Characterization of the antibody response to vaccination and or infection with porcine circovirus type 2 and identification and characterization of a decoy epitope in the capsid protein

by

Benjamin Ralph Trible

B.S., Kansas State University, 2007
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AN ABSTRACT OF A DISSERTATION

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DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine and Pathobiology
College of Veterinary Medicine

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Abstract

Chapter 1: Porcine circovirus-associated disease (PCVAD) encompasses a group of complex, multi-factorial syndromes, which are dependent on infection with porcine circovirus type 2 (PCV2). Current strains of PCV2 circulating in the field are phylogenically classified into two groups, termed PCV2a and 2b. Outbreaks of severe PCVAD in North America in 2005 are linked with a shift in the predominant circulating genotype, from PCV2a to PCV2b. Therefore genotype specific differences in pathogenesis and antigenicity have been suggested. Overall, evidence suggests pathogenicity is a function of the specific PCV2 isolate, regardless of genotype. In addition, only minor antigenic differences have been reported. In terms of immunopathogenisis, a genotypically conserved immune decoy epitope, located in the C-terminal region of the capsid protein, provides an explanation for the inability to identify pathogenic differences between genotypes. Finally, genetic variation of PCV2 and the resulting consequences with respect to vaccination and diagnostics is discussed.

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Chapter 2: Open reading frame 2 (ORF2) of porcine circovirus type 2 (PCV2) codes for the 233 amino acid capsid protein (CP). Baculovirus-based vaccines that express only ORF2 are protective against clinical disease following experimental challenge or natural infection. The goal of this study was to identify regions in CP preferentially recognized by sera from experimentally infected and vaccinated pigs, and compare these responses to pigs diagnosed with porcine circovirus-associated disease (PCVAD), including porcine multi-systemic wasting syndrome (PMWS) and porcine dermatitis and nephropathy syndrome (PDNS). The approach

was to react porcine sera with CP polypeptide fragments followed by finer mapping studies using overlapping oligopeptides that covered amino acids 141-200. The results showed that vaccinated pigs preferentially recognized only the largest CP(43-233) polypeptide fragment. A subset of experimentally infected pigs and pigs with PDNS showed strong reactivity against a CP oligopeptide, 169-STIDYFQPNNKR-180. Alanine scanning identified Y-173, F-174, Q-175 and K-179 as important for antibody recognition. The results from this study support the notion of PCV2 modulation of immunity, including antibody responses that may represent a precursor for disease. The recognition of CP(169-180) and other polypeptides provide opportunities to devise diagnostic tests for monitoring the immunological effectiveness of vaccination.

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Approved by:

Major Professor
Raymond R.R. Rowland

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Dedication

I dedicate this work to my wife Shelby. I know this has been a long and arduous journey. Thankk you for putting up with me.

Chapter 1 - variation of porcine circovirus type 2 (PCV2) and its relevance to vaccination pathogenesis and diagnosis

Introduction

Porcine circovirus-associated disease (PCVAD) was first described in the early 1990s and has since emerged as an economically important disease worldwide (Allan and Ellis, 2000). The onset and progression of PCVAD is linked to infection with porcine circovirus type 2 (PCV2). PCVAD encompasses a group of diverse multi-factorial syndromes, including porcine multi-systemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), porcine respiratory disease complex (PRDC), reproductive failure, and others (Chae, 2004, 2005; Opriessnig et al., 2007; Ramamoorthy and Meng, 2009). Even though PDNS was reproduced in gnotobiotic pigs in the absence of PCV2 (Krakowka et al., 2008), pigs with clinical PDNS possess high levels of PCV2-specific antibodies, which are implicated in disease progression (Thomson et al., 2002; Wellenberg et al., 2004). A more subtle manifestation of PCV2 infection is poor growth performance in apparently healthy herds (Horlen et al., 2008). More recently, a novel peracute syndrome has been described, termed acute pulmonary edema (APE), which appeared in vaccinated herds (Cino-Ozuna et al., 2011). Unlike previously described syndromes, which are slow and progressive, APE is characterized by acute respiratory distress in apparently healthy animals followed by almost immediate death.

PCV2 is placed in the family *Circoviridae*, which encompasses a group of small single-stranded DNA viruses that infect avian and swine species. Within the genus circovirus, PCV2 is closely related to porcine circovirus type 1 (PCV1). The 1.7kb ambisense genome of porcine circoviruses codes for at least two open reading frames (ORFs), which are essential for virus replication. The largest, ORF1, codes for the replicase proteins, Rep and Rep' (Mankertz and

Hillenbrand, 2001). ORF1 is oriented in the sense direction relative to the origin in the PCV2 genome. Rep is translated from the entire ORF1 transcript; whereas, Rep' is derived by alternative splicing of the ORF1 transcript. The C-terminal 68 amino acids (aa) of Rep' are derived from a different reading frame. Oriented in the antisense direction, ORF2 codes for the 233 or 234 aa virus capsid protein (CP). CP is involved in the formation of the homopolymer capsid and is likely involved in translocating the viral genome into the nucleus during virus replication (Liu et al., 2001; Nawagitgul et al., 2000). Recently, the crystal structure of a monomeric CP subunit and its orientation within a PCV2-like particle was reported (Khayat et al., 2011). In this model, 60 CP subunits form an icosahedron with T=1 symmetry. A computer reconstruction of the PCV2 CP monomer and its incorporation into the capsid are presented in Fig. 1-1.

Evolution and classification of PCV2 isolates.

Origins of circoviruses

The plant virus families, *Nanoviridae* and *Geminiviridae*, are considered the closest relatives to the *Circoviridae.* Overall, these families share a common stem loop structure, which contains the origin (Ori) of replication within the circular genome. Analysis of circovirus and nanovirus Rep peptide sequences led to a proposed mechanism for the evolutionary origin of circoviruses (Gibbs and Weiller, 1999). In this study, similarities between nanoviruses and circoviruses were found in the N-terminal region of Rep. However, the C-terminal region of PCV Rep was more closely related to an RNA binding protein, 2C, of a vertebrate calicivirus, an RNA virus. The proposed site of recombination was located between positions 129 and 178 of PCV1 Rep. The presence of the calicivirus 2C sequence suggests that PCV arose following a recombination event between a plant nanovirus and vertebrate calicivirus. Since nanovirus replication does not

rely on an RNA step and caliciviruses possess an RNA genome, the incorporation of the 2C sequence was likely mediated by a transcriptase via a retrovirus or retrotransposon intermediate. The exact timing of this event is unclear. One hypothesis is that circoviruses co-evolved in their respective avian and mammalian hosts, which would mean that circoviruses first appeared prior to the divergence of birds and mammals, approximately 300 million years ago. However, an analysis performed by Firth et al. (2009) suggests that circoviruses have been present for only the last 500 years.

In terms of the continued evolution of PCV2, the analysis of 160 full length PCV2 genomes revealed a mutation rate of $1.2X10^{-3}$ substitutions/site/year (s/s/y; Firth et al., 2009). Similar results were found in a recent study analyzing PCV2 sequences from Cuba ($3.1X10^{-3}$ to $6.6X10^{-3}$ s/s/y; Pérez et al., 2011). Overall, these data reveal that PCV2 possesses the highest mutation rate reported for any DNA virus; falling into the range of genetic change reported for most RNA viruses (Duffy et al., 2008).

The nature of diversity was evaluated through sequence analysis of six members of the circovirus family, including beak and feather disease virus, columbid circovirus, goose circovirus, muscovy duck circovirus, PCV1 and PCV2 (Hughes and Pointkivska, 2008). The results showed significantly increased synonymous versus nonsynonymous nucleotide diversity, which suggests PCV2 is undergoing purifying selection. Interestingly, PCV2 possessed a relatively large number of nonsynonymous nucleotide changes within Rep not found in the other five viruses. The authors conclude that the rare mutations were likely the result of a population bottleneck, followed by a population expansion.

Emergence of PCV2

In the early 1970s, Tischer et al. (1974) described a viral contaminant of the porcine kidney cell line, PK-15 (ATCC-CCL31). Biochemical analysis revealed a virus with a circular ssDNA genome, giving rise to the name porcine circovirus (PCV; Tischer et al., 1982). In early experimental infection studies, PK-15 contaminant was observed to cause no clinical signs of disease (Tischer et al., 1986). In Canada in the early 1990s, a new wasting disease of pigs emerged and was termed PMWS (Clark, 1997; Harding, 1997). Electron microscopy and immunohistochemistry utilizing PCV-specific monoclonal antibodies identifiedthe presence of a circovirus in tissues from affected pigs. PCR using PCV1-specific primers and analysis of resulting DNA sequence showed approximately 70% identity to the virus described by Tischer (Meehan et al., 1998). The designations, PCV1 and PCV2, were used to distinguish the non-pathogenic PK-15 contaminant virus from the new PMWS-associated isolates.

Retrospective analysis of archived tissues from Northern Germany identified PCV2-specific DNA sequences in tissues that were obtained as far back as 1962 (Jacobsen et al., 2009). The analysis included samples from pigs that possessed PMWS-like clinical signs. Using in situ hybridization and PCR, the prevalence of PCV2 in tissue samples between 1962 and 1984 was estimated to be 2.5%. Beginning in 1985, the prevalence of PCV2 sequence made a dramatic jump to over 30%. This increase correlated with the appearance of PCV2-associated tissue lesions. Analysis of ORF2 sequences from the 1985 samples showed similarities to the PCV2a genotypic group. Attempts to amplify PCV2 DNA from earlier samples produced only small PCR fragments from ORF1, which were not sufficient for making accurate comparisons with contemporary viruses. A study in the UK performed on archival tissues dating back to the 1980's

produced similar results, including the recovery of ORF2 sequences closely related to PCV2a (Grierson et al., 2004).

While PCV2a-like sequences are well-documented in the 1980's, several reports suggest a more recent appearance for viruses in the PCV2b genotype. For example, prior to 2005, PCV2a viruses were endemic in North American swine herds. However, in 2005, outbreaks of severe PCVAD were reported in Canada and later in the US. Diagnostic case submissions and field studies showed an epidemiological correlation between outbreaks of severe PCVAD and the emergence of viruses associated with the PCV2b genotype (Carman et al., 2008; Cheung et al., 2007; Horlen et al., 2007). This pattern for the appearance of PCV2b following a period of endemnic circulation with PCV2a, has been reported in other countries, including China (Wang et al., 2009), Thailand (Jantafong et al., 2011), Korea (Guo et al., 2010), Denmark (Dupont et al., 2008) and Switzerland (Wiederkehr et al., 2009). A Danish genotype, designated 2c, which was recovered from tissues archived in 1980, may represent a progenitor to the contemporary genotypes (Dupont et al., 2008). Based on these and other studies, Patterson & Opriessnig (2010) propose a detailed timeline for the emergence of PCV2 in Northern Germany and eventual spread throughout Europe, Asia, the Americas and Australia.

Classification and phylogenetic relationships of PCV2 genomes

Early sequence analysis revealed PCV2 isolates could be clustered into distinct subgroups or genotypes. Since the International Committee on Taxonomy of Viruses (ICTV) does not describe the classification of viruses below the species level, a variety of designations were proposed as a means to place PCV2 into distinct genotypic subgroupings, which are now known as PCV2a and 2b. A summary of the historical designations for PCV2a and 2b genotypes are presented in Table 1. In 2008, Segalés et al. proposed a unifying system of nomenclature,

designating each genotype within PCV2 by a lower case letter; i.e., PCV2a, 2b and 2c. GenBank accession numbers for representative isolates of current genotypes are listed in Table 2. The placement of an isolate into a genotypic group is based on performing pairwise sequence comparisons (PASC) to determine the degree of genetic variation (p), which is calculated by determining the number of base differences divided by the total number of positions between genomes. Currently, there are two classification schemes for distinguishing PCV2 genotypes. Based on complete PCV2 genome analysis, a cutoff value of p=0.02 is used to distinguish genotypic groups (Grau-Roma et al., 2008). Based on the large degree of genetic variation reported for ORF2 (Fenaux et al., 2000; Hamel et al., 2000; Larochelle et al., 2002; Mankertz et al., 2000), the cutoff value increases to p=0.035 (Segales et al., 2008). The common standard is to classify different genotypes based on ORF2 only. Indeed, a comparison of using the complete PCV2 genome or the nucleotide sequence of ORF2 for phylogenetic mapping revealed no significant differences (Olvera et al., 2007). A phylogenetic tree incorporating whole genome sequences from the three genotypes is presented in Fig. 1-2. The tree shows the clustering of the different PCV2 genotypes.

Recently, two additional genotypes, designated PCV2d and PCV2e, were suggested following sequence analysis of PCV2 isolates from China (Wang et al., 2009). However, a subsequent analysis of the sequence data failed to support the new classification (Cortey et al, 2011).

A classification scheme for further designation of isolates in groups below the genotype level or clades has been described (Olvera et al., 2007). Using this convention, PCV2a was further subgrouped into 5 clades, termed PCV group II A-E. PCV2b was subgrouped into 3 clades, termed PCV group I A and B. In this classification system, the distances between group II (2a)

clades was 0.0158 and 0.0234 for group I (2b) clades. However, the relevance of a clade designation is unclear.

The current classification scheme for grouping viruses is complicated by genetic recombination (Cai et al., 2011; Hesse et al., 2008; Lefebvre et al., 2009; Ma et al., 2007). The plasticity of the PCV2 genome is illustrated by the appearance of chimeric viruses that possess ORF1 from PCV1 and ORF2 from PCV2a (Gagnon et al., 2010). An underlying prerequisite for recombination involves co-infection of cells with more than one virus. Samples from PCVAD-affected pigs show the simultaneous co-infection of pigs with PCV2a and PCV2b (Horlen et al., 2007; Hesse et al., 2008). Viruses possessing both PCV2a and 2b sequences were first reported in Hong Kong (Ma et al., 2007). The analysis indicated that recombination was a relatively frequent event among Chinese viruses. Based on the presence of a phylogenetic incongruity, Hesse et al. (2008) described a virus that possessed ORF1 from PCV2a and ORF2 from PCV2b. Since then, several reports have confirmed that PCV2a/b chimeric viruses are a relatively common occurrence (Cheung, 2009; Kim et al., 2009; Lefebvre et al., 2009). While initial studies suggested that hot spots or designated breakpoints accounted for recombination (Ma et al., 2007), experimental models indicate that any position along the genome can be a site for the genetic exchange between viruses (Cai et al., 2011).

Molecular differences and PCV2 pathogenesis

The genome sizes for PCV1, PCV2a and PCV2b are 1,759, 1,768 (Tischer et al., 1986; Meehan et al., 1998). At the nucleotide level, PCV1 and PCV2 share approximately 70 percent sequence identity whereas, PCV2a and PCV2b genotypes share an identity of approximately 95%. The principal difference between the PCV2 genotypes occurs in ORF2, where both the nucleotide and peptide sequence identities are approximately 90%. It has been proposed that

peptide sequence difference accounts for a difference in pathogenesis, which resulted in the less pathogenic PCV2a being replaced by PCV2b. Sequence comparisons have revealed a pair of signature motifs in ORF2 that distinguish PCV2a from PCV2b isolates (Cheung et al. 2007). PCV2b has the sequence **T**C**A**/AAC/**CCC/CG** at position 1486–1472 of the viral genome; whereas, PCV2a has the sequence **A**C**C**/AAC/**AAA/AT** at position 1487-1473. The nucleotide sequences translate to the peptide sequences, 86-**S**N**PR**S**V** for PCV2b and 86-**T**N**KI**S**I** for PCV2a. While the particular motifs have been useful for rapid diagnostic approaches to distinguish the two genotypes, the capacity of the signature motif to function as a domain associated with difference in virulence has not been demonstrated.

Pathogenic differences between porcine circoviruses

Experimental infection studies characterized PCV1 as a non-pathogenic virus that was ubiquitous within the swine population (Tischer et al., 1986; Allan et al., 1995). However, a recent study involving inoculation of pig fetuses at day 55 with various PCV1 isolates reported virus replication and the appearance of lung lesions 21 days after infection (Saha et al., 2011).

The outbreak of PCVAD in late 2005 in North America focused on the appearance of a new PCV2 strain with enhanced pathogenesis (Horlen et al., 2007; Carman et al., 2008). Early assessment suggested that relatively non-pathogenic PCV2a were replaced with a more pathogenic virus, later described as PCV2b (Grierson et al., 2004; Horlen et al. 2007; Wiederkehr et al., 2009). In fact, early classification schemes designated the PCV2a genotype as "nonpathogenic" and the PCV2b genotype as "pathogenic".

To date, experimental infection models analyzing differences in virulence have reported mixed results. In one experimental study, involving inoculation of gnotobiotic pigs with infectious DNA clones derived from PCV2a or PCV2b, differences in the onset and overall

mortality were reported (Lager et al. 2007). Combined morbidity and mortality was 25% and 100% for PCV2a-infected and PCV2b-infected groups, respectively. Mortality for the PCV2b group occurred between 22 and 27 days after infection; whereas, mortality in the PCV2a group (a single pig) occurred on day 35. However, both genotypes produced similar lesions, lymphocyte depletion and similar amounts of PCV2 antigen in affected tissues. In an experimental challenge study evaluating PRRSV with PCV2a or PCV2b, no differences in the level of PCV2-specific antibody, PCV2 virus load in serum, or PCV2 shedding were detected between groups (Sinha et al., 2011). Following experimental challenge with different PCV2a and 2b isolates, pathogenicity was reported to be a function of the individual properties of an isolate and not related to genotype (Opriessnig et al., 2008b). Further evidence of isolate-specific differences were reported following the co-mingling of PCV2 naïve pigs with PCV2b-infected pigs from PMWS-affected and non-affected herds (Dupont et al. 2009). The results showed that virus spread and the onset of disease was associated with PCV2b strains from PMWS-affected herds and not PCV2b strains from non-affected herds. Overall, the results of experimental studies provide inconclusive support for the hypothesis that PCV2a and PCV2b differ in pathogenicity. However, this conclusion does not adequately explain outbreaks of severe PCVAD that coincided with the appearance of PCV2b. A more intriguing possibility is the experimental infection of germ-free pigs with combinations of PCV2a/2b, 2b/2a, 2a/2a or 2b/2b (Harding et al. 2010). The first virus administered seven days prior to the second virus. The combination 2a and 2b, regardless of order reproduced PCVAD. The results provide evidence for the interaction between PCV2a and PCV2b in the pathogenesis of PCVAD. In addition, they provide an explanation for the enhanced pathogenesis of 2b observed during the 2005 North American outbreak. Further evidence for this possibility was provided by a study

involving in situ hybridization on tissues from pigs with disease or with subclinical infection (Khaiseb et al. 2011). Tissues from diseased pigs showed cells co-infected with both genotypes; whereas, the subclinical pigs were infected with PCV2a or 2b. A mechanism that involves cooperative replication between PCV2a and PCV2b genomes in the development and progression of disease was subsequently proposed.

Contributions of ORF1 and ORF2 to pathogenesis

Elucidating the molecular mechanisms of PCV2 pathogenesis has proven difficult because infection with PCV2 alone does not generally result in overt clinical disease. With this limitation, measurements of PCV2 replication and histological changes are often used to quantify the virulence potential of an isolate. In the field, PCVAD is generally seen when PCV2 infection is complicated by cofactors that can modify and/or enhance disease. Similarly, experimental models that reproduce disease usually incorporate a dual challenge, e.g. PCV2 in combination PRRSV.

The first studies describing a molecular mechanism for PCV2 pathogenesis were based on sequence analysis of an attenuated virus. In vitro passage of a PCV2a virus in PK-15 cells 120 times (P120) resulted in a 1 log increase in virus replication in culture (Fenaux et al., 2004). Infection of pigs with P120 showed a decrease in viremia and lower scores for gross pathological and histopathological lesions compared to the parental virus. Sequence analysis showed only two nucleotide changes, both in ORF2. The mutations resulted in a proline to alanine substitution at position 110 and an arginine to serine change at 191. Neither mutation maps to the proposed heparin sulfate receptor binding domain, located between residues 98-103 (Misinzo et al., 2006) or the signature motif. It was subsequently suggested the two mutations resulted in a conformational change in the overall structure of CP. The possible participation of ORF1 in

pathogenesis was illustrated by experiments using a chimeric virus containing ORF1 from PCV1 and ORF2 from PCV2a (Fenaux et al., 2003). Even though the chimera was attenuated relative to the wild-type PCV2, a similar attenuation was observed when the chimera contained ORF1 from PCV2 and ORF2 from PCV1.

Antibody epitopes within CP

The results for PEPSCAN analysis incorporating overlapping oligopeptide from a PCV2b isolate and reacted with PCV1 and PCV2-specific sera showed that CP residues 65-87, 113-139, and 193-207were important for recognition. An epitope, 169-183, was recognized by both antisera. In a different study, conformational epitopes were analyzed by reacting chimeric ORF2 PCV1-2a infected cells with CP-specific monoclonal antibodies (Lekcharoensuk et al., 2004). The results identified CP residues 47-85, 165-200 and 200-233 as immunoreactive regions. Overall these results identify four antibody recognition domains; labeled epitopes A-D (see Fig. 1-3).

Solution of the crystal structure of the PCV2 capsid allowed for a more detailed analysis if the key binding residues within epitopes A-D (Khayat etal.. 2011). Position 70-Asp, 71-Met, 77-Asn and 78-Asp were identified as key residues within epitope A. Within epitopes B and D, CP 113-Glu, 115-Asp and 127-Asp and 203-Glu, 206-Ile and 207-Tyr were identified as essential for antibody recognition.

As discussed in more detail below, we identified CP(169-180), a region within epitope C (see Fig. 1-3), as an immunodominant epitope associated with disease (Trible et al., 2011). Alanine scanning identified Y-173, F-174, Q-175, and to a lesser extent, K-179 as important for antibody recognition.

Antigenic differences between PCV2 genotypes

The first report analyzing antibody binding differences between PCV2 genotypes involved reacting 16 monoclonal antibodies (mAbs) derived from infection with a PCV2a isolate, Stoon 1010. Antibodies were reacted with cells infected with four different PCV2a isolates and three different PCV2b isolates (Lefebvre et al. 2008). The results showed that 11 of the 16 mAbs reacted with all isolates; whereas, four mAbs were specific for only the PCV2a isolates. The 11 mAbs that reacted with both genotypes were able to neutralize four of the seven viruses, two from PCV2a and two from PCV2b. The results demonstrate antigenic differences between PCV2a and 2b genotypes, as well as antigenic differences within genotypes.

In another study, virus neutralization was performed with four PCV2a and four PCV2b isolates using mAb 8E4, an antibody produced against a PCV2a isolate (Huang et al. 2011). The mAb neutralized only the PCV2a isolates. The determinant of neutralization was investigated by testing neutralizing activity against viruses composed of chimeric PCV2a/2b CP sequences. The results showed that a single residue change in CP, alanine (found in PCV2a) to arginine (found in PCV2b) at position 59, eliminated virus neutralization activity. However, the substitution of an alanine for arginine in PCV2b failed to restore neutralization. Therefore alanine-59 is considered necessary but not sufficient for virus neutralization by 8E4.

Role of genetic variation in diagnostics

Differential diagnostics for PCV1 and PCV2

The standard diagnostic approaches for PCV and PCVAD have been extensively reviewed (Gillespie et al., 2009; Opriessnig et al., 2007). The incorporation of PCV1 and PCV2-specific primers can be used in polymerase chain reaction (PCR) and in situ hybridization (ISH) assays to distinguish PCV1 from PCV2 (Larachelle et al., 19999; Ouardani et al., 1999; Kim and Chae,

2003). Another method involved PCR amplification followed by restriction fragment length polymorphism (RFLP) analysis (Fenaux et al., 2000). Also described are PCV1 and PCV2-specific mAbs, which can be incorporated into antibody-based assays, such as indirect fluorescent antibody (IFA), Immunohistochemistry (IHC) and antigen capture enzyme-linked immunosorbant assay (ELISA; Allan and Ellis, 2000). Finally, there are methods for the detection of PCV1 or PCV2 specific antibodies, including IFA and ELISA (Allan and Ellis, 2000: Blanchard et al., 2003).

Differentiating PCV2a and PCV2b

One of the first approaches for rapidly differentiating PCV2a from PCV2b is RFLP mapping (Wen, et al., 2005; Carman et al. 2008). An example is the use of RFLP to identify the PCV2b genotype when it first appeared in North America (Carman et al., 2008). The assay incorporates the PCR amplification of a 902nt fragment containing all of ORF2. The PCR product is digested with *XbaI, EcoRI* and *SmaI* in separate reactions. The resulting patterns, 422 or 321, identify PCV2a or PCV2b, respectively. Rapid PCR-based approaches to differentiate PCV2a and PCV2b genotypes were initially based on developing probes against the genotype-specific signature motif in ORF2 (described in Section 3; Cheung et al., 2007). However, hybridization of probes to a specific signature motif often failed, because minor sequence variations within the signature motif. An alternative TaqMan-based assay, offered by the Kansas State Veterinary Diagnostic Laboratory (KSVDL), is based on a single nucleotide change located at position 872 within ORF1. At this position, PCV2a genotypes possess a C; whereas ,PCV2b genotypes possess a T. Even though this is a single mutation in a third codon position it is stable, and based on sequences in GenBank, consistently discriminates between 2a and 2b. Currently, there are no antibody based assays capable of distinguishing PCV2 genotypes.

Vaccination and Immunity

Efficacy of PCV2 vaccines

The efficacy of the first commercial two-dose baculovirus-expressed CP vaccine (Intervet) was tested in 2006 on a small farrow to finish PRRSV-negative farm in Kansas (Horlen et al., 2008). The history showed a severe outbreak of PCVAD in late 2005, which involved the appearance of PCV2b. Pigs in the vaccine group had reduced mortality and reduced viremia compared to controls. Furthermore, vaccinated pigs had significantly higher weights at the time of shipment for market (see Fig. 1-4). This study demonstrated that a PCV2a-based vaccine was protective in the field against PCV2b. Moreover, this was the first report to identify reduced growth performance as a non-overt syndrome of PCVAD. Additional field and experimental studies have confirmed these findings (Fachinger et al. 2008; Fort et al., 2008, 2009; Kixmöller et al., 2008; Martelli et al., 2011; Opriessnig et al., 2008a, 2011, 2009).

Currently, four commercial vaccines, based on the expression of an ORF2 antigen from PCV2a, are available for use in the field. Circumvent PCV (Intervet) and Ingelvac CircoFLEX, (Boehringer Ingelheim) consist of CP from PCV2 expressed by baculovirus and administered in two doses or one dose, respectively. A third vaccine, Fostera PCV2 (Pfizer), is comprised of a killed whole virus preparation prepared from a PCV1 backbone that expresses ORF2 from PCV2. The fourth, Circovac (Merial), contains inactivated whole PCV2 as the antigen. There is no evidence suggesting that the incorporation of a CP antigen from PCV2b overs enhanced protection over current vaccines.

Recently, experimental vaccine approaches have described inclusion of foreign tags, as a means for a positive marker (Beach et al., 2011; Huang et al., 2011). These vaccines would allow for the development of assays that can differentiate vaccinated from infected animals (DIVA).

Host immunity following vaccination versus infection

A key feature of PCVAD is the capacity for PCV2 to modulate the host immune response. For example, PMWS is characterized by an almost complete loss of lymphocytes (Chae, 2004). At the other extreme, pigs with PDNS possess a hyperimmune response, including the production of large quantities of PCV2-specific antibodies, which likely contribute to immune complex formation and disease progression (Wellenberg et al., 2004).

In order to develop a mechanistic understanding of PCV2 modulation of host immunity, we characterized the regions in CP recognized by sera from experimentally infected, vaccinated and clinically diseased pigs (Trible et al., 2011). Antibodies induced by vaccination primarily recognized the largest polypeptide, CP(43-233). In contrast, antibodies from PDNS pigs were primarily directed against small polypeptide regions including an immunodominant region represented by a short oligopeptide, 169-STIDYFQPNNKR-180. The CP(169-180) domain, located in Epitope C (see Fig. 1-3), is highly conserved among all PCV2 isolates. A qualitative difference in the antibody response was also found between vaccinated and PCV2-infected pigs. Although vaccinated and infected pigs possessed similar levels of PCV2-specific antibodies, vaccination resulted in an approximate 4-fold increase in PCV2 neutralizing activity (See Fig. 1-5). These results indicate that CP(169-180) may serve as a decoy, diverting the humoral response away from a protective epitope. A possible explanation lies in the antibody accessibility and immunogenicity of the monomer versus polymer forms of the CP. As discussed earlier, Khayat et al. (2011) solved the X-ray crystal structure of CP. The monomeric form contains an exposed CP(169-180), which is buried in the capsid. The exposure to the monomeric form during infection induces antibodies against the immunodominant CP(169-180)

domain. In addition, baculovirus-expressed CP has been reported to assemble into virus-like particles (Khayat et al., 2011).

Further evidence for this was demonstrated in an experimental challenge study (manuscript in preparation). Pigs were vaccinated with a monomeric form of *E. coli* expressed CP(43-233) or with baculovirus expressed CP. Vaccination with baculovirus-expressed protein induced high levels of anti-CP(43-233) antibodies and low levels of anti- CP(160-233) antibodies. After challenge with PCV2, no virus was detected in serum. Immunization with the CP(43-233) monomer also induced high levels of antibody against CP(43-233) as well as a highly elevated response to CP(160-233). Viremia was no different from non-vaccinated and PCV2-challenged pigs. As summarized in Fig. 1-6, we propose that protective antibodies are generated from epitopes formed by the PCV2 VLP and non-protective antibodies are produced by exposure to the CP monomer. As presented by the proposed model in Fig. 7, the antibody response to the decoy epitope plays a key role in the progression towards clinical PCVAD. Since the decoy epitope is highly conserved among all PCV2 isolates, this model predicts that there should be no distinction in the disease syndromes caused by PCV2a versus PCV2b.g

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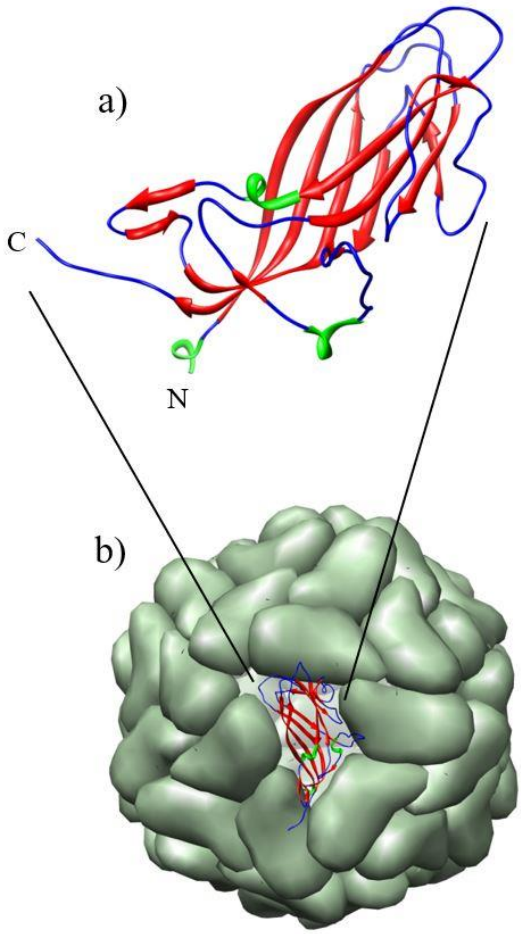

Figure 1-1. PCV2 CP subunit model structure and assembly into a viral capsid

The ribbon model of the CP subunit (a) with helices, loops and sheets shown in green, blue and red, respectively. Panel (b) shows a CP subunit placed in the context of the viral capsid. The remaining 59 CP subunits are depicted in grey. Both models are based on the data of Khayat et al. (2011) and reproduced using the UCSF Chimera computer program (PDB ID 3R0R; Petterson et al., 2004).

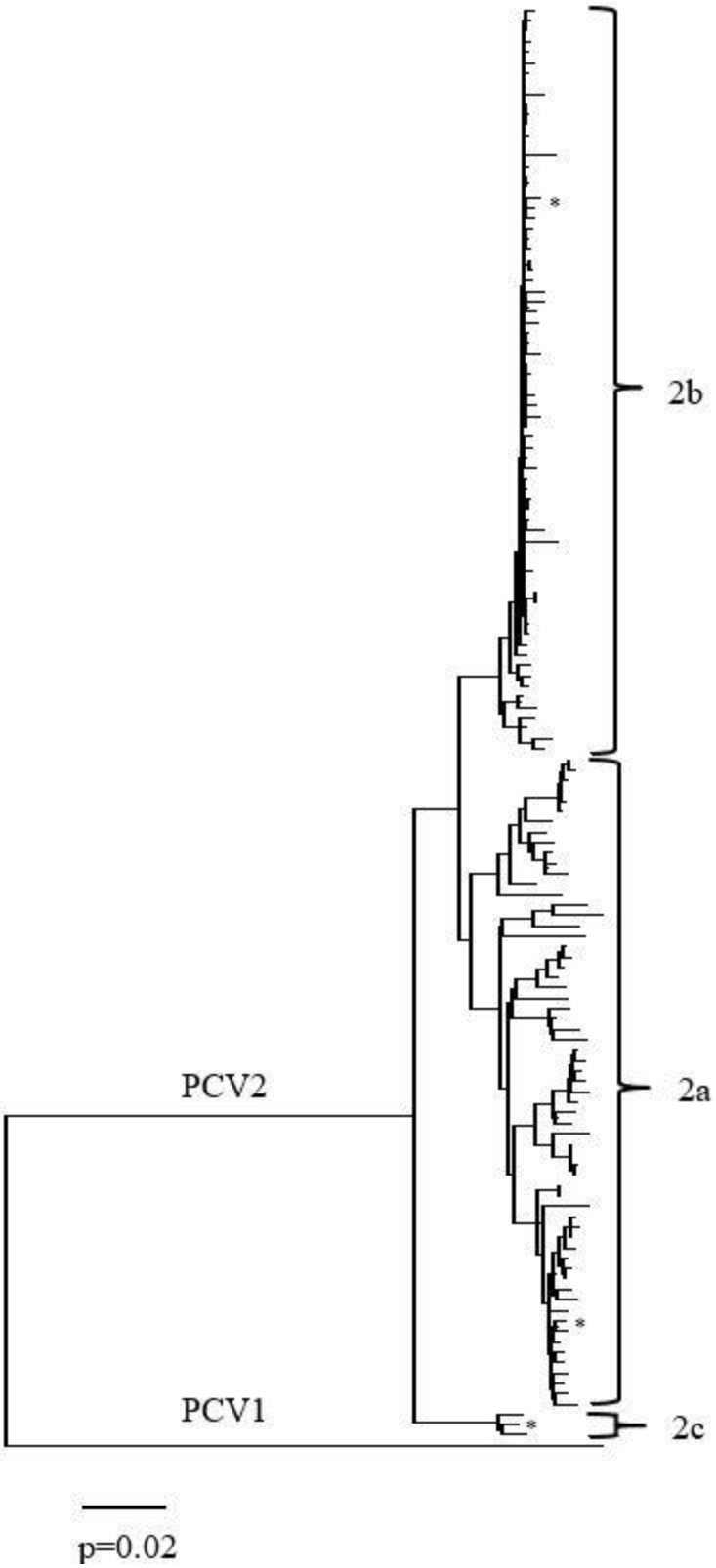
2b
PCV2
2a
PCV1
2c
p=0.02

Figure 1-2 Phylogenetic tree of 139 genomic PCV2 nucleotide sequences from GenBank

The phylogenetic analysis was performed using MEGA5 software (Tamura et al., 2011). The analysis includes 139 PCV2 sequences and a single PCV1 sequence as an outgroup. The tree was constructed by the neighbor joining method and the bootstrap test (1000 replicates). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances (p) which were computed using the Kimura 2-parameter method. The evolutionary distance scale is shown at the bottom.

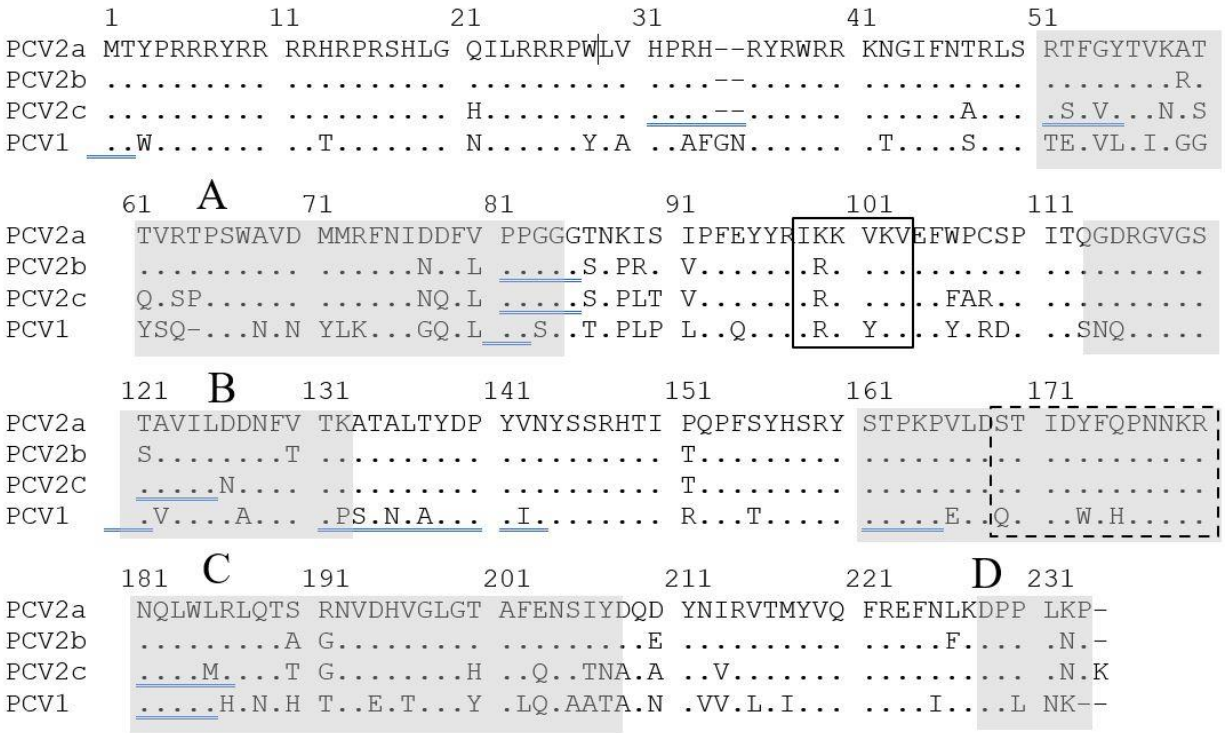

Figure 1-3 Alignment of PCV2 CP peptide sequences from representative PCV2 genotypes

Reference peptide sequences are from the GenBank accession numbers in Table 2, which are identified by asterisks in Fig. 1-2. The grey areas correspond to antibody recognition domains described in Trible et al. (2011). The solid line box shows the putative heparin sulfate binding receptor domain (Misinzo et al., 2006). The dashed line shows the location of an

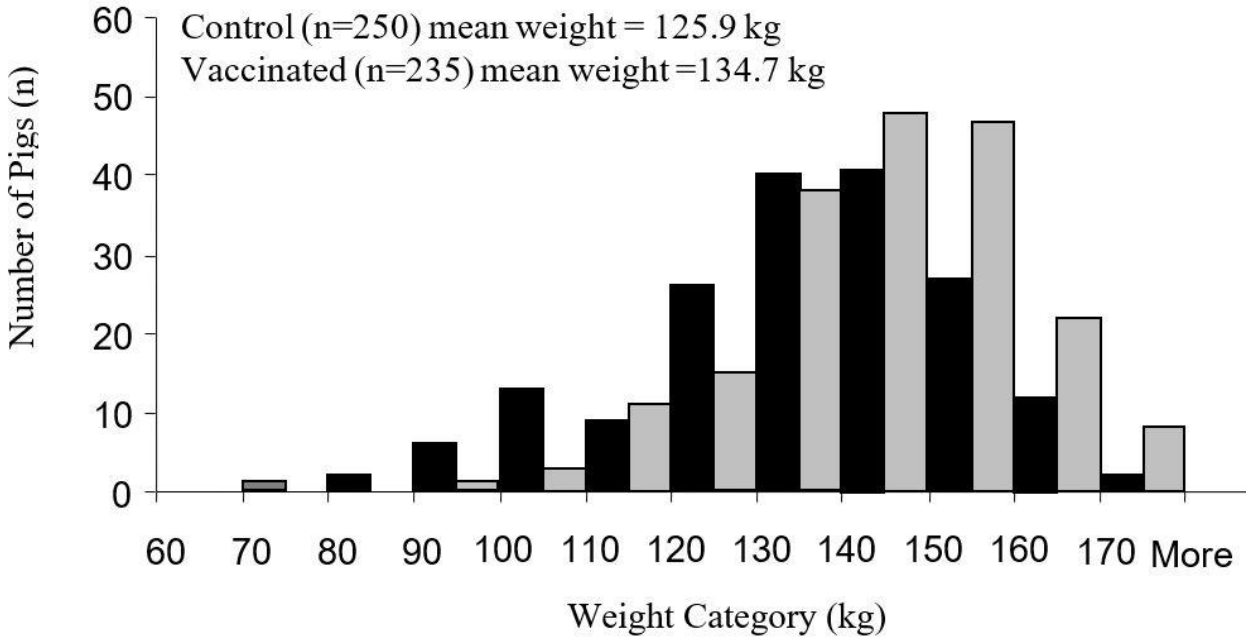

Figure 1-4 Weight distribution of vaccinated and control groups in a field vaccination trial

At the time of vaccination, vaccine and control group average weights were 6.4kg and 6.5kg, respectively. The figure shows weight distribution for vaccinates (dark grey bars) and controls (light grey bars) collected one day prior to sending the pigs to market.

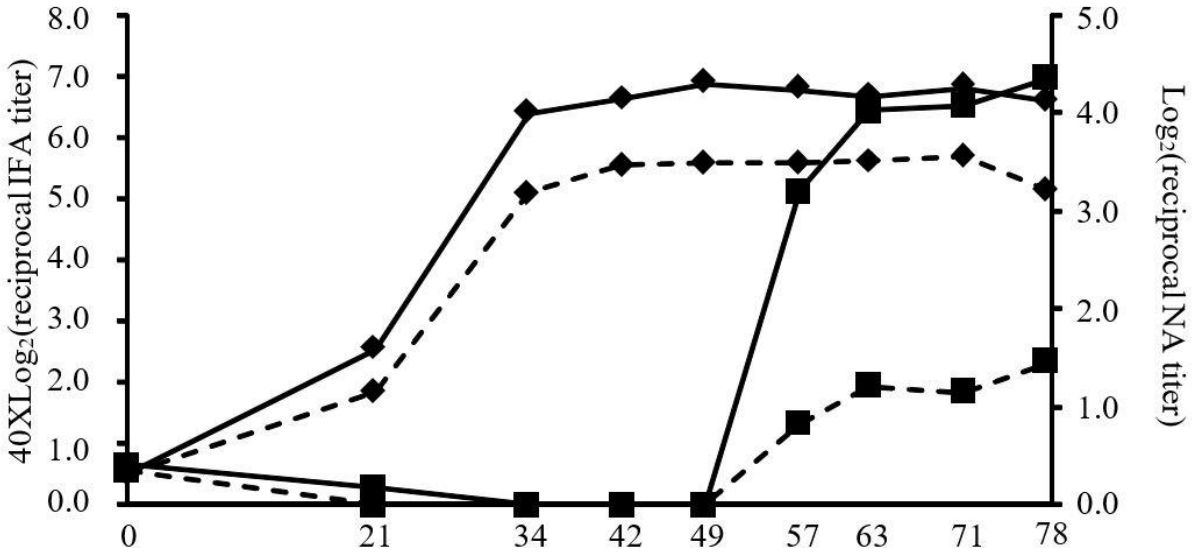

Figure 1-5 PCV2 immunofluorescent antibody (IFA) and neutralizing antibody (NA).

IFA and NA were measured similar to the methods described in Trible et al. (2011). The left axis represents values for IFA while the right axis shows the values for NA. Treatment Group Key: Control (closed circles), Vaccine (closed triangles), PCV2 (closed squares), PCV2/Vaccine (closed diamonds). Antibody Assay Key: IFA (solid line), NA (dashed line).

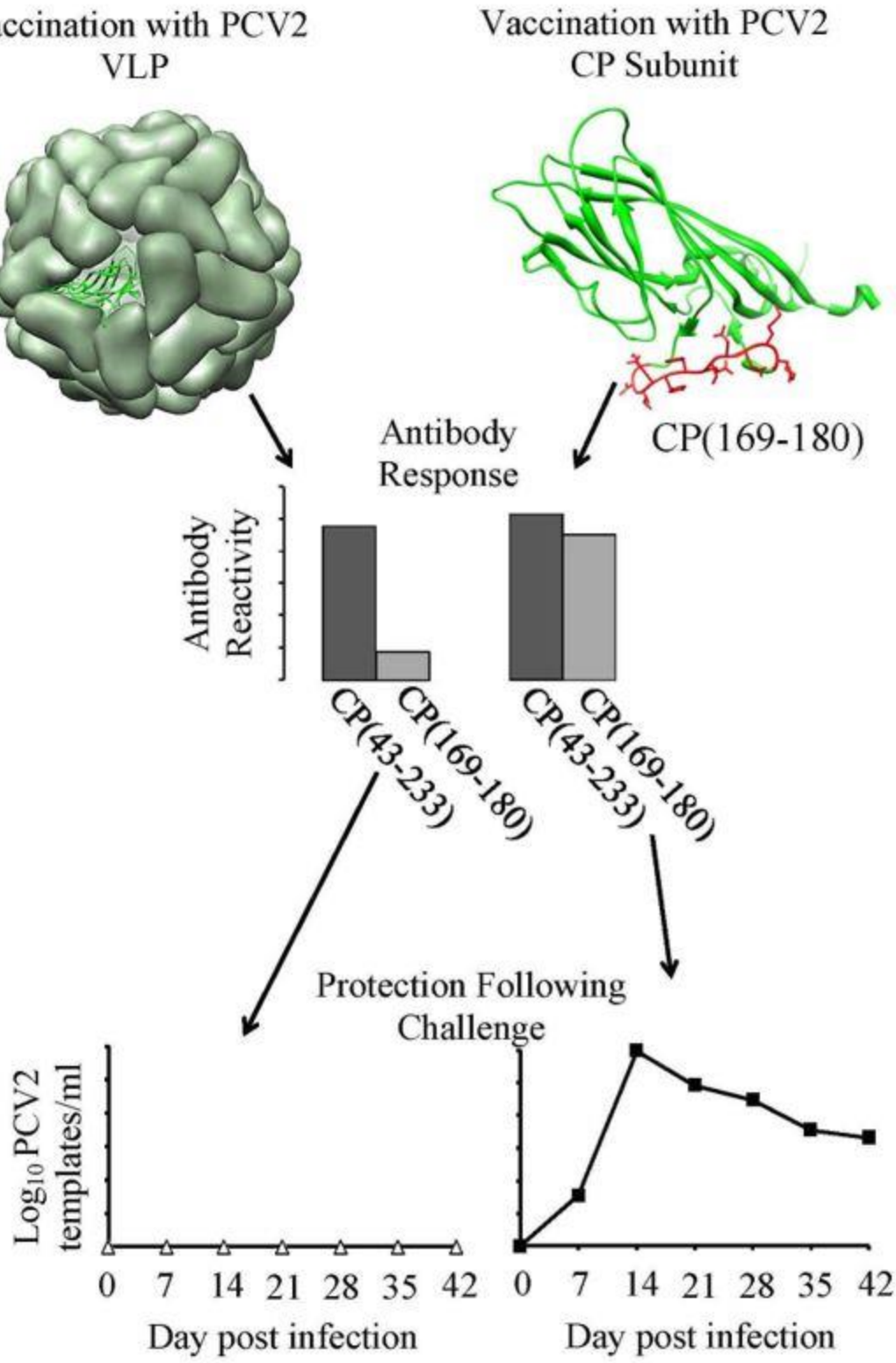
Vaccination with PCV2
VLP
Vaccination with PCV2
CP Subunit
CP(169-180)
Antibody
Response
Antibody
Reactivity
CP(43-233)
CP(169-180)
CP(43-233)
CP(169-180)
Protection Following
Challenge
Log10 PCV2
templates/ml
0 7 14 21 28 35 42
Day post infection
0 7 14 21 28 35 42
Day post infection

Figure 1-6 Immunity and protection following vaccination with VLP or CP monomer.

The figure illustrates result that occur following vaccination with baculovirus-expressed CP, which assembles into a VLP compared to the outcome following vaccination with CP monomer, expressed in bacteria.

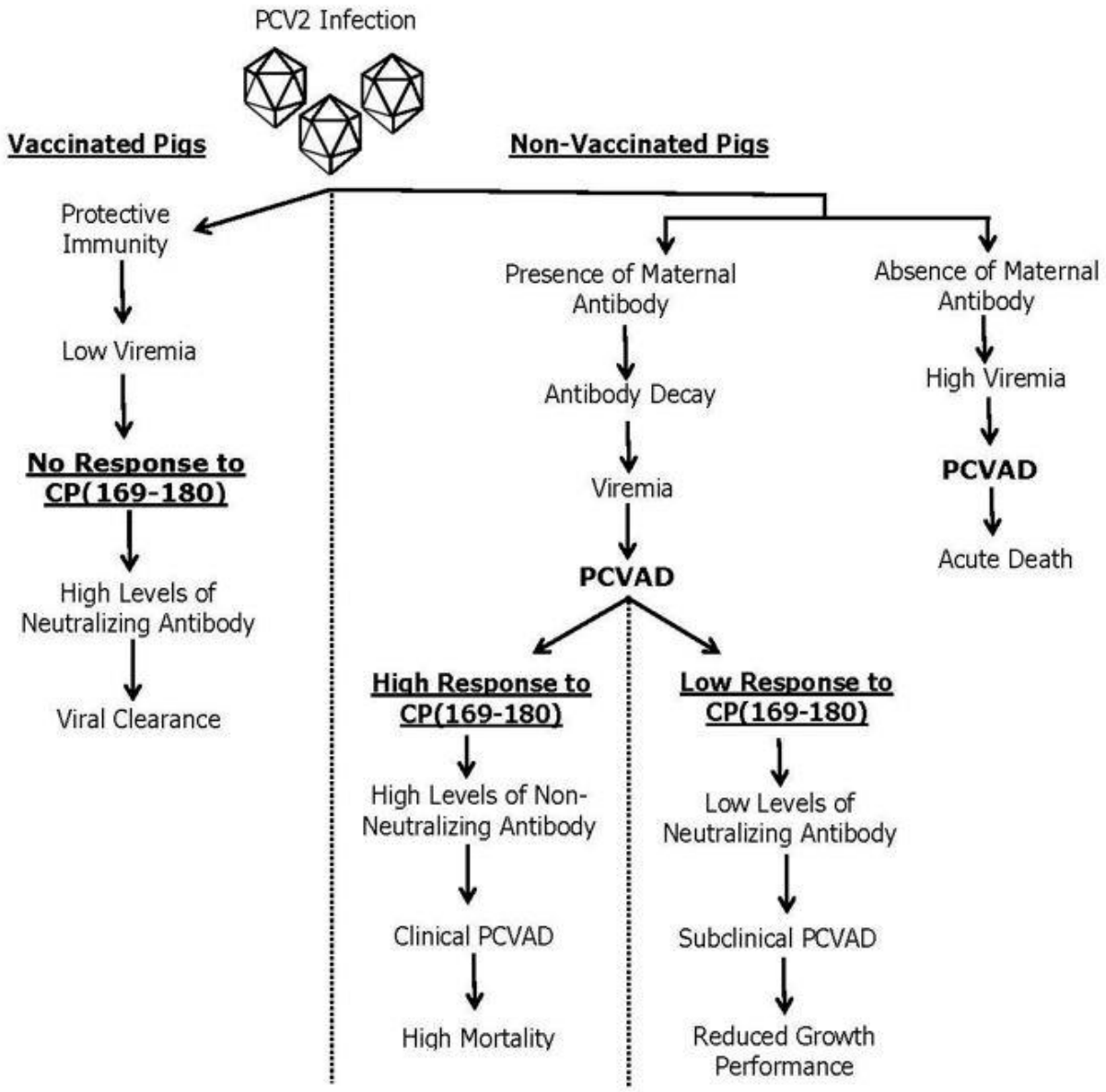

Figure 1-7 Immunity and disease following natural infection in vaccinated and non-vaccinated pigs.

Depicted are the different outcomes of PCV2 infection in vaccinated (left of the dashed line) or non-vaccinated pigs (right of the dashed line). In this model, vaccination prior to

infection results in protection against PCV2 infection, which is characterized by high levels of neutralizing antibodies. In non-vaccinated pigs, the presence or absence of maternal antibody impacts disease progression. The antibody response to the CP(169-180) decoy epitope predicts the outcome of PCV2 infection. High and low responders progress to clinical and subclinical forms of PCVAD.

Table 1-1. **Historical nomenclature for PCV2 genotypes**

PCV2a designation	PCV2b designation	Reference
PCV2 Genotype 2	PCV2 Genotypes 1	Grau-Roma et al., 2008
PCV2 Group 2	PCV2 Group 1	Olvera et al., 2007
PCV2 II	PCV2 I	de Boisséson et al., 2004
PCV2 SG 3	PCV2 SG 1/2	Timmusk et al., 2008
PCV2 B	PCV2 A	Martins Gomes de Castro et al., 2007
PCV2 RFLP 422	PCV2 RFLP 321	Carman et al., 2008

Table 1-2. **Prototypic members of current PCV2 genotypes**

Genotype	GenBank number	Country of origin	Reference
2a	AF055391	USA	Meehan et al., 1998
2b	AF055393	France	Meehan et al., 1998
2c	EU148503	Denmark	Dupont et al., 2008

Chapter 2 - Antibody recognition of porcine circovirus type 2 capsid protein epitopes after vaccination, infection, and disease

Introduction

First described in Canada in the early 1990s, porcine circovirus-associated disease (PCVAD) has emerged as an economically important disease worldwide (1, 48). A central feature of PCVAD is the involvement of porcine circovirus type 2 (PCV2). PCVAD encompasses a group of complex, multi-factorial diseases of which porcine multi-systemic wasting syndrome (PMWS) and porcine dermatitis and nephropathy syndrome (PDNS) are common syndromes (7, 49, 52, 53). Even though Krakowka et al. (25) reported the appearance of PDNS in gnotobiotic pigs in the absence of PCV2, pigs with clinical PDNS possess high levels of PCV2-specific antibodies, which are implicated in disease progression (53). In 2008, poor growth performance in herds without overt clinical signs was reported as another manifestation of PCVAD (20). Factors, such as host genetics, other infectious agents, and the pathogenic potential of the PCV2 isolate contribute to the disease (2, 3, 14, 23, 24, 40, 41, 43).

The majority of PCV2 isolates can be divided into one of two genotypes, known as PCV2a and PCV2b (11, 19, 39). A third genotype, designated PCV2c, describes a small group of historical isolates found in Denmark (48). The genotypic classification of PCV2 is complicated by field isolates composed of PCV2a and PCV2b sequences (18, 32). The PCV2 genome is dominated by three open reading frames (ORFs). The 233 amino acid capsid protein (CP), coded for by ORF2, forms a homopolymer that surrounds the single-stranded ambisense 1.7 kb DNA genome (38). The principal sequence differences between PCV2a and PCV2b genotypes locate to ORF2, where the nucleotide and peptide sequence identities are approximately 93%. Baculovirus-based vaccines that express PCV2 ORF2 are sufficient to offer protection from

disease (4, 15, 19, 31, 42, 51). ORF1 is 945 nucleotides (nt) in length and codes for two replicase proteins: Rep and Rep' (12, 34). A third gene, ORF3, is in a different reading frame embedded within ORF1 and codes for a protein associated with apoptosis (28). The significance of ORF3 with regard to the onset and severity of PCVAD is still unknown (9).

The current model for the virion capsid structure locates the arginine-rich N-terminal end of CP projecting inward, where it interacts with the viral genome. Based on studies of another circovirus, psittaccine beak and feather disease virus, the arginine-rich domain functions as a nuclear localization signal sequence, which shuttles the viral DNA across the nuclear pore complex and into the nucleus, the site of PCV2 replication (10, 17, 29, 37).

Previous studies describing the humoral response following PCV2 infection indicate that seroconversion occurs between 10 and 28 days post-infection or after vaccination (1, 35, 45). There are reports that pigs with PMWS seroconvert later, or produce a reduced antibody titer, compared to subclinically infected animals (5, 35). Another difference between subclinical infection and PMWS is reduced neutralizing antibody (NA) titers in PMWS pigs compared to subclinally infected or vaccinated pigs (16, 45). There is no PCV2-based model system that can reproduce PDNS. However, clinically moribund pigs show a hyperimmune response leading to significant antibody production, which may contribute to immune complex formation and PDNS (56).

For the purpose of mapping antibody epitopes in CP, Lekcharoensuk et al. (26) used a panel of seven anti-CP monoclonal antibodies (mAb's), which were reacted with cells transfected with infectious PCV chimeric DNA clones composed of different combinations of PCV1 and PCV2a ORF2 sequences. Immunoreactive regions were reported between residues 47-85, 165-200, and 200-233. Using overlapping oligopeptides and sera from infected and

immunized pigs, Mahe et al. (33) described antibody reactive regions between amino acids 23-43, 71-85, 117-131, and 171-202 in CP. In a follow-up study, Truong et al. (55) identified amino acids 117-131 as a dominant antibody-recognition region. Shang et al. (50) reported four regions in CP associated with mAb recognition; amino acids 156-162, 175-192, 195-202 and 228-233.

The purpose of this study was to test the hypothesis that sera from pigs experimentally infected, vaccinated or with clinically diagnosed PCVAD produce different responses to epitopes within PCV2 CP. The original intent was to identify specific epitopes that could offer protection versus those epitopes involved in immunopathogenesis. The epitope mapping methodology incorporated in this study is an extension of previous work demonstrating that bacterially expressed CP antigens react with antibody from PCV2-infected pigs (30, 38, 54). We extended this approach by evaluating the reactivity of individual polypeptide fragments different combinations of epitopes followed by finer epitope mapping using overlapping oligopeptides. The results describe several different recognition patterns within the different groups of pigs, including the identification of a single epitope, 169-STIDYFQPNNKR, which was preferentially recognized by pigs diagnosed with PDNS and a subset of pigs experimentally infected with PCV2. Alanine substitutions within CP(169-180) showed that Y-173, F-174, Q-175 and K-179 amino acid residues contribute to antibody recognition. The results from this study support the notion of immune dysregulation, characterized by a hyperimmune response during PDNS and a diminished response during PMWS. Furthermore, the methods incorporated in this study provide a means for characterizing the immune response upon vaccination, natural infection and disease.

Materials and Methods

Experimental PCV2 infection

All animal experiments were performed after review and approval by Kansas State University Institutional Animal Use and Biosafety Committees. PCV2-negative pigs were derived from sows with low PCV2 antibody titers (44). Upon entry into the challenge facility, pigs were confirmed negative for PCV2 DNA by PCR. At the time of virus challenge, pigs were confirmed negative for PCV2 DNA and PCV2 antibody as measured by PCR and IFA, respectively. Pigs were randomly assigned to one of seven groups, summarized in Table 1. The timeline for vaccination and infection is diagrammed in Fig. 2-1. PCV2 vaccination was performed using a two-dose commercial baculovirus-expressed PCV2 ORF2 product (Intervet) administered at four and seven weeks of age according to label instructions. Two weeks after the last vaccine dose, pigs were challenged with PCV2 or PCV2 plus porcine reproductive and respiratory syndrome virus (PRRSV). The PCV2 challenge inoculum consisted of a lymph node homogenate from a pig with PMWS. Virus from the homogenate was titrated on swine testicle (ST) cells in quadruplicate on a 96 well plate. Three days after infection, the cells were fixed and stained with FITC-labeled anti-PCV (VMRD). The 50% tissue culture infectious dose per ml ($TCID_{50}$/ml) was calculated according to the Reed-Muench method (47). The concentration of virus in the challenge homogenate was determined to be approximately 10^8 $TCID_{50}$/ml. The inoculum was negative for other common pathogens, such as porcine parvovirus and swine influenza virus, but was positive for PRRSV. The homogenate was filtered to remove bacteria. The rationale for using a homogenate was based on the inability to obtain significant quantities of PCV2b in culture. PRRSV and other heat labile infectious agents were removed by heat-inactivation at 60^o C for 30 minutes. Pigs were challenged intranasally with approximately 10^5

$TCID_{50}$ of PCV2. For dual challenge, 10^5 $TCID_{50}$ of PRRSV was added back to the PCV2 inoculum. All pigs were monitored for clinical signs and bled weekly. Sera were assayed for PCV2 and PRRSV nucleic acid and virus-specific antibodies by the Kansas State Veterinary Diagnostic Laboratory (KSVDL) using standard molecular and serological diagnostic techniques. The experiment was terminated at six weeks after virus challenge. Sera samples used for this study were obtained at the conclusion of the experiment or five weeks after infection and seven weeks after vaccination.

Selection of PCVAD pigs

PMWS and PDNS pigs were submitted as diagnostic cases to the KSVDL. The diagnosis of PDNS or PMWS was made as previously described (20). The PMWS pigs presented as emaciated with greatly enlarged superficial inguinal lymph nodes. Histologically, secondary lymphoid organs were almost completely depleted of lymphocytes. PCV2 antigen, as determined by diagnostic immunohistochemistry (IHC) and PCR, was present in histological lesions. PDNS pigs were identified by the presence of multi-focal erythematous lesions on the hindquarters. At the anatomical level, the kidneys were greatly enlarged with cortical petechiae covering the kidney surface. The glomeruli were swollen and fibrinous with necrosis of glomerular tufts. All pigs were confirmed to be infected with a PCV2b virus. For the purposes of this study, ten PDNS and ten PMWS sera samples were selected.

PCV2 IFA and measurement of virus neutralizing activity

Total PCV2 antibodies were measured by indirect fluorescent antibody (IFA) staining of infected cells. Rapidly dividing ST cells, maintained in MEM with 10% FBS and antibiotics, were infected with a laboratory isolate of PCV2 on 96 well plates. After three days, the plates were fixed for 10 min in 80% acetone. An initial dilution of 1:40 was followed by 1:2 dilutions

of each serum sample. Samples were diluted in PBS with 5% FBS (PBS-FBS) and incubated with antigen for two hr at room temp. After washing in PBS, bound antibody was detected with FITC-labeled anti-pig antibody (Jackson Labs) according to manufacturer's recommendations. Plates were read on an inverted fluorescence microscope, and titers calculated as the reciprocal of the last serum dilution that showed fluorescence staining. For measurement of virus neutralizing activity, serial dilutions of serum in 100 ul of cell culture medium were mixed with a constant quantity of PCV2 virus (50-300 TCID50), incubated for 1 hour at 37oC and placed onto four replicate wells of one day old ST cells in 96 well plates. Plates were incubated for three days at 37oC and then fixed and stained with FITC-labeled anti-PCV2 (VMRD, Inc.). The log2NA50 endpoint was calculated by the method of Spearman and Karber.

Cloning, expression and purification recombinant PCV2 polypeptides

Previous immunological studies identified several antibody recognition sites in CP, including at least one immunorelevant epitope (26, 33, 55). The epitope recognition regions from these previous studies were combined and summarized in Fig. 2-2. From these studies, we located four general antibody recognition regions, labeled A (51-84), B (113-131), C (161-207), and D (228-233). The CP amino acid sequence of the PCV2 virus used to infect pigs is the same PCV2b genotype as the CP used to prepare polypeptide fragments (GenBank accession# HQ713495). CP was 99% identical to the CP of the PCV2b used for experimental infection. There were two amino acid substitutions; a phenylalanine to asparagine at position 46 and a phenylalanine to aspartic acid at position 115. To prepare recombinant CP polypeptides, primers were designed to amplify regions of CP containing one or more of the antibody recognition regions. The 5' end of the forward and reverse primers contained additional Sac II and Hind III restriction sites, respectively. The primers used for amplification and cloning of the individual

ORF2 cDNA fragments are listed in Table 2. PCR products were cloned into PCR2.1 Topo (Promega) and plasmids propagated in *E. coli*. Plasmids were cut with Sac II and Hind III and the inserted DNA was cloned in frame into the Sac II and Hind III sites in the *E. coli* expression vector, pHUE (6). The pHUE vector is designed to yield a 6xHis ubiquitin fusion protein. For recombinant protein expression, *E. coli* were grown in LB plus ampicillin at 37°C until bacteria reached an OD_{600} = 0.4-0.6. Protein expression was induced by the addition of IPTG (1.0 ug/ml final concentration) and cultures grown for an additional 4 hr. Bacteria were harvested and proteins purified using a PrepEase His-Tagged Protein Purification Kit (USB) according to the manufacturer's protocol. A modification of the procedure included the addition of urea at a final concentration of 8 M to the bacteria disruption and Ni column elution buffers. After elution from the nickel column, the protein containing-fractions were concentrated on a 5,000 MW cut-off spin column (Millipore) and total protein measured using Protein Assay (Bio-Rad) according to the manufacturer's protocol. The purified proteins were visualized by SDS-PAGE.

CP polypeptide and oligopeptide ELISA

Commercially prepared 20-mer oligopeptides (21^{st} Century Biochemicals) were conjugated to BSA by the manufacturer. For the purpose of conjugation to BSA, a cysteine was added to the N or C-terminal end of the oligopeptide. Each CP polypeptide or BSA-conjugated oligopeptide was diluted to 4 μg/ml in 0.05 M carbonate binding buffer (pH 9.6) and 100 μl added to each well of an ELISA plate (Costar). Antigen-coated ELISA plates were incubated at 4^0C for approximately15 hours. Wells were washed 3X with PBS 0.01% Tween 20 (PBST) then blocked for at least 1 hr at room temp in PBS with 10% goat serum (PBS-GS). Next, 100 μl of 1:100 dilutions of pig sera in PBS-GS were added to the plates, then incubated for two hr at room temp. After washing with PBST, 100 μl of peroxidase-labeled goat anti-swine antibody

(Accurate Chemical & Scientific Corp.) diluted 1:2000 in PBS-GS was added to each well and incubation continued for one hr at room temp. After extensive washing with PBST, peroxidase activity was detected by addition of 100 µl of the chromagenic substrate ABTS (KPL). Following a 20 min incubation away from light, absorbance values were read at 405nm using a maxline microplate reader (Molecular Devices Corporation).

Binding ratio calculation

To compare results across ELISA plates, each ELISA plate included an internal positive control, which consisted of wells coated with the largest CP polypeptide (CP(43-233)) incubated with a serum from a PDNS pig with a high PCV2 antibody titer. The antibody binding ratio was calculated as the A405 value of the test sample minus background divided by the A405 value of the internal positive control minus background. Antibody binding ratios for samples and control were derived from a 1:100 dilution.

Field vaccination study

For the purpose of determining antibody responses to CP(43-233) and CP(169-180) before and after vaccination, samples were collected from a 150 head sow source herd that was being vaccinated for the first time. The source of the vaccine was a baculovirus-expressed ORF2 PCV2 vaccine: the same vaccine used to experimentally vaccinate pigs in the experimental study. Pigs were vaccinated at three and six weeks of age according the vaccine label instructions. Blood was collected from 33 randomly selected three week-old pigs derived from 10 different dams. The same pigs were bled a second time at 16 weeks of age or 10 weeks after administration of the last vaccine dose. Sera were reacted with CP(43-233) and CP(169-180) in an ELISA as previously described.

Results

Expression and immunoreactivity of CP polypeptides

The largest CP fragment contained the full-length protein minus the first 42 amino acid residues. The removal of the first 42 amino acids, which contains the arginine-rich domain, increases polypeptide expression in bacteria (54). Analysis of CP(43-233) on SDS PAGE showed a single protein band that migrated at the predicted molecular weight (data not shown). The remaining ORF2 constructs, CP(43-135), CP(43-160), CP(43-180), CP(91-160), CP(160-233), CP (135-233) and CP(91-233) were cloned into pHUE, expressed and affinity purified. Unlike CP (43-233), the smaller polypeptides were retained within the bacteria as insoluble inclusion bodies. Therefore, 8 M urea was added to the extraction and purification buffers. SDS PAGE confirmed that all recombinant polypeptides were expressed and possessed the predicted size (data not shown). CP polypeptides were coated directly onto ELISA plates as His-ubiquitin fusion proteins. As mentioned above, urea was required to solubilize the polypeptides with the exception of the largest CP(43-233). The synthesis of a truncated protein combined with the presence of urea likely disrupted conformational epitopes within CP; however, all polypeptides demonstrated the ability to react with sera from diseased and infected pigs (see below).

The first antibody measurements were performed on sera from experimentally infected pigs (described in Table 1). To reduce variation in results across ELISA plates, each ELISA plate included an internal positive control serum reacted with CP(43-233). The results in Fig. 2-3A showed only low binding ratios for the uninfected control pigs. The same serum samples were also negative for PCV2 antibody as measured by IFA and negative for PCV2 DNA by PCR (data not shown). The results confirmed that the control pigs remained negative for PCV2 infection during the experiment. The antibody binding ratios for pigs infected with PCV2

and PCV2-PRRSV are shown in Fig. 2-3B. Since there was no significant difference in the antibody responses between the pigs infected with PCV2 and dual infected with PCV2-PRRSV, the data for both groups were combined. All PCV2 challenged pigs were positive for PCV2 infection by PCR and for PCV2 antibody by IFA, which confirmed that all pigs were productively infected. PRRSV RT-PCR and serology confirmed that all dual-infected pigs were productively infected with PRRSV. Seven of the 11 pigs in this group showed no clinical evidence of PDNS or PMWS. Three pigs in the dual-challenged group died and were not included in the study. The remaining pigs showed some disease signs including reduced weight gain and accumulation of PCV2 antigen in lymph nodes (data not shown). As shown in Fig. 2-3B, the PCV2 group showed measurable antibody activity against all polypeptides by at least one pig; however, the highest mean binding ratio was obtained for the full-length CP(43-233) polypeptide and the lowest binding for CP(43-135), CP(43-160), and CP(91-160). The remaining polypeptides, CP(91-233), CP(135-233), CP(160-233), and CP(43-180) possesses mean ratios that were intermediate between CP(43-233) and the low-responding polypeptides. The responses for the seven pigs receiving only the PCV2 vaccine are shown in Fig. 2-3C. All vaccinated pigs were positive for PCV2 antibody by IFA and were confirmed to be negative for PCV2 by PCR (data not shown). The highest binding ratios were observed for the CP(43-233) polypeptide, with only background levels of binding against the smaller CP fragments. The only exception was CP(43-180), which showed a small, but significant increase in binding relative to the other small polypeptide fragments. In order to demonstrate that vaccinated pigs were protected, 14 vaccinated pigs were challenged with PCV2 (seven pigs) or PCV2 plus PRRSV (seven pigs). All challenged pigs remained clinically normal with no mortality and were negative for PCV2 DNA in serum (data not shown).

Serological response of pigs with PCVAD

Currently, there are no PCV2 experimental infection models that consistently reproduce all of the elements of PMWS or PDNS. Therefore, sera from pigs submitted to the KSVDL with a diagnosis of PCVAD were evaluated for recognition of CP polypeptides. Even though PCV2 may not be the proximal cause of PDNS (25), pigs with PDNS typically possess high levels of PCV2 antibodies (56). As shown in Fig. 2-4A, nine of ten PDNS pigs possessed high binding activity against CP(43-233). Overall, relatively high levels of antibody binding were observed for seven of the eight polypeptides, with the highest mean antibody binding ratio against the smallest polypeptide, CP(160-233). Significantly lower binding ratios were observed for CP(43-135) and CP(43-160). The results for sera from ten pigs diagnosed with PMWS are shown in Fig. 2-4B. Unlike the PDNS pigs, overall binding ratios against the CP fragments were relatively low for a majority of the pigs. Two of the ten pigs showed elevated binding ratios against CP(43-233), while four pigs exhibited only background activity against CP(43-233), and the remaining four showed intermediate activities. The overall low binding ratios against CP(43-233) likely reflect decreased antibodies as a result of the overall depletion of lymphocytes, which occurs during end-stage PMWS. Responses against the other oligopeptides were also variable, except for CP(43-135) and CP(43-160), which were negative for binding.

A summary of the antibody binding activities of the different groups of pigs against the different CP fragments is presented in Table 3. For PCV2-infected, PMWS, and PDNS pigs, the highest levels of antibody binding were primarily obtained for CP polypeptides that contained Epitope C (see Fig.

Pepscan mapping of the C-terminal region of CP

Further studies were performed to determine the smallest oligopeptide recognized by PCV2-infected pigs. Based on the results of Table 3, 20-mer oligopeptides, with 10 overlapping residues, spanning Epitope C and the flanking region (residues 141-200) were prepared and reacted with sera from PCV2-infected and PDNS pigs. The results for the experimentally infected PCV2 pigs showed a large variation in binding activity. Closer inspection of the data revealed that pigs could be divided into two groups. For instance, as presented in Fig. 2-5A, four of the 11 serum samples showed minimal binding activity against all oligopeptides (gray bars); similar to the response of vaccinated and uninfected control pigs (data not shown). The remaining PCV2-infected pigs exhibited a pattern shown by the black bars, with relatively high activity against the CP(161-180) and CP(169-188) oligopeptides and lower binding against the flanking oligopeptides. The combined region CP(161-188) is within the Epitope C region. Two 12-mer oligopeptides covering the region overlapped by CP(161-180) and CP(169-188) were prepared and tested for antibody binding. The BSA-conjugated oligopeptides, CP(169-180C) and CP(169-180N), were constructed with an aminohexonic acid (Ahx) spacer added to the C or N-terminal end, respectively. The spacer fragment was designed to increase antibody accessibility by extending the oligopeptide beyond the surface of the BSA molecule. Both oligopeptides showed binding activities similar to the CP(161-180) and CP(169-188) parent oligopeptides. Results for sera from PDNS pigs are shown Fig. 2-5B. Similar to the responses shown by black bars in Fig. 2-5A, the highest binding activities for the PDNS pigs were against CP(161-180), CP(169-188), and CP(169-180).

Alanine scanning mutagenesis in CP(169-180)

To identify the individual amino acids that contributed to antibody recognition within CP(169-180), single alanine substitutions were made along the CP(169-180) peptide sequence, 169-STIDYFQPNNKR-180. The results for the subset of experimentally infected PCV2 pigs that showed high binding against CP(169-180) in Fig. 2-5A are presented in Fig. 2-6A. The results showed reduced binding for oligopeptides with alanine substitutions at tyrosine-173, phenylalanine-174, glutamine-175 and lysine-179. Similar alanine scanning results were obtained for sera from PDNS pigs (Fig. 2-6B).

Four hundred and sixty-two CP(169-180) peptide sequences, obtained from GenBank and diagnostic lab submissions, were analyzed for amino acid differences. The results, presented in the lower portion of Fig 2-6A, showed that 45 of the 462 sequences possessed changes within CP(169-180): all were single amino acid substitutions. For those residues that contributed to binding, tyrosine-173, phenylalanine-174, glutamine-175 and lysine-179, there were only two amino acid substitutions. Therefore, the core peptide region that forms the epitope within CP(169-180) is highly conserved.

Virus neutralizing activity in PCV2 infected and vaccinated pigs

Virus neutralizing activity in serum samples from the same PCV2 pigs in Fig. 2-2B, and vaccinated pigs in Fig. 2-2C is shown in Fig. 2-7. The mean total antibody level for PCV2-infected pigs (IFA titer = 6.7), as measured by IFA, was almost twice the level of the mean value for the vaccinated pigs (IFA titer = 5.8). In contrast, the mean NA level for vaccinated pigs ($log2NA_{50}$ =2.8) was approximately four times higher than the mean level obtained from sera of PCV2-infected pigs ($log2NA_{50}$ =1.6).

Serological response to CP_169(180) following PCV2 vaccination in the field

We determined the antibody responses to CP(43-233) and CP(169-180) before and after vaccination of a small pig herd that had a previous history of clinically apparent PCV2 infection, which was confirmed by serology and PCR (data not shown). The results, summarized in Fig. 2-8, showed that the antibody responses of the three week-old pigs were directed against both CP(43-233) and the CP(169-180) oligopeptide. The presence of CP-specific antibody in pre-vaccination pigs is primarily the result of maternally-derived antibodies from dams that had been naturally infected with PCV2 (19). At 16 weeks of age (10 weeks after the second vaccine dose) the pattern of antigen recognition changed, with the response against the CP(169-180) greatly diminished relative to CP(43-233). The effectiveness of vaccination was confirmed by the disappearance of clinical disease from the herd and by the decreased prevalence of PCR positive serum samples. Together, the results illustrate a pattern showing the initial presence of antibodies derived from natural infection, which were replaced with CP(43-233)-specific antibodies generated in response to vaccination with CP.

Discussion

Previous studies of humoral immunity during PCV2 infection have primarily focused on mapping antigenic regions of CP, including the characterization of immurelevant epitopes (26, 33, 55). The present study describes differences in the antibody responses towards PCV2 CP immunoreactive regions following vaccination and experimental infection, and during severe disease. The results illustrate the complexity of the immune response during PCV2 infection, while providing information on the immunological basis for protection during vaccination and the initiation of immunopathogenesis. Analysis of antibody reactivity of infected, vaccinated and PCVAD pigs against individual CP polypeptides fragments and oligopeptides identified at

least four unique antibody recognition patterns, which are summarized in Table 3. The first pattern is illustrated by pigs with severe PMWS. Overall, there was a descreased reactivity to all CP polypeptide antigens (Fig. 2-4B). This outcome is consistent with the immune suppression associated with PMWS, a disease syndrome characterized by an almost complete depletion of lymphocytes with a corresponding loss or severe dysregulation of immune function (13). Immunohistochemical staining of PMWS lymph nodes typically shows large accumulations of PCV2 antigen (8). Presumably, dividing lymphocytes, activated in response to infection or other immune stimuli, become a primary target for PCV2 replication. Cytopathogenesis in lymphocytes is attributed to the function of PCV2 ORF3, which is not required for virus replication in culture, but has been linked to apoptosis (21, 22, 28). In sharp contrast, pigs with clinically apparent PDNS showed high reactivity to all CP polypeptide fragments (Fig. 2-4), including the oligopeptide CP(169-180; Fig. 2-5B). This outcome is consistent with hyperactive humoral response and immune complex formation. Pathogenesis is linked to the deposition of antigen-antibody complexes in the kidney and other organs followed by the activation of complement (56). The role that PCV2 plays in PDNS remains unclear. Krakowka et al. (25) reported the induction of PDNS in gnotobiotic pigs after infection with a group 1 torque teno virus (TTV) and PRRSV. To date, there are no models of PCV2 infection that reproduce PDNS. One likely possibility is PCV2 infection may not be the proximal cause of PDNS, but may play a role in the evolution of the disease process and expression of full-blown disease.

A third pattern of antibody recognition was found in the response of pigs experimentally infected with PCV2. The results showed the highest antibody response to CP(43-233) followed by reactivity with polypeptides that contained Epitope C. A dichotomy in the response to the Epitope C region was evident by the oligopeptide mapping results, in which

PCV2 pigs could be divided into two groups: those that produced a response similar to PDNS pigs and recognized CP(169-180), and those that responded similar to vaccinates. And finally, a fourth antibody response pattern was found in pigs vaccinated with a baculovirus-expressed CP antigen. Vaccinated pigs almost exclusively recognized the largest CP(43-233) polypeptide and much lower responses to smaller polypeptides, including those polypeptide fragments that contained Epitope C (Fig. 2-3C). This pattern of antibody recognition suggests that vaccination produces antibodies that primarily recognize a single large conformational epitope. Evidence for the protective nature of this response was found in the complete protection of vaccinated pigs challenged with PCV2 or PCV2 and PRRSV, and suggests that vaccination with only CP is sufficient to deliver sterilizing immunity.

The principal difference between PCVAD and vaccinated pigs located to CP(169-180), within Epitope C. PDNS and a subset of PCV2 pigs preferentially recognized CP(169-180). To further demonstrate the specific nature of the recognition, alanine scanning mutagenesis identified specific residues as important for antibody recognition. Furthermore, the key amino acid residues involved in antibody binding are highly conserved among PCV2 isolates. The significance of this epitope in disease progression is not entirely clear. However, the results suggest that antibodies directed against this epitope are not involved in immune protection. Protection from infection and disease is likely dependent on antibodies directed against a single, conformational epitope. The results support the hypothesis that antibodies preferentially directed against Epitope C are non-protective, and signal the initial immune defect that leads to disease. One possibility is that Epitope C functions as a decoy epitope, allowing PCV2 to evade humoral immunity by focusing the antibody response towards a non-protective epitope. Evidence for this possibility is found in the total and neutralizing antibody responses of PCV2-infected versus CP-

vaccinated pigs (see Fig.2- 7). Even though the total PCV2 antibody response of infected pigs was almost twice the response of vaccinated pigs, the mean neutralizing activity for the vaccinated group was almost four times that of the infected group. Therefore, the apparent paradox of decreased neutralizing activity in the face of an overall robust humoral response can be resolved if the response is directed towards non-neutralizing epitopes. The diversion of humoral immunity away from the larger neutralizing epitope is a strategy that has been proposed for HIV and PRRSV (27, 46).

One interesting aspect of PCV2 infection is that only a subpopulation of infected pigs go on to develop full-blown disease, while other infected pigs remain unaffected. The mixed antibody response of PCV2-infected pigs provides insight into a possible mechanism for differences in disease susceptibility within the same population of PCV2-infected pigs. For example, those pigs that respond to PCV2 in a manner similar to the response of pigs following vaccination, produce an effective antibody response that results in virus clearance and protection. In contrast, those pigs that produce a response similar to PDNS pigs; i.e., against non-protective epitopes, are more susceptible to prolonged virus replication and disease.

A practical application of the results from this study is illustrated by the data obtained for herd vaccination, shown in Fig. 2-8. Prior to vaccination, maternally derived antibody (MDA) acquired during natural infection, showed reactivity to both CP(43-233) and CP(169-180), a pattern similar to experimental PCV2 infection. After vaccination and decay of MDA, the antibody recognition pattern switched to recognition of only CP(43-233). Therefore, differential recognition of CP(43-233) and CP(169-180) provides the basis for diagnostic approaches that can assess the level of protective antibody in a vaccinated herd, as well as methods that can differentiate infected from vaccinated animals (DIVA).

Acknowledgements

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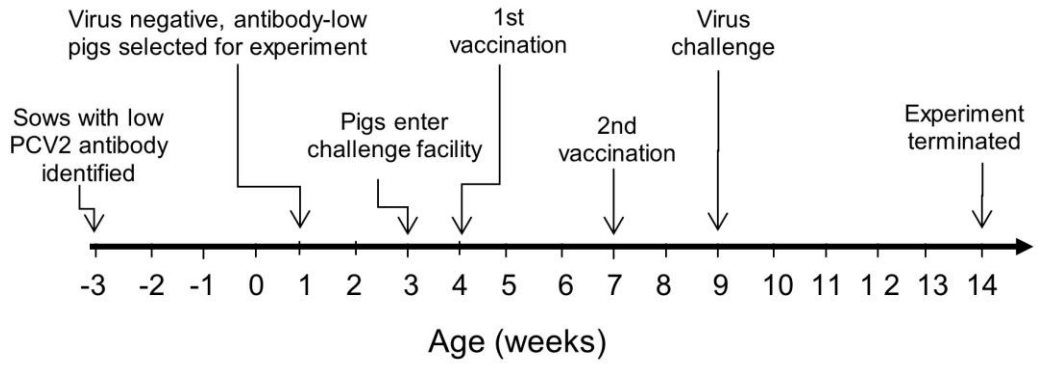


Figure 2-1. **Timeline for experimental PCV2 vaccination and infection.**

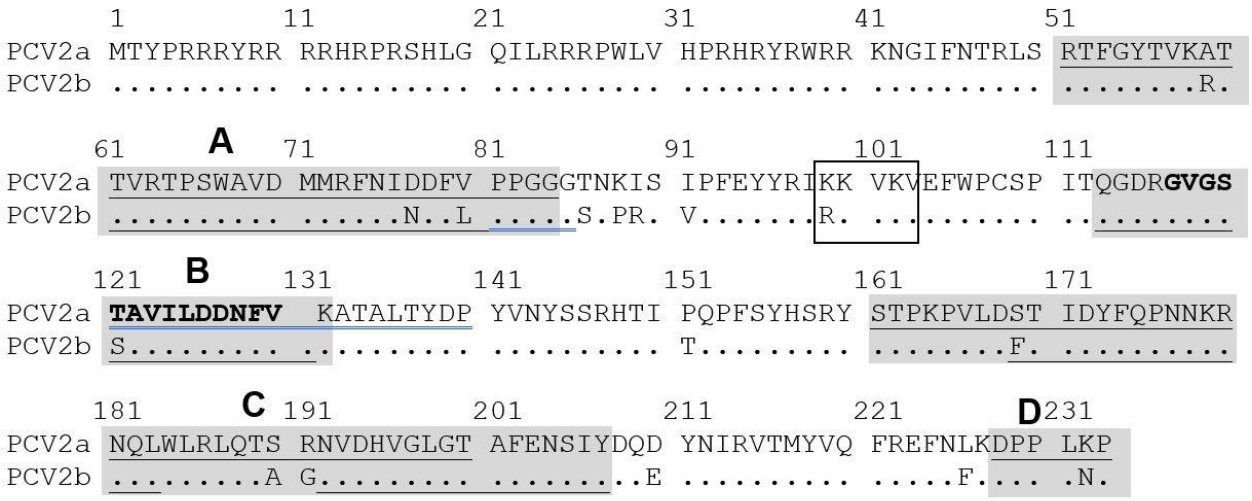


Figure 2-2 Location of immunoreactive regions in PCV2 CP.

Underlined regions in the PCV2a genotype peptide sequence show the locations of the immunoreactive regions described by Lekcharoensuk et al.,(25) using mAb's prepared against whole purified virus (strain ISU 31, GenBank accession number AJ223185). The underlined regions in the PCV2b genotype sequence (GenBank accession number AF201311) summarize the results obtained from the pepscan analysis by Mahe et al. (32). The epitope maps are

combined to yield the shaded peptide sequences, identified as epitope regions A, B, C and D. The box identifies the putative receptor binding region described in Misinzo et al. (35).

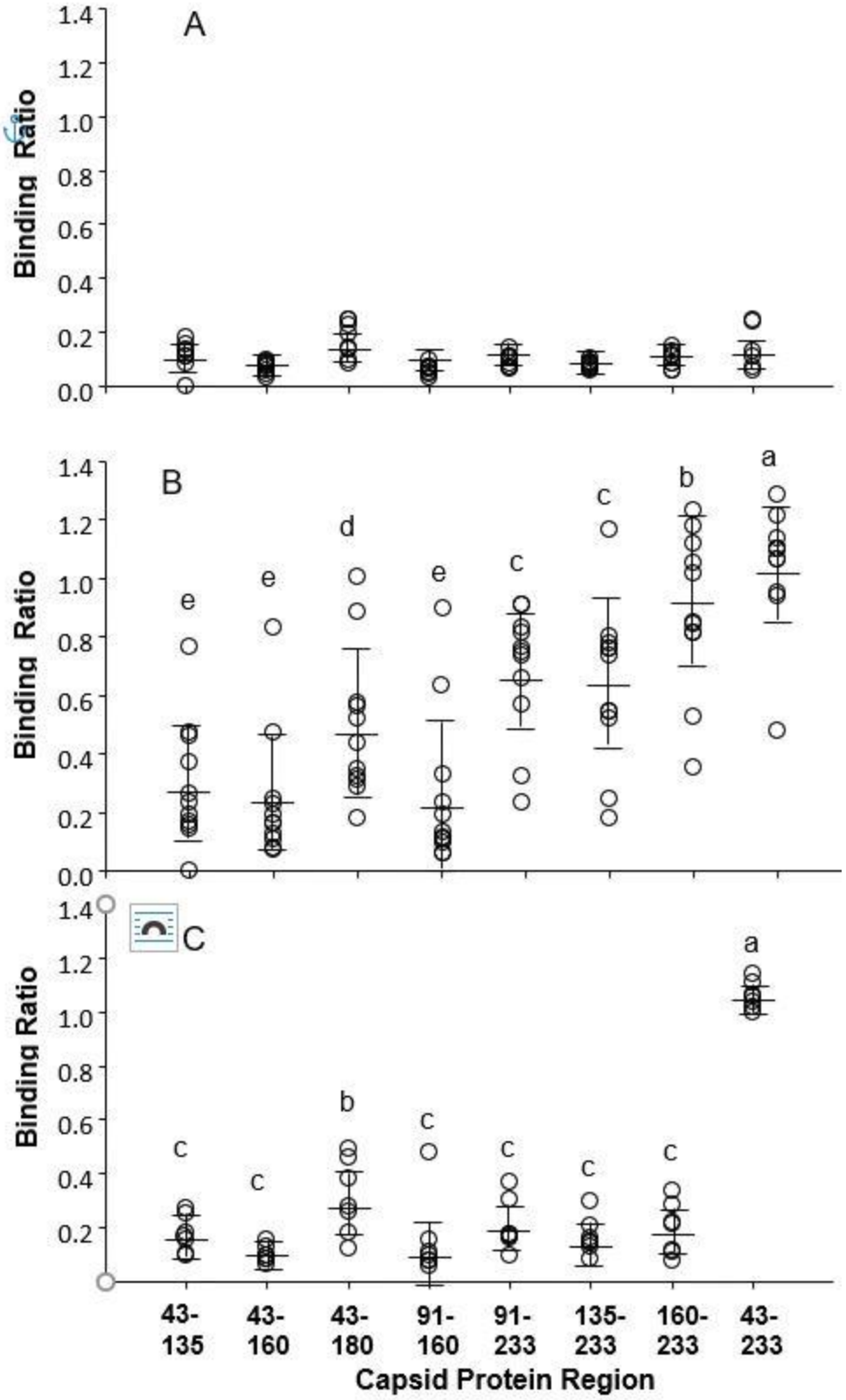

A
B
C
Binding Ratio
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
d
e
43-135
43-160
43-180
91-160
91-233
135-233
160-233
43-233
Capsid Protein Region

Figure 2-3 Immunoreactivity of bacteria-expressed polypeptides with sera from experimentally PCV2 infected and vaccinated pigs.

The ELISA results show the response of sera from PCV2-negative (A), PCV2-infected (B) and CP-vaccinated (C) pigs obtained at five weeks after experimental infection with PCV2, or seven weeks after vaccination as described in the timeline shown in Fig. 2-1. The antibody binding ratio was calculated as described in Materials and Methods. The open circles show the response of individual pigs. The means and standard deviations are represented by horizontal lines. Means with the same lower case letter do not significantly differ as determined by the Student-Newman-Keuls method ($P<0.05$).

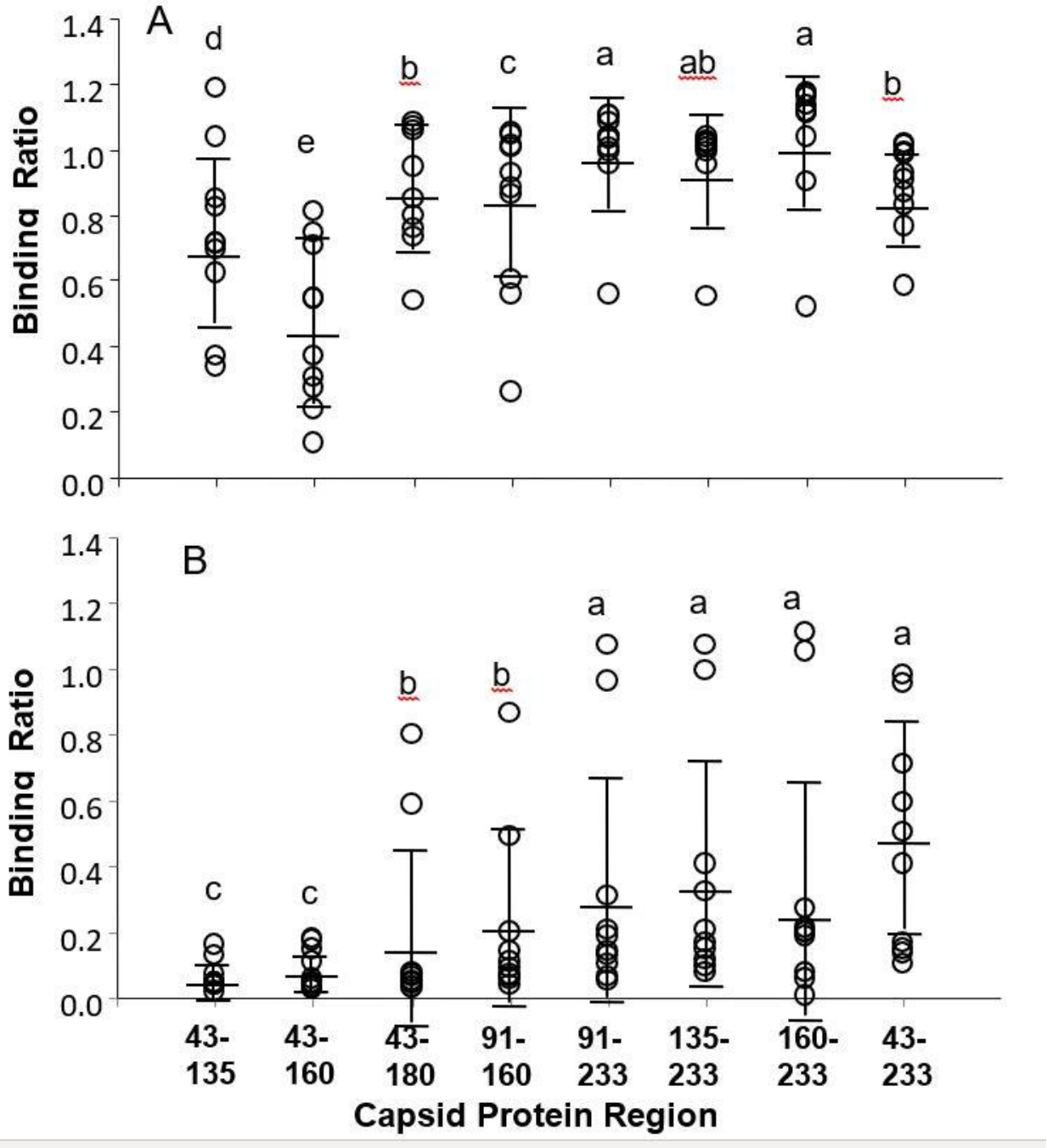


Figure 2-4 Immunoreactivity of PCV2 CP polypeptides with sera from pigs with PDNS and PMWS.

The same ELISA method described in Fig. 2-3, was used to assess antibody activity in sera from pigs diagnosed with PDNS (A) or PMWS (B). The open circles show the response of individual

pigs. The horizontal lines show means and standard deviations. Means with the same lower case letter do not significantly differ as determined by the Student-Newman-Keuls method ($P<0.05$).

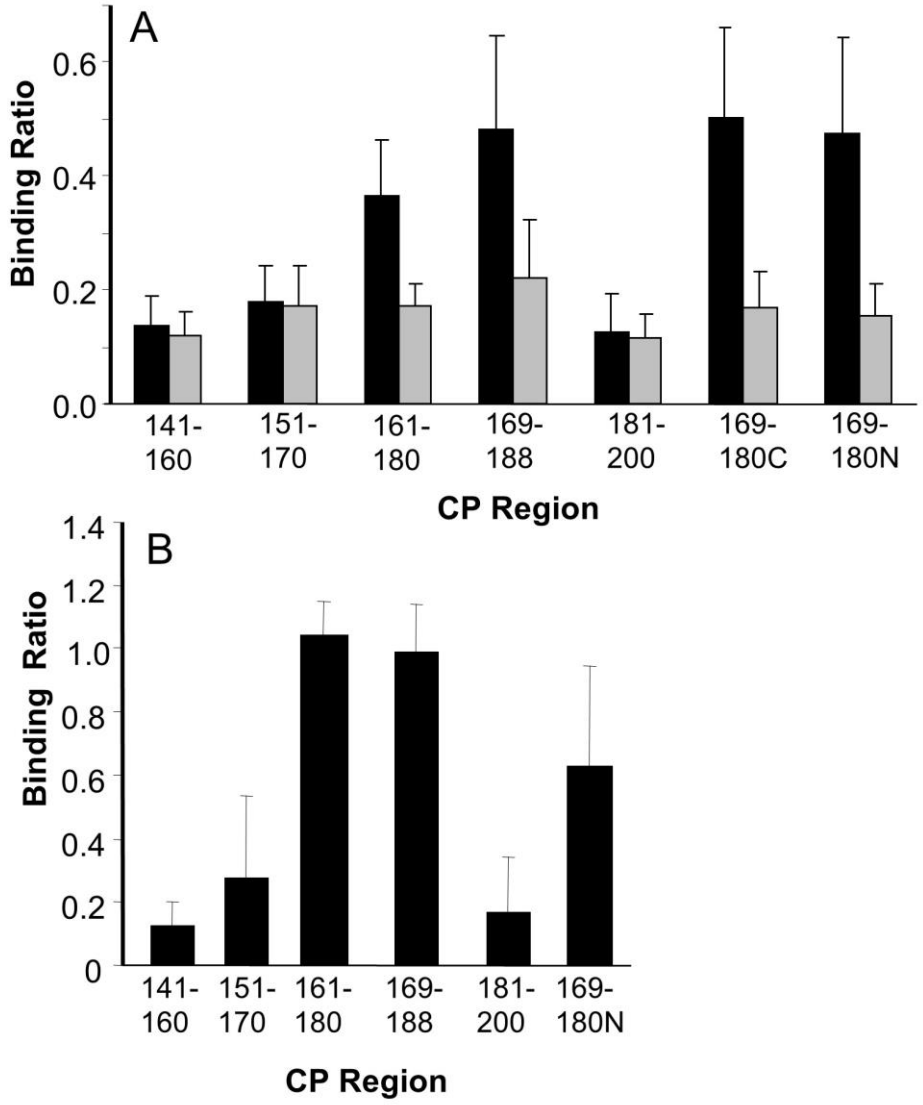


Figure 2-5 Immunoreactivity of pig sera with CP oligopeptides.

Panel A shows the antibody response of experimentally infected pigs against a series of 20mer overlapping oligopeptides that cover residues 141-200 of the PCV2 CP. The gray bars show the mean and standard deviation for a subset of four pigs that possessed low antibody levels of

binding against all oligopeptides. The black bars show the mean and standard deviation for the remaining six pigs. Panel B shows the response of sera from PDNS pigs. The results show the mean and one standard deviation of the antibody binding ratio for each group of pigs.

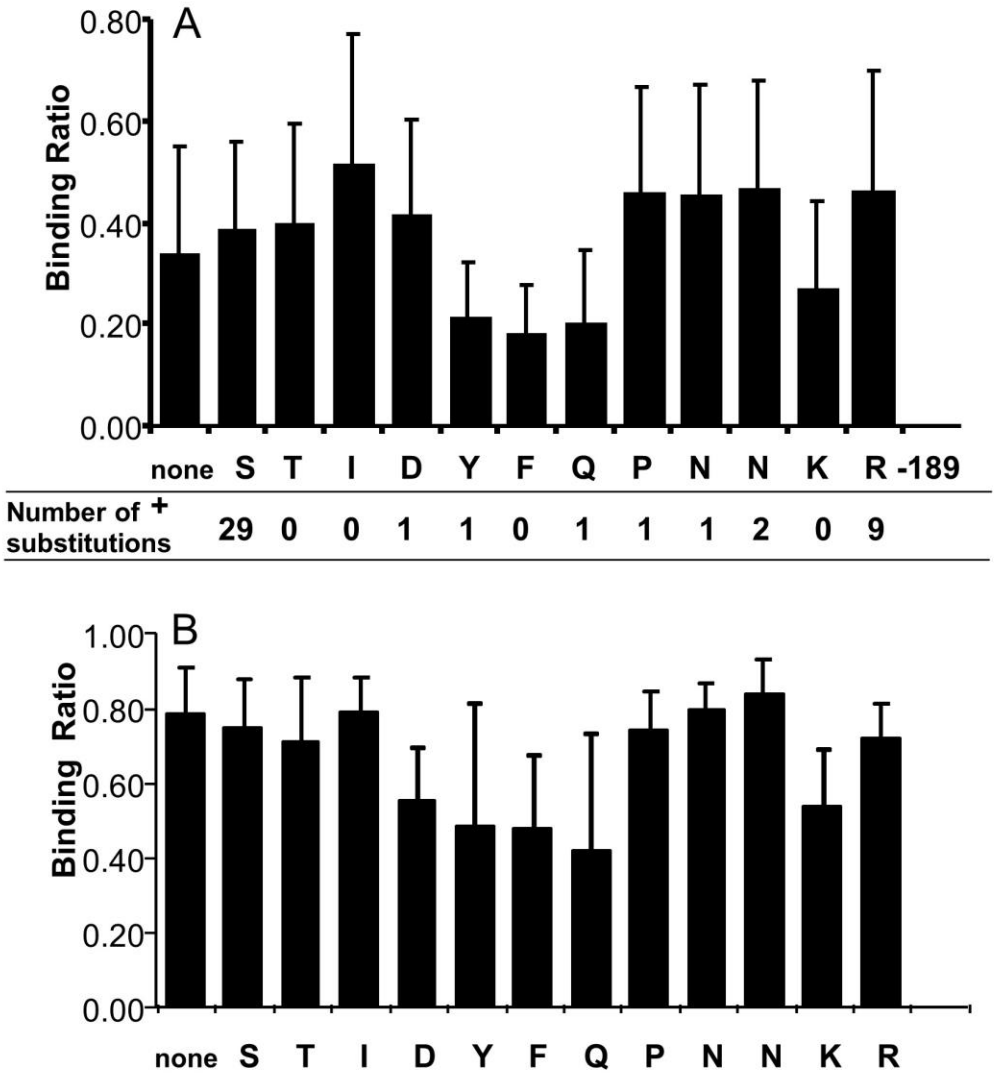


Figure 2-6 Alanine scanning mutagenesis.

Panel A shows the mean and standard deviation results for the same six pigs in Fig. 2-5A (black bars) that recognized CP(169-180). BSA-conjugated oligopeptides that possessed single alanine

substitutions were tested. The numbers below (A) show the number of amino acid substitutions found in a comparison of 462 peptide sequences obtained from GenBank and diagnostic lab submissions. Panel B shows the alanine scanning results for the same PDNS pigs shown in Fig. 2-5B.

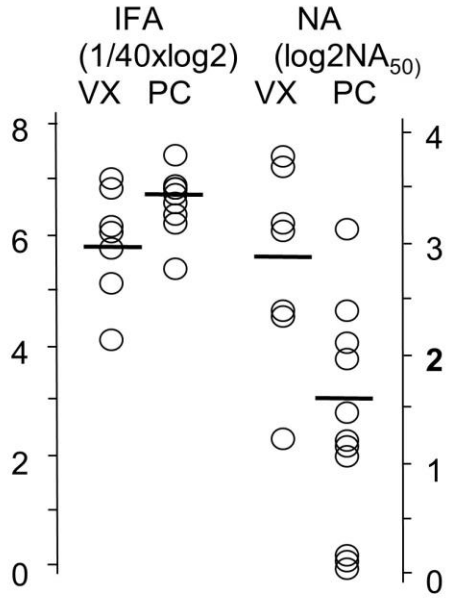


Figure 2-7 Total and neutralizing antibody responses following vaccination and infection.

Serum samples from PCV2-infected (PC) pigs (Fig. 2B) and PCV2 vaccinated pigs (VX; Fig. 2-2C) were tested for the presence of total antibody (IFA) and neutralizing activity (NA). Open circles represent values for individual pigs and the horizontal bar the mean value for each group.

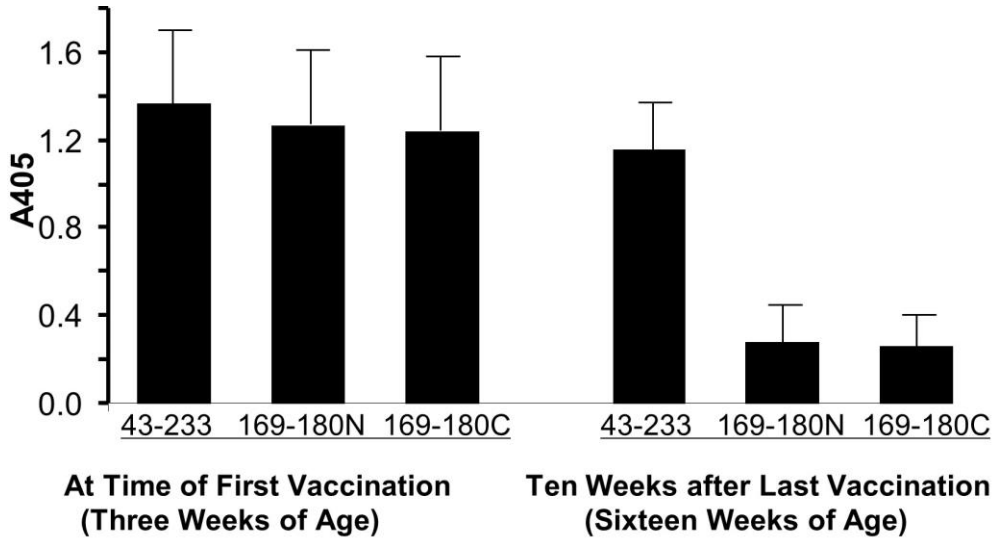


Figure 2-8 Recognition of CP(43-233) and CP(169-180) in a herd before and after PCV2 vaccination.

Results are from a herd vaccinated for PCV2 for the first time. Samples were tested when pigs received the first dose of vaccine (three weeks of age) and the same pigs tested at 16 weeks of age, or 10 weeks after the second vaccine dose. The results are shown as the mean and standard deviation for 33 pigs reacted with CP(43-233) and CP(169-180) oligopeptides. All samples were assayed on a single plate and results (vertical axis) are presented as A405 values. The oligopeptides CP(169-180N) and CP(169-180C) were linked to BSA via the N- or C-terminal end of the oligopeptide, respectively.

Table 2-1 Experimental infection /vaccination treatment groups

Grp	Description	n	Treatment		
			Vaccine	PCV2	PRRSV
1	Control	7	-	-	-
2	Vaccine	7	+	-	-
3	PRRSV	7	-	-	+
4	PCV2	7	-	+	-
5	PCV2-Vaccine	7	+	+	-
6	PCV2/PRRSV	7[a]	-	+	+
7	PCV2/PRRSV-Vaccine	7	+	+	+

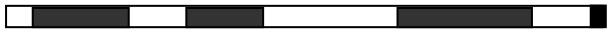
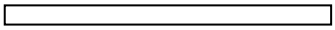
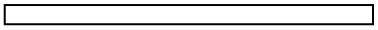
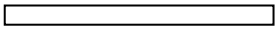
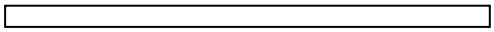
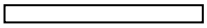
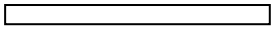
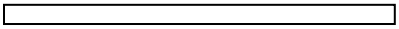
[a]Three of the seven pigs died following infection and were not subjected to further study

Table 2-2 Primer sequences used for preparing CP polypeptides[a]

CP Region (amino acids)	Forward Primer	Reverse Primer
43-135	5'CCGCGGTGGTAATGGCATCTTCAACA	5'AAGCTTTTAGGCTGTGGCCTTTGATA
91-233	5'CCGCGGTGGAGTGCCCTTTGAATACT	5'GCGCAAGCTTTTAAGGGTTAAGTGGC
136-233	5'CCGCGGTGGACTCACCTATGACCCCT	5'GCGCAAGCTTTTAAGGGTTAAGTGGC
91-160	5'CCGCGGTGGAGTGCCCTTTGAATACT	5'AAGCTTTTAGTAGCGGGTGTGGTAGC
160-233	5'CTCCGCGGTGGATACTTTACCCCCAA	5'GCGCAAGCTTTTAAGGGTTAAGTGGC
43-160	5'CCGCGGTGGTAATGGCATCTTCAACA	5'AAGCTTTTAGTAGCGGGTGTGGTAGC
43-180	5'CCGCGGTGGTAATGGCATCTTCAACA	5'GCGCAAGCTTTTAATCTTTTGTTGTT
43-233	5'CCGCGGTGGTAATGGCATCTTCAACA	5'GCGCAAGCTTTTAAGGGTTAAGTGGC

[a]Additional Sac II and Hind III restriction sites are underlined

Table 2-3 Summary of antibody responses to PCV2 capsid polypeptides

Name	Epitope Regions	PC[a]	Vx	PM	PD
	A B C D				
43-233		++++[I]	++++	++	+++
43-135		+	-	-	++
43-160		+	-	-	+
91-160		+	-	+	+++
43-180		++	+	+	+++
160-233		+++	-	+	+++
135-233		++	-	+	+++
91-233		++	-	+	+++

[a]Key: PC = PCV2-infected; Vx = vaccinated; PM = PMWS; PD = PDNS.

[b]Relative binding activity. Key: (-) no measureable binding activity; (+) low binding activity; (++) intermediate binding activity; (+++) high binding activity ; (++++) very high binding activity

Chapter 3 - Antibody responses following vaccination versus infection ina porcine circovirus type 2 (PCV2) disease model show distinct differences in virus neutralization and epitope recognition

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 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 the absence or decreased levels of PCV2 neutralizing antibodies [25–28]. Based on the hypothesis that the immune response 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.

Materials and Methods

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 immunoflourescent 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.

Virus 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 37oC and 5% CO2, the cells were fixed with acetone and stained with fluorescein isothiocyanate (FITC)-labeled porcine anti-PCV (Veterinary Medical Research and Development, Inc.; VMRD). The 50% tissue culture infection dose (TCID50) per gram of lymph node homogenate was calculated by the method of

Spearman and Karber [35]. The homogenate contained approximately 108 TCID50/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 0.22µm 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 -80oC. The quantity of PRRSV was 107 TCID50/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 37oC and 5% CO2; then analyzed for virus induced cytopathic effects. The TCID50/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 60oC for 30 minutes to inactivate PRRSV and other heat-labile viruses and bacteria. For dual challenge, PRRSV was added back to the heat-treated homogenate material. Pigs were challenged intranasally with 105 TCID50 of PRRSV and/or 105 TCID50 PCV2 in 3 ml of MEM.

Immuinohistochemistry 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.

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 1:2,000 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 log2(40x1/dilution).

Four replicate (100 ul each)1:2 serial dilutions of sera were mixed with 100 TCID50 of PCV2b and incubated for 1 hour at 37oC. Well contents were transferred onto day-old ST cells in 96-well plates, incubated for three days at 37oC, 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 log2

50% neutralizing activity per ml (NA) 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 E. coli. Ninety-six well ELISA plates (Costar) were coated with 100 µl of purified CP(43-135), CP(160-233) or CP(43-233) at a concentration of 4 µg/ml and incubated overnight at 4oC. After incubation, plates were washed with PBS containing 0.01% Tween-20 (Sigma-Aldrich; PBST) and blocked for a minimum of 1 hr with PBS containing 10% goat serum (Invitrogen; PBS-GS). After blocking, duplicate sera samples diluted in PBS-GS, were added to wells and incubated for 2 hr at room temp. Plates were then washed and 100 µl 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 hr, the plate was washed and 100 µl 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.

PCR for PCV2 and PRRSV nucleic acud'

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^{TM}-96 Viral RNA Isolation Kit (Applied Biosystems) according to the manufacturer's instructions. Master mixes were prepared using the AgPath ID^{TM} 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.

Statistical methods

Statistical analysis was performed using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, California). IFA titers were $40X\log_2$ 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 $P<0.05$.

Results

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 in 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 weighed at the beginning and end of the study. Mean ADWG for the group 1 (CN) and group 4 (PC) pigs was 0.76+/-0.10kg/day and 0.69+/-0.07 kg/day, respectively. The mean for group 6 (PC/PR) pigs was further decreased to 0.61+/-0.09 kg/day; however, only 4 of the 7 pigs survived to the end of the study. Vaccination prior to dual challenge (group7) showed an increase in ADWG for the group 7 pigs (PC/PR/VX), which was 0.71+/-0.14 kg/day.

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 3-1A. 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+/-0.4 log10 templates/rxn versus 4.7+/-1.1 log10 templates/rxn for group 6 (Fig. 3-1A). 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 with PCV2 (group 5) or PCV2 and PRRSV (group 7).

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. 3-2C). 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. 3-2B). Representative results for control and vaccinated pigs are presented in Fig. 3-2, panels A through 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 challenged with PRRSV remained negative for PRRSV throughout the study.

PCV2 antibody response

Mean PCV2 IFA endpoints for all treatment groups are shown in Fig. 3-3A. 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. 3-3B). The NA levels for all vaccinated groups was approximately 16 (log2=4/ml) compared to 4 (log2=2/ml) for the unvaccinated PCV2-infected groups Together, the results show that vaccination and infection result in similar levels of total antibody, but neutralizing antibody response 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).

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. 3-4A, 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. 3-4B, 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. 3-4C. Antibody reactivity was elevated against CP(160-233) and CP(43-233), but the results were not significantly different between the three polypeptides. Since there was not data on the three pigs that dies, only four pigs were included in the analysis However, vaccination prior to challenge showed a response similar to the vaccine only group.

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 (Fig.3-1 and Fig. 3-2), which support 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/or vaccination results in similar levels of total serum antibody (Fig. 3-3 A). However, vaccination generated approximately four times the amount of PCV2 virus neutralizing activity

compared to infection with PCV2 alone or PCV2 and PRRSV (Fig. 3-3 B). Therefore, increased NA in vaccine 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 and 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 levels (see Fig. 3-4). In contrast, pigs infected with PCV2 or PCV2-PRRSV showed reactivity to both CP(43-233) and CP(160-233) and relatively low NA. 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 fragments produced by PCV2-infected cells. In contrast VLP lack such an epitope. The locations of CP(169-180) within the X-ray crystal structure of the PCV2 CP monomer and virus like particle (VLP) [42] provide further support for this model. In the monomer form of CP, CP(169-180) is exposed on an outer loop allowing access to antibody; 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.

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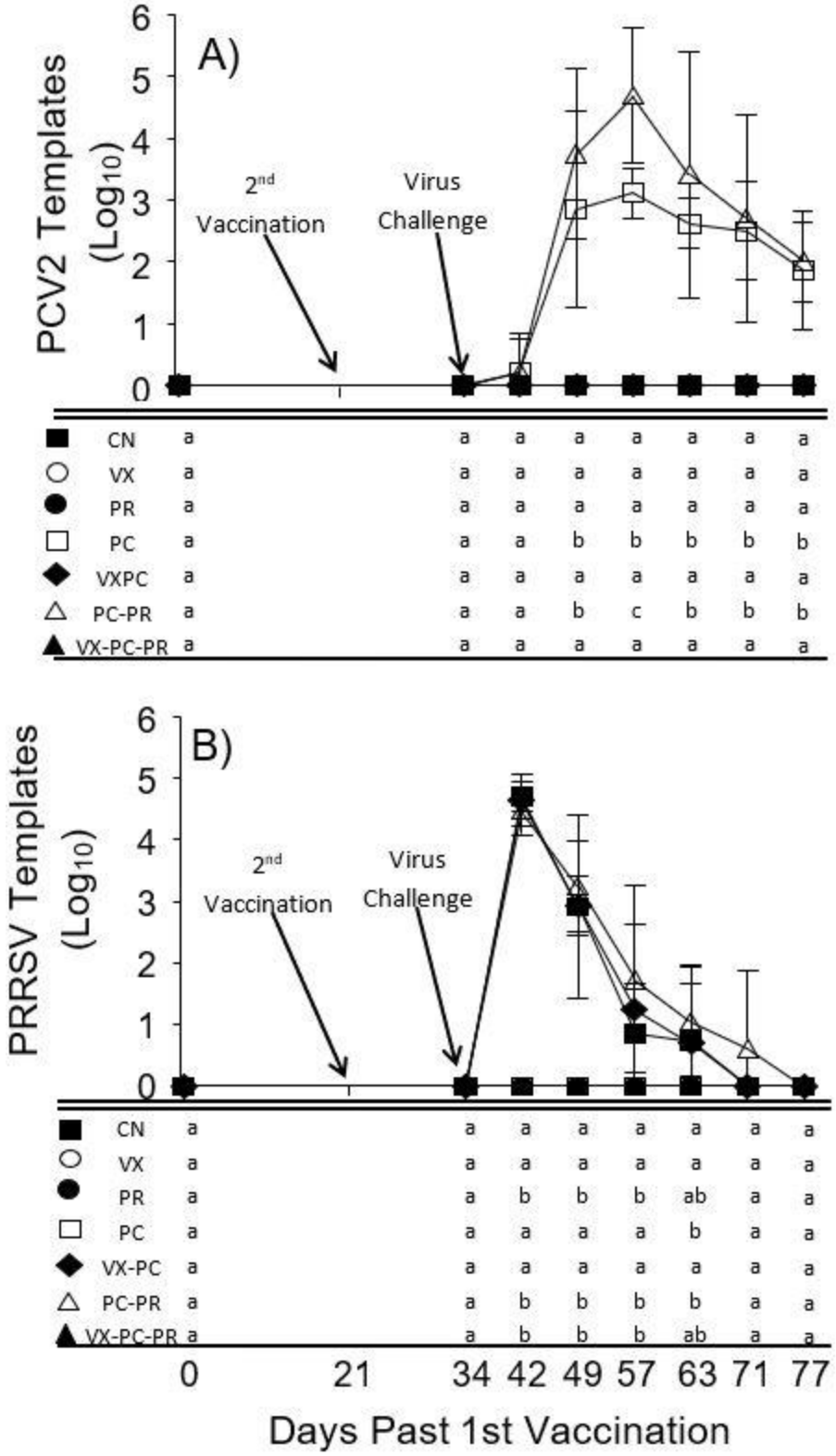

A)
PCV2 Templates (Log10)
2nd Vaccination
Virus Challenge
CN a a a a a a a a
VX a a a a a a a a
PR a a a a a a a a
PC a a a b b b b b
VXPC a a a a a a a a
PC-PR a a a b c b b b
VX-PC-PR a a a a a a a a
B)
PRRSV Templates (Log10)
2nd Vaccination
Virus Challenge
CN a a a a a a a a
VX a a a a a a a a
PR a a b b b ab a a
PC a a a a a b a a
VX-PC a a a a a a a a
PC-PR a a b b b b a a
VX-PC-PR a a b b b ab a a
0 21 34 42 49 57 63 71 77
Days Past 1st Vaccination

Figure 3-1 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).

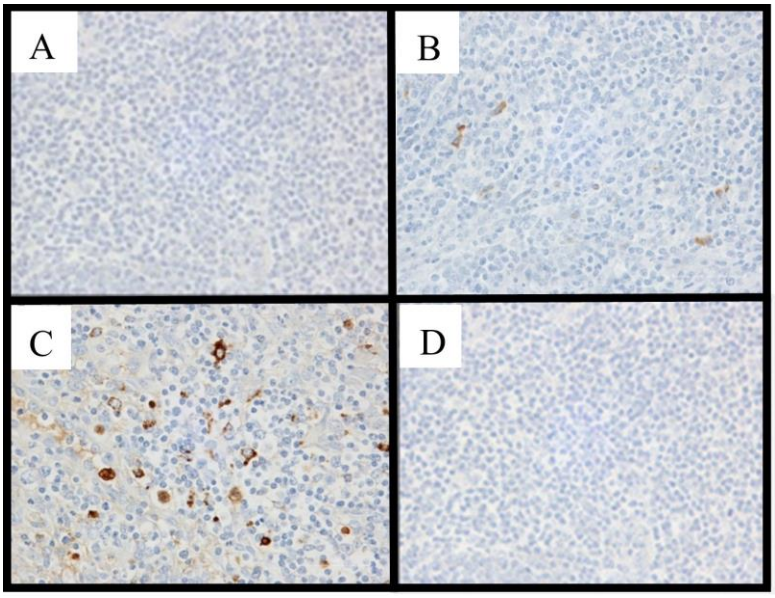


Figure 3-2 PCV2 antigen in lymph nodes.

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 photomicropgraph that is representative for all pigs in group 7 (PC/PR/VX). Except for (C), the results are from lymph nodes collected 44 days after virus challenge.

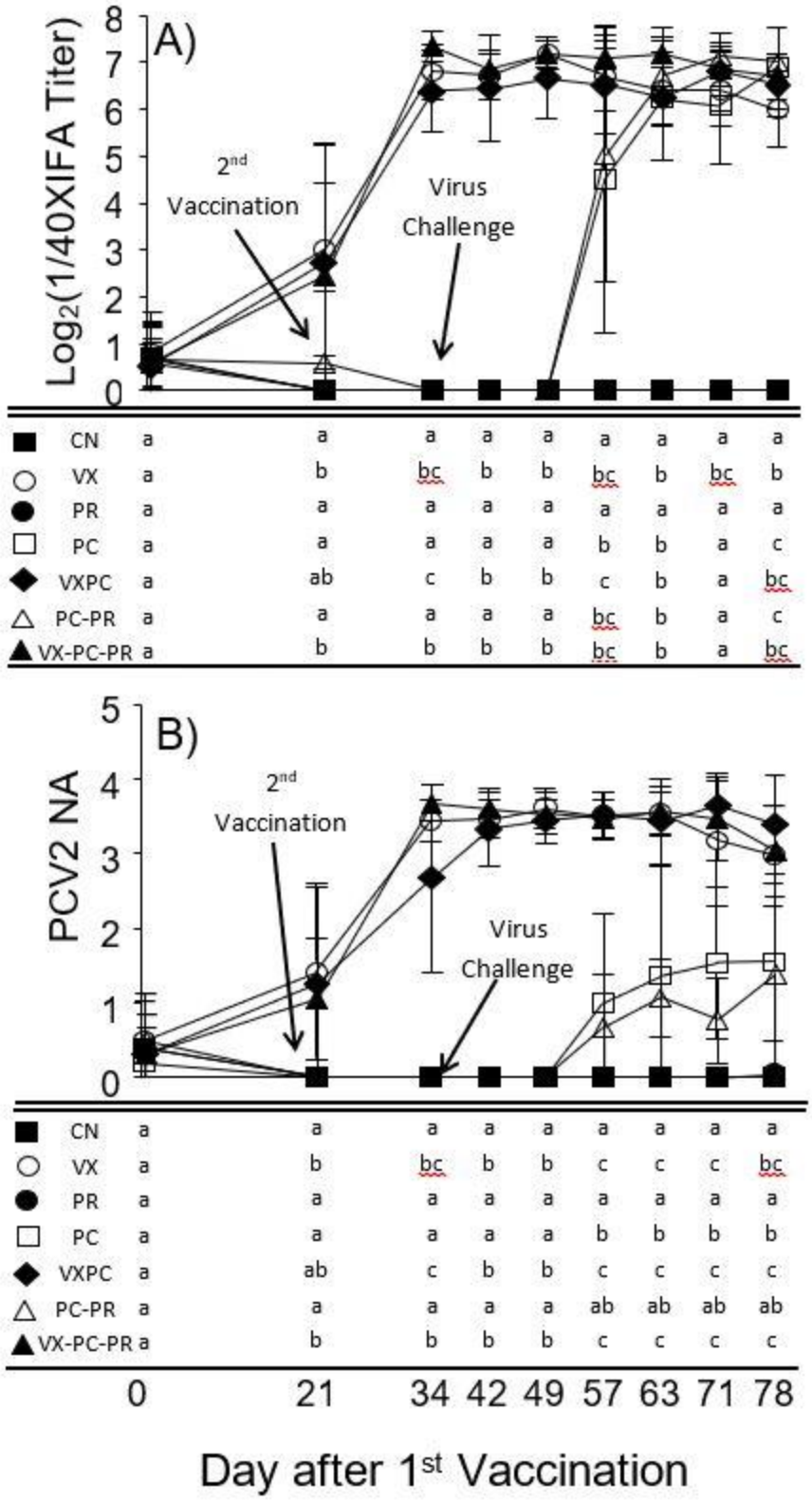
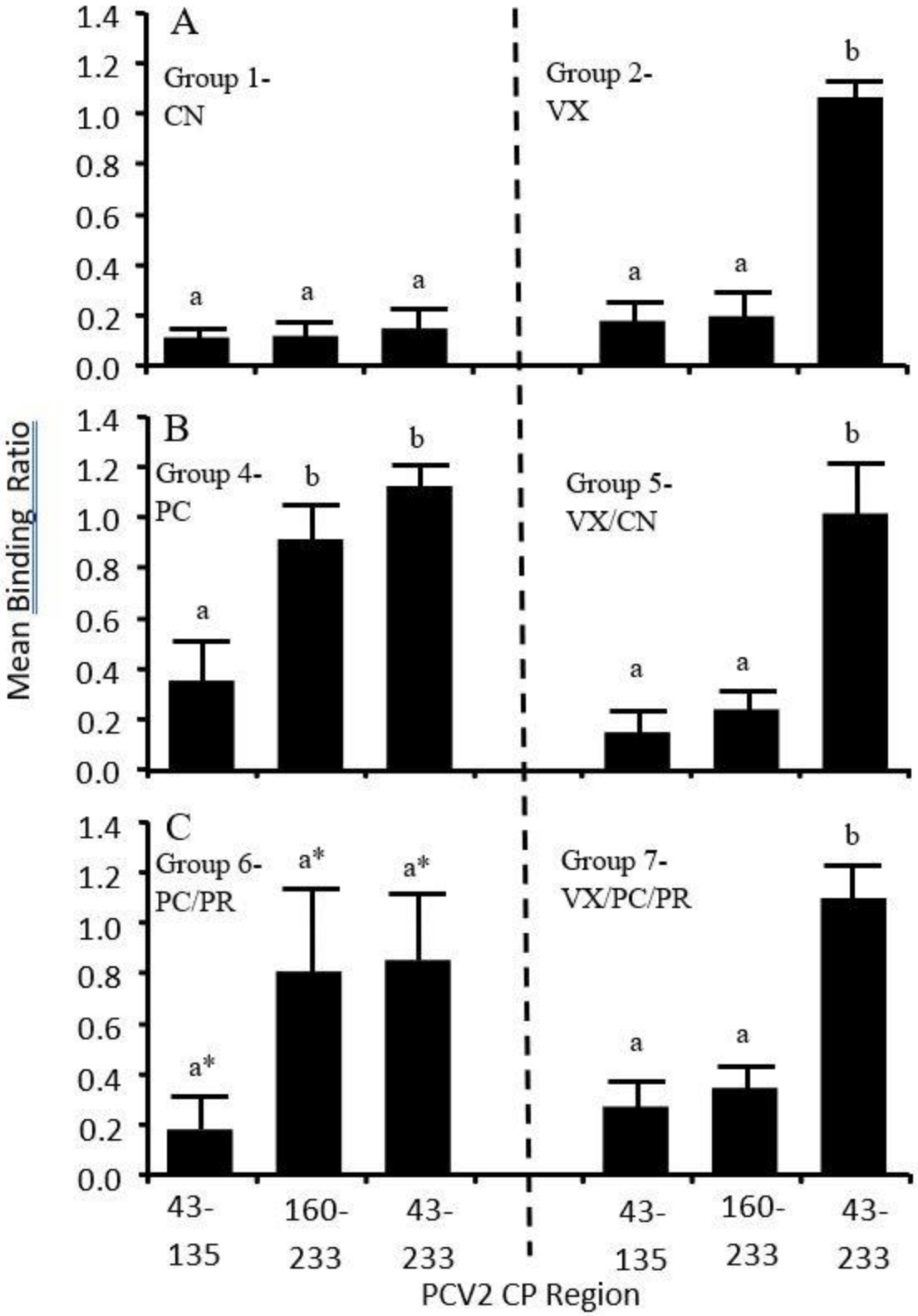

A)
Log2(1/40XIFA Titer)
2nd Vaccination
Virus Challenge
CN a a a a a a a a a
VX a b bc b b bc b bc b
PR a a a a a a a a a
PC a a a a a b b a c
VXPC a ab c b b c b a bc
PC-PR a a a a a bc b a c
VX-PC-PR a b b b b bc b a bc
B)
PCV2 NA
2nd Vaccination
Virus Challenge
CN a a a a a a a a a
VX a b bc b b c c c bc
PR a a a a a a a a a
PC a a a a a b b b b
VXPC a ab c b b c c c c
PC-PR a a a a a ab ab ab ab
VX-PC-PR a b b b b c c c c
0 21 34 42 49 57 63 71 78
Day after 1st Vaccination

Figure 3-3 PCV2 immunofluorescent antibody (IFA) and neutralizing activity (NA) responses.

IFA (A) and NA (B) was measured in serum samples as described in Materials and Methods. IFA results are reported as $\log_2$(40 x 1/dilution). 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 ($P>0.05$).


A
Group 1-
CN
Group 2-
VX
B
Group 4-
PC
Group 5-
VX/CN
C
Group 6-
PC/PR
Group 7-
VX/PC/PR
Mean Binding Ratio
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
a
a
a
a
b
a
b
b
a
a
b
a*
a*
a*
a
a
b
43-
135
160-
233
43-
233
43-
135
160-
233
43-
233
PCV2 CP Region

a

Figure 3-4 Antibody response of pigs to PCV2 CP polypeptides.

Serum samples were 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 ($P>0.05$). *Only 4 pigs were included in the analysis for group 6. All other groups included 7 pigs.

Table 3-1 Experimental groups and outcomes

Group		Description	n	Treatment			Mortality[b]
				Vaccine [a]	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	*PC/VX*	PCV2/Vaccine	7	+	+	-	0
6	*PC/PR*	PCV2/PRRSV	7	-	+	+	3 (15[c],24[d], 28[e])
7	*PC/PR/VX*	PCV2/PRRSV/Vaccine	7	+	+	+	0

[a] *Vaccinated with two doses of a recombinant baculovirus expressed PCV2 capsid protein product (Intervet/Schering-Plough Animal Health).*

[b] *Mortality = death or euthanasia of moribund pigs. The number in parenthesis is the day after virus challenge that death occurred.*

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

[d] *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.*

[e] *Euthanized. Lungs with neutrophilic bronchitis and atelectasis. Positive for PCV2 IHC staining in lymph nodes.*

Chapter 4 - Recognition of the different structural forms of the capsid protein (CP) determines the outcome following infection with porcine circovirus type 2 (PCV2)

Introduction

Porcine circovirus associated disease (PCVAD) encompasses a variety of progressive disease syndromes, that include a variety of clinical signs, such as respiratory distress, wasting, dermatitis, reduced growth performance, and reproductive failure (3, 4, 13). A new syndrome, acute pulmonary edema (APE), is characterized by the rapid onset of respiratory distress followed by death (6). Experimental studies demonstrate that the infection of pigs with porcine circovirus type 2 (PCV2) alone is necessary but not sufficient to reproduce PCVAD, but require additional co-factors, which can include infection with viruses or bacteria, immune stimulation following vaccination, and the genetics of the host (18, 21, 22, 28, 29, 34, 36). The contribution of co-factors in disease progression is likely related to immune modulation combined with increased numbers of proliferating lymphocytes, the primary targets of virus replication. One example is the experimental co-infection of pigs with PCV2 and porcine reproductive and respiratory syndrome virus (PRRSV). Co-infection results in increased PCV2 viremia and the appearance of clinical signs resembling PCVAD (1, 31, 37, 41, 44).

PCV2 isolates are placed in two major genotypes, termed PCV2a and PCV2b (38). A third genotype, PCV2c, was identified in archived tissues from Denmark (7). The virion is non-enveloped, with a 1.7 kb circular single-stranded DNA genome which is dominated by three open reading frames (ORFs) (11). The largest, ORF1, encodes the replicase proteins, Rep and Rep' (24). ORF3, which is embedded within ORF1, is reported to be involved in apoptosis.

However, a role for ORF3 in pathogenesis remains controversial (15, 19). ORF2 codes for the 233 or 234 amino acid capsid protein (CP), which is responsible for forming the homopolymer icosahedral capsid (27). In addition, CP participates in the attachment, entry, and shuttling of the viral genome across the nuclear pore complex and into the nucleus; the site of virus replication (26, 40). CP expressed in baculovirus or E. coli spontaneously forms a virus like particle (VLP) demonstrating that CP alone is sufficient for capsid formation (16, 20, 46, 47). Recombinant vaccines incorporating baculovirus-expressed CP (Bac-CP) are effective in reducing viremia, improving growth performance, and protecting against PCVAD (8, 13, 17, 25). Another vaccine approach is the expression of CP using a PCV1 backbone (9). We showed that sera from pigs vaccinated with Bac-CP preferentially react with a single CP polypeptide fragment, CP(43-233), and possess strong virus neutralizing activity. PCVAD-affected pigs and a subset of pigs experimentally infected with PCV2 recognize CP(43-233), but also recognize a group of truncated polypeptides that contain a single epitope, 169-STIDYFQPNNKR-180, which is located within the epitope C region of CP (43, 45). Mahe et al. (23) identified a similar immunodominant oligopeptide. Results of alanine scanning mutagenesis showed that 173-Tyr, 174-Phe, 175-Glu, and to a lesser extent, 179-Lys, are important for antibody recognition (43). Removal of a single key residue is sufficient to inhibit antibody recognition.

Recently, Khayat et al. (16) reported the crystal structure of CP (40-233). Maintenance of CP as a monomer required the presence of 20mM CAPS and 200mM L-Arg. The monomer structures ere assembled into a VLP model consisting of 60 CP subunits to form an icosahedron with T=1 symmetry, which was identical to a cryo-EM reconstruction of VLPs derived from Bac-CP. Representations of ribbon and space-filling structures for a single CP subunit are shown in Figure 4-1, panels A and B. The CP(169-180) domain forms an external loop structure, which

protrudes from the outer surface of the CP subunit. The key antibody binding residues, 173-Tyr, 174-Phe and 175-Glu, are located in the middle of a connecting loop domain and lie in a similar plane. The location and orientation of CP(169-180) within the VLP capsid is shown in Figure 4-1C. The CP(169-180) region is located near the interface of the icosahedral 3-fold axis. Close examination reveals that, of the key residues, only 173-Tyr (blue residue in Figure 4-1C) is visible on the surface of the VLP, but is located at the bottom of a cleft formed by the junction of three CP monomers. The remaining antibody binding residues are not visible on the surface.

Combining previous experimental observations with the models for the CP monomer and VLP provide a structural basis for the function of CP(169-180) as a decoy epitope; i.e., an immunodominant epitope not involved in protection and diverts the immune response away from protective epitopes. Therefore, we predicted that immunization of pigs with monomer CP should favor the production of antibodies against CP(169-180) and should not protect pigs following virus challenge. In this study, pigs were immunized with a stabilized form of CP monomer protein and then co-challenged with PCV2 and PRRSV. The results showed that immunization with the CP monomer induced high levels of PCV2 specific antibodies, but low levels of PCV2 neutralizing activity, and failed to protect pigs following virus challenge. Overall, the antibody response produced against the CP monomer was similar to that observed for pigs with PCVAD.

Materials and Methods

Cloning and expression of protein and polypeptides.

A PCV2b capsid gene fragment containing amino acid residues 43-233 was cloned and expressed in the *E.coli* vector, pHUE, as previously described (43). To obtain enhanced green fluorescent protein (EGFP), the coding region of EGFP was PCR amplified from the pEGFP:C3 vector using the forward primer, 5'-CCGCGGTGGTATGGTGAGCAAGGGCGAGG, and the

reverse primer, 5'-AAGCTTTTACTTGTACAGCTCGTCCATGC. SacII and HindIII restriction enzyme sites (underlined sequences) were added to 5' primer ends. The PCR product was double-digested with SacII and HindIII, cloned into pHUE (2), and then transformed into BL-21 *E. coli* (Invitrogen) according to the manufacturer's instructions. For protein expression, bacteria were grown in Luria-Bertani (LB) broth plus ampicillin (0.01 mg/ml) and incubated at 37°C with shaking. When the OD^{600} reached 0.4-0.6, protein expression was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG; 1 mM/ml final concentration) and bacteria harvested four hr later. Protein was purified using the USB PrepEase Histidine-tagged Protein Purification Kit (Affymetrix/USB) under non-denaturing conditions, according to the manufacturer's directions. Purity was assessed by SDS-PAGE and total protein measured using the Bio-Rad Protein Assay (BioRad Laboratories, Inc).

Size exclusion chromatography (SEC).

A 400 mm by 15 mm column (Fischer and Porter) was packed with Sephacryl G-200 high resolution gel filtration media (GE Healthcare) to a final bed volume of 60 ml. The column was equilibrated with elution buffer (EB; 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0; Affymetrix/USB), and calibrated for a 1 ml/min flow rate, as recommended by the manufacturer. The void volume (V_0) was determined using Blue Dextran (Sigma). Proteins of known size, including bovine serum albumin (BSA; New England Biolabs), lysozyme (Sigma), and Ub-GFP were included as standards. Proteins and Blue Dextran were diluted in EB to a final concentration of 1 mg/ml and loaded onto the column. Two ml fractions were collected. . Blue Dextran was measured by absorbance at 610 nm. Protein concentrations were measured in each fraction by protein assay (BioRad). The peak fraction containing Ub-GFP was confirmed by GFP fluorescence. In addition to total protein, the relative concentration of Ub-CP(43-233) was

estimated by immunoassay. Briefly, from an initial dilution of 1:2, serial 2-fold dilutions of each Ub-CP(43-233) containing fraction were prepared in 0.05 M carbonate binding buffer (pH 9.6), 100 µl of each was added to a 96-well ELISA plate (Costar) and the plate incubated overnight at 4^{o}C.After incubation, plates were washed with PBS containing 0.01% Tween-20 (PBST) and blocked for 2 hr at room temperature with PBS containing 10% goat serum (PBS-GS). Plate-bound antigen was detected with serum from a pig with PCVAD. Serum, diluted 1:400 in PBS plus 10% goat serum, was added to each well (GS; Colorado Serum Company). Following incubation and washing, 100 µl of peroxidase-labeled goat anti-swine antibody (Accurate Chemical & Scientific Corp.), diluted 1:2,000 in PBS-GS, was added to each well. After incubation at room temperature for one hr, plates were washed with PBST, and 100 µl of the chromogenic substrate ABTS (KPL) added to each well. Peroxidase activity was detected by measuring the absorbance at 405 nm. A standard curve was constructed by plotting elution volume (Ve) divided by Vo against the $\log_{10}$ of the formula weight of each standard.

Animal Experiments.

Experiments involving animals were performed after approval by Kansas State University Institutional Animal Use and Biosafety committees. PCV2-negative pigs were derived as described by Opriessnig et al. (30). Briefly, offspring from pregnant dams possessing indirect fluorescent antibody (IFA) titers of less than 320 were selected. At 3 weeks of age, 26 pigs, confirmed PCR negative for PCV2 and low IFA titer, were assigned into 3 treatment groups with 8 pigs in each group, plus a reference control group of 2 pigs. The seven pigs that possessed maternally derived antibodies were divided equally between treatment groups. Table 1 and Figure 4-2 show the experimental groups and the timeline for the experiment, respectively. Treatment groups included: pigs immunized with Ub-CP (group Ub-CP), pig immunized with

Bac-CP (group Bac CP) commercial vaccine, and pigs that were not immunized (Non-Im). The reference control group contained two pigs that were neither immunized nor challenged. The Ub-CP immunogen consisted of Ub-CP(43-233) at a concentration of 50 µg/ml emulsified in Emulsigen-D adjuvant (20% v/v; MVP Technologies), gentamycin (30 µg/ml), and thimerosol (0.01%) prepared in a 2 ml volume of MEM. Pigs were immunized intra-muscularly with a 2 ml dose at four and seven weeks of age. The Bac-CP immunogen consisted of a commercial vaccine (Intervet-Schering Plough) administered as a 2 ml dose at 4 and 7 weeks of age, according to the label instructions. The PCV2b-PRRSV preparation used for challenge was the same as described previously (43, 44). Pigs in each treatment group were intermingled and challenged with PCV2b-PRRSV at a dose of approximately 105 TCID50 for each virus. The 3 ml virus inoculum was administered intranasally, with 1.5 ml divided between nares. Following virus challenge, pigs were monitored daily for clinical signs and blood samples were collected weekly. Weights were measured at the beginning of the study, at virus challenge, and at the termination of the study. At the end of the study, lung, kidney and lymphoid tissues were collected for histopathology and PCV2 immunohistochemistry (IHC).

Immunohistochemistry for PCV2.

PCV2 antigen was detected by IHC staining on paraffin-embedded tissue sections. At necropsy, 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). PCV2 antigen was detected using a rabbit anti-PCV2 polyclonal antibody (Veterinary Medical Research and Development, Inc.) Bound antibody was detected with biotinylated goat anti-rabbit IgG followed by avidin-

horseradish peroxidase and DAB chromogen (Ventana Medical). Slides were counterstained with hematoxylin and eosin.

PCV2 PCR.

PCV2 in serum was measured using a semi-quantitative TaqMan PCR assay, performed as routine diagnostic tests by personnel in the Kansas Veterinary Diagnostic Laboratory (KSVDL). Briefly, total DNA was isolated from serum using a MagMAXTM-96 Viral DNA Isolation Kit (Applied Biosystems) according to the manufacturer's instructions. The 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 DNA were prepared and assayed concurrently with the samples. The assay results were reported as the Log10 of PCV2 DNA copy number per 50 μl reaction.

Measurement of PCV2 and PRRSV antibodies.

Total PCV2 antibody in serum was measured by IFA as previously described (44). Briefly, rapidly dividing ST cells, maintained in MEM with 7% FBS and Gentamycin (30 ug/ml; MEM-FBS-Gent) on 96-well plates, were infected with a laboratory isolate of PCV2b. Three days later, the plates were fixed in 80% acetone. Serum samples were added at an initial dilution of 1:40 followed by serial 1:2 dilutions in a total volume of 100 μl. Samples were diluted in PBS with 10% fetal bovine serum (PBS-FBS; Sigma) and incubated for two hr at room temperature. After washing with PBS, FITC-labeled anti-pig antibody (Jackson Labs) diluted 1:2,000 in PBS-FBS was added to each well. Plates were incubated for two hr at room temperature, washed, and viewed on an inverted fluorescence microscope. The results were reported as the reciprocal of the last serum dilution showing fluorescence.

PCV2 neutralizing activity was measured as previously described (44). Briefly, serial 1:2 dilutions of serum were prepared in MEM-FBS-Gent. Four 100 µl replicates of each dilution were mixed with 100 TCID50 of PCV2b (100 µl each) and incubated for 1 hr at 37oC. The well contents were transferred onto day-old ST cells in 96-well plates. Following incubation for four days at 37oC, wells were fixed in 80% acetone. Infection was detected with anti-PCV2 antibody (kindly provided by Dr. Ying Fang) diluted 1:2,000 in phosphate buffered saline (PBS)followed by incubation with FITC-labeled anti-mouse antibody (Jackson Labs). The log2 50% neutralizing antibody (log2NA50) endpoint was calculated according to the method of Spearman and Karber (10).

Anti-CP(169-180) antibody was measured by ELISA on 96-well plates coated with 100 µl of CP(169-180) oligopeptide (21st Century Biochemicals) at a concentration of 4 µg/ml (43, 44). Plates were incubated overnight at 4oC. After washing with PBS containing 0.05% Tween-20 (PBST), plates were blocked for 2 hr at RT with PBS containing 10% goat serum (PBS-GS). Serum, diluted 1:400 in PBS-GS, was added to each well and incubated for 2 hr at RT. Plates were washed with PBST and 100 µl of peroxidase-labeled goat anti-swine antibody (Accurate Chemical & Scientific Corp.), diluted 1:2,000 in PBS-GS, added to each well. After incubation at RT for 1 hr, plates were washed with PBST, and 100 µl of ABTS added to each well. Peroxidase activity was detected by measuring the absorbance at 405 nm on a FLUOstar Omega instrument (BMG Labtech). To compare results across experiments, each ELISA plate included an internal positive control as previously described (43, 44). Results were reported as the antibody binding ratio; the A405 value of the unknown sample minus background divided by the A405 value of the internal positive control minus background.

PRRSV-specific antibody was analyzed by a commercially available ELISA (PRRS X3, IDEXX). Samples were considered positive if the ratio of the sample to the positive control was greater than 0.39, as recommended by the manufacturer.

Statistical methods.

Statistical analysis was performed using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, California). Data incorporating repeated measures 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 were analyzed using the Kruskal-Wallis test. If differences were identified, measures were further assessed by Wilcoxon's test.

Results

Size exclusion chromatography (SEC) of Ub-CP(43-233).

In this study, the fusion of 76 amino acid ubiquitin to CP(43-233) was used as a means to maintain CP in a stable monomeric form. The removal of ubiquitin with the deubiquitylating enzyme, Usp-2cc (2), resulted in precipitation of CP(43-233) (data not shown). The expressed fusion protein was analyzed by SDS PAGE. As shown in Figure 4-3A, purified Ub-CP was present as a single 33 kDa protein, which is similar to the formula weight calculated for the 6xHis-Ub-CP fusion protein. The structural form of Ub-CP was analyzed by size exclusion chromatography on Sephacryl G-200. Monograms for total protein and immunoreactive Ub-CP are shown in Figure 4-3B. Total and immunoreactive protein monograms were similar.Immunoreactive CP showed minor peaks in fractions 14, 17, and 20, which suggested the presence of a small quantity of Ub-CP in multimeric forms. Plotting the major peak against the

standard curve showed that Ub-CP eluted as a 34.3 kDa molecule. Based on these results, the Ub-CP used for the immunization of pigs was present as a relatively pure monomer protein with only a small amount of Ub-CP present in a multimeric form.

Clinical disease and outcome.

The overall design of the experiment is shown in Figure4-2. At approximately seven days after virus challenge, pigs in all virus groups showed signs of mild respiratory distress, a clinical sign associated with acute PRRS. The two pigs in the non-challenged control group remained normal. Serology confirmed that all virus challenged pigs were positive for PRRSV (data not shown). At the end of the study, histopathology showed lesions in the lungs, including interstitial pneumonia with the infiltration of lymphocytes, macrophages, and neutrophils. IHC showed PCV2 antigen in lung tissue sections from all infected groups. PCV2 staining was localized in the bronchiolar epithelium. However, there was a difference in the number of pigs with antigen staining in lung. The greatest number of pigs positive for PCV2 antigen was in the Ub-CP group (6 of 8 pigs positive for PCV2 antigen), followed by the non-immunized group (4 of 8 pigs), and the Bac-CP group (2 of 8 pigs).

Antibody response to PCV2.

Serum samples collected before and after immunization were tested for total PCV2 antibody by IFA (see Figure 4-4). At the beginning of the study, low levels of PCV2-specific antibodies were detected in several pigs, likely the result of maternally-derived antibody. For the Ub-CP group, a detectable increase in mean Ab titer was observed at the time of the second immunization (day 14), that peaked by day 7 after virus challenge, and was followed by a second peak 28 days after challenge. Pigs immunized with the PCV2 vaccine, Bac-CP, showed a similar antibody response. For the Non-Im group, antibody was detected by 21 days after virus challenge

and peaked by 28 days. For days 21-42 after infection, the IFA titers for the three challenge groups were similar and not statistically different. PCV2 antibody titers for the two non-challenged control pigs remained below detectable levels throughout the study.

Serum samples were also reacted with the CP(169-180) oligopeptide. Significant amounts of anti-CP(169-180) binding activity were detected in the Ub-CP pigs at the time of virus challenge and remained elevated throughout the remainder of the study period (see Figure 4-4B). In contrast, anti-CP(169-180) activity in the vaccine group, Bac-CP, remained near background levels and similar to the levels for the two non-infected control pigs. Following virus challenge, the Non-Im group showed anti-CP(169-180) activity which first appeared at 21 days after infection and peaked at the same level of binding activity as the Ub-CP group.

Results for PCV2 neutralizing activity, measured at the time of the first vaccine dose, the virus challenge, seven days after challenge, and the termination of the study, are presented in Figure 4-5. Prior to the first vaccine dose, only background levels of PCV2 neutralizing activity were detected. After two doses of a baculovirus expressed CP product, significantly higher levels of PCV2 neutralizing activity was detected in the Bac-CP group compared to Non-Im and Ub-CP immunized groups at the time of virus challenge, seven days after challenge, and at the end of the study.

PCV2 viremia.

All pigs were negative for PRRSV and PCV2 by PCR at the beginning of the study. PCV2 DNA was first detected at 14 days after virus challenge in the Ub-CP and Non-Im groups (see Figure 4-6). Mean viremia levels for both groups were similar with no significant differences between groups on any day after infection. Viremia in the Bac-CP group remained below detectable levels throughout the study. After virus challenge, pigs from all three treatment

groups were intermingled. Even though the virus challenged Bac-CP pigs remained constantly exposed to viremic pigs, all Bac-CP pigs remained negative for the presence of PCV2 DNA in serum.

Discussion

Previous work showed that the antibody of pigs with PCVAD is primarily directed towards an immunodominant oligopeptide epitope, CP(169-180), located within the epitope C region of CP (43). In this study, the antibody response of PCVAD pigs was mimicked by immunizing pigs with a monomer form of CP. The outcomes following immunization with monomer CP included high levels of anti-PCV2 antibody, antibody recognition of CP(169-180), undetectable levels of virus neutralizing activity, and the absence of protection following virus challenge. Since baculovirus- and E. coli-expressed CP spontaneously form VLP, one of the first obstacles was to maintain the CP immunogen in a stable monomeric form. This was achieved by the fusion of CP to ubiquitin to form Ub-CP. In a similar manner, Yin et al. (47) incorporated SUMO for the expression of single CP subunits. Similar to the cleavage of Ub from Ub-CP, the removal of SUMO with the SUMO protease resulted in the spontaneous assembly of CP into VLPs. How fusion with Ub or SUMO functions to prevent the formation of a VLP is not clear. One possibility is that the fusion partner sterically blocks the interaction between CP subunits. Another possibility is that the addition of Ub or SUMO prevents subunit association through a conformational change in the CP monomer . Regardless of the mechanism for blocking VLP formation, the immunization of pigs with Ub-CP resulted in the production of antibodies against CP(169-180). As a positive control for these experiments, pigs were immunized with a commercial baculovirus-expressed CP vaccine. For the Bac-CP group, the total PCV2 antibody response followed a similar course as pigs immunized with Ub-CP (see Figure 4-4A). In

contrast, the Bac-CP vaccinated pigs possessed high levels of virus neutralizing activity, low levels of anti-CP(169-180) activity, and showed no evidence of viremia after virus challenge.

In this study, the infection of pigs with a combination PCV2 and PRRSV did not result in mortality or the appearance of full-blown PCVAD, which was observed previously (1, 31, 37, 41, 44). It is difficult to faithfully reproduce PCVAD, even in the presence of known infectious disease cofactors. The absence of PCVAD in this study is likely the result of other, yet-unknown factors that are involved in disease progression. The initial high health status of the pigs and high level of care may slow down disease progression and/or suppress clinical signs. Another possibility is that the experiment was terminated before PCVAD could develop. As shown in Figure 4-6, virus was still present in sera of Ub-CP and Non-Im pigs. Continued virus replication may have eventually culminated in disease.

A potential role for anti-CP(169-180) antibodies in the development of PCVAD was evident by the number of pigs with antigen in the lung. For the non-immunized (Non-Im) and vaccinated (Bac-CP) groups, 4 of 8 and 2 of 8 pigs were positive for antigen staining in the lungs. In contrast, 6 of 8 pigs in the Ub-CP group showed antigen staining in the lung. In all cases, PCV2 staining was localized in the bronchiolar epithelium. In PCVAD cases, the lung is one of the primary targets for PCV2 infection. For example, the APE syndrome is associated with massive quantities of PCV2 replication in the lung which results in pulmonary edema (5). PCV2 antigen in lung is associated with the onset of PRDC (3, 32). Further, Shen et al. (39) showed that PCV2 DNA was more prevalent in the lung compared to serum of pigs with PCVAD. One interesting observation in this study was the presence of PCV2 antigen in the lung epithelium of Bac-CP pigs, even in the absence of viremia. These results suggest that the lung represents a site for continuous shedding of virus, even in vaccinated populations.

The most intriguing feature of PCV2 infection is the dysregulation of the immune response, which culminates in a variety of disease outcomes. On one extreme is porcine multisystemic wasting syndrome (PMWS) which, in the end stage of the disease process, results in an almost complete elimination of B and T cells, and the eventual decay of anti-PCV2 antibody (4). On the other extreme, porcine dermatitis and nephropathy syndrome (PDNS) is characterized by hyperimmunoreactivity, including immune complex formation and deposition of antigen-antibody complexes in organs, such as kidney and skin (3). We propose that antibody recognition of CP(169-180) functions similar to an immunological decoy, which directs the humoral response away from protective epitopes. The decoy antibody response is the result of host recognition of CP monomer or fragments produced during virus replication. Classic examples of immunological decoys are found in lentiviruses, such as feline immunodeficiency virus (FIV) and the human immunodeficiency virus (HIV). As reviewed by Hosie et al. (14), the variable regions, V3, V4 and V5, of the envelope (Env) protein of FIV are essential for virus infection, immunodominant, and targets for neutralization by antibody. However, continuous variation, as a result of shortening, lengthening, and/or peptide sequence hypervariability produces large quantities of antibodies that are non-protective. A similar mechanism occurs in the HIV glycoprotein 120 (gp120) (42). PRRSV possesses a hypervariable region in GP5 that may function in a similar manner by continually recruiting B cells that do not produce neutralizing antibody (33). An example of a non-essential protein functioning as a decoy is proposed for gp150 of gammaherpesvirus-68. The protein is immunodominant and results in a non-protective antibody response (12). In sharp contrast to lenti- and herpes viruses, PCV2 possesses a rather small genome dominated by two relatively conserved proteins. Furthermore, CP is essential for virion integrity, infection and replication. Therefore, when employing a decoy

strategy, the virus does not possess the luxury of peptide sequence hypervariability or the production of a non-essential protein. As illustrated in the model, presented in Figure 4-7, we propose that PCV2 incorporates a unique strategy for employing a decoy epitope as a defense mechanism. Under normal circumstances, the immune response following PCV2 infection favors the production of antibodies directed against the whole virion. The resulting outcome is the control of virus replication and the generation of protective immunity. In contrast, the recognition of free CP and CP fragments, produced by infected cells, result in the production of antibodies against CP(169-180). Cofactors, such as co-infecting pathogens may increase PCV2 replication, and as a result may increase the level of non-VLP forms of CP. Recognition of CP(169-180) results in the production of non-protective antibodies, thus allowing for increased PCV2 replication and the progression towards disease. Host genetics or co-infections may further modulate the host response, favoring the recognition CP(169-180). This model explains why the amount of circulating antibody fails to correlate with the outcome following PCV2 infection and provides important considerations in the design of PCV2 vaccines.

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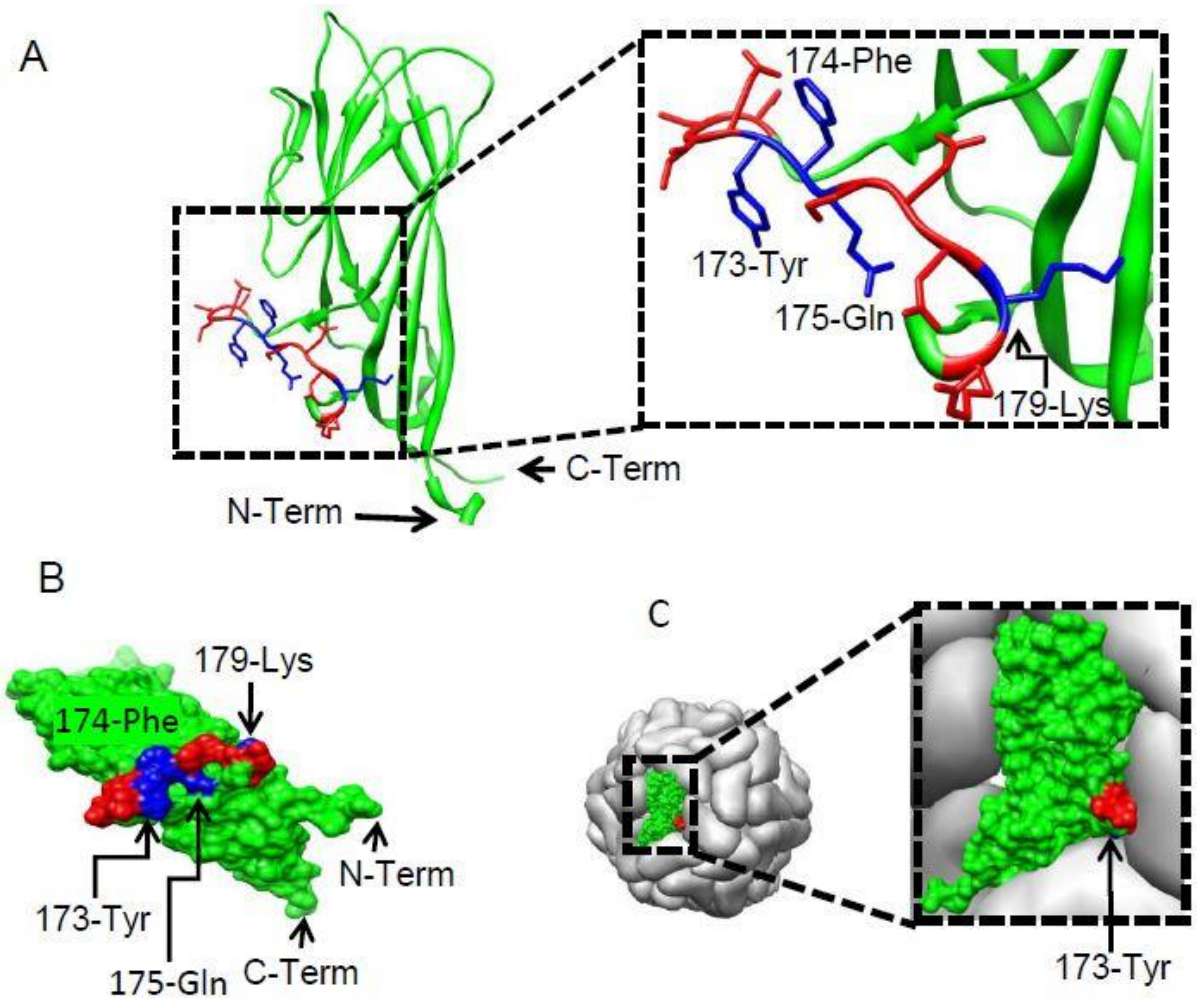

Figure 4-1 Location of the CP(169-180) epitope within a single CP sugunit and VLP.

Depicted are the ribbon (A) and surface (B) maps of a single CP subunit. The blue and red residues form the epitope, CP(169-180). The blue residues are important for antibody recognition (23). The VLP (C) shows the surface of the VLP with a single CP shown in green, red, and blue. The red and blue regions correspond to the same residues in (A). Coordinates for the PCV2 CP(41-233) subunit and VLP were accessed through the RCSB Protein Data Bank (PDB ID 3R0R: (2, 12) and loaded into the open source molecular visual program, Chimera (30).

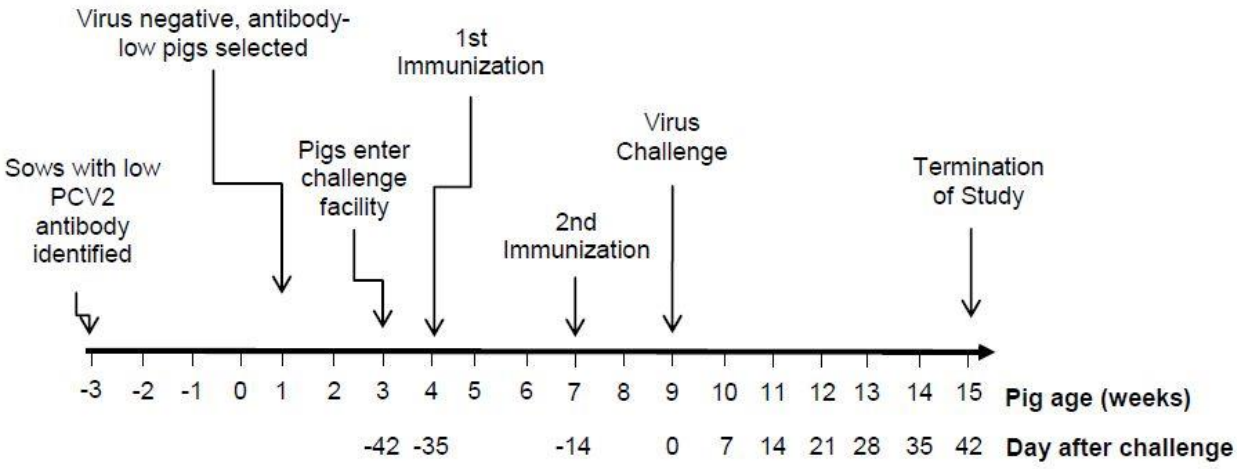

Figure 4-2 Study timeline.

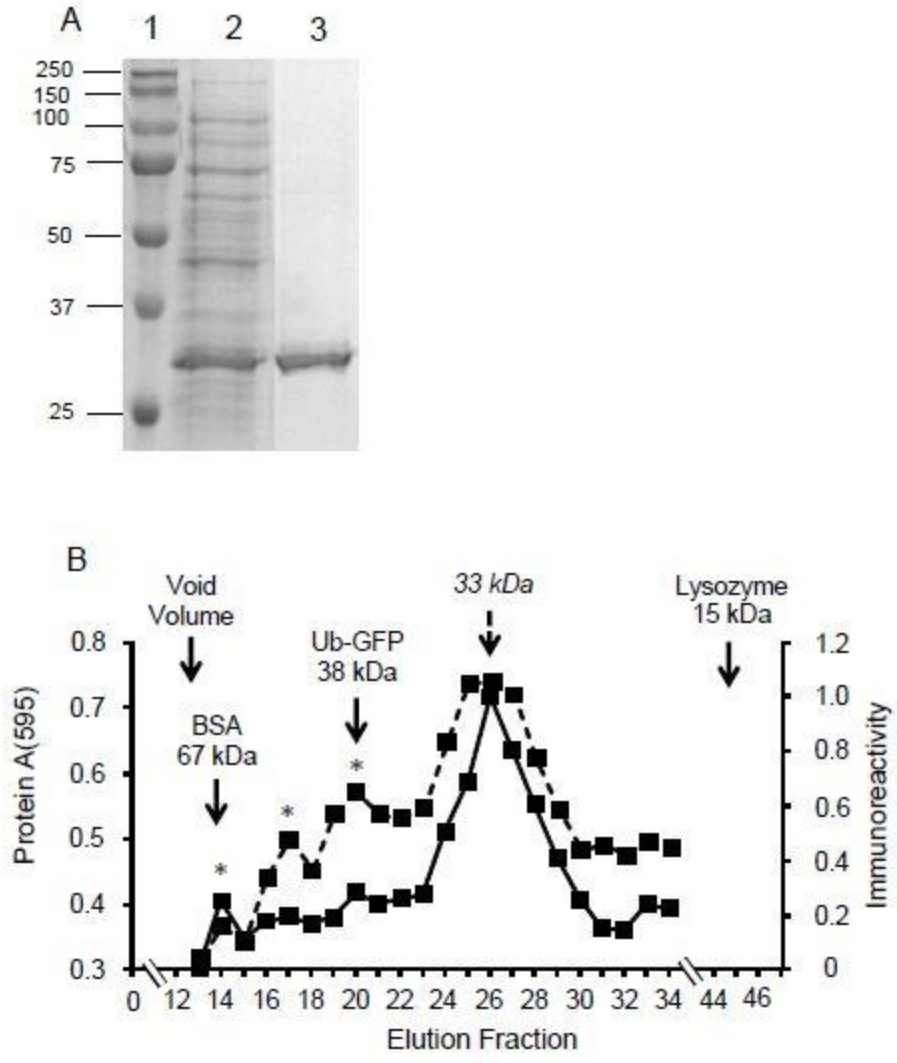

Figure 4-3 Size exclusion chromatography of CP(43-233).

(A) SDS-Page showing affinity purified Ub-CP. (B) Sandard curve and size estimation for Ub-CP. (C) Monogram of Ub-CP(43-233) eluted on a Sephacryl G-200 column was constructed by measuring total (solid line) and immunoreactive (dashed line) protein. Solid arrows show the location of the peaks for the BSA, Ub-GFP and lysozyme standards. The asterisks identify minor immunoreactive peaks, which may represent CP multimers. Each fraction contained 2 ml of elution buffer. Fraction volume was 2 ml.

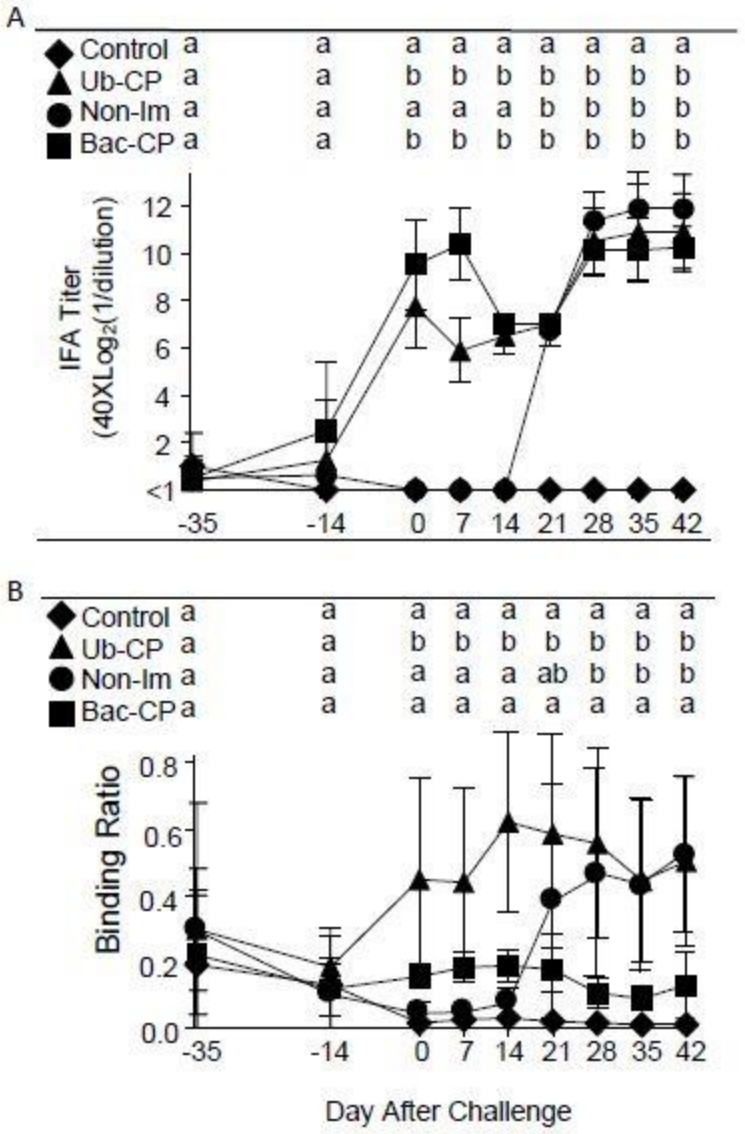

Figure 4-4 Total PCV2 and CP(169-180) oligopeptide antibody response.

Total antibody (A) was measured by indirect fluorescent antibody assay (IFA). CP(169-180) immunoreactivity, shown in (B), was determined by ELISA using plates coated with BSA conjugated with CP(169-180). Results show the mean value for each group. Means with the same letter are not significantly different (P>0.05).

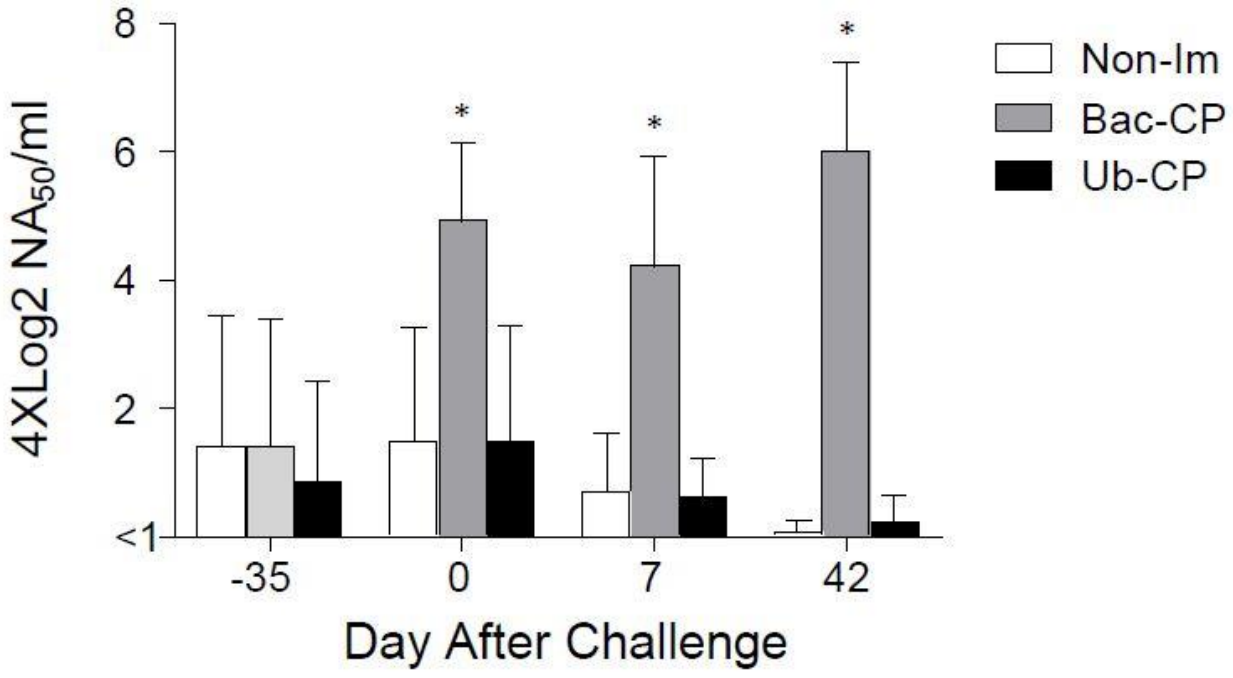

Figure 4-5 PCV2 neutralizing activity.

The 50%neutralizing activity (NA_{50})/ml was determined by performing serum neutralization assays as described in methods. The asterisk (*) shows means that are significantly different ($P<0.05$).

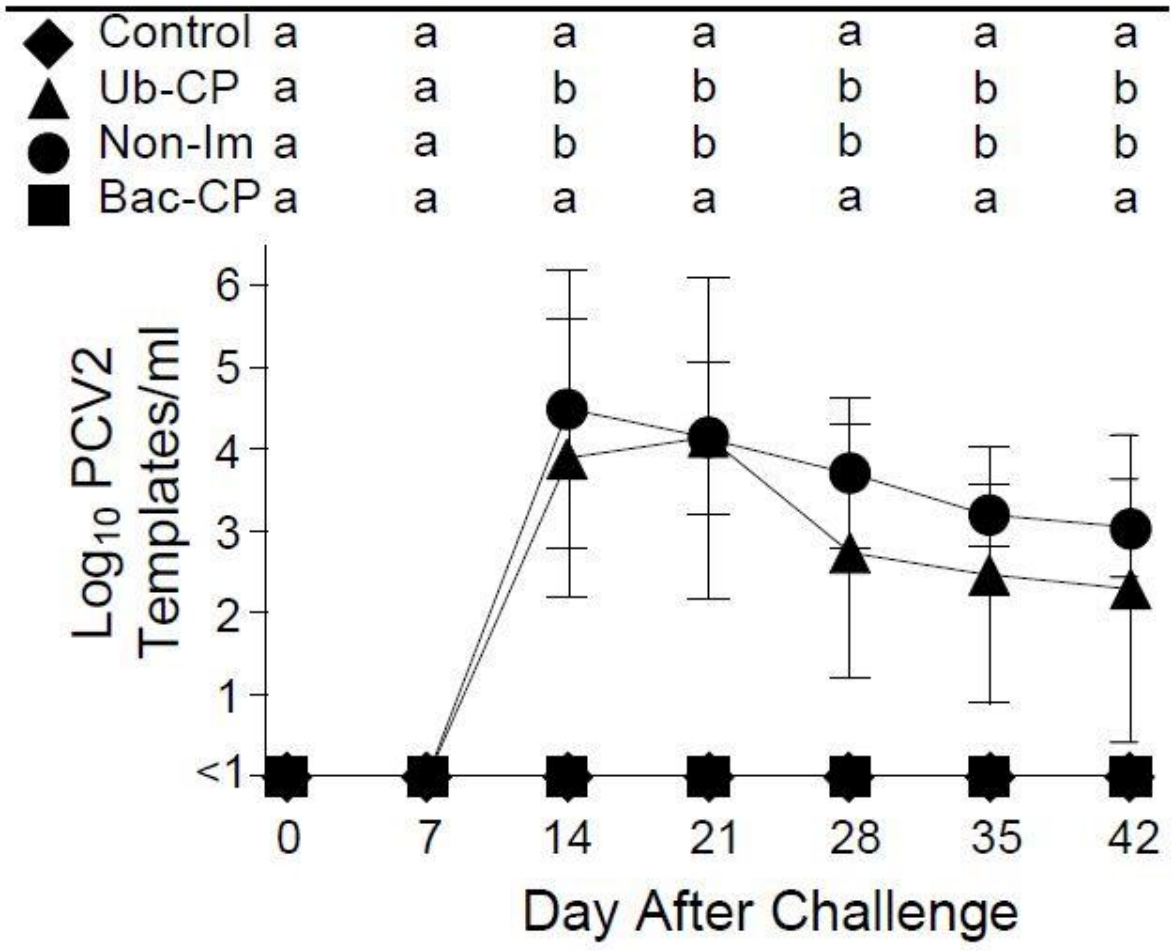

Figure 4-6 PCV2 viremia.

Data are shown as the mean +/- a single standard deviation. Means with the same letter are not significantly different (P>0.05).

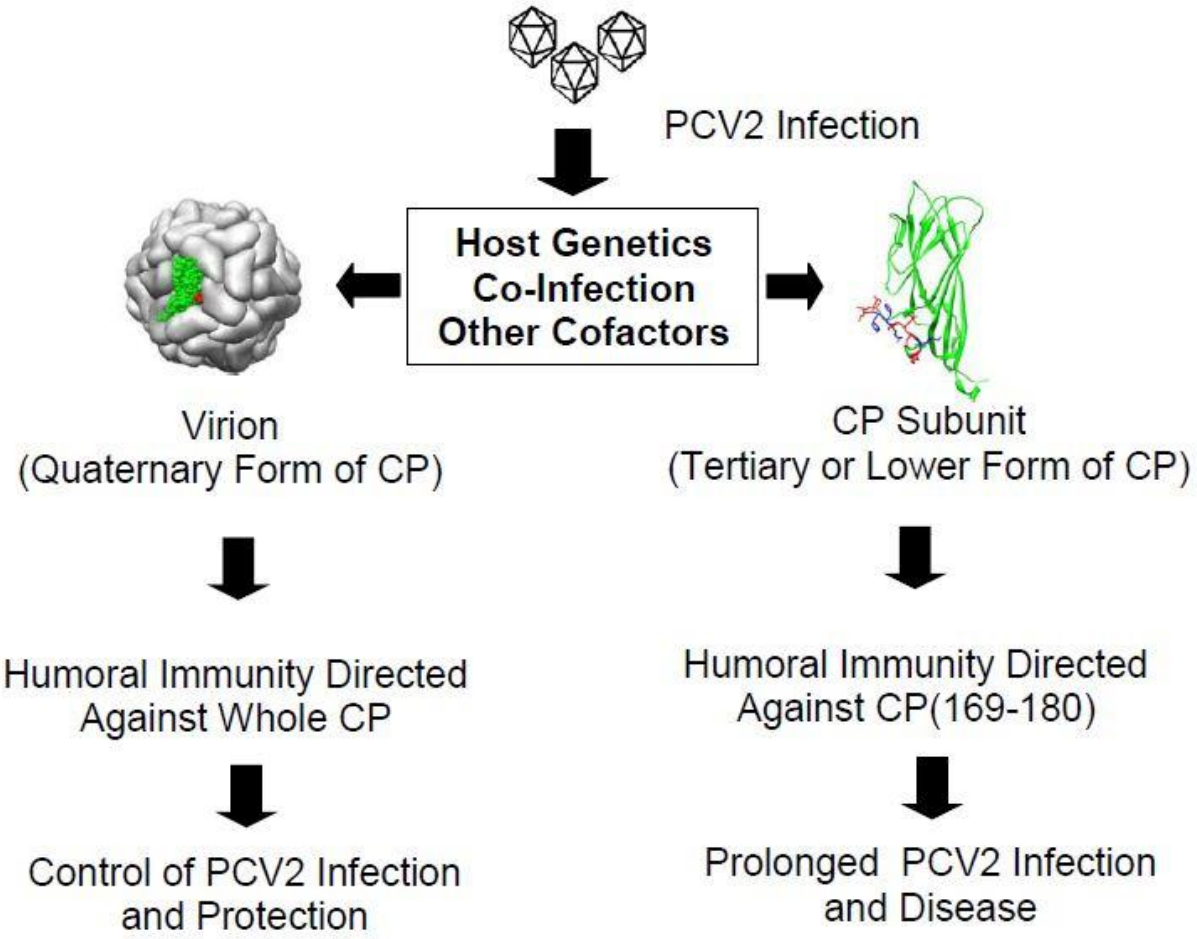

Figure 4-7 Structural form of immunogen recognized by the host and relationship to outcome following PCV2 infection.

Table 4-1 Treatment groups.

Group	n	Immunogen	PCV2/PRRSV
Non-IM	8	None	Yes
Bac-CP	8	Bac-CP vaccine	Yes
Ub-CP	8	Ub-CP	Yes
Control	2	None	No