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## Effect of route of inoculation on innate and adaptive immune responses to porcine epidemic diarrhea virus infection in suckling pigs

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### ABSTRACT

The effects of route of administration on systemic and gut mucosal immune responses induced by porcine epidemic diarrhea virus (PEDV) infection in suckling pigs were investigated. Twenty-four conventional 5-day-old suckling piglets were randomly divided into four groups and were inoculated orally, intranasally (I.N.), intramuscularly (I.M.) with PEDV or D MEM (mock). Pigs were monitored daily for clinical signs and fecal viral load. Blood samples were collected at 7, 14, 21 days post infection (dpi) and subjected for the analyses of serum antibody production, T cell and natural killer (NK) cell frequencies, NK cytotoxicity and serum cytokine levels. Oral inoculation led to higher levels of PEDV-specific IgA antibodies in both serum and gut mucosal sites than did other routes of inoculation. Intranasal inoculation elicited significantly higher titers of virus-specific IgG antibodies in serum. PEDV-infected pigs regardless of inoculation routes had significantly lower NK cell frequencies than those of the control pigs at 14 dpi. The orally inoculated pigs had significantly higher CD3 + CD8 + T cell frequencies as compared to I.N. or I.M. inoculated pigs at 14 dpi, while there was no significant difference among orally, I.N. or I.M. inoculated pigs and control pigs in CD3 + CD4 + T cell frequencies in peripheral blood. PEDV-infected and control pigs had low, but detectable NK cell activities at 14 and 21 dpi, however, NK cell activities were barely detectable at 7 dpi whether the pigs were infected or not. Serum IL-10 levels were induced drastically in orally infected pigs at 7 dpi and then gradually declined. Serum IL-12 levels followed a similar pattern while the fold-change was much lower. In conclusion, oral inoculation may generate more comprehensive immune responses.

### 1. Introduction

Porcine epidemic diarrhea virus (PEDV) is an enveloped, positive single-stranded RNA virus belonging to the family *Coronaviridae* (Song et al., 2015). It is the causative agent of porcine epidemic diarrhea (PED), which is characterized by severe enteritis, diarrhea, vomiting, dehydration, and high mortality rates especially among suckling piglets (Langel et al., 2016).

The disease was first recognized in Europe in 1971 (Pensaert and Martelli, 2016). PEDV was first identified in the 1980s in China (Xuan et al., 1984). Beginning in October 2010, a large-scale outbreak of PED caused by a highly pathogenic PEDV variant emerged in China (Sun et al., 2012). In May 2013, a highly virulent PEDV variant emerged in

the United States and has spread nationally thereafter (Stevenson et al., 2013). The disease has caused tremendous economic losses.

The development of an infectious disease involves complex interactions between the agent and the host (Fink and Campbell, 2018). Host defense against viral infection is mediated by the effector mechanisms of innate and adaptive immunity. Innate immunity is the initial response to prevent, control, or eliminate viral infection and stimulates and influences the types of adaptive immune responses that develop (Akira et al., 2006). Natural killer (NK) cells, the first and best described innate lymphoid cells, play important roles in innate immune responses. The effector functions of NK cells are to kill infected cells and to produce interferon gamma (IFN- $\gamma$ ). NK cells may also be important later in the course of viral infection by killing infected cells that have

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escaped cytotoxic T lymphocyte (CTL)-mediated immune attack by reducing expression of class I MHC molecules (Lam and Lanier, 2017). Adaptive immunity against viral infection is mediated by antibodies, which block virus binding and entry into host cells, and by CD8+ CTLs, which eliminate the infection by killing infected cells (Lu et al., 2018). The functions of CD4+ effector T cells are to recruit and activate phagocytes that destroy intracellular viruses and to help B lymphocytes to produce antibodies (Pepper and Jenkins, 2011).

The information on immune responses of suckling pigs to PEDV infection by different routes of infection is limited. In this study, we investigated PEDV-specific antibody production, T cell frequencies, NK cell frequencies and cytotoxicity and cytokine profiles of piglets inoculated orally, intranasally or intramuscularly with PEDV.

## 2. Materials and methods

### 2.1. Virus, cell lines and antibodies

The PEDV strain ZJ15XS0101 (GenBank accession No. KX550281.1) was isolated from clinically diseased pigs in Zhejiang, China. The cell line Vero E6 was cultured at 37 °C and 5% CO<sub>2</sub> in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12, Gibco, Grand Island, NY, USA) supplemented with 10% newborn calf serum (MHBIO, China), 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin. The virus was serially propagated for 120 passages and titrated in Vero E6 cells. Virus stocks were stored at –80 °C. Mouse monoclonal anti-S1 antibody 6G1 and anti-N antibody 1E4 were raised in our laboratory.

### 2.2. Immunofluorescence assay

At 16 h post infection (hpi), PEDV-infected and mock-infected Vero cells were fixed with 80% acetone, air dried and incubated with 1000 × diluted mouse monoclonal anti-S1 antibody 6G1 for 1 h at 37 °C, washed twice with PBS, followed by using 2000 × dilution of fluorescein-labeled donkey anti-mouse IgG (A31570, ThermoFisher Scientific, Marina, CA, USA) for 1 h at 37 °C protected from the light. Cell nuclei were counterstained with DAPI (1 µg/ml). Cell staining was examined on the inverted fluorescence microscope X81 (Olympus, Tokyo, Japan).

### 2.3. One-step growth curve

Confluent Vero E6 cells were washed with PBS three times and then infected with the 16th-, 35th-, or 120th-passaged ZJ15XS0101 at a MOI of 0.01, culture supernatant and cells were harvested at 12, 24, 48, 72, 96, and 120 hpi. The viral titration was determined according to the Reed and Muench method and expressed as 50% tissue culture infective dose (TCID<sub>50</sub>/ml).

### 2.4. PCR amplification and sequencing of the Spike (S) gene

Full-length S gene cDNAs were amplified from the parent, the 16th-, 35th-, and 120th-passaged ZJ15XS0101 by RT-PCR using the HiScript® II 1st Strand cDNA Synthesis Kit (Vazyme, China) and the Phanta Max Master Mix (Vazyme) with primers (data not shown). The resultant amplicons were excised from 1.0% agarose gels and purified using E.Z.N.A.® Gel Extraction Kit (Omega, Norcross, GA, USA) and cloned into the pEASY-Blunt cloning vector (Transgen, China) and sequenced by GENEWIZ (Suzhou, China).

### 2.5. Animal experiment

The study was carried out according to the guidelines of the Animal Care and Use Committee of Zhejiang University (Approval No. 2018011028). PEDV and TGEV-seronegative 5-day-old suckling piglets were obtained from a farm which had no outbreaks or vaccination of

PEDV and/or TGEV, and all sows that gave birth to the experimental piglets were serologically negative for PEDV or TGEV antibodies. Suckling pigs were exposed to 1 ml dose of 10<sup>6.5</sup> TCID<sub>50</sub>/ml PEDV ZJ15XS0101 P120 inoculum orally (n = 6), intranasally (I.N., n = 6), or by intramuscular inoculation (I.M., n = 6). The control pigs (n = 6) received DMEM orally. Each group was raised in individual sterile isolators, respectively. Pigs were monitored daily for clinical signs of the disease such as diarrhea, vomiting and anorexia until necropsy. Appearance and consistency of feces were recorded and assigned to a score as follows: 0 = normal; 1 = mild fluidic feces; 2 = moderate watery feces; 3 = severe watery and projectile diarrhea, with scores of 1 or more considered diarrheic. One pig was randomly selected from each group and sacrificed at 7 and 14 dpi for post-mortem examinations. At the end of the experiment (21 dpi), all pigs were euthanized for pathological examination. On day 0, 1, 2, 3, 4, 5, 7, 10, 12, and 14, rectal swabs were collected and assayed for viral load. On day 7, 14 and 21, peripheral blood lymphocytes (PBL) were isolated for flow cytometric analyses and NK cytotoxicity assays; blood samples were taken and serum were separated and aliquoted for cytokine assays as described below.

To assess the pathogenicity of PEDV ZJ15XS0101, viral stock at 16 passages were tested in 3-day-old piglets. Fifteen piglets were randomly allocated to three groups. Each group was maintained in a separate room. From 10<sup>4.0</sup> TCID<sub>50</sub>/ml to 10<sup>2.0</sup> TCID<sub>50</sub>/ml of PEDV P16 were inoculated into piglets through the oral route. The inoculated animals were observed for clinical symptoms of diarrhea and mortality for 10 days. The remaining piglets were euthanized at the end of the study.

### 2.6. Detection of PEDV antigen by immunohistochemistry (IHC) in the small intestines

At necropsy, jejunum tissue specimens were collected from each piglet and fixed with 4% paraformaldehyde for 24 h at 4 °C and embedded in paraffin. The paraformalin-fixed paraffin-embedded tissues were cut in 5 µm thick sections on a microtome. The sectioned tissues were then deparaffinized in xylene for 5 min and washed in decreasing concentrations of ethanol (100%, 95%, 90%, 80%, and 70%) for 3 min each. The deparaffinized intestinal tissue sections were subjected to immunohistochemistry (IHC) using 100 × diluted PEDV N-specific mAb. Briefly, the paraffin-embedded tissue sections were deparaffinized, treated with 0.01 M citrate buffer (pH 6.0) in a microwave oven for 5 min, chilled at room temperature (RT) for 20 min, and then incubated with 0.3% hydrogen peroxide in distilled water for 20 min to block endogenous peroxidase. The sections were washed three times in PBS and then incubated for 1 h at RT with N-specific mAb (100 × diluted). After rinsing in PBS, the samples were reacted for 45 min at RT with biotinylated goat anti-mouse secondary antibody (Abcam, Cambridge, UK) and then incubated with avidin-biotin peroxidase complex (VECTASTAIN ABC Kit, Vector Laboratories, Burlingame, CA, USA) for 45 min at RT, and developed using the DAB Substrate Kit (Vector Laboratories) according to the manufacturer's instructions. The slides were then counterstained with hematoxylin, dehydrated, cleared with xylene, and mounted on microscope glass slides in mounting buffer, and tissue staining was visualized by a microscope.

### 2.7. Quantification of viral RNA by real-time RT-PCR

One rectal swab from each pig was suspended in 1 ml PBS and centrifuged at 3000 rpm for 15 min. The viral RNA was extracted from 200 µl of the supernatant using a RNA extraction kit (Biotek, China). Viral RNA level was quantified via real-time RT-PCR in qPCR machine (BIO-RAD iQ<sup>™</sup>5, Berkeley, CA, USA) as follows: reverse transcription at 50 °C for 10 min and initial denaturation at 95 °C for 30 s, followed by 45 cycles (5 s at 95 °C and 40 s at 60 °C) with a final cooling at 40 °C for 30 s. Results were expressed as the mean of the logarithmic viral RNA copies per µl.

Primers and probe were:

PEDV-F: 5'-CGCAAAGACTGAACCCACTAACCT-3',

PEDV-R: 5'-TTGCCTCTGTTGTTACTTGGAGAT-3',

Probe: FAM-TGTTGCCATTACCACGACTCCTGC-TAMRA.

## 2.8. Antibody ELISA

IgG and IgA antibodies specific for PEDV were detected by indirect ELISA. Briefly, 96-well microplates were coated with purified S1 diluted in 100 mM carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C, after which the coating solution was discarded. The plates were blocked with 5% skim milk in 10 mM PBS containing 0.1% Tween-20 (PBST) and incubated at 37 °C for 30 min. The blocking buffer was then discarded. Serum samples were diluted 1:400 with PBST and 2% FBS (diluent buffer). Rectal swabs were suspended in 1 ml PBS supplemented with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA), and centrifuged at 3000 rpm for 15 min to yield supernatant. The contents of small intestine were scraped from 10 cm scissored small intestine and then macerated in 5 ml PBS supplemented with protease inhibitor cocktail, followed by centrifuging at 5000 rpm for 20 min at 4 °C. Diluted samples of serum, feces or small intestine tissue (100 µl per well) were added to the plates and incubated at 37 °C for 1 h. After incubation, the plates were washed three times with PBST, and horseradish peroxidase-conjugated goat anti-pig IgA (Novus, Littleton, CO, USA) or IgG (Proteintech, Rosemont, IL, USA) in diluent buffer was added at 100 µl per well and incubated at 37 °C for 30 min. The plates were washed three times with PBST and then incubated for 10 min with 100 µl per well of TMB solution (Sigma-Aldrich). The absorbance was measured at 450 nm after the addition of 50 µL stop solution (2 M H<sub>2</sub>SO<sub>4</sub>).

## 2.9. Isolation of peripheral blood lymphocytes

Porcine peripheral blood lymphocytes (PBL) were separated from EDTA-K2-treated peripheral blood by density gradient centrifugation (P8770, Solarbio, China) at 500 × g for 20 min, then PBL were washed and re-suspended in RPMI-1640 (Gibco) containing 10% heat-inactivated fetal bovine serum (Gibco).

## 2.10. T cell and NK cell frequencies

To determine the frequencies of T helper cells (CD3+CD4+), cytotoxic T cells (CD3+CD8+) and NK cells (CD3-CD4-CD8+), 100 µl of cell suspension in PBS (1.5 × 10<sup>6</sup> cells/ml, i.e., containing ≈ 150,000 cells) were stained with 1 µg FITC mouse anti-pig CD3<sub>ε</sub> (clone: BB23-8E6-8C8, BD Biosciences, San Jose, CA, USA), 1 µg PE mouse anti-pig CD4a (clone: 74-12-4, BD Biosciences), 1 µg Alexa Fluor 647 mouse anti-pig CD8a (clone: 76-2-11, BD Biosciences) and 0.1 µg 7-Amino-Actinomycin D (7-AAD, 559925, BD Biosciences) for 15 min in dark at 4 °C. 7-AAD was used for the exclusion of nonviable cells in flow cytometric assays. After incubation, the cells were washed with cold PBS three times at 400 × g for 5 min, suspended in 500 µl of ice cold PBS and subjected to flow cytometry. Generally, 1 × 10<sup>4</sup> gated events were counted for each sample. The frequencies of T cells or NK cells were expressed as percentage of lymphocytes expressing the respective markers. Cells stained with isotype antibodies were used as controls. The gating strategy is shown in Supplementary Fig. 1.

## 2.11. NK cytotoxicity assays

The ability of NK cells (effectors) to kill K562 target cells was analyzed using Pierce lactate dehydrogenase (LDH) cytotoxicity assay kit according to the manufacturer's instructions (88954, ThermoFisher Scientific). Various numbers of effector cells and a constant number of target cells (20,000 cells/100 µl media as previously optimized) were mixed at the ratios of 25:1, 12.5:1 and 6.25:1. Each effector: target cell

ratio was performed in triplicate. The plates were incubated at 37 °C, 5% CO<sub>2</sub> overnight. At the end of incubation, the plates were centrifuged at 250 × g for 3 min and 50 µl supernatant were then transferred to a 96-well flat bottom plate. Subsequently, 50 µl of the reaction mixture were added and incubated for 30 min at room temperature protected from light. The absorbance at 490 nm and 680 nm were recorded after the addition of 50 µl stop solution. To determine LDH activity, the 680 nm absorbance value (background signal from instrument) were subtracted from the 490 nm absorbance value. The cytotoxicity was calculated using the following equation with the corrected values: [(experimental value – effector cells spontaneous control – target cells spontaneous control)/(target cell maximum control – target cells spontaneous control)] × 100.

## 2.12. Cytokine assays

Serum IFN- $\gamma$ , TNF- $\alpha$ , IL-8, IL-10, IL-12, IL-17 A levels were analyzed using commercially available swine ELISA kits per manufacturer's recommendations (KSC4022, KSC3012, KSC0082, KSC0102, ESIL12 A, ESIL17 A, ThermoFisher Scientific).

## 2.13. Statistical analysis

All values are expressed as the means  $\pm$  standard error of the means (SEM). NK cell activity, NK cell numbers, T cell numbers and cytokine amounts were analyzed by one-way ANOVA using GraphPad Prism software.

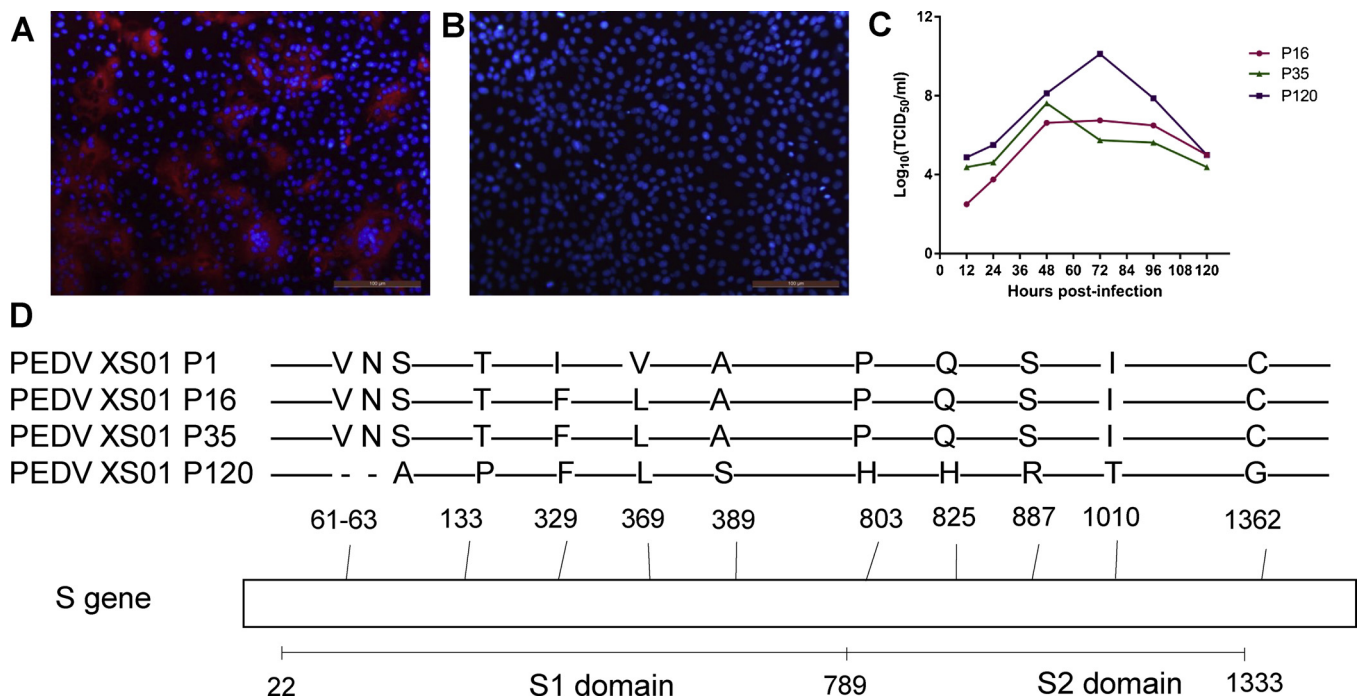
## 3. Results

### 3.1. PEDV ZJ15XS0101 strain at P120 replicated to a higher titer in cell culture

The PEDV strain ZJ15XS0101 was isolated from the intestine of a 1-day-old piglet. The virus isolate was serially passed in Vero E6 cells for 120 passages. PEDV-infected Vero E6 monolayer were fused and detached within 20 h, as compared to mock-infected cells. The PEDV S1 protein was distributed in the cytoplasm as shown by IFA (Fig. 1A). The one-step growth curves showed that the 120th generation of PEDVs (P120) replicated to a higher titer in Vero E6 cells than the earlier passages. The viral titers of P120, P35, and P16 peaked at 10.125 log<sub>10</sub> TCID<sub>50</sub>/ml at 72 hpi, 7.125 log<sub>10</sub> TCID<sub>50</sub>/ml at 48 hpi, 6.125 log<sub>10</sub> TCID<sub>50</sub>/ml at 48 hpi, respectively (Fig. 1C). The results indicate that P120 were culture adapted as compared with the earlier passages.

We analyzed the changes in the spike (S) gene sequence of the virus. The S gene of the parent, the 16th- and 35th-passaged virus consisted of 4,161 nucleotides encoding 1,386 amino acids, whereas the 120th-passaged virus had a deletion of 6 nucleotides at position 182-187 that resulted in Val<sub>61</sub> and Asn<sub>62</sub> deletions. Compared to the S gene of the parent virus, the 16th-, 35th-, and 120th-passaged virus had 2, 2, and 15 nucleotide changes, respectively. These mutations were predicted to change 2, 2, and 10 amino acids in the S protein of the 16th-, 35th-, and 120th-passaged virus, respectively (Table 1 and Fig. 1D). All the mutations were nonsynonymous, implying a positive selection of the mutations in the S gene of ZJ15XS0101 during the consecutive passages in Vero E6 cells.

The pathogenicity of PEDV P16 was tested in 3-day-old piglets. Three groups of 5 piglets were inoculated orally with PEDV P16 from 10<sup>4</sup> TCID<sub>50</sub>/ml to 10<sup>2</sup> TCID<sub>50</sub>/ml. The animals were observed for clinical signs of diarrhea and mortality for 10 days. All piglets developed symptoms of watery diarrhea in 24–48 h. In high and medium dose groups, 5/5 and 3/5 piglets died within 96 h. All piglets in low dose group survived till the end of the experiment (Table 2).



**Fig. 1.** Immunofluorescence assay of PEDV isolate in infected Vero E6 cells. Vero E6 cells were infected with PEDV ZJ15XS0101 isolate or mock-infected, cells were examined by IFA using PEDV-S1-specific monoclonal antibody at 16 h post infection (A and B, scale bar, 100 μm). C: one-step growth curve of P120, P35 and P16 in Vero E6 cells. Cells were infected with P120, P35 or P16 at a MOI of 0.01, culture supernatant and cells were harvested at 12, 24, 48, 72, 96, and 120 hpi. The viral titration was determined according to the Reed and Muench method and expressed as TCID<sub>50</sub>/ml. D: Schematic representation of mutations in the S protein of the parent, 16th-, 35th-, and 120th-passaged ZJ15XS0101.

**Table 1**  
Lengths and mutations in nucleotides and amino acids of the S gene of ZJ15XS0101 strain.

	Virus			
	P1	P16	P35	P120
Nucleotides	4161	4161	4161	4155
Amino acids	1386	1386	1386	1384
Nucleotide mutations	-	2	2	15
Synonymous substitutions	-	0	0	0
Nonsynonymous substitutions	-	2	2	10

**Table 2**  
Pathogenicity analysis of the 16th-passaged ZJ15XS0101 in 3-day-old piglets.

Virus	Ages (days)	No. of pigs	Inoculation		Diarrhea and No. of death
			Doses <sup>a</sup> (TCID <sub>50</sub> /ml)	route	
The 16th passage	3	5	10 <sup>4</sup>	oral	5/5, 5/5
			10 <sup>3</sup>	oral	5/5, 3/5
			10 <sup>2</sup>	oral	5/5, 0/5

<sup>a</sup> 1ml.

**3.2. Orally inoculated pigs had earlier onset of diarrhea and higher fecal PEDV RNA titers**

None of the control pigs developed diarrhea during the experiment. In orally inoculated group, one pig had diarrhea lasting from day 1 to day 3. Another two pigs had diarrhea at day 1 and day 2, respectively. All pigs exposed to PEDV intranasally had diarrhea at 2 and 3 dpi. Diarrhea was the most severe in I.M. group, starting from day 1 and lasting for 4 days. Since day 5, no diarrhea was observed in all PEDV-inoculated groups (Fig. 2A).

Fecal PEDV RNA titer peaked at 3 dpi in orally inoculated piglets,

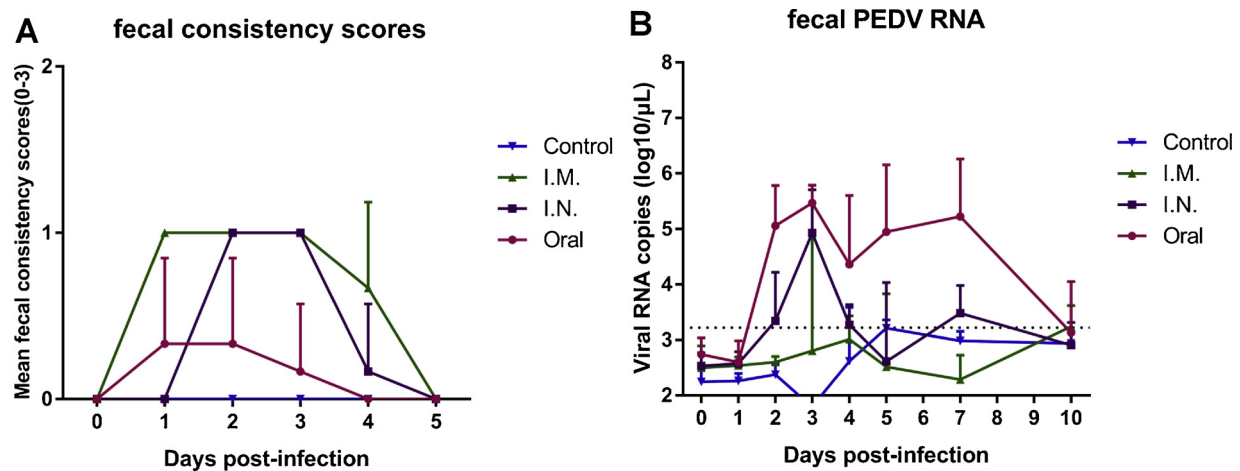
two days after the onset of diarrhea. Among the I.N. inoculated piglets, titer of PEDV RNA fecal shedding also reached the peak at 3 dpi, one day after the onset of clinical signs. The highest titer of PEDV RNA fecal shedding in orally inoculated group was about 10<sup>5.5</sup> copies/μl and gradually decreased over time. PEDV RNA was undetected in the rectal swab of orally inoculated piglets at 10 dpi. The highest titer of PEDV RNA fecal shedding of I.N. inoculated piglets was slightly lower than that of the orally inoculated group, and virus RNA shedding stopped at 4 dpi. No fecal virus shedding was found in I.M. inoculated groups. The DMEM inoculated control pigs had no detectable PEDV RNA in feces (Fig. 2B).

**3.3. No gross lesions were visible in PEDV-infected pigs regardless of inoculation routes**

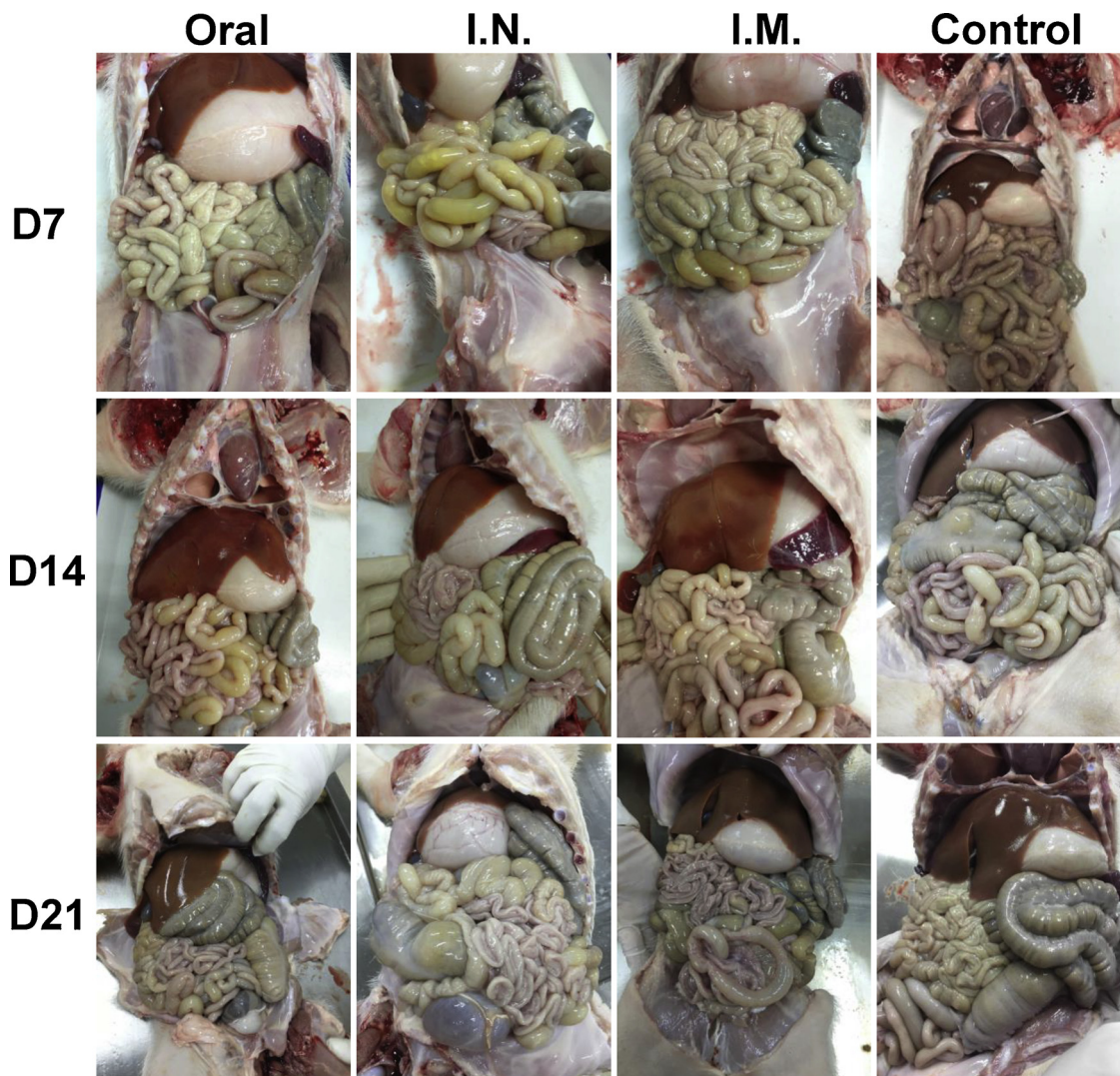
There were no observable changes in the small intestines and the internal organs of PEDV-inoculated pigs by all three routes as compared to the control pigs (Fig. 3). Immunohistochemistry staining against N protein showed that virus antigen was present in orally inoculated pigs only at 7 dpi. In I.N. or I.M. inoculated groups, antigen was observed at day 7 and 14. No virus antigen was present in the small intestines in any pigs in the control group (Fig. 4).

**3.4. Orally inoculated pigs had the highest serum, fecal and jejunal IgA titers**

Among the antibody titers of serum samples from the suckling pigs at 21 dpi, the orally inoculated group had the highest serum IgA titer (Fig. 5A) as compared to that of the I.N., I.M., or control groups. The IgA titers in sera from both the I.N. and I.M. groups were not statistically significant from the control group at all time points. Fecal IgA production followed a similar trend (Fig. 5B). At 21 dpi, all the experimental and control pigs were sacrificed and jejunal content were collected and subjected for IgA detection. The optical density (OD) value of jejunal IgA in orally inoculated group was markedly higher



**Fig. 2.** A: Fecal consistency score. After PEDV inoculation, piglets were monitored for clinical signs every day until necropsy. Diarrhea was assessed by scoring fecal consistency. Appearance and consistency of feces were recorded and assigned to a score as follows: 0 = normal; 1 = mild fluidic feces; 2 = moderate watery feces; 3 = severe watery and projectile diarrhea, with scores of 1 or more considered diarrheic. B: Significantly higher fecal viral shedding in orally inoculated piglets. Fecal shedding PEDV RNA titers were determined by qRT-PCR. The detection limit of qRT-PCR was 3.3 log<sub>10</sub> RNA copies per μL of rectal swab fluid. Data are reported as mean ± SEM.



**Fig. 3.** Pathological examination. Small (duodenum, jejunum, and ileum) and large (cecum and colon) intestinal tissues and other major organs were examined grossly at 7, 14, and 21 dpi.

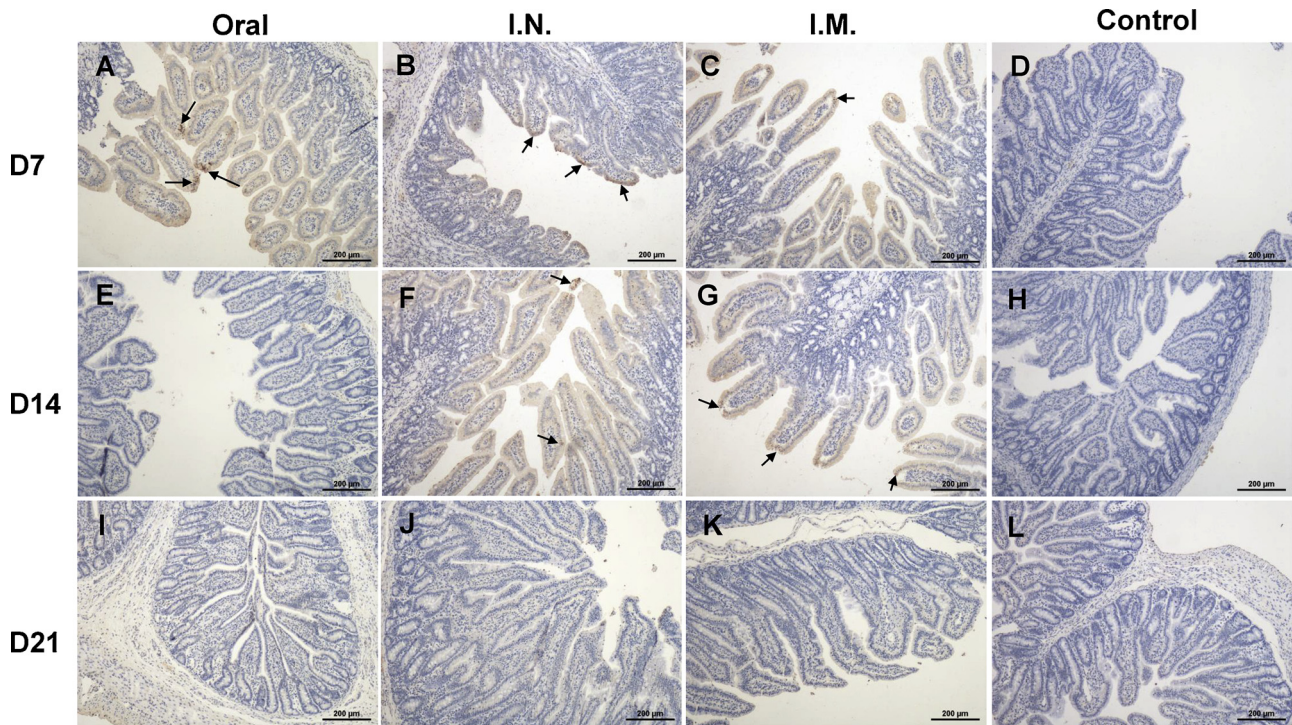


Fig. 4. Immunohistochemistry for the detection of PEDV antigen in the intestine of PEDV-inoculated pigs by different routes and control pigs at indicated time points. The arrow indicates PEDV antigen-positive cells.

than that of the I.N., I.M., or control groups. No major differences in jejunal IgA titer were observed among piglets from I.N., I.M., or control groups (Fig. 5C). In regard to serum IgG titers, the results showed that I.N. group had a higher OD value of serum IgG than that of the control group at 7, 14, 21 dpi. The serum IgG levels of orally or I.M. inoculated group had no significant difference from the control group at 7 and 14

dpi. At 21 dpi, three groups of different inoculation routes had comparable levels of serum IgG, which were significantly higher than that of the control group (Fig. 5D).

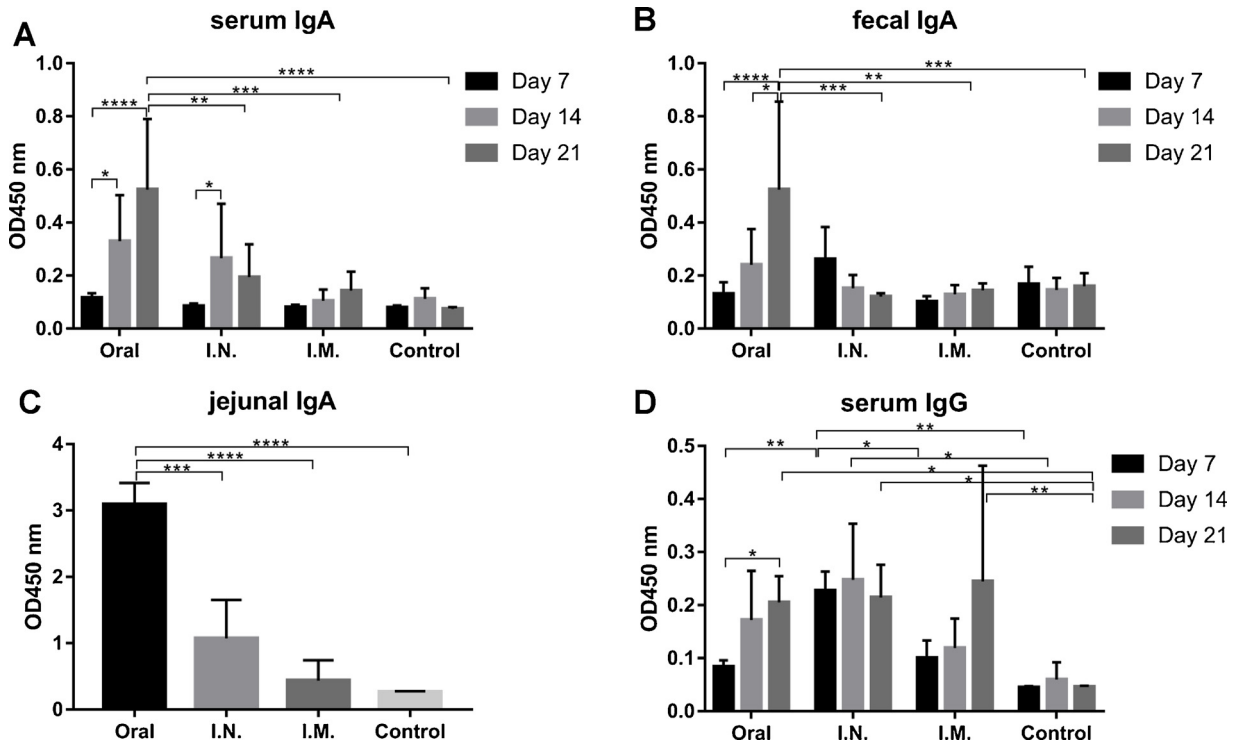
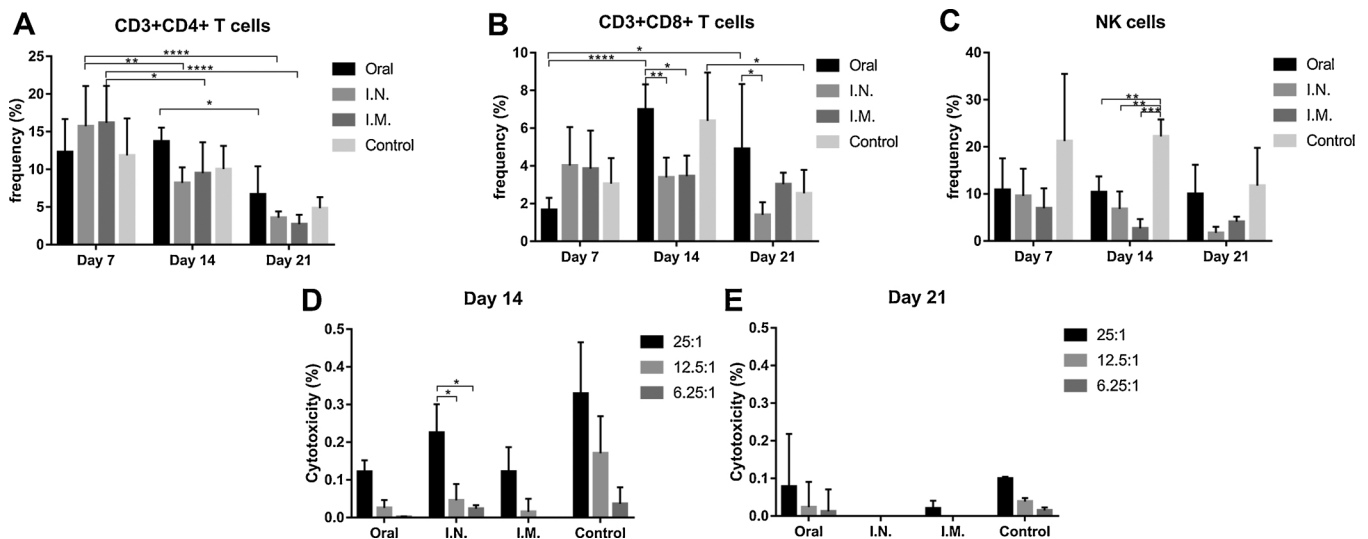


Fig. 5. PEDV-S1-specific serum IgA (A), fecal IgA (B), jejunal IgA (C), and serum IgG (D) antibody in treatment groups and control group were determined by ELISA. Data are reported as mean  $\pm$  SEM (\* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001, and \*\*\*\* $P$  < 0.0001).



**Fig. 6.** CD3+CD4+ (A) and CD3+CD8+ (B) T cell and NK cell (C) frequencies as well as NK cytotoxicity (E and F) of PEDV-inoculated pigs via oral, intranasal, or intramuscular route or control pigs at indicated time points. Porcine peripheral blood lymphocytes were stained with anti-pig CD3 $\epsilon$ , CD4 $\alpha$  and CD8 $\alpha$  for 15 min in dark at 4 °C and then subjected to flow cytometry. The ability of NK cells to kill K562 target cells was analyzed using Pierce lactate dehydrogenase (LDH) cytotoxicity assay kit. Data are reported as mean  $\pm$  SEM (\* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001, and \*\*\*\* $P$  < 0.0001).

### 3.5. CD3+CD8+ T cell frequencies were higher in orally inoculated pigs than in I.N. or I.M. inoculated pigs at 14 dpi

There was no difference between orally, I.N. or I.M. inoculated pigs and control pigs in CD3+CD4+ T cell frequencies in blood at day 7, 14 and 21. The CD3+CD4+ T cell frequencies were lower at 21 dpi as compared to 14 dpi in the blood of orally inoculated pigs. The I.N. or I.M. infected pigs had significantly reduced CD3+CD4+ T cell frequencies at 14 and 21 dpi as compared to 7 dpi (Fig. 6A).

The CD3+CD8+ T cell frequencies were significantly elevated in orally inoculated pigs at 14 and 21 dpi as compared to 7 dpi. There was no difference between orally, I.N. or I.M. inoculated and control pigs in CD3+CD8+ T cell frequencies in blood at day 7. The orally inoculated pigs had significantly higher CD3+CD8+ T cell frequencies as compared to I.N. or I.M. inoculated pigs at 14 dpi (Fig. 6B).

### 3.6. PEDV-infected pigs had lower NK cytotoxicity compared to control pigs

The infected pigs regardless of inoculation routes had similar NK cell frequencies compared to the control pigs at 7 and 21 dpi. The NK cell frequencies of orally, I.N., or I.M. inoculated pigs were lower than those of the control pigs at day 14 (Fig. 6C).

The piglets had no detectable NK cell activity in blood in neither PEDV infection groups nor control group at 7 dpi (data not shown). At 14 dpi, the control group had low but detectable NK cell activities, the piglets infected by three routes had lower NK cell activities as compared to the control group with no statistical difference (Fig. 6D). The NK cell activity of control piglets was decreased at 21 dpi as compared to 14 dpi. The piglets infected by oral inoculation had reduced NK cell activity at 21 dpi. The NK cell activities of I.N. and I.M. group were barely detectable (Fig. 6E).

### 3.7. Serum IL-12 and IL-10 levels were significantly elevated in orally inoculated pigs

Serum IL-12 levels were markedly induced in orally infected piglets at 7 dpi which declined thereafter. For the I.N. or I.M. inoculated groups, serum IL-12 levels were similar to those of the control group. Serum IL-12 levels of orally infected suckling pigs were the highest compared to the I.N., I.M., or control groups at all time points (Fig. 7A). Serum IL-10 levels followed a similar trend to those of IL-12. Serum IL-

10 levels in orally infected piglets were the highest at 7 dpi and gradually declined at 14 and 21 dpi. Serum IL-10 levels of orally inoculated piglets were about 89-fold higher than those of the control piglets at 7 dpi. There were no significant differences in serum IL-10 levels of I.N. or I.M. inoculated piglets compared to the control group at all time points (Fig. 7B).

Serum IL-17 levels were significantly higher in orally infected suckling pigs compared to the control group at 7 dpi. At 21 dpi, I.M. inoculated pigs had significantly higher serum IL-17 level compared to the control group (Fig. 7C). Serum IL-8 levels were elevated in I.N. infected piglets as compared to orally or I.M. infected piglets at 21 dpi. All the treatment and control piglets had similar IL-8 levels at all time points (Fig. 7D). Orally inoculated piglets had slightly higher levels of serum IFN- $\gamma$  than those of I.N. or I.M. inoculated piglets at 7 dpi. There were no differences in serum IFN- $\gamma$  levels among any groups at 14 and 21 dpi (Fig. 7E). The serum TNF- $\alpha$  levels were similar between the treatment groups and control group at all time points (Fig. 7F).

## 4. Discussion

The aim of the study is to investigate the immune responses to porcine epidemic diarrhea virus infection in suckling pigs by different routes. Porcine epidemic diarrhea is an acute viral enteric infection that results in high mortality and morbidity in suckling pigs (Jung and Saif, 2015). The fecal-oral route is the main route of PEDV transmission (Crawford et al., 2015), and airborne transmission is also considered a potential route for PEDV dissemination (Alonso et al., 2014; Li et al., 2018). Therefore, induction of localized immune responses at these mucosal sites is important in preventing disease (Neutra and Kozlowski, 2006). Several studies have shown that the degree of protection against various viral infections correlates better with the level of secretory than with serum antibodies (Gould et al., 2017; Habibi et al., 2015). Intramuscular injection is the most common route used in vaccination. However, this route usually does not induce immune response in the external secretions that bathe the mucosal membranes, the most common sites of entry of infectious agents. A more effective route that results in a generalized immune response manifested by the parallel appearance of specific antibodies in several external secretions, involves ingestion or inhalation of antigens.

The PEDV strain used in the study was continually propagated in Vero E6 cells up to 120 passages. The cytopathogenic effect is clear. It



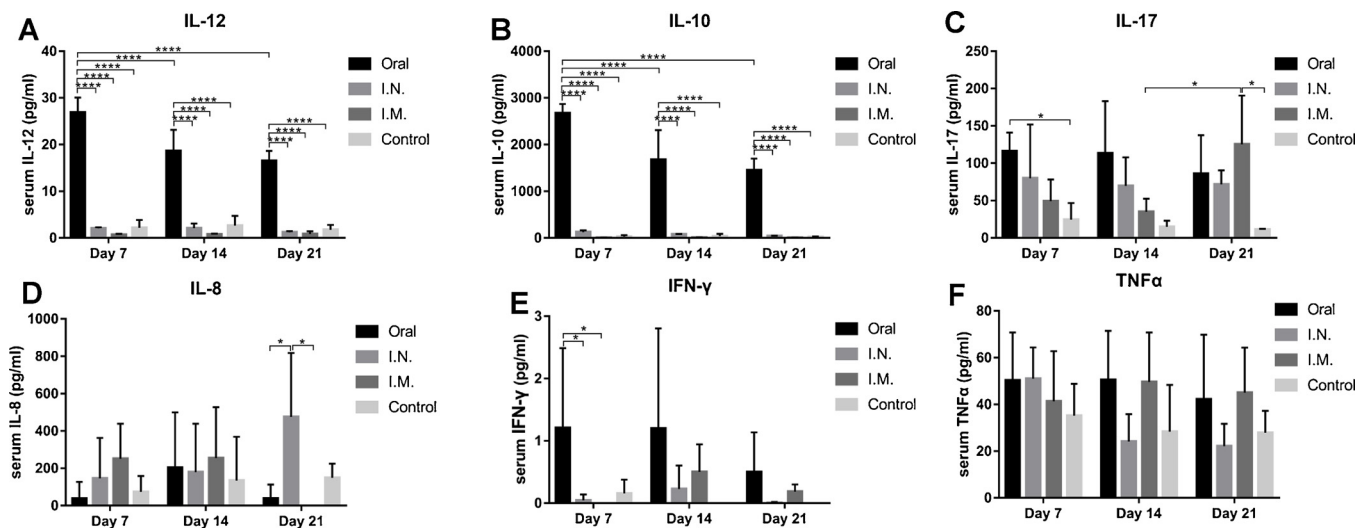


Fig. 7. Serum cytokine profiles of PEDV-inoculated pigs via oral, intranasal, or intramuscular route or control pigs at 7, 14 and 21 dpi. Blood samples were collected at indicated time points and separated sera were frozen at  $-20^{\circ}\text{C}$  until assayed. The assay of IL-12 (A), IL-10 (B), IL-17 (C), IL-8 (D), IFN- $\gamma$  (E), and TNF $\alpha$  (F) was performed by using commercial kits. Data are reported as mean  $\pm$  SEM (\* $P < 0.05$  and \*\*\*\* $P < 0.0001$ ).

has been shown that a serial passage of PEDV and other coronaviruses often results in growth adaptation *in vitro* and attenuation of virus virulence (Chen et al., 2015; Huo et al., 2016; Kweon et al., 1999). In our study, the 120th passage virus were cell-culture adapted compared to the earlier passages, and the viral titers of the 16th, 35th and 120th passage virus were continuously increasing. The nucleotide and amino acid changes of P120 may contribute to the cell culture adaption of the strain. In the virulence experiment, the 16th-passaged virus caused watery diarrhea and high mortality rate in pigs, while the P120-infected pigs showed only mild diarrhea and recovered in a few days. There were no gross lesions evident in any PEDV-inoculated pigs. These results indicated that the 120th-passaged virus was attenuated. In orally inoculated piglets, three piglets showed mild signs of diarrhea in two or three days after inoculation, and the signs seemed to be transient. The diarrhea coincided with the viral load in the feces, and orally infected pigs continued to shed virus for the next few days. All intranasally exposed piglets shed mild fluidic feces starting at 2 dpi, the syndrome lasted for 2 days and all piglets recovered in the next 2–3 days. The viral load peaked at 3 dpi and became undetectable in I.N. group. We observed about one day delay in viral shedding and onset of diarrhea in I.N. inoculated group as compared with the orally inoculated group. This is in general agreement with a recent paper (Li et al., 2018), which showed that PEDV could cause typical diarrhea in piglets through a nasal spray and illustrated the mechanism of its transport from the entry site via systemic route by T cells carrying the viral pathogen to the intestines where the infection occurs. In their model, the intranasally inoculated piglets began to exhibit typical PEDV symptoms at 60 hpi, about 14 h later than orally inoculated piglets. They detected viral RNA in the duodenum, jejunum and ileum at 24 hpi, but not before (3 or 12 hpi) in intranasally inoculated piglets. This delay represents the time required by the host cells to transfer the virus to the intestines through the blood circulation. In intramuscularly infected group, however, the diarrhea lasted for 4 days. It is interesting that fecal viral RNA copies were undetectable, so the diarrhea may partly be attributed to the stress caused by injection.

It is reported that mucosal immunization routes vary in their ability to induce serum antibodies (Kozłowski et al., 1997). In our study, intranasal inoculation induced serum IgG antibodies earlier and higher than oral inoculation, while, serum IgA antibodies were induced markedly when inoculated orally. Generally, the specific antibody levels induced by mucosal immunization does not reach the magnitude of those induced by intramuscular injection, however, our results showed

the levels of serum IgG antibodies induced by intranasal inoculation were comparable with those of intramuscular injection and were even slightly higher during the first two weeks. Almost no serum IgA antibodies against PEDV were detected in intramuscularly inoculated pigs.

Following oral inoculation, an increase in serum IgA antibodies was observed almost concomitantly with the appearance of fecal IgA antibodies. In contrast, almost no IgA antibodies against PEDV were detected in serum and feces after intramuscular inoculation. Nasal immunization in humans results in antibody responses in the upper airway mucosa and regional secretions (saliva, nasal secretions) without evoking an immune response in the gut (Holmgren and Czerkinsky, 2005). In our study, IgA antibodies in feces and jejunum were almost undetected in intranasally inoculated pigs. In accordance with the profiles of antibodies secreted in serum and feces, the jejunal contents of orally inoculated pigs showed remarkable increased IgA antibodies at 21 dpi. It has been reported that IgA presented in the intestine is related to the protection against gastroenteric infections (Blutt and Conner, 2013; Blutt et al., 2012). IgA and IgG antibodies in gut associated lymphoid tissues and blood were positively correlated with protection against PEDV challenge 21 day after the first infection (De Arriba et al., 2002). These results indicate that the immunization route affects the dominant distribution of specific antibodies in sera and secretions.

Cytokines play an important part in selecting the isotypes of antibody induced during the immune response. IL-10 is produced by many immune cell populations of the innate and adaptive immune system, including activated macrophages and dendritic cells, regulatory T cells, Th1, Th2 and Th17 cells (Saraiva and O'garra, 2010). Our results showed that serum IL-10 levels surged in orally inoculated pigs at 7 dpi and decreased at 14 and 21 dpi, but still were significantly higher as compared to those of the I.M., I.N., or control groups. In humans, transforming growth factor (TGF)- $\beta$  and IL-10 in concert with IL-4 have been shown to promote B-cell switch to IgA and differentiation into IgA-producing cells (Asano et al., 2004). Therefore, the high levels of serum IL-10 may partly contribute to increased IgA production in orally inoculated pigs.

Cytokine patterns elicited by infection are critical in the regulation of the adaptive immune response and may define the outcome of the infection (Ouyang et al., 2011). IL-10 is an inhibitor of activated macrophages and dendritic cells and is thus involved in control of innate immune reactions and cell-mediated immunity. IL-10 can inhibit the production of many cytokines including interferon gamma (IFN- $\gamma$ ), tumor necrosis factor alpha (TNF- $\alpha$ ) and IL-12. IL-12 is secreted by

dendritic cells and macrophages and stimulates IFN- $\gamma$  production by NK cells and T cells, enhances NK cell and CTL-mediated cytotoxicity (Saraiva and O'garra, 2010). Both serum IL-10 and IL-12 levels were elevated in orally inoculated pig and the pattern of production was similar, peaking at 7dpi and declining thereafter. The fold-change of IL-10 level was drastic. A study showed that IL-10 secretion did not change in both CV-777 infected immature and mature dendritic cells *in vitro*, while IL-12 and IFN- $\gamma$  secretions were significantly induced (Gao et al., 2015). The discrepancy may be due to the different strains and experimental conditions. Given the fact that IL-10 can decrease anti-viral associated IFN- $\gamma$  production, it is possible that the secretion of IL-10 could have substantially diminished the ability of the cells spontaneously secreting IFN- $\gamma$  to mediate protective immunity against the virus.

IL-17 is an important link between T cell-mediated adaptive immunity and the acute inflammatory response (Khader et al., 2009). IL-17 induces neutrophil-rich inflammation and stimulates the production of antimicrobial substances. Serum IL-17 levels were the highest in orally infected pigs, followed by I.N. and I.M. inoculated groups during the first two weeks. Serum levels of IL-8, a neutrophil chemotactic factor, were almost unchanged whether the pigs were infected or not. The serum levels of IL-8, IFN- $\gamma$ , and TNF $\alpha$  varied widely among individuals in the same treatment group. The huge standard deviation bars were due to the individual differences. Future studies with a larger sample size are warranted to further explore the effects on cytokine profiles of PEDV-infected piglets by different inoculation routes.

Porcine NK cells are defined as CD3-CD4-CD8a + cells (Gerner et al., 2009). Suckling pigs had no detectable NK cell activity in blood PBLs and the activity did not change upon PEDV infection with any routes at 7 dpi. These results corroborated the previous study showing that the PEDV-infected suckling pigs had no detectable NK cell activity in blood mononuclear cells at 1 and 5 dpi (Annamalai et al., 2015). At 14 dpi, control pigs showed detectable activity of NK cells in blood PBLs. Orally, I.N., or I.M. inoculated suckling pigs had lower NK cell activities compared to the control group with no statistical difference despite NK cell frequencies were significantly reduced.

CD4 + T lymphocytes are critical for phagocyte-mediated elimination of microbes, whereas CD8 + T effector cells are responsible for the eradication of viruses that infect and replicate inside all cells (Germain, 2002). There was no difference between orally, I.N. or I.M. inoculated pigs and control pigs in CD3 + CD4 + T cell frequencies in blood at day 7, 14 and 21. The CD3 + CD8 + T cell frequencies were significantly elevated in orally inoculated pigs at 14 and 21 dpi as compared to 7 dpi, while I.N. or I.M. inoculated pigs showed no difference in CD3 + CD8 + T cell frequencies. These results indicated that oral infection may induce CD8 + T cell response.

In summary, neonatal pigs are deficient in the cytotoxicity function of NK cells and PEDV infection further inhibits NK cytotoxicity. Serum IL-10 and IL-12 levels increased markedly in orally inoculated pigs. Mucosal and systemic inoculation routes affect the dominant distribution of specific antibodies in sera and secretions, with the oral inoculation route being the most effective in inducing IgA both in serum and mucosal sites.

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## Conflicts of interest

The authors declare no conflict of interest.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.vetmic.2018.11.019>.

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