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Review

Genetic variation of porcine circovirus type 2 (PCV2) and its relevance to vaccination, pathogenesis and diagnosis

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ABSTRACT

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

Contents

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1. Introduction

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

PCV2 is placed in the family *Circoviridae*, which encompasses a group of small single-stranded DNA viruses that infect avian and swine species. Within the genus circovirus, PCV2 is closely related to porcine circovirus type 1 (PCV1). The 1.7 kb ambisense genome of porcine circoviruses codes for at least two open reading frames (ORFs), which are essential for virus replication. The largest, ORF1, codes for the replicase proteins, Rep and Rep′ (Mankertz and Hillenbrand, 2001). ORF1 is oriented in the sense direction relative to the origin in the PCV2 genome. Rep is translated from the entire ORF1 transcript, whereas, Rep′ is derived by alternative splicing of the ORF1 transcript. The C-terminal 68 amino acids (aa) of Rep′ are derived from a different reading frame. Oriented in the antisense direction, ORF2 codes for the 233 or 234 aa virus capsid protein (CP). CP is involved in the formation of the homopolymer capsid and is likely involved in translocating the viral genome into the nucleus during virus replication (Liu et al., 2001; Nawagitgul et al., 2000). Recently, the crystal structure of a monomeric CP subunit and its orientation within a PCV2-like particle were reported (Khayat et al., 2011). In this model, 60 CP subunits form an icosahedron with $T = 1$ symmetry. A computer reconstruction of the PCV2 CP monomer and its incorporation into the capsid are presented in Fig. 1.

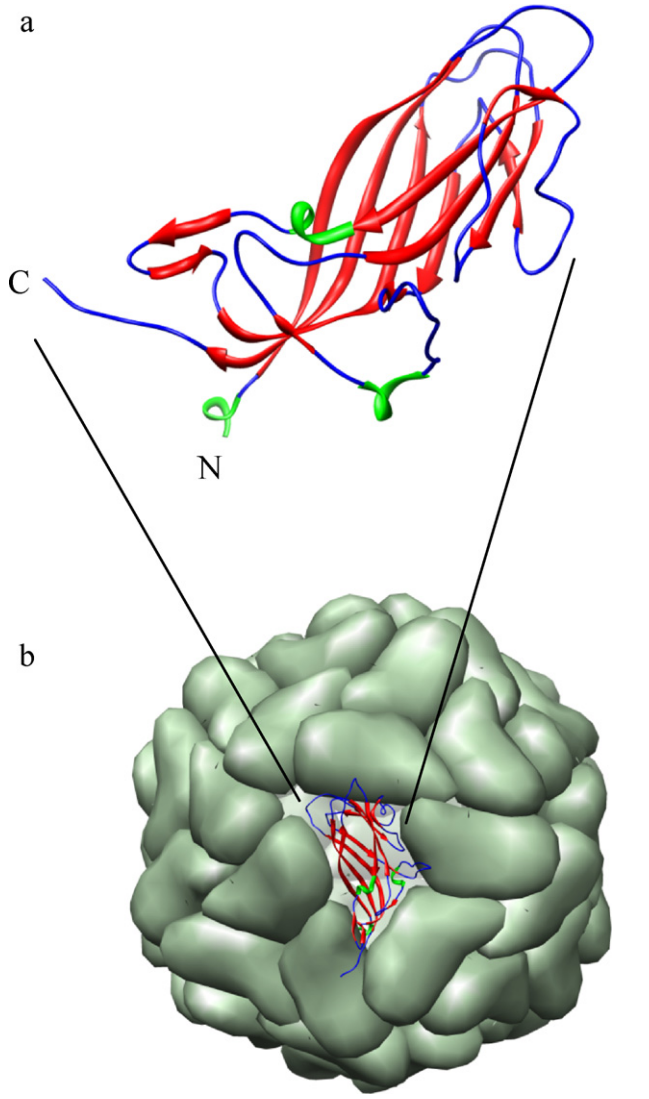

Fig. 1. PCV2 CP subunit model structure and assembly into a viral capsid. The ribbon model of the CP subunit (a) with helices, loops and sheets shown in green, blue and red, respectively. Panel b shows a CP subunit placed in the context of the viral capsid. The remaining 59 CP subunits are depicted in gray. Both models are based on the data of Khayat et al. (2011) and reproduced using the UCSF Chimera computer program (PDB ID 3R0R; Pettersen et al., 2004).

2. Evolution and classification of PCV2 isolates

2.1. Origin of circoviruses

The plant virus families, *Nanoviridae* and *Geminiviridae*, are considered the closest relatives to the *Circoviridae*. Overall, these families share a common stem loop structure, which contains the origin (Ori) of replication within the circular genome. Analysis of circovirus and nanovirus Rep peptide sequences led to a proposed mechanism for the evolutionary origin of circoviruses (Gibbs and Weiller, 1999). In this study, similarities between nanoviruses and circoviruses were found in the N-terminal region of Rep. However, the C-terminal region of PCV Rep was more closely related to an RNA binding protein, 2C, of a vertebrate calicivirus, an RNA virus. The proposed site of recombination was located between positions 129 and 178 of PCV1 Rep. The presence of the calicivirus 2C sequence suggests that PCV arose following a recombination event between a plant nanovirus and vertebrate calicivirus. Since nanovirus replication does not rely on an RNA step and caliciviruses possess an RNA genome, the incorporation of the 2C sequence was likely mediated by a transcriptase via a retrovirus or retrotransposon intermediate. The exact timing of this event is unclear. One hypothesis is that circoviruses co-evolved in their respective avian and mammalian hosts, which would mean that circoviruses first appeared prior to the divergence of birds and mammals, approximately 300 million years ago. However, an analysis performed by Firth et al. (2009) suggests that circoviruses have been present for only the last 500 years.

In terms of the continued evolution of PCV2, the analysis of 160 full length PCV2 genomes revealed a mutation rate of 1.2×10^{-3} substitutions/site/year (s/s/y; Firth et al., 2009). Similar results were found in a recent study analyzing PCV2 sequences from Cuba (3.1×10^{-3}–6.6×10^{-3} s/s/y; Pérez et al., 2011). Overall, these data reveal that PCV2 possesses the highest mutation rate reported for any DNA virus, falling into the range of genetic change reported for most RNA viruses (Duffy et al., 2008).

The nature of diversity was evaluated through sequence analysis of six members of the circovirus family, including beak and feather

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

2.2. Emergence of PCV2

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

Retrospective analysis of archived tissues from Northern Germany identified PCV2-specific DNA sequences in tissues that were obtained as far back as 1962 (Jacobsen et al., 2009). The analysis included samples from pigs that exhibited PMWS-like clinical signs. Using in situ hybridization and PCR, the prevalence of PCV2 in tissue samples between 1962 and 1984 was estimated to be 2.5%. Beginning in 1985, the prevalence of PCV2 made a dramatic jump to over 30%. This increase correlated with the appearance of PCV2-associated tissue lesions. Analysis of ORF2 sequences from the 1985 samples showed similarities to the PCV2a genotypic group. Attempts to amplify PCV2 DNA from earlier samples produced only small PCR fragments from ORF1, which were not sufficient for making accurate comparisons with contemporary viruses. A study in the UK performed on archival tissues dating back to the 1980s produced similar results, including the recovery of ORF2 sequences closely related to PCV2a (Grierson et al., 2004).

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

Table 1
Historical nomenclature for PCV2 genotypes.

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

2.3. Classification and phylogenetic relationships of PCV2 genotypes

Early sequence analyses revealed PCV2 isolates could be clustered into distinct subgroups or genotypes. Since the International Committee on Taxonomy of Viruses (ICTV) does not describe the classification of viruses below the species level, a variety of designations were proposed as a means to place PCV2 into distinct genotypic subgroupings, which are now known as PCV2a and 2b. A summary of the historical designations for PCV2a and 2b genotypes is presented in Table 1. Segalés et al. (2008) proposed a unifying system of nomenclature, designating each genotype within PCV2 by a lower case letter; i.e., PCV2a, 2b and 2c. GenBank accession numbers for representative isolates of current genotypes are listed in Table 2. The placement of an isolate into a genotypic group is based on performing pairwise sequence comparisons (PASC) to determine the degree of genetic variation (p), which is calculated by determining the number of base differences divided by the total number of positions between genomes. Currently, there are two classification schemes for distinguishing PCV2 genotypes. Based on complete PCV2 genome analysis, a cutoff value of $p = 0.02$ is used to distinguish genotypic groups (Grau-Roma et al., 2008). Based on the large degree of genetic variation reported for ORF2 (Fenaux et al., 2000; Hamel et al., 2000; Larochelle et al., 2002; Mankertz et al., 2000), the cutoff value increases to $p = 0.035$ (Segalés et al., 2008). The common standard is to classify different genotypes based on ORF2 only. A comparison using the complete PCV2 genome or the nucleotide sequence of ORF2 for phylogenetic mapping revealed no significant differences between classification schemes (Olvera et al., 2007). A phylogenetic tree incorporating whole genome sequences from the three genotypes is presented in Fig. 2. The tree shows the clustering of the different PCV2 genotypes.

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

A classification scheme for further differentiation of isolates in groups below the genotype level, or clades, has been described (Olvera et al., 2007). Using this convention, PCV2a was subgrouped into 5 clades, termed PCV group II A–E. PCV2b was subgrouped into 3 clades, termed PCV group I A, B and C. In this classification system, the distances between group II (2a) clades was 0.0158 and 0.0234 for group I (2b) clades. However, the relevance of a clade designation is unclear.

The current classification scheme for grouping viruses is complicated by genetic recombination (Cai et al., 2011; Hesse et al.,

Table 2
Prototypic members of current PCV2 genotypes.

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

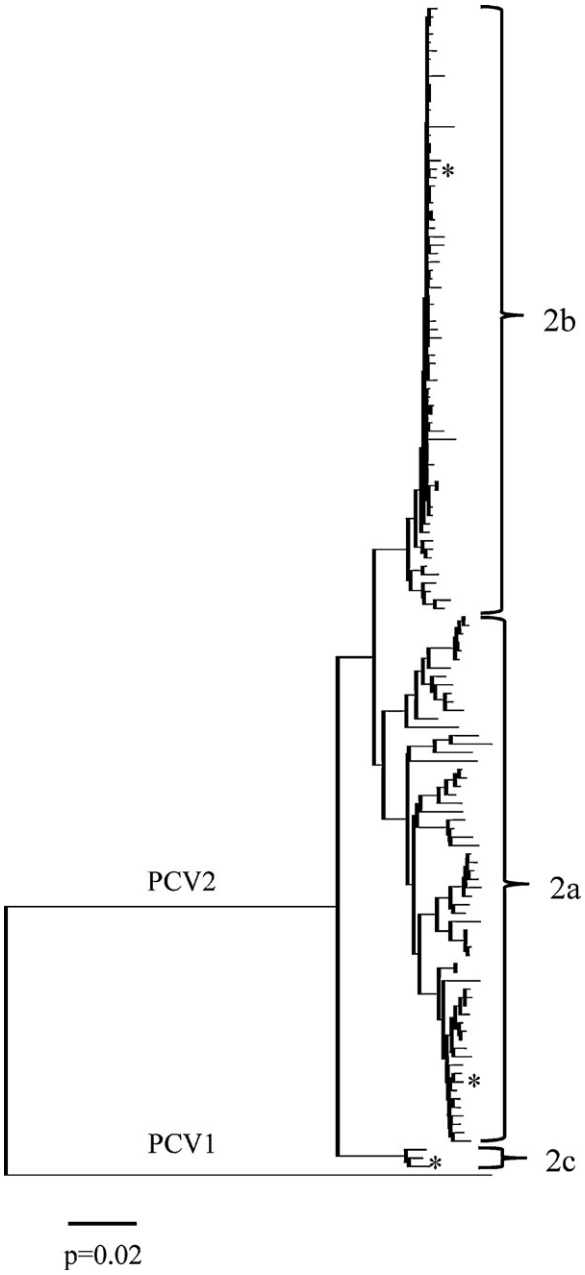

Fig. 2. Phylogenetic tree of 139 genomic PCV2 nucleotide sequences from GenBank. The phylogenetic analysis was performed using MEGA5 software (Tamura et al., 2011). The analysis includes 139 PCV2 sequences and a single PCV1 sequence as an outgroup. The tree was constructed by the neighbor joining method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

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

3. Molecular differences and PCV2 pathogenesis

The genome sizes for PCV1, PCV2a and PCV2b are 1759, 1768, and 1767 nucleiotides, respectively. (Tischer et al., 1986; Meehan et al., 1998). At the nucleotide level, PCV1 and PCV2 share approximately 70% sequence identity whereas, PCV2a and PCV2b genotypes share an identity of approximately 95%. The principal difference between the PCV2 genotypes occurs in ORF2, where both the nucleotide and peptide sequence identities are approximately 90%. It has been proposed that peptide sequence differences account for a difference in pathogenesis, which resulted in the less pathogenic PCV2a being replaced by PCV2b. Sequence comparisons have revealed a pair of signature motifs in ORF2 that distinguish PCV2a from PCV2b isolates (Cheung et al., 2007). PCV2b has the sequence **TCA**/AAC/**CCC/CG** at position 1486–1472 of the viral genome, whereas, PCV2a has the sequence **ACC**/AAC/**AAA/AT** at position 1487–1473. The nucleotide sequences translate to the peptide sequences, 86-**SNPRSV** for PCV2b and 86-**TNKISI** for PCV2a. While the particular motifs have been useful for rapid diagnostic approaches to distinguish the two genotypes, the capacity of the signature motif to function as a domain associated with virulence has not been demonstrated.

3.1. Pathogenic differences between porcine circoviruses

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

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

To date, experimental infection models analyzing differences in virulence have reported mixed results. In one experimental study involving inoculation of gnotobiotic pigs with infectious DNA clones derived from PCV2a or PCV2b, differences in symptom onset and overall mortality were reported (Lager et al., 2007). Combined morbidity and mortality was 25% and 100% for PCV2a-infected and PCV2b-infected groups, respectively. Mortality for the PCV2b group occurred between 22 and 27 days after infection, whereas, mortality in the PCV2a group (a single pig) occurred on day 35. However, both genotypes produced similar lesions, lymphocyte depletion, and similar amounts of PCV2 antigen in affected tissues. In an experimental challenge study evaluating PRRSV with PCV2a or PCV2b, no differences in the level of PCV2-specific antibody, PCV2 virus load in serum, or PCV2 shedding were detected between groups (Sinha et al., 2011). Following experimental challenge with different PCV2a and 2b isolates, pathogenicity was reported to be a function of the individual properties of an isolate and not related to genotype (Opriessnig et al., 2008b). Further evidence of

isolate-specific differences were reported following the co-mingling of PCV2 naïve pigs with PCV2b-infected pigs from PMWS-affected and non-affected herds (Dupont et al., 2009). The results showed that virus spread and the onset of disease were associated with PCV2b strains from PMWS-affected herds and not PCV2b strains from non-affected herds. Overall, the results of experimental studies provide inconclusive support for the hypothesis that PCV2a and PCV2b differ in pathogenicity. However, this conclusion does not adequately explain outbreaks of severe PCVAD that coincided with the appearance of PCV2b. A more intriguing possibility has been reported following experimental infection of pathogen-free pigs with combinations of PCV2a/2b, 2b/2a, 2a/2a, or 2b/2b (Harding et al., 2010). In this experiment, the first virus was administered seven days prior to the second virus. The combination 2a and 2b, regardless of order, reproduced PCVAD. The results provide evidence for the interaction between PCV2a and PCV2b in the pathogenesis of PCVAD. In addition, they provide an explanation for the enhanced pathogenesis of 2b observed during the 2005 North American outbreak. Further evidence for this possibility was provided by a study involving in situ hybridization on tissues from pigs with disease or with subclinical infection (Khaiseb et al., 2011). Tissues from diseased pigs showed cells co-infected with both genotypes; whereas, the subclinical pigs were infected with either PCV2a or 2b. A mechanism that involves cooperative replication between PCV2a and PCV2b genomes in the development and progression of disease was subsequently proposed.

3.2. Contributions of ORF1 and ORF2 to pathogenesis

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

The first studies describing a molecular mechanism for PCV2 pathogenesis were based on sequence analysis of an attenuated virus. In vitro passage of a PCV2a virus in PK-15 cells 120 times (P120) resulted in a 1 log increase in virus replication in culture (Fenaux et al., 2004). Infection of pigs with P120 showed a decrease in viremia and lower scores for gross pathological and histopathological lesions compared to the parental virus. Sequence analysis showed only two nucleotide changes, both in ORF2. The mutations resulted in a proline to alanine substitution at position 110 and an arginine to serine change at 191. Neither mutation maps to the proposed heparin sulfate receptor binding domain, located between residues 98 and 103 (Misinzo et al., 2006), or to the signature motif. It was subsequently suggested the two mutations resulted in a conformational change in the overall structure of CP. The possible participation of ORF1 in pathogenesis was illustrated by experiments using a chimeric virus containing ORF1 from PCV1 and ORF2 from PCV2a (Fenaux et al., 2003). Even though the chimera was attenuated relative to the wild-type PCV2, a similar attenuation was observed when the chimera contained ORF1 from PCV2 and ORF2 from PCV1.

3.3. Antibody epitopes within CP

The results for PEPSCAN analysis incorporating overlapping oligopeptides from a PCV2b isolate and reacted with PCV1 and PCV2-specific sera showed that CP residues 65–87, 113–139, and 193–207 were important for recognition (Mahé et al., 2000). An epitope, 169–183, was recognized by both antisera. In a different study, conformational epitopes were analyzed by reacting chimeric ORF2 PCV1–2a infected cells with CP-specific monoclonal antibodies (Lekcharoensuk et al., 2004). The results identified CP residues 47–85, 165–200 and 200–233 as immunoreactive regions. Overall these results identify four antibody recognition domains, labeled epitopes A–D (see Fig. 3).

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

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

3.4. Antigenic differences between PCV2 genotypes

The first report analyzing antibody binding differences between PCV2 genotypes involved reacting 16 monoclonal antibodies (mAbs) derived from infection with a PCV2a isolate, Stoon 1010. Antibodies were reacted with cells infected with four different PCV2a isolates and three different PCV2b isolates (Lefebvre et al., 2008). The results showed that 11 of the 16 mAbs reacted with all isolates, whereas, 4 mAbs were specific for only the PCV2a isolates. The 11 mAbs that reacted with both genotypes were able to neutralize 4 of the 7 viruses, 2 from PCV2a and 2 from PCV2b. The results demonstrate antigenic differences between PCV2a and 2b genotypes, as well as antigenic differences within genotypes.

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

4. Role of genetic variation in diagnostics

4.1. Differential diagnostics for PCV1 and PCV2

The standard diagnostic approaches for PCV and PCVAD have been extensively reviewed (Gillespie et al., 2009; Opriessnig et al., 2007). The incorporation of PCV1 and PCV2-specific primers can be used in polymerase chain reaction (PCR) and in situ hybridization (ISH) assays to distinguish PCV1 from PCV2 (Larochelle et al., 1999; Ouardani et al., 1999; Kim and Chae, 2002). Another method involved PCR amplification followed by restriction fragment length polymorphism (RFLP) analysis (Fenaux et al., 2000). Also described are PCV1 and PCV2-specific mAbs, which can be incorporated into antibody-based assays, such as indirect fluorescent antibody (IFA), immunohistochemistry (IHC) and antigen capture enzyme-linked immunosorbant assay (ELISA; Allan and Ellis, 2000). Finally, there are methods for the detection of PCV1 or PCV2 specific antibodies, including IFA and ELISA (Allan and Ellis, 2000; Blanchard et al., 2003).

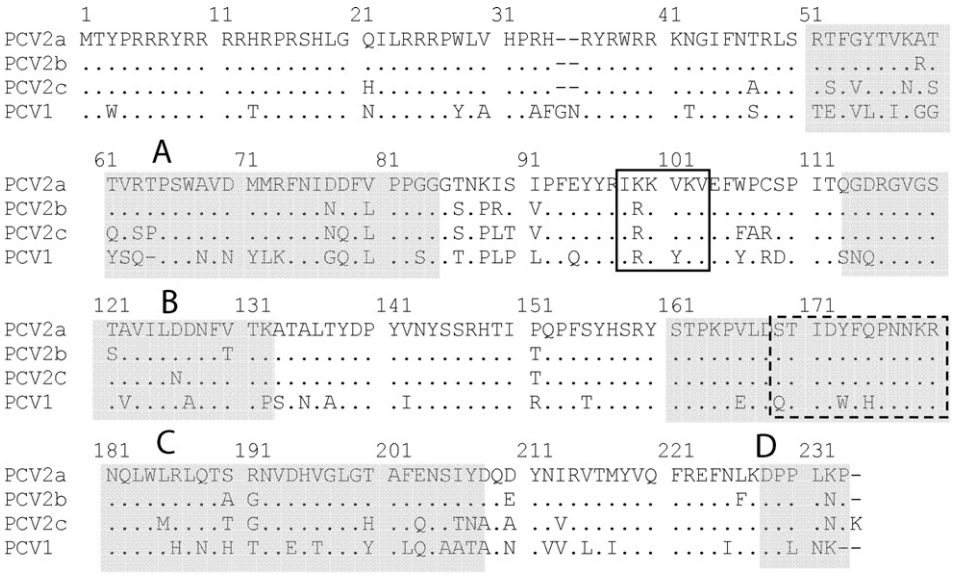

Fig. 3. Alignment of PCV2 CP peptide sequences from representative PCV2 genotypes. Reference peptide sequences are from the GenBank accession numbers in Table 2, which are identified by asterisks in Fig. 2. The gray areas correspond to antibody recognition domains described in Trible et al. (2011). The solid line box shows the putative heparin sulfate binding receptor domain (Misinzo et al., 2006). The dashed line shows the location of an immunodominant decoy epitope within the epitope C domain (Trible et al., 2011).

4.2. Differentiating PCV2a and PCV2b

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

5. Vaccination and immunity

5.1. Efficacy of PCV2 vaccines

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

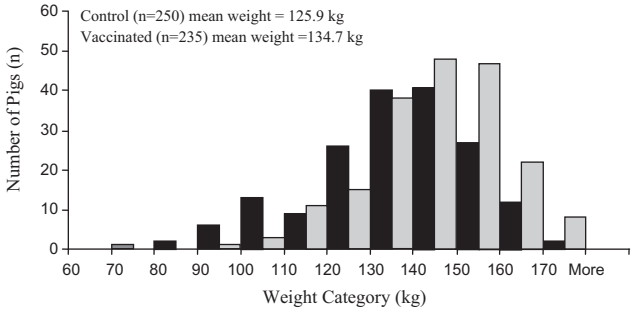

Fig. 4. Weight distribution of vaccinated and control groups in a field vaccination trial. At the time of vaccination, vaccine and control group average weights were 6.4 kg and 6.5 kg, respectively. The figure shows weight distribution for vaccinates (dark gray bars) and controls (light gray bars) collected one day prior to sending the pigs to market.

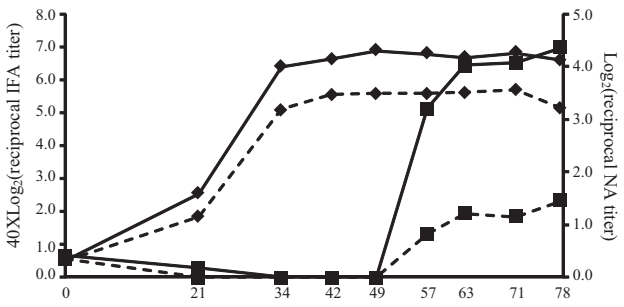

Fig. 5. PCV2 immunofluorescent antibody (IFA) and neutralizing antibody (NA). IFA and NA were measured similar to the methods described in Trible et al. (2011). The left axis represents values for IFA while the right axis shows the values for NA. Treatment group key: PCV2 challenged (closed squares), PCV2 vaccinated/PCV2 challenged (closed diamonds). Antibody assay key: IFA (solid line), NA (dashed line).

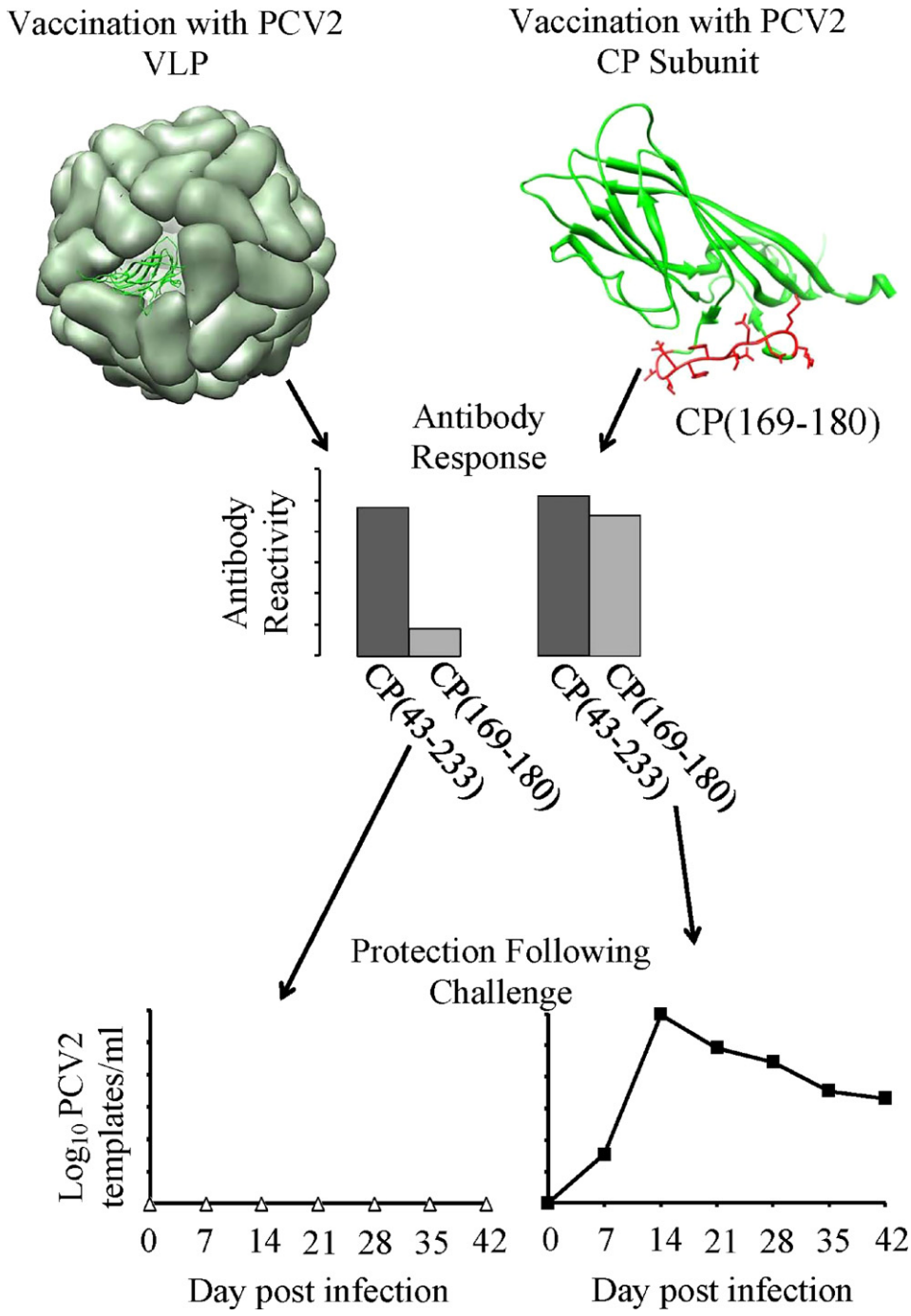

Fig. 6. Immunity and protection following vaccination with the PCV2 VLP or CP monomer. Vaccination with the VLP elicited antibodies that primarily recognize CP(43-233) and provided sterilizing immunity according to serum viremia. Vaccination with the CP monomer elicited antibodies that recognize both CP(43-233) and CP(169-180) but were unable to protect against PCV2 challenge.

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

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

5.2. Host immunity following vaccination versus infection

A key feature of PCVAD is the capacity for PCV2 to modulate the host immune response. For example, PMWS is characterized by an almost complete loss of lymphocytes (Chae, 2004). At the

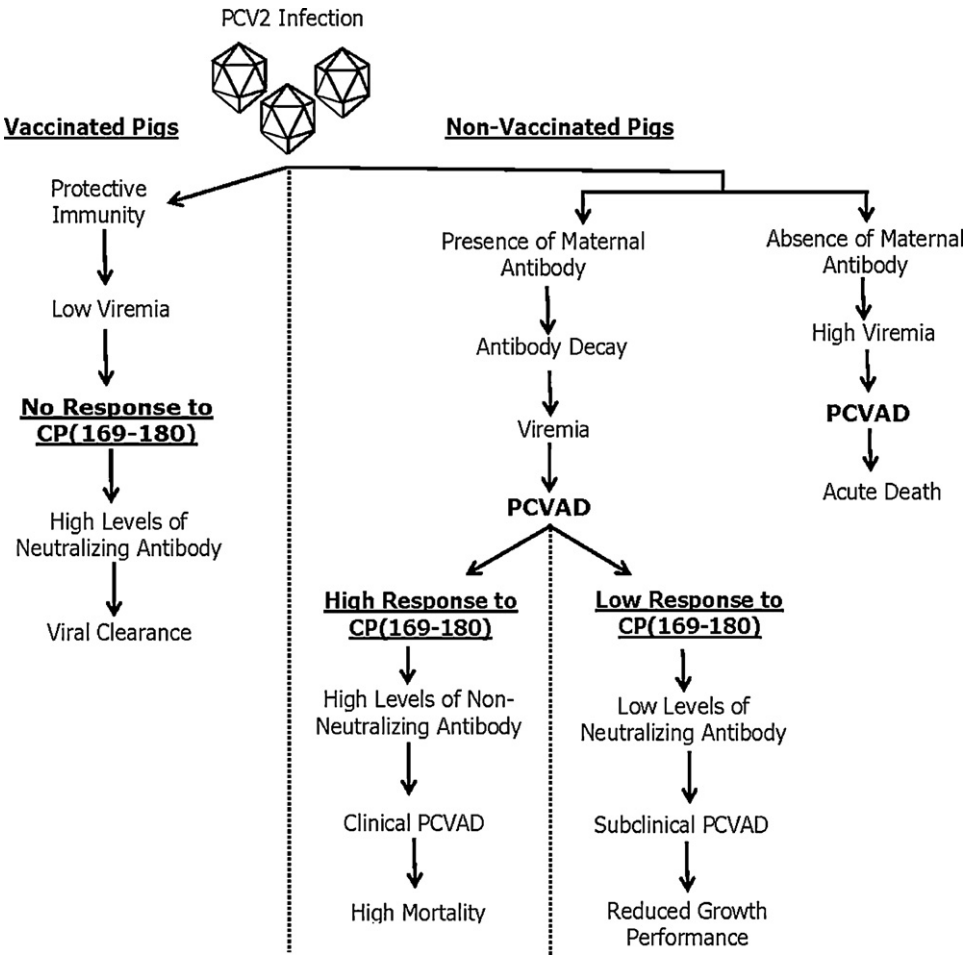

Fig. 7. Immunity and disease following natural infection in vaccinated and non-vaccinated pigs. Depicted are the different outcomes of PCV2 infection in vaccinated (left of the largest dashed line) or non-vaccinated pigs (right of the largest dashed line). In this model, vaccination prior to infection results in protection against PCV2 infection, which is characterized by high levels of neutralizing antibodies. In non-vaccinated pigs, the presence or absence of maternal antibody impacts disease progression. The antibody response to the CP(169–180) decoy epitope predicts the outcome of PCV2 infection. High and low responders progress to clinical and subclinical forms of PCVAD.

other extreme, pigs with PDNS possess a hyperimmune response, including the production of large quantities of PCV2-specific antibodies which likely contribute to immune complex formation and disease progression (Wellenberg et al., 2004).

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

Further evidence for this was demonstrated in an experimental challenge study (manuscript in preparation). Pigs were vaccinated with a monomeric form of *Escherichia coli* expressed CP(43–233) or with baculovirus expressed CP. Vaccination with baculovirus-expressed protein induced high levels of anti-CP(43–233) antibodies and low levels of anti-CP(169–180) antibodies. After challenge with PCV2, no virus was detected in serum of pigs vaccinated with the baculovirus expressed VLP. Immunization with the CP(43–233) monomer induced high levels of antibody against CP(43–233) as well as a highly elevated response to CP(160–180). Viremia of pigs immunized with the CP monomer was similar to that of non-vaccinated PCV2 challenged pigs (data not shown). As summarized in Fig. 6, we propose that protective antibodies are generated from epitopes formed by the PCV2 VLP and non-protective antibodies are produced by exposure to the CP monomer. As described in the proposed model in Fig. 7, the antibody response to the decoy epitope plays a key role in the progression towards clinical PCVAD. Since the decoy epitope is highly conserved among all PCV2 isolates, this model predicts that there should be no distinction in the disease syndromes caused by PCV2a versus PCV2b.

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