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## Natural co-infection of torque teno virus and porcine circovirus 2 in the reproductive apparatus of swine

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## ABSTRACT

This work aimed to detect and study natural co-infection of *Circoviridae* torque teno virus (TTV) and porcine circovirus 2 (PCV2) in the swine reproductive apparatus. Semen and organs from 17 boars were tested by nested and real-time PCR. PCV2 was amplified from semen (47%), lymph nodes (84.6%) and testicles (35.3%). TTV2 was amplified from 16/17 testis and 13/13 lymph nodes. TTV1 DNA was detected in fewer testicle samples (2/17), which were also TTV2 positive. Analyzed ovaries, follicular fluid and uteri of 83 culled sows showed TTV2, TTV1 and PCV2 from 49.3%, 30.1% and 6.0% of the sows, respectively. Sperm analysis indicated insignificant differences between PCV2 and TTVs positive and negative boars. The most frequent pathologic lesion in sows was endometritis (28.9%), but this was unassociated with PCV2 or TTVs detection. These findings question the importance of PCV2 and TTV2 natural co-infection in the pathology of porcine reproductive failures.

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### 1. Introduction

The *Circoviridae* torque teno virus (TTV) is a small, non-enveloped, circular, single stranded DNA virus, belonging to the 'floating' genus *Anellovirus* (Biagini, 2009). TTV was first identified in humans in 1997 from a Japanese patient that presented hepatitis of unknown etiology (Nishizawa et al., 1997). Up to this date, at least five different genogroups of human TTV have been identified, and more recently TTVs have also been isolated from domestic animals (Okamoto et al., 2002; Peng et al., 2002). Complete genome analysis of porcine TTVs has identified two distinct genogroups, TTV1 and TTV2, with genomes of approximately 2.8 kb in length (Niel et al., 2005). Recently, full-length genomic sequences of four porcine TTV strains representing different genotypes or subtypes in a serum sample of a single boar were reported (Huang et al., 2009). These TTV strains included two porcine TTV1 and two porcine TTV2 denominated TTV1a-VA and PTTV1b-VA, PTTV2b-VA and PTTV2c-VA (Huang et al., 2009).

Studies using PCR assays have determined that porcine TTV is widely distributed in the swine populations in Spain (Kekarainen

et al., 2006), France (Bigarre et al., 2005) and Italy (Martelli et al., 2006), with variable prevalence. Although TTV2 was found more frequently, retrospective studies have shown that both genogroups have been present in swine in Spain since 1985 (Segales et al., 2009).

Even though porcine TTVs are disseminated in swine, the pathogenesis is not clear (Kekarainen and Segales, 2009). No tissue culture system for TTVs propagation has been identified (Kekarainen and Segales, 2009). The great majority of those studies associated the co-infection of porcine TTV with another *Circoviridae*, the porcine circovirus type 2 (PCV2) (Kekarainen et al., 2006). TTV genogroup 2 (TTV2) is more frequently related to post-weaning multisystemic wasting syndrome (PMWS) caused by PCV2 when compared to TTV1 in Spain (77% prevalence) (Kekarainen et al., 2006). However, this co-infection appears to increase the severity of PCV2 associated diseases or PCVAD. In contrast, other studies have shown that TTV1 viral infection facilitates PCV2-induced PMWS (Ellis et al., 2008). The importance of porcine TTV and/or its co-infection with PCV2 infection in adult pigs or in reproductive function is unclear. The role of the sow in transmitting porcine TTV to piglets and the infection dynamics of both swine TTV genogroups (TTV1 and TTV2) during the lactation period has been studied (Sibila et al., 2009). However, that study focused on serum sample analysis of sows and piglets and no tissue detection or pathogenesis studies were conducted.

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While several reports have identified PCV2 in boar semen and serum (Hamel et al., 2000; Kim et al., 2001, 2003; Larochele et al., 2000; Madson et al., 2008, 2009b) and further analyzed its epidemiological importance in transmission to naïve sows and piglets (Gava et al., 2008; Madson et al., 2009a), few studies have investigated the prevalence of porcine TTV in boar semen and sera. An investigation of three boar studs in Spain reported the detection of porcine TTVs in 74% of boar sera and 72% in semen (Kekarainen et al., 2007). The prevalence of TTV1 in sera and semen was 64% and 55%, respectively, but a lower prevalence of TTV2 was observed in both sera (38%) and semen (32%) (Kekarainen et al., 2007). These results indicate a high prevalence of TTV genogroups in boar semen, suggesting that semen may be an important route of infection and viral persistence (Kekarainen et al., 2007). The importance of the porcine TTV infection in the reproductive system of boars and sows, specifically viral tropism and viral distribution has to be further investigated.

The objective of this work was to detect the natural infection by porcine TTV1 and TTV2 in reproductive organs of male pigs from boar studs and culled sows, aiming to verify by tissue distribution and prevalence, the importance of infection in those sites. Furthermore, sperm analysis from boars and reproductive tract exams of sows were also performed.

## 2. Materials and methods

### 2.1. Samples

#### 2.1.1. Boars

Seventeen healthy male pigs from six boar studs were used in this study. Those samples included adult boars and 11 young male pigs ( $\leq 30$  weeks old). The age of tested males varied from 171 to 1686 (average 695.87) days. All male pigs were serologically negative for pseudorabies virus (PRV), porcine reproductive and respiratory syndrome virus (PRRSV), porcine parvovirus, leptospirosis, brucellosis and tuberculosis. Semen was obtained by manual collection, centrifuged and seminal fluid DNA was extracted as described (Ciacci-Zanella et al., 2006; Kim et al., 2001). Boars were humanely euthanized and necropsied or castrated for organ sample collection. For both protocols, pigs were cared for in compliance with the Ethics and Experimentation Animal Committee (CEUA) of the Embrapa Swine and Poultry Research Center. When necropsies were not performed, boars were castrated; testis were collected, and the animals were later sent to slaughter. Thus, lymph nodes from some boars were not available for all analysis. (Table 1, ND = not done). For those on which a necropsy was performed, a pool of lymph nodes' (mesenteric, prostatic, preputial, penile, inguinal) DNA was used for nested PCR.

**Table 1**  
Detection of PCV2, TTV1 and TTV2 DNA in organs and semen samples of boars by nested-PCR.

Virus	Boar ID Sample	212	314 <sup>a</sup>	315 <sup>a</sup>	316 <sup>a</sup>	336 <sup>a</sup>	340 <sup>a</sup>	400 <sup>a</sup>	401 <sup>a</sup>	403 <sup>a</sup>	408 <sup>a</sup>	409 <sup>a</sup>	410 <sup>a</sup>	739	751	996	6834	46/07	Total	%
TTV1	Testicle	–	–	–	–	+	–	–	–	–	–	–	–	–	+	–	–	–	2/17	11.7
	Lymph node	–	–	–	–	–	–	+	–	–	–	–	–	ND	ND	ND	–	ND	1/13	7.7
	Semen	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0/17	0
TTV2	Testicle	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	16/17	94.1
	Lymph node	+	+	+	+	+	+	+	+	+	+	+	+	ND	ND	ND	+	ND	13/13	100
	Semen	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–	2/17	11.7
PCV2	Testicle	+	–	–	–	–	+	–	–	–	+	+	–	–	+	–	–	+	6/17	35.3
	Lymph node	+	–	+	+	+	+	+	–	–	+	+	+	ND	ND	ND	+	ND	11/13	84.6
	Semen	+	–	–	–	–	+	–	+	–	+	+	–	–	+	–	+	+	8/17	47

ND: not done.

<sup>a</sup> Young swine males ( $\leq 30$  weeks of age).

#### 2.1.2. Sperm analysis

An aliquot of the ejaculate was evaluated to check sperm motility, and formalin citrate solution in the ratio of 1:200 was added for sperm morphology analysis.

#### 2.1.3. Sows

Reproductive organs and ovarian follicular fluid (OFF) from 83 randomly culled sows, from four slaughterhouses of Santa Catarina State (3–5 sows per farm, total of 5 farms/slaughterhouse) were collected. The reason for their removal was not necessarily due to reproductive failures, thus the selection of studied sows was not based on a previous history of abortion, mummified fetuses, or other reproductive pathology. OFF was carefully collected by aspiration with a 19 G  $\times$  1 1/2" (1.1  $\times$  40 mm) gauge needle attached to a plastic disposable 2 mL syringe. From each ovary OFF was aspirated from all visible follicles and a pool of 2 mL from the ovary was analyzed. A total of 71 samples of OFF and 83 fragments of ovaries and 83 fragments of uterus were collected. Organ fragments were fixed in 10% formalin for histopathology examination or were submitted for DNA extraction followed by amplification of PCV2, TTV1 and TTV2 nucleic acids. Nested-PCRs were performed using specific primers as previously described (Kekarainen et al., 2006; Kim et al., 2001).

#### 2.1.4. Histopathology

Samples of tissues were embedded in paraffin, sectioned at 4  $\mu$ m and stained with hematoxylin and eosin (HE) for light microscopical examination (Luna, 1968).

#### 2.2. DNA extraction

Tissues were minced and treated with lysis buffer (200 mM NaCl, 100 mM Tris Base, pH 7.5, 20 mM EDTA 0.5 M, pH 8.0, 1% SDS), digested with 20  $\mu$ g/ $\mu$ L of proteinase K (Invitrogen<sup>®</sup>, Carlsbad, CA, USA) and incubated at 56 °C for 4 h. The same procedure was done for OFF, semen and serum. DNA was extracted twice with phenol (Invitrogen<sup>®</sup>, Carlsbad, CA, USA), chloroform (Sigma<sup>®</sup>, St. Louis, MO, USA) and isoamyl alcohol (Vetec Quimica Fina<sup>®</sup>, Duque de Caxias, RJ, Brazil) (25:24:1), precipitated with 3 M sodium acetate and cold 100% ethanol (twice the final volume) and kept at –20 °C for 20 h. DNA was washed in 70% ethanol, air dried and resuspended in TE (10 mM Tris Base, pH 7.5, 1 mM EDTA 0.5 M, pH 8.0) and stored at –70 °C (Sambrook and Russell, 2001).

#### 2.3. PCV2 nested-PCR

Semen and organs from boars, OFF and organs from sows were analyzed with nested-PCR amplifications carried out as described (Ciacci-Zanella et al., 2006; Kim et al., 2001). The primers used were those used by Kim et al. (2001), which were designed from

conserved sequences among PCV2 isolates of ORF2 (Kim et al., 2001). Positive and negative controls used on this work were a PCV2 reference DNA sample (Fernandes et al., 2006) and nuclease-free water (Qiagen®, Valencia, CA, USA), respectively.

#### 2.4. PCV2 real-time PCR

PCV2 quantification in semen samples was performed as described elsewhere (Olvera et al., 2004).

#### 2.5. Porcine TTV1 and TTV2 PCR methods

Primers used here were those used by Kekarainen et al. (2006), which were designed to detect nucleic acids of TTV1 and TTV2 and amplify a non-coding region of the swine TTV genome (Kekarainen et al., 2006). Nested-PCR amplifications for TTV1 and TTV2 were carried out as described previously with some adaptations for detection in tissues (Kekarainen et al., 2006). Briefly, PCRs were performed in a 25 µL final volume, containing 2 µL of DNA; 10 µmol of each primer; 0.4 mM DNTP (Invitrogen®, Carlsbad, CA, USA); 2 mM MgCl<sub>2</sub>, PCR buffer and 1 U Taq DNA polymerase (Invitrogen®, Carlsbad, CA, USA). Amplification was done in a thermocycler (Mastercycler Eppendorf®, Hamburg, Germany), using 35 cycles of 94 °C for 30 s; primers annealing at 54 °C for 20 s, extension at 72 °C for 30 s and a final extension for 10 min at 72 °C. From this reaction, 2 µL of the amplification products were used as templates for nested-PCR by using specific primers. The amplified products (260 bp for TTV1 and 230 bp for TTV2) were run in a 1% agarose gel with 5 µg/mL of ethidium bromide. The size of the amplified products was determined by comparison with a 100 pb DNA ladder (Invitrogen®, Carlsbad, CA, USA). Positive controls for the analyses included samples from clones of TTV1 and TTV2 (Virology Department, Fiocruz Laboratory, Rio de Janeiro, Brazil) (Niel et al., 2005) which were genomically identified by sequencing (data not shown). Nuclease-free water (Qiagen®, Valencia, CA, USA) was used as negative control in the reactions.

#### 2.6. Statistical analysis

Data were analyzed using Fisher's exact test, comparing the presence of virus type (PCV2, TTV1, TTV2) in each organ (lymph node, testicle) and vice versa. Statistical analyses were performed using the PROC FREQ of SAS (SAS, 2002–2003).

Analysis of variance (ANOVA) with a *p*-value of *p* < 0.05 considered significant, (GraphPad Prism®, GraphPad Software, La Jolla, CA, USA) was used to analyze average and motility percentages on boar sperm samples.

### 3. Results

#### 3.1. Detection of PCV2, TTV1 and TTV2 DNA in organs of boars

Semen and organs, such as lymph nodes (mesenteric, prostatic, preputial, penile, inguinal) and testicles were collected and analyzed by nested-PCR. PCV2 was amplified more frequently from lymph nodes (11/13 or 84.6%), followed by testicles (6/17 or 35.3%). Table 1 shows detailed results from the boar testicle and lymph node DNA. TTV1 was detected in 5 (29.4%) and TTV2 in 16 (94.1%) animals. For TTV2, specific PCR primers were able to amplify TTV2 sequences in 16/17 testis and 13/13 lymph node samples from the boars. TTV1 amplified products were also detected in some testis samples (2/17), which were also positive for TTV2, while only one lymph node sample tested positive for TTV1 (pig 400). Pigs in this study were naturally infected with TTV2 in the testicles more frequently than PCV2 or TTV1. This can be noticed

**Table 2**

Statistical analyses showing the percentages of PCV2, TTV1, or TTV2 positive boars in all studied organs.

Organ	Virus			<i>p</i> *
	PCV2	TTV1	TTV2	
Lymph node	84.6% (11/13) <sup>a</sup>	7.7% (1/13) <sup>b</sup>	100.0% (13/13) <sup>a</sup>	<0.0001
Testicle	35.3% (6/17) <sup>b</sup>	11.8% (2/17) <sup>b</sup>	94.1% (16/17) <sup>a</sup>	<0.0001
<i>p</i> *	0.0105	1.0000	1.0000	

Percentages followed by distinct letters in the same line differ significantly by Fisher's exact test (*p* < 0.05).

\* *p*-Value of the Fisher's exact test.

in Table 2, which shows a higher number of positive boars for both TTV2 and PCV2 in lymph nodes (>84%), while the percentage of TTV1 positive boars was lower (7.7%). For testis, specifically the percentage of positive boars was high for TTV2, but not for PCV2, which remained low, similar to TTV1 in this organ. Regarding viral tropism to a specific organ, it was observed that only PCV2 has a predilection for the lymph nodes, while the other two viruses have similar prevalence in both organs.

#### 3.2. Semen analysis and identification of PCV2, TTV1 and TTV2 DNA in semen of boars

All studied boars were in good health condition, showed no clinical signs of PCVAD and had been routinely used for semen collection and/or training. Swine semen samples were collected from six boar studs and from one farm on which natural breeding was performed. Semen samples were tested by nested and/or real-time PCR for PCV2 and by nested-PCR for TTV1 or TTV2. Table 1 shows the individualized PCR results from the semen sample of each pig. TTV2 was detected in 2/17 (11.7%) and PCV2 in 8/17 (47.0%) animals. TTV1 amplified products were undetected in all semen samples (0/17). PCV2 DNA was detected in 8/17 (47.0%) seminal fluid samples from male pigs. Young males (≤30 weeks of age), still in training for semen collection, also tested positive (4/11). Real-time PCR was used to quantify PCV2 in samples from positive boars. This test had detection limits (calculated by Probit test) of 4500 virus/mL, with a retest rate of 95% with positive results, and 1000 virus/mL, with 50% of retested samples showing positive results. Quantitative values (viral load) ranged from 2 × 10<sup>3</sup> to 3.9 × 10<sup>4</sup> PCV2 genomes/mL (data not shown).

Morphology and sperm motility analysis did not indicate significant differences between PCV2 positive or negative boars. All semen samples tested presented motility greater than 80%, which could allow their use for processing, dilution and artificial insemination. Morphology analysis was performed following the motility exam. For sperm morphology analysis the acrosome integrity, head, cytoplasmic droplets and tail were observed (data not shown).

Boar 6834 in particular, was sampled seven times with intervals of 15 days between each collection. Each semen sample was tested for all viral agents. TTV2 and PCV2 were both detected in 4/7 samplings individually, but coincidentally (PCV2 + TTV2) in just one of those (1/7). In addition to that, in one of the samplings boar 6834 presented 32% of total morphologic pathologies, one of them being 10% cytoplasm droplets. Nevertheless, in the same boar stud, 9.3% of boars (*n* = 4) presented sperm pathologies above 20% (including pig 6834) and 3/4 were negative for the viral agents investigated here (data not shown). No significant sperm pathologies were observed in the other semen samples analyzed here. Thus, the data suggest that the occurrence of PCV2 or TTV2 shows no relationship with sperm morphology changes. Although intermittent shedding was observed in one analyzed boar, PCV2 and/

or TTV2 DNA were detected more frequently than TTV1 DNA in pig semen.

### 3.3. Detection of PCV2, TTV1 and TTV2 DNA in organs of culled sows

Organs samples such as ovaries, uterus and ovarian follicular fluids (OFF) of 83 culled sows were tested individually by nested-PCR for DNA viral amplification of PCV2 and TTVs. Nucleic acids of PCV2 were amplified from 5/83 (6.0%) sows. TTV1 and TTV2 were detected in 25 (30.1%) and 41 (49.3%) sows, respectively. For TTV2 specifically, 27/71 samples (38%) tested positive in OFF, 19/83 (23%) in ovaries and 14/83 (17%) in uterine tissues. In some cases, the occurrence of co-infections with viral agents in sampled sows was observed (Table 3). The same samples were processed for PCV2 amplification by nested-PCR. From a total of 83 sows, PCV2 was amplified from 5 (6.02%) of them. Positive tissues included the uterus (3/5), ovaries (3/5) and only one (1/5) positive in the OFF. Two sows were positive in both ovarian and uterine samples. PCV2 and TTV2 co-infection was observed in two sows (OFF or ovary). The low detection of PCV2 DNA is not surprising, since previous findings also found negative results on ovaries and OFF (Madson et al., 2009b; Pogranichniy et al., 2008).

In addition to porcine TTVs and PCV2 viral detection, organ samples from reproductive tracts were evaluated macro and microscopically. Ovaries were examined and the estrous cycles of the sows were determined. The ovaries of the sows were classified as cycling, anestrus, or cystic ovaries according to the presence of growing follicles, corpora lutea following ovulation, or follicular cysts. All sows naturally infected with PCV2 were cycling, suggesting no interference by the virus on the estrous cycle. TTV2 positive included anestrus sows, but this was not statistically significant.

### 3.4. Histopathologic exam of sow uteri

Endometritis microscopic lesions (catarrhal, purulent, or chronic) were observed in 24/83 (28.9%) of sow uteri by histopathologic evaluation. None were PCV2 positive by PCR, indicating that the naturally infected sows presented normal uterine tissues.

## 4. Discussion

The present study evaluated the occurrence of natural infection of porcine *Circoviridae* family DNA viruses TTV and PCV2 in reproductive organs of male pigs from boar studs and culled sows. Due to the viral tropism to lymphoid cells, lymph nodes from those pigs were also analyzed (Krakowka and Ellis, 2008; Segales et al., 2005). The results presented here show a higher prevalence of porcine TTV2 in analyzed organ samples, indicating its importance, mainly in co-infections with PCV2. In swine, TTV alone has not presented to be pathogenic, but its role in co-infections with other pathogens has been investigated. Porcine TTV2 is more frequently related to PCV2 associated diseases (PCVAD) when compared to porcine TTV1 in Spain, (Kekarainen et al., 2006). Other reports indicate that TTV1 viral infection facilitates PCVAD (Ellis et al., 2008). Porcine TTV DNA can be detected in serum, plasma and feces of swine,

**Table 3**  
Nested-PCR results of 83 ovary/uterus samples and 71 ovarian follicular fluid samples from culled sows.

Samples	TTV1	TTV2	TTV1 and TTV2
Ovarian follicular fluid (n = 71)	13 (18.3)	27 (38.0)	5 (7.0)
Ovary (n = 83)	12 (14.4)	19 (22.8)	6 (7.2)
Uterus (n = 83)	7 (8.4)	14 (16.8)	1 (1.2)

Number and percentage of positive samples (%).

indicating that the fecal–oral route is important in TTV transmission (Brassard et al., 2008). Detection of porcine TTV in semen indicates that vertical transmission of swine TTV may occur (Kekarainen et al., 2007), but the effects of this on female reproductive tracts was not investigated. Transmission route studies have shown that porcine TTVs can be transmitted by colostrum and transplacental/intrauterine routes, demonstrating the importance of porcine TTV transmission from breeders to offspring (Martinez-Guino et al., 2009; Pozzuto et al., 2009). Interestingly, positive stillborn piglets were always positive with the same genogroup as their mothers, but sequencing analysis showed nucleotide diversity of TTV genomes from the sows and in stillborns (Martinez-Guino et al., 2009). It was hypothesized that stillborns may be infected with several strains of TTV, even from the semen, by the transplacental route (Martinez-Guino et al., 2009). The samples analyzed here, albeit presented with a low viral load, showed higher prevalence of PCV2 DNA in semen (47% of tested samples) and followed by TTV2 (11.7%). All tested semen samples were negative for TTV1. Other studies reported a higher prevalence of TTV1 and TTV2 in pig semen (55% and 32%, respectively), with no interference on semen quality (Kekarainen et al., 2007). This was also observed in our study. Although intermittent shedding was observed in one analyzed boar, PCV2 and/or TTV2 DNA were detected in pig semen more frequently than TTV1 DNA (all tested pigs were negative). No typical clinical signs indicate porcine TTV infection, thus infected boars, which are clinically healthy, can be maintained for semen production and breeding, increasing viral transmission. Multiple porcine TTV infections via different routes may generate distinct genotypes of TTV in pigs (Huang et al., 2009). Herein, PCV2 and TTV2 DNA were amplified by nested-PCR in a significant percentage of boars from boar studs, 47% and 11.7%, respectively, without demonstrating detrimental effects to sperm quality (motility or spermatic morphology). However, PCV2 and TTV2 shedding is sporadic (Larochelle et al., 2000), and requires a frequent monitoring of boar studs. PCV2 has been detected by nested-PCR in testes and accessory glands of boars (Opriessnig et al., 2006), suggesting a risk of PCV2 shedding throughout the semen.

Viral persistence and sporadic shedding may be a risk factor for dissemination of PCV2 or TTV to negative sows, potentially causing reproductive failures. This irregular pattern of shedding also draws attention to the fact that boars which tested negative on the first collection may be positive in subsequent ejaculates. A periodic monitoring for PCV2 or TTV2 must be established in boar studs. PCV2 and TTV2 presence in semen samples of younger animals may indicate a recent infection or may even be related to management or measures of stress. Morphology and sperm motility analysis did not indicate significant differences between PCV2 positive and negative boars. All semen samples tested presented motility superior to 80%, which could allow their use for processing, dilution and artificial insemination.

Several factors determine the removal of sows from a swine herd. Sows are culled when they are considered unsuitable for further production, reproductive failure being the predominant cause, being responsible for 13–49% of removals (D'Allaire and Drolet, 2006). Infectious causes of reproductive failures vary, and many can be due to viral agents (D'Allaire and Drolet, 2006). Here we investigated the occurrence of PCV2 and two genogroups of TTV. Other viral agents, for example DNA from porcine parvoviruses was detected in 7.2% of ovaries and 4.2% of OFF (data not shown). Porcine reproductive and respiratory virus (PRRSV) can cause significant losses, but is not present in Brazilian swine herds (Ciacci-Zanella et al., 2004). Another investigation on 49 abattoir-obtained pig ovaries detected PPV by PCR in 6 OFF samples (12.2%) (Pogranichniy et al., 2008). Other viruses known to cause abortion and embryonic losses in pigs (PCV1, PCV2, PRRSV, BVDV,

BDV) were not detected in the OFF samples analyzed in that study (Pogranichniy et al., 2008). Here we tested ovaries, OFF and uteri of 83 culled sows. TTV2, TTV1 and PCV2 DNA were detected by nested-PCR from 49.3%, 30.1% and 6.0% of the sows, respectively. Macroscopic and microscopic exams were also performed. The most frequent pathologic lesions on uterine tissues were endometritis (28.9%), but this was unassociated with sows naturally infected with PCV2. PCV2 naturally infected sows were cycling, suggesting no interference on the estrous cycle. Although TTV2 was detected in almost 50% of the sows studied here, the association with PCV2 co-infection and reproductive failure was statistically insignificant.

To our knowledge this is the first study investigating the natural co-infection of PCV2 and porcine TTVs in reproductive organs, semen and OFF of swine. In summary, both PCV2 and TTV2 were most prevalent during natural infection in those sites. No sign of reproductive failure was associated with this co-infection. These findings raise the question of the importance of these viral infections in the pathology of reproductive failures.

### Conflict of interest statement

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the EMBRAPA, Brazilian Agriculture Research Corporation.

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