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Fine mapping of antigenic epitopes on capsid proteins of porcine circovirus, and antigenic phenotype of porcine circovirus Type 2

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ABSTRACT

Type 2 porcine circovirus (PCV2) is associated with post-weaning multisystemic wasting syndrome in pigs. In this study, three monoclonal antibodies (mAbs) against the capsid protein (Cap) of PCV2, eight mAbs to Cap of type 1 porcine circovirus (PCV1) and five mAbs specific for Cap of both PCV1 and PCV2, were generated and used to finely map the antigenic sites of PCV1 and PCV2, and to identify the antigenic phenotype of PCV2 with different length of genome. Five linear B-cell epitopes, including the residues 231–233 and 195–202 specific for PCV2, residues 92–103 specific for PCV1, and residues 156–162 and 175–192 shared between PCV1 and PCV2, were finely defined with synthetic peptides, and the critical residue in epitope 231–233 and 156–162 was located at residues 233 (^{233}Proline) and 156 (^{156}Tyrosine), respectively. The conformational epitopes recognized by mAbs with neutralizing activity against both PCV1 and PCV2 were detected in transfected PK-15 and the residues 231–233 also participated in the formation of conformational epitopes. Analysis of antigenic diversity on these epitopes exhibited three antigenic phenotypes of PCV2, ^{1766}PCV2, ^{1767}PCV2 and ^{1768}PCV2 using mAbs. The results from this study first demonstrated the different antigenic phenotype between PCV2 isolates.

1. Introduction

Porcine circovirus (PCV), a member of the family Circoviridae, is a small, non-enveloped virus with a circular, single-stranded DNA genome (Tischer et al., 1982). Porcine circovirus type 1 (PCV1) was originally isolated from a porcine kidney cell line PK-15 as a persistent contaminant (Tischer et al., 1982) and thought to be nonpathogenic for experimentally infected pigs (Allan et al., 1995; Tischer et al., 1986). In contrast, porcine circovirus type 2 (PCV2) is pathogenic and believed to be the primary causative agent of a newly emerging multifactorial swine disease, post-weaning multisystemic syndrome (PMWS) (Allan et al., 1998; Allan and Ellis, 2000; Clark, 1997; Ellis et al., 1998). PCV2 has also been associated with porcine dermatitis and nephropathy syndrome (PDNS) (Meehan et al., 2001; Rosell et al., 2000), porcine reproductive disorders (Ladekjaer-Mikkelsen et al., 2001; Meehan et al., 2001; West et al., 1999) and other disease syndromes (Segales et al., 2005). However, serological surveys indicated that both PCV1 and PCV2 were widespread in swine populations (Allan and Ellis, 2000; Segales and Domingo, 2002; Tischer et al., 1995; Zhou et al., 2006).

The two types of PCV have 68 to 76% nucleotide identity (Cheung and Bolin, 2002) and a similar genomic organization containing two major open reading frames (ORFs) (Hamel et al., 1998; Meehan et al., 1998; Morozov et al., 1998). ORF1 encodes two replication-associated proteins (Rep and Rep') (Cheung, 2003; Mankertz and Hillenbrand, 2001; Mankertz et al., 1998), and ORF2 encodes a viral capsid protein (Cap) involved in the host immune responses (Mahe et al., 2000; Meehan et al., 1998; Nawagitgul et al., 2000). The capsid protein between PCV1 and PCV2 have 65% amino acid identity (Morozov et al., 1998) and contain a nuclear localization signal (NLS) at the N terminus (Liu et al., 2001a). Capsid protein of PCV2 (PCV2 Cap) expressed in baculovirus-infected insect cells and *Escherichia coli* was demonstrated to be immunoreactive with swine anti-PCV2 serum (Liu et al., 2001b; Mahe et al., 2000; Nawagitgul et al., 2002; Zhou et al., 2005). Monoclonal antibody (mAb) with neutralizing activity against PCV2 Cap (Lefebvre et al., 2008; Lekcharoensuk et al., 2004; McNeilly et al., 2001; Zhou et al., 2005) and against PCV1 Cap (Allan et al., 1994) has also been produced and characterized.

Recently, four linear immunodominant regions on the PCV2 Cap, including a common reactive region with PCV1 Cap (residues

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169–183), were revealed by PEPSCAN analysis (Mahe et al., 2000) and at least five overlapping conformational epitopes of PCV2 Cap, within residues 47–85, 165–200 and 230–233 were mapped by chimeric PCV1 and PCV2 (Lekcharoensuk et al., 2004). Using mAbs to PCV2 Cap, antigenic differences of various PCV-2 strains was also demonstrated (Lefebvre et al., 2008). However, to date, there is lack of mapping of fine antigenic epitopes of PCV2. Meanwhile, all antigenic epitopes present probably on PCV2 Cap still remain to map to understand capsid-induced protective immune response. Also, the cross-reactivity between PCV1 and PCV2 was still indefinite since the common epitope on capsid protein between PCV1 and PCV2 was explained to be inaccessible (Mahe et al., 2000). Until now, epitope mapping of antigenic differences between PCVs have not yet been accomplished.

In this study, mAbs to Cap protein of PCV1 and PCV2 were produced, characterized, used to finely map type-specific and common antigenic epitopes on Cap protein between PCV2 and PCV1 and to identify antigenic differences among different genotypic PCV2 strains. Finally, the immunodominant epitopes of PCV2 Cap were further evaluated by PCV2 antisera from experimentally infected pigs.

2. Materials and methods

2.1. Cells, virus and serum

PCV-free and PCV1-contaminated PK-15 cells were maintained in minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GibcoBRL Life Technologies, Grand Island, NY) at 37 °C with 5% CO_2. PCV2 isolates TZ0601(Genbank no. EU257516) and JH0602 (Genbank no. EU257512) with a genome of 1766 nucleotides (^{1766}PCV2), and HZ0201 (Genbank no. AY188355), HZ0301 (Genbank no. AY510375), NB0301 (Genbank no. AY391729) and SX0201 (Genbank no. AY536755) with a genome of 1767 nucleotides (^{1767}PCV2) were propagated in PK-15 cell as previously described (Zhou et al., 2006). PCV2 isolate ISU-31(Genbank no. AJ223185) with a genome of 1768 nucleotides (^{1768}PCV2) was provided kindly by Dr. Yoon from Iowa State University. Swine anti-PCV2 serum and mAb to VP1 protein of infectious bursal disease virus were stored in our laboratory.

2.2. Preparation of mAbs against PCV capsid protein

To generate mAbs to Cap protein of PCV2 and PCV1, three immunogens were prepared for the vaccination of mice. Recombinant PCV2 Cap protein was produced as previously described (Zhou et al., 2005), recombinant PCV1 Cap protein was expressed in *E. coli* by cloning truncated ORF2 of PCV1 into pET28a(+) vector (unpublished data), and PCV2 virions from infected PK-15 cells were purified through sucrose gradient.

For each antigen, five mice were immunized, and each was injected intraperitoneally three times at 3-week intervals with 50 μg of the antigen mixed with complete or incomplete Freund's adjuvant. The appearance of antibodies to the antigen in immunized mice was tested on PCV2-infected PK-15 cells by IFA as stated (Zhou et al., 2005). Three days prior to fusion, the mice were boosted intraperitoneally with 80 μg of the antigen. The mice were subsequently euthanized, the spleen was harvested and fused with SP2/O myeloma cells as described previously (McNeilly et al., 2001). The fused cells were cultured with RPMI 1640 medium containing hypoxanthine–aminoprerin–thymidine. Resulting hybridoma cells were maintained in RPMI 1640 medium containing hypoxanthine–thymidine and were cloned by limiting dilution. Hybridoma supernatants were screened for the presence of PCV Cap-specific antibodies by IFA. Furthermore, analysis of mAb IgG subtype was performed with standard procedures illuminated by the protocol of SBA Clonotyping™ System/HRP (Southern Biotechnology Associates, Inc., Birmingham, AL35260, USA). Finally, ascites containing mAb to PCV Cap protein were prepared and mAb titer was determined by PCV recombinant Cap-based ELISA assays as recently reported (Shang et al., 2008).

2.3. Western blot

Lysates of host cells containing parental vector, the purified recombinant Cap proteins of PCV1 and PCV2, purified PCV1 and PCV2 virions, were separated by SDS-PAGE on 12% polyacrylamide, and were transferred to a nitrocellulose membrane (Amersham). Then the membrane was blocked with 5% skim milk in PBS, and incubated with 1:10 hybridoma supernatants at 37 °C for 2 h, respectively. After three washes in PBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma, USA) at 37 °C for 1 h. Finally the blots were developed with tetramethylbenzidine (TMB) stabilized substrate after three washes as mentioned above.

2.4. Virus neutralization (VN) assay

A focus reduction assay was used to detect the VN activity of the mAbs as previously described (Lekcharoensuk et al., 2004). Briefly, PCV virus stock was diluted to an infectious dose of $10^{4.0}$ $TCID_{50}$/ml in MEM medium. 50 μl of the diluted virus stock was mixed with equal volume of the serial 10-fold dilution (10^{-1} to 10^{-6}) of the heat-inactivated mAbs, and then were incubated for 2 h at 37 °C. The mAb to VP1 of infectious bursal disease virus and anti-PCV2 swine serum were treated likewise as negative and positive controls, respectively. Afterwards, the virus–antibody mixtures were inoculated on the 80% confluent monolayer of PCV-free PK-15 cells. Three replications were performed for each mAb dilution and control. After further incubation for 72 h at 37 °C with 5% CO_2, cells were fixed and stained for PCV2 antigens by IFA technique. The average number of PCV2-infected cells per well was counted from selected five fields in each well and a mAb is considered as having neutralizing activity if it can reduce the average numbers of positive foci by more than 90% compared to the negative control.

2.5. Peptide ELISA and dot-ELISA to determine epitopes with overlapping peptides

Twenty overlapping 18-mer peptides shifted by 10 amino acids, spanning residues 25–233 of PCV2 Cap protein, were initially synthesized by the solid-phase peptide synthesis method using a Symphony Multiplex Peptide Synthesizer (Protein Technologies, Inc., USA). Peptide purity was greater than 90% as assessed by HPLC and mass spectrometry. During synthesis, a cysteine residue was added at the N-terminal of all peptides except those that have a cysteine residue in the position for conjugation. Peptides were conjugated to the carrier protein BSA using heterobifunctional cross-linker Sulfo-SMCC (Sigma). These BSA-conjugated peptides were tested for their reactivity with mAbs by ELISA as followed. After an immunoactive peptide was identified, its N-truncated, C-truncated and Ala-substituted derivatives were further synthesized and used to define the epitope motif by ELISA.

Peptide ELISA was performed to analyze the reactivity of epitope peptides with mAbs and swine anti-PCV2 serum. Microtiter plates (Nunc, Denmark) were coated with 100 μl of 1 μg/ml BSA-conjugated peptide in 0.05 M carbonate buffer (pH 9.6) at 4 °C for 24 h. The plates were washed and blocked as above. After incubation with 1:500 diluted mAb or anti-PCV2 swine serum at 37 °C for 2 h, the plates were washed five times with PBST and incubated

Table 1
The summary of the primers used in this study.

Primer name	Nucleotide sequence	Tm
Up1-230	CTTCTCGAGATGACGTATCCAAGGAG	65.5
Down230	CTTGTCGACTTATGGGGGGTCTTTAAG	66.7
Down192	CTTGTCGACTTAATTTCCAGCAGTTTGT	65.6
Down154	CTTGTCGACTTAGAAGGGCTGGGTTAT	66.5
Up61-233	CTTCTCGAGATGACAGTCAAAACGC	65.6
Down233	CTTGTCGACTTAAGGGTTAAGTGGGG	65.9
Up155-233	CTTCTCGAGATGTCCTACCACTCCC	65.6
Down204	CTTGTCGACTTAGTTTTCGAACGCAGT	66.7

Underlined text represents restriction enzyme digestion site.

with HRP-conjugated goat anti-mouse IgG at 37 °C for 1 h. Following five washes, the colorimetric reaction was developed using TMB chromogenic substrate (Sigma) for 10 min at 37 °C and stopped with 2 M H_2SO_4. The optical density at 450 nm (OD450) was recorded using universal Microplate Reader ELx800 (Bio-Tek Instruments, Inc., Winooski, VT, USA).

In peptide-dot ELISA, 1 μg of each BSA-conjugated peptide was dotted onto nitrocellulose membrane. Then the membrane was blocked with 5% skim milk in PBS and incubated with 1:1000 mAbs at 37 °C for 2 h, followed by incubation with HRP-conjugated goat anti-mouse IgG (Sigma) at 37 °C for 1 h. The membrane was washed at each incubation step with PBST. Color was developed by the 3-amino-9-ethylcarbazole staining system (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) according to the manufacturer's instruction. The carrier protein BSA was also included as negative controls.

2.6. Construction of eukaryotic expression vector for truncated Cap protein of PCV2

For mapping of conformational epitopes on PCV2 Cap protein, seven eukaryotic expression vectors containing truncated PCV2 Cap fragment as well as whole PCV2 Cap gene were constructed. Briefly, six nucleotide segments from PCV2 Cap gene, corresponding respectively, to amino acid residues 1–230, 1–192, 1–154, 61–233, 155–233, 155–204 of PCV2 Cap protein, were amplified with a panel of primers containing XhoI and SalI site (Table 1). PCR reaction condition was 30 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 30 s and extension at 72 °C for 45 s after predenaturation at 95 °C for 3 min, and a final extension at 72 °C for 10 min. The purified PCR products were digested with XhoI and SalI and inserted into the eukaryotic expression vector pEGFP-C3 (Clontech Laboratory, Inc., Mountain View, CA). Recombinant plasmid was transformed to *E. coli* Top10 strain (Invitrogen, Grand Island, NY), and confirmed by PCR, restriction enzyme digestion, and DNA sequencing.

2.7. In vitro transfection

PCV-free PK-15 cells were seeded in 96-well plates and grown to 80–90% confluence. After washing with OptiMEM medium (GibcoBRL), the cells were transfected with a mixture of recombinant plasmid and Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Each well contained 0.2 μg DNA and 0.5 μl lipofectamine 2000 in 50 μl medium. The cells were then covered with complete medium and the truncated Cap protein of PCV2 expressed in PK-15 cells 48 h after transfection was analysed by IFA as mentioned above.

2.8. Detection of antibodies against viral epitopes after swine PCV2 infection

For preparation of swine PCV2-positive serum, three 30-day-old PCV-free pigs were inoculated intranasally with 2 ml of PCV2 HZ0201 strain ($10^{6.6}$ $TCID_{50}$/ml). After inoculation, serum samples were collected at 1-week intervals from the pigs until 49 days postinoculation (dpi), and used to test the kinetic curves of the antibodies to the identified Cap epitopes with the above-mentioned peptide-based ELISA.

3. Results

3.1. Generation, characterization and reactivity of mAbs against PCV Cap protein

To prepare mAbs recognizing PCV virions, BALB/c mice were respectively, immunized with recombinant Cap protein of PCV2

Table 2
Characterization of monoclonal antibodies to Cap protein of PCV.

Antigen	mAb no.	Isotype, subclass	Indirect ELISA		Western-blot		IFA	
			PCV1 dCap	PCV2 dCap	PCV1 dCap	PCV2 dCap	PCV1	PCV2
Recombinant dCap protein of PCV2	8A12	IgG1,κ	–	+++	–	+++	–	+++
	1B3	IgG1,κ	–	+++	–	+++	–	+
	8B12	IgG1,κ	–	+++	–	+++	–	+++
	1B9	IgG1,κ	–	+++	–	+++	–	+
	8C12	IgG1,κ	–	+++	–	+++	–	+++
	1C7	IgG1,κ	–	+++	–	+++	–	+++
	5E11	IgG1,κ	–	+++	–	+++	+++	+++
	3F6	IgG1,κ	–	+++	–	+++	+++	+++
PCV2 virion	2B1	IgG1,κ	–	–	–	+	–	+++
	7F5	IgG2a,κ	–	–	–	+	+++	+++
	6H9	IgG1,κ	–	++	–	++	+++	+++
Recombinant dCap protein of PCV1	1A11	IgG1,κ	+++	–	++	–	+	–
	4A2	IgG1,κ	+++	–	++	–	+	–
	2D4	IgG1,κ	++	–	++	–	+	–
	1D11	IgG1,κ	+++	–	++	–	+	–
	3F11	IgG2b,κ	++	–	++	–	+	–
	2F5	IgG2a,κ	++	–	++	–	+	–
	2G9	IgG2b,κ	++	–	++	–	+	–
	3H1	IgG1,κ	+++	–	++	–	+	–
	4H7	IgG1,κ	+++	+++	++	++	+	+

Note: In ELISA, "+++", OD450 ≥ 1.0; "++", 0.5 ≤ OD450 < 1.0; "+", 0.1 < OD450 < 0.5; "–", OD450 ≤ 0.1. In Western, "+++", Highest dilution of mAb supernatant >1:512; "++", >1:128 and ≤1:512; "+", >1:16 and ≤128. "–", no reactivity. In IFA, "+++", strong reactivity; "++", moderate reactivity; "+", weak reactivity. "–", no reactivity.

(Zhou et al., 2005), purified PCV2 virions and recombinant Cap protein of PCV1 as antigens. After repeatedly cloning by limiting dilutions and screening by ELISA, 20 hybridoma cell lines secreting mAbs were developed and their IgG subclasses were identified (Table 2), None of these mAbs reacted with lysates of uninfected PK15 cells or *E. coli* BL21 strain.

Western blot and IFA assays were conducted showing that most of the mAbs originated from either Cap proteins of PCV1 or PCV2 have no cross-reactivity except for the mAbs 5E11, 3F6, 7F5, 4H7 and 6H9 that reacted with both PCV1 and PCV2 (Table 2). In the mAbs generated against PCV2 particles, only mAb 2B1 can react with recombinant PCV2 Cap protein and PCV2 virions, but mAbs 7F5 and 6H9 could react with PCV1 particles, indicating that mAb 2B1 are specific for PCV2. Although mAbs 3F6 and 4H7 could recognize PCV1 and PCV2 virions, mAb 3F6 could not recognize recombinant PCV1 Cap protein while 4H7 could, indicating that

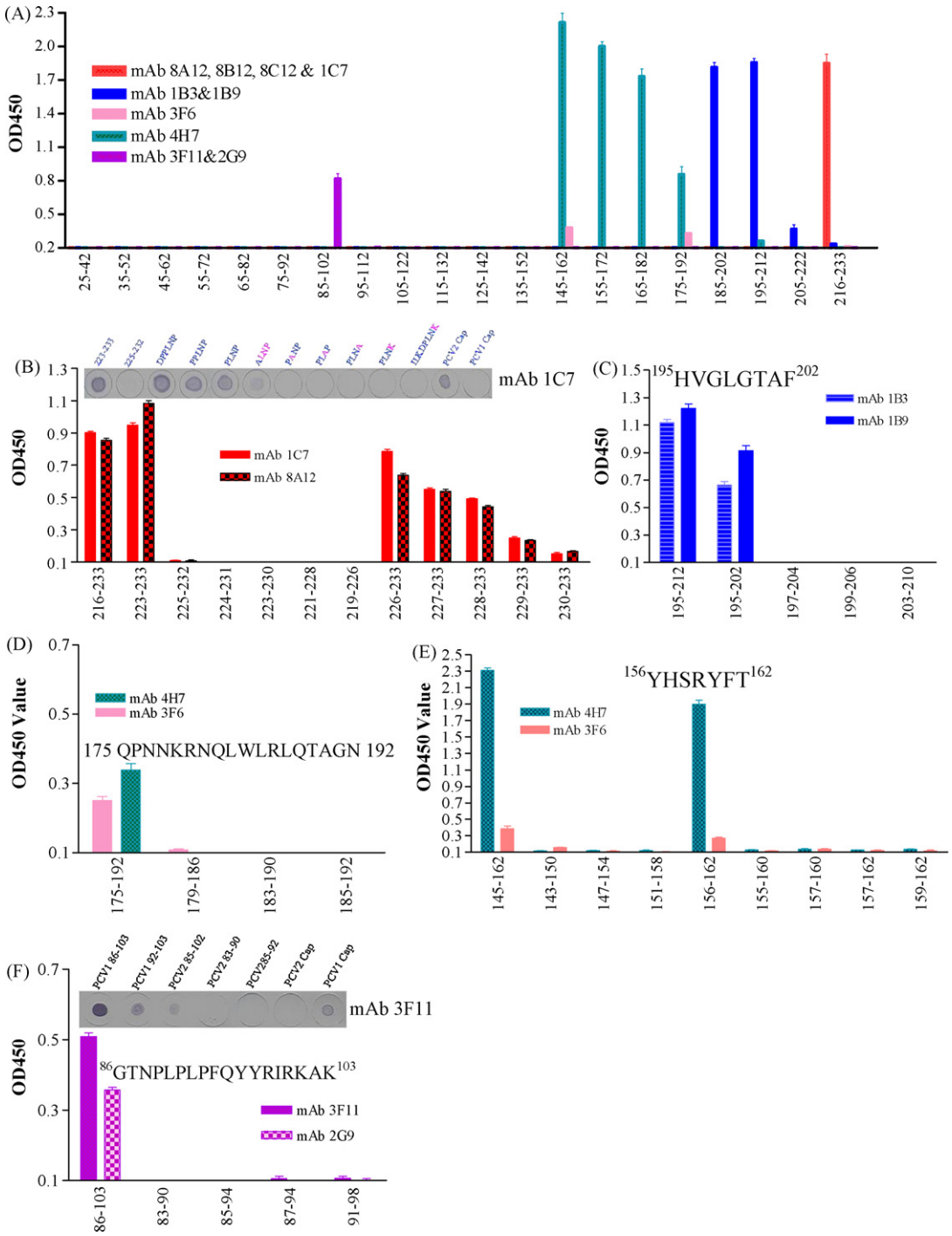

Fig. 1. Fine mapping of the linear B cell epitopes on cap protein of PCV with peptide-ELISA and peptide-dot ELISA. (A) Five immunoreactive peptides, respectively spanning residues 216–233, 195–212, 175–192, 145–162 and 85–102 were initially probed by 11 of 21 mAbs against PCV Cap. (B) Using the C- or N-terminal truncated or alanine-substituted derivatives, the epitope motif within the residues 216–233 was localized to three amino acids (^{231}LNP^{233}) and the ^{233}Pro was shown to be a type-specific and key amino acid. (C) The peptide of the residues 195–212 was finely defined to the residues ^{195}HVGLGTAF^{202}. (D) The residues ^{175}QPNNKRNQLWLRLQTAGN^{192} failed to be further located. (E) The peptide of the residues 145–162 was further detected to the residues ^{156}YHSRYFT^{162} and the ^{156}Tyr was a critical residue for this epitope. (F) The residues ^{86}GTNPLPLPFQYYRIRKAK^{103} were finally located to the residues ^{92}LPFQYYRIRKAK^{103} with the mAbs 3F11 and 2G9 to Cap of PCV1.

the mAbs 3F6 and 4H7 might recognize common conformational and linear epitopes exhibited within PCV1- and PCV2-infected cells. These mAbs were subsequently used in this study for fine mapping of the linear and conformational antigenic epitopes of PCV Cap protein.

3.2. Neutralizing activity of mAbs

Virus-neutralizing activity of these mAbs was determined with ^{1766}PCV2, ^{1767}PCV2, ^{1768}PCV2 and PCV1 using a focus reduction assay. Among the generated mAbs, mAbs 3F6, 7F5 and 6H9 to Cap protein of PCV had the neutralizing ability to both ^{1766}PCV2 and ^{1767}PCV2 with a neutralizing titer ranged from $10^{-3.3}$ to $10^{-4.0}$ (data not shown). The other 15 mAbs had no virus neutralizing activity (data not shown). The anti-PCV2 swine serum that served as a positive control showed strong virus neutralization (VN) activity.

3.3. Fine mapping of linear epitopes on capsid proteins between PCV2 and PCV1

For mapping and localization of the linear B-cell epitopes recognized by the mAbs shown in Table 2, the overlapping linear peptides of Cap protein were synthesized by PEPSCAN techniques. As shown in Fig. 1A, 5 of 20 18-mer peptides were recognized by 11 of 21 mAbs to PCV Cap and located, respectively in the amino acid residues 216–233 (designated as L-233) detected by PCV2-specific mAbs 8A12, 8B12, 8C12 and 1C7, 185–202 (designated as L-202) recognized by PCV2-specific mAbs 1B3 and 1B9, 175–192 (designated as L-192) and 145-162 (designated as L-162) recognized by mAbs 3F6 and 4H7 specific for PCV1 and PCV2, and the aa 85-102 (designated as L-102) recognized by PCV1-specific mAbs 3F11and 2G9.

Subsequently using shorter N- and C-terminal truncated peptides derived from these five 18-mer peptides, two PCV2 Cap linear epitopes, one PCV1 Cap linear epitope and one common linear epitope of Cap protein between PCV1 and PCV2 were finely defined and located, respectively within the residues 230–233 (L-233, Fig. 1B), 195-202 (L-202, Fig. 1C), 156–162 (L-162, Fig. 1E) and 92-103 (L-102, Fig. 1F). The epitope L-192 failed to be further defined for that no reaction of shorter peptide derivatives was observed with the corresponding mAbs (Fig. 1D). Further analysis of alanine substitution within the epitope L-233 (Fig. 1B) revealed that the proline at aa 230 is dispensable for the epitope, but the replacement of any residue within ^{231}LNP^{233} led to the loss of antigenicity of the peptide, indicating that ^{231}LNP^{233} is the core motif of the epitope. Although only one amino acid difference existed in the residues 230–233 between PCV2 Cap and PCV1 Cap, the peptide ^{230}PLNK^{233} from PCV1 Cap showed no reactivity with the mAb 1C7 recognizing ^{230}PLNP^{233} epitope of the PCV2 Cap, confirming that the proline at aa 233 (^{233}Pro) was a crucial and type-specific residue for PCV1 and PCV2. In addition, the deletion of tyrosine at aa 156 (^{156}Tyr) also resulted in the loss of the antigenicity of the corresponding peptide ^{156}YHSRYFT^{162}, showing ^{156}Tyr is also a critical residue for this epitope (Fig. 1E).

Table 3
Immunoactivity of mAbs to PCV Cap protein on the PK-15 cells transfected with truncated Cap gene fragment.

Peptide	mAb					Swine anti-PCV2 serum
	1C7	3F6	5E11	6H9	7F5	
$pep_{1–230}$	–	+++	+++	+++	–	++++
$pep_{1–192}$	–	–	–	–	–	++
$pep_{1–154}$	–	–	–	–	–	++
$pep_{61–233}$	+++	–	+	–	–	+++
$pep_{155–204}$	–	–	–	–	–	++
$pep_{155–233}$	+++	–	+++	–	–	+++
$Cap_{1–233}$	+++	+++	+++	+++	+++	+++

3.4. Detection of conformational epitopes on capsid protein

To identify the conformational antigenic sites, a set of peptide (pep) segments of PCV2 Cap partially containing or artificially deleting the linear epitopes were constructed and expressed in transfected PK-15 cells (Table 3). The IFA reactivities of these peps with the remaining mAbs which have no reactivity with linear peptides are shown in Table 3. All the remaining mAbs to PCV2 Cap were shown to recognize the entire capsid protein expressed in transfected PK-15 cells. However, the mAb 7F5 did not react with $pep_{1–230}$ and $pep_{61–233}$, indicating that the mAbs 7F5 recognize the conformational epitope composed of the motif ^{231}LNP^{233} and the aa 1–60 together. The mAbs 3F6 and 6H9 only reacted with $pep_{1–230}$ and Cap protein of PCV2, and not with other peptides expressed in the transfected PK15 cells, exhibiting that aa 1–230 contained a conformational epitope. The mAb 5E11 reacted strongly with $pep_{1–230}$ and pep $_{155–233}$, and weakly with $pep_{61–233}$, but not with $pep_{1–154}$, $pep_{1–192}$ and $pep_{155–204}$, indicating that aa 205–230 formed a conformational epitope.

3.5. Reactivity of mAbs with different genomic PCV2 isolates

To analyze antigenic diversity of the PCV2 isolates, the mAbs were further tested by IFA on PK-15 cells infected with different genotype of PCV2, including ^{1766}PCV2, ^{1767}PCV2 and ^{1768}PCV2. Data in Table 4 revealed that there were seven identical epitopes in Cap protein between ^{1766}PCV2 and ^{1767}PCV2, and four identical epitopes in Cap protein among ^{1766}PCV2, ^{1767}PCV2 and ^{1768}PCV2. ^{1766}PCV2 and ^{1768}PCV2 did not react with the mAb 1C7 to ^{1767}PCV2 Cap which recognized the L-233 epitope, indicating that the L-233 epitope recognized by the mAb 1C7 was a distinct marker of PCV2 with a genome of 1767 nucleotides. Meanwhile, ^{1768}PCV2 also did not react with the mAbs 2B1, 7F5 and 6H9 to Cap protein recognizing ^{1766}PCV2 and ^{1767}PCV2, indicating that the anti-PCV Cap mAbs 2B1, 7F5 and 6H9 were a differentiating marker of PCV2 with a genome of 1768 nucleotides. Therefore, using mAbs 1C7 and 2B1 or 7F5 or 6H9, PCV2 with different genome was divided into three antigenic phenotypes, designated as ^{1766}PCV2, ^{1767}PCV2 and ^{1768}PCV2.

Table 4
The antigenic phenotype of different genomic PCV2 identified with mAbs.

PCV2 isolate	Genome	mAb to PCV Cap						mAb 1C7 to PCV2 Cap
		2B1	5E11	3F6	7F5	4H7	6H9	
TZ0601	1766nt	+++	+++	+++	+++	+++	+++	–
JH0602	1766nt	+++	+++	+++	+++	+++	+++	–
HZ0201	1767nt	+++	+++	+++	+++	+++	+++	+++
HZ0301	1767nt	+++	+++	+++	+++	+++	+++	+++
NB0301	1767nt	+++	+++	+++	+++	+++	+++	+++
SX0201	1767nt	+++	+++	+++	+++	+++	+++	+++
ISU-31	1768nt	–	+++	+++	–	+++	–	–

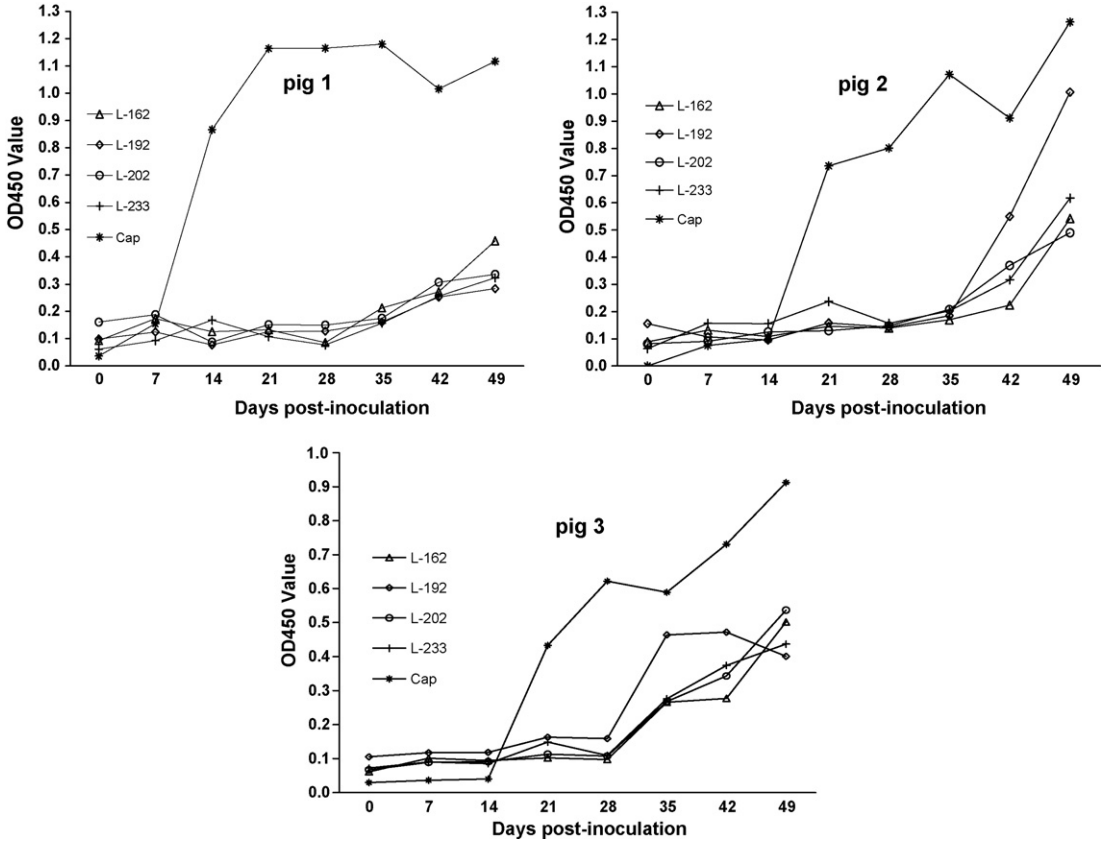

Fig. 2. Kinetic curves of antibodies against epitope peptides in PCV2-infected pigs. Four identified epitope peptides including aa145–162, 175–192, 195–212, 216–233, and PCV2 Cap were used as antigens to detect the corresponding antibody in swine anti-PCV2 sera collected from 0 to 49 days after inoculation. The antibodies to these epitope peptides increased apparently in the antisera after 28 days post-inoculation, but was delayed for 14 days as compared to antibody to Cap protein. The OD_{450nm} value indicates the values of the antiserum minus the background of negative serum.

3.6. Antibody response to the identified linear epitopes following PCV2 experimental infection

To investigate antigenicity of the identified linear epitopes in pigs, the kinetic changes of antibodies against the linear epitopes (L-233, L-202, L-192, L-162) were detected in swine anti-PCV2 serum by ELISA using the peptides as antigen. As shown in Fig. 2, swine anti-PCV2 Cap antibody began to be detected at 14 dpi and reached a peak at 28–35 dpi, however the antibodies to L-162, 192, 202 and 233 were detectable until 28 dpi, indicating that the induction of antibodies to the four linear epitopes are delayed during PCV2 infection. In addition, the antibodies to the four linear epitopes have a lower titer as compared to the antibody to PCV2 Cap. This implies that there may be more immunodominant epitopes within PCV2 Cap protein than the four epitopes.

4. Discussion

The Cap protein of PCV is major structural protein, immunogen and epidemiological marker for PCV2 (Blanchard et al., 2003; Fenaux et al., 2004; Olvera et al., 2007; Wang et al., 2006). Among the mAbs to Cap protein generated in this study (Table 2), eight mAbs generated against recombinant PCV2 Cap and three mAbs against purified PCV2 particles all reacted only with PCV2 Cap protein by Western blot analysis, but one mAb made against recombinant PCV1 Cap also recognized PCV2 Cap protein as well as PCV1 Cap protein. However, by IFA (Table 2), on PCV2- or PCV1-infected PK-15 cells, five mAbs were common for PCV1 and PCV2 particles, three mAbs were type-specific for PCV2 virions and eight mAbs were type-specific for PCV1 particles. Subsequent results of linear antigenic sites further demonstrated that the L-233 epitope only binds with the mAbs 8A12, 8B12, 8C12 and 1C7 specific for PCV2 Cap, and the L-202 epitope was solely recognized by the mAbs 1B3 and 1B9. Accordingly the L-102 epitope can react with mAbs 3F11 and 2G9 specific for PCV1 Cap. However, two linear epitopes L-162 and L-192 could react with mAbs 3F6 and 4H7specific for Cap protein of PCV1 and PCV2. Hence these data confirm that mAbs 8A12, 8B12, 8C12 and 1C7, and mAbs 1B3 and 1B9, respectively recognized two different linear epitopes, and the epitopes L-233 and L-202 are two type-specific linear epitopes for PCV2, and that the epitope L-102 is a type-specific linear epitope for PCV1.

Two linear epitopes were identified in the sequence of PCV2 Cap, the epitope L-202 had weak reaction with mAbs 1B3 and 1B9 to PCV2 Cap, but the epitope L-233 strongly reacted with mAbs 8A12, 8B12, 8C12 and 1C7 to PCV2 Cap in an IFA assay. The epitope L-202 failed to be further defined as no reaction of shorter peptide derivatives was observed with the corresponding mAbs. Moreover, the peptide of the epitope L-233 could be truncated to the residues 231–233 and keep the reactivity with the corresponding mAbs. This verified that the basic motif of the epitopes L-202 and L-233 was ^{195}HVGLGTAF^{202} and ^{231}LNP^{233}, respectively. In this study, the linear epitopes L-202 and L-233 were firstly finely defined in Cap protein of PCV2 though the peptide 193–207 was shown to be an immunoreactive region specific for swine anti-PCV2 antibody (Mahe et al., 2000).

Mahe et al. (2000) identified two immunoreactive peptides of the residues 157–183 and 193–207 on PCV Cap using swine anti-PCV2 antibody. To date, no cross-reactivity between mAbs against

the Cap proteins of PCV2 and PCV1 have been found (Lefebvre et al., 2008; Lekcharoensuk et al., 2004; McNeilly et al., 2001). Interestingly, in this study, three linear epitopes, aa156–162, aa179–192 and aa 195–202, were finely confirmed in two immunoreactive regions of the residues 157–183 and 193–207 identified in previous report (Mahe et al., 2000). Notably, only the residues 195–202 was detected to be type-specific for PCV2 Cap protein, but aa 156–162 and aa 179–192 were found to be common epitopes of Cap protein of PCV1 and PCV2. Moreover, the ^{156}Tyr deletion resulted in the loss of the antigenicity of the corresponding peptide ^{156}YHSRYFT^{162}, whereas the truncated epitope L-192 also lacked immunoreactivity to the corresponding mAbs. This showed that ^{156}Tyr is a critical residue for the linear epitope L-162 (Fig. 1E) and that the ^{175}QPNNKRNQLWLRLQTAGN^{192} was a basic motif of the linear epitope L-192 (Fig. 1D). Additionally, in the past, it was believed that PCV1 was widespread in swine population (Tischer et al., 1995). Whether this opinion resulted from existence of common antigenic sites of PCV1 and PCV2 will need further PCV1-specific serological investigation.

In our experiment, IFA reactivities of PK-15 cells transfected with a set of PCV2 Cap peptide (Table 3) displayed that three conformational epitopes were observed, that is, the residues 1–230 reacting with the mAbs 3F6 and 6H9, the residues 1–60 and 231–233 binding the mAb 7F5, and the residues 205–230 recognized by the mAb 5E11. These data indicate that the residues ^{231}LNP^{233} not only form a linear epitope L-233 specific for PCV2, but also participate in the formation of conformational epitope. In previous reports, the residue ^{230}PLNP^{233} at the C terminus of the PCV2 Cap protein were also demonstrated to participate in the formation of the conformational epitopes of PCV2 Cap (Lekcharoensuk et al., 2004). Correspondingly, the mAbs 3F6, 6H9 and 7F5 had the neutralizing activity to PCV2 virions, and the mAb 7F5 were also found to have neutralizing ability to PCV1 virions. Therefore, it was reasonable to believe that the conformational antigenic sites within the residue 1–60 and 231–233 are common neutralizing epitopes of both PCV1 and PCV2. These findings also imply that the residue ^{233}Pro is not closely related to the formation of conformational epitope. Although IFA analysis exhibits that the mAbs 6H9 and 7F5 could react with PCV1- and PCV2-infected PK-15 cells, the abovementioned data also shows that the conformational epitope within the residues 1–230 is a neutralizing antigenic site to PCV2, and non-neutralizing for PCV1.

Recently, two genotypes of PCV2 (1 and 2) were defined using the capsid protein as phylogenetic and epidemiological marker for PCV2 (Grau-Roma et al., 2008; Olvera et al., 2007), and the genome of PCV2 genotype 1 was believed to be 1767 nucleotides while PCV2 genotype 2 strains was 1768 nucleotides (Cheung et al., 2007). Meanwhile, antigenic difference on capsid protein for two genotype of PCV2 isolates was demonstrated using mAbs to PCV2 isolate Stoon-1010 (Lefebvre et al., 2008). However, the molecular characterization of antigenic difference on the capsid protein among PCV2 isolates is not directly demonstrated. In the present study, ^{1767}PCV2 and ^{1766}PCV2 could be distinguished from ^{1768}PCV2 using the mAb 2B1 or 7F5 or 6H9, while ^{1767}PCV2 could be differentiated from ^{1766}PCV2 using mAb 1C7 to the epitope L-233 of PCV2 (Table 4). Hence we are the first to find that ^{1766}PCV2, ^{1767}PCV2 and ^{1768}PCV2 are three different antigenic phenotypes within PCV2. However, whether the antigenic phenotypes accompanying genomic change of PCV2 results in variation of pathogenicity of PCV2 needs further evaluation.

Previous reports indicated that the B-133 epitope (the residues 117–133) was a serological marker for the late stage of PCV2 infection (Truong et al., 2001). In this report, by studying the antibody response in experimentally inoculated pigs, the antibodies to the four linear epitopes L-162, 192, 202 and 233 in PCV2-infected pigs were shown to be detectable although the appearance of the antibodies to four linear epitopes was postponed for 14 days compared to swine anti-PCV2 Cap antibody detected 14 dpi. These results indicate that the linear epitopes L-202 and L-233 may be used as a serologically type-specific marker of PCV2 infection. Furthermore, the biological significance of the delayed non-neutralizing antibodies to the four linear epitope needs further evaluation.

In present study, we have firstly clarified the fine linear epitopes specific for PCV2 Cap protein, and for PCV1 Cap protein, whilst the linear and conformational common epitopes between PCV2 and PCV1 were formulated. Furthermore, three antigenic phenotypes of PCV2 with different genome length were identified for the first time. These data provided further insight into antigenic differences between PCV2 isolates and a tool of differentiating the antigenic phenotypes of PCV2.

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