Single amino acid mutations in the capsid switch the neutralization phenotype of porcine circovirus 2

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Porcine circovirus 2 (PCV2) is the causative agent of porcine circovirus-associated diseases in pigs. Previously, it was demonstrated that mAbs 16G12, 38C1, 63H3 and 94H8 directed against the PCV2 capsid protein recognize PCV2 strains Stoon-1010 (PCV2a), 48285 (PCV2b), 1121 (PCV2a), 1147 (PCV2b) and II9F (PCV2b), but only neutralize Stoon-1010 and 48285. This points to the existence of two distinct PCV2 neutralization phenotypes: phenotype z (mAb recognition with neutralization; Stoon-1010 and 48285) and phenotype β (mAb recognition without neutralization; 1121, 1147 and II9F). In the present study, amino acids that are important in determining the neutralization phenotype were identified in the capsid. Mutation of T at position 190 to A in strain 48285 (phenotype z) resulted in a capsid resembling that of strain 1147 (phenotype β) and caused a loss of neutralization (switch from z to β). Mutations of P at position 151 to T and A at position 190 to T in strain II9F (phenotype β) resulted in a capsid resembling that of strain 48285 (phenotype z) and gave a gain of neutralization (switch from β to z). Mutations of T at position 131 to P and of E at position 191 to R in Stoon-1010 (phenotype z) changed the capsid into that of 1121 (phenotype β) and reduced neutralization (switch from z to β). This study demonstrated that single amino acid changes in the capsid result in a phenotypic switch from z to β or β to z.

INTRODUCTION

Porcine circovirus type 2 (PCV2) is a non-enveloped, ssDNA virus. It is the causative agent of several economically important porcine circovirus-associated diseases (PCVAD) (Gillespie et al., 2009), of which the post-weaning multisystemic wasting syndrome (PMWS) in weaned pigs (Kennedy et al., 2000; Albina et al., 2001; Bolin et al., 2001) and reproductive failure in sows (Park et al., 2005; Rose et al., 2007; Madson et al., 2009; Saha et al., 2010) have been reproduced experimentally with PCV2 alone.

The covalently closed circular PCV2 genome consists of 1766–1769 nt (Meehan et al., 1998; Huang et al., 2011) and contains 11 putative ORFs (Hamel et al., 1998). Protein expression has been described for three of these ORFs. ORF1 encodes the non-structural replication-associated protein Rep and its spliced, frameshifted variant Rep′ (Cheung 2003; Mankertz et al., 2003). ORF2 encodes the structural capsid protein (Nawagitgul et al., 2000). The non-structural ORF3 protein has been associated with apoptosis in vitro and virus pathogenesis in vivo (Liu et al., 2005, 2006), but these results are somewhat controversial because they have not yet been reproduced by other laboratories (as described and reviewed by Juhan et al., 2010).

The PCV2 capsid protein consists of 233–235 aa (Nawagitgul et al., 2000; Olvera et al., 2007; Huang et al., 2011) and there are three major regions of amino acid heterogeneity (aa 57–91, 121–136 and 180–191), two of which have a high immunogenic index (Larochelle et al., 2002; de Boisséson et al., 2004). Several antigenic domains have been identified on the PCV2 capsid protein by using porcine polyclonal antibodies (pAbs) (Mahe´ et al., 2000; Truong et al., 2001) or mouse mAbs (Lekcharoensuk et al., 2004; Shang et al., 2009). Recently, PCV2 strains have been divided into two major genotypes (PCV2a and PCV2b) and eight genetic clusters (PCV2b-1A to PCV2b-1C and PCV2a-2A to PCV2a-2E), based on their genomic sequences (Olvera et al., 2007; Grau-Roma et al., 2008; Segalés et al., 2008). Genotype- and cluster-specific
domains on the capsid protein have also been identified using mouse mAbs generated against genotype PCV2a or PCV2b (Cheung et al., 2007; Cheung & Greenlee, 2011; Saha et al., 2012a).

PMWS-affected pigs have significantly lower levels of PCV2-neutralizing antibodies compared with subclinically infected animals (Meerts et al., 2006; Fort et al., 2007), suggesting a crucial role for neutralizing antibodies in the prevention of PMWS. Several authors have described mAbs with neutralizing activity for the PCV2 capsid protein (McNeilly et al., 2001; Lekcharoensuk et al., 2004a, 2005; Zhou et al., 2004; Lefebvre et al., 2008a; Shang et al., 2009). The aim of the present study was to demonstrate which change(s) in the capsid of PCV2 cause(s) a switch from phenotype α (IPMA+ N+) to β (IPMA+ N⁻) and vice versa.

RESULTS

Construction of infectious clones from the PCV2 strains Stoon-1010 and 48285

In order to produce small change(s) in the PCV2 capsid, infectious clones were constructed from Stoon-1010 and 48285; the resulting clones are Stoon-1010-8 and 48285-24, respectively. Sequencing of Stoon-1010-8 revealed four amino acid differences from its sequence in GenBank (AF055392) at positions 71 (M to I), 72 (M to I), 191 (G to A) and 200 (A to T). Sequencing of clone 48285-24 showed one amino acid difference at position 200 (T to I) from its sequence in GenBank (AF055394). Van Doorselaere et al. (2010) previously constructed an infectious clone from II9F (II9F-13).

In order to produce infectious virus, the HindIII-digested plasmids (48285-24, Stoon-1010-8 and II9F-13) were transfected onto PCV-negative PK-15 cells and, subsequently, PCV2 was passaged several times on PK-15 cells. Indirect immunofluorescence staining of PCV2-infected PK-15 cells and titration of supernatant fluids from PCV2-infected PK-15 cells demonstrated that PCV2 virus was produced. PCV2 genomic sequences were obtained after five passages on PK-15 cells and the nucleotide sequences were identical to the genomes in the pCR-BluntII-TOPO vectors.

Mutagenesis of the cap from clones 48285-24, Stoon-1010-8 and II9F-13

As shown in Fig. 1, alignments of the capsid proteins of 48285-24 (α; IPMA+N⁺) and 1147 (β; IPMA+ N⁻) demonstrated two amino acid differences at positions 57 and 190. Alignment of the capsid proteins of II9F-13 (β; IPMA+ N⁻) and 48285 (α; IPMA+ N⁺) showed three amino acid differences at positions 63, 151 and 190. Alignment of the capsid proteins of Stoon-1010-8 (α; IPMA+ N⁺) and the 1121 (β; IPMA+ N⁺) revealed five amino acid differences at positions 71, 72, 131, 191 and 232. As strains Stoon-1010 and 48285 are neutralized by mAbs 16G12, 38C1, 63H3 and 94H8 using the immunoperoxidase monolayer assays (IPMA), only Stoon-1010 and 48285 were neutralized (N⁺). This points to the existence of two distinct PCV2 neutralization phenotypes: phenotype α (mAb recognition without neutralization; IPMA+ N⁺) and phenotype β (mAb recognition without neutralization; IPMA+ N⁻).

The aim of the present study was to demonstrate which change(s) in the capsid of PCV2 cause(s) a switch from phenotype α (IPMA+ N⁺) to β (IPMA+ N⁻) and vice versa.

<table>
<thead>
<tr>
<th>PCV2 strains</th>
<th>Phenotype</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>48285-24</td>
<td>α</td>
<td>MTTPAYGSRYSSRHLSSISLQILHELPWLVAFRYYSGDFSGIFPKWLLGPGILGTQTVVTVTTSEANMEISMPVDIPEFPGSGSNHNYFVTRYYFRKVEPFCPECFSTTTGQSDC</td>
</tr>
<tr>
<td></td>
<td>β</td>
<td>MTTPAYGSRYSSRHLSSISLQILHELPWLVAFRYYSGDFSGIFPKWLLGPGILGTQTVVTVTTSEANMEISMPVDIPEFPGSGSNHNYFVTRYYFRKVEPFCPECFSTTTGQSDC</td>
</tr>
<tr>
<td>1147</td>
<td>α</td>
<td>MTTPAYGSRYSSRHLSSISLQILHELPWLVAFRYYSGDFSGIFPKWLLGPGILGTQTVVTVTTSEANMEISMPVDIPEFPGSGSNHNYFVTRYYFRKVEPFCPECFSTTTGQSDC</td>
</tr>
<tr>
<td></td>
<td>β</td>
<td>MTTPAYGSRYSSRHLSSISLQILHELPWLVAFRYYSGDFSGIFPKWLLGPGILGTQTVVTVTTSEANMEISMPVDIPEFPGSGSNHNYFVTRYYFRKVEPFCPECFSTTTGQSDC</td>
</tr>
<tr>
<td>II9F-13</td>
<td>α</td>
<td>MTTPAYGSRYSSRHLSSISLQILHELPWLVAFRYYSGDFSGIFPKWLLGPGILGTQTVVTVTTSEANMEISMPVDIPEFPGSGSNHNYFVTRYYFRKVEPFCPECFSTTTGQSDC</td>
</tr>
<tr>
<td></td>
<td>β</td>
<td>MTTPAYGSRYSSRHLSSISLQILHELPWLVAFRYYSGDFSGIFPKWLLGPGILGTQTVVTVTTSEANMEISMPVDIPEFPGSGSNHNYFVTRYYFRKVEPFCPECFSTTTGQSDC</td>
</tr>
<tr>
<td>48285</td>
<td>α</td>
<td>MTTPAYGSRYSSRHLSSISLQILHELPWLVAFRYYSGDFSGIFPKWLLGPGILGTQTVVTVTTSEANMEISMPVDIPEFPGSGSNHNYFVTRYYFRKVEPFCPECFSTTTGQSDC</td>
</tr>
<tr>
<td></td>
<td>β</td>
<td>MTTPAYGSRYSSRHLSSISLQILHELPWLVAFRYYSGDFSGIFPKWLLGPGILGTQTVVTVTTSEANMEISMPVDIPEFPGSGSNHNYFVTRYYFRKVEPFCPECFSTTTGQSDC</td>
</tr>
<tr>
<td>Stoon-1010-8</td>
<td>α</td>
<td>MTTPAYGSRYSSRHLSSISLQILHELPWLVAFRYYSGDFSGIFPKWLLGPGILGTQTVVTVTTSEANMEISMPVDIPEFPGSGSNHNYFVTRYYFRKVEPFCPECFSTTTGQSDC</td>
</tr>
<tr>
<td></td>
<td>β</td>
<td>MTTPAYGSRYSSRHLSSISLQILHELPWLVAFRYYSGDFSGIFPKWLLGPGILGTQTVVTVTTSEANMEISMPVDIPEFPGSGSNHNYFVTRYYFRKVEPFCPECFSTTTGQSDC</td>
</tr>
</tbody>
</table>

Fig. 1. ORF2 amino acid alignment of three pairs of PCV2 strains with small amino acid differences (shown in green; mutated amino acids shown in red) and a different phenotype within each pair. Consensus key: * = fully conserved residue; := conservation of strong groups; . = conservation of weak groups.
48285(T190A), which resembles the capsid of strain 1147 (β; IPMA+N−). The hydrophobic amino acid valine (V) at position 57 was not mutated because, in strain 1147, another hydrophobic amino acid (isoleucine, I) is present at this position (Fig. 1).

The capsid of II9F-13 (β; IPMA+N−) was mutated from A to T at position 190 and/or from P to T at position 151. This resulted in mutants II9F(A190T), II9F(P151T) and II9F(A190T/P151T), and the capsid of the double mutant looks like that of 48285 (α; IPMA+N+). The basic amino acid arginine (R) at position 63 was not mutated because, in strain 48285, a conserved substitution (lysine, K) is present at this position (Fig. 1).

The capsid of Stoon-1010-8 (α; IPMA+N+) was mutated from E to R at position 191 and/or from T to P at position 131. These mutations of Stoon-1010-8 resulted in mutants 1010(E191R), 1010(T131P) and 1010(E191R/T131P), and the capsid of the double mutant resembles that of 1121 (β; IPMA+N−). The isoleucine (I) residues at position 71 and 72 of Stoon-1010-8 were not mutated into methionine (M) (Fig. 1), as both amino acid residues have hydrophobic characteristics.

The HindIII-digested plasmids of all the mutants were transfected onto PCV-negative PK-15 cells and the resulting PCV2 viruses were passaged on PK-15 cells as described above. After five passages, the mutant viruses were verified by sequencing and were found to be identical to the mutants in the plasmid.

**Reactivity of mAbs to different PCV2 strains in IPMA**

The reactivity of mAbs 16G12, 38C1, 63H3 and 94H8 with viruses 48285-24, Stoon-1010-8 and II9F-13 obtained from infectious clones and their mutant viruses was assessed by IPMA and compared with the reactivity of the mAbs with their original PCV2 strains (48285, Stoon-1010, II9F, 1121 and 1147) (Table 1). All four mAbs reacted with 48285, Stoon-1010, II9F, 1121, 1147, the viruses obtained from infectious clones and the mutants in a similar manner, although a small difference between strain II9F-13 and its mutants was observed.

**Sensitive neutralization (SN) assays**

SN assays were performed to determine the neutralizing activity of the mAbs with 48285, Stoon-1010, II9F, 1121, 1147, the viruses obtained from infectious clones and the mutant viruses.

PCV2 48285 and 48285-24 (α; IPMA+N+) were neutralized by mAbs 16G12, 38C1, 63H3 and 94H8, whereas 1147 (β; IPMA+N−) and the mutant 48285(T190A) were not neutralized. The mutant had the β phenotype (Table 2).

PCV2 II9F and II9F-13 (β; IPMA+N−) were not neutralized by mAbs 16G12, 38C1, 63H3 and 94H8, whereas 48285 (α; IPMA+N+) and the mutants [II9F(A190T), II9F(P151T) and II9F(A190T/P151T)] were. The mutants had the α phenotype (Table 2).

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**Table 1. Recognition of the different PCV2 strains and their mutants by mAbs 16G12, 38C1, 63H3 and 94H8 directed against the PCV2 capsid protein**

<table>
<thead>
<tr>
<th>PCV2 pair</th>
<th>Strain/mutant*</th>
<th>IPMA antibody titre with mAb:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>16G12</td>
</tr>
<tr>
<td>48285(α)/1147 (β)</td>
<td>48285</td>
<td>2560</td>
</tr>
<tr>
<td>48285-24IC</td>
<td>640</td>
<td>640</td>
</tr>
<tr>
<td>48285(T190A)</td>
<td>2560</td>
<td>2560</td>
</tr>
<tr>
<td>1147</td>
<td>2560</td>
<td>2560</td>
</tr>
<tr>
<td>Il9F (β)/48285 (α)</td>
<td>Il9F</td>
<td>640</td>
</tr>
<tr>
<td>Il9F(A190T)</td>
<td>2560</td>
<td>2560</td>
</tr>
<tr>
<td>Il9F(P151T)</td>
<td>2560</td>
<td>2560</td>
</tr>
<tr>
<td>Il9F(A190T/P151T)</td>
<td>2560</td>
<td>2560</td>
</tr>
<tr>
<td>48285</td>
<td>2560</td>
<td>2560</td>
</tr>
<tr>
<td>Stoon-1010 (α)/1121 (β)</td>
<td>Stoon-1010</td>
<td>640</td>
</tr>
<tr>
<td>Stoon-1010-8IC</td>
<td>2560</td>
<td>640</td>
</tr>
<tr>
<td>1010(E191R)</td>
<td>2560</td>
<td>2560</td>
</tr>
<tr>
<td>1010(T131P)</td>
<td>640</td>
<td>2560</td>
</tr>
<tr>
<td>1010(E191R/T131P)</td>
<td>2560</td>
<td>2560</td>
</tr>
<tr>
<td>1121</td>
<td>2560</td>
<td>2560</td>
</tr>
</tbody>
</table>

*IC indicates that a strain was derived from an infectious clone.
Table 2. Neutralization of the different PCV2 strains and their mutants by mAbs 16G12, 38C1, 63H3 and 94H8 directed against the PCV2 capsid protein.

A mean neutralizing activity of >32% was considered as neutralization (indicated in bold). Phenotype \( \alpha \), mAb recognition with neutralization (IPMA\(^+\)N\(^+\)); phenotype \( \beta \), mAb recognition without neutralization (IPMA\(^+\)N\(^-\)).

<table>
<thead>
<tr>
<th>PCV2 pair</th>
<th>Strain/mutant*</th>
<th>Neutralizing activity of mAbs [mean ± sd neutralization (%)]</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>16G12</td>
<td>38C1</td>
</tr>
<tr>
<td>48285 (( \alpha ))/1147 (( \beta ))</td>
<td>48285</td>
<td>77 ± 14</td>
<td>77 ± 10</td>
</tr>
<tr>
<td></td>
<td>48285-24( ^{\text{IC}} )</td>
<td>79 ± 8</td>
<td>75 ± 12</td>
</tr>
<tr>
<td></td>
<td>48285(T190A)</td>
<td>22 ± 18( ^{\dagger} )</td>
<td>23 ± 25</td>
</tr>
<tr>
<td></td>
<td>1147</td>
<td>29 ± 1</td>
<td>31 ± 1</td>
</tr>
<tr>
<td>I19F (( \beta ))/48285 (( \alpha ))</td>
<td>I19F</td>
<td>19 ± 8</td>
<td>6 ± 7</td>
</tr>
<tr>
<td></td>
<td>I19F-A190T</td>
<td>14 ± 14</td>
<td>5 ± 7</td>
</tr>
<tr>
<td></td>
<td>I19F(A190T)</td>
<td>60 ± 5( ^{\dagger} )</td>
<td>62 ± 8( ^{\dagger} )</td>
</tr>
<tr>
<td></td>
<td>I19F(A190T/P151T)</td>
<td>60 ± 7( ^{\dagger} )</td>
<td>61 ± 7( ^{\dagger} )</td>
</tr>
<tr>
<td></td>
<td>I19F(P151T)</td>
<td>60 ± 4( ^{\dagger} )</td>
<td>55 ± 8( ^{\dagger} )</td>
</tr>
<tr>
<td></td>
<td>48285</td>
<td>77 ± 14</td>
<td>77 ± 10</td>
</tr>
<tr>
<td>Stoon-1010 (( \alpha ))/1121 (( \beta ))</td>
<td>Stoon-1010</td>
<td>90 ± 1</td>
<td>91 ± 2</td>
</tr>
<tr>
<td></td>
<td>Stoon-1010-8( ^{\text{IC}} )</td>
<td>90 ± 1</td>
<td>91 ± 1</td>
</tr>
<tr>
<td></td>
<td>1010(E191R)</td>
<td>85 ± 4</td>
<td>83 ± 4</td>
</tr>
<tr>
<td></td>
<td>1010(T131P)</td>
<td>69 ± 4( ^{\dagger} )</td>
<td>74 ± 4( ^{\dagger} )</td>
</tr>
<tr>
<td></td>
<td>1010(E191R/T131P)</td>
<td>13 ± 6( ^{\dagger} )</td>
<td>56 ± 7( ^{\dagger} )</td>
</tr>
<tr>
<td></td>
<td>1121</td>
<td>0 ± 8</td>
<td>0 ± 10</td>
</tr>
</tbody>
</table>

\( ^{\text{IC}} \) indicates that a strain was derived from an infectious clone.

\( ^{\dagger} \) Significantly different (\( P < 0.05 \)) when the value of the mutant virus was compared with the value of the corresponding virus derived from the infectious clone.

PCV2 Stoon-1010 and Stoon-1010-8 (\( \alpha \); IPMA\(^+\)N\(^+\)) were neutralized equally by mAbs 16G12, 38C1, 63H3 and 94H8, whereas 1121 (\( \beta \); IPMA\(^+\)N\(^-\)) and the double mutant 1010(E191R/T131P) were not neutralized (except with mAb 38C1). This double mutant had the \( \beta \) phenotype. Single mutants 1010(E191R) and 1010(T131P) remained neutralized by mAbs 16G12, 38C1, 63H3 and 94H8; however, the neutralization was slightly reduced in mutant 1010(E191R) in comparison to that of Stoon-1010-8 and a significant drop in neutralization (except with mAb 94H8) was observed in mutant 1010(T131P). The single mutants had the \( \alpha \) phenotype (Table 2).

**DISCUSSION**

In this study, we demonstrated that single amino acid mutations in the PCV2 capsid protein result in a switch from phenotype \( \alpha \) (IPMA\(^+\)N\(^+\)) to \( \beta \) (IPMA\(^+\)N\(^-\)) and vice versa.

This study confirms the work of Lefebvre et al. (2008a) stating that strains 48285 and Stoon-1010 are recognized as well as neutralized by mAbs 16G12, 38C1, 63H3 and 94H8 (\( \alpha \); IPMA\(^+\)N\(^-\)), whereas strains 1147, 1121 and I19F are recognized but not neutralized (\( \beta \); IPMA\(^+\)N\(^-\)).

For the four mAbs tested, a mutation of T at position 190 to A of 48285-24 resulted in a complete loss of neutralization. This clearly shows that one single amino acid change in the capsid may switch the neutralization phenotype of PCV2. The amino acid at position 190 seems to play a critical role in the neutralization capacity of a mAb. The major difference between T and A consists of a hydroxyl group (on T); therefore this hydroxyl group might be important as part of the binding site of the mAbs. Another PCV2 strain, 1206, has also A at position 190 and is also not neutralized fully or partially by mAbs 16G12, 38C1, 63H3 and 94H8 (Lefebvre et al., 2008a); this is in agreement with the above findings. Also, it appears that the disappearance of a small part of the recognition area of a mAb does not change the binding of the mAb to the virus, but no longer allows the mAb to block infection. Probably, the amino acid at position 190 is close to the viral ligand that interacts with the cellular receptors, such as heparan sulphate and dermatan sulphate (Misinzo et al., 2006).

Mutation of A at position 190 to T or mutation of P at position 151 to T or mutations at both positions of I19F-13IC resulted in a complete loss of neutralization. This clearly demonstrates that amino acid change(s) at position 190 or 151, or both, in the capsid could switch the neutralization phenotype of PCV2. This suggests that amino acids at positions 190 and 151 appear to play an important role in determining the neutralization capacity of a mAb. As described above, the presence of a hydroxyl group on T...
could be critical as a part of the binding site of the mAbs, since A or P does not have this hydroxyl group. Previously, it was demonstrated that amino acid residues 145–162, 175–192 (Shang et al., 2009) and 165–200 (Lekcharoensuk et al., 2004) in the capsid were recognized by neutralizing mAbs. In our case, the amino acids at positions 151 and 190 in the capsid have been identified as critical for determining the neutralization capacity of the mAbs. No additive effect was observed in the double mutant II9F(A190T/P151T). The neutralization values of the double mutant II9F(A190T/P151T) were not different from the values of 48285, as their capsids are identical except at position 63, where a basic amino acid is present in both II9F(A190T/P151T) and 48285 (R and K, respectively). Amino acid residue 190 is situated on the exterior surface of the PCV2 capsid protein, whereas amino acid position 151 is located in the interior surface of the capsid protein (as shown in Fig. 2). Khayat et al. (2011) indicated that the interior surface of the PCV2 capsid can be antigenic if the capsid is externalized transiently for 'breathing' as described for a number of viruses, such as flock house virus (Bothner et al., 1998), human rhinovirus 14 (Lewis et al., 1998) and poliovirus (Li et al., 1994). Exposition of internal polypeptides was shown to be essential for the infectivity of these viruses. In addition, it was shown that poliovirus was only neutralized when the internal peptides of this virus were exposed externally (Li et al., 1994).

A mutation of E at position 191 to R of Stoon-1010-8 showed a slight drop in neutralization, but this drop was not statistically significant. A mutation of T at position 131 to P resulted in a significant loss of neutralization (P<0.05). Mutations at both positions 191 (from E to R) and 131 (from T to P) had a clear additive effect on the neutralization and the neutralization was similar to that of 1121 (β; IPMA⁺N−) (except that mAb 38C1 showed partial neutralization with the double mutant). This suggests that changes at both positions are necessary to switch the neutralization phenotype. The residual neutralization activities of the mAb 38C1 on the double mutant may be associated with the amino acid at position 232 (K in Stoon-1010-8 and N in 1121). Further mutagenesis at position 232 (from K to N) is needed. The neutralization results demonstrated clearly that position 131 (from T to P) has a greater impact in determining the neutralization capacity of the mAb than position 191 (from E to R). As both E and R have polar and hydrophilic characteristics, changes from E to R did not show any significant effect on the neutralization. The T at position 131 might be critical as a part of the binding site of the mAb. On the other hand, E at position 191 may not be a part of the binding site of the mAb; however, we can designate it as a 'helper' because an additive effect on the neutralization was produced when the amino acid at position 191 was mutated together with the amino acid at position 131. Position 191 is situated close to the mAbs' binding site (position 190, as shown above) and this could be the reason why this position has a

Fig. 2. Location of amino acid residues on the capsid proteins that are different between the two strains of each PCV2 pair with different phenotypes (α, IPMA⁺N⁺; β, IPMA⁺N⁻). Surface diagrams of the PCV2 capsid proteins of different PCV2 strains (Stoon-1010, 48285, 1121, 1147 and II9F) of two phenotypes were generated using the PCV2 crystal structure (PDB accession no. 3R0R) (Khayat et al., 2011) as a template with the swiss-model workspace (Guex & Peitsch, 1997; Schwede et al., 2003; Arnold et al., 2006). Figures were made using UCSF Chimera 1.5.3 (Pei & Grishin, 2001).
‘helper’ effect on the neutralization. Like amino acid residue 190, amino acid residues 131 and 191 are also located on the exterior surface of the PCV2 capsid protein (Fig. 2).

It remains to be determined why the phenotype α strains were recognized as well as neutralized by the mAbs, and why phenotype β strains were only recognized but not neutralized. Glaser et al. (1995) also pointed to the existence of two neutralization phenotypes in the case of equine arteritis virus (EAV). mAbs recognized all field isolates of EAV and all isolates were neutralized by mAbs except for one isolate, DL11, which remained non-neutralized. Absence of neutralizing activity of mAbs on phenotype β strains might be related to low binding strength or avidity of the mAbs. Changes of amino acid at positions 131, 151 and 190 in phenotype β strains might lead to a modification of the viral capsid conformation, which could be responsible for the lower avidity of mAbs. Further research is required to solve these issues.

This study demonstrates that single amino acid mutations in the PCV2 capsid protein switch the neutralization phenotypes of PCV2.

**METHODS**

**Viruses and cells.** Five different PK-15-adapted PCV2 strains, Stoon-1010 (GenBank accession no. AF055392), 48285 (AF055394), 1121 (AJ293868), 1147 (AJ293869) and I9F (EU909688), were included in this study.

PCV-negative PK-15 cells were grown in minimal essential medium (MEM) containing Earle’s salts and GlutaMAX-I (MEM + GlutaMAX-I; Gibco) and supplemented with 5% FCS, 100 U penicillin ml⁻¹, 0.1 mg streptomycin ml⁻¹ and 0.1 mg kanamycin ml⁻¹. Cell cultures were maintained at 37°C in the presence of 5% CO₂.

**PCV2 pairs.** Previously, alignment of the capsid proteins (Lefebvre et al., 2008a) has shown a very limited number of non-conserved amino acid differences between the neutralized strain 48285 (phenotype α) and the non-neutralized strains 1147 and I9F (both phenotype β), and between the neutralized strain Stoon-1010 (phenotype α) and the non-neutralized strain 1121 (phenotype β). Therefore, 48285 (α)/1147 (β), I9F (β)/48285 (α) and Stoon-1010 (α)/1121 (β) were chosen as PCV2 pairs and these non-conserved amino acid residues were selected for site-directed mutagenesis in order to switch the neutralization phenotype.

**Construction of PCV2 infectious clones.** The full-length genome of strain I9F has previously been cloned in pcR-BluntII-TOPO, producing I9F-13 (Van Doorselaere et al., 2010). Strain I9F has a similar sequence to strain VC2002-K39 (Lefebvre et al., 2012a). Amplification of the genomes of Stoon-1010 and 48285 was performed (as described above for I9F) with primers INFC1-PCV2-FW (5′-gggacctgtagtttatcatgaa-3′) and INFC1-PCV2-REV (5′-cttctcataccatacagcttc-3′), hereby introducing a HindIII site (underlined in the primer sequence) after the stop codon of the Rep gene. Infectious clones produced from Stoon-1010 and 48285 were designated Stoon-1010-8 and 48285-24, respectively.

**Mutagenesis of the capsid gene.** Mutagenesis of the capsid gene (Fig. 1) was performed using a QuickChange site-directed mutagenesis kit (Stratagene). The capsid gene of clone 48285-24 was mutated using primers 48285TtoA1147FW (5′-cctgagctcacaactgcttaggtcagc-3′) and 48285TtoA1147REV (5′-ggtgcttagctccagagttgtcgctc-3′) introducing a T to A change at position 190.

The capsid gene of I9F-13 was mutated at amino acid position 190 (from A to T) using primers I9FatoT48285FW (5′-ctgagctcacaactgcttaggtcagc-3′) and I9FatoI148285REV (5′-ggtgcttagctccagagttgtcgctc-3′) and at position 151 (from P to T) using I9FptoT48285FW (5′-ttctcctcataccatacagcttc-3′) and I9FptoT48285REV (5′-gtagagtctccagagttgtcgctc-3′). Mutagenesis at both positions was performed with all four primers.

The capsid gene of Stoon-1010-8 was mutated at amino acid position 191 (from E to R) using primers StoonetoR1121FW (5′-agactacaaacctagttgaccagcacta-3′) and StoonetoR1121REV (5′-taagcttgttcctcattcaggtttgtcctg-3′) and position 131 (from T to P) using StoonetoP1147FW (5′-tgatgatacttgctacaagagacgacgctc-3′) and StoonetoP1121REV (5′-taagcttggtctccttgctcagcctc-3′). Mutagenesis at both positions was performed with the four primers simultaneously.

**Transfection of PK-15 cells and production of PCV2 virus.** The HindIII-digested (48285-24, I9F-13 and Stoon-1010-8 and their mutants) plasmids were transfected onto PCV-negative PK-15 cells with Lipofectamine (Invitrogen), according to the manufacturer’s instructions. Seventy-two hours post-transfection, PCV2-transfected cells and supernatant fluids were collected. PCV2-transfected cells were smeared onto glass slides, fixed in methanol at −20°C for 10 min and stored at −20°C until use. Thereafter, cell smears were air-dried at room temperature for 10 min and stained by indirect immunofluorescence staining, adapted from Saha et al. (2012b). mAb F190 (McNeill et al., 2001) or biotinylated purified porcine pAbs were used as primary antibodies, and FITC-labelled goat anti-mouse pAbs or Texas red-labelled streptavidin (both from Molecular Probes) were used as secondary antibodies. Cells were visualized with a fluorescence microscope as described previously (Lefebvre et al., 2008b). Supernatant fluids were passed several times on PCV-negative PK-15 cells and the resulting supernatant fluids were titrated on PCV-negative PK-15 cells as described by Meerts et al. (2005).

**Sequence analysis.** The sequences of the PCV2 genomes (48285-24, I9F-13 and Stoon-1010-8 and their mutants) in the pcR-bluntII-TOPO vector were determined using M13FW, M13REV and PCV2-specific primers (Lefebvre et al., 2008a; Lefebvre et al., 2009) and PCV2 viruses produced from these plasmids were sequenced as described previously (Lefebvre et al., 2008a; Lefebvre et al., 2009).

**Reactivity of mAbs to different PCV2 strains in immunoperoxidase monolayer assay (IPMA).** The reactivity of the mAbs to different PCV2 strains was described previously (Lefebvre et al., 2008a; Saha et al., 2012a). In the present study, serial fourfold dilutions, starting from 1:10, of the four neutralizing mAbs 16G12, 38C1, 63H3 and 94H8 were made in PBS and used to test their reactivity to different PCV2 strains (original PCV2 viruses, the viruses derived from infectious clones and the mutant viruses) in an IPMA, similar to the technique described previously (Lefebvre et al., 2008a; Saha et al., 2012a). These assays were performed three times for each PCV2 strain.

**SN assay.** The neutralizing activity of mAbs 16G12, 38C1, 63H3 and 94H8 on different PCV2 strains was determined in an SN assay, identical to the technique described by Lefebvre et al. (2008a). The original PCV2 strains 48285 and Stoon-1010 (neutralized by the mAbs) and 1147, 1121 and I9F (not neutralized by the mAbs) (Lefebvre et al., 2008a) were enclosed as controls. These experiments were performed three times for each PCV2 strain.

The neutralizing activities of mAbs 13D12 and 1C11 were −2±15% and 13±19%, respectively. Because the mean ± SD neutralizing...
activity of mAb 1C11 was 13 + 19 = 32 %, a mAb was arbitrarily considered as neutralizing when its mean neutralizing activity was >32 %.

Statistical analysis. Neutralization percentages of the mAbs on the PCV2 viruses derived from infectious clones and the mutants were compared by applying an unpaired t-test with Welch’s correction e.g. the neutralization percentages of mAbs to 48285 were compared with that of 48285(T190A). Similarly, the neutralization percentages of mAbs to 1010-B were compared with those of 1010(E191R), 1010(E191R/T131P) and 1010(E191R/T131P). The neutralization percentages of mAbs to I9F-13 were compared with those of I9F(A190T), I9F(E191R/T131P) and I9F(E191R/T131P). Statistical analyses were performed using GraphPad Prism software version 5.0b (GraphPad Software, Inc.). Differences were considered significant when P < 0.05.

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