Infection and immune response to porcine hemagglutinating encephalomyelitis virus in grower pigs

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A R T I C L E   I N F O
Keywords:
Coronavirus
Betacoronavirus
Porcine hemagglutinating encephalomyelitis virus
Immune response
Subclinical infection
Grower-finisher pigs

A B S T R A C T
Porcine hemagglutinating encephalomyelitis virus (PHEV) is the cause of acute outbreaks of vomiting and wasting disease and/or encephalomyelitis in neonatal pigs, with naïve herds particularly vulnerable to clinical episodes. PHEV infections in older pigs are generally considered to be subclinical, but are poorly characterized in the refereed literature. In this study, twelve 7-week-old pigs were oronasally inoculated with 0.5 mL (1:128 HA titer) PHEV (Mengeling strain) and then followed through 42 days post inoculation (dpi). Fecal and oral fluid specimens were collected daily to evaluate viral shedding. Serum samples were tested for viremia, isotype-specific antibody responses, cytokine, and chemokine responses. Peripheral blood mononuclear cells were isolated to evaluate phenotype changes in immune cell subpopulations. No clinical signs were observed in PHEV inoculated pigs, but virus was detected in oral fluid (1–28 dpi) and feces (1–10 dpi). No viremia was detected, but a significant IFN-α response was observed in serum at 3 dpi, followed by the detection of IgM (dpi 7), and IgA/ IgG (dpi 10). Flow cytometry revealed a one-off increase in cytotoxic T cells at 21 dpi. This study demonstrated that exposure of grower pigs to PHEV results in subclinical infection characterized by active viral replication and shedding followed by an active humoral and cell-mediated immune response that attenuates the course of the infection and results in viral clearance.

1. Introduction

Porcine hemagglutinating encephalomyelitis virus (PHEV) was isolated in 1962 (Greig et al., 1962) and classified as a coronavirus in 1971 (Greig et al., 1971). PHEV is the only Betacoronavirus (family Coronaviridae, subfamily Orthocoronavirinae) that affects pigs, with one serotype described to date. Although PHEV showed neurotropism in mice and Wistar rats under experimental conditions (Hirano et al., 2004; Yagami et al., 1993), pigs are the only species naturally susceptible to PHEV; the virus does not pose a hazard to humans.

PHEV can cause outbreaks of vomiting and wasting disease (VWD) and/or encephalomyelitis in neonatal pigs, with mortality rates reaching up to 100 % in naïve herds (Cartwright et al., 1969; Pensaert and Callibaout, 1974; Quiroga et al., 2008). Lesions characteristic of PHEV include lymphoplasmacytic encephalitis and gastritis (Alexander et al., 1959; Andries and Pensaert, 1980a; Narita et al., 1989). The infection typically starts in the upper respiratory tract and tonsils and is usually characterized by non-specific clinical signs including sneezing, coughing, and transient fever of 1–2 days (Andries and Pensaert, 1980b; Hirahara et al., 1987). The virus can spread from the oronasal mucosal epithelium via peripheral nerves to the central nervous system (Meyvisch and Hoorens, 1978). Nerve tissues, especially trigeminal ganglia, are the primary site of viral replication and reservoir (Yagami et al., 1993). More PHEV-specific clinical signs, including VWD and/or neurological signs, appeared between 4–7 days post-infection in neonatal piglets (Alexander et al., 1959; Cartwright et al., 1969; Pensaert and Callibaout, 1974; Quiroga et al., 2008). PHEV is the only member of the family Coronaviridae with the ability to penetrate the central nervous system (neuroinvasion). The infection is normally restricted to the perikaryon and processes of neurons and glial cells (neurotropism) (Andries and Pensaert, 1980b).

Previous reports indicated that PHEV can also infect grower-finisher and adult pigs but most of these infections were subclinical (Appel et al., 1965), and animals developed a strong antibody response against the
virus (Quiroga et al., 2008). Older pigs occasionally showed mild transient respiratory signs (Hirahara et al., 1987; Lorbach et al., 2017). Only one study from Taiwan reported mild transient neurological signs in 30- to 50-day-old pigs (Chang et al., 1993). A recent study published by this group demonstrated that PHEV is endemic, highly prevalent, and circulates subclinically in commercial U.S. sow herds (Mora-Díaz et al., 2020a). However, currently, there are no official PHEV surveillance programs in the United States nor epidemiological data from other swine producing countries.

Overall, clinical outcomes are variable depending age, previous history of viral circulation, and likely viral strain (Appel et al., 1965; Mengeling and Cutlip, 1976). Although both VWD and encephalomyelitis have been reproduced in colostrum-deprived neonatal pigs under experimental conditions (Mengeling and Cutlip, 1976; Pensaert and Callebaut, 1974), there is sparse information related to PHEV infection in older animals. Thus, the objective of this study was to characterize the infection and immune response to PHEV in conventional grower pigs.

2. Material and methods

2.1. Virus culture and propagation

Swine kidney primary (SKP) cells (National Veterinary Services Laboratories (NVSL), United States Department of Agriculture (USDA), Ames, IA, USA) were seeded in 75 cm² tissue culture flasks (Thermo Scientific, Waltham, MA, USA) using growth medium (Minimum Essential Medium with Earle’s (EMEM) (Gibco™, Thermo Scientific) with 0.5 % lactalbumin enzymatic hydrolysate (Sigma-Aldrich, St. Louis, MO, USA) supplemented with heat inactivated 10 % fetal bovine serum (FBS; ATCC, Manassas, VA, USA), 0.15 % sodium bicarbonate (Sigma-Aldrich), 1 % l-glutamine (Gibco™, Thermo Scientific), 1 % sodium pyruvate (Gibco™, Thermo Scientific), 3 μg/mL amphotericin (Gibco™, Thermo Scientific), 25 μg/mL kanamycin (Gibco™, Thermo Scientific), and 75 μg/mL of gentamycin (Gibco™, Thermo Scientific) and incubated for 2 days at 37 °C with 5% CO₂. Once the cell monolayer reached a confluence of 80 %, the growth medium was decanted from the flask and the cells were rinsed with 5 mL of ATV trypsin (8 g of sodium chloride (Sigma-Aldrich), 0.40 g of potassium chloride (Sigma-Aldrich), 1 g of dextrose (Sigma-Aldrich), 0.58 g of sodium bicarbonate (Sigma-Aldrich), 0.50 g of trypsin (Sigma-Aldrich), 0.20 g of versine (Sigma-Aldrich), and 0.04 % phenol red (Sigma-Aldrich)) for 30 s. SKP cells were inoculated with 2 mL of ATV trypsin and incubated at 37 °C in 5% CO₂. After 5 min, 5 mL of PHEV 67 N or “Mengeling strain” (NVSL) diluted 1:10 in infection medium (growth medium without FBS) was added to the flask. The cell suspension was divided between two 75 cm² tissue culture flasks (Thermo Scientific) containing 22 mL of infection medium and incubated for 4 days at 37 °C in 5 % CO₂. Virus was harvested by three -80 °C freeze-thaw cycles followed by a centrifugation at 3000 × g for 20 min at 4 °C to remove cellular debris. The supernatant was aliquoted into cryovials and identified for further use.

The viral titer was obtained by hemagglutination assay (HA). Briefly, 50 μl of PBS (Gibco™, Thermo Scientific) were added to four columns of a V-bottom 96-well plate (Asygen, Corning®, Corning, NY, USA), and then 50 μl of PHEV was added to the first two wells of the row, and 50 μl of PBS (cell control) was added to second half of the row. Serial two-fold dilutions were done until the last row of the 96-well plate and then 50 μl of 0.5 % suspension of washed rooster erythrocytes were added to each well. The plate was sealed with a tape and incubated for 30 min at 20–25 °C until a distinct button formed in the cell control well. The plate was observed under a plate reading mirror and the endpoint, i.e., the highest dilution of the virus causing 100 % hemagglutination, was defined as 1 HA.

2.2. Experimental inoculation and specimen collection

The animal study was approved by the Institutional Animal Care and Use Committee of Iowa State University (IACUC log # 5-15-8017-S; approval date: March 15, 2016). Seven-week-old pigs (n = 24) negative for PHEV and other common swine coronavirus infections were pre-screened and selected based on molecular and serology testing previously described (Gimenez-Lirota et al., 2017). In brief, pig rectal swabs were tested by a porcine epidemic diarrhea virus (PEDV) N gene-based real-time RT-PCR (rRT-PCR) and porcine deltacoronavirus (PDCoV) M gene-based rRT-PCR, while both pig rectal and nasal swabs were tested by transmissible gastroenteritis virus (TGEV) S gene/PRCV N gene-based differential rRT-PCR. Moreover, pigs’ serum samples were tested using a PEDV IFA, PEDV whole virus ELISA, TGEV/PRCV differential ELISA, and PDCoV IFA. Animals were randomly divided into two groups (treatment and control). Each group (n = 12) was housed in a separate room equipped with six pens (two pigs per pen). Each pen was equipped with nipple drinkers and pigs were fed twice daily with an antibiotic-free commercial diet (Heartland Co-op, West Des Moines, IA, USA). Pigs in the treatment group were inoculated with 5 mL of inoculum (0.5 mL of PHEV 67 N at 1:128 HA in 4.5 mL of EMEM (Gibco™, Thermo Scientific)) oronasally (2.5 mL orally and 2.5 nasally; 1.25 mL per nostril). Likewise, control animals received 5 mL of EMEM (Gibco™, Thermo Scientific) oronasally.

Blood samples were collected from the jugular vein or cranial vena cava of each pig using a single-use collection system (Becton Dickinson, Franklin Lakes, NJ) and 10 mL serum separation tubes (Kendall, Mansfield, MA, USA) at 0, 3, 7, 10, 14, 17, 21, 28, 35, and 42 days post-inoculation (dpi). Serum was harvested by centrifugation at 1500 × g for 5 min, aliquoted into 2-mL cryogenic vials (Cryovial®, Greiner Bio-One, Monroe, NC, USA), stored at −80 °C.

Whole blood for plasma and peripheral blood mononuclear cells (PBMCs) was collected in 5 mL heparinized tubes (Thermo Scientific) at 0, 3, 7, 10, 14, 17, and 21 dpi and then PBMCs and plasma were recovered by Ficoll-Paque (GE Healthcare, Uppsala, Sweden) density gradient centrifugation. Whole blood was first diluted 1:1 with PBS pH 7.2 and then carefully layered over the density gradient medium and centrifuged at 400 × g for 40 min at 20–25 °C. The PBMCs were collected from the interface between the density gradient medium and the plasma. Isolated PBMCs were counted and the viability was evaluated by trypan blue dye exclusion using a Countess II FL Automated Cell Counter (Life Technologies, Carlsbad, CA, USA). PBMC concentration was adjusted to 2 × 10⁶ cells/mL in a volume 1:1 of growth medium and cryoprotective medium (Lanza, Basel, Switzerland), and then samples were stored in liquid nitrogen until use.

Floor fecal samples were collected daily from each pen from −7 to 42 dpi using 50 mL conical tubes. Oral fluid samples were collected from each pen twice a day (morning and afternoon combined) from −7 to 42 dpi using 3-strand 1.6 cm 100 % cotton rope (Web Rigging Supply, Inc., Carrollton, GA) hung from a bracket fixed to one side of each pen. After 30 min exposure to the pigs, the wet end of the rope was severed, placed in a plastic bag, and then passed through a clothes wringer (Dyna-Jet, Overland Park, KS) while still inside the bag. The oral fluid that accumulated in the bottom of the bag was decanted into 50 mL conical tubes (Corning®). Pen-based feces and oral fluids were aliquoted into 2 mL cryogenic tubes (Greiner Bio-One GmbH), and stored at −80 °C.

Pigs were clinically evaluated twice a day for general health, respiratory or neurological signs, vomiting or anorexia. At dpi 42, all pigs were euthanized using a penetrating captive bolt device (Accles and Shelvoke, Ltd., Sutton Coldfield, UK).

2.3. Real-time RT-PCR

A real-time PHEV rRT-PCR was used to test for viremia using pig serum samples (n = 264 total) collected at DPIs -7, 0, 3, 7, 10, 14, 17, 21, 28, 35, and 42 from the PHEV inoculated (n = 132) and control group (n = 132). The kinetics of viral shedding assessed using daily (DPIs –7 to 42) pen oral fluids (n = 294) and fecal (n = 294) samples collected from each group (n = 588 total).

Viral RNA extractions were performed using the MagMAX-96
Pathogen RNA/DNA kit (Applied Biosystems™, Waltham, MA, USA) with KingFisher™ Flex 96 Deep-Well Magnetic Particle Processor (Thermo Scientific) following the manufacturer’s instructions. The PHEV rRT-PCR assay used in the study was developed by Tetracore (Tetracore, Inc., Rockville, MD, USA) and the veterinary diagnostic laboratory at Iowa State University (ISU-VDL). The assay targeted the conserved regions of the nucleocapsid (N) gene using a cocktail of primers and probes (non-disclosed by manufacturer; Tetracore Inc.). In brief, each 25 μl real-time PCR reaction was set up by combining 19 μl of PHEV rRT-PCR master mix and 1.0 μl of the enzyme blend (reverse transcriptase and RNase inhibitor). An internal control (IC) was used as an extraction control, with 6 μl of the IC added to the lysis buffer. Then 5 μl of the extracted sample RNA with IC was added to the Master Mix. All rRT-PCR reactions were performed in duplicate, a negative extraction control (NEC), positive extraction control (PEC), and “no template” control (NTC) were included in each run. All PCRs were run on a Rotor-Gene Q (QIAGEN, Germantown, MD, USA) with cycling conditions, 48 °C for 15 min and 95 °C for 2 min holding; 45 cycles, 95 °C for 10 s denaturation and 60 °C for 40 s amplification. The PCR results were analyzed using Rotor-Gene Q–Pure Detection software (v 2.3.1). Samples with threshold cycle (Ct) above 40 were considered negative.

2.4. Isotype-specific antibody ELISAs

As previously reported, the amino-terminal receptor-binding (S1) portion of the PHEV S protein expressed in a mammalian expression system was used to develop a PHEV IgG serum ELISA (Mora-Díaz et al., 2020a). This assay was further adapted to detect PHEV isotype-specific (IgG, IgA, and IgM) antibody. The coding region of the S1 protein of PHEV VW572 strain (Genbank #DQ011855.1) was synthesized with the addition of a 5’-terminal eukaryotic native signal (MFFILLISL-PSAFAVIG), a 3’-terminal Tobacco etch virus (TEV) cysteine protease site (ENLYFQSQ), and the Fc portion of human IgG1 antibody (GenBank JX292764.2). After amplification by PCR, the amplicon was cloned in pNPMS expression vector (Novoprotein, Short Hills, NJ, USA) and the plasmid was transfected into HEK 293 cells (Thermo Scientific) using polyethyleneimine (PEI) (Thermo Scientific). Transfected HEK293 cells were grown in serum-free FreeStyle 293 Expression medium (Gibco™, Thermo Scientific) at 37 °C, 5 % CO₂, and shaking at 120 rpm. Five days after transfection, the culture supernatant was harvested by centrifugation at 3500 × g for 20 min. To soluble Fc-S1 fused protein (1005 aa; 112.3 kDa) was purified by protein A affinity chromatography (GE Healthcare, Pittsburgh, PA, USA) followed by Fc-tag cleavage and further purification by nickel (Ni)-chelating Sepharose Fast Flow affinity chromatography (GE Healthcare). Purified PHEV S1 (754 aa; 0.47 mg/mL yield) protein was dialyzed against phosphate-buffered saline (PBS) pH 8, and analyzed by 12 % dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot. PHEV S1 protein was coated (0.94 μg/mL in PBS pH 7.4) onto 96-well plates (Immuno Breakables Modules, Thermo Fisher Scientific, Agawam, MA, USA) and incubated at 4 °C for 16 h. Plates were then washed 5 times with PBS, pH 7.4, containing 0.1 % Tween 20 (PBST), blocked with a 1 % (wt/vol) bovine serum albumin solution (Jackson ImmunoResearch), incubated at 25 °C for 2 h, dried at 37 °C for 3 h, and stored at 4 °C and preserved from humidity until use.

To perform the test, serum samples (n = 264) and positive and negative controls (in duplicate) were tested at 1:100 (100 μl/well) in phosphate buffer pH 7.4 containing 50 % goat serum, and the plate incubated at 37 °C for 1 h. Then, plates were washed 5 times (350 μl/well) with PBST (PBS pH 7.4 and 0.1 % Tween 20), and 100 μl of peroxidase-conjugated goat anti-pig IgG (Fc) (1/30,000), IgA (1/2000) or IgM (1/3000) antibody (Bethyl Laboratories, Inc., Montgomery, TX, USA) were added to each well and incubated at 37 °C for 1 h. The reaction was visualized after 5 min incubation with 100 μl of tetramethylbenzidine-hydrogen peroxide (TMB) substrate solution per
well (SurModics IVD, Inc., Eden Prairie, MN, USA) and stopped with 100 μl of stop solution per well (SurModics). Optical density was measured at 450 nm using an ELISA plate reader (BioTek Instruments, Inc., Winooski, VT, USA) operated with commercial software (SoftPro 7; Molecular Devices, San Jose, CA, USA). Serum antibody (IgG, IgA, and IgM) responses were expressed as sample-to-positive (S/P) ratios. 

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\frac{S}{P} \text{ ratio} = \frac{\text{Sample OD}}{\text{Negative Control mean OD}} - \frac{\text{Positive Control mean OD}}{\text{Negative Control mean OD}}
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2.5. Multiplex porcine cytokine and chemokine immunoassay

A porcine cytokine and chemokine 9-plex Luminex® assay (ProcartaPlex Panel; Invitrogen, Thermo Scientific, Frederick, MD, USA) was used to test for IFN-α, IFN-γ, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12, and TNF-α in plasma samples (n = 240) collected at 0, 3, 7, 10, 14, 17, and 21 dpi from both PHEV and negative control groups. The assay was performed according to the manufacturer’s instructions. Results were acquired and analyzed with a Bio-Plex® 200 system using Bio-Plex manager software (Bio-Rad, Hercules, CA, USA). The fluorescence intensity of each sample was subtracted from the blank wells, and the concentration of each cytokine was calculated from the standard curve generated from kit’s internal standards and analyzed using GraphPad Prism® 8 (GraphPad Software Inc., La Jolla, CA, USA).

2.6. Immunofluorescent staining and flow cytometry analysis of PBMCs

Cryopreserved PBMCs were rapidly thawed at 37 °C, and transferred to 10 mL of complete RPMI medium [advance RPMI 1640 medium (Gibco™, Thermo Scientific), supplemented with 10 % FBS (Gibco™, Thermo Scientific), and 1% antibiotic-antimycotic (Gibco™, Thermo Scientific)]. Cells were washed by centrifugation at 670 × g for 5 min at 20–25 °C, and PBMCs were resuspended in 10 mL of complete RPMI medium. Because of cryopreservation could affect the expression of PBMC markers and cell viability, PBMCs were seeded in a flat-bottom 96-wells plate (200 μL/well; 2 × 10⁶ cells/mL) (Costar®, Corning®), cultured at 37 °C with 5% CO2 for 16 h before cell-specific staining for

![Fig. 2. PHEV S1-based ELISA IgM (A), IgA (B), and IgG (C) responses (mean S/P values, SE) overtime (49 days) in pigs (n = 12) experimentally inoculated with PHEV or mock inoculated (n = 12) with culture medium. Samples above the S/P cutoff value (0.6; dashed line) were considered positive. *Denoted statistical differences (p < 0.05).](image-url)
flow cytometry analysis. Specifically for swine leucocytes, PBMCs were stained with 1:25 of anti-CD3-PE/Cy-7 (clone BB23-8E6-8C8), 1:25 of anti-CD21-PE (clone B-ly4), 1:25 of anti-SWC3/CD172a-FITC (clone 74-22-15A) and 1:10 anti-CD4-PerCP/Cy5.5 (clone 74-12-4). Whereas, for T-cell subpopulation, cells were stained with 1:25 of anti-CD3-PE/Cy-7, 1:10 anti-CD4-PerCP/Cy5.5, 1:25 of anti-CD8-FITC (clone 76-2-11) and 1:10 anti-\(\gamma\delta\) TCR-APC (clone MAC320). All the antibodies and staining buffer for immunostaining were purchased from BD Biosciences (San Jose, CA, USA). Immunostaining reactions were performed at 4\(^\circ\)C for 30 min in the dark. Stained PBMCs were washed twice with 300 \(\mu\)l of staining buffer (FBS; BD Pharmingen, San Diego, CA, USA) and analyzed immediately thereafter. Otherwise, stained PBMCs were fixed with fixation buffer (BD Cytofix, San Diego, CA, USA) for 15 min at 4\(^\circ\)C, and preserved protected from light in 4\(^\circ\)C until analysis. The fluorescent minus one (FMO) staining controls were performed during the validation of the assay. The cells were analyzed using BD FACSCanto flow cytometry (BD Biosciences). Dead cell and debris exclusion and lymphocyte population sorting were performed using forward scatter (FSC) and side scatter (SSC) gating (Fig. S1; Fig. S2).

2.7. Data analysis

To analyze the differences in the detection of PHEV by rRT-PCR (Ct value) between oral fluids and feces, a mixed-effect linear model (PROC GLIMMIX) was applied with “specimen” (oral fluids and feces) and “dpi” as fixed effects and “pen” as the random effect (each pen was sampled repeatedly over time). Differences in the proportion of PHEV rRT-PCR positive oral fluid and feces were compared using Fisher’s exact test (PROC FREQ). A mixed-effect linear model (PROC GLIMMIX) was also used to study the differences in S/P values between PHEV-inoculated pigs and control pigs for PHEV isotype-specific (IgM, IgA, and IgG) antibody responses with “treatment” (PHEV vs mock inoculated) and ‘dpi’ as fixed effects and the pig is the random effect. Pearson correlation analysis (PROC CORR) was conducted to analyze the relationship between the changing patterns of the isotype-specific antibodies detection by PHEV iELISA from both groups (inoculated and control). To perform this test the time of study (7 to 42 dpi) was divided in three periods: 7 to 14, 17–42. Differences in serum cytokine concentration between PHEV-inoculated and control pigs by dpi were analyzed by two-way ANOVA. For all analyses, a p-value < 0.05 was considered statistically significant. Statistical analyses were performed using SAS version 9.4 (SAS, Cary, North Carolina/USA, SAS Institute, Inc.) and GraphPad Prism® 7 (GraphPad Software Inc.).

3. Results

3.1. Exposure of grower pigs to PHEV results in subclinical infection with oral-fecal shedding but absence of viremia

Both PHEV inoculated and negative control pigs appeared and
3.3. Increased serum levels of IFN-α early after PHEV infection

Serum samples collected on dpi 0, 3, 7, 10, 14, 17, and 21, were simultaneously assayed for IFN-α, IFN-γ, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12, and TNF-α using a multiplex Luminex™ assay. Distinct numeric but no statistically significant patterns or trends were observed in several of the parameters measured (Fig. 3). Although no significant between PHEV and control groups, the observed pattern for IL-6 in the control group was due to one single animal, clinically normal otherwise (Fig. 3I). The only statistically significant observation was an increase (p < 0.05) on dpi 3 in the average IFN-α plasma levels of PHEV infected pigs compared to pigs in the negative group (Fig. 3A).

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3.4. Significant increase in cytotoxic T cells in response to PHEV infection

The isolated PBMC fraction from whole blood collected at 0, 3, 7, 10, 14, 17, and 21 dpi was used to characterize subpopulations of T lymphocytes CD3⁺ [T cells (CD3⁺CD21 SWC3⁺)], helper T cells (CD3⁺CD4⁺CD8⁻), cytotoxic T cells (CD3⁺CD4⁻CD8⁺), memory T cells (CD3⁺CD4⁺CD8⁺), naive T cells (CD3⁺CD4⁺CD8⁻), and gamma-delta T cells (CD3⁺γδ⁺) (Fig. 4), and other immune cells, including NK cells (CD3⁻CD4⁻CD8⁺), B-lymphocytes (CD3⁺CD21⁺SWC3⁺), monocytes (CD3⁻CD21⁺SWC3⁺high), and dendritic cells (DC) (myeloid DC (CD3⁻CD21⁺SWC3⁺lowCD14⁺), and plasmacytoid DC (CD3⁻CD21⁺SWC3⁺lowCD14⁻)) (Fig. 5), using flow cytometry. Flow cytometry analysis revealed a significant increase (p < 0.05) in cytotoxic T cells (dpi 21) (Fig. 4B) population in response to PHEV infection. No other relevant changes on immune cell populations were observed.

4. Discussion

Following the first report in 1958, outbreaks of PHEV-associated disease were documented in neonatal and nursing pigs (Alexander et al., 1959; Roe and Alexander, 1958). Subsequently, Appel et al. (1965) demonstrated that active PHEV infection could occur if the virus were introduced into a naive herd between 2 weeks before and 10 days after the farrowing of litters not protected by maternal antibodies. In a
The purpose of this study was to expand the available information on patterns of viral shedding and immune responses to PHEV infection in grower pigs. Specifically for the PHEV 67 N or “Mengeling” strain used in the present study, there are not previous reports of clinical disease in grower or adult pigs. Consistent with previous observations (Appel et al., 1965), PHEV infected pigs appeared clinically healthy over the course of the study. The pathogenicity of the PHEV 67 N strain, virus dose and route of inoculation used in this study was confirmed in a separate study on caesarean-derived, colostrum-deprived (CDCD) neonatal pigs (Mora-Díaz et al., 2020b under review). Whether PHEV 67 N strain can cause clinical disease in grower pigs under certain conditions (considerably higher dose, co-infection with other respiratory pathogens, immunostimulation, etc) would need to be further investigated.

Regardless, this study demonstrated that inoculated grower pigs were infected and shed virus in oral fluid and feces. The presence of infectious PHEV in oral fluids was originally achieved by isolating virus in pig kidney cortical cells from oral fluid samples collected, shortly after infection (3–5 days) from 5-weeks-old pigs orally inoculated with HEV-1 strain (Appel et al., 1965). Although previously attempted (Appel et al., 1965; Pensaert and Callebaut, 1974), this is the first report of the isolation or detection of PHEV in feces. Notably, the time and duration of the detection and the concentration of PHEV RNA detected in feces (1–7 dpi) was significantly lower compared to oral fluids (dpi 1–22), perhaps reflecting viral tropism for tissues of the upper respiratory tract. Although of shorter duration, a similar pattern of viral shedding in oral fluids was described for PRCV (Magtoto et al., 2019). Specifically, Magtoto et al. (2019) detected PRCV RNA in oral fluids from 1 to 7 dpi by rRT-PCR, although not in feces. Like PHEV, PRCV replicates primarily in the upper respiratory tract and it circulates subclinically in most swine herds (Saif et al., 2019). Cumulatively, the detection of PHEV in oral/fecal specimens suggests that, in the field, the virus spreads pig-to-pig through secretions and excretions or contaminated fomites. Importantly, this study reinforces the potential of oral fluids for diagnostic and active surveillance of different coronaviruses circulating in swine populations (Bjistrom-Kraft et al., 2016; Magtoto et al., 2019).

Subclinical infection was also evidenced by detection of specific seroconversion in PHEV inoculated pigs. Indeed, this is the first study evaluating the dynamic of the humoral response against PHEV. Only a few exceptions, no major outbreaks of VWD and/or encephalomyelitis in neonatal pigs have been reported (Li et al., 2016; Quiroga et al., 2008; Sasseville et al., 2001) and the presence of PHEV is mainly revealed through passive diagnostic surveillance. In the aggregate, these observations could be explained by highly effective maternal immunity, i.e., high seroprevalence in sow herds could result in high levels of lactogenic immunity in piglets and protection against clinical disease. On the other hand, since the clinical presentation in piglets is variable, unspecific, and brief, and older pigs do not often develop clinical signs, failure to detect a widely endemic viral pathogen may also reflect the fact that PHEV is not considered to have an influential effect on the performance of pig farms and, therefore, it is not included on the list of differential diagnoses.
Information about antiviral activity of pro-inflammatory cytokines/chemokines in response to PHEV infection is scarce. Derbyshire (1989) demonstrated in vitro that PHEV was sensitive to porcine IFN-α and IFN-β in pig kidney cells treated with interferon before and/or after infection. A rapid type 1 IFNs response against viral infections is critical to suppress initial viral replication and promote the adaptive immune response to facilitate virus clearance (Chanappanavar et al., 2019). Compared to pigs in the control group, significantly higher IFN-α level in serum was observed at 3 dpi but decreasing right after. Similar pattern has been recently described in CDCD neonatal pigs infected with PHEV (using same strain, dose and route of exposure as described herein), with moderate inflammatory response characterized by increased levels of IFN-α in plasma by 5 dpi (Mora-Diaz et al., 2020b under review). Interestingly, PRCV, a self-limiting respiratory disease of pigs, induced only of IFN-α on BAL fluids, leading to a subclinical outcome (Van Reeth et al., 2002). Contrary to animals going on to become severely clinically affected, subclinical infections are often characterized by a much more muted increase in the cytokines, which allow a better clearance of the infection and may explain the subclinical outcome of the PHEV infection observed in this study. Unfortunately, the systemic cytokine response data alone does not reveal the driving force behind this muted yet effective response in subclinically infected growing pigs.

Furthermore, flow cytometry analysis of PBMCs cells revealed a one-off increase on the cytotoxic T cell populations at 21 dpi in response to PHEV infection results in virus shedding and leads to an active immune response timing relative to virus replication determines MERS coronavirus infection outcomes. J. Clin. Invest. 129, 3625–3639.


Mora-Diaz et al. 2020 (4 paper).


