UNIVERSIDADE FEDERAL DO PARANÁ

MARILIA DE OLIVEIRA KOCH

DETECTION OF *NEOSPORA CANINUM* AND *TOXOPLASMA GONDII* IN SEMEN OF NATURALLY INFECTED RAMS, AND DETECTION OF ANTIBODIES AGAINST *SARCOCYSTIS NEURONA, NEOSPORA CANINUM* AND *TOXOPLASMA GONDII* IN HORSES, DOGS AND CATS FROM PARANÁ STATE, BRAZIL

CURITIBA

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Tese apresentada ao Programa de Pós-Graduação em Ciências Veterinária, Setor de Ciências Agrárias, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Ciências Veterinárias.

Orientadora: Profa. Dra. Rosangela Locatelli Dittrich

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em CIÊNCIAS VETERINÁRIAS da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **MARILIA DE OLIVEIRA KOCH** intitulada: **Detection of *Neospora Caninum* and *Toxoplasma Gondii* in semen of naturally infected rams, and detection of antibodies against *Sarcocystis Neurona*, *Neospora Caninum* and *Toxoplasma Gondii* in horses, dogs and cats from Paraná state, Brazil**, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua Aprovação no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 22 de Março de 2019.

ROSANGELA LOCATELLI DITTRICH
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ODILEI ROGERIO PRADO
Avaliador Externo (UTP)

MARCIA KIYOE SHIMADA
Avaliador Externo (UFPR /DPB)

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RESUMO GERAL

A presente tese de doutorado é composta de dois artigos científicos distintos. O primeiro artigo refere-se à detecção de *Neospora caninum* e *Toxoplasma gondii* no sêmen de ovinos naturalmente infectados, onde o objetivo foi detectar o DNA de *N. caninum* e *T. gondii* em amostras de sêmen fresco e palhetas de sêmen congeladas de ovinos naturalmente infectados utilizando a reação em cadeia da polimerase (PCR). Os genes Nc-5 e B1 foram usados como regiões alvo para detectar DNA de *N. caninum* e *T. gondii*, respectivamente. Foi detectado o DNA de *N. caninum* em amostra de sêmen fresco, obtido na terceira coleta de um ovino soropositivo para *N. caninum* e *T. gondii*, e o DNA de *T. gondii* foi detectado em uma amostra de sêmen fresco de ovino soropositivo para *T. gondii*.

O segundo artigo estuda a prevalência de anticorpos contra *Sarcocystis neurona*, *Neospora caninum* e *Toxoplasma gondii* em cavalos, cães e gatos da região de Curitiba, no estado do Paraná, Brasil. O objetivo deste estudo foi investigar a ocorrência e distribuição de anticorpos contra os três protozoários em cavalos, cães e gatos de Curitiba no Paraná. Foram selecionadas amostras de soro de 100 cavalos, 100 cães e 100 gatos da rotina do Laboratório de Patologia Clínica Veterinária do Hospital Veterinário da Universidade do Paraná (UFPR). Os anticorpos contra *S. neurona, N. caninum* e *T. gondii* foram encontrados amplamente distribuídos entre cavalos, cães e gatos na região de Curitiba, estado do Paraná, Brasil.

Palavras-chave: Carneiros. Ovelhas. Ovinos. Taquizoítos. Palheitas de sêmen congeladas. Sêmen fresco. Sarcocistose. Neosporose. Toxoplasmose. Sinais neurológicos.

ABSTRACT

This thesis is composed of two distinct scientific articles. The first article refers to the detection of *Neospora caninum* and *Toxoplasma gondii* in semen of naturally infected rams, where the objective was to detect the DNA of *N. caninum* and *T. gondii* in fresh and frozen semen samples from naturally infected sheep using polymerase chain reaction (PCR). The Nc-5 and B1 genes were used as target regions to detect *N. caninum* and *T. gondii* DNA, respectively. *N. caninum* DNA was detected in a fresh semen sample obtained from the third collection of a seropositive ram for *N. caninum* and *T. gondii*, and *T. gondii* DNA was detected in a sample of fresh sheep semen seropositive for *T. gondii*.

The second article studies the prevalence of antibodies against *Sarcocystis neurona*, *Neospora caninum* and *Toxoplasma gondii* in horses, dogs and cats of the Curitiba region, in the state of Paraná, Brazil. The objective of this study was to investigate the occurrence and distribution of antibodies against the three protozoa in horses, dogs and cats of Curitiba, Paraná. Serum samples of 100 horses, 100 dogs and 100 cats from the routine of the Veterinary Clinical Pathology Laboratory of the Veterinary Hospital in the University of Paraná (UFPR) were selected. The antibodies against *S. neurona*, *N. caninum* and *T. gondii* were found widely distributed among horses, dogs and cats in the region of Curitiba, state of Paraná, Brazil.

Keywords: Male sheep. Ewes. Ovine. Tachyzoites. Frozen extended semen straws. Fresh semen. Sarcocystosis. Neosporosis. Toxoplasmosis. Neurological signs.

RESUMO DO ARTIGO I

O objetivo deste estudo foi detectar o DNA de *Neospora caninum* e *Toxoplasma gondii* em amostras de sêmen fresco e palhetas de sêmen congeladas de ovinos naturalmente infectados. Neste estudo foram avaliadas amostras de sêmen fresco e palhetas de sêmen congeladas de 38 ovinos de centros de inseminação artificial. Dos 38 ovinos, 11 eram soropositivos para *N. caninum* e/ou *T. gondii* e foram selecionados para coleta de sêmen fresco (coletas realizadas nos dias 1, 50, 55 e 58) com um total de 20 coletas. Dos outros 27 ovinos foram selecionadas 27 palhetas de sêmen congeladas, para análise. No total, 20 amostras de sêmen fresco e 27 amostras de sêmen congelado foram utilizadas para detectar a presença do DNA de *N. caninum* e *T. gondii* pela reação em cadeia da polimerase (PCR). Os genes Nc-5 e B1 foram usados como regiões alvo para detectar DNA de *N. caninum* e *T. gondii*, respectivamente. O DNA de *N. caninum* foi detectado no sêmen fresco, obtido na terceira coleta de um ovino soropositivo para *N. caninum* e *T. gondii*. O DNA de *T. gondii* foi detectado em uma amostra de sêmen fresco de ovino soropositivo para *T. gondii*. As seqüências nucleotídicas obtidas apresentaram 99% e 100% de identidade com o DNA de *N. caninum* e *T. gondii*, respectivamente, em comparação com outras seqüências depositadas no GenBank. *N. caninum* e *T. gondii* não foram detectados em nenhuma das 27 palhetas de sêmen congeladas usadas para inseminação artificial. Este estudo demonstrou a presença de DNA de *N. caninum* e *T. gondii* em amostras de sêmen fresco de ovinos naturalmente infectados.

Palavras-chave: Carneiros. Ovelhas. Ovinos. Taquizoítos. Palheitas de sêmen congeladas. Sêmen fresco.

ABSTRACT ARTICLE I

The aim of this study was to detect DNA of *Neospora caninum* and *Toxoplasma gondii* in fresh semen and frozen extended semen straws of naturally infected rams used for artificial insemination. Semen samples of 38 rams from artificial insemination centers were evaluated. Of the 38 rams, eleven were naturally infected (seropositive for anti-*N. caninum* and/or anti-*T. gondii* IgG) and were selected for fresh semen and blood collection (collections on days 1, 50, 55 and 58), with a total of 20 fresh semen samples and serum. 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 nucleotide sequences obtained presented 99% and 100% identity with *N. caninum* and *T. gondii* DNA, respectively, compared with other sequences deposited at GenBank. *N. caninum* and *T. gondii* were not detected in any of the 27 frozen extended semen straws used for artificial insemination. This study demonstrated the presence of *N. caninum* and *T. gondii* DNA in fresh semen samples of naturally infected rams.

Keywords: Male sheep. Ewes. Ovine. Tachyzoites. Frozen extended semen straws. Fresh semen.

RESUMO DO ARTIGO II

O objetivo deste estudo foi investigar a ocorrência e distribuição de anticorpos contra *Sarcocystis neurona, Neospora caninum* e *Toxoplasma gondii* em cavalos, cães e gatos de Curitiba, Paraná. Amostras de soro de 100 cavalos, 100 cães e 100 gatos da rotina do Laboratório de Patologia Clínica Veterinária do Hospital Veterinário da Universidade do Paraná (UFPR) foram selecionadas. As 100 amostras de soro de cães eram de 2 grupos: 35 amostras de cães com sinal neurológico (convulsão) e 65 amostras de cães sem sinais neurológicos. Os animais eram adultos de diferentes raças, machos e fêmeas. As amostras foram analisadas pelo teste de reação de imunofluorescência indireta (RIFI) para os protozoários *S. neurona, N. caninum* e *T. gondii* nas seguintes diluições de corte: cavalos: 1:50, 1:50 e 1:16; cães: 1:50, 1:50 e 1:16; gatos: 1:50, 1:50 e 1:50, respectivamente. Os resultados obtidos foram 42% dos cavalos, 7% dos cães e 5% dos gatos soropositivos para *S. neurona*; 58% dos cavalos, 68% dos cães e 42% dos gatos soropositivos para *N. caninum*; e 36% dos cavalos, 20% dos cães e 21% dos gatos soropositivos para *T. gondii*. Entre os cães com sinail neurológico, 8,6%, 68,6% e 25,7% deles foram soropositivos para *S. neurona, N. caninum* e *T. gondii*, respectivamente. E entre os cães sem sinais neurológicos, 6,2% 67,7% e 16,9% deles foram soropositivos para *S. neurona, N. caninum* e *T. gondii*, respectivamente. Não foi encontrada diferença estatística entre os grupos de cães soropositivos para os três protozoários com sinal neurológico e sem sinais neurológicos. Coinfecção e altos títulos de anticorpos foram detectados. Os anticorpos contra *Sarcocystis neurona, Neospora caninum* e *Toxoplasma gondii* foram encontrados amplamente distribuídos entre cavalos, cães e gatos na região de Curitiba, estado do Paraná, Brasil.

Palavras-chave: Sarcocistose. Neosporose. Toxoplasmose. Sinais neurológicos.

ABSTRACT ARTICLE II

The aim of this study was to investigate the occurrence and distribution of antibodies against *Sarcocystis neurona*, *Neospora caninum* and *Toxoplasma gondii* in horses, dogs and cats from Curitiba, Paraná state. Serum samples of 100 horses, 100 dogs and 100 cats from the routine of the Veterinary Clinical Pathology Laboratory in the Veterinary Hospital of the University of Paraná (UFPR) were selected. The 100 dogs' samples were divided into 2 groups: 35 samples from dogs with neurological sign (convulsion) and 65 samples from dogs without neurological signs. The animals were adults of different breeds, males and females. The samples were analyzed by indirect fluorescence antibody test (IFAT) for protozoa *S. neurona, N. caninum* and *T. gondii* at the following cut-off dilutions: horses: 1:50, 1:50 and 1:16; dogs: 1:50, 1:50 and 1:16; cats: 1:50, 1:50 and 1:50, respectively. The results obtained were 42% of horses, 7% of dogs and 5% of cats seropositive for *S. neurona*; 58% of horses, 68% of dogs and 42% of cats seropositive to *N. caninum*; and 36% of horses, 20% of dogs and 21% of cats seropositive for *T. gondii*. Among the dogs with neurological sign, 8.6%, 68.6% and 25.7% of them were found seropositive for *S. neurona, N. caninum* and *T. gondii*, respectively. And among the dogs without neurological signs 6.2% 67.7% and 16.9% of them were found seropositive for *S. neurona, N. caninum* and *T. gondii*, respectively. No statistical difference was found between groups of seropositive dogs for the three protozoa with neurological sign and without neurological signs. Co-infection and high antibody titers were detected. The antibodies against *Sarcocystis neurona, Neospora caninum* and *Toxoplasma gondii* were found widely distributed among horses, dogs and cats in the region of Curitiba, state of Paraná, Brazil.

Keywords: Sarcocystosis. Neosporosis. Toxoplasmosis. Neurological signs.

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ARTICLE I

Detection of *Neospora caninum* and *Toxoplasma gondii* in semen of naturally infected rams

1 INTRODUCTION

Natural infection of sheep by *Neospora caninum* was first identified in 1990 in a congenitally infected lamb with signs of ataxia and weakness after birth [1], and *Toxoplasma gondii* infection was described for the first time in 1942, in an ewe presenting nervous symptomology, increased temperature and muscular rigidity [2].

Neospora caninum and *Toxoplasma gondii* are closely related cyst-forming apicomplexan parasites identified as important causes of reproductive failure in cattle [3,4]. Moreover, abortion cases attributed to *N. caninum* [1,5,6] and *T. gondii* [7-10] infection have been occasionally reported in sheep. These parasites have already been identified in 77% of 74 brain samples from aborted sheep fetuses [11].

Sexual transmission (horizontal transmission) by contaminated semen has been studied, and the presence of *N. caninum* DNA in the semen of naturally infected bulls [12-18] and experimentally infected rams was reported [19].

Experimental studies showed the presence of *T. gondii* in the semen and/or reproductive tract of some species, such as dogs [20], goats [21,22], swine [23], cattle [24], and rams [25-28]. Studies performed with naturally infected animals have shown the detection of *T. gondii* only in the semen of rams [29,30] and dogs [31], suggesting a new route of elimination of the parasite that can cause reproductive damage and be sexually transmitted [32].

Studies on the elimination of *N. caninum* and its potential transmission via semen in rams are scarce. The recent experimental study by Syed-Hussain *et al*. [19] showed the intermittent detection of *N. caninum* DNA in the semen of rams up to five weeks post-inoculation, with the concentrations ranging from that equivalent to 1-889 tachyzoites per mL of semen, but none of the ewes mated with the experimentally infected rams seroconverted. This experimental study demonstrates the need to investigate the presence of the parasite in semen samples from naturally infected rams that are used for reproduction, including frozen extended semen straws used for artificial insemination.

Studies have only found *N. caninum* DNA in frozen extended semen straws samples from cattle [12,13,16], demonstrating the need to carry out the research for the detection of *N. caninum* in frozen extended semen straws from rams used for reproduction.

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*.

Due to the relatively scarce information on the molecular detection of *N. caninum* in the semen of naturally infected rams, the purpose of this study was to detect parasitic DNA in fresh semen samples and in frozen extended semen straws from male sheep from artificial inseminations centers in Southern Brazil.

2 MATERIALS AND METHODS

This Project was approved by the Animal Ethics Committee of the Department of Agricultural Sciences (CEUA SCA) under the protocol number 065/2016.

2.1 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.

Of the 38 rams, 11 were previously seropositive for *N. caninum* and/or *T. gondii* IgG antibodies and were selected for posterior collections of fresh semen and blood samples. A total of twenty semen samples and serum were collected from these 11 rams seropositive for *N. caninum* and/or *T. gondii* IgG antibodies (identified with numbers 1-11).

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 (Table 1). 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.

Table 1 – Twenty fresh semen samples obtained from 11 seropositive rams (identified with numbers 1-11) were collected on days 1, 50, 55 and 58:

11 Rams	Day 1	Day 50	Day 55	Day 58
1	fresh semen	fresh semen	fresh semen	nd
2	fresh semen	fresh semen	fresh semen	nd
3	fresh semen	fresh semen	fresh semen	nd
4	fresh semen	nd	nd	fresh semen
5	fresh semen	nd	nd	fresh semen
6	fresh semen	nd	nd	fresh semen
7	fresh semen	nd	nd	nd
8	fresh semen	nd	nd	nd
9	fresh semen	nd	nd	nd
10	fresh semen	nd	nd	nd
11	fresh semen	nd	nd	nd

‘‘nd’’ Denotes no sample was collected.

Samples of fresh semen were collected using an artificial vagina and according to the availability of ewes in estrus. Fifteen minutes 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 hours.

Sera from the 11 rams were obtained by centrifuging the blood at 3000 × *g* for 10 minutes 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 minutes, and the sediments of approximately 0.5 mL were frozen at -20°C before DNA extraction.

The 27 semen straws frozen (Table 2) 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 hours during the transport to the laboratory. Then, 0.25 mL of the sample transferred to micro-tubes and frozen at -20°C until processing.

Table 2 – Twenty-seven frozen extended semen straws samples from 27 other breeding rams with not known serological data (identified with numbers 12-38) were obtained on day 1:

27 Rams	Day 1	Day 50	Day 55	Day 58
12	semen straw	nd	nd	nd
13	semen straw	nd	nd	nd
14	semen straw	nd	nd	nd
15	semen straw	nd	nd	nd
16	semen straw	nd	nd	nd
17	semen straw	nd	nd	nd
18	semen straw	nd	nd	nd
19	semen straw	nd	nd	nd
20	semen straw	nd	nd	nd
21	semen straw	nd	nd	nd
22	semen straw	nd	nd	nd
23	semen straw	nd	nd	nd
24	semen straw	nd	nd	nd
25	semen straw	nd	nd	nd
26	semen straw	nd	nd	nd
27	semen straw	nd	nd	nd
28	semen straw	nd	nd	nd
29	semen straw	nd	nd	nd
30	semen straw	nd	nd	nd
31	semen straw	nd	nd	nd
32	semen straw	nd	nd	nd
33	semen straw	nd	nd	nd
34	semen straw	nd	nd	nd
35	semen straw	nd	nd	nd
36	semen straw	nd	nd	nd
37	semen straw	nd	nd	nd
38	semen straw	nd	nd	nd

‘‘nd’’ Denotes no sample was obtained.

2.2 INDIRECT FLUORESCENT ANTIBODY TEST (IFAT)

The indirect fluorescent antibody test was used to detect IgG antibodies against *N. caninum* and *T. gondii* in the 20 serum samples from eleven rams [33]. 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*. [34]. Serum samples with titers 1:25 for *N. caninum* and 1:40 for *T. gondii* were considered positive as described by Guimarães *et al.* [35]. Sera were analyzed until reaching the final titer. All IFAs 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 (F4891, Sigma-Aldrich Brazil) was used at a dilution of 1:100.

2.3 COLLECTION OF SEMEN

Fresh semen samples were collected from 11 seropositive rams on days 1, 50, 55, and 58 using an artificial vagina (Figure 1) 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 [36].

Figure 1 – Artificial vagina used to collect fresh semen samples:

2.4 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 (Invitrogen, Carlsbad, CA, USA).

2.5 HEMI-NESTED PCR TO DETECT *N. 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* [37], following the protocol previously published with some modifications [38]. 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® Safe DNA gel stain (Kasvi, K9-16C) and visualized under UV light.

2.6 NESTED PCR TO DETECT *T. 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 [39], following the protocol previously published with some modifications [40]. *T. gondii* DNA amplification was carried out in a 25-µL reaction volume containing 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. 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.

2.7 SEQUENCING ANALYSIS

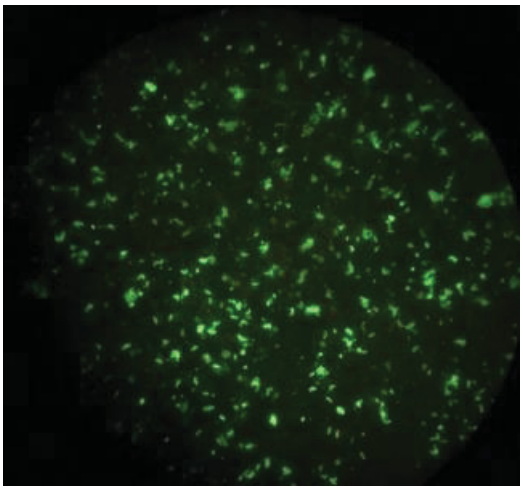
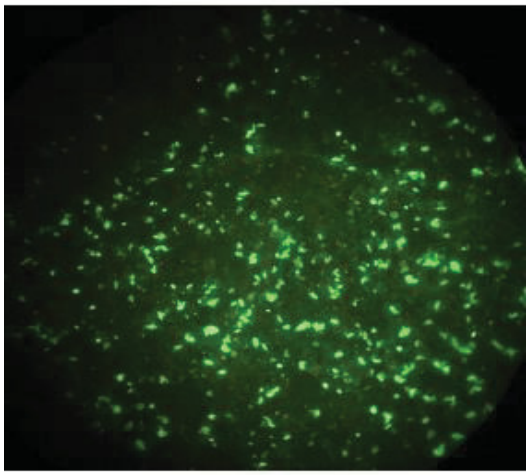
PCR products were purified using PureLink® Quick Gel Extraction Kit (Invitrogen) according to manufacturer's instructions, and sequence analysis was performed using primer Np7, 5'GGGTGAACCGAGGGAGTTG3', to identify the DNA sequence of *N. caninum* [38] and primer 5'TGCATAGGTTGCCAGTCACTG'3, which corresponds to the gene B1 nucleotides 853-831, to identify the DNA sequence of *T. gondii* [40]. 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).

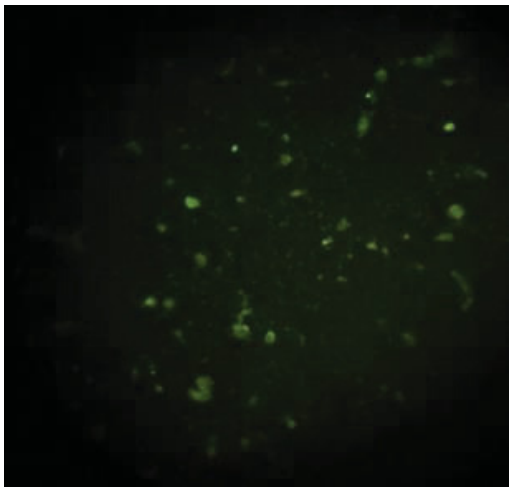
Sequences were, then, subjected to BlastN analysis using the GenBank database at NCBI to confirm that the PCR-amplified sequences were those of *N. caninum* and *T. gondii*.

3 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 (Figure 1).

Figure 1 – Positive and negative control with the indirect fluorescence antibody test (IFAT) in ram's serum samples:

a)  b) 

c) 

a) Tachyzoites of *N. caninum* with fluorescence = positive control (40X);

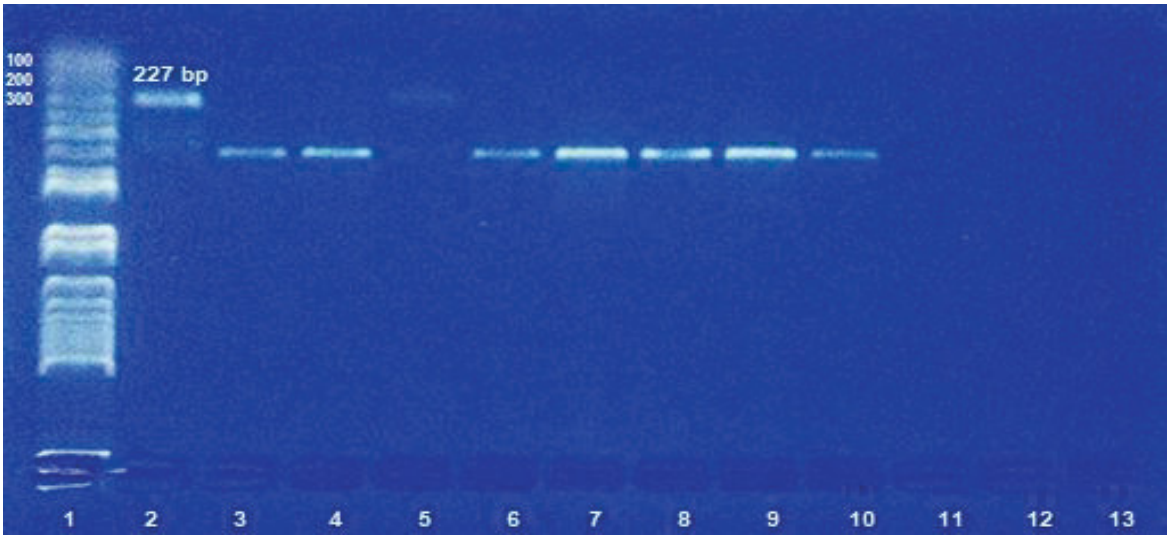
b) Tachyzoites of *T. gondii* with fluorescence = positive control (40 X);

c) Tachyzoites of *T. gondii* without fluorescence = negative control (40 X);

Among the 20 fresh semen samples from the 11 rams, *N. caninum* DNA was detected in the semen of ram 1 (Figure 2), collected on day 55 (Table 2). *T. gondii* DNA was detected in the fresh semen sample of ram 7 (Figure 3), on day 1 (Table 2).

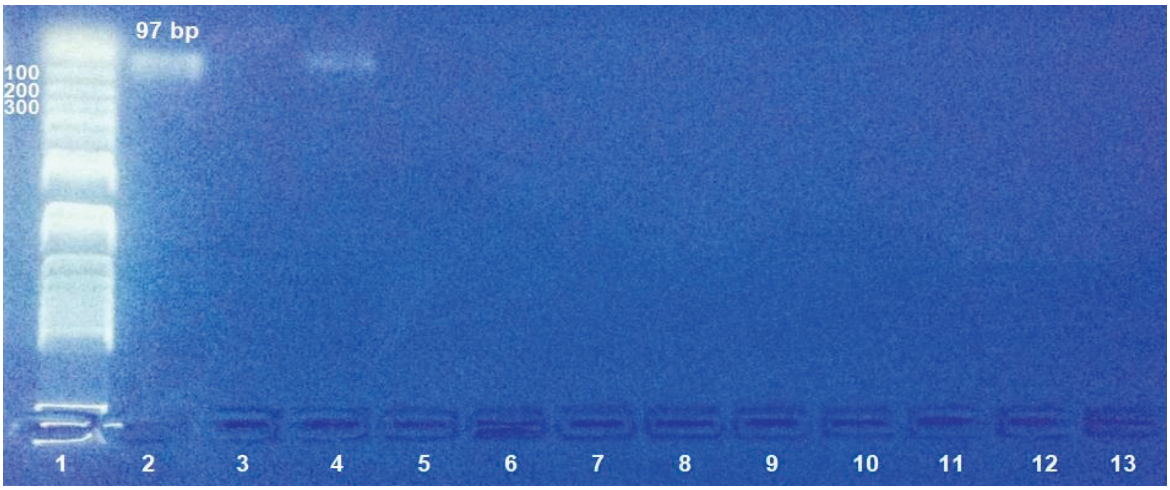
N. caninum and/or *T. gondii* DNA were not detected in any of the 27 frozen extended semen straws.

Figure 2 – Hemi-Nested PCR results showing the amplicon of 227 bp from *N. caninum* DNA:



1 - Molecular weight marker 1 kb plus DNA; 2- Positive control; 5 – Positive semen sample (ram 1); 3, 4, 6, 7, 8, 9, 10, 11, 12 - Negative semen samples; 13 - Negative control.

Figure 3 – Nested PCR results showing the amplicon of 97-bp from *T. gondii* DNA:



1 - Molecular weight marker 1 kb plus DNA; 2 - Positive control; 4 – Positive semen sample (ram 7); 3, 5, 6, 7, 8, 9, 10, 11, 12 - Negative semen samples; 13 - Negative control.

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

	Day 1		Day 50		Day 55		Day 58	
Rams	***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

‘‘-’’ Denotes negative; ‘‘nd’’ denotes no sample available.

Table 2 - Detection of *N. caninum* and *T. gondii* DNA in fresh semen samples from 11 rams using Hemi-Nested PCR and Nested PCR, respectively:

	Day 1	Day 50	Day 55	Day 58
Rams	**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

‘‘-’’ Denotes negative; ‘‘nd’’ denotes no sample available.

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.

4 DISCUSSION

In our study, *N. caninum* DNA was detected in the semen sample from the third collection from ram 1. Syed-Hussain *et al*. [19] detected *N. caninum* DNA in the semen samples of experimentally infected seropositive rams and 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*. [11], 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 [41] and 1:800 [42] 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 [29,30].

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*. [30], 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*. [12] 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*. [13] 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*. [11], 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* [43], transplacental transmission [44,45], and histopathological lesions [46]; however, no study has evaluated the horizontal transmission capacity (via semen) of different isolates. The experimental study of Syed-Hussain *et al*. [19] 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 GenBank 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.

In conclusion, this study demonstrated that naturally infected, *N. caninum* and *T. gondii* seropositive rams can eliminate the protozoa through semen.

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ARTICLE II

Detection of antibodies against *Sarcocystis neurona*, *Neospora caninum* and *Toxoplasma gondii* in horses, dogs and cats from Paraná state, Brazil

1 INTRODUCTION

Sarcocystis neurona, *Neospora caninum* and *Toxoplasma gondii* are protozoa of the *Sarcocystidae* family, that can cause systemic disease in many species of domestic and wild animals (DUBEY; LINDSAY, 1996; DUBEY et al., 2001; DUBEY, 2008; DUBEY et al., 2015).

S. neurona is often associated as the cause of equine protozoal mieloencephalitis (MEP) in the Americas (DUBEY et al., 2001). MPS-like disease occurs in other animals, including martens, raccoons, skunks, pacific seals, ponies, southern sea otters (DUBEY; HAMIR, 2000), dogs (DUBEY et al., 2006; COOLEY et al., 2007; DUBEY et al., 2014) and cats (DUBEY; HAMIR, 2000; DUBEY et al., 2003).

N. caninum is the etiological agent of neosporosis, an infectious disease considered to be the leading cause of reproductive loss in cattle and neuromuscular diseases in dogs around the world (DONAHOE et al., 2015). *N. caninum* has already been detected in horses, but *N. hughesi* has been identified as the leading cause of neurological disease in most horses (LINDSAY, 2001; DUBEY, 2003; LOCATELLI-DITTRICH et al., 2006a). In cats there are still no reports of naturally infected animals by *N. caninum*, however, the presence of antibody against *N. caninum* has been reported in domestic and wild cats (DUBEY et al., 2009; ONUMA et al., 2014).

T. gondii is widely prevalent in humans and animals in Brazil (DUBEY et al., 2012) and all over the world (DUBEY, 2008). *T. gondii* DNA has been detected in the retina, choroid and sclera of a 17-year-old pony (TURNER; SAVVA, 1991), in an equine placenta (TURNER; SAVVA, 1990) and in a foal (TURNER; SAVVA, 1992), showing that horses may be susceptible to *T. gondii* and that vertical transmission may occur in pregnant mares. However, horses are considered one of the less sensitive species to the pathogenic effect of *T. gondii* (TASSI, 2007). Primary toxoplasmosis is rare in dogs (DUBEY, 2010) and it is mainly observed in immunosuppressed dogs, often with canine morbillivirus infection (canine distemper virus) (DUBEY; BEATTIE, 1988). Cats infected with *T. gondii* have not shown any symptoms, but signs of the disease have been observed in immunosuppressed cats

especially infected with feline immunodeficiency virus or feline leukemia virus (PLATT et al., 2014).

The prevalence of antibodies against *S. neurona* in horses from different regions of Brazil ranges from 20.8% to 90% (HOANE et al., 2006; ANTONELLO et al., 2015; RIBEIRO et al., 2016; BORGES et al., 2017; OLIVEIRA et al., 2017; SPOHR et al., 2018), and there is a lower prevalence of antibodies against *Neospora spp.* and *T. gondii*, ranging from 2.5% to 47% for *Neospora spp.* (LOCATELLI-DITTRICH et al., 2006; HOANE et al., 2006; ABREU et al., 2014; LASKOSKI et al., 2015; RIBEIRO et al., 2016; BORGES et al., 2017; OLIVEIRA et al., 2017; SPOHR et al., 2018) and 0.9% to 25% for *T. gondii* (LOCATELLI-DITTRICH et al., 2006; LASKOSKI et al., 2015; RIBEIRO et al., 2016; BORGES et al., 2017; OLIVEIRA et al., 2017; SPOHR et al., 2018).

In dogs from Brazil, there is still no report of the presence of antibodies against *S. neurona*. A study carried out with research of anti-*S. neurona* antibody in 47 dogs by indirect fluorescence antibody test (IFAT), from the Paulicéia region in São Paulo, Brazil, did not detect the antibody in any of the dogs (OLIVEIRA et al., 2017). The prevalence of antibodies against *N. caninum* in dogs in the region of Curitiba have been demonstrated in some studies, and ranged from 10.52% to 25% (LOCATELLI-DITTRICH et al., 2008; FRIDLUND-PLUGGE et al., 2008; ABREU et al., 2014). In other studies, the prevalence of *T. gondii* antibodies in dogs from Curitiba was 30.7% (CONSTANTINO et al., 2016), and dogs with neurological signs showed 21.8% seropositivity (PLUGGE et al., 2011).

Cats may show elevated antibody titers when submitted to experimental infection by *S. neurona* (DUBEY et al., 2002b). In Brazil, antibodies against *S. neurona* were detected in 4% of serum samples from cats in the region of Bahia (MENESES et al., 2014), however, in a study with cats from São Paulo, antibodies against *S. neurona* were not detected in this species (DUBEY et al., 2002a). The prevalence of antibodies against *N. caninum* in cats from São Paulo in Brazil is 11.9% (DUBEY et al., 2002) and 24.5% (BRESCIANI et al., 2007). In Curitiba, Paraná, the seroprevalence of anti-*T. gondii* antibodies in cats is 16.3% (CRUZ et al., 2011).

There is no serological data about the frequency of antibodies against *Sarcocystis neurona* in horses, dogs and cats from Paraná state in southern Brazil, and serological data of antibodies against *Neospora caninum* in cats in the same region is unknown. Therefore, our study aimed to verify the frequency of antibodies and the presence of co-infection with *Sarcocystis neurona, Neospora caninum* and *Toxoplasma gondii* in horses, dogs and cats from the region of Curitiba in the state of Paraná, in southern Brazil.

2 MATERIAL AND METHODS

2.1 ANIMALS AND SAMPLES

Serum samples of 100 horses, 100 dogs and 100 cats from the routine of the Veterinary Clinical Pathology Laboratory of the Veterinary Hospital in the Federal University of Paraná were selected. These animals were of different breeds and ages (adults), males and females, from the urban and rural region of Curitiba (-25.429722 S, -49.271944 O and altitude of 934 m) in the state of Paraná, Brazil.

Of the 100 serum samples from dogs, 35 were from dogs with neurological sign (convulsion) and 65 from dogs without neurological signs but with other clinical signs. The serum samples from 100 horses and 100 cats were obtained from animals that did not showed neurological signs and were attended due to other clinical signs.

Blood samples (3-5 ml) were collected by jugular vein puncture in siliconized vacutainer tubes without anticoagulant and centrifuged at 3700 x g (relative centrifugal force) for 10 minutes. The serum samples obtained were transferred to microtubes and frozen at -20°C until analysis.

Blood samples were collected between September 2016 and March 2017, with the approval of the Ethics Committee on Animal Use (CEUA) of the Sector of Agricultural Sciences of UFPR under number 065/2016.

2.2 CULTIVATION OF PARASITES TO PRODUCE ANTIGEN

Merozoites of *Sarcocystis neurona* (SN37R) (SOFALY et al., 2002), *Neospora caninum* tachyzoites (NC-1) (DUBEY et al., 1988) and *Toxoplasma gondii* tachyzoites (RH) (NICOLLE; MANCEAUX, 1908) were cultured to perform indirect fluorescence antibody test.

All protozoa were cultured *in vitro* in Roux flasks containing Vero cell monolayers (African green monkey kidney cells) in Eagle's medium supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin and incubated at 37°C with 5% of CO_2 as described by Koch et al. (2016) when isolating and cultivating *T. gondii in vitro*.

Samples were collected twice a week. The collected medium was centrifuged at 1200 x g for 10 min, washed with sterile saline phosphate buffer (pH 7.2), passed through a 26G needle and filtered with a 5 µm filter-syringe.

Approximately 0.8 to 1 x 10^4 merozoites of *S. neurona* and tachyzoites of *N. caninum* and *T. gondii* diluted in 30 µl of phosphate-buffered saline (PBS) were added to the slides for

indirect fluorescence antibody test, and then dried outdoors for 6 to 12 hours. The slides containing the antigens were frozen at -20°C until analysis.

2.3 INDIRECT FLUORESCENCE ANTIBODY TEST (IFAT)

All the 300 serum samples were analyzed using indirect fluorescence antibody test to investigate the presence of circulating IgG antibodies specific for *S. neurona, N. caninum* and *T. gondii* antigens, at the respective dilutions and species-specific anti-IgGs conjugates, as below:

- horses: 1:50, 1:50 and 1:16; rabbit anti-horse IgG whole molecule FITC (F7759, Sigma-Aldrich®) at a 1:100 dilution;
- dogs: 1:50, 1:50 and 1:16; rabbit polyclonal anti-Dog IgG whole molecule FITC (F4012, Sigma-Aldrich®) at a 1:100 dilution;
- Cats: 1:50, 1:50 and 1:50; goat anti-cat IgG FITC (ab112800, ABCAM®) at a 1:200 dilution;

Only the samples that showed fluorescence of the whole surface of the parasite were considered positive. The positive samples were diluted until reaching the maximum titration. Previously positive and negative sera for *S. neurona, N. caninum* and *T. gondii* were included in each reaction as control, according to the species analyzed.

2.4 STATISTICAL ANALYSIS

The means of the group of 100 dogs (35 dogs with neurological sign and 65 dogs without neurological signs) were compared by Fisher's exact test for the three protozoa with a 95% confidence interval. $P<0.05$ was considered significant.

3 RESULTS AND DISCUSSION

Among the 100 horse serum samples, 42%, 58% and 36% were positive for anti-*S. neurona*, anti-*N. caninum* and anti-*T. gondii* antibody (IgG), respectively (Table 1). A high prevalence of IgG antibodies against *S. neurona* in horses (42%) was detected in our study, but within values already found in other regions of Brazil, ranging from 20.8% to 90% by IFAT (HOANE et al., 2006; ANTONELLO et al., 2015; RIBEIRO et al., 2016; BORGES et al., 2017 ; OLIVEIRA et al., 2017; SPOHR et al., 2018).

Table 1. Frequency of IgG antibodies against *Sarcocystis neurona, Neospora caninum* and *Toxoplasma gondii* in the sera of horses, dogs and cats, tested by indirect fluorescence antibody test (IFAT):

	Seropositive animals (%)		
Species	*Sarcocystis neurona*	*Neospora caninum*	*Toxoplasma gondii*
Horses (n=100)	42	58*	36
Dogs (n=100)	7	68	20
Cats (n=100)	5	42	21

* Denotes *Neospora spp*.

The prevalence of IgG against *N. caninum* (58%) detected in horses was higher than the percentage detected by Locatelli-Dittrich et al. (2006) in the same region, which was 47% in mares of rural properties in the state of Paraná. However, serological tests such as ELISA, indirect fluorescence antibody test (IFAT) and direct agglutination test (DAT) cannot differentiate *N. caninum* from *N. hughesi* (WALSH et al., 2000), so *Neospora spp*. was detected in the serum samples of the horses in this study. Little is known about the pathogenicity or prevalence of *neospora* antibodies in horses, and currently there are no serological tests to differentiate *N. hughesi* and *N. caninum* (LINDSAY, 2001).

The seroprevalence of *T. gondii* in the horses of this study was also higher, 36% of horses were detected seropositive, unlike other studies that detected 2.7% of seropositive mares in the same region (LOCATELLI-DITTRICH et al., 2006), and 19.9% of seropositive horses in southern state of Minas Gerais (RIBEIRO et al., 2016).

Of the 100 serum samples from dogs, 7%, 68% and 20% were positive for anti-*S.neurona*, anti-*N. caninum* and anti-*T. gondii* antibodies, respectively (Table 1). Our results demonstrated 7% of *S. neurona*-seropositive dogs with titers that reached 1:500, contrary to the work of Oliveira et al. (2017), who did not detect anti-*S. neurona* antibodies in any of the 47 dogs in the Paulicéia region, in São Paulo, Brazil. We also found a greater number of seropositive dogs for *N. caninum* (68%) than other surveys conducted in the same region, which obtained results between 10.52% and 25% of dogs positive for anti-*N. caninum* antibody (LOCATELLI-DITTRICH et al., 2008; FRIDLUND-PLUGGE et al., 2008; ABREU et al., 2014). The prevalence of seropositive dogs for *T. gondii* (20%) in this study was close to the results obtained in other studies, which were 21.8% (PLUGGE et al., 2011) and 30.7% (CONSTANTINO et al., 2016).

Of the 100 serum samples from cats, 5%, 42% and 21% were positive for anti-*S. neurona,* anti *N. caninum* and anti *T. gondii* IgG antibody, respectively (Table 1). We obtained a result of 5% of seropositive cats for *S. neurona* with a maximum titre of 1:100, close to the result

obtained in seropositive cats in the state of Bahia, which was 4% (MENESES et al., 2014) but with a higher titre of 1:800. A study carried out in the State of São Paulo did not detect any sample of seropositive cat for *S. neurona* (DUBEY et al., 2002a). The prevalence of seropositive cats for *N. caninum* was 42% in the region of Curitiba, above values found in regions of the state of São Paulo, which were 11.9% (DUBEY et al., 2002) and 24,5%, (BRESCIANI et al., 2007). In our study, 21% of cats were found positive to *T. gondii*, close to the value found by Cruz et al. (2011) in the same region.

The maximum titers of IgG antibodies against *S. neurona, N. caninum* and *T. gondii* detected in horse sera were 1:600 for all parasites (Table 2). In dogs, the maximum titers of IgG antibodies against *S. neurona, N. caninum* and *T. gondii* detected in serum were 1:500, 1:1200 and 1:100, respectively. In cats, the maximum titers of IgG antibodies against *S. neurona, N. caninum* and *T. gondii* detected in serum were 1:100, 1:600 and 1:1200, respectively (Table 2).

Table 2. Maximum titre of IgG antibodies against *Sarcocystis neurona, Neospora caninum* and *Toxoplasma gondii* detected in sera from horses, dogs and cats, tested by indirect fluorescence antibody test (IFAT):

	Maximum titre		
Species	*Sarcocystis neurona*	*Neospora caninum*	*Toxoplasma gondii*
Horses(n=100)	1:600 (n=3)	1:600* (n=6)	1:600 (n=3)
Dogs(n=100)	1:500 (n=2)	1:1200 (n=2)	1:100 (n=3)
Cats (n=100)	1:100 (n=2)	1:600 (n=2)	1:1200 (n=4)

* Denotes *Neospora spp*.

The frequency of co-infection by *S. neurona, N. caninum* and *T. gondii* in horses, dogs and cats is shown in Table 3.

Table 3. Frequency of co-infection detected by IgG antibodies against *Sarcocystis neurona, Neospora caninum* and *Toxoplasma gondii* in the sera of horses, dogs and cats, tested by indirect fluorescence antibody test (IFAT):

	Seropositive animals (%)			
Species	*S. neurona* and *N. caninum*	*N. caninum* and *T. gondii*	*S. neurona* and *T. gondii*	*S. neurona, T. gondii* and *N. caninum*
Horses (n=100)	15*	13*	5	11*
Dogs (n=100)	3	14	1	3
Cats (n=100)	1	8	1	0

* Denotes *Neospora spp*.

The serum IgG antibody frequency of the 35 dogs with neurological sign was 8.6%, 68.6% and 25.7% for *S. neurona, N. caninum* and *T. gondii*, respectively (Table 4). The frequency of IgG antibodies in the sera of the 65 dogs without neurological signs was 6.2%, 67.7% and 16.9% for *S. neurona, N. caninum* and *T. gondii*, respectively (Table 4).

Table 4. Frequency of IgG antibodies against *Sarcocystis neurona, Neospora caninum* and *Toxoplasma gondii* detected in the sera of 100 dogs (35 with neurological signs and 65 without neurological signs), tested by indirect fluorescence antibody test (IFAT):

Dogs	Seropositive dogs (%)		
	Sarcocystis neurona	*Neospora caninum*	*Toxoplasma gondii*
With neurological signs (n=35)	8,6	68,6	25,7
Without neurological signs (n=65)	6,2	67,7	16,9
P-value*	0.69	1.00	0.45

* Denotes p-value by Fisher's exact test with a 95% confidence interval where P<0.05 was considered significant

In the current study, the statistical difference for circulating IgG antibodies in the groups of 35 dogs with neurological sign and 65 dogs without neurological signs for specific *Sarcocystis neurona* (P=0.69)*, Neospora caninum* (P=1.00) and *Toxoplasma gondii* (P=0.45) antigens were not significant.

In the group of dogs with neurological sign, a frequency of 68.6% of antibodies against *N. caninum* and 25.7% against *T. gondii* was obtained, above the values obtained by Plugge et al. (2011), which were 11.56% and 21.08%, respectively, among dogs with neurological signs in the same region.

Antibodies against *S. neurona* were detected in dogs with and without neurological signs, in the percentage of 8.6% and 6.2%, respectively, indicating that dogs with neurological signs already had contact with the protozoan.

The maximum titre of IgG antibodies against *S. neurona, N. caninum* and *T. gondii* detected in sera from 35 dogs with neurological sign were 1:50, 1:1200 and 1:100, respectively (Table 5). The maximum titre of IgG antibodies against *S. neurona, N. caninum* and *T. gondii* detected in the sera of the 65 dogs without neurological signs were 1:500, 1:600 and 1:50, respectively (Table 5).

Table 5. Maximum titre of IgG antibodies against *Sarcocystis neurona, Neospora caninum* and *Toxoplasma gondii* detected in the sera of 100 dogs (35 with neurological signs and 65 without neurological signs), tested by indirect fluorescence antibody test (IFAT):

Dogs	Maximum titre		
	Sarcocystis neurona	*Neospora caninum*	*Toxoplasma gondii*
With neurological signs (n=35)	1:50 (n=1)	1:1200(n=2)	1:100(n=3)
Without neurological signs (n=65)	1:500(n=1)	1:600(n=4)	1:50(n=2)

4 CONCLUSION

This study was the first to report the presence of IgG antibodies against *S. neurona* and *N. caninum* in cats from Curitiba and detected IgG antibodies against *S. neurona* with high titers in dogs in Brazil.

No statistical difference was found between groups of seropositive dogs for *S. neurona, N. caninum* and *T. gondii* antigens with neurological sign and without neurological signs, but the titers were higher in seropositive dogs for *N. caninum* and *T. gondii* with neurological signs.

Our results indicate that horses, dogs and cats from Curitiba, in the state of Paraná, Brazil, are exposed to infection by *S. neurona, N. caninum* and *T. gondii*.

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SUPPLEMENT

UNIVERSIDADE FEDERAL DO PARANÁ
SETOR DE CIÊNCIAS AGRÁRIAS
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o protocolo número065/2016, referente ao projeto **"Soroprevalência para *Sarcocystis neurona, Toxoplasma gondii* e *Neospora caninum* em cães, gatos e equinos com sinais neurológicos e/ou miopatia e diagnóstico sorológico e molecular de *S. neurona, T. gondii* e *N. caninum* em ovinos e caprinos"**, sob a responsabilidade de **Rosangela Locatelli Dittrich**– que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de Outubro, de 2008, do Decreto nº 6.899, de 15 de julho de 2009,e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), efoi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DO SETOR DE CIÊNCIAS AGRÁRIAS DA UNIVERSIDADE FEDERAL DO PARANÁ - BRASIL, com grau 1de invasividade, em reunião de 10/08/2016.

Vigência do projeto	Setembro/2016 até Fevereiro/2018
Espécie/Linhagem	Cão; caprino; equídeo; gato; ovino
Número de animais	550 (100; 100; 100; 100; 150 respectivamente)
Peso/Idade	3 a 40 kg; 30 a 150 kg; 380 a 1.000 kg; 3 a 15 kg; 30 a 150 kg / Adulto
Sexo	Ambos
Origem	Rotina do Laboratório de Patologia Clínica Veterinária da Universidade Federal do Paraná e fazendas dos estados do Paraná e de Santa Catarina

CERTIFICATE

We certify that the protocol number 065/2016, regarding the project **"Seroprevalence of *Sarcocystis neurona, Toxoplasma gondii* and *Neospora caninum* in dogs, cats and horses with neurological signs and/or myopathy and serological and molecular diagnosis of *S. neurona, T. gondii* and *N. caninum* in sheep and goats"** under **Rosangela Locatelli Dittrich** supervision – which includes the production, maintenance and/or utilization of animals from Chordata phylum, Vertebrata subphylum (except Humans), for scientific or teaching purposes – is in accordance with the precepts of Law nº 11.794, of8 October, 2008, of Decree nº 6.899, of 15 July, 2009, and with the edited rules from Conselho Nacional de Controle da Experimentação Animal (CONCEA), and it was approved by theANIMAL USE ETHICS COMMITTEE OF THE AGRICULTURAL SCIENCES CAMPUS OF THE UNIVERSIDADE FEDERAL DO PARANÁ (Federal University of the State of Paraná, Brazil), with degree 1 of invasiveness, in session of10/08/2016.

Duration of the project	September/2016until February/2018
Specie/Line	Dog; caprine; equine; cat; ovine
Number of animals	550 (100; 100; 100; 100; 150 respectively)
Wheight/Age	3 to 40 kg; 30 to 150 kg; 380 to 1.000 kg; 3 to 15 kg; 30 to 150 kg / Adult
Sex	Both
Origin	Laboratory of Veterinary Clinic Pathology at the Federal University of Parana and farms in the states of Parana and Santa Catarina

Curitiba, 10 de agosto de 2016.

Simone Tostes de Oliveira Stedile

Presidente CEUA-SCA

Comissão de Ética no Uso de Animais do Setor de Ciências Agrárias - UFPR