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Genetic diversity of Brazilian strains of porcine circovirus type 2 (PCV-2) revealed by analysis of the *cap* gene (ORF-2)

A. M. Martins Gomes de Castro[1,4], **A. Cortez**[2], **M. B. Heinemann**[3], **P. E. Brandão**[2,4], and **L. J. Richtzenhain**[2,4]

[1] Faculdade de Jaguariúna, Curso de Medicina Veterinária, Jaguariúna, São Paulo, Brazil
[2] Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, Brazil
[3] Escola de Veterinária, Universidade Federal de Minas Gerais, Minas Gerais, Brazil
[4] Circovirus Research Group, São Paulo, Brazil

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Summary

Porcine circovirus 2 (PCV-2) is associated with a broad range of syndromes. In this study, 19 of 870 samples from pigs from different Brazilian states were found to be positive for PCV-2 by polymerase chain reaction (PCR). A fragment of 700 nt of the *cap* gene (ORF-2) from the 19 PCV-2-positive samples were sequenced using three pairs of primers (Fa/Ra, Fb/Rb and Fc/Rc). Maximum parsimony genealogy with a heuristic algorithm using the 19 field strain studied here, 21 sequences from GenBank and PCV-1 as an out-group showed the existence of two major clusters (A and B) and the Brazilian strains segregating in both of them. PCV-2 was found in pigs with various clinical signs. No association between clusters of PCV-2 and different states or clinical signs were observed, demonstrating that the exact role of PCV-2 in porcine circovirus diseases (PCVD) in Brazil still needs to be clarified. These results contribute to the molecular characterization of PCV-2, which serve as a basis for the epimiology of PCV-2 infection.

Author's address: Alessandra Marnie Martins Gomes de Castro, Departamento de Medicina Veterinária Preventiva e Saúde, Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Avenida Professor Doutor Orlando Marques de Paiva, 87 Cidade Universitária, CEP 05508-900 São Paulo, SP, Brazil. e-mail: almarnie@yahoo.com

Introduction

Porcine circovirus (PCV) is a small non-enveloped virus that contains a single-stranded circular DNA of 1.8 kb, belonging to the family *Circoviridae*, genus *Circovirus* [1]. Two genotypes of PCV are recognized. PCV type 1 (PCV-1), detected as a contaminant of the porcine kidney PK15 cell line (CCL-33), is widespread in the swine population of many countries based on past serological surveys and is not currently considered to be pathogenic. Porcine circovirus type 2 (PCV-2), so-named because of antigenic and genomic differences with PCV-1, is associated with various clinical conditions such as porcine dermatitis and nephropathy syndrome (PDNS) [32], congenital tremors [36], reproductive failure [13, 38], porcine respiratory

disease complex (PRDC) and mainly postweaning multisystemic wasting syndrome (PMWS) [1]. The terminology porcine circovirus diseases (PCVD) have been proposed to include the conditions that had been linked with PCV-2 infection [3].

PMWS is endemic in many swine-producing countries, including Brazil [8] and is characterized by progressive weight loss, respiratory signs and jaundice. The syndrome occurs in herds that are usually in good health, have a low morbidity but a relative high mortality rate among pigs in the 5- to 12-week-old age range. The precise role of PCV-2 in the pathogenesis of PMWS is poorly understood, since is known that PCV-2 is necessary but not sufficient. Others factors like viral and/or bacterial co-infections [2, 10, 11, 33], environment and management livestock system have been shown to be involved in the development of this syndrome [24]. Experimental inoculation of pigs with pure PCV-2 only reproduced mild PMWS clinical signs [15, 25], and PCV-2 antigen and/or DNA has been found in diseased as well as in healthy animals [4]. In spite of the high prevalence of PCV-2 in swine herds and its association with various clinical signs that have an important impact on the productivity of the swine herd, studies on the genetic diversity of PCV-2 remains to be carried out.

PCV-2 has a compact ambisense genomic structure composed of two intergenic regions flanked by two head-to-head-arranged open reading frames (ORFs), the *rep* (ORF-1) and *cap* (ORF-2) genes. The *rep* gene is the largest ORF located on the viral sense strand and encodes two replication proteins, Rep (312 aa and 35.6 kDa) and Rep' (168 aa and 19.2 kDa) [28]. The *cap* gene represents the second ORF located on the anti-sense strand and encodes the structural Cap protein (234 aa and 30 kDa) [29]. Genetic diversity studies have shown the existence of PCV-2 variants, and the extent of nucleotide variation was greater for the *cap* gene when compared with the *rep* gene. These results demonstrate the possible association of the structural Cap protein and pathogenicity, since alterations in the *cap* gene may alter domains involved in tissue tropism or virus-host interaction [14, 18, 27]. Furthermore, the Cap protein also harbors immunoreactive domains that are important targets for the development of diagnostic tests and vaccines [5, 26].

This article reports the genetic diversity of Brazilian PCV-2 isolates based on nucleotide sequencing analysis of the *cap* gene in order to better understand the molecular epidemiology of PCV-2 in Brazil.

Materials and methods

Samples and DNA extraction

Six hundred thirty-five tissue samples (lung or lymph nodes or pool of lung, liver and kidney) from swine with various clinical conditions originated from three Brazilian states (São Paulo, Minas Gerais and Santa Catarina) were tested for PCV-2 from April 2001 to October 2004. These samples were submitted to the Swine Pathology Laboratory of the Veterinary School of University of São Paulo for routine examination of PCV-2. In the same period, 235 lymph nodes were collected from finish pigs in one abattoir in São Paulo State and also included in this study (Fig. 1 and Table 1).

The tissue samples were homogenized by Stomacher 80 (Seward/Lab System) in 20% (v/w) TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and stored at −80 °C until DNA extraction. DNA extraction was carried out according to the procedure described by Chomkzynski [7].

Amplification

The primers used for PCV-2 detection in the 870 samples were F66/B67 [29] and Fa/Ra (Fig. 2). For the positive PCV-2 samples, two other pairs of primers (Fb/Rb and Fc/Rc) were used together with Fa/Ra to amplify three overlapping fragments encompassing the entire *cap* gene (Fig. 2).

Amplification conditions for the polymerase chain reaction (PCR) were performed as follows: 5 μL of extracted DNA in a final volume of 50 μL, containing 0.2 mM of each dNTP, 50 pmol of each primer (Fig. 2), 1.25 mM $MgCl_2$, 1 × PCR buffer (Gibco-BRL), 1.5 units *Platinum* Taq DNA polymerase (Gibco-BRL), and milliQ water QS. PCR amplification was performed in a MJ Research PTC-200 Thermo-Cycler under the following conditions: 1 cycle at 95 °C for 5 min, 40 cycles at 95 °C for 30 s, annealing temperature (Fig. 2) for 30 s and 72 °C for 30 s, and 1 cycle at 72 °C for 5 min. PCR products were electrophoresed in 2.0% agarose gels in standard TBE (0.045 M Tris–Borate, 1 mM EDTA) and stained with 0.5 μg/mL ethidium bromide [34].

Nucleotide sequencing and alignment

Amplified products with the expected molecular masses were excised from the gel and purified using a commercial kit (Concert, Gibco-BRL™). Bi-directional sequencing reactions were performed using the dideoxynucleotide chain-termination method with the BigDye™ Terminator kit (Applied Biosystems™), and sequences were determined

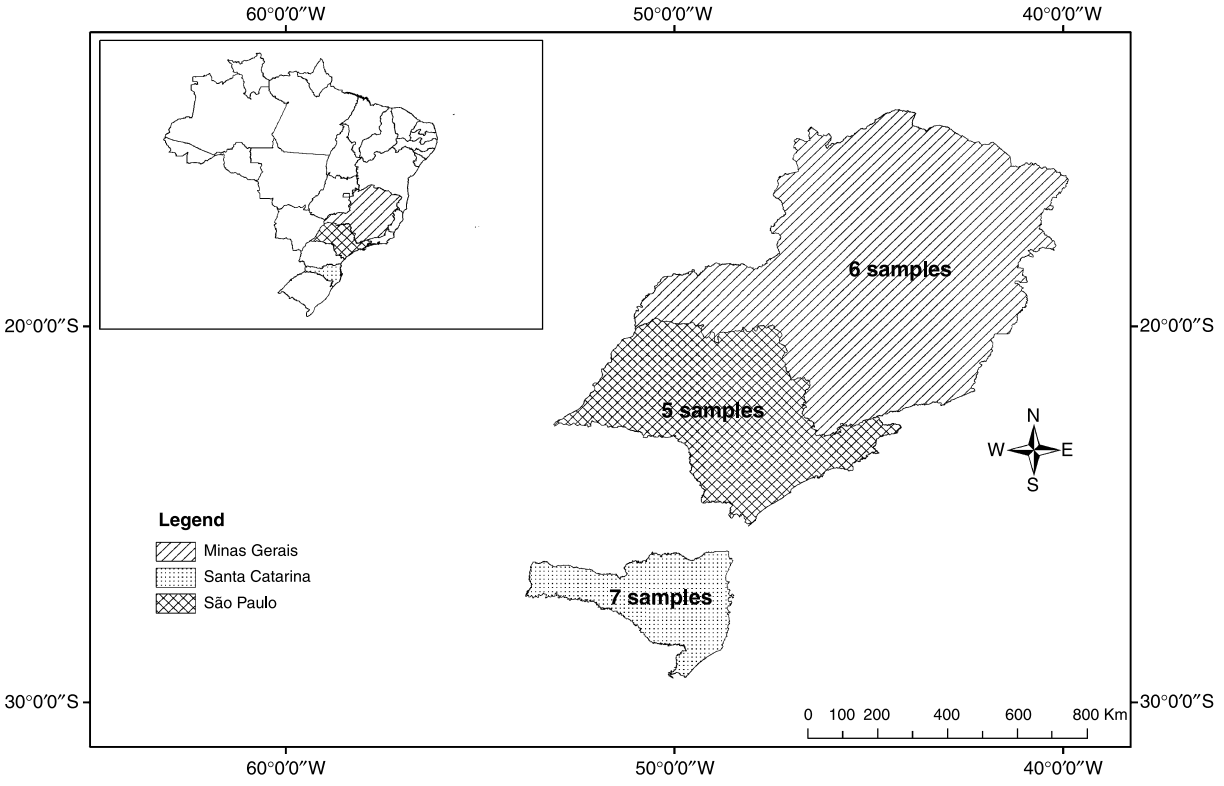

Fig. 1. Origin of PCV-2 samples from different Brazilian states

Table 1. Identification, origin, clinical history and age from the animals used in this study

Identification	Accession numbers	Origin[a]	Primers par	Age (days)[b]	Clinical signs
am2	DQ856575	–	F66/B67	–	respiratory disorders
am6	DQ856568	SC	F66/B67	70	respiratory disorders
am7	DQ856579	SC	F66/B67	70	respiratory disorders
am13R	DQ856571	SP	Fa2/Ra2	150	wasting
am22R	DQ856572	SP	Fa2/Ra2	150	wasting
am25R	DQ856573	SP	Fa2/Ra2	150	wasting
amC	DQ856569	SP	Fa2/Ra2	120	without clinical signals
amD	DQ856570	SP	Fa2/Ra2	120	without clinical signals
am3	DQ856581	SC	F66/B67	63	respiratory disorders
am4	DQ856578	SC	F66/B67	45	respiratory disorders
am5	DQ856567	SC	F66/B67	50	respiratory disorders
am9	DQ856576	MG	F66/B67	45–50	respiratory disorders
am10	DQ856563	MG	F66/B67	65	respiratory disorders
am39	DQ856566	SC	F66/B67	42	respiratory disorders
am13	DQ856565	MG	F66/B67	55–60	respiratory disorders
am15	DQ856564	MG	F66/B67	55–60	respiratory disorders
am16	DQ856574	SC	F66/B67	45	respiratory disorders
am21	DQ856577	MG	F66/B67	55–60	respiratory disorders
am22	DQ856580	MG	F66/B67	55–60	respiratory disorders

[a] Origin – State (*SP* São Paulo; *SC* Santa Catarina; *MG* Minas Gerais).
[b] 150 and 120 day-old-animals originated from abattoirs.
– Without information.

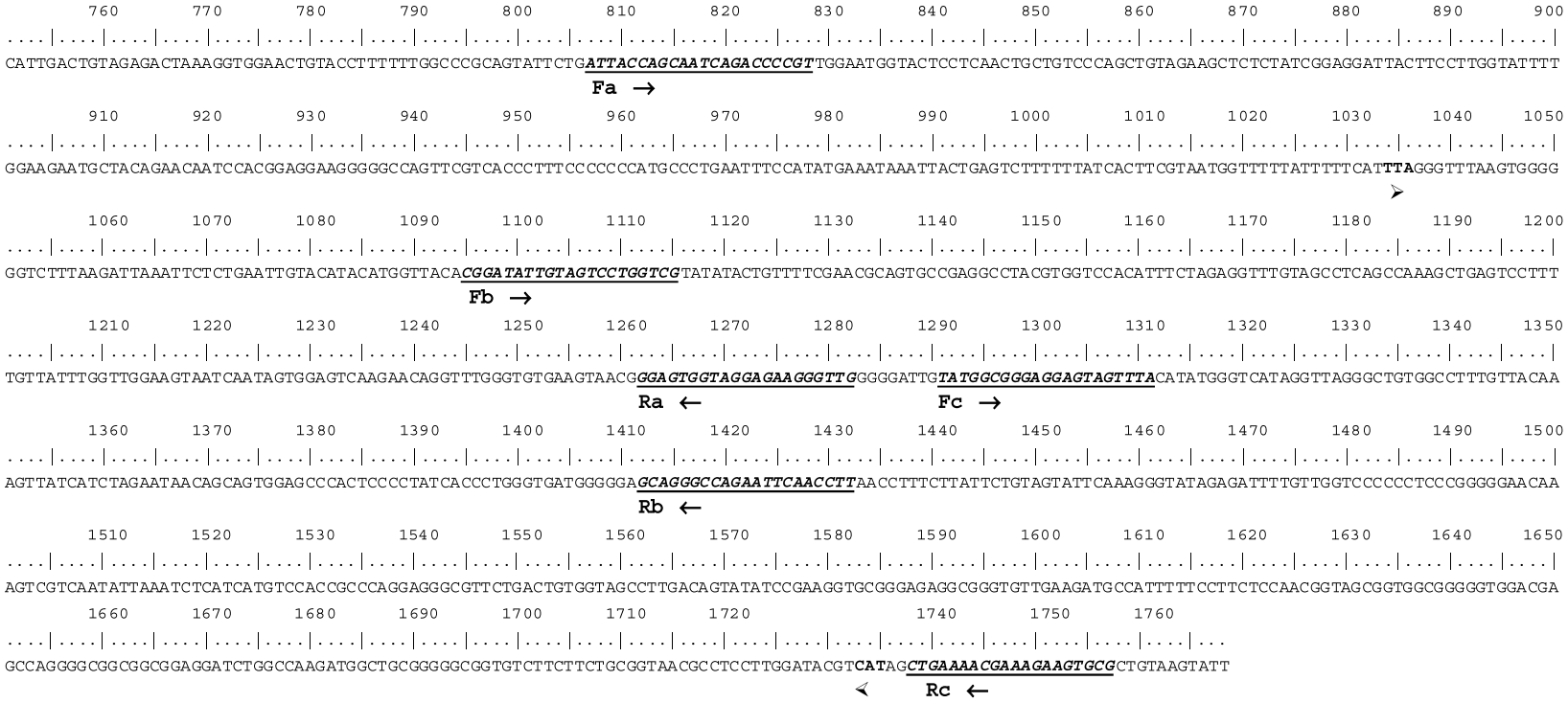

Fig. 2. Pars of primers used for PCV-2 *cap* gene sequencing. The localizations, sequence and positions are indicated relatively to a reference strain (GenBank accession number AF201311). Annealing temperature used was 51, 58 and 60 °C for the pars of primers Fa/Ra, Fb/Rb and Fc/Rc, respectively. → and ← indicated the direction 5′–3′. ➢ ➢ represents the beginning and ending from the *cap* gene

Table 2. PCV-2 strains included in the phylogenetic analysis

Accession numbers	Clinical signs	Country	Symbols	Authors
AB072301	PMWS	Japan	JA	Imai et al.*
AF109399	various clinical syndromes	Canada	CAN1	Hamel and Nayar*
AF112862	various clinical syndromes	Canada	CAN2	Hamel et al. [18]
AF381177	various clinical syndromes	China	CH1	Lu and Yang*
AF520783	PDNS	Korea	KO	Park et al.*
AY099498	PMWS	United States	USA1	Choi et al.*
AY181945	PMWS	China	CH2	Wang and Yang*
AY256457	PMWS	Hungary	HU1	Dan et al. [9]
AY256460	PDNS	Hungary	HU2	Dan et al. [9]
AY321984	PMWS	France	FRA1	Boisséson et al. [5]
AY322000	PMWS	France	FRA2	Boisséson et al. [5]
AY322004	Nephritis	France	FRA3	Boisséson et al. [5]
AY325495	PMWS	South Africa	SA	Drew et al.*
AY424402	PMWS	Austria	AU1	Exel et al.*
AY424404	PMWS	Austria	AU2	Exel et al.*
AY484414	PMWS	United Kingdom	UK	Grierson et al. [16]
AY885225	without information	Taiwan	TAI	Huang et al.*
DQ220734	PMWS	Canada	CAN3	Tremblay
DQ629127	without information	United States	USA2	Cheung*
DQ870484	without information	United States	USA3	Rowland*
EF067853	PMWS	China	CH3	Han*

* Unpublished.
PMWS postweaning multisystemic wasting syndrome; *PDNS* porcine dermatites and nephropathy syndrome.

with an automatic sequencer (ABI model 377, Applied Biosystems™) according to the manufacturer's instructions. Assembly of the complete sequence was done using the PHRED/PHRAP and CAP3 (http://bioinformatica.ucb.br/electro.html) program with an analyses quality point of 20. The obtained *cap* gene sequences were aligned using CLUSTAL X [37]. The degree of similarity among sequences at both the nucleotide and amino acid levels was determined using BioEdit v. 7.0.5.3 [17].

Genealogic analysis and nucleotide sequence diversity

Phylogenetic analysis was performed on the aligned data set, and a rooted tree was constructed using the distance-based neighbor-joining method in Mega v.2.1 [22] using the PCV-1 sequence as an out-group (GenBank number AY184287). The best evolutive model was determined by Model test [30]. Bootstrap values were calculated on 1000 repeats. The rooted tree was created using Tree Explorer from the Mega v.2 package [22]. The accession numbers of the sequences available in GenBank used for genealogical analysis are shown in Table 2.

Analysis of protein secondary structures

The secondary structure of the putative capsid protein of each strain detected in the present study was predicted with NNPredict at http://www.cmpharm.ucsf.edu/nomi/nnpredict.html and compared with those related to homologous sequences of PCV-2 from different pathotypes and geographic origins (Table 2) by manual alignment.

Results

Detection of PCV-2 by PCR

PCV-2 was detected in 19 (2.2%) out of the 870 tissue samples examined. Among the 19 PCV-2-positive samples, 15 were detected with F66/B67 and Fa2/Ra2 and five only with Fa2/Ra2 primers.

The signs that predominated in the affected pigs were respiratory disorders (14 samples) characterized by slow growth, decreased feed efficiency, lethargy, anorexia, fever, cough and dyspneia followed by an increase in the mortality rate, especially in finishing pigs. The other conditions observed were wasting (three samples from the abattoir) in which the animals presented slow growth (animals with 150 days weighing in average 85 kg), decreased feed efficiency, lethargy, emaciation and

Table 3. Polymorphic sites within PCV-2 *cap* gene sequences from Brazil. Dashes represent amino acid identity. Amino acid positions are numbered according to Mahé [26]. The sequence with 100% amino acid identity were excluded

Samples	Amino acid position																									
	10	38	46	47	57	59	60	63	72	76	77	80	86	88	89	91	123	131	136	151	185	190	191	206	210	232
am10	Y	W	N	T	I	R	T	K	M	I	N	L	S	P	R	V	V	T	A	T	L	A	G	I	E	N
am21	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	T	–	–	–	–
am25R	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	P	–	T	A	K	D	K
amC	F	K	T	–	V	A	S	T	L	–	–	V	T	K	I	I	–	–	–	P	–	T	A	K	D	K
am22R	F	K	T	–	V	A	S	T	L	–	–	V	T	K	I	I	–	–	–	P	–	T	A	K	D	K
am6	F	–	–	A	V	A	–	S	L	L	D	V	T	K	I	I	I	I	Q	P	M	S	R	K	D	K

*The sequences with 100% identity to am10 (am5, am2, am15, am13, am7 and am3) and to am21 (am22, am16, am9, am4, am39 and am15) were omitted from the table. Position of the immunogenic domain A: 69–83.

marked spine. The animals without clinical signs (two samples from the abattoir) had the expected growth (animals with 150 days weighing on average 85 kg) (Table 1).

Sequence analysis

The *cap* gene coding regions of 19 Brazilian PCV-2 samples were amplified and sequenced using the three primer pairs (Fig. 2). The sequence analysis showed that they contained 699 nt, and no gap was detected. The alignment of the 19 sequences showed nucleotide identities of 91.9–100%, with 100% observed in the following groups: i) am22, am9, am16 and am39; ii) am2, am3 and am7; iii) amD, amC and am13R and iv) am13 and am10. Over the 699 nt, 64 polymorphic sites were found scattered within nucleotide sequences: 27 transitions, 35 transversions and 2 with both. At the amino acid level, 26 polymorphic sites were detected (Table 3).

These sequences were aligned with *cap* gene sequences from PCV-2 strains available in the GenBank database from different countries (Table 2) and showed the following identities : i) 92.4 and 95.4% with the sequence from South Africa (SA); ii) 91.2 and 99.4% with the sequence from North America (CAN1, 2 and 3 and USA1, 2 and 3); iii) 91.4 and 99.7% with the sequence from Asia (CH1, 2 and 3, JA, KO and TAI) and iv) 94.8 and 100% with the sequence from Europe (FRA1, 2 and 3, AU1 and 2, HU1 and 2 and UK).

An alignment of *cap* gene amino acid sequences from the 19 strains studied herein showed that amino acid changes were scattered in the immunogenic domain A (69–83), previously identified by PEPSCAN [26], and regions above and below next to this domain.

Genealogic analysis

In an effort to understand the phylogenetic relationships of the viral isolates between the 11 (sequences with 100% amino acid identity were excluded) variants described herein, their sequences were compared to those from other countries (Fig. 3). Two main clusters A and B, supported

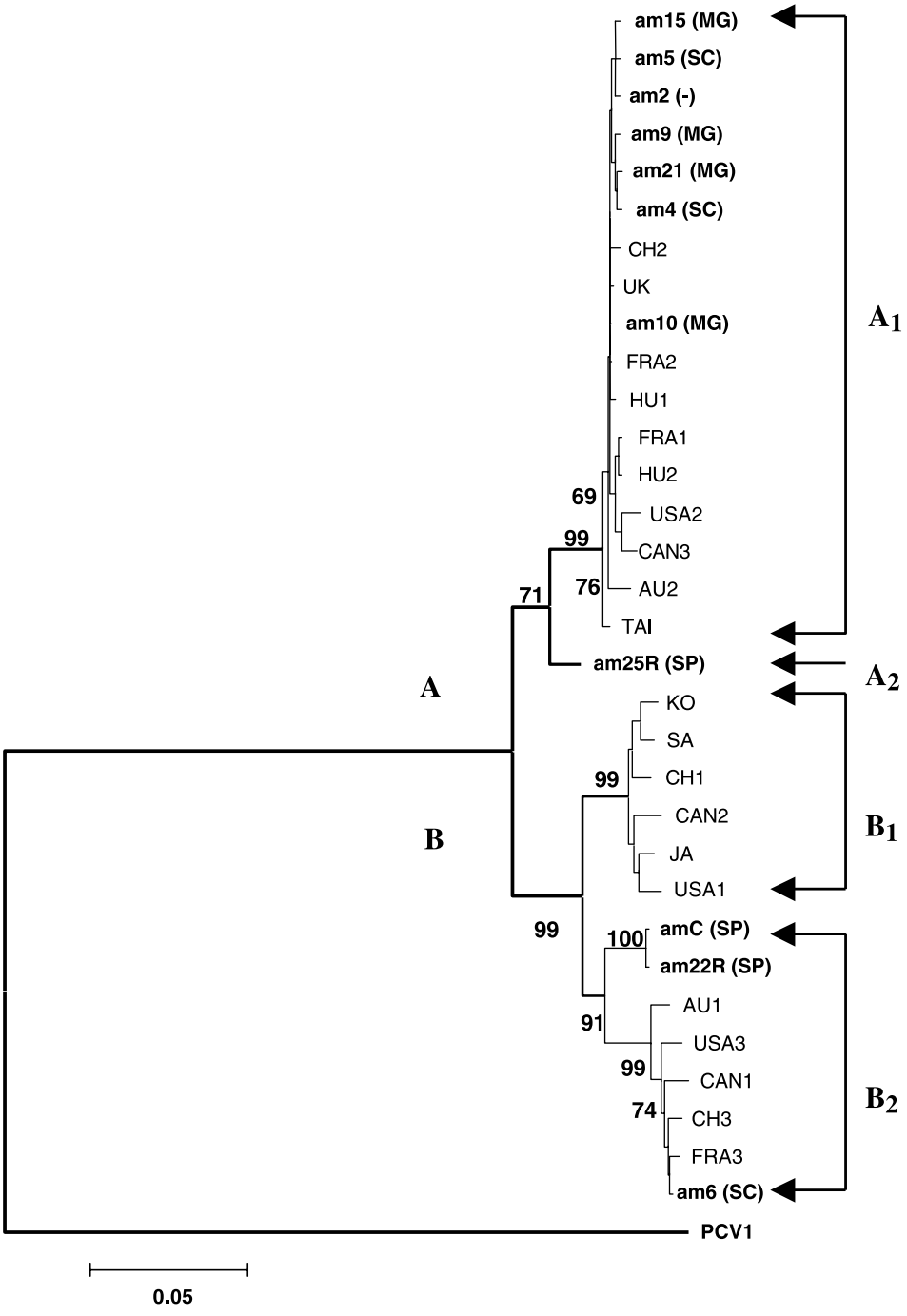

Fig. 3. Phylogenetic analysis of PCV-2 *cap* gene sequences. The tree is constructed by the neighbor-joining method using a corresponding PCV-1 (AY184287) sequence as outgroup. Numbers indicate bootstrap values calculated on 1000 repeats of the alignment with the heuristic method. Line indicated without information. The sequence with 100% amino acid identity were excluded. Geographical information from the sequences with 100% amino acid identity: **am9 (MG)** = am22 (MG) = am16 (SC) = am39 (SC); **am2 (-)** = am3 (SC) = am7 (SC); **amC (SP)** = amD (SP) = am13R (SP) and **am10 (MG)** = am13 (MG)

by significant bootstrap values, were obtained. Within cluster A, two subgroups were identified: A_1 composed of the almost complete PCV-2 *cap* gene sequence obtained in this study plus ten previously related sequences from Europe (FRA1 and 2, HU1 and 2, AU2, and UK), North America (USA2 and CAN3) and Asia (CH2 and TAI) and A_2 composed of only one sequence obtained in this study. The second main cluster (B) included subgroup B_1 composed of previously determined related sequences from America (CAN2 and USA1), South Africa (SA) and Asia (CH1, KO and JA). Three sequences (am6, am22R and am6) from this study plus five previously determined related sequences from Europe (AU1 and FRA3), North America (USA3 and CAN1) and Asia (CH3) compose the other subgroup (B_2).

Analysis of protein secondary structures

The analysis of the secondary structures for the predicted amino acid sequences of the capsid protein did not reveal any pattern that was exclusive to a given pathotype or to different pathotypes of geographic region.

Discussion

In previous studies, PCV-2 was widely found in non-affected and affected pigs, associated with a wide variety of clinical conditions and lesions [1, 5, 6, 12, 31, 35].

In the present study, the signs that predominated in the affected pigs were respiratory disorders followed by death, especially in finishing pigs. These findings were previously described as signals associated with PCV-2 infections [12, 21, 35] and suggest that PCV-2 infections in Brazil may occur in the absence of typical clinical signs of PMWS. The detection of PCV-2 in finishing pigs has also been reported previously in Europe, Asia, America, Australia [15, 23, 27, 31], and also in Brazil [8].

Despite PCV-2 being reported in respiratory disorders, it is known that other viral (such porcine respiratory and reproductive virus and swine influenza virus) and/or bacterial agents (*Mycoplasma hyopneumoniae* and *Pasteurella multocida*) could play a role in the clinical and pathological presentation of this syndrome [6, 10, 19, 21]. PRDC occurs in growing and finishing pigs aged around 6–22 weeks. The role of PCV-2 in PRDC involves interaction or synergism with the previously related agents, which results in an increase in the severity of lesions. Of the 14 swine with respiratory disorders in this investigation, three (21.4%) were found to be positive for *Mycoplasma hyopneumoniae* and *Bordetella bronchiseptica* and negative for porcine parvovirus (data not shown). However, other probable agents involved have not been tested.

The presence of PCV-2 acid nucleic in two animals without clinical signs was also observed in the present study. These two animals were also negative for porcine and other bacterial agents (data not shown). It should be noted, however, that they have not been tested for other agents. This results may be interpreted as a result of the lack of other unknown factors necessary for the outcome of symptoms.

In the present study, 15.7% (3/19) of the animals had presented wasting as the major clinical signal, suggesting PMWS. However, the final diagnosis of this syndrome is based on the presence of compatible clinical signs (wasting), presence of characteristic histopathological lesions in lymphoid tissues, and the presence of PCV-2 (antigen or nucleic acid) [35]. The absence of other criteria used in PMWS diagnosis does not allow a conclusive diagnosis of the syndrome to be made. The animals were also negative for PPV, and other agents were not tested, which revealed that other factors involved in the occurrence of clinical signs in Brazilian swine herds are still to be established.

The sequence of the *cap* gene was chosen for several reasons: i) it codes for the major structural protein of PCV-2 [29]; ii) it comprises the most important immunogenic domains and iii) it is the most variable region in the PCV-2 genome [5, 20, 26, 29]. The alignment of the 19 sequences analyzed in the present study showed a nucleotide identity of 91.9–100%, with 64 polymorphic sites. Among the sequences with 100% of identity, some originated from different Brazilian states, as observed within the am9 (MG) and am39 (SC).

The alignment of the Brazilian sequences and ones from other countries showed a nucleotide

identity of 91.2–100%. The 100% identity was observed between am10 (MG) and FRA2 and UK. This obervation could be explained by the introduction in the swine herds of Santa Catarina and Minas Gerais states genetic renewals through the importation of pigs of European ancestry. This can explain the PCV-2 identity with the European samples (mainly FRA2 and UK) since this virus could have been introduced in the coutry during this importation. However, the state of São Paulo maintains inbreeding without the introduction of new hybrids. This can explain the difference between most of the São Paulo samples from samples of other countries. However, more studies are necessary to confirm this interrelationship.

The nucleotide mutations identified in the *cap* gene resulted in amino acid substitutions, some of which localized in the imunogenic domain and others next to it. This imunodominant region of the capsid protein of PCV-2 exposed to selective immune pressures could represent potential candidate regions involved in the emergence of PCV-2 variants. However, no repeatable or characteristic amino acid motifs for these regions of PCV-2 capsid protein could be associated with strains identified from cases of respiratory disease or other clinical conditions, a finding also reported by Larochelle et al. [23].

The Brazilian sequences were divided into two major groups. Most of the samples from the state of São Paulo were grouped with sequences from Europe (AU1 and FRA3), North America (USA3 and CAN1), and Asia (CH2), except am25R. Despite the fact that the samples originated from the same Brazilian states, they are from different pig farms and these differences can explain the cluster grouping. However, these results show that in the states of São Paulo and Santa Catarina, two different subtypes of PCV-2 are circulating, which can make clinical diagnosis difficult when associated with other co-factors. Other authors have also observed the presence of more than one subtype in the same country [20].

The degree of conservation among the majority of Brazilian sequences described is strikingly high considering the large range of their geographical origins. These results can be related with the time of outbreak of the virus, low selection pressure for this virus strain in these animals, and the absence of introduction of new virus strains. It must be considered that swine herds from different regions can acquire animals from the same mother breeds.

As the secondary structures for the predicted capsid proteins of the PCV-2 strains studied herein revealed no major differences that could be the basis of the different syndromes associated with these strains, one can argue that the basis for divergent manifestations of PCV-2 pathogenesis lies in other regions of the genome, e.g., the ORF-1/*rep* gene. Nevertheless, if no difference exists in the secondary structures of different PCV-2 pathotypes, the wide range of symptoms could be attributed to the different ages of the pigs sampled, such as the level of expression of cellular receptors to PCV-2 in the different tissues according to the age, the transmission route, or the immune status of the animal.

Despite the high overall conservation of the cap gene sequences, the genealogical analysis presented here provide enough evidence to show two PCV-2 subtypes (A and B). These results also show no association between clusters of PCV-2 and different states or clinical signs, demonstrating that the exact role of PCV-2 in PCVD in Brazil still need to be clarified. This genetic drift is likely to generate new variants that have important consequences in the epimiology of PCV-2 infection.

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