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Seroprevalence rates of antibodies against *Leishmania infantum* and other protozoan and rickettsial parasites in dogs

Soroprevalência de anticorpos contra *Leishmania infantum* e outras espécies de protozoários e rickettsia em cães

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Abstract

Canine visceral leishmaniasis (CVL) is caused by the protozoan *Leishmania infantum,* which infects dogs and humans in many regions of Brazil. The present study involved an indirect fluorescent antibody test (IFAT) to analyze *L. infantum*, *Ehrlichia* spp., *Babesia canis*, *Toxoplasma gondii* and *Neospora caninum* infection rates in serum samples from 93 dogs in a rural settlement in Ilha Solteira, SP, Brazil. The seroprevalence rates of anti-*L. infantum*, anti-*Ehrlichia,* anti-*B. canis*, anti-*T. gondii* and anti-*N. caninum* antibodies were 37.6%, 75.3%, 72%, 47.3% and 6.4%, respectively. In addition to IFAT, direct microscopic examination of popliteal lymph node aspirates revealed 26.9% of CVL positive dogs. Serological tests revealed that 17.2% of the dogs were seropositive for a single parasite, 29% for two parasites, 33% for three, 16.1% for four, and 1.1% for five parasites, while 3.2% were seronegative for five parasites. The presence of antibodies against these parasites in serum samples from dogs confirmed their exposure to these parasites in this rural area. Because of the potential zoonotic risk of these diseases, mainly leishmaniasis, ehrlichiosis and toxoplasmosis, special attention should focus on programs for the improvement of diagnostic assays and control measures against these parasites.

Keywords: *Leishmania*, *Babesia, Ehrlichia*, *Toxoplasma*, *Neospora*, dogs.

Resumo

Leishmaniose Visceral Canina (LVC) é causada pelo protozoário *Leishmania infantum,* podendo infectar cães e humanos em várias regiões do Brasil. O presente estudo teve por objetivo realizar a reação de imunofluorescência indireta (RIFI) para analisar os índices de infecção parasitária para *L. infantum, Ehrlichia* spp., *Babesia canis*, *Toxoplasma gondii* e *Neospora caninum*, em 93 amostras de soro de cães de um assentamento rural no município de Ilha Solteira, SP, Brasil. A taxa de soroprevalência de cães com anticorpos anti-*L. infantum*, anti-*Ehrlichia*, anti-*B. canis*, anti-*T. gondii* e anti-*N. caninum* foi de 37,6%, 75,3%, 72%, 47,3% e 6,4%, respectivamente. Pelo exame microscópico direto dos parasitas nos esfregaços de aspirados de linfonodos poplíteos dos cães, a positividade para LVC foi de 26,9%. Pelos exames sorológicos, 17,2% dos cães estavam positivos com um único parasita, 29% com dois, 33% com três, 16,1% com quatro e 1,1% com cinco parasitas. Além disso, 3,2% eram soronegativos para todos os cinco agentes parasitários. A presença de anticorpos aos parasitos em amostras sorológicas confirmam a exposição dos cães às doenças parasitárias nesse assentamento rural. Devido ao potencial risco zoonótico destas doenças, principalmente leishmaniose, erliquiose e toxoplasmose, atenção especial deve ser dada aos programas que objetivam o aprimoramento de testes diagnósticos e de medidas de controle dessas parasitoses.

Palavras chave: *Leishmania, Ehrlichia, Babesia, Toxoplasma, Neospora,* cães.

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Canine Visceral Leishmaniasis (CVL) is a disease caused by a kinetoplastid protozoan of the genus *Leishmania* (ROSS, 1903). *Leishmania infantum* (syn. *L. chagasi*) is the causative agent of visceral leishmaniasis in the New World, with endemic regions extending from southern USA to northern Argentina, including Brazil (KUHLS et al., 2011), which is transmitted to humans and animals by the bite of the sandfly *Lutzomyia longipalpis* (Diptera: Psychodidae: Phlebotominae) (PORROZZI et al., 2006). The main factors that favor the evolution and dissemination of CVL are human invasion of natural endemic areas; migration from rural to urban areas, and social and economic deterioration, particularly in suburban areas (DESJEUX, 2004; GRAMICCIA; GRADONI, 2005; ALVAR et al., 2006).

Dogs can be infected naturally by a variety of parasites of wide geographical distribution (GUIMARÃES et al., 2009). *Toxoplasma gondii* is a protozoan that infects humans and wild and domestic animals worldwide, including domestic dogs (SOUZA et al., 2003; AZEVEDO et al., 2005; DUBEY et al., 2007; LOPES et al., 2011). *Neospora caninum* is an intracellular protozoan causing a serious disease in cattle and dogs worldwide (DUBEY et al., 1988). Recent Brazilian surveys of foxes found seropositivity for *N. caninum, T. gondii* and *L. infantum* in 3.2%, 18.0% and 78.1%, respectively (CATENACCI et al., 2009). Coinfection has already been described with *L. infantum* and *N. caninum* (TARANTINO et al., 2001; CRINGOLI et al., 2002; ANDREOTTI et al., 2006) and with these parasites and *T. gondii* in dogs (GENNARI et al., 2006; RIBEIRO et al., 2011). In addition, some studies suggest that immunosuppression caused by *Leishmania* spp. can enhance the susceptibility of dogs to coccidian parasites (CRINGOLI et al., 2002; GENNARI et al., 2006).

Babesiosis and Ehrlichiosis are tick-borne diseases that are very common in dogs, the former caused by an intraerythrocytic parasite, *Babesia canis* (LOBETTI, 1998), and the latter by an obligate intracytoplasmic rickettsia, *Ehrlichia* spp. (GUILLÉN LLERA et al., 2002). Some studies have reported cross-reaction between *Leishmania* and *E. canis* or *Leishmania* and *B. canis* in serological tests (GOMES; CORDEIRO, 2004). However, Oliveira et al. (2008) examined serum samples from dogs living in indemic and non-endemic areas for CVL and found only coinfection, but not antibody cross-reaction between anti-*Leishmania* and anti-*B. canis* or anti-*Leishmania* and anti-*E. canis.*

The purpose of this study, therefore, was to analyze the seroprevalence rates of antibodies against *L. infantum, Ehrlichia* spp., *B. canis, T. gondii* and *N. caninum* in serum samples from dogs living in a rural settlement in an area where CVL is endemic.

This rural settlement of about three thousand hectares is located in the municipality of Ilha Solteira, São Paulo, Brazil (20° 25' 36.47" S and 51° 20' 26.47" W) close to an urban area, and is divided into small land holdings of about 14 ha each, where about 209 families live. An estimated 600 dogs live in this settlement.

This study involved dogs (n = 93) of different breeds and ages (from 5 months to 18 years old) and both sexes from this rural settlement. Serum samples were collected from these dogs and subjected to an indirect fluorescent antibody test (IFAT) to detect antibodies against four protozoan and one rickettsial parasite. The study was approved by the Animal Ethics Committee of UNESP at Jaboticabal, under Protocol no. 010208.

To detect anti-*L. infantum* antibodies, the IFAT was performed according to Oliveira et al. (2008) and is briefly described below.

Leishmania parasites were obtained from bone marrow of naturally infected dogs and maintained in RPMI-1640 medium at 25 °C. Promastigotes of *Leishmania* maintained in culture medium were placed on slides and used as antigen for the IFAT procedure. Double serum dilutions starting at 1:40 (cutoff point) were placed on the antigens, followed by incubation at 37 °C for 30 minutes. The slides were then incubated with anti-dog IgG serum conjugated with fluorescein isothiocyanate (KPL, USA) diluted at 1:30 in PBS containing 1% of Evans Blue and washed in PBS. After adding buffered glycerin, the slides were examined under a fluorescent microscope. Reference sera were included as negative and positive controls. Samples that displayed a bright-green peripheral stain with a dull fluorescence of the parasite cytoplasm were considered positive.

IFAT for *B. canis* was performed as described by Furuta et al. (2009), and serum samples were positive starting at dilutions of 1:40 and higher. *E. canis* IFAT was performed using IFAT test kits according to the manufacturer's instructions (VMRD Inc., USA), and serum samples tested at a dilution of 1:40 were considered positive.

Tachyzoites were used as antigen for anti-*T. gondii* antibodies (RH strain), as described by Camargo (1974). For anti-*N. caninum* antibody detection, IFAT was performed using culture-derived NC-1 isolate tachyzoites (DUBEY et al., 1988). Rabbit anti-canine IgG conjugate (Sigma, USA) was used in both tests and sera were tested in 2-fold serial dilutions, starting at 1:16 for *T. gondii* and 1:50 (cutoff point) for *N. caninum.* Positive and negative control sera were added to all the reactions.

The presence of amastigote forms of *Leishmania* was examined under a light microscope (1000× magnification) in smears of popliteal lymph node aspirates fixed in methanol and stained with Giemsa.

For statistical analyses, the chi-square test with a significance level of 5% was performed using BioEstat® 5.0 software to assess the associations between the presence of antibodies against *Ehrlichia* spp., *B. canis, T. gondii* and *N. caninum* in *L. infantum* positive and negative dogs.

Ilha Solteira is a municipality well known to be endemic for CVL (NORONHA JUNIOR et al., 2007; ASSIS et al., 2010; QUEIROZ et al., 2010), where the prevalence rate of CVL in dogs living in urban areas is reportedly 23% (NORONHA JUNIOR et al., 2007).

This paper reports the occurrence of CVL in a rural settlement in the municipality of Ilha Solteira, located close to an urban area (southeastern Brazil). Thirty-five of the 93 dogs (37.6%) were positive in serological tests (Table 1) and 25 out of 93 (26.9%) in parasitological tests. In addition, the IFAT indicated that 17.1% of the dogs showed high levels of antibodies (titer ≥ 1280). This prevalence rate was lower than that observed by Queiroz et al. (2010) in urban areas of the same municipality (55.9%), but the occurrence of the disease represents a risk in both rural and urban areas because the presence of *L. longipalpis* that contributes for parasite dispersion (PAULAN et al., 2012). Four percent of these CVL positive dogs were symptomatic and the most frequent clinical signs were weight loss, onycogryphosis, hypertrophic popliteal lymph nodes, alopecia and other dermatological alterations.

Table 1. Antibody detection of *Leishmania infantum*, *Ehrlichia* spp., *Babesia canis*, *Toxoplasma gondii* and *Neospora caninum* in serum samples of 93 dogs from a rural settlement, Ilha Solteira, SP, Brazil, by IFAT.

Parasites	Numbers and percentages of serum positive dogs by IFAT		
	Positive		IFAT(cut-off)
	Nº	% (N = 93)	
L. infantum	35	37.6	1:40
Ehrlichia spp.	70	75.3	1:40
B. canis	67	72.0	1:40
T. gondii	44	06.5	1:50
N. caninum	06	47.3	1:16

Nº - number; % percentage.

In addition to *L. infantum*, dogs had antibodies reactive to multiple parasites, including other protozoans (*B. canis*, *T. gondii* and *N. caninum*) and rickettsial (*Ehrlichia* spp.) parasites (Table 1). In this study, 95.7% of the dogs (89/93) had serum reactive to 1 to 5 parasites: 4.3%, 17.2%, 28%, 33.3%, 16.1% and 1.1% of the dogs with antibodies targeted to zero, one, two, three, four and five parasites, respectively, which was indicative of coinfections. In addition, 13.8%, 8.6% and 5.7% of the dogs were serum positive for only one parasite, as follow: *Ehrlichia*, *Babesia* and *Leishmania*, respectively. However, one dog (1.1%) had serum reactive to five parasites, and most of the dogs (61.3%) showed antibody reactivity from 2 to 3 parasites with the following associations: *Leishmania* × *Babesia* × *Toxoplasma* or *Leishmania* × *Ehrlichia* × *Toxoplasma* (48.6%) or *Leishmania* × *Babesia* × *Ehrlichia* (31.4%). The association between two parasites ranged from 62.9% to 74.3% (Table 2).

For hemoparasites, 75.3% and 72% of the dogs were serum positive for *Ehrlichia* spp. and *B. canis,* respectively, which represented the most prevalent parasites (Table 1). Similarly high prevalence rates of *B. canis* (OLIVEIRA et al., 2008; FURUTA et al., 2009) and *Ehrlichia* spp. (OLIVEIRA et al., 2008) were reported in dogs from urban areas in Brazil. It is well known that both parasites (*Ehrlichia* spp. and *B. canis*) are transmitted by the brown dog tick *Rhipicephalus sanguineus* in endemic areas in Brazil (LABRUNA; PEREIRA, 2001; DANTAS-TORRES, 2008). The dogs in this rural settlement were frequently infected with these ticks, which explains the high prevalence of these hemoparasites.

Serum antibody reactivity was also detected simultaneously for *L. infantum* × *Ehrlichia* spp. or *L. infantum* × *B. canis* in 74.3% of the dogs, for *B. canis* × *Ehrlichia* spp. in 50%, and for *L. infantum* × *B. canis* × *Ehrlichia* spp. in 31.4%. We agree with Oliveira et al. (2008) that the high prevalence rates of these two hemoparasites in a CVL endemic area is much more suggestive of coinfections than of antibody cross reaction among these three parasites.

T. gondii and *N. caninum* are two coccids that are important for animal and human public health (DUBEY et al., 1988). In the present study (southeastern Brazil), 47.3% of the dogs from a rural area were positive for the presence of anti-*T. gondii* (Table 1), with antibody titers ranging from 16 to 2048. In contrast, the prevalence rate of *T. gondii* based on serological tests in dogs living in urban areas of northeastern Brazil was only 18% (LOPES et al., 2011).

Table 2. Antibody positivity against *Leishmania infantum* (L); *Ehrlichia* spp. (E); *Babesia canis* (B); *Toxoplasma gondii* (T) and *Neospora caninum* (N) in different parasitic associations in serum samples from dogs in a rural settlement in Ilha Solteira, SP, Brazil.

Parasite association	*Leishmania* positive dogs (N = 35)	
	No.	%
L × B × E × T × N	01	02.9
L × B × E × T	13	37.1
L × B × E	11	31.4
L × B × T	17	48.6
L × B × N	01	02.9
L × B × E × N	02	05.7
L × B × T × N	00	00.0
L × E × T × N	00	00.0
L × T × N	01	02.9
L × E × N	01	02.9
L × E × T	17	48.6
L × B	26	74.3
L × E	26	74.3
L × T	22	62.9
L × N	02	05.7
L	02	05.7

Nº - number; % percentage.

Dogs are considered sentinels for *T. gondii* environmental contamination, and the high prevalence of this parasite in dogs in this rural settlement demonstrates its wide distribution in that region. The owners of these dogs allowed them to roam outside home, which presumably placed them in closer contact with other sources of animal products and higher exposure to *T. gondii* oocysts from cats roaming freely in the area. It is well known that the presence of cats and their close contact with dogs are important factors in the epidemiology of the disease (DUBEY; BEATTIE, 1988). Thus, the presence of cats in this rural settlement may have favored the dispersion of oocysts and increased the risk of environmental contamination.

In contrast, these dogs showed a very low seroprevalence of anti-*N. caninum* antibody, which reached only 6.5% (Table 1), with antibody titers from 100 to 6400. Azevedo et al. (2005) reported a similarly low seroprevalence in dogs in northeastern Brazil (8.4%), which was much lower than that reported by Gennari et al. (2002) for dogs in the city of São Paulo, SP (18%) and by Souza et al. (2002) for dogs in a rural area in the state of Paraná (21.6%). In fact, this low prevalence was surprising due to the presence of cattle on most of the properties in the rural settlement, which could contribute to increase the risk of transmission of neosporosis; however, there was no data about *Neospora* infection in cattle in that area.

Simultaneous seroreactivity was also detected against *Leishmania* and coccidial parasites (*Toxoplasma* and *Neospora*) in addition to *Ehrlichia* and *Babesia* in different degrees of association (Table 2) suggestive of coinfection, which require further studies.

Some protozoan parasites are known to be immunosuppressive and may predispose the host to the invasion and proliferation of

Table 3. Occurrence in numbers (Nº) and percentages (%) of anti *B. canis*, anti-*Ehrlichia* spp., anti-*T. gondii* and anti-*N. caninum* antibodies in positive (+) and negative (–) dogs for *Leishmania infantum*, by IFAT and parasitological tests in a rural settlement, Ilha Solteira, SP, Brazil.

Parasites		*Leishmania* positive dogs (N = 35)		*Leishmania* negative dogs (N = 58)	
		Nº	%	Nº	%
Ehrlichia	(+)	26	74.3	44	75.8
	(–)	09	25.7	14	24.1
B. canis	(+)	26	74.3	41	70.7
	(–)	09	25.7	17	29.3
T. gondii	(+)	22*	62.9	22	37.9
	(–)	13	37.1	36	62.1
N. caninum	(+)	02	5.7	04	6.9
	(–)	33	94.3	54	93.1

*Statiscally significant association ($X^2 = 6.47$; $p = 0.01$) between parasite infection ($L^+ \times T^+$). Nº - number; % percentage.

other concomitant parasites or bacteria (PINELLI et al., 1994). Although antibody reactivity between *L. infantum* and other parasites was observed (Table 3), a significant association was observed only between *L. infantum* and *T. gondii* ($X^2 = 6.47$; $p = 0.01$), suggesting immunosuppression caused by *Leishmania* spp., which could enhance the susceptibility of dogs to coccidian parasites. However, this hypothesis lacks scientific corroboration. In contrast, no statistically significant parasitic association was detected between *L. infantum* and *B. canis* or *N. caninum* and *E. canis* (Table 3). This is consistent with studies of *N. caninum* (GRECA et al., 2010; ANDREOTTI et al., 2006) that revealed no parasitic association as well as correlation in dogs naturally infected with *Leishmania* spp. Nevertheless, it is important to emphasize that these positive dogs for *T. gondii* and *Leishmania* shared the same habitat marked by poor sanitary conditions, the presence of sand flies, coccidian oocysts, ticks and animals positive for different parasitic diseases, and were therefore equally exposed to many risk factors for the infection.

In conclusion, this study reports the occurrence of antibody seroreactivity against *L. infantum, Ehrlichia* spp., *B. canis*, *T. gondii* and *N. caninum* in dogs from a rural settlement in Ilha Solteira, SP, Brazil. The dogs had antibodies targeting simultaneously for two to five of these parasites, suggesting coinfections in different degrees of parasitic association. Due to the zoonotic potential of the canine diseases caused by these parasites, particularly leishmaniasis, ehrlichiosis and toxoplasmosis, special attention should focus on improving diagnostic assays and on implementing adequate control measures.

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