

# Antigenic Determinants of Possible Vaccine Escape by Porcine Circovirus Subtype 2b Viruses



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**ABSTRACT:** Currently available commercial vaccines against porcine circovirus strain 2 (PCV2) solely target the PCV2a genotype. While PCV2 vaccines are highly effective in preventing clinical signs, PCV2b has dominated over the PCV2a genotype in prevalence, corresponding with the introduction of PCV2a vaccines. A recently emerged PCV2b recombinant with an additional amino acid in the capsid protein, designated the mutant PCV2b (mPCV2b), is cause for concern due to its increased virulence and rapid spread. The accumulation of recent evidence for the increased genetic diversity in PCV2 suggests that current vaccines against PCV2a may be inducing selection pressure and driving viral evolution. In this study, the hypothesis that differences in key immune epitopes between the PCV2a vaccine strains, a classical PCV2b strain called PCV2b 41513 obtained from a vaccine-failure case, and mPCV2b strains could promote vaccine escape was tested using immuno-informatic tools. In the major viral proteins, 9 of the 18 predicted swine leukocyte antigens (SLA) class-I epitopes, 8 of the 22 predicted SLA class-II epitopes, and 7 of the 25 predicted B cell epitopes varied between the vaccine and field strains. A majority of the substitutions in both the T- and B-cell epitopes were located in the capsid protein. Some B- and T-cell epitopes that were identified as immunogenic in the vaccine strain were not identified as epitopes in the field strains, indicating a subtle shift in the antigenic profile of the field strains. Several nonconserved epitopes had both predicted B- and T-cell functions. Therefore, substitutions in the dual epitopes could affect both arms of the immune response simultaneously, causing immune escape. Our findings support further rational design of PCV2 vaccines to increase the current threshold of protection.

**KEYWORDS:** porcine circovirus strain 2 (PCV2), post-weaning multisystemic wasting syndrome (PMWS), epitope

**SUPPLEMENT:** Current Developments in Veterinary Bioinformatics

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## Introduction

Porcine circovirus (PCV) is a 1.7 kb, nonenveloped virus belonging to the *Circoviridae* family with a circular, single-stranded DNA genome. Porcine circoviruses consist of two major types, PCV1 and PCV2. PCV1 is considered to be nonpathogenic.<sup>1</sup> However, PCV2 is a substantial problem for the global swine industry as the cause of post-weaning multisystemic wasting syndrome (PMWS),<sup>2</sup> which manifests as severe wasting in weaning piglets. With time, several other disease manifestations, which include cutaneous, reproductive, and respiratory signs, have emerged and are now collectively known as porcine circovirus-associated disease (PCVAD).<sup>3–5</sup>

The circular genome of PCV2 contains three major open reading frames: ORF1, ORF2, and ORF3. ORF1 encodes the replicase proteins that are necessary for the replication of the virus. The sequence for the binding sites of Rep and Rep' are located within the origin of replication.<sup>6</sup> ORF2 encodes the capsid protein that is responsible

for viral structure and protective immunity. Thus, ORF2 is often used as a phylogenetic and epidemiological marker for PCV2.<sup>7</sup> ORF3, while not essential for replication, has been found to have a role in apoptotic activity and may potentially regulate virulence.<sup>8,9</sup> Recently, ORF 4 was discovered within an overlapping region of the ORF3. Experimental analysis has proposed that ORF4 plays a role in suppressing caspase activity as well as regulating the production of CD4+ and CD8+ T cells.<sup>10</sup>

There are two major subtypes of PCV2 that are commonly prevalent in swine: PCV2a and PCV2b.<sup>11,12</sup> From the late 1990s to about 2006, PCV2a was predominant in the United States, until commercial vaccines against PCV2 were introduced in 2006. All of the current vaccines contain the PCV2a capsid protein as the primary immunogen. Corresponding with the introduction of the PCV2a vaccines, there was a global shift in the prevalence of genotypes from PCV2a to PCV2b, associated with severe clinical manifestations in vaccinated herds.<sup>13</sup> PCV2b is now the predominant



subtype all over the world.<sup>14–16</sup> It is well recognized that coinfections with other pathogens such as swine influenza virus and the porcine reproductive and respiratory syndrome virus exacerbate PCVAD.<sup>17</sup> Additionally, over 90% of farmed swine are coinfecting with both PCV2a and b subtypes.<sup>18,19</sup> Coinfection can promote homologous recombination between PCV2a and PCV2b strains.<sup>20</sup> Mutation also plays a role in viral evolution. Mutated forms of the viral antigens, including extension of the capsid by one or two amino acids, have been described.<sup>7</sup> PCV2 has evolved rapidly since its discovery. Viral variants that are composed of recombined genomes containing new mutations have increased the probability of altered immunogenicity.<sup>21–25</sup> The recent emergence of a virulent recombinant form of PCV2b with an additional amino acid in the C terminus of the capsid protein, called the mutant PCV2b (mPCV2b), is of additional concern, as it was isolated from vaccine-failure cases all over the world.<sup>26–29</sup> Similar to the previous type-switching event with the classical PCV2b, the new variant is spreading rapidly and globally, and is believed to be a new subtype that could be designated as PCV2d.<sup>30</sup>

Accumulating evidence points toward the increased virulence of the emerging subtypes and vaccine-induced selection pressure in driving viral evolution. However, experimental evidence for the same remains variable,<sup>26,31,32</sup> possibly due to the limitations of experimental models in reproducing virulence. The demonstrated cross-protection induced by PCV2a vaccines indicates that the commonality in shared epitopes between PCV2a and PCV2b allows protection against clinical signs induced by both subtypes. However, it is also likely that suboptimal immunity against subtype-specific epitopes could induce selection pressure and account for the recent acceleration in PCV2's genetic diversification. The sensitivity of current serological methods precludes the detection of epitope-specific immunity. Continued evolution of PCV2 could allow the evasion of vaccine immunity as well as increase in virulence, resulting in economic losses to the pork industry.

To determine whether subtle changes to T- or B-cell epitopes could cause vaccine escape, we have predicted and compared putative antigenic epitopes in the PCV2a vaccine strain, a classical PCV2b strain isolated and characterized in this study, as well as the newly recognized mPCV2 strains, using immuno-informatic tools. We have also compared our findings with published information about experimentally validated PCV2 epitopes. We expect that our findings will advance PCV2 vaccine design at the epitope, rather than protein, level.

## Materials and Methods

**Clinical samples.** Lung and liver samples were obtained from two piglets from a case of PCV2 vaccine failure, which was submitted to the North Dakota State Veterinary Diagnostic Laboratory. All samples were confirmed as PCV2 positive by immunohistochemistry and polymerase chain

reaction (PCR) using standard diagnostic lab protocols. Samples were processed and stored at  $-80^{\circ}\text{C}$  until needed.

**Infectious clone.** Genomic DNA was extracted from the lung tissue of one piglet using the QIAamp DNA mini kit (Qiagen) following the manufacturer's protocol. PCV2 genomic DNA was amplified and cloned as previously described.<sup>33</sup> Briefly, the forward primer (5'-GAACCGCGGGCTG-GCTGAACTTTTCAAAGT-3') and the reverse primer (5'-GCACCGCGGAAATTTCTGACAAACGTTACA-3') with flanking unique SacII restriction sites were used to amplify the PCV2 genome with a product of size 1.7 kb. The PCR protocol consisted of an initial denaturation step at  $95^{\circ}\text{C}$  for two minutes, followed by denaturation at  $95^{\circ}\text{C}$  for 30 seconds, annealing at  $50^{\circ}\text{C}$  for 30 seconds, extension at  $72^{\circ}\text{C}$  for two minutes, and final extension at  $72^{\circ}\text{C}$  for 10 minutes. The PCR product was purified using QIAamp gel extraction kit (Qiagen), subjected to restriction digestion by the Sac-II enzyme (New England Biolabs), and ligated into a commercial plasmid pBluescript SK II+ (Agilent Technologies). One genomic clone was sequenced and identified as a classical PCV2b strain. It was named as PCV2b 41513. Sequences were assembled using the Lasergene 9 suite (DNASTAR Inc.). The sequence information was deposited in GenBank with the accession number KR816332. The cloned genome was extracted from pBlueScript SK II+ and self-ligated at  $37^{\circ}\text{C}$  for 7 minutes to form tandem dimers. The dimers were ligated into pBluescript SK II+ and used for transfection.<sup>33</sup>

**Rescue and immunoreactivity of the infectious clone.** The dimerized infectious clones were used to transfect PK15 N (PK15 cells free of PCV1 contamination) (NVSL labs). Liopfectamine LTX (Life technologies) was used for transfections, according to the manufacturer's instructions. After 24 hours of incubation at  $37^{\circ}\text{C}$  in a  $\text{CO}_2$  incubator, the cells sheets were fixed using a mixture of methanol/acetone in the ratio of 1:1. The immunoreactivity of the cloned PCV2b capsid protein to anti-PCV2a anti-serum was verified by an indirect immunofluorescence assay (IFA). The fixed cell sheets were stained with polyclonal and swine anti-PCV2a serum from experimentally infected pigs,<sup>34</sup> followed by an FITC-conjugated anti-swine IgG secondary antibody (KPL) and counter-stained with DAPI (Life technologies). The stained cells were viewed using a fluorescent microscope for evidence of PCV2 infection.

**Phylogenetic analysis.** Pairwise comparisons of the PCV2b 41513 isolate with a prototype PCV2b strain NC 16845 (EU340258.1)<sup>31</sup> and a PCV2a strain representing the vaccine strain PCV2a 40895 (AF264042.1)<sup>33</sup> were carried out using the Clustal-W program. The Jalview program and Clustal web service<sup>35</sup> was used for multiple sequence alignment. For phylogenetic analysis, ORFs 1, 2, and 3 of PCV2b 41513 were aligned against representative sequences of PCV2a, PCV2b, and mPCV2b strains obtained from GenBank. The MegAlign Pro software (DNASTAR Inc.) of the LaserGene core suite 9.0 was used to generate phylogenetic trees by the neighbor-joining method. Corresponding sequences from PCV1 were used as the "outgroup".

**Prediction of swine leukocyte class I (SLA Class-I) epitopes.** The NetMHCpan server version 2.8<sup>36,37</sup> was used to predict SLA Class-I epitopes in PCV2's ORF1, 2, and 3 proteins. Alleles SLA1:-0401, SLA2:-0401, and SLA3:-0401 were selected for analysis, as they represent the most common SLA class-I haplotypes present in the United States.<sup>38</sup> Prediction results for 9 mer epitopes were ranked according to binding affinity, with the cutoff for a strong binder being a% rank of 0.5 or an  $IC_{50}$  of 50. The cutoff for weak binders was set at the default value of a% rank of 2 or  $IC_{50}$  of 500. Strong binding peptides with a score of 0.5 or less are listed in Table 2. PCV2a strain 40895 (AF264042.1) was used to represent the vaccine strain for comparison of changes to the predicted epitopes in the field strain PCV2b 41513 (KR816332). The mPCV2b strain was represented by GenBank accession number JX535297.

**Prediction of swine leukocyte class II (SLA Class-II) epitopes.** As SLA class-II prediction servers for swine are not available, potential epitopes were predicted using the PRO-PRED MHC II prediction server for all the 51 DRB1 or DRB 5 alleles listed on the server.<sup>39</sup> The predicted SLA class-II epitopes in ORF1, 2, and 3 of the field strains were compared to the vaccine strain (accession numbers as described above) to determine sequence differences that could lead to vaccine escape. A default threshold setting of 3 was used to reduce the chances of obtaining false positives. Results were narrowed by default to peptides to the top 10% scored peptides. Anchor residues M, F, I, L, V, W, and Y, which are essential at the first position for high-affinity binding,<sup>40</sup> are identified by small letters in Table 3.

**Prediction of B cell epitopes.** To determine whether possible changes in the B-cell epitopes of the field strains could lead to immune escape, the Kolaskar and Tongaonkar

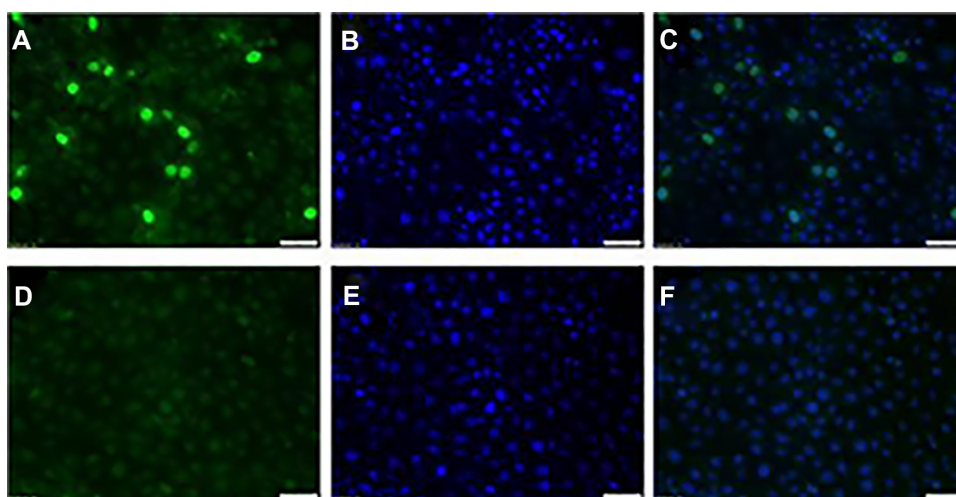
antigenicity index tool from the Immune Epitope Database (IEDB) was used to predict and compare potential B-cell epitopes.<sup>41</sup> Since antibody responses to ORF3 have not been detected in infected animals thus far, the B-cell epitope analysis was confined to ORF1 and 2 of the three selected PCV2 subtypes (accession numbers as described above).

**Experimentally validated epitopes.** Published biomedical literature on PCV2 epitopes was mined for information about experimentally validated PCV2 epitopes using the NCBI PubMed database and the Boolean search terms "PCV2" and "epitope". Findings from published studies were compared to the immuno-informatic predictions to discuss the validity and functionality of the predictions.

## Results

**Phenotypic characterization of the PCV2b 41513 field isolate.** Recombinant PCV2 virus was successfully rescued upon transfection of PK-15 cells with the infectious clone (Fig. 1). As expected, the rescued virus was clearly detected using anti-serum from pigs infected with a PCV2a strain,<sup>34</sup> indicating a strong cross-reactivity with vaccine-induced antibodies.

**Genotypic characterization of the PCV2b 41513 field isolate.** To determine whether the field strain isolated from the vaccine failure case was unique, its genetic and amino acid sequences were compared to the vaccine strain PCV2a 40895 and a classical prototype PCV2b strain. The field strain PCV2b 41513 shared a total genome identity of 95.7% with the PCV2a vaccine strain. The genome length of the PCV2b strain (1767 bp) was 1 bp shorter than that of the PCV2a vaccine isolate.<sup>11</sup> ORF1 was 98.73% similar, and the ORF2 showed the greatest number of changes, with a nucleotide identity of 93.13% and 16 amino acid changes, some of which were



**Figure 1.** Indirect Immunofluorescence (IFA) image of the rescued PCV2b 41513 field virus. PK-15 N cells transfected with the dimerized infectious clones of PCV2b 41513. Cells were stained at 24 hours post transfection with anti-sera from swine infected with PCV2a strain and detected by an FITC-tagged secondary anti-swine IgG antibody. The nuclei were counter-stained with DAPI (blue). (A) Transfected PK-15 cells stained with an FITC-tagged secondary anti-swine IgG antibody. (B) Transfected PK-15 cells stained with DAPI. (C) Overlay of (A) and (B). (D) Untransfected PK-15 cells stained with an FITC-tagged secondary anti-swine IgG antibody. (E) Untransfected PK-15 cells stained with DAPI. (F) Overlay of (D) and (E). Positive cells show an apple green nuclear fluorescence, typical of PCV2.





in the previously identified immunogenic regions.<sup>42,43</sup> ORF3 differed by seven nucleotides and six amino acids including the C-terminal amino acids PNK 102–104 LHQ. ORF4s differed by two bases and one amino acid C34Y (Table 1).

When compared to the prototype PCV2b 16845 sequence, the overall nucleotide identity was 99.7% with three and two point mutations in the ORF2 and ORF1 genes,

respectively. The changes to the ORF2 gene resulted in two amino acid changes, D79A and E210G, while in ORF1 the change was manifested as N41D. Changes to the nucleotide sequences of ORF3 and ORF4 did not result in amino acid changes (Table 1). Therefore, the PCV2b 41513 isolate was largely comparable to circulating classical PCV2b strains.

**Identification of variable amino acid residues.** Amino acid sequence differences in the capsid proteins of the three subtypes indicated that substitutions were present in all the four experimentally characterized major immunogenic regions.<sup>43,44</sup> Conserved residues, which are likely responsible for cross-protection, were also identified. Differences between the vaccine strain and the classical PCV2b strains were present at residues S57I, I89R, T121S, S190A, and K232 N. The PCV2b 41513 isolate had two unique changes at residues D80A and E210G, which distinguished it from all the other strains examined. While change at residue 80 was located in the first immunogenic region, the change at residue 210 was not, although it was adjacent to predicted SLA class-II and B-cell epitopes (Tables 3 and 4). Differences between the vaccine strain and the newly evolved mPCVb strain (Fig. 2) were present at residues F55I, A68N, I89L, S90T, T134N, S169R, V215I, and the additional K at position 234.

As expected, the replicase gene was largely conserved. Differences that were conserved between the all the classical PCV2b strains examined and the vaccine strains were not

**Table 1.** Pairwise comparison of percentage nucleotide and amino acid identity.

ORF	PCV1 <sup>c</sup>	40895-PCV2a <sup>a</sup>	16845-PCV2b <sup>b</sup>
<b>A. Nucleotide sequence comparison</b>			
NDSU 41513-ORF1	82.95%	97.88%	99.68%
NDSU 41513-ORF2	67.52%	92.31%	99.57%
NDSU 41513-ORF3	80.95%	98.10%	100.00%
NDSU 41513-ORF4	86.67%	98.33%	100.00%
<b>B. Amino acid sequence comparison</b>			
NDSU 41513-ORF1	82.80%	98.73%	99.36%
NDSU 41513-ORF2	66.67%	93.13%	99.14%
NDSU 41513-ORF3	80.95%	97.12%	100.00%
NDSU 41513-ORF4	86.67%	98.31%	100.00%

**Notes:** <sup>a</sup>Representative vaccine strain PCV2a 40895 (AF264042.1). <sup>b</sup>Prototype PCV2b field strain (EU340258.1). <sup>c</sup>Prototype PCV1 strain in (GU799575, U49186.1).

**Table 2.** Identification of predicted conserved and variable MHC-I epitopes in the major PCV2 proteins.

ALLELE	POSITION	VACCINE STRAIN PCVa AF264042.1	PCV2b 41513 KR816332	mPCV2b JX535297
<b>ORF1</b>				
SLA-1:0401	87		GTDQQNKEY	
	186		FADPETTYW	
SLA-2:0401	67		QTFNKVKWY	
SLA-3:0401	274	YRRITFLVF	YRRITSLVF	YRRITSLVF
	275	RRITFLVFW	RRITSLVFW	RRITSLVFW
<b>ORF2</b>				
SLA-1:0401	132	ATALTYDPY	ATALTYDPY	ANALTYDPY <sup>a</sup>
	165	VLDSTIDYF	VLDSTIDYF	VLDRTIDYF
	198		GTAFENSIY	
SLA-2:0401	129	VTKATALTY	VTKATALTY	VTKANALTY <sup>a</sup>
	132	ATALTYDPY	ATALTYDPY	ANALTYDPY
	147	HTIPQPFSY	HTIIQPFSY	HTITQPFSY
SLA-3:0401	46	TRLSRTFGY	TRLSRTFGY	TRLSRTIGY <sup>a</sup>
	48	LSRTFGYTV	LSRTFGYT	LSRTIGYTV <sup>b</sup>
	58	ATTVRTPSW <sup>b</sup>	RTTVRTPSW	KTTVRTPSW <sup>a</sup>
	145	SRHTIPQPF	SRHTIIQPF	SRHTIIQPF
	178		KRNQLWMRL	
<b>ORF3</b>				
SLA-3:0401	86	SRQVTPLSL	SRQVTPLSL	SREVTPLSL <sup>b</sup>
	95	RSRSSTFNK	RSRSSTLNQ <sup>@</sup>	RSRSSTFYQ <sup>b</sup>

**Notes:** <sup>a</sup>Weak binders with a% greater than 0.5 and less than 2.0. <sup>b</sup>Not identified –% rank greater than 2.0.

**Table 3.** Identification of predicted conserved and variable MHC-II epitopes in the major PCV2 proteins.

START	VACCINE STRAIN PCV2a AF264042.1 <sup>a</sup>	PCV2b 41513 KR816332 <sup>a</sup>	mPCV2b JX535297 <sup>a</sup>
<b>ORF1</b>			
17		wVFTLNNPS	
37		iSLFDYFIV	
64		fVKKQTFNKvKWYIGARCHIE	
143		fVRNfRGLAELIKVSGKMQK	
206		yHGEEVvVIDDFYGW	
217		fYGWLPWDDIIRLCDRYPL	
246		fLARSIIITSNQTPLEwYSSSTAVPA	
274		lyRRITFLVfWKNATEQS	
<b>ORF2</b>			
8		yRRRRHRPR	
22		iRRRPWLVHPRHR yRwRRKNGI	
45	fNTRLSRTF	fNTRLSRTF	fNTRLSRTI
74	fNIDDFVPP	fNINAF <sup>l</sup> LPP	fNIND <sup>f</sup> LPP
95		yYRIRKVKVE	
123		vILDDNFVT	
141		yVNYSSRHT	
161	fTPKPVLDs	fTPKPVLDs	fTPKPVLD <sup>r</sup>
174	fQPNNKRNQI wmRLQTSRNV	fQPNNKRNQL wIRLQTAGNV	yfQPNNKRNQL wIRLQT <sup>g</sup> GNV
211	yNIRVTMYVqf refNLKDPPLK	yNIRVTMYVqf refNLKDPPL <sup>n</sup>	yNIRITMYVqf refNLKDPPL <sup>n</sup>
<b>ORF3</b>			
1		mVTIPPIvSRWfPVCGRVCKISSP	
40	iGLPITLLHFFPAH fQKFSQPAE	iSLPITPLHFFPAH fQKFSQPAE	iRLPITLLHFFPAH fQKFSQPAE
67		yRVLLCNGH	
90	vTPISIRSRsSTFN	vTPISLIRSRsSTL <sup>h</sup>	vTPISIRSRsSTF <sup>y</sup>

**Note:** <sup>a</sup>Anchor residues that are essential for high affinity binding are represented in small letters.

detected. The selected mPCVb isolates showed conserved differences at residues 6 (S) and 77 (F) when compared to the other two subtypes (Fig. 3).

### Phylogenetic analysis

When the amino acid sequences of the capsid protein were analyzed, the PCV2b 41513 isolate clustered in the same branch as the other classical PCV2b strains. The mPCV2b strains clustered together in a distinct branch and showed the maximum distance from the vaccine PCV2a strain (Fig. 4). As expected, the analysis with the replicase protein did not show a distinct clustering of the PCV2a and classical PCV2b isolates, due to the more conserved nature of the replicase gene. However, the mPCV2b strains clustered in a separate branch (Fig. 5).

**Variable SLA class-I epitopes predicted by NetMHCpan.** When SLA class-I epitopes for alleles SLA1-: 0401, SLA2-: 0401 and SLA3-: 0401 were predicted for the replicase proteins of the three subtypes using the NetMHCpan tool, five strong binding peptides were identified. Two adjacent peptides at positions 274 and 275 varied from the vaccine strain by one amino acid in both the field strains, while all the other peptides were conserved (Table 2).

In ORF2, 11 strong binding peptides were predicted for the above-mentioned alleles. Only two peptides at position 178 and 198 were conserved in all three strains. Of the 11 predicted SLA class-I epitopes, 4 and 7 epitopes varied between the PCV2a vaccine strain and PCV2b 41513 or mPCV2b strain, respectively. All variations comprised single amino acid changes. Interestingly, of the seven predicted SLA class-I epitopes that varied between the vaccine strain and mPCV2b, four were identified as weak binders and one was not identified as a binder. Two peptides at position 132 and 147 were identified as binding to both SLA1-: 0401 and SLA2-: 0401 (Table 2).

In ORF3, two strong binding peptides were identified for the SLA3-:0401 allele. One of them was conserved between the PCV2b 41513 and PCV2a vaccine strains. Neither of them was conserved in the mPCV2b strain, nor were they identified as binders in the analysis of the mPCV2b ORF3 protein (Table 2).

**Variable SLA class-II epitopes predicted by ProPred MHC-II.** Eight, 10, and 4 SLA class-II epitopes were predicted by the ProPred MHC-II server in the ORFs 1, 2, and 3 respectively; with some of them overlapping two or three other possible epitopes. In ORF1, only one of the predicted epitopes varied between the vaccine strain and the mPCV2b field strain. In ORF2, three predicted epitopes varied between the PCV2b 41513 and the vaccine strain, while five epitopes varied in the mPCV2b strain. One substitution at position 174 was conserved between the two field strains. In ORF3, the predicted SLA class-II epitopes at positions 41 and 90 varied between all three strains. The epitope at position 90 included the last three C-terminal amino acids, which vary between all the three strains (Table 3). A majority of the epitopes were highly promiscuous, as they were predicted as binding to several of the 51 alleles examined.

**Variable B-cell epitopes predicted by the Kolaskar and Tongaonkar antigenicity index tool.** In ORF1, 14 linear B-cell epitopes were predicted for the three PCV2 viral strains examined. Except for one amino acid change at position 34, all epitopes were conserved among the vaccine and field strains. Eleven linear epitopes were predicted in the ORF2 proteins. Four of them varied between the vaccine strain and the PCV2b 41513 strain, while five varied between the mPCV2b strain and the vaccine strain. None of the varying epitopes was conserved between the two PCV2b strains, indicating that existing immunity against the circulating classical PCV2b may not guarantee complete protection against the

**Table 4.** Predicted differences in linear B-cell epitopes using the Kolaskar and Tongaonkar antigenicity index tool.

START <sup>a</sup>	VACCINE STRAIN PCV2a AF264042.1	PCV2b 41513 KR816332	mPCV2b JX535297
<b>ORF1</b>			
15		KRWVFTL	
33	RELPISLFDYFIVG	RQLPISLFDYFIVG	RELPISLFDYFIVG
56		PHLQGFANFVKKQ	
73		GARCHIE	
94		KEYCSKE	
102		NLLIECGAP	
120		STAVSTLLESGLVTVAEQHPVTFV	
150		LAELLKVS	
168		NVHVIVGPPGCG	
208		GEEVVIDD	
223		WDDLRLCDRYPLTVE	
243		TVPFLARSILITS	
265		STAVPAVEALYRRITFLVFWK	
298		FVTLSPPCPEFP	
<b>ORF2</b>			
16		RSHLGQI	
26		RPWLHPRH	
54	GYTVKAT	Not detected	GYTVKKT
62	VRTPSWA	RTPSWA	VRTPSWN <sup>b</sup>
77	DDFVPPG	NAFLPPG	NDFLPPG <sup>b</sup>
90	SIPFEYYRIRKVKVEFWPCSPI	RSVPFEYYRIRKVKVEFWPCSPI	PLTVPFEYYRIRKVKVEFWPCSPI
120	STAVILDDN	GSSAVILDDN	STAVILDDN
130	VTKATALTYDPYVNYS		
147	RHTIPQPFYSHSRYFTPKPVL DSTIDYF	ITQPFSYHSRYFTPKPVL DSTIDYF	ITQPFSYHSRYFTPKPVL DRT
193		VDHVGLG	
212		NIRVTMYVQFR	

**Notes:** <sup>a</sup>Start position in the vaccine strain. <sup>b</sup>Not predicted.

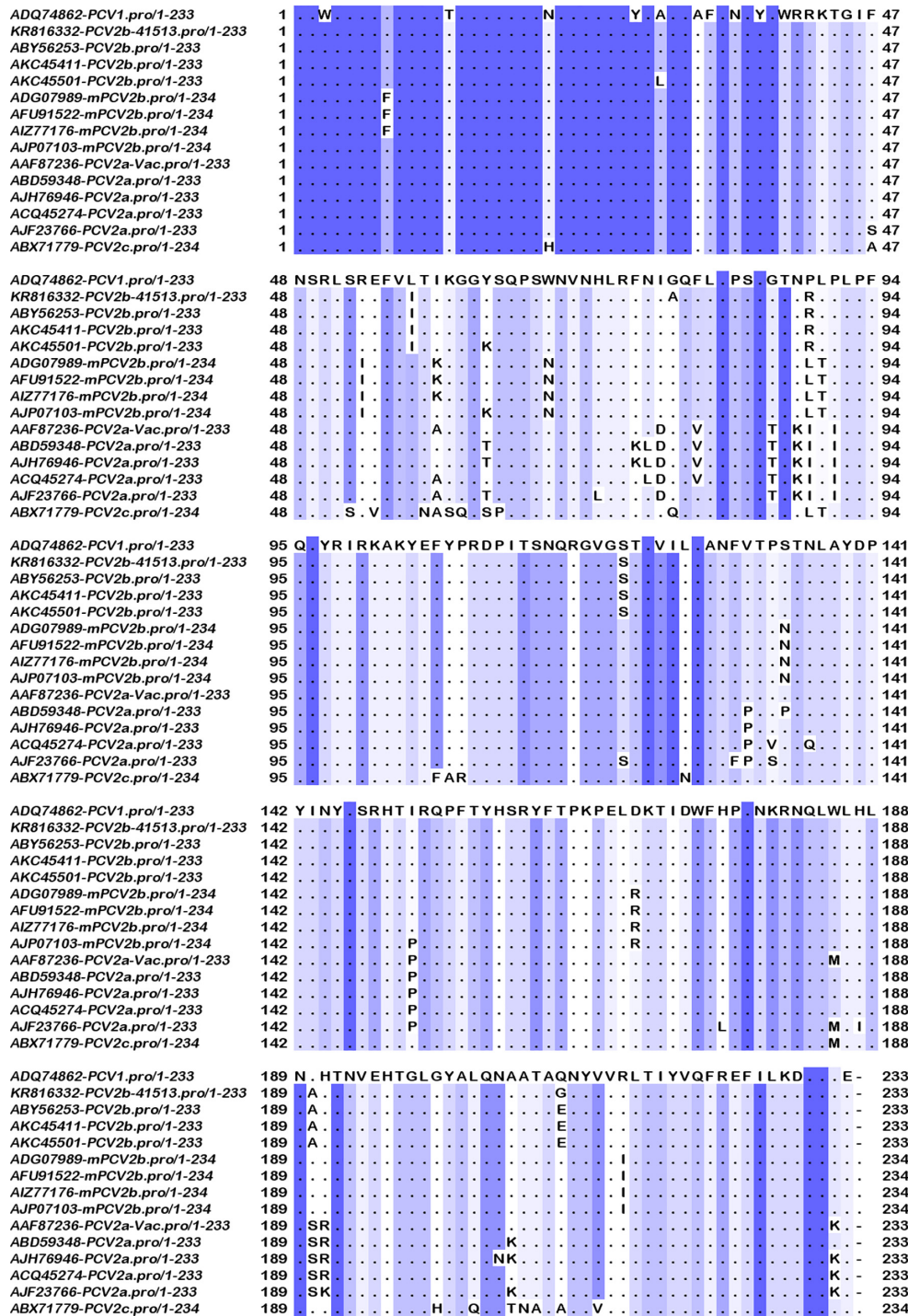
mPCV2b. One epitope that was present in the vaccine strain at position 54 was not predicted for the PCV2b 41513 strain, and two epitopes at positions 62 and 77 were not predicted in the mPCV2b strain ORF2 (Table 4). The positions of the amino acids that differed between the strains were plotted on the crystal structure 3R0R from the protein database (Fig. 6). Except for the residue in position 120, all other changes were located on the surface.

**Experimentally validated epitopes.** Information about experimentally validated T-cell epitopes was limited to one publication<sup>45</sup> describing two epitopes in ORF1 and one epitope in ORF3. In the study by Stevenson et al, although the authors tested several peptides from each protein, only three showed broad immunogenicity in the test system. The major immune-dominant regions depicted in Figure 2 and the epitopes within these regions are described by several authors.<sup>42–44,46,47</sup> Additionally, one epitope was identified in the N-terminal nuclear localization signal of the ORF2,<sup>48</sup> which does not fall under the experimentally

validated immunogenic regions. Functionally, conformational neutralizing residues and epitopes have been identified in positions 59 and 60 as well as the last four C-terminal amino acids.<sup>42,44,49,50</sup> An immunodominant decoy epitope is reported to span residues 169–180<sup>51</sup> and hypothesized to play a role in immune subversion.

## Discussion

Current commercial vaccines against PCV2 have demonstrated success in preventing clinical signs induced by both the PCV2a and b subtypes. However, PCV2 has continued to evolve rapidly after the introduction of commercial vaccines. Although the PCV2b 41513 strain described in this study was isolated from a case of vaccine failure, it was unexpectedly very similar to circulating PCV2b strains, both antigenically and genetically (Table 1). While there is a possibility that improper adherence to vaccination protocols, rather than genetic diversity, could have accounted for the failure, the mechanisms of immune escape for PCV2 or vaccine-induced



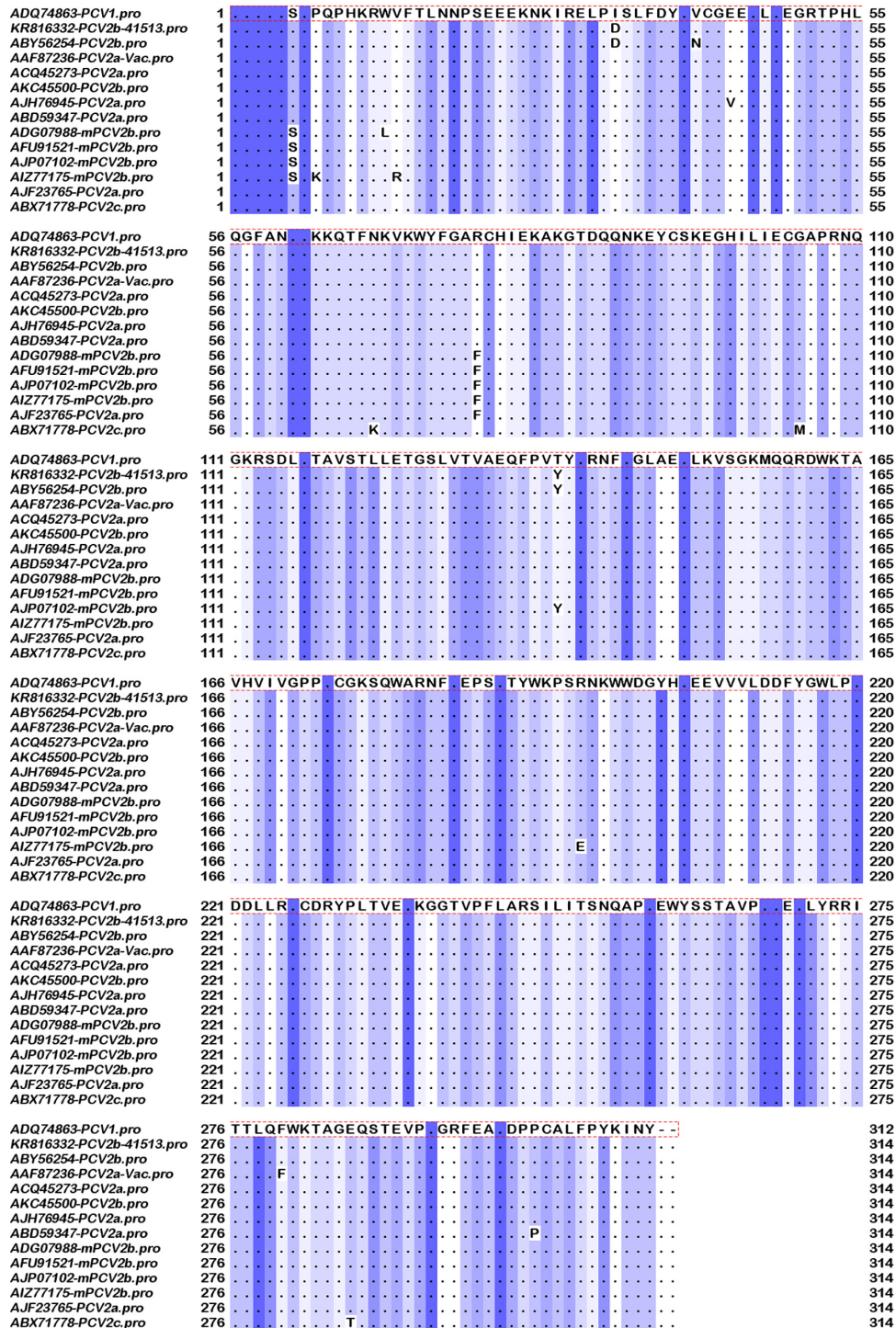
**Figure 2.** Multiple sequence alignment of the capsid proteins. Depiction of conserved and variable residues in the capsid protein of PCV2 isolates representative of the major subtypes PCV2a, classical PCV2b, and the recently identified mutant PCV2b. The diagram was generated using Jalview 2.4 software. Conserved residues are represented by dots and a sliding blue color scale, with the darkest residues being the most conserved. The experimentally validated antigenic regions are demarcated by solid boxes.

selection pressure in driving viral evolution most likely involve very subtle changes to the immunogenic epitopes due to the small size of the virus. Indeed, it has been shown that even a minor change of two amino acids can result in a phenotypic change,<sup>52</sup> prompting the detailed examination of differences in the putative B- and T-cell epitope sequences of

the three circulating PCV2 subtypes in this study, with the ultimate goal of understanding the possible antigenic basis of vaccine escape.

Although the replicase and capsid proteins of the selected strains had a high level of amino acid sequence identity overall, conserved substitutions between the PCV2a vaccine strain



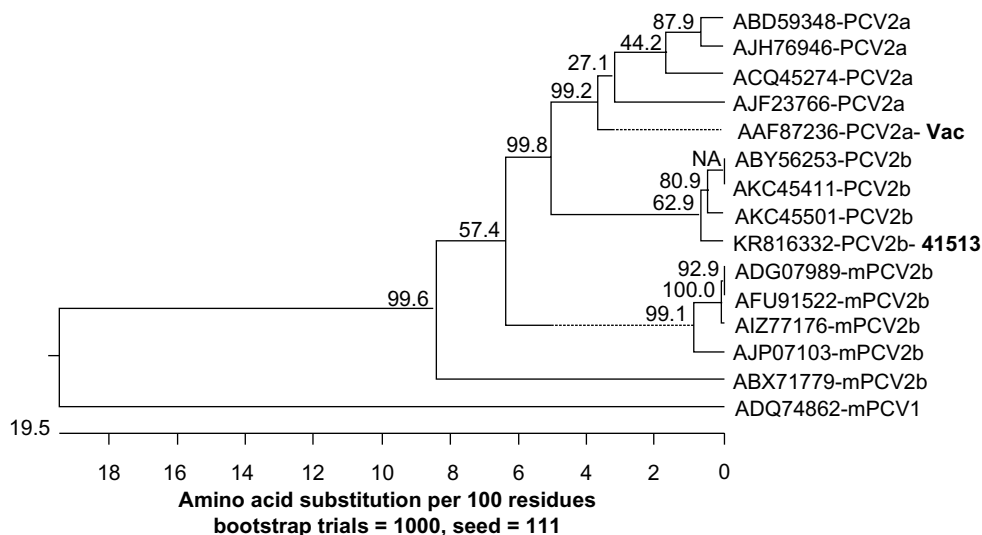


**Figure 3.** Multiple sequence alignment of the replicase proteins. Depiction of conserved and variable residues in the replicase protein of PCV2 isolates representative of the major subtypes PCV2a, classical PCV2b, and the recently identified mutant PCV2b, generated using Jalview 2.4 software. Conserved residues are represented by dots and a sliding blue color scale, with the darkest residues being the most conserved.

and the field strains, which could translate to subtle antigenic changes, were predicted by the immuno-informatic tools used. ORF1 and ORF3 are well-characterized nonstructural proteins of PCV2. While there were no major amino acid sequence differences between the ORF1 of the vaccine and PCV2b 41513 strains, two conserved changes in

the mPCV2b strains at positions 6 and 77 distinguished the mPCV2b strains from the circulating PCV2a and b strains and led to clustering on a separate branch on the phylogenetic tree (Figs. 3 and 5). The change at position 77 was a part of a predicted MHC-II epitope and an overlapping B-cell epitope located close to an experimentally validated T-cell

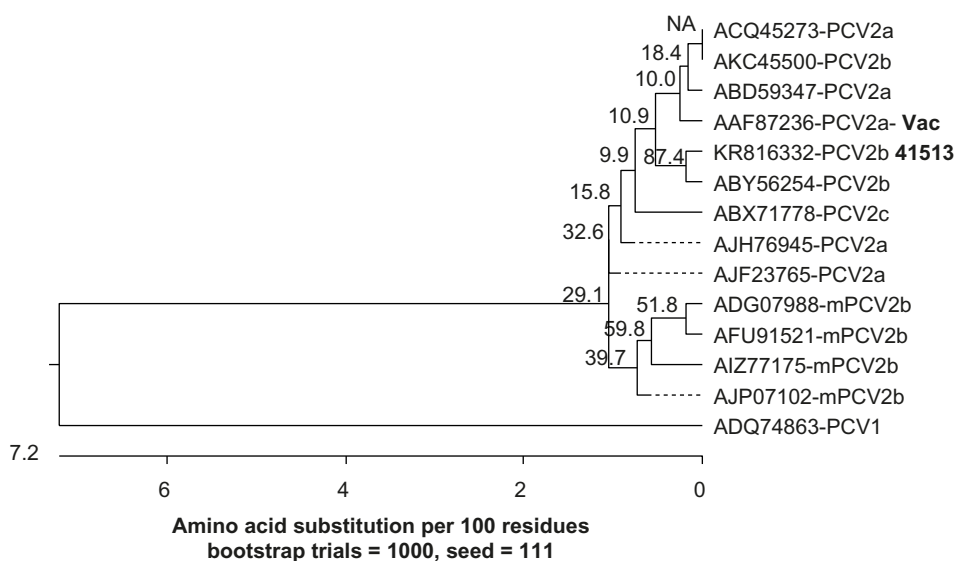




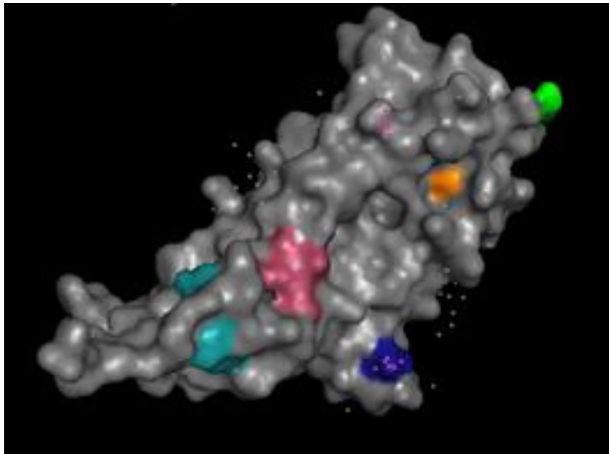
**Figure 4.** Phylogenetic analysis of the PCV2 capsid protein. Amino acid sequences representative of the PCV2a vaccine strain, classical PCV2b strains, and the newly identified mutant PCV2b isolates are represented. The tree was constructed using the MegAlign software by the neighbor-joining method with 1000 bootstrap replicates. Bootstrap values are indicated above the branches. The length of the branches denotes the extent of differences between sequences. A PCV1 capsid sequence was used as the outgroup.

epitope at position 81.<sup>45</sup> While further experimental work is required to determine the significance of this change, it could be speculated that it plays a role in the increased virulence or immune escape of the mPCV2b. Since antibody responses to ORF3 have not been detected in infected animals so far, B-cell epitope analysis for ORF3 has not been carried out. However, overlapping MHC-I and MHC-II epitopes were predicted at the C-terminal amino acids of ORF3 (Tables 2 and 3). These epitopes vary between the three subtypes and could therefore play a role in virulence and T-cell-mediated

immunity. Moreover, a predicted MHC-II epitope in the ORF3 at position 40 corresponded to an experimentally validated epitope.<sup>45</sup> The role of ORF1 and 3 in inducing protective immunity against PCV2 is not well characterized. Attempts to map T-cell epitopes in the three major ORFs revealed that only three peptides in ORF1 or 3 were immunodominant in a majority of the animals from which primed peripheral blood mononuclear cells (PBMCs) were derived for the analysis.<sup>45</sup> The lack of reactivity can be justified by the genetic diversity of conventional outbred pigs as well as by the fact that PCV2 is



**Figure 5.** Phylogenetic analysis of the PCV2 replicase proteins. Amino acid sequences representative of the PCV2a vaccine strain, classical PCV2b strains, and the mPCV2b strains are depicted. The tree was constructed using the MegAlign software by the neighbor-joining method with 1000 bootstrap replicates. Bootstrap values are indicated above branches. The length of the branches denotes the extent of differences between sequences. A PCV1 replicase protein sequence was used as the outgroup.



**Figure 6.** Location of amino acid changes in B-cell epitopes. The amino acids that differed between the vaccine PCV2a strain and the field strains within the predicted B-cell epitopes listed in Table 4 are indicated on the crystal structure. The diagram was generated using Pymol 1.3, and structure ID 3R0R from the protein database. Numbers indicate the positions and the color the differing residue: 54 – yellow, 62 – orange, 77 – pink, 90 – cyan, 120 – not exposed, 147 – dark blue.

largely immunosuppressive.<sup>4</sup> However, based on our in silico analysis, ORF1 and 3 likely influence the T-cell-mediated immune response to PCV2 and should be considered in vaccine design.

Viruses commonly achieve increased fitness due to beneficial mutations in their receptor binding sites.<sup>53</sup> The putative receptor binding site of the PCV2 capsid protein is believed to be located between residues 99 and 104.<sup>54</sup> However, the strict conservation of the putative receptor binding site between the three subtypes examined in this study precludes the possibility that the newly emerging PCV2 variants achieve increased fitness due to positive selection in their receptor binding site. As expected, several conserved differences in sequence were detected between the vaccine strain and the field strains in the capsid protein sequences (Fig. 2). Of the four conserved differences between the vaccine strain and the PCV2b 41513 strain detected in ORF2, three were located in the experimentally validated immunogenic regions. A motif of six amino acids in length, which distinguishes the PCV2 subtypes, is located at positions 86 to 91.<sup>11</sup> However, the motif region has not been experimentally recognized as immunogenic thus far, except for the last three residues,<sup>55</sup> but could form as-yet-unidentified conformational epitopes. The motif region was predicted as a possible B-cell epitope in our analysis (Table 4). One conserved change at position 89 also overlapped a predicted SLA class-II epitope. Interestingly, the epitope including residue 89 was not recognized as immunogenic in mPCV2b by the prediction algorithm, indicating a possible change in the antigenicity of mPCV2b (Table 3) when compared to the vaccine and classical PCV2b strains.

Although computational prediction of B-cell epitopes is known to be less reliable than that of T-cell epitopes,<sup>56</sup>

the Kolaskar and Tongaonkar antigenicity index tool is reported to have a 70% accuracy in prediction, as it is trained using experimentally validated epitopes.<sup>41</sup> Of the eight conserved differences in mPCV2b ORF2 when compared to the vaccine, strain 6 localized to the experimentally validated immunogenic regions and epitopes (Fig. 2, Table 5). Overlapping and nonconserved MHC-I, MHC-II, and B epitopes were predicted at residues 53, 134, and 169 for the mPCV2b ORF2. The differences could affect both B- and T-cell immunity against the field strains and promote immune escape. The last four amino acids of ORF2 have been experimentally validated as neutralizing residues<sup>42,44</sup> and differ among all three subtypes. In fact, the additional K at the C terminus of the mPCV2b strain is hypothesized to play a role in its increased virulence.<sup>26,57</sup> However, the C-terminal amino acids were not predicted as immunogenic by the Kolaskar and Tongaonkar antigenicity index tool, nor were they predicted to contain T-cell epitopes. A decoy B-cell epitope located at position 169–180<sup>51</sup> was completely conserved in all the three subtypes, and hence is probably not involved in differential immunity.

Based on the analysis with monoclonal antibodies, residue 59 is believed to form a conformational epitope, which

**Table 5.** Experimentally validated B- and T-cell epitopes.

ORF	POSITION	SEQUENCE	REFERENCE
<b>B-cell epitopes</b>			
1	39–46	LFDYFIVG	46
1	99–106	KEGNLLIE	46
2	69–83	VDMMRFNINDFLPPG	43
2	113–127	QGDRGVGSSAVILDD	43,47
2	169–183	FTIDYFWPNKRNQL	43
2	169–180	STIDYFQPNNKR <sup>a</sup>	51
2	156–162	YHSRYFT	44
2	175–192	QPNNKRNQLWLRLLQTAGN	44,42
2	195–202	HVGLGTAF	44
2	231–233	LNP <sup>b</sup>	44
2	62–73	VRTPSW AVDMMR	55
2	79–84	FLPPGG	55
2	86–93	SNPRSVPF	55
2	102–107	KVEFWP	55
2	119–128	GSSXXXLDDN	55
2	229–223	PPLNP <sup>b</sup>	55,42
2	59, 60	A/R, A/T <sup>c</sup>	49,50,42
2	26–36	RPWLVHPRHRY	48
<b>T-cell epitopes</b>			
1	81–200	CHIEKAKGTDQQNKEYCSKE	45
1	201–220	KWWDGYHGEEVVVIDDFYGW	45
3	31–50	PRWPHNDVYIGLPITLLHFP	45

**Notes:** <sup>a</sup>Decoy epitope. <sup>b</sup>Neutralizing epitope. <sup>c</sup>Critical residues in a type-specific, neutralizing, conformational epitope.

confers type-specific neutralizing properties.<sup>49,50</sup> Multiple sequence alignment confirmed that residue 59 was substituted from an A to R in classical PCV2b strains when compared to the vaccine strains and from an A to K in mPCV2b strains (Fig. 2). Hence, it is very likely that the substitution at residue 59 plays an important role in antibody-mediated immune escape. Residue 59 was also predicted as part of a linear B-cell epitope (Table 4, Fig. 6). However, the corresponding epitope was not predicted as immunogenic for the mPCV2b. Similarly, other MHC-I and B-cell epitopes, which were predicted to be immunogenic in the vaccine strain, were either identified as weak binders or nonbinders in the field strains (Tables 2 and 4), especially for the mPCV2b, indicating subtle changes in antigenicity of the mPCV2b. Over 95% of conventional swine are infected with the classical PCV2b and very likely have immunity against it.<sup>16</sup> However, based on our *in silico* analysis, the number of subtle differences predicted between the T- and B-cell epitopes of the classical PCV2b and mPCV2b (Tables 2–4) suggest that natural immunity against PCV2b is unlikely to completely protect against the mPCV2b. Indeed, the recent and rapid spread of the mPCV2b<sup>30</sup> lends credence to our findings. The importance of the capsid protein in inducing protective antibody responses is generally well accepted. However, our *in silico* analysis suggests that ORF2 plays an equally important role in differential T-cell-mediated immunity due to the sequence diversity and presence of predicted T-cell epitopes (Tables 2 and 3).

While the limitations of immuno-informatic tools for epitope analysis are acknowledged, it is not within the scope of this study to examine specific weaknesses that may prevent obtaining a highly correlated experimental validation, should such an analysis be attempted. The lack of freely available SLA class-II epitope prediction tools is an additional limitation of this study. However, it is known that the SLA class-II and HLA-II regions are very similarly organized. SLA class-II genes exhibit a higher homology to their HLA counterparts than with other SLA class-II genes.<sup>58,59</sup> Experimental validation is key to verifying that the predicted findings can translate into practice and also to improve current computational methods. However, our findings emphasize the importance of computational vaccinology in reducing the lead development time by detecting strain-specific differences, which can aid in the development of universal vaccine technologies for genetically diverse pathogens.<sup>60</sup>

## Conclusions

The wide-spread deployment of commercial vaccines after the discovery of PCV2 is associated with the proportionally increasing genetic diversity of PCV2, all over the world. Our analysis of the antigenic basis for possible immune escape from vaccine-derived immunity by the predominant circulating PCV2b subtypes showed three major trends. The first was the presence of conserved single amino acid substitutions in the predicted and experimentally validated immune

epitopes, which could lead to partial immune derecognition in vaccine-primed animals. Secondly, some predicted immunodominant T- and B-cell epitopes in the vaccine strain were not identified as epitopes in the field strains by the immuno-informatic tools, indicating a difference in their antigenicity or binding affinity to receptors, which, in turn, would affect the quality of the downstream immune cascade. The third was the presence of several epitopes with overlapping B- and T-cell functions, wherein changes in sequence could cause possible deleterious effects on protective immunity in both the cell-mediated and humoral arms of the host immune response. In conclusion, our findings support the rational design of next-generation PCV2 vaccines at the epitope level.

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## Author Contributions

Conceived and designed the experiments: SR. Analyzed the data: MC, SR, MS, OK. Wrote the first draft of the manuscript: SR. Contributed to the writing of the manuscript: MC, MS, OK. Agree with manuscript results and conclusions: MC, MS, OK, SR. Jointly developed the structure and arguments for the paper: MC, MS, OK, SR. Made critical revisions and approved final version: SR. All authors reviewed and approved of the final manuscript.

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