

Differential recognition of ORF2 protein from type 1 and type 2 porcine circoviruses and identification of immunorelevant epitopes

Dominique Mahé, Philippe Blanchard, Catherine Truong, Claire Arnaud, Pierre Le Cann, Roland Cariolet, François Madec, Emmanuel Albina and André Jestin

Agence Française de Sécurité Sanitaire des Aliments, Laboratoire de Biologie Moléculaire, BP 53, 22440 Ploufragan, France

Two types of porcine circovirus (PCV) have been isolated and are referred to as PCV1 and PCV2. PCV1 represents an apathogenic virus, whereas PCV2 is associated with post-weaning multi-systemic wasting syndrome. The two PCVs are related, since they display about 70% identity based on nucleotide sequences. In order to discriminate between common and type-specific antigens, an immunocytological approach was used following transfections with cloned circovirus DNAs, as well as recombinant proteins expressed by either baculovirus or plasmid vectors. The ORF1-encoded proteins in the two viruses were shown to be antigenically related, whereas the ORF2 proteins were recognized differentially by polyclonal anti-PCV2 antibodies. Furthermore, PEPSCAN analysis performed on overlapping fragments of the genes encoding part of ORF1 and the entire ORF2 and ORF3 led to the identification of five dominant immunoreactive areas, one located on ORF1 and four on ORF2. However, only some ORF2 peptides proved to be immunorelevant epitopes for virus type discrimination. The potential use of ORF2-derived antigens as diagnostic tools is demonstrated.

Introduction

Porcine circovirus type 1 (PCV1) was identified as a persistent non-cytopathic contaminant of the continuous porcine kidney cell line PK-15 and was characterized as a small, non-enveloped virus with a capsid size of 17 nm (Tischer *et al.*, 1982). This small virus was classified in a newly recognized virus family, the *Circoviridae* (Lukert *et al.*, 1995), along with other animal viruses including beak and feather disease virus of psittacine birds (Bassami *et al.*, 1998; Niagro *et al.*, 1998) and chicken anaemia virus (Todd *et al.*, 1990). Whereas PCV was reassigned as the type member of the genus *Circovirus*, chicken anaemia virus has been proposed to represent the type member of a new genus, *Gyrovirus*, within this family (Pringle, 1999). Common characteristic features of the circoviruses are their small size and their circular single-stranded DNA genomes. The PCV1 genome consists of a circular single-stranded DNA of 1759 nt (Meehan *et al.*, 1997). The replicative form contains seven putative ORFs with the potential to encode proteins

larger than 5 kDa. Two of them represent major ORFs; ORF1, which encodes a protein of 35·7 kDa involved in virus replication (Mankertz *et al.*, 1998*a*), and ORF2, whose product of 27·8 kDa is suspected to represent the major structural capsid protein. Serological surveys have demonstrated a high prevalence of anti-PCV1 antibodies in the swine population (Dulac & Afshar, 1989; Edwards & Sands, 1994; Hines & Lukert, 1995; Tischer *et al.*, 1995), although no disease could be assigned to this virus either in PCV1-positive farms or after experimental transmission (Allan *et al.*, 1995; Tischer *et al.*, 1986). PCV1 was therefore considered to be non-pathogenic.

Recently, a new situation has appeared with the emergence, in France and other European countries as well as in North America, of a new porcine disease known as post-weaning multisystemic wasting syndrome (PMWS). The disease is characterized clinically by fever and progressive weight loss, as well as respiratory and digestive disorders (Allan *et al.*, 1998*a, b*; Kennedy *et al.*, 1998; Kiupel *et al.*, 1998; Le Cann *et al.*, 1997; Morozov *et al.*, 1998; Nayar *et al.*, 1997; Segalés *et al.*, 1997). Morbidity rates ranging from 5 to 30% have been reported in affected batches of pigs (Madec *et al.*, 2000). Virus isolation from the tissues of pigs affected with PMWS led to

Author for correspondence: Dominique Mahé.

Fax +33 2 96 01 62 83. e-mail d.mahé@ploufragan.afssa.fr

the identification of PCV-like antigens and nucleic acids (Ellis *et al.*, 1998; Nayar *et al.*, 1997). These viruses were found to form a closely related group, exhibiting more than 96% sequence identity (Allan *et al.*, 1998*b*; Meehan *et al.*, 1998). However, they were significantly different from the PCV1 strain, thus representing a new type of porcine circovirus, which has been termed PCV2. Although they display similar genomic organization, their genomes share only about 70% sequence identity. Higher sequence conservation could be observed in the replicative ORF1-encoded protein (Rep protein).

PCV2s are believed to be of aetiological importance in PMWS (Allan *et al.*, 1999*a*; Balasch *et al.*, 1999; Ellis *et al.*, 1999) and the need for type-specific serological assays is therefore significant in examining the prevalence of PCV2 infection. The aim of the present study was to characterize the expression of the major viral proteins, to analyse their immunological properties and thereby to determine the extent of cross-reactivity between viral antigens from PCV types 1 and 2.

Methods

■ PCR amplification and cloning

DNA extraction. Lymph nodes from PMWS-affected piglets were kept frozen at -80°C prior to DNA extraction. DNA was extracted by using the Qiagen tissue kit according to the manufacturer's recommendations and used for all subsequent PCV1 and PCV2 PCR assays.

PCR amplification and cloning of PCV genomes. The PCV1 genome was cloned by utilizing an inverse PCR approach with forward (5' GGCGGCCATCTGTAACGGTTT 3') and reverse (5' GATGGCGCCGAAAGACGGGTATC 3') primers, each containing a *NarI* restriction endonuclease site (underlined). These primers were designed according to the PCV1 sequence presented in the accession number AF012107 (Mankertz *et al.*, 2000) except that the forward primer was modified by two nucleotides in its 5' end without affecting the PCV1 sequence between the *NarI* sites. The PCV2 genome was cloned in a same inverse PCR approach by using forward (5' GTGGAGCTCCTAGATC-TCAGGG 3') and reverse (5' TAGGAGCTCCACTCCATCAG 3') primers deduced from the PCV2 sequence presented in the accession number AF201311 (Mankertz *et al.*, 2000). Each primer contains a *SacI* restriction endonuclease site (underlined).

Amplifications were performed with $0.4\ \mu\text{M}$ of each primer, $0.1\ \text{mM}$ of each dNTP and $2.5\ \text{U}$ *Taq* DNA polymerase (Boehringer) with the following cycling parameters: denaturation at 94°C , 5 min; 35 cycles (94°C , 30 s; annealing at 60°C , 30 s; 72°C , 90 s); final elongation at 72°C , 10 min. PCR products were then cloned into the pPCR-Script SK vector by using the pCR-Script Amp cloning kit (Stratagene). The clones were sequenced on both strands with an ABI 373 sequencer by using the M13 forward and reverse universal primers as well as circovirus genome-specific primers and the ABI Prism Dye terminator cycle sequencing kit (PE Biosystems).

Cloning of PCV genes. The genes encoding ORF1 and ORF2 from both PCVs were amplified by PCR prior to cloning into the pcDNA3.1 mammalian expression vector (Invitrogen) and into either pVL1393 or pAcGHLT baculovirus transfer vectors (PharMingen), the latter allowing the expression of glutathione S-transferase (GST)- and $6 \times \text{His}$ -fused proteins. The following PCR primer pairs were used for

PCV gene amplifications: for PCV1, ORF1 was amplified with ORF1.1 forward (5' CGGGATCCCAGTGGAAAATGCCAAGCAAGAAAAG 3') and reverse (5' CGGGATCCCGATGTGATAACAAAAAAGACTCAG 3') primers, whereas ORF2 was amplified with ORF2.1 forward (5' CGGGATCCCTTTTTTGTATACATCGTAATGG 3') and reverse (5' CGGGATCCTCTTTCACTTTTATAGGATGACGTG 3') primers. For PCV2 gene amplification, ORF1.2 forward (5' ATGGATCCAGCAGACACATGCCAGCAAG 3') and reverse (5' GGGGATCCGAAGTGATAAAAAAGACTCAG 3') primers were used to generate the ORF1 gene whereas ORF2 was amplified by using ORF2.2 forward (5' CCGGATCCTCCATTAAGGGTTAAGTGGG 3') and reverse (5' CCGGATCCTCAGATATGACGTATCCAAG 3') primers. All primers included a *Bam*HI cloning site in their 5' end. Amplifications were performed as already mentioned except for the annealing temperature, which was 55°C , and the elongation time, which was shortened to 45 s.

PCR products corresponding to the PCV2 genes were cloned into the pPCR-Script SK plasmid as indicated above. Recombinant clones were sequenced to ensure that the sequences did not contain mutations. The recombinant plasmids were then digested with *Bam*HI. The purified fragment encoding the ORF1-PCV2 protein was subcloned into *Bam*HI-cut pcDNA3.1 and pVL1393 plasmids, whereas the purified fragment encoding the ORF2-PCV2 protein was subcloned into *Bam*HI-digested pcDNA3.1 and *Bgl*II-digested pAcGHLT.

For the PCV1 genes, PCR products were cloned directly into pcDNA3.1 vector after digestion with *Bam*HI. Sequencing was performed for both constructs. The fragments encoding ORF1-PCV1 and ORF2-PCV1 were then purified and cloned into *Bam*HI-digested pVL1393 and *Bgl*II-digested pAcGHLT, respectively.

■ Antisera

Anti-PCV2 antisera. Lymph nodes obtained from PMWS-affected pigs and checked by PCR for the presence of PCV2 and the absence of PCV1 genomes were homogenized and ultrafiltered ($0.45\ \mu\text{m}$) before intramuscular and intratracheal inoculations to either specific-pathogen-free (SPF) or conventional 6-week-old pigs. Sera were collected before virus inoculation and weekly for a maximum of 6 weeks post-infection (p.i.).

Hyperimmune sera. Hyperimmune serum against ORF2-PCV2 was generated in SPF pigs that received two intramuscular injections of $200\ \mu\text{g}$ of each pcDNA3.1 plasmid encoding ORF2-PCV2 and porcine granulocyte/macrophage colony-stimulating factor at 3 week intervals. Animals were then boosted 3 weeks later with $1\ \text{ml}$ Sf9 cells (5×10^6) infected by a recombinant baculovirus encoding the ORF2-PCV2 protein and $1\ \text{ml}$ Montanide adjuvant (Seppic). Blood was harvested 3 weeks after the last injection.

Polyclonal antibodies to ORF1-PCV1 were generated by immunizing mice (Agro-Bio). BALB/c mice received four injections of $50\ \mu\text{g}$ of the corresponding recombinant pcDNA3.1 vector at 2 week intervals and two additional injections at 8 and 10 weeks.

■ Transfection/infection assays. The permanent PK-15 cell line, which was free of PCV, was maintained in minimal essential medium (E-MEM, Biowhittaker) supplemented with 5% heat-inactivated foetal calf serum, penicillin and streptomycin. Cells were seeded in 24-well plates (3.5×10^4 cells per well) and grown to 60–70% confluence. After one wash with OptiMEM medium (Gibco BRL), cells were transfected for 8 h with $0.1\ \mu\text{g}$ DNA (either plasmids encoding viral genes or viral genomes) and $2.25\ \mu\text{g}$ Transfectam reagent (Promega), according to the manufacturer's protocol, except that OptiMEM was used for material dilution instead of serum-free E-MEM. For genome transfections, *NarI*-digested PCV1 and *SacI*-digested PCV2, obtained from the corresponding cloned

genomes, were gel-purified and recircularized in the presence of T4 DNA ligase (Boehringer) overnight at room temperature before being transfected. Cells were then overlaid with complete medium and viral protein expression was analysed by an immunoperoxidase monolayer assay (IPMA) as described below, 48 h after gene transfections or 72 h after genome transfections. In the case of genome transfections, cells were additionally treated 24 h after transfection with 300 mM D-glucosamine as described previously (Tischer *et al.*, 1987).

For the infection test, genome-transfected cells were subjected to three successive freeze–thaw cycles. Following a centrifugation step at 2000 g for 5 min, lysate supernatants were collected and used to infect PCV-free PK-15 cells. Freshly trypsinized cells were seeded into 96-well plates and, once semi-confluent, they were inoculated for 1 h at 37 °C with lysate supernatant. They were then subjected to glucosamine treatment as described above and analysed by IPMA 72 h after infection.

For IPMA analysis, cells were fixed and permeabilized at –20 °C with 80% cold acetone and washed with PBS. The cells were then incubated for 1 h at 37 °C with 4% dry milk (Bio-Rad) in PBS containing 0.05% Tween 20 (PBS/Tw) and 0.1% Triton X-100 and incubated for a further 90 min at 37 °C with antisera diluted 100 times in PBS/Tw containing 2% dry milk. After three washes with PBS containing 0.25% Tween 20, cells were incubated for 90 min at 37 °C with HRP-conjugated rabbit anti-mouse or anti-swine IgG (Dako) in PBS/Tw containing 2% dry milk and washed again. Staining was developed in the presence of 9-aminoethyl carbazole (Sigma) in dimethylformamide as well as hydrogen peroxide and was stopped by substrate removal.

■ **Generation and production of recombinant baculoviruses.** Sf9 cells, maintained in TC100 medium (Gibco BRL) supplemented with 5% foetal calf serum, were co-transfected with 500 ng linearized Baculogold baculovirus DNA (Pharmingen) and 5 µg recombinant baculovirus transfer vector by using the DOTAP transfection reagent (Boehringer) according to the supplier's instructions. Recombinant baculoviruses were plaque-purified and amplified.

■ **Expression and identification of recombinant proteins.** Sf9 cells were infected with wild-type *Autographa californica* multiple nuclear polyhedrosis virus or recombinant baculoviruses at an m.o.i. of 10 p.f.u. per cell. The cells were harvested 72 h p.i. and protein expression was analysed by immunoblot. Total proteins from 10⁵ cells were then separated on a 10% SDS–polyacrylamide gel and transferred onto a nitrocellulose membrane (Bio-Rad). The proteins were identified by using either an anti-GST polyclonal antibody (Pharmingen) or an anti-PCV antiserum and a peroxidase-labelled rabbit anti-mouse or anti-swine IgG conjugate (Dako).

■ **PEPSCAN analysis.** The simultaneous synthesis of peptide sequences was carried out on a cellulose membrane by Fmoc amino acid chemistry (SYNT:EM). Overlapping peptides covering the last 214 and 213 amino acids, respectively, of ORF1 from PCV1 and PCV2 (102 peptides) as well as the complete sequence of ORF2 (112 peptides) and ORF3 (73 peptides) from both PCVs were synthesized on the membrane. The peptides, which were 15 amino acids in length, overlapped by 11 amino acids and differed by a four amino acid shift. Antibody reactivity to the membrane-bound peptides was analysed by an indirect colorimetric immunoassay according to the manufacturer's recommendations (SYNT:EM).

Briefly, the membranes were incubated overnight in blocking buffer at 4 °C. After washing once in 50 mM Tris–HCl, pH 7, containing 150 mM NaCl, 30 mM KCl and 0.05% Tween 20, the membranes were incubated with serum diluted 100 times in blocking buffer. Development was

performed in the presence of BCIP, MTT and MgCl₂. Spots corresponding to peptides with antibody reactivity produced a positive blue signal, which was quantified by using the NIH 3.1 Image software.

Results

Antigenic profile study following transfection with cloned PCV genomes

PK-15 cells transfected with the replicative form of either PCV1 or PCV2 were screened by IPMA for the presence of viral antigens by using experimental antisera (Fig. 1). Parallel infection assays were performed after transfection to ensure that the cloned PCV genomes used were functional in producing infectious virus particles. As shown in Fig. 1(a), the serum generated in SPF pig after PCV2 infection revealed the presence of immunoreactive proteins in PCV1- and PCV2-transfected cells, thus indicating the existence of serological cross-reactivity between proteins from the two types of PCV. Hyperimmune sera generated against the major ORF1- and ORF2-encoded viral proteins were used to define better the extent of this serological cross-reaction. The serum generated against ORF1-PCV1 protein revealed a nuclear signal in PCV1- and also in PCV2-transfected cells (Fig. 1a), thus demonstrating the existence of common antigenic determinants between ORF1-encoded proteins. However, hyperimmune serum generated against ORF2-PCV2 could only detect the protein in PCV2-transfected cells, whereas no signal was observed in cells transfected with PCV1 genome (Fig. 1a). These results were confirmed further by IPMA on cells infected with PCV1 and PCV2 (Fig. 1b).

Immunoreactivity study on recombinant proteins

In order to check the potential absence of cross-reactivity between the two ORF2 proteins and to confirm the presence of common immunoreactive epitopes on the ORF1 proteins, the corresponding coding sequences were cloned into either plasmid or baculovirus expression vectors and expressed as recombinant proteins in mammalian and insect cells, respectively. When tested on PK-15 cells transfected with the recombinant vector expressing ORF2 protein from PCV1 or PCV2, both anti-PCV2 antiserum and ORF2-PCV2 hyperimmune antiserum could detect ORF2 from PCV2, whereas they both failed to detect ORF2 from PCV1 (Fig. 2). The same proteins were also expressed as GST-fused proteins in the baculovirus system. The synthesis of the recombinant proteins in infected Sf9 cells was monitored by Western blot with a GST-specific antibody. In each case, a band of the expected size (GST plus ORF) was identified (Fig. 3b), thus attesting to the correct expression of both proteins. However, whereas the ORF2-PCV2 protein was recognized by a serum generated in pig following PCV2 infection, no reactivity could be observed for the ORF2-PCV1 protein (Fig. 3a). The absence of serological cross-reactivity when experimental anti-PCV2 antisera were used was further confirmed with sera collected at different times p.i. (Table 1). Indeed, ORF2 immunoreactivity

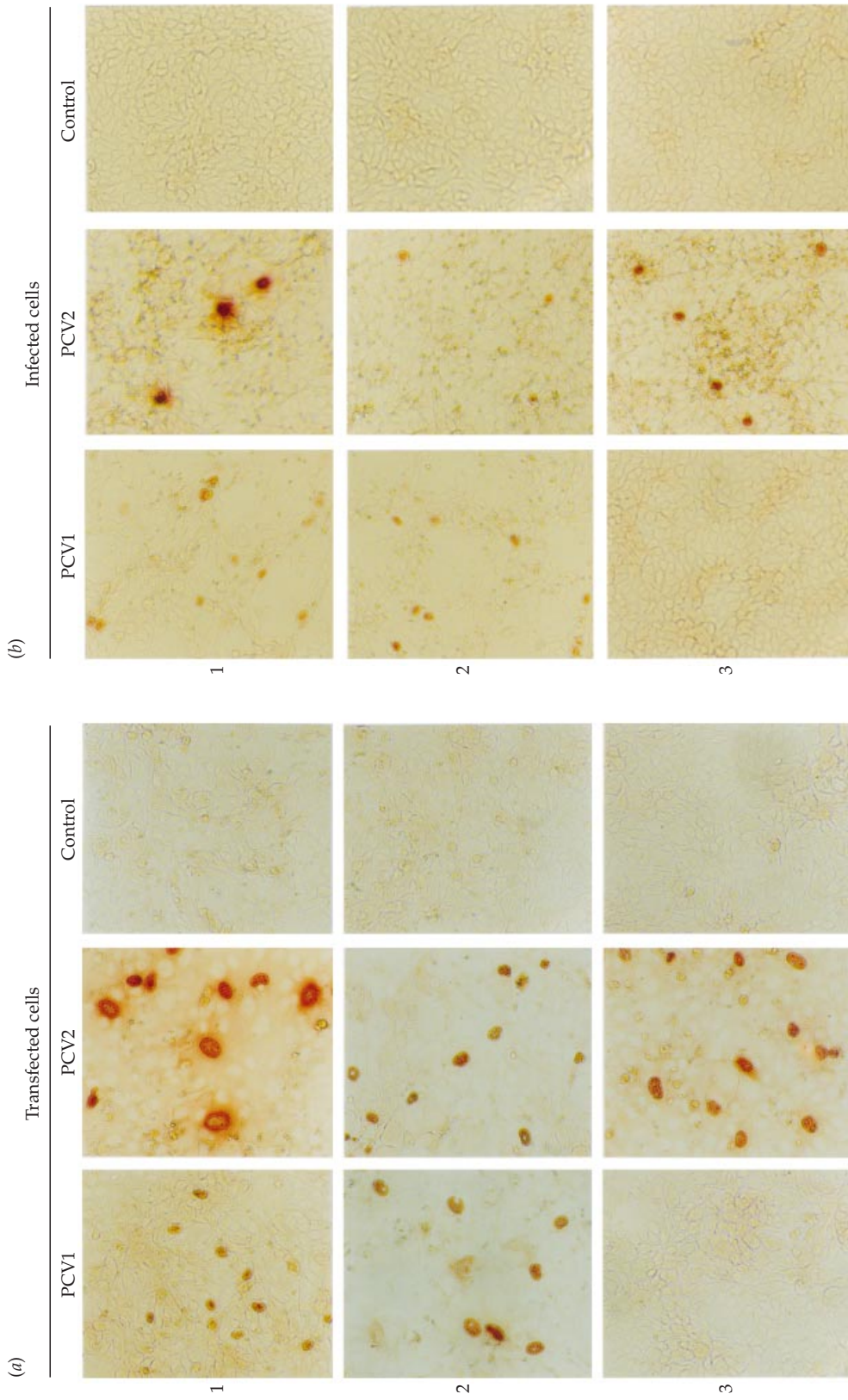


Fig. 1. PK-15 cells were either (a) transfected with cloned genomic DNA or (b) infected. In each case, PCV1 (left panels), PCV2 (middle panels) or the absence of PCV material (control, right panels) was used. Viral antigens were detected by IPMA with either experimental anti-PCV2 antiserum generated in SPF piglet and collected 6 weeks p.i. (row labelled 1) or polyclonal antibodies raised in mouse against PCV1-ORF1 (row 2) and in swine against PCV2-ORF2 (row 3).

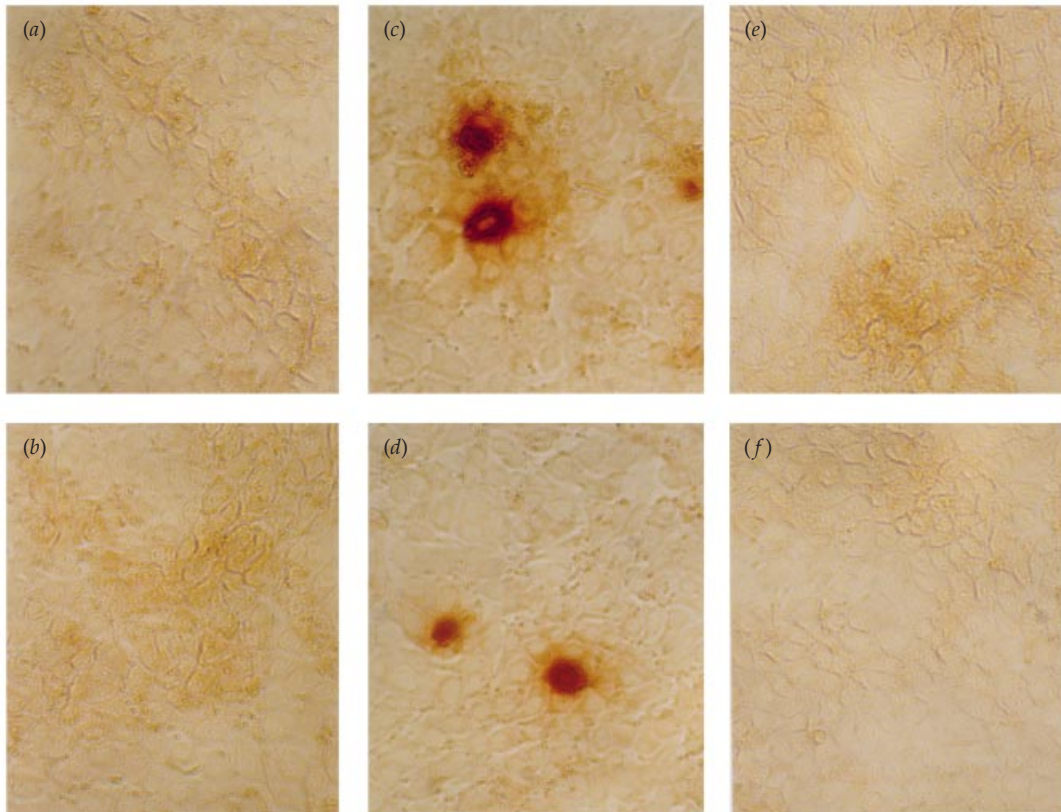


Fig. 2. PK-15 cells were transfected with expression vectors encoding ORF2-PCV1 (a, b) or ORF2-PCV2 (c, d) or were mock transfected (e, f). Cells were fixed 48 h post-transfection and analysed by IPMA with anti-PCV2 antiserum generated in SPF piglet and collected 6 weeks p.i. (a, c, e) or with hyperimmune serum generated against ORF2-PCV2 (b, d, f).

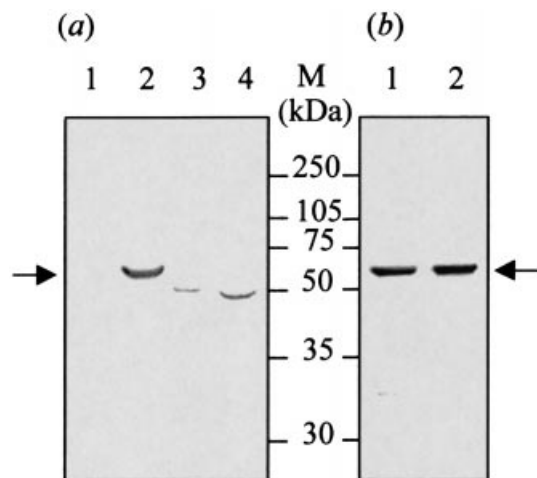


Fig. 3. Western blot analysis of GST-fused ORF2 proteins from PCV1 and PCV2. Total lysates of Sf9 cells infected with pAcGHLT-A recombinant baculovirus expressing a GST-fused ORF2-PCV1 (lanes 1) or ORF2-PCV2 (lanes 2) or wild-type baculovirus (lane 3) and a total lysate of non-infected Sf9 cells (lane 4) were incubated with a swine anti-PCV2 antiserum (a) or with an anti-GST antibody (b). The positions of molecular mass markers are shown.

Table 1. Reactivity of experimental anti-PCV2 sera collected at different times p.i.

PK-15 cells were transfected separately with plasmids encoding the indicated ORFs from PCV type 1 or 2 and were assayed by IPMA for the reactivity of experimental sera collected at different times following experimental infection with PCV2. The intensity of the signal detected in IPMA was graded visually from - to ++.

Animal	Transfection		Time p.i. (days)						
	ORF	PCV type	0	7	14	21	28	35	42
1	2	1	-	-	-	-	-	-	-
	2	2	-	-	+	++	++	++	++
	1	2	-	-	-	±	+	+	++
2	2	1	-	-	-	-	-	-	-
	2	2	-	-	-	+	++	++	++
	1	2	-	-	-	±	+	+	++
3	2	1	-	-	-	-	-	-	-
	2	2	-	-	+	++	++	++	++
	1	2	-	-	-	+	++	++	+

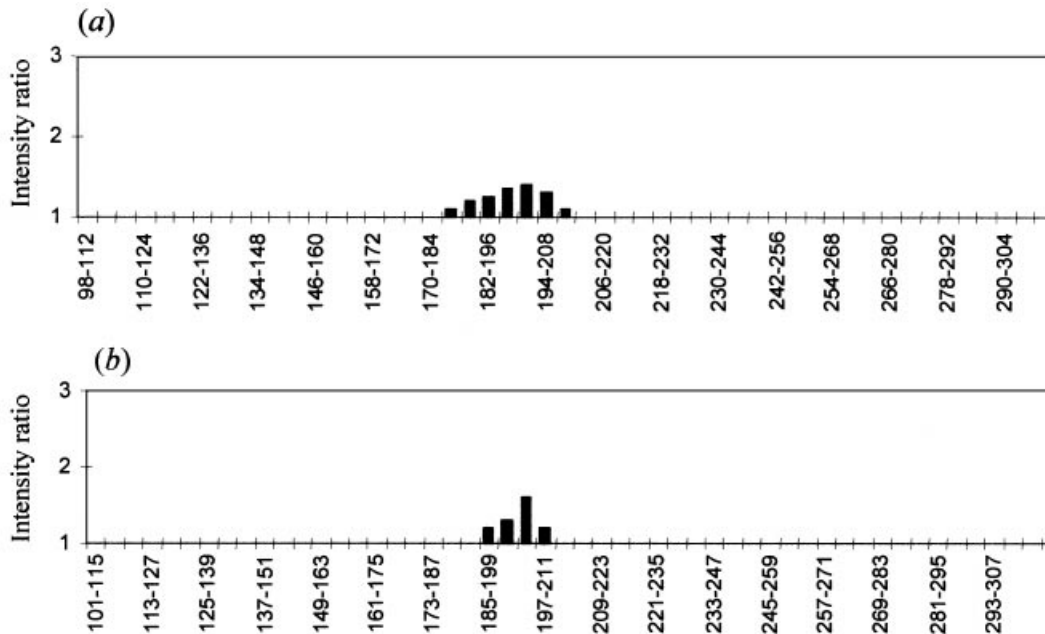


Fig. 4. Immunoreactivity of anti-PCV2 antiserum generated in experimentally inoculated SPF piglet with overlapping peptides spanning residues 98–304 of ORF1-PCV1 (a) or residues 101–307 of ORF1-PCV2 (b). The intensity ratio indicates the ratio between values obtained for the serum collected 6 weeks post-inoculation and the serum collected before experimental inoculation. Peptides are indicated by their amino acid locations.

could be detected only for PCV2 antigens and appeared from 2 weeks p.i. As expected for ORF1-encoded proteins, the proteins from both viruses could be detected by anti-PCV2 and anti-ORF1 antisera (not shown). Moreover, the kinetic analysis of antisera following experimental infection, which was performed on ORF1 from PCV2 alone, indicated a later appearance of anti-ORF1 antibodies after infection compared with the anti-ORF2 antibodies (Table 1).

Epitope mapping with anti-PCV2 antibodies

In order to characterize better the antigenic domains recognized by the anti-PCV2 antibodies, PEPSCAN analysis was performed on ORF1-, ORF2- and ORF3-encoded proteins with experimental anti-PCV2 antisera. This study was carried out on PCV2 and PCV1 sequences so as to identify common and/or discriminating PCV epitopes. For the ORF1 proteins, the first 100 amino acids were excluded from this study, as they display 12% amino acid divergence in this sequence area. Only the C-terminal 212 amino acid sequences, which differ by 16.5% between the two PCV types, have been investigated. As detected with an anti-PCV2 antiserum on peptides derived from the ORF1 amino acid sequence (Fig. 4), reactive peptides were shown to span the common residues (residues 185–211) of PCV1 and PCV2. However, the serum reacted weakly with these peptides, since the intensity ratio did not exceed 1.6. In contrast, as shown in Fig. 5(a, b), the same serum reacted more strongly with the ORF2 peptides. Moreover, it allowed the identification of ORF2-reactive peptides derived from PCV1

and localized between amino acids 25–43 and 169–187 (Fig. 5a). When tested on ORF2 overlapping peptides from PCV2, this serum revealed not only the same areas encompassing residues 25–43 and 169–183, but also additional reactive peptides localized between amino acids 65–87, 113–147 and 193–207. Another anti-PCV2 antiserum generated in a conventional pig was also tested for its reactivity on ORF2 peptides (Fig. 5c, d). While only one reactive peptide could be detected between amino acids 169 and 183 derived from PCV1, four areas of ORF2 derived from PCV2 were shown to be immunoreactive. These included residues 65–87, 113–147, 157–183 and 193–207.

Comparison of the results obtained for these two anti-PCV2 antisera thus allowed the identification of common and discriminating reactive peptides on ORF2-PCV2 alone. Indeed, whereas a common reactive peptide could be detected in the C-terminal part of the ORF2 proteins (residues 169–183), three groups of ORF2 peptides located between amino acids 65–87, 113–139 and 193–207 were recognized specifically by both anti-PCV2 antisera. The peptide sequences representative of each group are indicated in Table 2. Three of these peptides, namely B-121, B-133 and B-146, allowed the detection of anti-PCV2 antibodies. The B-133 peptide was more effective for PCV2 discrimination, since its A-189 counterpart was not recognized by experimental anti-PCV2 antisera. However, the B-121 peptide, which gave discriminating results with experimentally infected conventional piglets, was not recognized by the anti-PCV2 antisera generated in SPF pigs.

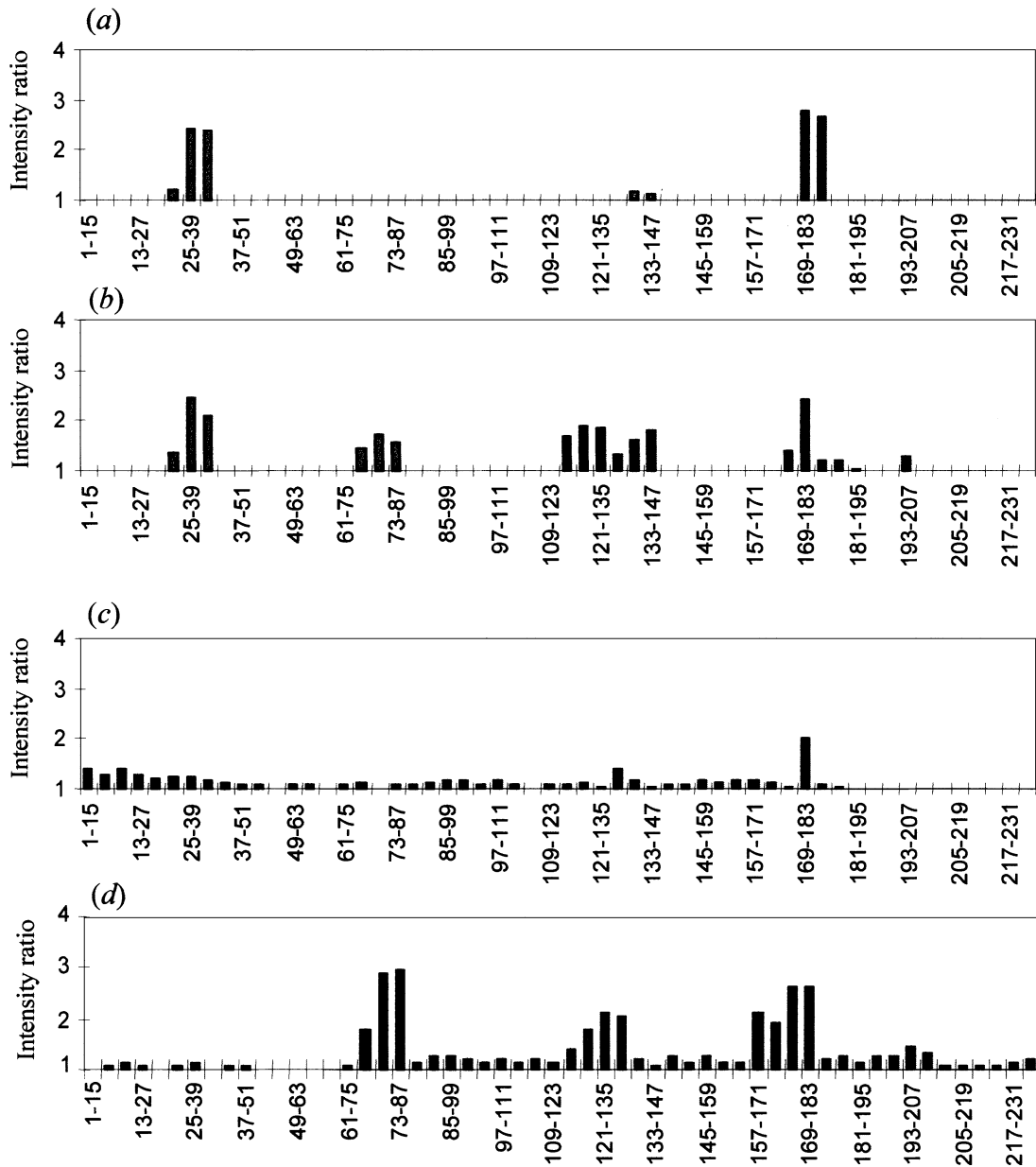


Fig. 5. Immunoreactivity of anti-PCV2 antiserum with ORF2 peptides. Overlapping peptides spanning the ORF2-encoded protein from PCV1 (a, c) or PCV2 (b, d) were analysed for their reactivity with experimental antisera generated in SPF (a, b) and conventional (c, d) piglets. The intensity ratio indicates the ratio between values obtained for the serum collected 6 weeks post-inoculation and the serum collected before experimental inoculation. Peptides are indicated by their amino acid locations.

PEPSCAN analysis was also performed on ORF3-encoded protein, but no reactivity was detected (not shown), thus indicating the lack of immunogenicity of this predicted product.

Discussion

The PCV1 and PCV2 genomes used in this study were isolated from PMWS-affected animals (Le Cann *et al.*, 1997; Mankertz *et al.*, 2000). Sequence comparison of ORF-

encoded proteins revealed significant similarities ranging from about 80 to 60% amino acid identity for the two major proteins, ORF1 and ORF2. Moreover, these levels of similarity were shown to be relatively well conserved between different PCV isolates from both types (Mankertz *et al.*, 2000; Meehan *et al.*, 1998; Morozov *et al.*, 1998). For this reason, the existence of antigenic cross-reactivities between viral products of the two genotypes has been suggested. To address this issue, we first used a system involving cells transfected with specific genomes. The replicative forms of cloned PCV1 and PCV2

Table 2. Sequence of PCV ORF2 peptides and their serological reactivities with experimental anti-PCV2 antisera

Names of peptides corresponding to PCV2 sequences begin with B and their respective counterparts on PCV1 have names beginning with A. The amino acid sequences and positions are reported for each peptide.

Peptide	PCV type	Position	Sequence	PEPSCAN reactivity	Serum reactivity*	
					SPF	Farm
B-121	2	69–83	VDMMRFNINDFLPPG	+	±	++
A-177	1	69–83	NVNELRFNIGQFLPP	–	–	–
B-132	2	113–127	QGDRGVGSSAVILDD	+	–	–
A-188	1	113–127	TSNQRGVGSTVVILD	–	–	–
B-133	2	117–131	GVGSSAVILDDNFVT	+	+	++
A-189	1	117–131	RGVGS TVVILDANFV	–	–	–
B-146	2	169–183	FTIDYFQPNNKRNL	+	+	+
A-202	1	169–183	DQTIDWFQPNNKRNL	+	++	++
B-152	2	193–207	VDHVGLGTAFENSIY	+	–	–
A-208	1	193–207	NVEHTGLGYALQNT	–	–	–

* Reactivity, which indicates seroconversion, was analysed by ELISA with antisera collected from SPF and farm piglets before and 6 weeks after experimental infection with PCV2.

genomes used as the DNA source in transfection assays were shown to represent functional genomes, as demonstrated by their ability to generate infectious virus particles in an infection test. This system can thus be used to mimic the different processes of virus formation, including viral protein synthesis for replication and virus assembly. By using this model, serological cross-reaction could be identified here with anti-PCV2 antisera generated after experimental infection. We next attempted to determine the nature of the antigens responsible for these common but also different immunoreactivities, in order to identify common and discriminating serological markers of PCV infection.

By using ORF1-PCV1 hyperimmune serum, we were able to demonstrate that the ORF1 proteins were antigenically related in the two PCV types. This was also confirmed by using recombinant ORF1 proteins from PCV1 and PCV2, which were both recognized by anti-PCV2 antisera. The presence of anti-ORF1 antibodies after PCV2 infection was also shown in this way. In contrast, the absence of cross-reaction that was reported by Balasch *et al.* (1999) with experimental anti-PCV2 sera indicated the absence of anti-ORF1 antibodies. This could be explained by the time after experimental infection that the sera were collected. Indeed, ORF1 antibodies, which would be absent or present at a very low titre at least up to 2 weeks p.i. (Balasch *et al.*, 1999), might appear later after infection (this study).

Further analysis of the antigenic regions of ORF1-encoded proteins with synthetic peptides showed that, in the last 200 amino acids, a common antigenic determinant could be detected between residues 185 and 211 of the proteins. Furthermore, since the first 100 amino acids, which display

strong identity between the two proteins, were excluded from this study, we cannot exclude the presence of common antigenic domains in this area. In contrast, no discriminating epitope could be identified. The demonstration of serological cross-reactivity between the ORF1 proteins correlates well with the high degree of sequence identity observed between the two types of PCV and is fully consistent with its essential conserved function for virus replication (Mankertz *et al.*, 1998a).

Immunoreactivity against the putative capsid protein encoded by the major ORF2 was also investigated. In contrast to ORF1, a hyperimmune serum generated against ORF2 from PCV2 did not allow the detection of viral protein in PCV1-transfected cells. Hence, the question was whether this absence of signal resulted from the lack of serological reaction between ORF2-PCV2 antiserum and ORF2-PCV1 protein, since it could also be explained by the absence of ORF2 expression by PCV1. The identification of ORF2 transcript expression in PCV1-infected cells (Mankertz *et al.*, 1998b) does not support this latter hypothesis. Moreover, ORF2 proteins fused to GST were expressed and used to demonstrate here that, in spite of their 56% sequence identity, no detectable cross-reactivity could be shown between ORF2 proteins. Antigenic differences have already been reported between PCV1 and PCV2 isolates, as demonstrated by the differences in anti-PCV antibody titres on infected cells. These differences might thus be explained by the differential antigenicity of ORF2-encoded structural proteins (Allan *et al.*, 1999b; Ellis *et al.*, 1998). Further analysis of immunodominant regions determined by using ORF2 synthetic peptides led, as was expected, to the identification of antigenic domains in only the ORF2-PCV2 protein (residues

65–87, 113–139 and 193–207). However, a few reactive peptides common to ORF2 from PCV1 and PCV2 could also be identified in this way, although variations could be observed depending on the serum used. The absence of cross-reactivity observed in transfected cells or after immunoblot assays might then be explained by the lack of accessibility of this peptide sequence in the whole protein.

The differential immunoreactivity of ORF2-PCV2 peptides or proteins with anti-PCV2 antibodies is of particular interest, since such reagents could be used to develop an immunoassay such as ELISA for the detection of PCV2 infection in herds. Of the reactive peptides chosen to represent the antigenic domains of ORF2 protein, one, B-133, could be selected for such ELISA development, since it allowed anti-PCV2 antibody detection and discrimination in both SPF and farm antisera (unpublished results). This peptide shows 25% amino acid sequence divergence from its counterpart, the A-189 peptide.

As demonstrated by PEPSCAN analysis, greater immunoreactivity was observed for the ORF2 protein, which encodes the putative capsid protein. A sequence comparison of this protein from the different strains of PCV2 revealed some variations in the selected peptides. Among them, the B-133 peptide sequence differs by one amino acid between European and North American strains. Whether the anti-PCV2 antiserum generated from a French PCV2 strain could recognize the same epitopes in strains from other countries is not yet known and would provide information about epitope variability among PCV2 strains.

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