The porcine circovirus 3 humoral response: characterization of maternally derived antibodies and dynamic following experimental infection

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ABSTRACT Since Porcine Circovirus 3 (PCV3) was first identified in 2016, our understanding of the humoral response is still relatively scarce. Current knowledge of the PCV3 humoral response is primarily based on field studies identifying the seroprevalence of PCV3 Cap-induced antibodies. Studies on the humoral response following experimental PCV3 infection have conflicting results where one study reports the development of the Cap IgG response 7 days postinfection with no concurrent Cap IgM response, while a second study shows a Cap IgM response at the same time point with no detection of Cap IgG. The dynamics of the PCV3 Cap and Rep IgG following maternal antibody transfer and experimental infection have not been well characterized. Additionally, the cross-reactivity of convalescent serum from PCV2 and PCV3 experimentally infected animals to serologic methods of the alternate PCV has limited evaluation. Here, we show that maternally derived antibodies were detectable in piglet serum 7–9 weeks postfarrowing for the Cap IgG and 5-weeks-post farrowing for the Rep IgG using Cap- and Rep-specific enzyme linked immunosorbent assays (ELISA) and immunofluorescent assays (IFA) methods. Following experimental inoculation, Cap IgG was detected at 2-weeks-post inoculation and Rep IgG detection was delayed until 4-weeks-post inoculation. Furthermore, convalescent serum from either PCV2 or PCV3 methods displayed no cross-reactivity by serological methods against the other PCV. The information gained in this study highlights the development of both the Cap- and Rep-specific antibodies following experimental infection and through the transfer of maternal antibodies. The increased understanding of the dynamics of maternal antibody transfer and development of the humoral response following infection gained in the present study may aid in the establishment of husbandry practices and potential application of prophylactics to control PCV3 clinical disease.

IMPORTANCE Research on Porcine Circovirus 3 (PCV3) immunology is vital for understanding and controlling this virus. Previous studies primarily relied on field observations, but they have shown conflicting results about the immunological response against PCV3. This study helps fill those gaps by looking at how antibodies develop in pigs, especially those maternal-derived, and their impact in neonatal pigs preventing PCV3-associated disease in piglets. In addition, we look at the dynamics of antibodies in experimental infections mimicking infection in pigs in the grower-phase condition. Understanding this process can help to develop better strategies to prevent PCV3 infection. Also, this research found that PCV2 and PCV3 do not cross-react, which is crucial for serological test development and results interpretation. Overall, this work is essential for improving swine health and farming practices in the face of PCV3 infections.

KEYWORDS Porcine Circovirus 3, humoral response, maternal immunity

Circoviruses are small, circular, single-stranded DNA viruses in the family *Circoviridae* and in the genus *Circovirus* (1). Currently, four species of porcine circoviruses (PCV) have been identified that naturally infect pigs. PCV1 was identified in 1974 as a cell culture contaminant in the porcine kidney (PK-15) cell line (1) and is nonpathogenic to pigs (2, 3). In contrast, PCV2 was identified in the 1990s and is the primary etiological agent of porcine circovirus-associated disease (PCVAD), and since that time, PCV2 has become one of the most economically impactful and important swine diseases globally (4–6). PCV3 was identified in 2016 and has most commonly been associated with multisystemic inflammation, reproductive failure, and subclinical infection (7, 8). Most recently, PCV4 was identified in 2019 in pigs with respiratory and enteric clinical signs (9); however, the role of PCV4 as a causative agent remains controversial due to the presence of coinfections and limited studies using direct detection methods (7, 10).

The PCV3 genome comprises 2,000 base pairs (bp) with two major open reading frames (ORFs) oriented in opposite directions. ORF1 encodes the 297 amino acid (aa) Replicase (Rep) protein, which is involved in viral replication. Additionally, ORF2 encodes for the 214 aa Capsid (Cap) protein, which is the sole component of the viral capsid and gives the virus many of its antigenic properties (8, 11, 12). Similarly, the PCV2 genome is 1,766–1,768 bp (13) with a 315 aa Rep and 233–234 aa Cap protein (14, 15). PCV2 and PCV3 have relatively low nucleotide homology at the whole genome (46.8%) and ORF2 (26%–36%) levels (8, 12, 16). The PCV4 genome is 1,770 bp with a 296 aa Rep and 228 aa Cap protein (9). PCV4 has relatively low nucleotide homology to the PCV2 whole genome (51.5%) and ORF2 (12.7%–45.0%) as well as the PCV3 whole genome (42.9%–45.0%) and ORF2 (23.2%–24.8%) (9, 17).

Several enzyme-linked immunosorbent assays (ELISA) and indirect immunofluorescent assays (IFA) have been developed to detect PCV3 Cap-specific antibodies. The antigens platforms reported for the development of PCV3 ELISAs include the full length, codon optimized Cap protein expressed in a baculovirus (18) and *Escherichia coli* expression systems (19). The 33 N-terminus aa of the Cap protein composes a nuclear localization signal (NLS) with a high arginine content and rare codons, resulting in decreased protein expression in prokaryotic expression systems (20). Thus, most ELISAs utilize a truncated Cap protein, which excludes the NLS sequence expressed in *E. coli* (21–23). IFA assays have been developed by expressing the Cap in baculovirus (24) and other eukaryotic expression systems (25).

Current knowledge of the PCV3 humoral response is primarily based on field studies identifying the seroprevalence of PCV3 Cap-induced antibodies (21). Retrospective seroprevalence studies have reported the PCV3 antibody positivity rate ranging from 20% to 80% (18, 21). Notably, serological evaluations conducted in sow farms did not find significant differences within farm seroprevalence rate or antibody titer measured by baculovirus-expressed capsid protein-based indirect ELISA regardless of the presence of clinical signs. Additionally, the antibody positivity rate was significantly higher in sows with high levels of viremia compared to sows with low level of viremia (18). Transfer of maternal antibodies has been hypothesized based on the absence of viremia in animals of 3–4 weeks of age (26) and increased detection of viremia in animals 6–24 weeks of age (27). However, the dynamics of PCV3-maternal antibodies have not been characterized. Studies on the humoral response following experimental PCV3 infection have reported conflicting results. One study reported the development of the Cap IgG response 7 days postinfection with no concurrent Cap IgM response (28), while a second study showed a Cap IgM response at the same time point with no detection of Cap IgG (22). Moreover, experimental studies have not characterized the Rep antibody dynamic or the protective role conferred by the humoral response. Given the genetic similarity of PCV2 and PCV3 (8, 12, 16), the potential of antibody cross-reactivity has been speculated following natural infection and vaccination. PCV3 convalescent serum has shown no cross-reactivity to PCV2 virus-like particles (21); however, the cross-reactivity of PCV2 and PCV3 serum to serological methods of the alternate PCV has not been reported.

The humoral response to following the transfer of maternal antibodies and development of antibodies after natural infection has been well characterized resulting in the development of specific vaccination schedules to greatly reduce PCVAD. Both PCV2 Cap and Rep maternally derived antibodies have been detected in piglets with the highest titers observed at 1 week of age (29), and the mean half-life of PCV2 Cap maternally derived antibodies is 19 days. In 3-week old specific pathogen free pigs experimentally inoculated with PCV2, Cap IgG, and Rep antibodies were detectable 7–28 days postinfection (dpi) with neutralizing titers detectable 10 dpi (30). Furthermore, experimental inoculated cesarean-derived colostrum-deprived (CD/CD) 8-week-old pigs produced Cap antibodies earlier and at a higher titer compared to Rep antibodies (31).

The understanding of the PCV3 humoral response remains limited. Thus, an increased understanding of the dynamics of maternal antibody transfer and development of the humoral response following infection may aid in the establishment of husbandry practices and potential application of prophylactics to control PCV3 clinical disease. Therefore, the objectives were to develop PCV3 Cap and Rep IFA and ELISA methods to characterize the PCV3 IgG antibody dynamics following the transfer of maternal antibodies and experimental infection. Furthermore, the cross-reactivity of convalescent serum from PCV2 and PCV3 experimentally infected animals to serologic methods of the alternate PCV was evaluated. The information gained in this study highlights the development of both the Cap- and Rep-specific antibodies following experimental infection and through the transfer of maternal antibodies. Additionally, convalescent serum from either PCV2 or PCV3 methods displayed no cross-reactivity by serological methods against the other PCV.

MATERIALS AND METHODS

Study 1: Maternal transfer of antibodies from naturally infected sows

A cross-sectional evaluation of pregnant sows 2 weeks before farrowing was performed in a gestational unit located in Iowa, USA. Assuming default 50% seroprevalence with 95% confidence interval and 17% absolute error, the sample size was calculated to be 30 sows ($n = 30$) as previously described (32, 33). The farm had a PCV3 history characterized by an abortion storm with a 40% abortion rate for approximately 1 month. The presence of PCV2, porcine reproductive and respiratory syndrome virus (PRRSV), and porcine parvovirus 1 (PPV1) was ruled out by PCR, and presence of PCV3 was confirmed by PCR conducted on fetal tissue by routine diagnostic submission at the ISU-VDL. Sow sera were evaluated by a PCV3 ORF2 recombinant protein-based indirect ELISA (22). Three litters consisting of a total of 31 piglets (litter 1, $n = 8$; litter 2, $n = 11$; and litter 3, $n = 12$) were arbitrarily selected for the longitudinal study from litters of sow with low, mid, and high pre-farrow antibodies levels relative to the distribution of ELISA S/P ratios of 30 sows selected for pre-farrow prescreening. Each piglet was individually identified with plastic ear-tags. All piglets remained with the dams for 21 days (±3 days), and no cross-fostering was performed during lactation to ensure specific antibody transfer from dams. At weaning, all piglets were reallocated into a nursery barn and commingled with piglets from the same farm and an additional sow farm. Blood samples were collected once a week from 1 to 9 weeks of post farrowing using a single‑use blood collection system (Vacutainer SST, BD, Franklin Lakes, NJ, USA). Blood samples were kept on ice during transport to the ISU-VDL. The serum was separated immediately upon arrival to the laboratory by centrifugation at 2,000 g for 15 min, aliquoted, and stored at −80℃ until final serological and molecular testing.

Study 2: PCV3 antibody dynamic following experimental inoculation

The inoculation study was carried out as previously described (28). Briefly, 18 5-week-old CD/CD pigs were randomly assigned to four treatment groups: PCV3 infectious material (PCV3, $n = 6$), PCV3 infectious material+adjuvant (keyhole limpet hemocyanin)

(PCV3+KLH, n = 6), adjuvant control (KLH control, n = 3), and mock-inoculated negative control (negative control, n = 3). The PCV3 and PCV3+KLH groups were inoculated bilaterally and intramuscularly in the neck with 2 mL of tissue homogenate with Ct = 8 (gc = 3.38×10^{12} mL^{-1}), and inoculated intranasally with 2 mL of tissue homogenate with Ct = 13 (gc = 1.04×10^{11} mL^{-1}), and re-inoculated 7 days later following the same protocol. Both control groups were inoculated similarly to the challenged groups with 2 mL of Minimum Essential Medium Eagle (MEM) at 0 dpi, and reinoculated at 7 dpi. Immunostimulation was performed in the PCV3+KLH and KLH Ctrl groups by subcutaneous administration of 2 mL of keyhole limpet hemocyanin (1 mg mL^{-1}) (KLH, Sigma-Aldrich, St. Louis, MO, USA) emulsified in incomplete Freund's adjuvant (ICFA, Sigma-Aldrich) into the right hip and right shoulder (1 mL/site) at 3 dpi and reinoculated in the left hip and left shoulder at 7 dpi with the same KLH dose. One pig from the PCV3 and PCV3+KLH groups was randomly euthanized at 11 dpi. Furthermore, one animal per treatment group was euthanized at 21 dpi, and all remaining animals were euthanized at 42 dpi. Blood was collected from all pigs at 0, 3, 7, 10, 14, 17, 21, 24, 28, 31, 35, and 42 dpi in 5 mL serum separator tubes (Thermo Fisher Scientific, Waltham, MA, USA). The blood was centrifuged at 2,000 g for 10 min, and serum aliquots were stored at −80°C until testing.

Study 3: PCV2 infection study

Ten 10-day-old CD/CD pigs were sham inoculated with an adjuvant carrier for a PCV2 vaccine efficacy trial 49 days pre-challenge (pre chal). All pigs were challenged at 59 days of age with PCV2 tissue homogenate (2 mL intranasal and 1 mL intramuscular) on 0 days post challenge (dpc). Blood was collected from all pigs on 49 days pre chal and 0, 14, and 28 dpc in 5 mL serum separator tubes (Thermo Fisher Scientific, Waltham, MA, USAThermo Fisher Scientific). The blood was centrifuged at 2,000 g for 10 min, and serum aliquots were stored at −80°C until testing.

PCV3 qPCR

In study 1, viremia was evaluated by litter where serum samples were pooled with a maximum of five animals per pool. In study 2, viremia was evaluated individually. PCV3 detection was performed by quantitative-PCR (qPCR) as previously described (34) according to standard operating procedure at the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL). Briefly, MagMAX-96 Pathogen RNA/DNA kit (Applied Biosystem, Waltham, MA, USA) with a KingFisher Flex 96 Deep-Well Magnetic Particle Processor (Thermo Fisher Scientific) was used to extract the DNA from pooled serum according to the manufacturer's instructions. A conserved region of the PCV3 replicase (ORF1) gene was detected from the extracted DNAs by qPCR using the TaqMan Fast Virus 1-Step Master Mix (Life Technologies, Carlsbad, CA, USA) with forward primer 5′-TGTWCGGGCACACAGCCATA-3′ and reverse primer 5′-TTTCCGCATAAGGGTCGTCTT-3′ with the probe 5′-/5SUN/ACCACAAAC/ZEN/ACTTGGCTC/31ABkFQ/−3′ previously described (34). qPCR was performed on 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the following cycling conditions: one cycle at 50°C for 5 min, one cycle at 95°C for 20 s, 40 cycles at 95°C for 3 s, and 60°C for 30 s. All samples were controlled appropriately with positive, negative, and internal controls according to validated ISU-VDL standard operating procedures. Samples with Ct < 37.0 were considered positive.

Production of PCV3 recombinant Cap and Rep proteins

Recombinant truncated Cap and full-length Rep proteins were used as antigens for the indirect ELISAs based on the ORF1 and ORF2 sequences of the PCV3 29160 (GenBank KT869077). A truncated Cap gene (bp 90–645) was used as previous studies have shown exclusion of the nuclear localization sequence improves prokaryotic expression (20). The truncated Cap and full-length Rep sequence were codon optimized for prokaryotic

expression and synthesized with flanking *BamHI* and *XhoI* endonuclease restriction sites in the pET-28b expression vector, which includes a 6× histidine tag on the N terminus (GenScript, Piscataway, NJ, USA). The synthesized plasmids were transformed into BL21(DE3) *E. coli* cells (New England Biolabs, Ipswich, MA, USA). Bacterial clones were grown in Luria-Bertani (LB) broth (Thermo Fisher Scientific, Waltham, MA, USA) containing 50 µg/mL kanamycin at 37℃ with shaking at 250 rpm. At an A_{600} of 0.5, protein expression was induced using 1.0 mM isopropyl β-D-1 thiogalactopyranoside (IPTG, Thermo Fisher Scientific, Waltham, MA, USA) and incubated for an additional 8 h at 37℃. Bacterial cultures were harvested by centrifugation at 12,000 *g* for 10 min at 4℃ and were resuspended in solution (B-PER, Thermo Fisher Scientific, Waltham, MA, USA). The suspension was centrifuged at 12,000 *g* and the insoluble Rep protein was denatured using 8 M urea. Both Cap and Rep proteins were purified using nickel-nitrilotriacetic acid (NTA) affinity column chromatography (Qiagen, Hilden, GER) (35). The purified proteins were analyzed for purity and linear integrity by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). To confirm specificity, the proteins were recognized by Western blotting using an anti-histidine monoclonal antibody (Thermo Fisher Scientific, Waltham, MA, USA).

Indirect PCV3 Cap ELISA

An Immulon 2HB, 96-well plate (Thermo Fisher Scientific, Waltham, MA, USA) was coated with 100 µL the truncated Cap protein diluted 1:1,000 in antigen coating buffer (15 mM sodium carbonate 35 mM sodium bicarbonate, pH 9.6) and incubated for 1 h at 37℃ and overnight at 4℃. The next morning, plates were washed three times with PBST (phosphate-buffered saline containing 0.1% Tween 20) and were blocked with 200 µL per well of blocking buffer (PBS containing 1% Casein and 0.05% Tween 20) for 1 h at 37℃. Plates were washed three times with PBST and 100 µL of swine serum samples tested in duplicated were diluted 1:50 in blocking buffer was added and incubated for 1 h at room temperature. Next, plates were washed three times with PBST and 100 µL per well goat anti-pig IgG horseradish peroxidase (HRP) secondary antibody (Bethyl Laboratories, Inc., Montgomery, TX, USA) diluted 1:2,500 in blocking buffer was added and incubated for 1 h at room temperature. Plates were washed three times with PBST, and 100 µL per well of tetramethylbenzidine-hydrogen peroxide (TMB) substrate-solution (Invitrogen, Waltham, MA, USA) was added and incubated for 7 min at room temperature. 100 µL per well ELISA stop solution (Thermo Fisher Scientific, Waltham, MA, USA) was added, and reactions were measured for optical density (OD) at 450 nm using as ELISA plate reader (BioTek Instruments, Inc., Winooski, VT, USA) operated with commercial software (Gen5, BioTek Instruments, Inc.).

Each plate contained a positive control (Pig 8, 28 dpi, Study 1) and negative control (Pig 1, 0 dpi, Study 1) tested in duplicate. Serum antibody responses were expressed as a sample-to-positive (*S/P*) ratios: *S/P* ratio = (sample OD – negative control mean OD)/(positive-control mean OD – negative control mean OD). For study 2, the estimated positive cutoff *S/P* ratio was calculated as the mean *S/P* ratios of the negative controls' plus three standard deviations for each time point and then taking the mean of the individual time points (28, 36).

Indirect PCV3 Rep ELISA

An Immulon 1B, 96-well plate (Thermo Fisher Scientific, Waltham, MA, USA) was coated with 100 µL the Rep protein diluted 1:1,000 in antigen coating buffer (15 mM sodium carbonate 35 mM sodium bicarbonate, pH 9.6) and incubated for 1 h at 37℃ and overnight at 4℃. The next morning, plates were washed three times with PBST and were blocked with 200 µL per well of blocking buffer (PBS containing 1% Casein and 0.05% Tween 20) for 1 h at 37℃. Plates were washed three times with PBST and 100 µL of swine serum samples tested in duplicated were diluted 1:50 in blocking buffer was added and incubated for 1 h at room temperature. Next, plates were washed three times with PBST and 100 µL per well goat anti-pig IgG HRP secondary antibody (Bethyl

Laboratories, Inc.) diluted 1:10,000 in blocking buffer was added and incubated for 1 h at room temperature. Plates were washed three times with PBST and 100 µL per well of TMB substrate-solution (Invitrogen) was added and incubated for 7 min at room temperature. 100 µL per well ELISA stop solution (Thermo Fisher Scientific, Waltham, MA, USA) was added and reaction OD was measured at 450 nm using as ELISA plate reader (BioTek Instruments, Inc.) operated with commercial software (Gen5, BioTek Instruments, Inc.).

Each plate contained a positive control (Pig 17 at 35 dpi, Study 1) and negative control (Pig 3, 0 dpi, Study 1) performed in duplicate. Serum antibody responses were expressed as S/P ratios as described in previous section. For study 2, the estimated positive cutoff S/P ratio was calculated as the mean S/P ratios of the negative controls' plus three standard deviations for each time point and then taking the mean of the individual time points (28, 36).

PCV3 Cap and Rep IFAs

The full-length Cap and Rep sequences from PCV3 29160 (KT869077) were cloned into a pcDNA3.1(+)-N-6His expression vector using the *BamHI* and *XhoI* endonuclease restriction sites (GenScript). The plasmids were transformed into and purified from DH5α *E. coli* cells (New England Biolabs).

The Cap-pcDNA3.1 and Rep-pcDNC3.1 expression vectors were transfected into Human Embryonic Kidney (HEK) 293 cells (ATCC, Manassas, VA, USA) using Lipofectamine 3000 (Invitrogen) according to manufacturers recommended instructions. After 48 h incubation, cells were fixed with 80% acetone. Transfection and expression of the Cap and Rep proteins were confirmed by staining with an anti-histidine monoclonal antibody diluted 1:1,000 in PBS (Thermo Fisher Scientific) followed by a Fluorescein isothiocyanate (FITC) conjugated goat anti-mouse secondary antibody diluted 1:100 in PBS (Thermo Fisher Scientific, Waltham, MA, USA). For the Cap IFA, swine serum samples were tested in duplicate and were diluted two-fold starting at 1:40 (Study 1) or 1:4 (Study 2) in blocker (PBS containing 1% bovine serum albumin) at 50 µL per well and were incubated for 1 h at 37°C. For the Rep IFA, swine serum samples were tested in duplicate and were diluted two-fold starting at 1:10 (Study 1) or 1:4 (Study 2) in blocker at 50 µL per well and were incubated for 1 h at 37°C. Plates were washed three times with PBS and 50 µL per well of FITC conjugated anti-swine IgG secondary (Bethyl Laboratories, Inc.) diluted 1:100 in blocker was added and incubated for 1 h at 37°C. Plates were washed three times with PBS and staining was observed by fluorescent microscopy (Olympus IX83 Research Inverted Microscope, Olympus Life Science, Waltham, MA, USA). The IFA titer was expressed as the highest dilution where fluorescent signal was detected.

PCV2 qPCR, ELISA, and IFA

PCV2 qPCR, ELISA, and IFA were conducted according to standard operating procedure at the ISU-VDL. For the PCV2 qPCR, MagMAX-96 Pathogen RNA/DNA kit (Applied Biosystem, Waltham, MA, USA) with a KingFisher Flex 96 Deep-Well Magnetic Particle Processor (Thermo Fisher Scientific, Waltham, MA, USA) was used to extract the DNA from pooled serum according to the manufacturer's instructions. DNA extracts were used to detect the conserved region of the PCV2 replicase (ORF1) gene using TaqMan Fast Virus 1-step Master Mix (Life Technologies, MA, USA) with forward primer 5′-GACTGTW-GAGACTAAAGGTGGAACTGTA-3′ and reverse primer 5′-GCTTCTACACCTGGGACAGCA-3′ with the probe 5′-/56-FAM/-CCCGTTGGAATGGT/3MGBEc/−3′. The qPCR was performed (7500 Fast Real-Time PCR System, Applied Biosystems, Foster City, CA, USA) with the following cycling conditions: one cycle at 50°C for 5 min, one cycle at 95°C for 20 s, 40 cycles at 95°C for 3 s and 60°C for 30 s. Samples with Ct values < 37 were considered positive.

The Ingezim PCV2 IgG ELISA kit (Gold Standard Diagnostics, Budapest, Hungary) was utilized for PCV2 ELISA testing. Briefly, 100 µL per well of diluted serum samples were added to the ELISA plate and incubated for 1 h at room temperature. Plates were washed four times with wash solution, and 100 µL per well of conjugate was added

and incubated for 30 min at room temperature. Plates were washed six times with wash solution, and 100 µL per well of substrate was added. Plates were incubated for 10 min at room temperature, and 100 µL of stop solution was added. Sample OD was measured at 450 nm within 5 min after the addition of the stop solution. Positive and negative cutoffs and sample S/P ratios were calculated according to manufacture formulas.

For the PCV2 IFA, PK-15 cells were mixed with PCV2 and seeded in a 96-well plate. Following 72 h incubation, plates were fixed with 80% acetone and stored at −70℃. Serum samples were diluted two-fold from 1:20 to 1:2,560 in PBS and 90 µL of each serum dilution per well was added to warmed plate. Plates were incubated for 1 h at 37℃, and plates were washed three times with PBS. 50 µL per well of conjugate was added and incubated for 30–60 min at 37℃. The IFA titer was expressed as the highest dilution where fluorescent signal was detected compared to the positive control.

Statistical analysis

Differences in IFA titers and ELISA S/P ratios for studies 1 and 2 were analyzed by 2-way ANOVA with Tukey's correction for multiple comparisons. Statistical significance was set with an alpha value of 0.05. Data analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

RESULTS

Expression of PCV3 Cap and Rep recombinant proteins

The truncated Cap and full-length Rep of PCV3 were cloned and expressed in *E. coli* as a polyhistidine fusion protein. Protein purity was analyzed using SDS-PAGE, where both the truncated Cap and Rep proteins migrated to the predicted molecular masses of 23 kDa (truncated Cap) and 35 kDa (Rep) by Coomassie brilliant blue 250 staining (Fig. 1A). Further analysis by Western blot using anti-histidine monoclonal antibody showed specificity and proteins with the predicted molecular masses (Fig. 1B).
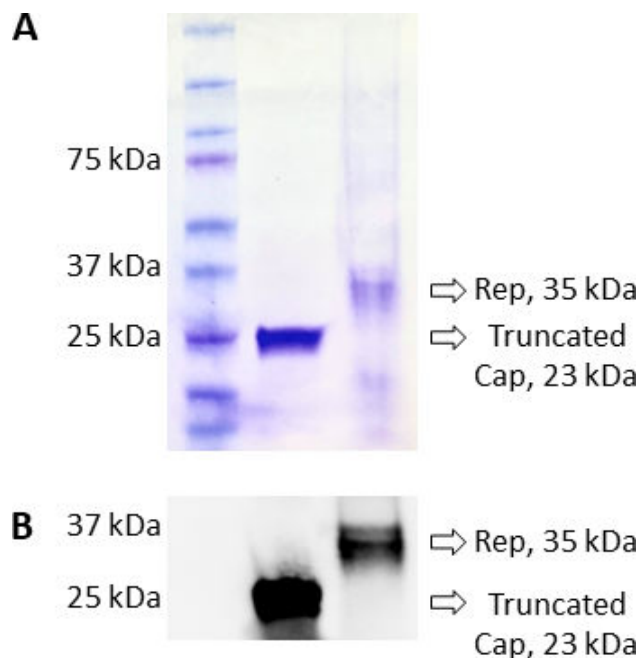

FIG 1 Purification and specificity of the truncated Cap and full-length Rep proteins of PCV3. (A) Coomassie blue staining of *E. coli* expressed and Nickel-NTA affinity column chromatography purified truncated Cap (left lane, 23 kDa) and full-length Rep protein (right lane, 35 kDa). (B) Western blotting confirming specificity and predicted molecular masses of the truncated Cap (left lane) and full-length Rep (right lane) polyhistidine tagged proteins by staining with an anti-histidine monoclonal antibody.
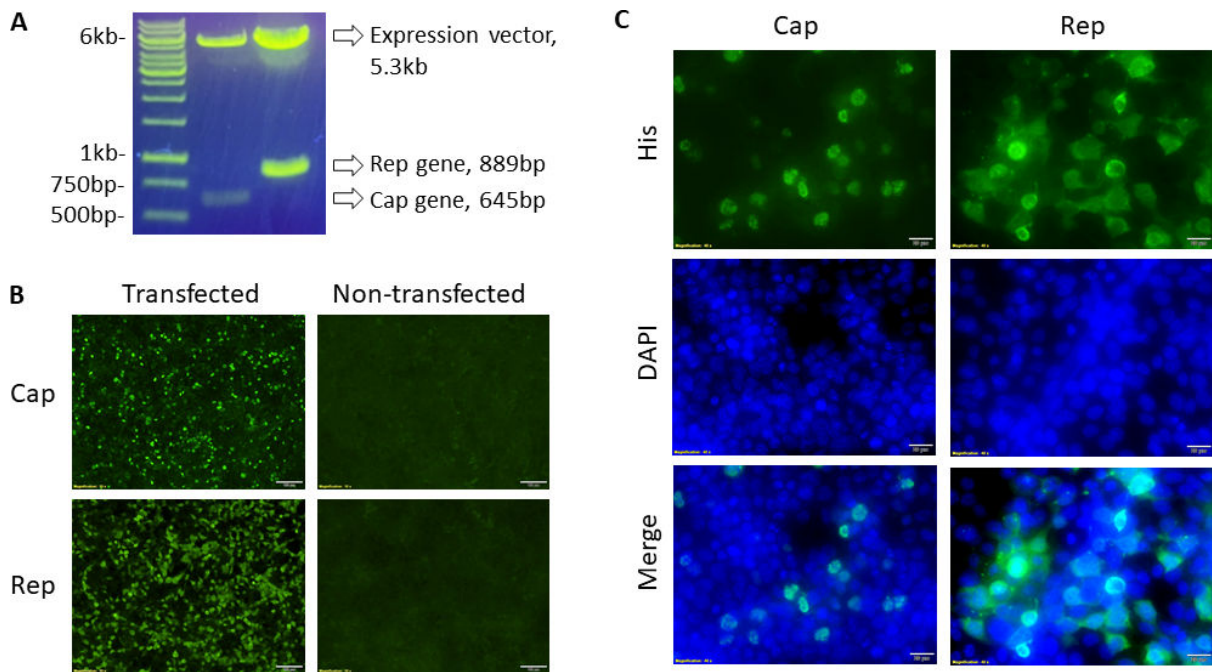
FIG 2 Molecular cloning and *in vitro* expression of the full-length Cap and Rep proteins of PCV3. (A) 1% agarose gel of Cap-pcDNA3.1(+)-N-6His (left lane) and Rep-pcDNA3.1(+)-N-6His (right lanes) plasmids digested with *BamHI* and *XhoI* endonuclease restriction enzymes. Band sizes appear as predicted: Cap (645 bp), Rep (889 bp), and expression vector (5.3 kb). (B) *In vitro* expression of Cap and Rep proteins in transfected HEK 293T cells by staining with an anti-histidine monoclonal primary antibody followed by an FITC conjugated goat anti-mouse secondary observed under ultraviolet fluorescence at 10× magnification. (C) Characterization of Cap and Rep proteins in transfected HEK 293T cells by staining with an anti-histidine monoclonal primary antibody followed by an FITC conjugated goat anti-mouse secondary antibody and DAPI observed under ultraviolet fluorescence and DAPI filter at 40× magnification.

In vitro expression of PCV3 Cap and Rep proteins

The full-length Cap and Rep genes of PCV3 were cloned into the pcDNA3.1(+)-N-6His expression vector. Enzyme restriction analysis by *BamHI* and *XhoI* endonuclease of the Cap-pcDNA3.1(+)-N-6His and Rep-pcDNA3.1(+)-N-6His plasmids on 1% Agarose gel confirmed successful cloning of the Cap (645 bp) and Rep (889 bp) genes into the expression vector (5.3 kb) (Fig. 2A). *In vitro* expression of the Cap and Rep proteins in transfected HEK 293T cells was confirmed by detection of green-fluorescent intranuclear and intracytoplasmic staining, respectively (Fig. 2B). The signal/background discrimination from transfected cell versus cell-control was evaluated for both the Cap and Rep proteins, and the transfection rate was approximately 70% (Fig. 2B). Dual staining with anti-histidine and DAPI (4′,6-diamidino-2-phenylindole) in transfected cells revealed expression of the Cap protein in nucleus while the Rep protein was predominately expressed in the cytoplasm (Fig. 2C).

Characterization of PCV3 Cap and Rep maternal antibody transfer

Thirty sows from a commercial sow farm with a history of PCV3 had a positive Cap antibody status 2 weeks prior to before farrowing by ORF2 recombinant protein-based indirect ELISA (data not shown) (22). The humoral response was evaluated longitudinally in three litters of piglets (31 total piglets) 1–9 weeks post farrowing using Cap and Rep indirect ELISA and IFA. All piglets from all litters showed detectable Cap IgG antibodies by IFA through 6 weeks post farrowing. Furthermore, Cap IgG antibodies were detectable in approximately 60% of litter 2 piglets by 9 weeks post farrowing while no Cap IgG antibodies were detected in piglets of litters 1 and 3 at the same time point (Fig. 3A and B). The highest Cap IgG IFA antibody titers and ELISA *S/P* ratios occurred 1–2 weeks post-farrowing in all piglets and declined to nearly undetectable levels between 7 and
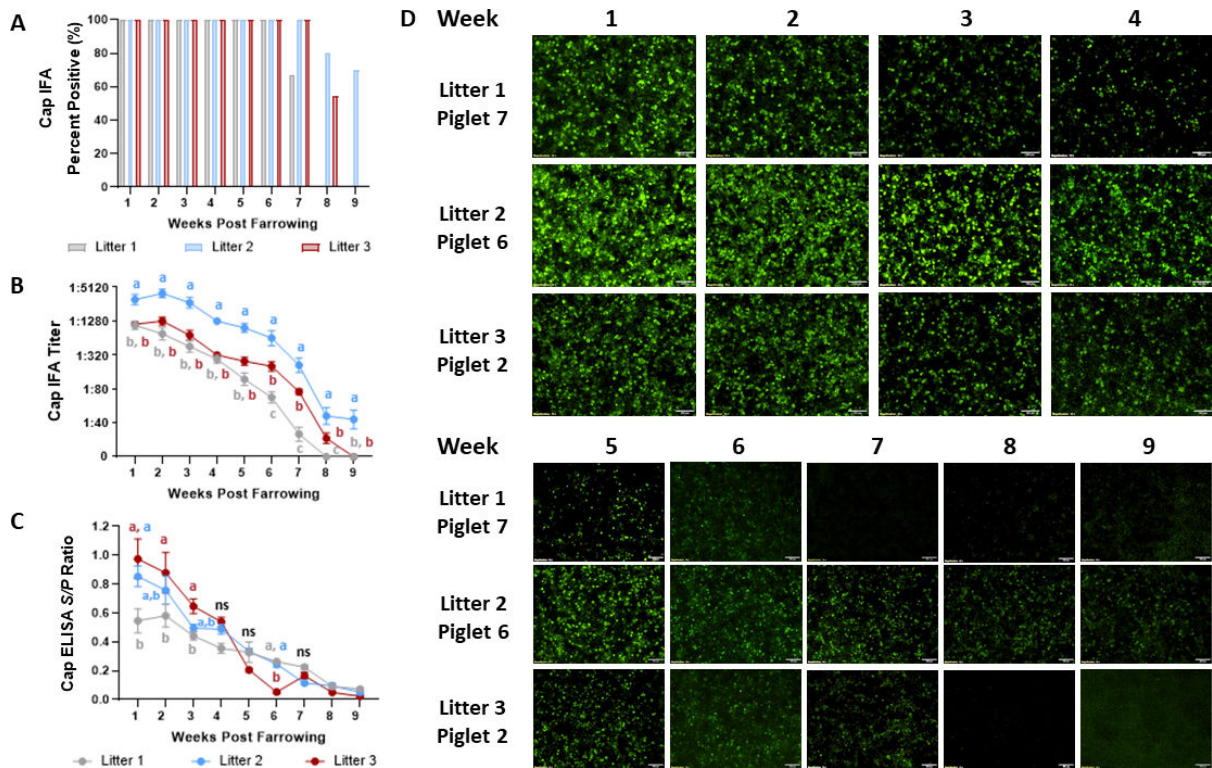
FIG 3 Kinetic of the maternally transferred serum Cap IgG antibodies by IFA and ELISA in piglets 1–9 weeks post farrowing. Three litters of piglets born from commercial sows naturally exposed to PCV3 were selected for study. The Cap IgG antibody status was evaluated by the percentage of piglets per litter positive (A), average IFA titer by litter (B), and average ELISA S/P ratios (C). IFA and ELISA methods concurred maximal Cap IgG titers occurred 1–2 weeks post farrowing, which waned in the following weeks. Antibodies were detectable 9-weeks post farrowing by IFA in litter 2 piglets but were nondetectable in litter 1 and 3 piglets. (D) The Cap IgG IFA staining from one representative piglet per litter at a serum dilution of 1:20 and 4× magnification, where the bright and diffuse immunofluorescent staining decreased over time correlating to lower IFA titers. Bars represent the SE of the mean, and significance was established at P < 0.05.

9 weeks of age (Fig. 3B and C). Litter 2 had significantly higher IFA titers (P < 0.05) compared to litters 1 and 3 from 1 to 9 weeks post farrowing with litter 2 IFA titers reaching 1:5,120 at 1-week post farrowing. No significant differences in IFA titer for litters 1 and 3 were observed between 1 and 5 weeks post farrowing with maximum titers of 1:1,280 at 1-week post farrowing. The Cap IgG IFA staining of one representative piglet per litter is shown in Fig. 3D, where positive antibody detection is characterized by nuclear green-fluorescent staining of transfected cells.

For the Rep IgG response, no antibodies were detected in litter 1 piglets by IFA. However, Rep IgG antibodies were detectable by IFA in 100% of piglets in litters 2 and 3 1-week post farrowing (Fig. 4A). Rep IgG antibody levels declined more rapidly compared to Cap IgG antibodies, with no detectable Rep IgG antibodies by 3-weeks post farrowing for litter 3 and 6-weeks post farrowing for litter 2 (Fig. 4A through C). The highest Rep IgG IFA antibody titers and ELISA S/P ratios were observed at 1-week post farrowing. IFA titers and ELISA S/P ratios in litter three more rapidly declined to a negative antibody status 2-weeks post farrowing. In contrast, the IFA titer and ELISA S/P ratios remained significantly higher until 5-weeks post farrowing (Fig. 4B and C). The highest Rep IgG IFA titer was 1:640 for piglets in litter 2 1-week post farrowing, which was approximately eight-fold lower than the maximum Cap IgG titer observed at the same time point. The Rep IgG IFA staining of one representative piglet per litter is shown in Fig. 4D. No immunofluorescent staining was observed in piglet 7 from litter 1. Furthermore, the cytoplasmic green-fluorescent staining of transfected cells in the piglets from litters 2 and 3 rapidly decreases and is not visible after 6 weeks post farrowing (Fig. 4D).
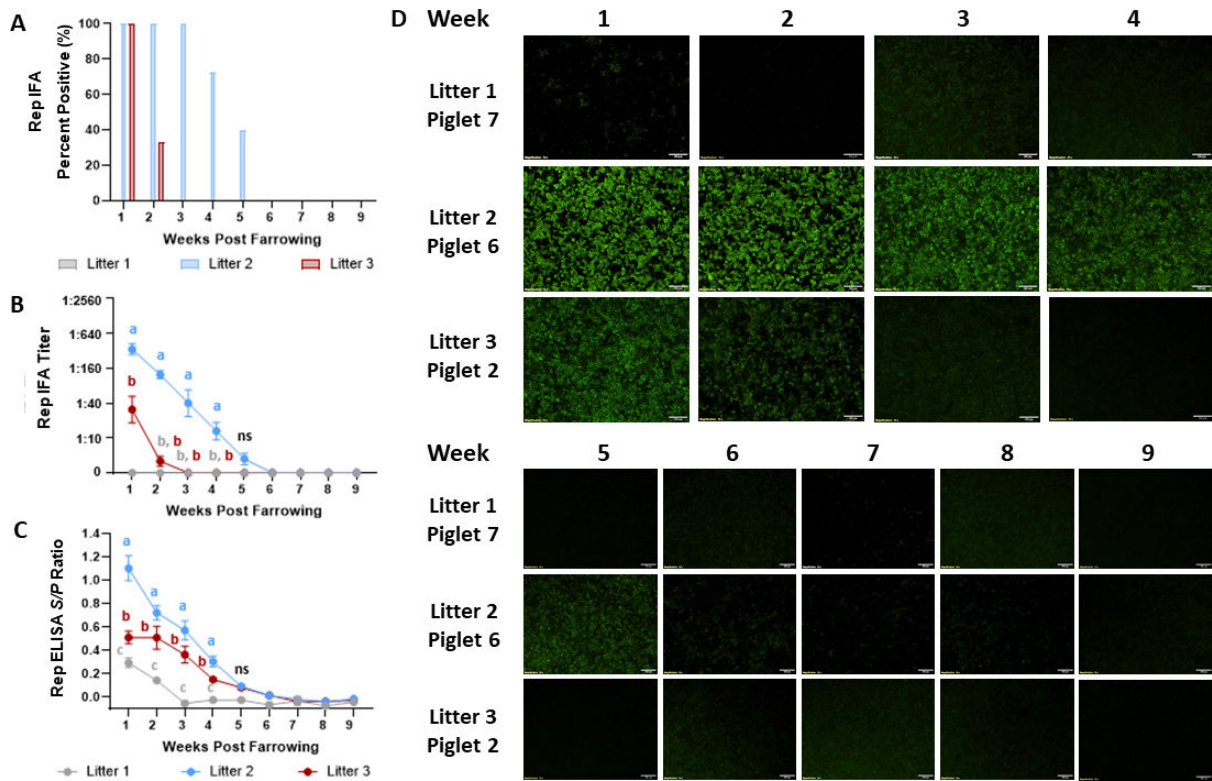
FIG 4 Kinetics of the maternally transferred serum Rep IgG antibodies by IFA and ELISA at 1–9 weeks post farrowing. Three litters of piglets born from commercial sows naturally exposed to PCV3 were selected for the study. The Rep IgG antibody status was evaluated by the percentage of positive piglets per litter by IFA (A), average IFA titer by litter (B), and average ELISA S/P ratios (C). Rep IgG antibodies were nondetectable in litter 1 piglets. For litter 2 and 3 piglets, Rep IgG antibodies were at maximal levels by IFA and ELISA 1-week post farrowing and rapidly declined to nondetectable levels by 3-weeks post farrowing for litter 3 piglets and 6-weeks for litter 2 piglets. (D) The Rep IgG IFA staining from one representative piglet per litter at a serum dilution of 1:10 and 4× magnification, where no staining is evident in piglet 7 from litter 1 and the bright and diffuse immunofluorescent staining is not visible past 6 weeks post farrowing for piglet 6 from litter 2. Bars represent the SE of the mean, and significance was established at $P < 0.05$.

The PCV3 viremia, as detected by qPCR, was evaluated by litter from 1 to 9 weeks post farrowing. No detectable viremia was observed in litter 1 throughout the duration of the study. From 1 to 4 weeks post farrowing, litters 2 and 3 had intermittent detection of PCV3 DNA at relatively low amounts with a Ct values ranging from 32.3 to 36.9. Interesting, litter 3 had a low amount of PCV3 (Ct = 36.7) detected in the serum 9-weeks post farrowing after six consecutive weeks of having no detectable viremia (Table 1).

Characterization of PCV3 Cap and Rep antibody dynamics in grower pigs following experimental infection

The evaluation of the antibody dynamic in experimentally infected pigs showed a Cap IgG antibody response, detectable in a single pig as early as 14 dpi in the PCV3+KLH group and two pigs at 21 dpi in the PCV3 group. All the pigs from both PCV3-inoculated groups were positive by IFA at 35 dpi (Fig. 5B). All pigs in both PCV3

TABLE 1 Detection of PCV3 DNA in the serum of piglets born from naturally infected sows 1–9 weeks post farrowing[a]

Litter	Weeks of age (serum) (Ct-value)								
	1	2	3	4	5	6	7	8	9
1	–	–	–	–	–	–	–	–	–
2	+ (36.8)	+(36.4)	–	+ (36.9)	–	–	–	–	–
3	+ (35.5)	–	+ (32.3)	–	–	–	–	–	+ (36.7)

[a]Serum samples were pooled by litter with a maximum of five animals per pool. The mean of the pooled Ct values by litter were calculated to yield the litter Ct value.
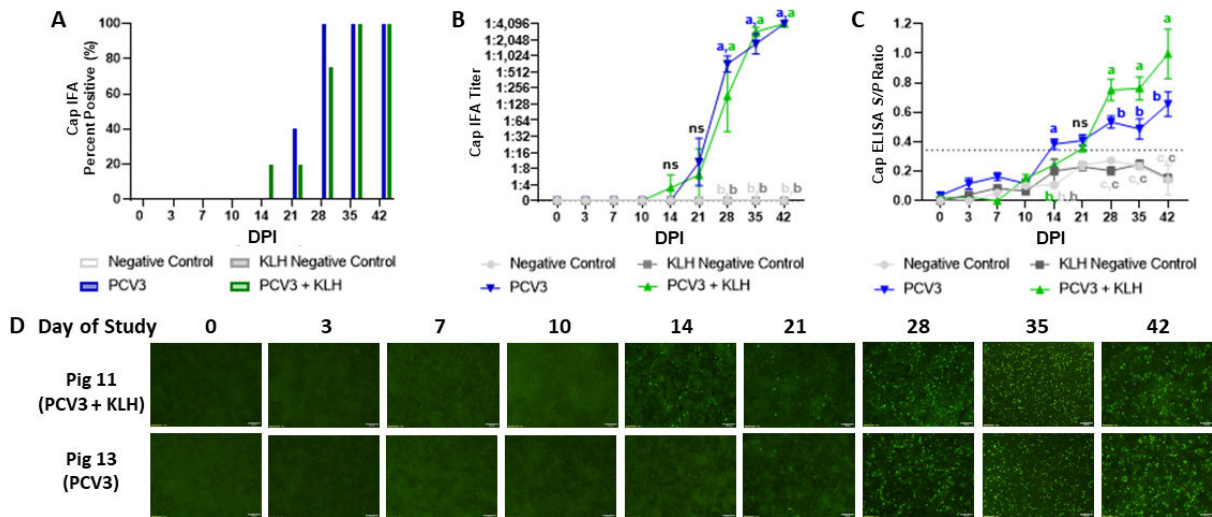
FIG 5 Dynamics of Cap IgG antibodies by IFA and ELISA in CD/CD pigs experimentally inoculated with PCV3 tissue homogenate with and without immunostimulation. PCV3 and PCV3+KLH groups were inoculated intranasally with 2 mL of PCV3 tissue homogenate (1.04×10^{11} genome copies mL^{-1}) and intramuscularly with 2 mL of PCV3 tissue homogenate (3.38×10^{12} genome copies mL^{-1}) then reinoculated 7 days after the first inoculation. Immunostimulation was performed in the PCV3+KLH and KLH negative control groups through subcutaneous administration of 1 mL of 1 mg mL^{-1} of KLH in ICFA. Cap IgG antibodies were evaluated by the percentage of pigs per group (A), average IFA titer by group (B), and average ELISA S/P ratios by group (C). Seroconversion was detected at 14 dpi with significantly antibody higher levels in the PCV3 and PCV3+KLH groups by IFA and ELISA occurring at 28–42 dpi. (D) The Cap IgG IFA staining from one representative pig in the PCV3 and PCV3+KLH groups at a serum dilution of 1:4 and 4× magnification, where relatively low amounts of immunofluorescent staining are present at 14 dpi for pig 11 in the PCV3+KLH group and much greater amounts of bright and diffuse immunofluorescent staining from both pigs are present 28–42 dpi, correlating to the increase in IFA titers. Bars represent the SE of the mean, and significance was established at $P < 0.05$.

and PCV3+KLH-inoculated groups had significantly higher Cap IgG antibody titers by IFA, compared with negative control groups, by 28 dpi and reached a maximum IFA titer of 1:4,096 by 42 dpi (Fig. 5A and B). The IFA antibody titers and ELISA S/P ratios for the PCV3 and PCV3+KLH group were significantly higher ($P < 0.05$) than the negative control and KLH negative control groups 28–42 dpi (Fig. 5B and C). No statistical difference between the PCV3 and PCV3+KLH group IFA titers was observed. However, the statistical analysis of the ELISA S/P ratios showed that the PCV3+KLH group had significantly higher levels of antibodies ($P < 0.05$) compared to the PCV3 group at 28–42 dpi (Fig. 5C). All the pigs in the mock-inoculated negative control and adjuvant control groups tested negative for the presence of Cap IgG by IFA and ELISA throughout the study duration (Fig. 5B and C). The Cap IgG IFA staining of one representative pig from the PCV3 and PCV3+KLH groups is shown in Fig. 5D. Relatively low amounts of nuclear green-fluorescent staining were present at 14 dpi for pig 11 in the PCV3+KLH group and 21 dpi for pig 13 in the PCV3 group. The nuclear green-fluorescent staining was more intense in both pigs between 28 and 42 dpi, correlating to the increase in IFA titers and ELISA S/P ratios (Fig. 5D).

For the Rep IgG antibody response, a single pig in the PCV3 group had detectable antibodies at 28 dpi by IFA (Fig. 6A and B). All pigs in both PCV3 and PCV3+KLH groups had detectable antibodies by 35 dpi reaching antibody titers detectable by IFA as high as 1:256, which is comparatively 16-fold lower than the maximum Cap IgG IFA titer observed. Furthermore, maximum Rep IgG IFA titers were approximately 30-fold lower than Cap IgG IFA titers at 35 dpi. The IFA titers and ELISA S/P ratios for the PCV3 and PCV3+KLH group were significantly higher ($P < 0.05$) than the negative control and KLH negative control groups 35–42 dpi (Fig. 6B and C). No significant differences in Rep IgG antibody titers were observed between PCV3 and PCV3+KLH groups by IFA and ELISA at 35 and 42 dpi (Fig. 6B and C). All the pigs in the mock-inoculated negative control and adjuvant control groups were negative for the presence of Rep IgG by IFA and ELISA throughout the study (Fig. 6B and C). The Rep IgG IFA staining of one representative pig from the PCV3 and PCV3+KLH groups is shown in Fig. 6D. Moderate amounts of
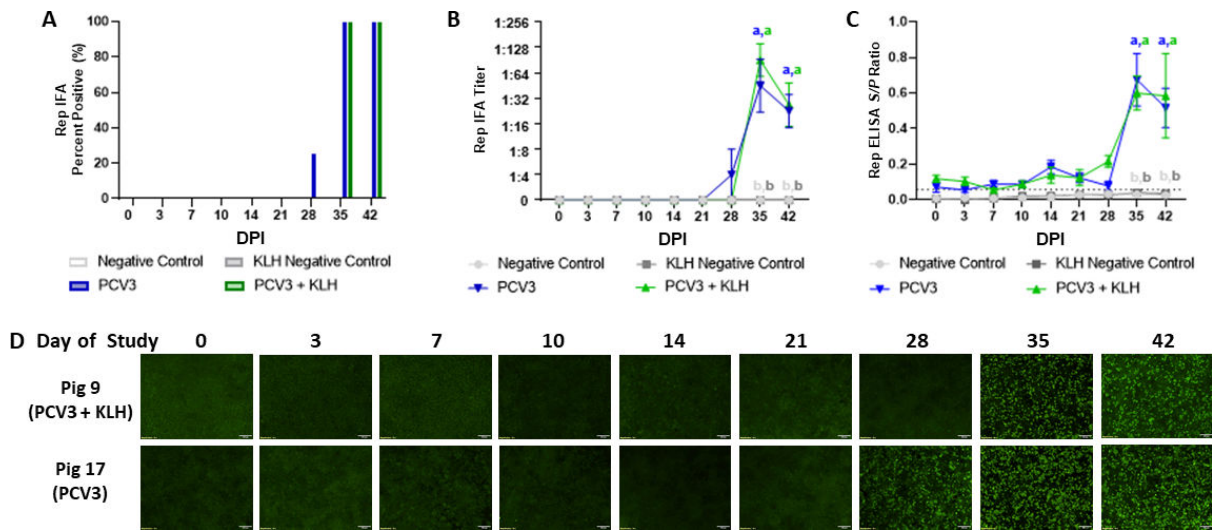
FIG 6 Dynamic of Rep IgG antibodies by IFA and ELISA in CD/CD pigs experimentally in inoculated with PCV3 tissue homogenate with and without immunostimulation. PCV3 and PCV3+KLH groups were inoculated intranasally with 2 mL of PCV3 tissue homogenate (1.04×10^{11} genome copies mL^{-1}) and intramuscularly with 2 mL of PCV3 tissue homogenate (3.38×10^{12} genome copies mL^{-1}) then reinoculated 7 days after the first inoculation. Immunostimulation was performed in the PCV3+KLH and KLH negative control groups through subcutaneous administration of 1 mL of 1 mg mL^{-1} of KLH in ICFA. Rep IgG antibodies were evaluated by the percentage of pigs per group (A), average IFA titer by group (B), and average ELISA *S/P* ratios by group (C). Seroconversion was detected at 28 dpi with significantly higher antibody levels in the PCV3 and PCV3+KLH groups by IFA and ELISA occurring at 35–42 dpi. (D) The Rep IgG IFA staining from one representative pig in the PCV3 and PCV3+KLH groups at a serum dilution of 1:4 and 4× magnification, where moderate amounts of immunofluorescent staining are present at 28 dpi for pig 17 in the PCV3 group and greater amounts of bright and diffuse immunofluorescent staining from both pigs are present 35–42 dpi, correlating to the increase in IFA titers. Bars represent the SE of the mean, and significance was established at *P* < 0.05.

intracytoplasmic green-fluorescent staining were present at 28 dpi for pig 17 in the PCV3 group. Greater amounts of bright and diffuse immunofluorescent staining from both pigs were present 35–42 dpi, correlating to the increase in IFA titers and ELISA *S/P* ratios (Fig. 5D).

Detection and quantitation of PCV3 DNA were assessed throughout the study (data not shown). Briefly, prolonged viremia was observed in the PCV3 and PCV3+KLH groups from 3 dpi to the end of study with no significant changes in viral load regardless of immunostimulation. Peak viremia occurred at 3 dpi with similar average genomic copy numbers per mL^{-1} between the PCV3 (2.20×10^8 copies mL^{-1}) and PCV3+KLH groups (1.42×10^8 copies mL^{-1}) (28).

Evaluation of cross-reactivity between PCV3 and PCV2 seropositive sera by IFA and ELISA

Sera obtained from PCV3 experimentally infected pigs were evaluated for potential cross-reactivity with PCV2 antigens by different PCV2 serological methods. From study 2, serum samples from all animals at 0, 28, and 42 dpi were evaluated for the presence of viremia and PCV2 antibodies by qPCR, IFA, and ELISA. All animals displayed no PCV2 viremia throughout the study duration (28). Additionally, PCV2 IFA titers were <1:20 for all animals at each time point, consistent with a negative antibody status (Fig. 7A). Furthermore, PCV2 ELISA *S/P* ratios fell below the positive cutoff for all animals at each timepoint (Fig. 7B).

The presence of PCV2 antibodies following experimental PCV2 infection was evaluated for potential cross-reactivity on PCV3 serological methods developed in this study. For study 3, 10 CD/CD pigs were challenged with PCV2, and serum was collected pre-challenge (49 days before challenge), 0 , 14, and 28 dpc. No PCV2 viremia or PCV2 antibodies were observed in samples collected during pre-challenge (Fig. 8A and B). After challenge, PCV2 viremia was detectable 14 and 28 dpc by qPCR (Fig. 8A), and seroconversion was detected at 28 dpc by ELISA in all animals (Fig. 8B). PCV3 viremia
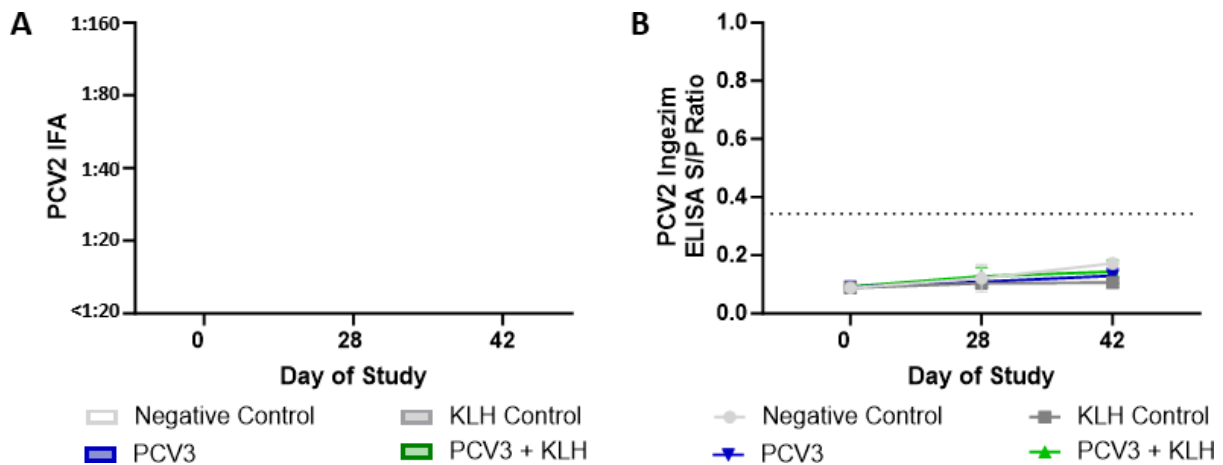
FIG 7 Evaluation of PCV3 antibody cross-reactivity to PCV2 serological methods from pigs experimentally inoculated with PCV3 tissue homogenate. PCV3 and PCV3+KLH groups were inoculated intranasally with 2 mL of PCV3 tissue homogenate (1.04×10^{11} genome copies mL^{-1}) and intramuscularly with 2 mL of PCV3 tissue homogenate (3.38×10^{12} genome copies mL^{-1}) then reinoculated 7 days after the first inoculation. Immunostimulation was performed in the PCV3+KLH and KLH negative control groups through subcutaneous administration of 1 mL of 1 mg mL^{-1} of KLH in ICFA. Serum samples were evaluated by PCV2 IFA (A) and Ingezim ELISA (B) conducted at the ISU-VDL. All pigs had a PCV2 IFA titer of <1:20 and PCV2 ELISA *S/P* ratios below the positive cutoff, suggesting a negative PCV2 antibody status. Bars represent the SE of the mean.

was not detected in all animals throughout the study duration (data not shown). PCV3 Cap and Rep IgG ELISA *S/P* ratios were <0.1 at 28 dpc (Fig. 8C and D), supporting the lack of cross-reactivity of PCV2 positive sera on PCV3 ELISA. In addition, PCV3 Cap and Rep IgG IFA titers were <1:20 and <1:10, respectively (Fig. 8E and F), further demonstrating a lack of cross-reactivity of PCV2 antibodies to PCV3 serologic methods. All PCV2 experimentally infected pigs lacked the nuclear green fluorescent staining by the Cap IgG IFA (Fig. 8G) and cytoplasmic green fluorescent staining by the Rep IgG IFA (Fig. 8H) to suggest a positive IFA titer.

DISCUSSION

Since PCV3 was identified in 2016, the virus has most commonly been associated with multisystemic inflammation, reproductive failure, and subclinical infection (7, 8). Despite the advancements in PCV3 detection and the availability of serological tools for PCV3 serological diagnosis, a significant gap remains in understanding the dynamics of antibodies in grower-finisher pigs, the duration of maternal antibodies, and the potential cross-reactivity with PCV2 antibodies. Field studies have hypothesized the transfer of maternally derived antibodies due to increased detection of viremia in pigs older than 3–4 weeks of age (26, 27). More recently, an experimental infection study in pregnant gilts during mid and late gestation reproduced transplacental infection resulting in piglets with viremia at birth, decreased weaning weight, and lymphohistiocytic arteritis and periarteritis in piglets. However, antibody dynamics were not assessed due to the lack of validated antibody tests (37). Retrospective field studies have reported variable PCV3 seroprevalence ranging from 20% to 80% in varying production phases. However, these studies do not describe the antibody dynamics of natural infection or the transfer of maternally derived antibodies (18, 21). Experimental infection studies have reported conflicting results in the humoral dynamics (22, 28). Furthermore, information regarding the presence and dynamics of PCV3 Rep antibodies remains limited. While cross-reactivity between PCV3 and PCV2 has been explored by *in vitro* analyses with field samples, controlled experiments utilizing experimentally infected animals to elucidate this diagnostic inquiry are lacking. Thus, this study aims to address these three primary knowledge gaps concerning the humoral response to PCV3 and the serological diagnosis of PCV3.
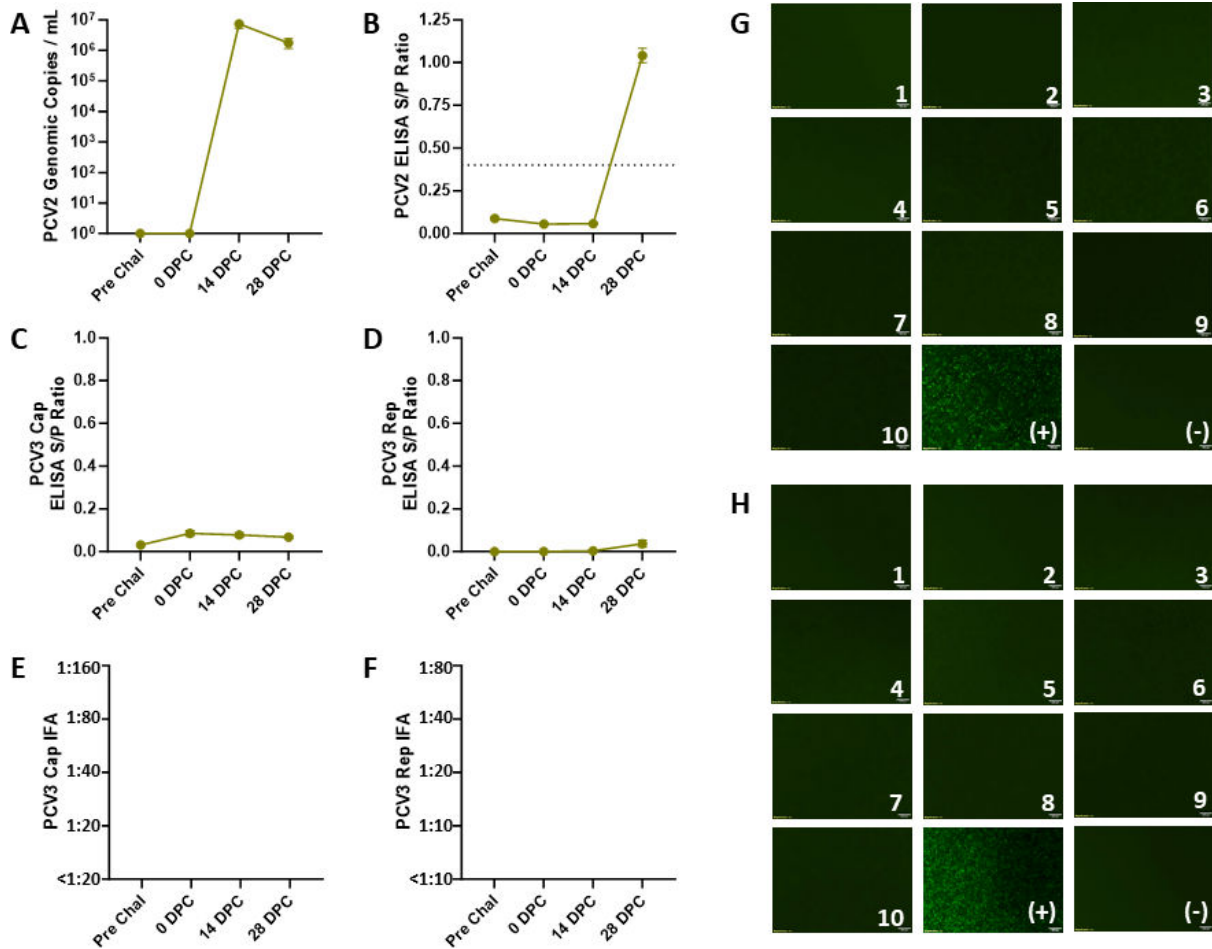
FIG 8 Evaluation of PCV2 antibody cross-reactivity to PCV3 serologic methods from pigs experimentally challenged with PCV2. All pigs were challenged with PCV2 tissue homogenate (2 mL intranasal and 1 mL intramuscular) on 0 dpc. Serum samples were analyzed by PCV2 qPCR (A) and Ingezim ELISA (B) conducted at the ISU-VDL to confirm successful challenge and seroconversion of PCV2 antibodies. Additionally, all pigs had no detectable PCV3 viremia throughout the study duration (data not shown). Serum samples were further evaluated by PCV3 Cap IgG ELISA (C), Rep IgG ELISA (D), Cap IgG IFA (E), and Rep IgG IFA (F). ELISA *S/P* ratios remained relatively low, and Cap and Rep IFA titers were <1:20 and <1:10, respectively, suggesting a negative PCV3 antibody status. None of the experimentally treated animals showed staining by PCV3 Cap IgG IFA (G) or Rep IgG IFA (H), in contrast to the positive controls. . Bars represent the SE of the mean.

The transfer of PCV2 maternal antibodies has been well characterized where both PCV2 Cap and Rep maternally derived antibodies can be detected in piglets (29). The half-life of PCV2 Cap maternally derived antibodies is approximately 19 days (38). However, the duration of PCV2 Cap antibodies in piglets can vary depending on the maternal antibody levels. Thus, based on the immunological status of the sow, the dynamics of low, mid, and high maternal antibody transfer can last approximately 4–6, 6–10, and 8.5–13 weeks of age, respectively (38). The presence of PCV3 maternal antibodies has yet to be fully evaluated. The presence of PCV3 maternally derived antibodies has been hypothesized due to a lack of detectable viremia during the lactation phase and increased viral detection in pigs older than 3–4 weeks (26, 27). In the present study, 30 sows from a commercial farm with a history of PCV3 were evaluated for the presence of Cap antibody 2 weeks before farrowing. Three litters from PCV3 antibody positive sows were selected for longitudinal evaluation of the Cap and Rep IgG humoral response 1–9 weeks post-farrowing. All piglets from all litters displayed the highest levels of PCV3 Cap IgG antibodies 1-week post-farrowing. These antibodies waned to nondetectable levels by approximately 6–8 weeks post-farrowing for litters 1 and 3, while
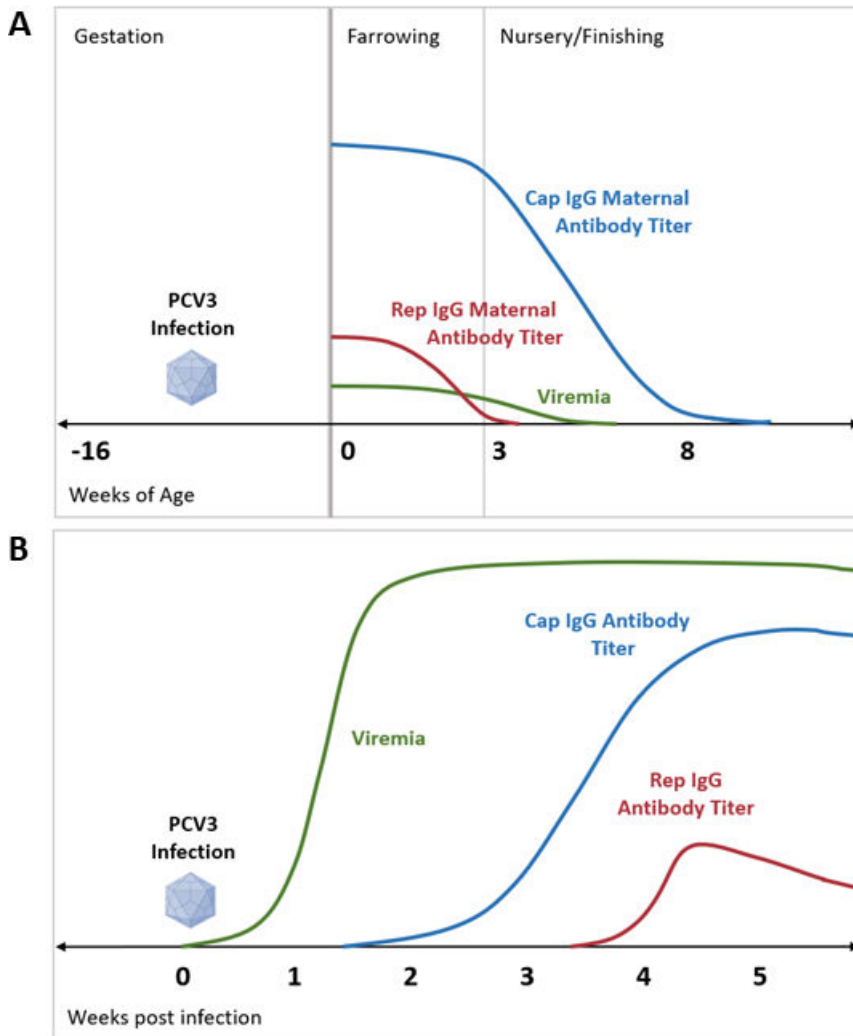
FIG 9 Summary of PCV3 viremia, Cap IgG, and Rep IgG dynamics following maternal antibody transfer in naturally PCV3 infected sows (A) and experimental PCV3 infection (B).

antibodies were detectable in approximately 60% of the litter 2 piglets by IFA at 9 weeks post-farrowing (Fig. 9A). Multiple factors influence maternal antibody transfer including piglet milk intake, milk availability, the immune status of the sows, sow parity, and husbandry practices aimed at enhancing colostrum intake (39, 40). As per these findings, the dynamic of PCV3 Cap maternally derived antibodies was similar to the transfer of a moderate quantity of PCV2 Cap-specific maternal antibodies (38). Further studies are necessary to evaluate how differences in sow antibody levels may affect the duration of the Cap antibody dynamic in piglets.

In accordance with previous studies assessing the PCV2 Rep IgG maternal antibodies, maternally derived PCV2 Rep IgG antibodies have been detected until 11 weeks of age in piglets (29). However, the approximate duration of PCV2 Rep antibodies has not been characterized in terms of sow antibody levels. In this study, PCV3 Rep IgG antibody detection was more variable between litters. Maternally derived PCV3 Rep IgG antibodies were not detected in litter 1, while antibodies waned by approximately 5-weeks post-farrowing in litters 2 and 3. Although the duration of PCV3 Rep IgG antibodies can also be associated with several factors similar to those reported affecting PCV2 Cap antibody duration (39, 40), our results demonstrate that lower amounts of maternally derived PCV3 Rep antibodies were transferred to the piglets compared to Cap antibodies. Additionally, our results may suggest that PCV3 Rep antibodies have a shorter half-life

compared to PCV3 Cap antibodies and the PCV3 Rep antibody dynamic may be shorter than PCV2 Rep antibodies (29).

During PCV2 infection, clinical PCVAD is generally not observed before 4 weeks of age due to the presence of maternally derived antibodies (41–44). Thus, PCV3 maternally antibodies may have a similar role in protecting against infection in the lactation and nursery phase. In previous field studies, pigs 3–4 weeks of age displayed no PCV3 viremia (26). Furthermore, the proportion of PCV3 viremia detection increased in pigs from 6 to 24 weeks of age (27). The present study did not examine whether piglets developed lesions compatible with PCV3 infection. Thus, the study cannot confirm that the presence of maternally derived antibodies provided complete protection against infection. However, piglets did not develop apparent clinical signs suggestive of PCV3 infection, indicating that maternally derived antibodies may have aided in the clearance of viremia during 1–4-weeks post–farrowing, resulting in subclinical infection. Interestingly, viremia was detected in litter 3 at 9-weeks post-farrowing, in concordance with the absence of maternally derived antibodies, potentially suggesting the detection of an early infection. Future studies should examine when the development of viremia occurs in relationship to low, mid, and high levels of PCV3 maternal antibody transfer.

The antibody response following PCV2 infection has also been well characterized where seroconversion occurs 7–28 dpi and neutralizing antibodies are detectable beginning at 10 dpi (30). Furthermore, Cap antibodies are produced earlier and reach higher titer compared to Rep antibodies (31). In the present study, the Cap and Rep IgG responses were evaluated following experimental inoculation with PCV3 tissue homogenate with and without immunostimulant. Regardless of immunostimulation, the PCV3 Cap IgG response was detectable in both inoculated PCV3 groups at 14 dpi reaching highest levels detected either by IFA or by ELISA by 28–42 dpi (Fig. 9B). ELISA S/P ratios from 28 to 42 dpi showed that immune stimulation with KLH induced significantly higher levels of antibodies ($P < 0.05$) compared to the PCV3 group. These significant differences in antibody titers between inoculated groups were not observed by IFA. This is perhaps due to intrinsic factors of the IFA technique such as the two-fold serial dilution of sera, potentially resulting in a less precise determination of titer at higher dilutions. Several previous PCV2 studies have shown immunostimulation by a variety of methods such as adjuvants, vaccination, or coinfection agents triggering the development of PCVAD (45). Thus, based on our results, PCV3 immunostimulation may enhance the Cap IgG humoral response, suggesting co-factors such as coinfections may also exacerbate the PCV3 humoral response. Additional studies evaluating the humoral response to PCV3 and other coinfections are necessary to not only to evaluate the PCV3 antibody dynamics but also to characterize the potential effect of PCV3 infection on the immune response against other pathogens.

In the present study, PCV3 Rep IgG antibodies were detectable 28 dpi with significantly higher IFA titers and ELISA S/P ratios occurring at 35–42 dpi (Fig. 9B). Interestingly, the highest Rep IgG IFA titers were approximately 30-fold lower than Cap IgG IFA titers at 35 dpi. These results show PCV3 Cap antibodies are produced earlier and at much greater amounts compared to PCV3 Rep antibodies, which coincided with the levels and the dynamic previously reported for PCV2 humoral response (31). The PCV2 Rep protein is a weak immunogen. Vaccination with Rep DNA vaccines followed by a booster with recombinant protein did not result in protection against clinical disease. In comparison, a DNA Cap vaccine following the same vaccination and challenge model abrogated the development of clinical disease, confirming the Cap protein of PCV2 is the major protective immunogen (46). Similar to PCV3, the PCV2 Cap is the sole component of the virion. Therefore, the PCV2 Rep protein is expressed following initial viral replication mediated by host cell DNA replication machinery (47). Much is still unknown regarding the PCV3 life cycle. However, given the similarities of PCV2 and PCV3, the intracytoplasmic nature of the Rep protein and reliance on the host cell for expression may explain the delayed development of Rep antibodies at lower levels, which may potentially provide

limited protection. The biological activity of Rep maternally derived antibodies and Rep antibodies developed following infection should be further evaluated.

Interestingly, prolonged PCV3 viremia was observed in the PCV3 and PCV3+KLH groups from 3 dpi to the end of study with no significant changes in viral load regardless of immunostimulation (28). The development and maintenance of prolonged viremia while pigs produce an antibody response is a similar feature of infection for PCV2 and PCV3. (48–50). Following PCV2 infection, virus-specific neutralizing antibodies are detectable at approximately 21 dpi (41, 45, 51, 52). Virus-neutralizing antibodies appear to limit viral replication *in vitro* (30, 53) and the development of PCVAD *in vivo* (54). However, protective immunity following vaccination can be achieved with low levels of humoral immunity (55), suggesting both the humoral and cell-mediated immune responses contribute to protection (56). Pigs naturally infected with PCV2 develop a robust Interferon‐gamma (IFN‐γ) response (57). Furthermore, PCV2-specific IFN-γ production has been correlated to lower levels of viremia (58, 59), demonstrating the cell-mediated response is vital for clearance of PCV2 infection (56, 60–63). Future studies should aim to determine the duration of the PCV3 antibody response, development of neutralizing antibodies, and potential correlation of total and virus-specific neutralizing antibody titers to the reduction of viremia. Additionally, future research should elucidate the development of PCV3-specific INF-γ production.

Because of limited sample volume, the current study was limited to focusing on characterizing the Cap and Rep IgG dynamics. However, characterizing the IgM response warrants further investigation. Previous experimental PCV3 inoculation studies have conflicting results on the presence of Cap IgM (22, 28). For PCV2 infection, the presence of the Cap IgM isotype seems to be an indicator of the development of clinical disease. In pigs affected with postweaning multisystemic wasting syndrome (PMWS), IgM remained at low or nondetectable titers and IgG titers were significantly lower compared to healthy group mates (30). Furthermore, the IgM isotype is correlated to low levels of neutralizing activity and has no correlation to viremia reduction within the first 2 weeks of PCV2 infection (64). Therefore, we hypothesize that the presence of IgM may be correlated to PCV3 clinical disease and the persistence of IgM in the absence of class switching to the IgG isotype could result in the development of PCV3 clinical disease.

The genetic homology of PCV2 and PCV3 is approximately 46.8% at the whole genome level, 26%–36% for the ORF2, and 48% for the ORF1 (8, 12, 16). Despite the low genomic similarity, potential antibody cross-reactivity between PCV2 and PCV3 has been proposed, and information generated based on *in vitro* studies is nonconclusive. Convalescent rabbit serum generated by the immunization of recombinant PCV2-, PCV3-, and PCV4-truncated Cap proteins showed cross-reactivity among all the three PCVs using viral-based IFAs using virus rescued from infectious clones. Cross-reactivity was hypothesized due to the presence of a conserved epitope within the PCV2, PCV3, and PCV4 Cap genes (65). However, several studies have failed to recapitulate the *in vitro* cross-reactivity of PCV2 and PCV3 antibodies using swine convalescent serum. PCV2 antibody positive serum was not cross reactive to a PCV3 IFA by expression of the PCV3 Cap in a baculovirus system (24). Furthermore, PCV3 convalescent serum has shown no cross-reactivity to PCV2 virus-like particles (21). The current study further characterizes the cross-reactivity of PCV2 and PCV3 antibodies in convalescent serum from experimentally infected animals. No cross-reactivity of PCV3 antibodies was observed by PCV2 IFA and ELISA. Furthermore, no cross-reactivity of PCV2 antibodies was displayed by PCV3 Cap and Rep IFA and ELISA. These findings may indicate that natural infection may not confer protection against the alternate PCV through the humoral response. Future investigations ought to validate the absence of cross-protection offered by either natural infection or vaccination against the alternate PCV.

Characterization of the PCV2 protective immunity provided by maternal antibody transfer, and dynamic following natural infection has resulted in the development of highly implemented vaccination schedules to greatly reduce PCVAD and subclinical infection (66). While no vaccines are currently commercially available for PCV3, further

research into the neutralizing antibody response and the protection provided following maternal antibody transfer and natural infection may aid in the establishment of husbandry practices and potential future application of prophylactics to control PCV3 clinical disease.

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ETHICS APPROVAL

Experimental sample were collected under the experimental protocol approved by the Iowa State University Institutional Animal Care and Use Committee (log number: 1-17-8422-S; approval date: 27 January 2017).

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