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BRIEF REPORT

Identification of three new type-specific antigen epitopes in the capsid protein of porcine circovirus type 1

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Abstract Porcine circovirus type 1 (PCV1) has been identified as a contaminant of porcine kidney cell line (PK-15). Serological evidence and genetic studies have suggested that PCV1 is widespread in domestic pigs. In this study, monoclonal antibodies (mAbs) and polyclonal antibodies (pAbs) were generated against a recombinant PCV1 Cap protein (PCV1-Cap), which was expressed using the baculovirus system. PEPSCAN analysis was used to identify epitopes on the PCV1-Cap with mAbs and pAbs. Three linear B-cell epitopes, including residues $_{85}$GGTNPLP$_{91}$, $_{162}$FTPKPELDKTIDWFHPNNK$_{180}$ and $_{219}$YVQFREFILKDPLNK$_{233}$, specific for PCV1-Cap, were finely defined. These results will facilitate future investigations into antigenic differences and differential diagnosis between PCV1 and PCV2.

Porcine circovirus type 1 (PCV1) has been identified as a persistent non-cytopathic contaminant of the continuous porcine kidney cell line (PK-15) and is characterized as a small, non-enveloped virus with a capsid diameter of 17 nm [17]. This small virus is classified as a member of the family *Circoviridae,* genus *Circovirus*, along with porcine circovirus type 2 (PCV2), which is associated with several clinical manifestations collectively known as porcine circovirus-associated diseases (PCVAD) with post-weaning multisystemic wasting syndrome (PMWS) as the most significant manifestation [4]. Serological surveys have demonstrated a high prevalence of anti-PCV1 antibodies in the swine population [2, 3, 5, 19], although no disease could be assigned to this virus either in PCV1-positive farms or after experimental inoculation [1, 18]. PCV1 has therefore been considered non-pathogenic to pigs. But recently, PCV1 has also been described as pathogenic to porcine foetuses [14].

The PCV1 genome consists of a circular single-stranded DNA of 1759 nt [13]. It has a genomic organization similar to that of PCV2, containing two major open reading frames (ORFs): ORF1, which encodes two proteins (Rep and Rep′) that are involved in virus replication [11, 12], and ORF2, whose product is the major structural capsid protein. PCV1 and PCV2 are related to each other, since they have about 70% nucleotide sequence identity. The ORF1-encoded proteins of the two viruses, with about 80% amino acid (aa) identity, have been shown to be antigenically related, whereas the ORF2 proteins, with about 60% aa identity, are recognized differentially by polyclonal anti-PCV2 antibodies [10]. However, common epitopes also are found in the capsid proteins of PCV1 and PCV2 [10, 15].

For epitopes of PCV1, two common reactive regions (residues 25–43 and 169–183) in the PCV1 capsid protein (PCV1-Cap) have been revealed by PEPSCAN analysis using anti-PCV2 antiserum [10], and one specific epitope (residues 92–103) along with two common epitopes (residues 156–162 and 175–192) have been mapped with monoclonal antibodies (mAbs) against PCV1 and PCV2 by PEPSCAN analysis [15]. However, to date, there is lack of fine mapping of type-specific epitopes of PCV1. The present study aims to map type-specific linear epitopes on PCV1-Cap.

For that purpose, mAbs and polyclonal antibodies (pAbs) against PCV1-Cap were prepared. To obtain the mAbs against PCV1-Cap, a recombinant baculovirus

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expressing recombinant capsid protein derived from a PCV1/G strain was produced and identified as described by Liu et al. [8] (data not shown). The production of mAbs against the recombinant PCV1-Cap (PCV1-rCap) was performed following a similar procedure as for PCV2 mAbs [6]. Two indirect ELISAs based on PCV1-rCap and the recombinant PCV2-rCap were used as detection methods as described previously [8]. The hybridoma was screened when the result of the PCV1-ELISA was positive while that of the PCV2-ELISA was negative. Five positive hybridomas secreting PCV1 mAbs were selected and cloned by limiting dilution to harvest three stable hybridomas. These mAbs were named as 2C10, 3A8 and 5D1. The supernatant titers of mAbs 2C10, 3A8 and 5D1 were 1:640, 1:640 and 1:1,280, respectively. The isotypes of the mAbs were determined using a Mouse MonoAb-ID Kit (HRP) (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The isotype of mAbs 2C10 and 3A8 was IgG1, and that of mAb 5D1 was IgG3. The light chains of these three mAbs were of the κ type. The reactivity of mAbs to PCV1/G and PCV2/LG was determined by western blot analysis as described elsewhere [6]. MAb 6F10, directed against PCV2-Cap, was used as a positive control [6]. MAb 4D1, directed against nonstructural protein 1 of West Nile virus, was used as a negative control [16]. All three mAbs gave a strong and specific reaction with the 28-kDa capsid protein of PCV1/G (Fig. 1). No reaction was observed with PCV2/LG except for mAb 6F10 (Fig. 1). MAb 4D1 did not show any reaction with either PCV1-Cap or PCV2-Cap (Fig. 1). This indicates that these three mAbs produced against PCV1-Cap had no cross-reaction with PCV2-Cap.

Polyclonal antibodies to PCV1-rCap were generated by immunizing a New Zealand white rabbit (from the Laboratory Animal Center of Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences). The rabbit received three subcutaneous injections of a 1 ml mixture containing 200 μg of purified PCV1-rCap and a 10% volume of the oil-in-water adjuvant ISA 15A VG (Seppic, Shanghai, China) at three-week intervals. Blood was harvested two weeks after the last injection. The polyclonal antibodies were named rabbit anti-PCV1/rCap serum. Rabbit anti-PCV2/rCap was generated using PCV2-rCap [8] following the same method as described above. Rabbit anti-PCV1/rCap serum and rabbit anti-PCV2/rCap serum were tested by immunoperoxidase monolayer assay (IPMA) as described previously by Liu et al. [9], and their antibody titers were 1:3,200 and 1:1,600, respectively.

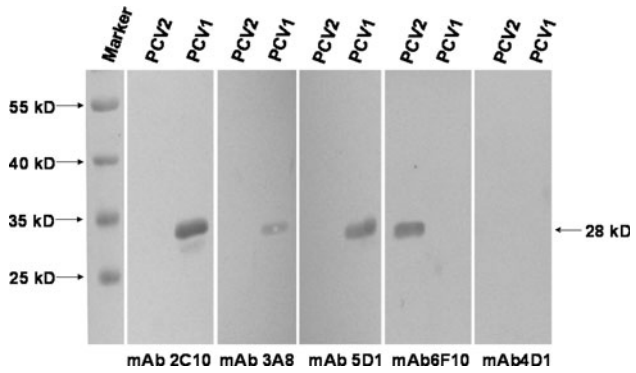

Fig. 1 Analysis of the immunoreactivity of mAbs by western blot analysis. Purified virions of PCV1 (G strain) and PCV2 (LG strain) were separated by SDS-PAGE, transferred to nitrocellulose membranes, and incubated with mAb 2C10, 3A8, 5D1, 6F10 (against PCV2-Cap [6]) as a positive control and 4D1 as a negative control. Lane M: protein molecular weight markers

To identify epitopes against mAbs and pAbs, 12 peptides of 25 aa spanning the capsid protein deduced from the PCV1/G strain were designed and synthesized (GL Biochem, Shanghai, China). Adjacent peptides had six aa residues in common and were designated as aa 1–25, aa 20–44, aa 39–63, aa 58–82, aa 77–103, aa 96–120, aa 115–139, aa 134–158, aa 153–177, aa 172–196, aa 191–215 and aa 210–233. The peptides were screened by an indirect ELISA. Briefly, an ELISA plate was coated with the synthesized peptides (30 μg per well) and incubated overnight at 4°C. The plate was then blocked with 1% bovine serum albumin (BSA) for 1 h at 37°C. The plate was then washed three times with PBS containing 0.05% Tween 20 (PBS-T), and 100 μl of undiluted hybridoma supernatant or diluted pAb (1:100) was added to each well and incubated at 37°C for 1 h. Following three washes with PBS-T, the bound mAbs and pAbs were detected with a 1:5,000 dilution of horseradish peroxidase (HRP)-conjugated protein A (Invitrogen). After three further washes with PBS-T, the color was developed by adding 100 μl of 2, 2-azino-di [3-ethylbenzthiazoline sulfonic acid] (0.21 mg/ml) in 0.1 mol/L citrate (pH 4.2) containing 0.003% hydrogen peroxide (ABTS substrate), and the reaction was terminated by adding 50 μl of 1% NaF solution. The $OD_{405\ nm}$ value of each well was read using a microplate reader. An irrelevant mAb 6F10 (against PCV2-Cap), a PCV-negative rabbit serum and a PCV-negative pig serum were used as controls. The cutoff value for the ELISA was determined as the mean $OD_{405\ nm}$ values of the negative control plus three standard deviations (SD). All samples were analyzed in triplicate, and the results are presented as the average of these readings ± SD. The data for the mAbs and the rabbit anti-PCV1/rCap serum specifically reactive with the overlapping peptides are summarized in Fig. 2A. The peptide of aa 172–196 was recognized by mAb 2C10. The peptide of aa 153–177 was recognized by mAb 3A8. Both of these peptides were recognized by mAb 5D1. The peptides of aa 77–103, 134–158, 153–177 and 210–233 were recognized by rabbit anti-PCV1/rCap serum. Based on these results, 29 other

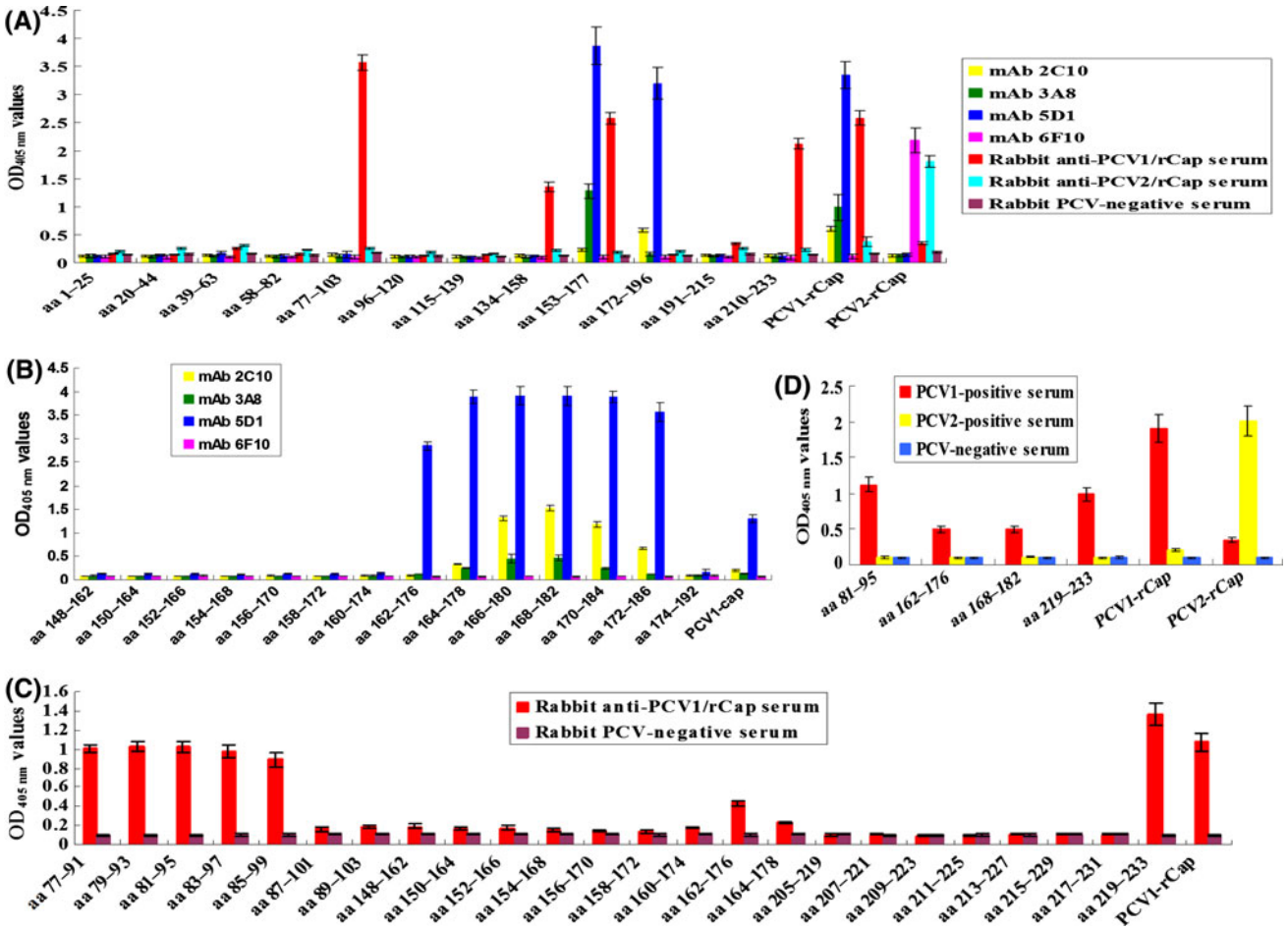

Fig. 2 Fine mapping of the linear B-cell epitopes on the capsid protein of PCV1 by peptide ELISA. (**A**) Overlapping peptides (25 aa) spanning PCV1-Cap were analysed for their reactivity with mAbs and rabbit anti-PCV1/rCap serum. (**B**) Overlapping peptides (15 aa) spanning aa 148 to 192 of PCV1-Cap were analysed for their reactivity with three mAbs. (**C**) Overlapping peptides (15 aa) spanning aa 77 to 103, 148 to 178 and 205 to 233 of PCV1-Cap were analysed for their reactivity with rabbit anti-PCV1/rCap serum. (**D**) Detection of aa 81–95, aa 162–176, aa 168–182 and aa 219–233 with PCV1-positive, PCV2-positive and PCV-negative sera by peptide-ELISA

overlapping 15-mer peptides spanning aa 77–103, 148–186 and 205–233 were subsequently synthesized (GL Biochem, Shanghai, China). Adjacent peptides had 13 aa residues in common and were designated as aa 77–91, aa 79–93, aa 81–95, aa 83–97, aa 85–99, aa 87–101, aa 89–103, aa 148–162, aa 150–164, aa 152–166, aa 154–168, aa 156–170, aa 158–172, aa 160–174, aa 162–176, aa 164–178, aa 166–180, aa 168–182, aa 170–184, aa 172–186, aa 174–192, aa 205–219, aa 207–221, aa 209–223, aa 211–225, aa 213–227, aa 215–229, aa 217–231 and aa 219–233. These peptides were used for further mapping of epitopes and were screened using the ELISA method described above. The peptide ELISA results are showed in Fig. 2B and C. Peptides of aa 164–178, 166–180, 168–182, 170–184 and 172–186 were recognized by mAb 2C10, and the common aa sequence among those peptides was aa $_{172}$IDWFHPN$_{178}$. Peptides of aa 164–178, 166–180, 168–182 and 170–184 were recognized by mAb 3A8, and the common aa sequence among those peptides was $_{168}$LDKTIDWFHPNNK$_{182}$. Peptides of aa 162–176, 164–178, 166–180, 168–182, 170–184, and 172–186 were recognized by mAb 5D1, and the common aa sequence among those peptides was $_{172}$IDWFH$_{176}$. Therefore, epitopes identified with mAbs were $_{172}$IDWFHPN$_{178}$, $_{168}$LDKTIDWFHPNNK$_{180}$ and $_{172}$IDWFH$_{176}$. Peptides of aa 77–91, 79–93, 81–95, 83–97, 85–99, 162–176 and 219–233 were recognized by rabbit anti-PCV1/rCap serum (Fig. 2C). The common aa sequence among aa 77–91, 79–93, 81–95, 83–97 and 85–99 was $_{85}$GGTNPLP$_{91}$, the lack of two of these aa residues ($_{85}$GG$_{86}$) in aa 87–101, resulted in no reactivity with rabbit anti-PCV1/rCap serum. This indicates that the core aa sequence comprising the epitope was $_{85}$GGTNPLP$_{91}$ and that the dipeptide $_{85}$GG$_{86}$ is important for this epitope. The peptide $_{162}$FTPKPELDKTIDWFH$_{176}$, which reacted with rabbit anti-PCV1/rCap serum, was identified as an epitope of PCV1-Cap. Another epitope identified with rabbit anti-PCV1/rCap serum was peptide

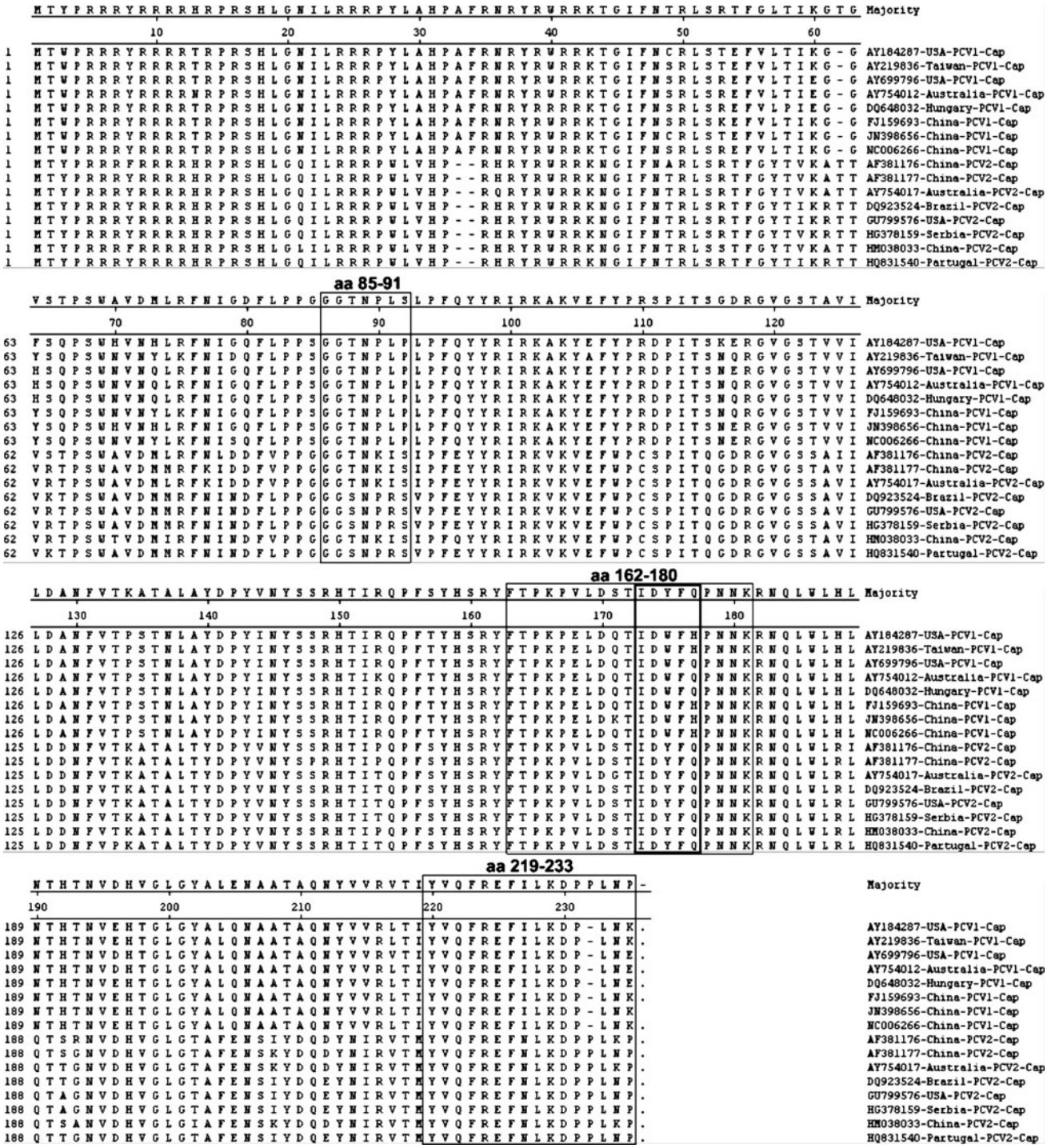

Fig. 3 Alignment of three epitopes with the capsid protein sequences of PCV1 and PCV2. A total of eight PCV1 strains and eight PCV2 strains were used in the analysis. The sequence motif recognized by mAbs or rabbit anti-PCV1/rCap is boxed

$_{219}$YVQFREFILKDPLNK$_{233}$. Interestingly, the peptide ELISA result with aa 217–231 was negative, while the result with aa 219–233 was positive. This demonstrates that the critical aa residues in epitope 219–233 were $_{232}$NK$_{233}$. The immunoreactivity of the artificial peptides (aa 81–95, aa 162–176, aa 168–182 and aa 219–233) containing epitopes of PCV1-Cap was confirmed using PCV1-positive, PCV2-positive and PCV-negative serum from pigs [6] by a peptide ELISA similar to that mentioned above. From the results shown in Fig. 2D, we could conclude that these four peptides were reactive with the PCV1-positive sera but not with the PCV2-positive sera, confirming that those

type-specific epitopes were recognized by the immune system of swine.

Multiple alignments of amino acid sequences in the capsid protein of eight PCV1 isolates (GenBank accession numbers: AY184287, AY219836, AY699796, AY754012, DQ648032, FJ159693, NC006266 and JN398656) and eight PCV2 isolates (GenBank accession numbers: AF381176, AF381177, AY754017, DQ923524, GU799576, HG378159, HM038033 and HQ831540) were performed using the Clustal W method within the DNASTAR software (version 7.0). Analysis of capsid protein sequences demonstrates that the epitope $_{85}$GGTNPLP$_{91}$ is highly conserved (100% aa identity), while the other two epitopes are not conserved among the PCV1 strains used in this study (Fig. 3).

The unique structural protein of PCV1 is the capsid protein. It is the main target for vaccine design and for the development of immunity-based diagnostic techniques for controlling PCV1. To date, identification of B-cell antigenic epitopes for PCV1-Cap has been limited, although four common epitopes (aa 25–43, aa 169–183, aa 156–162 and aa 175–192) and only one type-specific epitope (aa 92–103) have been identified [10, 15]. In this study, we identified three novel linear B-cell epitopes (aa 85–91, aa 162–180 and aa 219–233) specific for PCV1-Cap. To our knowledge, they are new finely mapped B-cell epitopes in PCV1-Cap. Furthermore, we found that the epitope of $_{85}$GGTNPLP$_{91}$ is highly conserved (100% aa identity) among the PCV1 strains used in this study (Fig. 3). Residues 89–91 may be responsible for discriminating between PCV1 from PCV2. For epitope $_{219}$YVQFREFILKDPLN*K*$_{233}$, the last two residues, $_{232}$NK$_{233}$, are very important for rabbit anti-rCap serum binding to this epitope (Fig. 3C). Similar results have been reported for the epitopes of PCV2-Cap [7, 15]. For the epitope of mAb 5D1, the core motif is $_{172}$IDWFH$_{176}$. The residue tryptophan, a hydrophobic amino acid, at position 174 in the PCV1-Cap is highly conserved and different from the corresponding tyrosine residue (hydrophylic) of PCV2-Cap. Therefore, residue 174 may be responsible for the difference in binding of mAb 5D1 to PCV1 versus PCV2 in this epitope. From Fig. 3B we can conclude that mAbs 2C10, 3A8 and 5D1 bind to overlapping epitopes, $_{172}$IDWFHPN$_{178}$ for 2C10, $_{168}$LDKTIDWFHPNNK$_{180}$ for 3A8, and $_{172}$IDWFH$_{176}$ for 5D1. There are two possible explanations for this result: 1) The region of $_{168}$LDKTIDWFHPNNK$_{180}$ may be one of the predominant domains in the PCV1-Cap. 2) Those three mAbs may have different affinity and contribute to overlapping epitopes.

In present study, we have identified three novel type-specific linear epitopes for PCV1-Cap. The results of this study will facilitate future investigations of the antigenic differences and differential diagnosis of PCV1 and PCV2.

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