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## Communication

[Comunicação]

### Identification of Porcine parvovirus from wild boars by partial sequencing of the VP-2 coding gene

[Identificação de parvovírus suíno em javalis a partir do sequenciamento parcial do gene VP-2]

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Porcine parvovirus (PPV) is one of the most frequent pathogens associated with reproductive disorders in swine, even though few cases have been described in wild boars (*Sus scrofa*), all of these based on antibody detection, mainly by ELISA and hemagglutination inhibition tests. In 1994, blood samples were collected from 108 wild hogs from North Carolina and Tennessee (USA) and antibody titers to PPV were found in 15 (14%) samples (New et al., 1994). By 1998, Saliki et al. (1998) tested 120 blood samples from wild swine in Oklahoma, USA, and had found antibody against porcine parvovirus in 17% of them. Later, 20 feral hogs from a newly discovered population from Kansas, USA, were shot and examined. Those hogs were generally healthy, although serological evidence indicated that 70% of the animals were exposed to PPV (Gipson et al., 1999). Antibodies were also detected in 10% of the serum samples from 78 European wild boars harvested during the 1999-2000 hunting season in south-central Spain (Vicente et al., 2002). Serologic evidence of exposure to porcine parvovirus was investigated in 219 wild boars from a commercial farm in Croatia and 41.6% of the samples showed positive results both in ELISA and hemagglutination inhibition test (Ročić et al., 2005). Serum samples collected from 178 shot wild boars were tested for the presence of antibodies against PPV by ELISA throughout Slovenia during the hunting season 2003-2004,

demonstrating antibodies in 87 sera (49%) (Vengust et al., 2006).

PPV and anti-PPV antibodies are widely spread among Brazilian swine herds (Gouveia et al., 1984; Bersano et al., 1995; Soares et al., 1999), but only one antibody investigation on wild boars has been made so far in Brazil (Bersano et al., 1999), where wild boars commercial population and its contact with swine herds have increased in the last few years.

Although the occurrence of parvovirus antibodies in feral swine in various parts of the world is well documented, it is important carrying out direct detection of the virus to confirm the infection in wild boars. The aim of this study was to survey for PPV DNA in organ samples from wild boars bred for commercial purposes by PCR (NS-1 and VP-2 coding gene) and DNA sequencing targeting the VP-2 coding gene.

Pool of organs (spleen, kidney, liver, lymph nodes, and tonsil) from three adults and asymptomatic wild boars (bred for commercial purposes) of Paraguaçu Paulista, SP, were submitted to PPV detection. The DNA from homogenates was extracted following a method described elsewhere (Chomczynski, 1993) and stored at -20°C until required. These samples were submitted to PPV detection by a screening nested-PCR directed to the highly conserved NS-1 coding sequence and the enzymatic

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amplification for NS-1 coding gene was carried out in 50µL of PCR mixture containing 0.5mM of outer primers (P1 and P6; Table 1), 2.5U DNA polymerase<sup>1</sup>, 1x PCR reaction buffer, 1.5mM MgCl<sub>2</sub>, 0.2mM of each dNTPs, 5µL of extracted DNA, and ultrapure water as diluent (Soares et al., 1999).

PCR amplification was performed in thermo cycler<sup>2</sup> under the following conditions: 1 cycle at 95°C for five minutes, 35 cycles of 95°C for 45 seconds, 55°C for 60 seconds, 72°C for 90 seconds and followed by a final heating at 72°C for 10 minutes for the extension. An identical amplification procedure was adopted in the nested-PCR, except for the use of a different set of primers (inner primers, P2 and P5; Table 1) and a volume of 5µL from the first round amplification products as sample (Soares et al., 1999).

In order to confirm these results, a nested-PCR for VP-2 coding gene was carried out. Amplification conditions for VP-2 coding gene nested-PCR were performed as follows: 5µL of extracted DNA in a final volume of 50µL, containing 50pmol each outer primer (P3 and P8; Table 1), 2U DNA polymerase<sup>1</sup>, 1x PCR reaction buffer, 1.5mM MgCl<sub>2</sub>, 0.2mM of each dNTP and ultrapure water as diluent (Soares et al., 2003).

After an initial heating at 94°C for three minutes, 35 cycles at 94°C for 45 seconds, 55°C for 60 seconds, and 72°C for 90 seconds, and one cycle at 72°C for 10 minutes were carried out. Nested-PCR was performed with 5µL of the primary amplification template and inner primers (P4 and P7; Table 1). The thermal cycles for the nested assay were the same, except for an amplification phase of 25 cycles (Soares et al., 2003).

PCR products were resolved in 2.0% agarose gels electrophoresis with standard TBE buffer and stained with 0.5µg/mL ethidium bromide.

Amplified products with the expected sizes of VP-2 gene were excised from the gel and purified using a commercial kit<sup>3</sup>. Sequencing reactions were performed using the

dideoxynucleotide chain-termination method<sup>4</sup> and sequences were determined with an automated sequencer<sup>5</sup>, according to instructions of the manufacturer. Nucleotide sequencing was performed in both directions using the primers P4 and P7 for the VP-2 nested-PCR product and generated sequences were aligned using CLUSTAL W software (Thompson et al., 1994).

The degree of similarity among sequences at both nucleotide and amino acid levels were compared to NADL-2 (accession number NC 001718) and Kresse (accession number U44978) strains of PPV using BIOEDIT v. 7.0.9 software (Hall, 1999). BIOEDIT was also used calculate the identity matrix of VP-2 sequences between Brazilian PPV (accession number AY145472 to AY145500) and sequences from the present study (accession number EF026981 and EF026982).

The nested-PCR directed to the highly conserved NS-1 coding sequence resulted in two positive samples. These samples were then submitted to a nested-PCR using primers directed to the structural protein VP-2 coding sequence in order to amplify the last 492 nucleotides of the 3'-end of this ORF, corroborating the first results. The entire VP2-coding sequence of PPV comprises 1,740 nucleotides (Bergeron et al., 1993), over which it was amplified a 492bp fragment and analyzed a 331 nucleotide consensus area, in terms of identity with homologous sequences. These sequences (accession number EF026981 and EF026982) were compared to homologous sequences from the NADL-2 strain and Kresse strain, demonstrating 100% of nucleotide identity.

Comparing the nucleotide sequences obtained in this study with others previously published, it is possible to notice that both Brazilian wild boar strains are included in genotypic group A, as stated by Soares et al. (2003). This classification is not associated with clinical presentation or geographical origin of the samples.

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<sup>1</sup>Invitrogen - São Paulo, Brazil.

<sup>2</sup>Eppendorf Mastercycler® Gradient Thermal Cycler - Hamburg, Germany.

<sup>3</sup>Concert™ Rapid Gel Extraction System, Gibco-BRL® - Rochville, USA.

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<sup>4</sup>Big Dye® Terminator v3.1 Cycle Sequencing kit, Applied Biosystems - Foster City, USA.

<sup>5</sup>ABI Prism® 377 DNA Sequencer, Applied Biosystems - Foster City, USA.

Table 1. Oligonucleotide primers used for PCR amplification of porcine parvovirus DNA

Target (coding gene)	Reaction	Primer	Sequence (5'-3')	Fragment size
NS-1	PCR	P1	ATA CAA TTC TAT TTC ATG GGC CAG C	330bp
		P6	TAT GTT CTG GTC TTT CCT CGC ATC	
	Nested-PCR	P2	TTG GTA ATG TTG GTT GCT ACA ATG C	127bp
		P5	ACC TGA ACG TAT GGC TTT GAA TTG G	
VP-2	PCR	P3	GGC ACC ACT AAA CCT AGA AAA TAC A	558bp
		P8	CAA TGA TAG TAG TAC ATG ATT AAC CAA	
	Nested-PCR	P4	CAC TTT TAC CTT CAG ATC CAA TAG G	492bp
		P7	CTG AGT TTT TAT TTA CAG AGT TAT TT	

<sup>1</sup>Soares et al. (1999, 2003).

Using the NADL-2 Porcine parvovirus complete genome sequence (accession number NC\_001718) as reference for the nucleotide positions, it was observed one to three substitutions depending on the Brazilian strain which has been compared to the generated sequences (position 4462: A→C / 4472: A→C / 4473: A→G). When those sequences were translated, these substitutions showed non-silent mutations in the VP-2 residues 551 (Glu→Asp) and 555 (Asn→His or Asn→Arg), all polar to polar amino acid substitutions (Alnahi, 1991) in the surface area of the domain 1 of the protein, as observed in VP-2 PDB identification 1K3V (Simpson et al., 2002) using Cn3D 4.1 software.

The close genetic identity among swine and wild boar-derived PPV strains suggests that both host species share a common variant of the virus that freely circulates between them, posing a risk to the control of PPV-caused diseases in herds in which these are kept in close proximity.

These results show for the first time the direct detection of porcine parvovirus in wild boars from a commercial farm.

Keywords: wild boar, nucleotide sequence, PCR, PPV

## RESUMO

*Este estudo descreve a detecção e a identificação de DNA de parvovírus suíno (PVS) em amostras de órgãos de dois javalis, por PCR e sequenciamento direcionado ao gene VP-2. Pools de órgãos (baço, rins, fígado, linfonodos e tonsila) de três javalis adultos e assintomáticos de Paraguaçu Paulista, SP, criados com propósitos comerciais, foram submetidos à detecção de PVS, resultando em duas amostras positivas após reações de nested-PCR direcionadas aos genes NS-1 e VP-2. Os fragmentos parciais de VP-2 foram sequenciados e comparados a sequências homólogas de cepas NADL-2 e Kresse, demonstrando identidade nucleotídica de 100%. Com relação a 29 cepas de PVS previamente isoladas no Brasil, o grau de identidade nucleotídica variou de 99 a 100% (uma a três substituições de nucleotídeos). Estes resultados demonstram, pela primeira vez, a detecção direta por PCR de parvovírus suíno em javalis, confirmada por análise de sequenciamento genético.*

*Palavras-chave: javali, sequência nucleotídica, PCR, PVS*

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