The *Cap* gene of porcine circovirus type 2 (PCV2) evolves by positive selection *in vitro*

O gene Cap de circovírus suíno tipo 2 (PCV2) evolui por seleção positiva in vitro

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Abstract

This article reports the genetic divergence of PCV2 after passages in VERO and SK-RST cell lines. Fisher's exact test indicated a trend for positive selection on the *cap* gene. These results allow insights on the development of PCV2 vaccines and the evolution of the genus *Circovirus*.

Keywords: PCV2. Isolation. Capsid. Positive selection.

Resumo

Este artigo relata a divergência genética de PCV2 após passagens em células das linhagens VERO e SK-RST. O teste exato de Fisher indicou tendência para seleção positiva no gene *cap*. Estes resultados permitem inferências relativas ao desenvolvimento de vacinas contra PCV2 e sobre a evolução do gênero *Circovirus*.

Palavras-chave: PCV2. Isolamento. Capsídeo. Seleção positiva.

Porcine cirvorirus (PCV) is a non-cytophatogenic non-enveloped virus with icosahedral symmetry and a single-stranded circular DNA as genome[1] that codes for the capsid protein (*cap* gene in ORF2) and replicase (*rep* gene in ORF1)[2]. Type 1 porcine circovirus (PCV1) is classically associated to permanent infections in PK-15 while type 2 (PCV2) might have a role on some diseases of the swine such as post-weaning multi-systemic wasting syndrome and reproductive failures[3].

Currently, little is known on the genetic stability of PCV2 *in vitro*, mainly due to the tricky nature of PCV2 isolation in cell culture and the need for immune reagents for the confirmation of virus isolation.

The aim of this investigation was to assess the genetic divergence of PCV2 after low passage in VERO and SK-RST cell lines and to suggest the detection of evolutionary signal as a tool for the confirmation of virus isolation.

Two pools (12-04 and 17-04) of spleen/liver/ lymph nodes from two different adult swine collected at a slaughterhouse, previously positive for PCV2[4] were used for cell culture isolation. The pools were prepared as 20% suspensions (w/v) in TE buffer (10 mMTris–HCl, 1 mM EDTA, pH 8.0) and stored at -80 ºC.

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The two suspensions were filtered through 0.22 µm membranes (Millipore™) and a volume of 0.5 mL was inoculated in 24-hour-old 50% confluent monolayers of both VERO (green monkey kidney) and SK-RST (swine kidney) cells in 25 cm^2 flasks. Minimum essential medium (MEM) was used as negative control inoculum. After one hour at 37 ºC, the inocula were discarded, the monolayers were rinsed with 5 mL of MEM and next 7 mL of MEM plus 5% fetal bovine serum were added. Each passage was kept at 37 ºC for up to one week and observed daily under light microscope. For the second, third and forth passages of each sample in each of the two cell lines, the previous passage was frozen at -80 ºC and thawed at room temperature and the same procedures described above were used.

Each inoculated monolayer (n = 16) and the mock-infected monolayers were monitored for the presence of PCV2 genomic DNA with a PCR targeted to the region between ORFS 1 and 2 as described by De Castro et al.[4], with a 476-bp amplicon containing both *Cap* and *Rep* partial sequences. The 476-bp amplicons were purified from agarose gels with GFX™ kit (GE Healthcare) and submitted to bi-directional sequencing with BigDye 3.1 (Applied Byosystems™) according to manufacturer's instructions and sequences were resolved in a ABI-377 automatic sequencer (Applied Byosystems™).

Putative amino acid sequences were aligned using the BLOSUM62 matrix with CLUSTAL/W in Bioedit v. 5.0.9[5]. Nucleotide and amino acid identities were calculated with Bioedit v. 5.0.9 and amino acid similarities were calculated according to amino acids classification by Alnahi[6].

Codon-based Fisher's exact test was applied to sequences from each sample, cell line and partial gene, *i. e.*, *rep* and *cap*, for the detection of positive selection (if $p < 0.05$) or, instead, purifying selection (if $p = 1$), using the software Mega 4[7].

After four serial passages in VERO and SK-RST cells, samples 12-04 and 17-04 produced no alteration in the monolayers and culture media and no cythopatic effect after seven days of incubation.

For the first passage of strain 17-04 compared to the second and third ones in VERO cells, nucleotide and amino acids identities for the *Cap* region were 99.3 and 98.1% respectively (Table 1), with amino acid similarity of 100% as a result of a G197C polar-to-polar amino acid substitution corresponding to a G589T nucleotide substitution.

Between the first and second passages of strain 12-04 in VERO, polymorphism occurred at the *Rep* region, with nucleotide and amino acids identities of 96.7 and 96.5%, respectively, (Table 1), with amino acids similarity of 96.50% resulted from a Q297P polar-to-non-polar amino acids substitution due to A890C and G891C nucleotides substitutions at this region. Furthermore, a synonymous G876C nucleotide substitution was also detected.

No variation was detected (nucleotide identities = 100%) neither at the *Cap* region for the first, second and third passages of strain 17-04 in SK-RST cells and first and second passages of strain 12-04 in both VERO and SK-RST nor at the *Rep* region of the first,

Table 1 - Nucleotide (nt) and amino acids (aa) identities for the replicase (Rep) and capsid (Cap) regions of PCV2 for serial passages of samples 17-04 and 12-04 in VERO and SK-RST cells

Sample	Cell line	Passages	*Rep* nt	Rep aa	*Cap* nt	Cap aa
17-04	VERO	$1^{st} \rightarrow 2^{nd}$	100%	100%	99.3%	98.1%
17-04	SK-RST	$1^{st} \rightarrow 2^{nd}$ and 3^{rd}	100%	100%	100%	100%
12-04	VERO	$1^{st} \rightarrow 2^{nd}$	96.7%	96.5%	100%	100%
12-04	SK-RST	$1^{st} \rightarrow 2^{nd}$	100%	100%	100%	100%

second and third passages of strain 17-04 in both cell lines and 12-04 in SK-RST (Table 1).

Passages 3 and 4 of samples 12-04 and 17-04 in SK-RST and VERO and passage 4 of sample 17-04 in SK-RST resulted in no amplification after PCR and thus no sequences are available for these. The nucleotide sequences generated were assigned GenBank accession numbers EU909392 to EU909400.

Regarding the *Cap* gene, one can notice nucleotide and amino acids diversity amongst the first and the following passages as in the case of strain 17-04 in VERO (Table 1), a suggestion that the nucleotide plesiomorphic states found in the original virus were under a substitution process due to selective pressure caused by the cell line used.

As the capsid protein plays a major role in receptor binding[8], the polymorphism in the primary structure of this protein as reported here might be interpreted as a result of the adaptation of the viral population to the membrane receptors of VERO cells.

Amino acids substitutions such as P110A and R191S in the capsid protein are molecular markers to *in vitro* PCV2 attenuation[9]. Nonetheless, amino acids substitutions at the replicase protein of PCV2 grown *in vitro*, as detected for strain 12-04 in VERO cells, have not been described so far.

Nonetheless, despite the suggestion of a cell-driven selection on these PCV2 strains, it must be considered that the polymorphisms described herein might have been originated not from selection, but genetic drift.

The only p value lower than 1 resulted from Fisher's exact test was 0.772 for the capsid protein of strain 17-04 in VERO cells (Table 2), leading to the conclusion that there was a trend for positive selection regarding this PCV -2 strain in VERO cells, while in SK-RT no PCV2 was isolated and the sequences retrieved might be a residue of the original sequence present in the inoculum.

PCV2 isolation has been achieved in a range of cell lines with variable success frequencies[10,11], but the methods reported hitherto for the confirmation of virus isolation did not include evolutionary signal detection as proposed herein.

The demonstration that, after low *in vitro* passage, PCV2 strains might not be genetically stable is of major importance for the understanding for the development of cell-culture-derived circovirus vaccines.

Also, polymorphisms in the replicase protein of PCV2, a fact previously not reported, shall now on affect developments on the knowledge of evolutive trends in the genus *Circovirus*.

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Table 2 - Fisher's exact test p values for passages of strains 17-04 and 12-04 in VERO and SK-RST cells for capsid (Cap) and replicase (Rep) proteins of PCV2

Strain	Cell line	Passages	P value	
			Cap	Rep
17-04	VERO	1a → 2a	0.772	1.000
17-04	SK-RST	1a → 2a and 3a	1.000	1.000
12-04	VERO	1a → 2a	1.000	1.000
12-04	SK-RST	1a → 2a	1.000	1.000

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