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Serological detection of *Toxoplasma gondii, Leishmania infantum* and *Neospora caninum* in cats from an area endemic for leishmaniasis in Brazil

Detecção sorológica de *Toxoplasma gondii, Leishmania infantum* e *Neospora caninum* em gatos de uma área endêmica para leishmaniose no Brasil

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Abstract

An investigation was made into the occurrence of antibodies to *Toxoplasma gondii, Leishmania infantum* and *Neospora caninum* in 151 domestic cats, based on the indirect fluorescent antibody test (IFAT). Serum samples were collected from 151 domestic cats (65 free-roaming and 86 domiciled cats; 55 males and 96 females) in Campo Grande, Mato Grosso do Sul, Brazil between January and April 2013. IgG antibodies to *T. gondii*, *L. infantum* and *N. caninum* were found, respectively, in 49 (32.5%), 34 (22.5%) and 10 (6.6%) sampled cats. A positive correlation was found between *T. gondii* and *N. caninum*, *T. gondii* and *L. infantum*, and *N. caninum* and *L. infantum* ($p < 0.05$) infections. Also, a significant interaction was identified between gender and area of activity on the probability of *T. gondii* ($p = 0.0324$) infection. However, no significant interaction was observed between gender and area of activity on infections by either *N. caninum* or *L. infantum*. This study showed that cats from an area endemic for visceral leishmaniasis in Brazil are exposed to three different protozoans, two of which are causal agents of important zoonosis.

Keywords: Cats, *Leishmania infantum, Neospora caninum, Toxoplasma gondii,* serology, Brazil.

Resumo

O presente estudo teve como objetivo investigar a ocorrência de anticorpos anti-*Toxoplasma gondii, Leishmania infantum* e *Neospora caninum,* em 151 gatos, por meio da Reação de Imunofluorescência Indireta (RIFI). Entre os meses de janeiro e abril de 2013, amostras de soro foram coletadas de 151 gatos domésticos (65 gatos errantes e 86 gatos domiciliados; 55 machos e 96 fêmeas), de Campo Grande, Mato Grosso do Sul, Brasil. Anticorpos IgG anti-*T. gondii*, anti-*L. infantum* e anti-*N. caninum* foram encontrados em 49 (32,5%) , 34 (22,5%) e 10 (6,6%) gatos amostrados, respectivamente. Verificou-se uma associação estatisticamente significativa entre as infecções por *T. gondii* e *N. caninum*, *T. gondii* e *L. infantum* e *N. caninum* e *Leishmania infantum* ($p < 0,05$). Além disso, foi observada uma interação significativa entre sexo, área de atividade na probabilidade de infecção por *T. gondii* ($p = 0,0324$). No entanto, não foi observada interação significativa entre sexo e área de atividade nas infecções por *N. caninum* e *L. infantum.* Este estudo mostrou que os gatos de uma área endêmica brasileira para leishmaniose visceral são expostos a três diferentes protozoários, sendo dois deles importantes agentes zoonóticos.

Palavras-chave: Gatos, *Leishmania infantum, Neospora caninum, Toxoplasma gondii,* sorologia, Brasil.

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Introduction

Toxoplasma gondii and *Neospora caninum* are apicomplexan intracellular protozoal parasites that affect a wide range of animal species, including humans (DUBEY, 1986; DUBEY et al., 1988). Both organisms cause serious reproductive diseases as well as economic losses, mainly in ruminants (DUBEY et al., 1988; MASALA et al., 2003). Felines play an important role in the epidemiology of *T. gondii,* because they are the only animal group that excrete resistant oocysts into the environment (LINDSAY et al., 1997). On the other hand, the definitive hosts of *N. caninum* are domestic dogs, coyotes and Australian dingoes, which shed oocysts (DUBEY & Schares, 2011). Due to the importance of these parasites to public health, many surveys have been conducted worldwide showing the seroprevalence of *T. gondii* in felines (GARCIA et al., 1999; LANGONI et al., 2001; SILVA et al., 2002; DUBEY et al., 2004; PENA et al., 2006; LOPES et al., 2008; ESTEVES et al., 2014). However, there a few reports about the seroprevalence of *N. caninum* in cats (DUBEY et al., 2002; BRESCIANI et al., 2007; BRAGA et al., 2012).

Leishmaniasis, an infectious disease that affects humans and wild and domestic mammals worldwide, is caused by a kinetoplastid flagellate protozoan parasite of the genus *Leishmania*, transmitted mainly by sand flies of the genera *Lutzomyia* spp. and *Phlebotomus* spp. (SOLANO-GALLEGO et al., 2007). Although dogs are considered the main reservoirs of *Leishmania* spp. in urban areas of Brazil, the increase in cases of leishmaniasis among cats suggests the possibility that these animals participate in the epidemiology of the disease (MAROLI et al., 2007; VIDES et al., 2011). Recently, *Leishmania* spp. infection in domestic cats has been reported in several countries, including Brazil, where this zoonosis is endemic (POLI et al., 2002; SOLANO-GALLEGO et al., 2007; SOUZA et al., 2005; SOBRINHO et al., 2012).

This study was conducted to evaluate the serological occurrence of *T. gondii*, *N. caninum* and *L. infantum* in domestic cats living in an area endemic for leishmaniasis in the municipality of Campo Grande, Mato Grosso do Sul, Brazil. The risk factors associated with seropositivity for these selected agents in cats were also evaluated.

Materials and Methods

Sample collection

Between January and April 2013, whole blood samples were collected by convenience from 151 cats (54 males, 95 females and two without gender registration) in the city of Campo Grande, capital of the state of Mato Grosso do Sul, Brazil. Free-roaming non-domiciled cats (n = 65) were caught by technical staff from the local zoonosis control center (CCZ). These cats shared the same type of food and water, and received no health check-ups or vaccinations. Domiciled cats without outdoor access (n = 86) were sampled during preoperative procedures in a castration project at the CCZ; these animals were returned to their homes after surgery. Overall, the physical status of domiciled cats was better than that of the non-domiciled animals. Cat blood samples (6 mL) were collected by jugular venipuncture and stored at -20°C. The project was approved by the Ethics Committee on Animal Use of São Paulo State University – UNESP at Jaboticabal, under Protocol no. 004987/13.

Serological tests for T. gondii, N. caninum and L. infantum

The presence of *T. gondii*, *N. caninum* and *L. infantum* antibodies in the serum of each sampled animal was detected by the Indirect fluorescent Assay (IFAT), as described previously (ANDRÉ et al., 2010; OLIVEIRA et al., 2008). *N. caninum* NC-1 strain tachyzoites were used as an antigen (DUBEY et al., 1988; MINEO et al., 2009). *Toxoplasma gondii* RH strain tachyzoites were also used as an antigen, as described by Domingues et al. (1998). In this study, a *L. infantum* strain was used that was isolated in Jaboticabal (OLIVEIRA et al., 2008), in the state of São Paulo, and characterized as *donovani* complex, probably *L. infantum*, using molecular techniques described by Cortes et al. (2004). Promastigotes of *L. infantum* were maintained on RPMI-1640 medium (Sigma-Aldrich, St. Louis, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Canyon City, USA) at 25°C. This medium was then subjected to seven freeze-thaw cycles, after which the medium was centrifuged (3000 × g for 30 min at 4°C). The resulting supernatant was harvested and used as crude antigen, and was used to prepare the crude antigens for IFAT (OLIVEIRA et al., 2009). Antigen slides were removed from storage and allowed to thaw at room temperature for 30 min. Ten microliters of sera at dilution of 1:50 (cut-off for *N. caninum*), 1:40 (cut-off for *T. gondii*) and 1:40 (cut-off for *L. infantum*) were placed in wells on antigen slides. Cat serum samples positive and negative for *T. gondii*, *N. caninum* and *L. infantum* (BRAGA et al., 2012), obtained from the serum bank of the Laboratory of Immunoparasitology, Department of Veterinary Pathology of UNESP at Jaboticabal, SP, were also used in the serological reactions. Slides were incubated at 37°C in a moist chamber for 45 min, washed three times in phosphate-buffered saline (pH 7.2) for 5 min, and air-dried at room temperature. Immunoglobulin G (IgG) anti-cat conjugate (whole molecule with fluorescein isothiocyanate, dilution of 1:32; Sigma®, St. Louis, Missouri) for domestic feline samples were diluted according to the manufacturer's instructions and added to each well. These slides were incubated again, washed, dried, and overlaid with buffered glycerin (pH 8.7), covered with glass coverslips, and examined using an epifluorescence microscope (Olympus, Japan). The parasite strains used as antigens in the present study were also used in earlier serological surveys among wild and domestic animals in Brazil (MINEO et al., 2009; ANDRÉ et al., 2010; JUSI et al., 2011).

Statistical analysis

Logistic regression models were employed to assess the effect of the putative predictor variables (i.e., gender, area of activity and the interaction between them) on the logit of the probability of infection by each agent. The significance of co-infection by the different parasites was assessed using Pearson's Chi-squared test with

Yates continuity correction. All the analyses were performed using R 3.0.2 software (R DEVELOPMENT CORE TEAM, 2013).

Results

IgG antibodies to *T. gondii*, *N. caninum* and *L. infantum* were detected, respectively, in 49 (32.5%), 34 (22.5%) and 10 (6.6%) sampled cats. IgG antibody titers ranged from 1:40 (cut-off) to 1:20480 for *T. gondii,* 1:50 (cut-off) to 1:200 for *N. caninum,* and 1:40 (cut-off) to 1:1280 for *L. infantum* (Table 1). Five cats (3.3%) showed IgG antibodies to *T. gondii*, *N. caninum* and *L. infantum*. Seventeen cats (11.2%) were seropositive only for *T. gondii* and *N. caninum;* 2 (1.3%) were seropositive only for *T. gondii* and *L. infantum,* and 3 (1.9%) were seropositive for *N. caninum* and *L. infantum*. Twenty-five cats (16.5%) showed antibodies to *T. gondii,* and 9 (5.9%) were seropositive for *N. caninum*. None of the cats was seropositive only for *L. infantum*. Ninety cats (59.6%) were seronegative for all the parasites. The results showed a statistically positive correlation between infections by *T. gondii* and *N. caninum, T. gondii* and *L. infantum*, and *N. caninum* and *L. infantum* ($p < 0.05$) (Table 2).

Logistic regression results revealed a significant interaction between gender and area of activity on the probability of infection by *T. gondii* ($p = 0.0324$) (Table 3), while neither of the predictor variables fitted in the logistic regression models had a significant effect on *N. caninum* and *L. infantum* infections ($p = 0.7$; $p = 0.6$ respectively) (Table 3). In the case of probability of infection by *T. gondii*, the results of subsequent regressions are presented owing to the significant interaction between gender and area of activity, and these analyses are stratified by either gender or area of activity (Table 4). When the animals were stratified according to gender, male cats from the local CCZ exhibited a significantly higher probability of infection by *T. gondii* than domiciled cats, although this stratification showed no significant difference between females from the CCZ and domiciled females (Table 4). The results of the analyses stratified according to area of activity revealed that, among the domiciled cats, females showed a significantly higher probability of becoming infected by this agent than males, while the cats from the local CCZ showed no significant difference between males and females.

Table 1. Distribution of reciprocal titers measured by IFAT for *T. gondii*, *N. caninum* and *L. infantum* in 151 cats of the city of Campo Grande, MS, Brazil.

	Titer	Number of positive cats	(%)
T. gondii	1:40	49	32.45
	1:80	44	29.13
	1:160	35	23.17
	1:320	30	19.86
	1:640	20	13.24
	1:1280	15	9.93
	1:2560	8	5.29
	1:5120	4	2.64
	1:10240	2	1.32
	1:20480	1	0.66
N. caninum	1:50	34	22.51
	1:100	13	8.60
	1:200	3	1.98
L. infantum	1:40	10	6.62
	1:80	5	3.31
	1:160	2	1.32
	1:320	1	0.66
	1:640	1	0.66
	1:1280	1	0.66

Discussion

This study revealed the occurrence of *L. infantum* antibodies in 6.6% of the sampled cats, which is higher than that found in previous studies conducted in another areas endemic for leishmaniasis in the state of São Paulo, Brazil, where seroprevalence ranging from 0.5 to 4.64% has been reported, using the same serological technique (IFAT) and cut-off (≥ 40) (SOBRINHO et al., 2012; CARDIA et al., 2013; BRESCIANI et al., 2010). On the other hand, Vides et al. (2011) reported a higher seroprevalence by IFAT (10.9%) among cats sampled in the city of Araçatuba, state of São Paulo, which was higher than that found in this study, probably because all the cats sampled in the former study showed dermatologic lesions, one of the most suggestive clinical signs of feline leishmaniasis (SOLANO-GALLEGO et al., 2007).

Sousa et al. (2013) recently found high seroprevalence (100%) and high levels of *L. infantum* antibody titers ranged from 1:40 to 1:20480 among dogs sampled in the same area where this study was conducted. Lower seropositivity to *Leishmania* spp. among cats compared to dogs in areas endemic for leishmaniasis have been reported in previous studies (SOLANO-GALLEGO et al., 2007; BRESCIANI et al., 2010; SOBRINHO et al., 2012; CARDIA et al., 2013). According to Solano-Gallego et al. (2007), the immune response in cats, mainly cellular immunity, is effective enough to control the infection and confer some natural resistance

Table 2. Association between infections by *T. gondii* and *N. caninum, T. gondii* and *L. infantum* and *N. caninum* and *L. infantum.*

Agents				Chi-square	P-value
		N.caninum			
		-	+		
T. gondii	–	90	12	18.9716	0.0000133*
	+	27	22		
		L.infantum			
		-	+		
T.gondii	–	99	3	5.1762	0.0229*
	+	42	7		
		L.infantum			
		-	+		
N.caninum	–	115	2	16.908	0.000039*
	+	26	8		

*(P<0.05).

Table 3. Risk factors affecting the probability of infection by *Toxoplasma gondii*, *Neospora caninum* and *Leishmania infantum**.

T.gondii			
Effect	**Estimate**	**SE**	***P*-value**
Intercept	–0.511	0.327	0.118
Gender (G)	0.329	0.539	0.542
Area of activity (A)	–0.210	0.435	0.630
G x A	–1.842	0.861	0.032
N.caninum			
Effect	**Estimate**	**SE**	***P*-value**
Intercept	–1.099	0.365	0.003
Gender (G)	0.336	0.586	0.566
Area of activity (A)	–0.405	0.506	0.423
G x A	–0.260	0.820	0.752
L.infantum			
Effect	**Estimate**	**SE**	***P*-value**
Intercept	–2.512	0.600	<0.001
Gender (G)	0.667	0.864	0.440
Area of activity (A)	–0.765	0.938	0.415
G x A	–0.791	1.516	0.602

*Estimates and respective standard errors (SE) of logit coefficients obtained in multiple logistic regression analyses are presented, followed by the associated *P-values* based on the Wald z-statistic.

Table 4. Estimated odds ratio of the probability of infection by *Toxoplasma gondii* according to gender and area of activity*.

Grouping variable	**Risk factor**	**OR**	**2.5%**	**97.5%**
Gender	Area of activity			
Males	Domiciled	1	-	-
	CCZ	7.778	1.986	39.564
Females	Domiciled	1	-	-
	CCZ	1.233	0.523	2.900
Area of activity	Gender			
Domiciled	Males	0.220	0.048	0.731
	Females	1	-	-
CCZ	Males	1.389	0.479	4.019
	Females	1	-	-

*Estimated odds ratio (OR) and associated thresholds for the 95% confidence interval (2.5% and 97.5% represent lower and upper threshold, respectively) are presented for each level of the risk factors, obtained from stratified analyses, considering only one level of the grouping variable at each time. Levels of risk factor variables with OR values equal to 1 represent the reference level employed to compute the odds ratio in each situation. CCZ - Zoonosis control center.

to the parasite when immunosuppressive events are not involved. Due to immunosuppression caused by coinfections with Feline Immunodeficiency Virus (FIV), Feline Leukemia Virus (FeLV) and opportunistic pathogens (SOLANO-GALLEGO et al., 2007; SOBRINHO et al., 2012; SUKHUMAVASI et al., 2012), cats infected with *Leishmania* may develop clinical signs and act as a source of infective amastigotes to sand flies. Although infection of sand flies by a cat naturally infected with *L. infantum* has been reported (MAROLI et al., 2007), studies to evaluate the role of cats as potential sources of *L. infantum* infection for phlebotomine sand flies are sorely needed. A positive association between FIV (PENNISI et al., 2004; VIDES et al., 2011; SOBRINHO et al., 2012) or FeLV (SHERRY et al., 2011) and *Leishmania* spp. infection has been reported in cats.

The occurrence of *T. gondii* antibodies in 32.4% of the sampled cats (49/151) was higher than previously reported in the Brazilian states of São Paulo (25%) (BRESCIANI et al., 2007), Rio de Janeiro (24.39%) (GONÇALVES NETTO et al., 2003), and Santa Catarina (14.33%) (DALLA ROSA et al., 2010), but lower than those reported in the states of Maranhão (50.5%) (BRAGA et al., 2012), Rio Grande do Sul (37.9%) (PINTO et al., 2009) and Paraná (73%) (GARCIA et al., 1999)., Differences in specificity and sensitivity among different techniques used in earlier studies are likely the cause of disparate seroprevalence rates.

Although the production of immunoglobulins is not associated with oocyst shedding (DUBEY, 1986), the seroprevalence found in sampled felines may be an alternative to measure the spread of *T. gondii* in the environment (BRAGA et al., 2012). Titers lower than 1:64 in IFAT without clinical symptoms suggest chronic infection by *T. gondii*; on the other hand, titers exceeding 1024 usually indicate acute infection with or without clinical signs (MCKINNEY, 1973).

Several authors reported that they found no significant influence of gender on *T. gondii* infection (BRESCIANI et al., 2007; COELHO et al., 2011). In this study, we found that the we found that the correlation between gender and area of activity significantly influenced the probability of *T. gondii* infection (p = 0.0324). Male cats from the CCZ showed a significantly higher probability of becoming infected with *T. gondii* (OR= 7.77) than male domiciled cats, although no significant difference in terms of probability of *T. gondii* infection was found between female cats from different areas of activity (i.e., CCZ vs. domiciled). Environmental conditions and behavioral effects may have contributed to the animals' exposure to *T. gondii,* since they live together in the same location (CCZ), share the same food and water, and may be in contact with other intermediate hosts, in addition to the fact that male cats fight with each other.

Among domiciled cats, females showed a higher probability of becoming infected with *T. gondii* than males, while among the cats from the CCZ, gender did not have a significant effect. Several studies have reported that gender does not significantly influence the probability of infection by *T. gondii* (LOPES et al., 2008; ESTEVES et al., 2014), although, unlike the present study, they did not investigate interactions between gender and other risk factors. As a general rule, males are more prone to acquiring infected parasites than females, owing to several endocrinal and behavioral differences (KLEIN, 2000). However, this seems to disagree with the higher probability of infection found for domiciled females in the present study. This discrepancy may be explained by the fact that some confounders (e.g., age, feeding habits, breed and contact with other intermediate hosts) were not controlled in this study, due to lack of information.

Although previous studies found no significant association between *T. gondii* and *Leishmania* spp. in cats (SHERRY et al., 2011; SOBRINHO et al., 2012), a positive statistical association between infections by *T. gondii* and *L. infantum* and *N. caninum* and *L. infantum*, which was indicative of coinfections, was detected in this study. Numerous studies have reported immunosuppression caused by *Leishmania* spp., which could enhance the susceptibility

of dogs to coccidian parasites (GENNARI et al., 2006). However, future studies are sorely needed to elucidate the real role of *L. infantum* in the epidemiology of *N. caninum* and *T. gondii* in cats. A positive correlation between the presence of *N. caninum* and *T. gondii* antibodies was also found in the present study, which corroborates the findings of previous studies, due to the feeding habits of cats (FRENKEL, 1973; HUANG et al., 2004; BRESCIANI et al., 2007). Recently, several authors reported that small birds and rodents living in synanthropic areas could act as intermediate hosts for *T. gondii* and *N. caninum* (MINEO et al., 2009; HUANG et al., 2004). Moreover, because of the mechanical transmission of oocysts, environmental contamination may have contributed to increase the exposure of cats to both agents.

There are few reports about the occurrence of *N. caninum* in cats. In this study, we found a seroprevalence to *N. caninum* of 22.5%, which is similar to previous reports in cats in the states of São Paulo (24.5%) and Maranhão in Brazil (27%) (BRESCIANI et al., 2007; BRAGA et al., 2012), and in Italy (24.8%) (FERROGLIO et al., 2005), but higher than that found by Dubey et al. (2002) among cats sampled in the cities of São Paulo and Guarulhos, Brazil (11.9%). The possibility of cross-reactions between *T. gondii* and *N. caninum* are remote, because cross-reactivity is observed only when soluble antigens are used, while surface antigens such as those used in IFA usually do not cause cross-reactions (DUBEY et al., 1996; HIGA et al., 2000).

The importance of *N. caninum* as a zoonotic agent remains unknown, but *N. caninum* antibodies have been detected in humans in the United States (TRANAS et al., 1999) and Brazil (LOBATO et al., 2006; BENETTI et al., 2009). In Brazil, IgG antibodies against *N. caninum* were detected in patients infected with human immunodeficiency virus (HIV) and in patients with neurological disorders, suggesting the possibility that neosporosis could be an opportunistic parasite in immunocompromised patients (LOBATO et al., 2006). Furthermore, the authors found a positive correlation between infections by *N. caninum* and *T. gondii*, and suggested that further studies are needed to evaluate *N. caninum* infections in humans.

Although the clinical status of the animals in this study was not evaluated, Dubey et al. (1990) observed encephalitis and myositis in tissues of cats experimentally infected with neosporosis similar to those of feline toxoplasmosis. Therefore, *N. caninum* should be included in the differential diagnosis of cats exhibiting neurological clinical symptoms.

The gender of cat was not a factor that could be correlated with *N. caninum* ($p>0.05$) infection, as also reported by other authors (FERROGLIO et al., 2005; BRESCIANI et al., 2007). Although environmental conditions seem to be an important factor for neosporosis infection, owing to the higher probability of contact with reservoirs of infection and final hosts (HORNOK et al., 2008), the results of this study did not reveal a significant correlation between the area of activity and the probability of infection with *N. caninum.*

In conclusion, this paper reported the occurrence of *T. gondii, N. caninum* and *L. infantum* antibodies in cats in an area endemic for canine leishmaniasis in Brazil. The real impact of infection by multiple pathogens in cats on the shedding of *T. gondii* oocysts or the acquisition of amastigotes by sand flies feeding on *L. infantum*-infected cats is still unknown. The data presented herein may be useful in pointing out possible biological and/or epidemiological interactions between these parasites in cats. Further studies should be conducted to investigate the real role of cats in the epidemiological cycle of leishmaniasis in Brazil. Preventive measures and improvements in the diagnostic assays for *Leishmania* spp. and *T. gondii* among cats should also be adopted, in view of their importance to public health. Lastly, it is advisable for veterinarians to include *N. caninum* in the differential diagnosis of cats displaying neurological clinical symptoms.

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