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Detection of *Neospora caninum* and *Toxoplasma gondii* in Semen of Naturally Infected Rams

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ABSTRACT

***Background*:** *Neospora caninum* and *Toxoplasma gondii* are closely related cyst-forming apicomplexan parasites identified as important causes of reproductive failure in cattle. Moreover, abortion cases attributed to *N. caninum* and *T. gondii* infection have been occasionally reported in sheep. Due to the relatively scarce information on the molecular detection of *N. caninum* in the semen of naturally infected rams, this study aimed to detect parasitic DNA in fresh semen samples and in frozen extended semen straws from male sheep from artificial inseminations centers in Southern Brazil.

***Materials, Methods & Results*:** Semen samples of 38 rams from artificial insemination centers were evaluated. Eleven rams were naturally infected (seropositive for anti-*N. caninum* and/or anti-*T. gondii* IgG) and were selected for fresh semen collection. We tested all the samples for the closely related protozoan *T. gondii* to detect a possible cross-reaction and co-infection, due to the close similarity with *N. caninum*. The indirect fluorescent antibody test was used to detect IgG antibodies in the 11 serum samples from rams. Fresh semen samples were collected from 11 rams on days 1, 50, 55, and 58 using an artificial vagina and ewe in estrus. Other 27 rams had their frozen extended semen straws analyzed. A total of 20 fresh semen samples and 27 frozen extended semen straws samples were used to detect the presence of *N. caninum* and *T. gondii* DNA by polymerase chain reaction (PCR). Nc-5 and B1 genes were used as target regions to detect *N. caninum* and *T. gondii* DNA, respectively. The presence of *N. caninum* DNA was confirmed in the third collection of a fresh semen sample of one seropositive ram. *T. gondii* DNA was detected in a fresh semen sample of one seropositive ram. The DNA sequences of 186 bp from *N. caninum* (GenBank accession: MH806393) and 492 bp from *T. gondii* (GenBank accession: MH793503) were obtained by sequencing, and analysis revealed 99% and 100% identity, respectively, compared with other sequences deposited at GenBank. *N. caninum* and *T. gondii* DNAs were not detected in any of the 27 frozen extended semen straws used for artificial insemination.

***Discussion*:** This study demonstrated the presence of *N. caninum* and *T. gondii* DNA in fresh semen samples of naturally infected rams. The non-detection of *N. caninum* and *T. gondii* DNA in frozen semen samples of rams could be due to the dilution that was used to prepare the semen straws (GGL diluent and 5% glycerol), since fresh semen samples were not diluted prior to the test. Moreover, in our study, the volume of frozen semen samples (0.25 mL) used for PCR was lower than the volume of sediment obtained from fresh semen (0.5 mL), and the fresh semen centrifugation to obtain the sediment may have grouped the tachyzoites, increasing the sensitivity of the technique employed. No high IgG serological titers were detected in the rams at the time they were eliminating the parasite through fresh semen. The final titer of anti-*N. caninum* and anti-*T. gondii* IgGs in serum was 1:100, suggesting chronic infection. It is suggested that a new parasite elimination pathway is occurring among rams used for reproduction, due to the presence of *N. caninum* and *T. gondii* DNA in fresh semen samples from seropositive animals. Although the detection of genomic DNA of *N. caninum* and *T. gondii* in semen does not necessarily imply the presence of infectious stages of the parasites and does not determine their viability, these results demonstrate the need for further studies. Our study also indicates the need to reinforce preventive measures for sheep in artificial insemination centers until the risks are evaluated, by performing serological examinations with anti-*N. caninum* and anti-*T. gondii* antibodies, for instance, to select the rams that will be used for breeding.

Keywords: male sheep, ewes, ovine, tachyzoites, frozen extended semen straws, fresh semen.

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INTRODUCTION

Natural infection of sheep by *Neospora caninum* was first identified in 1990 [12], and *Toxoplasma gondii* infection was described for the first time in 1942 [32]. *Neospora caninum* and *Toxoplasma gondii* are closely related cyst-forming apicomplexan parasites identified as important causes of reproductive failure in cattle [13,14]. Abortion cases attributed to *N. caninum* [12,21,35] and *T. gondii* [10,20,28,44] infection have been occasionally reported in sheep. These parasites have already been identified in 77% of 74 brain samples from aborted sheep fetuses [23].

The presence of *N. caninum* DNA in the semen of naturally infected bulls [7,11,17,18,24,33,40] and experimentally infected rams was reported [42]. Experimental studies showed the presence of *T. gondii* in the semen and/or reproductive tract of dogs [2], goats [15,38], swine [31], cattle [39], and rams [1,27,41,43]. Studies performed with naturally infected animals have shown the detection of *T. gondii* only in the semen of rams [4,29] and dogs [25], suggesting a new route of elimination of the parasite that can cause reproductive damage and be sexually transmitted [9]. Studies have only found *N. caninum* DNA in frozen extended semen straws samples from cattle [7,33,40].

Due to the relatively scarce information on the molecular detection of *N. caninum* in the semen of naturally infected rams, this study aimed to detect parasitic DNA in fresh semen samples and in frozen extended semen straws from male sheep from artificial inseminations centers in Southern Brazil. We tested all the samples for the closely related protozoan *T. gondii* to detect a possible cross-reaction and co-infection, due to the close similarity with *N. caninum*.

MATERIALS AND METHODS

Animals and samples

Thirty-eight rams aged 1-6 years (Dorper, Texel, White Dorper, Suffolk, and crossbreed), with no history of reproductive problems, from three rural properties with sporadic occurrences of abortions in pregnant ewes, in the state of Paraná, Southern Brazil, were included in this study to detect *N. caninum* and *T. gondii* DNA in fresh and frozen semen samples.

Twenty semen samples were collected from 11 rams seropositive for *N. caninum* and/or *T. gondii* IgG antibodies (identified with numbers 1-11) that had been selected to detect *N. caninum* and *T. gondii* DNA in fresh semen samples. Twenty-seven frozen extended semen straws samples from 27 other breeding rams belonging to the same rural properties were used to detect *N. caninum* and *T. gondii* DNA. Since we did not have access to these animals, serological data is not known.

Samples of fresh semen were collected using an artificial vagina and according to the availability of ewes in estrus. Fifteen min after collecting semen samples, blood samples were collected without anticoagulant by puncturing the jugular vein. Blood and semen samples from the 11 rams were collected on days 1, 50, 55 and 58. Samples were refrigerated at 4°C and transported to the laboratory within 2 h.

Sera from the 11 rams were obtained by centrifuging the blood at 3000 × *g* for 10 min and, then, stored at -20°C until processing. The 20 fresh semen samples (the total volume of ejaculates ranged from 1.0-2.0 mL) were centrifuged at 1000 × *g* for 10 min, and the sediments of approximately 0.5 mL were frozen at -20°C before DNA extraction.

The 27 semen straws frozen in 5% glycerol with 0.25 mL of extender GGL (Glycine-Yolk-Milk), and approximately 100 million spermatozoa per dose (400×10^6/mL) were refrigerated at 4°C for approximately 4 h during the transport to the laboratory. Then, 0.25 mL of the sample transferred to micro-tubes and frozen at -20°C until processing.

Indirect fluorescent antibody test (IFAT)

The indirect fluorescent antibody test was used to detect IgG antibodies against *N. caninum* and *T. gondii* in the 11 serum samples from rams [30]. Slides were prepared containing tachyzoites of *N. caninum* (NC-1 strain) and *T. gondii* (RH strain), obtained through cell culturing as described by Locatelli-Dittrich *et al*. [26]. Serum samples with titers 1:25 for *N. caninum* and 1:40 for *T. gondii* were considered positive as described by Guimarães *et al*. [22]. Sera were analyzed until reaching the final titer. All IFATs included known positive and negative ram serum samples as control. All samples were processed twice to ensure the quality of the results. The Monoclonal Anti-Goat/Sheep IgG-FITC antibody[1] (F4891) was used at a dilution of 1:100.

Collection of semen

Fresh semen samples were collected from 11 seropositive rams on days 1, 50, 55, and 58 using an

artificial vagina and ewe in estrus. The artificial vagina consisted in a 20 cm × 6 cm rigid tube and a flexible rubber tube. The space between the rigid and the flexible tube was filled with warm water at 55°C, so that the inside of the artificial vagina reached a temperature between 42°C and 45°C. The artificial vagina membrane was lubricated with aqueous base gel. When the ram jumped over the ewe, his penis was gently diverted into the artificial vagina, and the ejaculate was deposited in the collection tube [16].

DNA extraction

The DNA extraction from 20 fresh semen samples (containing 0.5 mL of semen sediment) and 27 frozen semen straws (containing 0.25 mL of sample) was performed using the PureLink® Genomic DNA Mini Kits[2].

Hemi-Nested PCR to detect Neospora caninum *DNA*

Hemi-nested PCR was performed to detect the presence of *N. caninum* DNA by using the primer pairs Np4/Np7 and Np6/Np7 of the pNC-5 gene of *N. caninum* [46], following the protocol previously published with some modifications [3]. The 25-μL PCR mixture contained 80 ng of target DNA, 1.5 mM of $MgCl_2$, 0.2 mM of dNTPs, 1 U/reaction of Taq polymerase, 1× buffer, 0.5 pmol/μL of primers, and Milli-Q Water qsp. PCRs using Np4/Np7 were performed in a thermocycler for 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. For the second round, with primers Np6/Np7, 1 μL of amplicon solution from the first-round Np4/Np7 PCR was used as target DNA, with the same PCR mixture, with 25 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 30 s. The secondary amplification products had a size of 227 bp. Positive (purified *N. caninum* tachyzoite DNA of the NC-1 strain) and negative (no DNA) controls were included in each PCR run. Amplicons were resolved on a 1.5% agarose gel, which was stained with SYBR®[3] Safe DNA gel stain (Kasvi, K9-16C) and visualized under UV light.

Nested PCR to detect Toxoplasma gondii *DNA*

The presence of *T. gondii* DNA in the samples was addressed by a Nested PCR in which the target was part of the sequence of the repetitive gene B1 [5], following the protocol previously published with some modifications [19]. *T. gondii* DNA amplification was carried out in a 25-μL reaction volume containing 80 ng of target DNA, 1.5 mM of MgCl2, 0.2 mM of dNTPs, 1 U/reaction of Taq polymerase, 1× buffer, 0.5 pmol/μL of primers, and Milli-Q Water qsp. The primers used in the first round were those corresponding to gene B1 nucleotides 694-714 and 887-868. PCR was performed for 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s. The primers used in the second round corresponded to gene B1 nucleotides 757-776 and 853-831 and were used in a reaction with 0.5 μL of the amplicon solution from the first-round PCR as target DNA, with the same PCR mixture, with 20 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 35 s, and extension at 72°C for 30 s. The secondary amplification products had a size of 97 bp. Positive (DNA from *T. gondii* tachyzoites of the RH strain) and negative (no DNA) controls were included in each PCR run. Amplicons were resolved on a 1.5% agarose gel, which was stained with SYBR® Safe DNA gel stain (Kasvi, K9-16C) and visualized under UV light.

Sequencing analysis

PCR products were purified using PureLink® Quick Gel Extraction Kit[2] according to manufacturer's instructions, and sequence analysis was performed using primer Np7, 5'GGGTGAACCGAGGGAGTTG3', to identify the DNA sequence of *N. caninum* [3] and primer 5'TGCATAGGTTGCCAGTCACTG'3, which corresponds to the gene B1 nucleotides 853-831, to identify the DNA sequence of *T. gondii* [19]. Sequence analysis was performed using the Big Dye Terminator Kit (Applied Biosystems) according to manufacturer's instructions in an ABI DNA Model 3500 Series Genetic Analyzer (Applied Biosystems).

Sequences were, then, subjected to BlastN analysis using the GenBank database at NCBI to confirm that the PCR-amplified sequences were those of *N. caninum* and *T. gondii*.

RESULTS

The anti-*N. caninum* IgG titer ranged from 1:25 to a maximum titer of 1:100 in the ram serum samples. The anti-*T. gondii* IgG titer ranged from 1:40 to a maximum titer of 1:100 (Table 1). Serum samples were considered positive to the presence of *N. caninum* and *T. gondii* if the entire surface of the tachyzoites was fluorescent.

Among the 20 fresh semen samples from the 11 rams, *N. caninum* DNA was detected in the semen of ram 1, collected on day 55 (Table 2). *T. gondii* DNA was detected in the fresh semen sample of ram 7, on day 1 (Table 2).

N. caninum and/or *T. gondii* DNA were not detected in any of the 27 frozen extended semen straws.

The DNA sequences of 186 bp from *N. caninum* (GenBank accession: MH806393) and 492 bp from *T. gondii* (GenBank accession: MH793503) were obtained by sequencing, and analysis revealed 99% and 100% identity with sequences deposited at GenBank, respectively.

Table 1. Titers of anti-*Neospora caninum* and anti-*Toxoplasma gondii* IgGs detected by means of indirect fluorescence test (IFAT) in the serum of 11 rams.

Ram	Day 1		Day 50		Day 55		Day 58	
	T.gondii	*N. caninum*	*T.gondii*	*N. caninum*	*T.gondii*	*N. caninum*	*T.gondii*	*N. caninum*
1	-	1:50	1:40	1:50	1:40	1:50	nd	nd
2	1:40	-	1:40	-	-	-	nd	nd
3	1:40	-	1:100	-	1:100	-	nd	nd
4	-	1:100	nd	nd	nd	nd	-	1:50
5	-	1:25	nd	nd	nd	nd	-	1:25
6	1:40	-	nd	nd	nd	nd	-	-
7	1:40	-	nd	nd	nd	nd	nd	nd
8	1:100	-	nd	nd	nd	nd	nd	nd
9	1:40	-	nd	nd	nd	nd	nd	nd
10	-	1:25	nd	nd	nd	nd	nd	nd
11	-	1:50	nd	nd	nd	nd	nd	nd

Negative: -; nd: no sample available.

Table 2. Detection of *Neospora caninum* and *Toxoplasma gondii* DNA in fresh semen samples from 11 rams using Hemi-Nested PCR and Nested PCR.

Ram	Day 1	Day 50	Day 55	Day 58
	PCR	PCR	PCR	PCR
1	-	-	*N. caninum* DNA	nd
2	-	-	-	nd
3	-	-	-	nd
4	-	nd	nd	-
5	-	nd	nd	-
6	-	nd	nd	-
7	*T. gondii* DNA	nd	nd	nd
8	-	nd	nd	nd
9	-	nd	nd	nd
10	-	nd	nd	nd
11	-	nd	nd	nd

Negative: -; nd: no sample available.

DISCUSSION

In our study, *N. caninum* DNA was detected in the semen sample from the third collection from ram 1, in agreement with Syed-Hussain *et al*. [42] that detected *N. caninum* DNA in the semen samples of experimentally infected seropositive rams and also demonstrated sporadic parasite elimination between the first and fourth week after infection.

The ram in which semen *N. caninum* DNA was detected was seropositive for anti-*N. caninum* (1:50) and anti-*T. gondii* (1:40) IgGs. Co-infection in sheep has been reported in abortion cases by Hughes *et al*. [23], who detected the simultaneous presence of both parasites in 12.2% of brain samples from aborted sheep fetuses.

No high IgG serological titers were detected in the rams at the time they were eliminating the parasite through semen. The final titer of anti-*N. caninum* and anti-*T. gondii* IgGs in serum was 1:100, suggesting chronic infection. Studies on serological titration of ewes that presented abortion showed titers of 1:1024 [6] and 1:800 [45] for infection with *T. gondii* and *N. caninum*, respectively, and high titration of IgG suggests acute toxoplasmosis.

The DNA of *T. gondii* was detected in the semen of ram 7, in agreement with other studies that detected *T. gondii* DNA in samples of fresh and frozen semen from naturally infected rams used for reproduction [4,29].

In our study, *T. gondii* and *N. caninum* DNAs were not detected in the 27 frozen extended semen straws used for artificial insemination. This result is different from that in the study from Bezerra *et al*. [4], where *T. gondii* DNA was detected in 22.2% of frozen semen samples and in 100% of fresh semen samples from seropositive rams commercialized in artificial insemination centers in Northeastern Brazil.

The non-detection of *N. caninum* and *T. gondii* DNA in frozen semen samples of rams could be also due to the dilution that was used to prepare the semen straws (GGL diluent and 5% glycerol), since fresh semen samples were not diluted prior to the test. Moreover, in our study, the volume of frozen semen samples (0.25 mL) used for PCR was lower than the volume of sediment obtained from fresh semen (0.5 mL), and the fresh semen centrifugation to obtain the sediment may have grouped the tachyzoites, increasing the sensitivity of the technique employed.

Ortega-Mora *et al*. [33] investigated the presence of *N. caninum* DNA in fresh non-extended semen straws and frozen extended semen straws of eight bulls and detected a smaller number of positive samples in frozen semen than in fresh, indicating that positive results may be considered as confirmatory, but negative results cannot be considered as true negative.

Caetano-Da-Silva *et al*. [7] detected *Neospora* DNA sporadically in frozen semen samples from seropositive cattle. They also demonstrated that the non-detection of protozoa DNA in frozen semen samples can be explained by the low number of parasites in the sample, which is undetectable by the technique used, or by the fact that, when testing frozen semen samples with a low concentration of target molecules, it is likely that *Neospora* DNA will be absent in each tube of the reaction, resulting in a false negative PCR result.

The nucleotide sequence obtained from the semen sample where *N. caninum* DNA was detected presented 99% identity with sequences deposited in GenBank, and, among them, a sequence deposited by Hughes *et al*. [23], who detected the presence of *N. caninum* in 18.9% of brain samples from aborted lambs, but not in other tissues, such as heart and umbilical cord samples, from these fetuses.

Our results indicate that further studies are needed to evaluate the real potential of *N. caninum* transmission via semen, like using one protozoa isolated from semen of naturally infected rams to inseminate ewes. There is a variation in degrees of pathogenicity among different protozoan isolates that has already been demonstrated experimentally for the *N. caninum* parasite, such as different viability *in vitro* [37], transplacental transmission [8,36], and histopathological lesions [34]; however, no study has evaluated the horizontal transmission capacity (via semen) of different isolates. The experimental study of Syed-Hussain *et al*. [42] demonstrated that the transmission of *N. caninum* via semen in sheep is unlikely, since ewes that had copulated with rams experimentally infected with the NcNZ1 isolate and that had *N. caninum* in semen did not present seroconversion. However, further studies with different isolates are required to confirm the non-transmission of *N. caninum* via semen.

The nucleotide sequence obtained from the *T. gondii* DNA-positive semen sample showed 100% identity with many sequences deposited in the Gen-

Bank database, and, among them, *T. gondii* sequences obtained from sheep tissue samples, confirming the result obtained by PCR.

It is suggested that a new parasite elimination pathway is occurring among rams used for reproduction, due to the presence of *N. caninum* and *T. gondii* DNA in fresh semen samples from seropositive animals. Although the detection of genomic DNA of *N. caninum* and *T. gondii* in semen does not necessarily imply the presence of infectious stages of the parasites and does not determine their viability, these results demonstrate the need for further studies.

Our study also indicates the need to reinforce preventive measures for sheep in artificial insemination centers until the risks are evaluated, by performing serological examinations with anti-*N. caninum* and anti-*T. gondii* antibodies, for instance, to select the rams that will be used for breeding.

CONCLUSION

This study demonstrated that naturally infected rams with *Neospora caninum* and *Toxoplasma gondii* can eliminate the protozoa through semen.

MANUFACTURERS

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***Declaration of interest*.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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