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Short communication

Caprine arthritis-encephalitis virus (CAEV) detection in semen of endangered goat breeds by nested polymerase chain reaction

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ABSTRACT

Semen and peripheral blood mononuclear cells (PBMCs) samples of four naturally infected, four experimentally infected (endangered breeds) and four non-infected bucks (endangered breeds) were evaluated for the presence of CAEV proviral-DNA by nested polymerase chain reaction (n-PCR). Three out of the eight PBMC samples from infected bucks were positive for CAEV-DNA and four out of the eight semen samples were positive for CAEV proviral-DNA. This is the first report describing the presence of CAEV proviral-DNA in semen from seropositive Anglo-Nubian, Moxotó and Canindé bucks, providing useful information towards the design of efficient methods to prevent CAEV dissemination in the endangered goat livestock genetic resources in Brazil.

1. Introduction

Brazil has various species of domestic animals, which were developed from breeds brought by the Portuguese settlers soon after the discovery. For five centuries, these breeds have been subjected to natural selection in specific environments. Today, they present characteristics adapted to the specific Brazilian environmental conditions. These breeds developed in Brazil are known as "Crioulo," "local," or naturalized (Mariante and Egito, 2002). From the beginning of the 20th century, some exotic breeds, selected in temperate regions, have been imported. Although more productive, most of these breeds do not have adaptive traits, such as resistance to disease and parasites found in the naturalized breeds. Even so, they are gradually replacing the native breeds, to such an extent that the latter, are in most of cases in danger of extinction. In 1983, to avoid the loss of this important genetic material, the National Research Center for Genetic Resources and Biotechnology (Cenargen) of the Brazilian Agricultural Research Corporation (Embrapa) decided to include conservation of animal genetic resources in its research Conservation and Utilization of Genetic Resources program (Mariante et al., 2009). This involves the storage of semen and embryos from those native breeds. The Brazilian Animal Germplasm Bank is kept at Cenargen, which is responsible for the semen storage of various endangered native goat breeds such as Moxotó, Canindé, Marota and Repartida.

However, many animal viruses are disseminated via semen, but there is little information on how to protect the male reproductive tract and semen from viral infection. Semen contaminated with viral agents may cause an enormous spread of certain diseases that could eventually infect numerous farms, areas, or even countries in a short period of time (van Rijn et al., 2004). Recently, the presence of caprine arthritis-encephalitis virus (CAEV) was detected in the semen and genital tract from naturally infected bucks, and these findings support the first step in proving sexual

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Table 1
Analysis of semen and peripheral mononuclear cells from 12 bucks (eight CAEV seropositive numbered 1–8, and four seronegative numbered 9–12, tested by agar gel immunodiffusion for the presence of *gag* sequence of CAEV proviral-DNA using polymerase chain reaction (nested-PCR)).

Buck	CAEV gag[a]	Breed	Semen	PBMC	Serum
1	NI	Anglo-Nubian	–	–	+
2	NI	Anglo-Nubian	+	+	+
3	NI	Saanen	+	+	+
4	NI	Anglo-Nubian	–	+	+
5	EI	Canindé	+	–	+
6	EI	Moxotó	–	–	+
7	EI	Moxotó	–	–	+
8	EI	Moxotó	+	–	+
9	C	Canindé	–	–	–
10	C	Canindé	–	–	–
11	C	Moxotó	–	–	–
12	C	Moxotó	–	–	–

[a] NI: naturally infected; EI: experimentally infected; C: control; +: positive; –: negative.

transmission of CAEV (Andrioli et al., 1999; Ali Al Ahmad et al., 2008; Peterson et al., 2008).

Caprine arthritis-encephalitis virus is a lentivirus that causes arthritis, leukoencephalomyelitis, interstitial pneumonia, and indurative mastitis with decreased milk production in adult goats (Smith and Cutlip, 1988). Besides affecting production efficiency, the sanitary and economic impact of CAEV infection is associated with interference in the international movement of goats and their germplasm, due to sanitary restriction imposed by countries that have control programs. Furthermore, there is a potential risk of viral dissemination by infected semen from animal germplasm (Andrioli et al., 2006).

The most widely used serological test for caprine arthritis-encephalitis (CAE) is the agar gel immunodiffusion (AGID) recommended by the World Animal Health Organization (OIE, 2008). However, this seems to underestimate infection incidence especially in animals with delayed seroconversion (Johnson and Zink, 1992). This can impede early or precise serological diagnosis in animals, which could be an efficient infection source. For successful preventive CAEV dissemination other laboratory diagnostic resources for detecting viruses are indispensable such as PCR (Rimstad et al., 1993; Leroux et al., 1997). Utilization of molecular biology techniques specifically PCR, to detect the presence of CAEV provirus in semen should facilitate certification of germplasm as being free of contaminating virus (Choi et al., 2002).

The objective of this work was to evaluate a nested-PCR assay to detect the presence of proviral-DNA of CAEV in semen, in order to have an optimal diagnostic tool with high specificity and sensitivity.

2. Materials and methods

Twelve 2–3-year-old bucks, from four breeds (Moxotó, Canindé, Anglo-Nubian and Saanen) were studied (Table 1). Four bucks were naturally infected with CAEV, and four others were inoculated with 5 mL of infected cell culture supernatant containing 10^6 TCID50 CAEV strain Cork. A third group of four bucks were inoculated with supernatant fluid from uninfected goat synovial membrane (GSM) cells and kept as the CAEV-negative control. CAEV inoculations were given intravenously.

Blood (4 mL in EDTA) and serum samples were collected from the jugular vein and semen (0.8–1.0 mL) by artificial vagina. Presence of CAEV proviral-DNA in blood and semen was determined by nested-PCR. Serum antibodies to CAEV were identified using a commercial (Biovetech, Pernambuco, Brazil) AGID test.

Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation on a ficoll gradient (Histopaque 1.077, Sigma–Aldrich, Steinheim, Germany) at $400 \times g$ for 30 min, washed twice with PBS (0.01 M, 0.05 M NaCl, pH 7.2) and freeze-dried at −80 °C.

DNA extraction from PBMC cells was done using a commercial blood nucleic acid extraction kit (Biosystems, Curitiba, Paraná, Brazil) using the manufacturer's protocol. Semen samples (100 μL) were passed through a Sephacryl S400 (Pharmacia, Uppsala, Sweden) chromatography column (Santurde et al., 1996), before being submitted to DNA extraction. CAEV proviral-DNA was obtained from the filtered semen by a boiling procedure using Chelex 100 (Sigma–Aldrich, USA) resin (Walsh et al., 1991). In a 1.5 mL tube, a 3 μL of filtered semen was added to 200 μL of a solution containing 5% Chelex 100 (w/v) in ultrapure water, 2 μL of 10 mg mL^{-1} proteinase K (Invitrogen, São Paulo, Brazil) and 7 μL of DTT. The mixture was incubated at 56 °C for 45 min, followed by centrifugation at $13{,}000 \times g$ for 10 s, incubation in a boiling water bath for 8 min, and subsequent centrifugation at $13{,}000 \times g$ for 3 min.

The n-PCR technique was performed as described previously by Barlough et al. (1994). Two rounds of PCR amplification were used to detect the gag sequence of the CAEV genome. In the first round, viral detection was done via the amplification of a fragment of proviral-DNA, located between nucleotide 953 and nucleotide 1226, using external primers GAG EX 5 (5′-CAAGCAGCAGGAGGGAGAAGCTG-3′) and GAG EX3 (5′-TCCTACCCCCATAATTTGATCCAC-3′).

This round was immediately followed by a second round, amplifying the fragment located between nucleotide 997 and nucleotide 1154, using internal primers GAG IN5 (5′-GTTCCAGCAACTGCAAACAGTAGCAA-3′) and GAG IN3 (5′-ACCTTTCTGCTTCTTCATTTAATTTCCC-3′) as described by Andrioli et al. (1999). Oligonucleotide primers specific to the fourth exon of the human β-actin gene (ES30, ES32, ES31, and ES33) were used as an internal control for the integrity of the DNA (Ali Al Ahmad et al., 2008).

For the first round of amplification, 5 μL of DNA (containing 100–200 ng) were added to 45 μL of an amplification solution, or "mix1" containing: 5 μL of reaction buffer 10× (670 mM Tris/HCl (pH 8.8), 160 mM $(NH_4)_2SO_4$, 0.1% Tween-20), 1.5 μL of $MgCl_2$ (50 mM), 1 μL of dNTP (25 mM of each oligonucleotide triphosphate: dATP, dGTP, dCTP, dTTP), 0.2 μL of TAQ (5 U/μL, Platinum® Taq DNA polymerase) (Invitrogen, São Paulo, Brazil), 1.5 μL of each primer GAG EX3, GAG EX5, ES30 and ES32 (10 mM, Custom primers, Invitrogen, São Paulo, Brazil) and 31.3 μL of DEPC-treated water. For the second round, 1 μL of the first round were added to 49 μL of a second amplification solution, or"mix2" containing the same reagents as the solution in mixture 1, except that internal primers GAG IN5, GAG IN3, ES31, and ES33 were used to replace primers GAG EX5, GAG EX3, ES30, and ES32. For each round, following an initial denaturation at 94 °C for 5 min, the samples were submitted to a series of 35 cycles comprising, successively, a further 1-min denaturation phase at 94 °C, a 90 s hybridization phase at 46 °C and a 2.5-min extension phase at 60 °C. Each round was followed by a final extension at 60 °C for 15 min.

Amplification products were visualized using electrophoresis on 1.5% agarose gel containing ethidium bromide in 1× TBE (0.045 M Tris–boric acid, 1 mM EDTA, pH 7.2) buffer. Two controls were performed for each gel: a positive control (CAEV proviral-DNA from infected goat synovial membrane) and a negative control (distilled water). Following electrophoretic separation, the bands were visualized using trans-illumination with ultraviolet light. Samples analyzed for CAEV proviral-DNA using n-PCR were considered positive when a 185 bp band, corresponding to the positive control, was seen in the agar gel electrophoresis under ultraviolet light between 100 and 200 bp molecular weight bands.

3. Results and discussion

The objective of this study was to evaluate a PCR method to detect CAEV in semen samples in order to asses the possibility of using non-infected semen from seropositive animals of endangered breeds in germplasm banks.

Samples analyzed for CAEV proviral-DNA using n-PCR were considered positive when a 185 bp band, correspond-

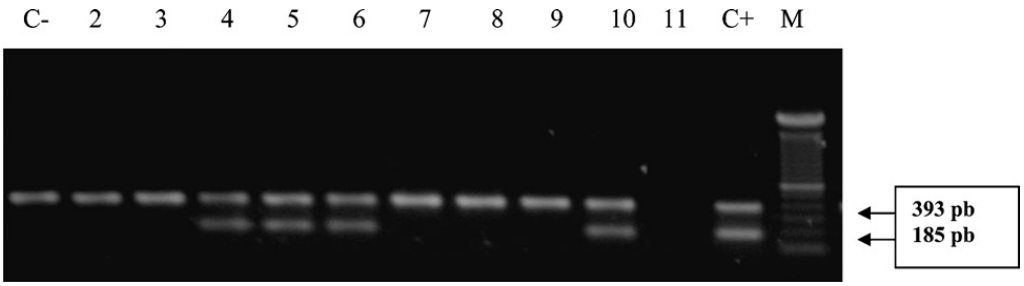

Fig. 1. Example of nested-PCR amplification of proviral-DNA. DNA from blood cells and semen from male goats were used to perform nested-PCR using specific sets of oligonucleotide primers to amplify both the 185-pb CAEV *gag* and 393-pb-actin fragments. Following nested-PCR reactions, each PCR product was separeted on 1, 2% agarose gel and the bands visualized by staining with ethidium bromide. M: smart ladder used as a molecular weight standart. C+: positive control (CAEV proviral-DNA from GSM infected cells). C−: negative control. Lanes 2 and 3: blood cells-negative; Lanes 4–6, semen positive. Lanes 7–9: semen negative. Lane 10: blood-cell positive. Lane 11: distilled water.

ing to the positive control, was amplified. Whereas the 393 bp band, generated from amplification of the endogenous actin gene, was present in both non-infected and CAEV-infected samples. An example of the results is displayed in Fig. 1, showing the analysis of DNA isolated from blood cells (PBMC) and semen. Four (4/12) semen and three (3/12) PBMC samples, respectively presented n-PCR positive. Results displayed in Table 1, show the analysis of the serum antibodies and CAEV proviral detection from blood cells and semen in infected and non-infected bucks. Rimstad et al. (1993) found 17/72 (23.6%) milk samples and 24/72 (33.3%) blood samples were PCR positive using *gag* primers.

Several studies have described the use of PCR methods in blood and other tissues, though few estimates of sensitivity and specificity have been made (Rimstad et al., 1993; Barlough et al., 1994; Travassos et al., 1999). The target sequences for primers range across the whole genome and include the LTR, and *gag*, *pol*, and *env* genes. The oligonucleotides evaluated in this study corresponded to the region *gag* of CAEV genome from DNA sequence of strain Cork (Saltarelli et al., 1990). Positive bands were detected covering a range from $10^{4.5}$ to $10^{-4.5}$ $TCID_{50}$/50 μL when a second round of PCR (n-PCR) was performed both in tenfold dilutions of supernatants of CAEV-inoculated cell culture and artificially inoculated semen. RFLP analyses were performed on the second round of PCR products with *Bal* I resulting in the expected fragments of 116 and 69 pb. Barlough et al. (1994) developed double-nested PCR using primers for both *gag* and *pol* in a single tube and results showed that the PCR had a sensitivity of 94.7% and 87.5%, respectively, relative to a recombinant ELISA. Travassos et al. (1999) found that primers from *env* region detected 14/15 (93.2%) infected goats, while primers from *gag* detected 9/15 (60%) virus load in vivo.

Recent studies (Ali Al Ahmad et al., 2008) demonstrated the presence of proviral CAEV-DNA in semen and reproductive tissue of male goats by PCR based on *gag* gene region. There are two main difficulties in developing suitable PCR tests: strain variation and virus load.

The presence of the virus was not necessary concordant in semen and PBMC samples. Our data indicated that in those animals the virus population's reservoirs in semen can be distinct from that in blood and probably there was no correlation between the presence of virus in blood and shedding in semen. It could be argued that PCR tests require a certain level of viral replication in the host for proviral-DNA to be present in a specific blood sample, which contends the diagnostic sensitivities of serological and PCR assays should be in the same range. It is also possible that macrophage-tropic CAEV variants are more likely to be found in, and thereby detected from, the genital compartment, since in contrast to balance in the blood, a number of macrophages are found in semen. A number of HIV studies indirectly suggest that the free viral particles and infected contaminated semen are produced within the male genital tract (Gupta et al., 2000; Le Tortorec and Dejucq-Rainsford, 2007). Therefore, the PCR detection of proviral-DNA in the blood monocytes is not a reliable method of selecting animals with CAEV-free semen.

Potential cellular reservoirs of CAEV in ejaculates include non-sperm cells, such as macrophages, immature germ cells, and epithelial lining cells (Peterson et al., 2008). It is important to investigate methods of preventing infection in the male reproductive tract, or at least shortening the shedding duration and lowering the viral load in semen to prevent widespread viral dissemination. There is little information on how to protect the male reproductive tract and semen from CAEV infection. Despite the resistance of naturalized breeds to diseases, this study demonstrated that CAEV proviral-DNA was shed into the semen of moxotó and canindé breeds. Since there is no vaccine or treatment available, eradication and control of the disease is exclusively based on early diagnostic and segregation of infected animals. Culling of seropositive animals, with or without their progenies, has been applied with some success in control programs. However, slaughtering cannot be applied to endangered species or livestock with relatively low number of animals in conservation programs of genetic resources.

Utilization of molecular biology techniques, especially PCR to detect the presence of CAEV provirus in semen, should facilitate certification of germplasm as being free of the contaminating virus. Previous data suggests that semen infected with CAEV can be potentially infectious. However, to prove sexual transmissibility of CAEV, it is necessary to conduct in vivo insemination of positive ejaculates in non-infected individuals, and subsequently confirm signs of the infection in those individuals. The use of proper AI collection techniques, microscope evaluation of semen to evaluate the presence of contaminating leukocytes and application of PCR to detect CAEV in representative straws

of semen from each ejaculate, should provide enough confirmation of CAEV-free status. This study is the first to demonstrate the presence of CAEV proviral-DNA in semen of Canindé and Moxotó breeds. The results show the critical importance to develop a CAEV-diagnostic PCR regiontailored assays for semen samples, in order to certify the germplasm as being CAEV-free, and, thus enabling CAEV-free semen to be obtained from infected male goats in conservation livestock genetic resources.

4. Conclusions

Shedding of proviral CAEV-DNA in semen has been proven in Moxotó and Canindé breeds. PCR prove to be useful in detecting proviral-DNA in blood as well as in semen. Blood, serum and semen should be sampled and an association of serology and PCR might be optimal for detecting infected animals. Application of PCR to detect CAEV in representative straws of semen from each ejaculate should provide enough confirmation of CAEV-free status.

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