RESEARCH REPORT
SWINE HEALTH

Title: Pathogenesis of the S-INDEL PEDV in nursing piglets and cross-protection against the US original highly virulent PEDV strains - **NPB #14-265**

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Industry Summary:
Although the original US porcine epidemic diarrhea virus (PEDV) has been studied and known to cause mortality in suckling piglets, strains with a different genetic profile have not been studied. To discover whether the spike-insertion deletion (S-INDEL) PEDV strains have similar morbidity and mortality, we inoculated 3-4 day-old conventional suckling piglets with virus. Six litters of age-matched piglets were inoculated with either S-INDEL Iowa106 (4 litters), or original US PEDV PC21A (1 litter, positive control) or medium (1 litter, negative control). After 21-29 days, we challenged the piglets with original US PEDV PC21A to assess cross-protection. After the first virus inoculation, all piglets that received the virus developed diarrhea. The severity of the disease and mortality among the four S-INDEL Iowa106-inoculated litters varied greatly. Compared to original PC21A, piglets euthanized/died from S-INDEL Iowa106 infection had relatively milder disease and more limited intestinal infection. Two of four S-INDEL Iowa106-infected sows and the original PC21A-infected sow showed anorexia and watery diarrhea for 1-4 days. Our results suggest that the disease from S-INDEL PEDV Iowa106 strain was less than from the original US PEDV PC21A strain in suckling pigs, with 100% morbidity and 18% (6/33) overall (0 to 75%) mortality, depending on viral dose. We found that PEDV disease in suckling piglets depends on factors such as the sow's health and lactation and the piglets' birth weight. Prior infection by S-INDEL Iowa106 did provide partial cross-protection to piglets inoculated with the original PEDV PC21A strain.

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Scientific Abstract
Although the original US porcine epidemic diarrhea virus (PEDV) was confirmed as highly virulent by multiple studies, the virulence of spike-insertion deletion (S-INDEL) PEDV strains is undefined. In this study, 3-4 day-old conventional suckling piglets were inoculated with S-INDEL PEDV Iowa106 (4 litters) to study its virulence. Two litters of age-matched piglets were inoculated with either original US PEDV PC21A or mock as positive and negative controls, respectively. Subsequently, all pigs were challenged with original US PEDV PC21A on 21-29 post-inoculation-day (PID) to assess cross-protection. All S-INDEL Iowa106- and original US PC21A-inoculated piglets developed diarrhea. However, the severity of clinical signs, mortality (0-75%) and fecal PEDV RNA shedding titers varied among the four S-INDEL Iowa106-inoculated litters. Compared to original PC21A, piglets euthanized/died acutely from S-INDEL Iowa106 infection had relatively milder villous atrophy, lower antigen scores and more limited intestinal infection. Two of four S-INDEL Iowa106-infected sows and the original PC21A-infected sow showed anorexia and watery diarrhea for 1-4 days. After original PC21A challenge, a subset (13/16) of S-INDEL Iowa106-inoculated piglets developed diarrhea, whereas no pigs in the original PC21A-inoculated pigs had diarrhea. Our results suggest that the virulence of S-INDEL PEDV Iowa106 was less than the original US PEDV PC21A in suckling pigs, with 100% morbidity and 18% (6/33) overall (0 to 75%) mortality in suckling pigs depending on factors such as the sow's health and lactation and the piglets' birth weight. Prior infection by S-INDEL Iowa106 provided partial cross-protection to piglets against original PC21A challenge at 21-29 PID.

Introduction
Porcine epidemic diarrhea (PED) is a highly contagious swine enteric disease resembling transmissible gastroenteritis (TGE). It was first recognized among English feeder and fattening pigs in 1971 (1). Experimental inoculation with the Belgian isolate, the PED virus (PEDV) prototype CV777 strain, revealed that PEDV is enteropathogenic for both nursing and fattening pigs (2). Subsequently, the etiological agent of PED was identified as PEDV, belonging to the *Alphacoronavirus* genus within the *Coronaviridae* family. Before the end of 2010, endemic PED had been reported in many European and Asian countries, but with low impact. The subsequent PED pandemic outbreaks started in China (3) and spread to other Asian counties, inducing high piglet mortality (4). In April 2013, PED outbreaks occurred suddenly in US swine (5). Piglets up to 7 days old developed vomiting and diarrhea that led to dehydration, rapid weight loss, and death in 2 to 4 days, with mortality reaching 100% in suckling piglets (6). PEDV infection also impaired the performance of surviving pigs (7), resulting in significant economic losses to the US pork industry.

Sequence analysis of complete PEDV genomes revealed that original US PEDV strains were closest to one recent Chinese strain AH2012 (8). The high virulence of the original US PEDV strains was experimentally confirmed in gnotobiotic (Gn) piglets (9), cesarean-derived and colostrum-deprived (CDCD) piglets (10, 11) and conventional piglets (12). Also, compared to neonatal piglets, conventional 3-4 weeks old pigs were less susceptible to original US PEDV infection (12, 13). Concurrently, several US variant PEDV strains, characterized by insertions and deletions (INDELs) in the spike (S) gene, and designated as S-INDEL PEDV were found to be circulating in US swine farms (8, 14). When compared to three other original US PEDV strains (USA/IN/2013/19338P7, USA/NC/2013/35140P7, and USA/NC/2013/49469P7), 5-day-old non-suckling piglets inoculated with an S-INDEL PEDV strain (USA/IL/2014/20697) developed no clinical signs and mild histopathologic lesions (15). However, the virulence of the S-INDEL PEDV in pigs in the field varied. In one report, S-INDEL PEDV OH851 strain infected pigs showed minimal to no clinical signs in pigs in the field (14). However, recent reports described that European S-INDEL PEDV strains caused high mortality in suckling piglets in southern Germany (16) and southern Portugal (17). Sequence analysis of partial S1 gene revealed that the Portugal PEDV strain shared 99% and 100% nucleotide identities with US S-INDEL PEDV OH851 strain and the German strains (GER/L00719/2014 and GER/L00721/2014), respectively (17). Factors contributing to the contradictory clinical signs have not yet been clarified (18).

We recently reported that convalescent antisera obtained from S-INDEL-infected pigs cross-reacted with original US PEDV PC22A strain in two-way cell culture immunofluorescence and viral neutralization assays (19). Others reported that sows recovered from natural infection by an S-INDEL PEDV, 7 months prior to farrowing and orally boosted with an original US PEDV strain around day 109 of gestation provided

lactogenic immunity and partial protection to their piglets from a subsequent challenge by an original US PEDV (20). These *in vivo* and *in vitro* antigenicity and cross-protection studies suggest that S-INDEL PEDV strains may serve as vaccine candidates to protect pigs from highly virulent original US PEDV strains (19, 20), if the S-INDEL PEDV strains are confirmed as naturally attenuated strains. Our aims were to evaluate the pathogenicity of S-INDEL PEDV Iowa106 strain in conventional suckling piglets and to examine whether infection of the piglets with this S-INDEL PEDV strain induces cross protection against diarrhea caused by a subsequent (3-4 weeks later) challenge with the original US PEDV PC21A strain.

Objectives: To study the pathogenesis of S INDEL PEDV Iowa106 strain in nursing piglets and cross-protection to the highly virulent PEDV PC21A strain.

Materials and Methods

PEDV inoculum

Pig intestinal contents containing the S-INDEL PEDV Iowa106 (GenBank accession No. KJ645695) were collected from a pig during a mild diarrhea outbreak (8). The original sample tested negative for group A, B and C rotaviruses at the Veterinary Diagnostic Laboratory, University of Minnesota and TGEV/porcine respiratory coronavirus, porcine deltacoronavirus in our laboratory as described previously (9, 21). The virus was passed once in one conventional pig litter (litter A). The intestinal contents collected from the piglets at PID 3 were stored in aliquots at -80 ^{o}C and used to prepare inocula for the following 3 litters (litters B, C and D). The intestinal contents were suspended in cell culture grade phosphate buffered saline (PBS; pH 7.4; Sigma-Aldrich, St. Louis, MO) followed by vortexing and centrifugation at 2,095 ×g for 30 min. The supernatant was collected and diluted further or filtered through 0.22 μm-pore size filters before using as inocula. The original US PEDV PC21A was collected from the intestinal contents of a field 1-day-old diarrheic pig and passaged twice in Gn piglets (Jung et al., 2014). Based on prior experience, the viral infectious titers of cell culture adapted PEDV decreased about 1 log after a freeze-thaw cycle or filtration (unpublished data). Therefore, the doses of each inoculum were adjusted to the comparable titers of the 10 log_{10} genomic equivalents (GE) (frozen and thawed once, without filtration) according to the inoculum preparation process (**Table 1**). The PBS was used as mock control (litter F). In addition, no cross-contamination between original US PEDV PC21A and S-INDEL PEDV Iowa106 in each inoculum was confirmed by conventional differential RT-PCR for original US and S-INDEL strains (Liu and Wang, unpublished data).

Animals

Six 93 to 100-day, Large White × Duroc crossbred, pregnant sow (A) or gilts (B, C, D, E, F) were sourced from a specific pathogen free swine herd of The Ohio State University. The sows/gilts tested seronegative for PEDV by CCIF (19) and ELISA (Annamalai, Saif and Wang, unpublished). All sows/gilts arrived at least two weeks before farrowing for adaption to the facility. The sows/gilts farrowed naturally in our biosafety level-2 animal facility. Each pig litter (sow and her piglets) was housed in a separate room. All piglets were evaluated and were healthy at the day of inoculation.

Experimental design

All animal-related experimental protocols were approved by The Ohio State University Institutional Animal Care and Use Committee. Six conventional sows and their litters (litter A-F) were assigned randomly to three groups: 1) S-INDEL PEDV Iowa106 inoculation (litters A-D); 2) Original US PEDV PC21A inoculation (litter E); and 3) Mock inoculation (litter F). Neonatal suckling piglets were inoculated at 3-4-days of age. Piglets were observed 3 times daily for the first 7 post-inoculation days (PID) and twice daily thereafter until the end of the study. Clinical signs, including vomiting, diarrhea, anorexia and depression, were evaluated. Rectal swabs were collected and scored daily for the first 9 PIDs and every other day thereafter. Fecal consistency was scored as follows: 0, solid; 1, pasty; 2, semi-liquid; 3, liquid, respectively. The rectal temperatures and body weights were recorded daily for each piglet at 0 (pre-inoculation) to 7 PIDs and then weekly thereafter. Sows were considered as anorexic when their feed consumption was reduced ≧ 50%. If anorexia persisted for more than 2 days, the sows were medically treated with Flunixin meglumine (Banamine®, Merck; 10 ml, IM) and Pepto-bismol (P&G Everyday, 60 ml,

PO) by the clinician to improve their appetite.

One piglet in each litter was randomly selected and euthanized for histopathology evaluation at PID 3; others, unless they were moribund and fit early removal criteria, were retained to evaluate the duration of clinical signs, mortality and fecal viral shedding.

On the day before virulent original US PEDV PC21A challenge [post-challenge day (PCD) -1], one pig in each litter was euthanized to observe any histopathological lesions in the pigs recovered from the primary acute infection. At PID 21-29, all pigs were challenged with original US PEDV PC21A. The clinical parameters as described earlier were measured/recorded daily. All piglets were euthanized at PCD 7 / PID 28-36 for necropsy examination. The piglets were bled weekly to collect serum and serum and milk of sows were collected weekly for immunological examinations.

Gross and histopathological examination

At necropsy, both intestine and other major organs were examined. Duodenum (5 cm distal to the pylorus), jejunum (three samples taken at 40-60 cm intervals), ileum (5 cm anterior to the ileo-caecal valve), cecum, the middle segment of colon and mesenteric lymph nodes were collected. After 48 h fixation in 10% neutral buffered formalin, tissue sections were trimmed, processed, and embedded in paraffin. Four micron sections were cut and routinely stained with hematoxylin and eosin. For each jejunum section, at least ten villi and crypts were measured using a computerized image system with villous height and crypt depth (VH:CD) ratios calculated as previous described (9).

Immunohistochemistry (IHC) staining

The IHC staining was optimized as described previously (11, 22) using a non-biotin polymerized horseradish peroxidase system (BioGenex Laboratories, San Ramon, CA). The IHC signal of PEDV antigen was scored as 0–3 according to the percentage of villous enterocytes within the section showing a positive signal. Score 0 denotes no positive cells; scores 1 to 3 denote less than 30%, 30 to 60% and more than 60% of villous enterocytes showing a positive signal, respectively.

Analysis of PEDV RNA fecal shedding titers

Two rectal swabs were suspended in 4 ml Minimum Essential Media (Invitrogen, Carlsbad, CA, USA) as a 10% fecal suspension. The RNA was extracted from 50 µl of clarified (centrifugation at 2,095 × g for 30 min at 4 ºC) fecal suspensions using MagMax^{TM}-96 Viral Isolation kit (Ambion, Austin, TX) according to the manufacturer's instructions. The PEDV fecal shedding titers were determined by TaqMan real-time reverse transcription-PCR (RT-qPCR) with the primers and probe targeting the conserved nucleocapsid (N) protein region of PEDV as described previously (23). The detection limit was 10 GE per 20 µl of reaction, corresponding to 4.8 $\log_{10}$ GE per ml of original fecal samples.

Enzyme-linked immunosorbent assays (ELISA) for the detection of IgG and IgA antibodies

To evaluate levels of PEDV-specific IgA and IgG antibodies of pig serum and milk samples, IgA-ELISA and IgG-ELISA using PEDV PC22A strain were developed. The 96-well plates (Nunc-Immuno, Denmark) were coated with home-made guinea pig hyperimmune serum against PEDV in coating buffer (bicarbonate/carbonate buffer, pH 9.6). Afterwards, Vero cell cultured PEDV supernatants (PC22A strain, 7 log10 PFU/mL) (19) or mock Vero cell cultured supernatants were added as positive-coated and mock-coated wells. Serially diluted (4-fold) serum and milk samples were tested in both positive-coated and mock-coated wells. For the detection of IgA antibodies, horseradish peroxidase (HRP) conjugated anti-pig IgA (AbD Serotec, Raleigh, NC) was used before adding substrate [2, 2'-Azino-di (3-ethylbenzthiazoline-6-sulfonate) (ABTS, KPL, Gaithersburg, MD)]. For the detection of IgG antibodies, biotinylated anti-pig IgG (KPL, Gaithersburg, MD) followed by HRP-conjugated streptavidin (Roche, Nutley, NJ) was used before adding substrate. Absorbance was measured at 405 nm using a spectrometer (SpectraMax 340PC384, Sunnyvale, CA). The titer of a sample was defined as the reciprocal of the highest dilution giving positive results. Cut-off value was the mean of negative serum samples plus 3 times of standard deviation (M+3SD).

Plaque reduction virus neutralization (VN) assay

PEDV VN antibody titers in serum and milk samples were determined by a plaque reduction VN assay

in 6-well plates, which was developed based on the published protocol for transmissible gastroenteritis virus (2). The serum samples were serially diluted 4-fold starting at 1:8. Each serum dilution was mixed with an equal volume of PEDV PC22A virus (35 PFU per well) and incubated at 37°C for 90 min. Then 500 µl of virus-serum mixture/well was transferred to Vero cell monolayers in duplicate wells and incubated for 1 h at 37℃. After incubation, the virus-serum mixture was removed and the cell monolayers were washed twice. Afterwards, 3 ml of equal volume of 1.5% Seaplaque agarose (Lonza, Rockland, ME) and 2X MEM containing tryptose phosphate broth (0.04%), penicillin-streptomycin (200 units/mL penicillin, 200 µg/mL streptomycin) and trypsin (20 µg/mL) were overlaid. Once the agarose solidified, plates were covered, inverted and incubated at 37°C. The plates were stained on the 3rd day with 0.15% neutral red (Sigma, St.louis, MO) solution in PBS, incubated at 37°C, and plaques were counted. Virus only, mock and negative serum controls were included. The neutralizing antibody titer of a serum sample was defined as the reciprocal of the highest serum dilution that resulted in 80% reduction in plaque numbers as compared to the virus only control.

Statistical analysis

Comparison of piglets' rectal temperatures before and after inoculation was conducted by paired T test. The body weight, duration of diarrhea, fecal PEDV RNA shedding titers among litters, and antibody titers between groups were compared using one way analysis of variance (ANOVA) followed by Duncan's multiple range test. The continuous variables between group 1 (S-INDEL PEDV Iowa106 inoculation) and group 2 (US original PEDV PC21A inoculation) were compared by student's t test. To compare the weekly body weight gain among pig litters, analysis of covariance (ANCOVA) was applied to adjust initial body weights. In addition, correlations between continuous variables were calculated using Pearson correlation coefficients. Statistical analyses were done using SAS (Statistical Analysis System; SAS for windows 9.12; SAS Institute Inc., Cary, NC, USA). A *P* value of less than 0.05 was considered statistically significant.

Results

Clinical signs

The general information and the clinical signs for 6 pig litters are summarized in **Table 1**. After inoculation with S-INDEL PEDV Iowa106, all piglets developed watery diarrhea (RS = 3) within 3 days. Transient vomiting was noted in one and two piglets in litters B and C, respectively. However, the magnitude and duration of diarrhea differed significantly among these litters. Piglets in litters A and D showed watery diarrhea (RS = 3) for only 1-2 days and diarrhea (RS ≧ 2) subsided within 5 days. Piglets in litters B and C had longer duration (6.33 ± 0.57 and 5.78 ± 0.97 days, respectively) of diarrhea. In addition, for all the piglets, the duration of diarrhea correlated negatively with the piglet body weight measured at the day of inoculation (PID 0) ($r = -0.26$, $P < 0.01$). The most hypothermia and mortality were seen only in litter B: piglet body temperature dropped from 39.02 ± 0.36 ºC at PID 1 to 36.50 ± 0.84 ºC at PID 3 ($P < 0.01$); and 75% (6/8) of the piglets died or were moribund and were euthanized. In litter C, a slight decrease of body temperatures (about 1 ºC) was observed only on the day of onset of clinical signs (PID 2) and no piglets died. Piglets in both litters B and C had a decrease in mean body weight gain (-0.28 ± 0.16 kg and -0.1 ± 0.20 kg) between PID 0 and 7. The piglets in litter D gained 0.50 ± 0.30 kg between PID 0 and 7, which was significantly higher than those of litters B and C, but still lower than that of the mock-inoculated litter F (0.95 ± 0.16 kg). Sows B and C, but not sows A and D, had anorexia and watery diarrhea, lasting 2 and 4 days, respectively.

In original US PEDV PC21A-inoculated litter E, all piglets showed watery diarrhea within PID 1. Transient vomiting was also observed in two piglets. The body temperature of piglets dropped dramatically from 39.1 ± 0.2 ºC at PID 0 to 37.2 ± 0.9 ºC at PID 1 ($P < 0.01$). Mortality was 55% (6/11). The surviving piglets had diarrhea for 7.20 ± 0.45 days, which was significant longer than that in the S-INDEL-inoculated piglets (**Tables 1 and 3**). Compared to non-surviving piglets (n = 6), the surviving piglets (n = 5) had significantly higher body weight (1.84 ± 0.36 kg v.s. 1.36 ± 0.40 kg) at 4-days of age (PID 0) ($P < 0.05$). However, they did not gain body weight by PID 7. In addition, sow E had anorexia for 2 days and transient diarrhea for 5 days. No clinical signs were observed in piglets of the mock group (litter F).

Fecal PEDV RNA shedding profiles

Neither pre- nor mock-inoculated piglets (litter F) shed PEDV RNA in the feces. Among 4 S-INDEL PEDV Iowa106-inoculated litters, the first and also the highest peak titer of PEDV RNA fecal shedding was detected on the day of onset of clinical signs, at 1-3 PIDs (**Figure 1a**). The means of the highest fecal PEDV RNA shedding titers were all above 11 $\log_{10}$ GE/ml in all S-INDEL Iowa106-inoculated litters (A-D) (**Table 1**). Subsequently, the titer gradually decreased but increased again every 3 to 6 days. For example, piglet No.2 of litter B had relatively higher (12.8 and 10.8 $\log_{10}$ GE/ml) titers at 2 and 5 PIDs but lower titers (7.9 and 6.3 $\log_{10}$ GE/ml) at 4 and 8 PIDs (**Figure 1c**). Overall, continuous fecal PEDV RNA shedding beyond PID 21 was detected in a majority (21/26) of surviving piglets.

In litter E inoculated with original US PEDV PC21A, the highest fecal PEDV RNA shedding titer (11.80 ± 0.89 log GE/ml) was detected on PID 1 (**Figure 1a**). Subsequently, the titers gradually dropped to 7.09 ± 0.44 $\log_{10}$ GE/ml at PID 7 and were consistently maintained around 8.0-8.5 $\log_{10}$ GE/ml during 9 to 17 PIDs (**Figures 1a and d**).

In both S-INDEL Iowa106- and US original PC21A-inoculated litters, all sows were infected with PEDV by direct contact with their piglets (**Figure 1b**). Generally, higher titers (> 8 $\log_{10}$ GE/ml) were detected before PID 14. Thereafter, the titer dropped in the 4 S-INDEL Iowa106-infected sows (A-D). However, the original US PC21A-infected sow maintained higher fecal virus shedding titers (>8 $\log_{10}$ GE/ml) to PID 19. In addition, the intestinal contents obtained from euthanized piglets and RS obtained from sows of each litter were confirmed as the corresponding PEDV strains by conventional RT-PCR with PEDV strain-specific primers.

Gross lesions, histopathology and immunohistochemistry staining

During the acute stage of infection (PID 2 to 4), 10 S-INDEL PEDV Iowa106- and 4 original US PEDV PC21A-infected piglets were necropsied. The lesions incurred by S-INDEL Iowa106 and original US PC21A could not be distinguished by gross pathological examination. All piglets died/euthanized were emaciated with yellow feces coating the skin and hair. In some piglets, the intestinal lumens were filled with large amounts (approximately 50-70 mL) of yellowish foamy fluid. In other piglets, the walls of the small intestine were transparent and thin and the intestinal lumens were empty. No significant gross lesions were observed in other major organs (lung, kidney, liver and heart).

Microscopic examination revealed subacute, moderate to severe, extensive, atrophic enteritis in S-INDEL Iowa106-inoculated piglets. Shortening, blunting and fusion of the villi, and occasionally, vacuolization and exfoliation of enterocytes were noted. The VH:CD ratios in jejunum ranged between 2.00 ± 0.12 and 5.04 ± 0.58 (**Figures 2a, 3a**). IHC staining showed brown signal of PEDV N proteins were located in the cytoplasm of villous epithelial cells. Except for one piglet (No.2 of litter B) that had a PEDV antigen score of 3 in jejunum, all piglets (n = 10) had scores equal or below 2 in jejunum (**Figures 2a and 3a**) and ileum. No PEDV antigen was detected in the crypt cells. Sporadically, PEDV antigens (score = 1) were observed in duodenum in 50% (5/10) of S-INDEL Iowa106-infected piglets. Weak PEDV antigen signal was scattered in the colon of one piglet (No. 7 in litter B) (**Figure 3**).

Microscopic lesions and viral antigen distribution patterns observed in original US PEDV PC21A-infected piglets were more severe and extensive than those observed in S-INDEL Iowa106-inoculated piglets (**Figures 2b and 3b**). The VH:CD ratios ranged between 0.85 ± 0.36 to 1.98 ± 0.22 in 4 original PC21A-inoculated piglets (**Figures 2b, 2c and 3a**). The signal of original US PEDV antigen was mainly located in epithelial cells covering the villi and, occasionally, in some crypt cells (**Figure 2b**). The virus antigen scores of 3 in jejunum and 2 in ileum were observed in all piglets infected with the original US PEDV- (**Figure 2b**), with the exception of one piglet (No. 3 in litter E) that had clustered PEDV antigen signal in crypt cells, but less frequently in villous epithelial cells (**Figures 2c and 3b**).

No significant gross and microscopic lesions or PEDV IHC antigens were noted in pre-inoculation (PID 0) or mock-inoculated piglets (litter F). The VH:CD ratios were 6.88 ± 0.12 and 6.26 ± 0.34 in two piglets, respectively (**Figure 3**).

S-INDEL PEDV Iowa106 infection induced partial cross-protection in piglets against original US PEDV PC21A challenge

The clinical signs of pigs after challenge with original US PEDV PC21A are summarized in **Table 2**.

The PEDV naïve pigs (litter F) were challenged with 10 $\log_{10}$ GE/pig of original US PC21A at 29-days of age. However, no clinical signs were observed by 3 days after challenge, probably due to older pigs being less sensitive to PEDV as reported previously (12, 13, 24). Therefore, these piglets were challenged again with a 2-$\log_{10}$ higher dose (12 $\log_{10}$ GE/pig) at 32-days of age. Moderate to severe diarrhea, as well as the peaks of fecal PEDV RNA shedding (10.57 ± 0.81 $\log_{10}$ GE/ml) occurred at 2 or 3 post challenge days (PCD). Diarrhea lasted 3.80 ± 0.84 days and completely subsided by PCD 7 in all pigs.

Similarly, the dose of 10 $\log_{10}$ GE/pig of original US PEDV PC21A caused no disease in pigs recovered from S-INDEL PEDV Iowa106 infection (litter A). In litter B, only two piglets (Nos.8 and 9) completely recovered from S-INDEL Iowa106 infection. Under the same schedule and doses as the mock-inoculated litter (litter F), pigs of litter B were challenged twice at 29 and 32 day-old. No clinical signs, but a slight increase of PEDV fecal RNA titer (7.44 ± 0.10 $\log_{10}$ GE/mL), was noted after the second challenge, but not after the first. In another two S-INDEL Iowa106-inoculated litters (litter C, n =7; litter D, n = 7), pigs were challenged with 12 $\log_{10}$GE/pig of original US PC21A. Those pigs developed diarrhea, lasting 2.29 ± 1.60 (litter C) and 2.71 ± 1.11 (litter D) days, respectively. Their body temperatures did not change after challenge. The median fecal PEDV RNA shedding titers increased from 5.10 to 10.80 ± 0.76 $\log_{10}$ GE/ml in litter C and from < 4.80 (detection limit) to 9.61 ± 1.64 in litter D at PCD 1 to 2.

Pigs recovered from original US PEDV PC21A were challenged with 12 $\log_{10}$ GE/pig of homologous strain at 25 day-old (PID 21). No clinical signs were observed. The PEDV fecal RNA shedding titers increased slightly from 6.60 ± 0.50 (PCD 0/ PID 21) to 7.06 ± 0.82 $\log_{10}$ GE/mL during PCD 3-5/ PID 24-26.

After piglets were challenged with original US PC21A, only the PEDV naïve sow F showed clinical signs, including anorexia for 2 days and diarrhea for 4 days. Her highest fecal PEDV RNA shedding titer was 10.44 $\log_{10}$ GE/mL. No clinical signs and lower (< 8 $\log_{10}$ GE/mL) fecal PEDV RNA shedding titers were noted in the PEDV pre-exposed sows A-E.

All pigs were euthanized at PCD 7 or 9. No significant gross and microscopic lesions were observed. The VH:CD ratio ranged between 3.41 and 5.50 in jejunum and no significant differences were observed among litters. By IHC staining, PEDV N proteins were detected in individual mononuclear cells in intestinal submucosa/Peyer's patches and mesenteric lymph nodes in 75-100% of all pigs. On the other hand, only a few PEDV-positive epithelial cells at the villous tips (score = 1) were detected in the jejunum of pig No. 2 of litter E and in the ileum of pig No. 1 of litter F.

Piglet and sow serum and sow milk antibody responses.

The serum IgA and IgG antibody levels of original US PEDV PC21A-inoculated piglets were significantly higher than those of S-INDEL Iowa106-inoculated piglets on 7 and 21 PIDs, whereas the titers were similar between the groups on 14 PID and on 28-36 PID/7 PCD (Fig. 4). There was no statistical difference in serum VN antibody levels of piglets among the two groups. Both PEDV strains induced IgA and VN antibody responses in sow serum and sow milk. IgG antibody was detected from sow serum samples, but not from sow milk samples. No statistics analysis was done for sow samples because of the low numbers.

Discussions

The first US S-INDEL PEDV strain, OH851, was identified in conventional pigs with minimal clinical signs and no mortality (14). In the present study, the morbidity was consistently 100% in all S-INDEL PEDV Iowa106-inoculated piglets and 50% (2/4) in the contact exposed sows. High mortality (75%) was seen in one litter (litter B). The sows used in the present study were PEDV naïve until their piglets were inoculated. In the field observations, piglets in the farms may have been protected by PEDV-specific maternal antibodies since the sows were previously infected with S-INDEL PEDV OH851 strain (14). Therefore, the different results between natural and experimental infections were likely due to the presence or lack of lactogenic immunity. Compared to a current study with one US S-INDEL PEDV strain, USA/IL/2014/20697 (15), piglets in our study usually showed more pronounced clinical signs. Wild type or cell culture-adapted virus, doses of inoculum (11), environmental/animal conditions (18) and single nucleotide polymorphisms (SNPs) among US S-INDEL strains (8) could contribute to the variations observed among studies.

The S-INDEL PEDV strains that emerged in southern Germany showed high nucleotide identity (99.54 %) with US PEDV S-INDEL strains (16). These German S-INDEL PEDV strains resulted in large variations in mortality (67.7% and 5.5%) in different ages of suckling piglets in two farms (16). Most recently, outbreaks of S-INDEL PEDV in Portugal were reported, causing severe diarrhea and high mortality (17). Similarly, litter variations on the severity of PEDV infection were observed in our study, despite similar background of sows and environmental factors. Among the S-INDEL PEDV Iowa106-inoculated piglets, the body weight of piglets measured on the day of inoculation (PID 0) correlated negatively with the duration of diarrhea. In the original US PEDV PC21A-inoculated litter, five surviving piglets had significantly higher body weight at PID 0 than their non-surviving littermates. In an large scale swine farm surveillance, lower piglet birth weight and higher within-litter variability of birth weight were the factors associated with higher losses from birth to weaning (25). During PEDV infection, it is likely that the stronger piglets obtained more milk than their smaller littermates and were more likely to survive until intestinal villi regenerated and immunity developed. In a gnotobiotic mouse model, neonatal mice with better nutritional condition and higher body weight had higher enterocyte proliferation activity, more intensive response to probiotics and shorter duration of rotavirus-induced diarrhea (26). In the present study, milk of sows provided the only food source for the piglets. Two of four sows of S-INDEL PEDV Iowa106-inoculation group showed diarrhea and anorexia, whereas the other two were asymptomatic. Since the sows' health condition has a direct impact on the amount and quality of colostrum/milk (18, 25), it is critical to the infection outcome of piglets. Based on our results, the severity of PED was associated with virus strain, piglet birth weight and sow health/lactation status. The impact of other factors, such as pig genetic background and gut microflora, requires further investigation. Previous histopathology studies demonstrated that a high percentage of villous epithelial cells in the small intestine was infected and destroyed by virulent PEDV strains shortly after clinical signs appeared (2, 5, 9, 27). In both prototype PEDV CV777- and US original PEDV US/Iowa/18984/2013A-inoculated CDCD piglets, PEDV antigen-positive enterocytes decreased from PID1-2 and then increased at PID 3-4 (2, 10). In agreement with the above results, the first and also the highest peak of PEDV RNA fecal shedding titer was detected on the day of onset of clinical signs in both PEDV S-INDEL Iowa106- and US original PC21A-inoculated litters. Afterward the titers of fecal PEDV RNA shedding decreased rapidly and then rebounded (**Figure 1**). Interestingly, the intervals (3 to 6 days) between fecal PEDV RNA shedding peaks were compatible with the reported typical replacement time of small intestinal villous epithelium in suckling piglets (28). Since the replication of PEDV is sustained in enterocytes, this observation provided indirect evidence that both S-INDEL PEDV Iowa106 and original US PEDV PC21A severely damaged the infected enterocytes and may spread to infect regenerating new enterocytes.

In the present study, inoculation of original US PEDV PC21A to one piglet litter (n = 11) reproduced the results as described in our (9, 12, 29) and others' (5, 10, 13) studies. Although piglet infection by S-INDEL PEDV Iowa106 also caused severe clinical signs in two litters (litter B and C), generally, the virulence of S-INDEL PEDV Iowa106 was lower than that of original US PEDV strains as evident by: a longer incubation time, a shorter duration of diarrhea, relatively higher VH:CD ratios, a lower percentage of PEDV-positive enterocytes, more limited regions of virus infection (crypt not involved) and overall lower piglet mortality (18% vs 55%) **(Table 3)**. The infection of S-INDEL is less severe than original US PEDV strains but varies, depending upon whether the sow also becomes ill with reduced milk production. These findings suggest that the replication kinetics of S-INDEL PEDV Iowa106 in piglets may be slower than that of original US PEDV. Decreased pathogenicity of S-INDEL PEDV Iowa106 allowed time for the damaged intestinal villi to be repopulated with new enterocytes and facilitated survival of infected piglets.

In agreement with previous studies (2, 5, 10, 29), the original US PEDV infection was not restricted to epithelial cells covering the villi but, less frequently, also spread to some epithelial cells lining the crypts (**Figure 2b**). In one piglet, infection by original US PEDV PC21A was restricted to an individual crypt, but not observed in the adjacent villous epithelial cells (**Figure 2c**). Factors affecting the cell tropism of PEDV are still an important topic and need to be investigated in the future.

It is clearly established that the severity of PEDV infection is highly dependent on the age of pigs (12, 24). In the present study, one litter of mock-inoculated piglets was challenged with original US PEDV PC21A (10 and 12 $\log_{10}$ GE/pig) at 29- and 32-days of age, respectively. However, only the repeated challenge with a 2 $\log_{10}$-higher dose induced mild clinical signs. In contrast, in the 3-day-old piglets (litter

E), a lower dose (10 $\log_{10}$ GE/pig) of original US PEDV PC21A could cause severe clinical signs. The age-dependent resistance to PED (12, 24) was also confirmed in this study. A longer time for replacement of villous epithelial cells (12, 28), a higher abundance of viral receptor expression (30) and the immature innate immune system in piglets less than 1-week of age have been proposed to explain the fatal disease in young piglets.

Current *in vitro* and *in vivo* studies suggest antigenic cross reactivity between original US and S-INDEL PEDV strains (19, 20). However, vaccines based on European and historic PEDV strains failed to control the more recent virulent PEDV outbreaks in Asia (31), suggesting the possibility of antigenic variation among different PEDV strains (18, 19). In the present study, a high dose (12 $\log_{10}$ GE/pig) of original US PEDV challenge induced clinical signs in two S-INDEL PEDV Iowa106-inoculated pig litters (litter C and D), but not in the homologous strain-inoculated pig litter (litter E). [Two litters in the S-INDEL PEDV Iowa106 inoculation group, either challenged with lower dose of original US PEDV PC21A (litter A) or at another older age (litter B), were excluded.] Our results suggest that the immunity induced by S-INDEL PEDV Iowa106 infection only partially protected pigs from original US PEDV disease, which may result from antigenic variation between original US and S-INDEL PEDV strains (11). However, other factors also need to be considered: 1) All piglets in litters C and D recovered from S-INDEL PEDV Iowa106 infection and, subsequently, were challenged with original US PEDV PC21A at 27-days of age. However, only 45% of piglets in litter E recovered from original US PEDV PC21A inoculation. These piglets had higher birth weights and may have been less affected by PEDV infection than their non-surviving littermates. 2) The effect of material antibodies may be affected by the titer of protective antibody and the amount of milk, which sows provide to their piglets. In addition, the level of acute immunity developed in the piglets can affect their susceptibility to repeated PEDV infection. 3) The enterocyte turnover time is related to the age of pigs (28) and enteric viral infections (12). Newly replaced villous enterocytes were speculated to be less susceptible to repeated PEDV or TGEV infection because innate and adaptive immune responses were elicited (32).

At the end of the study (PID 28-30/PCD 7-9), the clinical signs and intestinal lesions subsided completely in all pigs. PEDV antigens were detected mainly in mucosal lymphoid tissues and mesenteric lymph nodes, but rarely in the villous epithelia. These PEDV IHC positive-stained mononuclear cells in lymph nodes were interpreted to be macrophages in recent studies (10, 11). It is reported that piglets developed adaptive immunity around PID 7 (13). Our previous study showed that PEDV infection impaired the tight junctions of the villous epithelium (33). PEDV-induced enteritis could attract macrophages to the gut. Development of mucosal immunity and increased permeability of the intestinal barrier could facilitate uptake of PEDV by macrophages and/or dendritic cells from the intestinal lumen.

In conclusion, our study suggests that S-INDEL PEDV Iowa106 is milder in virulence compared to original US PEDV PC21A, but it is still capable of causing mortality in some litters. The severity of clinical signs induced by PEDV is associated with multiple factors, such as the birth weight of the piglets and the sow's health/lactation status. In addition, a minority (19%, 3/16) of the piglets recovered from S-INDEL PEDV Iowa106 infection were fully protected from disease after a high challenge dose of original US PEDV PC21A. Although the serum VN antibody levels of piglets were similar in the S-INDEL and original US PEDV groups, prior infection with S-INDEL does not provide full protection against original US PEDV infection and disease in pigs. Studies of the detailed kinetics of PEDV humoral and cellular immune responses and the factors influencing the susceptibility of pigs to PEDV infection need to be further investigated. Considering the safety and effectiveness, S-INDEL PEDV Iowa106 is not currently a suitable live vaccine to protect piglets from the highly virulent original US PEDV strains, but would require further development and testing.

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Table 1. General litter information and the clinical signs of piglets and sows after PEDV inoculation (before challenge)

Litter No.	Litter size; stillborn; lost to injury	Age (day); body weight (kg) at inoculation	Inoculum strain (passage)[#]; dose (log_{10} GE/pig); processing method	Piglet condition								Sow condition		
				morbidity (%)[*]	mortality (%)[*]	Highest fecal PEDV shedding titer (log_{10} GE/ml)	Onset of diarrhea (PID)	Duration of diarrhea (days) [+] RS = 3 [†]	Duration of diarrhea (days) [+] RS ≧ 2 [†]	Duration of diarrhea (days) [+] RS ≧ 1 [†]	Duration of hypothermia (days) [+]	First week mean body weight gain (kg) [+]	Anorexia	Diarrhea (RS ≧ 2)	Highest fecal PEDV shedding titer (log_{10} GE/pig)
A	7; 0; 1	3; NA	Iowa106 (P0); 12; F&T 2x, filtrated	100 (6/6)	0 (0/5)	11.24 ± 1.80 [a,b]	1	1.80 ± 0.45 [b]	4.20 ± 0.84 [c]	7.00 ± 0.71 [a, b]	NA	NA	0	0	9.46
B	14; 3; 1	3; 1.67 ± 0.31 [b]	Iowa106 (P1); 10; F&T 1x,	100 (10/10)	75 (6/8)	11.08 ± 0.05 [b]	2	4.67 ± 0.58 [a]	6.33 ± 0.57 [a, b]	8.33 ± 1.53 [a]	6.75 ± 1.71 [a]	-0.28 ± 0.16 [c]	4	4	11.08
C	12; 1; 1	4; 1.8 ± 0.10 [b]	Iowa106 (P1); 12; F&T 2x	100 (10/10)	0 (0/10)	12.67 ± 0.48 [a]	2	4.00 ± 1.11 [a]	5.78 ± 0.97 [b]	6.56 ± 0.73 [b]	1.11 ± 0.93 [c]	-0.1 ± 0.20 [c]	1	2	11.60
D	11; 0; 1	4; 2.2 ± 0.30 [a]	Iowa106 (P1); 12; F&T 2x	100 (10/10)	0 (0/10)	12.17 ± 0.47 [a]	2-3	1.56 ± 0.73 [b]	3.44 ± 1.01 [c]	6.00 ± 1.41 [b]	0.00 ± 0.00 [d]	0.5 ± 0.30 [b]	0	0	9.40
E	13; 2; 0	4; 1.60 ± 0.42 [b]	PC21A (P2); 10; F&T 1x	100 (11/11)	55 (6/11)	11.80 ± 0.89 [a]	1	5.25 ± 0.96 [a]	7.20 ± 0.45 [a]	8.25± 1.26 [a]	3.22 ± 2.17 [b]	0.03 ± 0.31 [c]	2	5	9.67
F	10; 2; 1	4; 1.80 ± 0.16 [b]	Mock	0 (0/7)	0 (0/7)	ND	ND	0.00 ± 0.00 [c]	0.00 ± 0.00 [d]	0.00 ± 0.00 [c]	0.00 ± 0.00 [d]	0.95 ± 0.16 [a]	0	0	ND

[a, b, c, d] Different letters in each column mean significant different levels among litters ($P < 0.05$).

[†] RS score: 0 = normal, 1 = pasty, 2 = semi-liquid, 3 = liquid feces.

[*] Piglets injured by their sow or euthanized during acute infection phase for histopathology examination were excluded.

[+] Piglets died or euthanized from physical trauma or PEDV were excluded.

[#] Virus were passaged in gnotobiotic piglets (US original PEDV PC21A) or conventional piglets (S-INDEL PEDV Iowa106)

PID: post-inoculation day; RS: rectal swab; ND: not detectable; NA: data not available; GE: genomic equivalents; F&T: frozen and thaw (once 1x, twice 2x); P1: passage level 1; P2: passage level 2; filtrated: filtrated through 0.22 μm-pore size.

Table 2. Clinical signs of pigs after challenge with original US PEDV strain PC21A

Litter No.	Inoculation piglets at 3-4- days of age; dose (log_{10} GE/pig)	Challenge strain (passage)[#]; dose (log_{10} GE/pig)	Age (day) at challenge (PID)	Piglet condition								Sow condition		
				morbidity (%)	mortality (%)	Highest fecal PEDV RNA shedding titer (log_{10} GE/pig)	Onset of diarrhea (PCD)	Duration of diarrhea (day)			Body weight gain during 0 to 7 PCD (kg)	Anorexia	Diarrhea (RS ≧ 2) [†]	Highest fecal PEDV RNA shedding titer (log_{10} GE/pig)
								RS = 3 [†]	RS ≧ 2 [†]	RS ≧ 1 [†]				
A	Iowa106; 12	PC21A (P2); 10	24 (21)	0 (0/4)	0 (0/4)	6.21 ± 0.99	- [*]	0.00 ± 0.00 [*]	0.00 ± 0.00 [*]	0.00 ± 0.00 [*]	NA [*]	0 [*]	0 [*]	5.44
B	Iowa106; 10	PC21A (P2, P4); 10 [*], 12	29, 32 (25, 29)	0 (0/2) [*]	0 (0/2) [*]	7.44 ± 0.10 [*]	-	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.85 ± 0.35	0	0	7.65
C	Iowa106; 12	PC21A (P4); 12	27 (23)	86 (6/7)	0 (0/7)	10.8 ± 0.76 [a]	1-2	1.00 ± 1.15 [a]	2.29 ± 1.60	4.00 ± 1.29	0.93 ± 0.36 [c]	0	0	8.00
D	Iowa106; 12	PC21A (P4); 12	27 (23)	100 (7/7)	0 (0/7)	9.61 ± 1.64 [a]	1-2	0.29 ± 0.49 [b]	2.71 ± 1.11	4.00 ± 0.81	1.53 ± 0.24 [b]	0	0	7.50
E	PC21A; 10	PC21A (P4); 12	26 (20)	0 (0/4)	0 (0/4)	7.24 ± 0.79 [b]	-	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	2.47 ± 0.47 [a]	0	0	6.61
F*	Mock	PC21A (P2, P4); 10 *, 12	29, 32 (25, 29)	100 (5/5) *	0 (0/5) *	10.57 ± 0.81 *	1-2 *	2.00 ± 0.71 a *	3.80 ± 0.84 *	5.4 ± 0.55 *	2.56 ± 1.51 *	1-2 *	4 *	10.44

[*] The first challenge on 29-day-old did not induce clinical signs and viral shedding in naïve control pigs (litter F), so a higher dose was used. Evaluation of clinical signs of litters B and F was based on the second challenge at 32-days of age.

[†] RS score: 0, 1, 2, and 3 corresponded to normal, pasty, semi-liquid and liquid feces, respectively.

[#] Virus passaged in gnotobiotic piglets (PC21A), frozen and thaw once, no filtration.

[a, b, c, d] Different letters in each column mean different levels among litters ($P < 0.05$). Pigs challenged with lower dose of original PEDV (litter A) or at older ages (litter B and F) were excluded from statistical analysis.

PCD: post-challenge day, RS: rectal swab score, NA: not available, GE: genomic equivalents

Table 3. Comparison between S-INDEL PEDV Iowa106- and US original PEDV PC21A-infection in conventional suckling piglets

	PEDV strain	
	S-INDEL Iowa106	original US PC21A
Piglet morbidity	100%	100%
Piglet mortality	18% (0-75%)	55% (NA)
Onset of diarrhea (PID)	2.06 ± 0.63 (1-3) *	1.00 ± 0.00 (1-1)
Duration of diarrhea (RS ≧ 2; days)	4.75 ± 1.52 (2-7) *	7.20 ± 0.45 (7-8)
Highest fecal PEDV RNA shedding titer (log_{10} GE/ mL) [a]	11.67 ± 1.07 (9.46-13.40)	11.76 ± 0.91 (10.03-13.13)
VH:CD ratio in jejunum [b]	2.90 ± 1.24* (1.36-5.40)	1.40 ± 0.47 (0.85-1.98)
PEDV antigen score in jejunum [b]	1.40 ± 0.70* (1-3)	2.50 ± 1.00 (1-3)

Data are showed as mean ± standard deviation (full range). *Significant difference between Iowa106 and PC21A by student t-test ($P < 0.05$).

PID: post-inoculation day; GE: genomic equivalent; VH : CD: the ratio of villous height : crypt depth; NA: not available

[a] It was detected on the same day of onset of diarrhea.

[b] Piglets died or euthanized between 2-6 PIDs.

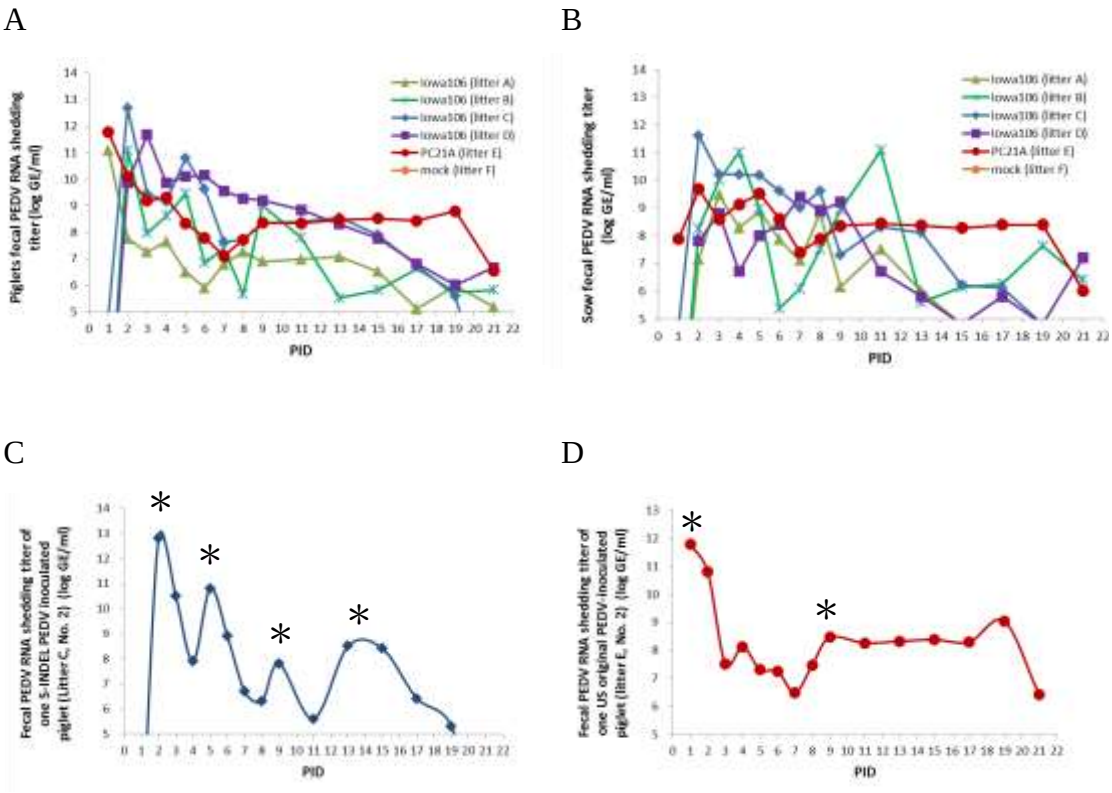

Figure 1. Fecal PEDV RNA shedding profiles of piglets (A) and their sows (B) after oral inoculation of piglets at 3-4 days of age. Data were shown as mean of piglets (A) or individual sow (B) of each litter. Representative PEDV RNA fecal shedding pattern of one S-INDEL PEDV Iowa106- (C) and one US original PEDV PC21A- (D) inoculated piglets were shown. A biphasic curve with 3-6 days of intervals between peaks (C) or a time-dependent, gradual down-sloping curve (D) was observed. A dominant "peak" of fecal PEDV RNA shedding titer was defined when the titer difference between the peak and the lowest values was > 1.5 log10 (~5 Ct) and was marked with ∗. Four and two peaks were counted in (C) and (D), respectively.

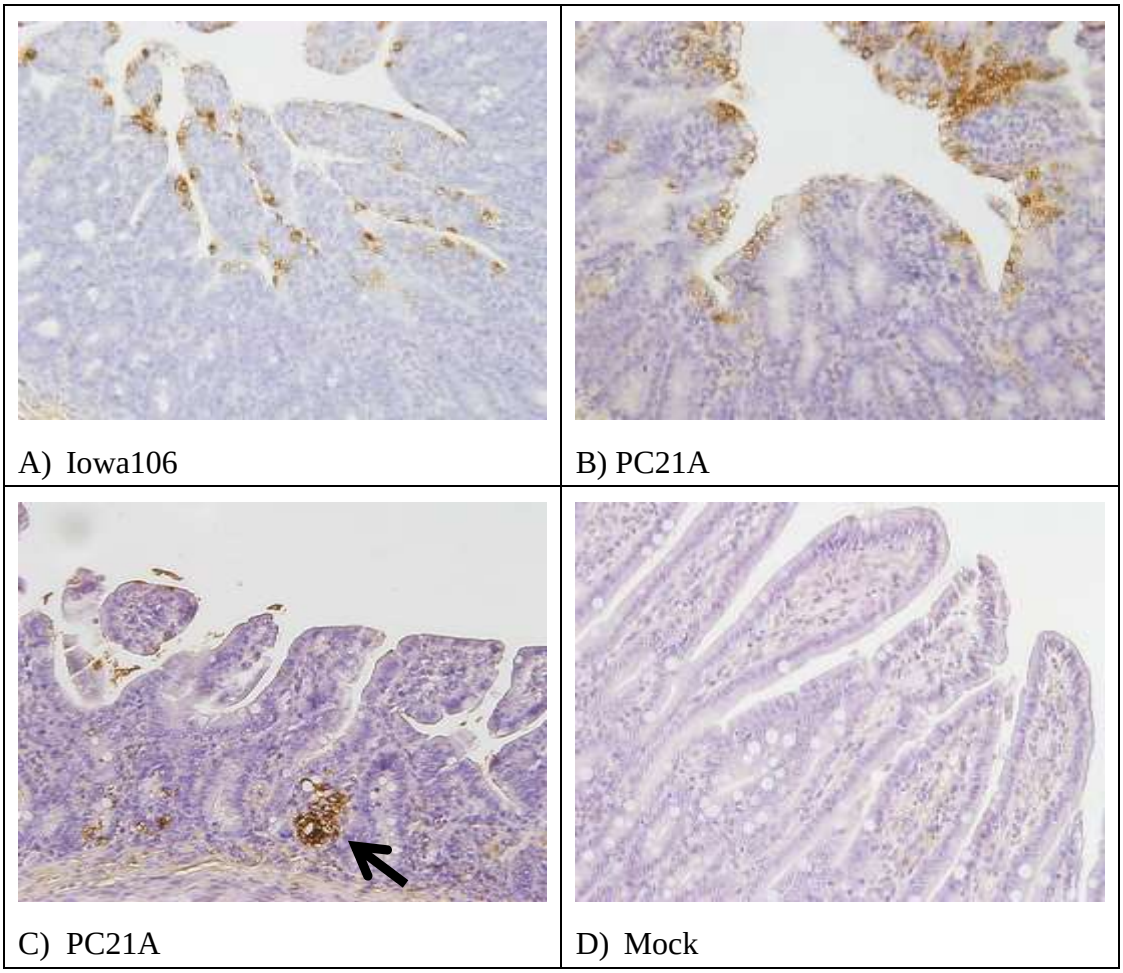

Figure 2. Antigen distribution pattern of S-INDEL PEDV Iowa106 strain (A), US original PEDV PC21A strain (B and C), and mock (D) in jejunum. PEDV nucleocapsid proteins were detected by immunohistochemistry staining (brown) using monoclonal antibody SD6-29 against the N protein of PEDV. Both S-INDEL PEDV Iowa106 (A) and US original PEDV PC21A (B and C) antigens were mainly detected in villous epithelial cells. Severe villous atrophy was observed in US original PEDV PC21A-inoculated pigs (B). Incidentially, dominant villous atrophy along with US original PEDV PC21A antigen located in crypts (arrow) were noted in one piglet (litter E, No. 3) (C).

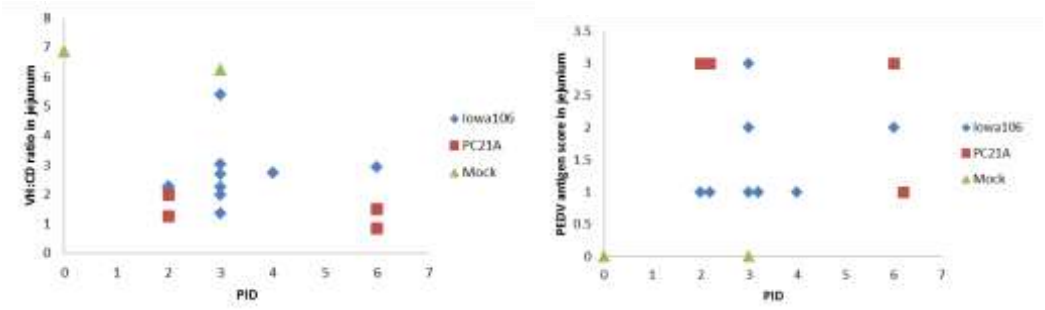

Figure 3. Histopathology and immunohistochemistry results of piglets that died or were euthanized by 7 post-inoculation day (PID). The intensities of villous atrophy and PEDV infection in jejunum were expressed as (A) villous high: crypt depth ratios (VH:CD) and (B) antigen scores, respectively. Score 0 denotes no positive cells; scores 1 to 3 denote less than 30%, 30 to 60% and more than 60% of villous enterocytes showing a positive signal, respectively.

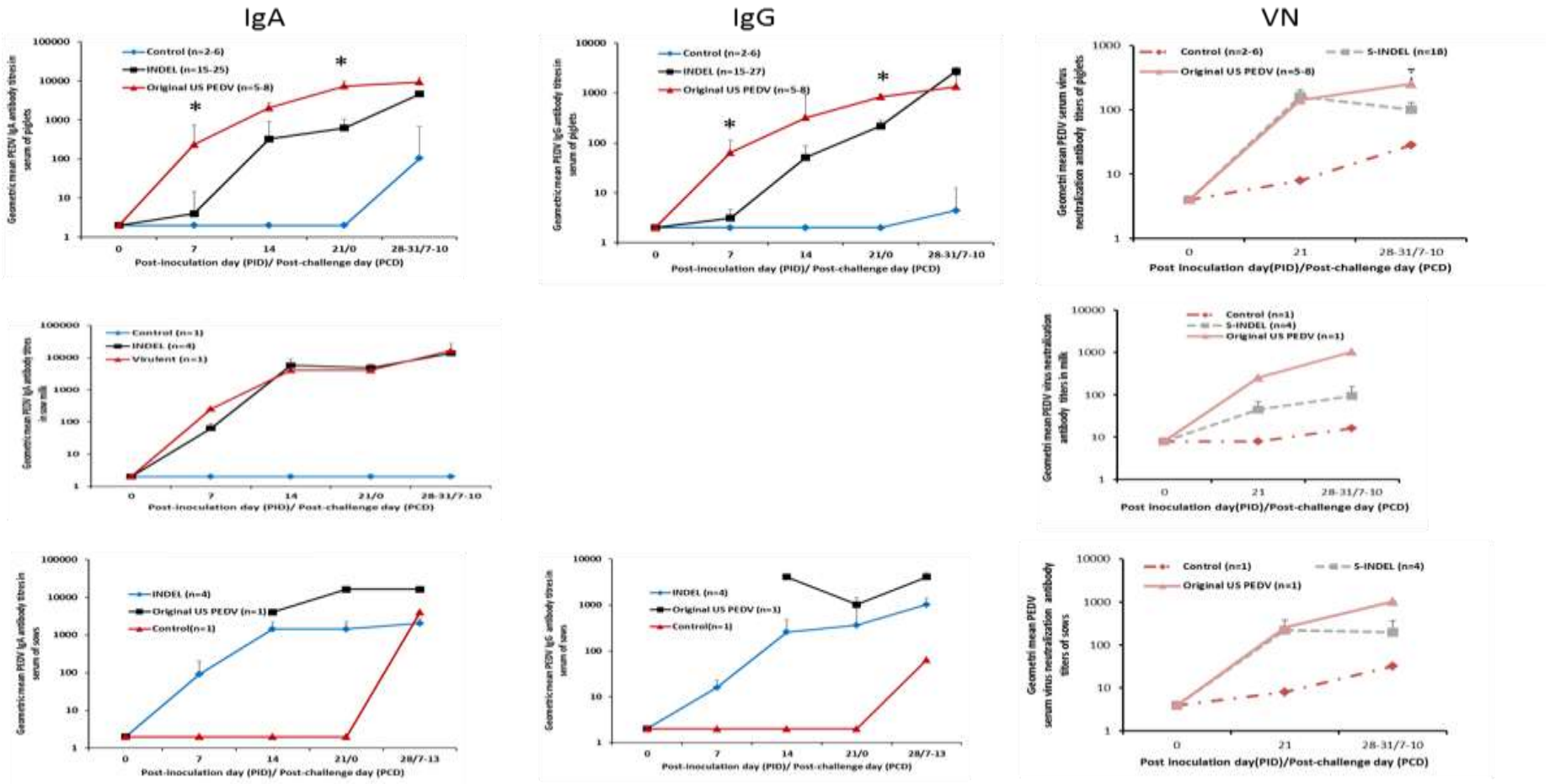

Figure. 4. Piglet and sow serum and sow milk IgA and virus neutralization (VN) antibody responses. Asterisks symbols indicate significant differences between S-INDEL Iowa106- and original US PEDV PC21A-groups at the same time-point for the same antibody.