Characterization and cross-protection of experimental infections with SeCoV and two PEDV variants.

Authors

Héctor Puente[1], Ivan Díaz[2], Héctor Arguello[1,3], Óscar Mencía-Ares[1], Manuel Gómez-García[1], Lucía Pérez-Perez[1], Clara Vega[1], Martí Cortey[4], Margarita Martín[4], Pedro Rubio[1,3], Ana Carvajal[1,3]

Affiliations

[1]Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad de León, León, Spain.

[2]IRTA, Centre de Recerca en Sanitat Animal (CReSA, IRTA-UAB), Universitat Autònoma de Barcelona, Bellaterra, Spain.

[3]INDEGSAL, Instituto de Desarrollo Ganadero, Universidad de León, León, Spain.

[4]Departament de Sanitat i Anatomia Animals, Facultat de Veterinària, Universitat Autònoma de Barcelona, Bellaterra, Spain.

Correspondence

Héctor Puente, Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad de León, 24071 León, Spain. Email: hpuef@unileon.es. Phone number: +34 987291306.

Abstract

The aim of this study was to characterize the infection of weaned pigs with *swine enteric coronavirus* (SeCoV) -a chimeric virus most likely originated from a recombination event between *porcine epidemic diarrhea virus* (PEDV) and *transmissible gastroenteritis virus*, or its mutant *porcine respiratory coronavirus*-, and two PEDV G1b variants, including a recently described recombinant PEDV-SeCoV (rPEDV-SeCoV), as well as to determine the degree of cross-protection achieved against the rPEDV-SeCoV.

For this purpose, forty-eight 4-week-old weaned pigs were randomly allocated into four groups of 12 animals; piglets within each group were primary inoculated with one of the investigated viral strains (B: PEDV; C: SeCoV and D: rPEDV-SeCoV) or mock-inoculated (A), and exposed to rPEDV-SeCOV at day 20 post-infection; thus, group A was primary challenged (-/rPEDV-SeCoV), groups B and C were subjected to a heterologous re-challenge (PEDV/rPEDV-SeCoV and SeCoV/rPEDV-SeCoV, respectively), and group D to a homologous re-challenge (rPEDV-SeCoV/rPEDV-SeCoV), Clinical signs, viral shedding, microscopic lesions and specific humoral and cellular immune responses (IgG, IgA, neutralizing antibodies and IgA and IFN-γ-secreting cells) were monitored.

After primo-infection all three viral strains induced an undistinguishable mild-to-moderate clinical disease with diarrhea as the main sign and villus shortening lesions in the small intestine. In homologous re-challenged pigs, no clinical signs or lesions were observed, and viral shedding was only detected in a single animal. This fact may be explained by the significant high level of rPEDV-SeCoV-specific neutralizing antibodies found in these pigs before the challenge. In contrast, prior exposure to a different PEDV G1b variant or SeCoV only provided partial cross-protection, allowing rPEDV-SeCoV replication and shedding in feces.

Keywords

Porcine epidemic diarrhea virus, *swine enteric coronavirus*, swine coronavirus, recombinant, immunity

1. Introduction

Porcine epidemic diarrhea virus (PEDV) is an enveloped, single-stranded, positive-sense RNA virus belonging to the order *Nidovirales*, family *Coronaviridae*, subfamily *Coronavirinae*, and genus *Alphacoronavirus* (Lefkowitz et al., 2017). It is the etiological agent of a highly contagious disease known as porcine epidemic diarrhea (PED), characterized by watery diarrhea and vomiting due to enterocyte destruction and villous atrophy, causing up to 80-90% mortality in neonatal piglets (Saif et al., 2019). The disease was initially described in Europe in the 1970s (Pensaert and de Bouck, 1978) and spread throughout Europe and Asia. Its incidence decreased markedly in the nineties and subsequent years in Europe, while in Asia the virus remains as a major cause of diarrhea (Carvajal et al., 2015). In 2013, PEDV emerged in America unleashing a major epidemic that caused substantial economic losses (Schulz and Tonsor, 2015). Soon after, PEDV re-emerged in Europe (Antas and Woźniakowski, 2019), becoming a major concern for swine industry worldwide.

Two PEDV genogroups, named G1 or INDEL and G2 or non-INDEL, are recognized based on insertions-deletions in the S1 subunit of the spike (S) gene. Both genogroups show differences in virulence and transmissibility (Chen et al., 2016; Gallien et al., 2018), leading to intensive research to better understand the prevailing PEDVs in different countries and regions. Whole genome or S gene sequencing of isolates recovered from different European farms demonstrated that all recent European PEDV strains are G1b (Grasland et al., 2015; Hanke et al., 2015; Mesquita et al., 2015; Theuns et al., 2015; Boniotti et al., 2016; Puente et al., 2021), with the only exception of a G2b isolate from Ukraine (Dastjerdi et al., 2015). In contrast, both genogroups have been detected on infected farms in Asia and America (Lin et al., 2016).

In addition, a chimeric virus known as *swine enteric coronavirus* (SeCoV) has been described across Europe between 1993 and 2016 (Akimkin et al., 2016; Belsham et al., 2016; Boniotti et al., 2016; de Nova et al., 2020). This recombinant, which causes a PED-like disease, has the S gene from PEDV and the backbone from *transmissible gastroenteritis virus* (TGEV) or *porcine respiratory coronavirus* (PRCV) (Belsham et al., 2016; Boniotti et al., 2016). In Europe, SeCoV has not been recently detected, although different PEDV variants within the G1b genogroup have been associated with diarrhea outbreaks. Among them, a recombinant PEDV-SeCoV (rPEDV-SeCoV) resulting from the substitution of a ~400 nt fragment at the 5′ end of the S gene with SeCoV (Valkó et al., 2017; de Nova et al., 2020) is reported frequently in large producing countries such as Spain (Puente et al., 2021) or Poland (Antas et al., 2021).

The aim of the present study was to characterize the infection of weaned pigs by SeCoV and two variants of PEDV G1b, including rPEDV-SeCoV, as well as to determine the degree of cross-protection provided in a re-infection with rPEDV-SeCoV.

2. Materials and methods

2.1. Ethical issues

All experiments involving pigs were done under the approval of the University of León Committee on Animal Care and Supply (OEBA-ULE-006-2019 and OEBA-ULE-013-2020). Pigs were handled by veterinarians and trained personnel who fulfilled the Spanish and European Union requirements. Animals were clinically examined upon arrival and monitored throughout the experiments.

2.2. Experimental design

Forty-eight commercial three-week-old weaned female pigs were purchased from a PEDV-free farrow-to-wean herd. On arrival, all pigs were confirmed to be free of PEDV,

TGEV, SeCoV, *porcine deltacoronavirus*, Rotavirus (A, B, C and H) and several enteropathogenic bacteria (*Salmonella* spp., *Lawsonia intracellularis*, enterotoxigenic *E. coli* and *Brachyspira* spp.) using PCR (viral infections and *L. intracellularis*), microbiological culture (*Salmonella* spp.) or a combination of culture and PCR (enterotoxigenic *E. coli* and *Brachyspira* spp.) on fecal samples. In addition, all pigs were seronegative to PEDV (INgezim PEDV ELISA, INGENASA). Pigs were randomly distributed into four groups (12 animals each) and housed in separated rooms of a biosafety level 2 animal facility. Animals were housed in a single solid floor pen with straw bedding and fed *ad libitum* with an antibiotic-free diet. Room temperature was set at 26ºC.

After a week of acclimation, the study was carried out in two stages. During the first stage, piglets in groups B, C and D were orally inoculated -day post-inoculation (dpi) 0-, using a gastric cannula, with 3 mL of a viral inoculum (10^6 $TCID_{50}$/mL) of PEDV G1b (strain 2330-Orense), SeCoV (strain 1480-Murcia-Lorca) or rPEDV-SeCoV (strain 1931-1-Valladolid-Molpeceres), respectively (Figure 1). Animals in group A were mock-inoculated on the same day with phosphate buffered saline solution (PBS). The second stage started twenty days later (dpi 20); all groups were orally inoculated with 3 mL of the viral inoculum containing rPEDV-SeCoV at 10^6 $TCID_{50}$/mL as described above. Hence, during this stage group D was subjected to a homologous re-challenge, groups B and C to a heterologous re-challenge (PEDV/rPEDV-SeCoV and SeCoV/rPEDV-SeCoV) and group A was primary challenged.

2.3. Viral inocula

Each inoculum was obtained from two three-day-old piglets that were intragastrically inoculated with 3 mL of viral positive feces (PEDV G1b 2330-Orense, SeCoV 1480-Murcia-Lorca and rPEDV-SeCoV 1931-1-Valladolid-Molpeceres) collected from

diarrheic pigs on infected farms in Spain. After 48 hours, piglets developed severe diarrhea and were euthanized. Small intestinal content and mucosal scrapings were collected, diluted 1/5 in PBS, filtered through a 0.22 μm syringe filter (GE Healthcare), tested by qPCR for viral quantification and subsequently stored at −80°C. Also, the suspensions were confirmed as PCR negative for porcine coronavirus (PEDV, TGEV, SeCoV and PDCoV, excluding the virus corresponding to each of the inocula) and Rotavirus (A, B, C and H) before being used.

2.4. Clinical monitoring and sample collection

Figure 1 summarizes the animal clinical monitoring and sampling strategy. Weight and rectal temperatures were daily measured. Clinical signs were scored considering four relevant parameters: (a) fecal consistency (0 = normal feces, 1 = soft stools, 2 = watery diarrhea); (b) general condition (0 = normal, 1 = slightly depressed, 2 = depressed, 3 = lethargic); (c) appetite (0 = hungry, 1 = partial anorexia, 2 = total anorexia); (d) vomiting (0 = no, 1 = yes). Using these clinical scores, a maximum value of 8 could be assigned to an individual pig on a single day. Fecal samples were collected from all piglets daily between dpi 0 to 7 and dpi 20 to 26 (days post-re-inoculation or dpri 0 to 6) as well as at dpi 9, 11, 13, 15, 17 and 19. Serum samples were collected weekly during the first stage of the experiment and each 3 days during the second stage. To obtain peripheral blood mononuclear cells (PBMC), blood samples were collected using lithium heparin tubes immediately before re-inoculation (dpi 20) and three days later. Finally, three animals from each group were randomly selected and euthanized at dpi 3, 6, 23 (dpri 3) and 26 (dpri 6). Duodenum, mid jejunum, and ileum were collected at necropsy and immediately fixed in formalin for further histological evaluation.

2.5. Virus isolation

147 The rPEDV-SeCoV isolate was propagated in cell culture as previously described (Díaz
148 et al., 2021) and used in viral neutralization test (VNT) and ELISPOT. Briefly, a confluent
149 monolayer of VERO cells (ATCC CCL-81) was inoculated with a clarified and trypsin-
150 treated (10 µg/mL of Trypsin 1:250, Gibco) suspension of viral inoculum (small intestinal
151 content and mucosal scrapings from infected three-day-old piglets). After 2 hours of
152 adsorption at 37°C, 5 mL of freshly prepared medium including trypsin was added. After
153 being cultured for 3 days at 37ºC and 5% CO_2, cytopathic effect characterized by round
154 syncytia was observed, and cultures were frozen and thawed to recover the virus. A single
155 virus stock was used for the immunological analysis (4.5 $\log_{10}$ $TCID_{50}$/mL, passage 3).

156 2.6. Sequence analysis

157 Viral inocula used for experimental challenge, together with cell-culture adapted rPEDV-
158 SeCoV isolate, and qPCR positive fecal samples yielding Ct < 20 (n = 15) were sequenced
159 by next generation sequencing (Cortey et al., 2019). The amino acid sequences in
160 neutralizing B-cell epitopes described by Okda et al. (2017) and Kong et al. (2020) were
161 visualized using BioEdit 7.2.5. Strain CO13 (GenBank accession number KF272920) was
162 used as reference (Okda et al., 2017).

163 2.7. Quantification of PEDV and SeCoV in fecal samples

164 Feces were diluted 1:2 in sterile PBS, homogenized by vortex mixing and centrifuged for
165 10 min at 20,000 g. The RNA was extracted from 140 µl of the supernatant using QIAMP
166 Viral RNA Mini Kit (QIAGEN), following the manufacturer's instructions. RT-qPCRs
167 with the primers and probes targeting the M protein gene of PEDV described by Zhou et
168 al. (2017), and the N protein gene of TGEV described by Masuda et al. (2016) were used
169 for quantification of PEDV and SeCoV, respectively. Both RT-qPCRs were carried out
170 using a PrimeScript TM RT-PCR Kit (TAKARA) and following the manufacturer's
171 recommendations in a QuantStudio 1 thermal cycler (Applied Biosystems). Cycling

conditions were as follows: reverse transcription at 42°C for 5 min, inactivation at 95°C for 10 s, followed by 40 cycles of denaturing at 95°C for 5 s and annealing and extension at 60°C for 35 s. Each RNA sample was analyzed in duplicate.

Ct values were converted into viral titers using a standard curve generated with samples of known PEDV concentration ($TCID_{50}$/mL). Thus, results were expressed as equivalent $TCID_{50}$/mL or the corresponding adjusted $TCID_{50}$/g.

2.8. Histology

Tissue samples fixed 48 h in 10% formalin were dehydrated, embedded, sectioned (4 µm thick), mounted onto glass slides and stained with hematoxylin-eosin. To measure villous length and crypt-depth of duodenum, mid jejunum and ileum, three sections of each tissue were blindly evaluated by a veterinary pathologist using a computerized image system (Leica LAS EZ 3.4 digital imaging software).

2.9. Specific PEDV IgG and IgA

Kinetics of specific-PEDV IgG in sera were determined using a commercially available ELISA based on the S glycoprotein (Ingezim PEDV, INGENASA). Results were expressed as sample/positive ratio (S/P).

The same commercial kit was used to measure specific-PEDV IgA as previously described (Díaz et al., 2021), substituting the anti-pig IgG conjugate by a goat anti-pig IgA HRP conjugate (Bethyl Laboratories). Results were expressed as optical densities (ODs).

2.10. Viral neutralization test (VNT)

Neutralizing antibodies (NA) were evaluated as described by Thomas et al. (2015), with minor modifications (Díaz et al., 2021). Mixtures (1:1) of the cell-culture adapted rPEDV-SeCoV containing 200 $TCID_{50}$ and serum (dilutions 1:4 to 1:256) were

inoculated onto confluent monolayers of Vero cells. Negative controls (mock-infected), viral infection controls (200 $TCID_{50}$ of rPEDV-SeCoV) and positive controls (200 $TCID_{50}$ of rPEDV-SeCoV plus positive sera) were included on each set of plates. Plates were read after 48 h of incubation by staining with a FITC labelled anti-PEDV monoclonal antibody (SD-1F-1 8D6-29PED-NP, Medgene Labs) (1:200). Titres were calculated as the reciprocal of the highest dilution resulting in ≥ 90% reduction of fluorescent foci compared to viral infection controls. As previously proposed, NA titres below 8 were considered negative (Thomas et al., 2015).

2.11. IgA and IFN-γ ELISPOT

rPEDV-SeCoV-specific IgA-secreting cells (SC) were measured by means of a commercial ELISPOT kit (Pig IgA single-color ELISPOT, CTL), as previously described (Jahnmatz et al., 2013; Díaz et al., 2021), while rPEDV-SeCoV-specific IFN-γ-SC were measured using a tailor-made IFN-γ ELISPOT (Díaz et al., 2021). PBMC were recovered from blood samples as described by Diaz et al. (2021), mock-stimulated or stimulated with rPEDV-SeCoV at a multiplicity of infection (moi) of 0.01. All tests were run in duplicate. Results were expressed as responding cells (counts of spots in stimulated cells minus counts of spots in unstimulated ones)/10^6 PBMC.

2.12. Statistical analysis

Proportions of diarrheic pigs and PEDV positive pigs were compared among groups using the $\chi 2$ test (Fisher's exact test). Numerical data were tested for normality (Kolmogorov-Smirnov test) and statistical differences among groups were evaluated using either ANOVA or Kruskal-Wallis test (Connover-Imman method for multiple comparisons). Friedman test was used for comparisons inside the same group. The area under the curve (AUC) for viral shedding in feces was calculated using the trapezoidal approach (Schäfer

et al., 2001). The analyses were carried out with IBM SPSS Statistics v26 and StatsDirect v 2.7.7 at the 5% significance level.

3. Results

3.1. Clinical assessment

No significant differences were found in daily rectal temperatures among groups. During the first stage (primo-infection), the highest clinical scores were recorded in challenged groups (B, C and D) between dpi 2 and 5 (Figure 2). Significant differences were observed when the three challenged groups were compared with the control ($p<0.05$), but not when compared among them (Figure 2). Liquid diarrhea was the main clinical sign and was recorded in 66.6% of the piglets of group B and 83.3% of groups C and D (Appendix Figure 1).

In the second stage of the experiment, clinical scores were significantly higher in group A (primo-infection) as compared with group D (homologous re-challenge) -between dpri 2 and 5-, group B (heterologous re-challenge PEDV/rPEDV-SeCoV) -between dpri 2 and 4-, and group C (heterologous re-challenge SeCoV/rPEDV-SeCoV) -only in dpri 3- ($p<0.05$) (Figure 2). No differences were observed when clinical and diarrhea scores were compared among groups B, C and D.

Average daily gain (ADG) during the first week post-infection was significantly lower in groups B, C and D ($p<0.05$) compared to control group (A) (Appendix Table 1). In the second stage of the experiment, ADG in group A was significantly lower than in group B and D ($p<0.05$). We also observed that differences in ADG between groups C and D and groups C and A were close to statistical significance ($p=0.059$ and $p=0.076$, respectively).

3.2. Quantification of PEDV and SeCoV in fecal samples

243 Results of viral detection and quantification, as well as statistical comparisons among
244 groups, are shown in Figure 3. None of the mock-inoculated pigs (group A) shed PEDV
245 or SeCoV RNA in their feces during the first stage of the experiment, while all pigs in the
246 challenged groups shed virus in their feces (Figure 3A). Maximum shedding was reached
247 at dpi 2 in group B (5.9 $\log_{10}$ $TCID_{50}$/g), dpi 3 in group C (6.7 $\log_{10}$ $TCID_{50}$/g) and dpi 5
248 in group D (5.7 $\log_{10}$ $TCID_{50}$/g) (Figure 3B). After peaking, viral shedding in feces was
249 progressively reduced. However, in group C, challenged with SeCoV, a second shedding
250 wave started at dpi 9 and extended until dpi 17. Accordingly, viral shedding measured as
251 AUC was significantly higher for group C from dpi 0 to dpi 20 (C > B and D; $p<0.05$)
252 (Figure 3C).

253 In the second stage of the experiment, rPEDV-SeCoV RNA was detected from dpri 2 in
254 all pigs of group A, reaching a maximum of 6.6 $\log_{10}$ $TCID_{50}$/g on dpri 3. On the contrary,
255 only one piglet of group D (homologous re-challenge) shed virus for two consecutive
256 days (dpri 2 and 3). Between dpri 2 and 6, rPEDV-SeCoV was detected in 66.7% of the
257 piglets from group B (up to 2.2 $\log_{10}$ $TCID_{50}$/g) and 100% from group C (up to 4.6 $\log_{10}$
258 $TCID_{50}$/g). AUC was significantly lower for group D (homologous re-challenge) as
259 compared with groups C (heterologous re-challenge SeCoV/rPEDV-SeCoV) and A
260 (primo-infection).

261 3.3. Histopathology and morphometry

262 Microscopic lesions consisting of shorted and fused villi were observed in all challenged
263 animals euthanized in the first stage of the experiment, particularly in the duodenum and
264 mid jejunum at dpi 3 (Figure 4). During the second stage, piglets of group A showed more
265 evident microscopic lesions, followed by group C.

Mean villous height to crypt-depth ratios for each intestinal segment and group were compared (Table 1). At dpi 3, piglets in groups B, C and D showed lower ratios than mock-infected animals for all small intestine segments. During the second stage of the study (dpri 3 and 6), villous shortening was more evident in primo-infected pigs (group A), which showed a significant reduction in these ratios, compared to groups B and D (heterologous and homologous challenge, respectively). A reduction of villous height to crypt-depth ratio was also evident in duodenum and mid jejunum in group C, although significant differences with groups A or D were not observed.

3.4. Sequence comparison

A total of 25 RT-qPCR positive fecal samples (Ct< 20) recovered throughout the experiment (n=4, n=7, n=8 and n=6 for groups A, B, C and D, respectively) were sequenced, together with the cell-culture adapted virus used for the immunological assays. Whole genome nucleotide identity was higher than 99.6% compared to the original inoculum for all samples.

Among the five known neutralizing B-cell epitopes described by Okda et al. (2017), 14 changes were observed between rPEDV-SeCoV and SeCoV, while only 3 were observed when PEDV was compared to rPEDV-SeCoV (Table 2).

3.5. Detection of specific IgGs and IgAs

No IgG antibodies against PEDV S glycoprotein were detected in any of the pigs at dpi 0. Mock-infected pigs (group A) remained negative during the first stage of the experiment. At dpi 6, one piglet in group C (11%) and two from group D (22%) seroconverted (Figure 5A), while at dpi 13, the percentage of seropositive piglets increased to 83% in groups B and C (5 out of 6) and 100% in group D (6 out of 6). In the second stage of the experiment, 2 out of 3 piglets in group A (66.7%) were seropositive

290 at dpri 6. Once seroconverted, all piglets remained positive by ELISA during the
291 remaining days of the study.

292 IgG kinetics based on mean S/P ratios are shown in Figure 5B. An increase was observed
293 in groups B, C and D when comparing S/P ratio before (dpi 20) and after (dpri 6) re-
294 challenge (booster effect), with no statistical differences.

295 IgA kinetics based on mean OD values are shown in Figure 5C. A significant booster
296 effect was observed in groups B and C ($p<0.05$), when results obtained before (dpi 20)
297 and after (dpri 6) heterologous challenge were compared. In contrast no booster effect
298 was observed in group D (homologous challenge).

299 3.6. Detection of specific neutralizing antibodies (NA)

300 NA against rPEDV-SeCoV were detected in all challenged pigs (groups B, C and D) at
301 dpi 20, reaching group D the highest values (D > B and C; $p<0.05$) (Table 3). NA dropped
302 in all infected groups after re-challenge. Thus, at dpri 3 only two animals in groups B and
303 C (33.3%) and five in group D (83.3%) showed NA titers ≥ 8. Finally, NA increased again
304 at dpri 6 in all re-challenged groups, being all animals positive.

305 3.7. IgA and IFN-γ ELISPOT

306 Mean numbers of specific-rPEDV-SeCoV IgA-SC were significantly higher in
307 challenged pigs when compared to group A at dpi 20 ($p<0.05$) (Table 3). Moreover, a
308 significant booster was observed at dpri 3 in groups B, C and D ($p<0.05$), without
309 differences among them.

310 Also, mean numbers of specific-rPEDV-SeCoV IFN-γ-SC were higher in groups B, C
311 and D when compared to group A at dpi 20 and dpri 3 ($p<0.05$) (Table 3). Moreover,
312 group D showed higher values for both time points when compared with groups B and C

313 ($p<0.05$). Again, a significant booster was observed at dpri 3 for groups B, C and D
314 ($p<0.05$).

315 **4. Discussion**

316 PEDV genetic diversity through mutations and recombinations has been demonstrated
317 (Wang et al., 2019). Also, clinical and epidemiological differences, in terms of virulence
318 and transmissibility, have been described among PEDV G1b and G2b strains (Chen et al.,
319 2016; Gallien et al., 2018). Nonetheless, potential differences in clinical signs, viral
320 shedding, lesions, or intensity of the induced immunity by different variants of PEDV
321 G1b or SeCoV, a PEDV/TGEV chimeric virus, have not been well characterized. In this
322 sense, although SeCoV has been identified in pig fecal samples from several European
323 countries (Akimkin et al., 2016; Belsham et al., 2016; Boniotti et al., 2016; de Nova et
324 al., 2020), its virulence had not been experimentally assessed . The present study is the
325 first comparative characterization of two PEDV G1b experimental infections, including
326 a rPEDV-SeCoV isolate that has recently reported as predominant in Europe (Antas et
327 al., 2021; Puente et al., 2021), plus a SeCoV strain. Cross-protection provided by these
328 PEDV variants or SeCoV against the challenge with the rPEDV-SeCoV strain was also
329 investigated.

330 In agreement with previous reports in weaned pigs infected by PEDV G1b (Gallien et al.,
331 2018; Díaz et al., 2021) or G2b (Madson et al., 2014; Crawford et al., 2015; Jung et al.,
332 2015; Gerber et al., 2016; Krishna et al., 2020), the clinical disease induced in primo-
333 infected animals was mild-to-moderate. This fact was probably associated to the already
334 described age-dependent disease severity (Stevenson et al., 2013; Carvajal et al., 2015),
335 irrespective of the high dose used for the challenge. Although signs were not severe, the
336 infection clearly impacted animal growth as observed by weight daily gain during the first
337 week, as previously described in pigs exposed to both PEDV genogroups (Madson et al.,

2014; Gallien et al., 2018). Clinical course was indistinguishable among all viruses, although a slightly prolonged duration of clinical illness was observed in SeCoV infected piglets that were already affected at dpi 1 and showed diarrhea until dpi 11.

Although extended viral shedding, up to 42 days, has been described in weaned pigs exposed to PEDV (Crawford et al., 2015; Gallien et al., 2018; Díaz et al., 2021), the design of our experiment did not allow to monitor prolonged shedding. However, viral shedding was still detected in a single animal (16.6%) from both PEDV exposed groups at dpi 15. At that time both pigs were asymptomatic, fact that could facilitate the maintenance and transmission of the infection on swine farms. This fact was even more obvious in SeCoV infected animals, which showed a clear viral shedding reactivation, with all pigs positive in feces at dpi 13 and 15. A similar shedding profile was described in PEDV infected piglets challenged at 3-4 days of age (Lin et al., 2015) or at weaning (Madson et al., 2014; Thomas et al., 2015), which has been associated with PEDV replication in new regenerated enterocytes (Lin et al., 2015). Both maximal and total shedding load (AUC) were higher in SeCoV compared to PEDV infected pigs. This result suggests an increased ability of this chimeric virus for replication in the enterocytes of the intestinal villi, compared to PEDV. Further studies based on immunohistochemistry assays are required to elucidate the differences in intestinal PEDV and SeCoV replication.

Microscopic lesions characterized by villous atrophy and fusion were also identical among the three infected groups. Villus height to crypt depth ratio was used to evaluate the degree of microscopic lesions as previously described (Madson et al., 2014; Jung et al., 2015; Thomas et al., 2015). Although this ratio can vary depending on several factors such as pig genetics or diet, usually it is about 3:1 in weaned piglets (Moon, 1971). In our work, this ratio varied between 1.58 and 2.71 in control pigs, but was significantly reduced in PEDV or SeCoV infected pigs (range 0.88 to 1.19). To the best of our

knowledge, this is the first research which evaluates microscopic lesions in weaned pigs exposed to PEDV G1b or SeCoV. Our results suggest that, both the location within the small intestine and the degree of villi shortening, are like previously reported lesions in weaned pigs exposed to PEDV G2b isolates (Madson et al., 2014; Jung et al., 2015).

In the evaluation of the serological response, PEDV specific IgG and IgA antibodies were detected in pigs exposed to SeCoV using a commercial ELISA based on the S-protein, confirming that indirect diagnostic methods based on this particular protein can lead to misidentification of SeCoV, as it occurs with direct detection (de Nova et al., 2020). According to previous reports (Lin et al., 2015; Thomas et al., 2015; Gerber et al., 2016; Krishna et al., 2020; Díaz et al., 2021), specific IgG antibodies were detected in most PEDV infected pigs by dpi 14, with the highest increase in S/P ratio between dpi 7 and 14. SeCoV infected pigs showed a more intense and slightly delayed specific IgG response, reaching its maximum at dpi 20. Particularities of SeCoV infection which could affect the time-lapse to specific IgG response establishment should be further studied.

Despite the amino acid substitutions observed in known neutralizing B-cell epitopes between SeCoV versus rPEDV-SeCoV or PEDV versus rPEDV-SeCoV, it is worth noting that rPEDV-SeCoV-specific NA were detected in 100% of the challenged pigs at dpi 20. As expected, mean NA titer was significantly higher in rPEDV-SeCoV as compared to PEDV or SeCoV infected pigs.

The degree and duration of cross-protection against subsequent infections, particularly heterologous, are aspects of great practical interest to design PEDV control strategies (Gerdts and Zakhartchouk, 2017). Thus, full protection against disease and sterilizing immunity have been reported in the short-term (few weeks after primo-infection), for both homologous (Crawford et al., 2015; Gerber et al., 2016) and heterologous PEDV challenges (Krishna et al., 2020). On the contrary, only partial protection has been

described in the long-term (Díaz et al., 2021). In our study, as it would be expected, piglets subjected to a homologous challenge three weeks after primo-infection did not show any clinical signs or lesions. Also, only one animal shed a low amount of virus (1.37 log_{10} $TCID_{50}$/g) in feces on dpri 2 and 3. In contrast, heterologous PEDV challenge led to fecal shedding in 66% of the piglets for 4 days, with no relevant clinical disease or lesions. Considering the minimal infectious dose proposed for PEDV (Thomas et al., 2015), the PEDV titers observed in the heterologous infected animals suggests their potential to transmit the infection. On the contrary, the single animal shedding virus in the homologous challenge, despite being positive, would not be considered infectious. Finally, some degree of diarrhea, shortening of the villi and reduction in daily weight gain were observed in the piglets primo-infected with SeCoV, suggesting a lower level of protection. Among these piglets, 100% shed rPEDV-SeCoV during heterologous challenge, although viral titers were reduced up to 2 log_{10} compared to primo-infected animals (group A). Also, shedding titers were clearly above the minimal infectious dose for PEDV. Altogether, our results suggest that there is only a partial level of cross-protection, against clinical disease and viral shedding, after heterologous infection. From a practical point of view, recurrent PEDV infections in farms can occur, even when the introduction takes place few weeks apart. This phenomenon emphasizes the need to maintain high levels of external biosecurity on swine farms.

Serum titers of IgG and IgA antibodies against PEDV S glycoprotein were similar in the three exposed groups before second challenge. This result suggests a lack of correlation with protection. In agreement with previous studies (Gerber et al., 2016; Krishna et al., 2020), no significant increase in serum PEDV-specific IgG, IgA and NA levels after homologous short-term re-challenge were observed. On the contrary, Diaz et al. (2021) demonstrated a strong serological anamnestic response (IgG, IgA and NA) after

homologous re-challenge 5 months apart. A high titer of specific NA in the gut will probably be able to limit viral replication and will not allow for a significant booster after a short-term re-exposure (Krishna et al., 2020). In our study, minimal viral shedding observed in a single piglet among those animals subjected to homologous infection supports this hypothesis. Moreover, piglets previously exposed to PEDV or SeCoV showed a significant lower NA response at dpi 20. These animals had higher viral shedding after re-infection and showed an anamnestic response for IgA and NA in serum. A similar pattern was observed for rPEDV-SeCoV-specific IgA-SC, also in the homologous challenge group, pointing to the presence of effector or memory cells.

Concerning rPEDV-SeCoV-specific IFNγ-SC, a significant booster was detected in all groups, increasing 3-4 times after the homologous or heterologous challenge. Before the challenge, the highest value was observed in rPEDV-SeCoV. The leading role of NA regarding protection has been stablished (Krishna et al., 2020; Díaz et al., 2021), in agreement with the results of this study. However, since the precise role of cell-mediated immunity, measured as IFNγ-SC, is not well known, it may not be ruled out a certain involvement of cellular response in protection.

To sum up, an experimental challenge of 4-week-old pigs with two PEDV G1b variants and one SeCoV strain induced an undistinguishable mild-to-moderate clinical disease, characterized by diarrhea and microscopic lesions of shorter and fused villi. Viral shedding was slightly higher on SeCoV infected pigs and exceeds clinical disease recorded in the three viral strains tested. This could explain the ability of these enteric coronaviruses to easily spread. Protection against clinical disease and viral shedding after a short-term re-challenge was strain-dependent, a fact which should be taken into consideration when immunizing pigs against PEDV. Finally, great diversity of PEDV

isolates, together with this limited cross-protection, makes necessary a continuous monitoring of novel PEDV variants that may emerge locally or globally.

Declaration of competing interest

None of the authors of this study has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Ethics approval and consent to participate

All procedures involving animals were approved by the institutional bioethical committee (Reference Number OEBA-ULE-006-2019 and OEBA-ULE-013-2020), and performed according to European regulations regarding animal welfare and protection of animals used for experimental and other scientific purposes.

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Data availability statement

Data are available in the GenBank database and by direct contact with the correspondence author.

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622 Table 1. Villous height to crypt depth ratio (μm/μm): mean and standard deviation
623 (SD). At day post-inoculation (dpi) 0, pigs from group A were mock-infected while pigs
624 from groups B, C and D were challenged with PEDV, SeCoV and rPEDV-SeCoV,
625 respectively. At dpi 20, all pigs were challenged with rPEDV-SeCoV. Three animals
626 were euthanized per group at dpi 3, 6, 23 and 26. Letters show significant differences
627 between groups for each particular day ($p<0.05$).

628

dpi (total number of pigs euthanized)	Group	Duodenum (mean ± SD)	Mid jejunum (mean ± SD)	Ileum (mean ± SD)
3 (12)	A	2.36±0.07^{a}	2.20±0.26^{a}	2.06±0.40^{a}
	B	1.01±0.17^{b}	0.90±0.17^{b}	0.96±0.14^{b}
	C	0.92±0.38^{b}	0.96±0.38^{b}	0.99±0.51^{b}
	D	0.99±0.23^{b}	0.83±0.18^{b}	0.85±0.43^{b}
6 (12)	A	2.57±0.28^{a}	2.72±0.14^{a}	1.58±0.11^{a}
	B	1.20±0.19^{b}	1.32±0.54^{b}	1.24±0.41^{ab}
	C	1.32±0.01^{b}	1.56±0.20^{b}	1.31±0.12^{ab}
	D	1.36±0.39^{b}	1.25±0.42^{b}	0.96±0.05^{b}
23 (12)	A	0.91±0.14^{b}	0.93±0.01^{b}	0.89±0.10^{b}
	B	1.98±0.19^{a}	2.05±0.09^{a}	1.97±0.26^{a}
	C	1.66±0.11^{ab}	1.32±0.05^{ab}	1.46±0.06^{a}
	D	2.12±0.55^{a}	2.25±0.31^{a}	1.86±0.14^{a}
26 (12)	A	1.16±0.20^{b}	1.21±0.16^{b}	1.27±0.14^{b}
	B	1.99±0.03^{a}	2.04±0.35^{a}	1.85±0.12^{a}
	C	1.51±0.16^{ab}	1.57±0.25^{ab}	1.80±0.12^{a}
	D	1.81±0.29^{a}	2.30±0.05^{a}	1.74±0.08^{a}

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630

Table 2. Amino acid substitutions (red) determined in neutralizing B-cell epitopes (NE) in the isolates used in the experiment as compared with strain CO13 as reference (Okda et al., 2017). The corresponding amino acid positions are detailed beside the amino acid code.

	Amino acid substitution and its position in each strain		
	PEDV[1]	**SeCoV[2]**	**rPEDV-SeCoV[3]**
NE 499-600			
	Ser517	Ala517	Ser517
	Ile527	Val527	Ile527
	Leu536	Phe536	Phe536
	Thr537	Ser537	Ser537
	Asp542	Glu542	Asp542
	Ser549	Thr549	Ser549
	Asp566	Thr566	Asp566
	Ser583	Asn583	Ser583
	Val587	Ile587	Val587
	Gly594	Gly594	Ser594
NE 722-731			
	Ser719	Asn179	Ser719
	Ser724	Asn724	Ser724
NE 744-759			
	Lys755	Thr755	Lys755
NE 747-774			
	Lys755	Thr755	Lys755
	Ser764	Tyr764	Ser764
	Ser766	His766	Ser766
NE 1371-1377		**No changes**	

[1] Strain 2330-Orense, GenBank accession nr. MN692791.

[2] Strain 1480-Murcia-Lorca, GenBank accession nr. MN692770.

[3] Strain 1931-1-Valladolid-Molpeceres, GenBank accession nr. MN692784.

642 Table 3. Detection of specific neutralizing antibodies (NA), IgA secreting cells (SC) and
643 IFN-γ-SC against rPEDV-SeCoV. Letters show significant differences between groups
644 ($p<0.05$). Booster effect shows the comparison of results obtained immediately before
645 and after the re-challenge (dpi 20 versus dpri 3 or dpri 6) within each group (* indicates
646 statistically significant differences; $p<0.05$).

647

Group	dpi 0	dpi 20	dpri 3	dpri 6	Booster
NA: Percentage of positive animals (number positive/number animals) Mean titer ± standard deviation					
A	0%	0% (0/6)	0% (0/6)	100% (3/3) 18.7 ± 4.6	-
B	0%	100% (6/6) 19.3 ± 7.4[b]	33.3% (2/6) 8.0 ± 0.0	100% (3/3) 30.7 ± 28.9	-
C	0%	100% (6/6) 24.7 ± 13.5[b]	33.3% (2/6) 8.0 ± 0.0	100% (3/3) 56.0 ± 36.6	-
D	0%	100% (6/6) 48.0 ± 14.3[a]	83.3% (5/6) 13.6 ± 6.7	100% (3/3) 21.3 ± 9.2	-
IgA SC: Mean ± standard deviation					
A		4.0 ± 0.0[b]	2.6 ± 2.1[b]		-
B		18.1 ± 11.8[a]	32.1 ± 14.2[a]		*
C		16.9 ± 8.3[a]	33.3 ± 16.7[a]		*
D		16.2 ± 7.7[a]	25.0 ± 5.8[a]		*
IFN-γ-SC: Mean ± standard deviation					
A		1.3 ± 2.3[c]	3.0 ± 2.1[c]		-
B		8.1 ± 4.7[b]	25.3 ± 10.6[b]		*
C		11.1 ± 6.0[b]	41.4 ± 25.0[b]		*
D		26.4 ± 10.5[a]	103.3 ± 53.6[a]		*

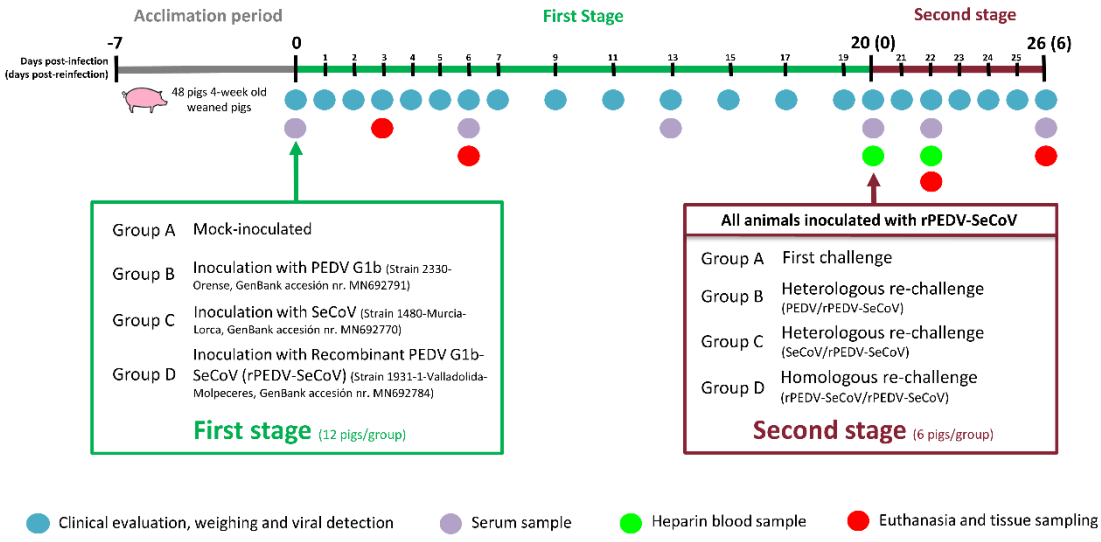
648 Pigs from group A were mock-infected while pigs from groups B, C and D were
649 challenged with PEDV, SeCoV and rPEDV-SeCoV, respectively. At day post-
650 inoculation (dpi) 20, all pigs were challenged with rPEDV-SeCoV.

Appendix Table 1. Average daily gain (ADG): mean and standard deviation for each of the groups throughout the experiment. At day 0, pigs from group A were mock-infected while pigs from groups B, C and D were challenged with PEDV, SeCoV and rPEDV-SeCoV, respectively. At the start of the 4th week (day 20) all pigs were challenged with rPEDV-SeCoV. Letters show significant differences between groups for a particular week ($p<0.05$).

ADG (Kg)	A (mock-infected)	B (PEDV)	C (SeCoV)	D (rPEDV-SeCoV)
	Mean ± standard deviation			
1st week	0.198±0.060[a]	0.065±0.049[b]	0.075±0.076[b]	0.098±0.057[b]
2nd week	0.338±0.092	0.276±0.051	0.248±0.075	0.257±0.097
3rd week	0.367±0.058	0.331±0.070	0.357±0.084	0.262±0.088
4th week	0.029±0.038[b]	0.338±0.022[a]	0.190±0.128[ab]	0.362±0.016[a]

659 Figure 1: Experimental design, clinical evaluation and sampling throughout the

660 experiment.



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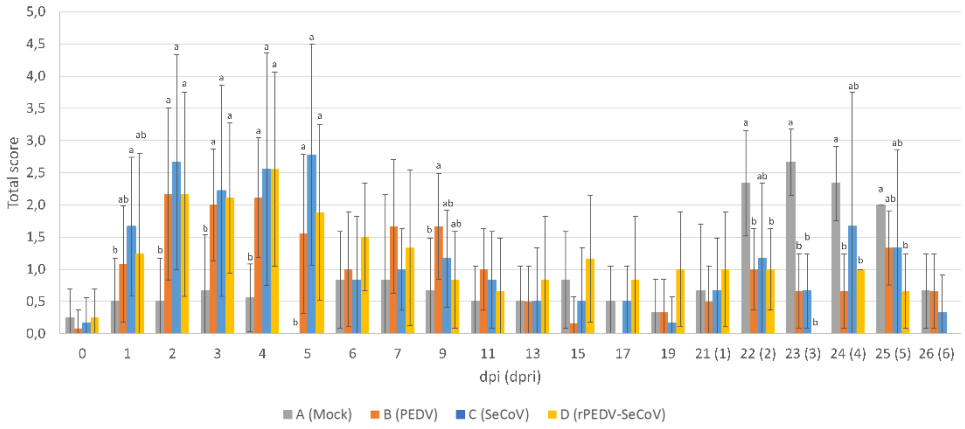
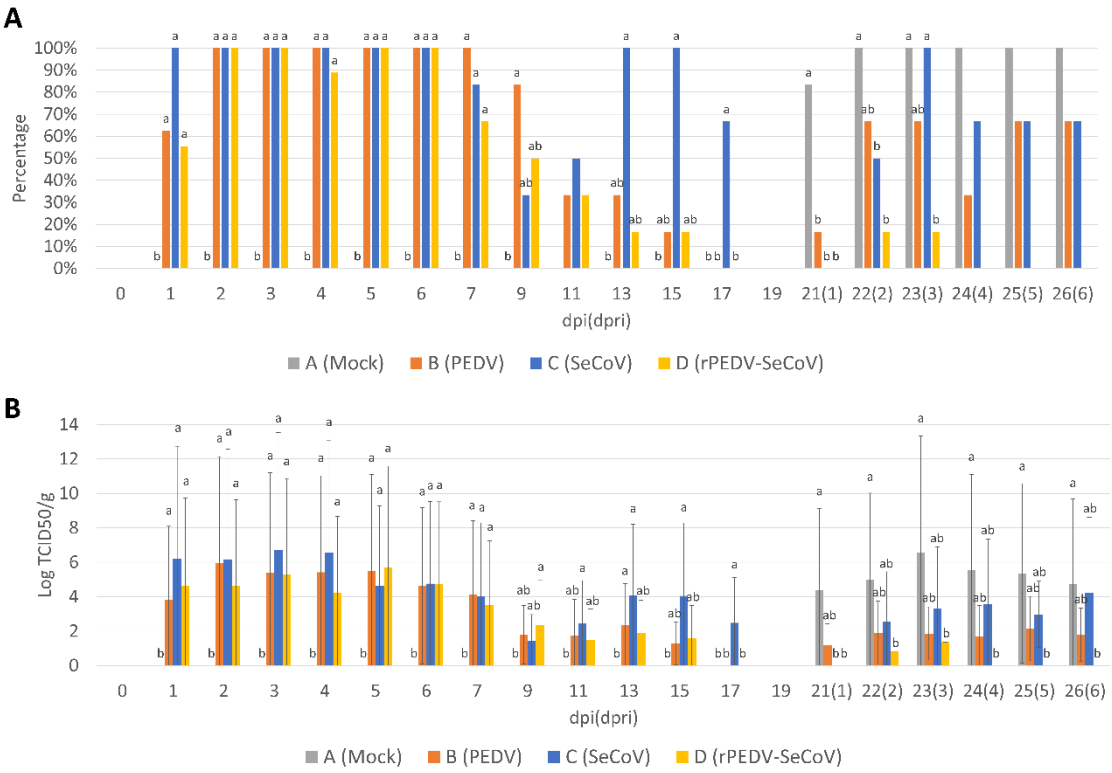
662

Figure 2. Clinical score (fecal consistency: 0-2 + general condition: 0-3 + appetite: 0-2 + vomiting: 0-1): mean and standard deviation (error bars) for each group throughout the experiment. Letters show significant differences between groups for each particular day ($p<0.05$).



At day post-inoculation (dpi) 0, pigs from group A were mock-infected while pigs from groups B, C and D were challenged with PEDV, SeCoV and rPEDV-SeCoV, respectively. At dpi 20, all pigs were challenged with rPEDV-SeCoV.

Figure 3. Viral detection in fecal samples throughout the experiment. (A) Percentage of RT-qPCR positive animals. (B) Average viral quantification (log_{10} $TCID_{50}$/g). (C) Area under the curve (AUC) for RNA viral shedding. Letters show significant differences between groups for each particular day ($p<0.05$).

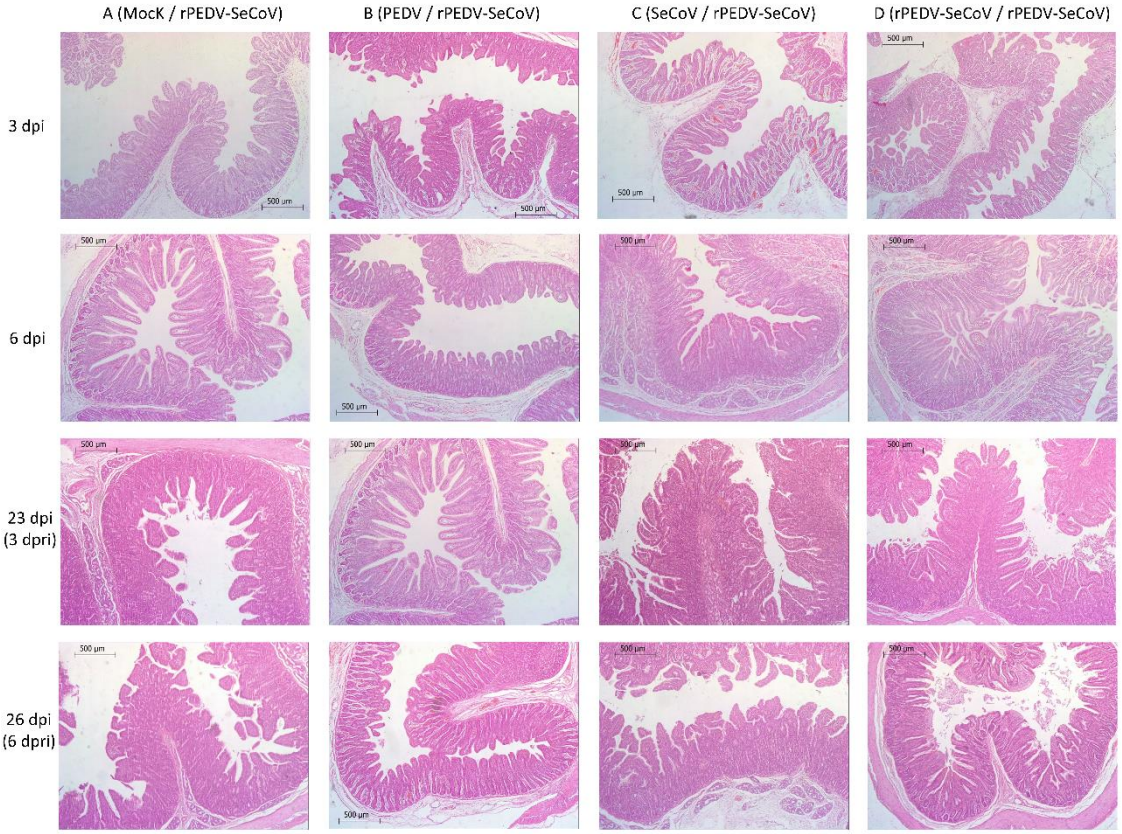


C

AUC	A (Mock)	B (PEDV)	C (SeCoV)	D (rPEDV-SeCoV)
1st stage (0-20 dpi)	0.0±0.0^{c}	47.2±5.19^{b}	70.1±9.13^{a}	49.2±9.84^{b}
2nd stage (20-26 dpi)	27.0±1.00^{a}	9.1±1.36^{bc}	14.5±5.72^{b}	2.2±1.84^{c}

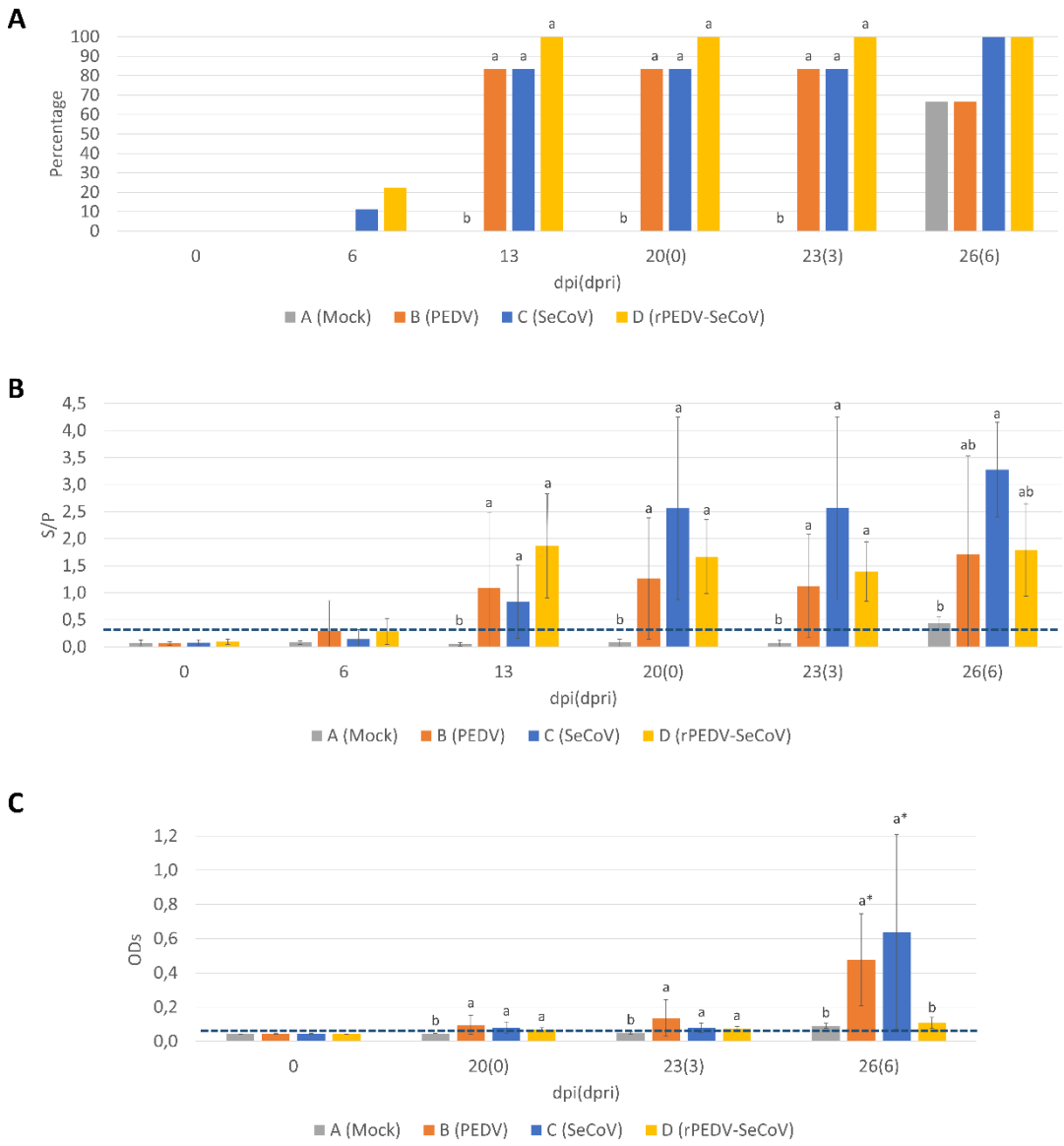
At day post-inoculation (dpi) 0, pigs from group A were mock-infected while pigs from groups B, C and D were challenged with PEDV, SeCoV and rPEDV-SeCoV, respectively. At dpi 20, all pigs were challenged with rPEDV-SeCoV.

Figure 4. Photomicrographs revealing severe villous atrophy and fusion at days post-infection (dpi) 3 and 6 in the duodenum of pigs challenged with PEDV (groups B and D) and SeCoV (group C). After re-challenge, lesions were also evident in pigs challenged for the first time (group A) and in those previously exposed to SeCoV (group C), but were absent in pigs from groups B and D previously exposed to two variants of PEDV.



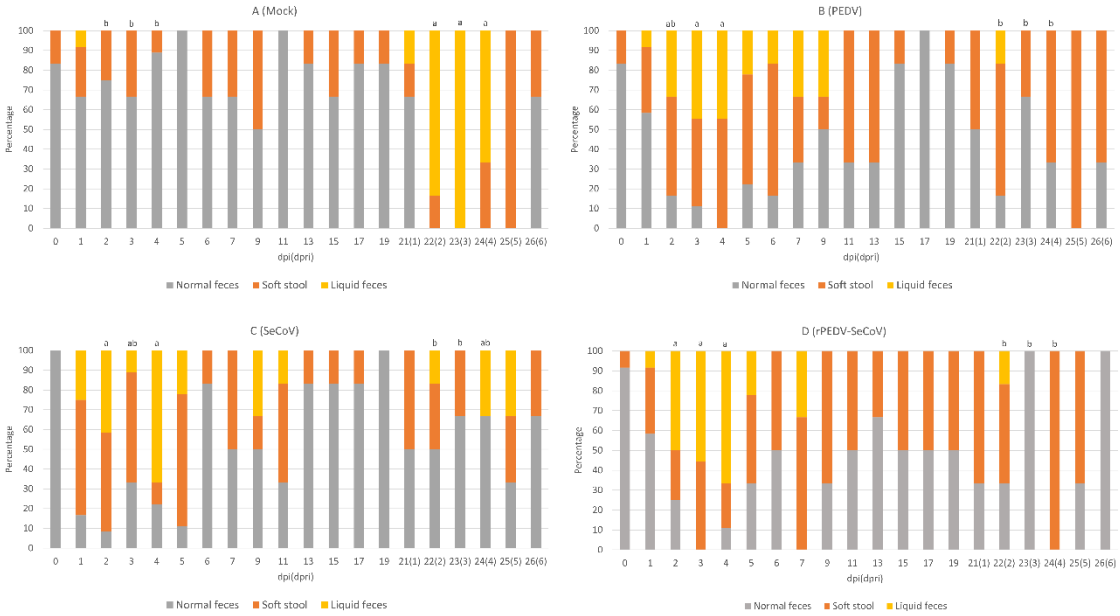
688 Figure 5. PEDV-specific IgG and IgA kinetics determined using a commercial ELISA.
689 (A) Percentage of IgG positive animals. (B) Mean S/P ratios and standard deviation
690 (error bars) of IgG detection per group. The dotted line shows the cut-off proposed by
691 the manufacturer (0.3). (C) Mean ODs and standard deviation (error bars) of IgA
692 detection. The dotted line shows the average OD of control pig sera plus two times
693 standard deviation used to discriminate positive results (0.05). Letters show significant
694 differences between groups for each particular day ($p<0.05$). Booster effect shows the
695 comparison of results obtained immediately before and after the re-challenge (dpi 20
696 versus dpri 3 or dpri 6) within each group (* indicates statistically significant

differences).



At day post-inoculation (dpi) 0, pigs from group A were mock-infected while pigs from groups B, C and D were challenged with PEDV, SeCoV and rPEDV-SeCoV, respectively. At dpi 20, all pigs were challenged with rPEDV-SeCoV.

Appendix Figure 1. Fecal consistency: percentage of animals with normal feces (grey), soft stools (orange) and liquid feces (yellow) for each group. Letters show significant differences between groups for each particular day ($p<0.05$).



At day post-inoculation (dpi) 0, pigs from group A were mock-infected while pigs from groups B, C and D were challenged with PEDV, SeCoV and rPEDV-SeCoV, respectively. At dpi 20, all pigs were challenged with rPEDV-SeCoV.