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Rapid Communication

Low levels of diversity among genomes of Porcine circovirus type 1 (PCV1) points to differential adaptive selection between Porcine circoviruses

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ABSTRACT

Several features related with the evolutionary patterns among all the PCV1 genomes available at GenBank have been analyzed in the present work (diversity, number of genotypes, recombination, saturation, selection, evolutionary rate). The reported results point to low levels of nucleotide and amino acid diversity, low number of positively selected codons and a slow evolution rate. Compared with the other species of the *Circoviridae* family, the diversity is the lowest reported. This can be related with the fact that PCV1 is the single non-pathogenic member of the family. Overall, differential levels of adaptive evolution between PCV1 and PCV2 may explain the different diversity levels, and the different evolutionary and selection rates observed.

Porcine circoviruses (PCVs) are small, non-enveloped, single-stranded DNA viruses showing icosahedral symmetry, which are classified into the family *Circoviridae*, genus *Circovirus*. PCVs are the smallest animal viruses known, with a genome length of only 1.7–1.8 kb. Two distinct PCVs species have been defined so far: PCV types 1 (PCV1) and 2 (PCV2); both show high levels of nucleotide identity and similar genomic organizations (Olvera et al., 2007). PCVs contain two major ambisense open reading frames (ORF). ORF1 (*rep* gene) is transcribed in a clockwise direction, and encodes two viral replication-associated proteins Rep and Rep'. ORF2 (*cap* gene) is located in the complementary viral strand and follows an anti-clockwise transcription, encoding the immunogenic capsid protein. Between the ORFs there is an intergenic region that comprises the origin of replication and characterized by a putative stem-loop structure (Mankertz et al., 2004). Both PCV1 and PCV2 infections are common in pig populations all over the world (Allan and Ellis, 2000; Beach et al., 2010; Fenaux et al., 2004), despite lower prevalences for PCV1 have been reported compared with those of PCV2 (Calsamiglia et al., 2002; Puvanendiran et al., 2011).

PCV2 has been related to a number of disease syndromes in pigs (Segalés et al., 2004), mainly postweaning multisystemic wasting syndrome (PMWS). On the contrary, PCV1 was discovered as a contaminant of the PK-15 cell line (Tischer et al., 1982), and is considered non-pathogenic as long as experimental inoculation of pigs does not produce clinical disease (Allan et al., 1995; Tischer et al., 1986). Consequently, PCV1 has attracted much less attention of researchers compared with PCV2. However, PCV1 has recently gained notoriety because of its presence as contaminant in live human vaccines (Baylis et al., 2011; Beach et al., 2011). Therefore, in order to fill one of the gaps existing in relation to PCV1 research, the main objective of this work was to analyze the evolutive patterns and relationships among PCV1 genomes published worldwide.

All PCV1 complete genomes (n = 36) available at the GenBank (Table 1) were downloaded, aligned with ClustalW (Thompson et al., 1994), and analyzed in the present study. Partial sequences of PCV1 also existing in the GenBank were not considered due to the lack of homogeneity to perform corresponding analyses. Complete genomes, and cap and rep genes separately did not present evidences of saturation or recombination, according to the analyses performed with packages DAMBE (Xia and Xie, 2001) and RDP3 (Martin et al., 2010), respectively. Therefore, both genes and the complete genome were used for phylogenetic inference. Neighbor-Joining trees of the complete genome, and the cap and the rep genes were constructed in MEGA4 (Tamura et al., 2007) based on the p-distance and re-sampled with 1000 bootstrap replicates. All trees exhibited very low levels of polymorphism, together with the lack of a clear clustering structure (Fig. 1). The lowest p-distance reported among the most divergent PCV1 genomes was as high as 98%. The low diversity levels

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Table 1
Summary of complete PCV1 genomes used in the study. Strain name, accession number, origin, year of isolation and reference (N/A, non-available).

Strain	Accession number	Origin	Year	Reference
NMB	GU799575	USA	2008	Beach et al. (2010)
HRB-09	GQ449671	PR of China	2009	Unpublished
Unspecified	NC_001792	USA	N/A	Niagro et al. (1998)
Tian Jin	GU371908	PR of China	2009	Unpublished
PCV3_Rotarix_con1	HM143844	USA	2009	Victoria et al. (2010)
PCU49186	U49186	UK	N/A	Meehan et al. (1997)
IL	AY219836	Taiwan	2003	Unpublished
Unspecified	AY184287	N/A	N/A	Cheung (2003)
Aust 4	AY754015	Australia	2005	Muhling et al. (2006)
Aust 3	AY754014	Australia	2005	Muhling et al. (2006)
Aust 2	AY754013	Australia	2005	Muhling et al. (2006)
Aust 1	AY754012	Australia	2005	Muhling et al. (2006)
Jiangsu	GU722334	PR of China	2003	Unpublished
PK	DQ650650	PR of China	2006	Unpublished
sc-5	DQ472016	PR of China	2006	Unpublished
sc-6	DQ472015	PR of China	2006	Unpublished
sc-4	DQ472014	PR of China	2006	Unpublished
sc-3	DQ472013	PR of China	2006	Unpublished
sc-2	DQ472012	PR of China	2006	Unpublished
Unspecified	AY660574	PR of China	N/A	Unpublished
Unspecified	AY699796	USA	N/A	Fenaux et al. (2004)
Unspecified	AY193712	N/A	N/A	Unpublished
BJ-1	FJ475129	PR of China	2008	Unpublished
Unspecified	AF071879	USA	N/A	Niagro et al. (1998)
Unspecified	Y09921	N/A	N/A	Mankertz et al. (1997)
CT-PCV-P7	AY099501	N/A	N/A	Choi et al. (2002)
Unspecified	AF012107	France	1997	Unpublished
NT70719	FJ159693	PR of China	2007	Unpublished
TZ70717	FJ159692	PR of China	2007	Unpublished
YZ70719	FJ159691	PR of China	2007	Unpublished
YZ70721	FJ159690	PR of China	2007	Unpublished
YZ70722	FJ159689	PR of China	2007	Unpublished
HZ2006	EF533941	N/A	N/A	Unpublished
SC-11	EF493843	PR of China	2006	Unpublished
WB-H-8	DQ648032	Hungary	N/A	Unpublished
SC-1	DQ358813	PR of China	N/A	Unpublished

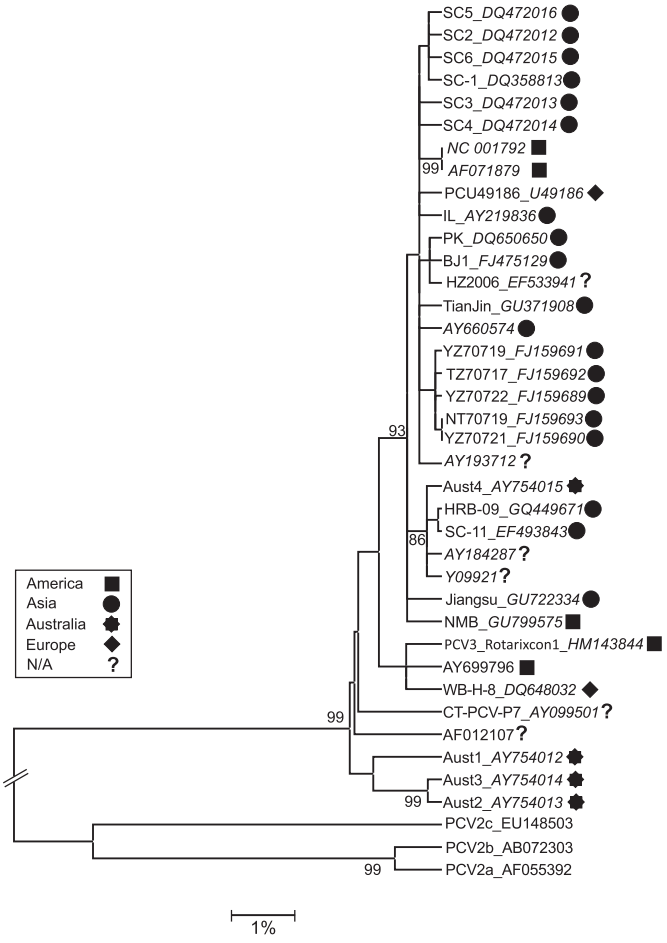

Fig. 1. Phylogenetic tree based on the Neighbor-Joining method and the p-distance for the 36 PCV1 genomes rooted with PCV2a, PCV2b and PCV2c types (with GenBank reference numbers AF055392, AB072303 and EU148503, respectively). Only bootstrap values higher than 85% are shown.

were observed also when mutations were analyzed; 138 (7.8%) mutations in the whole genome, 84 mutations (12%) in the cap and 52 mutations (5.5%) in the rep genes. In addition, more than half (87 out of 138, 63%) of these mutations were point mutations reported in a single PCV1 sequence. Likewise, most amino acid mutations were reported in single sequences (with no synapomorphic value), both in the cap (58%) and the rep (74%) genes. Accordingly, the nucleotide diversity estimation for the complete genome ($\pi = 0.0083$) was one order of magnitude lower compared with PCV2 ($\pi = 0.0348$; Olvera et al., 2007). Actually, the diversity levels for PCV1, both the number of mutations and nucleotide diversity estimations, were the lowest reported in the whole *Circoviridae* family (Table 2). The selection analyses (Fig. 2) strengthen the lack of variation observed within PCV1 genes: a vast majority of the amino acid positions were neutral or negatively selected in the cap (68% and 15%, respectively) and rep (85% and 8%, respectively) genes. Interestingly, most (16 out of 38, 41%) of the positively selected codons in the cap gene were located between residues 30 and 78, which roughly coincide with one of the epitopes described for PCV1 (Mahé et al., 2000).

Histograms of pairwised differences among sequences (PASC) have been used in several ssDNA viruses to define genotypes at the intraspecific level (Bao et al., 2008). The PASC analysis performed to the PCV1 sequences using the program Arlequin v3 (Excoffier et al., 2005) did not report any clear cut-off value (Fig. 2) for genotype definition, pointing to the lack of internal structuring for the species.

Finally, the rate of substitution was estimated with the BEAST package (Drummond and Rambaut, 2007) performing three independent runs of Bayesian Markov Chain Monte Carlo (MCMC). The posterior probability distribution of the chain was sampled every 100 generations until convergence, using HKY85 + Γ (Hasegawa et al., 1985) as a substitution model, and the default parameters in the prior's panel. The mean genomic substitution rate for PCV1 was estimated to be $1.15 \cdot 10^{-5}$ nucleotide substitutions per site per year (subs·site^{-1}·year^{-1}), two orders of magnitude lower than PCV2 ($1.2 \cdot 10^{-3}$ subs·site^{-1} year^{-1}; Firth et al., 2009).

A number of PCV1 studies have been focused on the infection and replication mechanisms (i.e. Beach et al., 2010; Finsterbusch et al.,

Table 2
Summary of diversity in the *Circoviridae* family.

Genus/species	N	Length	Variable positions (%)	Nucleotide diversity
Circovirus				
Beak and feather disease virus	87	1981–2019	956 (46.1%)	$\pi = 0.0808$
Canary circovirus	2	1952	0	–
Duck circovirus	50	1988–1996	515 (25.7%)	$\pi = 0.0974$
Finch circovirus	2	1962	0	–
Goose circovirus	25	1820–1821	325 (17.8%)	$\pi = 0.0609$
Gull circovirus	2	2035	0	–
Pigeon circovirus	12	2032–2040	486 (23.7%)	$\pi = 0.0897$
Porcine circovirus type 1	36	1758–1760	138 (7.8%)	$\pi = 0.0083$
Porcine circovirus type 2	772[a]	1767–1768	479 (27.1%)	$\pi = 0.0348$
Starling circovirus	2	2063	0	–
Swan circovirus	2	1783–1785	249 (13.9%)	$\pi = 0.1399$
Gyrovirus				
Chicken anemia virus	47	2286–2319	354 (15.2%)	$\pi = 0.0229$

[a] 772 PCV2 complete genomes are nowadays available at the GenBank, but diversity calculations are based on the 148 full-length genomes published by Olvera et al. (2007).

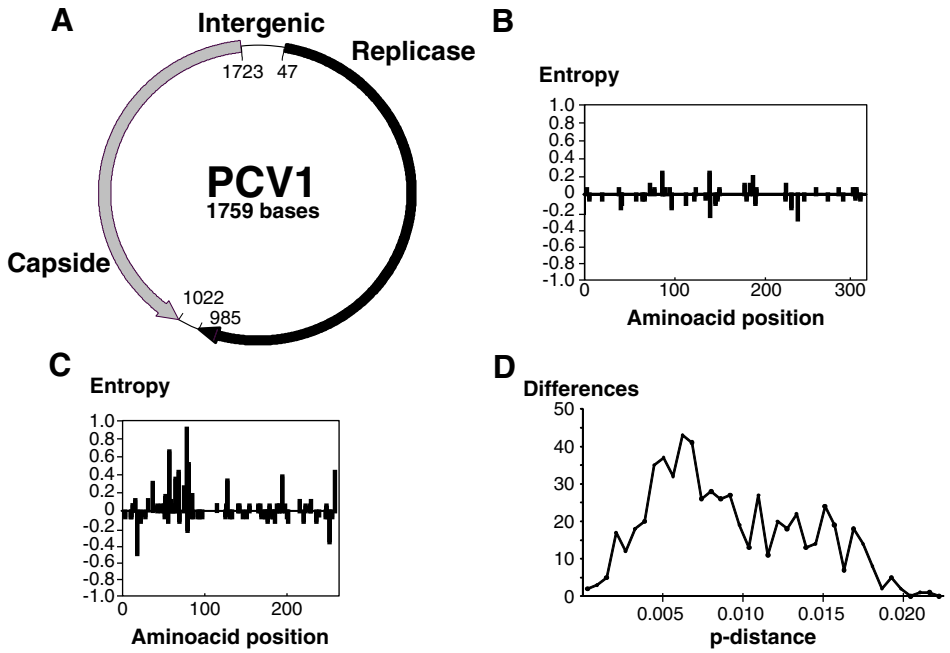

Fig. 2. A, genomic organization of PCV1 genome; the origin of replication is located at position 1. B, selective pressure per codon in the replicase gene. C, selective pressure per codon in the capside gene. D, histogram of pairwised differences among PCV1 sequences.

2009), prevalence and diagnostic studies (i.e. Calsamiglia et al., 2002; Puvanendiran et al., 2011), and lately on vaccine development and contamination (i.e. Baylis et al., 2011; Beach et al., 2011; Quintana et al., 2006; Tanzer et al., 2011) and presence in food of pork origin (Li et al., 2010). On the contrary, few evolutionary and phylogenetic topics have been analyzed (Cságola et al., 2008; Muhling et al., 2006).

In the present study, saturation and recombination levels for PCV1 have been evaluated, pointing to useful sequences for phylogeny. Very low levels of variability have been detected at the nucleotide and amino acid level, the lowest among the *Circoviridae* family. Hence, no clear clusters or groups have been identified in the phylogenetic inferences and the PASC analysis. Also, most codons are under neutral or negative selection, and the estimations of the substitution rate are low, around 10^{-5} subs·site^{-1} year^{-1}.

The *Circoviridae* family comprises 12 species organized in two genera: *Circovirus* (11 species) and *Gyrovirus* (1 species). Infection with circoviruses is associated with immune-suppression or immune-compromisation (Faurez et al., 2009), with the single exception of PCV1, which is considered non-pathogenic. Genetic diversity is one of the most important features that allow a population evolving in an ever-changing environment with shifting selecting pressures (Sanz-Ramos et al., 2008). Higher variability may let viral populations adapt and survive in the different intra-host environments, including the selective pressures generated by the host immune response (Pfeiffer and Kirkegaard, 2005). For instance, a higher level of genetic heterogeneity within the capsid gene explains evolution to pathogenicity in mice *Parvovirus* (López-Bueno et al., 2008). The maintenance of advantageous replacement mutations to evade the immune system of the host is defined as adaptive selection or adaptive evolution. Codons under adaptive evolution are positively selected. In this work, very low levels of diversity and few positively selected codons have been reported for PCV1. In contrast, PCV2 shows higher levels of diversity, and about 30% of the codons are positively selected. Positively selected codons in the PCV2 capsid were concentrated in the four proposed epitopes (Olvera et al., 2007), but only in a single region for PCV1.

A growing number of substitution rate estimations for ssDNA viruses indicate that they can evolve as fast as ssRNA viruses (reviewed by Duffy et al., 2008), despite their use of host's DNA polymerase, which is less error prone than other polymerases. Reasons for these fast rates of evolution in ssDNA viruses (Duffy and Holmes, 2008; van der Walt et al., 2008) are still unclear and challenging. The rate of evolution for PCV2 has been estimated to be as high as $1.2 \cdot 10^{-3}$ subs·site^{-1} year^{-1} (Firth et al., 2009). Intriguingly, PCV1 estimations are two orders of magnitude lower than PCV2, closer to dsDNA estimations. The reported results in the *Circoviridae* family, and specifically in the *Circovirus* genus, seem to relate pathogenicity with a higher genome diversity, as long as all pathogenic members of the family show at least ten times more variability than the non-pathogenic PCV1.

In summary, the non-pathogenic PCV1 has lower diversity levels, slower substitution rate and fewer positively selected codons when compared with the pathogenic PCV2. Overall, differential levels of adaptive evolution between PCV1 and PCV2 may explain the different diversity levels, and the different evolutionary and selection rates observed. Also, these differences between PCV1 and PCV2 may account for their differential pathogenicity.

Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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