

**Identification of an immunorelevant ORF2 epitope from porcine circovirus type 2 as a serological marker for experimental and natural infection**

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Accepted September 18, 2000

**Summary.** Post-weaning multisystemic wasting syndrome (PMWS) is a recently identified disease of pigs linked to the emergence of a new porcine circovirus (PCV2). We report here the characterization of immunorelevant linear B-cell epitopes of the Open Reading Frame 2-encoded protein (Orf2) from PCV2 by an enzyme-linked immunosorbent assay (ELISA) using experimental antisera collected from pigs inoculated with a PCV2 isolate. Two epitopes spanning residues 69 to 83 and 117 to 131 were specific to PCV2. Antibodies to the 117 to 131 epitope (B-133) were detected in 22% and 100% of specific pathogen-free (SPF) pig sera 6 and 11 weeks post inoculation, respectively. Cross-sectional studies performed with field sera collected from PMWS-affected herds showed B-133 antibodies in 5% of 8 to 10 week-old pigs, 38% of 13–14 week-old pigs, 62% of 16 to 19 week-old pigs, 56% of 20 to 25 week-old pigs and 45% of 26 to 31 week-old pigs. All these data suggest that epitope B-133 is a serological marker of PCV2 infection that could be used for the detection of PCV2 antibody response.

**Introduction**

PMWS is now a well-established disease of swine herds in France, other European countries, North America and Asia. Clinical signs are unthriftiness, pallor, fever and progressive wasting with associated respiratory and digestive disorders [8, 12, 29]. A clinical outcome is observed in 8 to 13 week-old pigs. Mortality attained 10% in some French farms [18].

A new porcine circovirus (PCV2) has been isolated from PMWS-affected piglet lesions [2, 3, 15–17, 21–23]. PCV2 is a small non-enveloped circular single-stranded DNA virus in the *Circoviridae* family which also includes other animal viruses such as Porcine Circovirus type 1 (PCV1), Psittacine Beak and Feather Disease Virus (BFDV) and Chicken Anemia Virus (CAV). PCV1 was first

described in 1974 as a persistent noncytopathic contaminant of the porcine kidney cell subline PK15 ATCC-CCL33 [30]. Although serological surveys indicated that PCV1 antibodies were common in the swine population, disease was not observed either in PCV1-seropositive farms [9, 10, 13, 31, 32] or after experimental PCV1 inoculation [1]. Porcine circoviruses PCV1 and PCV2 share 70% nucleotide sequence homology with mutations throughout the viral DNA. Orf1-encoded protein is more conserved between the two viruses than Orf2-encoded protein (86% and 56% homologies, respectively [11, 22]). Orf1-encoded protein (Rep protein) is essential for circoviral replication [20] whereas the function of Orf2-encoded protein has not yet been elucidated.

PCV2 infection diagnosis is currently based upon immunohistochemistry [28, 24] and *in situ* hybridization [7, 24, 28] in porcine lymphoid tissues. PCV2 antibodies can be successfully detected using Immunoperoxidase Monolayer Assay (IPMA) [5] or Indirect Immunofluorescent Assay (IFA) [4, 27] of PCV2 infected PK15 cells. Serotyping is achieved by comparative analysis of the titers obtained in PCV2 and PCV1 IPMAs [4, 5, 26].

We have previously demonstrated antigenic cross-reactions between PCV1 and PCV2 viruses in Orf1-encoded protein and Orf2-encoded protein has been evidenced as a good candidate for serotyping [19]. Here, we describe the characterization of an Orf2 peptide as an immunorelevant PCV2 specific linear B-cell epitope by ELISA, using sera from pigs experimentally inoculated with a PCV2 isolate, and its potential use as a serological marker to detect PCV2 antibodies following natural infection in swine herds.

## Materials and methods

### *Pig sera*

#### (i) Experimental PCV2 antisera

Four PMWS experimental transmission studies (A, B, C, D) were performed on 6 week-old specific pathogen-free piglets (SPF) or on conventional farm piglets in our experimental facilities [6].

The pigs were challenged with tissue homogenates prepared from sick piglets. Enlarged lymph nodes collected from a PMWS-affected field pig were crushed in Minimal Essential Medium (E-MEM from Biowhittaker, Belgium) then centrifuged (2000 g, 15 min, 4 °C). The supernatant was ultrafiltered on 0.22 µm and further subjected to differential PCV1 and PCV2 polymerase chain reaction (PCR) using two sets of type-specific primers: PCV1 forward (5'-GGCGGCGGCATCTGTAACGGTTT-3') and PCV1 reverse (5'-GATGGCGCCGAAAGACGGGTATC-3'), PCV2 forward (5'-GTGGAGCTCCTAGATCTCAGGG-3') and PCV2 reverse (5'-TAGGAGCTCCACACTCCATCAG-3') [20].

In this inoculum, the PCV2 titer was  $10^{4.5}$  TCID<sub>50</sub>/ml as determined by IPMA of PK15 cells according to Kärber [14]. For IPMA analysis,  $8 \times 10^4$  PK15 PCV-free cells were seeded into 96 microplate wells before infection with 10 µl of tissue homogenate ( $10^{4.5}$  TCID<sub>50</sub>/ml). Circoviral replication was boosted 4 hours post-infection with 100 µl of 300 mM d-glucosamine (Sigma, USA) in Earle's balanced salt solution (Sigma, USA) and the cells further grown in E-MEM with 10% fetal bovine serum for 36 h at 37 °C in a 5% CO<sub>2</sub> atmosphere. The cells were then fixed and permeabilized with 100 µl of 80% cold acetone in phosphate-

buffered saline (PBS) for 45 min at  $-20^{\circ}\text{C}$ . The wells were quenched with 200  $\mu\text{l}$  of PBS containing 5% of blotting grade blocker non-fat dry milk for 90 min at  $37^{\circ}\text{C}$ . The plates were then washed three times with 100  $\mu\text{l}$  of PBS containing 0.25% Tween 20 (PBS-Tw). 100  $\mu\text{l}$  of serum sample diluted 1:100 in PBS containing 3% blotting grade blocker non-fat dry milk and 0.05% Tween 20 (PBS-M-Tw) was added and further incubated for 60 min at  $37^{\circ}\text{C}$ . After three washes with PBS-Tw, 50  $\mu\text{l}$  of horseradish peroxidase (HRP)-conjugated rabbit anti-swine IgG (Dako, Denmark) diluted 1:1000 in PBS-M-Tw was added and further incubated for 60 min at  $37^{\circ}\text{C}$ . The plates were washed again with 100  $\mu\text{l}$  of PBS-Tw. Staining was developed with 9-aminoethyl carbazole (Sigma, USA) in dimethylformamide and hydrogen peroxide and was stopped by substrate removal.

The animals were inoculated intramuscularly and intratracheally once with 1 ml and 5 ml, respectively of either the above inoculum (infected piglets) or E-MEM (control piglets). Serial blood samples were taken weekly.  $D_0$  and  $D_{\text{end}}$  correspond to the pig serum samples collected before experimental inoculation and at the end of the experiment, respectively. Circovirus genotyping was also assessed by PCR in tissue samples from necropsied pigs. Trials A, B, and C were carried out for 6 weeks. Trial A involved 30 SPF piglets 6 of which were controls. Trial B involved 16 SPF and 8 conventional farm piglets of which 8 SPF piglets were controls. Trial C involved 24 conventional farm piglets 8 of which were controls. Study D was performed over a period of 11 weeks on 13 SPF piglets which included 6 controls.

#### (ii) Control sera

The negative control was collected from a piglet in our SPF herd. This serum was previously shown by IPMA to be free of PCV antibodies. The positive control was a PCV2-specific immune antiserum collected from a pig inoculated with a tissue homogenate containing PCV2 only, as assessed by differential PCR. PCV2 antibodies were previously detected in this serum by IPMA. The variability of the ELISA results was evaluated by testing the positive control serum on an entire plate in two different assays. A normal distribution of the positive control OD values was observed. This allowed determination of the coefficient of variation (CV%) corresponding to the ratio between standard deviation (SD)/mean (M)  $\times 100$ .

#### (iii) Antisera to other pig viruses

Antisera were raised against the following pig viruses: (i) three pestiviruses: bovine viral diarrhoea (BVD), border disease (BD) and classical swine fever (CSF) viruses (ii) two herpesviruses: pseudorabies virus (PRV) and cytomegalovirus (PCMV) (iii) three coronaviruses: transmissible gastroenteritis virus (TGEV), epidemic diarrhoea virus (PEDV), respiratory coronavirus (PRCV) (iv) influenza viruses (v) arterivirus: porcine reproductive and respiratory syndrome virus (PRRSV) (vi) cardiovirus: encephalomyocarditis virus (EMCV) (vii) enterovirus: talfan disease virus (viii) porcine parvovirus (PV). All these antisera were generated from 16- to 18-week-old SPF piglets.

#### (iv) Field serum samples

1445 pig sera were collected from 38 French herds. Seventeen of these 38 herds were selected for typical clinical signs of PMWS while the others were clinically free of the disease. Seven hundred and fifty blood samples were taken from the PMWS-affected herds and 695 samples from the PMWS-free herds. The pigs were 8 to 31 weeks of age.

#### *Peptides for ELISA*

Pepscan was assessed on nitrocellulose membranes with a panel of peptides overlapping the sequences of Orf1 PCV1, Orf1 PCV2, Orf2 PCV1 and Orf2 PCV2 using an indirect im-

munoassay with experimental antisera according to the manufacturer's guidelines. Pepsin-selected PCV2 peptides were used in ELISA to determine their reactivities with PCV2 experimental antisera collected from SPF or/and conventional farm pigs before and at various times after inoculation. PCV1 counterparts were also analyzed with the same antisera in order to identify cross-reacting PCV1 peptides.

#### *ELISA procedure*

Forty-eight wells of the microtiter plate (Nunc Maxisorp) were coated with 100  $\mu$ l of one PCV2 peptide (10  $\mu$ g/ml) in 0.05 M bicarbonate buffer pH 9.6 and incubated overnight at 37 °C while the other 48 wells were similarly coated with the PCV1 counterpart peptide. The unoccupied sites of the wells were then blocked with 200  $\mu$ l of PBS containing 3% of blotting grade blocker non-fat dry milk after incubation for 90 min at 37 °C. The plates were rinsed twice with 200  $\mu$ l of PBS-Tw. The serum samples were diluted 1:100 in PBS-M-Tw. Each serum sample was tested against the PCV1 and PCV2 peptides. Diluted serum sample (100  $\mu$ l) was added to a well and incubated for 60 min at 37 °C. The plates were then washed three times with 200  $\mu$ l of PBS-Tw. Fifty microliters of HRP-labeled rabbit anti-swine immunoglobulins diluted in PBS-M-Tw were added and incubated for 60 min at 37 °C after which the plates were washed three times with PBS-Tw. Hydrogen peroxide *o*-phenylenediamine (100  $\mu$ l) (OPD, Sigma, USA) was added and incubated for 30 min at 37 °C. Color development was stopped by the addition of 50  $\mu$ l of 1 N H<sub>2</sub>SO<sub>4</sub>. The optical densities (OD) were read at 490 nm.

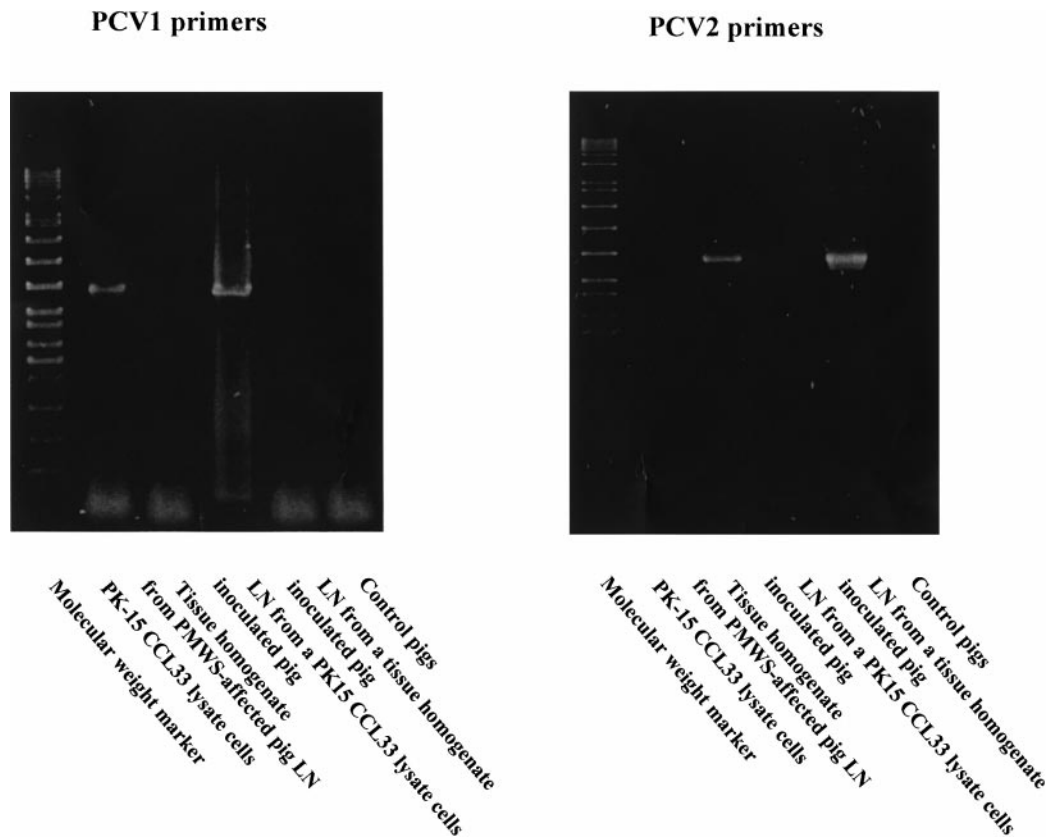
#### *Western-blot assay*

Orf2 gene from PCV1 and PCV2 was amplified and cloned into baculovirus transfer vectors as described elsewhere [19]. Proteins were expressed in *Spodoptera frugiperda* cells following infection with previously selected recombinant baculovirus. Expression control was checked by western-blot analysis. Briefly, 10<sup>5</sup> cells were separated on a 10% sodium dodecyl sulfate polyacrylamide gel and then transferred onto a nitrocellulose membrane. The recombinant Orf2 protein was identified using the experimental positive control sera as a 28 kDa product and further used for the screening of PCV2 antibodies from field sera.

## **Results**

### *Genotyping of the inoculum in relation to elicitation of PCV2 antibodies*

To ensure that the experimental sera had been raised against one strain of porcine circovirus, PCV1 and PCV2 DNAs were screened by PCR in the tissue homogenate inoculum as well as in tissue samples from necropsied pigs. As shown in Fig. 1, no PCV1 or PCV2 DNAs were detected in tissue samples from the control piglet (lanes 5a and 5b). No detectable antibody response was evidenced in either preimmune serum or time-end serum from this control pig as assessed by IPMA. PCV2 DNA was demonstrated in the tissue homogenate inoculum (lane 2b) but no PCV1 DNA was present in this sample (lane 2a). Only PCV2 was detected in the tissue sample from the necropsied pig inoculated with tissue homogenate (lanes 4a and 4b). No detectable PCV antibodies were evidenced by IPMA in the preimmune sera from this inoculated pig but seroconversion was observed at the end of the experiment (Fig. 2). PCV1 DNA was detected both in the PK15 ATCC-CCL33 lysate cell (lane 1a) and in PK15-PCV1 inoculated

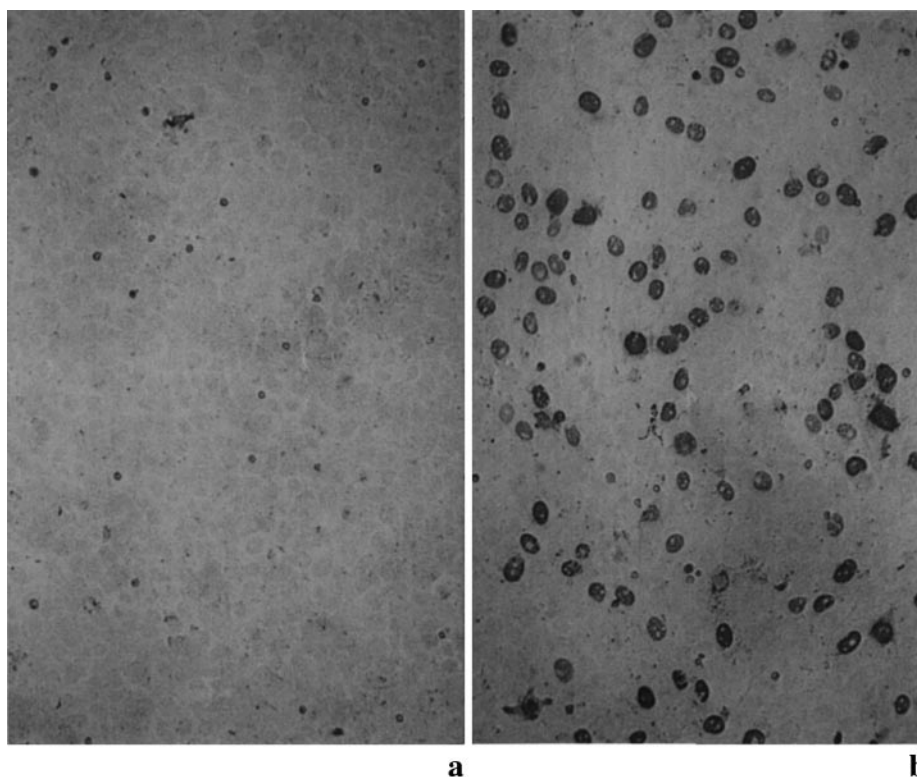


**Fig. 1.** Circoviral genotyping of tissue homogenate inoculum and tissue samples from necropsy. Agarose gel electrophoresis showing DNA products amplified from indicated samples with specific PCV1 or PCV2 primers. Kilobase pair markers ranging from 48.5 kbp to 0.234 kbp

pig lymph nodes (lane 3a) but no PCV2 DNA was detected in any of these samples (lanes 1b, 3b). The tissue homogenate inoculum contained only PCV2 DNA suggesting that the elicited antibodies were relevant to the PCV2 serotype.

#### *Characterization of PCV2 epitopes by ELISA*

Orf1- and Orf2-encoded protein Pepscan analysis was carried out with PCV2 experimental antisera in comparison with screening of PCV1 sequences [19]. All the Orf1 peptides from PCV2 that reacted with PCV2 antisera had counterparts in the PCV1 sequence which were also recognized by the same PCV2 antisera. Five immunoreactive domains were identified on Orf2 which were further analyzed by ELISA. The reactivities of the PCV2 peptides, namely B-121, B-132, B-133, B-146 and B-152, as well as their respective PCV1 counterparts A-177, A-188, A-189, A-202 and A-208 are summarized in Table 1. None of the pre-immune sera ( $D_0$ ) had detectable PCV2 antibodies that reacted with any of the 10 peptides. Time-end serum samples ( $D_{end}$ ) had antibodies that reacted with peptides B-121,



**Fig. 2.** IPMA of PCV2-infected PK15 cells showing the reactivity of (a) the preimmune sera of an experimental SPF pig inoculated with tissue homogenate (b) the immune sera of this experimental SPF pig 6 weeks post-inoculation. Infected-cell bound antibodies are detected by an indirect immunoassay using a colorimetric method. Positive staining is visualized as dark red cells

B-133, B146 and A202 as indicated by an absorbance value three times higher than that of the negative control ( $OD_{490nm} = 0.1$ ). At the end of the experiment, the levels of antibody of these three PCV2 peptides, as depicted by the ratio  $D_{end}/D_0$ , was 9 to 30 times higher than those from the preimmune samples. These peptides allowed the detection of circovirus seroconversion after inoculation with the tissue homogenate thereby confirming the immunorelevance of these epitopes during experimental infection with PCV2.

*Specificity of the antibody response of PCV2 infected pigs to defined peptides*

The respective PCV1 counterparts A-177 and A-189 of the PCV2 peptides, B-121 and B-133, were not recognized by post-infection serum from the two experimentally infected pigs as shown by the ratio value  $D_{end B}/D_{end A} > 1$ , indicating that these latter two peptides are PCV2 discriminant (Table 1). Two out of the three Orf2 immunorelevant epitopes were thus PCV2 specific. The B-133 epitope was selected for further ELISA since a seroconversion was more consistently

**Table 1.** Identification of immunorelevant ORF2 epitopes for serological diagnosis by ELISA of PCV2 infection

Peptide	PCV Strain	Amino-acids position	OD value with experimental PCV2 antisera from		Conventional farm piglets				
			SPF piglets	Conventional farm piglets	$D_{end}$	$D_{end}/D_0$	$D_{endB}/D_{endA}$	$D_{end}/D_0$	$D_{endB}/D_{endA}$
B-121	2	69-83	0.07	0.24	<b>3.4</b>	0.10	3.00	30	33
A-177	1	69-83	0.06	0.10	1.7	0.16	0.09	0.6	
B-132	2	113-127	0.06	0.12	2	0.12	0.13	0.1	1.4
A-188	1	113-127	0.07	0.11	1.6	0.10	0.09	0.1	
B-133	2	117-131	0.07	1.08	<b>15.4</b>	0.07	1.47	<b>21</b>	11.3
A-189	1	117-131	0.07	0.19	2.7	0.11	0.13	1.2	
B-146	2	169-183	0.09	0.81	<b>9</b>	0.07	0.72	<b>10.3</b>	0.3
A-202	1	169-183	0.12	2.67	<b>22.3</b>	0.11	2.45	<b>22.3</b>	
B-152	2	193-207	0.07	0.11	1.6	0.09	0.16	1.8	1.3
A-208	1	193-207	0.10	0.10	1.4	0.09	0.12	1.3	

Peptides corresponding to PCV2 are indicated as B-121, B-132, B-133, B-146 and B-152 while their PCV1 respective counterparts are A-177, A-188, A-189, A-202 and A-208. The amino acid positions are also reported for each peptide. The reactivity was analyzed by an indirect ELISA using experimental PCV2 pig antisera collected from SPF and conventional farm piglets before experimental inoculation ( $D_0$ ) and before euthanasia i.e. 6 weeks post-inoculation ( $D_{end}$ ). The ratio  $D_{end}/D_0$  which indicates the experimentally-induced seroconversion is reported in bold characters for significant values. The ratio  $D_{endB}/D_{endA}$  which indicates the circovirus type-discrimination is reported in black boxes for significant values

**Table 2.** Specificity of B-133 peptide for swine pathogens

Hyperimmune sera to		OD <sub>490 nm</sub>
Circovirus type 2	PCV2	0.550
Pestivirus	BVDV	0.22
	BDV	0.13
	CSFV	0.17
Herpesvirus	PRV	0.14
	PCMV	0.16
Coronavirus	TGEV	0.23
	PEDV	0.20
	PRCV	0.24
Influenzavirus	H1N1	0.11
	PRRSV	0.17
Enterovirus	EMCV	0.18
	Talfan	0.28
Parvovirus	PPV	0.28

ODs are reported for a panel of hyperimmune antisera collected from pigs inoculated with *BVDV* bovine viral disease virus, *BDV* border disease virus, *CSFV* classical swine fever virus, *PRV* pseudorabies virus, *CMV* cytomegalovirus, *TGEV* transmissible gastroenteritis virus, *PEDV* porcine epidemic diarrhea virus, *PRCV* porcine respiratory coronavirus, *H1N1* influenza virus, *PRRSV* respiratory and reproductive syndrome virus, *EMCV* encephalomyocarditis virus, *PPV* porcine parvovirus

evidenced with PCV2 experimental SPF as well as with conventional farm pig antisera. Non-specific serological cross-reactions between several other porcine virus antigens and the B-133 epitope were evaluated with a panel of antisera produced in SPF pigs by inoculation with 15 other swine viruses. None of these antisera reacted with our PCV2 peptide (Table 2), indicating a lack of cross-reactivity between PCV2 and the other viruses under study.

#### *Antibody response to the B-133 epitope following PCV2 experimental infection*

Sera collected from trials A, B, C and D were analyzed by ELISA (Fig. 3). No B-133 antibodies were detected in the preimmune sera (D<sub>0</sub>) from control and infected pigs. Preimmune serum samples from control piglets in experimental studies A, B, C and D were used as a source of PCV2 negative sera to define a positive threshold. Using our B-133 ELISA, the OD<sub>490nm</sub> values of these negative sera ranged from 0.062 to 0.25 and showed a normal distribution. This indicates that 99% of the negative sera have OD values ranging from the mean OD (M) minus

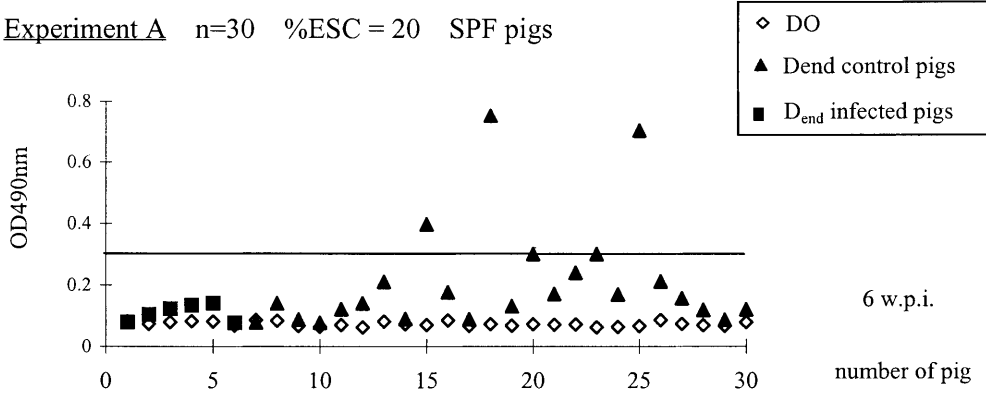


3 standard deviations (SD) to  $M + 3 \text{ SD}$ . The positive threshold determined from  $M = 0.12$  and  $\text{SD} = 0.06$ , was set at 0.30. All control piglets were seronegative at the end of the experiment ( $D_{\text{end}}$  control piglets) i.e. after 6 weeks for trials A, B, C and 11 weeks for trial D. None of the pigs had detectable antibodies during the first two weeks post-inoculation although signs of disease were observed 10 to 14 days post-inoculation. These signs consisted of a febrile phase which lasted for about 10 days and retarded growth (Albina, manuscript submitted). At six weeks post-inoculation 21% (5 out of 24) and 25% (2 out of 8) of the SPF antisera from trials A and B, respectively had detectable B-133 antibodies whereas 100% (7 out of 7) of the challenged piglets were seropositive 11 weeks post-inoculation (trial D), thus demonstrating that the B-133 epitope was a serological marker of PCV2 infection. In contrast to SPF pigs, 75% (6 out of 8) and 62% (10 out of 16) of the conventional farm pigs from trials B and C, respectively had detectable B-133 antibodies six weeks post-inoculation which suggests that the kinetics of PCV2 seroconversion vary according to the pig's health status. The CVs for our positive control were 9.8% and 8.5% respectively, in two different assays, indicating that the intravariability of this peptide-based ELISA was acceptable.

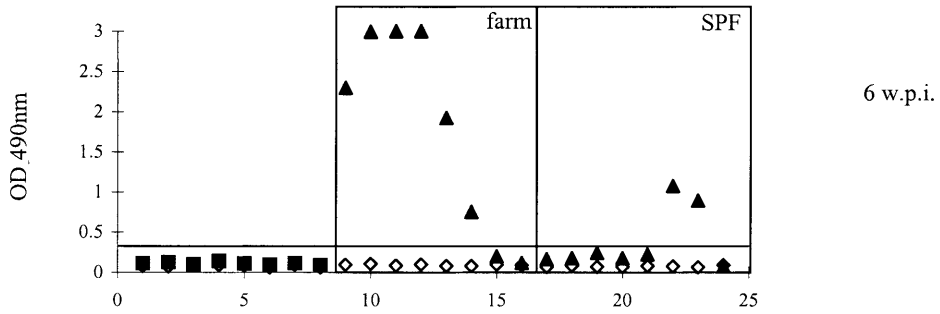
#### *Antibodies to the B-133 epitope during natural infection*

Serum samples were collected from post-weaning piglets and growing pigs from 8 to 31 weeks of age in 38 herds located in 5 areas of France (Bretagne, Vallée de la Loire, Nord, Champagne-Ardennes and Aquitaine). Seventeen of these 38 herds showed typical clinical signs of PMWS (750 serum samples) whereas the other 21 were clinically free of the disease (695 serum samples). The distribution of OD values in Fig. 4, based on the above-described positive threshold, shows two serological populations within the PMWS-affected porcine population. B-133 antibodies could be detected in forty-five percent (338 samples out of 750) of the total serum population collected from PMWS-affected herds. These serological analyses performed on post-weaning and growing pigs were used to determine the percentage seropositivities of the different age groups. Cross-sectional studies showed that B-133 antibodies were detected in 5% (6 out of 115) of the sera from 8 to 10 week-old pigs, 38% (18 out of 44) of sera from 13–14 week-old pigs, 62% (166 out of 267) of sera from 16 to 19 week-old pigs, 56% (65 out of 115) of sera from 20 to 25 week-old pigs and 45% (21 out of 46) of sera from 26 to 31 week-old pigs (Table 3), suggesting that the B-133 epitope was a serological marker for the later steps of PCV2 infection. Four hundred and six serum samples collected from 11 PMWS-free herds were then serologically screened for PCV2 antibodies. The OD distribution is shown in Fig. 4. Surprisingly, two serological populations emerged despite the absence of clinical PMWS, thus indicating that some asymptomatic pigs were infected by PCV2. Twenty-six percent (104 out of 406) of the pigs in the PMWS-free herds had detectable B-133 antibodies. These seropositivities were further confirmed by western-blot assay for all the 33 tested sera and supported the occurrence in swine herds of subclinical PCV2 infection.

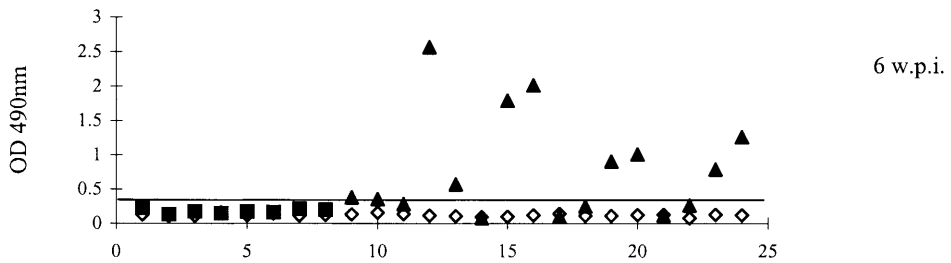
**Experiment A** n=30 %ESC = 20 SPF pigs



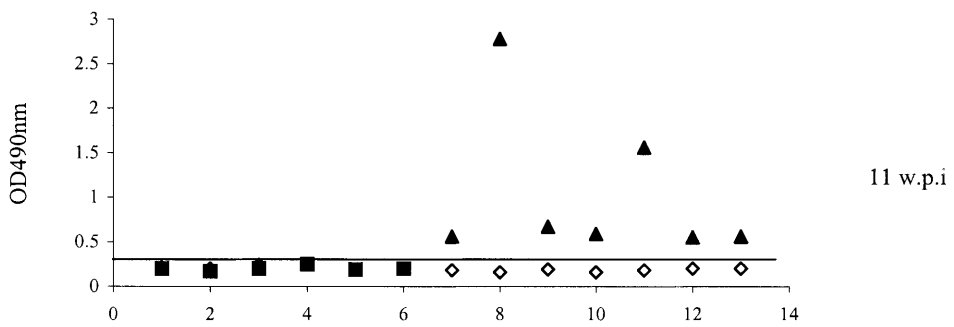
**Experiment B** n=24 %ESC = 50



**Experiment C** n=24 %ESC = 62.5 farm pigs



**Experiment D** n=13 %ESC = 100 SPF pigs



## Discussion

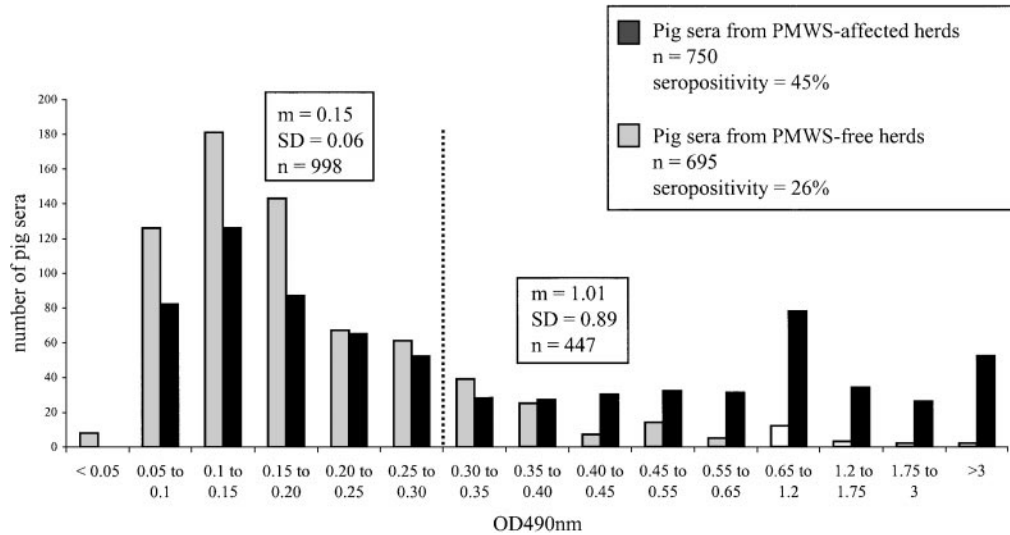
We have previously demonstrated that porcine circovirus PCV1 and PCV2 have common antigenic determinants located on the Orf1-encoded protein which are fully consistent with their high degree of sequence similarity while the Orf2-encoded protein has been characterized as a good candidate for serotyping due to its lack of antibody cross-reactivity between the two viruses under study [19].

In this report, we show that a peptide based on the amino acid sequence of the Orf2-encoded protein (residues 117 to 131) from PCV2 binds antibodies from pigs experimentally inoculated with tissue homogenates prepared from PMWS-affected pigs. Its specificity for PCV2 antibodies appears to be high since pig blood samples seropositive for several common porcine viruses did not react with the PCV2 peptide. Moreover this peptide is not a PCV1 cross-reacting epitope since PCV2 antibodies did not react with its PCV1 counterpart which shared 80% of sequence identity. This peptide-based ELISA was thus highly specific to PCV2 in contrast to the differential IPMA or IFA reported by others [4, 5, 27] which used cross-reacting virion subunits as detection antigens.

By studying the antibody response in experimentally inoculated pigs over a period of 11 weeks, we established that the B-133 peptide was a serological marker for PCV2 infection since all pigs had PCV2 antibodies at the end of the experiment. This serological marker is thus produced or detected relatively late since anti-B-133 antibody levels were undetectable during the first 2 weeks post-challenge although PCV2 antibodies were reported with IPMA of PCV2-infected cells or Orf2 western blot analyses [5, 27] in all inoculated pigs from the 14<sup>th</sup> day post-inoculation onwards. These data suggest a lower sensitivity of the peptide-based ELISA which is assumed to be linked to the availability of antigenic sites. Actually virion proteins may exhibit several linear or conformational antibody binding sites compared to the 15-mer peptide. Kinetic studies showed a variability in seroconversion rate dependent on the pig's initial health since 66% of the conventional farm animals were seropositive 6 weeks post-inoculation compared to only 22% of the SPF animals. These differential antibody kinetics could be explained by the individual backgrounds of the pigs in terms of previous immunological stimulations. We have already observed, for example, that conventional farm pigs generally exhibit higher cellular immune responses than SPF pigs (unpublished data).



**Fig. 3.** Serological analysis of four experimental trials by peptide B-133 based indirect ELISA. ODs are indicated for pig antisera collected before inoculation ( $\diamond$ ) and at the end of the experiment for control pig ( $\blacksquare$ ) and tissue homogenate inoculated pig ( $\blacktriangle$ ). The status of the animals is reported as SPF or conventional farm pigs. n corresponded to the number of pigs involved in each trial. The positive threshold (OD > 0.3) is represented as a full line. The percentage of experimental seroconversion (% ESC) given for each trial was determined using the following formula (% ESC = number of seropositive pigs in the experiment/number of challenged pigs in the experiment). Also indicated is the date of bleeding i.e. 6 weeks post-inoculation for studies A, B, C and 11 weeks for studies D



**Fig. 4.** Serological analysis of field sera by peptide B-133 based indirect ELISA. The positive threshold is represented as a dotted line. The distribution of ODs is reported for 1445 serum samples collected from 17 PMWS-affected herds (■) and 11 PMWS-free herds (□). n is the number of sera analyzed. The percentage of seropositivity given for both types of herds was determined using the following formula (% seropositivity = number of positive sera/n). The mean OD (m) as well as the standard deviation (SD) is also indicated for both negative and positive serological populations

**Table 3.** Seropositivity detection and pig age

Age of the pigs (weeks)	n. of positive sera/n. of total sera (%)
8 to 10	6/115 (5)
13 to 14	17/44 (38)
16 to 19	166/267 (62)
20 to 25	65/115 (56)
26 to 31	21/46 (45)

The number (n) of positive sera and the % of seropositive individuals in each group of pig age are reported

By studying the reactivity of the B-133 peptide with field pig sera, we established that it was also a serological marker for natural PCV2 infection since farm pigs kept in PMWS-affected herds had detectable PCV2 antibodies. Furthermore 26% of the farm pigs in PMWS-free herds were also seropositive and no reactivity was ever observed with PCV1 peptide. This absence of correlation between PCV2 seropositivity and the outcome of PMWS is fully consistent with previous reports [25, 26]. It can be assumed that other factors such as housing or management conditions, concurrent infections or even genetic factors are involved in the onset

of clinical symptoms in herds. In particular, recent studies have shown that some management failures [18] or simultaneous infection with porcine parvovirus [4] may influence disease outcome.

To date, the serodiagnosis of PCV2 infection necessitates cumbersome differential assays based on cell-cultured antigens. To our knowledge, no ELISA has so far been developed mainly because of the absence of immunological data concerning the virus. We have characterized certain of the linear B-cell epitopes of the Orf2-encoded protein from PCV2 and focused on an epitope designated B-133 to develop an ELISA. The most interesting feature of this easy-to-use assay is its PCV2 specificity since it seems to be less sensitive in antibody detection than a multiepitopic antigen. We have also identified two other relevant epitopes: a discriminant epitope (B-121) and B-146 which is apparently common to both circoviral types although no cross-reactivity has ever been observed from Orf2-encoded proteins. B-121 and its PCV1 counterpart share 80% amino acid identity while B-146 and A-202 share more than 86% sequence identity. These other epitopes could also be of interest in the serodiagnosis of PCV2 infection for example, in combination with the B-133 peptide or with the Orf2-encoded protein.

### Acknowledgements

The authors thank all the farmers, technicians and veterinarians who contributed to this study by collecting serum samples and particularly Eric Eveno, Pierre Morvan, Jean-Luc Jobert, Nicolas Rose and Pierre-Alexandre Beloeil. We also thank Roland Cariolet, Bernard Beaurepaire, Gérard Bennevent, André Keranflec'h and Jean-Pierre Jolly for their daily assistance in our experimental facilities. This work was supported by funds from the "Comité Régional Porcin", the "Région Bretagne" and the "FEOGA".

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Received May 5, 2000