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Intermittent elimination of Porcine Parvovirus 4 (PPV4) in naturally infected swine semen: first report

Primeiro relato de eliminação intermitente de Parvovírus suíno 4 (PPV4) em sêmen de suíno infectado naturalmente

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Abstract

Porcine parvovirus 4 (PPV4) has been reported in several countries and the high rate of concurrent PCV2 infection with PPV4 may trigger the "porcine circovirus disease" (PCVD). This has awakened the interest in how PPV4 virus behaves. The aim of this study was to show that, like other swine viruses, the elimination of PPV4 in semen is intermittent. The study was conducted in the Unidade de Pesquisa e Desenvolvimento de Itapeva da Agência Paulista de Tecnologia dos Agronegócios Regional (Apta Regional). Over a period of six months, four boars were monitored and the semen was collected every 10 days, totalizing 74 semen samples. Through PCR testing, PPV4 was detected in 32/74 (43.2%) semen samples. Three sequences of 284 nt, showed identity varying from 98.2% to 100%, confirming the detection of the virus in all boars. Semen volume and sperm motility did not indicate significant difference between PPV4 positive or negative semen samples ($p > 0.05$). There was no significant difference between the monthly distributions of positive and negative samples. This is the first study showing intermittent shedding of PPV4 in naturally infected boars. Absence of typical clinical signs and/or influence in semen quantity was also observed.

Key words: Animal PPV. PCR. Seminal plasma. Viral shedding.

Resumo

O Parvovírus suíno 4 (PPV4) foi descrito em vários países e a alta taxa de infecção simultânea entre PCV2 e PPV4 está associada ao desencadeamento da Circovirose. Isso despertou o interesse em estudar o comportamento do PPV4. O objetivo deste estudo foi mostrar que, assim como outros vírus suínos, a eliminação de PPV4 no sêmen é intermitente. O estudo foi realizado na Unidade de Pesquisa e Desenvolvimento de Itapeva da Agência Paulista de Tecnologia dos Agronegócios Regionais. Durante um período de seis meses, quatro cachaços foram monitorados e o sêmen foi coletado a cada 10 dias, totalizando 74 amostras de sêmen. Através de testes de PCR, o PPV4 foi detectado em 43,2% (32/74)

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das amostras de sêmen. Três sequências de 284 nt apresentaram 100% de identidade entre si, e quando, comparadas com sequências de PPV4 disponíveis no GenBank, mostraram uma identidade de 98.2% a 100%, confirmando a detecção do PPV4 nas amostras de sêmen dos cachaços. O volume de sêmen e a motilidade espermática não mostraram diferença significativa entre amostras positivas ou negativas de PPV4 ($p > 0,05$). Não houve diferença significativa entre as distribuições mensais de amostras positivas e negativas. Este é o primeiro estudo que mostra a eliminação intermitente de PPV4 em cachaços infectados naturalmente. Ausência de sinais clínicos típicos e / ou influência na quantidade de sêmen também foi observada.
Palavras-chave: Excreção viral. PCR. Plasma seminal. Vírus animal.

Porcine parvovirus 4 (PPV4), recently classified as *Ungulate copiparvovirus* 2, belongs to the *Parvoviridae* family, *Parvovirinae* subfamily and *Copiparvovirus* genus (NI et al., 2014).

PPV4 was found in 2010 in lung lavage from a sick pig that was co-infected with *Porcine circovirus* 2 (PCV2) (CHEUNG et al., 2010). After that, the virus was found in different countries and biological samples. In Chinese herds, PPV4 was described in serum and tissues of different organs of sick and weanling pigs (ZHANG et al., 2011). In Hungary, the virus was detected in feces, fetuses and semen (CSÁGOLA et al., 2012) and, in the USA, in lungs, fecal and serum samples in pigs of different ages (XIAO et al., 2013; OPRIESSNIG et al., 2014). The overall prevalence of PPV4 in herds worldwide varies from 1 to 44% (OPRIESSNIG et al., 2014; SUN et al., 2015; SAEKHOW et al., 2016). Beyond domestic pigs, PPV4 has been detected in serum of bush pigs in Uganda (BLOMSTRÖM et al., 2012) and in pooled tissue from various organs in wild boars in Romania (CADAR et al., 2012). A high rate of concurrent PCV2 infection with PPV4 was demonstrated in several geographic regions ranging from 2.0% to 26.6% (OPRIESSNIG et al., 2014; SUN et al., 2015; SAEKHOW et al., 2016) and its co-infection may have an impact in triggering the "porcine circovirus disease" (PCVD). The impact of these viruses for the pig industry remains unknown, and the aim of this study was to show that, like others swine viruses, the elimination of PPV4 in semen is intermittent.

The study was conducted in the Unidade de Pesquisa e Desenvolvimento de Itapeva da Agência Paulista de Tecnologia dos Agronegócios Regional (Apta Regional). This institution maintains animals for regular semen collection and analysis, which provided the samples used in the current study. Over a period of six months, four of the regularly sampled boars were monitored and semen was collected every 10 days, totalizing 74 samples. The four boars received 2.2 kg of diet/day containing 15% protein and 0.8% lysine, kept in a 9 m^2 pain with water *ad libitum*. All animals were previously tested for PPV4 e PCV2 using feces and blood samples. The PCR protocol for PPV4 testing is described further ahead and PCR protocol for PCV2 followed MONFERDINI et al. (2017 - DOI: 10.11606/issn.1678-4456.bjvras.2017.116672). The sperm-rich fraction without preliminary secretion and gel phase of each ejaculate was obtained by using the "gloved-hand" method. The semen was contained in a pre-warmed, insulated glass collection container (36° C). Semen volume was measured; sperm motility (percentage of motile spermatozoa) was visually assessed and the ejaculates were diluted to obtain a final sperm concentration of approximately 3×10^9 spermatozoa per 80 mL. An aliquot of 200µL of semen was treated with lysis buffer (200 mM NaCl, 100 mM Tris Base, pH 7.8, 20 mM EDTA 0.5 M, pH 8.0, 1% SDS), digested with 20 mg/L of proteinase K (Invitrogen, USA) and incubated at 56 °C for two hours. DNA was extracted using phenol-chloroform-ethanol protocols and stored at -20°C (CHOMCZYNSKI, 1993).

The extracts were subsequently used to detect PPV4 DNA using DreamTaq™ Green PCR Master Mix (2 X) (Fermentas, EUA), with the primer pair

PAV (5' TCA TAG CAC TAT GGC GAG C 3') and PBV (5' AGC ATT CTG CGT TGG ACA 3') used to amplify a 284 nucleotides (nt) fragment (CSÁGOLA et al., 2012). Amplification was performed in a PTC-100 Thermo Cycler (MJ Research, USA) with cycle conditions of 94°C for 5 minutes, followed by 34 cycles of 95° C for 30 seconds, 52° C for 45 seconds and 72° C for 45 seconds with a final extension at 72 °C for seven minutes. Finally, 10 µL of PCR products was electrophoresed in 2.0% agarose gel in standard TBE buffer (0.045 M of Tris-borate and 1 mM of ethylene diamine tetra acetic acid) and stained with GelRed (Biotium, USA). The DNA was tested in parallel for the amplification of β-actin as a PCR control (HUI et al., 2004).

One amplified product in the PCR assay of each animal was randomly selected for sequencing. The amplificon was purified with a commercial kit (Concert; Gibco-BRL) and bidirectionally sequenced in an ABI 3500 Genetic Analyzer with BigDye™.

Sequence quality analyses and consensus nt sequences were assembled using the PHRED/PHRAP/CAP3 program (http://asparagin.cenargen.embrapa.br/phph/). The obtained sequences were aligned by means of CLUSTAL X (17) with GenBank sequences and the sequence identity matrix was constructed with the use of BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). GenBank sequences used in this study were: KC701356, KC701338, JQ868710 and JX869095 from Romania; HQ910448 and HQ910449 from China, KY586146 from Brazil and NC_014665 from United State.

The Chi-square statistical analysis was used to compare the rates of positive and negative semen samples per month. Inferential statistical analysis was conducted to compare the data distributions of semen characteristics in terms of volume, motility, appearance and agglutination. The variables related to volume and motility were initially tested using the Kolgomorov-Smirnov test, to check for Normal distribution ($\alpha = 0.05$). Due to the lack of normality ($p < 0.05$), we opted for the nonparametric Mann Whitney U test.

The boars (Specimen number/Farm coding: A1/1565; A2/DR358; A3/MS14; A4/MS130) used in the study were in good health and had been routinely used for semen collection. Before the beginning of the study, all boars were tested positively by PCR for PPV4 infection. All semen samples were positive for the β-actin PCR indicating the absence of false negative results.

PPV4 was detected in 32/74 (43.2%) of the semen samples. Table 1 shows the individualized PCR results from the semen of each pig in different months showing that all eliminate PPV4 in semen at some point. The Chi-Square test showed no significant difference between the distributions of positive and negative samples throughout the months.

Table 1. Frequency of PPV4 positive semen samples per number of tested samples from four boars (A1; A2; A3; A4) over six months.

	A1	A2	A3	A4	Total
June	1/2	2/2	1/2	0/2	4/8
July	1/2	2/3	1/2	2/3	6/10
August	2/3	0/2	1/3	1/3	4/11
September	1/2	3/3	0/3	2/2	6/10
October	1/2	0/3	1/3	2/3	4/11
November	0/3	1/3	2/3	1/3	4/12
December	0/3	2/3	1/3	1/3	4/12

Semen volume and sperm motility did not indicate significant difference between PPV4 positive or negative semen samples ($p > 0,05$). Positive samples presented mean semen volume of 221.8 mL (± 115.4 mL), while mean semen volume of negative samples was 242.2 (±103.5 mL). Sperm motility means of positive and negative semen samples were 89.6% (± 1.3%) and 88.8 % (± 2.9%), respectively (Table 2).

Table 2. Descriptive statistics of semen volume (mL) and motility values for PPV4 positivity.

	Semen volume			Sperm motility		
Statistics	**Positive**	**Negative**	**P value**	**Positive**	**Negative**	**P value**
Mean	221.8	242.2	0.6162	89.64	88.75	0.3599
Standard deviation	115.4	103.5		1.34	2.87	
Median	210	221.5		90	90	
Interquartile range	137.5	120		0	0	

Statistical analysis of the qualitative, appearance and agglutination variables was not possible. There was no variability in appearance results and the agglutination data set did not meet the minimum conditions for conducting the chi-square test, since more than 25% of the expected values were less than five.

Three sequences of 284 nt, named PPV4 SE1 (GenBank accession number MH697556), PPV4 SE2 (GenBank accession number MH697557) and PPV4 SE3 (GenBank accession number MH697558), of suitable quality for analyses were obtained and showed identity varying from 98.2% to 100%, confirming our agent identity as PPV4.

The Romanian sequence KC701338 showed the lowest sequence identity. Identity of 100% was obtained in 2/3 Genbank sequences [China (HQ910448 and HQ910449); Romania (JQ868710 and JX869095), Brazil and United States (data not shown)].

The present study evaluated the occurrence of natural infection of PPV4 virus in boar semen and provided evidence of intermittent elimination as happens with other porcine DNA-viruses, such as porcine circovirus and Torque Teno Sus Virus Species k2 (TTSuVk2) (MADSON; OPRIESSNIG, 2011; ROSE et al., 2012; GERBER et al., 2010). The results also revealed viral tropism toward spermatozoa. The study design did not allow us to determine the effects of PPV4 infection itself on reproduction in boars because the four boars were positive and no PPV4-free animals were used. Additionally, values for the various semen characteristics were within normal ranges. The PPV4 detections had no negative effect on measurements of semen quality in boars.

Previous results showed the detection of PPV4 in different tissue samples, feces and serum with a prevalence varying from 6.4% to 13.2% (CSÁGOLA et al., 2012; GAVA et al., 2015). Only a few studies tested semen for PPV4, with a positivity ranging from 38.5% (GAVA et al., 2015) to 50% (CSÁGOLA et al., 2012), with only two samples tested in latter. However, none showed intermittent elimination. The absence of typical clinical signs and/or influence in semen quantity and quality showed that PPV4 is maintained during semen production and breeding, increasing viral transmission. Viral persistence and sporadic shedding may be a risk factor for dissemination of PPV4 to negative sows, since the real impact of this virus in reproductive failure remains unknown. This irregular pattern of shedding requires the periodic monitoring for PPV4 in boar studies, as proposed for others viruses (MADSON; OPRIESSNIG, 2011;

ROSE et al., 2012; GERBER et al., 2010).

In conclusion, to our knowledge, this is the first study that showed intermittent shedding of PPV4 in naturally infected boars. The absence of typical clinical signs and/or influence in semen quantity and quality suggest that PPV4 is maintained during semen production and breeding.

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