RESEARCH REPORT
SWINE HEALTH

Title: Pathogenicity and antibody responses of different U.S. PEDV strains in pigs of different ages **- #17-184- IPPA**

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Industry Summary:

Porcine epidemic diarrhea virus (PEDV) is the causative agent of porcine epidemic diarrhea (PED), an enteric disease affecting the global swine industries. In the U.S., PEDV was detected for the first time in 2013. The PEDVs associated with the initial outbreak in the U.S. in April 2013 are highly virulent and belong to the Emerging North American non-S INDEL clade; these PEDVs were also called "U.S. original PEDV strain" or "U.S. prototype PEDV strain". A clinically milder PEDV variant strain identified in January 2014 in the U.S. was called "S-INDEL PEDV strain". Recently, new variants of PEDV with large deletions (194aa-204aa deletions) in the N-terminal domain of the spike protein have been identified in the U.S. swine samples and referred to as "S1 NTD-del variant". However, these S1 NTD-del PEDV variant did not cause severe diarrhea in experimentally infected young pigs and its prevalence seems to be low. Diagnostics and research are mainly focused on U.S. prototype PEDV and S-INDEL PEDV strains.

Experimental infection studies have demonstrated that the U.S. S-INDEL PEDV isolates overall had lower pathogenicity than the U.S. prototype PEDV isolates in conventional neonatal piglets at 3-4 days of age or 5-6 days of age. However, pathogenicity and antibody responses of these two PEDV strains in older pigs have not been well characterized. This study aimed to compare pathogenicity and antibody responses of these two PEDV strains in three ages of pigs.

Thirty 3-week-old ("weaned"), thirty 8-week-old ("grower"), and thirty 23-week-old ("finisher") pigs totaling 90 PEDV naïve pigs were included. Thirty pigs of each age were divided into 3 groups (10 pigs/group) and orogastrically inoculated with prototype PEDV (10^5 TCID50/pig), S-INDEL PEDV (10^5 TCID50/pig), or virus-negative medium. Half the pigs in each group were randomly selected for necropsy at 4 DPI and remaining pigs were necropsied at 28 DPI. Virus loads in rectal swabs, oral fluids, and various tissues collected at 4 DPI necropsy were determined by a quantitative PEDV N gene-based real-time RT-PCR. Tissues collected at 4 DPI necropsy were also subject to histopathological and immunohistochemistry examinations. Five pigs in each group that went through 28 DPI were compared for antibody responses. Serum neutralizing antibody was measured by a fluorescent focus neutralization assay using prototype PEDV as the indicator virus. Serum IgG and oral fluid IgA antibodies were measured

by PEDV fluorescent microsphere immunoassay (FMIA) based on the N-terminal portion (S1) of the prototype PEDV spike protein.

In “weaned” pigs, prototype PEDV had longer duration of fecal shedding and significantly higher fecal and oral fluid virus loads than S-INDEL PEDV. In “grower” pigs, S-INDEL PEDV had longer fecal shedding than prototype PEDV; S-INDEL fecal virus load was significantly higher than prototype PEDV at 7 and 14 DPI but was opposite at 10 DPI. In “finisher” pigs, the onset of prototype PEDV fecal shedding was earlier and fecal virus load was higher than S-INDEL PEDV. For prototype PEDV, the onset of fecal virus shedding was earliest and shedding level was highest in “weaned”, followed by “grower” and “finisher” pigs. S-INDEL PEDV trended similarly when compared across age groups. The data suggest that pathogenicity of PEDV is pig age-dependent (more severe in younger pigs) and virus strain-dependent. Prototype PEDV appeared to be more pathogenic than S-INDEL PEDV in “weaned” and “finisher” pigs, but pathogenicity difference of two viruses was less distinct in “grower” pigs.

Neutralizing antibody, serum IgG and oral fluid IgA responses indicated that prototype PEDV induced greater antibody responses than S-INDEL PEDV in both “weaned” and “finisher” pigs, while the difference of antibody responses induced by two PEDV strains in “grower” pigs was not a clear cut and it depends on antibody assay. Prototype PEDV induced similar neutralizing antibody responses in three ages of pigs, stronger serum IgG responses in “weaned” pigs than in “grower” and “finisher” pigs, and stronger oral fluid IgA responses in “finisher” pigs than in “grower” and “weaned” pigs. Interestingly, S-INDEL PEDV appeared to consistently induce stronger antibody responses in “grower” pigs than in “weaned” and “finisher” pigs.

In summary, this study suggests that pathogenicity and antibody response of PEDV is both virus strain-dependent and pig age dependent. The data provide some guidance on selecting appropriate PEDV strain to induce antibody response in different age of pigs. It is noteworthy that different antibody assays may not always give the consistent results. For PEDV, mucosal immunity is critical for protection. However, at this point, it is unclear which antibody assay can better reflect mucosal and protective immunity although secretory IgA in small intestines is believed to be associated with mucosal and protective immunity. Nonetheless, it is not easy to routinely measure secretory IgA in small intestines. Work is in progress to assess the correlation of PEDV neutralizing antibody, serum IgG, and oral fluid IgA with secretory IgA in small intestines with the hope of finding an antibody assay that can be routinely conducted to reflect mucosal immunity.

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Keywords:
Porcine epidemic diarrhea virus; U.S. prototype PEDV; U.S. S INDEL PEDV; pig age; pathogenicity; antibody response

Scientific Abstract: This should be a scientific description limited to one page in length to describe your project and its results.

Currently, two main strains of porcine epidemic diarrhea virus (PEDV) i.e. U.S. prototype and S-INDEL PEDVs, circulate in U.S. swine but their pathogenicity and antibody responses in different ages of pigs have not been well characterized. This study aimed to compare pathogenicity and antibody responses of these two PEDVs in three ages of pigs. Thirty 3-week-old (“weaned”), thirty 8-week-old (“grower”), and thirty 23-week-old (“finisher”) pigs were included with each age divided into 3 groups (10

pigs/group) and orogastrically inoculated with PEDV isolate USA/IN19338/2013 (prototype), USA/IL20697/2014 (S-INDEL), or virus-negative medium. Half the pigs in each group were randomly selected for necropsy at 4 DPI and remaining pigs were necropsied at 28 DPI. Virus load was determined by a quantitative PEDV N gene-based real-time RT-PCR. Five pigs in each group that went through 28 DPI were compared for antibody responses. Serum neutralizing antibody was measured by a fluorescent focus neutralization assay using prototype PEDV as the indicator virus. Serum IgG and oral fluid IgA antibodies were measured by PEDV fluorescent microsphere immunoassay (FMIA) based on the N-terminal portion (S1) of the prototype PEDV spike protein. In "weaned" pigs, prototype PEDV had longer duration of fecal shedding and significantly higher fecal and oral fluid virus loads than S-INDEL PEDV. In "grower" pigs, S-INDEL PEDV had longer fecal shedding than prototype PEDV; S-INDEL fecal virus load was significantly higher than prototype PEDV at 7 and 14 DPI but was opposite at 10 DPI. In "finisher" pigs, the onset of prototype PEDV fecal shedding was earlier and fecal virus load was higher than S-INDEL PEDV. For prototype PEDV, the onset of fecal virus shedding was earliest and shedding level was highest in "weaned", followed by "grower" and "finisher" pigs. S-INDEL PEDV trended similarly when compared across age groups. The data suggest that pathogenicity of PEDV is pig age-dependent (more severe in younger pigs) and virus strain-dependent. Prototype PEDV appeared to be more pathogenic than S-INDEL PEDV in "weaned" and "finisher" pigs, but pathogenicity difference of two viruses was less distinct in "grower" pigs. Neutralizing antibody, serum IgG and oral fluid IgA responses indicated that prototype PEDV induced greater antibody responses than S-INDEL PEDV in both "weaned" and "finisher" pigs, while the difference of antibody responses induced by two PEDV strains in "grower" pigs was not a clear cut and it depends on antibody assay. Prototype PEDV induced similar neutralizing antibody responses in three ages of pigs, stronger serum IgG responses in "weaned" pigs than in "grower" and "finisher" pigs, and stronger oral fluid IgA responses in "finisher" pigs than in "grower" and "weaned" pigs. Interestingly, S-INDEL PEDV appeared to consistently induce stronger antibody responses in "grower" pigs than in "weaned" and "finisher" pigs. In summary, this study suggests that pathogenicity and antibody response of PEDV is both virus strain-dependent and pig age dependent. The data provide some guidance on selecting appropriate PEDV strain to induce antibody response in different age of pigs.

Introduction:

Porcine epidemic diarrhea virus (PEDV) is the causative agent of porcine epidemic diarrhea (PED) that was first recorded in England in the early 1970s (1). Since then, PED had been reported in European countries and several Asian countries such as Japan, South Korea, China, Thailand, and Vietnam (2, 3). In North America, PEDV was detected for the first time in the U.S. in April 2013 (4) and subsequently PEDV was reported in Canada (5) and Mexico (6). In January 2014, a clinically milder PEDV variant strain was identified in the U.S. (7). Since 2013, US-like PEDVs have also emerged in Taiwan, South Korea, Japan, Germany, Belgium, France, Portugal, Austria, and Ukraine (8). PEDV remains a significant challenge to the global swine industries.

Global PEDVs exhibit significant genetic diversity. There have been some discrepancies in the literatures regarding how to categorize different PEDV strains worldwide. Lin et al (9) proposed to phylogenetically classify PEDV strains into classical clade, S-INDEL clade, Emerging Asian non-S INDEL clade, and Emerging North American non-S INDEL clade. The PEDVs associated with the initial outbreak in the U.S. in April 2013 are highly virulent and belong to the Emerging North American non-S INDEL clade; these PEDVs were also called "U.S. original PEDV strain" (10) or "U.S. prototype PEDV strain" (11, 12). A clinically milder PEDV variant strain identified in January 2014 in the U.S. (7) was called "S-INDEL strain" (13) because it has characteristic insertions and deletions in the spike (S) gene compared to the U.S. PEDV strains originally identified in April 2013. Recently, new variants of PEDV with large deletions (194aa-204aa deletions) in the N-terminal domain of the spike protein have

been identified in the U.S. swine samples (14, 15) and referred to as "S1 NTD-del variant" (15) but these S1 NTD-del PEDV variant did not cause severe diarrhea in experimentally infected pigs (16). Interestingly, co-infection of U.S. prototype PEDV and S1 NTD-del PEDV has been found in clinical swine samples in the U.S. (15) and replication of U.S. PEDV prototype strain appeared to be enhanced during co-infection with S1 NTD-del PEDV (17). The prevalence of S1 NTD-del has not been thoroughly investigated but seems to be low. Diagnostics and research are mainly focused on U.S. prototype PEDV and S-INDEL PEDV strains.

Previous studies showed that the antibodies against U.S. prototype PEDV and S-INDEL strains can cross-react and cross-neutralize both strains *in vitro* (12, 18). An *in vivo* study (19) showed that sows exposed to S-INDEL PEDV infection 7 months ago could provide partial protection to newborn piglets challenged with a U.S. prototype PEDV strain. Another *in vivo* study (20) demonstrated that 3-4 days old piglets exposed to S-INDEL PEDV could partially protect against subsequent challenge with a U.S. prototype PEDV. We also have unpublished data that demonstrates both U.S. prototype and S-INDEL PEDV strains can provide homologous and heterologous protection against two virus strains in a weaned pig model. These data suggest that at least partial cross-protective immunity exist between the two U.S. PEDV strains.

Experimental infection studies have demonstrated that the U.S. S-INDEL PEDV isolates overall had lower pathogenicity than the U.S. prototype PEDV isolates in conventional neonatal piglets at 3-4 days of age or 5-6 days of age (11, 20). However, comparison of pathogenicity of these two PEDV isolates in pigs of older ages have not been conducted. Experimental infection studies in neonatal and nursery pigs have shown that the pathogenicity of the U.S. prototype PEDV isolates is age dependent (21-24) although the pathogenicity of the U.S. prototype PEDV in pigs >4 weeks has not been included for comparison. In addition, whether the pathogenicity of the U.S. S-INDEL PEDV is pig age-dependent has not been investigated. Also, it is important to determine which strain of PEDV induces better immune responses in pigs of different ages; such information is not available but will be important for choosing appropriate PEDV strains for vaccine development.

In the current study, we aimed to characterize the virus strain-dependent and pig age-dependent pathogenicity and antibody responses of PEDV. Specifically, we evaluated the pathogenicity and antibody responses of U.S. prototype and S-INDEL PEDV strains in 3-week-old pigs, 8-week-old pigs, and 23-week-old pigs.

Objectives:

Objective 1. Comparison of the pathogenicity differences of U.S. prototype PEDV and S-INDEL PEDV strains in 3-week-old pigs, 8-week-old pigs, and 23-week-old-pigs.

Objective 2. Evaluation of the antibody response of U.S. prototype PEDV and S-INDEL PEDV strains in 3-week-old pigs, 8-week-old pigs, and 23-week-old-pigs.

Materials & Methods:

Virus and cells. A U.S. prototype PEDV strain cell culture isolate USA/IN19338/2013 and U.S. S-INDEL PEDV strain cell culture isolate USA/IL20697/2014 were isolated, propagated, and titrated in Vero cells (ATCC CCL-81) in our lab as previously described (12, 25). Virus stocks at the 9th passage in cell culture were prepared and used in this study for both PEDV isolates.

RNA extraction. Nucleic acids were extracted from rectal swabs, sera, oral fluids, and tissue homogenates using a MagMAX Pathogen RNA/DNA Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) on a Kingfisher Flex instrument (Thermo Fisher Scientific) following the instructions of the manufacturer. One hundred microliter

(µl) of the samples were used for nucleic acid extraction and eluted into 90 µl of Elution buffer.

Quantitative real-time RT-PCR for PEDV. A PEDV nucleocapsid (N) gene-based real-time RT-PCR previously developed at the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) (24, 26) was used in this study. Any cycle threshold (C_T) values <37 were considered positive and the genomic copies/ml was calculated based on the standard curve generated using the *in vitro* transcribed PEDV N gene RNA as previously described (24).

Ethics statement. The experimental protocol for the pig studies was approved by the Iowa State University Institutional Animal Care and Use Committee (IACUC, Approval No. 11-17- 8650-S; approved on 6th of December 2017).

Pig study. In order to ensure that the pigs used in this study had the same genetic background, the same source of pigs at three different ages were reserved and used. On April 24, 2018, thirty 3-week-old pigs were delivered to the Iowa State University Laboratory Animal Resources (ISU LAR) and virus inoculation was conducted on April 27, 2018 (pigs were 3 weeks old). On May 30, 2018, the same batch of pigs were 8 weeks old. Thirty pigs were delivered to the ISU LAR and virus inoculation was conducted on Jun 4, 2018 (pigs were 8-9 weeks old). On September 5, 2018, the same batch of pigs were 22 weeks old. Thirty pigs were delivered to the ISU LAR and virus inoculation was conducted on September 10, 2018 (pigs were 23 weeks old).

Thirty 3-week-old pigs, thirty 8-week-old-pigs, and thirty 23-week-old pigs totaling 90 animals were all confirmed negative for PEDV, porcine deltacoronavirus (PDCoV), transmissible gastroenteritis virus (TGEV), and porcine rotaviruses (groups A, B, & C) by virus-specific PCRs on rectal swabs and negative for PEDV antibody by a virus-specific fluorescent microbead-based immunoassay (FMIA) on serum samples, prior to the start of the respective study. Pigs received an intramuscular injection of Excede® (Zoetis, Florham Park, New Jersey, USA) per label instructions prior to arrival at the ISU LAR. Each age group of pigs were randomized by weight into three groups of 10 pigs each, one room per group. Pigs were fed a complete swine diet mash and had free access to water. After three to five days of acclimation, pigs were orogastrically inoculated with either prototype PEDV stock virus at a concentration of 10^4 TCID50/mL, S-INDEL PEDV stock virus at 10^4 TCID50/mL, or virus-negative cell culture medium (10ml/pig) (**Table 1**). Size 12 French feeding tubes were used for orogastrical inoculation of 3-week-old pigs and size 18 French feeding tubes for 8-week-old and 23-week-old pigs.

Pigs were evaluated for clinical signs of vomiting, diarrhea, lethargy, and body condition daily for the first week, every other day for the second week and then once weekly until 28 days post inoculation (DPI). Diarrhea severity was assessed with the following criteria by both visual observation and rectal swabbing: 'normal'=no diarrhea, 'mild diarrhea'=soft (cowpie), 'moderate diarrhea'=liquid with some solid content, 'watery diarrhea'=watery with no solid content. Rectal swabs were collected at 0, 2, 4, 7, 10, 14, 21 and 28 DPI and were submerged into 1ml phosphate buffered saline (PBS, 1× pH 7.4) immediately after collection. Two ropes per room were hung and oral fluids were collected at 0, 2, 4, 7, 10, 14, 21 and 28 DPI. Serum was collected at -2, 2, 4, 7, 14, 21 and 28 DPI. For 3-week-old and 8-week-old pigs, body weight was recorded at -2, 2, 4, 7, 14, 21 and 28 DPI. For 23-week-old pigs, body weight was recorded at -2 DPI and at necropsy. Five of the ten pigs in each group were randomly selected for necropsy at 4 DPI (due to the late onset of virus shedding, five pigs in each group of the 23-week-old pigs were necropsied at 9 DPI) with the remaining five pigs being necropsied at 28 DPI. At necropsy, pigs were rinsed with water to remove feces. Formalin-fixed and fresh tissues were collected: ham, shoulder, loin, tonsil, heart, lung, liver, diaphragm, spleen, and kidney, proximal jejunum, middle jejunum, distal jejunum, ileum, cecum and colon. In addition, enteric tissues were evaluated for gross lesions.

Rectal swabs, serum, tissues (10% tissue homogenates), and oral fluids were tested by aforementioned quantitative PEDV N gene-based rRT-PCR. In addition, serum and oral fluid samples were tested for PEDV antibody using virus neutralization (VN)

test and PEDV S1 protein-based FMIA assay (see below). Formalin-fixed tissues were submitted to the ISU VDL for histopathology and immunohistochemistry examinations.

Histopathology and immunohistochemistry. All sections of formalin-fixed tissues were microscopically evaluated by a veterinary pathologist blinded to individual animal identifications and treatment groups. Three representative villi and crypts with integrated longitudinal sections were randomly selected from ileum of each pig for measurement of villus heights and crypt depths using a computerized image system following the previously described procedures (26). Villus-height-to-crypt-depth ratio of each tissue was calculated as the quotient of the average villus length divided by the average crypt depth. At 4 DPI, a section of ham, shoulder, loin, tonsil, heart, lung, liver, diaphragm, spleen, and kidney, proximal jejunum, middle jejunum, distal jejunum, and serial sections of ileum were evaluated for PEDV antigen by immunohistochemistry (IHC) using a PEDV-specific monoclonal antibody (BioNote, Hwaseong-si, Gyeonggi-do, Korea) as previously described (26). The IHC antigen detection was semi-quantitatively scored as previously described (27) with the following criteria: 0 = no staining; 0.5 = approximately <0.5% enterocytes/tissue with positive staining; 1 = approximately 1-10% enterocytes/tissue with positive staining; 2 = approximately 10%-25% enterocytes/tissue with positive staining; 3 = approximately 25%-50% enterocytes/tissue with positive staining; 4 = approximately 50%-100% enterocytes/tissue with positive staining.

Virus neutralization (VN) test. Serum samples were tested for PEDV neutralizing antibodies. Serum samples were first inactivated at 56°C for 30 min, then 2-fold serially diluted from 1:4 to 1:512 dilutions in 96-well plates with a volume of 75µl per well after dilution. Subsequently 75µl of 1.2×10^3 TCID50/ml of the U.S. prototype PEDV strain USA/IN19338/2013 was mixed with the equal volume of diluted sera and incubated for 1 h at 37°C with 5% CO2. Each serum sample was tested in duplicate. Vero cell monolayers grown in 96-well plates were washed twice with post-inoculation media (minimum essential medium supplemented with 0.3% tryptose phosphate broth, 0.02% yeast extract, 5µg/ml trypsin 250, 100 unit/ml penicillin, 100µg/ml streptomycin, 0.05 mg/ml gentamicin, and 0.25µg/ml amphotericin B). Then 100µl of the serum-virus mixture (containing 60 $TCID_{50}$ of virus) was transferred to prewashed Vero cell monolayers. After the plates were incubated for 1 h at 37°C, the cells were washed twice with post-inoculation media and incubated with 100µl/well of such media for 24 h at 37°C with 5% CO2. Then cells were fixed with cold 80% acetone and stained with PEDV N protein-specific monoclonal antibody SD6-29 conjugated to FITC (Medgene, Brookings, South Dakota) at a 1:100 dilution for 40 min. The staining was examined under a fluorescent microscope. The reciprocal of the highest serum dilution resulting in >90% reduction of staining as compared to the negative serum control was defined as the VN titer of the serum sample. A VN titer of ≥8 was considered positive.

Multiplex fluorescent microbead-based immunoassay (FMIA) for PEDV antibody detection. PEDV multiplex fluorescent microbead-based immunoassay (FMIA) was performed as previously described (28). In brief, magnetic microspheres (Luminex Corp.) were covalently coupled to the amino terminal portion (S1 portion) of the U.S. prototype PEDV spike protein expressed and purified under native conditions using a mammalian expression system and protein A-based affinity chromatography. Serum samples (50µl/sample), pre-diluted to 1/50 in assay buffer, and undiluted oral fluid samples (50µl/sample) were mixed with 50µl of the PEDV S1 coupled beads (2,500 beads per well) in each well (Bio-Rad Laboratories, Inc.). Each serum and oral fluid sample was individually tested for IgG and IgA. The plates were incubated for 30 min (serum) or 2h (oral fluids) on a microplate shaker at 400 rpm and were washed three times with 200µl of 0.1M PBST (pH7.4; 0.1% Tween-20). All incubations were performed at 22°C in a dark environment. Next, 50µl of biotin-labeled goat anti-pig IgG (Fc) (Bethyl Laboratories Inc.) diluted at 1/7,000 (for serum samples testing) or 1/500 (for oral fluid

samples testing), or 50µl of biotin-labeled goat anti-pig IgA (Bethyl Laboratories Inc.) diluted at 1/2,000 (for serum) or 1/1000 (for oral fluid) in assay buffer was added to each corresponding well, followed by a 30 min (serum) or 1h (oral fluid) incubation. After a washing step, 50µl of streptavidin phycoerythrin (SAPE; Moss Inc.) at a 1/100 dilution in assay buffer was added to each well and the plates were incubated for 30 min. After an additional wash step, the microspheres were resuspended in 100µl of assay buffer and analyzed using a dual-laser Bio-Plex 200 instrument (Bio-Rad Laboratories, Inc.). The antibody response, reported as fluorescent intensity (FI), was expressed as the sample-to-positive (S/P) ratios. The S/P ratio <0.1 was considered Negative, 0.1-0.2 was considered Suspect, and >0.2 was considered Positive.

Statistics. A generalized linear model with mixed effects (GLIMMIX) was used to analyze virus shedding titer Log10 (genomic copies/mL), VN antibody titer, fecal scores, average daily gain, histopathology, IHC, and ELISA titer differences among groups with SAS version 9.4 (SAS Institute, Cary, NC). Age, treatment, and DPI were designated as fixed effects and pig was the random effect. Simple effect comparisons were conducted for both treatment which were sliced at DPI-age, and for age which were sliced at DPI-treatment; all in a pairwise manner with Tukey adjustment. For oral fluid analysis, the fixed effects remained the same as pig level analysis; however, there was no random effect in GLIMMIX. When an oral fluid record was missing, its replicate within the same pen at the same DPI was used for imputation. When both records are missing, data within the same pen at the following dpi were used for imputation. Correlation analysis was conducted to study antibody correlations among five variables (VN, serum IgG, serum IgA, OF IgG, and OF IgA) and a Pearson correlation was calculated in SAS 9.4 by PROC CORR. In all analysis, p value < 0.05 was defined as statistically significant.

Results:

Objective 1: Comparison of the pathogenicity differences of U.S. prototype and S-INDEL PEDV strains in 3-week-old pigs, 8-week-old pigs, and 23-week-old-pigs.

1. Clinical assessment
    1) Clinical fecal scores: In 3-week-old prototype-PEDV-inoculated pigs, there was some mild to watery diarrhea at 2 DPI (9/10 pigs) and 4 DPI (5/10 pigs) that returned to normal by the end of the first week. In 3-week-old S-INDEL-PEDV-inoculated pigs, mild diarrhea in most pigs (9/10 pigs) occurred at 4 DPI after which fecal scores returned to normal for all but one pig by 7 and 10 DPI. All 3-week-old viral-inoculated pigs had normal fecal scores by 14 DPI to the end of the study. Most viral-inoculated pigs from 8-week-old and 23-week-old groups had normal fecal scores throughout the study. All negative control pigs of all ages were active and had no observed clinical signs or diarrhea.
    2) We did not observe depression, vomiting, dehydration, or loss of body condition in any pigs regardless of the treatment or age groups.
    3) Average daily gain (ADG).
        - For each age of pigs, five pigs in each group (Neg Ctrl, S-INDEL PEDV, and prototype PEDV) going through 28 DPI were used to calculate ADG in the intervals of -2 to 4 DPI, -2 to 7 DPI and/or -2 to 28 DPI, as shown in Fig 1A, B and C. In 3-week-old pigs, the ADG of prototype PEDV group was numerically lower than those of the Neg Ctrl and S-INDEL PEDV groups but the differences across three groups were not significant regardless of the intervals -2 to 4, -2 to 7, or -2 to 28 DPI (Fig 1A). In 8-week-old pigs, the ADG across three groups was not significantly different regardless of the intervals -2 to 4, -2 to 7, or -2 to 28 DPI (Fig 1B). In 28-week-old pigs, weight data were only available at -2 and 28 DPI and the ADG across three groups was not significantly different during this interval (Fig 1C).
        - For 3-week-old and 8-week-old pigs, ADG in the interval of -2 to 4 DPI based on 10 pigs per group (5 pigs/group were euthanized at 4 DPI) was

also calculated (Fig 1D and E). In 3-week-old pigs, there was no significant difference in ADG between the S-INDEL PEDV and Neg Ctrl groups; however, ADG of the Prototype PEDV group was significantly lower than those of the Neg Ctrl and S-INDEL PEDV groups (Fig 1D). In 8-week-old pigs, there was no significant difference in ADG across three groups (Fig 1E).

2. Fecal virus shedding (Fig 2)
   1) Fecal shedding of PEDV in prototype and S-INDEL-inoculated 3-week-old pigs is summarized in Fig 2A. For prototype PEDV-inoculated pigs, 10/10, 10/10, 5/5, 5/5, 1/5, 1/5, and 0/5 pigs shed detectable viral RNA in rectal swabs at 2, 4, 7, 10, 14, 21, and 28 DPI, respectively. Prototype PEDV shedding started at 2 DPI, lasted through 21 DPI, and decreased to zero at 28 DPI. For S-INDEL-inoculated pigs, 6/10, 9/10, 5/5, 1/5, 0/5, 0/5, and 0/5 pigs were PCR positive in rectal swabs at 2, 4, 7, 10, 14, 21, and 28 DPI, respectively. S-INDEL PEDV shedding started from 2 DPI, reached a peak at 7 DPI, and then decreased to zero at 14 DPI. Overall, in 3-week-old pigs, prototype PEDV had longer duration of fecal shedding and significantly higher fecal virus load at 2 DPI and 10 DPI, compared to S-INDEL PEDV.
   2) Fecal shedding of PEDV in prototype and S-INDEL-inoculated 8-week-old pigs is shown in Fig 2B. For prototype PEDV-inoculated pigs, 2/10, 4/10, 4/5, 5/5, 0/5, 0/5, and 0/5 pigs shed detectable viral RNA in rectal swabs at 2, 4, 7, 10, 14, 21, and 28 DPI, respectively. Prototype PEDV shedding reached a peak level at 10 DPI and then sharply decreased to zero by 14 DPI. For S-INDEL-inoculated pigs: 1/10, 5/10, 4/5, 3/5, 2/5, 0/5, and 0/5 pigs shed virus at 2, 4, 7, 10, 14, 21, and 28 DPI, respectively. S-INDEL viral titer peaked at 7 DPI and gradually decreased to zero at 21 DPI. Overall, in 8-week-old pigs, S-INDEL PEDV had longer duration of fecal shedding than prototype PEDV; S-INDEL fecal virus load was significantly higher than prototype PEDV at 7 DPI and 14 DPI but was opposite at 10 DPI.
   3) Fecal shedding of PEDV in prototype and S-INDEL-inoculated 23-week-old pigs is shown in Fig 2C. For prototype PEDV-inoculated pigs, 0/10, 3/10, 7/10, 3/5, 0/5, 0/5, and 1/5 pigs shed detectable viral RNA at 2, 4, 7, 10, 14, 21, and 28 DPI, respectively. Prototype PEDV shedding reached a peak level at 7-10 DPI and then gradually decreased. For S-INDEL-inoculated pigs, 0/10, 0/10, 2/10, 3/5, 0/5, 0/5, and 0/5 pigs had detectable viral RNA in rectal swabs at 2, 4, 7, 10, 14, 21, and 28 DPI, respectively. S-INDEL viral titer peaked at 10 DPI before decreasing to zero by 14 DPI to the end of the study. Overall, in 23-week-old pigs, the onset of prototype PEDV shedding in rectal swabs was earlier than S-INDEL PEDV and prototype PEDV had higher fecal virus load than S-INDEL PEDV.
   4) None of the Neg Ctrl pigs had fecal virus shedding throughout the study as seen in Fig 2D.
   5) Virus shedding in rectal swabs of all ages of S-INDEL PEDV-inoculated pigs is summarized in Fig 2E. The onset of fecal virus shedding was earlier in 3-week-old and 8-week-old pigs than in 23-week-old pigs. S-INDEL PEDV shedding levels in rectal swabs were significantly higher in 3-week-old pigs than in 8-week-old and 23-week-old pigs at 2-4 DPI. Fecal virus loads of S-INDEL PEDV at 7 DPI were similar between 3-week-old and 8-week-old pigs but were both significantly higher than 23-week-old pigs. The duration of S-INDEL PEDV shedding in rectal swabs was longer in 8-week-old pigs than in 3-week-old and 23-week-old pigs.

6) Virus shedding in rectal swabs of all ages of prototype PEDV-inoculated pigs is shown in Fig 2F. The onset of fecal virus shedding was earlier in 3-week-old and 8-week-old pigs than in 23-week-old pigs. Prototype PEDV shedding levels in rectal swabs were significantly higher in 3-week-old pigs than in 8-week-old and 23-week-old pigs during 2-7 DPI. Fecal virus loads of prototype PEDV in 8-week-old pigs were significantly higher than in 23-week-old pigs at 10 DPI.

3. Viral shedding in oral fluids (Fig 3)
   1) Oral fluid shedding of PEDV in prototype and S-INDEL-inoculated 3-week-old pigs is summarized in Fig 3A. For prototype PEDV-inoculated pigs, 2/2, 2/2, 2/2, 2/2, 1/2, 2/2, and 0/2 ropes for oral fluids were PCR positive at 2, 4, 7, 10, 14, 21, and 28 DPI, respectively. Prototype PEDV shedding in oral fluids peaked to a titer of 10^9.1 genomic copies/ml at 2 DPI, lasted through at least 21 DPI, and became negative at 28 DPI. For S-INDEL PEDV-inoculated pigs, 2/2, 2/2, 2/2, 2/2, 2/2, 0/2, and 0/2 ropes for oral fluids had detectable PEDV RNA at 2, 4, 7, 10, 14, 21, and 28 DPI. S-INDEL PEDV shedding in oral fluids peaked to a titer of 10^7.4 genomic copies/ml at 4 DPI and gradually decreased to zero at 21 DPI. Prototype PEDV-inoculated pigs overall had higher level of virus shedding in oral fluids as compared to S-INDEL PEDV-inoculated pigs.
   2) Oral fluid shedding of PEDV in prototype and S-INDEL-inoculated 8-week-old pigs is summarized in Fig 3B. For prototype PEDV-inoculated pigs, 2/2 ropes for oral fluids were PCR positive during 2-21 DPI, and 1/2 rope was PCR positive at 28 DPI. Prototype PEDV shedding in oral fluids had a titer ranging from 10^6.3 to 10^7.6 genomic copies/ml during 4-10 DPI. For S-INDEL PEDV-inoculated pigs, 2/2 ropes for oral fluids were PCR positive during 2-14 DPI, 0/2 ropes were PCR positive at 21 DPI, 1/2 rope was PCR positive at 28 DPI. S-INDEL PEDV shedding in oral fluids had a titer ranging from 10^5.6 to 10^6.9 genomic copies/ml during 4-10 DPI. Prototype PEDV-inoculated pigs overall had similar viral shedding levels in oral fluids when compared to S-INDEL PEDV-inoculated pigs.
   3) Oral fluid shedding of PEDV in prototype and S-INDEL-inoculated 23-week-old pigs is summarized in Fig 3C. For prototype PEDV-inoculated pigs, 1/2, 2/2, 2/2, 2/2, 2/2, 1/2, and 0/2 ropes for oral fluids were PCR positive at 2, 4, 7, 10, 14, 21, and 28 DPI, respectively. Prototype PEDV shedding in oral fluids peaked to 10^7.2 genomic copies/ml at 7 DPI and then gradually declined to zero at 28 DPI. For S-INDEL PEDV-inoculated pigs, 0/2 ropes for oral fluids were PCR positive at 2, 4, 21 and 28 DPI, and 2/2 ropes were positive at 7, 10, and 14 DPI. S-INDEL PEDV shedding in oral fluids also peaked at 7 DPI to a titer of 10^7.8 genomic copies/ml and then gradually declined to zero at 21 DPI. The onset of virus shedding in oral fluids was earlier in prototype PEDV-inoculated pigs than in S-INDEL PEDV-inoculated pigs but during 7-28 DPI two groups of pigs had similar levels of virus shedding in oral fluids.
   4) None of the Neg Ctrl pigs shed virus in oral fluids throughout the study as seen in Fig 3D.
   5) Virus shedding in oral fluids of all ages of S-INDEL PEDV-inoculated pigs is shown in Fig 3E. The onset of virus shedding in oral fluids was earlier in 3-week-old and 8-week-old pigs than in 23-week-old pigs. S-INDEL PEDV shedding levels in oral fluids of 3-week-old pigs were significantly higher at 4 DPI when compared to 8-week-old and 23-week-old pigs. During 7-14 DPI, S-INDEL PEDV shedding levels in oral fluids were similar in 8-week-old and 23-week-old pigs.
   6) Virus shedding in oral fluids of all ages of prototype PEDV-inoculated pigs is summarized in Fig 3F. Prototype PEDV shedding levels in oral fluids were

significantly higher in 3-week-old pigs than in 8-week-old and 23-week-old pigs at 2-4 DPI. Prototype PEDV shedding levels in oral fluids were overall similar in 8-week-old and 23-week-old pigs.

4. Viral loads in serum samples (Fig 4)

Both prototype and S-INDEL PEDVs only caused transient (2-7 DPI) and low level viremia in 3-week-old (Fig 4A), 8-week-old (Fig 4B), and 23-week-old (Fig 4C) pigs. In 3-week-old pigs, only 3 to 4 out of 10 pigs inoculated with either prototype or S-INDEL PEDV developed transient viremia. The viremia of prototype PEDV and S-INDEL PEDV occurred at 2-4 DPI and the average viremia levels were similar (Fig 4A). In 8-week-old pigs, 2/10 pigs inoculated with prototype PEDV developed low level of viremia at 2 DPI and 2/10 pigs inoculated with S-INDEL PEDV developed low level of viremia at 7 DPI (Fig 4B). In 23-week-old pigs, 1/10 pig inoculated with prototype PEDV developed low level of viremia at 4 DPI; S-INDEL PEDV viremia was negligible (Fig 4C). When S-INDEL PEDV viremia was compared in pigs of different ages (Fig 4E), viremia occurred earlier in 3-week-old pigs than in 8-week-old pigs; viremia peaked at 2 DPI in 3-week-old pigs and at 7 DPI in 8-week-old pigs; viremia level was significantly lower in 23-week-old pigs than in 3-week-old and 8-week-old pigs. Prototype PEDV viremia was slightly higher in 3-week-old pigs than in 8-week-old and 23-week-old pigs (Fig 4F).

5. Virus distribution in tissues examined at 4 DPI (Table 2 and Fig 5)

1) Tissue PCR results from prototype and S-INDEL PEDV-inoculated 3-week-old pigs at 4 DPI is summarized in Table 2 and Fig 5A. Prototype PEDV RNA was detected from all (5/5) duodenums, jejunums, ileums, ceca, colons, and mesenteric lymph nodes with mean virus titers ranging from 10^6.5 to 10^8.3 genomic copies/ml. S-INDEL PEDV RNA was detected from all (5/5) jejunums and colons and from most (4/5) duodenums, ileum, ceca, and mesenteric lymph nodes (MLN) with mean virus titers ranging from 10^4.7 to 10^6.7 genomic copies/ml. Either virus could be detected in low quantities in the tonsil, heart, liver, spleen, diaphragm, shoulder, loin, and ham. The viral loads of prototype PEDV were numerically higher than S-INDEL PEDV in most tissues but significant differences were only observed in the cecum, MLN, stomach, tonsil, heart, and muscles (shoulder, loin and ham).
2) Tissue PCR results from prototype and S-INDEL PEDV-inoculated 8-week-old pigs at 4 DPI is summarized in Table 2 and Fig 5B. Prototype PEDV RNA was detected in duodenums, jejunums, ileums, ceca, colons, and mesenteric lymph nodes from 0-2 out of 5 pigs with very low level of viral loads. S-INDEL PEDV RNA was detected in duodenums, jejunums, ileums, ceca, colons, and mesenteric lymph nodes from 1-3 out of 5 pigs with low level of viral loads although the average viral loads were relatively higher than prototype PEDV. Either virus was hardly detected from the stomach, tonsil, heart, liver, spleen, kidney and diaphragm of 8-week-old pigs. S-INDEL PEDV RNA was hardly detected in shoulder, loin, and ham muscles. Prototype PEDV RNA was detected in shoulder, loin, and ham muscles from 2-5 out of 5 pigs with low quantities which, however, were higher than those of S-INDEL PEDV.
3) Tissue PCR results from prototype and S-INDEL PEDV-inoculated 23-week-old pigs at 4 DPI is summarized in Table 2 and Fig 5C. Prototype PEDV RNA was detected in duodenums, jejunums, ileums, ceca, colons, and mesenteric lymph nodes from 1-3 out of 5 pigs with very low level of viral loads. S-INDEL PEDV RNA was detected in duodenums, jejunums, ileums, ceca, colons, and mesenteric lymph nodes from 0-1 out of 5 pigs with very low level of viral

loads. Either virus was hardly detected from the stomach, tonsil, heart, liver, spleen, kidney, diaphragm, shoulder, loin, and ham muscles of 23-week-old pigs.

4) None of the negative control pigs had detectable virus in collected tissues as seen in Fig 5D.
5) Comparison of S-INDEL PEDV RNA quantity in various tissues of 3-week-old, 8-week-old, and 23-week-old pigs is shown in Fig 5E. 3-week-old pigs had significantly higher viral titers in enteric tissues (duodenum, jejunum, ileum, cecum, and colon) and mesenteric lymph nodes than 8-week-old and 23-week-old pigs. 8-week-old pigs had higher viral RNA quantities in enteric tissues and mesenteric lymph nodes than 23-week-old pigs. Differences were not distinct in other tissues (stomach, tonsil, heart, liver, spleen, kidney, diaphragm, shoulder, loin, and ham muscles) across 3 ages of pigs.
6) Comparison of prototype PEDV RNA quantity in various tissues of 3-week-old, 8-week-old, and 23-week-old pigs is shown in Fig 5F. 3-week-old pigs had significantly higher viral titers in enteric tissues (duodenum, jejunum, ileum, cecum, and colon) and mesenteric lymph nodes than 8-week-old and 23-week-old pigs. Differences were not distinct in other non-enteric tissues across 3 ages of pigs.

6. Immunohistochemistry examination

Immunohistochemistry (IHC) examination was conducted in various tissues of 3-week-old and 8-week-old pigs at 4 DPI and 23-week-old pigs at 9 DPI. The results are summarized in Table 3.

1) PEDV IHC positive staining was mainly detected in jejunum and ileum tissues, some positive staining was detected in cecum, colon, mesenteric lymph node, and duodenum tissues, and no positive staining was detected in non-enteric tissues (e.g. stomach, tonsil, heart, lung, liver, spleen, kidney, diaphragm, shoulder, loin, and ham).
2) IHC staining in jejunum and ileum was used to compare two PEDV strains and three ages of pigs (see below).
3) In 3-week-old pigs, both prototype and S-INDEL PEDV antigens were detected from most pigs but the average IHC scores were not significantly different.
4) In 8-week-old pigs, prototype PEDV antigen was not detected from any of five pigs whereas S-INDEL PEDV antigen was detected from 2/5 pigs.
5) In 23-week-old pigs, neither prototype nor S-INDEL PEDV antigen was detected.
6) S-INDEL PEDV antigen was detected from 4/5 pigs of 3-week age and 2/5 pigs of 8-week age, but was not detected from any 23-week-old pigs.
7) Prototype PEDV antigen was detected from 5/5 pigs of 3-week age, but was not detected from any 8-week-old or 23-week-old pigs.

Objective 2. Evaluation of the antibody response of U.S. prototype and S-INDEL PEDV strains in 3-week-old pigs, 8-week-old pigs, and 23-week-old-pigs.

In this 3 x 3 study (Neg Ctrl, prototype PEDV, S-INDEL PEDV x 3-week-old pigs, 8-week-old pigs, 23-week-old pigs), there were totally nine groups. The serum samples from five pigs in each group that went through 0-28 DPI were tested for PEDV neutralizing antibody by VN assay, and for serum IgG and serum IgA by PEDV FMIA assay. In addition, the oral fluid samples collected from each group during 0-28 DPI (two oral fluids for each group at each time point) were tested for PEDV IgG and IgA by PEDV FMIA assay.

1. PEDV neutralizing antibody responses in serum samples (Fig 6)

1) PEDV neutralizing antibody titers in serum samples of prototype and S-INDEL PEDV-inoculated 3-week-old pigs are summarized in Fig 6A. Both

prototype and S-INDEL PEDV-inoculated pigs developed neutralizing antibodies from 7 DPI and lasted through 28 DPI. The VN antibodies were similar between two viruses during 7-14 DPI but were significantly higher in prototype PEDV-inoculated pigs at 21-28 DPI.

2) PEDV neutralizing antibody titers in serum samples of prototype and S-INDEL PEDV-inoculated 8-week-old pigs are summarized in Fig 6B. Neutralizing antibodies were detected from 5/5 prototype PEDV-inoculated pigs and from 4/5 S-INDEL PEDV-inoculated pigs during 7-28 DPI. The VN antibodies were similar between two viruses for the duration of the study.
3) PEDV neutralizing antibody titers in serum samples of prototype and S-INDEL PEDV-inoculated 23-week-old pigs are summarized in Fig 6C. It was found that 4/5 prototype PEDV-inoculated pigs and 2/5 S-INDEL PEDV-inoculated pigs consistently developed neutralizing antibodies during 7-28 DPI. Both groups overall had similar average neutralizing antibody titers throughout the study except at 14 and 21 DPI.
4) None of the negative control pigs had positive PEDV neutralizing antibody (Fig 6D).
5) For S-INDEL PEDV-inoculated pigs, the average neutralizing antibody titers were higher in 3-week-old pigs than in 8-week-old and 23-week-old pigs at 14 DPI, whereas the average neutralizing antibody titers were higher in 8-week-old pigs than in 3-week-old and 23-week-old pigs at 21-28 DPI (Fig 6E).
6) For prototype PEDV-inoculated pigs, the average neutralizing antibody titers were overall similar in 3 ages of pigs for the duration of the study except that the antibody titers in 23-week-old pigs were lower than 3-week-old and 8-week-old pigs at 14 DPI (Fig 6F).

2. Serum IgG and IgA antibody responses as measured by PEDV FMIA assay (Fig 7 and Fig 8)
   1) In 3-week-old pigs, 5/5 of prototype and 3/5 of S-INDEL PEDV-inoculated pigs developed PEDV IgG antibody in serum samples. The average serum PEDV IgG antibody titers were significantly higher in prototype than S-INDEL PEDV-inoculated pigs (Fig 7A). In 3-week-old pigs, 5/5 of prototype and 1/5 of S-INDEL PEDV-inoculated pigs developed PEDV IgA antibody in serum samples. The average serum PEDV IgA antibody titers were significantly higher in prototype than S-INDEL PEDV-inoculated pigs (Fig 7D). However, the serum IgA antibody titers were lower than serum IgG antibody titers (Fig 7A and D).
   2) In 8-week-old pigs, 5/5 of prototype and 4/5 of S-INDEL PEDV-inoculated pigs developed PEDV IgG antibody in serum samples. The average serum PEDV IgG antibody titers were significantly higher in prototype than S-INDEL PEDV-inoculated pigs at 14-21 DPI (Fig 7B). In 8-week-old pigs, 2/5 of prototype and 4/5 of S-INDEL PEDV-inoculated pigs developed PEDV IgA antibody in serum samples. The average serum PEDV IgA antibody titers were significantly higher in S-INDEL than prototype PEDV-inoculated pigs (Fig 7E).
   3) In 23-week-old pigs, 4/5 of prototype and 0/5 of S-INDEL PEDV-inoculated pigs developed PEDV IgG antibody in serum samples. The average serum PEDV IgG antibody titers were significantly higher in prototype than S-INDEL PEDV-inoculated pigs (Fig 7C). In 23-week-old pigs, 4/5 of prototype and 2/5 of S-INDEL PEDV-inoculated pigs developed PEDV IgA antibody in serum samples. The average serum PEDV IgA antibody titers were similar in S-INDEL and prototype PEDV-inoculated pigs (Fig 7F).
   4) PEDV IgG and IgA antibodies were not detected from any serum samples of negative control groups regardless of pig ages (Fig 8A and D).

5) For S-INDEL PEDV-inoculated pigs, serum PEDV IgG was detected in 3/5 of 3-week-old pigs, 4/5 of 8-week-old pigs, and 0/5 of 23-week-old pigs. The average serum PEDV IgG antibody levels were significantly higher in 8-week-old pigs than in 3-week-old pigs followed by 23-week-old pigs (Fig 8B). For S-INDEL PEDV-inoculated pigs, serum PEDV IgA was detected in 1/5 of 3-week-old pigs, 4/5 of 8-week-old pigs, and 2/5 of 23-week-old pigs. The average serum PEDV IgA antibody levels were significantly higher in 8-week-old pigs than in 23-week-old pigs followed by 3-week-old pigs (Fig 8E).
6) For prototype PEDV-inoculated pigs, serum PEDV IgG was detected in 5/5 of 3-week-old pigs, 5/5 of 8-week-old pigs, and 4/5 of 23-week-old pigs. The average serum PEDV IgG antibody levels were similar between 8-week-old and 23-week-old pigs, both of which were significantly lower than in 3-week-old pigs (Fig 8C). For prototype PEDV-inoculated pigs, serum PEDV IgA was detected in 5/5 of 3-week-old pigs, 2/5 of 8-week-old pigs, and 4/5 of 23-week-old pigs. The average serum PEDV IgA antibody levels were overall similar across three ages of pigs during the study (Fig 8F). The serum IgA antibody titers were lower than serum IgG antibody titers (Fig 8C and F).

3. Oral fluid IgG and IgA antibody responses as measured by PEDV FMIA assay (Fig 9 and Fig 10)
1) In 3-week-old pigs, PEDV IgG antibody was not detected from any oral fluid samples collected from either prototype or S-INDEL PEDV-inoculated pigs (Fig 9A). In 3-week-old pigs, PEDV IgA antibody was consistently detected from oral fluid samples collected from either prototype or S-INDEL PEDV-inoculated pigs. The average oral fluid PEDV IgA antibody titers were significantly higher in prototype than S-INDEL PEDV-inoculated pigs (Fig 9D).
2) In 8-week-old pigs, PEDV IgG antibody was detected from prototype PEDV-inoculated pigs only at 28 DPI whereas from S-INDEL PEDV-inoculated pigs during 14-28 DPI (Fig 9B). In 8-week-old pigs, PEDV IgA antibody was consistently detected from oral fluid samples collected from either prototype or S-INDEL PEDV-inoculated pigs. The average oral fluid PEDV IgA antibody titers were significantly higher in S-INDEL than prototype PEDV-inoculated pigs (Fig 9E).
3) In 23-week-old pigs, PEDV IgG antibody was consistently detected from prototype and S-INDEL PEDV-inoculated pigs during 14-28 DPI. The average oral fluid PEDV IgG antibody titers were significantly higher in prototype than S-INDEL PEDV-inoculated pigs (Fig 9C). In 23-week-old pigs, PEDV IgA antibody was consistently detected from oral fluid samples collected from either prototype or S-INDEL PEDV-inoculated pigs. The average oral fluid PEDV IgA antibody titers were significantly higher in prototype than S-INDEL PEDV-inoculated pigs (Fig 9F).
4) PEDV IgG and IgA antibodies were not detected from any oral fluid samples of negative control groups regardless of pig ages (Fig 10A and D).
5) For S-INDEL PEDV-inoculated pigs, PEDV IgG was detected in oral fluids collected from 8-week-old and 23-week-old pigs but not from 3-week-old pigs. The average oral fluid PEDV IgG antibody levels were significantly higher in 23-week-old pigs than in 8-week-old pigs followed by 3-week-old pigs (Fig 10B). For S-INDEL PEDV-inoculated pigs, PEDV IgA was detected in oral fluids from all three ages of pigs. The average oral fluid PEDV IgA antibody levels were significantly higher in 8-week-old pigs than in 23-week-old pigs followed by 3-week-old pigs (Fig 10E).
6) For prototype PEDV-inoculated pigs, PEDV IgG was detected in oral fluids from 8-week-old and 23-week-old pigs but not from 3-week-old pigs. The average oral fluid PEDV IgG antibody levels were significantly higher in 23-week-old pigs than in 8-week-old and 3-week-old pigs (Fig 10C). For prototype PEDV-inoculated pigs, oral fluid PEDV IgA was detected in oral fluids from all three

ages of pigs. The average oral fluid PEDV IgA antibody levels were similar between 3-week-old and 8-week-old pigs but were higher in 23-week-old pigs (Fig 10F).

Discussion: Explain your research results and include a summary of the results that is of immediate or future benefit to pork producers.

Previous experimental infection studies have demonstrated that the U.S. S-INDEL PEDV isolates overall had lower pathogenicity than the U.S. prototype PEDV isolates in conventional neonatal piglets at 3-4 days of age or 5-6 days of age (11, 20). However, differences in pathogenicity and antibody responses of these two PEDV isolates in pigs of older ages have not been investigated. In this study, we compared infection outcomes of U.S. prototype PEDV and S-INDEL PEDV in three ages of conventional pigs: 3-week-old or "weaned', 8-week-old or "grower", and 23-week-old or "finisher" pigs.

Mild to watery diarrhea was observed in prototype PEDV-inoculated 3-week-old pigs in the first week post inoculation. Mild diarrhea was observed in S-INDEL PEDV-inoculated 3-week-old pigs in the first week. No apparent diarrhea or other clinical signs were observed in 8-week-old and 23-week-old pigs inoculated with either PEDV strain. These suggest that clinical presentations of PEDV infection were pig age dependent, with more severe outcomes in younger pigs.

Previous studies indicated that prototype PEDV infection can have adverse effect on average daily gain (ADG) of weight during 0-3 DPI and 0-7 DPI in 5-day-old pigs (11) and during 0-7 DPI in 3-week-old pigs (23), while S-INDEL PEDV infection had adverse effect on ADG only during 0-3 DPI instead of 0-7 DPI in 5-day-old pigs (12). In the current study, adverse effect on ADG was only observed in prototype PEDV-inoculated 3-week-old pigs (10 pigs/group) during 0-4 DPI and was not observed in other groups regardless of virus strain and pig ages. The data suggest that PEDV infection may not have significant adverse impact on ADG in "weaned", "grower" and "finisher" pigs.

In order to further assess the pathogenicity differences of U.S. prototype and S-INDEL PEDVs in "weaned", "grower", and "finisher" pigs, parameters such as virus shedding in rectal swabs and oral fluids, virus load in various tissues, and histopathological & IHC examinations were compared.

In 3-week-old pigs, prototype PEDV-inoculated pigs had longer duration of fecal shedding and significantly higher fecal virus load at some time points, compared to S-INDEL PEDV. In addition, prototype PEDV-inoculated pigs overall had higher level of virus shedding in oral fluids as compared to S-INDEL PEDV-inoculated pigs. In 8-week-old pigs, S-INDEL PEDV had longer duration of fecal shedding than prototype PEDV; S-INDEL fecal virus load was significantly higher than prototype PEDV at 7 DPI and 14 DPI but was opposite at 10 DPI. Prototype PEDV-inoculated pigs overall had similar viral shedding levels in oral fluids when compared to S-INDEL PEDV-inoculated pigs. In 23-week-old pigs, the onset of prototype PEDV shedding in rectal swabs was earlier than S-INDEL PEDV and prototype PEDV had higher fecal virus load than S-INDEL PEDV. The onset of virus shedding in oral fluids was earlier in prototype than S-INDEL PEDV-inoculated pigs but during 7-28 DPI two groups of pigs had similar levels of virus shedding in oral fluids. Virus load, microscopic changes, and IHC scores in tissues collected at 4 DPI necropsy trended similarly to fecal virus shedding results. Taken together, these data suggest that U.S. prototype PEDV appeared to be more pathogenic than S-INDEL PEDV in both 3-week-old and 23-week-old pigs. Interestingly, the pathogenicity difference of the two PEDV strains was less distinct in 8-week-old pigs.

When prototype PEDV was compared in three ages of pigs, the onset of fecal virus shedding was earlier in 3-week-old and 8-week-old pigs than in 23-week-old pigs. Prototype PEDV shedding levels in rectal swabs were significantly higher in 3-week-old pigs than in 8-week-old and 23-week-old pigs during 2-7 DPI. Fecal virus loads of prototype PEDV in 8-week-old pigs were significantly higher than in 23-week-old pigs at 10 DPI. Prototype PEDV shedding levels in oral fluids were significantly higher in 3-week-old pigs than in 8-week-old and 23-week-old pigs at 2-4 DPI. Prototype PEDV shedding levels in oral fluids were overall similar in 8-week-old and 23-week-old pigs. Prototype PEDV viral loads in enteric tissues and mesenteric lymph nodes were significantly higher in 3-week-old pigs than in 8-week-old and 23-week-old pigs. Taken collectively, these data suggest that pathogenicity of U.S. prototype PEDV is pig age-dependent with more severe outcomes in younger pigs: “weaned” > “grower” > “finisher” pigs.

When S-INDEL PEDV was compared in three ages of pigs, the onset of fecal virus shedding was earlier in 3-week-old and 8-week-old pigs than in 23-week-old pigs. S-INDEL PEDV shedding levels in rectal swabs were significantly higher in 3-week-old pigs than in 8-week-old and 23-week-old pigs at 2-4 DPI. Fecal virus loads of S-INDEL PEDV at 7 DPI were similar between 3-week-old and 8-week-old pigs but were both significantly higher than 23-week-old pigs. The duration of S-INDEL PEDV shedding in rectal swabs was longer in 8-week-old pigs than in 3-week-old and 23-week-old pigs. The onset of virus shedding in oral fluids was earlier in 3-week-old and 8-week-old pigs than in 23-week-old pigs. S-INDEL PEDV shedding levels in oral fluids of 3-week-old pigs were significantly higher at 4 DPI when compared to 8-week-old and 23-week-old pigs. During 7-14 DPI, S-INDEL PEDV shedding levels in oral fluids were similar in 8-week-old and 23-week-old pigs. S-INDEL PEDV viral loads in enteric tissues and mesenteric lymph nodes were significantly higher in 3-week-old pigs than in 8-week-old pigs followed by 23-week-old pigs. Taken together, these data suggest that pathogenicity of S-INDEL PEDV is also pig age-dependent with more severe outcomes in younger pigs.

Both rectal swabs and oral fluid samples have been commonly used to test PEDV shedding. Based on the PEDV PCR shedding data in this study, it was found that duration of virus shedding was overall longer in oral fluids than in rectal swabs, and the proportion of PCR positive oral fluid specimens was higher than rectal swabs at multiple time points, regardless of PEDV strains and pig ages. In addition, there were variations of virus shedding status in rectal swabs among pigs (both virus amount and percentage of positive samples) even though the pigs were exposed to the same virus simultaneously. Therefore, it is advantageous to use oral fluid samples instead of individual rectal swabs if the purpose is to monitor the PEDV shedding in the whole population. Surely, viral shedding in oral fluid samples cannot reliably reflect the shedding status on individual pigs. Rectal swabs will still be the choice if it is important to assess viral shedding status in individual pigs.

It is also important to understand the antibody responses induced by each PEDV strain in pigs of different ages. In this study, serum virus neutralizing antibody, serum PEDV IgG and IgA antibodies, and oral fluid PEDV IgG and IgA antibodies were determined and compared. However, previous studies indicated that the FMIA assay based on prototype PEDV S1 protein and PEDV whole virus ELISA are more reliable for measuring serum IgG and oral fluid IgA (28, 29). Therefore, the discussion here focuses on serum neutralizing antibodies, serum PEDV IgG and oral fluid PEDV IgA antibodies.

The neutralizing antibody induced by prototype PEDV inoculation was overall higher than that induced by S-INDEL PEDV, in both 3-week-old and 23-week-old pigs. In contrast, neutralizing antibodies induced by two viruses were not significantly different in 8-week-old pigs. In prototype PEDV-inoculated pigs, the average neutralizing antibody titers were overall similar in 3 ages of pigs for the duration of the study except that the antibody titers

in 23-week-old pigs were lower than 3-week-old and 8-week-old pigs at 14 DPI. In S-INDEL PEDV-inoculated pigs, the average neutralizing antibody titers were higher in 3-week-old pigs than in 8-week-old and 23-week-old pigs at 14 DPI, whereas higher in 8-week-old pigs than in 3-week-old and 23-week-old pigs at 21-28 DPI.

Prototype PEDV inoculation consistently induced significantly higher level of serum IgG antibody than S-INDEL PEDV in all of the three ages of pigs examined. For pigs inoculated with prototype PEDV, the magnitude of serum IgG antibody was significantly higher in 3-week-old pigs than in 8-week-old and 23-week-old pigs whereas there was no significant difference between 8-week-old and 23-week-old pigs. For pigs inoculated with S-INDEL PEDV, the magnitude of serum IgG antibody was significantly higher in 8-week-old pigs than in 3-week-old pigs followed by 23-week-old pigs.

Prototype PEDV inoculation induced significantly higher level of oral fluid IgA antibody than S-INDEL PEDV in 3-week-old and 23-week-old pigs. In contrast, S-INDEL PEDV inoculation induced significantly higher level of oral fluid IgA antibody than prototype PEDV in 8-week-old pigs. For pigs inoculated with prototype PEDV, the magnitude of oral fluid IgA antibody was significantly higher in 23-week-old pigs than in 8-week-old and 3-week-old pigs. For pigs inoculated with S-INDEL PEDV, the magnitude of oral fluid antibody was significantly higher in 8-week-old pigs than in 23-week-old pigs followed by 3-week-old pigs.

Neutralizing antibody, serum IgG and oral fluid IgA responses clearly suggest that prototype PEDV induced greater antibody responses than S-INDEL PEDV in both “weaned” and “finisher” pigs, while the difference of antibody responses induced by two PEDV strains in “grower” pigs was not a clear cut and it depends on antibody assay. When the antibody responses induced by prototype PEDV were compared in three ages of pigs, differences were not always consistent; for example, similar neutralizing antibody responses in three age groups, stronger serum IgG responses in “weaned” pigs than in “grower” and “finisher” pigs, and stronger oral fluid IgA responses in “finisher” pigs than in “grower” and “weaned” pigs. Interestingly, S-INDEL PEDV appeared to consistently induce stronger antibody responses in “grower” pigs than in “weaned” and “finisher” pigs. It is noteworthy that all of the serum samples were tested for neutralizing antibody using a prototype PEDV isolate as the indicator virus in this study. The PEDV FMIA assay was based on prototype PEDV S1 protein. It remains to be investigated whether different antibody results will be obtained if S-INDEL PEDV is used as an indicator virus in VN test and if S-INDEL PEDV S1 protein in used in PEDV FMIA assay.

In summary, this study investigated the pathogenicity and antibody responses of two PEDV strains (U.S. prototype PEDV and S-INDEL PEDV) in three ages of pigs (“weaned”, “grower”, and “finisher” pigs). Pathogenicity of PEDV appears to be both pig age dependent (more severe in younger pigs) and virus strain-dependent. Prototype PEDV appeared to be more pathogenic than S-INDEL PEDV in “weaned” and “finisher” pigs, but pathogenicity difference of two virus strains was less distinct in “grower” pigs. Antibody responses also appear to be virus strain-dependent and pig age dependent. These data provide some guidance on selecting appropriate PEDV strain to induce antibody response in different age of pigs. It is noted that different antibody assays may not always give the consistent results. For PEDV, mucosal immunity is critical for protection. However, at this point, it is unclear which antibody assay can better reflect mucosal and protective immunity although secretory IgA in small intestines is believed to be associated with mucosal and protective immunity. Nonetheless, it is not easy to routinely measure secretory IgA in small intestines. Work is in progress to assess the correlation of PEDV neutralizing

antibody, serum IgG, and oral fluid IgA with secretory IgA in small intestines with the hope of finding an antibody assay that can be routinely conducted to reflect mucosal immunity.

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Table 1. Experimental Design

Group (number of pigs)	**Pig Age**	**0 DPI (Inoculation)***	**4 DPI (first necropsy)ǂ**	**28 DPI (second necropsy)**
G1 (N=10)	3 weeks old	Prototype PEDV; 10^4 TCID50/ml; 10 ml	N=5	N=5
G2 (N=10)	3 weeks old	S INDEL PEDV; 10^4 TCID50/ml; 10 ml	N=5	N=5
G3 (N=10)	3 weeks old	Virus-negative medium; 10 ml	N=5	N=5
G4 (N=10)	8 weeks old	Prototype PEDV; 10^4 TCID50/ml; 10 ml	N=5	N=5
G5 (N=10)	8 weeks old	S INDEL PEDV; 10^4 TCID50/ml; 10 ml	N=5	N=5
G6 (N=10)	8 weeks old	Virus-negative medium; 10 ml	N=5	N=5
G7 (N=10)	23 weeks old	Prototype PEDV; 10^4 TCID50/ml; 10 ml	N=5	N=5
G8 (N=10)	23 weeks old	S INDEL PEDV; 10^4 TCID50/ml; 10 ml	N=5	N=5
G9 (N=10)	23 weeks old	Virus-negative medium; 10 ml	N=5	N=5

* Prototype PEDV strain: USA/IN19338/2013 P9; S INDEL strain: USA/IL20697/2014 P9.

ǂ The first necropsy was conducted on 4 DPI for the 3-week-old and 8-week-old pig groups and on 9 DPI for the 23-week-old pig groups.

Table 2. Summary of PEDV PCR on various tissues of PEDV S-INDEL, Prototype-inoculated and negative control pigs at 4 or 9 days post inoculation (DPI)*

Tissue	3-week-old pigs			8-week-old pigs			23-week-old pigs		
	Neg	S-INDEL	Prototype	Neg	S-INDEL	Prototype	Neg	S-INDEL	Prototype
	PCR positive and Mean Ct†			PCR positive and Mean Ct†			PCR positive and Mean Ct†		
Duodenum	0/5; Ct>37	4/5; Ct 27.1	5/5; Ct 25.2	0/5; Ct>37	2/5; Ct 21.6	0/5; Ct>37	0/5; Ct>37	1/5; Ct 28.3	1/5; Ct 33.0
Jejunum	0/5; Ct>37	5/5; Ct 24.5	5/5; Ct 19.0	0/5; Ct>37	3/5; Ct 27.9	0/5; Ct>37	0/5; Ct>37	1/5: Ct 31.8	2/5: Ct 26.8
Ileum	0/5; Ct>37	4/5; Ct 19.0	5/5; Ct 20.1	0/5; Ct>37	2/5; Ct 26.3	1/5; Ct 35.1	0/5; Ct>37	1/5: Ct 29.8	3/5: Ct 26.7
Cecum	0/5; Ct>37	4/5; Ct 24.0	5/5; Ct 20.7	0/5; Ct>37	2/5; Ct 29.4	0/5; Ct>37	0/5; Ct>37	0/5: Ct>37	2/5: Ct 35.0
Colon	0/5; Ct>37	5/5; Ct 24.2	5/5; Ct 20.2	0/5; Ct>37	2/5; Ct 26.4	0/5; Ct>37	0/5; Ct>37	1/5: Ct 32.8	1/5: Ct 34.1
MLN	0/5; Ct>37	4/5; Ct 27.5	5/5; Ct 23.7	0/5; Ct>37	1/5; Ct 24.6	2/5; Ct 34.2	0/5; Ct>37	1/5: Ct 28.3	2/5: Ct 25.8
Stomach	0/5; Ct>37	0/5; Ct>37	3/5; Ct 34.3	0/5; Ct>37	0/5; Ct>37	0/5; Ct>37	0/5; Ct>37	0/5: Ct>37	0/5: Ct>37
Tonsil	0/5; Ct>37	0/5; Ct>37	5/5; Ct 33.3	0/5; Ct>37	0/5; Ct>37	0/5; Ct>37	0/5; Ct>37	0/5: Ct>37	1/5: Ct 30.4
Heart	0/5; Ct>37	0/5; Ct>37	1/5; Ct 36.7	0/5; Ct>37	0/5; Ct>37	0/5; Ct>37	0/5; Ct>37	0/5: Ct>37	0/5: Ct>37
Lung	0/5; Ct>37	0/5; Ct>37	0/5; Ct>37	0/5; Ct>37	0/5; Ct>37	0/5; Ct>37	0/5; Ct>37	0/5: Ct>37	1/5: Ct 36.7
Liver	0/5; Ct>37	2/5; Ct 35.1	2/5; Ct 35.5	0/5; Ct>37	1/5; Ct 33.2	0/5; Ct>37	0/5; Ct>37	0/5: Ct>37	0/5: Ct>37
Spleen	0/5; Ct>37	3/5; Ct 33.3	4/5; Ct 31.8	0/5; Ct>37	1/5; Ct 36.0	0/5; Ct>37	0/5; Ct>37	1/5: Ct 36.6	2/5: Ct 30.0
Kidney	0/5; Ct>37	0/5; Ct>37	1/5; Ct 35.0	0/5; Ct>37	0/5; Ct>37	0/5; Ct>37	0/5; Ct>37	0/5: Ct>37	0/5: Ct>37
Diaphram	0/5; Ct>37	0/5; Ct>37	1/5; Ct 36.3	0/5; Ct>37	0/5; Ct>37	0/5; Ct>37	0/5; Ct>37	0/5: Ct>37	1/5: Ct 35.4
Shoulder muscle	0/5; Ct>37	2/5; Ct 36.5	5/5; Ct 31.9	0/5; Ct>37	0/5; Ct>37	3/5; Ct 33.1	0/5; Ct>37	0/5: Ct>37	0/5: Ct>37
Loin muscle	0/5; Ct>37	1/5; Ct 36.5	5/5; Ct 30.6	0/5; Ct>37	1/5; Ct 36.4	5/5; Ct 33.7	0/5; Ct>37	0/5: Ct>37	0/5: Ct>37
Ham muscle	0/5; Ct>37	1/5; Ct 36.8	5/5; Ct 31.0	0/5; Ct>37	0/5; Ct>37	2/5; Ct 36.7	0/5; Ct>37	0/5: Ct>37	0/5: Ct>37

*Five pigs from each group of 3-week-old and 8-week-old pigs were euthanized at 4 DPI. Five pigs from each group of 23-week-old pigs were euthanized at 9 DPI.

†Mean Ct was mean Ct value of PCR-positive pigs (pigs with PCR Ct<37).

Table 3. PEDV PCR on small intestines and IHC staining on various tissues of PEDV S-INDEL, Prototype-inoculated and negative control pigs at 4 or 9 days post inoculation (DPI)

Inoculum group	Pig age	Necropsy (DPI)	PCR on enteric tissues and MLN			IHC positive pigs (Average positive IHC scores)						
			PCR positive	Ct range of Pos	Mean Ct of Pos	Duodenum	Jejunum	Ileum	Cecum	Colon	Mesenteric lymph node (MLN)	Various tissues*
Control	3-week-old	4	0/5	>37	>37	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)
	8-week-old	4	0/5	>37	>37	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)
	23-week-old	9	0/5	>37	>37	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)
S-INDEL	3-week-old	4	5/5	17.2 - 33.6	24.4	0/5 (0)	4/5 (1.5)	4/5 (2.8)	0/5 (0)	2/5 (0.8)	3/5 (0.5)	0/5 (0)
	8-week-old	4	3/5	20.2 - 36.5	26.0	0/5 (0)	1/5 (1.0)	1/5 (4.0)	0/5 (0)	1/5 (1.0)	0/5 (0)	0/5 (0)
	23-week-old	9	1/5	28.3 - 32.8	30.2	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)
Prototype	3-week-old	4	5/5	18.0 - 30.0	21.5	1/5 (2.0)	5/5 (3.0)	5/5 (3.0)	3/5 (0.5)	1/5 (1.0)	5/5 (0.5)	0/5 (0)
	8-week-old	4	3/5	33.1 - 35.2	34.6	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)
	23-week-old	9	3/5	24.4 - 36.2	30.2	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)

* IHC was conducted on various tissues individually and these tissues include the shoulder muscle, loin muscle, ham muscle, stomach, tonsil, heart, lung, liver, spleen, kidney, and diaphragm.

PCR Ct < 37 is considered positive.

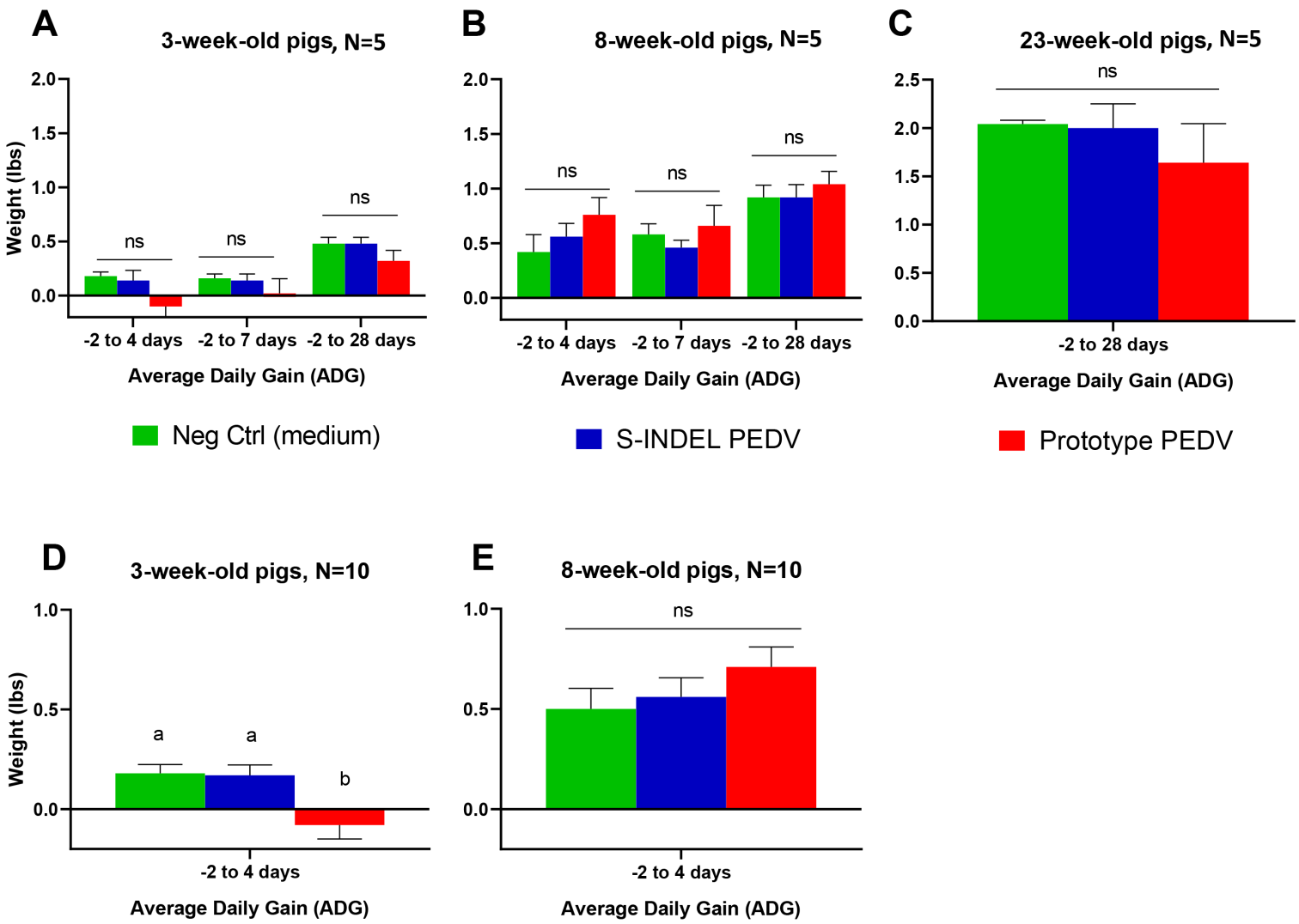

Fig 1. Average daily gain (ADG) of weight. Five pigs in each group (Neg Ctrl, S-INDEL PEDV, and Prototype PEDV) going through 28 DPI were used to calculate ADG in the interval of -2 to 4 DPI, -2 to 7 DPI and/or -2 to 28 DPI, as shown in A, B and C. For 3-week-old and 8-week-old pigs, ADG in the interval of -2 to 4 DPI based on 10 pigs per group (5 pigs/group were euthanized at 4 DPI) was also calculated (D, E).

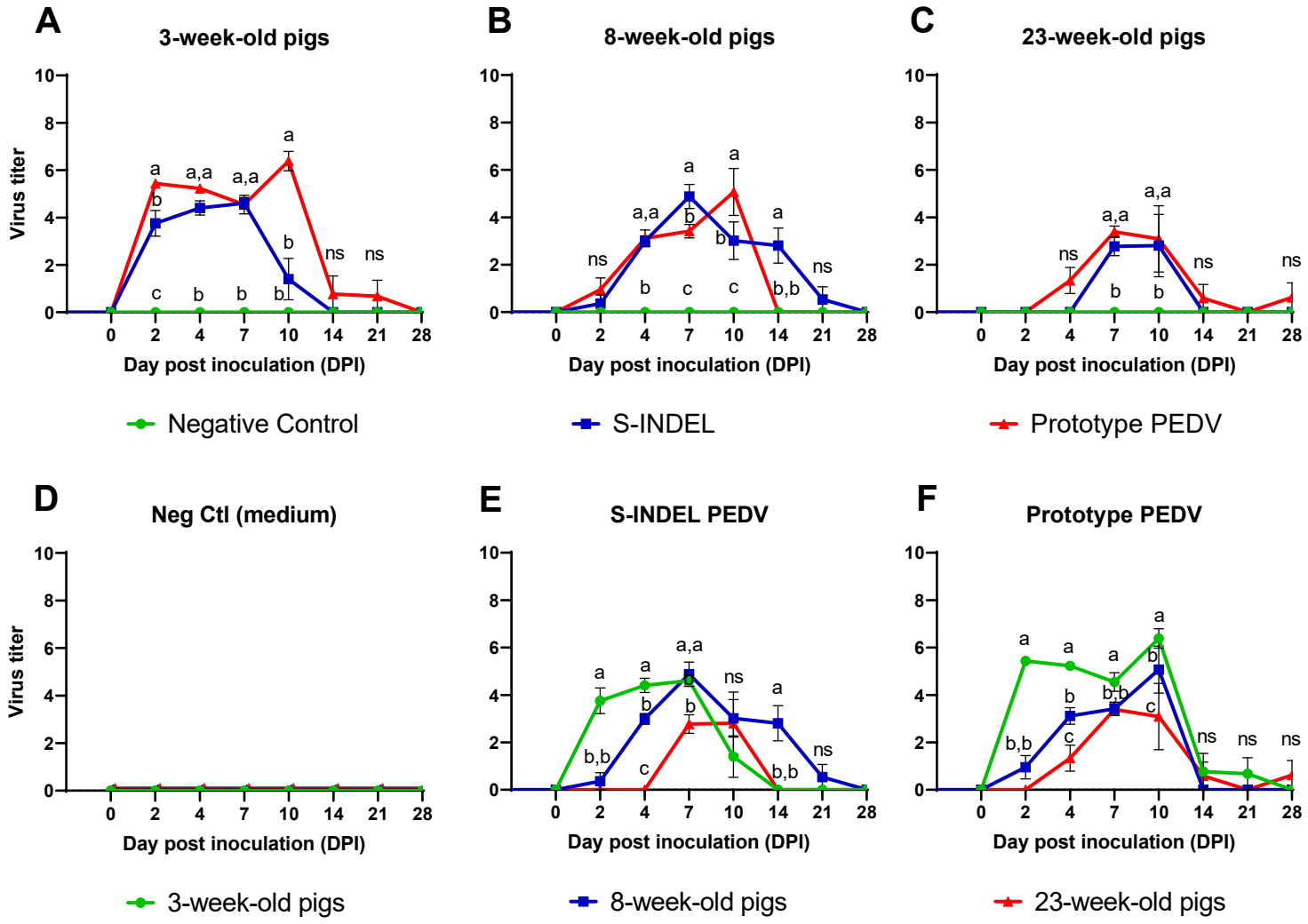

Fig 2. Virus shedding in rectal swabs of pigs at different ages inoculated with different US PEDV strains. Virus load was determined by a quantitative PEDV N gene-based real-time RT-PCR. The virus titers (log_{10}[genomic copies/ml]) at each time point were the mean values of all available pigs (both PCR-positive and PCR-negative pigs) in each group. The virus shedding was plotted in the category of pig ages (A: 3-week-old pigs, B: 8-week-old pigs, and C: 23-week-old pigs) and inoculum (D: Neg Ctrl [culture medium], E: S-INDEL PEDV, and F: Prototype PEDV), respectively. Standard error bars are shown in each figure. Statistical analysis was performed between different groups at each time point.

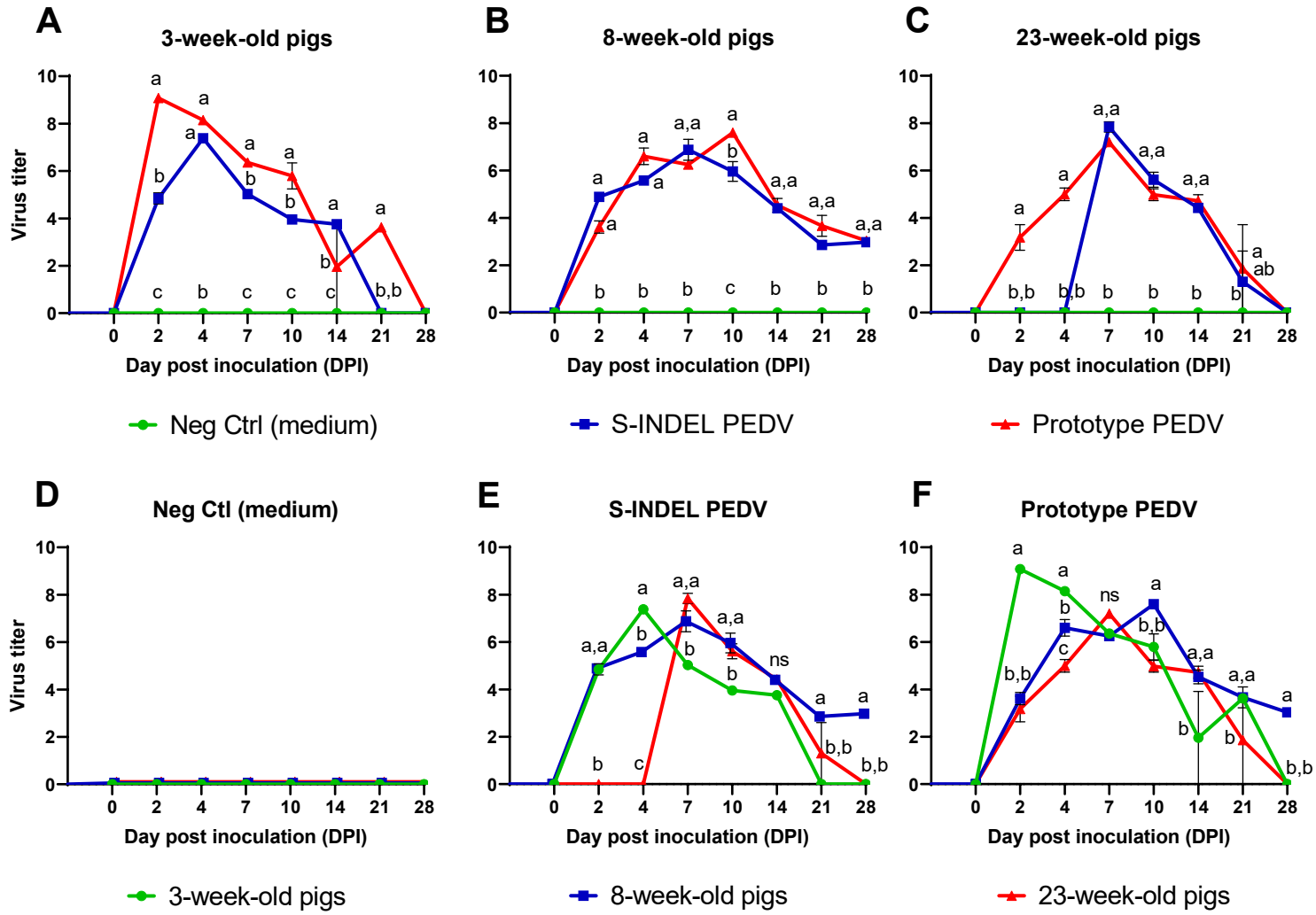

Fig 3. Virus shedding in oral fluids of pigs at different ages inoculated with different US PEDV strains. Virus load was determined by a quantitative PEDV N gene-based real-time RT-PCR. The virus titers (log_{10}[genomic copies/ml]) at each time point were the mean values of all available pigs (both PCR-positive and PCR-negative pigs) in each group. The virus shedding was plotted in the category of pig ages (A: 3-week-old pigs, B: 8-week-old pigs, and C: 23-week-old pigs) and inoculum (D: Neg Ctrl [culture medium], E: S-INDEL PEDV, and F: Prototype PEDV), respectively. Standard error bars are shown in each figure. Statistical analysis was performed between different groups at each time point.

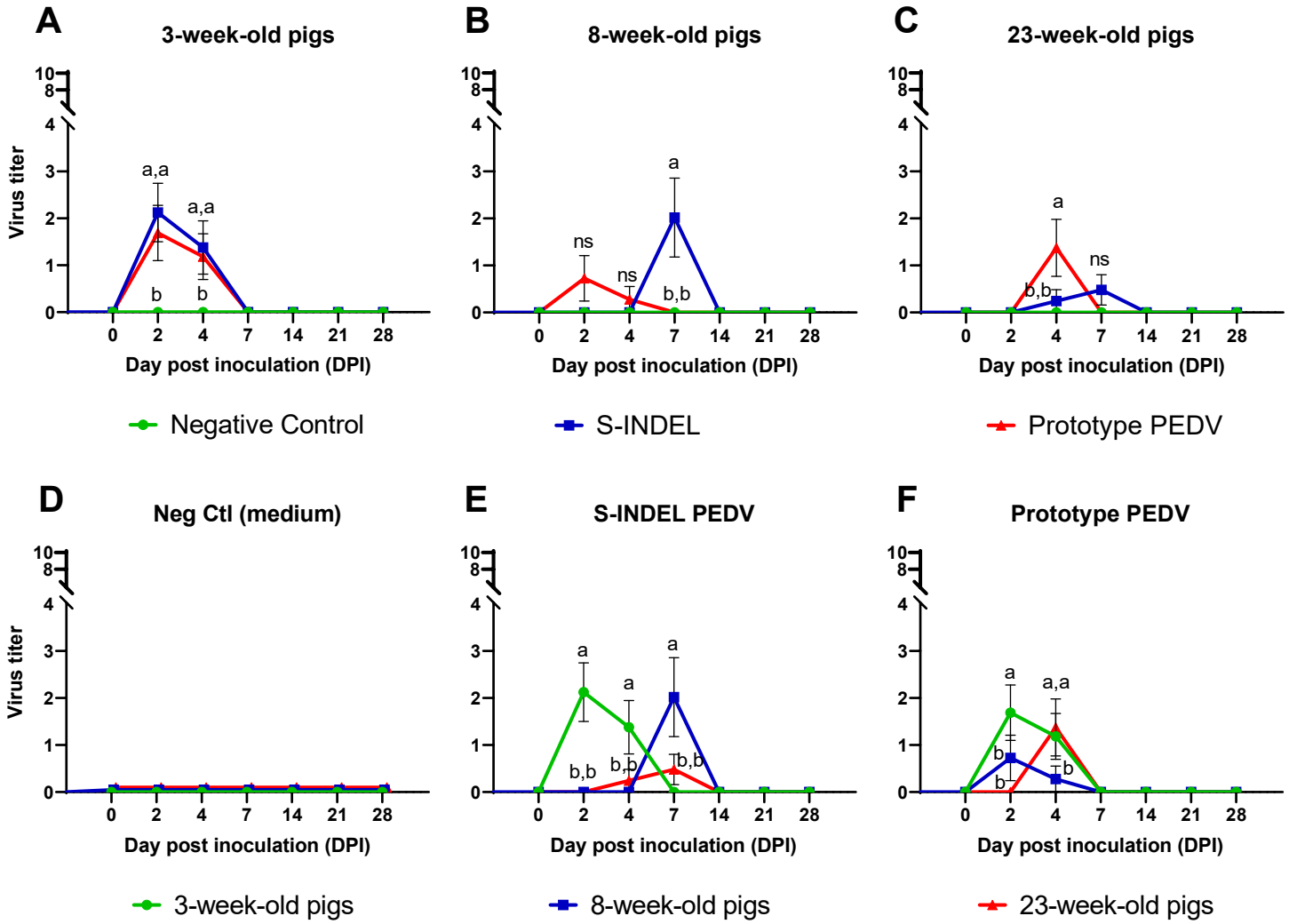

Fig 4. Virus loads in serum samples of pigs at different ages inoculated with different US PEDV strains. Virus load was determined by a quantitative PEDV N gene-based real-time RT-PCR. The virus titers (log_{10}[genomic copies/ml]) at each time point were the mean values of all available pigs (both PCR-positive and PCR-negative pigs) in each group. The virus shedding was plotted in the category of pig ages (A: 3-week-old pigs, B: 8-week-old pigs, and C: 23-week-old pigs) and inoculum (D: Neg Ctrl [culture medium], E: S-INDEL PEDV, and F: Prototype PEDV), respectively. Standard error bars are shown in each figure. Statistical analysis was performed between different groups at each time point.

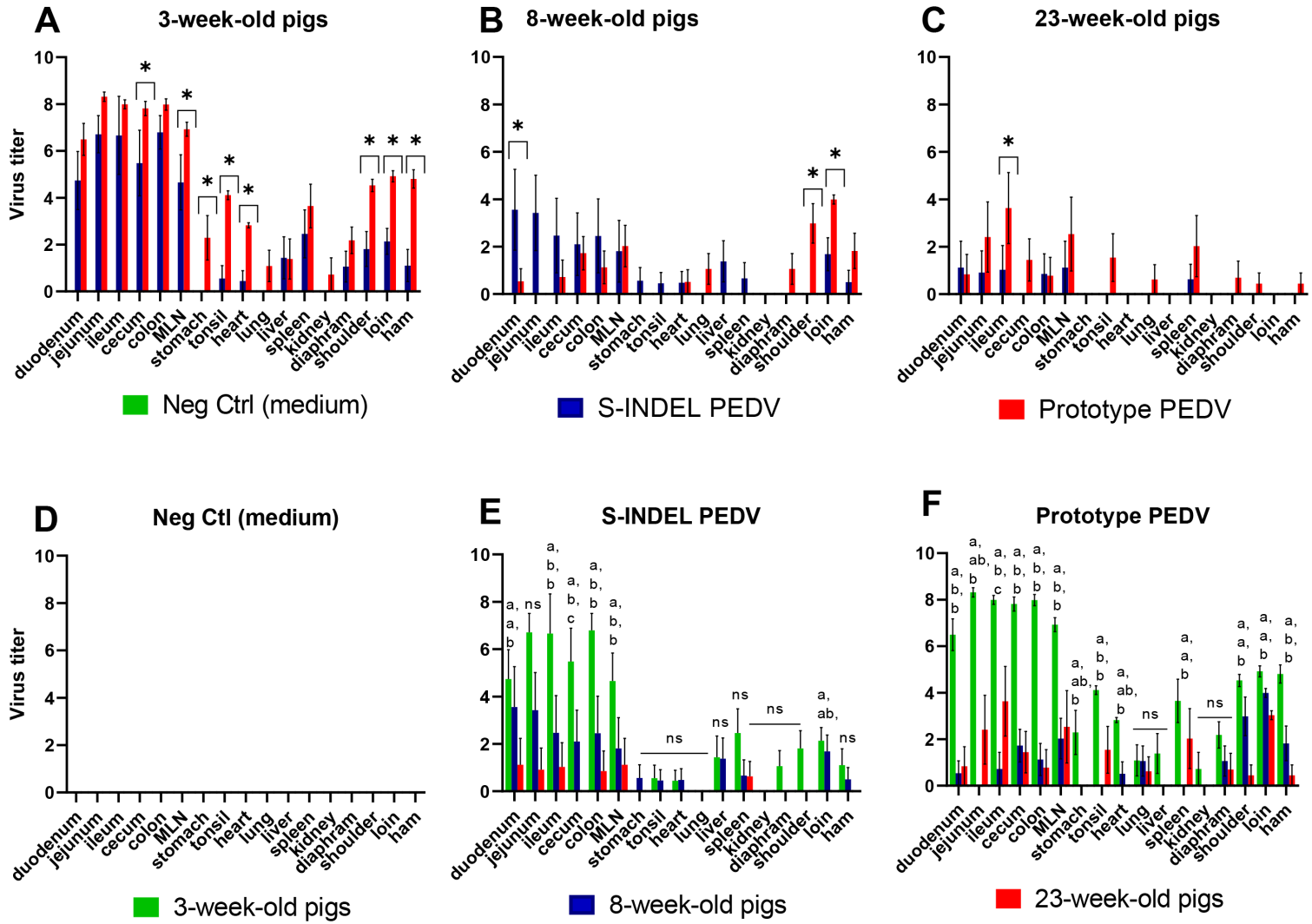

Fig 5. Virus loads in various tissues of pigs at different ages inoculated with different US PEDV strains (4 DPI). Virus load was determined by a quantitative PEDV N gene-based real-time RT-PCR. The virus titers (log_{10}[genomic copies/ml]) were the mean values of all 5 pigs necropsied at 4 DPI (both PCR-positive and PCR-negative pigs) in each group. The virus shedding was plotted in the category of pig ages (A: 3-week-old pigs, B: 8-week-old pigs, and C: 23-week-old pigs) and inoculum (D: Neg Ctrl [culture medium], E: S-INDEL PEDV, and F: Prototype PEDV), respectively. Standard error bars are shown in each figure. Statistical analysis was performed between different groups at each time point.

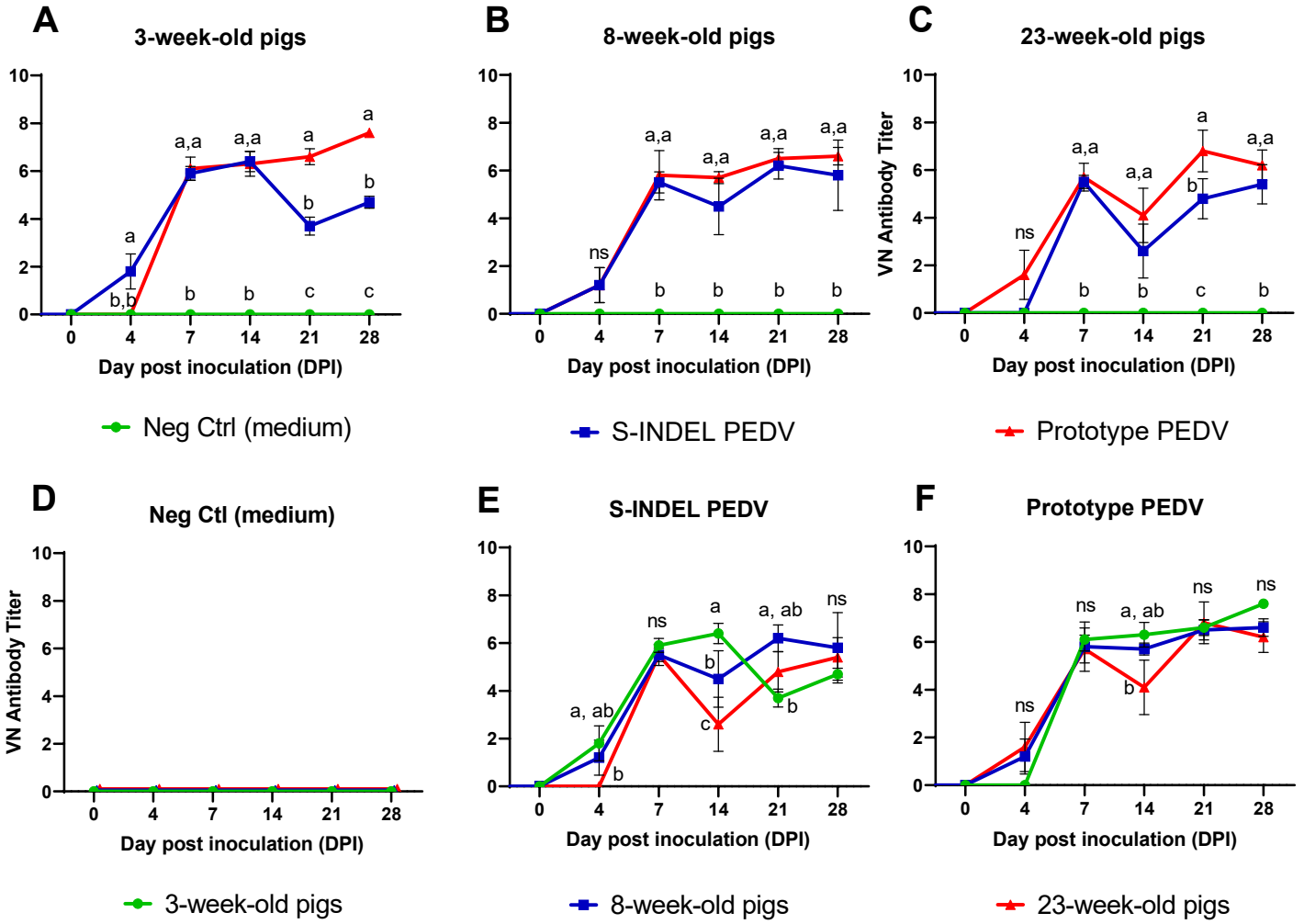

Fig 6. Serum virus neutralizing antibody titers in pigs at different ages inoculated with different US PEDV strains. Neutralizing antibody titer was determined using a US prototype PEDV strain (USA/IN19338/2013) as the indicator virus. The virus neutralizing antibody titers were shown as the mean values of $\log_2$ [Reciprocal VN Antibody Titer] of serum samples of 5 pigs in each group that went through 28 days post inoculation. The serum virus neutralizing antibody titers were plotted in the category of pig ages (A: 3-week-old pigs, B: 8-week-old pigs, and C: 23-week-old pigs) and inoculum (D: Neg Ctrl [culture medium], E: S-INDEL PEDV, and F: Prototype PEDV), respectively. Standard error bars are shown in each figure. Statistical analysis was performed between different groups at each time point.

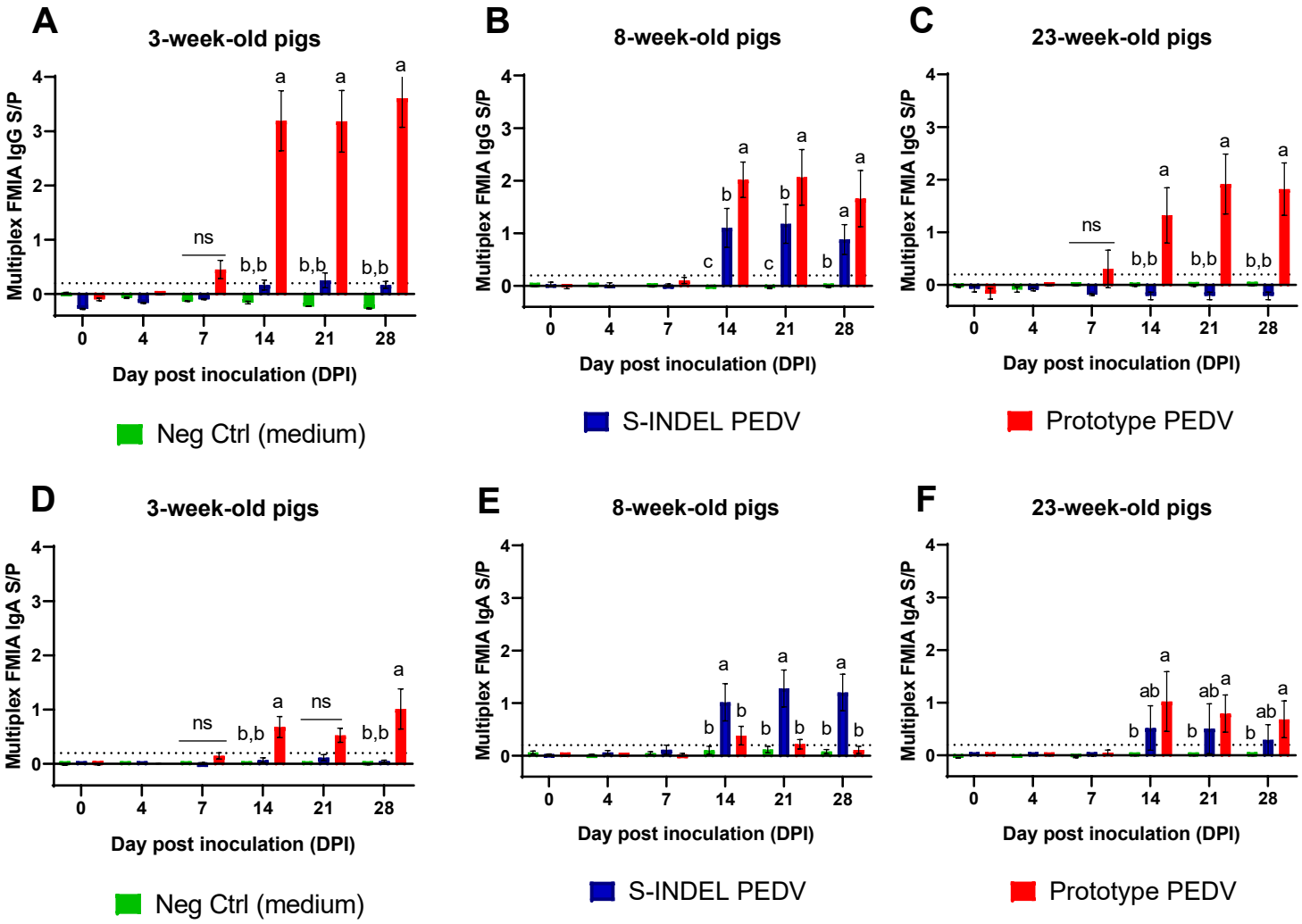

Fig 7. Serum anti-PEDV IgG (A, B, and C) and IgA (D, E, and F) antibody as measured by a PEDV S1-based FMIA antibody assay and plotted in the category of pig ages (3-week-old pigs, 8-week-old pigs, and 23-week-old pigs). The mean values of S/P ratios were calculated on serum samples of 5 pigs in each group that went through 28 days post inoculation. Standard error bars are shown in each figure. Statistical analysis was performed between different groups at each time point.

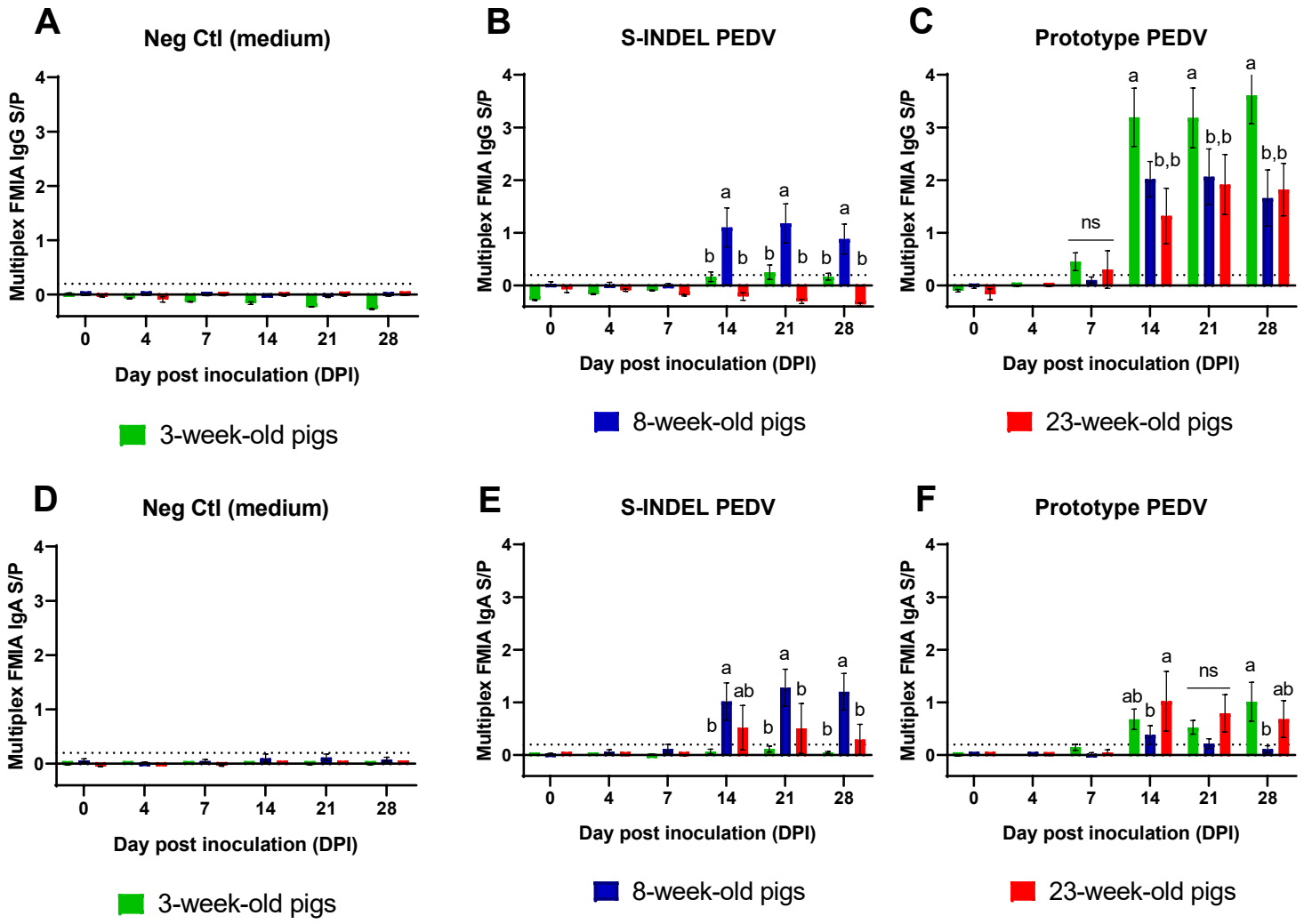

Fig 8. Serum anti-PEDV IgG (A, B, and C) and IgA (D, E, and F) antibody as measured by a PEDV S1-based FMIA antibody assay and plotted in the category of inoculum (Neg Ctrl [culture medium], S-INDEL PEDV, and Prototype PEDV), respectively. The mean values of S/P ratios were calculated on serum samples of 5 pigs in each group that went through 28 days post inoculation. Standard error bars are shown in each figure. Statistical analysis was performed between different groups at each time point.

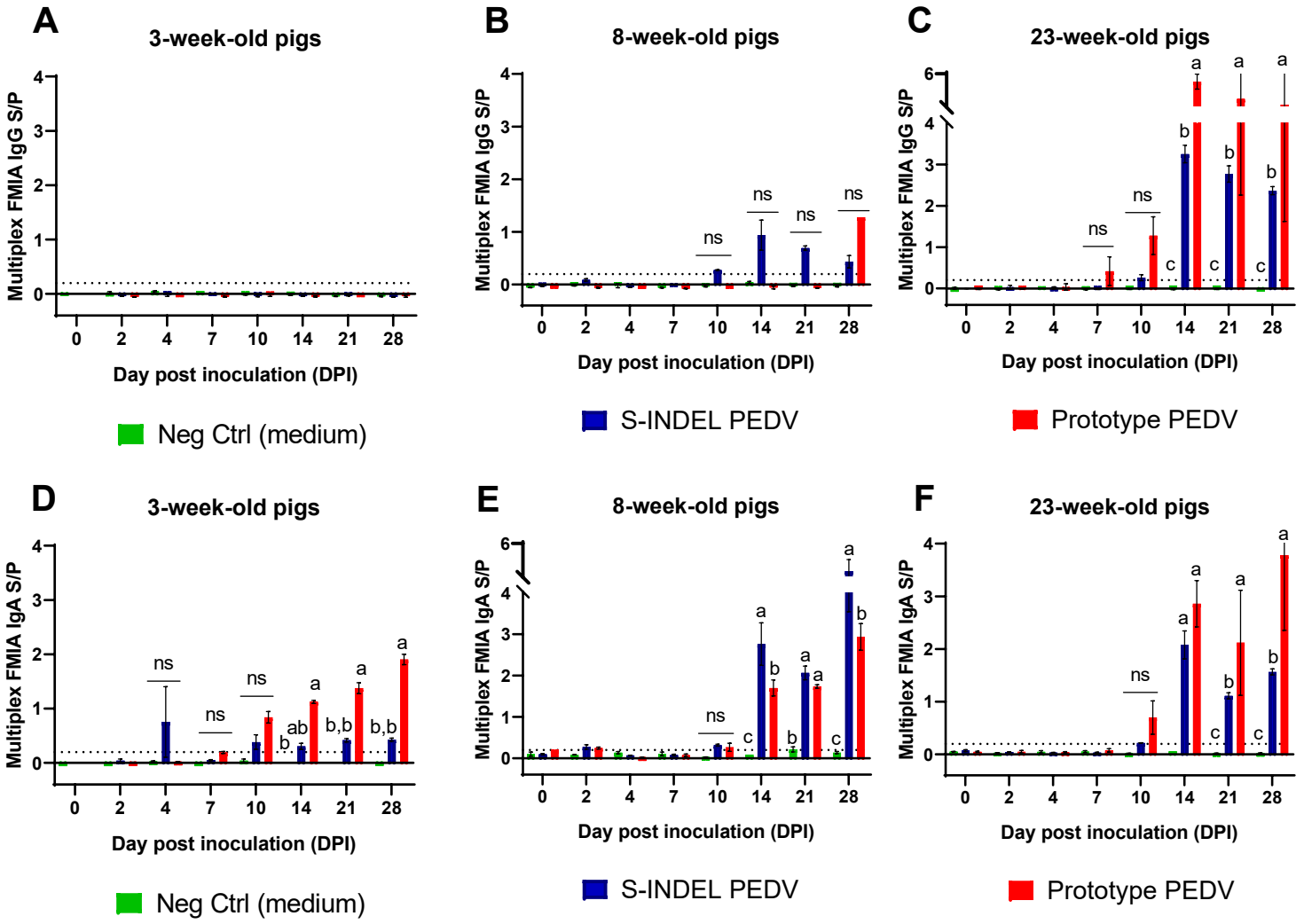

Fig 9. Oral fluid anti-PEDV IgG (A, B, and C) and IgA (D, E, and F) antibody as measured by a PEDV S1-based FMIA antibody assay and plotted in the category of pig ages (3-week-old pigs, 8-week-old pigs, and 23-week-old pigs). Standard error bars are shown in each figure. Statistical analysis was performed between different groups at each time point.

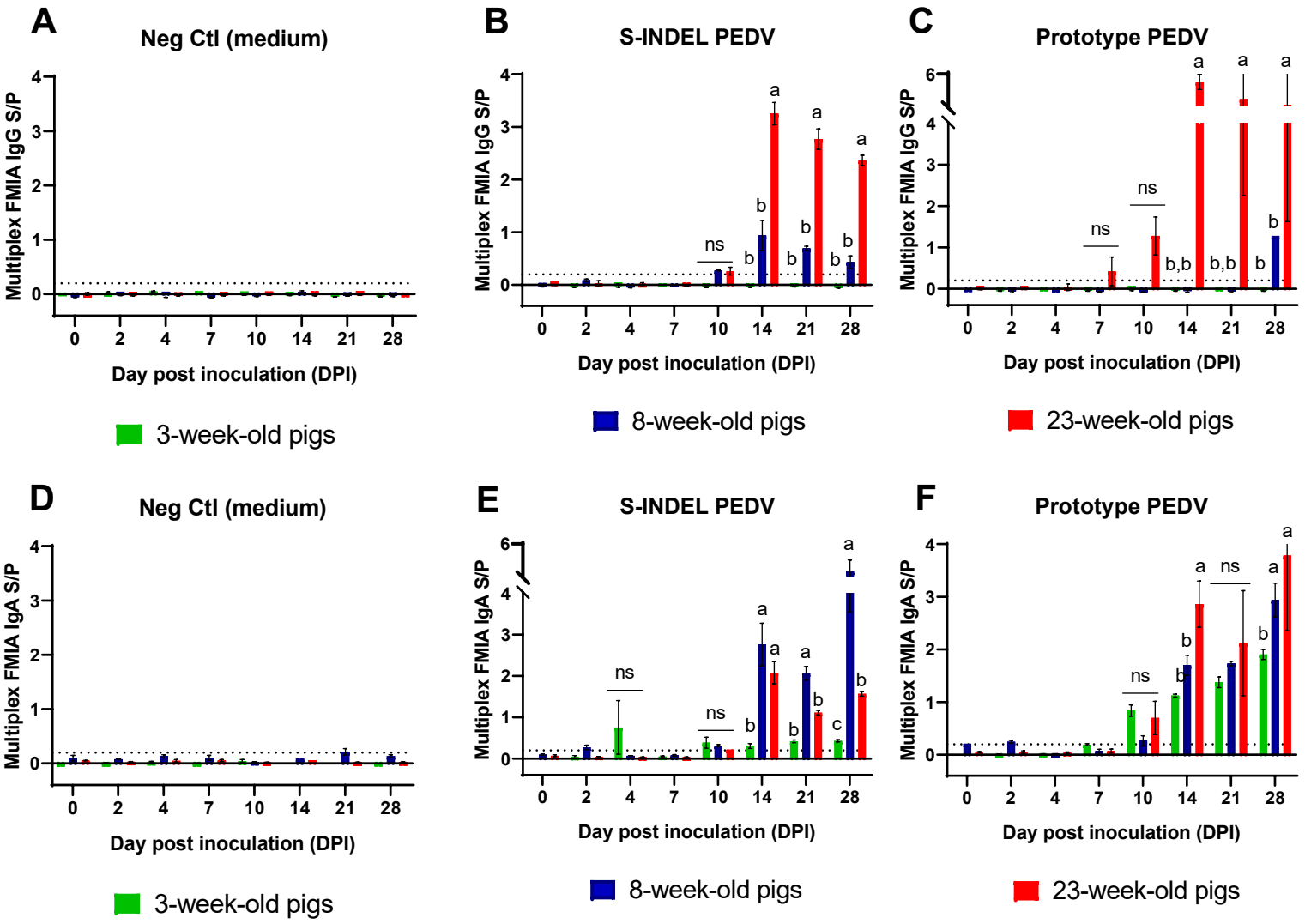

Fig 10. Oral fluid anti-PEDV IgG (A, B, and C) and IgA (D, E, and F) antibody as measured by a PEDV S1-based FMIA antibody assay and plotted in the category of inoculum (Neg Ctrl [culture medium], S-INDEL PEDV, and Prototype PEDV), respectively. Standard error bars are shown in each figure. Statistical analysis was performed between different groups at each time point.