Protective Immunity vs. Immunopathogenesis:
Recognition of the Structural form of the PCV2 Capsid Determines the Outcome

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1 Introduction

Pathogens employ multiple strategies to evade host defenses. One strategy involves deploying epitopes that are immunodominant, but not protective, also known as “decoy” epitopes. Infection with porcine circovirus type 2 (PCV2), the smallest known virus to autonomously replicate, contributes to a wide variety of syndromes, which are collectively termed porcine circovirus associated disease (PCVAD). One hallmark of PCVAD is dysregulation of host immunity, including the production of large quantities of non-neutralizing antibody. The limitations placed on a small genome, with a limited number of genes, means that PCV2 and other small viruses must employ unique strategies to subvert innate and acquired immune responses.

2 Porcine Circovirus Associated Disease

PCVAD forms a group of complex multi-factorial syndromes, which can include respiratory distress, diarrhea, reproductive failure, wasting, and dermatitis (reviewed in Segales, 2012). Common syndromes include porcine multi-systemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), poor growth performance, and acute pulmonary edema (APE). Even though these and other PCV2-associated syndromes can be mimicked by other types of infections, for the purpose of this review we describe those syndromes that are linked with PCV2. Under some circumstances, mortality can reach 50%. The common component of all syndromes is a large quantity of PCV2 and/or anti-PCV2 antibody (Meehan et al., 1998; Rossell et al., 2000; Wellenberg et al., 2004). Infection with PCV2 alone results in detectable viremia, but without overt clinical signs. The etiology of PCVAD, is linked to co-infection with viral or bacterial pathogens, such as porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), porcine parvovirus (PPV), Haemophilus parasuis, Streptococcus suis, Mycoplasma hypopneumoniae, Actinobacillus pleuropneumoniae, and others (reviewed by Opriessnig and Halbur, 2012). While the exact mechanistic basis for how co-infection contributes to PCVAD is unclear, experimental studies involving the co-infection of PCV2 with PRRSV, show increased PCV2 viremia and deposition of PCV2 antigen in lymph nodes in pigs infected with PCV2 and PRRSV (Allan et al., 2000; Rovira et al., 2002; Sinha et al., 2011; Trible et al., 2012a). However, PCV2 infection does not result in a corresponding increase in PRRSV replication. Co-infecting pathogens may function to alter the overall immune responsiveness of the host, thus preventing the induction of an effective anti-PCV2 response. Another possibility is that lymphoproliferation, in response to the co-infecting pathogen, may result in increased numbers of dividing lymphocytes, the primary targets of PCV2 replication (Darwich, et al., 2004).

2.1 Porcine Multi-systemic Wasting Syndrome (PMWS)

The most common PCVAD syndrome, PMWS, was first described in high health swine herds in Canada in the early 1990’s (Clark, 1997; Harding, 1997). The syndrome primarily affects pigs between 5-16 weeks of age with the greatest frequency of onset at 8-12 weeks. Mortality typically ranges between 10 and 25% (Harding & Clark, 1997; Horlen et al., 2007). Clinical signs can include lethargy, diarrhea, lymphadenopathy, discoloring of the skin, jaundice, and progressive weight loss (Harding and Clark, 1997). Gross histopathology shows enlargement of the submandibular, inguinal, and bronchial lymph nodes, as well as lungs that are non-collapsed and wet. At the microscopic level, lymph nodes show granuloma-
tious inflammation and the presence of intracytoplasmic inclusion bodies characterized by multinucleated giant cells. By the end stage of the disease, lymphoid cells in tissues are depleted and replaced by macrophages and multinucleated giant cells along with a large accumulation of PCV2 antigen (Harding and Clark, 1997).

2.2 Porcine Dermatitis and Nephropathy Syndrome (PDNS)

PDNS was first described in the United Kingdom in 1993 (Smith et al., 1993). The syndrome primarily affects pigs 12-14 weeks of age (Smith et al., 1993, Helie et al., 1995; Horlen et al., 2007). The predominant clinical sign is the presence of skin lesions, ranging in color from red to black, which primarily cover the hind legs (Smith et al., 1993; Helie et al., 1995; Horlen et al., 2007). Gross pathology is characterized by enlarged and hemorrhagic renal and inguinal lymph nodes with increased fluid in the pleural and peritoneal cavities. Kidneys appear wet and enlarged with pinpoint hemorrhages along the capsule (Smith et al., 1993; Helie et al., 1995; Horlen et al., 2007). Microscopically, PDNS is characterized by dermal/epidermal necrosis, fibrinous glomerulonephritis and systemic vasculitis. PDNS includes features characteristic of a type 3 hypersensitivity reaction, including the deposition of antigen-antibody complexes within capillary glomerular and vascular walls (Wellenberg et al., 2004).

Even though PDNS is characterized by large quantities of anti-PCV2 antibodies, the exact etiology of the syndrome remains unclear. PDNS was successfully reproduced after the dual challenge of gnotobiotic pigs with only porcine reproductive and respiratory syndrome virus (PRRSV) and group 1 toquevirus (TTV; Krakowka et al., 2008). However, cases of PDNS in the field are linked with PCV2 infection.

2.3 Poor Growth Performance

One of the first field studies to evaluate the efficacy of a baculovirus-expressed PCV2 capsid-based vaccine is described in Horlen et al. (2008). The herd had a previous history of PMWS and PDNS; however, at the time of vaccination, clinical PCVAD was no longer present. The study design involved 485 pigs, which were randomly assigned to vaccine or non-vaccine groups. Pigs were vaccinated at three weeks of age and boosted three weeks later. In addition to significant decreases in mortality and PCV2 viremia, vaccination resulted in a higher average daily weight gain. The average increase in weight at market was approximately 8.8 kg. The results demonstrated increased weight gain as a benefit in the vaccination of herds that did not possess overt clinical signs of PCVAD. Other vaccine studies have reported similar findings (Fachinger et al., 2008; Fort et al., 2008, 2009; Kixmöller et al., 2008; Martelli et al., 2011; Opriessnig et al., 2008, 2011).

2.4 Acute Pulmonary Edema (APE)

Typically, PCVAD syndromes, such as wasting, are slow and progressive. In 2009, a peracute syndrome, termed APE, was reported in PCV2-vaccinated herds (Cino-Ozuna et al., 2011). The principal clinical feature is the rapid onset of respiratory distress in apparently healthy pigs followed by death. Gross pathology shows diffusely wet and heavy lungs and a large volume of clear fluid in the thoracic cavity. Microscopically, APE is characterized by diffuse distension of intralobular septa, and diffuse interstitial infiltration of macrophages and lymphocytes. Blood vessel walls show fibrinoid necrosis with pulmonary edema in the surrounding areas. Similar to other PCVAD syndromes, APE is associated with a large
amount of PCV2 virus in blood, lymphoid depletion, and the deposition of viral antigen in lung and lymphoid tissues. The association between APE with PCV2 vaccination remains unclear.

3 Porcine Circovirus Type 2 (PCV2)

In the early 1970’s, a viral contaminant of a PK-15 cell line was first described (Tischer et al., 1974). Electron microscopy revealed virus particles with picornavirus-like morphology. However, based on the presence of a circular ssDNA genome, the virus was classified as a porcine circovirus (PCV). Serology identified pigs as the natural host of the virus, but infection with PCV could not be linked to clinical disease (Tischer et al., 1982; Tischer et al. 1986). In the early 1990’s, a new wasting syndrome, termed PMWS, was reported in high-health pigs in western Canada (Clark, 1997; Harding, 1997). Analysis of viral antigens and DNA from North American and European isolates revealed a new PCV, which was only 70% identical at the nucleotide level to the PK-15 contaminant (Meehan et al., 1998). The terminology, PCV1 and PCV2, were used to distinguish the PK-15 contaminant from the virus linked with PMWS. PCV2 isolates are further grouped into two main genotypes, termed PCV2a (GenBank accession #AF055392 as a prototype sequence) and PCV2b (GenBank accession #AF055394 as a prototype sequence; Mehan et al., 1998). A third genotype, termed PCV2c (GenBank accession #EU148503), was identified in archived pig tissues collected in Denmark during the 1980’s (Dupont et al., 2008). Initially, PCV2a and PCV2b were linked with disease outbreaks in North America and Europe, respectively (Segalés et al., 2008). However, outbreaks of PCVAD in Canada and the U.S. in 2005 were associated with the first appearance of PCV2b in the U.S. (Horlen et al., 2007). Since then, both genotypes circulate worldwide. Pigs can be co-infected with both genotypes and viruses composed of sequences from both PCV2a and PCV2b are present in the field (Hesse et al., 2008).

PCV possesses 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. 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% (Fenaux et al., 2004). The genome possesses at least three open reading frames (ORFs; Figure 1). The largest open reading frame, ORF1, codes for two proteins termed Rep and Rep’. Rep and Rep’ are translated from differentially spliced transcripts. The replicase proteins are essential for rolling circle replication (Mankertz & Hillenbrand, 2001). Oriented in the opposite direction, ORF2 is translated into a 233 or 234 amino acid capsid protein (CP; Nawagitungl et al., 2000). The PCV2 virus-like particle (VLP) contains 60 CP monomers, which forms an icosahedral capsid (Khayat et al., 2011). CP is essential for viral attachment and entry of the virion into cell. The basic amino acids on the N-terminus of CP are involved in shuttling the viral genome into the nucleus; the site of virus replication (Liu et al., 2001; Misinzo et al., 2006; Shuai et al., 2008). A third gene, ORF3, is in a different reading frame embedded within ORF1 and codes for a protein associated with apoptosis (Liu et al., 2005). The contribution of ORF3 to PCV2 pathogenesis remains controversial (Chaiyakul et al., 2010).
4 Humoral Immunity

4.1 PCV2 Vaccines

PCV2 vaccination is the most effective strategy for the control and prevention of PCVAD. Currently, four commercial vaccines, based on the expression of an ORF2 antigen from PCV2a, are available for use in the field. Circumvent PCV (Intervet/Merck) and Ingelvac CircoFLEX, (Boehringer Ingelheim) consist of CP expressed by baculovirus. A third vaccine, Fostera PCV (Pfizer Animal Health), is composed of a virus prepared from a PCV1 backbone that expresses ORF2 from PCV2. The fourth vaccine, Circovac (Merial), contains inactivated whole PCV2a as the antigen. The primary mechanism for vaccine protection is the production of PCV2 neutralizing antibodies (reviewed in Kekarainen et al., 2010; Trible et al., 2012c).

4.2 Epitope Mapping Studies

Studies to identify antibody epitopes within CP were originally performed by Lekcharoensuk et al. (2004) and Mahe et al. (2000). Mahe et al. (2000) identified linear epitopes by reacting sera from infected and immunized pigs and hyperimmunized rabbits with overlapping 12-mer oligopeptides covering all of CP. Antibody reactive regions were identified between amino acids 23-43, 71-85, 117-131, and 171-202 in PCV2b CP. Using a different approach, Lekcharoensuk et al. (2004) identified conformational epitopes by reacting seven anti-CP monoclonal antibodies (mAb’s) with cells transfected with infectious PCV chimeric DNA clones comprised of different combinations of PCV1 and PCV2a ORF2 sequences. The results showed immunoreactive regions between residues 47-85, 165-200, and 200-233. The results of both studies were combined to identify four immunoreactive regions, termed epitopes A-D (Trible et al., 2011), which are illustrated in Figure 2.
Figure 2: Alignment of PCV2 CP peptide sequences from representative PCV2 genotypes. The underlined regions within PCV2a and PCV2b represent immunoreactive regions reported by Lekcharoensuk et al. (2004) and Mahe et al. (2000), respectively. Results from both studies were combined to identify four antibody immunoreactive epitopes, termed epitopes A-D (grey boxes).

4.3 Immunodominant CP Epitopes Following Vaccination, Infection, or PCVAD

In order to characterize immunodominant epitopes within CP, sera from experimentally PCV2-infected, PCV2 vaccinated, or PCVAD pigs were reacted with CP polypeptides expressed in E. coli (Trible et al., 2011). As summarized in Table 1, sera from vaccinated pigs recognized only the largest polypeptide, CP(43-233). In contrast, PCVAD or experimentally infected pigs showed reactivity to the largest polypeptide, as well as reactivity against smaller fragments located in the C-terminal region. All reactive polypeptides contained the epitope C region.

To finer map the immunodominant epitope within the C region, sera from experimentally infected and PCVAD pigs were reacted with overlapping 15mer oligopeptides covering the epitope C region. As depicted in Figure 3, CP(169-180) was identified as the smallest immunoreactive oligopeptide. Further analysis incorporating alanine scanning mutagenesis identified amino acid residues 173-tyrosine, 174-phenylalanine, 175-glutamine, and 179-lysine as important for antibody recognition. Peptide sequence analysis of 462 PCV2 sequences from GenBank and other sources showed that this region is highly conserved among all PCV2 isolates.

As illustrated in Figure 4, the x-ray structure of CP shows that the CP(169-180) domain forms an external loop structure, which protrudes from the outer surface of the CP subunit (panel A and B). The key antibody binding residues, 173-Tyr, 174-Phe and 175-Glu, are located in the middle of a connecting loop domain and lie in a similar plane. This provides a structural basis for the accessibility of CP(169-180) to antibody, as well as its immunodominance. However, within the context of the virus-like particle, CP(169-180) is located near the interface of the icosahedral 3-fold axis with most of the key residues no longer exposed on the virion surface. Only 173-Tyr (blue residue in Figure 3C) is visible on the surface.
Table 1: Summary of antibody responses to PCV2 capsid polypeptides.

<table>
<thead>
<tr>
<th>Name</th>
<th>Epitope Regions</th>
<th>PC</th>
<th>Vx</th>
<th>PM</th>
<th>PD</th>
</tr>
</thead>
<tbody>
<tr>
<td>43-233</td>
<td>A B C D</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>43-135</td>
<td></td>
<td>+</td>
<td>-</td>
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<td>++</td>
</tr>
<tr>
<td>43-160</td>
<td></td>
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<td>+</td>
</tr>
<tr>
<td>91-160</td>
<td></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+++</td>
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<tr>
<td>43-180</td>
<td></td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
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<tr>
<td>160-233</td>
<td></td>
<td>+++</td>
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<td>+</td>
<td>+++</td>
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<td>135-233</td>
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<td>91-233</td>
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\( ^a \text{Key: PC = PCV2-infected; Vx = vaccinated; PM = PMWS; PD = PDNS.} \\
\( ^b \text{Relative binding activity. Key: (-) no measureable binding activity; (+) low binding activity; (++) intermediate binding activity; (+++) high binding activity; (++++) very high binding activity.} \\

Figure 3: Summary of PEPSCAN analysis of the epitope C region. As illustrated above, overlapping oligopeptides were reacted with sera from pigs experimentally infected with PCV2 and from pigs with PCVAD. The grey rectangles show oligopeptides with positive reactivity. The results from alanine scanning mutagenesis identified key residues (underlined) as important for antibody recognition.

Of the VLP, but is located at the bottom of a cleft formed by the junction of three CP monomers. Therefore, in the context of the VLP, CP(169-180) is not accessible to antibody.

To further characterize the properties of CP(169-180), immune responses and protection after virus challenge were studied in pigs immunized with a monomeric form of CP (Trible et al., 2012c). To prevent assembly into VLP, CP was maintained in a stable monomer form by expressing CP as a ubiquitin (Ub) fusion protein, Ub-CP. For the purpose of comparison, pigs were immunized with baculovirus-expressed CP (Bac-CP). Baculovirus and bacteria-expressed CP spontaneously assemble to form VLP (Khayat et al., 2011). As predicted, immunization of pigs with Bac-CP resulted in high levels of anti-PCV2 antibodies, including high levels of PCV2 neutralizing activity, and low amounts of antibodies against CP(169-180). After challenge with PCV2, no virus was detected in the serum. In contrast, immunization with the CP monomer, Ub-CP, also induced high levels of antibody against PCV2, including
**Figure 4**: Location of the CP(169-180) epitope within a single CP subunit and VLP. Depicted are the ribbon (A) and surface (B) maps of a single CP subunit. The blue and red residues form the immunodominant epitope, CP(169-180). The blue residues are important for antibody recognition. The VLP (C) shows the surface of the VLP with a single CP shown in green, red, and blue. The red and blue regions correspond to the same residues in (A). Coordinates for the PCV2 CP(41-233) subunit and VLP were accessed through the RCSB Protein Data Bank (PDB ID 3R0R: (Berman et al., 2000; Khayat et al., 2011) and loaded into the open source molecular visual program, Chimera (Pettersen et al., 2004).

significant amounts of antibody directed against CP(169-180). PCV2 neutralizing activity was not detected. Furthermore, after virus challenge, the level of viremia was no different from pigs infected with virus alone. High levels of anti-CP(169-180) without the control of virus replication reproduced the antibody response observed in pigs with PCVAD.

5 **The Role of PCV2 CP Epitope C as An Immunological Decoy**

A common feature of many pathogens, which establish a prolonged infection, is the ability to evade the host’s immune response by deploying immunodominant, non-protective epitopes, which are termed decoy epitopes. For viruses, the most common method for diverting immunity involves antigenic variation. A classic example is glycoprotein (gp) 120, one of the major structural proteins of
human and feline immunodeficiency viruses (Garrity et al., 1997; Hosie et al., 2011). Gp120 of HIV possesses five conserved (C1-C5) and five hypervariable (V1-V5) regions. V3 is immunodominant and possesses a neutralizing epitope. However, over the course of infection, peptide sequence hypervariability allows the continuous escape from antibody. Antigenic variation results in the continued recruitment, activation, and proliferation of new B-cells. The overall down-regulation of antibody production in response to continuous B cell activation results in fewer antibodies directed against conserved neutralizing epitopes. Another decoy strategy involves directing host immunity towards immunodominant, but non-essential proteins. For example, during murine gammaherpesvirus-68 (MHV-68) infection establishes a persistent infection despite a robust antibody response. The primary target of the humoral immune response is gp150, a protein dispensable for virus replication (Gillet et al., 2007).

PCV2 possesses one of the smallest known viral genomes, which limits the number and size of viral genes. As a result, all peptide sequence domains in CP are likely required to maintain the structure and function of CP necessary for virus replication and virion integrity. Conservation of structure and function places constraints on the extent of peptide sequence hypervariability. We propose a unique decoy mechanism for PCV2, which is based on the recognition of the different structural forms of CP. As illustrated in the model, presented in Figure 5, the outcome following infec-

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**Figure 5:** Structural form of PCV2 immunogen recognized by the host and relationship to outcome.
tion can follow two pathways. Under normal circumstances antibodies are directed against the whole virion, which results in the production of neutralizing antibody. The immunodominant epitope, CP(169-180) is buried and inaccessible to recognition by antibody. The outcome is the control of virus replication and the generation of protective immunity. In contrast, the recognition of free CP and CP fragments, likely produced by infected cells, results in the production of antibodies against the immunodominant epitope, CP(169-180). Cofactors, such as co-infecting pathogens increase PCV2 replication and the production of CP monomers and fragments by infected cells. The genetics of the host and other co-factors may further skew immunity towards the recognition of CP(169-180). Recognition of the CP(169-180) results in the production of non-neutralizing antibodies, thus allowing for continue PCV2 replication, release of more monomer CP, and the progression towards disease. Overall, the model explains why the amount of circulating antibody fails to correlate with the outcome following PCV2 infection and provides a model for understanding the immunopathogenesis caused by viruses with small genomes.

References


