The immune response does not prevent homologous *Porcine epidemic diarrhoea virus* reinfection five months after the initial challenge.

Short running title

Duration of PEDV immunity

Authors

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SUMMARY

The aim of the present study was to evaluate the duration of protective immunity against *Porcine epidemic diarrheoa* virus (PEDV). To that, a two phases study was performed. In the first phase, 75 four-week-old pigs (group A) were orally inoculated (0 days post-inoculation; dpi) with a European PEDV G1b strain and 14 were kept as controls (group B). The second phase started five month later (154 dpi), when animals in group A were homologous challenged and animals in group B were challenged for first time. Clinical signs, viral shedding and immune responses were evaluated after each inoculation, including the determination of antibodies (ELISA and viral neutralisation test, IgA and IgG ELISPOTs using peripheral blood mononuclear cells and lymph node cells) and the frequency of interferon-gamma (IFN-γ) secreting cells. During the first phase, loose stools/liquid faeces were observed in all group A animals. Faecal shedding of PEDV occurred mostly during the first 14 days but, in some animals, persisted until 42 dpi. All inoculated animals seroconverted for specific-PEDV IgG and IgA, and for neutralizing antibodies (NA). At 154 dpi, 77% of pigs were still positive for NA. After that, the homologous challenge resulted in a booster for IgG, IgA, NA, as well as specific-PEDV IgG, IgA and IFN-γ secreting cells. In spite of that, PEDV was detected in faeces of all pigs from group A, indicating that the immune response did not prevent reinfection although the duration of the viral shedding and the total load of virus shed was significantly lower for previously challenged pigs ($p<0.05$). Taken together, the results indicated that, potentially, maintenance of PEDV infection within an endemic farm may occur by transmission to and from previously infected animals and also indicates that sterilising immunity is shorter than the productive life of pigs.

KEYWORDS: Coronavirus**,** *Porcine epidemic diarrhoea virus,* immunity.

1. INTRODUCTION

Porcine epidemic diarrhoea (PED) was first described in Europe in the 1970s (Pensaert & de Bouck, 1978; Wood, 1977). The disease spread in Europe and Asia, where remained endemic, while America stayed PED-free. The causative agent of PED, PED virus (PEDV) is classified as an *Alphacoronavirus*, together with other coronaviruses of pigs such as the *Transmissible gastroenteritis virus*, the *Porcine respiratory coronavirus* and other porcine enteric coronaviruses (Antas & Woźniakowski, 2019; ICTV, 2011). PEDV strains are often divided in "classical", namely, those arising before 2010, and "emerging", arising after 2010. Emerging strains are subdivided in S INDEL and non-S INDEL based on the presence of insertions and deletions in the spike protein (Lee, 2015).

In 2013 PED emerged in America, causing a major epidemic that spread over the continent (Schulz & Tonsor, 2015). Two different PEDV are currently identified in the USA: G1b (S INDEL) and G2b (non-S INDEL). G1b strains, which can be also found in Europe and Asia, usually presenting low or moderate virulence, while G2b -isolated only in Asia, North America and Ukraine- always seems to be highly virulent (Carvajal et al. 2015; Dastjerdi et al., 2015).

PEDV infection causes an acute enteritis affecting the small bowel. There is an intense shortening of the villi that results in impaired absorption capabilities (Debouck et al., 1981). The fatality rate in newborns can reach almost 100%, but mortality decreases with age being almost nil in fatteners or adults (Lee, 2015; Shibata et al., 2000). After a PEDV introduction in a naïve farm, the infection spreads very rapidly, and most animals become infected in a matter of weeks. A clinical outbreak lasts 2-3 weeks, although can be longer in some cases (Martelli et al. 2008). As most animals became immunized the clinical disease mostly establishes in nurseries, where weaners can be infected after losing maternally derived antibodies.

Infection control is most often based on immunising sows before the first parturition. Since it has been demonstrated that colostral/lactogenic immunity is effective protecting piglets (Chattha et al., 2015; de Arriba et al., 2002; Goede et al., 2015; Langel et al., 2019), the rationale behind that strategy is to provide the piglets with a sufficient level of maternally derived antibodies. By this means, the piglet is

protected during the riskiest period and, if infected later, consequences are expected to be of lesser importance. Since fully effective vaccines have not been released yet and the commercialized ones are not available everywhere (Li et al., 2020), immunisation of gilts and sows is most often achieved by letting them to enter in contact with contaminated materials (for example faeces of diarrhoeic animals) during early gestation (Niederwerder & Hesse, 2018).

A question of major importance is how long protective immunity persists after an infection. In PEDV, previous studies indicated that neutralizing antibodies (NA) could be present 6 months after the initial infection (Clement et al., 2016; Ouyang et al. 2015). Piglets challenged at 30 days after an initial contact with the virus had some level of clinical and virological protection related to NA, but also to cell-mediated immunity (Krishna et al., 2020). However, although some data are available about the duration of antibodies, less is known about protection against a new challenge at longer periods. This can be of importance to understand PEDV epidemiology and control. Also, silent infections in adults may be present, potentially causing PEDV re-introduction in maternities. The aim of the present study was to evaluate the extent and duration of immunity in a model of infection in piglets and homologous challenge five months later. Humoral and cell-mediated responses were assessed along with the examination of viral shedding.

2. MATERIAL AND METHODS

2.1 Experimental design

Eighty-nine three-week old piglets were selected from a PEDV-negative farm, as determined by RT-qPCR (VetMAX Swine Enteric Panel TGEV/PEDV/PRV-A kit; Thermo Fisher Scientific Inc., Madrid, Spain) and ELISA (INgezim PEDV; 11.PED.K1; Eurofins INGENASA, Madrid, Spain). Animals (only males) from 24 different litters (3-4 animals per litter) were transported to the experimental facilities and ear tagged. The study was divided in two phases (Table 1). During the first phase, pigs were randomly distributed and placed separately in two groups: A (n=75) and B (n=14). At arrival, all animals were intramuscularly injected with Ceftiofur (3 mg/kg; EXCENEL, Zoetis, Hostalnou de Bianya, Spain) to prevent diarrhoea by *E.coli*. Piglets were left to acclimatize for one week. At 0 days post-inoculation (dpi), animals in group A were

inoculated orally using a gastric cannula with 2 mL of intestinal content of a diseased piglet containing the European G1b PEDV Calaf-1 (GenBank accession number MT602520), at a dose of cycle threshold (Ct) = 14.7, while animals in group B were mock-infected with PBS. One hundred and fifty-four days later (154 dpi), the second phase started. All pigs included in the study were inoculated orally with 5 mL of Calaf-1 PEDV as described above at a Ct=23.83. This second inoculum was adjusted to a lower load in order to mimic Ct values determined in faeces of the animals infected in the first phase. By doing this, group A was subjected to a homologous challenge, while group B was inoculated with the virus for the first time.

All experiments involving pigs were done under the approval of Ethical Committee of IRTA and authorized by the Catalan Government (Ref. CEO-H/9450). Animals were kept in approved experimental facilities and were subjected to veterinary supervision for health and welfare. Handling of pigs was done by veterinarians and trained personnel that fulfilled the Spanish and European Union requirements. Animals were clinically examined on arrival and supervised during all the experiment.

2.2 Clinical follow-up and sampling

The appearance of faeces was scored individually during both phases of the study using a scale with four categories: 0 (firm and shaped), 1 (soft/loose), 2 (semi-liquid faeces), and 3 (liquid faeces). Individual faecal and serum samples were collected weekly during the first six weeks after inoculation and then at 56, 78, 105, 133 and 154 dpi (just before the second phase). After that, faecal and serum samples were collected at 157, 161, 164 and 168 dpi.

Immediately before the start of the second phase (154 dpi), 30 piglets from group A and nine from group B were randomly selected (random ear tag numbers) and blood samples were collected in heparin tubes to obtain peripheral blood mononuclear cells (PBMC). Thirteen of those animals, 10 from group A and three from group B, were euthanised and mesenteric lymph nodes were collected to obtain lymph node mononuclear cells (LC). At 157 dpi, the remaining 26 pigs were bled again and thirteen of them (10 from A and 3 from B) were euthanised to collect mesenteric lymph nodes (Table 1).

2.3 Viral inoculum

The inoculum was obtained from four 3-day-old piglets intragastrically inoculated with 2 mL of the intestinal content of a pig with PEDV diarrhoea from a commercial farm (European G1b PEDV Calaf-1). By the second day, piglets developed severe diarrhoea and they were euthanized. Intestinal content and mucosal scrapings of duodenum and jejunum were collected, diluted 1/100 in PBS and stored at -80ºC. This suspension was found to be negative to *Transmissible gastroenteritis virus, Rotavirus A* (VetMAX Swine Enteric Panel TGEV/PEDV/PRV-A kit) and to *Porcine Circovirus 2* (VetMAX™ Porcine PCV2 Quant Kit, Thermo Fisher Scientific) and was used as experimental inoculum.

2.4 Virus isolation

An isolate of the PEDV inoculum strain (Calaf-1; GenBank accession number MT602520) was used in viral neutralization test (VNT) and ELISPOT analyses. Briefly, intestinal contents of the inoculated piglets were centrifuged to 15,000 g for 15 min, diluted 1:10 in DMEM-high w/glutamax (Thermo Fisher Scientific), with 300 UI/mL penicillin and 300 µg/mL streptomycin (Thermo Fisher Scientific), 50 µg/mL nystatin (Merck, Madrid, Spain), 0.02% yeast extract (Thermo Fisher Scientific) and, 0.3% tryptone phosphate broth (Merck). The suspension was filtered through a 0.22 µm filter (Merck Millipore, Madrid, Spain) and trypsin was added to a final concentration of 10 µg/mL (Trypsin solution from porcine pancreas, Sigma). Then, 0.5 mL of the trypsin-treated suspension was inoculated onto VERO cells (ATCC CCL-81) on 25cm^2 flasks. After 2h of adsorption at 37 ºC, 6 mL of the dilution medium was added, and cultures were incubated at 37 °C. After being cultured for 5 days a cytopathic effect (CPE) was observed and cultures were frozen and thawed to recover the virus. A single virus stock was used for the immunological analysis ($10^{5.3}$ $TCID_{50}$/mL, passage 22).

2.5 RT-qPCR for the detection of PEDV in experimentally inoculated animals

Collected faeces were initially diluted 1/10 in sterile PBS. After vortex, samples were centrifuged at 4,000 g for 10 min and the supernatant was recovered, aliquoted and frozen at -80 ºC until needed. Viral RNA from faecal suspension supernatants and sera was extracted with the MagMAX pathogen RNA/DNA kit (Thermo Fisher Scientific) and the BioSprint 96 workstation (Qiagen Iberia, Barcelona, Spain), according to the manufacturer's instructions. The presence of PEDV was determined with a real time

RT-qPCR commercial kit (VetMAX Swine Enteric Panel TGEV/PEDV/PRV-A kit, with Path-ID Multiplex One-Step RT-PCR kit; Thermo Fisher Scientific). Positive and negative controls (serial log_{10} dilutions of PEDV strain CV777 or from negative samples) were included in each RNA extraction and RT-qPCR reaction batch. Results of the RT-qPCR were expressed as Ct values.

2.6 Sequencing

The inoculum strain PEDV Calaf-1 was sequenced using the Illumina Miseq Platform applying the protocol described for RNA viruses by Cortey et al. (2019). The method applied did not include any PCR amplification step. The pipeline included: i) the construction of a genomic library for Illumina NGS sequencing, ii) the trimming of low quality reads (those showing quality scores lower than 20) with Trimmomatic (Bolger et al., 2014), iii) the mapping of reads against the PEDV reference genome available at NCBI (Accession Number NC_003436) and the Burrows-wheeler aligner (Li & Durbin, 2010), and iv) the assembly of a consensus genome sequence using the program Consensus (https://www.hiv.lanl.gov/content/sequence/CONSENSUS/consensus.html).

Genotyping of the Calaf-1 was determined by comparing its genome with two representatives of the genotypes 1a (GenBank accession numbers GU937797 and EF185992) and 1b (GenBank accession numbers KJ645635 and LT900502).

Virus stock used for immunological assays, as well as faecal samples yielding Ct<20 (n=13) were directly sequenced applying the same protocol described above.

2.7 Specific-PEDV IgG and IgA

A commercially available ELISA based on the spike glycoprotein was used to measure specific-PEDV IgG in sera (Ingezim PEDV; 11.PED.K1, Eurofins INGENASA). According to the manufacturer instructions, sample to positive control S/P ratios > 0.35 were considered as positive. All samples were tested using plates and reagents from the same kit batch. Samples yielding doubtful results were retested to discard any potential error attributable to the laboratory processing.

Kinetics of specific-PEDV IgA in serum were measured using a modification of the abovementioned commercial kit, in which the anti-pig IgG conjugate was

substituted by a goat anti-pig IgA HRP conjugate (1mg/mL; Bethyl Laboratories, Montgomery, USA). Final serum samples dilution (1:100) and anti-pig A concentration (1:150,000) were chosen after preliminary titration tests, which were done according to the manufacturer's instructions and previous reports (Gerber & Opriessnig, 2015).

2.8 Viral neutralization test

Selected serum samples were tested for the presence of NA at 0, 14, 42, 154, 157, 161 and 168 dpi for those animals in group A and B from which PBMC were obtained and were not euthanized before the end of the second phase (n=10 and 3 for A and B groups, respectively) (Table 1). NA were also analysed at 154 dpi for the remaining animals from which PBMC were obtained (26 additional animals).

VNT was performed according to the procedures described by Thomas et al. (2015), with minor modifications. Samples were serially diluted with DMEM from 1:4 to 1:512; 100 µl of the virus/serum mixture were inoculated onto Vero cells (200 $TCID_{50}$ in 100 µl of virus) and the neutralization was read after 24 h of incubation. Cells were fixed with absolute ethanol and stored at -20ºC. Staining was performed by incubating plates (1h at 37 ºC), with the anti-PEDV monoclonal antibody (SD-1F-1 8D6-29PED-NP, Medgene Labs, Brookings, USA) conjugated with FITC (1:200). Negative controls (cell cultures mock-stimulated and plus SFB), viral infection control (200 $TCID_{50}$) and positive controls (field samples from PEDV-positive farms) were added on each daily set of plates. Plates were read under the fluorescence microscope, taking the titre as the reciprocal of the highest dilution resulting in ≥ 90 % reduction of fluorescent foci compared to negative controls. Titres were expressed as $\log_2$. VNT titres below 1:8 was not considered significant as in Thomas et al. (2015).

2.9 PBMC AND LC

Frequencies of PEDV-specific IgG, IgA and IFN-γ secreting cells (SC) in PBMC and LC were determined by ELISPOT assays. PBMC were separated from whole blood by density-gradient centrifugation with Histopaque 1.077 (Sigma), whereas LC were obtained according to Aasted et al. (2002) with minor modifications. Mesenteric lymph nodes were surgically removed and transported to the laboratory in DMEM supplemented with antibiotics (300 U/mL penicillin, 300 µg/mL streptomycin, 150 U/mL nystatin, and 50 µg/mL gentamicin). After removing the adjacent fatty issue,

lymph nodes were diced and crushed. To obtain single-cell suspensions, they were filtered through a gauze filter and a stainless-steel mesh. Cell clumps and aggregates were removed by two consecutive filtering steps through 70 and 40 μm pore size filters (Corning). Finally, LC were washed by consecutive centrifugations with DMEM plus antibiotics and separated by density-gradient centrifugation as described above.

PBMC and LC were stored in cryovials at -150 ºC at a density of $2x10^7$/ml using a cryopreservation medium (Cryostor CS10, Stemcell, Grenoble, France). When needed, cells were thawed and resuspended in RPMI + 10% SFB. Viability was assessed using trypan blue. Samples were only used when cell viability was higher than 90%.

2.10 IgG and IgA ELISPOT

IgG and IgA-SC were measured by means of commercial ELISPOT kits (Porcine IgG ELISPOT BASIC, Mabtech, Nacka Strand, Sweden; Pig IgA single-color ELISPOT, CTL, Cleveland, USA). For both PBMC and LC, cells from the cryovials were separated in aliquots ($1x10^7$ in each tube). One of the aliquots was stimulated for 72 h (37 ºC, 5% CO_2 with the polyclonal activator R848 (Mabtech) and recombinant porcine IL-2 (R&D Systems, Abingdon, UK) at 1 μg/mL and 10 ng/mL, respectively (Jahnmatz et al., 2013). The other tube remained as an unstimulated control. The day before the assay, nitrocellulose bottomed plates (MultiScreen-HA plates, Merck Millipore, for IgG and PVDF plates, CTL, for IgA) were coated with Calaf-1 PEDV strain at 10^4 $TCID_{50}$/mL. This concentration was chosen after a preliminary dose-response test, as advised by the manufacturer of the kit, and by previous reports (Jahnmatz et al., 2013; Kesa et al., 2012). After three days of stimulation, cells in each tube were re-counted and adjusted to 250,000 and 500,000 cells/well. All tests were run in triplicates. By using the virus as a coating antigen in the plates, only PEDV-specific antibodies were detected. Plates were revealed following the manufacturer's instructions. Frequencies of PEDV-specific IgG or IgA-SC were calculated by subtracting counts of spots in unstimulated cells, from counts in stimulated ones. PEDV-specific number of IgG and IgA-SC were expressed as responding cells/10^6 PBMC or LC.

2.11 IFN-γ ELISPOT

Cell-mediated immune responses were measured by using the IFN-γ ELISPOT. The technique was performed as previously described (Diaz et al., 2005) using MultiScreen-HA filter plate (Merck Millipore), commercial monoclonal antibodies (porcine IFN-γ P2G10 and biotin P2C11, BD Biosciences Pharmingen, San Jose, USA) and TMB substrate for ELISPOT (Mabtech). PBMC and LC were adjusted to 250,000 and 500,000 cells/well. To evaluate PEDV-specific IFN-γ-SC, cells were stimulated with Calaf-1 isolate at a multiplicity of infection of 0.1. The viral dosage was determined according to preliminary dose-response tests. Unstimulated cells and phytohaemagglutinin (PHA)-stimulated cells (10 μg/mL) were used as negative and positive controls, respectively. All tests were run in triplicates. Frequencies of PEDV-specific IFN-γ-secreting cells (IFN-γ-SC) were calculated by subtracting counts of spots in unstimulated wells from counts in virus-stimulated wells. Results were expressed as responding cells/10^6 PBMC or LC.

2.12 Statistical analysis

Statistics were performed using StatsDirect v2.7.7. Mann-Whitney U and Kruskal-Wallis test (Dwass-Steel-Chritchlow-Fligner method for multiple comparisons) non-parametric tests were used for comparisons of means between two or more set of data, respectively. Comparison of the proportions of positive animals was determined by the $\chi 2$ test (Fisher's exact test). The area under the curve (AUC) for shedding in faeces was calculated using the trapezoidal approach (Schäfer et al., 2001). A survival analysis for detection of PEDV genome in faeces and IgG in sera from group A was done by means of the Kaplan-Meier survival test.

3. RESULTS

3.1 Clinical follow-up

Figure 1 shows the clinical scores for the different groups and timepoints. In phase one, all inoculated animals had at least one day of loose stools/liquid faeces. The highest proportion of animals (88%) and the highest clinical scores (120 out of a potential maximum of 225, 3 points x 75 animals) were recorded at 4 dpi. The last day that the animals showed loose stools or diarrhoea was 21 dpi (9% of the animals). In the second phase, loose stools/liquid faeces were observed in 36% of the animals in group A at 3 days after the homologous challenge (157 dpi), declining afterwards. At seven

and ten days (161 and 164 dpi), the percentage of animals with loose stools/liquid faeces in group B was significantly higher than in group A (62 and 4% for both dates, respectively; $p<0.05$) (Figure 1). The accumulated incidence of animals with loose stools/liquid faeces in the homologous challenged group A (36%) was significantly lower than the accumulated incidence in group B (100%; $p<0.05$).

3.2 Virological analysis

All faecal and serum samples from both groups were negative for PEDV at 0 dpi as determined by RT-qPCR. All pigs (100%) from group A shed PEDV in faeces at 7 dpi (Figure 2) with an average Ct=24.3±4.0. Afterwards, both the proportion of positive samples and the viral load significantly declined until day 21 dpi, when only 28.4% ($CI_{95\%}$: 18.1-38.6%) of the inoculated pigs were still positive (Ct=35.2±3.00). The survival analysis revealed that the time needed for the shedding animals declining to 50% was between 21 and 28 days. At 35 dpi, two animals were PEDV positive in faeces and one was still shedding at 42 dpi (1/75; 1.3% $CI_{95\%}$: 0.0-8.2%; Ct=36.7).

During the second phase, PEDV was detected in all pig faeces in group A, although the percentage of positive animals never reached 100% in any of the examined days. The highest percentage, as well as the lowest Ct values were detected at seven days, namely 161 dpi (87% $CI_{95\%}$ 77.7-95.9%; Ct=27.3±5.6). By day 14, the proportion of positive animals in group A decreased until 27.8% ($CI_{95\%}$ 15.2-39.7%; $p<0.05$).

Regarding the pigs infected for the first time at 154 dpi (group B), all were positive three to ten days after the inoculation (157 to 164 dpi). The lowest Ct values were observed at seven days post-challenge (23.1±5.6). Ten days after the challenge (164 dpi), the proportion of PEDV positive pigs was 100% ($p<0.05$ compared to group A). At the end of the experiment (168 dpi), 51% ($CI_{95\%}$ 35.3-62.7%) of pigs in group B were still shedding virus in faeces (Ct=32.0±4.2).

Figure 2 also summarizes the results of viral shedding for days 1-14 after the first and second challenges. Comparison of average Ct values showed that, at the shedding peak (7 days after the challenge), the viral load in faeces was similar in older naïve animals compared to naïve young piglets (Ct 23.1 vs. 24.3, respectively; non-significant), although the total shedding load (area under the curve) was higher for

younger naïve animals. Average viral loads, as well as total shedding load in faeces were significantly lower for animals challenged for second time.

3.2 Sequence comparison

Positive PEDV samples sequenced at any of the examined times (up to 161 dpi) or the virus used for the immunological assays were >99.8% similar (spike gene) to the original inoculum used at day 0.

3.3 PEDV-specific IgG

All pigs were seronegative for PEDV antibodies at 0 dpi. Control animals (B) remained negative during the first phase. Regarding group A, 96% of the animals were classified as seropositive at 14 dpi (72/75; $CI_{95\%}$=91.5-100%) (Figure 3). One week later, all piglets had seroconverted and remained positive until 56 dpi, when seropositivity lowered to 92% (69/75; $CI_{95\%}$=85.9-98.1%). Afterwards, the proportion of seropositive pigs steadily decreased until day 154, when only 27% of seropositive animals was determined by ELISA (20/75; $CI_{95\%}$=16.0-37.9%). The survival analysis for IgG revealed that half of the pigs were seronegative at 105 days. Regarding the antibody S/P ratios, the peak was reached at 21 dpi, declining after 42 dpi (Figure 3).

By day 7 after the homologous challenge (161 dpi), seroconversion reached 74% for group A pigs ($CI_{95\%}$=61.7-85.4%), whereas by day 10 (164 dpi) all had seroconverted. The average S/P ratio showed a significant increase by day 7 after inoculation (161 dpi), from 0.3±0.3 to 1.9±1.7 at 154 and 161 dpi, respectively ($p<0.05$), reaching a maximum at 168 dpi (average S/P = 4.0±0.9). For group B, the inoculation at 154 dpi resulted in seroconversion for all animals by day 10 (164 dpi), with an average S/P ratio of 1.7±1.0.

When results at fourteen days after the first and the second phase of the study were compared, S/P ratios of animals inoculated in the homologous challenge were significantly higher than those of naïve animals inoculated at 154 dpi, as well as animals inoculated at 0 dpi: $3.6\pm0.9^{a} > 2.3\pm1.3^{b}$ and 1.8 ± 0.9^{b}, respectively ($p<0.05$).

3.4 PEDV-specific IgA

Figure 4 shows the evolution in the optical densities (ODs) of PEDV-specific IgA in group A during the first and the second phase. Significant differences ($p<0.05$)

between the optical densities (ODs) of inoculated and non-inoculated pigs were observed from 7 dpi until 56 dpi ($p<0.05$), when some pigs in group A were no longer differentiable from uninoculated pigs.

After the homologous challenge, the average ODs in group A showed a significant increase by day 7 (from 0.1±0.0 at 154 dpi to 0.6±0.6 at 161 dpi; $p<0.05$), further increasing until the end of the experiment (1.5±0.6 and 1.4±0.4 at 164 and 168 dpi, respectively). For pigs in group B, seroconversion was observed in all animals ten days after the inoculation (164 dpi; 0.3±0.1), reaching an average S/P of 0.6±0.3 at 168 dpi. During this second phase, average S/P values in group A were always higher than average values of group B animals ($p<0.05$).

When comparing results obtained at fourteen days after the first and the second phase of the study, average ODs from pigs in group A after the homologous challenge were higher than those from group B and, in turn, the latter were higher than those in group A after the first challenge (1.4±0.4 > 0.6±0.3 > 0.1±0.1, respectively; $p<0.05$).

3.5 Viral neutralization test

Results of the viral neutralization test are summarized in Figure 5. Samples from both groups were negative at 0 dpi. Control animals (B) remained negative during the first phase. In regards of group A, NA were firstly detected at 14 dpi ($\log_2$=3.9±0.6). The two animals shedding PEDV at 35 dpi were below the positive threshold for NA ($\leq 3 \log_2$). All analysed animals were positive at 42 dpi ($\log_2$=4.3±0.5).

Immediately before the homologous challenge (154 dpi), NA were detected in 23/30 animals (77%) from group A ($\log_2$=4.9±0.6). At 168 dpi all were positive ($\log_2$=6.8±0.9). In group B, NA were firstly detected at the end of the study (100%; $\log_2$=4.8±0.6 at 168 dpi). When NA titres at fourteen days after the inoculation in the first or the second phase were compared (namely 14 dpi vs. 168 dpi) the highest titres were observed for group A at 168 dpi (6.8 ± 0.9^{a} for A at 168 dpi; 3.9 ± 0.6^{b} for A at 14 dpi and, 4.8 ± 0.6^{b} for B at 168 dpi; $p<0.05$).

3.6 IgG and IgA ELISPOTs

Frequencies of specific-PEDV-SC for IgG and IgA in PBMC and LC collected at 154 dpi and 157 dpi are shown in Figure 6. At 154 dpi, the highest frequencies of

PEDV-specific IgG-SC were detected for LC of group A (LC A: 15.6±4.5^{a} > PBMC A: 6.8±2.6^{b} > PBMC and LC B: 0.7±0.6^{c} and 1.0±0.0^{c}, respectively; $p<0.05$) (Figure 6a). Similar figures were observed for PEDV-specific IgA (figure 6b). Three days later, a clear memory response was observed for PEDV-specific IgG and IgA-SC in PBMC and LC of animals in group A. In contrast, for naïve animals inoculated at 154 dpi (group B), no significant increase was observed, confirming that they had had no previous contact with the virus (figures 6a and 6b).

3.7 IFN-γ ELISPOT

At 154 dpi, the highest frequencies of specific-PEDV IFN-γ-SC were detected for LC in group A (LC A: 11.4±6.6^{a} > PBMC A: 7.5±3.2^{b} > PBMC and LC B: 0.2±0.3^{c} and 0.5±0.5^{c}, respectively; $p<0.05$). Similar figures were observed three days later; LC A 28.2±13.9^{a} > PBMC A: 18.0±9.9^{b} > PBMC and LC B: 1.7±0.6^{c} and 2.0±1.0^{c}, respectively ($p<0.05$). Compared to the average frequencies at 154 dpi, significant increases were observed in group A for both PBMC and LC after the challenge ($p<0.05$). No significant increases were observed neither for PBMC, nor for LC in group B.

3.8 Correlation between specific-PEDV-SC from PBMC and LC

Correlation between frequencies of specific-PEDV-SC for IgG, IgA and IFN-γ by PBMC and by LC are summarized in table 2. The correlation was only significant for IFN-γ and IgA ($p<0.05$).

4. DISCUSSION

The first introduction of PEDV in a naïve farm usually results in an epidemic with high mortality among suckling piglets (Antas & Woźniakowski, 2019). Afterwards, the infection becomes endemic, with recurrent episodes of diarrhoea in nurseries, but a considerable lesser impact in maternities or other phases (Carvajal et al., 2015; Stevenson et al., 2013). This pattern is related to the development of immunity in sows that transfer colostral/lactogenic immunity that protect suckling piglets (Clement et al., 2016). Some evidence suggests that recurrent epidemics, in the whole farm, may also happen up to two years after the original introduction of the virus (Diep et al., 2017 and 2018; Jang et al., 2019). Those new outbreaks can be caused by variants of the

strain detected in the first case. Understanding how the infection is maintained in a farm is of importance; to understand how to control it and to figure out the feasibility of PEDV eradication o in that farm.

Recurrence of PEDV in a farm can be also the result of several factors: from periodic lateral introductions of different strains, to persistence of the virus in the facilities (environmental source of contagion), or to the existence of subclinical infections in animals of different ages, constantly reintroducing the virus in different production phases. In the present study, we aimed to test whether immunity after infection may last enough to protect pigs throughout the first six-months of life, representing the productive life of a fattening pig, or a gilt before entering the reproductive cycle.

The results of the present study clearly showed that, under the conditions of the experiment, 154 days after the initial infection all pigs could be infected, although pre-existing immunity probably resulted in a lower total viral shedding compared to naïve pigs of the same age or younger. Actually, at the shedding peak (day 7 post-inoculation), the average Ct values for PEDV in faeces of naïve or immunized pigs was Ct=23.1 and Ct=27.3, respectively (in our case, equivalent to $10^{3.2}$ – $10^{2.2}$ $TCID_{50}$/gr). Considering that the minimal infectious dose for PEDV has been established around 10^{1}-10^{3} $TCID_{50}$ (Schumacher et al., 2016; Thomas et al., 2015), our results would indicate that, potentially, PEDV infection might persist in a farm by transmission to and from older animals. Moreover, in our model of infection, if sterilising immunity was present, it seemed to be shorter than the productive life of pigs. In other reports, a second challenge of animals previously inoculated with PEDV (up to 7 weeks before the second challenge) was mostly unsuccessful, suggesting that for the first weeks after the initial infection, immunity is sterilising (Crawford et al. 2015; Gerber et al., 2016; Krishna et al., 2020).

Similar to other reports (de Arriba et al., 2002; Krishna et al., 2020; Thomas et al., 2015), the development of antibodies against PEDV was fast both in young and older animals, and seroconversion was clear 14 days after the inoculation. Interestingly, a strong anamnestic response was observed after the second inoculation for IgG, IgA and neutralizing antibodies. This observation is at odds with Krishna et al. (2020), who reported that no significant increase in IgA or IgG levels were observed in previously

exposed pigs after challenge and partially disagree with Gerber et al. (2016), who showed that re-challenge of 8-week-old pigs resulted in an increase in IgG but not in serum IgA. There could be several reasons for those discrepancies. A high titre of NA in the gut could have neutralized the virus before replication occurred and therefore, the potential booster would have been less potent than if replication happened. In any case, the pattern of humoral response observed in the present study for homologous challenged animals was canonical and represented a typical anamnestic response. Moreover, the pattern of anamnestic humoral response was also observed in the IgG and IgA ELISPOTs using PBMC or LN. The results for IgA, using PBMC or LN were significantly related, suggesting that PBMC may be potentially used as a subrogate sample for examining PEDV-specific IgA responses in live animals (de Arriba et al., 2002).

It is worth to note that NA were present in serum of most homologous challenged animals at 154 dpi, but those titres were not correlated with sterilising immunity, as shown by the fact that all challenged animals were infected. Interestingly, NA titres drop immediately after the challenge (see Figure 5) and rose afterwards, probably indicating that a part of the NA was exhausted in the neutralization of the inoculum and the first viral replication, but then an anamnestic response took place. In any case, AUC for viral shedding was significantly lower for homologous challenged pigs (group A) compared to naïve pigs. This is a clear indication that immunity played a role in controlling the infection.

Regarding the IFN-γ results, it was evident that the anamnestic response also existed and, again, there was a correlation between PBMC and LC. However, the magnitude of the response was low and is difficult to assess the biological significance regarding the control of the infection.

Besides the immune response, differences were also observed in the clinical outcome of the infection and the viral shedding. Infection in younger animals (4-weeks of age) caused a mild disease, while when animals were homologous challenged at 154 dpi, clinical signs were almost absent. In previous experiments, the use of the strain Calaf-1 caused a serious diarrhoea in 2-day-old animals (not shown). Moreover, in the present study, the Ct values of naïve animals inoculated at 4 or 24 weeks of age were similar, but the 14-day AUC was lower for older animals, suggesting as indicated

before, that the susceptibility of pigs to PEDV is related to age, being older pigs less susceptible (Carvajal et al., 2015; Stevenson et a., 2013).

In summary, five months after the initial infection with the PEDV strain Calaf-1 (G1b), sterilising immunity was absent, and all animals could be re-infected. This result indicates that in an endemic farm, older animals may contribute to the maintenance of the infection as recipients, but also as transmitters of the virus. This fact also emphasizes the need to maintain high levels of immunity in the gilts and sows, to minimize the chances of transmission to piglets and to increase colostral/lactogenic immunity.

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6. CONFLICT OF INTEREST STATEMENT

The Authors declare no conflict of interest with respect to the research, authorship, and/or publication of this article.

7. DATA AVAILABILITY STATEMENT

Data are available by direct contact with the correspondence author.

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	1ST PHASE			2ND PHASE				
	0 Days post-infection (dpi) **(4 weeks of age)**			**154 dpi (+0)** **(24 weeks of age)**				**168dpi** **END**
	GROUP A (n=75) G1b PEDV inoculation **GROUP B** (n=14) Mock-inoculation	Clinical follow-up Viral detection Specific-PEDV IgG and IgA		**GROUP A** G1b PEDV homologous inoculation **GROUP B** G1b PEDV inoculation	Clinical follow-up Viral detection Specific-PEDV IgG and IgA			
	0 dpi	**14 dpi**	**42 dpi**	**154 dpi** **(+0)**	**157 dpi** **(+3)**	**161 dpi** **(+7)**	**164 dpi** **(+10)**	**168 dpi** **(+14)**
Serum sample (VNT[1])	GROUP A (n=10) GROUP B (n=3)	GROUP A (n=10) GROUP B (n=3)	GROUP A (n=10) GROUP B (n=3)	GROUP A (n=10+20) GROUP B (n=3+6)	GROUP A (n=10) GROUP B (n=3)	GROUP A (n=10) GROUP B (n=3)	GROUP A (n=10) GROUP B (n=3)	GROUP A (n=10) GROUP B (n=3)
Heparin tubes (PBMC[2])				GROUP A (n=10+20) GROUP B (n=3+6)	GROUP A (n=10+10) GROUP B (n=3+3)			
Euthanised (LC[3])				GROUP A (n=10) GROUP B (n=3)	GROUP A (n=10) GROUP B (n=3)			

Table 1. Experimental design.

[1]VNT: Viral neutralization test; [2]PBMC: Peripheral blood mononuclear cells; [3]LC: Mesenteric lymph node mononuclear cells

Table 2. Correlation between frequencies of specific-PEDV IgG, IgA and IFN-γ-SC from PBMC and LC.

IgG: PBMC – LC	LC =0.23 PBMC + 17.70 r^2=0.02	non-significant
IgA: PBMC – LC	LC=0.68 PBMC + 18.47 r^2=0.34	p<0.05
IFN-γ: PBMC – LC	LC =1.60 PBMC + 0.86 r^2=0.42	p<0.05

Figure 1. Clinical scores. The appearance of faeces was scored in a scale ranging from 0 (firm and shaped) to 3 (liquid faeces). Figure shows the percentage of animals with loose stools/liquid faeces (scores 1-3) in the different groups throughout the experiment (phase 1 and 2). Animals in group A were infected with PEDV at day 0 (phase 1) and then homologous challenged at day 154 (phase 2). Animals in group B were kept as uninoculated controls until day 154 (phase 1) when they were challenged with PEDV (phase 2).

* Significant differences between groups comparing percentages of animals with loose stools/liquid faeces in a particular day from phase 2 ($p<0.05$).

Figure 2. RT-qPCR Detection of PEDV in faeces. PEDV detection in faeces by RT-qPCR. Bars show percentages of positives and lines (solid or dashed) show the average Ct ± standard deviation of positive animals. The table attached below show the results for the first 14 days after each challenge (average Ct ± standard deviation; % pos: percentage of positives ± $CI_{95\%}$), as well as the area under the curve (AUC) for the total faecal shedding ± standard deviation.

* Significant differences when comparing percentages of positive animals between groups for a particular day ($p<0.05$).

Figure 3. PEDV-specific IgG as determined in a commercial ELISA. Bars show the percentage of positive animals at each timepoint; lines (solid and dashed) show average S/P ratios ± standard deviation. The dotted line shows the cut-off value of the test (0.35).

* Significant increase in S/P ratios compared to 154 dpi ($p<0.05$).

^{a,b} Superscript letters show significant differences among S/P ratios fourteen days after each inoculation ($p<0.05$).

Figure 4. PEDV-specific IgA antibodies. Box and whisker plots for the ODs in the PEDV-specific IgA ELISA, showing the minimum, lower quartile, median, upper quartile, and maximum values, as well as average (red cross) for animals in group A. "Neg" shows the average ODs for all samples in group B from 0 to 154 dpi (naïve pigs).

* Significant difference between the 14-56 dpi period and the average OD of uninoculated animals ($p<0.05$).

** Significant increase in the average OD comparing sampling days ($p<0.05$).

Figure 5. Viral neutralization test. Neutralizing antibodies titres: percentage of positive samples and average titres (log_2) ± standard deviation for positive results. The dotted line shows the cut-off of the test (positive result $log_2>3$).

[a,b] Superscript letters show significant differences among all groups fourteen days after the inoculation, namely 14 and 168 dpi ($p<0.05$).

Figure 6. IgG and IgA ELISPOTs. Average frequencies (by 10^6 PBMC or LC) of specific-PEDV IgG-secreting cells (6a) and of specific-PEDV IgA-secreting cells (6b) (± standard deviation) before and after inoculation at 154 dpi. Blue bars correspond to group A; dark blue for PBMC and light blue for LC. Brown bars correspond to group B; dark brown for PBMC and light brown for LC.

* Significant differences comparing results before and after the challenge ($p<0.05$).

[a,b,c] Different superscript letters indicate significant differences in a given day ($p < 0.05$).