FULL ARTICLE

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

Detecção de anticorpos contra Sarcocystis neurona, Neospora caninum e Toxoplasma gondii em cavalos, cães e gatos do estado do Paraná, Brasil

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ABSTRACT

The occurrence and distribution of antibodies against *Sarcocystis neurona*, *Neospora caninum* and *Toxoplasma gondii* was investigated in horses, dogs and cats from Curitiba, Paraná state, Brazil. Serum samples were selected from 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). The 100 dog samples were divided into two 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. Samples were analyzed by indirect fluorescent 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 obtained results 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 signs, 8.6%, 68.6% and 25.7% were seropositive for *S. neurona, N. caninum* and *T. gondii*, respectively. Among the dogs without neurological signs, 6.2% 67.7% and 16.9% were 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 signs 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.

RESUMO

O presente trabalho investigou a ocorrência e distribuição de anticorpos contra *Sarcocystis neurona, Neospora caninum* e *Toxoplasma gondii* em cavalos, cães e gatos de Curitiba, estado do Paraná, Brasil. Amostras de soro de 100 cavalos, 100 cães e 100 gatos da rotina do Laboratório de Patologia Clínica Veterinária do Hospital Veterinário da Universidade Federal do Paraná (UFPR) foram selecionadas. As 100 amostras de cães foram divididas em dois grupos: 35 amostras de animais com sinal neurológico (convulsão) e 65 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 sinal neurológico, 8.6%, 68.6% e 25.7% deles foram soropositivos para *S. neurona, N. caninum* e *T. gondii*, respectivamente. Entre os cães sem sinais neurológicos, 6.2% 67.7% e 16.9% 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.

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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).

S. neurona is often identified 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 natural infection 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 worldwide (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 those infected with feline immunodeficiency virus or feline leukemia virus (Platt & Olby, 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., 2006b; 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., 2006b; 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 fluorescent 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 performed

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., 2002a) 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.

Material and Methods

Animals and samples

Serum samples from 100 horses, 100 dogs and 100 cats were collected during the routine of the Veterinary Clinical Pathology Laboratory of the Veterinary Hospital in the Federal University of Paraná. 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 show any 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 min. 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.

Cultivation of parasites for antigen production

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

Indirect Fluorescent Antibody Test (IFAT)

All 300 serum samples were examined by indirect fluorescent 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;

Samples that showed fluorescence of the whole surface of the parasite were considered positive. The positive samples were diluted until reaching their titer. 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.

Statistical analysis

The means of the group of 100 dogs (35 dogs with neurological signs 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.

Results and Discussion

Among the 100 horse examined, 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

Table 1 - Frequency of IgG antibodies (IFAT*) against *Sarcocystis neurona, Neospora caninum* and *Toxoplasma gondii* in the sera of horses, dogs and cats, attended in a university veterinary hospital, from Curitiba, Paraná state, Brazil. Blood collections performed from September 2016 to March 2017

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

*IFAT = indirect fluorescent antibody test; **Denotes *Neospora spp*.

(42%) was detected in the present 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).

The prevalence of IgG against *N. caninum* (58%) detected in horses was higher than the percentage detected by Locatelli-Dittrich et al. (2006b) in the same region, which was 47% in mares of rural properties in the state of Paraná. However, serological tests such as ELISA, indirect fluorescent 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 present investigation in the horse serum samples tested. Little is known about the pathogenicity or prevalence of *neospora* antibodies in horses, and there are currently no serological tests to differentiate *N. hughesi* and *N. caninum* (Lindsay, 2001).

The seroprevalence of *T. gondii* in the horses examined in the present investigation was very high, with 36% of horses detected seropositive, unlike other studies that detected 2.7% of seropositive mares in the same region (Locatelli-Dittrich et al., 2006b), and 19.9% of seropositive horses in southern state of Minas Gerais (Ribeiro et al., 2016).

Of the 100 dogs examined, 7%, 68% and 20% were positive for anti-*S.neurona*, anti-*N. caninum* and anti-*T. gondii* antibodies, respectively (Table 1). The obtained results demonstrated 7% of *S. neurona*-seropositive dogs with titers that reached 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. A greater number of seropositive dogs for *N. caninum* (68%) was also found 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 the present investigation was close to the results obtained in other studies, such as 21.8% (Plugge et al., 2011) and 30.7% (Constantino et al., 2016).

Of the 100 cats examined, 5%, 42% and 21% were positive for anti-*S. neurona,* anti-*N. caninum* and anti-*T. gondii* IgG antibody, respectively (Table 1). A result of 5% of seropositive cats for *S. neurona* with a maximum titer of 100 was obtained, close to the result obtained in seropositive cats in the state of Bahia, which was 4% (Meneses et al., 2014), but with a titer of 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., 2002a) and 24.5%, (Bresciani et al., 2007). In the present investigation, 21% of cats were positive for *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 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 500, 1200 and 100, respectively. In cats, the maximum titers of IgG antibodies against *S. neurona, N. caninum* and *T. gondii* detected in serum were 100, 600 and 1200, respectively (Table 2).

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

The serum IgG antibody frequency of the 35 dogs with neurological signs 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).

In the current study, the statistical difference for circulating IgG antibodies in the groups of 35 dogs with neurological signs 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 signs, a frequency of 68.6% of antibodies against *N. caninum* and 25.7% against *T. gondii* was obtained, above the values obtained

Table 2 - Maximum titer of IgG antibodies (IFAT*) against *Sarcocystis neurona, Neospora caninum* and *Toxoplasma gondii* detected in sera from horses, dogs and cats attended in a university veterinary hospital from Curitiba, Paraná state, Brazil. Blood collections performed from September 2016 to March 2017

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

*IFAT= indirect fluorescent antibody test; **Denotes *Neospora spp*.

Table 3 - Frequency of co-infection detected by IgG antibodies (IFAT*) against *Sarcocystis neurona, Neospora caninum* and *Toxoplasma gondii* in the sera of horses, dogs and cats, attended in a university veterinary hospital, from Curitiba, Paraná state, Brazil. Blood collections performed from September 2016 to March 2017

Species	Seropositive animals (%)			
	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

*IFAT = indirect fluorescent antibody test; **Denotes *Neospora spp*.

Table 4 - Frequency of IgG antibodies (IFAT*) against *Sarcocystis neurona, Neospora caninum* and *Toxoplasma gondii* detected in the sera of dogs attended in a university veterinary hospital, from Curitiba, Parana state, Brazil, according to the presence of neurological signs. Blood collections performed from September 2016 to March 2017

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

*IFAT = indirect fluorescent antibody test; **Denotes p-value by Fisher's exact test with a 95% confidence interval where $P<0.05$ was considered significant.

Table 5 - Maximum titer of IgG antibodies (IFAT*) against *Sarcocystis neurona, Neospora caninum* and *Toxoplasma gondii* detected in the sera of dogs attended in a university veterinary hospital, from Curitiba, Paraná state, Brazil according to the presence of neurological signs. Blood collections performed from September 2016 to March 2017

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

*IFAT = indirect fluorescent antibody test.

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

Conclusion

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

No association was found with the seropositivity of dogs for *S. neurona, N. caninum* and *T. gondii* antigens and the presence of neurological signs, but the titers were higher in seropositive dogs for *N. caninum* and *T. gondii* with neurological signs. Seropositive dogs should have their serums titrated for a better diagnosis.

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

Conflict of Interest

The authors state they have no conflicts of interest to declare.

Ethics Statement

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

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