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Molecular characterization of *Porcine circovirus* type 2 isolates from post-weaning multisystemic wasting syndrome-affected and non-affected pigs

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Porcine circovirus type 2 (PCV2) is recognized as a primary cause in post-weaning multisystemic wasting syndrome (PMWS). In this study, both PCV1 and PCV2 types were studied in pigs originating from PMWS-affected (+) and non-affected (−) herds from Brittany. PCV2 was identified by PCR in 100 % of animals from PMWS(+) herds and in 76 % from PMWS(−) herds, while PCV1 was not detected. The complete sequences of 38 PCV2 isolates were determined and 23 new variants were identified, displaying between 94·6 and 99·9 % nucleotide identity with one another. Although highly related to all the PCV2 sequences available in databases, the isolates from France gathered in a distinct subcluster. Compared with the 13 PCV2 from PMWS(+) farms, the 10 PMWS(−) sequences exhibited a slightly higher variability. No viral molecular marker specific to a pathogenic state could be identified, even by including other PCV2 variants isolated from PMWS-suffering animals from other countries. We concluded that the PMWS outbreaks in Brittany are most likely not due to the emergence of a new genotype of circovirus.

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INTRODUCTION

Post-weaning multisystemic wasting syndrome (PMWS) was initially described in North America (Clark, 1997; Harding, 1997) and subsequently observed in most pig-producing countries of Europe (LeCann *et al.*, 1997; Segales *et al.*, 1997; Allan *et al.*, 1998, 1999a; Kiss *et al.*, 2000; Wellenberg *et al.*, 2000; Saoulidis *et al.*, 2002) and Asia (Kiupel *et al.*, 1998; Onuki *et al.*, 1999; Choi *et al.*, 2000). PMWS is clinically characterized by weight loss, respiratory or digestive disorders and enlarged lymph nodes. Lymphocyte depletion and histiocytic infiltration of lymphoid tissues appear to be the most typical lesions. The disease affects pigs in both post-weaning and fattening units. A sporadic incidence within pig units is also reported as characteristic of the syndrome (Segales & Domingo, 2002).

Porcine circovirus type 2 (PCV2), a member of the *Circoviridae* family, is now recognized as a major aetiological agent of PMWS (Allan *et al.*, 2002; Pogranichniy *et al.*, 2002; Rodriguez-Arrioja *et al.*, 2002). However, its precise role in the pathogenesis is poorly understood, since PCV2 infection of pigs does not necessarily lead to PMWS. Indeed, experimental inoculations of pigs with pure PCV2 only reproduced mild PMWS symptoms (Magar *et al.*, 2000; Fenaux *et al.*, 2002), and serological evidence of PCV2 infection has been found in diseased as well as in healthy herds (Blanchard *et al.*, 2003). Furthermore, various viral co-infections (Allan *et al.*, 1999b; Ellis *et al.*, 2000; Rovira *et al.*, 2002) and environmental and management factors (Madec *et al.*, 2000; Labarque *et al.*, 2000; Rose *et al.*, 2003) have been shown to be involved in PMWS.

The PCV2 genome is a circular single-stranded DNA molecule of about 1·77 kb. The two main viral genes, ORF1 (replication-associated, *rep*) and ORF2 (capsid, *cap*) represent 93 % of the genome. PCV2 is highly related to, yet distinct from, the first reported swine circovirus, *Porcine circovirus* type 1 (PCV1), originally identified as a contaminant of a cell line (Tischer *et al.*, 1974). PCV1 infection in swine is not pathogenic and is widespread, as reported in a number of serological surveys (reviewed by Allan & Ellis, 2000).

In France, the first PMWS outbreaks were described in 1996 (Madec *et al.*, 2000). From 1999 onwards, the incidence of the disease regressed in response to improvements in rearing practices. At the time of PMWS emergence, both

The GenBank accession numbers of the 23 sequences reported in this paper are AY321982–AY322004.

PCV1 and PCV2 were detected by PCR in affected-pig samples, and a few isolates were genetically characterized (LeCann *et al.*, 1997; Mankertz *et al.*, 2000).

A large number of PCV2 isolates from different countries and from pigs of various health states have been characterized; all viral genomes shared a high nucleotide identity (range 95–100 %). No molecular marker associated with a particular disease condition has yet been identified (Fenaux *et al.*, 2000; Choi *et al.*, 2002; Larochelle *et al.*, 2002; Pogranichniy *et al.*, 2002).

The present study aimed to draw up an inventory of the PCV1 and PCV2 sequences isolated in Brittany during the PMWS outbreaks. In addition, we focused on the comparison of PCV2 strains obtained from PMWS affected- and non-affected herds to shed light on the relationship between the pool of circulating viruses and the onset of the syndrome.

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METHODS

Sample collection. All farms were located in Brittany, in the western part of France, except for two, which were situated eastwards at a distance of nearly 700 km from Brittany. PMWS(+) herds were currently affected farms, characterized by increased mortality percentages (5 % in comparison with the situation before PMWS was observed), typical PMWS lesions at necropsy and positive PCV2 *in situ* hybridization recorded for at least two pigs. PMWS(−) herds had no history of PMWS expression and lacked one or several of the above criteria (Rose *et al.*, 2003).

One pig (m1) raised in a PMWS(−) herd was considered as a PMWS sporadic case, since it exhibited typical lesions at necropsy. Thus, the strain isolated from this animal was reclassified with those from the PMWS(+) herds (Table 1).

From July 2000 to January 2002, 31 pigs originating from 13 PMWS(+) herds and 25 pigs from 10 PMWS(−) herds were selected on the basis of two to five animals per herd and necropsied. Tissue samples (lung, tonsil, ileum, and bronchial, inguineal and mesenteric lymph nodes) were collected and stored at −20 °C until used, which was within 2 weeks. The age of animals, clinical data and lesions are recorded in Table 1.

PCV detection and sequencing. Total DNA was extracted using a DNeasy Tissue kit (Qiagen), according to the manufacturer's instructions. In the first PCR step, specific PCV1 and PCV2 sequences were detected using two sets of previously described primers (Larochelle *et al.*, 1999): PF2/PR2, specific for a 349 bp fragment located in the ORF1 of PCV1, and CF8/CR8, specific for a 263 bp fragment in the ORF2 of PCV2. A high-fidelity DNA polymerase (DyNAzyme EXT DNA Polymerase; Ozyme) was used to amplify virus DNA with the following cycling programme: 2 min at 94 °C, 35 cycles consisting of 30 s at 94 °C, 30 s at 62 °C (PF2/PR2) or 60 °C (CF8/CR8) and 30 s at 72 °C, and a final extension step at 72 °C for 5 min. The PCR products were purified using a Qiaquick PCR purification kit (Qiagen). Secondly, amplification of the near-whole circular PCV2 genomes detected in the first step was performed with primers 64CA2 and 1723CA2, which resulted in a 1660 bp fragment overlapping the CF8/CR8 fragment. The Platinum *Taq* DNA polymerase High Fidelity (Gibco Life Technologies) was used and the following cycling programme was applied: 2 min at 94 °C, 10 cycles consisting of 30 s at 94 °C, annealing for 30 s at 67 °C decreased by 1 °C for each cycle (touch-down PCR) and 2 min at 68 °C, and finally 20 cycles consisting of 30 s at 94 °C, 30 s at 57 °C and 2 min at 68 °C.

For some samples, another more sensitive set of two primer pairs was used: 98F/721R and 665F/220R, which allowed the amplification of two overlapping fragments encompassing the entire PCV2 genome (644 bp and 1343 bp, respectively). These primers were used with Platinum *Taq* DNA polymerase High Fidelity under the following conditions: for 98F/721R, denaturation for 2 min at 94 °C and 35 cycles consisting of 30 s at 94 °C, 30 s at 52 °C and 1 min at 68 °C; for 665F/220R, denaturation for 2 min at 94 °C, 10 cycles consisting of 30 s at 94 °C, annealing for 30 s at 65 °C decreased by 1 °C for each cycle and 2 min at 68 °C, and finally 25 cycles consisting of 30 s at 94 °C, 30 s at 55 °C and 2 min at 68 °C. PCR products were subjected to electrophoresis and gel purified using a Qiaquick gel extraction kit (Qiagen). Sequencing reactions were carried out on both strands using an ABI Prism Dye Terminator Cycle Sequencing kit (Applied Biosystems). The sequencing was performed on a 373 DNA sequencer (Applied Biosystems). All PCR and sequencing primers are given in Table 2.

Amplification and sequencing of the complete PCV2 genome were performed for 19 pigs from seven PMWS(+) herds and 19 pigs from seven PMWS(−) herds. Sequences were obtained from the lung extracts of the 38 pigs, except three for which inguineal (m3) and bronchial lymph nodes (m6, m8) were used. To evaluate viral diversity within an individual, the six tissue extracts of two animals (m11 and m12) were subjected to analysis.

In one case (m3), the PCR product encompassing the intergenic sequence between ORF2 and ORF1 (primers 220R/665F) was cloned using the TopoTA cloning kit (Invitrogen, Life Technologies). Nine positive clones were sequenced.

Sequence analysis. Multiple alignments were obtained using CLUSTALW on-line version 1.8.2 (http://www.pasteur.fr). Phylogenetic analysis was performed using the PHYLIP version 3.573 package (http://evolution.genetics.washington.edu/phylip.html). Briefly, the SEQBOOT program generated 1000 pseudo-datasets from the original alignment by the bootstrap method. Distance matrices were calculated from each dataset using the DNADIST program. Trees were built by the neighbour-joining method with Kimura's modified two-parameter distance (NEIGHBOR) program. Parsimony analysis was performed by running DNAPARS. The CONSENSE program was used from both methods to produce consensus trees, which were finally plotted using TREEVIEW version 1.6.6 (http://taxonomy.zoology.gla.ac.uk). Amino acid sequences were analysed running PROTDIST with Dayhoff PAM matrix and PROTPARS in place of DNADIST and DNAPARS, respectively.

Pairwise comparison of nucleotide and amino acid sequence identities was performed using MEGALIGN version 1.13 (DNASTAR).

Mean similarity along the sequences of the PMWS(+) and PMWS(−) groups was determined by the PLOTSIMILARITY program of the GCG Wisconsin Package version 10.3 (Accelrys).

RESULTS

PCV1 and PCV2 detection

To estimate the prevalence of porcine circoviruses during the PMWS outbreaks in Brittany, we tested for the presence of PCV1 and PCV2 in herds by PCR. Attempts to detect PCV1 sequences failed, whatever the PMWS status of the

Table 1. Identification of herds, symptoms and lesions in animals, PCV2 strain designations and GenBank accession numbers

Only the samples identified as positive by PCR and subjected to PCV2 sequencing are shown. Location of herds is given in district numbers (22, 29, 35, 56, Brittany; 01, east of France). PDNS, porcine dermatitis and nephropathy syndrome; Fd, France PMWS diseased; Fh, France healthy.

Herd (district) PMWS (+/−)	Pig identification	Age (weeks)	Clinical signs, lesions and associated pathogens	PCV2 strain designation	Accession no.
A (22) PMWS(+)	**p1**	11	Typical PMWS	**Fd1**	AY322000
	p2	11	Typical PMWS	**Fd2**	AY321999
	p3	11	Typical PMWS	**Fd1**	AY322000
B (22) PMWS(+)	**p4**	13	Typical PMWS	**Fd3**	AY321984
	p5	13	Typical PMWS, signs of PDNS	**Fd3**	AY321984
	p6	12	Signs of PMWS	**Fd3**	AY321984
	p7	16	Typical PMWS	**Fd4**	AY321986
	p8	16	Bronchopneumonia, interstitial nephritis, oedema of legs	**Fd3**	AY321984
C (35) PMWS(+)	**p9**	13	Signs of PMWS	**Fd5**	AY321997
	p10	13	Wasting, pneumonia, pleurisy	**Fd5**	AY321997
	p11	13	Pleurisy, peritonitis, pericarditis	**Fd6**	AY321998
D (29) PMWS(+)	**p12**	12	Typical PMWS	**Fd7**	AY321990
	p13	12	Typical PMWS	**Fd7**	AY321990
E (01) PMWS(+)	**p14**	11	Typical PMWS, signs of PDNS	**Fd8**	AY321995
	p15	11	Wasting, pericarditis, peritonitis, pleuropneumonia	**Fd9**	AY321996
F (22) PMWS(+)	**p16**	15	PMWS, signs of PDNS	**Fd10**	AY321991
	p17	15	Signs of PMWS, pericarditis, pleurisy, perihepatitis, peritonitis	**Fd10**	AY321991
G (29) PMWS(+)	**p18**	15	Typical PMWS	**Fd11**	AY321988
	p19	15	PMWS, signs of PDNS	**Fd12**	AY321989
H (22) PMWS(−)	**m1**	18	Wasting, signs of PDNS	**Fd13**	AY321985
	m2	14	Hernia and abscess of umbilicus	**Fh14**	AY321982
I (22) PMWS(−)	**m3**	14	Multifocal lymphosarcoma	**Fh15**	AY321992
				Fh16	AY321993
	m4	14	Wasting, necrosis and proliferous ileitis associated with *Lawsonia* and *Pasteurella multocida*	**Fh16**	AY321993
J (22) PMWS(−)	**m5**	14	Nephritis, inguineal hernia	**Fh17**	AY322004
	m6	14		**Fh17**	AY322004
	m7	14		**Fh17**	AY322004
K (29) PMWS(−)	**m8**	17	Mild interstitial pneumonia	**Fh18**	AY321987
	m9	17	Mild interstitial pneumonia	**Fh18**	AY321987
	m10	17	Fibrous pleurisy and pericarditis, interstitial pneumonia associated with *P. multocida* and *Streptococcus suis* 2	**Fh18**	AY321987
L (56) PMWS(−)	**m11**	15		**Fh19***	AY322003
	m12	15		**Fh20***	AY321983
	m13	15		**Fh19**	AY322003
M (29) PMWS(−)	**m14**	16		**Fh21**	AY322001
	m15	16		**Fh22**	AY322002
	m16	16		**Fh22**	AY322002
N (29) PMWS(−)	**m17**	15		**Fh23**	AY321994
	m18	15		**Fh23**	AY321994
	m19	15		**Fh23**	AY321994

*All six tissue extracts of animals m11 and m12 were subjected to complete PCV2 amplification and sequencing.

herd. In contrast, PCV2-specific PCR (using CF8/CR8 primers) was positive in 100 % (31/31) of animals from PMWS(+) herds and in 76 % (19/25) of animals from PMWS(−) herds. Some of the latter 19 positive pigs had only two or three positive organs, most frequently the lymph nodes (data not shown).

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Table 2. Sequences of the PCR and sequencing primers

Primer positions are indicated relatively to a reference strain (GenBank accession no. AF201311) and numbered from the origin of replication (Hamel *et al.*, 1998).

	Position (nt)	Sequence (5′→3′)
PCR		
PF2	610–629	TTGCTGAGCCTAGCGACACC
PR2	955–936	TCCACTGCTTCAAATCGGCC
CF8	1322–1341	TAGGTTAGGGCTGTGGCCTT
CR8	1585–1566	CCGCACCTTCGGATATACTG
64CA2*	1536–1555	AGGAGGGCGTTCTGACTGTG
1723CA2*	1428–1409	GTTGAATTCTGGCCCTGCTC
98F*	98–119	GTGGGTGTTCACTCTGAATAA
721R*	741–721	CACACAGTCTCAGTAGATCAT
665F*	665–685	TTACCATGGTGAAGAAGTGGT
220R*	240–220	AATTAGCGAACCCCTGGAGGT
Sequencing		
pcv1	7–26	GCACTTCGGCAGCGGCAGCA
361F	129–147	GACGAGCGCAAGAAAATAC
570F	338–357	CTGCAGTAAAGAAGGCAACT
665F	665–685	TTACCATGGTGAAGAAGTGGT
1644CAF	860–879	TGTAGAAGCTCTTTATCGGA
1180F	960–979	CCATGCCCTGAATTTCCATA
1312F	1092–1111	CACGCATATTGTATTCCTGG
1476F	1256–1275	TAGCGGGAGTGGTAGGAGAA
1336	1327–1347	GAGGGCTGTGGCCTTTGTTAC
1336·2	1328–1348	AGGGCTGTGGCCTTTATTACG
64CA3	1536–1555	AGGAGGGCGTTTTGACTGTG
64CA4	1536–1555	AGGAGGGCGTGCTGACTGTG
313R	81–62	GTCCGCTTCTTCCATTCTTC
1209CAR	425–406	GGTACTCACAGCAGTAGACA
1062R	830–813	CAACGGGGTCTGATTGCT
1147R	927–908	CCGTGGATTGTTCTGTAGCA
1334R	1114–1095	CGACCAGGAATACAATATCC
1527R	1307–1288	ACTACTCCTCCCGCCATACC
260FCR	1581–1561	ACCTTCGGATATACTGTCAAG
260FCR2	1581–1561	ACCTTCGGATATACTGTGAAG
1696R	1714–1695	TTACCGGAGAAGAAGACACC

*These PCR primers were also used for sequencing.

Molecular characterization of PCV2 strains

A total of 23 distinct PCV2 sequences were identified, 13 originating from PMWS(+) herds and 10 from PMWS(−) herds (Table 1). All sequences were 1767 nt in length, except Fh17 and Fh16 (1768 and 1778 nt, respectively). Fh16 had an 11 nt insertion at position 42, nine bases upstream from the initiation codon of the ORF1 (Table 3). This insertion introduced successively a fourth hexamer (CGGCAG) and a third pentamer (CACCT), respectively identical with the repetitions and the spacers described by Steinfeldt *et al.* (2001) in the case of PCV1. The two variants Fh15 and Fh16 were identical at each position except that the 11 nt insertion was only found in Fh16. In one farm, only the variant Fh16 was isolated from pig m4, while both Fh15 and Fh16 were isolated from pig m3 (Table 1). Due to the mix of variants Fh15 and Fh16 in this case, the cloning of the PCR product encompassing the origin of replication was necessary to obtain readable sequences. Of nine clones, seven carried the insertion while the other two lacked it.

The intra-individual variability was evaluated by sequencing of PCR products obtained from several samples of two animals. In all six of the tested tissues, a unique variant was identified for each of the two pigs m11 and m12.

Each of the 23 identified variants was found in only one herd. A maximum of two variants was found in each herd (Table 1).

All the complete PCV2 sequences, pairwise compared, shared a nucleotide identity of 94·6–100 % (Table 4). This variation was mainly due to the variability within ORF2 (91·2–100 % nucleotide identity), the ORF1 being highly conserved (97·8–100 %). The amino acid identity of ORF1 (99–100 %) was shown to be greater than the nucleotide sequence identity, confirming conservation of the protein sequence. In contrast, the amino acid identity of ORF2 (90·1–100 %) was larger than the nucleotide sequence identity and reflected a lower level of structural constraints for this protein (Table 4).

An alignment of ORF2 amino acid sequences of 82 variants collected from this study, GenBank and the study of Larochelle *et al.* (2002) showed that changes were scattered throughout the protein, although three domains, A (57–91), B (121–136) and C (180–191) were more prone to variation (Fig. 1). Domains A and B were situated within an immunoreactive region previously identified by PEPSCAN (Mahé *et al.*, 2000), while domain C was at the border of such a region. Only domains A and C were within regions with a high immunogenic index (Fig. 1).

Phylogenetic analysis

The phylogenetic relationships between the 23 variants described herein and sequences from other countries were assessed (Fig. 2). Two main clusters supported by significant bootstrap values were obtained. Cluster I contained almost all the PCV2 sequences obtained in this study, plus three previously reported isolates from France and two sequences from the UK (AJ293869) and The Netherlands (AF201897). These sequences were all closely related one to another (>99 % nucleotide identity). Within cluster I, two subgroups were identified: subgroup Ia composed of three isolates from PMWS(−) herds and subgroup Ib in which 19 PCV2 sequences from this study scattered, irrespective of their PMWS status. The second main cluster (II) included a subgroup (IIa) composed of one isolate from Germany (AF201305), one isolate from Canada (AF109399) and a single isolate from France, Fh17. All other cluster II subgroups included sequences from countries distant from one another. For individual ORF1

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Table 3. Most frequent mutations of PCV2 sequences from the present study relative to a reference strain (GenBank accession no. AF201311)

The given nucleotide positions (ref. nt position) are numbered from the origin of replication (Hamel *et al.*, 1998) and underlined in the related codon. Nucleic (upper table) and amino acid (lower table) mutations are qualitatively and quantitatively (nbv, number of variants) reported for both groups of PCV2 sequences from diseased (Fd) and healthy (Fh) animals. A comparison with disease-associated strains (named as in Fig. 2) from other countries is included.

ref. nt position	42	152	161	254	365	872	1039	1118	1164	1167	1182	1284	1546	1558	1566
ref. genomic strand ref. complementary strand	A	GAT	ATA	CAG	ATG	CTT	AAC	ATA	GGA	GCT	CTG	ACC	AGA	CGA	GTC
Fd1 to Fd13 (nbv)						**--C (1)**							**-A- (8)**	**A-- (1)**	**A-- (8) --G (2)**
Fh14 to Fh23 (nbv)	**GGCAGCACCTC- (1)**	**--G (4)**	**--C (4)**	**--A (4)**	**--T (2) --A (1)**	**--C (4)**	**--A (2)**	**-A- (2)**	**A-- (1)**	**AG- (1)**	**A-- (4)**	**C-- (2)**	**-A- (4) --C (1)**	**GCT (4)**	**A-- (6)**
AJ 293869 (UK)					--C								-AG		A--
AF 201897 (NL)															
AF 055394 (FR)										A--			-A-		
AF 201305 (GR)		--G	--C	--A	--T	--C	--A	-A-	A--	AG-	A--	C--	--C	GCT	
AF 109399 (CN)		--G	--C	--A	--T	--C	--A	-A-	A--	AG-	A--	C--	--C	GCT	
AB 072301 (JP)		--G	--C	--A	--T	--C	--A	-A-	A--	T--		C--	-C-	GCT	
AF 264038 (US)		--G	--C	--A	--T	--C	--A	-A-	A--	T--		C--	-C-	--T	
AF 201310 (SP)		--G	--C	--A		--C	--A	-A-	-C-	T--		C--		GCT	
AF 364094 (TW)		--G	--C	--A	--C	--C	--A	-A-	--C	T--		C--	C--	GCT	--G

ref. peptide position	34	37	68	105	274	232	206	191	190	185	151	63	59	57
ORF1 ORF2	D	I	Q	M	L	N	I	G	A	L	T	R	R	V
Fd1 to Fd13 (nbv)												**K (8)**		**I (8)**
Fh14 to Fh23 (nbv)	**E (4)**			**I (3)**		**K (2)**	**K (2)**	**R (1)**	**S (1)**	**M (4)**	**P (2)**	**K (4) S (1)**	**A (4)**	**I (8)**
AJ 293869 (UK)				I								K		I
AF 201897 (NL)														
AF 055394 (FR)									T			K		
AF 201305 (GR)	E			I		K	K	R	S	M	P	S	A	
AF 109399 (CN)	E			I		K	K	R	S	M	P	S	A	
AB 072301 (JP)	E			I		K	K	R	S		P	T	A	
AF 264038 (US)	E			I		K	K	R	S		P	T		
AF 201310 (SP)	E					K	K	A	S		P		A	
AF 364094 (TW)	E			I		K	K		S		P		A	

and ORF2 sequences, the phylogenetic analysis was restricted to 15 strains representative of the subgroups obtained in the phylogenetic analysis of the whole PCV2 genomes. It revealed that the general clustering observed for complete genomes was maintained for the individual ORFs at the nucleic and amino acid levels, except for the ORF1 protein tree (Fig. 3).

Comparison of PCV2 strains from PMWS(+) and PMWS(−) herds

A wider nucleotide diversity in the 10 PMWS(−) sequences (94·6–99·9 %) was observed compared with the 13 PMWS(+) (99·5–99·9 %). This tendency was observed for the two main viral genes at both the nucleic and amino

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Table 4. Sequence identity ranges of PCV2 isolates from PMWS affected and non-affected herds

Pairwise comparison of PCV2 isolates within PMWS(+) and PMWS(−) herds are given as identity percentages for whole genome and for nucleotide (nt) and amino acid (aa) sequences of ORF1 and ORF2.

		PMWS(+)	PMWS(−)
Genome	nt	99·5–100	94·6–100
ORF1	nt	99·7–100	97·8–100
	aa	99·7–100	99·0–100
ORF2	nt	99·0–100	91·2–100
	aa	98·7–100	90·1–100

acid levels and was particularly obvious for ORF2 (Table 4). Similarity plots along the sequences of the PMWS(+) and PMWS(−) groups confirmed the above result (Fig. 4). The strong peak of dissimilarity before the beginning of ORF1 was due to the 11 nt insertion at position 42 in variant Fh16 (Fig. 4b). This peak exceeded the plot range of the graph, which was set between 0·75 and 1 for a better visualization of the differences. The variations in the similarity score were scattered along the whole PCV2 genome for both groups. As strain Fh17 was the most genetically distant from the others (Fig. 2), we excluded that strain from the PMWS(−) group and repeated the analysis. A profile very close to the one presented in Fig. 4(b) was obtained (data not shown), indicating a limited contribution of Fh17 to the variability between PMWS(−) isolates.

Interestingly, the two consensus sequences obtained from each of the PMWS(+) and PMWS(−) groups of complete PCV2 genomes were identical at every position (data not shown).

The most frequent nucleic and amino acid substitutions between PCV2 sequences of this study and the strain AF201311 from France, taken as a reference, are shown in Table 3. As expected, most were identified in the PMWS(−) sequences. All mutations detected in the PMWS(−) animals from the present study were also found in PCV2 sequences extracted from GenBank and obtained from animals suffering from PMWS.

DISCUSSION

PCV2 is now recognized to be of pivotal importance in PMWS, since it is always identified in sick animals. Nevertheless, its exact role remains questionable because natural or experimental PCV2 infection of pigs does not necessarily result in PMWS. A molecular survey of viral genomes circulating during the recent outbreaks in Brittany could help to clarify the relationship between the virus and the disease.

At the time of PMWS emergence in Brittany, both PCV1 and PCV2 sequences were detected by PCR in a few samples (LeCann *et al.*, 1997; Mankertz *et al.*, 2000), but, interestingly, PCV1 was no longer detected in any sample in the present study. This result confirms the general very low PCV1 incidence (3–5 %) reported by others (Mankertz *et al.*, 2000; Calsamiglia *et al.*, 2002). In addition, it strengthens the assumption by Calsamiglia *et al.* (2002) that the seroprevalence of PCV1 may have been overestimated in previous serological studies due to epitopes common to both PCV1 and PCV2. The low prevalence of PCV-1 is puzzling. As a non-pathogenic virus, a widespread subclinical propagation might be expected, as is the case for TT virus (TTV), another mammalian circovirus, widely disseminated in the human population (Khudyakov *et al.*, 2000). A possible explanation is the low efficiency of PCV1 in producing sufficient inoculum for dissemination, which contrasts with TTV whose daily production in chronically infected patients has been estimated to be at least $3{\cdot}8 \times 10^{10}$ virions (Maggi *et al.*, 2001). Another factor to consider is possible competition between PCV1 and PCV2. The decrease in PCV1 prevalence during the outbreaks in

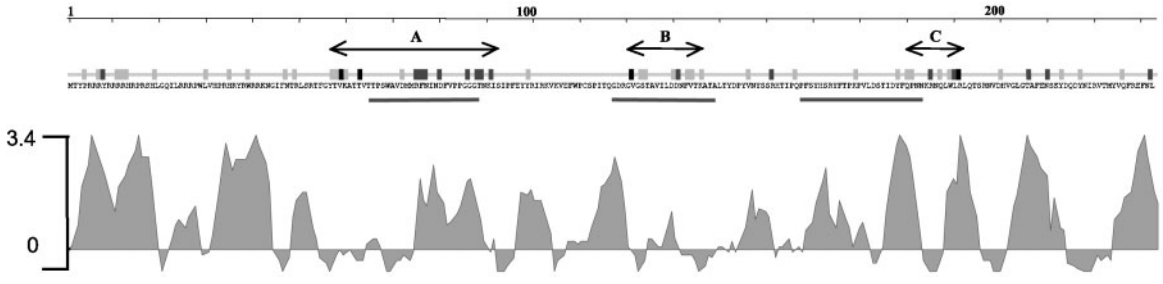

Fig. 1. Variability of the amino acid sequence of ORF2 (Cap). The indicated sequence is a consensus from an alignment of 82 variants extracted from GenBank, the study of Larochelle *et al.* (2002) and the present work. Light grey, medium grey and black blocks represent residues that are slightly, moderately or highly variable, respectively. Other residues are perfectly conserved. Variable domains A, B and C are indicated by arrows. The three horizontal bars represent immunogenic regions identified by PEPSCAN (Mahé *et al.*, 2000). The graph shows the antigenic index of Jameson–Wolf (DNASTAR) calculated on the consensus sequence.

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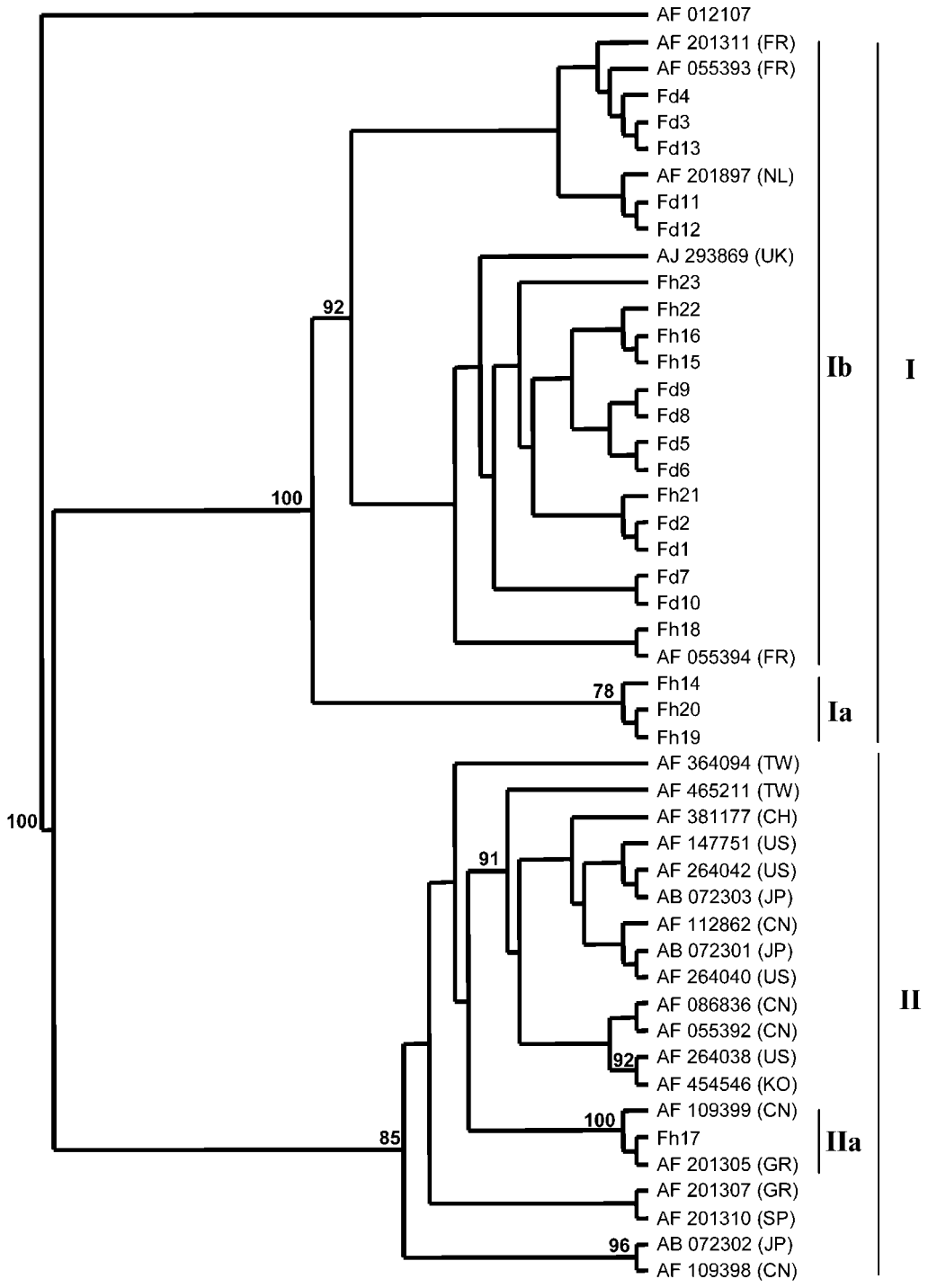

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Fig. 2. Phylogenetic relationships between 47 full-length PCV2 genomes. Twenty-three sequences from the present study were compared with 24 PCV2 from GenBank (accession nos and geographic origin: FR, France; NL, The Netherlands; UK, United Kingdom; TW, Taiwan; CH, China; US, USA; JP, Japan; CN, Canada; SP, Spain; KO, Korea; GR, Germany). The neighbour-joining algorithm based on the Kimura two-parameter distance estimation method was used. A similar result was obtained by the parsimony method (not shown). Significant bootstrap values are indicated as a percentage for 1000 replicates. A sequence of PCV1 (AF012107) was used as an outgroup. Main clusters (I, II) and subgroups of interest (Ia, Ib, IIa) are indicated.

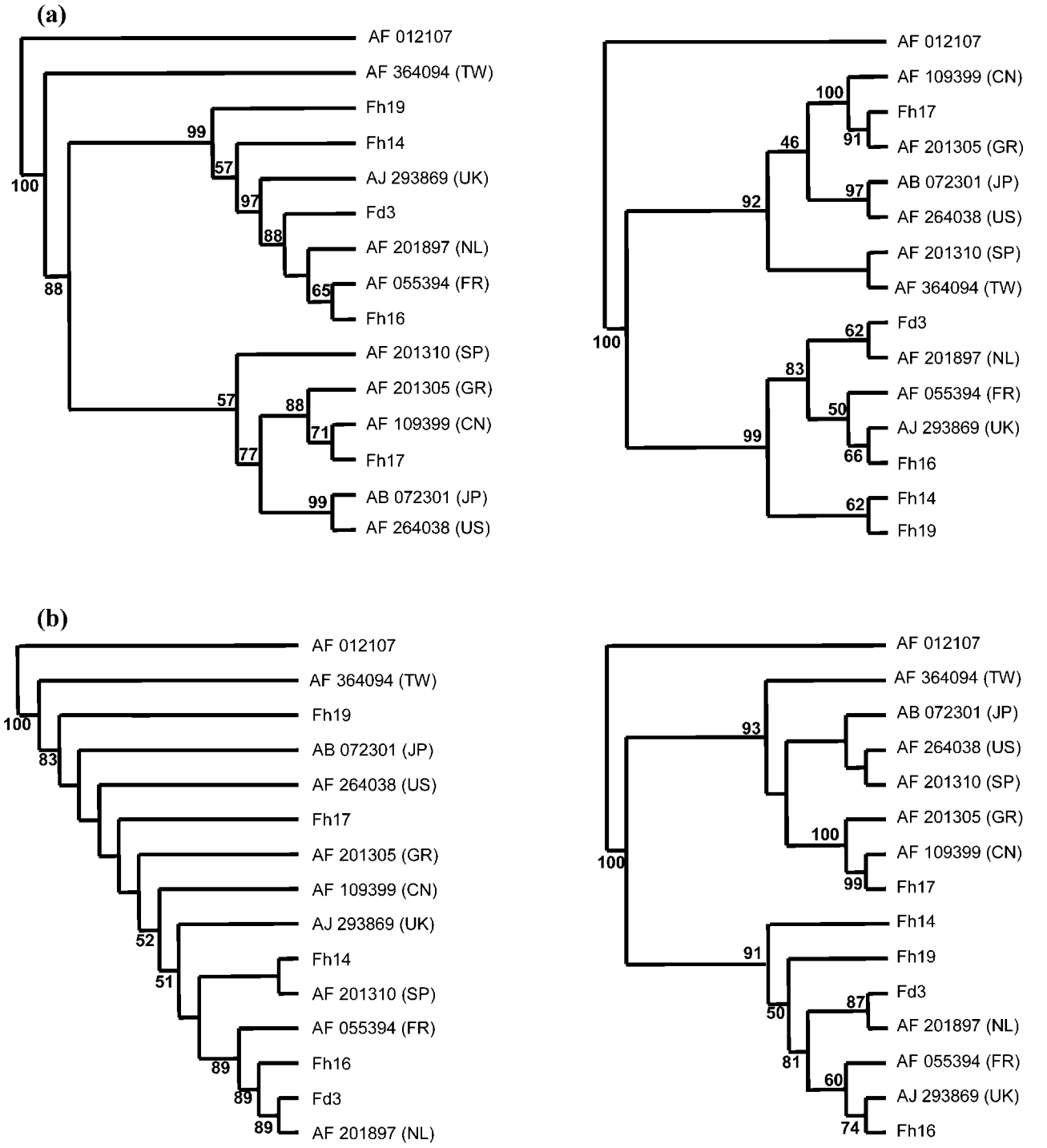

Fig. 3. Cladograms obtained for the two main genes (ORF1 and ORF2) of 15 PCV2 isolates. Sequences representative of main subclusters were selected from Fig. 2. ORF1 (left) and ORF2 (right) sequences were compared at the nucleic acid (a) and amino acid (b) levels. The neighbour-joining algorithm based on the Kimura two-parameter distance estimation method for nucleotides and on the Dayhoff PAM Matrix for amino acids was used. Similar results were obtained by the parsimony method. Significant bootstrap values are indicated as a percentage for 1000 replicates. Sequences from GenBank are named as in Fig. 2. A sequence of PCV1 (AF012107) was used as an outgroup. Except for the ORF1 amino acid sequences (b, left), the phylogenetic trees had topologies similar to that observed with full-length genomic sequences.

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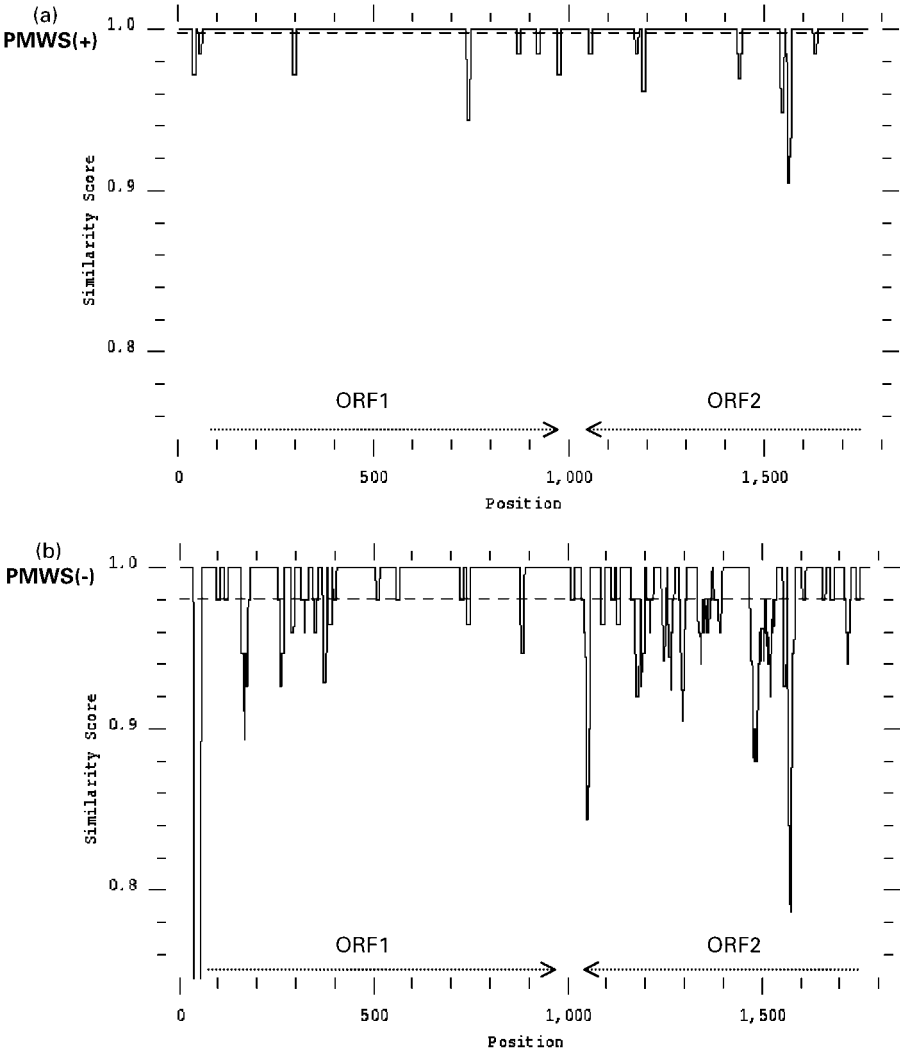

Fig. 4. Similarity plots along PCV2 sequences from PMWS(+) and PMWS(−) herds. The PLOTSIMILARITY program (GCG Wisconsin package) was used to draw similarity score curves by means of a 10-nucleotide sliding window. (a) 13 PCV2 sequences from PMWS(+) herds; (b) 10 PCV2 sequences from PMWS(−) herds. Dashed lines represent the mean similarities along the entire sequences. ORF1 and ORF2 positions are indicated by dotted arrows.

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Brittany may be a consequence of the massive multiplication of PCV2. *In vitro* experiments are needed to test this hypothesis.

From the present phylogenetic analysis, the most striking observation was the distribution of PCV2 in two highly related yet distinct clusters (Fig. 2): strains from France, the UK and The Netherlands (cluster I) versus all the other PCV2 sequences from North America, Asia, Germany and Spain (cluster II). Sequences from cluster I had a single nucleotide deletion in the C-terminal part of the ORF2 resulting in an amino acid change (K232N) previously described (Mankertz *et al.*, 2000). The strains Fh19 and Fh20 were an exception, since the K→N change was restored (N→K) by a G→T transversion at position 1039 (Table 3). Another particular strain within cluster I was the strain Fh16, which had an 11 nucleotide motif inserted downstream from the origin of replication. To our knowledge, this is the first description of such an insertion in a PCV2 genome from a virus isolated in the field. The influence of this particular motif within a domain crucial for virus replication needs to be studied.

All the changes identified in the *rep* gene (ORF1) were located at the third position of codons, leading to silent mutations or to conservative amino acid substitutions (Table 3). This is not surprising, since the *rep* protein of most DNA viruses is not prone to variation because of a strong selection pressure exerted by its functions. In contrast, the nucleotide mutations identified in the *cap* gene (ORF2) induced amino acid substitutions, some of which were non-conservative (Table 3). As a multifunctional protein, Cap plays a key role in virulence for some *Circoviridae* (Todd *et al.*, 2002) and other DNA icosahedral

viruses (Truyen *et al.*, 1995). We have examined the ORF2 product of all strains sequenced to date, including the Canadian strains of Larochelle *et al.* (2002) and ours. Three domains within or near immunogenic regions exhibit polymorphism, suggesting that regions at the external surface of the capsid may be variable. Assuming that the structure of the Cap protein respects the β-barrel structure common to all single-stranded DNA viruses, these three domains might be localized in the loops between β-coils, which are highly constrained structures. In contrast, the domain 137–177 is strongly conserved among PCV1 and PCV2 sequences (not shown), suggesting a key region for virus–host interactions or virus assembly, such as receptor binding or interactions between capsid monomers.

When comparing the two groups of PCV2 sequences from PMWS(+) and PMWS(−) herds, a slight difference in variability was noticed, PMWS(−) strains being more heterogeneous than their counterparts in PMWS(+) herds. However, several lines of evidence suggest that this difference does not reflect a correlation between a restricted virus genetic pool and disease symptoms. First, the intra-group heterogeneity among PMWS(−) sequences appeared to be mainly due to the three strains of the subgroup Ia that branch out from all the other sequences from France (Figs 2 and 3) and to the strain Fh17, which clusters with foreign strains. The six other strains from the PMWS(−) group proved to be closely related to all the PMWS(+) sequences (subgroup Ib, Fig. 2). Secondly, the consensus sequences obtained from each of the two PMWS(+) and PMWS(−) groups were identical. Thirdly, although several residues were shown to be specific to PMWS(−) strains relative to PMWS(+) (Table 3), they were also found in PMWS(+) strains. Thus, their direct implication in the virulence was rejected. Consequently, we concluded that the possibility that the disease is associated with a few variants particularly well suited for virulence within all strains from France should be eliminated. In other words, we could not identify any molecular marker of virulence in PMWS(+) strains that could explain the sudden outbreaks. This latter result is consistent with previous reports (Choi *et al.*, 2002; Larochelle *et al.*, 2002; Pogranichniy *et al.*, 2002).

The degree of conservation among all the PCV2 sequences described so far is strikingly high considering the large range of their geographical origins and the general prevalence of the virus. In contrast, TTV and TTV-like viruses exhibit a much greater variability (reviewed by Simmonds, 2002). Within this narrow frame of PCV2 diversity, the variants from France, except Fh17, still form a distinct lineage with inter-isolate identities greater than 98 %. This indicates that these variants are derived from a very restricted genetic pool of PCV2 disseminated throughout the country. In this context, the unique genetic profile of strain Fh17, clustered with foreign strains, suggests a different introduction of the virus to France.

A likely hypothesis to explain why PCV2 is so homogeneous is that porcine circoviruses could have been submitted to one or several genetic bottlenecks. These as yet unidentified events could be linked to the history of the host. In particular, molecular evidence suggests that the domestication of the pig occurred 9000 years ago, and there was a massive introduction of Asian pigs into European breeds during the 18th and 19th centuries (Giuffra *et al.*, 2000).

All studies, including the present one, converge on the conclusion that the PMWS epidemics do not originate in the appearance of a new PCV2 variant. In addition, the very high prevalence of PCV2 among healthy animals suggests that this virus is well adapted to its host, and that both organisms may have coexisted for a long time. Our work shows that the PCV2 mutation rate is very low, since a single virus clone was detected by PCR in each animal. The mix of clones Fh15 and Fh16 in one individual is a noticeable exception, but these sequences clearly derived from each other, based on the phylogenetic analysis. However, the capacity for evolution of PCV2 at a geographically small-region scale is evident, since all farms studied in Brittany are infected with highly related, yet distinct, clones. The biological or environmental factor(s) disrupting the equilibrium between host and parasite during PMWS is still to be uncovered.

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