Evaluation of the use of non-pathogenic porcine circovirus type 1 as a vaccine delivery virus vector to express antigenic epitopes of porcine reproductive and respiratory syndrome virus

Evaluation of the use of non-pathogenic porcine circovirus type 1 as a vaccine delivery virus vector to express antigenic epitopes of porcine reproductive and respiratory syndrome virus

Pablo E. Piñeyro[a,b], Scott P. Kenney[a], Luis G. Giménez-Lirola[b], Tanja Opriessnig[b,c], Debin Tian[a], C. Lynn Heffron[a], and Xiang-Jin Meng[a,*]

[a]Department of Biomedical Sciences & Pathobiology, Virginia-Maryland College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA 24060, USA.

[b]Department of Veterinary Diagnostic and Production Animal Medicine, Iowa State University College of Veterinary Medicine, Ames, Iowa 5001, USA.

[c]The Roslin Institute and The Royal (Dick) School of Veterinary Studies, University of Edinburgh, Edinburgh, United Kingdom.

Running title: PCV1 as a vaccine delivery virus vector

*Corresponding author:
X.J. Meng, M.D., Ph.D., University Distinguished Professor
Department of Biomedical Sciences & Pathobiology,
Virginia Polytechnic Institute and State University,
1981 Kraft Drive, Blacksburg, VA 24061-0913, USA.
Phone: 1-515-231-6912. E-mail: xjmeng@vt.edu

Word counts: Abstract: 250 Text: 4237 Figures: 6 Tables: 2

Abstract

We previously demonstrated that the C-terminus of the capsid gene of porcine circovirus type 2 (PCV2) is an immune reactive epitope displayed on the surface of virions. Insertion of foreign epitope tags in the C-terminus produced infectious virions that elicited humoral immune responses against both PCV2 capsid and the inserted epitope tags, whereas mutation in the N terminus impaired viral replication. Since the non-pathogenic porcine circovirus type 1 (PCV1) shares similar genomic organization and significant sequence identity with pathogenic PCV2, in this study we evaluated whether PCV1 can serve as a vaccine delivery virus vector. Four different antigenic determinants of porcine reproductive and respiratory syndrome virus (PRRSV) were inserted in the C-terminus of the PCV1 capsid gene, the infectivity and immunogenicity of the resulting viruses are determined. We showed that an insertion of 12 (PRRSV-GP2 epitope II, PRRSV-GP3 epitope I, and PRRSV-GP5 epitope I), and 14 (PRRSV-GP5 epitope IV) amino acid residues did not affect PCV1 replication. We successfully rescued and characterized four chimeric PCV1 viruses expressing PRRSV linear antigenic determinants (GP2 epitope II: aa 40–51, ASPSHVGWWSFA; GP3 epitope I: aa 61–72, QAAAEAYEPGRS; GP5 epitope I: aa 35–46, SSSNLQLIYNLT; and GP5 epitope IV: aa 187–200, TPVTRVSAEQWGRP). We demonstrated that all chimeric viruses were stable and infectious *in vitro* and three chimeric viruses were infectious *in vivo*. An immunogenicity study in pigs revealed that PCV1-VR2385$_{EPI}$ chimeric viruses elicited neutralizing antibodies against PRRSV-VR2385. The results have important implications for further evaluating PCV1 as a potential vaccine delivery vector.

Keywords: Vaccine delivery vector; Porcine circovirus type 1 (PCV1); Porcine reproductive and respiratory syndrome virus (PRRSV); Antigenic epitopes; Porcine circovirus type 2 (PCV2)

1. Introduction

Porcine circoviruses (PCV) belong to the genus *Circovirus* of the family *Circoviridae* (Finsterbusch and Mankertz, 2009). The viral genome is packaged in an icosahedral capsid approximately 17 nm in diameter, and PCV is the smallest virus infecting mammals. Two types of PCV, PCV1 and PCV2, have been identified thus far. PCV1 was first described in 1974 as a contaminant of the porcine kidney cell line, PK-15, and is non-pathogenic in pigs (Tischer et al., 1982). PCV2 is pathogenic and causes an economically-important porcine circovirus-associated diseases (PCVAD) in swine worldwide (Allan et al., 1998; Allan et al., 1999; Ellis et al., 1998). Both PCV1 and PCV2 are non-enveloped, single-stranded circular DNA molecules of 1,759 (PCV1) and 1,768 (PCV2) kb in size (Finsterbusch and Mankertz, 2009).

The non-pathogenic PCV1 shares similar genomic organization with the PCVAD-associated PCV2 (Chae, 2005), which is characterized by 11 potential open reading frames (ORFs) with predicted protein sizes ranging from 2 to 36 kDa (Hamel et al., 1998). However, thus far only two major ORFs are believed to be essential for completing the basic functions of the virus: ORF1 encodes the replicase (Rep) (314 aa) and the truncated, spliced Rep' (178 aa), whereas the ORF2 encodes the immunogenic capsid protein (233 aa). Sequence analyses revealed that PCV1 shares a 76% nucleotide sequence identity with its pathogenic counterpart PCV2. The ORF1-encoded replicase protein has approximately 80% amino acid sequence identity between the two viruses, whereas the ORF2 capsid protein has about 60% amino acid sequence identity (Mahé et al., 2000; Trible and Rowland, 2012). The ORF1 and ORF2 genes are oriented in opposite directions, resulting in an ambisense orientation. Between the 5' end of ORF1 and ORF2, there exists an intergenic region that contains the origin of virus replication characterized by a stem-loop structure (Mankertz et al., 2004).

Since the initial identification of PCV2 (Ellis et al., 1998), several genotypes have now been described (Jantafong et al., 2011; Wang et al., 2009) and demonstrated to co-exist in pigs (Allan et al., 2012; Zhai et al., 2011). For a single-stranded DNA virus, PCV2 has been shown to have the highest DNA mutation rate that is comparable to single-stranded RNA viruses (Firth et al., 2009). In contrast, the non-pathogenic PCV1 has been demonstrated to have a low mutation rate and low genetic diversity worldwide (Cortey and Segalés, 2012; Tombácz et al., 2014). While PCV2 is highly prevalent in most swine-producing countries and is associated with clinical PCVAD, PCV1 is non-pathogenic and has a low prevalence in swine herds (Allan et al., 1994; Allan et al., 1995; Calsamiglia et al., 2002; Dulac and Afshar, 1989; Edwards and Sands, 1994; Kim and Chae, 2001; Krakowka et al., 2000; Magar et al., 2000; Tischer et al., 1986). A recent survey demonstrated that, while PCV2 DNA and PCV2-specific antibodies are present in more than 80% of the samples evaluated, the molecular and serological prevalence of PCV1 is less than 2.4% (Puvanendiran et al., 2011).

We previously demonstrated that a genetically modified infectious PCV2 can tolerate up to a 27 aa insertion in the C-terminus of the ORF2 capsid gene (Beach et al., 2011). We showed that insertion of single, dimeric, and trimeric hemagglutinin (HA) tags, a GLu-GLu epitope tag of a mouse polyomavirus, and the KT3 epitope tag of the simian virus 40 in the C-terminus of PCV2 capsid gene resulted in infectious chimeric viruses that induce both PCV2-neutralizing antibodies and anti-epitope tag antibodies (Beach et al., 2011). Another study reported that insertion of a VP1 epitope region (aa 141–160, LTNVRGDLQVLAQKAARPLP) of the foot and mouth disease virus (FMDV) in PCV2 produced infectious virus *in vitro* and in a mouse model, and the PCV2-FMDV chimera elicited dual immunity against PCV2 and FMDV (Huang et al., 2014). We recently demonstrated that chimeric PCV1-2a vaccine can tolerate the insertion of

93 linear PRRSV epitope and induce dual immunity again PCV2 and PRRSV as a potential bivalent
94 vaccine (Pineyro et al., 2015)

95 Because of the low prevalence of PCV1 in swine herds, the non-pathogenic nature, the
96 low mutation rate, and the systemic tropisms of PCV1 for multiple tissues and organs, it is
97 logical to explore the potential use of PCV1 as a vaccine delivery virus vector. Therefore, in this
98 study, as a proof-of-principle, we evaluated whether PCV1 can express known antigenic
99 determinants of porcine reproductive and respiratory syndrome virus (PRRSV), an
100 economically-important swine pathogen. Generation of chimeric viruses containing neutralizing
101 antigenic epitopes of PRRSV in the backbone of the non-pathogenic PCV1 could potentially
102 elicit protective immunity against PRRSV with the benefit of a live virus-vectored vaccine, but
103 without the risk of pathogenicity or reversion to virulence often associated with the traditional
104 modified live-attenuated vaccines.

105

106 **2. Materials and methods**

107 **2.1. Construction of chimeric PCV1-PRRSV$_{EPI}$ (epitope) infectious clones:**

108 Four different known antigenic epitopes derived from PRRSV strain VR2385, including
109 GP2 epitope II (aa 40–51, ASPSHVGWWSFA), GP3 epitope I (aa 61–72, QAAAEAYEPGRS),
110 GP5 epitope I (aa 35–46, SSSNLQLIYNLT), and GP5 epitope IV (aa 187–200,
111 TPVTRVSAEQWGRP), were each cloned individually in frame into the C-terminus of the
112 PCV1 capsid gene (GenBank accession number GU799575). Chimeric viruses were constructed
113 by overlapping extension and fusion PCR following a method previously described (Beach et al.,
114 2011). Briefly, a pCR2.1-PCV1 infectious clone plasmid containing the full-length PCV1
115 genome was used as the template to generate two amplicons of 200 bp and 1,800 bp with

complementary overhangs containing individual PRRSV antigenic epitope sequences (**Table 1**). A second round of fusion PCR was performed to assemble the previously synthesized amplicons. The PCR product was digested with *Kpn*I and inserted into the pCR2.1TOPO vector (Invitrogen) (**Fig. 1**). Recombinant plasmids containing the insert were transformed into the alpha-select strain of *E. coli* (Bioline). Positive clones were selected, and insertion of each specific PRRSV epitope was confirmed by DNA sequencing. The viral genomic DNA was excised from the plasmid by enzymatic digestion with *KpnI* and concatemerization was carried out through a ligation reaction with T4 DNA ligase (Invitrogen), overnight, at room temperature. The infectious chimeric virus was generated by transfection of the concatemerized genomic DNA into the PCV1-free PK15 cells at 30%–40% confluency with lipofectamine ltx (Invitrogen). After 72 h post-transfection, the infectious virus was harvested by three cycles of freezing and thawing of the cells.

2.2. *In vitro* infectivity, epitope expression, and titration of chimeric PCV1-PRRSV$_{EPI}$ viruses:

PCV1-free PK-15 cells were seeded at a concentration of 2×10^5 cells/well in a 48-well plate. After reaching approximately 40%–50% confluency, cells were washed once with Hank's Balanced Salt Solution (Gibco). The cells were then incubated with 100 µL of 1:10 serial dilution of the virus stock for 1 h at 37°C in 5% CO_2, after which the cells were washed once with 200 µL of minimum essential media (MEM) (Gibco). Infection was carried out in 300 µL MEM supplemented with 10% FBS (Invitrogen) and 1% antibiotic/antimycotic (Fisher) at 37°C in 5% CO_2. After 72 h post-infection, the cells were fixed with 80% acetone and the infectivity was assessed by an immunofluorescence assay (IFA).

Briefly, infected cells were incubated with 100 µL of mouse anti-PCV1-Cap monoclonal antibody, followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG (KPL, Kirkegaard & Perry Laboratories, Inc.). Expression of PRRSV-specific antigenic epitopes was also confirmed by IFA using custom polyclonal rabbit antibodies (Biomatik) against each synthetic PRRSV epitope followed by secondary goat anti-rabbit IgG (DyLight 550). Cells positive for both PCV1 Cap and PRRSV epitopes were visualized using a Zeiss LSM 880 confocal microscope (Zeiss, Pleasanton, CA). Serial ten-fold dilutions of the virus stock were performed in order to determine the 50% tissue culture infectious dose ($TCID_{50}$) of the virus stocks according to the method described by of Reed and Muench (Reed and Muench, 1938).

2.3. *In vivo* characterization of the infectivity and immunogenicity of four PCV1-$PRRSV_{EPI}$ chimeric viruses

2.3.1. Experimental design for the animal study

A total of 21 5-weeks-old specific-pathogen-free (SPF) pigs were randomly assigned into seven groups of three pigs each, including two positive control groups (PCV1 and PRRSV), a negative control (MEM-treated group), and four groups for each of the PCV1-$PRRSV_{EPI}$ chimeric viruses. Pigs in each of the PCV1-$PRRSV_{EPI}$ chimeric virus groups and the PCV1-positive control group were intramuscularly inoculated with 5 mL (4.64×10^2 $TCDI_{50}$/mL) of the respective viruses. Pigs in the PRRSV-VR2385-positive control group were each inoculated with 5 mL (2×10^5 $TCDI_{50}$/mL) of PRRSV-VR2385. Serum samples were collected from each pig prior to inoculation and weekly thereafter for a period of 7 weeks.

2.3.2. Quantification of viral DNA loads in sera and lung tissues

The viral DNA was extracted from serum samples at 0, 7, 14, 21, 28, 35, and 42 days post-inoculation (dpi) and from tissues (lung and tracheobronchial lymph node) at 42 dpi, using Ambion MagMAX-96 Viral DNA Isolation kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The DNA standards used for the qPCR were virus stock used for inoculation as well as plasmids containing the full-length PCV1 infectious clone. In order to rule out potential cross-contamination, DNA extracted from PCV2a and PCV2b virus stocks, and empty pCR2.1TOPO vector (Invitrogen), were included as the negative control. The PCV1 DNA copy numbers in sera or tissues were quantified by a TaqMan® Fast Virus 1-Step Master Mix (Life Technologies Corp.) according to the manufacturer's protocol. The PCR primers and probes (PCV1 P9/PCV1 P10/PCV1 probe) (**Table 1**) used in the qPCR assay were designed to target a specific amplicon of 97 bp in ORF2. The qPCR assay was conducted using the ABI 7500 (RT) PCR system (Life Technologies Corp). The PCR conditions included denaturation at 95°C for 20 s, annealing at 95°C for 3 s, amplification at 60°C for 30 s, and a final extension at 72°C for 5 min, with a total number of 40 cycles. Each reaction was performed in triplicate.

2.3.3. Serological evaluation of anti-PCV1 antibodies and anti-$PRRS_{EPI}$ antibodies:

Specific antibodies against PCV1-Cap were tested by an indirect immunofluorescence assay (IIFA). For the IIFA, the PCV1-free PK-15 cells were inoculated with 100 μL of PCV1 and incubated for 72 h at 37°C, and fixed in 80% acetone. Sera from pigs in each of the chimeric PCV1-$PRRSV_{EPI}$-infected groups as well as from pigs in both the positive and negative control groups were serially diluted and incubated for 1 h at 37°C. Cells were washed three times with PBS, followed by addition of 100 μL (1:100) of fluorescent-labeled secondary anti-swine IgG

antibody (KPL, Kirkegaard & Perry Laboratories, Inc.). The cells were then washed again, and positive cells were detected using a fluorescence microscope. The virus titer was defined as the highest positive dilution and expressed as a mean geometric titer. The anti-PRRSV N antibody response was also evaluated using the IDEXX HerdCheck X3 ELISA kit according to the manufacturer's instructions.

Four different PRRSV KLH-conjugated synthetic antigenic peptides (GP2 epitope II: aa 40–51, ASPSHVGWWSFA; GP3 epitope I: aa 61–72, QAAAEAYEPGRS; GP5 epitope I: aa 35–46, SSSNLQLIYNLT; and GP5 epitope IV: aa 187–200, TPVTRVSAEQWGRP) were used as the antigen for the four PRRSV peptide-based antibody ELISAs. Each vial of lyophilized peptide (5 mg) was resuspended in 1 mL of UltraPure™ Distilled Water (Gibco®, Life Technologies) to a final stock concentration of 5 mg/mL, aliquotted, and stored at –80°C. Following titration and optimal dilution, 96-well microtitration plates (Nunc, Thermo Fisher Scientific) were manually coated with 100 µL per well of each peptide at a concentration of 5 µg/mL in phosphate-buffered saline (PBS) at pH 7.4 (Gibco®, Life Technologies) and incubated at 4°C overnight. The ELISA conditions, including coating and blocking, buffers, sample and conjugate dilutions, and incubation conditions (time and temperature), were identical for the four different peptide-based ELISAs. Serum samples were diluted at 1:50, after which plates were loaded with 100 µL of the diluted sample per well. Plates were incubated at 37°C for 1 h and washed five times with PBS containing 0.1% Tween 20. Subsequently, 100 µL of peroxidase-conjugated goat anti-pig IgG (Fc) antibody (Bethyl Laboratories Inc.), diluted at 1:15,000, were added to each well and the plates were incubated at 37°C for 1 h. After a washing step, the reaction was visualized by adding 100 µL of tetramethylbenzidine-hydrogen peroxide (Dako North America, Inc.) substrate solution to each well. After 10 min incubation at room

temperature, the reaction was stopped by the addition of 50 μL of a stop solution (1 M sulfuric acid) to each well. Reactions were measured according to the optical density at 450 nm using an ELISA plate reader (Biotek® Instruments Inc.) operated with commercial software (GEN5TM, Biotek® Instruments Inc.).

2.3.4. Serum virus neutralization assay to evaluate the neutralizing activity against PRRSV VR2385:

The neutralizing antibody titers against PRRSV-VR2385 were determined by a serum virus neutralization assay essentially as previously described (Zhou et al., 2012). Briefly, two-fold diluted serum samples collected at 28, 35, and 42 dpi from each pig were mixed with an equal volume of the PRRSV VR2385 virus at an infectious titer of 2×10^3 $TCID_{50}$/mL and incubated at 37°C for 1 h. The mixtures were then inoculated onto MARC-145 cells in 96-well plates and incubated for 1 h at 37°C. After washing with PBS, the cells were maintained in DMEM with 2% FBS. At approximately 20 hpi, the cells were assayed by IFA for virus infection. The neutralizing antibody titers were expressed as the highest dilution that showed a 90% or above reduction in the number of fluorescent foci compared to that of antisera from negative control pigs. Samples were evaluated in triplicate and three independent tests were performed for each serum sample.

2.4. Statistical analysis

The Student's *t*-test (unpaired) was used to evaluate the differences ($P < 0.05$) between the samples in the two groups. Repeated measure two-way ANOVA with Tukey's correction was calculated for multiple comparison. Statistical significance was set to alpha = 0.05. All analyses

were performed using commercially available software GraphPad Prism® 6 (GraphPad Software, Inc, CA).

3. Results

3.1. Chimeric PCV1 viruses containing PRRSV VR2385 antigenic epitopes inserted in the C-terminus of the PCV1 capsid are infectious *in vitro*:

Each of the chimeric PCV1-PRRS$_{EPI}$ clones was verified by full-length genomic sequencing for the presence in frame of each of the inserted PRRSV antigenic epitopes in the C-terminus of the PCV1 capsid gene. Transfection of each full-length chimeric virus DNA clone in PK-15 cells resulted in the production of infectious virions. Confocal microscopy revealed that each of the PCV1-PRRS$_{EPI}$ chimeric viruses expressed PCV1 Cap as well as the respective PRRSV antigenic determinant (**Fig. 2**). Infected cells showed dual nuclear staining with both anti-PCV1 monoclonal antibodies and anti-PRRSV epitope peptides (GP2 II, GP3I, GP5I, and GP5IV) mono-specific antibodies. There was no significant difference in the genomic copy numbers between wild-type PCV1 and PCV1-PRRS$_{EPI}$ chimeric viruses at 96 hpi (data not shown). The stability of chimeric viruses was confirmed after five successful serial passages in PK-15 followed by dual IFA staining of the PCV1 capsid and respective PRRSV antigenic determinant, as well as by sequence confirmation of the chimeric viruses harvested after the five passages (data not shown).

3.2. PCV1-VR2385$_{EPI}$ chimeric viruses are viremic and replicate in tissues of experimentally inoculated pigs:

All serum samples, evaluated by TaqMan® qPCR for the presence of PCV1 prior to inoculation at day 0, were negative. Viremia was detected at as early as 7 dpi for PCV1-

253 VR2385$_{EPI}$GP3I and PCV1-VR2385$_{EPI}$GP5IV viruses, and at 21 dpi for PCV1-VR2385$_{EPI}$GP2II
254 and PCV1-VR2385$_{EPI}$GP5I viruses (**Table 2**). The frequency of pigs showing viremia in each
255 group varied during the trial. The number of animals used and the frequency variability do not
256 allow for robust statistical analysis; however, the average DNA viral loads in serum samples
257 from each chimeric virus were within one $\log_{10}$ difference: 1.39×10^5 genomic copies/mL for
258 PCV1-VR2385$_{EPI}$GP2II; 1.87×10^5 genomic copies/mL for PCV1-VR2385$_{EPI}$GP3I; 4.22×10^5
259 genomic copies/mL for PCV1-VR2385$_{EPI}$GP5I; and 1.96×10^5 genomic copies/mL for PCV1-
260 VR2385$_{EPI}$GP5IV, and had at least two-$\log_{10}$ lower genomic copies/mL than the parental PCV1
261 (1.26×10^7 genomic copies/mL) (**Fig. 3A**). After 42 dpi, all animals were necropsied and no
262 significant pathological lesions were observed. However, all infected groups had detectable viral
263 DNA in the tracheobronchial lymph nodes and lungs (**Table 2**), indicating virus replication in
264 tissues. No significant differences in viral genomic copy number/gram of tissue were observed
265 between parental PCV1 and PCV1-VR2385$_{EPI}$ chimeric viruses in tracheobronchial lymph nodes
266 and lungs (**Fig. 3B**). No evidence of PCV1 replication was observed in the PRRSV-VR2385 and
267 MEM control groups.
268
269 **3.3. PCV1-VR2385$_{EPI}$ chimeric viruses induce both PCV1-specific and PRRSV antigenic**
270 **epitope-specific antibodies in pigs:**
271 Anti-PCV1 IgG antibodies were detected in the sera of the wild-type PCV1 control group
272 as well as all the PCV1-VR2385$_{EPI}$ chimeric viruses-inoculated groups. IgG anti-PCV1
273 antibodies were detected from 14 dpi in wild-type PCV1-infected pigs and remained seropositive
274 at 42 dpi. Anti-PCV1 IgG antibodies were detected in PCV1-VR2385$_{EPI}$GP3I- and PCV1-
275 VR2385$_{EPI}$GP5IV-infected groups at 14, 21, and 28 dpi, followed by a significant titer reduction

276 compared to the wild-type PCV1 at 35 and 42 dpi. The remaining chimeric viruses-infected
277 groups, PCV1-VR2385$_{EPI}$GP2II and PCV1-VR2385$_{EPI}$GP5I, showed a delayed seroconversion to
278 anti-PCV1 IgG antibodies (35 dpi) and significantly lower levels of anti-PCV1 IgG antibodies,
279 compared to the wild-type PCV1-infected group, at 35 and 42 dpi (**Fig. 4A**). Anti-PCV1 IgG
280 antibodies were not detected in PRRSV or MEM control groups. As expected, anti-PRRSV N
281 antibodies were only detected in the PRRSV 2385 infected group (**Fig. 4B**).

282 Antibody responses against the inserted PRRSV antigenic epitopes were detected by
283 specific epitope-based ELISA. Antibody response against PRRSV-GP2 epitope II was not
284 detected in pigs experimentally infected with the PCV1-VR2385$_{EPI}$GP2II chimeric virus (**Fig.**
285 **5A**). However, specific antibodies against PRRSV-GP3 epitope I were detected in the PCV1-
286 VR2385$_{EPI}$GP3I chimeric virus group at 28 dpi and remained positive at 42 dpi (**Fig. 5B**).
287 Specific antibodies against PRRSV-GP5 epitope I were detected at 21 dpi in the wild-type
288 PRRSV VR2385-infected group, and at 35 dpi in the PCV1-VR2385$_{EPI}$GP5I chimeric virus-
289 infected group at 35 dpi, and remained positive at 42 dpi (**Fig. 5C**). The presence of anti-
290 PRRSV-GP5 epitope IV antibodies was detected at 21 dpi in the PCV1-VR2385$_{EPI}$GP5IV
291 chimeric virus group and at 28 dpi in wild-type PRRSV VR2385 group, and remained at a high
292 level in the PCV1-VR2385$_{EPI}$GP5IV chimeric virus group at 35 and 42 dpi (**Fig. 5D**). The low
293 number of animals per group, as well as individual variation amongst animals may play a role in
294 the different levels of antibodies response observed amongst groups.
295

3.4. PCV1-PRRSV$_{EPI}$ chimeric viruses-infected pigs develop neutralizing antibodies against the PRRSV VR2385:

To investigate whether PCV1-VR2385$_{EPI}$ chimeric viruses can induce neutralizing antibodies against PRRSV, a serum virus neutralization assay against PRRSV VR2385 strain was performed. Anti-PRRSV-VR2385 neutralizing antibodies were detected in the PCV1-VR2385$_{EPI}$GP3I, PCV1-VR2385$_{EPI}$GP5I, and PCV1-VR2385$_{EPI}$GP5IV chimeric viruses-infected groups at 28 dpi and remained detectable at 42 dpi. No statistical difference in neutralizing antibody titers were observed throughout the experiment between wild-type PRRSV VR2385 and PCV1-VR2385$_{EPI}$GP3I, PCV1-VR2385$_{EPI}$GP5I, and PCV1-VR2385$_{EPI}$GP5IV, except for PCV1-VR2385$_{EPI}$GP5I at 42 dpi (**Fig. 6**). PRRSV 2385 neutralizing antibodies were not detected in PCV1 or MEM control groups.

4. Discussion

PCV1 is a non-pathogenic virus in pigs infecting multiple tissues and organs (Allan et al., 1995; Calsamiglia et al., 2002; Krakowka et al., 2000). Early field studies reported a high serological prevalence of anti-PCV1 antibodies in the swine population (Dulac and Afshar, 1989; Edwards and Sands, 1994; Tischer et al., 1995), although no disease could be associated with the presence of this virus either naturally or experimentally (Allan et al., 1995; Tischer et al., 1986). However, more recent field studies have demonstrated that the serological prevalence of anti-PCV1 antibodies as well as virus circulation in the swine population are very low (Puvanendiran et al., 2011). Sequence and phylogenetic analyses have also demonstrated a low mutation rate and low genetic diversity of the PCV1 strains worldwide (Tombácz et al., 2014). Thus, the low prevalence of PCV1, lack of evidence of pathogenicity, low mutation rate, and systemic tropisms

for multiple tissues and organs make PCV1 an attractive candidate for a potential live vaccine vector.

Previous studies have successfully used the non-pathogenic PCV1 as the genomic backbone for the development of PCV2 vaccines (Fenaux et al., 2004). Cloning of PCV2 ORF2 capsid gene into the backbone of PCV1 proved to be viable *in vivo* and conferred full protection against PCV2, whilst still retaining the non-pathogenic nature of PCV1 (Fenaux et al., 2004; Fenaux et al., 2003). Additionally, pigs experimentally infected with a PCV2-1 reciprocal chimeric virus, containing PCV1 ORF2 in a PCV2 backbone, showed specific anti-PCV1 IgG antibody response (Fenaux et al., 2003). Herein in this study, we further demonstrated that insertion of known PRRSV antigenic determinants in the C-terminus of PCV1 ORF2 capsid gene produced infectious chimeric viruses and did not impair the humoral immune response against PCV1. Although different levels of anti-PCV1 IgG were detected, all PCV1-VR2385$_{EPI}$ chimeric viruses were capable of induce anti-PCV1 IgG antibodies in infected SPF pigs.

The different levels of anti-PCV1 IgG antibodies might be associated with a different replication timeline and viral DNA load observed amongst the various PCV1-VR2385$_{EPI}$ chimeric viruses compared with wild-type PCV1. The extension of the C-terminus of the PCV1 capsid gene through the addition of PRRSV antigenic epitopes might also affect the structural conformation and antibody induction capability of the PCV1 capsid. Indeed, previous studies demonstrated that mutation of the last four amino acid residues of the PCV2 ORF2 (-PLKP) to three amino acid residues of the PCV1 ORF2 (-LNK) reduces viral antibody recognition (Lekcharoensuk et al., 2004). Therefore, in the current study, anti-PCV1 IgG antibodies generated against PCV1-VR2385$_{EPI}$ chimeric viruses may not completely bind to the full PCV1 Cap expressed by the wild-type PCV1 that was used for the IIFA serology test. Further studies

342 will be necessary to demonstrate whether the insertion of a foreign amino acid sequence at the C-
343 terminus may alter the conformation of the PCV1 capsid protein.

344 It has been previously demonstrated that the C-terminus of the PCV2 capsid is a type-
345 specific immune reactive epitope that is displayed on the surface of the virion capsid
346 (Lekcharoensuk et al., 2004; Shang et al., 2009). In two separate studies, following the insertion
347 of epitope tags in the C-terminal region, PCV2 and chimeric PCV1-2a vaccine were generated
348 and shown to elicit dual immunity against both PCV2 capsid and the inserted epitopes, whereas
349 mutation of the N-terminus of PCV2 capsid impaired viral replication (Beach et al., 2011;
350 Pineyro et al., 2015). Herein in the present study, we inserted four different known PRRSV
351 antigenic epitopes into the C-terminus of the non-pathogenic PCV1 capsid gene, and
352 demonstrated that the insertions did not significantly affect virus infectivity *in vitro* or viral
353 replication *in vivo*. We previously showed that epitopes as large as 27 amino acids can be
354 inserted in the PCV2 capsid gene without impairing viral viability (Beach et al., 2011). In the
355 present study, we demonstrated that insertions, varying from 12 aa for PRRSV-GP3 epitope I
356 and PRRSV-GP5 epitope I, 12 aa for PRRSV-GP2 epitope II, and 14 aa for PRRSV-GP5 epitope
357 IV, did not affect viral infectivity *in vitro* or replication *in vivo*. Future study is necessary to
358 determine the tolerance of maximal length of amino acid insertion in PCV1 capsid without
359 affecting the viability of the virus.

360 Although the PCV1 chimeric viruses generated in this study were capable of replicating
361 *in vivo*, the rate of replication appears to be low. It has previously been demonstrated that PCV1
362 can replicate to a higher titer *in vitro*, compared to PCV2 (Beach et al., 2010a). However, to our
363 knowledge, there is no information regarding the minimal infectious dose required for PCV1
364 infection *in vivo*. Previous studies using PCV1-2 chimeric virus in the backbone of PCV1

365 showed positive results with variable titers (Beach et al., 2010b; Fenaux et al., 2004; Pineyro et
366 al., 2015). We therefore speculate that the low replication rate of the chimeric viruses observed
367 in this study might be due to a low virus titer that was used for inoculation. Propagation of
368 PCV1, PCV2 as well as chimeric viruses to higher infectious titers have been very challenging
369 thus far.

370 In the current study, PCV1 chimeric viruses expressing four known B-cell linear epitopes
371 of PRRSV, previously demonstrated to be immunogenic against PRRSV (de Lima et al., 2006;
372 Plagemann, 2004b; Vanhee et al., 2011), were generated. It has been reported that GP5 plays a
373 major role in PRRSV neutralization (Plagemann, 2004a; Plagemann, 2004b; Plagemann et al.,
374 2002). PRRSV-GP5 epitope IV is an important immunogenic epitope (P^{188}LTR (V/T)
375 SAEQW^{197}) that has also been proved to be reactive with sera raised against European PRRSV
376 strains. Despite a few amino acid changes, this epitope seems to be well conserved amongst type
377 2 PRRSV strains (Oleksiewicz et al., 2001). We showed in this study that PCV1-
378 VR2385$_{EPI}$GP5IV chimeric virus induced neutralizing antibody levels comparable to those
379 induced by the PRRSV VR2385 virus. The PRRSV-GP5 epitope I neutralizing epitope,
380 ^{37}SHLQLIYNL, for the PRRSV VR2332 is located in the GP5 ectodomain sequence and is
381 considered as the primary neutralizing epitope for the type 2 PRRSV isolates (Plagemann,
382 2004b). The PCV1-VR2385$_{EPI}$GP5I chimeric virus in this study induced similar neutralizing
383 antibody levels compared to those induced by PRRSV VR2385 virus at 28, and 35 dpi. However,
384 the neutralizing antibody titers induced by the PCV1-PRRSV$_{EPI}$ viruses appeared to decline more
385 rapidly compared to the PRRSV VR2385, thus causing a significant reduction of neutralizing
386 antibodies titer at 42 dpi.

PRRSV ORF3 is considered as the second most variable PRRSV structural protein, with four consecutive peptides from aa 61–105, all of which are considered as important immunodominant domains of GP3 (de Lima et al., 2006; Zhou et al., 2006). The PCV1-VR2385$_{EPI}$GP3I chimeric virus generated in this study contains the aa 61–72 (QAAAEAYEPGRS) and was shown to induce similar levels of neutralizing antibodies compared to the PRRSV VR2385. Despite the fact that PCV1-VR2385EPIGP2II chimeric virus is infectious *in vitro*, no viral DNA were detected in tissues of inoculated pigs, probably due to the short duration of viremia not being detectable with the current sampling scheme. This explanation was also supported by the presence of anti-PCV1 IgG at 14 dpi, indicative of virus replication. Furthermore, the chimeric virus also failed to induce antibodies against PRRSV-GP2 epitope II. Therefore, we have successfully demonstrated that three of the four PCV1-VR2385$_{EPI}$ chimeric viruses generated in this study induced PRRSV epitope-specific antibodies and neutralizing antibodies against PRRSV VR2385 at a level comparable to those induced by wild-type PRRSV VR2385.

In summary, we successfully generated and rescued four PCV1 chimeric viruses expressing different known PRRSV linear-B epitopes (GP2 epitope II: aa 40–51, ASPSHVGWWSFA; GP3 epitope I: aa 61–72, QAAAEAYEPGRS; GP5 epitope I: aa 35–46, SSSNLQLIYNLT; and GP5 epitope IV: aa 187–200, TPVTRVSAEQWGRP). We further showed that three of these chimeric viruses were infectious *in vitro* and in pigs, and genetically stable. Importantly, we found that three PCV1-VR2385$_{EPI}$ chimeric viruses elicited neutralizing antibodies against PRRSV-VR2385. Therefore, the results from the present study provided a proof of concept for further exploring the use of the non-pathogenic PCV1 as a live virus vector for vaccine delivery.

Acknowledgements

We would like to thank Dr. Gordon Allan and Dr. John Ellis for providing the PCV1 monoclonal antibody, and Dr Nathan Beach for the construction of the original PCV1 infectious clone. We also would like to thanks Sarah Abate, Dr. Phillip Gauger, and Dr Karen Harmon for assistance with molecular diagnosis, and Nicholas Catanzaro for assistance with confocal microscopy. The authors also thank Diane McDonald for assistance and coordination of the animal work. The project is funded by Virginia Tech internal funds.

Conflict of interest statement:

The corresponding author is the inventor of the chimeric PCV1-2a virus which is the basis for the Zoetis Inc's Fostera® PCV commercial vaccine. There is no other apparent conflict of interest for the authors.

5. References

Allan, G., Krakowka, S., Ellis, J., Charreyre, C., 2012. Discovery and evolving history of two genetically related but phenotypically different viruses, porcine circoviruses 1 and 2. Virus Res. 164(1–2), 4-9.

Allan, G., Meehan, B., Todd, D., Kennedy, S., McNeilly, F., Ellis, J., Clark, E.G., Harding, J., Espuna, E., Botner, A., Charreyre, C., 1998. Novel porcine circoviruses from pigs with wasting disease syndromes. Vet. Rec. 142(17), 467-468.

Allan, G.M., Kennedy, S., McNeilly, F., Foster, J.C., Ellis, J.A., Krakowka, S.J., Meehan, B.M., Adair, B.M., 1999. Experimental reproduction of severe wasting disease by co-infection of pigs with porcine circovirus and porcine parvovirus. J. Comp. Pathol. 121(1), 1-11.

Allan, G.M., Mackie, D.P., McNair, J., Adair, B.M., McNulty, M.S., 1994. Production, preliminary characterisation and applications of monoclonal antibodies to porcine circovirus. Vet Immunol Immunop 43(4), 357-371.

Allan, G.M., McNeilly, F., Cassidy, J.P., Reilly, G.A.C., Adair, B., Ellis, W.A., McNulty, M.S., 1995. Pathogenesis of porcine circovirus; experimental infections of colostrum deprived piglets and examination of pig foetal material. Vet. Microbiol. 44(1), 49-64.

Beach, N.M., Juhan, N.M., Cordoba, L., Meng, X.J., 2010a. Replacement of the Replication Factors of Porcine Circovirus (PCV) Type 2 with Those of PCV Type 1 Greatly Enhances Viral Replication In Vitro. J. Virol. 84(17), 8986-8989.

Beach, N.M., Ramamoorthy, S., Opriessnig, T., Wu, S.Q., Meng, X.-J., 2010b. Novel chimeric porcine circovirus (PCV) with the capsid gene of the emerging PCV2b subtype cloned in the genomic backbone of the non-pathogenic PCV1 is attenuated in vivo and induces

445 protective and cross-protective immunity against PCV2b and PCV2a subtypes in pigs.
446 Vaccine 29(2), 221-232.
447 Beach, N.M., Smith, S.M., Ramamoorthy, S., Meng, X.-J., 2011. Chimeric Porcine Circoviruses
448 (PCV) Containing Amino Acid Epitope Tags in the C Terminus of the Capsid Gene Are
449 Infectious and Elicit both Anti-Epitope Tag Antibodies and Anti-PCV Type 2
450 Neutralizing Antibodies in Pigs. J. Virol. 85(9), 4591-4595.
451 Calsamiglia, M., Segalés, J., Quintana, J., Rosell, C., Domingo, M., 2002. Detection of Porcine
452 Circovirus Types 1 and 2 in Serum and Tissue Samples of Pigs with and without
453 Postweaning Multisystemic Wasting Syndrome. J. Clin. Microbiol. 40(5), 1848-1850.
454 Chae, C., 2005. A review of porcine circovirus 2-associated syndromes and diseases. Vet. J.
455 169(3), 326-336.
456 Cortey, M., Segalés, J., 2012. Low levels of diversity among genomes of Porcine circovirus type
457 1 (PCV1) points to differential adaptive selection between Porcine circoviruses. Virology
458 422(2), 161-164.
459 de Lima, M., Pattnaik, A.K., Flores, E.F., Osorio, F.A., 2006. Serologic marker candidates
460 identified among B-cell linear epitopes of Nsp2 and structural proteins of a North
461 American strain of porcine reproductive and respiratory syndrome virus. Virology
462 353(2), 410-421.
463 Dulac, G.C., Afshar, A., 1989. Porcine circovirus antigens in PK-15 cell line (ATCC CCL-33)
464 and evidence of antibodies to circovirus in Canadian pigs. Can. J. Vet. Res. 53(4), 431-
465 433.
466 Edwards, S., Sands, J.J., 1994. Evidence of circovirus infection in British pigs. Vet. Rec.
467 134(26), 680-681.

Ellis, J., Hassard, L., Clark, E., Harding, J., Allan, G., Willson, P., Strokappe, J., Martin, K., McNeilly, F., Meehan, B., Todd, D., Haines, D., 1998. Isolation of circovirus from lesions of pigs with postweaning multisystemic wasting syndrome. Can. Vet. J. 39(1), 44-51.

Fenaux, M., Opriessnig, T., Halbur, P.G., Elvinger, F., Meng, X.J., 2004. A Chimeric Porcine Circovirus (PCV) with the Immunogenic Capsid Gene of the Pathogenic PCV Type 2 (PCV2) Cloned into the Genomic Backbone of the Nonpathogenic PCV1 Induces Protective Immunity against PCV2 Infection in Pigs. J. Virol. 78(12), 6297-6303.

Fenaux, M., Opriessnig, T., Halbur, P.G., Meng, X.J., 2003. Immunogenicity and Pathogenicity of Chimeric Infectious DNA Clones of Pathogenic Porcine Circovirus Type 2 (PCV2) and Nonpathogenic PCV1 in Weanling Pigs. J. Virol. 77(20), 11232-11243.

Finsterbusch, T., Mankertz, A., 2009. Porcine circoviruses—Small but powerful. Virus Res. 143(2), 177-183.

Firth, C., Charleston, M.A., Duffy, S., Shapiro, B., Holmes, E.C., 2009. Insights into the Evolutionary History of an Emerging Livestock Pathogen: Porcine Circovirus 2. J. Virol. 83(24), 12813-12821.

Hamel, A.L., Lin, L.L., Nayar, G.P., 1998. Nucleotide sequence of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. J. Virol. 72(6), 5262-5267.

Huang, L., Zhang, F., Tang, Q., Wei, Y., Wu, H., Guo, L., Fu, Y., Liu, C., 2014. A recombinant porcine circovirus type 2 expressing the VP1 epitope of the type O foot-and-mouth disease virus is infectious and induce both PCV2 and VP1 epitope antibodies. Appl. Microbiol. Biotechnol. 98(22), 9339-9350.

490 Jantafong, T., Boonsoongnern, A., Poolperm, P., Urairong, K., Lekcharoensuk, C.,
491 Lekcharoensuk, P., 2011. Genetic characterization of porcine circovirus type 2 in piglets
492 from PMWS-affected and -negative farms in Thailand. Virol J. 8(1), 88.
493 Kim, J., Chae, C., 2001. Differentiation of porcine circovirus 1 and 2 in formalin-fixed, paraffin-
494 wax-embedded tissues from pigs with postweaning multisystemic wasting syndrome by
495 in-situ hybridisation. Res. Vet. Sci. 70(3), 265-269.
496 Krakowka, S., Ellis, J.A., Meehan, B., Kennedy, S., McNeilly, F., Allan, G., 2000. Viral Wasting
497 Syndrome of Swine: Experimental Reproduction of Postweaning Multisystemic Wasting
498 Syndrome in Gnotobiotic Swine by Coinfection with Porcine Circovirus 2 and Porcine
499 Parvovirus. Vet. Pathol. 37(3), 254-263.
500 Lekcharoensuk, P., Morozov, I., Paul, P.S., Thangthumniyom, N., Wajjawalku, W., Meng, X.J.,
501 2004. Epitope Mapping of the Major Capsid Protein of Type 2 Porcine Circovirus
502 (PCV2) by Using Chimeric PCV1 and PCV2. J. Virol. 78(15), 8135-8145.
503 Magar, R., Muller, P., Larochelle, R., 2000. Retrospective serological survey of antibodies to
504 porcine circovirus type 1 and type 2. Can. Vet. J. 64(3), 184-186.
505 Mahé, D., Blanchard, P., Truong, C., Arnauld, C., Le Cann, P., Cariolet, R., Madec, F., Albina,
506 E., Jestin, A., 2000. Differential recognition of ORF2 protein from type 1 and type 2
507 porcine circoviruses and identification of immunorelevant epitopes. J. Gen. Virol. 81(7),
508 1815-1824.
509 Mankertz, A., Çaliskan, R., Hattermann, K., Hillenbrand, B., Kurzendoerfer, P., Mueller, B.,
510 Schmitt, C., Steinfeldt, T., Finsterbusch, T., 2004. Molecular biology of Porcine
511 circovirus: analyses of gene expression and viral replication. Vet. Microbiol. 98(2), 81-
512 88.

513 Oleksiewicz, M.B., Bøtner, A., Toft, P., Normann, P., Storgaard, T., 2001. Epitope Mapping
514 Porcine Reproductive and Respiratory Syndrome Virus by Phage Display: the nsp2
515 Fragment of the Replicase Polyprotein Contains a Cluster of B-Cell Epitopes. J. Virol.
516 75(7), 3277-3290.
517 Pineyro, P.E., Kenney, S.P., Gimenez-Lirola, L.G., Heffron, C.L., Matzinger, S.R., Opriessnig,
518 T., Meng, X.J., 2015. Expression of antigenic epitopes of porcine reproductive and
519 respiratory syndrome virus (PRRSV) in a modified live-attenuated porcine circovirus
520 type 2 (PCV2) vaccine virus (PCV1-2a) as a potential bivalent vaccine against both
521 PCV2 and PRRSV. Virus Res. 210, 154-164.
522 Plagemann, P.G.W., 2004a. GP5 ectodomain epitope of porcine reproductive and respiratory
523 syndrome virus, strain Lelystad virus. Virus Res. 102(2), 225-230.
524 Plagemann, P.G.W., 2004b. The primary GP5 neutralization epitope of North American isolates
525 of porcine reproductive and respiratory syndrome virus. Vet Immunol Immunop 102(3),
526 263-275.
527 Plagemann, P.G.W., Rowland, R.R.R., Faaberg, K.S., 2002. The primary neutralization epitope
528 of porcine respiratory and reproductive syndrome virus strain VR-2332 is located in the
529 middle of the GP5 ectodomain. Arch. Virol. 147(12), 2327-2347.
530 Puvanendiran, S., Stone, S., Yu, W., Johnson, C.R., Abrahante, J., Jimenez, L.G., Griggs, T.,
531 Haley, C., Wagner, B., Murtaugh, M.P., 2011. Absence of porcine circovirus type 1
532 (PCV1) and high prevalence of PCV 2 exposure and infection in swine finisher herds.
533 Virus Res. 157(1), 92-98.
534 Reed, L.J., Muench, H., 1938. A simple method of estimating fifty per cent endpoints. Am. J.
535 Epidemiol. 27(3), 493-497.

536 Shang, S.-B., Jin, Y.-L., Jiang, X.-t., Zhou, J.-Y., Zhang, X., Xing, G., He, J.L., Yan, Y., 2009.
537 Fine mapping of antigenic epitopes on capsid proteins of porcine circovirus, and
538 antigenic phenotype of porcine circovirus Type 2. Mol. Immunol. 46(3), 327-334.
539 Tischer, I., Bode, L., Peters, D., Pociuli, S., Germann, B., 1995. Distribution of antibodies to
540 porcine circovirus in swine populations of different breeding farms. Arch. Virol. 140(4),
541 737-743.
542 Tischer, I., Gelderblom, H., Vettermann, W., Koch, M.A., 1982. A very small porcine virus with
543 circular single-stranded DNA. Nature 295(5844), 64-66.
544 Tischer, I., Mields, W., Wolff, D., Vagt, M., Griem, W., 1986. Studies on epidemiology and
545 pathogenicity of porcine circovirus. Arch. Virol. 91(3-4), 271-276.
546 Tombácz, K., Patterson, R., Grierson, S.S., Werling, D., 2014. Lack of Genetic Diversity in
547 Newly Sequenced Porcine Circovirus Type 1 Strains Isolated 20 Years Apart. Genome
548 Announc 2(2).
549 Trible, B.R., Rowland, R.R.R., 2012. Genetic variation of porcine circovirus type 2 (PCV2) and
550 its relevance to vaccination, pathogenesis and diagnosis. Virus Res. 164(1–2), 68-77.
551 Vanhee, M., Van Breedam, W., Costers, S., Geldhof, M., Noppe, Y., Nauwynck, H., 2011.
552 Characterization of antigenic regions in the porcine reproductive and respiratory
553 syndrome virus by the use of peptide-specific serum antibodies. Vaccine 29(29–30),
554 4794-4804.
555 Wang, F., Guo, X., Ge, X., Wang, Z., Chen, Y., Cha, Z., Yang, H., 2009. Genetic variation
556 analysis of Chinese strains of porcine circovirus type 2. Virus Res. 145(1), 151-156.

557 Zhai, S.-L., Chen, S.-N., Wei, Z.-Z., Zhang, J.-W., Huang, L., Lin, T., Yue, C., Ran, D.-L.,
558 Yuan, S.-S., Wei, W.-K., Long, J.-X., 2011. Co-existence of multiple strains of porcine
559 circovirus type 2 in the same pig from China. Virol J. 8(1), 517.
560 Zhou, L., Ni, Y.-Y., Piñeyro, P., Sanford, B.J., Cossaboom, C.M., Dryman, B.A., Huang, Y.-W.,
561 Cao, D.-J., Meng, X.-J., 2012. DNA shuffling of the GP3 genes of porcine reproductive
562 and respiratory syndrome virus (PRRSV) produces a chimeric virus with an improved
563 cross-neutralizing ability against a heterologous PRRSV strain. Virology 434(1), 96-109.
564 Zhou, Y.-J., An, T.-Q., He, Y.-X., Liu, J.-X., Qiu, H.-J., Wang, Y.-F., Tong, G., 2006. Antigenic
565 structure analysis of glycosylated protein 3 of porcine reproductive and respiratory
566 syndrome virus. Virus Res. 118(1–2), 98-104.

567

568 **Figure legends**

569 **Fig. 1. A schematic diagram for the construction of the PCV1-PRRSV$_{EPI}$ chimeric DNA**
570 **clones**. The epitope insertion was accomplished by two rounds of overlapping extension PCR.
571 The first amplicon of 200 bp containing an overhanging GPxx$_{EPI}$ region (xx denote different
572 inserted epitopes, GP2II, G3I, GP5I and GP5IV) was generated with M13-F and GPxx$_{EPI}$-R
573 primers (black arrows). The second amplicon of 1778 bp containing a complementary GPxx$_{EPI}$
574 overhanging region was generated with GPxx$_{EPI}$-F and M13-R primers (empty arrow heads). The
575 full-length PCV1-PRRSV epitopes chimeric clones were assembled by a fusion PCR using
576 previously generated amplicons as templates, and with M13-F and M13-R primers.

577

578 **Fig. 2. Confocal microscopy of PK-15 cells infected with wild-type PCV1 as well as with**
579 **four different PCV1-PRRSV$_{EPI}$ chimeric viruses.** PCV1-PRRSV$_{EPI}$ chimeric viruses (PCV1-
580 VR2385$_{EPI}$GP2II, PCV1-VR2385$_{EPI}$GP3I, PCV1-VR2385$_{EPI}$GP5I, and PCV1-VR2385$_{EPI}$GP5IV)
581 and wild-type PCV1 were assayed by dual immunofluorescence staining. Infected cells were
582 dually labeled with a mixture of mouse anti-PCV1 capsid monoclonal antibody (1:1000) (Mab)
583 and PRRSV epitope-specific polyclonal antibodies (1:500) (Pab). In order to determine cross
584 reactivity, cells infected with each specific chimeric virus group were tested against each
585 respective PRRSV-specific epitope antibody. After incubation with the primary antibody, a
586 mixture of fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG (1:100) (KPL,
587 Kirkegaard & Perry Laboratories, Inc.) and goat anti-rabbit IgG-DyLight (1:500) (Thermo
588 Scientific) were added. Dually infected cells were visualized using a Zeiss LSM 880 confocal
589 microscope (Zeiss, Pleasanton, CA) with a 40X objective, using the argon 488 and helium-neon
590 594 lasers.

591 **Fig. 3. Detection and quantification of PCV1 viral DNA loads in serum, lymphoid tissues**
592 **and lung samples in specific-pathogen-free (SPF) pigs experimentally infected with PCV1-**
593 **PRRSV$_{EPI}$ chimeric viruses**. Pigs were experimentally infected with wild-type PCV1 as well as
594 each of the four different PCV1-PRRSV$_{EPI}$ chimeric viruses (PCV1-VR2385$_{EPI}$GP2II, PCV1-
595 VR2385$_{EPI}$GP3I, PCV1-VR2385$_{EPI}$GP5I, and PCV1-VR2385$_{EPI}$GP5IV). Determination of viral
596 DNA loads in serum and tissues was performed using TaqMan® qPCR. The number of animals
597 used and the frequency variability in each time point, do not allow for robust statistical analysis.
598 **(A)** Group mean log viral genomic copies/ml of serum is plotted for each treatment group, and
599 the error bars indicate standard errors. **(B)** Mean viral DNA loads in tracheobronchial lymph
600 node and lung were determined for each treatment group. Mean log viral genomic copies/gram
601 of tissue is plotted for each treatment group, and the error bars indicate standard errors.
602
603 **Fig. 4. Anti-PCV1 IgG antibodies and anti-PRRSV N antibodies in specific-pathogen-free**
604 **(SPF) pigs experimentally infected with PCV1-PRRSV$_{EPI}$ chimeric viruses.** Pigs were
605 infected with wild-type PCV1 as well as with each of the four different PCV1-PRRSV$_{EPI}$
606 chimeric viruses. **(A)** Anti-PCV1 IgG antibodies were detected by an indirect
607 immunofluorescence assay (IIFA). PCV1-free PK15 cells were infected with 1 MOI of wild-type
608 PCV1. Immunoreactivity against PCV1 was evaluated in serum samples generated in pigs
609 infected by wild-type PCV1, PCV1-PRRSV$_{EPI}$ chimeric viruses, PRRSV-VR2385 and MEM
610 control. Detectable anti-PCV1 IgG antibodies were seen as early as 14 dpi in wild-type PCV1,
611 PCV1-VR2385$_{EPI}$GP3I, and PCV1-VR2385$_{EPI}$GP5IV, followed by PCV1-VR2385$_{EPI}$GP2II, and
612 PCV1-VR2385$_{EPI}$GP5I at 21 dpi. Treatments with different letters represent statistically
613 significant differences on that day. **(B)** The anti-PRRSV N antibody titers at indicated time

614 points were detected using the IDEXX HerdCheck X3 ELISA kit. The level of antibody was
615 expressed as a sample/positive (S/P) value ratio. The dash line shows the cutoff threshold (S/P
616 value ≥0.4). Each plot represents the mean value of 3 pigs per infected group at each time point.
617 Statistical comparison was performed using repeated-measures analysis of variance, followed by
618 Tukey's post-hoc procedure for multiple comparisons. Statistical significance was set to alpha =
619 0.05.
620
621 **Fig. 5. PRRSV antigenic epitope-specific ELISAs for detection of the inserted PRRSV**
622 **epitope antibodies induced by PCV1-PRRSV$_{EPI}$ chimeric viruses.** Specific-pathogen-free
623 pigs were infected with wild-type PCV1, as well as with each of the four PCV1-PRRSV$_{EPI}$
624 chimeric viruses containing respective PRRSV antigenic epitopes. PRRSV epitope-specific
625 antibody responses were tested in serum samples of pigs infected with PCV1-VR2385$_{EPI}$GP2II,
626 PCV1-VR2385$_{EPI}$GP3I, PCV1-VR2385$_{EPI}$GP5I, and PCV1-VR2385$_{EPI}$GP5IV, wild-type PCV1,
627 and PRRSV VR2385. All infected groups were tested individually against each epitope peptide.
628 **(A)** PCV1-VR2385$_{EPI}$GP2II; **(B)** PCV1-VR2385$_{EPI}$GP3I; **(C)** PCV1-VR2385$_{EPI}$GP5I; and **(D)**
629 PCV1-VR2385$_{EPI}$GP5IV. The average of three animals is plotted for each time point, and
630 standard errors are indicated. Asterisks indicate significant differences on that day for each of the
631 PCV1-PRRSV$_{EPI}$ chimeric viruses compared to PRRSV VR2385 infected group. The dotted
632 horizontal line indicates the cutoff of each assay. Statistical comparison was performed using
633 two-way ANOVA followed Tukey's correction for multiple comparison. Statistical significance
634 was set to alpha = 0.05.
635

Fig. 6. Kinetics of anti-PRRSV neutralizing antibody response in pigs experimentally infected with each of the four PCV1-VR2385$_{EPI}$ chimeric viruses as well as with the PRRSV VR2385 virus. Neutralizing antibody (NA) titers induced against the PRRSV VR2385 by each of the four PCV1-VR2385$_{EPI}$ chimeric viruses as well as by the parental PRRSV VR2385 virus were detected as early as 28 dpi. NA antibodies titers observed in the PCV1-VR2385$_{EPI}$GP3I, PCV1-VR2385$_{EPI}$GP5I, and PCV1-VR2385$_{EPI}$GP5IV groups against PRRSV VR2385 were comparable to those observed in the PRRSV VR2385-infected group at 28 and 35 dpi. At 42 dpi, the NA antibodies titers in PCV1-VR2385$_{EPI}$GP5IV group were significantly lower than those observed in the PRRSV-VR2385-infected group. The NA titers against parental strain PRRSV VR2385 and each of the PCV1-VR2385$_{EPI}$ chimeric viruses were expressed as the highest dilution (2n) that showed a 90% or above reduction in the number of fluorescent foci compared to that of serum from negative control group. The NA titers against PCV1-VR2385$_{EPI}$ GP2 II was not shown because of undetectable NA titer. Three independent experiments were performed for each test, and the error bars indicate standard errors. The P value shows whether one chimeric virus group had significant differences in NA titers compared to the parental PRRSV VR2385 group.

Table 1. Primer sequences used in the construction and detection of the PCV1-PRRSV$_{EPI}$ chimeric viruses

Chimeric clone	Primer	Position	Primer sequence (5'-3')
PCV1-PRRSV$_{EPI}$GP2II	GP2II$_{EPI}$-P1	F47-59	CGCAAAGCTCCACCAGCCCACATGGCTCGGGCTCGCaggtctttagg
	GP2II$_{EPI}$-P2	R29-46	GCGAGCCCGAGCCATGTGGGCTGGTGGAGCTTTGCGctaaatgaataaaaataa
PCV1-PRRSV$_{EPI}$GP3I	GP3I$_{EPI}$-P3	F47-59	GCTACGGCCCGGTTCATACGCTTCCGCCGCCGCCTGaggtctttagg
	GP3I$_{EPI}$-P4	R29-46	CAGGCGGCGGCGGAAGCGTATGAACCGGGCCGTAGCctaaatgaataaaaataa
PCV1-PRRSV$_{EPI}$GP5I	GP5I$_{EPI}$-P5	F47-59	GGTCAGGTTATAAATCAGCTGCAGGTTGCTGCTGCTaggtctttagg
	GP5I$_{EPI}$-P6	R29-46	AGCAGCAGCAACCTGCAGCTGATTTATAACCTGACCctaaatgaataaaaataa
PCV1-PRRSV$_{EPI}$GP5IV	GP5IV$_{EPI}$-P7	F47-59	CGGACGGCCCCACTGTTCCGCGCTCACACGGGTCACCGGGGTagggtctttagg
	GP5IV$_{EPI}$-P8	R29-46	ACCCCGGTGACCCGTGTGAGCGCGGAACAGTGGGGCCGTCCG ctaaatgaataaaaataa
pCR2.1 TOPO	M13-R	R207-225	GGAAACAGCTATGACCATG
pCR2.1 TOPO	M13-F	F390-406	ACTGGCCGTCGTTTTAC
PCV1	P9	F505-525	CGATGTTGAATCTGAGGTGGT
PCV1	P10	R581-602	AGAAAGGCGGGAATTGAAGATA
PCV1	Probe	R528-553	ACATTCCAAGATGGCTGCGAGTATCC

Table 2. Detection of viremia and virus replication in tissues of pigs infected by parental PCV1, and each of the four PCV1-PRRSV$_{EPI}$ chimeric viruses

Treatments	No. of pigs with detectable viremia and viral genome in tissues (positive/total no. of pigs)									
	dpi									
	0	7	14	21	28	35	42	Total	Lung	TBLN
PCV1-VR2385$_{EPI}$GP2II	0/3	0/3	0/3	**1/3**	**2/3**	**2/3**	0/3	**2/3**	**1/3**	**1/3**
PCV1-VR2385$_{EPI}$GP3 I	0/3	**1/3**	0/3	0/3	**1/3**	0/3	0/3	**2/3**	**1/3**	**1/3**
PCV1-VR2385$_{EPI}$GP5 I	0/3	0/3	0/3	**2/3**	**1/3**	**3/3**	**1/3**	**3/3**	**1/3**	**2/3**
PCV1-VR2385$_{EPI}$GP5IV	0/3	**1/3**	**1/3**	0/3	**1/3**	**1/3**	0/3	**2/3**	**3/3**	**2/3**
PCV1	0/3	**2/3**	**1/3**	**1/3**	**1/3**	**1/3**	**2/3**	**2/3**	**2/3**	**2/3**
PRRSV-VR2385	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Mock (MEM)	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3

dpi: days post infection, TBLN tracheobronchial lymph nodes

Fig 1

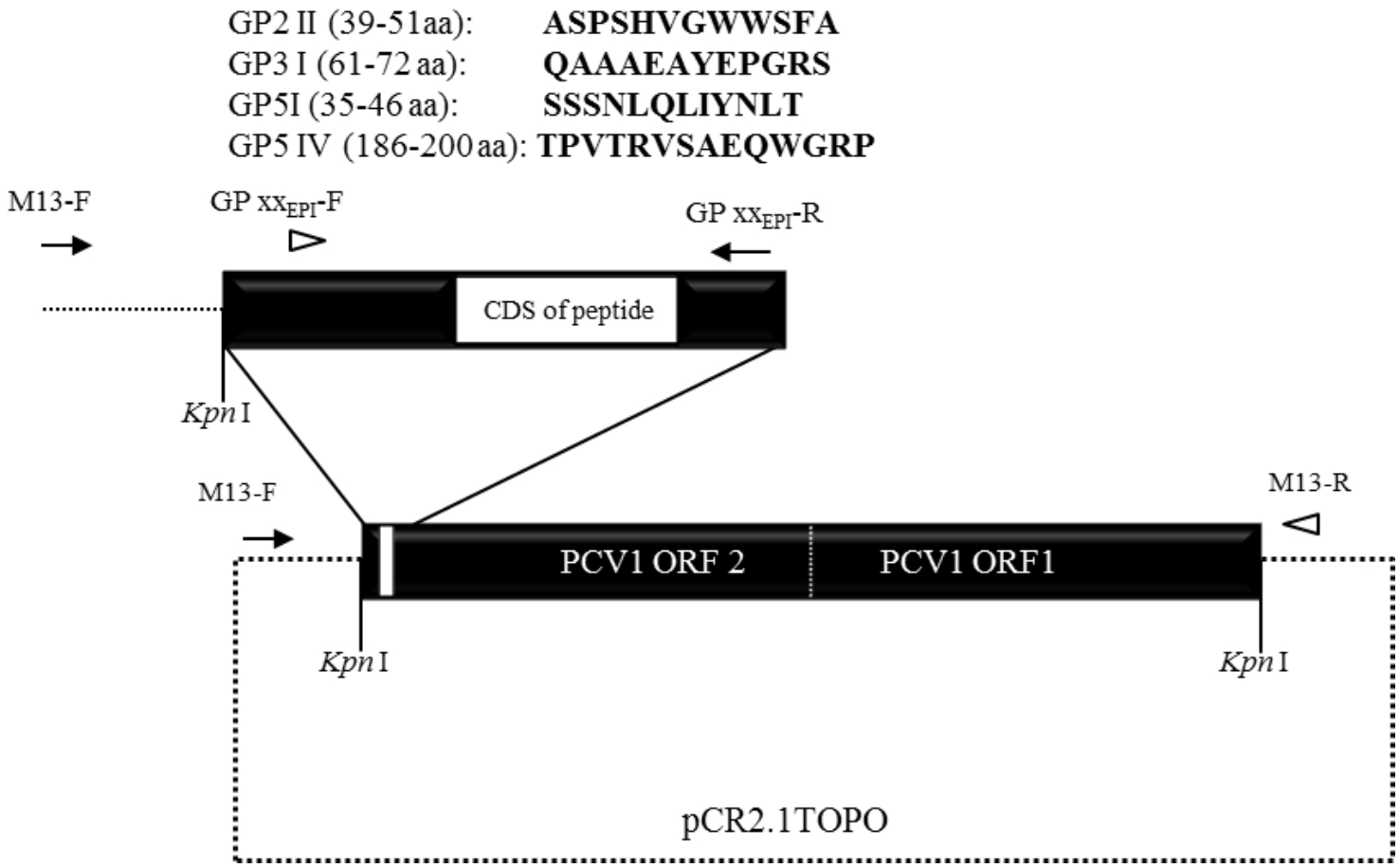

GP2 II (39-51aa): ASPSHVGWWSFA
GP3 I (61-72 aa): QAAAEAYEPGRS
GP5I (35-46 aa): SSSNLQLIYNLT
GP5 IV (186-200 aa): TPVTRVSAEQWGRP
M13-F
GP xx$_{EPI}$-F
GP xx$_{EPI}$-R
CDS of peptide
Kpn I
M13-F
M13-R
PCV1 ORF 2
PCV1 ORF1
Kpn I
Kpn I
pCR2.1TOPO

Fig 2

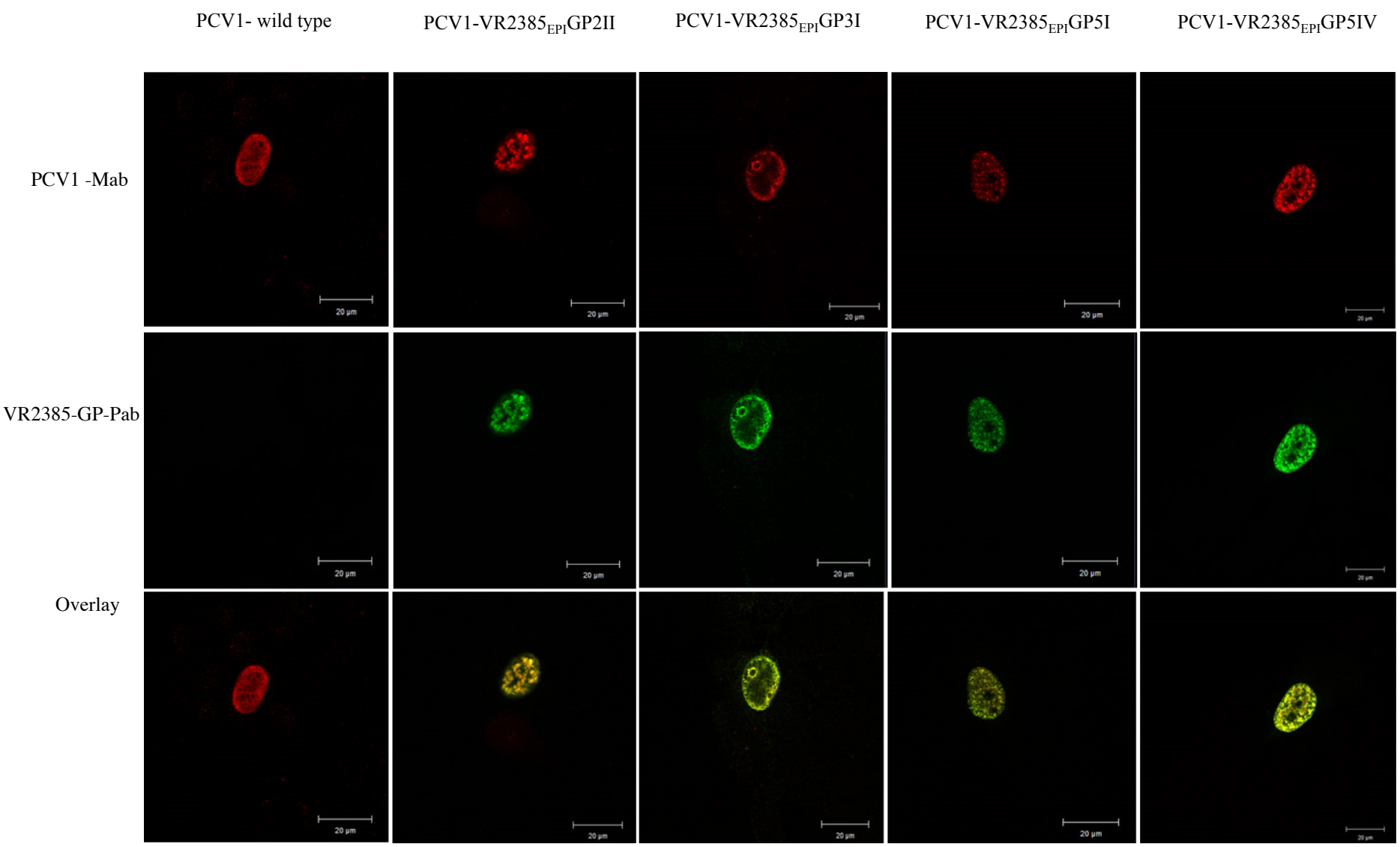

PCV1- wild type
PCV1-VR2385EPIGP2II
PCV1-VR2385EPIGP3I
PCV1-VR2385EPIGP5I
PCV1-VR2385EPIGP5IV
PCV1 -Mab
VR2385-GP-Pab
Overlay
20 µm

Fig 3

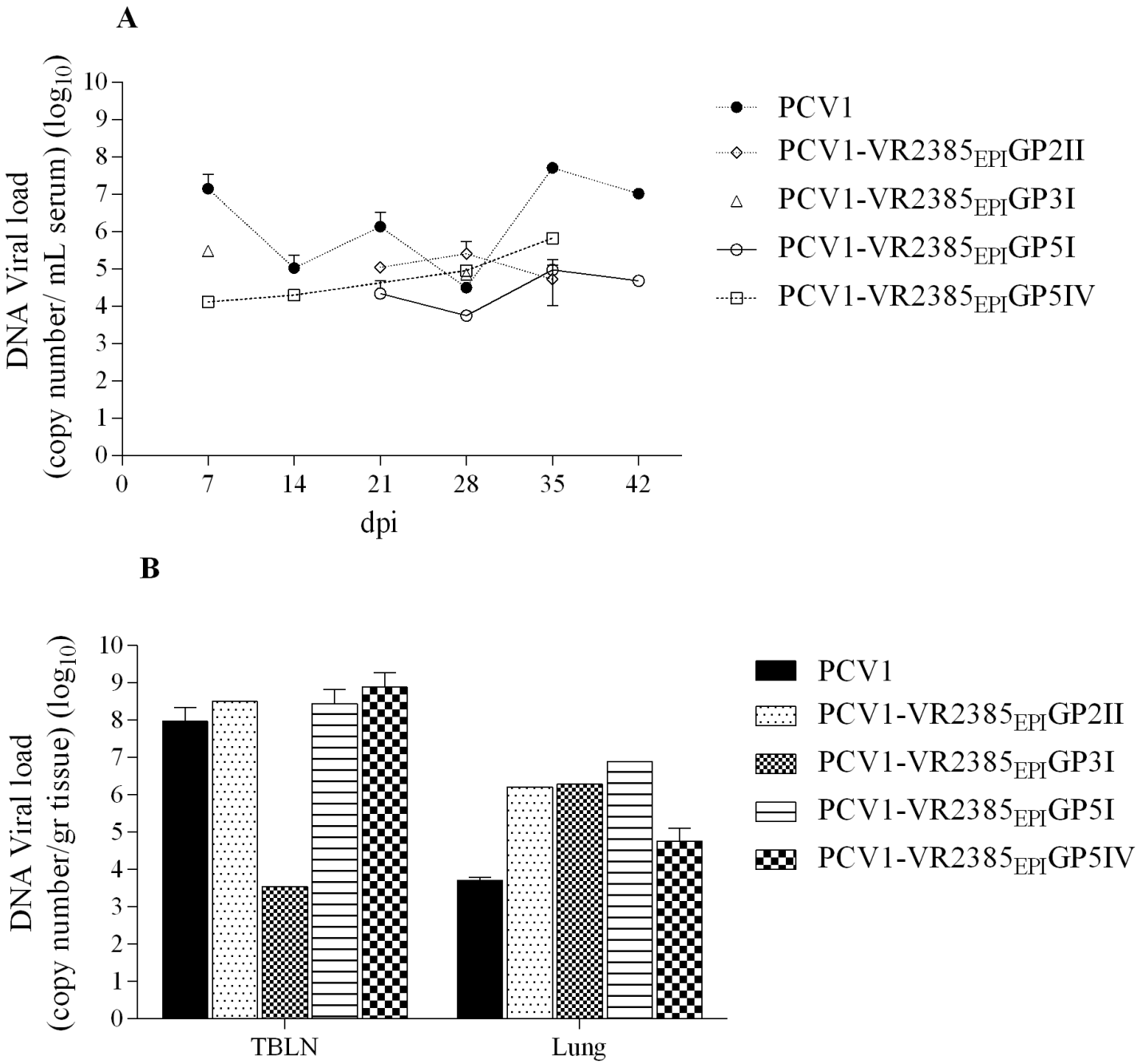

A
DNA Viral load
(copy number/ mL serum) (log10)
10
9
8
7
6
5
4
3
2
1
0
0
7
14
21
28
35
42
dpi
PCV1
PCV1-VR2385EPIGP2II
PCV1-VR2385EPIGP3I
PCV1-VR2385EPIGP5I
PCV1-VR2385EPIGP5IV
B
DNA Viral load
(copy number/gr tissue) (log10)
10
9
8
7
6
5
4
3
2
1
0
TBLN
Lung
PCV1
PCV1-VR2385EPIGP2II
PCV1-VR2385EPIGP3I
PCV1-VR2385EPIGP5I
PCV1-VR2385EPIGP5IV

Fig 4

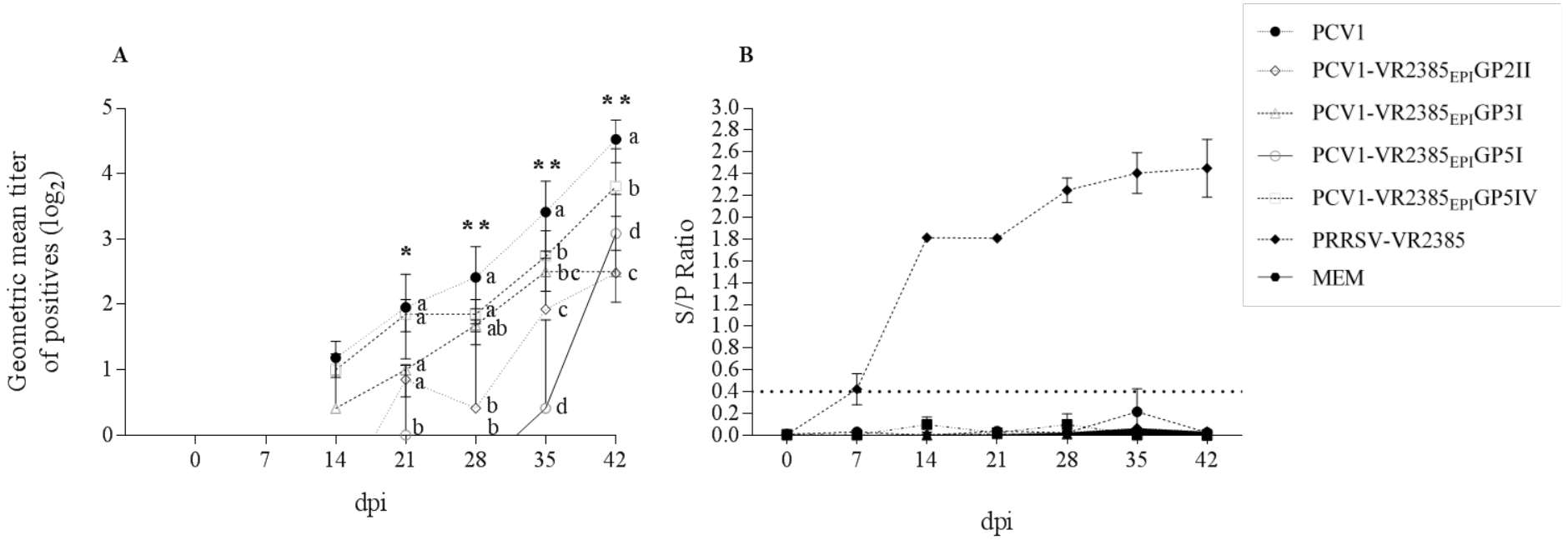

A
Geometric mean titer of positives (log_2)
dpi
B
S/P Ratio
dpi
PCV1
PCV1-VR2385$_{EPI}$GP2II
PCV1-VR2385$_{EPI}$GP3I
PCV1-VR2385$_{EPI}$GP5I
PCV1-VR2385$_{EPI}$GP5IV
PRRSV-VR2385
MEM

Fig 5

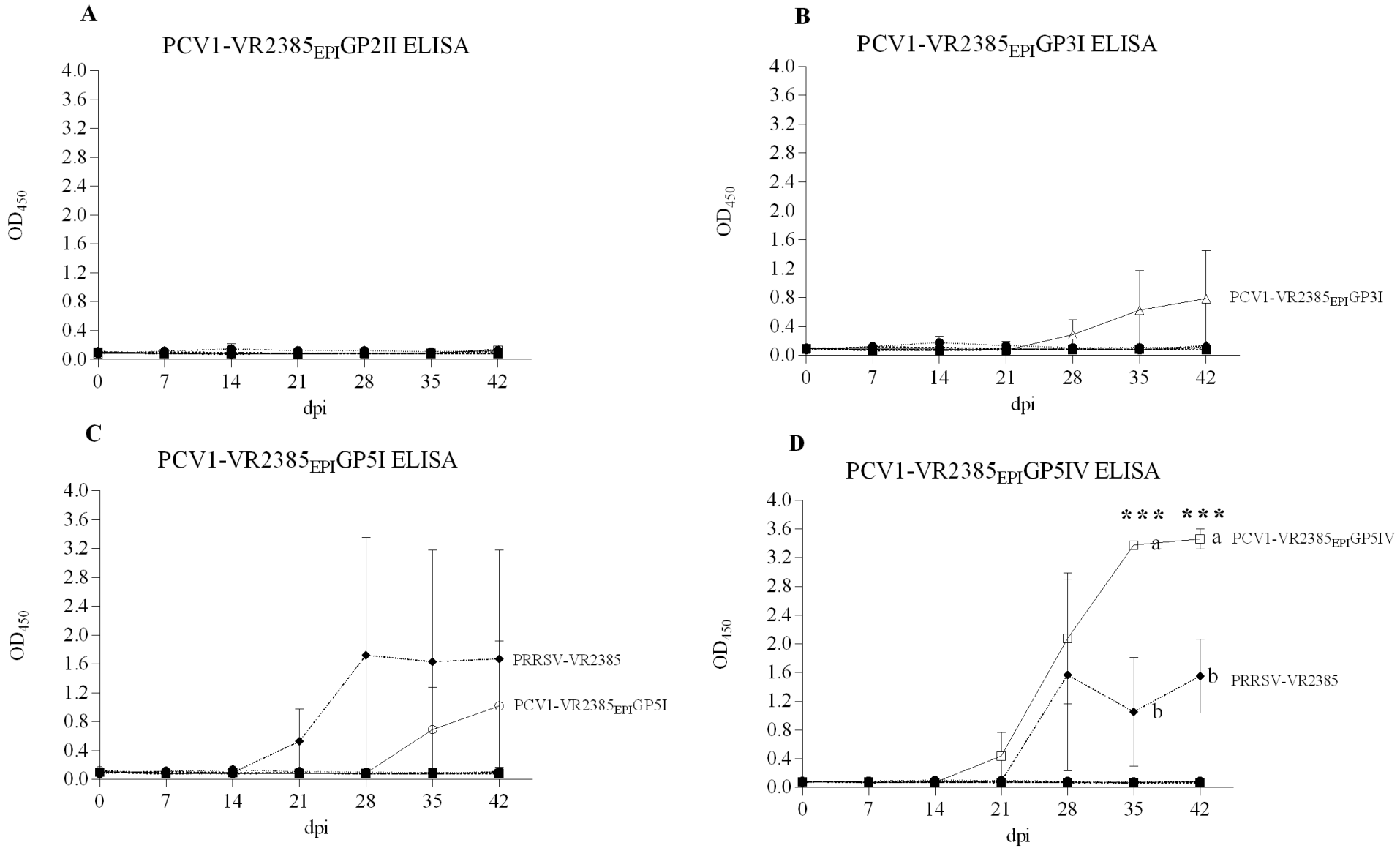
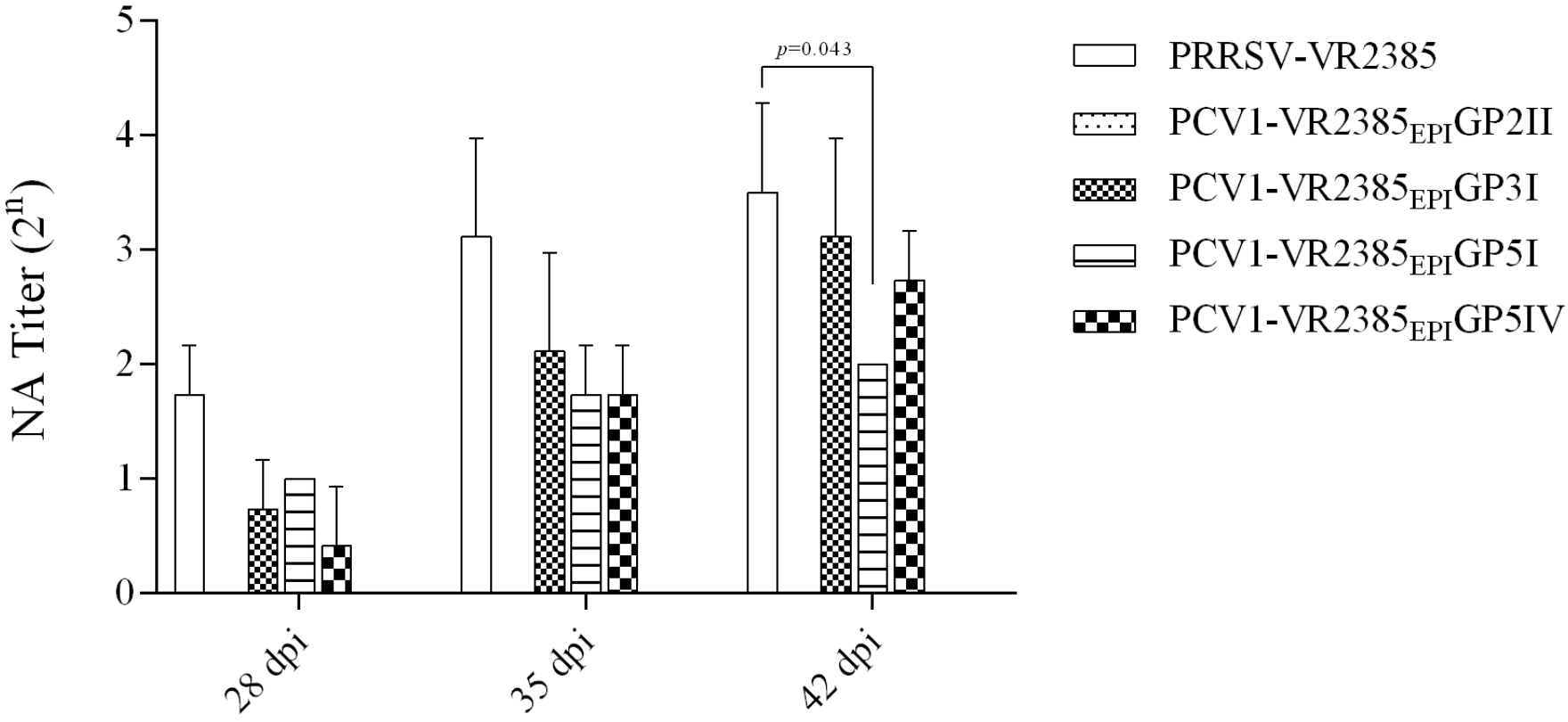

A
PCV1-VR2385EPIGP2II ELISA
OD450
dpi
B
PCV1-VR2385EPIGP3I ELISA
PCV1-VR2385EPIGP3I
C
PCV1-VR2385EPIGP5I ELISA
PRRSV-VR2385
PCV1-VR2385EPIGP5I
D
PCV1-VR2385EPIGP5IV ELISA
* * * * * *
a
b
PCV1-VR2385EPIGP5IV
PRRSV-VR2385

Fig 6


NA Titer (2^n)
5
4
3
2
1
0
p=0.043
28 dpi
35 dpi
42 dpi
PRRSV-VR2385
PCV1-VR2385EPIGP2II
PCV1-VR2385EPIGP3I
PCV1-VR2385EPIGP5I
PCV1-VR2385EPIGP5IV