

6-8-2012

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## Abstract

Porcine circovirus associated disease (PCVAD) encompasses a group of syndromes linked to infection with porcine circovirus type 2 (PCV2). Based on the hypothesis that the immune responses to vaccination versus infection are quantitatively and qualitatively different, the objective of this study was to evaluate immunity, virus replication and disease protection in pigs vaccinated with PCV2 capsid protein (CP) and during infection. The disease model included dual infection with PCV2 and porcine reproductive and respiratory syndrome virus (PRRSV), a virus known to enhance disease progression and severity. The principal effect of PRRSV infection was to increase peak PCV2 viremia by almost 40-fold; however, PCV2 failed to show a reciprocal effect on PRRSV. In vaccinated pigs, there was no evidence of disease or PCV2 replication following dual virus challenge. Immunity following vaccination favored PCV2 neutralizing activity; whereas, PCV2 infection and disease produced high levels of non-neutralizing antibody, primarily directed against a polypeptide in the C-terminal region of CP. These results support the notion that the magnitude of the total antibody response cannot be used as a measure of protective immunity. Furthermore, protection versus disease lies in the immunodominance of specific epitopes. Epitope specificity should be taken into consideration when designing PCV2 vaccines.

## Keywords

PCV2, PRRSV, PCVAD, PCV2 vaccine, Antibody response

## Disciplines

Large or Food Animal and Equine Medicine | Veterinary Infectious Diseases | Veterinary Preventive Medicine, Epidemiology, and Public Health

## Comments

This article is published as Tribble, Benjamin R., Alejandro Ramirez, Andrew Suddith, Alexandra Fuller, Maureen Kerrigan, Richard Hesse, Jerome Nietfeld, Baoqing Guo, Eileen Thacker, and Raymond R.R. Rowland. "Antibody responses following vaccination versus infection in a porcine circovirus-type 2 (PCV2) disease model show distinct differences in virus neutralization and epitope recognition." *Vaccine* 30, no. 27 (2012): 4079-4085. DOI: [10.1016/j.vaccine.2012.04.022](https://doi.org/10.1016/j.vaccine.2012.04.022).

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## Antibody responses following vaccination versus infection in a porcine circovirus-type 2 (PCV2) disease model show distinct differences in virus neutralization and epitope recognition

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### ARTICLE INFO

#### Article history:

Received 17 December 2011

Received in revised form 13 March 2012

Accepted 3 April 2012

Available online 19 April 2012

#### Keywords:

PCV2

PRRSV

PCVAD

PCV2 vaccine

Antibody response

### ABSTRACT

Porcine circovirus associated disease (PCVAD) encompasses a group of syndromes linked to infection with porcine circovirus type 2 (PCV2). Based on the hypothesis that the immune responses to vaccination versus infection are quantitatively and qualitatively different, the objective of this study was to evaluate immunity, virus replication and disease protection in pigs vaccinated with PCV2 capsid protein (CP) and during infection. The disease model included dual infection with PCV2 and porcine reproductive and respiratory syndrome virus (PRRSV), a virus known to enhance disease progression and severity. The principal effect of PRRSV infection was to increase peak PCV2 viremia by almost 40-fold; however, PCV2 failed to show a reciprocal effect on PRRSV. In vaccinated pigs, there was no evidence of disease or PCV2 replication following dual virus challenge. Immunity following vaccination favored PCV2 neutralizing activity; whereas, PCV2 infection and disease produced high levels of non-neutralizing antibody, primarily directed against a polypeptide in the C-terminal region of CP. These results support the notion that the magnitude of the total antibody response cannot be used as a measure of protective immunity. Furthermore, protection versus disease lies in the immunodominance of specific epitopes. Epitope specificity should be taken into consideration when designing PCV2 vaccines.

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### 1. Introduction

First described in Canada in the early 1990s, porcine circovirus-associated disease (PCVAD) has emerged as an economically important disease worldwide [1,2]. A central feature of PCVAD is the involvement of porcine circovirus type 2 (PCV2). PCVAD includes a set of syndromes characterized by a variety of clinical disease signs, which present alone or in combination, including wasting, diarrhea, respiratory distress, dermatitis, and reproductive failure (reviewed in [3–5]). The most common PCV2 syndrome is porcine multi-systemic wasting syndrome (PMWS), which is characterized by wasting, lymphadenopathy, immune suppression, and lymphoid depletion. In 2008, we identified PCV2 as the source of reduced growth in apparently asymptomatic herds [6]. Although PCVAD is generally considered to be slow and progressive, a peracute syndrome, known as acute pulmonary edema (APE), has appeared in

some vaccinated herds [7]. The clinical manifestation and severity of PCVAD are linked to a variety of co-factors, such as the disease potential of the PCV2 isolate [8,9], the presence of pathogenic or opportunistic infections [10], host genetics [11–13], and use of immunostimulating agents, such as vaccines [14,15].

PCV2 is a small DNA virus possessing a 1.7 kb circular, single-stranded genome surrounded by a homopolymer capsid [16]. PCV2 isolates are divided into two main genotypes, known as PCV2a and PCV2b [6,17,18]. A third genotype, designated PCV2c, includes a small group of historical isolates identified in Denmark [19]. The genotypic classification of PCV2 is complicated by the appearance of field isolates possessing both PCV2a and PCV2b sequences [20,21]. The ambisense PCV2 genome is dominated by three open reading frames (ORFs). The 233 amino acid capsid protein (CP) is coded for by ORF2.

Recombinant vaccines containing only PCV2a CP are effective in reducing morbidity and mortality and improve overall growth performance, even in pigs without overt clinical signs [6,22–24]. Even though PCV2-infected pigs produce high levels of CP-specific antibody, the onset and severity of PCVAD is correlated with

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**Table 1**  
Experimental groups and outcomes.

Group	Description	n	Treatment			Mortality <sup>b</sup>	
			Vaccine <sup>a</sup>	PCV2	PRRSV		
1	CN	Control	7	–	–	–	0
2	VX	Vaccine only	7	+	–	–	0
3	PR	PRRSV only	7	–	–	+	0
4	PC	PCV2 only	7	–	+	–	0
5	VX-PC	Vaccine; PCV2	7	+	+	–	0
6	PC-PR	PCV2+PRRSV	7	–	+	+	3 (15 <sup>c</sup> , 24 <sup>d</sup> , 28 <sup>e</sup> )
7	VX-PC-PR	Vaccine; PCV2+PRRSV	7	+	+	+	0

<sup>a</sup> Vaccinated with two doses of a recombinant baculovirus expressed PCV2 capsid protein product (Intervet/Schering-Plough Animal Health).

<sup>b</sup> Mortality = death or euthanasia of moribund pigs. The numbers in parenthesis indicate the day after virus challenge that deaths occurred.

<sup>c</sup> Lungs showed diffuse interstitial inflammation (clinical severity score of 4, on a scale of 1–4). Pathology was similar to PRRS with the likely contribution of a bacterial infection. Lymph nodes were positive for PCV2 IHC staining.

<sup>d</sup> Lungs showed acute edema, hemorrhage and accumulation of macrophages in alveoli. Marked lymphocyte depletion in lymph nodes with scattered multinucleated giant cells. Lymph nodes were positive for PCV2 IHC staining.

<sup>e</sup> Euthanized. Lungs with neutrophilic bronchitis and atelectasis. Positive for PCV2 IHC staining in lymph nodes.

the absence or decreased levels of PCV2 neutralizing antibodies [25–28]. Based on the hypothesis that the immune responses to vaccination versus infection are quantitatively and qualitatively different, the objective of this study was to evaluate immunity, virus replication, and disease protection in pigs vaccinated with PCV2 capsid protein (CP). Experimental challenge models, which incorporate PCV2 alone, produce virus replication, but with only mild or subclinical disease [29–32]. The unique aspect of this study was the incorporation of a PCV2 disease model which included dual infection with a “matched set” of PCV2b and PRRSV isolates, derived from a pig with PMWS.

## 2. Materials and methods

### 2.1. Pigs and experimental challenge

All animal experiments were performed after approval by the Kansas State University and Iowa State University institutional animal use and biosafety committees. PCV2-negative pigs were derived from conventional sows as described in Opriessnig et al. [33]. Briefly, prior to farrowing, sows were selected on the basis of a PCV2 IFA titer less than 320. Approximately two weeks after farrowing, piglets were screened and selected from four different sow litters based on low antibody titers (less than 320 immunofluorescent assay titer) to PCV2 and negative results by PCR for PCV2 DNA in serum. Upon arrival at the challenge facility, the four week-old pigs were confirmed negative for PCV2 and PRRSV by PCR and then assigned to one of seven treatment groups (see Table 1). Groups were balanced according to sex, sow litter, and weight. At five weeks of age (study day 0), groups 2, 5, and 7 were vaccinated according to label instructions using a commercial baculovirus-expressed PCV2 ORF2 product (Intervet/Schering-Plough Animal Health). As per instructions, the second vaccine dose was administered three weeks later. Groups 1, 3, 4, and 6 were left unvaccinated. Two weeks after the second vaccine dose (study day 34), the pigs were challenged with PCV2 alone (groups 4 and 5), PRRSV alone (group 3), PCV2 plus PRRSV (groups 6 and 7) or mock-challenged (groups 1 and 2).

After virus challenge, all pigs were monitored daily for clinical signs and blood samples were collected weekly. Body weights were measured at the time of challenge and at the termination of the study. At 44 days after challenge (study day 78), all pigs were humanely euthanized with an intravenous overdose of sodium pentobarbital. At necropsy, lung, kidney and lymphoid tissues were collected for PCV2 immunohistochemistry (IHC) and histopathology.

### 2.2. Viruses and infection

The PCV2/PRRSV challenge inoculum originated from a pig that succumbed to severe PMWS. Titration of PCV2 was performed on swine testicle (ST) cells [34]. Briefly, serial 10-fold dilutions of lymph node homogenate were plated in quadruplicate onto rapidly dividing ST cells in a 96-well tissue culture plate (Falcon). Dilutions were made in EMEM (Sigma–Aldrich) containing 7% fetal bovine serum (Sigma–Aldrich; FBS) and 50 µg/ml of gentamicin (Lonza; EMEM-FBS-Gent). Following a 3 day incubation at 37 °C and 5% CO<sub>2</sub>, the cells were fixed with acetone and stained with fluorescein isothiocyanate (FITC)-labeled porcine anti-PCV2 (Veterinary Medical Research and Development, Inc.; VMRD). The 50% tissue culture infection dose (TCID<sub>50</sub>) per gram of lymph node homogenate was calculated by the method of Spearman and Karber [35]. The homogenate contained approximately 10<sup>8</sup> TCID<sub>50</sub>/g of PCV2. Sequence analysis showed the isolate to be of the PCV2b genotype (Genbank accession #JQ692110). The homogenate material was tested and confirmed negative for common viruses including influenza and parvovirus, but was positive for PRRSV. PRRSV was recovered from the homogenate by isolation on MARC-145 cells, as previously described [36]. The homogenate was filtered through a 0.22 µm filter (Fisherbrand) to remove bacteria. To prepare a PRRSV challenge stock, the virus was passaged an additional two times on MARC-145 cells and stored at –80 °C. The quantity of PRRSV was 10<sup>7</sup> TCID<sub>50</sub>/ml, as determined by titration on MARC-145 cells. Briefly, serial 1:10 dilutions of virus stock were prepared in EMEM-FBS-Gent and added, in quadruplicate, to confluent MARC-145 cells in a 96 well plate. The cells were incubated for 3 days at 37 °C and 5% CO<sub>2</sub>, then analyzed for virus induced cytopathic effects. The TCID<sub>50</sub>/ml of homogenate was calculated by the method of Spearman and Karber [35].

For challenge with PCV2 alone, the filtered homogenate was heat-treated at 60 °C for 30 min to inactivate PRRSV and other heat-labile viruses. For dual challenge, PRRSV was added back to the heat-treated homogenate material. Pigs were challenged intranasally with 10<sup>5</sup> TCID<sub>50</sub> of PRRSV and/or 10<sup>5</sup> TCID<sub>50</sub> PCV2 in 3 ml of MEM.

### 2.3. Immunohistochemistry for PCV2 antigen

IHC staining for PCV2 antigen was performed on paraffin-embedded tissue sections as routine diagnostic assays performed by personnel within the Kansas State Veterinary Diagnostic Laboratory (KSVDL). Upon collection, tissues were immediately placed in 10% buffered formalin. After processing, paraffin-embedded sections were mounted on slides, deparaffinized and stained using an automated procedure (NexES IHC Staining Module,

Ventana Medical). A rabbit anti-PCV2 polyclonal antibody was used for the detection of PCV2 antigen. Bound rabbit antibody was detected with biotinylated goat anti-rabbit (H+L) IgG (Ventana Medical) followed by avidin-horseradish peroxidase and DAB chromogen (Ventana Medical). Slides were counterstained with hematoxylin.

2.4. Measurement of PCV2 and PRRSV antibody

Antibody assays for detection of total and neutralizing antibody were performed as routine diagnostic assays within the KSVDL. Total PCV2 antibody in serum was measured by indirect fluorescent antibody assay (IFA). Briefly, rapidly dividing ST cells, maintained in EMEM-FBS-Gent on 96 well plates, were infected with a laboratory isolate of PCV2b. Three days later, the plates were fixed for 10 min in 80% acetone. Serum samples were added at an initial dilution of 1:40 followed by serial 1:2 dilutions. Samples were diluted in PBS with 10% FBS (PBS-FBS) and incubated for two hours at room temperature. After washing with PBS, FITC-labeled goat anti-pig (H+L) antibody (Jackson Labs) diluted 1:2000 in PBS-FBS was added to each well. Plates were incubated for two hours at room temperature, washed, and viewed on an inverted fluorescence microscope. The antibody titer for each sample was calculated as the reciprocal of the last serum dilution that exhibited fluorescence staining and reported as  $\log_2(40 \times 1/\text{dilution})$ .

For the measurement of virus neutralizing activity (NA), four replicate (100  $\mu\text{l}$  each) 1:2 serial dilutions of sera were mixed with 100 TCID<sub>50</sub> of PCV2b and incubated for 1 h at 37 °C. Well contents were transferred onto day-old ST cells in 96-well plates, incubated for three days at 37 °C, fixed and stained with undiluted FITC-labeled anti-PCV2 (VMRD). A positive control from a vaccinated pig and negative control from an antibody negative cesarean-derived colostrum-deprived (CDCD) pig were included with each assay. Wells were considered positive for PCV2-specific neutralization if greater than 90% reduction in PCV2-specific fluorescence was detected. The results were reported as the  $\log_2$  NA<sub>50</sub> per ml as determined by the method of Spearman and Karber [35].

PRRSV antibody was measured using a commercially available ELISA (PRRS X3, IDEXX). The results were reported as a sample to positive (S/P) ratio. A S/P ratio greater than 0.39 was considered positive for PRRSV antibody.

PCV2 capsid polypeptide ELISA was performed as previously described [37]. Briefly, CP polypeptides were cloned from a PCV2b isolate (Genbank accession# HQ713495) and expressed in *Escherichia coli*. Ninety-six well ELISA plates (Costar) were coated with 100  $\mu\text{l}$  of purified CP(43-135), CP(160-233), or CP(43-233) at a concentration of 4  $\mu\text{g/ml}$  and incubated overnight at 4 °C. After incubation, plates were washed with PBS containing 0.01% Tween-20 (Sigma-Aldrich; PBST) and blocked for a minimum of 1 h with PBS containing 10% goat serum (Invitrogen; PBS-GS). After blocking, duplicate serum samples diluted in PBS-GS were added to wells and incubated for 2 h at room temperature. Plates were then washed and 100  $\mu\text{l}$  of peroxidase-labeled goat anti-swine antibody (Accurate Chemical & Scientific Corp.) diluted 1:2000 in PBS-GS was added to each well. After incubation at room temperature for one hour, the plate was washed and 100  $\mu\text{l}$  of the chromagenic substrate ABTS (KPL) added to each well. Peroxidase activity was detected by measuring absorbance at 405 nm using a Maxline microplate reader (Molecular Devices Corporation). To compare results across experiments, each ELISA plate included an internal positive control consisting of a high IFA antibody titer serum reacted with the CP(43-233) polypeptide. Results are reported as an antibody binding ratio, which was calculated as the A405 value of the unknown sample minus background divided by the A405 value of the internal positive control minus background.

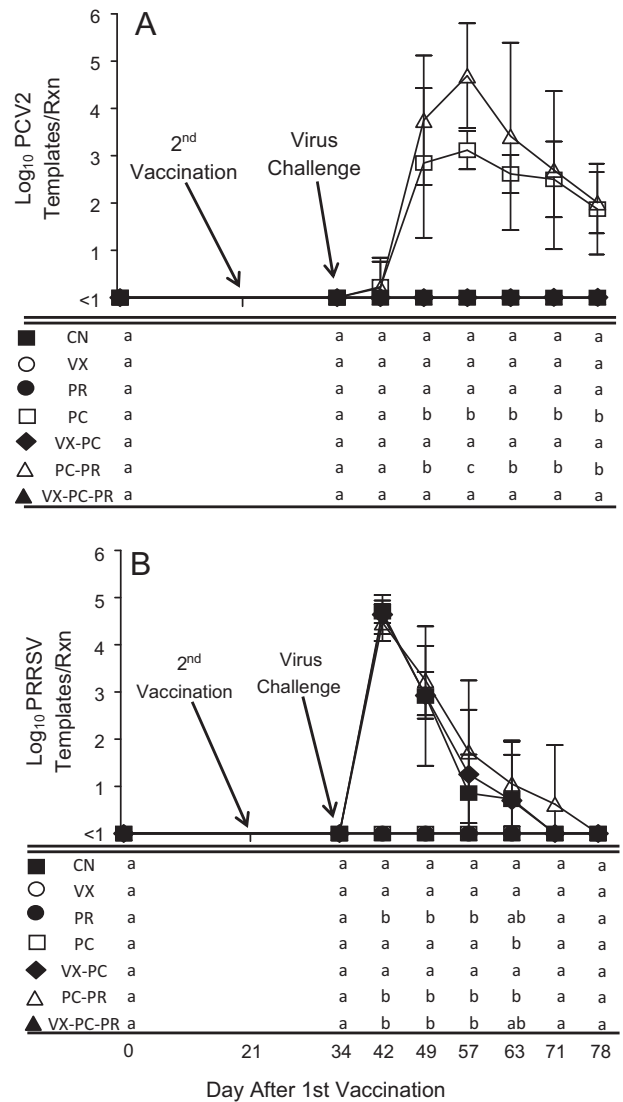
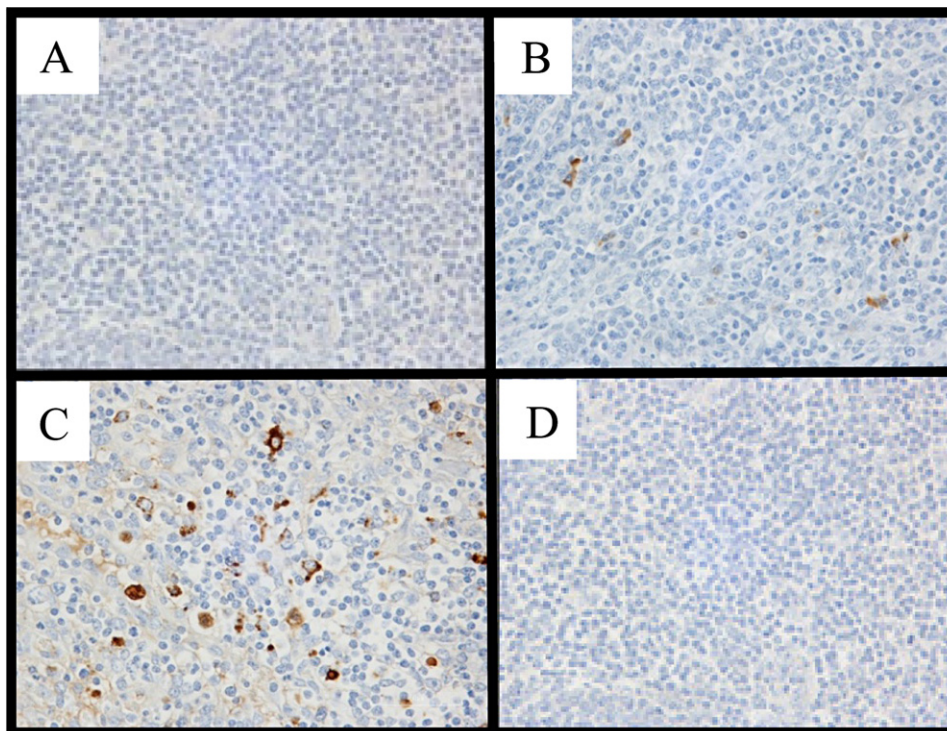


Fig. 1. PCV2 and PRRSV viremia. PCR for PCV2 (A) and PRRSV (B) was performed as described in Materials and methods. Key: Group 1 – CN (closed squares), Group 2 – VX (open circles), Group 3 – PR (closed circles), Group 4 – PC (open squares), Group 5 – VX-PC (closed diamonds), Group 6 – PC-PR (open triangles), Group 7 – VX-PC-PR (closed triangles). Groups with the same letters at specific time points indicate means that are not significantly different ( $P > 0.05$ ).

2.5. PCR for PCV2 and PRRSV nucleic acid

Viremia was measured using semi-quantitative TaqMan PCR assays for PRRSV RNA and PCV2 DNA. PCR assays were performed as routine diagnostic tests by personnel in the KSVDL. For PRRSV, total RNA was isolated from serum using a MagMAX™-96 Viral RNA Isolation Kit (Applied Biosystems) according to the manufacturer's instructions. Master mixes were prepared using the AgPath ID™ NA & EU PRRSV kit (Applied Biosystems) and assays setup as a one-step reverse transcriptase (RT) PCR reaction, according to the kit instructions. The RT-PCR reactions were carried out on a QST 7500 Real-Time PCR System (Applied Biosystems) in a 96-well format. For the construction of a standard curve, dilutions of template RNA, supplied by the manufacturer, were prepared and assayed concurrently with the samples. PCV2 DNA was assayed by PCR using the same nucleic acid isolation method and was performed using PCV2 specific primers and probes. The assay results are reported as the  $\log_{10}$  of PCV2 DNA or PRRSV RNA copy number per reaction.



**Fig. 2.** PCV2 antigen in lymph nodes. IHC staining in paraffin-embedded sections shows the presence of PCV2 antigen by the red staining. (A) shows a representative result for tissues from pigs in group 1 (CN). (B) shows evidence of positive, but weak staining in a single pig in group 4 (PC). (C) is a photomicrograph representative of the three pigs in Group 6 (PC-PR) that died prior to end of the study. Panel D shows a photomicrograph that is representative for all pigs in group 7 (VX-PC-PR). Except for (C), the results are from lymph nodes collected 44 days after virus challenge.

## 2.6. Statistical methods

Statistical analysis was performed using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, California). IFA titers were  $40 \times \log_2$  transformed prior to analysis. Repeated measures data were analyzed by one way analysis of variance followed by the Tukey post-test. Differences at specific time points were analyzed by the Kruskal–Wallis test. If significant differences were detected, specific groups at time points were assessed using Wilcoxon's test. Non-repeated measures, such as antibody reactivity to CP polypeptides, were assessed using the Kruskal–Wallis test. If differences were detected, measures were further assessed by Wilcoxon's test. Significant differences were accepted if  $P < 0.05$ .

## 3. Results

### 3.1. Clinical signs and pathology

Clinical signs and pathology were primarily restricted to the dual-challenge (group 6) and PRRS only (group 3) groups. Both groups showed signs of acute PRRS, including mild respiratory distress. The clinical signs were more pronounced following dual challenge, which resulted in the death of three pigs (see Table 1). Histopathology indicated that death was the result of pneumonia with the infiltration of neutrophils, an indicator of bacterial infection. One of the dead pigs showed marked lymphocyte depletion in lymph nodes, an indication of PCVAD. The remaining groups showed no clinical signs and appeared normal throughout the study period. Histopathology, performed at the end of the study did not identify lymphocyte depletion in pigs from the other groups.

The impact of infection on growth was determined by measuring the average daily weight gain (ADWG). All groups were balanced according to weight and sex and pigs were weighed at the beginning and end of the study. Mean ADWG for the

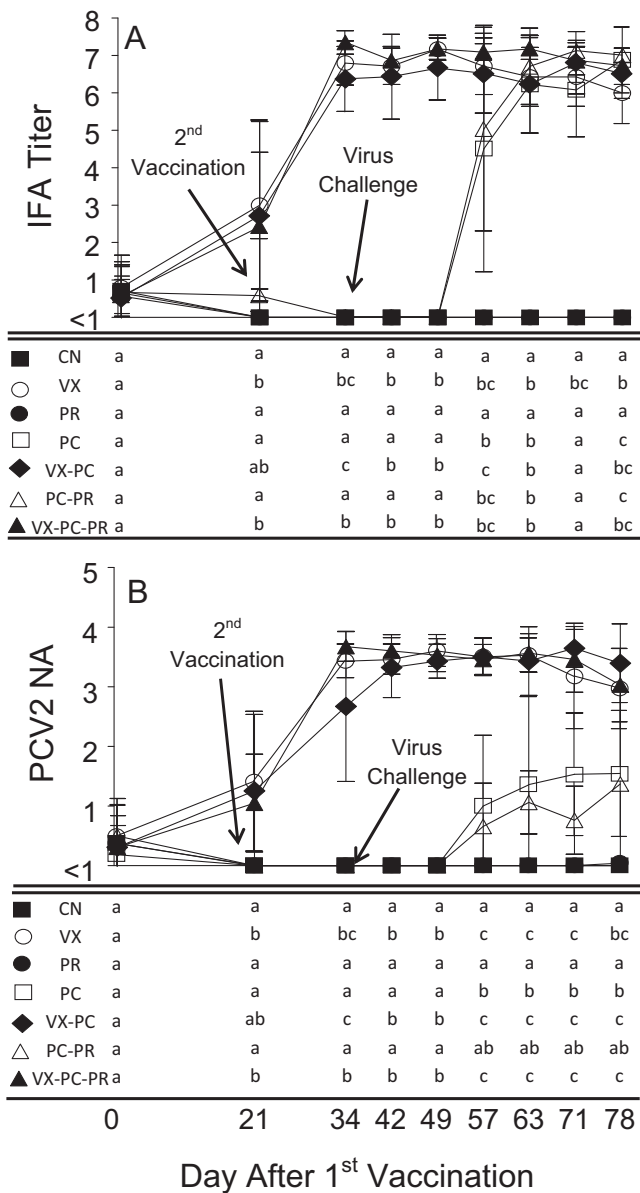
group 1 (CN) and group 4 (PC) pigs was  $0.76 \pm 0.10$  kg/day and  $0.69 \pm 0.07$  kg/day, respectively. The mean for group 6 (PC-PR) pigs was  $0.61 \pm 0.09$  kg/day; however, only 4 of the 7 pigs survived to the end of the study. For pigs vaccinated prior to dual challenge (VX-PC-PR), ADWG was  $0.71 \pm 0.14$  kg/day.

### 3.2. PCV2 and PRRSV viremia

All pigs were negative for PCV2 and PRRSV nucleic acid at the beginning of the study and at the time of challenge. The results for PCV2 viremia are summarized in Fig. 1A. By 15 days after infection, all pigs in groups 4 (PC) and 6 (PC-PR) were positive for PCV2 nucleic acid in serum. The principal difference between group 4 and group 6 was observed 23 days after challenge (study day 57), when mean viremia for group 4 was  $3.1 \pm 0.4 \log_{10}$  templates/rxn versus  $4.7 \pm 1.1 \log_{10}$  templates/rxn for group 6 (Fig. 1A). The difference in viremia was significant ( $P = 0.0042$ ). By the end of the study, PCV2 nucleic acid was still detected in all pigs in groups 4 and 6. For all other groups, PCV2 DNA was below detectable levels, including pigs that were vaccinated prior to challenge, i.e. group 5 (PC-VS) and group 7 (PC-PR-VS).

Increased viremia in group 6 (PC-PR) pigs was supported by the presence of IHC staining for PCV2 antigen in lymphoid tissues in those pigs that succumbed to infection (see Fig. 2C). All other pigs were negative for PCV2 antigen staining except for a single pig in group 4 (PC) that showed areas of weak staining in a single lymph node (see Fig. 2B). Representative results for control and vaccinated pigs are presented in Fig. 2, panels A and D.

The results for PRRSV viremia are shown in Fig. 1B. Mean PRRSV viremia peaked at approximately 8 days after challenge for groups 3, 6 and 7. By 44 days, PRRSV nucleic acid was below detectable levels in all groups. There was no statistical difference between the PRRSV-infected groups at any day after infection. Pigs not



**Fig. 3.** PCV2 immunofluorescent antibody assay (IFA) and virus neutralizing activity (NA) responses. IFA (A) and NA (B) were measured in serum samples as described in Materials and Methods. IFA results are reported as  $\log_2(40 \times 1/\text{dilution})$  and NA as the  $\log_2 \text{NA}_{50}$  per ml. Key: group 1 – CN (closed squares), group 2 – VX (open circles), group 3 – PR (closed circles), group 4 – PC (open squares), group 5 – VX-PC (closed diamonds), group 6 – PC-PR (open triangles), group 7 – VX-PC-PR (closed triangles). Groups with the same letter at a specific time point indicate means that are not significantly different ( $P > 0.05$ ).

challenged with PRRSV remained negative for PRRSV throughout the study.

**3.3. PCV2 antibody response**

Mean PCV2 IFA endpoints for all treatment groups are shown in Fig. 3A. At the time of entry into the challenge facility, most pigs possessed detectable levels of PCV2 antibody, the likely result of maternally-derived antibody (MDA) acquired during suckling. By the time of virus challenge, PCV2 antibody IFA titers were below detectable levels in the non-vaccinated groups. Pigs in group 1 (CN) and group 3 (PR) remained PCV2 IFA negative throughout the remainder of the study. For the vaccinated groups, PCV2 antibody was present by 21 days after the first vaccine dose and peaked by

day 34. At 21 days after PCV2 challenge (study day 42), the IFA titers in group 4 (PC) and group 6 (PC-PR) reached detectable levels. Peak antibody levels for infected pigs were the same as the vaccinated groups.

In contrast to total antibody, there were distinct differences in PCV2 neutralizing activity between the vaccinated pigs (groups 2, 5, and 7) and unvaccinated PCV2-infected pigs (groups 4 and 6; see Fig. 3B). The NA for all vaccinated groups was approximately 16 ( $\log_2 = 4$ ) compared to 4 ( $\log_2 = 2$ ) for the unvaccinated PCV2-infected groups. Together, the results show that vaccination and infection result in similar levels of total antibody, but neutralizing activity during natural infection is significantly impaired.

PRRSV antibody ELISA, performed at the end of the study, confirmed that all pigs challenged with PRRSV (groups 3, 6 and 7) were seropositive for PRRSV, while pigs in the remaining groups remained seronegative (data not shown).

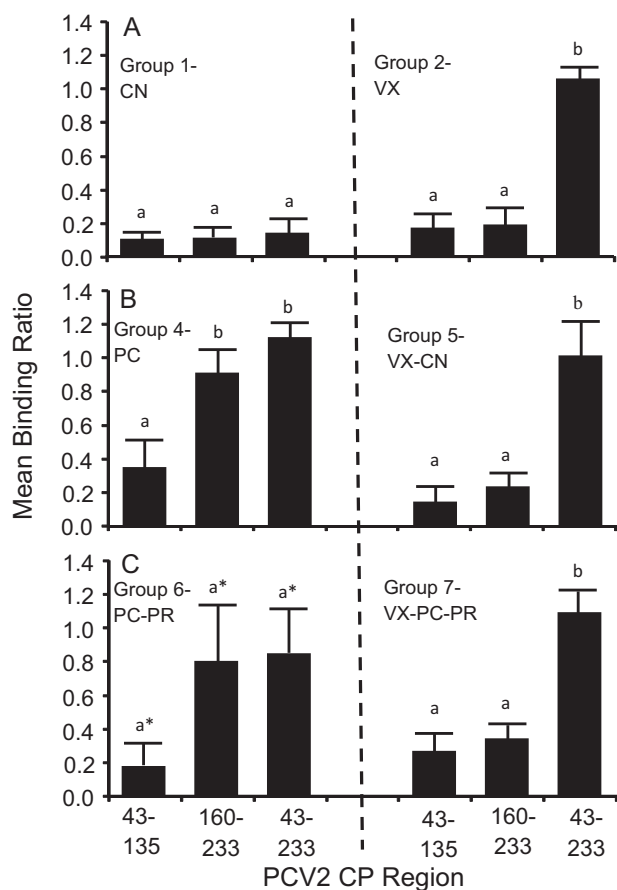
**3.4. Recognition of PCV2 CP polypeptides**

Previously, we demonstrated that vaccination resulted in antibody reactivity towards CP(43-43-233). In contrast, PCVAD pigs recognized small CP polypeptide fragments, primarily located in the C-terminal region. Finer mapping with synthesized 20mer oligopeptides identified a small epitope, CP(169-180) recognized by PCVAD pigs but not vaccinated pigs [37]. Therefore, the pattern of reactivity against the three polypeptides was used to characterize the nature of the antibody response; i.e. consistent with vaccination versus disease. For the purpose of this study, ELISA was performed using an N-terminal polypeptide, CP(43-135), C-terminal fragment, CP(160-233), and CP(43-233). As shown in Fig. 4A, the mean response for group 1 (CN) pigs showed only background levels of binding to all polypeptides fragments. The vaccine only group, group 2, showed high binding activity against CP(43-233) and background binding for CP(43-135) and CP(160-233). As shown in Fig. 4B, infection with PCV2 alone showed reactivity against CP(43-233) and CP(160-233) with minimal reactivity towards CP(43-135). Vaccination prior to PCV2 challenge resulted in response similar to vaccine. The response of dual infected pigs is shown in Fig. 4C. Antibody reactivity was elevated against CP(160-233) and CP(43-233), but the results were not significantly different between the three polypeptides. However, vaccination prior to challenge showed a response similar to the vaccine only group.

**4. Discussion**

In this study, the PCV2 vaccine response was evaluated in the context of a PCVAD challenge model. Dual challenge with PCV2 and PRRSV resulted in high mortality and the presence of clinical signs and pathology associated with PCVAD. One effect of PRRSV was increased PCV2 infection as determined by the presence of increased PCV2 nucleic acid in the blood and deposition of PCV2 antigen in lymph nodes (Figs. 1 and 2), supporting previous observations following PCV2-PRRSV infection [38–41]. The mechanistic role of co-factors, such as PRRSV, in the onset of PCVAD has remained largely unclear. One possibility relates to PRRSV modulation of host immunity, by increasing the number of PCV2-permissive lymphocytes through PRRSV-induced blastogenesis, or by suppressing anti-PCV2 immune responses (reviewed by Opriessnig and Halbur [38]).

Experimental and field studies have clearly demonstrated the efficacy of PCV2 vaccines in reducing viremia, eliminating PCVAD, and increasing growth performance [6,22–24]. The data from this study provide further insights regarding the nature of the antibody response during infection and after vaccination. The IFA results showed that PCV2 infection and vaccination result in similar levels



**Fig. 4.** Antibody response of pigs to PCV2 capsid polypeptides. Serum samples from non-vaccinated and vaccinated pigs were reacted with polypeptides that covered amino acids 43–135, 160–233 and 43–233 of CP. Results for non-infected groups are in panel A, groups infected with PCV2 only in (B), and groups infected with PCV2 and PRRSV in (C). The dotted line separates vaccinated groups (right side) from non-vaccinated groups (left side). The assay was performed with sera collected at 44 days after virus challenge. Mean antibody ratios with the same letter are not significantly different ( $P > 0.05$ ). \*Due to mortality, only 4 pigs were included in the analysis for group 6. All other groups included 7 pigs.

of total serum antibody (Fig. 3A). However, vaccination generated approximately four times the amount of virus neutralizing activity compared to infection with PCV2 alone or with PCV2 and PRRSV (Fig. 3B). Therefore, increased NA in vaccinated pigs represents a quantitative difference between vaccine and natural infection. This study also provides evidence for a qualitative difference in the antibody response following infection versus vaccination. Previously, we identified an immunodominant epitope in the C-terminal region of CP that is recognized by sera from PCVAD affected pigs. The results from this study showed that all vaccinated groups recognized only the large CP(43–233) polypeptide and possessed relatively high NA (see Fig. 3, panel B and Fig. 4, right side of dashed line). In contrast, PCV2 pigs infected showed reactivity to both CP(43–233) and CP(160–233) and exhibit relatively low NA (see Fig. 3, panel B and Fig. 4, left side of dashed line). We propose a model in which immune modulation during active PCV2 infection leads to the production of antibodies primarily directed towards CP(169–180), located in the CP(160–233) fragment. Antibodies specific to this region are non-neutralizing. Response to the CP(169–180) epitope is likely generated by the immune response to free CP monomer and/or smaller CP fragments produced by PCV2-infected cells. In contrast, the PCV2 virus like particle (VLP) does not display this epitope. The locations of CP(169–180) within the X-ray crystal structure of the PCV2 CP monomer and VLP [42]

provide further support for this model. In the monomer form of CP, CP(169–180) is exposed on an outer loop where it induces a specific antibody response, whereas, in the context of the VLP, CP(169–180) is buried within the CP structure and therefore, hidden [43]. We propose that protective antibodies induced by vaccination are generated in response to the VLP. Baculovirus-expressed CP, which is incorporated into two of the five currently available commercial PCV2 vaccines, likely expresses CP in the form of a VLP [42]. Therefore, the form of the CP antigen should be taken into consideration when developing PCV2 vaccines.

## Acknowledgements

This work was supported by National Pork Board Grant #06-073, #07-208 and USDA AFRI Grant #2009-35204-05290.

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