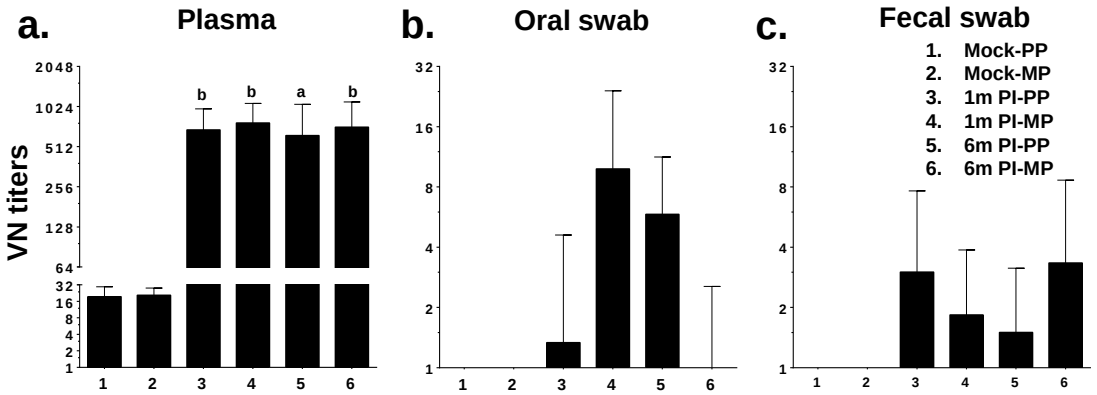

Fig. 2. Virus neutralizing antibody titers in PEDV infected sows. (a) Plasma, (b) oral swab and (c) fecal swab samples were analyzed for the VN titers against PEDV by immunofluorescence assay. Each bar represents mean VN titers ± SEM from 6 sows. Lower case alphabet indicates a statistically significant difference ('a' $P<0.05$ and 'b' $P<0.01$) between mock-uninfected and PEDV-infected corresponding primiparous and multiparous sow groups or between the two indicated PEDV-infected sow groups.

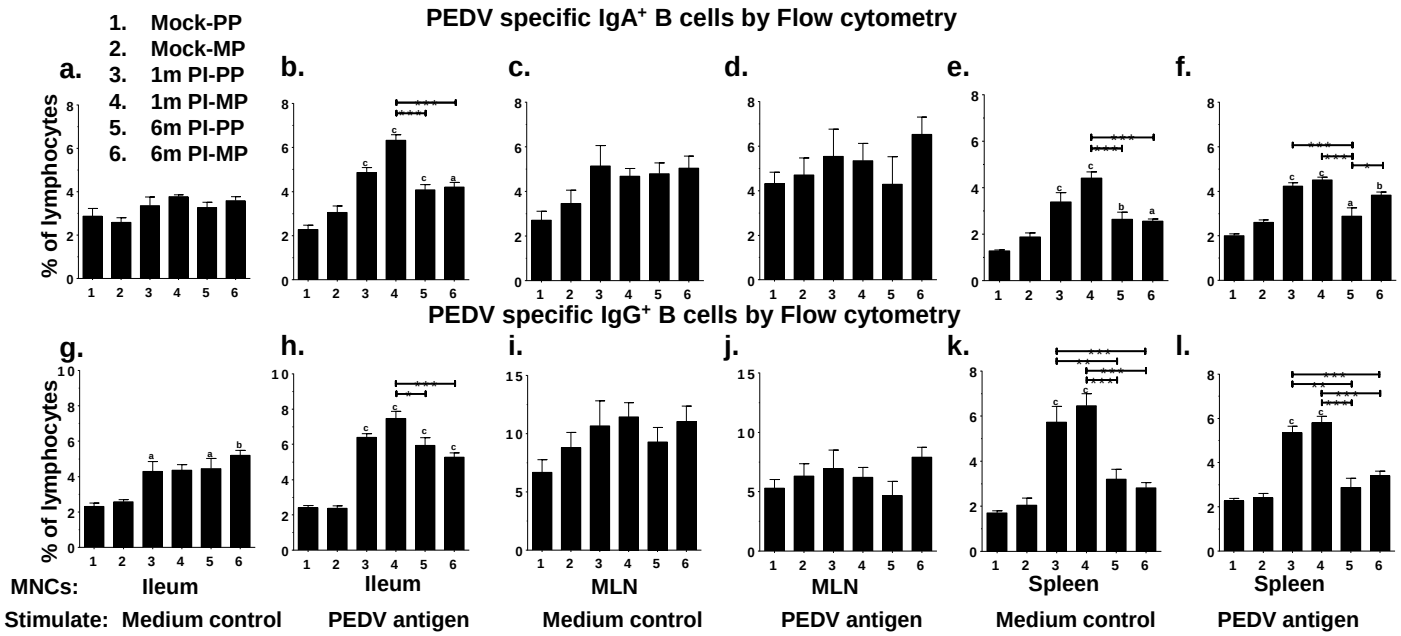

Fig. 3. Flow cytometry analysis to determine the frequency of IgA$^+$ and IgG$^+$ B cells in mucosal tissues of PEDV infected sows. Mononuclear cells were isolated from ileum (a, b, g, h), mesenteric lymph nodes (c, d, i, j) and spleen (e, f, k, l), and stimulated *ex vivo* with PEDV antigen (b, d, f, h, j, l) or medium control (a, c, e, g, i, k) for 6 days. The frequency of B cells positive for IgA (a-f) and IgG (g-l) antibodies were determined by flow cytometry. Each bar represents the mean of percent of lymphocytes positive for IgA or IgG positive B cells ± SEM from 6 sows. Lowercase alphabet and asterisk indicates a statistically significant difference ('a' or * $P<0.05$, 'b' or ** $P<0.01$ and 'c' or * $P<0.001$) between mock-uninfected and PEDV-infected corresponding primiparous and multiparous sow groups or between the two indicated PEDV-infected sow groups.

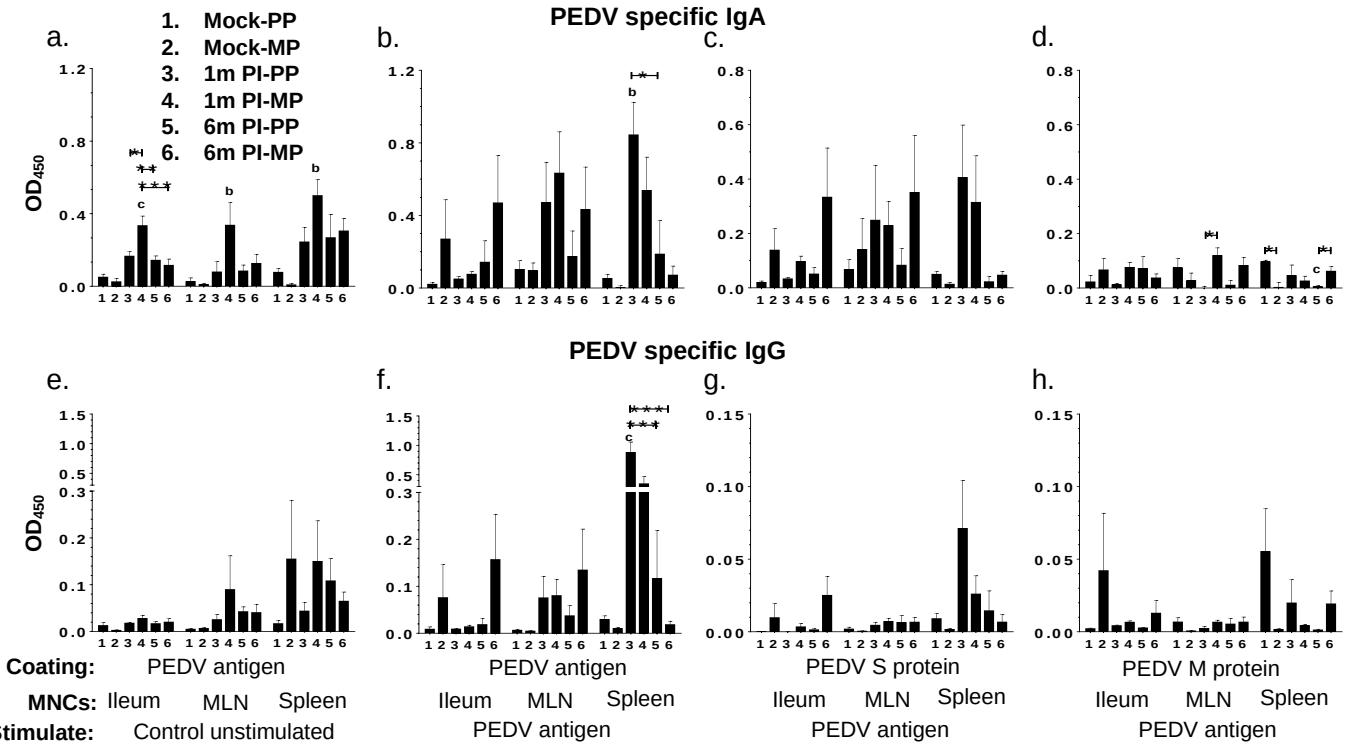

Fig. 4. Analysis of PEDV specific IgA and IgG antibodies secreted by stimulated MNCs of ileum, mesenteric lymph nodes and spleen. Culture supernatants were harvested on day 6 from MNCs stimulated *ex vivo* with PEDV antigen (b-d, f-h) or control unstimulated (a, e). PEDV specific IgA (a-d) and IgG (e-h) levels were determined by ELISA, with plates coated either with PEDV antigen (a-b and e-f), S protein (c, g) or M protein (d, h). Each bar represents mean OD_{450} value ± SEM from 6 sows. Lowercase alphabet and asterisk indicates a statistically significant difference ('a' or * $P<0.05$, 'b' or ** $P<0.01$ and 'c' or * $P<0.001$) between mock-uninfected and PEDV-infected corresponding primiparous and multiparous sow groups or between the two indicated PEDV-infected sow groups.

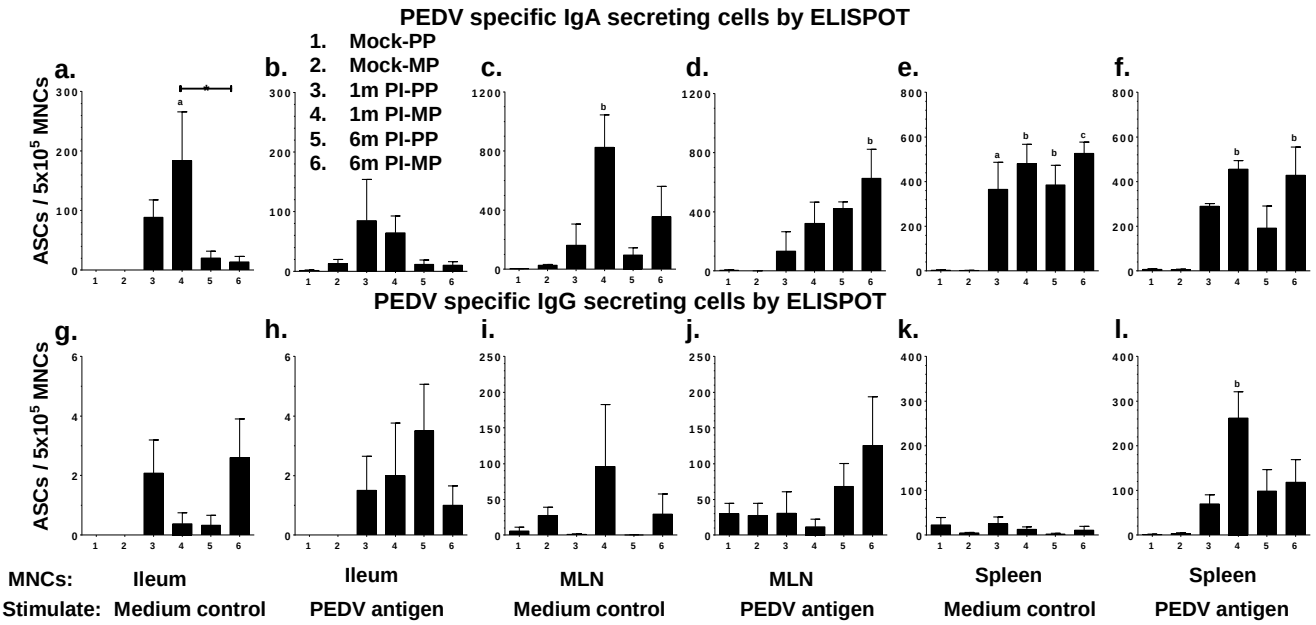

Fig. 5. ELISPOT analysis to quantify PEDV specific IgA and IgG antibody secreting cell population in ileum, mesenteric lymph nodes and spleen of PEDV infected sows. MNCs isolated from ileum (a, b, g, h), MLN (c, d, I, j) and spleen (e, f, k, l) were stimulated *ex vivo* with PEDV antigen (b, d, f, h, j, l) or medium control (a, c, e, g, i, k) for 6 days. PEDV specific IgA (a-f) and IgG (g-l) antibody secreting cells (ASCs) were detected by ELISPOT. Each bar represents mean number of PEDV-specific ASCs per 5×10^5 MNCs from 6 sows. Lowercase alphabet and asterisk indicates a statistically significant difference ('a' or * $P<0.05$, 'b' or ** $P<0.01$ and 'c' or * $P<0.001$) between mock-uninfected and PEDV-infected corresponding primiparous and multiparous sow groups or between the two indicated PEDV-infected sow groups.