EXPRESSION OF RECOMBINANT PORCINE CIRCOVIRUS 2 (PCV2) CAPSID POLYPEPTIDES FOR MAPPING ANTIBODY EPITOPES FOLLOWING VACCINATION, INFECTION, AND DISEASE

by

BENJAMIN R TRIBLE

B.S., KANSAS STATE UNIVERSITY, 2007

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Diagnostic Medicine and Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2012

Approved by:

Major Professor
Dr.Raymond R.R. Rowland

Abstract

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). The approach was to react porcine sera with different CP polypeptide fragments that each contained one or more immunoreactive regions. Expression of polypeptides was performed using *E.coli*. Initial results showed that sera from vaccinated pigs preferentially recognized only the largest CP(43-233) polypeptide fragment and showed low levels of binding to other CP polypeptide fragments. The results of sera from pigs diagnosed with PMWS showed only minimal reactivity with CP polypeptide fragments, including the largest CP(43-233). PCV2 infected or PDNS diagnosed pigs reacted to all CP polypeptides: however, the strongest reactivity was primarily directed towards CP polypeptides containing residues in the 160-180 region. For this purpose, finer mapping studies were performed. These experiments involved reacting sera from experimentally infected PCV2 pigs and PDNS pigs with overlapping oligopeptides that covered amino acids 141-200. Overall, the results showed a subset of experimentally infected pigs and pigs with PDNS preferentially recognized the 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 results from this study support the notion of PCV2 modulation of immunity.

Furthermore, the methods incorporated in this study provide a means for characterizing the immune response upon vaccination, natural infection and disease.

Table of Contents

List of Figures viii

List of Tables ix

Acknowledgements x

CHAPTER 1 - Porcine Circovirus Type 2 Literature Review 1

Introduction 1

The Circoviridae Family 1

Porcine Circoviruses 2

History 3

Genomic Organization and Proteins 4

The Viral Proteins 5

Virus Replication 7

The “Cruciform” RCR mechanism 8

The “melting pot” RCR model 9

Infection and the Virus Life Cycle 10

Biological and Physico-chemical Properties of PCV 14

Geographic Virus Distribution and Prevalence 14

Transmission 15

Detection and Quantification of PCV2 Antigen, DNA and Antibodies 16

PCVAD 18

PMWS 19

PDNS 21

Control and Prevention of PCV2 and PCVAD 23
The Host Immune Response Following PC2 Infection 24
Innate Immune response 24
The Adaptive Immune Response 27
Cell-Mediated Response 27
The Humoral Response 27
Epitope Mapping of PCV2 Proteins 30
T-cell epitope mapping 30
B-cell epitope mapping 31
Purpose 32
Figures 34
CHAPTER 2 - Materials and Methods 48
Cloning, expression and purification of recombinant PCV2 polypeptides 48
Selection of the virus 48
Construction of CP polypeptides 48
Expression of CP polypeptide fusion proteins 50
Full Length CP(1-233) Expression Optimization 50
Purification of CP(43-233)under native conditions 50
Purification of CP polypeptides under denaturing conditions 51
Protein purification using detergents 52
CP polypeptide dialysis 52
CP polypeptide “hybrid” purification protocol 52
Sequential buffer exchange/purification protocol 53

Determining protein purity and concentrations 53
CP Oligopeptides 53
PCV2 experimental infection/vaccination 54
Selection of PCVAD pigs. 55
CP polypeptide and oligopeptide ELISA’s 56
Binding Ratio Calculation 57
PCV2 IFA and measurement of virus neutralizing activity 57
Figures and Tables 58
CHAPTER 3 - Results 67
Cloning, expression and purification of CP polypeptides 67
PCV2 virus selection 67
Cloning and expression of CP(1-233) 67
Cloning and expression of other segmented CP polypeptides 69
Attempts to return small CP polypeptides to non-denaturing buffers 70
Experimental PCV2 infection 71
Clinical outcome and histopathology 71
Immunoreactivity of PCV2 CP polypeptides 72
Comparative ELISA 72
Immunoreactivity of experimentally infected/vaccinated pigs 73
Immunoreactivity of pigs with PCVAD 74
Pepscan mapping of the CP C-terminus 75
Alanine scanning of the CP(169-180) region 76
Virus neutralizing activity in PCV2-infected and vaccinated pigs 77

Figures and Tables 78

Chapter 4 – Discussion and Conclusions 95

Chapter 5 - References 102

List of Figures

Figure 1-1 Genomic DNA sequence alignment of PCV2a, PCV2b, and PCV2c. 34

Figure 1-2 Map of the PCV2 genome 37

Figure 1-3 Three dimensional structure alignment of PCV2 and FBNYV endonuclease domain 39

Figure 1-4 RCR models 42

Figure 1-5 The virus life cycle 46

Figure 2-1 Immunoreactive Regions within PCV2 Capsid Protein 58

Figure 2-2 Experimental Infection and Vaccination Timeline 60

Figure 3-1 pHUE expression product map 78

Figure 3-2 Expression and Purification of CP(43-233) 79

Figure 3-3 PCV2 Capsid Protein Polypeptide Fragment Digestions 80

Figure 3-4 Purified CP Polypeptides 81

Figure 3-5 CP(160-233) fractions after buffer exchange 82

Figure 3-6 Hybrid protocol for purification of CP(160-233) 83

Figure 3-7 Comparative ELISA results 84

Figure 3-8 Reactivity of Sera from Experimentally Infected and Vaccinated Pigs to CP Polypeptides 85

Figure 3-9 Reactivity of Sera from PCVAD Pigs to CP Polypeptides 87

Figure 3-10 Reactivity of Sera from Experimentally PCV2 Infected and Pigs Diagnosed with PDNS to CP Oligopeptides 89

Figure 3-11 Alanine Scanning of the CP(169-180) Oligopeptide 91

Figure 3-12. Total and neutralizing antibody responses following vaccination and infection. ... 93

List of Tables

Table 2-1. Primers for whole genome PCV2 sequencing 61

Table 2-2. Summary of CP polypeptides 62

Table 2-3 Primer Sequences Used for Preparing Capsid Protein Polypeptides* 63

Table 2-4 Synthesized BSA Conjugated Oligopeptides 64

Table 2-5 PCV2 Experimental Infection Study Groups 65

Table 2-6 Identification of PDNS and PMWS Serum Samples 66

Table 3-1 Summary of Antibody Responses to PCV2 Capsid Protein Polypeptides 94

Acknowledgements

I would like to thank my major professor, Dr. Bob Rowland for his wisdom and expertise, motivational skills and probably most importantly, his patience. Thanks to my committee members, Dr. Richard Hesse and Dr. Carol Wyatt for their expertise. Thanks to Maureen Kerrigan for all of her technical advice and expertise. Thanks to all of the members of the Rowland lab and other members of the KSU CVM DMP for all of their help.

This work was supported by National Pork Board Grant #06-073 and USDA NRI Grant# 2009-35204-05290.

CHAPTER 1 - Porcine Circovirus Type 2 Literature Review

Introduction

Porcine circovirus associated disease (PCVAD) was first described in the 1990's and continues to economically impact the global pork industry. Within the U.S. alone, PCVAD was reported to cost pork producers around 3-4 dollars per pig with some losses peaking at 20 dollars per pig in 2006 (32). The term PCVAD describes a group of complex syndromes that vary in the way they manifest and the overall clinical outcome. The factor linking the syndromes is the presence of porcine circovirus (PCV) type 2. Research over the past decade has been directed towards understanding PCV2 and its involvement in the onset of disease. A significant outcome of this research includes the production and availability of commercial vaccines which have proved effective in the prevention of PCVAD. However, little is known regarding the mechanism of PCV2 immunopathogenesis or the protection offered by commercial PCV2 vaccines.

The Circoviridae Family

The family *Circoviridae* describes some of the smallest known viruses, with genomes ranging from 1 to 4 kb. The most recent taxonomy list from the International Committee on Taxonomy of Viruses (ICTV) (137) classifies two genera of animal viruses, *Circovirus* and *Gyrovirus*, within in the family *Circoviridae*. The genus *Circovirus* contains eleven species, including *PCV1, PCV2, Canary circovirus, Duck circovirus, Finch circovirus, Goose circovirus, Gull circovirus, Pigeon Circovirus, Starling circovirus, Swan Circovirus, and Beak and feather disease virus (BFDV. Chicken anemia virus* is the only member of the *Gyrovirus* genus. *Chicken anemia virus*, is characterized by a negative sense genome and larger virion compared to

Circovirus family members. Recently, a new genus, termed *Cyclovirus*, has been proposed for inclusion in the *Circoviridae* family (61). This genus includes recently discovered circovirus like genomes from a variety of vertebrates including humans, chimpanzees, cattle, goats, sheep, camels, and birds (61, 62). Furthermore, a *Cyclovirus* has been described in dragonflies, which is the first report of a circular ssDNA virus identified in insects (103).

The plant virus families *Nanoviridae* and *Geminiviridae* are considered the closest relatives to the *Circoviridae.* Overall, these families share a common stem loop structure (discussed below) as well as homologous sequences in the N-terminal region of their replicase proteins (Rep) (75). Gibbs et al. (31) proposed a mechanism for the origin and evolution of *Circoviruses* based on analysis of *Circovirus* and *Nanovirus* Rep protein sequences. They report similarities in the N-terminal region of *Circovirus* and *Nanovirus* Rep. However, the C-terminal region of PCV Rep is closely related to an RNA binding protein (protein 2C) sequence encoded by *Caliciviruses*. These findings led to the following proposal for recombination and evolution of *Circovirus*. First, a plant *Nanovirus* changed kingdoms and infected a vertebrate host. This could have happened by exposure to sap, through a wound or ingestion, or through an intermediate host such as an arthropod. This was followed by a recombination event with a *Calicivirus*, which added the 2C like protein region to the Rep protein. Due to *Nanoviruses* not having an RNA stage or *Caliciviruses* not having a DNA stage during replication, the recombination event was likely mediated by a retrovirus or retrotransposon.

Porcine Circoviruses

1 ***History***

2 In the early 1970's, a stable viral contaminant of the porcine PK-15 cell line (ATCC-CCL31)
3 was described (123). In order to identify the contaminant, purified supernatant from cell culture
4 was analyzed by electron microscopy. This revealed viral particles with picornavirus like
5 morphology. Biochemical and serological assays showed the virus had a circular ssDNA
6 genome and identified pigs as the host of the virus (120). Subsequently, the novel virus was
7 termed porcine circovirus (PCV) (120). Analysis of serum from pigs revealed PCV was
8 ubiquitous within the swine population and caused no clinical signs of disease (121). In Canada
9 in the early 1990's, a new wasting disease of pigs emerged and was termed postweaning multi-
10 systemic wasting syndrome (PMWS) (15, 38). Analysis of viral antigens and DNA from North
11 American and European diseased pigs revealed a new genotype of PCV (76). The terms PCV1
12 and PCV2 were adapted to distinguish the cell culture contaminant from the genotype associated
13 with disease, respectively.
14
15 Sequence analysis revealed PCV2 isolates could be clustered into two main groups or genotypes.
16 Due to the ICTV not defining anything below the species level, the scientific community came
17 up with a variety of names for the genotypes. These included the following: PCV2 genotype 1
18 and 2;,PCV2 groups 1 and 2; PCV2 I and II; PCV2 SG3 and SG1/2; PCV2 A and B; PCV2 b
19 and a; and restriction fragment length polymorphism patterns 321 and 422 (106). Since that
20 time, a new genotype of PCV2 has been identified, which led to the proposal of a unifying
21 system of nomenclature (106). Genotypes are now classified as PCV2a (Genbank accession
22 #AF055392), PCV2b (Genbank accession #AF055394) and PCV2c (Genbank accession
23 #EU148503) with the first sequences recorded in Genbank demarked as the prototypic virus (See

Fig. 1-1 for an alignment of the sequences). The most recently identified genotype, PCV2c, is from archived pig tissues in Denmark during the 1980's that were not associated with disease (19). In contrast, PCV2a and PCV2b were associated with disease outbreaks in North America and Europe, respectively (106). More recently, outbreaks of PCVAD in Kansas and other US states in 2005 were shown to be associated with PCV2b (45). Since then, both genotypes have been identified worldwide. Interestingly, analysis of isolates from the Kansas State Veterinary Diagnostic Lab (KSVDL) identified isolates composed of sequences from both PCV2a and PCV2b (42). These results suggest the possibility of recombination between genotypes (i.e. a PCV2a/b hybrid).

Genomic Organization and Proteins

Porcine circoviruses possess an ambisense ss-DNA genome in the form of a covalently closed circle. The PCV1 genome is 1,759 nucleotides (nt) in length; whereas; genomes of PCV2a, PCV2b, and PCV2c are 1,768, 1,767 and 1,767 nt, respectively. A map of the PCV2 genome is shown in Figure 1-2. The genome sequences of PCV1 and PCV2 share an identity of 68-76%. Sequences of PCV2a, PCV2b, and PCV2c share an identity of approximately 95% (22).

The PCV genome codes for two main open reading frames (ORF). The gene products, Rep and CP, perform the most elementary functions of a virus, including copying and packaging of the viral genome. The rep and cp genes are oriented head to head, creating two intergenic regions (IR; see Fig. 1-2). The larger 109 nt IR locates between the 5' ends of rep and cp, while a smaller 37 nt IR is located between the 3' ends of rep and cp. The 109 nt IR posesses the origin of replication (Ori) which is characterized by a stem loop (hairpin) structure that contains the nonamer sequence 5' AAGTATTAC at its apex, flanked by 11nt inverted repeats (73). Four

hexanucleotide sequences (H1, H2 and H3: CGGCAG and H4: CAGCAG) are located downstream of the stem loop structure. The 3' portion of the stem loop and the most proximal hexamers, H1 and H2 make up the minimal binding site (MBS) for the replication proteins Rep and Rep' (described below) (118). The stem loop structure and the nonamer sequence 5'(A/T)AxTAxTAC ("x" represents positions that can be substituted without the loss of function) are conserved in viruses, plasmids and bacteriophages that perform rolling circle replication (RCR, described below) (73).

The Viral Proteins

The largest open reading frame, ORF1, is located on the positive strand of the genome and codes for two proteins associated with replication termed Rep (PCV1: 312 amino acids (aa); PCV2: 314 aa) and Rep' (PCV1: 168 aa; PCV2: 178 aa). Rep and Rep' are translated from differentially spliced transcripts. Rep is produced from the full length transcript of ORF1 whereas Rep' is translated from a truncated and C-terminal frame-shifted transcript (72). Compared to other circular ss-DNA viruses, a factor that is unique to circoviruses is the requirement of both Rep and Rep' for replication. Three conserved RCR motifs (See Fig. 1-3: motif I, FTLNN; motif II. HxQ and motif III. YxxK) as well as a dNTP binding motif (P-loop), GKS, are located within the N-terminus of Rep (72, 118). Functions of motifs I-II as well as the GKS motif are described below. Aside from these motifs, Rep and Rep' contain three nuclear localization signals (NLS) within their N-terminus. While NLS1 and NLS2 are required for recruitment to the nucleus, NLS3 functions as an enhancer for localization (24). The promoter of rep (Prep), located within the 109 nt IR, is negatively regulated by Rep, whereas, Rep' and Cap show no regulation capabilities (74). The structure of the catalytic domain of Rep (aa 1-116) of PCV2 ORF1has been resolved by NMR (126). In addition, the NMR structure of the

1 corresponding domain in the *Faba Bean Necrotic Yellow Virus* (FBNYV) Rep protein (127),
2 which is in the *Nanovirus* family has been resolved. Figure 1-3 shows the three dimensional
3 (3D) structure alignment of the two regions from each virus. Interestingly, although the
4 sequence homology is ~35% for this region, the 3D structures are similar. Furthermore, the
5 amino acid sequences and locations of the three conserved RCR motifs are almost identical (see
6 Fig. 1-3 panels A-D). Overall, the conservation of both the amino acid sequence and the
7 structural location demonstrate the significance of these motifs for RCR and likely the survival
8 of these viruses. Additionally, the similarities in the positions of the RCR motifs support the
9 hypothesis that PCV evolved from a nanovirus.
10
11 Located on the minus strand of the PCV genome, ORF2 is translated into the 232 (PCV1) or 233
12 (PCV2) aa capsid protein (CP) (68). In contrast to Prep, the promoter of cap (Pcp), located
13 within ORF1, is not regulated by any of the PCV gene products (Rep, Rep' or CP). Aside from
14 being the only major structural protein, CP is the main antigenic determinant of PCV. Similar to
15 other circoviruses, PCV CP contains an arginine rich basic N-terminus responsible for nuclear
16 localization (68, 86). In BFDV, the N-terminal residues are also responsible for binding viral
17 DNA after entry into the cell, providing evidence that PCV CP likely functions to target the viral
18 genome to the nucleus for replication (83).
19
20 The smallest ORF, ORF3, maps within ORF1 and is transcribed from the negative sense strand
21 of the PCV2 genome. ORF3 of PCV2 codes for a 105 aa protein whereas, in PCV1, ORF3 is 207
22 aa. The corresponding regions of PCV1 and PCV2 ORF3 share ~60% sequence homology. To
23 date, the function of ORF3 remains highly debatable. Liu et al. (66) reported the gene product

1 of ORF3 was responsible for induction of apoptosis in PCV2 infected PK-15 cells. Follow up
2 studies from the same group reported an increase in PCV2 associated lesions in BALB-c mice
3 infected with WT PCV2 compared to a mutant virus lacking ORF3 (11) and that abrogation of
4 the function of ORF3 attenuated PCV2 infection in pigs (48). These results have led to
5 speculations that the difference in pathogenicity between PCV1 and PCV2 is due to ORF3. In a
6 recent study, Juhan et al. (47) reported delayed PCV2 seroconversion and lower PCV2 serum
7 titers in pigs infected with ORF3 mutant PCV2 compared to serum titers in pigs infected with the
8 wild type (WT) virus. However, in the same study, no significant differences were reported in
9 the gross lesions, amount of PCV2 specific antigen in tissues, and average scores of histological
10 or gross lesions in WT- or ORF3 mutant-PCV2 infected pigs. Overall, it is unclear whether
11 ORF3 plays a major role in the PCV2 virulence. One possibility is that apoptosis associated with
12 ORF3 plays a part, although, is not the sole factor for PCV2 pathogenesis. Further research is
13 needed to determine the exact role of ORF3 in terms of pathogenicity in pigs.

14 ### *Virus Replication*

15 Based the structure of the stem loop as well as the three conserved RCR motifs within Rep, it has
16 been proposed that PCV replicates by the rolling circle replication model. To date, two RCR
17 mechanisms have been proposed. The first is the "cruciform" mechanism. This model describes
18 replication of the PCV genome from a single 'leading strand.' The second is the "melting pot"
19 mechanism. This model describes replication from both a leading and lagging strand. For the
20 purpose of this report, a general outline of each mechanism will be described. Both models are
21 described and depicted in extensive detail in Figure 1-4. Both models have been extensively
22 reviewed and summarized by Faurez et al. (21), and Finsterbusch et al. (25).

1 ***The "Cruciform" RCR mechanism***

2 As summarized by Faurez et al. (21), once PCV has infected a cell, the ssDNA genome of the
3 virus is likely converted by host enzymes into a supercoiled dsDNA replicative form (RF).
4 However, at this time, neither the viral DNA sequence, or the host proteins involved in the
5 production of the RF are known. Upon formation of the RF, the PCV replication proteins, Rep
6 and Rep', form a replication complex (RC) that binds to the origin of DNA replication. As
7 previously mentioned, the MBS for the Rep complex was mapped to the 3' portion of the stem
8 loop and the most proximal hexamers, H1 and H2 within the 109 nt IR. Binding of the RC
9 destabilizes and unwinds the dsDNA at the origin which leads to the exposure of the nonamer
10 sequence as ssDNA and the formation of a cruciform. The exposed nonamer sequence is then
11 recognized and cleaved by the RC between the position 7-T and position 8-A (i.e.
12 TAGTATT'AC). Cleavage of the ssDNA nonamer is dependent upon the three conserved RCR
13 motifs located within Rep and Rep'. Although the exact function of motif I is unclear, it is
14 speculated that this motif serves as a catalyst. Motif II is required for coordination of divalent
15 metal cations, which are required for nicking the viral DNA for unwinding. Motif III contains a
16 tyrosine which performs the cleavage of the phosphodiester bond by nucleophilic attack (25).
17 Cleavage by tyrosine causes the RC to be covalently attached to the 5' end of the viral genome
18 and generates a 3'-hydroxyl that serves as a primer for DNA synthesis by the host DNA
19 polymerase. Upon completion of a single genome, termination occurs when the newly formed
20 leading strand displaces the positive sense coding strand and the RC covalently attaches the 5'
21 and 3' ends of the genome, forming a circle. The positive circular ss parental DNA is then
22 released leaving a ds-circular DNA molecule composed of the negative parental strand and the
23 newly synthesized positive strand. At this point, the newly synthesized ssDNA molecule can

1 either be encapsidated or be involved in a second round of replication (21). An extensive
2 diagram of this model is presented and described in Figure 1-4 panel I.

3 ***The "melting pot" RCR model***

4 The significance of inverted repeats, located within Ori, was reported by Cheung (13). The
5 analysis included PCV clones engineered with mutations within the Ori region followed by
6 transfection of the parental viruses and analysis of the of progeny viruses. From the results it
7 was concluded that the "cruciform" RCR model could not account for all of the progeny viruses
8 that were produced. Therefore, a novel mechanism of RCR, which was termed the "melting pot"
9 model, was proposed (see Figure 1-4 panel II). In this model events are exactly the same as in
10 the cruciform model up until the binding of the RC and formation of the cruciform. Instead of
11 forming a cruciform, all four strands of the inverted repeats are in a melted state with no
12 hydrogen bonding between the plus and minus strands. However the strands remain in close
13 proximity and are positioned in a four-stranded tertiary structure (See Fig. 1-4 panel I A.) Upon
14 nicking of the nonamer by the RC, elongation proceeds into the palindromic region of the
15 melting pot (through the right arm of the stem loop) displacing the old strand (y in Fig. 1-4 panel
16 I A). Due to the positioning of the strands in the melting pot, both the complementary strand (y')
17 and the palindromic strand (x) are available as templates. Upon completion of a single round of
18 genomic replication, termination occurs when the leading strand ascends into the melting pot
19 (along the left arm of the stem loop) and displaces the old strand (x). At this point in replication,
20 both the newly synthesized strand (y_n) and the complementary strand (x') are available as
21 templates. After this, events involving closure and release of the ssDNA viral genome are
22 similar to the "cruciform" model.

1 ***Infection and the Virus Life Cycle***

2 The oro-nasal route is considered the primary route of entry for PCV2 (7, 135). Upon entering
3 the host, PCV2 replicates within the tonsils and lymph nodes (32). From there, the infection of
4 B-cells or dendritic cells has been suggested as the mechanism of dissemination throughout the
5 host (17, 33). PCV2 then establishes infection in a wide range of cell types. Antigen from the
6 virus has been found in multinucleated giant cells, dendritic cells, histiocytes, as well as other
7 cells of the monocyte macrophage lineage (17). Other cells harboring PCV2 include kidney and
8 respiratory epithelial cells, lymphocytes, vascular endothelial cells, enterocytes, hepatocytes,
9 smooth muscle cells and pancreatic acinar and ductular cells (83).
10
11 The first phase of a viral infection involves binding and entry of the virus into the host cell,
12 which often dictates the cell and tissue tropism and can affect pathogenesis. Entry into the host
13 cell can occur by direct penetration through the plasma membrane or through endocytic
14 pathways preceding interaction with cell-surface receptors. Traditional techniques to analyze
15 viral entry include the use of chemical inhibitors that block pathways of endocytosis as well as
16 co-localization of entering viruses with components of the cellular endocytosis machinery.
17 Using these techniques, Misinzo et al. (82) analyzed the route of entry for PCV2 into the
18 monocytic cell line 3D4/31. The results of their studies showed that entry of PCV2 virus like
19 particles (VLP) could be inhibited by methods that disrupted or inhibited: 1) clathrin-mediated
20 enocytosis, 2) actin and 3) endosomal acidification. Furthermore, they used fluorescent confocal
21 microscopy to show that clathrin co-localizes with PCV2 VLPs. From these results, they
22 concluded that entry of PCV2 into monocytic cells occurs predominantly through clathrin-
23 mediated endocytosis and that endosomal acidification is important for PCV2 infection.

1

2 Following this study, Misinzo et al. (83) analyzed the possible role of glycosaminoglycan's
3 (GAG) as PCV2 receptors. GAGs were chosen because they are a common receptor for many
4 viruses, PCV2 infects a wide range of cell types and GAGs are expressed in a wide variety of
5 cells. In addition, the sequence IRKVKV is conserved within PCV2 CP and is classified as a
6 heparin sulfate (HS; a glycosominoglycan) binding motif (XBBXBX: B=basic aa,
7 X=neutral/hydrophobic aa). To analyze whether GAG plays a role in attachment, they
8 performed direct binding measurements, competition assays with soluble GAG and infection
9 analysis of cells with GAGs enzymatically removed. The results of their assays showed that
10 pre-incubating PCV2 virus with soluble heparin, heparin sulfate (HS), or chondroitin sulfate-b
11 (CS-B) significantly decreased PCV2 infection of PK-15 cells or 3D4/31 cells. Similar results
12 were found upon enzymatic removal of the aforementioned GAGs from 3D4/31 cells.
13 Furthermore, PCV2 infection of CHO cells with mutant or absent GAG was significantly
14 reduced compared to WT CHO cells. From these results, they concluded HS and CS-B are
15 involved in attachment of PCV2. Aside from this, the inability to completely block PCV2
16 infection in CHO cells lacking GAG led to the conclusion that PCV2 requires additional cellular
17 receptor(s).

18

19 Other cells that support the replication of PCV2 in vitro include epithelial cells, including PK-15
20 cell lines (77). While it was demonstrated that PCV2 uses the same receptor for attachment to
21 epithelial cells and monocyte/macrophage cells (83), it was unknown whether factors such as
22 endosome-lysosome acidification was important for PCV2 replication in epithelial cells. To
23 analyze this, Misinzo et al. (84) incorporated the same techniques as previously described (82),

1 with the exception of incorporating epithelial cells (primary porcine kidney epithelial, swine
2 testicle (ST), PK-15, and porcine kidney (PK) rather than 3D4/31 cells in culture. Experimental
3 results showed that inhibition of endosome/lysosome acidification caused in increase in the
4 number of PCV2 infected ST and primary porcine kidney epithelial cells. Further analysis
5 revealed that inhibition of endosome/lysosome acidification affected the disassembly stage of
6 PCV2 infection. Next, they used double immunofluorescent labeling for PCV2 virus like
7 particles (VLP) and markers of early endosomes, lysosomes, the Golgi apparatus, and the
8 endoplasmic reticulum to identify the intracellular compartment in which PCV2 is transported
9 following internalization in epithelial cells. Results of these assays showed that PCV2 VLPs co-
10 localized with the early endosome and lysosome. Furthermore, they showed that the addition of
11 a serine protease, a protease often involved in viral disassembly, abolished disassembly of PCV2.
12 Overall, these results show that inhibition of endosome/lysosome acidification enhanced PCV2
13 replication in endothelial cells and that the effects of inhibitors were at the level of PCV2 capsid
14 disassembly. Additionally, a serine protease mediates disassembly of the PCV2 capsid.
15
16 Results showing the different acidification requirements for endosomes/lysosomes in epithelial
17 and monocyte/macrophage cell lines prompted the investigation of the mechanism for
18 internalizing PCV2 into epithelial cells. Using similar techniques as previously described,
19 Misinzo et al. (81) analyzed PCV2 binding and internalization in PK-15, SK, and ST epithelial
20 cells. Initial co-localization results showed that clathrin co-localizes with PCV2 VLPs upon
21 entry. However, inhibitors of clathrin mediated endocytosis had no effect on PCV2 infection,
22 which prompted analysis of clathrin and caveon independent pathways (CCIP) for
23 internalization. Results from these experiments showed that the addition of small GTPase

1 inhibitors as well as inhibition of actin polymerization significantly reduced PCV2 infection.
2 Interestingly, depletion of plasma membrane cholesterol significantly enhanced PCV2 infection.
3 Overall, the results of these experiments led to the following conclusions: 1) PCV2 can enter
4 epithelial cells by either clathrin mediated endocytosis or by a small GTPase-regulated CCIP, 2)
5 a small GTPase-regulated CCIP rather than the clathrin mediated endocytosis is more effective
6 for PCV2 infection, 3) the internalization of PCV2 is dependent on actin, and 4) PCV2 infection
7 is strongly enhanced by removing membrane cholesterol.
8

9 The next step in PCV2 replication is transport of the DNA genome into the nucleus.
10 Circoviruses depend on the host replication machinery for de novo DNA synthesis (77). Aside
11 from the replication complex, continuation of viral replication depends on cellular enzymes
12 expressed during S phase and therefore, commences only after the host cell has proceeded
13 through mitosis (122). Prior to replication, the viral DNA must pass through the nuclear
14 envelope. Due to size limitations, the viral genome is unable to cross the nuclear envelope by
15 passive diffusion (41). Therefore, macromolecules such as proteins or viral DNA must be
16 actively transported through protein-lined aqueous channels known as nuclear pore complexes
17 (NPC) (41). The transport of proteins through the nuclear pore complex is signal mediated. As
18 mentioned, both Rep and Cap contain NLS sequences. However, the way in which the PCV
19 genome reaches the nucleus is unclear. In a different circovirus, beak and feather disease virus,
20 targeting the viral DNA to the nuclease was carried out by the CP (65). Therefore, it is likely
21 that the PCV CP interacts with the viral genome and is transported through the nuclear pore
22 complex into the nucleus. Upon entry into the nucleus, host cell factors convert the ssDNA into
23 a dsDNA replicative form. This is followed by commencement of RCR (previously described).

Currently, there is little knowledge of how the newly synthesized ssDNA genome is packaged, how the virus assembles or how progeny viruses exit the host cell. A complete summary of the PCV2 life cycle is described in Fig. 1-5.

Biological and Physico-chemical Properties of PCV

PCV is characterized as a non-enveloped, single stranded-DNA virus with icosahedral symmetry. The outer protein of a PCV2 particle spans ~17nm and is composed of 60 capsid protein molecules arranged into 12 pentameric units (3). Biochemical characteristics of PCV1 include a buoyant density of 1.37g/ml in a CsCl gradient, stability at a pH of 3.0, and stability at temperatures of 56°C or 70°C for up to 15 minutes. PCV1 is also incapable of hemagglutinating erythrocytes from a wide range of species (2, 37, 49, 55, 64, 85, 89, 105). Disinfectants aimed at dissolving lipids such as those based on alcohol, chlorhaxidine, iodine and phenol have no effect on PCV2. Inactivation of PCV2 requires alkaline disinfectants (sodium hydroxide), oxidizing agents (sodium hypochlorite) or quaternary ammonium compounds (35).

Geographic Virus Distribution and Prevalence

After the identification of PCV1 and PCV2, the two viruses have been found worldwide. Jacobson et al. (46) performed a retrospective study and reported PCV2 infection in pigs as early as 1962. Furthermore, they reported the characteristic histopathological lesions of PMWS together with PCV2 antigen in archived tissues from 1985. The prevalence of PCV2 and PCVAD has been reported in multiple countries including the United States, Canada, Germany, the Netherlands, Hungary, Ireland, Greece, Spain, Croatia, the United Kingdom, Japan, Taiwan, Korea, countries in South America, and more recently in Australia (98). Interestingly, PCV2

1 viral infection has been identified in Australia without the onset of PCVAD (35). PCVAD is
2 now considered enzootic in the majority of the world and can become epizootic when the
3 mortality increases significantly compared to the previous mortality status (98).
4
5 In 1986, 77-96% of serum samples from pigs were reported seropositive for PCV (121). After
6 identifying PCV2 as a factor for the onset of PMWS, subsequent studies identified PCV2 as the
7 predominant circulating strain (98). Overall, the prevalence of antibodies to PCV2 ranges from
8 around 40-80% in in PCV2 affected countries such as Spain, Taiwan, Canada and the United
9 States. The prevalence of PCV2 viral antigen or DNA ranges around 23% in Japan, 8% in
10 Korea, 35% in the UK, 10% in the USA, and 50% in Taiwan (3). Due to the low sensitivity of
11 detection methods and the cross identification of PCV1 and PCV2 in early studies, obtaining
12 exact data is challenging. Overall, morbidity associated with PCV2 is generally low; however,
13 mortality can peak as high as 50% in some affected herds (98).

14 ***Transmission***

15 Within an environment, the virus is very stable. Within a pig, PCV2 has been demonstrated to
16 cause a persistent infection causing viremia in semen, blood, and tissues. One study involving
17 250 pigs from a herd with a history of PMWS reported PCV2 nucleic acid (PCR) in serum
18 obtained at 7, 12, and 28 weeks of age, demonstrating the ability of the virus to persist within the
19 host. (7). Experimentally, it was demonstrated that point source exposure to the virus was
20 enough to seroconvert naïve pigs (112). A different experimental infection study demonstrated
21 that co-mingling of naïve pigs with pigs experimentally inoculated with PCV2 42 days prior led
22 to infection of all naïve pigs (7). The virus has been detected by way of PCR in oro-nasal swabs,
23 urine, blood, and feces in experimentally infected pigs (53, 59, 111). Additionally, the virus has

1 been detected in both semen and colostrum, although there is no evidence that the virus is spread
2 by insemination or by ingestion of colostrum (95, 96, 119). Vertical transmission of the virus
3 has also been reported in an experimental infection study. Park et al. (95) demonstrated that
4 infection of a sow 6 weeks prior to farrowing caused reproductive failure. Additionally, PCV2
5 antigen as well as infectious virus were detected in fetal tissues. However, this occurrence is
6 believed to be rare in natural farm settings (10). Overall, the stability of the virus in the open
7 environment and within the pig, as well as the highly infectious nature of the virus, indicate the
8 horizontal route as the primary means by which the virus is spread.
9

10 ***Detection and Quantification of PCV2 Antigen, DNA and Antibodies***

11 Two common methods for identification of PCV2 infection in tissues are immunohistochemistry
12 and in situ hybridization (115). Both techniques are performed on paraffin-embedded, formalin-
13 fixed tissues. For IHC, both polyclonal and monoclonal antibodies are commonly used for
14 detection of PCV2 antigens. Virus specific probes are used for detection of PCV2 DNA.
15 Additionally, PCV2 antigens can be detected by indirect immunofluorescence assay (IFA),
16 immunoperoxidase monolayer assay (IPMA), and antigen capture enzyme linked immunosorbant
17 assay (ELISA) (100). Other methods for detecting PCV2 infection include virus isolation and
18 PCR. Virus isolation, usually performed by titrating serum or tissue homogenate on PK-15 cells,
19 is more time consuming and not as sensitive as IHC or ISH (115). On the other hand, PCR is
20 one of the more widely used assays for PCV2 detection (36, 43, 94). This assay can be used to
21 detect and quantify viral DNA in fixed tissues, semen, blood/serum/plasma, and other excretions.
22 Additionally, PCR assays have been developed to distinguish PCV2 genotypes (45).
23

1 Serological assays for detecting both PCV1 and PCV2 antibodies have been developed (2, 116).
2 As discussed below, due to the presence of PCV2 in clinically moribund PVCAD pigs as well as
3 clinically normal pigs, serological assays are of little use in the diagnosis PCVAD. The most
4 common methods for identifying the presence of PCV2 antibodies are the indirect
5 immunofluorescence assay (IFA), the immunoperoxidase monolayer assay (IPMA) and the
6 enzyme linked immunosorbant assays (ELISA). Both IFA and IPMA involve measuring the
7 ability of serum antibodies to bind to a fixed monolayer of PCV2 infected cells in culture. The
8 principle difference between the two assays is IFA incorporates a secondary fluorescein-
9 conjugated antibody, whereas, IPMA incorporates a secondary peroxidase-conjugated antibody
10 for detection. Additionally, IFA incorporating cells expressing PCV2 ORF2 have been described
11 (97). Results of comparing assays with virus infected or PCV2 ORF2 expressing cells showed
12 that assays with ORF2 were more sensitive that virus infected cells.
13
14 As described by Nawagitgul et al. (88), pitfalls for both IPMA and IFA include the requirement
15 for experienced technicians for preparation of infected cells and interpretation of staining results.
16 Furthermore, reading the results of plates can become tedious and time consuming. One assay
17 that can be alleviate these issues is the PCV2 specific ELISA. The first PCV2 ELISA described
18 involved a monoclonal antibody based competitive ELISA (132). Since then, indirect ELISAs
19 incorporating antigen from PCV2 infected cells or recombinant PCV2 ORF2 expressed either in
20 bacteria or mammalian cells have been described (6, 87, 88, 97, 110, 136). The diagnostic
21 sensitivity and specificity of ELISAs incorporating PCV2 antigen from infected cells has been
22 reported (88). Similar to IFA and IPMA, these assays require the time consuming and labor
23 intensive task of cultivating virus from PCV2 infected cells. A more successful approach is the

1 use of recombinant PCV2 ORF2 using the baculovirus expression system (6, 88). In order to
2 produce antigen, recombinant baculovirus containing ORF2 of PCV2 is inoculated onto insect
3 cells. In general, whole cell lysates are used as antigen in ELISAs based on this system. One
4 major drawback is the costs associated with the production of recombinant proteins in any
5 eukaryotic system. A much more cost effective approach is the expression of ORF2 in bacteria.
6 The successful production of ORF2 fused to either maltose binding protein or glutathione-S-
7 transferase has been reported (69, 136). However, expression assays produced only low levels of
8 ORF2 fusion proteins. The low level expression of recombinant ORF2 protein can likely be
9 attributed to the Arg-rich, NLS located at the N-terminus of ORF2. To increase protein
10 expression, Zhou et al. (136) produced an ORF2-fusion protein void of the N-terminal 40 amino
11 acids (NLS). Western blot analysis demonstrated the ability of PCV2 positive pig serum to
12 recognize NLS-deleted ORF2-fusion proteins. Using a different approach, Trundova et al. (124)
13 reported high yields of ORF2 expressed in bacteria following codon optimization of the ORF2
14 sequence. Immunoblots incorporated the codon optimized and expressed ORF2 demonstrated
15 the ability of pig anti-PCV2 serum to recognize the recombinant protein. In summary, bacterial
16 expression of ORF2 is a cost effective system for producing PCV2 capsid protein antigen.
17 Furthermore, assays incorporating recombinant bacterial expressed capsid protein provide for an
18 effective means to measure antibodies specific for PCV2.

19 ## PCVAD

20 The term PCVAD describes a group of complex multi-factorial syndromes. Traditionally, the
21 etiology of a syndrome is based on the identification of a single infectious agent. Due to the fact
22 that PCV2 can be isolated from normal healthy pigs, it was difficult to link PCV2 in the etiology
23 of PCVAD. . The diversity of the syndromes classified as PCVAD's lead to the hypothesis that

individual viruses were responsible for each syndrome (1, 4, 40, 90). Furthermore, PCV2 alone could not reproduce all of the PCVAD syndromes.. Although some success at reproducing PMWS has been reported (32, 39, 45), most knowledge regarding PCVAD is obtained from cases in the field.

Herds with PCVAD usually have a mortality around 10%, however, mortalities reaching 50% have occurred (70, 98). PCVAD syndromes include porcine multi-systemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), porcine respiratory disease complex (PRDC), reproductive failure, granulomatious enteritis, exudative epidermitis, necrotizing lymphadenitis, and congenital tremors. For the purpose of this study, PDNS and PMWS will be discussed in detail below. For a review of other syndromes, see Chae (2005) (15, 38).

PMWS

PMWS was first described in specific pathogen free swine herds in Canada in the early 1990's (10). Chae (10) proposed three main criteria for diagnosing a pig with PMWS: (I) the presence of compatible clinical signs, (II) the presence of microscopic lesions characteristic of PCV2 infection, and (III) the presence of PCV2 within the microscopic lesions (10, 70, 108). PMWS primarily affects pigs between 5-16 weeks of age with the greatest frequency onset at 8-12 weeks of age (10, 39, 45). Mortalities in post weaned herds range from 10-25%. Clinical signs of PMWS are somewhat non-specific and variable. Signs from experimentally infected as well as field cases include lethargy, diarrhea, lymphadenopathy, discoloring of the skin, jaundice, and wasting characterized by progressive weight loss (45, 92). Necropsy shows enlargement of the submandibular, inguinal, and bronchial lymph nodes as well as non-collapsed wet lungs. In

certain cases, the lungs, liver, kidney and heart can be found to have granulomatious lesions (10, 45). Histopathological characteristics of PMWS include granulomatious inflammation and the presence of intracytoplasmic inclusion bodies (10). Intracytoplasmic inclusion bodies are characterized as large basophilic or amphophilic structures that are often found in the cytoplasm of multinucleated giant cells and histiocytic cells. Granulomatious inflammation is a lesion characterized by infiltrates of epitheliod cells and multinucleated giant cells. These lesions are seen in the liver, spleen, tonsil, lymph nodes, thymus, and Peyer's patches. During PMWS, lymphoid cells and tissues are often depleted and replaced by macrophages and multinucleated giant cells (10).

There are multiple viral and bacterial pathogens that have been identified with PCV2 in cases of PMWS. Examples include porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus, porcine parvovirus (PPV), *Haemophilus parasuis, Streptococcus suis, Mycoplasma hypopneumoniae, and Actinobacillus pleuropneumoniae* (57). The presence of other pathogens with PCV2 complicates the diagnosis of PMWS due to presentation of different clinical signs with different co-infecting pathogens.

Multiple hypotheses have been formed to explain why so many different pathogens are associated with PCV2 in causing PMWS. First, it may be possible that a variety of pathogens share a similar mechanism in affecting the immune system. This then allows PCV2 infection to progress into PMWS (17). Another possibility involves PCV2 initiating lymphoid depletion. This would allow for opportunistic infection from other viruses or bacteria. In an experimental infection study involving g1-TTV and PCV2, the development of lesions consistent with PMWS

1 were reported following infection with g1-TTV prior to infection with PCV2 (114). These
2 results provide evidence supporting the hypothesis that pathogens may weaken the immune
3 system and allow PCV2 infection to progress to PMWS.
4

5 ***PDNS***

6 PDNS was first described in the United Kingdom in 1993 (104). PCV2 was subsequently
7 identified in tissues of pigs with PDNS (9, 32). The syndrome primarily affects pigs that are 12-
8 14 weeks of age, however, the syndrome can affect pigs as young as 5 weeks of age (45, 114).
9 Interestingly, cases of PDNS are sporadic within a herd. Clinical signs of PDNS include
10 lethargy, fever, severe weight loss, and anorexia. A more obvious clinical sign is the presence of
11 skin lesions, ranging in color from red to purple to black, covering the hind legs, or other areas of
12 the body (9, 45). One of the striking features of PDNS is that pigs displaying clinical signs often
13 die within 3 days of the onset and overall mortality is approximately 20%. At necropsy, renal
14 and inguinal lymph nodes are enlarged and hemorrhagic. The pleural and peritoneal cavities
15 generally have an increase in fluid. Kidneys appear wet, enlarged and have pinpoint
16 hemorrhages along the capsule (32, 45). Microscopically, PDNS is characterized by
17 dermal/epidermal necrosis, fibrinous glomerulonephritis and systemic vasculitis (92). In
18 addition, deposits of antigen-immune complex within capillary glomerular and vascular walls
19 have been identified, characteristic of a type 3 hypersensitivity reaction (133).
20

21 Similar to PMWS, multiple pathogens have been identified in conjunction with PCV2 in pigs
22 diagnosed with PDNS. To date, the overall cause of PDNS is unclear, although there are
23 multiple hypotheses that attempt to explain the onset. Wellenberg et al. (2004) (58) reported

1 relatively increased levels of IgG and IgM antibodies directed towards PCV2 in pigs diagnosed
2 with PDNS. Furthermore, they describe the deposition of the antibodies IgG1, IgG2 and IgM
3 and the complement components C1q and C3 within the renal glomeruli of PDNS affected pigs.
4 Although they were unable to detect PCV2 antigen within immune complexes, they hypothesize
5 that the high levels of PCV2 antibodies trigger the deposition of immune complexes in the
6 kidney. They further speculate that the histopathological features of PDNS, including, vasculitis
7 and glomerulonephritis are likely the effect of a systemic immune complex disorder.
8 Interestingly, Krakowka etal. (2008) (9) reported the induction of PDNS in gnotobiotic pigs after
9 infection with only group 1 torque teno virus and PRRSV. In contrast to Wellenberg et al
10 (2004), they reported the presence of the plasma glycoproteins fibrin and fibrinogen in deposits
11 within renal glomeruli. They subsequently hypothesize that PDNS is the direct result of an acute
12 systemic coagulation defect or disseminated intravascular coagulation. The exact mechanism of
13 disease onset remains to be determined.
14
15 There have been multiple reports of single outbreaks of either PDNS or PMWS as well as
16 outbreaks of concurrent PMWS and PDNS (14). Although PDNS and PMWS are both
17 associated with PCV2, there has been no evidence of a direct relationship between the two
18 syndromes. Pigs with PMWS never progress to PDNS and vice versa. It was reported that
19 PCV2 viral DNA was more abundant in kidneys from pigs with PDNS than in pigs with PMWS.
20 In addition, PCV2 viral DNA was more abundant in lymph nodes from pigs with PMWS than in
21 pigs with PDNS (54). Although it is clear that there are multiple factors contributing to the onset
22 of PDNS or PMWS, these results may indicate that the progression of pigs to either PMWS or
23 PDNS is due to the tissue tropisms of the specific PCV2 virus infecting the host.

Control and Prevention of PCV2 and PCVAD

Currently, the most effective protection from PCV2 and PCVAD are commercial vaccines. Before the advent of vaccines, multiple measures were incorporated with varying effects. To this day, proper housing and management is critical in the prevention of disease. Studies have demonstrated that proper hygiene, stress reduction, practicing an all in all out policy, and the prevention of age mixing can reduce disease and its spread (131). Previous methods that were used with minimal success include antibiotics to control secondary infections, serum therapy and depopulation (16, 20, 44, 56, 107).

The most successful way to control PCV2 and prevent the onset of disease is the administration of PCV2 vaccines. In the United States, it is estimated that 99% of pigs are vaccinated for PCV2. There are multiple commercial vaccines available that are all based on PCV2a. The first ever commercially produced PCV2 vaccine was CIRCOVAC (Merial). This contained inactivated PCV2 in an oil adjuvant. More recent vaccines include Circumvent PCV/Porcilis (Intervet-Schering Plough). Circumvent PCV is a two dose recombinant vaccine incorporating ORF2 (CP) of PCV2 expressed by baculovirus infected insect cells. A similar recombinant vaccine is Ingelvac CircoFLEX (Boehringer). This vaccine differs from Circumvent PCV in that it requires only 1 dose. The fourth major vaccine is a chimeric inactivated virus that contains ORF2 of PCV2 carried in the backbone of PCV1. This is a one dose vaccine called Suvaxyn PCV2 One Dose (Fort Dodge/Pfizer). Multiple studies have been performed to demonstrate the efficacy of PCV2 vaccines. In vaccine studies conducted in the field, the most common results reported were significant decreases in mortality rates, reduction in costs of antibiotic treatments,

increases in average daily weight gain, as well as decreases in overall PCV2 virus load in serum and decreases clinical and microscopic signs of PCVAD (30).

The Host Immune Response Following PC2 Infection

Innate Immune response

The innate immune response is a critically vital defense to infection by any pathogen. It is likely that the adaptive and innate immune responses have co-evolved, and between them, there is a significant degree of interaction and interdependence. An effective adaptive response is predicated on recognition by the innate immune response (50). Recently, it has been hypothesized that the way PCV2 influences the innate immune response can determine the disease outcome of viral infection (17). Cells such as monocytes/macrophages and dendritic cells are key activators of the immune system and have been reported to be associated with PCV2 infection (54).

One of the major leukocytes of the immune system is the dendritic cell (DC). These cells are the major antigen presenting cells of the immune system. Aside from this, they are involved in the production of reactive oxygen species, and production of cytokines (128, 130). Since DCs play a pivotal role in the immune system, they are an ideal target for viral evasion from the immune response. Experiments performed by Vincent et al. (128) regarding the interaction of PCV2 and DCs has indicated that PCV2 interacts and persists within bone marrow derived and monocyte derived DCs. Interestingly, although PCV2 resides within these cells, neither an increase nor decrease in infectious virus was detected after incubation for 5 days. Further experiments showed that PCV2 infection caused no apparent effects on the overall immune function of these

1 cells (i.e. antigen presentation and co-stimulation of lymphocytes). Additionally, no viral
2 transmission was detected in assays involving lymphocytes interacting with dendritic cells.
3 These results indicate that DCs may provide a "safe haven" for PCV2 and serve as a vehicle to
4 traffic PCV2 throughout the host. Other reports from the same lab group have identified a subset
5 of DCs that are impacted by infection with PCV2. In a study analyzing activation of natural
6 interferon producing cells, it was reported that PCV2 infection impairs induction of interferon
7 (IFN)-α and tumor necrosis factor (TNF)-α, which are both essential for maturation of
8 conventional DCs (130). A subsequent study revealed that this inhibition was caused by the viral
9 DNA. Furthermore, PCV2 DNA was shown to affect immune responses induced by toll-like
10 receptor (TLR)-7 and TLR9 agonist, as well as viruses such as classical swine fever virus and
11 psuedorabies virus (129). Overall, these results indicate that modulation of of the immune
12 response by PCV2 could render the host more susceptible to secondary infections.
13
14 Other integral cells of both the adaptive and innate immune response include
15 macrophages/monocytes. These cells are involved in phagocytosis, antigen presentation,
16 mediation of inflammatory responses, production of reactive oxygen and nitrogen species, and
17 production of cytokines and complement proteins (34). Similar to DCs, studies involving the
18 interaction of PCV2 with monocyte derived macrophages (MdM), alveolar macrophages (AM),
19 and monocytes) showed AM and MdM internalize and carry PCV2 (12, 18). However, there is
20 little or no PCV2 replication within these cells. To evaluate the effects of PCV2 infection on
21 macrophages, Chang et al. (12) performed in vitro assays analyzing microbicidal activity and
22 modulation of cytokine production. Their results showed a decrease in phagocytosis and the
23 production of reactive oxygen species in PCV2 infected macrophages. Modulation of cytokines

1 in macrophages included an increase in the production of tumor necrosis factor-α (TNF-α),
2 macrophage derived colony stimulating factor-II, granulocyte colony stimulating factor (GM-
3 CSF), monocytes chemotactic protein-1 (MCP-1), and interleukin-8 (IL-8) compared to non-
4 infected macrophages.
5
6 Peripheral blood mononuclear cells (PBMCs) have also been reported to be modulated by PCV2
7 infection. In contrast to DCs and macrophages/monocytes, PCV2 has not been found to infect or
8 reside within PBMCs. However, in an assay involving stimulation of PBMCs isolated from pigs
9 infected with PCV2, PBMCs were reported to have a reduced production of IL-2 and interferon-
10 γ (IFN-γ) compared to PBMCs from healthy pigs (52).
11
12 With regard to immunosuppression, PCV2 infection of bone marrow derived dendritic cells,
13 PBMCs and $CD172a^{+}$ cells has been reported to increase the production of the
14 immunosuppressant cytokine IL-10 (52, 128, 130, 134). In summary, active replication within
15 cells of the innate immune system is not necessary for PCV2 to modulate the immune response.
16 In addition, PCV2 is capable of interfering with response pathways such as TLRs as well as
17 down regulate antimicrobial responses such as reactive oxygen species. Furthermore, PCV2
18 infection leads to the modulating cytokine profiles. Overall, modulation of the innate immune
19 response likely increases the host susceptibility to infection from other pathogens. This may
20 provide an explanation for the presence of wide variety of co-infecting pathogens during the
21 onset of PCVAD. Aside from this, inhibiting the production of IFN and TNF could reduce the
22 number of circulating DCs. Considering the role they play in antigen presentation, this could
23 significantly impact the activation of the adaptive immune response.

The Adaptive Immune Response

Cell-Mediated Response

Clearance of PCV2 likely occurs by activation of both the humoral and cell-mediated branches of the adaptive immune response. Unfortunately, there is little information regarding the cell mediated response and PCV2. A factor that has received attention is INF-γ secreting cells. Three experimental PCV2 infection studies have reported the significance of INF-γ secreting cells in developing the adaptive immune response (26, 27, 117). Co-infection of specific pathogen free (SPF) pigs with PCV2 and PPV led to the production of INF-γ secreting cells five days post inoculation. In contrast, the production of INF-γ secreting cells occurred 21 days after infection with PCV2 alone (117). Conventionally reared farm pigs infected pigs developed INF-γ secreting cells 14 days after PCV2 infection (26). In an assay for INF-γ secreting cells, treatment with anti-CD4+ or CD8+ antibodies significantly reduced the number of INF-γ secreting cells (27). This indicates both types of T-cells were involved in the anti-PCV2 response. Currently, there are no reports analyzing the cell mediated immune response during PCVAD. Studies involving pigs that develop PCVAD and pigs subclinically infected with PCV2 would increase knowledge of how the cell mediated response differs between these groups of pigs. However, it was reported that both PCV2 specific IFN-γ and neutralizing antibodies (NA) were important for reducing the load of PCV2 in serum (27). From these results, Kekarainen et al. (51) hypothesized that failure of one of the branches of the adaptive immune response could prevent the host from clearing PCV2 from the system. In turn this could lead to the development of PMWS.

The Humoral Response

1 The antibody response of both experimentally infected pigs and naturally infected pigs in the
2 field have been reported. Experimental studies describing the humoral response following PCV2
3 infection indicate that seroconversion occurs between 10 and 28 days post-infection, regardless
4 of the presence of clinical disease (28, 78). The primary immunoglobulin isotypes include IgG1,
5 IgG2, IgA, and IgM. IgG1, IgG2, and IgA generally follow the course of total antibody titers.
6 IgM was reported to develop between 7 and 14 days post infection and peak around day 21 (28).
7 In the same study, IgGs developed between days 14-21 and titers were found to increase through
8 69 days post infection. A different study showed that two pigs with PMWS had increased levels
9 of IgM antibodies 10 days post infection and decreased IgM 21 days post infection compared to
10 subclinically infected pigs (79). It was subsequently hypothesized that the presence of IgM is an
11 indication of viremia.
12
13 Two studies analyzing the production of neutralizing antibodies reported that NA production
14 follow a similar course as the total antibody production in subclinically infected pigs (28, 79).
15 Following isotype specific ELISAs and virus neutralization assays, Fort et al. (28) reported IgG
16 as the primary antibody isotype responsible for virus neutralization. In addition, increases in the
17 titer of NA were found to correlate with the reduction of PCV2 load in serum. In pigs with
18 PMWS, NA titers were reported to be low or non-existent (79).
19
20 In the field, the presence of maternal antibody can impact the seroconversion of pigs. Colostral
21 antibodies have been reported to last until late nursery or early fattening periods (101). The
22 observation that pigs younger than 4 wks of age do not develop PVCAD led Kekarainen et al.
23 (51) to speculate that maternal antibody is likely an important for the prevention of PCVAD.

1 Under field conditions, seroconversion in pigs generally occurs around 7-15 weeks in age. In a
2 field study analyzing the dynamics of PCV2 infection in a herd with PMWS, Rodriquez-Arrioja
3 et al. (102) reported that anti-PCV2 antibodies in pigs decreased from farrowing to 7 wks in age,
4 increased from 3 to 7wks in age, then slowly decreased until 28wks in age. Similar to
5 experimental infection conditions, the predominant ant-PCV2 antibody isotypes are IgG1, IgG2,
6 IgA, and IgM (79). Presently, there is no PCV2-based model system that can reproduce PDNS.
7 However, clinically moribund pigs show a hyperimmune response leading to significant antibody
8 production, which may contribute to immune complex formation and PDNS (133).
9
10 As discussed below, commercial vaccines for PCV2 are available. In general, the efficacy of
11 PCV2 vaccines is based on the induction of a protective antibody response. Vaccine trials in the
12 field have indicated that seroconversion usually occurs 4 weeks after vaccination with an ORF2
13 subunit vaccine (56). Horlen et al. (44) reported differences in the dynamics of the antibody
14 response in vaccinated and unvaccinated pigs. In this field study, at the time of vaccination
15 (3wks of age) all pigs in the test were positive for PCV2 antibodies and at a similar titer. By
16 9wks of age, the titer of non-vaccinated pigs had decreased, with some pigs showing negligible
17 levels of PCV2 specific antibodies. In contrast, the overall titer in vaccinated pigs had increased
18 by 9 wks of age. By 17wks in age, the antibody titer in unvaccinated pigs peaked, surpassing
19 titers of vaccinated pigs, whereas in vaccinated pigs, titers remained at levels similar to those
20 measured at 9wks of age.
21
22 In summary, the dynamics of the humoral immune response is different during subclinical PCV2
23 infection, the onset of disease and after vaccination. Further complicating matters, the antibody

response during PVCAD is inconsistent. For example, pigs diagnosed with PDNS generate significant levels of antibodies to PCV2. In contrast, pigs diagnosed with PMWS generally have a reduced level of antibodies to PCV2. Overall these insights emphasize the significance of understanding the immune dysregulation caused by PCV2 infection.

Epitope Mapping of PCV2 Proteins

Epitope mapping is an important technique used to determine regions within a protein that can be recognized by the immune system. Determining immunoreactive regions can facilitate the development of diagnostic tests as well as therapeutic procedures. Since the onset of PCVAD, there have been multiple reports over both B-cell and T-cell epitope mapping of the proteins of PCV2.

T-cell epitope mapping

To date, there has only been one report describing T-cell epitope mapping of PCV2 proteins. Stevenson et al. (2006) isolated PBMCs from two different sets (n=4 and n=3 respectively) of pigs experimentally infected with PCV2 (71). PBMCs were then treated with 20mer oligopeptides that overlapped by 10 residues and covered PCV2 ORFs 1 2, and 3. Reactivity was determined in a lymphocyte proliferation assay. The authors reported two regions within PCV2 ORF1 located at residues 81-100 and 201-220 and one region within PCV2 ORF3 located at residues 31-50 that were immunodominant T-cell epitopes.

B-cell epitope mapping

Antibody epitope mapping of PCV2 proteins was first reported in 2000. Mahe et al (2000) used a PEPSCAN approach to map linear epitopes within residues 101-307 of ORF1, as well as the entire sequences of ORFs 2 and 3 (125). In order to map epitopes within ORF1, 15mer oligopeptides overlapping by 11 residues were constructed and reacted with serum from SPF pigs experimentally infected with PCV2b. A similar approach was used to map epitopes within ORFs 2 and 3. One exception included the incorporation of serum from conventional farm pigs experimentally infected with PCV2 in addition to serum from SPF pigs experimentally infected with PCV2 in their assay. Their results showed one weak immunoreactive region in ORF1 located at residues 185-211. No immunoreactive regions were reported in ORF3, however, immunoreactive regions in ORF2 were reported at residues 69-83, 113-127, 117-131, 169-183, and 193-207. In a follow up study, Truong et al. (2001) incorporated sera from experimentally infected SPF pigs and field sera from PCVAD affected herds into an ELISA using oligopeptides spanning residues 69-83 and 117-131 (60). They reported the 117-134 region as the ORF2 immunodominant epitope.

Mapping conformational epitopes within PCV2 ORF2 was first accomplished by Lekcharoensuk et al. (2004) (109). For this, seven monoclonal antibodies (MAbs) specific to PCV2a ORF2 were generated by immunizing mice with PCV2 virions purified from infected PK-15 cells. These mAbs were then reacted in an immunofluorescence assay (IFA) with cells transfected with chimeric PCV viruses that contained differing segments of ORF2 from PCV2a and PCV1. Their results showed conformational immunoreactive regions located at residues 47-85, 165-200, and 200-233.

Using a different approach to map epitopes, Shang et al. (2009) generated mAbs against, recombinant PCV1 and PCV2 ORF2, as well as purified PCV2 virions from infected PK-15 cells. These MAbs were then reacted with 18mer or smaller oligopeptides in an ELISA to determine the location of linear epitopes (80). They also reacted MAbs with cells transfected with different segments of PCV2 ORF 2 cloned into expression plasmids to determine the location of conformation epitopes. They report conformational epitopes formed from residues 1-60 and 231-233, 1-230, and 205-230. Linear epitopes were reported at residues 156-162, 179-192, 195-202 and 231-233.

In a more recent study, Meng et al. (2010) mapped the linear epitopes located within the N-terminal region of ORF1 of PCV2 that is shared by Rep and Rep' (60, 71, 125). For this, mAbs were generated that were either specific to PCV2 ORF1 N-terminal residues or both PCV2 and PCV1 ORF1 N-terminal residues. The mAbs were then reacted in a Western blot with different fragments of the PCV2 or PCV1 ORF1 N-terminal residues. Their results showed a linear epitope specific to PCV2 ORF1 at residues 39-46 and a linear epitope specific to PCV1 and PCV2 at ORF1 residues 99-106.

Purpose

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 (91). We extended this approach by evaluating the reactivity of individual polypeptide fragments comprising 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.

Figures

Figure 1-1 Genomic DNA sequence alignment of PCV2a, PCV2b, and PCV2c.

PCV2a (Genbank accession #AF055392), PCV2b (Genbank accession #AF055394) and PCV2c (Genbank accession #EU148503) sequences were aligned using ClustalW software. The number at the end of each alignment indicates nucleotide position within the genome. Stars (*) and blank spaces, located on the bottom row of each alignment group indicate consensus and non-consensus positions, respectively.

```
PCV2a           AATTCAACCTTAACCTTTCTTATTCTGTAGTATTCAAAGGGTATAGAGATTTTGTTGGTC 60
PCV2b           AATTCAACCTTAACCTTTCTTATTCTGTAGTATTCAAAGGGCACAGAGCGGGGGTTTGAG 60
PCV2c           AATTCCACTTTAACCTTTCTTATTCTGTAGTATTCAAAGGGCACAGTGAGGGGGTTTGAG 60
                ***** ** ******************************** * ** *     *** *

PCV2a           CCCCCTCCCGGGGGAACAAAGTCGTCAATTTTAAATCTCATCATGTCCACCGCCCAGGAG 120
PCV2b           CCCCCTCCTGGGGGAAGAAAGTCATTAATATTGAATCTCATCATGTCCACCGCCCAGGAG 120
PCV2c           CCCCCTCCTGGGGGAAGAAATTGGTTAATATTAAATCTCATCATGTCCACCGCCCAAGAG 120
                ******** ******* *** *  * *** ** *********************** ***

PCV2a           GGCGTTGTGAC-TGTGGTACGCTTGACAGTATATCCGAAGGTGCGGGAGAGGCGGGTGTT 179
PCV2b           GGCGTTTTGAC-TGTGGTTCGCTTGACAGTATATCCGAAGGTGCGGGAGAGGCGGGTGTT 179
PCV2c           GGTGGTGAGACCTGTGAGGCA-TTAACGGTATAAACAAAGGAGCGGGAGAGGCGGGCATT 179
                ** * *  *** ****   *  ** ** *****  * **** **************  **

PCV2a           GAAGATGCCATTTTTCCTTCTCCAACGGTAGCGGTGGCGGGGGTGGACGAGCCAGGGGCG 239
PCV2b           GAAGATGCCATTTTTCCTTCTCCAGCGGTAACGGTGGCGGGGGTGGACGAGCCAGGGGCG 239
PCV2c           GAAGATTCCATTTTTCCTTCTCCAACGGTAGCGGTGGCGGGGGTGGACGAGCCAGGGGCG 239
                ****** ***************** ***** *****************************

PCV2a           GCGGCGGAGGATCTGGCCAAGATGGCTGCGGGGGCGGTGTCTTCTTCTGCGGTAACGCCT 299
PCV2b           GCGGCGGAGGATCTGGCCAAGATGGCTGCGGGGGCGGTGTCTTCTTCTCCGGTAACGCCT 299
PCV2c           GCGGCGGAGGATATGGCCAAGATGGCTGCGGGGGCGGTGTCTTCTTCTCCGGTAACGCCT 299
                ************ *********************************** ***********

PCV2a           CCTTGGATACGTCATAGCTGAAAACGAAAGAAGTGCGCTGTAAGTATTACCAGCGCACTT 359
PCV2b           CCTTGGATACGTCATATCTGAAAACGAAAGAAGTGCGCTGTAAGTATTACCAGCGCACTT 359
PCV2c           CCTTGGATACGTCATATCTGAAAACGAAAGAAGTGCGCTGTAAGTATTACCAGCGCACTT 359
                *************** ********************************************

PCV2a           CGGCAGCGGCAGCACCTCGGCAGCACCTCAGCAGCAACATGCCCAGCAAGAAGAATGGAA 419
PCV2b           CGGCAGCGGCAGCACCTCGGCAGCACCTCAGCAGCAACATGCCCAGCAAGAAGAATGGAA 419
PCV2c           CGGCAGCGGCAGCACCTCGGCAGCACCTCAGCAGCAATATGCCCAGCAAGAAGAATGGAA 419
                ************************************* **********************

PCV2a           GAAGCGGACCCCAACCACATAAAAGGTGGGTGTTCACGCTGAATAATCCTTCCGAAGACG 479
PCV2b           GAAGCGGACCCCAACCCCATAAAAGGTGGGTGTTCACTCTGAATAATCCTTCCGAAGACG 479
PCV2c           GAAGCGGACCCCAACCACATAAAAGGTGGGTGTTCACGCTCAATAATCCTTCCGAAGACG 479
                **************** ******************** ** *******************

PCV2a           AGCGCAAGAAAATACGGGAGCTCCCAATCTCCCTATTTGATTATTTTATTGTTGGCGAGG 539
PCV2b           AGCGCAAGAAAATACGGGATCTTCCAATATCCCTATTTGATTATTTTATTGTTGGCGAGG 539
PCV2c           AGCGCAAGAAAATACGGGAGCTCCCAATCTCCCTATTTGATTATTTTATTGTTGGCGAGG 539
                ******************* ** ***** *******************************

PCV2a           AGGGTAATGAGGAAGGACGAACACCTCACCTCCAGGGGTTCGCTAATTTTGTGAAGAAGC 599
PCV2b           AGGGTAATGAGGAAGGACGAACACCTCACCTCCAGGGGTTCGCTAATTTTGTGAAGAAGC 599
PCV2c           AGGGTAATGAGGAAGGACGAACACCCCACCTCCAGGGGTTCGCTAATTTTGTGAAGAAGA 599
                ************************* *********************************
```

```
PCV2a                AAACTTTTAATAAAGTGAAGTGGTATTTGGGTGCCCGCTGCCACATCGAGAAAGCCAAAG 659
PCV2b                AGACTTTTAATAAAGTGAAGTGGTATTTGGGTGCCCGCTGCCACATCGAGAAAGCGAAAG 659
PCV2c                AAACTTTTAATAAAGTGAAGTGGTATTTGGGTGCCCGCTGCCACATCGAGAAAGCGAAAG 659
                     * ***************************************************** ****

PCV2a                GAACTGATCAGCAGAATAAAGAATATTGCAGTAAAGAAGGCAACTTACTTATTGAATGTG 719
PCV2b                GAACAGATCAGCAGAATAAAGAATACTGCAGTAAAGAAGGCAACTTACTGATGGAGTGTG 719
PCV2c                GAACTGATCAGCAGAATAAAGAATACTGCAGTAAAGAAGGCAACTTACTTATGGAGTGTG 719
                     **** ******************** *********************** ** ** ****

PCV2a                GAGCTCCTCGATCTCAAGGACAACGGAGTGACCTGTCTACTGCTGTGAGTACCTTGTTGG 779
PCV2b                GAGCTCCTAGATCTCAGGGACAACGGAGTGACCTGTCTACTGCTGTGAGTACCTTGTTGG 779
PCV2c                GAGCTCCGAGATCTCAAGGACAACGGAGTGACCTGTCTACTGCTGTGAGTACCTTGTTGG 779
                     *******  ******* *******************************************

PCV2a                AGAGCGGGAGTCTGGTGACCGTTGCAGAGCAGCACCCTGTAACGTTTGTCAGAAATTTCC 839
PCV2b                AGAGCGGGAGTCTGGTGACCGTTGCAGAGCAGCACCCTGTAACGTTTGTCAGAAATTTCC 839
PCV2c                AGAGCGGGAGTCTGGTGACCGTTGCAGAGCAGCACCCTGTAACGTTTGTCAGAAATTTCC 839
                     ************************************************************

PCV2a                GCGGGCTGGCTGAACTTTTGAAAGTGAGCGGGAAAATGCAGAAGCGTGATTGGAAGACCA 899
PCV2b                GCGGGCTGGCTGAACTTTTGAAAGTGAGCGGGAAAATGCAGAAGCGTGATTGGAAGACTA 899
PCV2c                GCGGGCTGGCTGAACTTTTGAAAGTGAGCGGGAAAATGCAGAAGCGTGATTGGAAGACCA 899
                     ********************************************************** *

PCV2a                ATGTACACGTCATTGTGGGGCCACCTGGGTGTGGTAAAAGCAAATGGGCTGCTAATTTTG 959
PCV2b                ATGTACACGTCATTGTGGGGCCACCTGGGTGTGGTAAAAGCAAATGGGCTGCTAATTTTG 959
PCV2c                ATGTACACGTCATTGTGGGGCCACCTGGGTGTGGTAAAAGCAAATGGGCTGCTAATTTTG 959
                     ************************************************************

PCV2a                CAGACCCGGAAACCACATACTGGAAACCACCTAGAAACAAGTGGTGGGATGGTTACCATG 1019
PCV2b                CAGACCCGGAAACCACATACTGGAAACCACCTAGAAACAAGTGGTGGGATGGTTACCATG 1019
PCV2c                CAGACCCGGAAACCACATACTGGAAACCACCTAGAAACAAGTGGTGGGATGGTTACCATG 1019
                     ************************************************************

PCV2a                GTGAAGAAGTGGTTGTTATTGATGACTTTTATGGCTGGCTGCCGTGGGATGATCTACTGA 1079
PCV2b                GTGAAGAAGTGGTTGTTATTGATGACTTTTATGGCTGGCTGCCCTGGGATGATCTACTGA 1079
PCV2c                GTGAAGAAGTGGTTGTTATTGATGACTTTTATGGCTGGCTGCCGTGGGATGATCTACTGA 1079
                     ******************************************* ****************

PCV2a                GACTGTGTGATCGATATCCATTGACTGTAGAGACTAAAGGTGGAACTGTACCTTTTTTGG 1139
PCV2b                GACTGTGTGATCGATATCCATTGACTGTAGAGACTAAAGGTGGAACTGTACCTTTTTTGG 1139
PCV2c                GACTGTGTGATCGATATCCATTGACTGTAGAGACTAAAGGTGGAACTGTACCTTTTTTGG 1139
                     ************************************************************

PCV2a                CCCGCAGTATTCTGATTACCAGCAATCAGACCCCGTTGGAATGGTACTCCTCAACTGCTG 1199
PCV2b                CCCGCAGTATTCTGATTACCAGCAATCAGACCCCGTTGGAATGGTACTCCTCAACTGCTG 1199
PCV2c                CCCGCAGTATTCTGATTACCAGCAATCAGACCCCGTTGGAATGGTACTCCTCAACTGCTG 1199
                     ************************************************************

PCV2a                TCCCAGCTGTAGAAGCTCTCTATCGGAGGATTACTTCCTTGGTATTTTGGAAGAATGCTA 1259
PCV2b                TCCCAGCTGTAGAAGCTCTTTATCGGAGGATTACTTCCTTGGTATTTTGGAAGAATGCTA 1259
PCV2c                TCCCAGCTGTAGAAGCTCTCTATCGGAGGATTACTTCCTTGGTATTTTGGAAGACTGCTA 1259
                     ******************* ********************************** *****

PCV2a                CAGAACAATCCACGGAGGAAGGGGGCCAGTTCGTCACCCTTTCCCCCCCATGCCCTGAAT 1319
PCV2b                CAGAACAATCCACGGAGGAAGGGGGCCAGTTCGTCACCCTTTCCCCCCCATGCCCTGAAT 1319
PCV2c                CAGAACAATCCACGGAGGAAGGGGGCCAGTTCGTCACCCTTTCCCCCCCATGCCCTGAAT 1319
                     ************************************************************

PCV2a                TTCCATATGAAATAAATTACTGAGTCTTTTTTATCACTTCGTAATGGTTTTTATTATTCA 1379
PCV2b                TTCCATATGAAATAAATTACTGAGTCTTTTTTATCACTTCGTAATGGTTTTTATTATTCA 1379
PCV2c                TTCCATATGAAATAAATTACTGAGTCTTTTTTATCACTTCGTAATGGTTTTTATTATTCA 1379
                     ************************************************************

PCV2a                TTTAGGGTTTAAGTGGGGGGTCTTTAAGATTAAATTCTCTGAATTGTACATACATGGTTA 1439
PCV2b                TTAAGGGTT-AAGTGGGGGGTCTTTAAGATTAAATTCTCTGAATTGTACATACATGGTTA 1438
PCV2c                CTTAGGGTT-AAGTGGGGGGTCTTTAAGATTAAATTCTCTGAATTGTACATACATGGTTA 1438
                      * ****** **************************************************
```

```
PCV2a           CACGGATATTGTAGTCCTGGTCGTATTTACTGTTTTCGAACGCAGCG-CCGAGGCCTACG 1498
PCV2b           CACGGATATTGTATTCCTGGTCGTATATACTGTTTTCGAACGCAGTG-CCGAGGCCTACG 1497
PCV2c           CACGGACATTGTAGGCCTGGGCATTTGTACTGTTTTGAAAGGC-GTGTCCGAGGCCTACA 1497
                ****** ******  ***** * * * *********  ** ** * * ***********

PCV2a           TGGTCCACATTTCCAGAGGTTTGTAGTCTCAGCCAAAGCTGATTCCTTTTGTTATTTGGT 1558
PCV2b           TGGTCTACATTTCCAGTAGTTTGTAGTCTCAGCCACAGCTGATTTCTTTTGTTGTTTGGT 1557
PCV2c           TGGTCTACATTTCCAGTAGTTTGTAGTCTCATCCACAGCTGATTTCTTTTGTTATTTGGT 1557
                ***** **********  ************* *** ******** ******** ******

PCV2a           TGGAAGTAATCAATAGTGGAGTCAAGAACAGGTTTGGGTGTGAAGTAACGGGAGTGGTAG 1618
PCV2b           TGGAAGTAATCAATAGTGGAATCTAGGACAGGTTTGGGGGTAAAGTAGCGGGAGTGGTAG 1617
PCV2c           TGGAAGTAATCAATAGTGGAATCAAGGACAGGTTTGGGGGTAAAGTAGCGGGAGTGGTAG 1617
                ******************** ** ** *********** ** ***** ************


PCV2a           GAGAAGGGTTGGGGGATTGTATGGCGGGAGGAGTAGTTTACATATGGGTCATAGGTTAGG 1678
PCV2b           GAGAAGGGCTGGGTTATGGTATGGCGGGAGGAGTAGTTTACATAGGGGTCATAGGTGAGG 1677
PCV2c           GAGAAGGGTTGGGTTATGGTATGGCGGGAGGAGTAGTTTACATAGGGGTCATAGGTTAGG 1677
                ******** ****  ** ************************** *********** ***

PCV2a           GCTGTGGCCTTTGTTACAAAGTTATCATCTAGAATAACAGCAGTGGAGCCCACTCCCCTA 1738
PCV2b           GCTGTGGCCTTTGTTACAAAGTTATCATCTAGAATAACAGCACTGGAGCCCACTCCCCTG 1737
PCV2c           GCTGTGGCCTTTGTTACAAAGTTATCATTTAGAATAACAGCAGTGGAGCCCACTCCCCTG 1737
                **************************** ************* ****************

PCV2a           TCACCCTGGGTGATGGGGGAGCAGGGCCAG 1768
PCV2b           TCACCCTGGGTGATCGGGGAGCAGGGCCAG 1767
PCV2c           TCACCTTGGGTGATGGGGGATCTTGCAAAG 1767
                ***** ******** ***** *  *   **
```

Figure 1-2 Map of the PCV2 genome

The PCV2 genome (1,767nt for PCV2b and 1,768nt for PCV2a and PCV2c) contains three open reading frames (ORFs). ORF1 is oriented in the positive sense direction and codes for the replicase proteins, Rep and Rep'. ORF2 is oriented in the negative sense direction and codes for the capsid protein. ORF3, coded within ORF1 and oriented in the negative sense direction, codes for a protein associated with apoptosis. Two intergenic regions lay between the 5' and 3' ends of ORFs 1 and 2. Within the 109nt IR, located between the 5' ends of ORFs 1 and 2, is the origin of replication (Ori). The Ori is characterized by a stem loop structure, three conserved hexamer repeats (boxed and labeled H1-H3) and a fourth semi-conserved hexamer repeat (boxed and labeled H4). The nonamer sequence, conserved in viruses that perform RCR, is shown in the grey box within the single-stranded loop. The site where Rep nicks the genome at the start of RCR is indicated by the arrow.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23

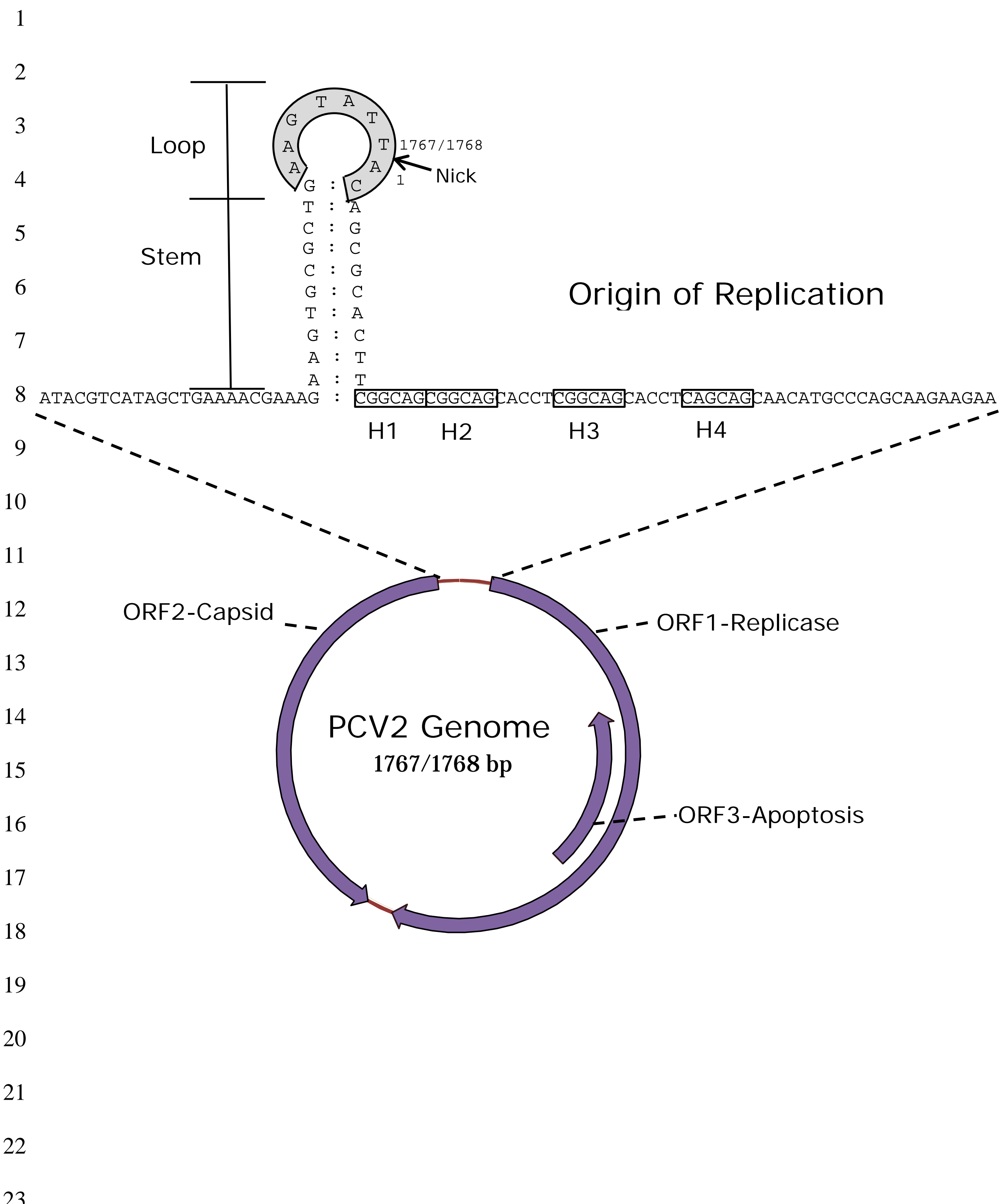
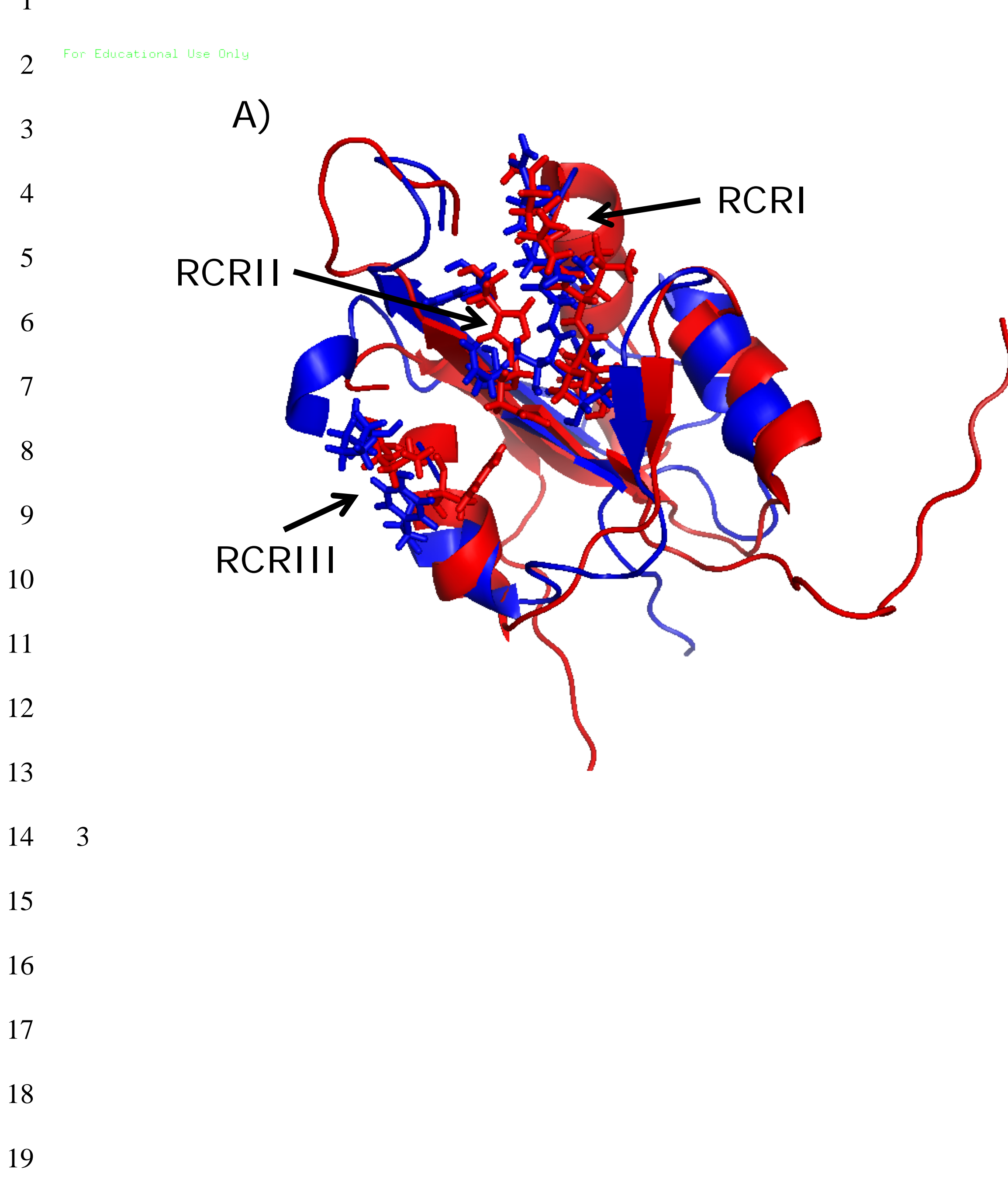


Figure 1-3 Three dimensional structure alignment of PCV2 and FBNYV endonuclease domain

The endonuclease domains of PCV2 Rep (126) and FBNYV (127) have been solved. Alignment of the 3D structures was performed using Pymol. PDB files were downloaded from the Protein Data Bank (PDB accession: 2hw0 for PCV2 and 2hwt for FBNYV). The endonuclease structure of PCV2 and FBNYV are shown in red and blue, respectively, for panels A-D. A) Alignment and location of the three conserved motifs involved in RCR. Stick figures of the residues important for the function of each motif are shown. Only the backbone of the remaining structure is shown with spirals representing alpha helices and flattened arrow representing beta sheets. The location of each RCR motif (I-III) are indicated by the arrows. B) Location of the RCRI motif (FTLNN). The arrows point to the location of important residues. C) Alignment of the RCRII motif (HxQ). The arrows point to the location of important residues. D) Alignment of the RCRIII motif (YxxK). The arrows point to the location of important residues.



3

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23

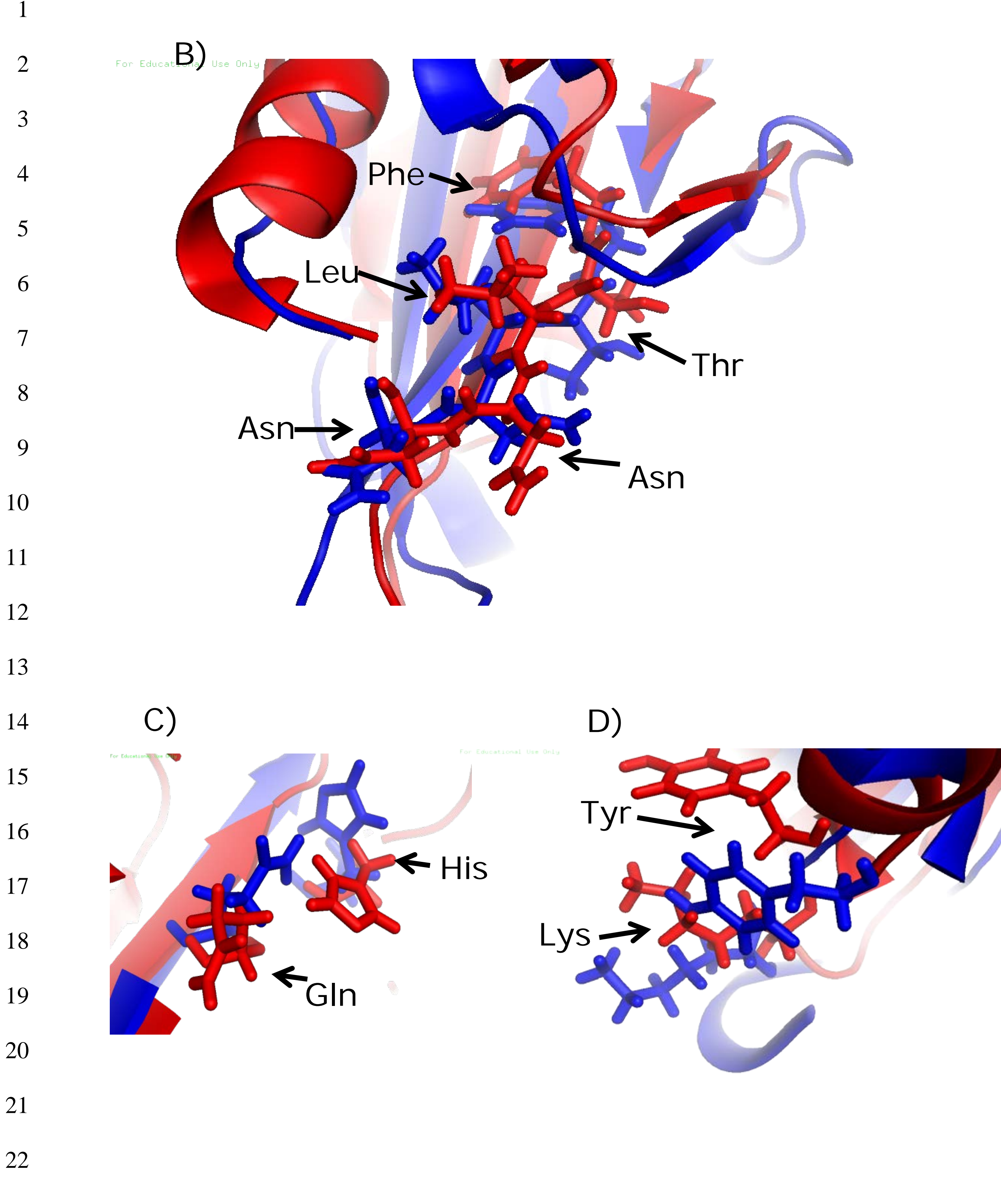
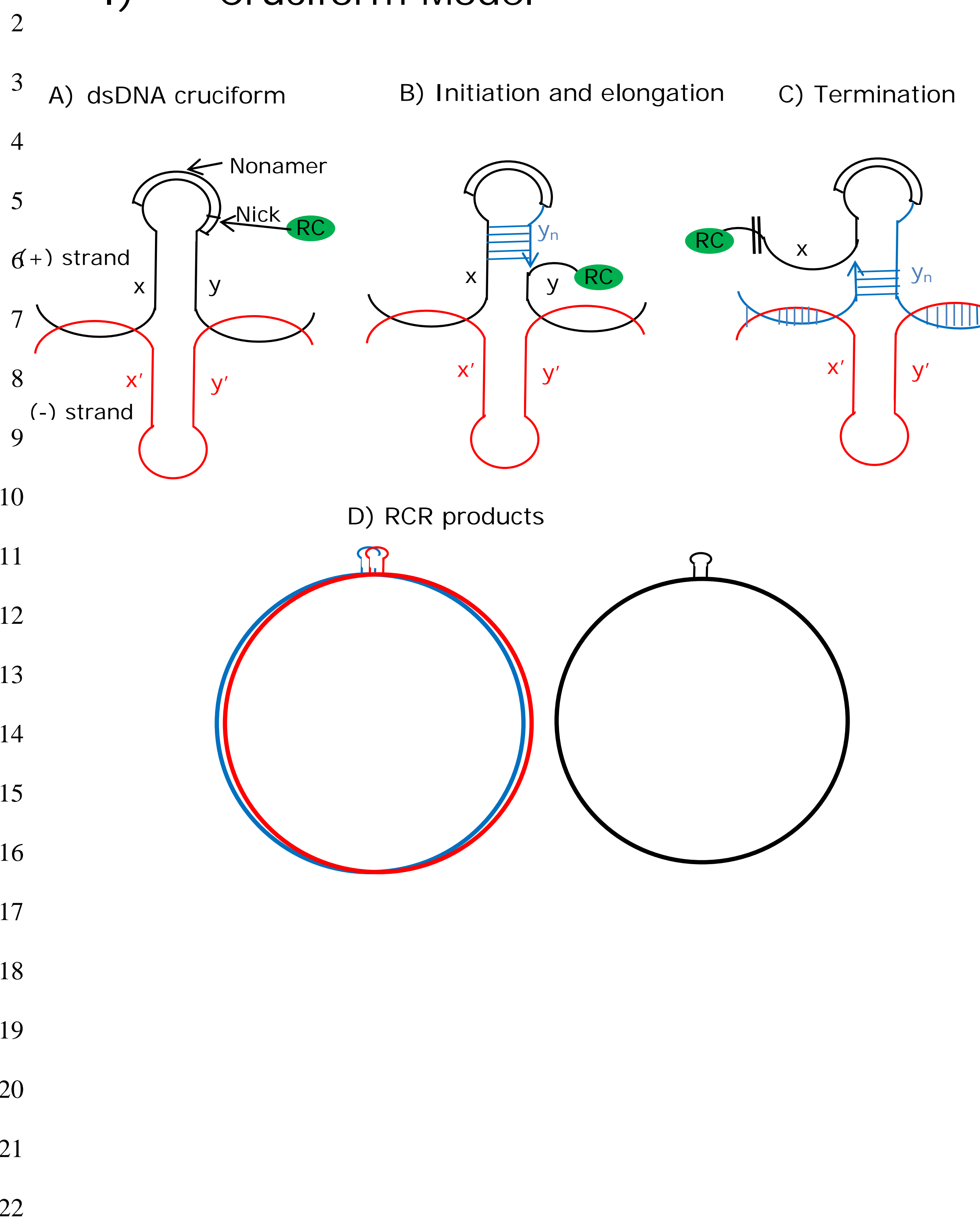


Figure 1-4 RCR models

Based on the identification of features conserved in viruses, bacteriophages and plasmids, PCV2 is considered to replicate via rolling circle replication. Two models have been proposed for the RCR mechanism. I) The cruciform model. A) Upon binding of the replication complex (RC) to the Ori, the RC nicks the dsDNA within the nonamer and the Ori takes on the cruciform shape. B) Upon nicking, Rep attaches to the 5' end of strand y, and the host DNA polymerase (pol) recognizes the free 3'-OH. DNA pol then proceeds to elongate strand y_n (new) using strand x as a template and at the same time, displacing strand y. C) Termination occurs when DNA pol proceeds through the stem loop, displacing strand y, using the newly synthesized strand y_n as a template. D) After one round of replication, products include a ssDNA copy of the genome covalently closed by the RC and a dsDNA composed of a (-) sense parental strand the newly synthesized (+) sense strand. II) The melting pot model. A) Upon binding of the replication complex (RC) to the Ori, the RC nicks the dsDNA within the nonamer and the Ori leading to a destabilized environment (melting pot) where the plus and minus strands are in close proximity to each other but not hydrogen bonded. The area of instability is circled by a dotted line. B) Upon nicking, Rep attaches to the 5' end of strand y, and the host DNA polymerase (pol) recognizes the free 3'-OH. DNA pol then proceeds to elongate strand y_n (new). As pol moves down the right arm of the stem loop, the conformation allow for use of either strand y or strand x' as a template. C) Termination occurs when DNA pol proceeds up the left arm of the stem loop, displacing strand y. Due to the conformation, pol can use either the newly synthesized strand y_n or strand x' as a template. D) After one round of replication, products include a ssDNA copy of the genome covalently closed by the RC and a dsDNA composed of a (-) sense parental strand the newly synthesized (+) sense strand. Black lines show the parental (+) sense

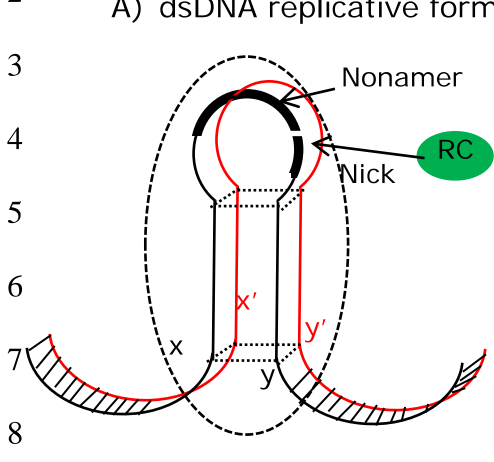
strand. Red lines show the (-) sense parental strands). Blue lines show newly synthesized (+) sense strands. The small blue lines between strands show potential base pairings in the process of replication. Parallel lines running through a strand indicate a break.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23

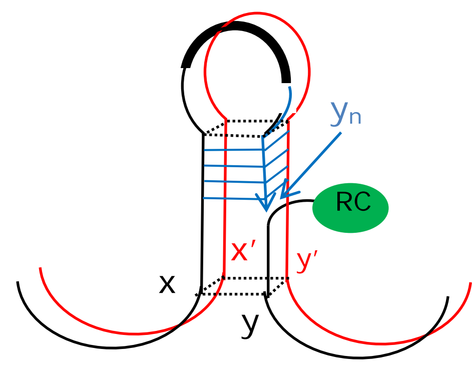


II) Melting Pot Model

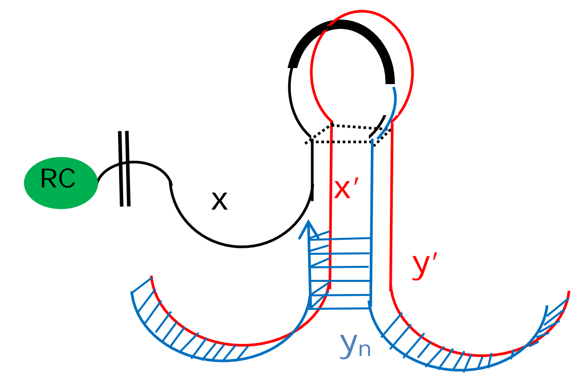
A) dsDNA replicative form



B) Initiation and elongation



C) Termination



D) RCR products

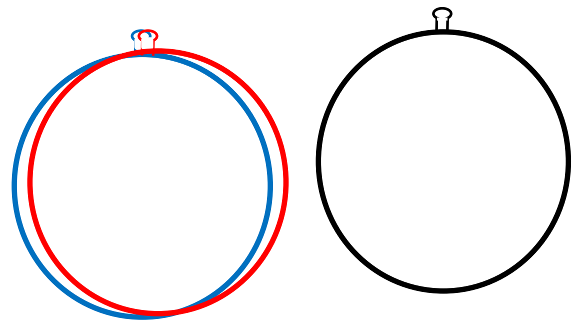
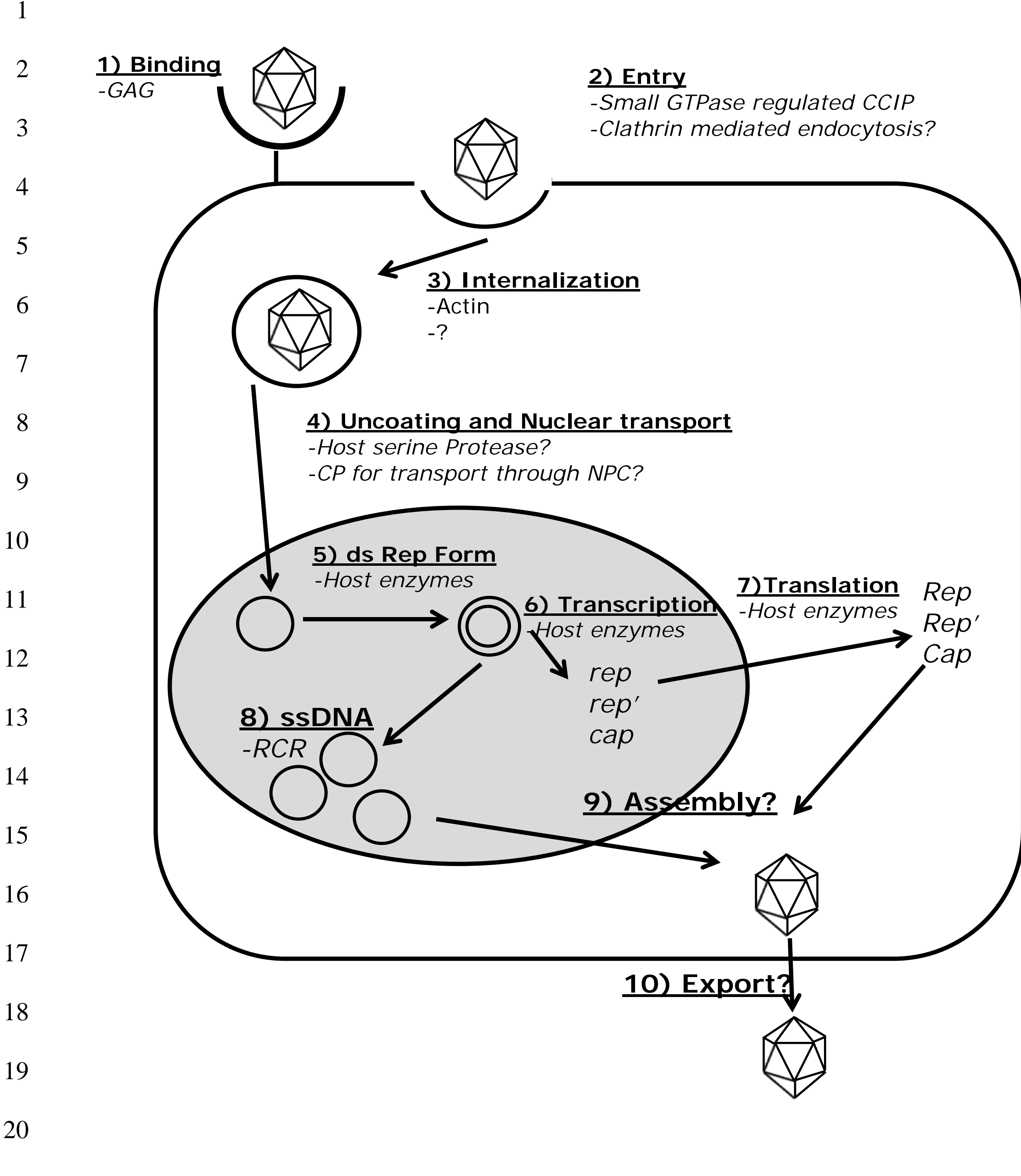


Figure 1-5 The virus life cycle

Once PCV2 has entered the host, the virus commonly infects multiple cell types, with the most common being epithelial cells or monocyte/macrophage cells. Steps in the virus lifecycle are as follows. 1) Attachment of the virus to cells occurs by binding of its IRKVKV motif to the GAGs heparin sulfate or chondroitin sulfate-B. 2) Entry into epithelial cells likely occurs by a small GTPase regulated clathrin and caveolin independent pathway (CCIP). Entry into monocytes/macrophages may proceed through clathrin mediated endocytosis. 3) Internalization occurs through an actin required process but is not well understood. 4) Uncoating the viral genome possibly occurs with the aid of a serine protease. This is followed by interaction of the CP with the viral genome, and transport through the nuclear pore complex (NPC) into the nucleus. 5) The single stranded viral genome is then made into a ds replicative form (RF) by the hosts enzymes. 6) Host enzymes then transcribe rep, rep' and cap into mRNA. 7) The mRNA is then translated by the hosts enzymes into Rep, Rep' and Cap. 8) Covalently closed circular ssDNA copies of the viral genome are produced by RCR (see Fig. 1-4). 9) Virus assembly likely occurs by encapsidation of the viral genome by Cap in a process that is not well understood. 10) Completed progeny viruses are then exported from the cell in a process that is not well understood.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22



CHAPTER 2 - Materials and Methods

Cloning, expression and purification of recombinant PCV2 polypeptides

Selection of the virus

PCV2 virus challenge stock was propagated from a sample collected from a pig diagnosed with PMWS and submitted to the Kansas State Veterinary Diagnostic Lab (KSVDL accession# 05-55004#7). To characterize the virus, sequence was obtained from the entire genome by amplification in two PCR reactions. The first reaction included primers CV1F and CV2R while, the second reaction included primers CV3F and CV4R (see Table 2-1 for primer sequences). Sequences analysis confirmed the genotype to be PCV2b. The sequence was subsequently submitted to GenBank (GenBank accession# HQ713495).

Construction of CP polypeptides

Previous work has identified both conformational and linear antibody recognition regions within the capsid protein of PCV2a and PCV2b (60, 71, 125). A closer look reveals at least four common areas antibody recognition regions, which are labeled A-D and presented in Figure 2-1. Based on these results, we constructed CP polypeptides comprised of one or more of these antibody recognition regions (see Table 2-2). To prepare recombinant CP polypeptides, primers were designed with 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 2-3. The PCR reaction mixture consisted of 10.5µL of nuclease free water, 12.5µL of GoTaq (Promega), 0.5uL of each primer, and 1µL of genomic viral DNA. PCR reactions were carried out by an initial denaturing step at 95°C for 2 minutes, followed by 40 rounds of the following: denaturing at 94°C for 45 seconds, annealing at

1 54°C for 45 seconds, and extension at 72°C for 1 minute/kb of product. A final step included
2 incubation at 72°C for 10 minutes. PCR products were then visualized on a 1% agarose gel.
3 Upon confirmation of purity and size, CP PCR products were cloned into the PCR2.1 TOPO
4 vector (Promega) according to the manufacturer's instructions. Plasmids containing CP
5 polypeptide sequences were then transformed into the *E.coli* strain Top 10F' (Invitrogen).
6 Transformed cells were plated on LB agar plates with antibiotics. After colony selection and
7 propagation, plasmid DNA was isolated and purified using the Wizard plus SV miniprep DNA
8 purification system (Promega) according to manufacturer's instructions. Next, restriction
9 enzyme digestion was carried out with the following reaction mixture: 1μL of Sac II
10 (20,000U/mL) (New England Biolabs), 1μL of Hind III (100,000U/mL) (New England Biolabs),
11 2μL of NEBuffer #4 (New England Biolabs), 1μg of the respective CP polypeptide plasmid, then
12 Q.S. to 20μl with nuclease free water. Incubation was performed for 1 hour at 37°C. Digestion
13 products were next visualized on a 1% agarose gel, followed by excision and purification using
14 the Wizard SV gel and PCR cleanup system (Promega) according to the company protocol. The
15 purified digestion products were then ligated, in frame into Sac II and Hind III sites of the
16 histidine-tagged ubiquitin expression vector (pHUE) *E coli* expression vector (60, 71, 125).
17 Ligation mixtures consisted of the following: 1ul of T4 DNA ligase (New England Biolabs), 1ul
18 of T4 DNA ligase buffer, 50ng of digested vector (pHUE), a threefold molar excess of insert
19 compared to the vector (pHUE), and Q.S. to 10 ul. Ligated plasmids were then transformed and
20 propagated in Top10 F' cells as previously described. Cloning fidelity was assessed by sequence
21 analysis.
22

1 ***Expression of CP polypeptide fusion proteins***

2 Initial experiments were aimed at expressing the full length capsid protein polypeptide (CP(1-
3 233). For this, the pHUE vector containing the PCV2 ORF2 was transformed into the following
4 E.coli cell lines: BL-21(DE:3), BL-21(DE:3)pLysS, BL-21(DE:3)pLysE and BL-21(DE:3)RIPL
5 (Invitrogen). All other CP polypeptides were transformed into the BL-21(DE:3) E. coli cell line.
6 For the purpose of recombinant protein expression, *E. coli* were grown in LB plus ampicillin at
7 30°C or 37°C until they reached an OD600 of 0.4-0.6. Induction of protein expression occurred
8 by addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of
9 1.0ug/ml. After addition of IPTG, bacteria were grown for an additional 4 hours and then
10 harvested by centrifugation at 4,000*g* for 10 minutes.

11 ***Full Length CP(1-233) Expression Optimization***

12 The sequence of the full length polypeptide was submitted to Blue Heron for optimization. The
13 rational for performing this was to increase overall protein expression by replacing rare codons
14 with ones that appear at a higher prevalence in bacteria. Optimizing the DNA sequence has no
15 effect on the overall peptide sequence. Upon receiving the optimized DNA sequence, restriction
16 digestion and cloning into pHUE was carried out as described above.
17

18 ***Purification of CP(43-233)under native conditions***

19 Protein purification was carried out using a PrepEase His-Tagged Protein Purification Kit (USB).
20 The largest CP polypeptide fragment (CP(43-233)) was purified using the "purification of his-
21 tagged proteins from *E. coli* under native conditions" protocol. For this, the bacterial pellet was
22 subjected to three freeze thaw cycles at -80°C and RT, respectively. This was followed by

suspension of the pellet in 5mL of 1X lysis, equilibration, and wash (LEW) buffer (USB) and 50ul of EDTA free protease inhibitor cocktail (THERMO scientific). Next, lysozyme was added to a final concentration of 1mg/ml and the mixture was incubating on ice for 30 min. Sonication was then performed six times for ten seconds, with 30 sec incubations on ice between bursts. The bacterial lysate was then ultra-centrifuged at 20,000Xg for 30 min. The soluble lysate fraction was then filtered through a 0.45um PVDF filter (Fisher). Next, gravity flow, affinity chromatography was performed by the addition of the filtered lysate to a Ni-TED mini column(USB) to capture the 6X-His tagged CP polypeptides. After washing with 3 column volumes of 1X LEW, the CP polypeptide-UBQ fusion protein was eluted in four 1mL aliquots using 1X elution buffer (USB).

Purification of CP polypeptides under denaturing conditions

All other CP polypeptides (CP(43-160,), CP(43-180), CP(91-160), CP(91-233), CP(135-233) and CP(160-233)) were purified using the "purification of his-tagged proteins from *E. coli* under denaturing conditions" protocol. The protocol for purification under denaturing conditions is the same as the aforementioned "native" protocol through the ultracentrifugation step. After ultra-centrifugation of the bacterial lysate, the soluble lysate fraction was discarded and the insoluble fraction was re-suspended in 1X LEW with urea added (final concentration of 8M). The re-suspended pellets were then incubated on ice for 30 min followed by ultra-centrifugation as described above. Ni-TED column purification was carried out the same as previously described with the exception of the addition of urea to the 1XLEW and elution buffers to a final concentration of 8M.

Protein purification using detergents

Attempts were made to purify CP(43-160,), CP(43-180), CP(91-160), CP(91-233), CP(135-233) and CP(160-233) under non-denaturing conditions. This involved inclusion of the detergents CAPS(3-(Cyclohexylamino)-1-propanesulfonic acid) and/or Sarkosyl (sosium lauroyl sarcosinate) rather than urea for the "purification under denaturing conditions described above. For this, 0.5M Caps and or 0.3% Sarkosyl were added to LEW and Elution buffers.

CP polypeptide dialysis

After purification of CP polypeptides under denaturing conditions, dialysis was performed to remove the 8M urea from the buffer. For this, 3ml of purified CP(161-233) in LEW plus 8M urea was added to 3,500MW cutoff dialysis tubing (SnakeSkin Pleated Dialysis Tubing, Pierce). After clamping each end, the tube was submerged into 1 liter of LEW-0.5M CAPS-0.3% Sarkosyl, LEW-0.5M CAPS, LEW-0.3% Sarkosyl or LEW. The dialysis reactions were carried out for 8 hours, repeated and then assayed for protein as described below.

CP polypeptide "hybrid" purification protocol

A hybrid protocol was designed to recover CP(160-233) fusion protein in LEW without urea. Similar to purification under denaturing conditions, 8M urea was incorporated into the initial steps of protein purification, including running the lysate (with 8M urea) through the USB column. After this step, sequential washes with 3mL of LEW-0.5M CAPS-0.3% Sarkosyl, LEW-0.5M CAPS and LEW, respectively, were performed. Following these, the CP fusion protein was eluted from the column in four 1mL aliquots with Elution buffer without urea.

1 ### ***Sequential buffer exchange/purification protocol***

2 A hybrid protocol was designed to remove urea from buffers containing CP(160-233). This
3 protocol involved an initial buffer exchange into LEW/CAPS/Sarkosyl followed by isolation and
4 purification into Elution buffer without urea. For this, CP(161-233) was purified using buffers
5 containing urea up until the point of loading onto the Ni-column. At this point, a column
6 containing a 3ml bed volume of Sephodex G10 was prepared and equilibrated with
7 LEW/CAPS/Sarkosyl buffer. The purified CP(161-233) in elution buffer-8M urea was then
8 added to the column, and ten, 1.5 ml fractions were collected and analyzed by SDS-PAGE.
9 Fractions containing the most protein were combined and then loaded onto the USB Ni columns
10 and eluted in the same way described previously for purification under native conditions.

11 ### ***Determining protein purity and concentrations***

12 After elution of CP polypeptides from nickel columns, the proteins were concentrated on a 5,000
13 MW cut-off spin column (Millipore) and the concentrations were measured using Protein Assay
14 (Bio-Rad) according to the manufacturer's instructions. Briefly, purified polypeptides were
15 mixed in duplicate with 200uL of 1X dye reagent in a flat bottom 96 well plate (Fisher).
16 Absorbance values of each well were determined by reading at 595nm on a precision microplate
17 reader (Molecular Devices). Protein concentrations were then determined by comparison to
18 BSA standard protein concentrations. Purified proteins were visualized by SDS-PAGE on a 15%
19 acrylamide gel.

20 ## CP Oligopeptides

21 Oligopeptides spanning residues 141-200 of PCV2 CP were prepared commercially (21st
22 Century Biochemicals). CP oligopeptides the length of 20 amino acids were prepared with a

cysteine added to the N or C-terminal end for the purpose of conjugation to BSA. In order to further characterize an immunoreactive region within CP, oligopeptides 12 amino acids in length were prepared in the same manner as previously described. One difference in the preparation of these oligopeptides was the addition of a aminohexonic acid (Ahx) spacer added to the C or N-terminal end. Table 2-4 summarizes the oligopeptides that were prepared.

PCV2 experimental infection/vaccination

Experiments involving animals were performed after review and approval of the Kansas State University Institutional Animal Use and Biosafety Committees. For the experiment, pigs were selected from sows with low PCV2 antibody titers according to the method described in Opriessnig et al. (99). Upon entry into the challenge facility, three week old pigs were confirmed negative for PCV2 DNA via PCR, and randomly assigned to seven groups as summarized in Table 2-5. Pigs in the vaccinated groups were given the commercial two dose baculovirus-expressed PCV2 ORF2 product (Intervet- Schering Plough) at four and seven weeks of age. Prior to challenge, pigs were confirmed negative for PCV2 antibody by IFA. Two weeks after the second vaccine dose, pigs were challenged with either PCV2 or PCV2 and PRRSV. A timeline of the experiment is shown in Figure 2-2. The inoculum for PCV2 consisted of lymph node homogenate from a PMWS affected pig. 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. 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%

1 tissue culture infectious dose per ml ($TCID_{50}$/ml) was calculated according to the Reed-Muench
2 method (45). The concentration of PCV2b in the challenge homogenate was determined to be
3 approximately 10^8 $TCID_{50}$/ml. The inoculum was negative for other common pathogens
4 including porcine parvovirus and swine influenza virus, however, was positive for PRRSV.
5 While virus isolated and grown in cell culture would be more desirable, the rationale for using a
6 homogenate was based the inability to grow significant quantities of PCV2 in cell culture.
7 PRRSV and other heat labile agents were inactivated by heat treatment at 60^oC for 30 minutes.
8 Pigs were challenged by intranasal inoculation with approximately 10^5 $TCID_{50}$/mL of PCV2.
9 Prior to heat inactivation, PRRSV was recovered by titration on MARC-145 cells. Following
10 two additional passages on MARC 145 cells, dual challenge was performed by the addition of
11 10^5 $TCID_{50}$/m of PRRSV to the PCV2 inoculum. Pigs were monitored daily for clinical signs of
12 disease. Blood samples were collected weekly. Experimental termination occurred six weeks
13 after virus challenge. Sera from all groups of pigs were assayed for PCV2 and PRRSV nucleic
14 acid and virus-specific antibodies using standard molecular and serological diagnostic techniques
15 (PCV2, ELISA, IHC and IFA) by the Kansas State Veterinary Diagnostic Laboratory (KSVDL).
16

17 **Selection of PCVAD pigs.**

18 Samples from pigs with PVCAD (PMWS and PDNS) came from diagnostic cases submitted to
19 KSVDL. Table 2-6 shows the case number for each serum sample selected for this study.
20 Diagnosing pigs with either PDNS or PMWS was performed as previously described (8).
21 Briefly, pigs diagnosed with PMWS were emaciated with enlarged superficial inguinal lymph
22 nodes. Histological analysis of lymph nodes found significantly depleted levels of lymphocytes.
23 Using diagnostic immunohistochemisty (IHC), large quantities of PCV2 antigen was found in

1 histological lesions. Pigs Diagnosed with PDNS were identified by multi-focal erythematous
2 lesions on their hindquarters. Kidneys were found to be greatly enlarged with cortical petechiae
3 over the surface. Glomeruli were swollen and fibrinous with necrosis of glomerular tufts. All
4 pigs were confirmed to be infected with a PCV2b virus. For the purpose of this study, ten PDNS
5 and ten PMWS sera samples were selected.

6 CP polypeptide and oligopeptide ELISA's

7 CP polypeptides or oligopeptides were diluted to a concentration of 4ug/mL in 0.05 M carbonate
8 coating buffer (pH 9.6). Diluted CP peptides were coated by addition of 100ul to all wells of a
9 96 well ELISA plate (Costar). Coated plates were then incubated overnight (approximately 15
10 hrs) at 4°C. Plates were then washed three times with PBS and 0.01% Tween 20 (PBST) and
11 blocked by a two hour incubation at room temperature with PBS-10% goat serum (PBS-GS).
12 Following incubation, plates were again washed with PBST. Next, pig sera samples, diluted
13 1:100 in PBS-GS, were added in duplicate to 96 well plates. To determine the background
14 reactivity, a single row was incubated with PBS-GS. Diluted sera samples were incubated at
15 room temperature for 2 hours then washed with PBST. Next, 100 µl of peroxidase labeled goat
16 anti-swine antibody (Accurate Chemical and Scientific Corp.), diluted 1:2,000 in PBS-GS, was
17 added to each well and incubated for an additional two hours at room temperature. Following
18 extensive washing, peroxidase activity was determined using the ABTS (2,2'-azino-bis(3-
19 ethylbenzthiazoline-6-sulphonic acid) chromogenic substrate kit (KPL). For this, 100ul of
20 ABTS substrate solution was added to each well and incubated, away from light at room
21 temperature for 20 minutes. Reactions were stopped by the addition of 100ul of a 1% SDS
22 solution. Reactivity was determined by reading the absorbance values of each well at 405 nm on
23 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 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.

PCV2 IFA and measurement of virus neutralizing activity

To measure the total amount of antibody in pig serum specific to PCV2, indirect fluorescent antibody (IFA) was performed. For this, 96 well plates with rapidly dividing ST cells maintained with EMEM with 10% FBS and antibiotics, were infected with a laboratory isolate of PCV2. After a three day incubation period, the cells were fixed in 80% acetone for 10 minutes. Next, 1:2 serial dilutions of swine sera, diluted in 5% fetal bovine serum in PBS (PBS-FBS), were added to the plates, then incubated for two hours at room temperature. Plates were then washed extensively with PBS. Bound antibody was detected with a FITC-labeled anti-pig antibody (Jackson Labs) according to the manufacturer's instructions. After further incubation and washing, 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 $TCID_{50}$), incubated for 1 hour at 37^oC, and placed onto four replicate wells of one day old ST cells in 96 well plates. Plates were incubated for three days at 37^oC and then fixed and stained with FITC-labeled anti-PCV2 (VMRD, Inc.). The $log2NA_{50}$ endpoint was calculated by the method of Spearman and Karber (23).

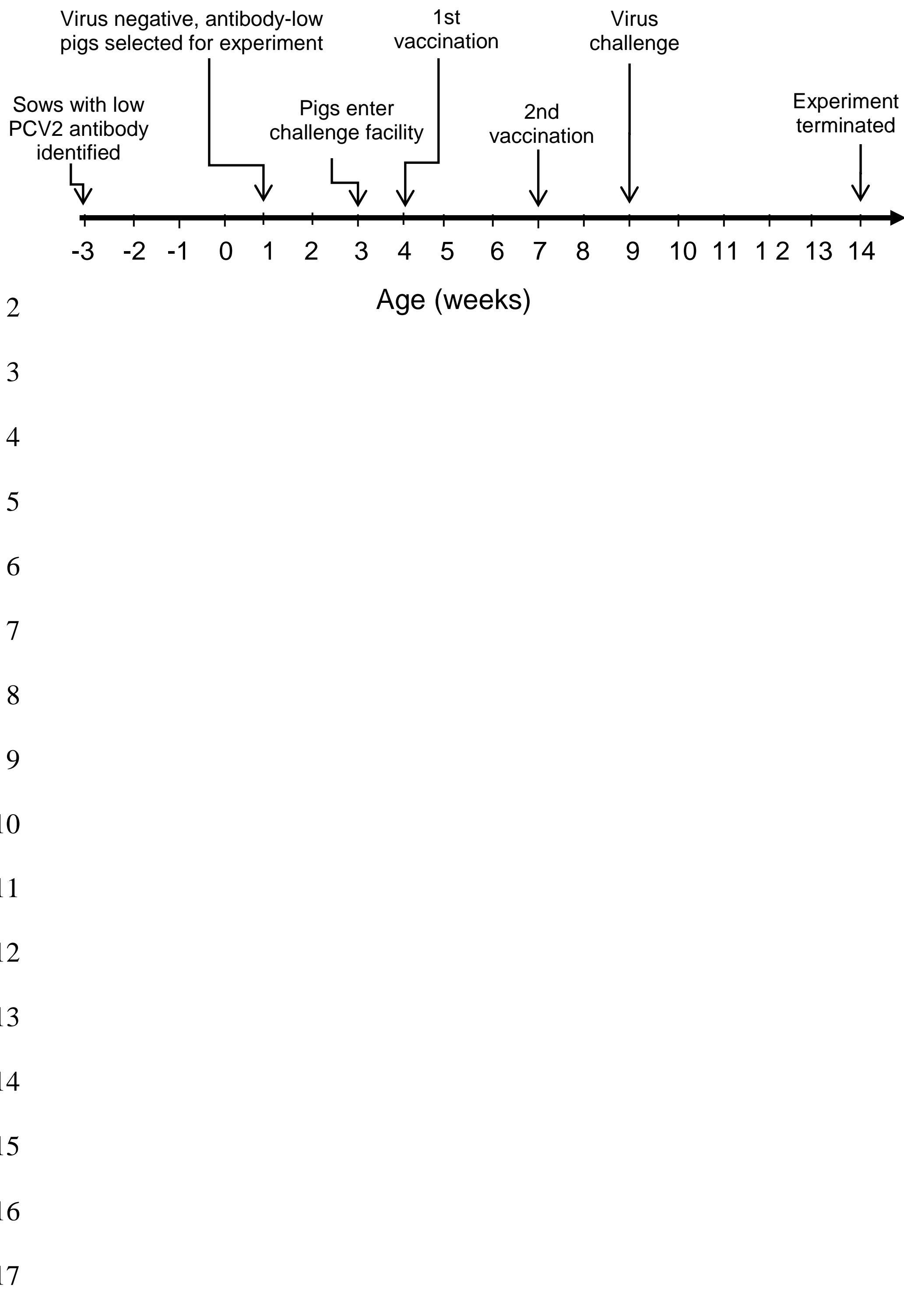
Figures and Tables

Figure 2-1 Immunoreactive Regions within PCV2 Capsid Protein

Peptide sequences of PCV2a, PCV2b and PCV2c are from genbank accession #s AF055392, AF055394 and EU148503 respectively. Lekcharoensuk et al., (2004) used mAb's that had been prepared against whole PCV2a virus (strain ISU 31, GenBank accession number AJ223185) and used those to locate immunoreactive regions within CP (71). These regions are underlined in the PCV2a sequence. Using pepscan analysis of the PCV2b sequence (GenBank accession number AJ223185) Mahe et al. reported the immunoreactive regions underlined in the PCV2b sequence (125). The shaded regions represent the combined sequences, identified as reactive regions A, B, C, and D. The boldface residues (121-131) represent an immunodominant oligopeptide reported by Truong et al. (2002) (83). Within the box lies the putative receptor binding region reported by Misinzo et al. (2006) (8).

```
1         1          11         21         31         41         51
2  PCV2a MTYPRRRYRR RRHRPRSHLG QILRRRPWLV HPRHRYRWRR KNGIFNTRLS RTFGYTVKRT
3  PCV2b .......... .......... Q......... .......... ......T... .T.G...KRT
4  PCV2c .......... .......... H......... .......... ......A... .S.V...NAS
5
6        61    A     71         81         91         101        111
7  PCV2a TVTTPSWAVD MMRFKIDDFV PPGGGTNKIS IPFEYYRIKK VKVEFWPCSP ITQGDRGVGS
8  PCV2b T.KT...... ....N.ND.L .....S.PRS V.......R. .....WPC.. ..........
9  PCV2c Q.SP...... ....N.NQ.L .....S.PLT V......... .....FAR.. ..........
10
11       121   B     131        141        151        161        171
12 PCV2a TAVILDDNFV TKATALTYDP YVNYSSRHTI PQPFSYHSRY STPKPVLDST IDYFQPNNKR
13 PCV2b S....D.... .......... .......... T......... .......... ..........
14 PCV2C T....N.... .......... .......... T......... .......... ..........
15
16       181     C   191        201        211        221     D  231
17 PCV2a NQLWLRLQTS GNVDHVGLGA AFENSKYDQD YNIRVTMYVQ FREFNLKDPP LKP-
18 PCV2b ....L....T .........T ..E..IYD.E ..I....... .....F.... .N.-
19 PCV2c ....M....T .........H ..Q..TNA.. ..V....... .......... .N.K
20
21
22
23
24
25
26
27
```

1 **Figure 2-2 Experimental Infection and Vaccination Timeline**



2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18

1 **Table 2-1. Primers for whole genome PCV2 sequencing**

Primer Name	Sequence
CV1F	AGGGCTGTGGCCTTTGTTAC
CV2R	TCTTCCAATCACGCTTCTGC
CV3F	TGGTGACCGTTGCAGAGCAG
CV4R	TGGGCGGTGGACATGATGAG

2

3

4

5

6

7

8

9

10

11

12

13

14

15

16

17

18

19

20

21

Table 2-2. Summary of CP polypeptides

Name	Epitope Regions
	A B C D
1-233	
43-233	
43-135	
43-160	
91-160	
43-180	
160-233	
135-233	
91-233	

Table 2-3 Primer Sequences Used for Preparing Capsid Protein Polypeptides*

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
1-233	5’GAACCGCGGGCTGGCTGAACTTTTGAAAGT	5'GCGCAAGCTTTTAAGGGTTAAGTGGC

*Additional Sac II and Hind III restriction sites are underlined

1 **Table 2-4 Synthesized BSA Conjugated Oligopeptides**

PCV2 CP Oligopeptide	*Amino Acid Sequence*
141-160	YVNYSSRHTITQPFSYHSRY
151-170	TQPFSYHSRYFTPKPVLDST
161-180	FTPKPVLDSTIDYFQPNNKR
169-188	STIDYFQPNNKRNQLWLRLQ
181-200	NQLWLRLQTAGNVDHVGLGT
169-180	STIDYFQPNNKR
S169A	ATIDYFQPNNKR
T170A	SAIDYFQPNNKR
I171A	STADYFQPNNKR
D172A	FTPKPVLDSTIAYFQPNNKR
Y173A	STIDAFQPNNKR
F174A	STIDYAQPNNKR
Q175A	STIDYFAPNNKR
P176A	STIDYFQANNKR
N177A	STIDYFQPANKR
N178A	STIDYFQPNAKR
K179A	FTPKPVLDSTIDYFQPNNAR
R180A	FTPKPVLDSTIDYFQPNNKA

2

3

4

5

6

7

8

9

10

11

12

13

14

1 **Table 2-5 PCV2 Experimental Infection Study Groups**

			Treatment		
Group Number	*Group Name*	*n*	*Vaccine*	*PCV2*	*PRRSV*
1	Control	4	-	-	-
2	Vaccine	7	+	-	-
3	PRRSV	7	-	-	+
4	PCV2	7	-	+	-
5	PCV2/Vaccine	7	+	+	-
6	PCV2/PRRSV	7*	-	+	+
7	PCV2/PRRSV/Vaccine	7	+	+	+

*Three of the seven pigs within this group died following infection and were therefore no longer subjected to further study.

2

3

4

5

6

7

8

9

10

11

12

13

14

15

16

17

1 **Table 2-6 Identification of PDNS and PMWS Serum Samples**

Clinical Diagnosis	*KSUVDL Case #*	*n*	*Total*
PDNS	06-35002	6	10
	06-46421	1	
	06-13540	1	
	06-29286	2	
PMWS	06-14550	5	10
	06-14552	1	
	06-16686	1	
	06-16687	1	
	05-60804	1	
	06-14593	1	

2

3

4

5

6

7

8

9

10

11

12

13

14

15

16

CHAPTER 3 - Results

Cloning, expression and purification of CP polypeptides

PCV2 virus selection

Initial efforts were directed towards the cloning and bacterial expression of the entire amino acid sequence of the PCV2 CP. At this time, severe outbreaks of PCVAD were occurring in farms around Kansas (45). Sequence analysis revealed the majority of PCVAD cases were associated with the PCV2b genotype. For this purpose, ORF2 from a PCV2b isolate was selected for inclusion into this research.

Cloning and expression of CP(1-233)

For amplification and cloning of PCV2 ORF2, forward and reverse primers containing SacII or HindIII sites in their 5' end, respectively, were constructed for PCR. Agarose gel electrophoresis analysis of PCR reactions revealed a single band of the correct size for CP(1-233) (data not shown). Following isolation and purification, the PCR product was cloned into the pCR2.1-TOPO vector (Invitrogen). The rationale for using this as a shuttle vector include the following: 5'-A and 3'-T overhangs for direct ligation of Taq-polymerase amplified PCR products, ligation speed and efficiency (reactions in 5 minutes at room temperature), EcoRI sites flanking the PCR product insertion site for easy scanning of clones, inclusion of both kanamycin and ampicillin resistance genes for selection in E. coli, as well as the ability to do blue/white colony screening. Inserts were then digested from pCR2.1, and inserted in frame into the SacII and HindIII sites of pHUE (8). Within the multiple cloning site of pHUE, SacII and HindIII are the most proximal restriction sites. These two restriction sites were selected because the PCV2b ORF2 nucleotide

1 sequence is void of either SacII or HindIII restriction sites. The pHUE expression vector was
2 incorporated into this study for multiple purposes. Most importantly, the products of expression
3 include a 6X-His tag and ubiquitin (see Figure 3-1). The 6X-His tag allowed for simple affinity
4 column purification, whereas, fusion of cloned peptides to ubiquitin increased bacterial
5 expression and solubility. After cloning, fidelity was confirmed by digestion and agarose gel
6 electrophoresis as well as sequence analysis (data not shown).
7
8 To express the UBQ-CP(1-233) fusion protein, pHUE containing the CP(1-233) gene was
9 initially transformed into the *E.coli* strain BL21(D:E3) (Invitrogen). This cell line was selected
10 for its ability to efficiently express proteins from the T7 promoter. SDS-PAGE analysis of
11 expression assays showed no products of the correct size for the UBQ-CP(1-233) fusion protein
12 (data not shown). A closer look at the sequence of PCV2b CP revealed an Arg-rich region in the
13 N-terminus of the protein which has been identified as a nuclear localization signal (68). For this
14 purpose, the pHUE vector was transformed into BL-21(DE3) cell lines pLysS and RIPL.
15 Initially, it was hypothesized that the presence of a Arg-rich region may be toxic. Therefore, the
16 *E.coli* strain BL21(DE3)plysS was employed based on the ability to provide tighter control of the
17 expression of toxic proteins. Results of expression assays again were negative. Next, we
18 hypothesized that the *E. coli* lacked significant transfer RNA's (tRNA's) for the abundance of
19 Arg located in the N-terminus of CP. For this reason, we transformed the pHUE vector into the
20 E.coli BL21-CodonPLus(DE3)-RIPL (Invitrogen) cell line. This cell line carries extra copies of
21 Arg tRNA genes. Once again, results of protein expression were negative. As previously
22 mentioned, the successful bacterial expression of large quantities of immunoreactive CP after
23 deletion of the NLS has been reported (124, 136). For this purpose we re-amplified and cloned

1 the region of CP coding for residues 43-233 into pHUE. Transformation, expression and
2 purification resulted in a single band of the predicted molecular weight in the elution from the
3 USB protocol described in Materials and Methods (see Figure 3-2). From the results of
4 Trundova et al. (124) showing codon optimization increased bacterial expression, the sequence
5 of the NLS truncated CP was optimized (Blue Heron). In general, this process involves matching
6 the codon utilization of the amino acid sequence (PCV2 CP) to the frequency of the host
7 organism (in this case, E.coli). Next, the codon optimized gene was synthesized, cloned into
8 pHUE and transformed into E.coli. Results of expression showed a moderate increase in
9 production of the truncated CP (data not shown).

10 ***Cloning and expression of other segmented CP polypeptides***

11 Studies performed in the past have identified immunoreactive sites in CP from both PCV2a and
12 PCV2b. Further, one site was reported as immunodominant (8). The CP sequences and
13 immunoreactive sites from these studies are shown in Figure 2-1. In general, antibody
14 recognition can be broken into four main regions, labeled A(51-84), B(113-131), C(161-207),
15 and D(228-233) in Figure 2-1. For the purposes of this study, one or more reactive regions were
16 cloned in frame into the Sac II and Hind III sites of the pHUE *E. coli* expression vector. As
17 demonstrated in Figure 3-3, all polypeptide fragment DNA sequences cloned into pHUE
18 migrated to the correct size on an agarose gel after digestion with Sac II and Hind III. In order to
19 optimize expression, aliquots of expression competent *E. coli* were taken at the time of induction
20 with IPTG, as well as every hour after for a total of six hours. Analysis of samples using SDS-
21 PAGE found peak expression at four hours after induction (data not shown). The largest
22 polypeptide was constructed with the entire CP sequence except the arginine rich, N-terminal 42
23 residues. ELISA results showed no reactivity between PCV2 positive sera samples and

1 oligopeptides consisting of CP(1-21) or CP(21-42) (data not shown), and therefore, no further
2 experiments involving the NLS were performed. Protein extraction and purification of CP(43-
3 233) was carried out under native conditions. Attempts to purify smaller CP polypeptide
4 fragments under native condition failed, leading to the idea that these proteins formed insoluble
5 inclusion bodies. As indicated by Trundova et al., formation of inclusion bodies may be
6 prevented by performing bacterial expression at 30°C rather than 37 ^{o}C. However, expression at
7 30 did not increase CP polypeptides in the soluble fraction (data not show). Therefore, 8M urea
8 was incorporated into the extraction and purification buffers, allowing for sulubilization and
9 purification of the remaining CP polypeptides. SDS-PAGE demonstrated that all purified
10 polypeptides were of the predicted size (Figure 3-4).

11 ***Attempts to return small CP polypeptides to non-denaturing buffers***

12 Multiple assays were designed and carried out in an attempt to obtain CP polypeptides into
13 buffers without urea. Initial approaches included dialysis of purified (under denaturing
14 conditions) CP(160-233) into LEW. After dialysis for ~8 hours, analysis of the dialysis tubing
15 revealed a white precipitate. SDS-PAGE analysis of the precipitate, solubilized in 8M urea,
16 revealed a single band of predicted size for CP(160-233) (data not shown). Next, dialysis was
17 performed into the LEW containing the detergents CAPS and sarkosyl. After dialysis for ~8
18 hours, analysis of the dialysis tubing again revealed CP(160-233) had precipitated in the LEW
19 buffer plus detergents. A different approach, which we previously termed the hybrid purification
20 protocol in the materials and methods, involving the initial use of urea in the purification
21 process, followed by sequential washing also resulted in the loss of protein (data not shown). A
22 different protocol, involving the use of Sephadex G10 for buffer exchange was then performed
23 as described in materials and methods. SDS-PAGE analysis following the passage of CP(160-

233) through the Sephadex G10 column showed protein present in elutions 2-5, with elutions 3 and 4 being the most concentrated (see Figure 3-5). For this purpose, elutions 3 and 4 were combined and purified using the USB Ni column according to the manufacturer's instructions for purification under native conditions. SDS-PAGE analysis revealed a single band of predicted size for the CP(160-233) fusion protein to be present in elutions 2 and 3 (Figure 3-6), however, proteins assays determined the concentration to be <0.1mg/ml. In an attempt to increase the protein concentration, elutions 2 and 3 were combined and concentrated on a 5,000 MW cut-off spin column (Millipore). Unfortunately, protein concentration assays revealed a complete loss of protein following concentration.

Overall, the results show that even after codon optimization, the E.coli strain BL-21DE3 RIPL are unable to express the entire CP(1-233) polypeptide. Furthermore, urea is required for solubilization and purification of CP polypeptides with the exception of CP(43-233).

Experimental PCV2 infection

Clinical outcome and histopathology

Approximately one week after challenge, groups that were challenged with PRRSV or PCV2/PRRSV dual-infected pigs exhibited respiratory signs consistent with acute PRRSV infection. Mortality results showed that three of the seven dual-challenge pigs (Group 6) died (two pigs) or were euthanized (because of severe respiratory distress). The results of histopathology indicated that death was likely the result of PRRSV infection combined with a secondary bacterial infection. One of the dead pigs showed a marked depletion of lymphocytes in lymph nodes.

Prior to virus challenge, all pigs were negative for PRRSV and PCV2 nucleic acid in serum. At the time of entry into the challenge facility, most pigs possessed some PCV2 antibody; however, by the time of challenge, the level of antibody decayed to below detectable levels in the non-vaccine groups. The presence of PCV2 antibody was likely the result of small amounts of maternally-derived antibody (MDA), acquired during nursing. (The initial source of MDA is the prior exposure of dams to vaccine or virus.) The control and PRRSV-only groups remained negative for PCV2 antibody and PCV2 nucleic acid throughout the remainder of the study, confirming that pigs were PCV2-free prior to entry into the study. After challenge, the PCV2-only pigs were negative for PRRSV by serology and PCR throughout the study, demonstrating that heat inactivation had removed all viable PRRSV from the challenge inoculum. The results of IHC showed that all pigs in the PCV2-only or PCV2/PRRSV group were positive for PCV2 antigen in lymph nodes. Two of the remaining four pigs in the dual-challenged group possessed mild to severe lymphocyte depletion in one or more lymph nodes. Together, these results demonstrate that dual infection incorporating PCV2 and PRRSV reproduces several features of PCVAD, including severe disease, lymphocyte depletion, wasting and increased morbidity/mortality. Furthermore, the requirement of PCV2 was demonstrated by the absence of clinical signs in dual-challenged pigs vaccinated for PCV2.

Immunoreactivity of PCV2 CP polypeptides

Comparative ELISA

To determine if urea had a significant impact on the binding of PCV2 specific antibodies, a comparative ELISA was performed using the exact same methods described for CP polypeptide.

In this ELISA serum samples from two PDNS pigs, two experimentally infected pigs, two vaccinated pigs and two negative control pigs was reacted with CP(43-233) either purified under denaturing conditions (8M urea) or purified under native conditions. Results of the ELISA showed no significant difference in the capacity to bind sera groups for CP(43-233) purified under denaturing conditions (8M urea) or native conditions (see Figure 3-7).

Immunoreactivity of experimentally infected/vaccinated pigs

Initial experiments were carried out using sera from experimentally infected animals (described in Table 2-5). The serum samples used were obtained at the end of the experiment, five weeks after infection and seven weeks after vaccination (see Figure 2-2 for a timeline). To reduce variation in results across ELISA plates, each ELISA plate included an internal positive control serum reacted with CP(43-233). The results are shown in Figure 3-8. Uninfected control pigs showed only background levels of response to all CP polypeptides (Figure 3-8 panel A). IFA and PCR on the same samples as well as samples obtained throughout the study found no antibodies specific to PCV2 as well as no PCV2 DNA (data not shown). Overall, these results demonstrate that the pigs remained negative for PCV2 throughout the study. Panel B of Figure 3-8 shows the binding ratio of pigs experimentally infected with PCV2 or PCV2 and PRRSV. In contrast to negative control pigs, IFA and PCR found antibodies specific to PCV2 as well as PCV2 DNA in these samples (data not shown). PRRSV RT-PCR as well as PRRSV serological assays found that only duel infected pigs were productively infected with PRRSV (data not shown). With regard to assays for PCV2, no significant differences were found so the data for both groups was combined. 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

1 accumulation of PCV2 antigen in lymph nodes (data not shown). As shown in panel B of Figure
2 3-8, the PCV2 group showed measurable antibody activity against all polypeptides by at least
3 one pig. However, the highest mean binding ratio was obtained for the largest CP(43-233)
4 polypeptide and the lowest mean binding ratio for CP(43-135), CP(43-160), and CP(91-160)
5 polypeptides. The remaining polypeptides, CP(91-233), CP(135-233), CP(160-233), and CP(43-
6 180) possessed mean ratios that were intermediate between CP(43-233) and the low-responding
7 polypeptides. The response for the seven pigs receiving only the PCV2 vaccine is shown in
8 panel C of Figure 3-8. All vaccinated pigs were positive for PCV2 antibody by IFA and were
9 confirmed to be negative for PCV2 by PCR (data not shown). The highest binding ratios were
10 observed for the CP(43-233) polypeptide, with only background levels of binding against the
11 smaller CP fragments. The only exception was CP(43-180), which showed a small, but
12 significant increase in binding relative to the other small polypeptide fragments. In order to
13 demonstrate that vaccinated pigs were protected, 14 vaccinated pigs were challenged with PCV2
14 (seven pigs) or PCV2 plus PRRSV (seven pigs). All challenged pigs remained clinically normal
15 with no mortality and were negative for PCV2 DNA in serum (data not shown).

16 ### *Immunoreactivity of pigs with PCVAD*

17 Although multiple attempts have been made, there currently is no model to effectively reproduce
18 PCVAD by experimental infection with PCV2 or PCV2 with other pathogens. For this reason,
19 sera samples from pigs with PCVAD came from diagnostic clinical cases from the field. Sera
20 samples were picked from cases sent to KSVDL with a diagnosis of either PDNS or PMWS and
21 then tested for their reactivity with CP polypeptides. Although PDNS has been reproduced
22 without the presence of PCV2 experimentally (133), in the majority of PDNS cases from the
23 field, pigs possess high levels of antibodies directed towards PCV2 (60). The results of reacting

sera from pigs with PDNS with the CP polypeptides are shown in Figure 3-9 panel A. 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 10 pigs diagnosed with PMWS are shown in Figure 3-9 panel B. 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). The remaining four showed intermediate activities against CP(43-233). 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 polypeptides were also variable, except for CP(43-135) and CP(43-160), which were nearly 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 3-1. For PCV2-infected, PMWS, and PDNS pigs, the highest levels of antibody binding were primarily obtained for CP polypeptides that contained residues located in the immunoreactive region labeled Epitope C in Fig.2-1).

Pepscan mapping of the CP C-terminus

Further studies were performed to determine the smallest oligopeptide recognized by PCV2-infected pigs. Based on the results of Table 3-1, 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. A closer look at the results found that pigs could be divided into two groups. For instance, as presented in Figure 3-10 panel A, four of the 11 serum samples showed minimal binding activity against all oligopeptides (gray bars), which is 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. The binding reactivity of these oligopeptides with sera from experimentally infected pigs was similar to that of the CP(161-180) and CP(169-188) oligopeptides (Fig. 3-10A right portion). Serum samples from PDNS pigs were also reacted with the oligopeptides. As shown in Figure 3-10B, the response of PDNS pigs was similar to the high responding experimentally infected PCV2 pigs (black bars in Figure 3-10A), and the highest binding activity was directed towards the CP(161-180), CP(169-188) and CP(169-180N) oligopeptides.

Alanine scanning of the CP(169-180) region

In order to identify individual residues within the CP(169-180) region that contributed to antibody binding, oligopeptides were constructed that contained single alanine substitutions at each position of the sequence 169-STIDYFQPNNKR-180. These oligopeptides were then

1 reacted with the high responding experimentally infected pigs represented by the black bars in
2 Figure 3-10B. The results showed a reduction in binding for oligopeptides that had alanine
3 substitutions for tyrosine-173, phenylalanine-174, glutamine-175, and lysine-179 (Figure 3-11
4 panel A). Reactivity of PDNS sera with the same oligopeptides was similar (Figure 3-11 panel
5 B).
6
7 Four hundred and sixty-two CP(169-180) peptide sequences, obtained from GenBank and
8 diagnostic lab submissions, were analyzed for amino acid differences. The results showed that
9 only 45 of the 462 sequences showed mutations within the region as shown in the lower portion
10 of Figure 3-11A. Each mutation was a single residue change. For those residues that contributed
11 to binding, tyrosine-173, phenylalanine-174, glutamine-175 and lysine-179, there were only two
12 amino acid substitutions. Therefore, the core peptide region that forms the epitope within
13 CP(169-180) is highly conserved.

14 Virus neutralizing activity in PCV2-infected and vaccinated pigs

15 Virus neutralizing activity in serum samples from the same PCV2 pigs in Fig. 3-8 panel B, and
16 vaccinated pigs in Fig. 3-8 panel C is shown in Fig. 3-12. The mean total antibody level for
17 PCV2-infected pigs (IFA titer = 6.7), as measured by IFA, was almost twice the level of the
18 mean value for the vaccinated pigs (IFA titer = 5.8). In contrast, the mean NA level for
19 vaccinated pigs ($log2NA_{50}$ =2.8) was approximately four times higher than the mean level
20 obtained from sera of PCV2-infected pigs ($log2NA_{50}$ =1.6).
21
22

1

Figures and Tables

3

4 **Figure 3-1 pHUE expression product map**

5

6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23

1

2 **Figure 3-2 Expression and Purification of CP(43-233)**

3 As described in Materials and Methods, the CP(43-233) polypeptide was cloned in pHUE,

4 expressed in *E. coli* and purified using a Ni-TED column (USB). Lane 1 shows the bacterial

5 lysates supernatant after treatment with lysozyme and sonication. Lane 2 shows the flow

6 through after loading the contents of lane 1 onto the Ni-TED column. Lanes 3-5 show the flow

7 through after washing with 1XLEW buffer. Lanes 6-9 show the fractions collected after eluting

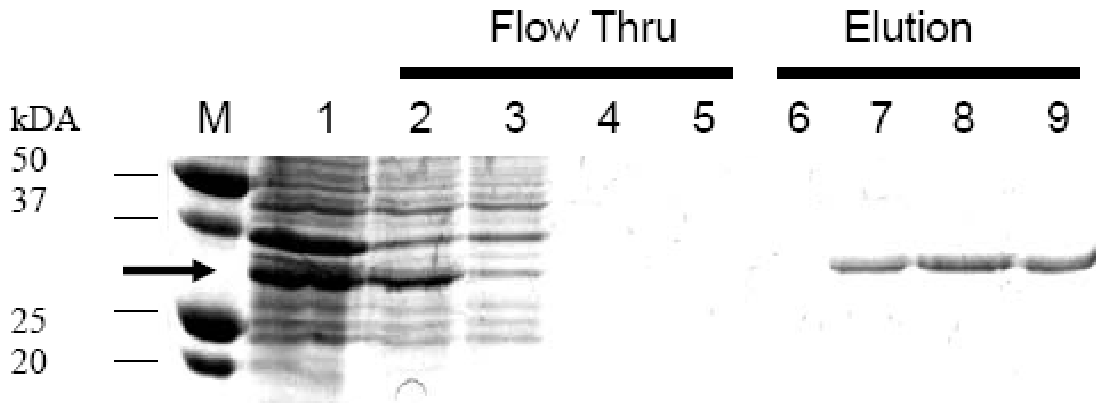
8 the 6X-His tagged protein with elution buffer. Proteins were visualized by SDS-PAGE on a 15%

9 acrylamide gel. Staining was performed using Simply Blue Safe Stain (Invitrogen). The lane

10 labeled M is the standards. The arrow points to the target CP(43-233) polypeptide.

11

12



13

14

15

16

17

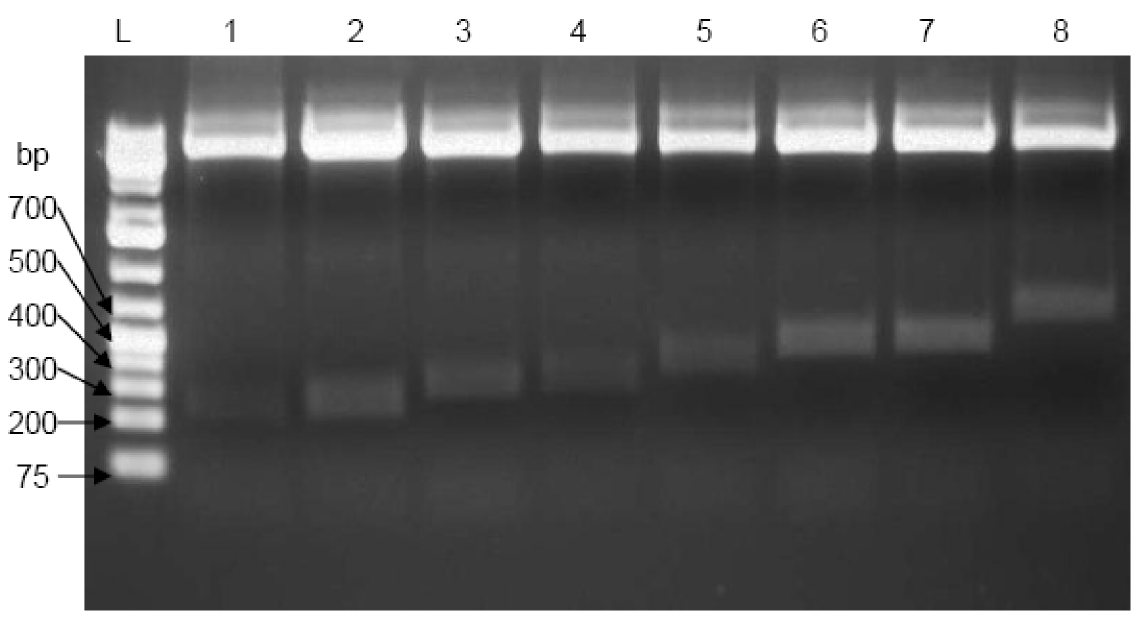
18

1

2 **Figure 3-3 PCV2 Capsid Protein Polypeptide Fragment Digestions**

3 Capsid protein polypeptides were amplified from a PCV2b sequence with forward and reverse
4 primers containing SacII or HindIII sites at their respective 5' or 3'ends. After PCR
5 amplification, fragments were cloned into pHUE ((60, 71, 125) and transformed into *E. coli*.
6 Double restriction digestion was carried out on purified plasmid DNA using the enzymes SacII
7 and HindIII. L represents the DNA ladder (Fermentus). Lanes 1-8 are as follows: CP(91-160),
8 CP(160-233), CP(43-135), CP(135-233), CP(43-160), CP(43-180), CP(91-233), and CP(43-233)
9 respectively. Plasmid digestions were separated on a 1% agarose gel followed by UV ethidium
10 bromide visualization at 302nm.

11



12

13

14

15

16

1

2 **Figure 3-4 Purified CP Polypeptides**

3 CP Polypeptides in lanes 1-7 were purified using USB's denaturing conditions protocol while the

4 polypeptide in lane 8 was purified using USB's purification under standard conditions protocol

5 as described in materials and methods. Lanes 1-8 are as follows: CP(91-160), CP(160-233),

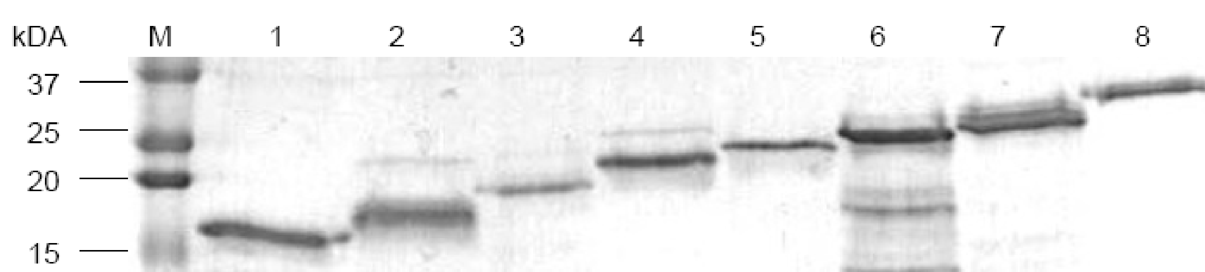
6 CP(43-135), CP(135-233), CP(43-160), CP(43-180), CP(91-233), and CP(43-233) respectively.

7 Lane M is the standards. Proteins were visualized by SDS-PAGE on a 15% acrylamide gel.

8 Staining was performed using Simply Blue Safe Stain (Invitrogen).

9

10



11

12

13

14

15

16

17

18

19

20

1

2 **Figure 3-5 CP(160-233) fractions after buffer exchange**

3 In an attempt to remove urea from the buffer, the bacterial expression cell lysate containing 160-

4 233 was passed through a column containing sephadex G10 in LEW/CAPS/Sarkosyl. Fractions

5 were collected and analyzed by SDS-PAGE on a 15% gel as described in Materials and Methods.

6 The numbers along the left side of the gel indicate the size of the standards in kDa. Labels are as

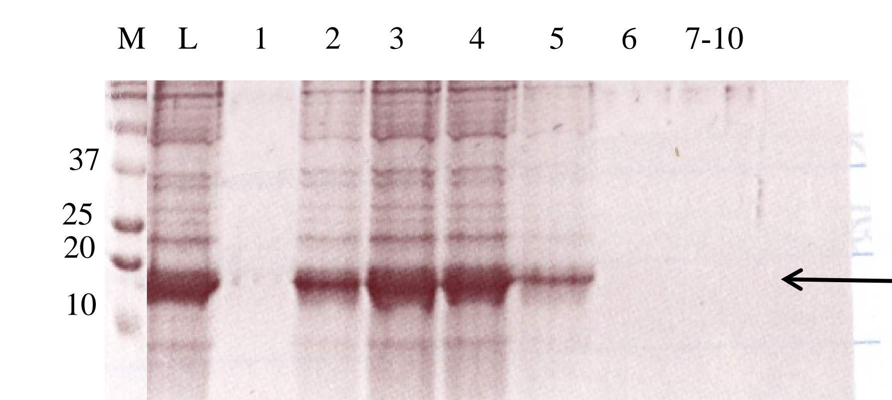
7 follows: M, standard markers; L, lysate in LEW/8M urea containing CP(160-233) after bacterial

8 expression; 1-10, elutions from the column in LEW/CAPS/Sarkosyl. The arrow indicates the

9 location of CP(160-233).

10

11



12

13

14

15

16

17

18

19

20

21

22

23

1

2 **Figure 3-6 Hybrid protocol for purification of CP(160-233)**

3 Following buffer exchange described in Materials and Methods and Fig. 3-5, CP(160-233) was

4 passed through the Ni-column (USB) as described in Materials and Methods for purification

5 under native conditions. The numbers along the left side of the gel indicate the size of the

6 standards in kDa. Labels are as follows: M, Standard markers; L, pooled fractions 3 and 4

7 which contain the bacterial expressed lysate in LEW/CAPS/Sarkosyl buffer containing CP(160-

8 233); F, flowthrough fraction; W, wash fraction; 1-4, elutions in native elution buffer. The arrow

9 indicates the location of CP(160-233).

10

11

12

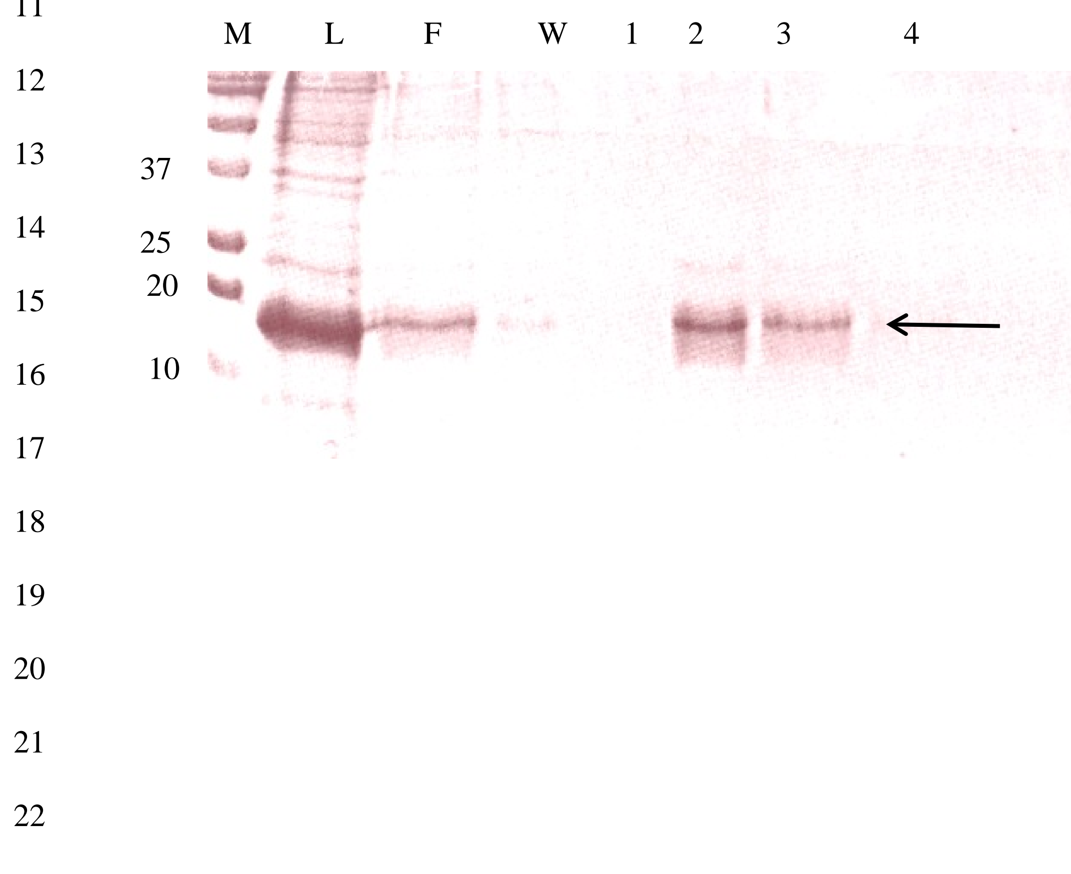
13

14

15

16

17



18

19

20

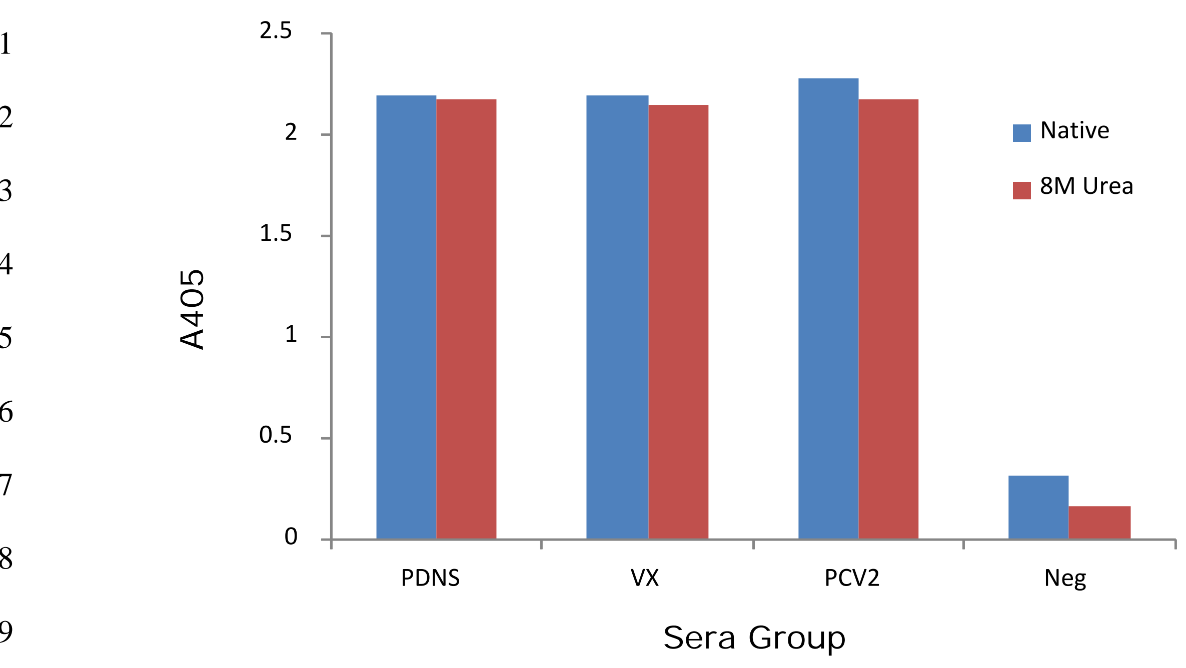
21

22

23

Figure 3-7 Comparative ELISA results

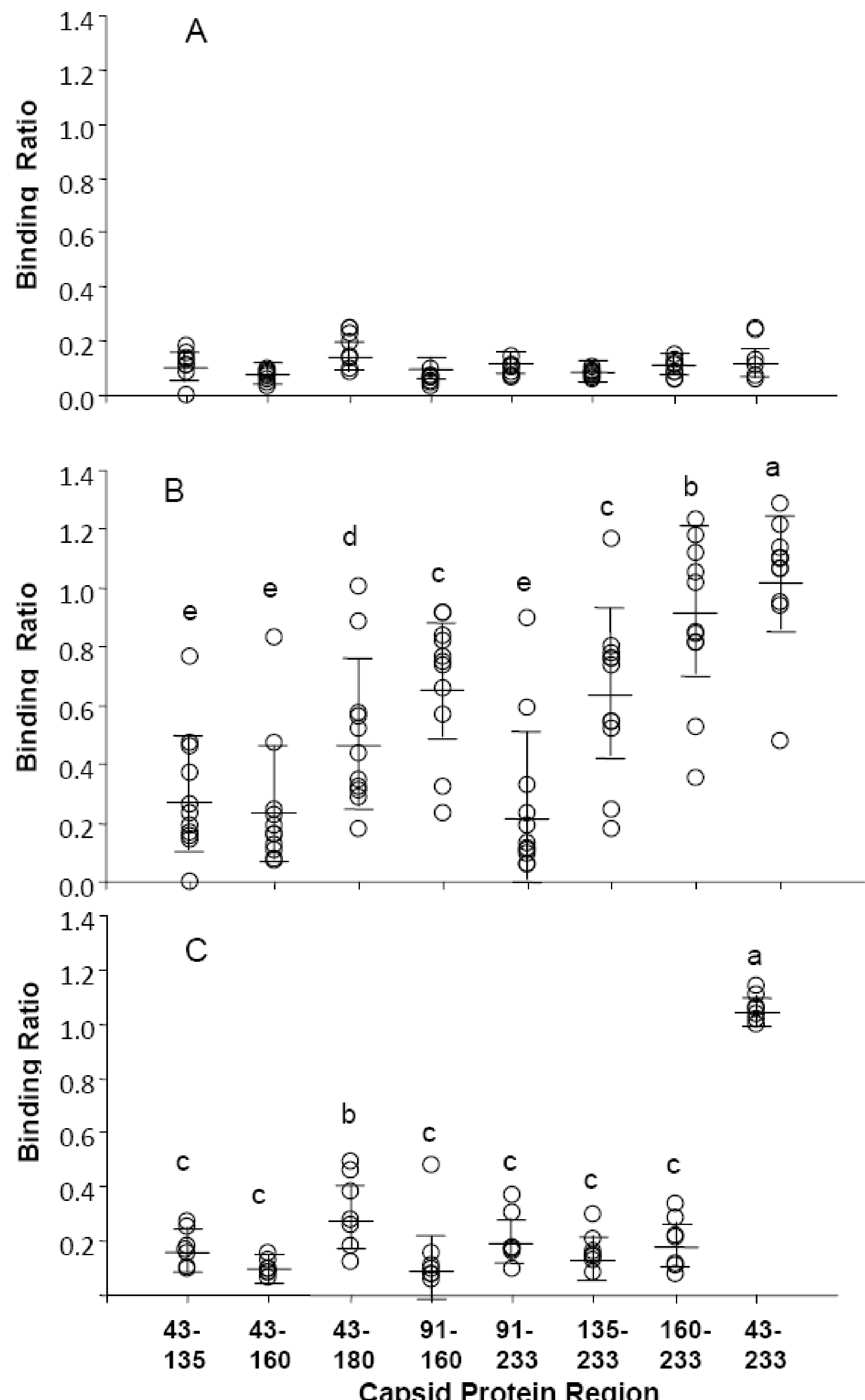
To compare the immunoreactivity of different sera groups with CP(43-233) purified under native or denaturing (in the presence of 8M urea) conditions, a comparative ELISA was performed as described in materials and methods. Two serum samples from PDNS diagnosed pigs, PCV2 experimentally infected pigs (PCV2), pigs vaccinated with a two dose PCV2 vaccine (VX) or negative control pigs were selected and analyzed in the ELISA. Antibody binding was measured at A405 and each sera groups reactivity against CP(43-233) purified under native or denaturing conditions was averaged.



1 **Figure 3-8 Reactivity of Sera from Experimentally Infected and Vaccinated Pigs to CP**

2 **Polypeptides**

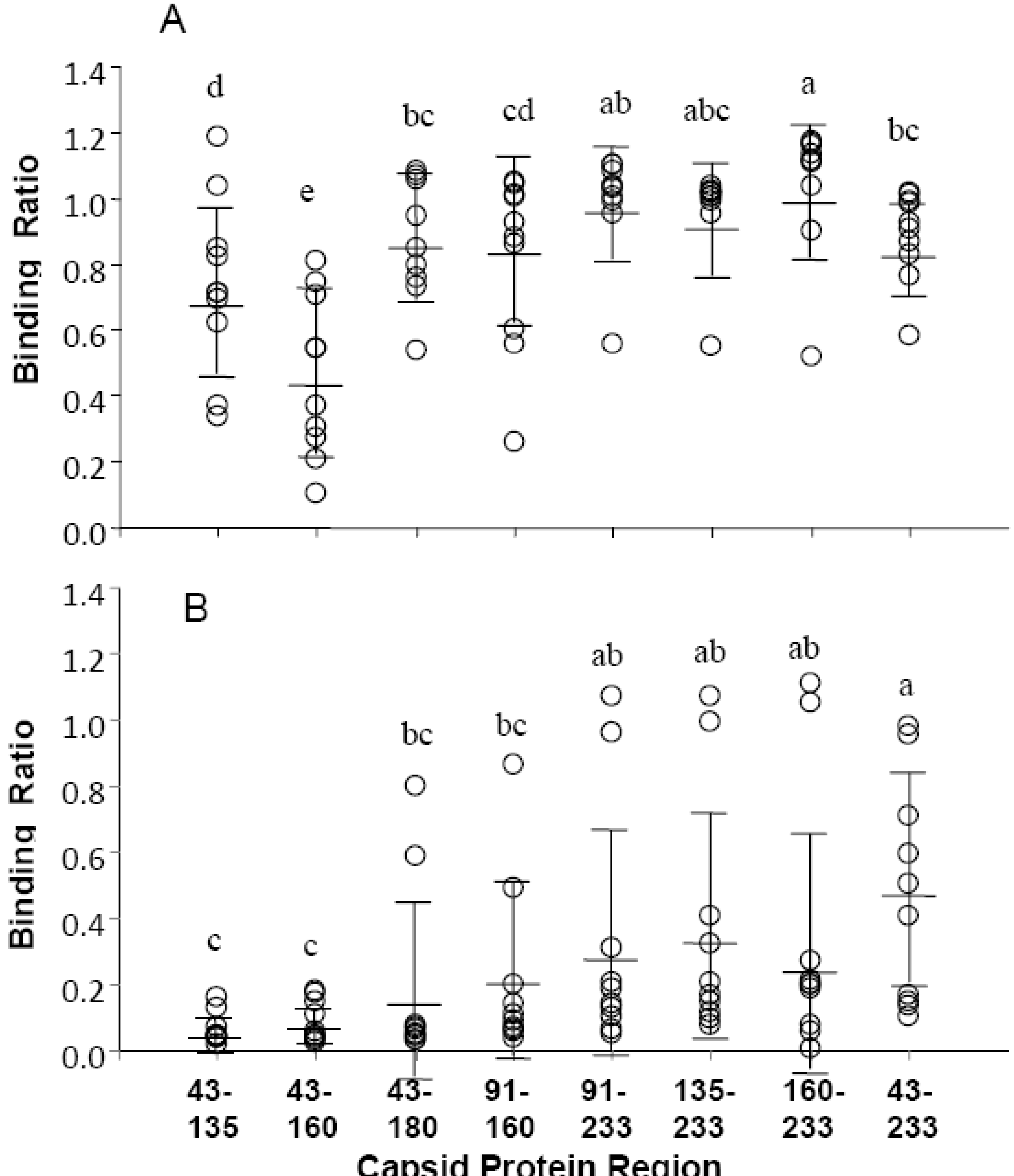
3 ELISA's were performed as described in Materials and Methods. Sera from PCV2 negative
4 (panel A), PCV2-infected (panel B) and vaccinated pigs was obtained at the end of the
5 experimental infection/vaccination study shown in Figure 2-2 or five weeks post infection and 7
6 weeks post vaccination. Binding ratios were calculated as the absorbance value at 405nm
7 (A405) of test sample minus background divided by the A405 value of the internal positive
8 control minus background. The antibody binding ratios were derived from a 1:100 dilution of
9 each serum sample. The open circles show the response of each individual pig while the lines
10 show the mean and standard deviation. Similar letters represent pigs with a statistically similar
11 response as calculated by the Student Newman Keuls method.
12



1

Figure 3-9 Reactivity of Sera from PCVAD Pigs to CP Polypeptides

The same methods described Figure 3-8 were used to determine antibody reactivity of pigs submitted to the Kansas State Veterinary Diagnostic Laboratory with a diagnosis of PDNS (panel A) or PMWS (panel B)



1

2

3

4

5

6

1 **Figure 3-10 Reactivity of Sera from Experimentally PCV2 Infected and Pigs Diagnosed**

2 **with PDNS to CP Oligopeptides**

3 Serum from PCV2 experimentally infected (panel A) and dignosed PDNS (panel B) pigs was

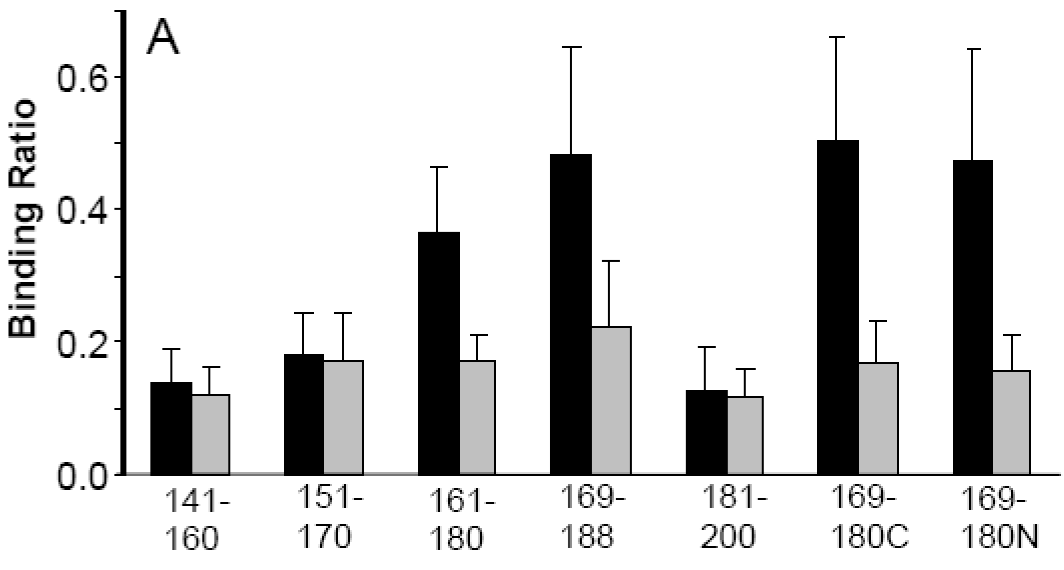
4 reacted with CP oligopeptides in an ELISA as described in Materials and methods. Binding

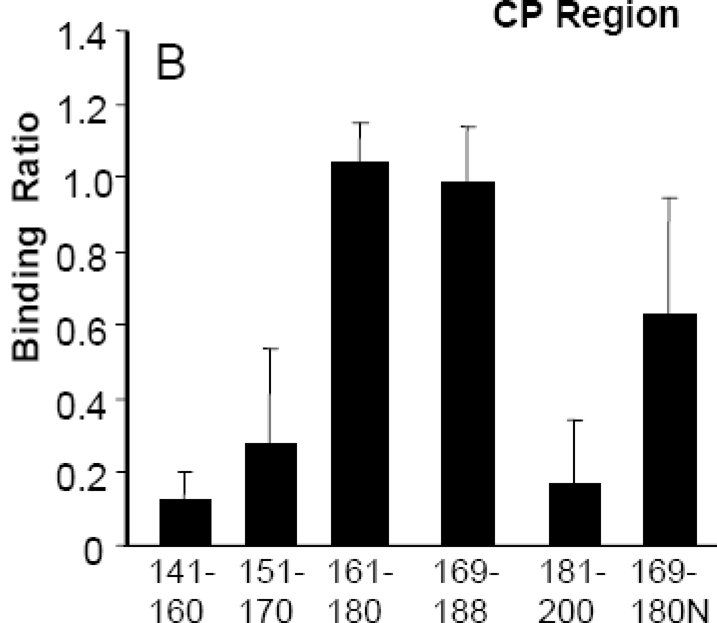
5 ratios were calculated as previously described. Panel A shows pigs could be divided into two

6 groups based on their antibody response: high responders (black bars) and low responders (grey

7 bars). Bars show the mean while lines above the bars show the standard deviation.

8





1

2

3

4

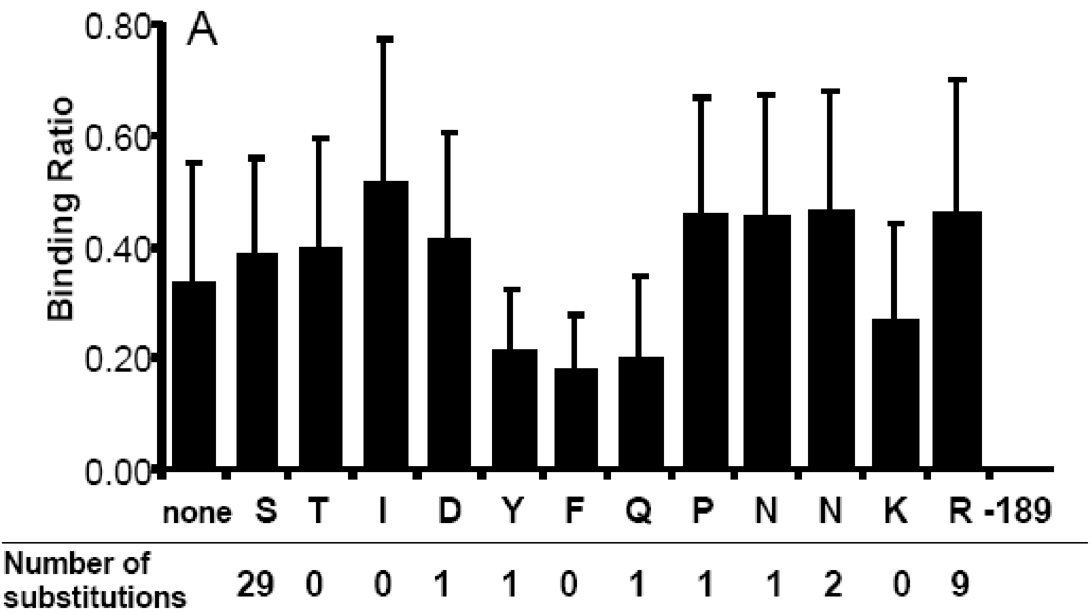
5

6

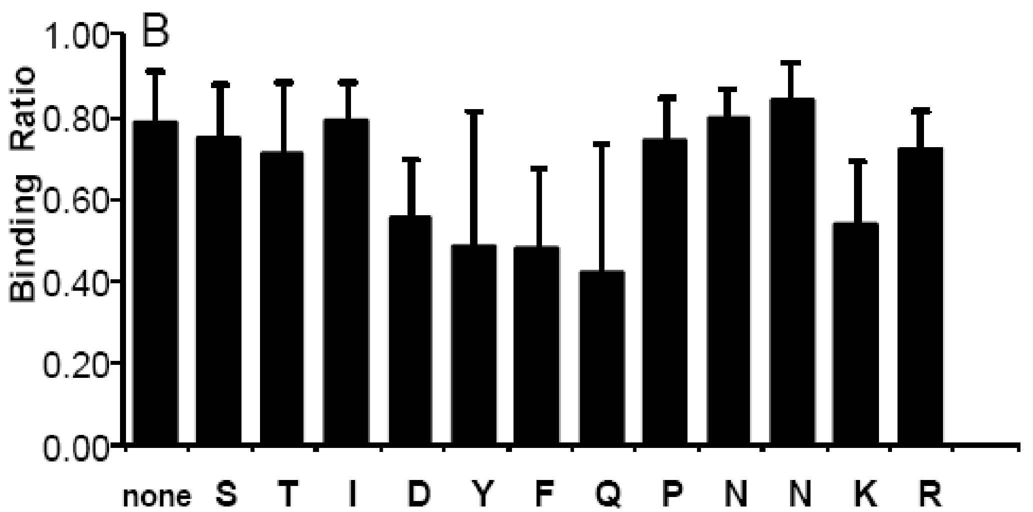
7

Figure 3-11 Alanine Scanning of the CP(169-180) Oligopeptide

Oligopeptides were synthesized containing alanine substitutions at each position of the CP(169-180) region. Oligopeptides were then reacted with serum from high responding experimentally infected pigs (represented by the black bars in Figure 3-10 and shown in panel A) and diagnosed PDNS pigs (panel B). Bars show the mean of the response from pigs while lines above the bars represent the standard deviation. The CP(169-180) region of 462 PCV2a and PCV2b sequences was analyzed for position substitutions. The total number of substitutions at each residue is shown between panel A and panel B.



A
Binding Ratio
0.80
0.60
0.40
0.20
0.00
none S T I D Y F Q P N N K R -189
Number of substitutions
29 0 0 1 1 0 1 1 1 2 0 9



B
Binding Ratio
1.00
0.80
0.60
0.40
0.20
0.00
none S T I D Y F Q P N N K R

1 **Figure 3-12**. **Total and neutralizing antibody responses following vaccination and**

2 **infection.**

3 Serum samples from PCV2-infected (PC) pigs (Fig. 8B) and PCV2 vaccinated pigs (VX; Fig.

4 8C) were tested for the presence of total antibody (IFA) and neutralizing activity (NA). Open

5 circles represent values for individual pigs and the horizontal bar the mean value for each group.

6

7

8

9

10

11

12

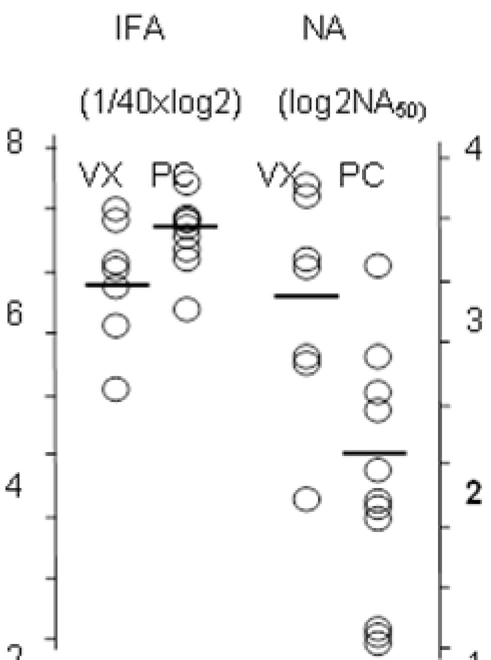
13

14

15

16

17



18

19

20

21

22

23

Table 3-1 Summary of Antibody Responses to PCV2 Capsid Protein Polypeptides

Name	Epitope Regions	PC*1	Vx	PM	PD
43-233	A B C D	++++*2	++++	++	+++
43-135		+	-	-	++
43-160		+	-	-	+
91-160		+	-	+	+++
43-180		++	+	+	+++
160-233		+++	-	+	+++
135-233		++	-	+	+++
91-233		++	-	+	+++

*1. Key: PC; PCV2; Vx, vaccine; PM, PMWS; PD, PDNS.
*2. Relative binding activity. key; (-) no measureable binding activity; (+) low binding activity; (++) intermediate bind activity; (+++) high binding activity ; (++++) very high binding activity

Chapter 4 – Discussion and Conclusions

In this study, CP polypeptides were expressed in E.coli using the pHUE expression vector. Products of expression, shown in Figure 3-1, include a 6X-His tag, followed by UBQ fused to the target gene of interest. The 6X-His tag is important for Ni-column affinity purification. Ubiquitin serves to increase solubility and expression of proteins. Aside from this, pHUE incorporates the T7 RNA polymerase promotor (pT7) and the lacI gene upstream from the His-UBQ-CP polypeptide sequence. The T7 RNA polymerase, from the T7 bacteriophage, catalyzes formation of mRNA in the 5'-3' direction. Furthermore, it only transcribes DNA that is downstream of its promoter and is extremely promoter specific. Further upstream of the pT7 is the lacI gene. This gene codes for a repressor that inhibits transcription from the pT7. Inhibition can only be abolished in the presence of lactose or other lactose derivatives. One convenient derivative is IPTG. IPTG mimics the function of lactose, however, is not metabolized by E. coli. Therefore, the concentration of IPTG remains constant after addition *to E.coli* in growth media.

Previous work has demonstrated the ability of bacterial expressed PCV2 CP protein to be recognized by sera from PCV2 infected pigs (69, 124, 136). The bacterial expression and purification of PCV2 ORF2 fused to either glutathione-S-transferase or maltose binding protein has been reported (69, 136). Furthurmore, Trundova et al. (124) reported expression of the full length CP(1-233) after codon optimization. In this study, attempts to express the full length polypeptide as a CP-UBQ fusion protein were met with failure, even after codon optimization of the sequence. The reason the entire CP(1-233) would not express in bacteria in our experiments is unknown. One posibility may be the difference in fusion protein used in assays. Its possible that fusion to either glutathione-S-transferase or maltose binding protein increases the solubility

1 of CP(1-233) compared to fusion to UBQ. A different possibility could be the specifity of assays
2 used to detect expressed protein. The use of SDS-PAGE in our study compared to assays in
3 previous reports (Western blot and immunoblot) could account for our inablility to identify
4 bacterially expressed CP(1-233). Reports also indicated high levels of expression following the
5 deletion of the NLS from CP (124, 136). Indeed, after deletion of the N-terminal, Arg-rich NLS
6 (CP(1-42)), bacterial expression of CP(43-233) was successful and resulted in large quantities of
7 purified protein (See Figure 3-2).
8
9 Overexpression of recombinant proteins in *E.coli* can lead to the formation of insoluble inclusion
10 bodies (113). Solubiliation and recovery from inclusion bodies is often complex and protein
11 specific. Results of protein expression from this study showed that only CP(43-233) could be
12 purified under native conditions. Significant efforts were made to either prevent the formation of
13 inclusion bodies (expression assays at 30°C instead of 37°C) or retern CP polypeptides into non-
14 denaturing buffers (See Materials and Methods for description of assays). In particular, the the
15 successful use of sarkosyl for purification of many proteins that form insoluble inclusion bodies
16 has been reported (29, 113). In addition, CAPS and sarkosyl are common in many commercially
17 available protein purification and refolding kits such as the Protein Refolding Kit (Novagen).
18 Ultimately, the results from this study inicated the incorporation of 8M urea was the only method
19 to obtain significant quantities of the smaller CP polypeptides. At this time, the basis for
20 chemical denaturation of proteins by urea is not well understood. One possibility proposed by
21 Bennion and Daggett (5) is that urea alters the structure and dynamics of water, diminishing the
22 hydrophic effect caused by water. This leads to solvation of hydrophobic groups and at the same
23 time, frees up water molecules to compete with intra-protein interactions or to interact with polar

residues. Ultimately, these interactions would lead to the stabilization of the nonnative conformation of the protein. Overall, 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 Figs 3-8 and 3-9). Interestingly, the results of the comparative ELISA showed no difference in the ability of the different sera groups to recognize CP(43-233) purified under native or denaturing (8M urea) conditions (Fig. 3-7). One possibility for this may be that dilution of the CP polypeptide to 4ug/ml, prior to coating ELISA plates, abolishes the ability of urea to unfold proteins. Therefore, CP polypeptides, originally purified under denaturing conditions, return to their native conformation upon dilution in the ELISA coating buffer.

Previous studies of humoral immunity during PCV2 infection have focused on mapping antigenic regions of CP, including the characterization of immurelevant epitopes (17). 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 3-1. The first pattern is illustrated by pigs with severe PMWS. Overall, there was a descreased reactivity to all CP polypeptide antigens (Fig. 3-9B). This outcome is consistent with the immune suppression associated with PMWS, a disease syndrome characterized by an almost complete depletion of lymphocytes with

1 a corresponding loss or severe dysregulation of immune function (10). Immunohistochemical
2 staining of PMWS lymph nodes typically shows large accumulations of PCV2 antigen (47, 48,
3 67). Presumably, dividing lymphocytes, activated in response to infection or other immune
4 stimuli, become a primary target for PCV2 replication. Cytopathogenesis in lymphocytes is
5 attributed to the function of PCV2 ORF3, which is not required for virus replication in culture,
6 but has been linked to apoptosis (133). In sharp contrast, pigs with clinically apparent PDNS
7 showed high reactivity to all CP polypeptide fragments (Fig. 3-9A), including the oligopeptide
8 CP(169-180; Fig. 3-10B). This outcome is consistent with a hyperactive humoral response and
9 immune complex formation. Pathogenesis is linked to the deposition of antigen-antibody
10 complexes in the kidney and other organs followed by the activation of complement (58). The
11 role that PCV2 plays in PDNS remains unclear. Krakowka et al.(63, 93) reported the induction
12 of PDNS in gnotobiotic pigs after infection with a group 1 torque teno virus (TTV) and PRRSV.
13 To date, there are no models of PCV2 infection that reproduce PDNS. While the reason for the
14 experimental production of PDNS without PCV2 is unknown, one likely possibility is PCV2
15 infection may not be the proximal cause of PDNS, but may play a role in the evolution of the
16 disease process and expression of full-blown disease.
17
18 A third pattern of antibody recognition was found in the response of pigs experimentally infected
19 with PCV2. The results showed the highest antibody binding to CP(43-233) followed by
20 reactivity with polypeptides that contained Epitope C. A dichotomy in the response to the
21 Epitope C region was evident by the oligopeptide mapping results, in which PCV2 pigs could be
22 divided into two groups: those that produced a response similar to PDNS pigs and recognized
23 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 with a much lower responses to smaller polypeptides, including those polypeptide fragments that contained Epitope C (Fig. 3-8C). 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. 3-12). Even though the total PCV2 antibody response of infected pigs was almost

1 twice the response of vaccinated pigs, the mean neutralizing activity for the vaccinated group
2 was almost four times that of the infected group. Therefore, the apparent paradox of decreased
3 neutralizing activity in the face of an overall robust humoral response can be resolved if the
4 response is directed towards non-neutralizing epitopes. The diversion of humoral immunity away
5 from the larger neutralizing epitope is a strategy that has been proposed for HIV and PRRSV
6 (63, 93).
7
8 One interesting aspect of PCV2 infection is that only a subpopulation of infected pigs go on to
9 develop full-blown disease, while other infected pigs remain unaffected. The mixed antibody
10 response of PCV2-infected pigs provides insight into a possible mechanism for differences in
11 disease susceptibility within the same population of PCV2-infected pigs. For example, those pigs
12 that respond to PCV2 in a manner similar to the response of pigs following vaccination, produce
13 an effective antibody response that results in virus clearance and protection. In contrast, those
14 pigs that produce a response similar to PDNS pigs; i.e., against non-protective epitopes, are more
15 susceptible to prolonged virus replication and disease.
16
17 With the application of PCV2 vaccines for preventing the onset of disease arises the need for a
18 diagnostic assay capable of distinguishing vaccinated animals from infected animals. Results
19 from this study, illustrated in Figure 3-10, demonstrate animals that have been infected with
20 PCV2 produce antibodies that react with the CP(169-180) oligopeptides. In contrast, animals
21 which have been vaccinated with a PCV2 CP based vaccine produce antibodies that primarily
22 react with the full length CP(43-233) polypeptide (Figure 3-8C). Therefore, differential

1 recognition of CP(43-233) and CP(169-180) could provide the basis for diagnostic approaches

2 that that can differentiate infected from vaccinated animals (DIVA).

3

Chapter 5 - References

1. **Allan G. M., S. Kennedy, F. McNeilly, J. C. Foster, J. A. Ellis, S. J. Krakowka, B. M. Meehan, and B. M. Adair**. 1999. Experimental reproduction of severe wasting disease by co-infection of pigs with porcine circovirus and porcine parvovirus. J. Comp. Pathol **121**:1-11.
2. **Allan G. M., F. McNeilly, S. Kennedy, B. Daft, E. G. Clarke, J. A. Ellis, D. M. Haines, B. M. Meehan, and B. M. Adair**. 1998. Isolation of porcine circovirus-like viruses from pigs with a wasting disease in the USA and Europe. J. Vet. Diagn. Invest **10**:3-10.
3. **Allan G. M., K. V. Phenix, D. Todd, and M. S. McNulty**. 1994. Some biological and physico-chemical properties of porcine circovirus. Zentralblatt Veterinarmedizin Reihe B **41**:17-26.
4. **Allan G., F. McNeilly, B. Meehan, I. McNair, J. Ellis, S. Krakowka, C. Fossum, E. Wattrang, P. Wallgren, and B. Adair**. 2003. Reproduction of postweaning multisystemic wasting syndrome in pigs experimentally inoculated with a Swedish porcine circovirus 2 isolate. J. Vet. Diagn. Invest **15**:553-560.
5. **Bennion B. J., and V. Daggett**. 2003. The molecular basis for the chemical denaturation of proteins by urea. Proceedings of the National Academy of Sciences of the United States of America **100**:5142 -5147.
6. **Blanchard P., D. Mahé, R. Cariolet, C. Truong, M. Le Dimna, C. Arnauld, N. Rose, E. Eveno, E. Albina, F. Madec, and A. Jestin**. 2003. An ORF2 protein-based ELISA for porcine circovirus type 2 antibodies in post-weaning multisystemic wasting syndrome. Vet. Microbiol **94**:183-194.
7. **Bolin S. R., W. C. Stoffregen, G. P. Nayar, and A. L. Hamel**. 2001. Postweaning multisystemic wasting syndrome induced after experimental inoculation of cesarean-derived, colostrum-deprived piglets with type 2 porcine circovirus. J. Vet. Diagn. Invest **13**:185-194.
8. **Catanzariti A.-M., T. A. Soboleva, D. A. Jans, P. G. Board, and R. T. Baker**. 2004. An efficient system for high-level expression and easy purification of authentic recombinant proteins. Protein Sci **13**:1331-1339.
9. **Chae C.** 2005. A review of porcine circovirus 2-associated syndromes and diseases. Vet. J **169**:326-336.
10. **Chae C.** 2004. Postweaning multisystemic wasting syndrome: a review of aetiology, diagnosis and pathology. Vet. J **168**:41-49.
11. **Chaiyakul M., K. Hsu, R. Dardari, F. Marshall, and M. Czub**. 2010. Cytotoxicity of ORF3 proteins from a nonpathogenic and a pathogenic porcine circovirus. J. Virol **84**:11440-11447.
12. **Chang H.-W., C.-R. Jeng, T.-L. Lin, J. J. Liu, M.-T. Chiou, Y.-C. Tsai, M.-Y. Chia, T.-R. Jan, and V. F. Pang**. 2006. Immunopathological effects of porcine circovirus type 2 (PCV2) on swine alveolar macrophages by in vitro inoculation. Vet. Immunol. Immunopathol **110**:207-219.
13. **Cheung A. K.** 2004. Detection of template strand switching during initiation and termination of DNA replication of porcine circovirus. J. Virol **78**:4268-4277.

1 14. **Choi J., G. W. Stevenson, M. Kiupel, B. Harrach, L. Anothayanontha, C. L. Kanitz,
2 and S. K. Mittal**. 2002. Sequence analysis of old and new strains of porcine circovirus
3 associated with congenital tremors in pigs and their comparison with strains involved with
4 postweaning multisystemic wasting syndrome. Can. J. Vet. Res **66**:217-224.
5 15. **Clark E. G.** 1997. Post-weaning wasting syndrome. Proceedings of the American
6 Association of Swine Practitioners **28**:499-501.
7 16. **Cline G., V. Wilt, E. Diaz, and R. Edler**. 2008. Efficacy of immunising pigs against
8 porcine circovirus type 2 at three or six weeks of age. Vet. Rec **163**:737-740.
9 17. **Darwich L., J. Segalés, and E. Mateu**. 2004. Pathogenesis of postweaning multisystemic
10 wasting syndrome caused by Porcine circovirus 2: An immune riddle. Arch. Virol
11 **149**:857-874.
12 18. **Darwich L., S. Pié, A. Rovira, J. Segalés, M. Domingo, I. P. Oswald, and E. Mateu**.
13 2003. Cytokine mRNA expression profiles in lymphoid tissues of pigs naturally affected
14 by postweaning multisystemic wasting syndrome. J. Gen. Virol **84**:2117-2125.
15 19. **Dupont K., E. O. Nielsen, P. Baekbo, and L. E. Larsen**. 2008. Genomic analysis of
16 PCV2 isolates from Danish archives and a current PMWS case-control study supports a
17 shift in genotypes with time. Vet. Microbiol **128**:56-64.
18 20. **Fachinger V., R. Bischoff, S. B. Jedidia, A. Saalmüller, and K. Elbers**. 2008. The
19 effect of vaccination against porcine circovirus type 2 in pigs suffering from porcine
20 respiratory disease complex. Vaccine **26**:1488-1499.
21 21. **Faurez F., D. Dory, B. Grasland, and A. Jestin**. 2009. Replication of porcine
22 circoviruses. Virol. J **6**:60.
23 22. **Fenaux M., T. Opriessnig, P. G. Halbur, Y. Xu, B. Potts, and X.-J. Meng**. 2004.
24 Detection and in vitro and in vivo characterization of porcine circovirus DNA from a
25 porcine-derived commercial pepsin product. J. Gen. Virol **85**:3377-3382.
26 23. **Finney D. J.** 1964. The Spearman-Karber method, p. 524-530. *In* D.J. Finney (ed.),
27 Statistical Method in Biological Assay, 2nd ed. Charles Griffin, London.
28 24. **Finsterbusch T., T. Steinfeldt, R. Caliskan, and A. Mankertz**. 2005. Analysis of the
29 subcellular localization of the proteins Rep, Rep' and Cap of porcine circovirus type 1.
30 Virology **343**:36-46.
31 25. **Finsterbusch T., and A. Mankertz**. 2009. Porcine circoviruses--small but powerful.
32 Virus Res **143**:177-183.
33 26. **Fort M., M. Sibila, E. Pérez-Martín, M. Nofrarías, E. Mateu, and J. Segalés**. 2009.
34 One dose of a porcine circovirus 2 (PCV2) sub-unit vaccine administered to 3-week-old
35 conventional piglets elicits cell-mediated immunity and significantly reduces PCV2
36 viremia in an experimental model. Vaccine **27**:4031-4037.
37 27. **Fort M., L. T. Fernandes, M. Nofrarias, I. Díaz, M. Sibila, J. Pujols, E. Mateu, and J.
38 Segalés**. 2009. Development of cell-mediated immunity to porcine circovirus type 2
39 (PCV2) in caesarean-derived, colostrum-deprived piglets. Vet. Immunol. Immunopathol
40 **129**:101-107.
41 28. **Fort M., A. Olvera, M. Sibila, J. Segalés, and E. Mateu**. 2007. Detection of neutralizing
42 antibodies in postweaning multisystemic wasting syndrome (PMWS)-affected and non-
43 PMWS-affected pigs. Vet. Microbiol **125**:244-255.
44 29. **Frankel S., R. Sohn, and L. Leinwand**. 1991. The use of sarkosyl in generating soluble
45 protein after bacterial expression. Proc Natl Acad Sci U S A **88**:1192-1196.

1 30. **Gagnon C. A., N. Music, G. Fontaine, D. Tremblay, and J. Harel**. 2010. Emergence of
2 a new type of porcine circovirus in swine (PCV): A type 1 and type 2 PCV recombinant.
3 Vet Microbiol.
4 31. **Gibbs M. J., and G. F. Weiller**. 1999. Evidence that a plant virus switched hosts to infect
5 a vertebrate and then recombined with a vertebrate-infecting virus. Proc. Natl. Acad. Sci.
6 U.S.A **96**:8022-8027.
7 32. **Gillespie J., T. Opriessnig, X. J. Meng, K. Pelzer, and V. Buechner-Maxwell**. 2009.
8 Porcine Circovirus Type 2 and Porcine Circovirus-Associated Disease. Journal of
9 Veterinary Internal Medicine **23**:1151-1163.
10 33. **Gillespie J., T. Opriessnig, X. J. Meng, K. Pelzer, and V. Buechner-Maxwell**. 2009.
11 Porcine Circovirus Type 2 and Porcine Circovirus-Associated Disease. Journal of
12 Veterinary Internal Medicine **23**:1151-1163.
13 34. **Gilpin D. F., K. McCullough, B. M. Meehan, F. McNeilly, I. McNair, L. S. Stevenson,**
14 **J. C. Foster, J. A. Ellis, S. Krakowka, B. M. Adair, and G. M. Allan**. 2003. In vitro
15 studies on the infection and replication of porcine circovirus type 2 in cells of the porcine
16 immune system. Vet. Immunol. Immunopathol **94**:149-161.
17 35. **Grau-Roma L., L. Fraile, and J. Segalés**. 2011. Recent advances in the epidemiology,
18 diagnosis and control of diseases caused by porcine circovirus type 2. Vet. J **187**:23-32.
19 36. **Grau-Roma L., C. K. Hjulsager, M. Sibila, C. S. Kristensen, S. López-Soria, C. Enøe,**
20 **J. Casal, A. Bøtner, M. Nofrarías, V. Bille-Hansen, L. Fraile, P. Baekbo, J. Segalés,**
21 **and L. E. Larsen**. 2009. Infection, excretion and seroconversion dynamics of porcine
22 circovirus type 2 (PCV2) in pigs from post-weaning multisystemic wasting syndrome
23 (PMWS) affected farms in Spain and Denmark. Vet. Microbiol **135**:272-282.
24 37. **Gresham A., N. Giles, and J. Weaver**. 2000. PMWS and porcine dermatitis nephropathy
25 syndrome in Great Britain. Vet. Rec **147**:115.
26 38. **Harding J. C. S.** 1997. Post-weaning multisystemic wasting syndrome (PMWS):
27 Preliminary epidemiology and clinical presentation. Proceedings of the American
28 Association of Swine Practitioners **28**:503.
29 39. **Harding J. C. S., and E. G. Clark**. 2007. Recognizing and diagnosing
30 postweaningmultisystemic wasting syndrome (PMWS). J Swine Health Prod **5**:201-203.
31 40. **Harms P. A., S. D. Sorden, P. G. Halbur, S. R. Bolin, K. M. Lager, I. Morozov, and P.**
32 **S. Paul**. 2001. Experimental reproduction of severe disease in CD/CD pigs concurrently
33 infected with type 2 porcine circovirus and porcine reproductive and respiratory syndrome
34 virus. Vet. Pathol **38**:528-539.
35 41. **Heath L., A.-L. Williamson, and E. P. Rybicki**. 2006. The capsid protein of beak and
36 feather disease virus binds to the viral DNA and is responsible for transporting the
37 replication-associated protein into the nucleus. J. Virol **80**:7219-7225.
38 42. **Hesse R., M. Kerrigan, and R. R. R. Rowland**. 2008. Evidence for recombination
39 between PCV2a and PCV2b in the field. Virus Res **132**:201-207.
40 43. **Hjulsager C. K., L. Grau-Roma, M. Sibila, C. Enøe, L. Larsen, and J. Segalés**. 2009.
41 Inter-laboratory and inter-assay comparison on two real-time PCR techniques for
42 quantification of PCV2 nucleic acid extracted from field samples. Vet. Microbiol **133**:172-
43 178.
44 44. **Horlen K. P., S. S. Dritz, J. C. Nietfeld, S. C. Henry, R. A. Hesse, R. Oberst, M. Hays,**
45 **J. Anderson, and R. R. R. Rowland**. 2008. A field evaluation of mortality rate and

1 growth performance in pigs vaccinated against porcine circovirus type 2. J. Am. Vet. Med.
2 Assoc **232**:906-912.
3 45. **Horlen K. P., P. Schneider, J. Anderson, J. C. Nietfeld, S. C. Henry, L. M. Tokach,**
4 **and R. R. R. Rowland**. 2007. A cluster of farms experiencing severe porcine circovirus
5 associated disease: Clinical features and association with the PCV2b genotype. JSHAP
6 **15**:270-278.
7 46. **Jacobsen B., L. Krueger, F. Seeliger, M. Bruegmann, J. Segalés, and W.**
8 **Baumgaertner**. 2009. Retrospective study on the occurrence of porcine circovirus 2
9 infection and associated entities in Northern Germany. Vet. Microbiol **138**:27-33.
10 47. **Juhan N. M., T. LeRoith, T. Opriessnig, and X. J. Meng**. 2010. The open reading frame
11 3 (ORF3) of porcine circovirus type 2 (PCV2) is dispensable for virus infection but
12 evidence of reduced pathogenicity is limited in pigs infected by an ORF3-null PCV2
13 mutant. Virus Res **147**:60-66.
14 48. **Karuppannan A. K., M. H. Jong, S.-H. Lee, Y. Zhu, M. Selvaraj, J. Lau, Q. Jia, and**
15 **J. Kwang**. 2009. Attenuation of porcine circovirus 2 in SPF piglets by abrogation of
16 ORF3 function. Virology **383**:338-347.
17 49. **Kawashima K., K. Katsuda, and H. Tsunemitsu**. 2007. Epidemiological investigation
18 of the prevalence and features of postweaning multisystemic wasting syndrome in Japan.
19 J. Vet. Diagn. Invest **19**:60-68.
20 50. **Kekarainen T., K. McCullough, M. Fort, C. Fossum, J. Segalés, and G. M. Allan**.
21 2010. Immune responses and vaccine-induced immunity against Porcine circovirus type 2.
22 Vet Immunol Immunopathol **136**:185-193.
23 51. **Kekarainen T., K. McCullough, M. Fort, C. Fossum, J. Segalés, and G. M. Allan**.
24 2010. Immune responses and vaccine-induced immunity against Porcine circovirus type 2.
25 Vet Immunol Immunopathol.
26 52. **Kekarainen T., M. Montoya, J. Dominguez, E. Mateu, and J. Segalés**. 2008. Porcine
27 circovirus type 2 (PCV2) viral components immunomodulate recall antigen responses.
28 Vet. Immunol. Immunopathol **124**:41-49.
29 53. **Kim J., D. U. Han, C. Choi, and C. Chae**. 2001. Differentiation of porcine circovirus
30 (PCV)-1 and PCV-2 in boar semen using a multiplex nested polymerase chain reaction. J.
31 Virol. Methods **98**:25-31.
32 54. **Kindt T. J., B. A. Osborne, and R. A. Goldsby**. 2006. Kuby ImmunologySixth Edition.
33 W. H. Freeman.
34 55. **Kiss I., S. Kecskeméti, T. Tuboly, E. Bajmócy, and J. Tanyi**. 2000. New pig disease in
35 Hungary: postweaning multisystemic wasting syndrome caused by circovirus (short
36 communication). Acta Vet. Hung **48**:469-475.
37 56. **Kixmöller M., M. Ritzmann, M. Eddicks, A. Saalmüller, K. Elbers, and V.**
38 **Fachinger**. 2008. Reduction of PMWS-associated clinical signs and co-infections by
39 vaccination against PCV2. Vaccine **26**:3443-3451.
40 57. **Krakowka S., J. A. Ellis, F. McNeilly, S. Ringler, D. M. Rings, and G. Allan**. 2001.
41 Activation of the immune system is the pivotal event in the production of wasting disease
42 in pigs infected with porcine circovirus-2 (PCV-2). Vet. Pathol **38**:31-42.
43 58. **Krakowka S., C. Hartunian, A. Hamberg, D. Shoup, M. Rings, Y. Zhang, G. Allan,**
44 **and J. A. Ellis**. 2008. Evaluation of induction of porcine dermatitis and nephropathy
45 syndrome in gnotobiotic pigs with negative results for porcine circovirus type 2. Am. J.
46 Vet. Res **69**:1615-1622.

1 59. **Larochelle R., A. Bielanski, P. Müller, and R. Magar**. 2000. PCR detection and
2 evidence of shedding of porcine circovirus type 2 in boar semen. J. Clin. Microbiol
3 **38**:4629-4632.
4 60. **Lekcharoensuk P., I. Morozov, P. S. Paul, N. Thangthumniyom, W. Wajjawalku, and**
5 **X. J. Meng**. 2004. Epitope mapping of the major capsid protein of type 2 porcine
6 circovirus (PCV2) by using chimeric PCV1 and PCV2. J. Virol **78**:8135-8145.
7 61. **Li L., A. Kapoor, B. Slikas, O. S. Bamidele, C. Wang, S. Shaukat, M. A. Masroor, M.**
8 **L. Wilson, J.-B. N. Ndjango, M. Peeters, N. D. Gross-Camp, M. N. Muller, B. H.**
9 **Hahn, N. D. Wolfe, H. Triki, J. Bartkus, S. Z. Zaidi, and E. Delwart**. 2010. Multiple
10 diverse circoviruses infect farm animals and are commonly found in human and
11 chimpanzee feces. J. Virol **84**:1674-1682.
12 62. **Li L., T. Shan, S. B. Oderinde, A. M. Masroor, T. Kunz, Z. S. Zaidi, and E. Delwart**.
13 2010. Possible cross-species transmission of circoviruses and cycloviruses in farm
14 animals. J Gen Virol.
15 63. **Lin G., and P. L. Nara**. 2007. Designing immunogens to elicit broadly neutralizing
16 antibodies to the HIV-1 envelope glycoprotein. Curr. HIV Res **5**:514-541.
17 64. **Lipej Z., J. Segalés, I. Toplak, B. Sostarić, B. Roić, M. Lojkić, P. Hostnik, J. Grom,**
18 **D. Barlic-Maganja, K. Zarković, and D. Oraić**. 2005. Postweaning multisystemic
19 wasting syndrome (PMWS) in pigs in Croatia: detection and characterisation of porcine
20 circovirus type 2 (PCV2). Acta Vet. Hung **53**:385-396.
21 65. **Liu H., M. I. Boulton, K. J. Oparka, and J. W. Davies**. 2001. Interaction of the
22 movement and coat proteins of Maize streak virus: implications for the transport of viral
23 DNA. J. Gen. Virol **82**:35-44.
24 66. **Liu J., I. Chen, Q. Du, H. Chua, and J. Kwang**. 2006. The ORF3 protein of porcine
25 circovirus type 2 is involved in viral pathogenesis in vivo. J. Virol **80**:5065-5073.
26 67. **Liu J., I. Chen, and J. Kwang**. 2005. Characterization of a previously unidentified viral
27 protein in porcine circovirus type 2-infected cells and its role in virus-induced apoptosis. J.
28 Virol **79**:8262-8274.
29 68. **Liu Q., S. K. Tikoo, and L. A. Babiuk**. 2001. Nuclear localization of the ORF2 protein
30 encoded by porcine circovirus type 2. Virology **285**:91-99.
31 69. **Liu Q., P. Willson, S. Attoh-Poku, and L. A. Babiuk**. 2001. Bacterial expression of an
32 immunologically reactive PCV2 ORF2 fusion protein. Protein Expr. Purif **21**:115-120.
33 70. **Madec F., N. Rose, B. Grasland, R. Cariolet, and A. Jestin**. 2008. Post-weaning
34 multisystemic wasting syndrome and other PCV2-related problems in pigs: a 12-year
35 experience. Transbound Emerg Dis **55**:273-283.
36 71. **Mahé D., P. Blanchard, C. Truong, C. Arnauld, P. Le Cann, R. Cariolet, F. Madec,**
37 **E. Albina, and A. Jestin**. 2000. Differential recognition of ORF2 protein from type 1 and
38 type 2 porcine circoviruses and identification of immunorelevant epitopes. J. Gen. Virol
39 **81**:1815-1824.
40 72. **Mankertz A., and B. Hillenbrand**. 2001. Replication of porcine circovirus type 1
41 requires two proteins encoded by the viral rep gene. Virology **279**:429-438.
42 73. **Mankertz A., F. Persson, J. Mankertz, G. Blaess, and H. J. Buhk**. 1997. Mapping and
43 characterization of the origin of DNA replication of porcine circovirus. J. Virol **71**:2562-
44 2566.
45 74. **Mankertz J., H.-J. Buhk, G. Blaess, and A. Mankertz**. 1998. Transcription Analysis of
46 Porcine Circovirus (PCV). Virus Genes **16**:267-276.

75. **Meehan B. M., J. L. Creelan, M. S. McNulty, and D. Todd**. 1997. Sequence of porcine circovirus DNA: affinities with plant circoviruses. J. Gen. Virol **78 (Pt 1)**:221-227.

76. **Meehan B. M., F. McNeilly, D. Todd, S. Kennedy, V. A. Jewhurst, J. A. Ellis, L. E. Hassard, E. G. Clark, D. M. Haines, and G. M. Allan**. 1998. Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. J. Gen. Virol **79 (Pt 9)**:2171-2179.

77. **Meerts P., G. Misinzo, F. McNeilly, and H. J. Nauwynck**. 2005. Replication kinetics of different porcine circovirus 2 strains in PK-15 cells, fetal cardiomyocytes and macrophages. Arch. Virol **150**:427-441.

78. **Meerts P., G. Misinzo, D. Lefebvre, J. Nielsen, A. Bøtner, C. S. Kristensen, and H. J. Nauwynck**. 2006. Correlation between the presence of neutralizing antibodies against porcine circovirus 2 (PCV2) and protection against replication of the virus and development of PCV2-associated disease. BMC Vet. Res **2**:6.

79. **Meerts P., G. Misinzo, D. Lefebvre, J. Nielsen, A. Bøtner, C. S. Kristensen, and H. J. Nauwynck**. 2006. Correlation between the presence of neutralizing antibodies against porcine circovirus 2 (PCV2) and protection against replication of the virus and development of PCV2-associated disease. BMC Vet. Res **2**:6.

80. **Meng T., Q. Jia, S. Liu, A. K. Karuppannan, C.-C. Chang, and J. Kwang**. 2010. Characterization and epitope mapping of monoclonal antibodies recognizing N-terminus of Rep of porcine circovirus type 2. J. Virol. Methods **165**:222-229.

81. **Misinzo G., P. L. Delputte, D. J. Lefebvre, and H. J. Nauwynck**. 2009. Porcine circovirus 2 infection of epithelial cells is clathrin-, caveolae- and dynamin-independent, actin and Rho-GTPase-mediated, and enhanced by cholesterol depletion. Virus Res **139**:1-9.

82. **Misinzo G., P. Meerts, M. Bublot, J. Mast, H. M. Weingartl, and H. J. Nauwynck**. 2005. Binding and entry characteristics of porcine circovirus 2 in cells of the porcine monocytic line 3D4/31. J. Gen. Virol **86**:2057-2068.

83. **Misinzo G., P. L. Delputte, P. Meerts, D. J. Lefebvre, and H. J. Nauwynck**. 2006. Porcine circovirus 2 uses heparan sulfate and chondroitin sulfate B glycosaminoglycans as receptors for its attachment to host cells. J. Virol **80**:3487-3494.

84. **Misinzo G., P. L. Delputte, and H. J. Nauwynck**. 2008. Inhibition of endosome-lysosome system acidification enhances porcine circovirus 2 infection of porcine epithelial cells. J. Virol **82**:1128-1135.

85. **Morozov I., T. Sirinarumitr, S. D. Sorden, P. G. Halbur, M. K. Morgan, K. J. Yoon, and P. S. Paul**. 1998. Detection of a novel strain of porcine circovirus in pigs with postweaning multisystemic wasting syndrome. J. Clin. Microbiol **36**:2535-2541.

86. **Nakai K., and M. Kanehisa**. 1992. A knowledge base for predicting protein localization sites in eukaryotic cells. Genomics **14**:897-911.

87. **Nawagitgul P., I. Morozov, S. R. Bolin, P. A. Harms, S. D. Sorden, and P. S. Paul**. 2000. Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. J. Gen. Virol **81**:2281-2287.

88. **Nawagitgul P., P. A. Harms, I. Morozov, B. J. Thacker, S. D. Sorden, C. Lekcharoensuk, and P. S. Paul**. 2002. Modified indirect porcine circovirus (PCV) type 2-based and recombinant capsid protein (ORF2)-based enzyme-linked immunosorbent assays for detection of antibodies to PCV. Clin. Diagn. Lab. Immunol **9**:33-40.

1 89. **Olvera A., M. Cortey, and J. Segalés**. 2007. Molecular evolution of porcine circovirus
2 type 2 genomes: phylogeny and clonality. Virology **357**:175-185.
3 90. **Opriessnig T., E. L. Thacker, S. Yu, M. Fenaux, X.-J. Meng, and P. G. Halbur**. 2004.
4 Experimental reproduction of postweaning multisystemic wasting syndrome in pigs by
5 dual infection with Mycoplasma hyopneumoniae and porcine circovirus type 2. Vet.
6 Pathol **41**:624-640.
7 91. **Opriessnig T., S. Yu, and E. L. Thacker**. 2004. Derivation of porcine circovirus type 2-
8 negative pigs from positive breeding herds. Journal of Swine Health and Production
9 **12**:186-191.
10 92. **Opriessnig T., X.-J. Meng, and P. G. Halbur**. 2007. Porcine circovirus type 2 associated
11 disease: update on current terminology, clinical manifestations, pathogenesis, diagnosis,
12 and intervention strategies. J. Vet. Diagn. Invest **19**:591-615.
13 93. **Ostrowski M., J. A. Galeota, A. M. Jar, K. B. Platt, F. A. Osorio, and O. J. Lopez**.
14 2002. Identification of neutralizing and nonneutralizing epitopes in the porcine
15 reproductive and respiratory syndrome virus GP5 ectodomain. J. Virol **76**:4241-4250.
16 94. **Pal N., Y. W. Huang, D. M. Madson, C. Kuster, X. J. Meng, P. G. Halbur, and T.
17 Opriessnig**. 2008. Development and validation of a duplex real-time PCR assay for the
18 simultaneous detection and quantification of porcine circovirus type 2 and an internal
19 control on porcine semen samples. J. Virol. Methods **149**:217-225.
20 95. **Park J.-S., J. Kim, Y. Ha, K. Jung, C. Choi, J.-K. Lim, S.-H. Kim, and C. Chae**. 2005.
21 Birth abnormalities in pregnant sows infected intranasally with porcine circovirus 2. J.
22 Comp. Pathol **132**:139-144.
23 96. **Pensaert M. B., R. E. Sanchez, A. S. Ladekjaer-Mikkelsen, G. M. Allan, and H. J.
24 Nauwynck**. 2004. Viremia and effect of fetal infection with porcine viruses with special
25 reference to porcine circovirus 2 infection. Vet. Microbiol **98**:175-183.
26 97. **Racine S., A. Kheyar, C. A. Gagnon, B. Charbonneau, and S. Dea**. 2004. Eucaryotic
27 expression of the nucleocapsid protein gene of porcine circovirus type 2 and use of the
28 protein in an indirect immunofluorescence assay for serological diagnosis of postweaning
29 multisystemic wasting syndrome in pigs. Clin. Diagn. Lab. Immunol **11**:736-741.
30 98. **Ramamoorthy S., and X.-J. Meng**. 2009. Porcine circoviruses: a minuscule yet
31 mammoth paradox. Anim Health Res Rev **10**:1-20.
32 99. **Reed J. L., and H. Muench**. 1938. A simple method of estimating fifty percent
33 endpoints. The American Journal of Hygiene **27**:493-497.
34 100. **Rodríguez-Arrioja G. M., J. Segalés, M. Balasch, C. Rosell, J. Quintant, J. M. Folch,
35 J. Plana-Durán, A. Mankertz, and M. Domingo**. 2000. Serum antibodies to porcine
36 circovirus type 1 and type 2 in pigs with and without PMWS. Vet. Rec **146**:762-764.
37 101. **Rodríguez-Arrioja G. M., J. Segalés, M. Calsamiglia, A. R. Resendes, M. Balasch, J.
38 Plana-Duran, J. Casal, and M. Domingo**. 2002. Dynamics of porcine circovirus type 2
39 infection in a herd of pigs with postweaning multisystemic wasting syndrome. Am. J. Vet.
40 Res **63**:354-357.
41 102. **Rodríguez-Arrioja G. M., J. Segalés, M. Calsamiglia, A. R. Resendes, M. Balasch, J.
42 Plana-Duran, J. Casal, and M. Domingo**. 2002. Dynamics of porcine circovirus type 2
43 infection in a herd of pigs with postweaning multisystemic wasting syndrome. Am. J. Vet.
44 Res **63**:354-357.
45 103. **Rosario K., M. Marinov, D. Stainton, S. Kraberger, E. J. Wiltshire, D. A. Collings,
46 M. Walters, D. P. Martin, M. Breitbart, and A. Varsani**. 2011. Dragonfly cyclovirus, a

novel single-stranded DNA virus discovered in dragonflies (Odonata: Anisoptera). J Gen Virol.

104. **Rosell C., J. Segalés, J. A. Ramos-Vara, J. M. Folch, G. M. Rodríguez-Arrioja, C. O. Duran, M. Balasch, J. Plana-Durán, and M. Domingo**. 2000. Identification of porcine circovirus in tissues of pigs with porcine dermatitis and nephropathy syndrome. Vet. Rec **146**:40-43.
105. **Rosell C., J. Segalés, A. Rovira, and M. Domingo**. 2000. Porcine circovirosis in Spain. Vet. Rec **146**:591-592.
106. **Segalés J., A. Olvera, L. Grau-Roma, C. Charreyre, H. Nauwynck, L. Larsen, K. Dupont, K. McCullough, J. Ellis, S. Krakowka, A. Mankertz, M. Fredholm, C. Fossum, S. Timmusk, N. Stockhofe-Zurwieden, V. Beattie, D. Armstrong, B. Grassland, P. Baekbo, and G. Allan**. 2008. PCV-2 genotype definition and nomenclature. Vet. Rec **162**:867-868.
107. **Segalés J., A. Urniza, A. Alegre, T. Bru, E. Crisci, M. Nofrarías, S. López-Soria, M. Balasch, M. Sibila, Z. Xu, H.-J. Chu, L. Fraile, and J. Plana-Duran**. 2009. A genetically engineered chimeric vaccine against porcine circovirus type 2 (PCV2) improves clinical, pathological and virological outcomes in postweaning multisystemic wasting syndrome affected farms. Vaccine **27**:7313-7321.
108. **Segalés J., G. M. Allan, and M. Domingo**. 2005. Porcine circovirus diseases. Anim Health Res Rev **6**:119-142.
109. **Shang S.-B., Y.-L. Jin, X.-tao Jiang, J.-Y. Zhou, X. Zhang, G. Xing, J. L. He, and Y. Yan**. 2009. Fine mapping of antigenic epitopes on capsid proteins of porcine circovirus, and antigenic phenotype of porcine circovirus type 2. Mol. Immunol **46**:327-334.
110. **Shang S.-B., Y.-F. Li, J.-Q. Guo, Z.-T. Wang, Q.-X. Chen, H.-G. Shen, and J.-Y. Zhou**. 2008. Development and validation of a recombinant capsid protein-based ELISA for detection of antibody to porcine circovirus type 2. Res. Vet. Sci **84**:150-157.
111. **Shibata I., Y. Okuda, K. Kitajima, and T. Asai**. 2006. Shedding of porcine circovirus into colostrum of sows. J. Vet. Med. B Infect. Dis. Vet. Public Health **53**:278-280.
112. **Shibata I., Y. Okuda, S. Yazawa, M. Ono, T. Sasaki, M. Itagaki, N. Nakajima, Y. Okabe, and I. Hidejima**. 2003. PCR detection of Porcine circovirus type 2 DNA in whole blood, serum, oropharyngeal swab, nasal swab, and feces from experimentally infected pigs and field cases. J. Vet. Med. Sci **65**:405-408.
113. **Singh S. M., and A. K. Panda**. 2005. Solubilization and refolding of bacterial inclusion body proteins. J. Biosci. Bioeng **99**:303-310.
114. **Smith W. J., J. R. Thomson, and S. Done**. 1993. Dermatitis/nephropathy syndrome of pigs. Vet. Rec **132**:47.
115. **Sorden S. D.** 2000. Update on porcine circovirus and postweaning multisystemic wasting syndrom (PMWS). Swine Health Prod. **8**:133-138.
116. **Sorden S. D.** 2000. Update on porcine circovirus and postweaning multisystemic wasting syndrome (PMWS). Swine Health and Production **8**:133-136.
117. **Steiner E., C. Balmelli, H. Gerber, A. Summerfield, and K. McCullough**. 2009. Cellular adaptive immune response against porcine circovirus type 2 in subclinically infected pigs. BMC Vet. Res **5**:45.
118. **Steinfeldt T., T. Finsterbusch, and A. Mankertz**. 2001. Rep and Rep' protein of porcine circovirus type 1 bind to the origin of replication in vitro. Virology **291**:152-160.

1 119. **Stevenson G. W., M. Kiupel, S. K. Mittal, J. Choi, K. S. Latimer, and C. L. Kanitz**.
2 2001. Tissue distribution and genetic typing of porcine circoviruses in pigs with naturally
3 occurring congenital tremors. J. Vet. Diagn. Invest **13**:57-62.
4 120. **Tischer I., H. Gelderblom, W. Vettermann, and M. A. Koch**. 1982. A very small
5 porcine virus with circular single-stranded DNA. Nature **295**:64-66.
6 121. **Tischer I., W. Mields, D. Wolff, M. Vagt, and W. Griem**. 1986. Studies on
7 epidemiology and pathogenicity of porcine circovirus. Arch. Virol **91**:271-276.
8 122. **Tischer I., D. Peters, R. Rasch, and S. Pociuli**. 1987. Replication of porcine circovirus:
9 induction by glucosamine and cell cycle dependence. Arch. Virol **96**:39-57.
10 123. **Tischer I., R. Rasch, and G. Tochtermann**. 1974. Characterization of papovavirus-and
11 picornavirus-like particles in permanent pig kidney cell lines. Zentralbl Bakteriol Orig A
12 **226**:153-167.
13 124. **Trundova M., and V. Celer**. 2007. Expression of porcine circovirus 2 ORF2 gene
14 requires codon optimized E. coli cells. Virus Genes **34**:199-204.
15 125. **Truong C., D. Mahe, P. Blanchard, M. Le Dimna, F. Madec, A. Jestin, and E. Albina**.
16 2001. Identification of an immunorelevant ORF2 epitope from porcine circovirus type 2 as
17 a serological marker for experimental and natural infection. Arch. Virol **146**:1197-1211.
18 126. **Vega-Rocha S., I.-J. L. Byeon, B. Gronenborn, A. M. Gronenborn, and R. Campos-**
19 **Olivas**. 2007. Solution structure, divalent metal and DNA binding of the endonuclease
20 domain from the replication initiation protein from porcine circovirus 2. J. Mol. Biol
21 **367**:473-487.
22 127. **Vega-Rocha S., B. Gronenborn, A. M. Gronenborn, and R. Campos-Olivas**. 2007.
23 Solution structure of the endonuclease domain from the master replication initiator protein
24 of the nanovirus faba bean necrotic yellows virus and comparison with the corresponding
25 geminivirus and circovirus structures. Biochemistry **46**:6201-6212.
26 128. **Vincent I. E., C. P. Carrasco, B. Herrmann, B. M. Meehan, G. M. Allan, A.**
27 **Summerfield, and K. C. McCullough**. 2003. Dendritic cells harbor infectious porcine
28 circovirus type 2 in the absence of apparent cell modulation or replication of the virus. J.
29 Virol **77**:13288-13300.
30 129. **Vincent I. E., C. Balmelli, B. Meehan, G. Allan, A. Summerfield, and K. C.**
31 **McCullough**. 2007. Silencing of natural interferon producing cell activation by porcine
32 circovirus type 2 DNA. Immunology **120**:47-56.
33 130. **Vincent I. E., C. P. Carrasco, L. Guzylack-Piriou, B. Herrmann, F. McNeilly, G. M.**
34 **Allan, A. Summerfield, and K. C. McCullough**. 2005. Subset-dependent modulation of
35 dendritic cell activity by circovirus type 2. Immunology **115**:388-398.
36 131. **Waddilove A., and E. Marco**. 2002. Assessing serotherapeutic control of PMWS in the
37 field. Proc Intern Pig Vet Soc Conf, Ames, Iowa, **17**:34.
38 132. **Walker I. W., C. A. Konoby, V. A. Jewhurst, I. McNair, F. McNeilly, B. M. Meehan,**
39 **T. S. Cottrell, J. A. Ellis, and G. M. Allan**. 2000. Development and application of a
40 competitive enzyme-linked immunosorbent assay for the detection of serum antibodies to
41 porcine circovirus type 2. J. Vet. Diagn. Invest **12**:400-405.
42 133. **Wellenberg G. J., N. Stockhofe-Zurwieden, M. F. de Jong, W. J. A. Boersma, and A.**
43 **R. W. Elbers**. 2004. Excessive porcine circovirus type 2 antibody titres may trigger the
44 development of porcine dermatitis and nephropathy syndrome: a case-control study. Vet.
45 Microbiol **99**:203-214.

1 134. **Wikström F. H., B. M. Meehan, M. Berg, S. Timmusk, J. Elving, L. Fuxler, M.
2 Magnusson, G. M. Allan, F. McNeilly, and C. Fossum**. 2007. Structure-dependent
3 modulation of alpha interferon production by porcine circovirus 2
4 oligodeoxyribonucleotide and CpG DNAs in porcine peripheral blood mononuclear cells.
5 J. Virol **81**:4919-4927.
6 135. **Yu S., T. Opriessnig, P. Kitikoon, D. Nilubol, P. G. Halbur, and E. Thacker**. 2007.
7 Porcine circovirus type 2 (PCV2) distribution and replication in tissues and immune cells
8 in early infected pigs. Vet. Immunol. Immunopathol **115**:261-272.
9 136. **Zhou J.-Y., S.-B. Shang, H. Gong, Q.-X. Chen, J.-X. Wu, H.-G. Shen, T.-F. Chen, and
10 J.-Q. Guo**. 2005. In vitro expression, monoclonal antibody and bioactivity for capsid
11 protein of porcine circovirus type II without nuclear localization signal. J. Biotechnol
12 **118**:201-211.
13 137. 2010. ICTV Virus Taxonomy: 2009 Release. International Committee on Taxonomy of
14 Viruses.

15