Pesq. Vet. Bras. 31(6):527-532, junho 2011

Occurrence of anti-*Neospora caninum* and anti-*Toxoplasma gondii* antibodies in dogs with visceral leishmaniasis[1]

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ABSTRACT.- Ribeiro R.R., Silva M.E., Silva S.M., Fulgêncio G.O., Pena H.F.J., Frézard F., Michalick M.S.M. & Gennari S.M. 2011. **Occurrence of anti-*Neospora caninum* and anti-*Toxoplasma gondii* antibodies in dogs with visceral leishmaniasis.** *Pesquisa Veterinária Brasileira 31(6):527-532.* Centro de Ciências Agrárias Ambientais e Biológicas, Universidade Federal do Recôncavo da Bahia, Campus Universitário Cruz das Almas s/n, Cruz das Almas, BA 44380-000, Brazil. E-mail: raul@ufrb.edu.br

Uninfected dogs and those naturally infected with *Leishmania chagasi* exhibiting different clinical forms of disease were evaluated for the presence of anti-*Neospora caninum* and anti-*Toxoplasma gondii* antibodies. Blood samples were collected from 110 mongrel dogs. Sera were tested using the indirect fluorescent antibody test (IFAT), and the animals with visceral leishmaniasis (VL) (*n*=60) were classified clinically. Out of the 110 sera investigated, 5 (4.5%) were positive for *N. caninum* (IFAT≥50) and 36 (32.7%) for *T. gondii* (IFAT≥16). Anti-*L. chagasi* antibody titers in asymptomatic dogs (*n*=10) were found to be significantly lower (*P*<0.05) than those in oligosymptomatic ones (*n*=22), which were in turn significantly lower (*P*<0.05) than those in symptomatic ones (*n*=28). No association between *Leishmania* and *N. caninum* infections was observed. Among dogs infected with *L. chagasi*, a tendency (*P*=0.053) towards an association between the infection with *T. gondii* and the appearance of VL symptoms was observed, suggesting that the clinical manifestation of VL in dogs may enhance their susceptibility to *T. gondii.* The possible influence of the immunosuppressive status of canine leishmaniasis in the different clinical forms of the disease is discussed.

INDEX TERMS: Canine visceral leishmaniasis, *Leishmania chagasi, Toxoplasma gondii, Neospora caninum*, co-infection, clinical forms.

RESUMO.- [**Ocorrência de anticorpos anti-*Neospora caninum* e anti-*Toxoplasma gondii* em cães com leishmaniose visceral.**] A presença de anticorpos anti-*Neospora caninum* e anti-*Toxoplasma gondii* foi avaliada em cães não infectados e naturalmente infectados com *Leishmania chagasi* manifestando diferentes formas clínicas da enfermidade. Amostras de sangue foram coletadas de 110 cães sem raça definida. Os soros foram avaliados por meio da reação de imunofluorescência indireta (RIFI) e os animais com leishmaniose visceral (LV) (*n*=60) foram classificados clinicamente. Dos 110 soros analisados, 5 (4,5%) foram reativos para *N. caninum* (RIFI≥50) e 36 (32,7%) para *T. gondii* (RIFI≥16). Os títulos de anticorpos anti-*L. chagasi* em cães assintomáticos (*n*=10) foram significativamente (*P*<0,05) mais baixos que aqueles verificados em oligossintomáticos (*n*=22), que por sua vez foram significativamente menores (*P*<0,05) que em cães sintomáticos

[1] Received on April 27, 2010.
Accepted for publication on November 2, 2010.

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(*n*=28). Não foi observada associação entre infecções por *Leishmania* e *N. caninum*. Entre os cães infectados com *L. chagasi*, verificou-se uma tendência de associação (*P*=0.053) entre infecção com *T. gondii* e aparecimento de sinais clínicos da LV, o que sugere que a manifestação clínica da LV em cães pode aumentar sua susceptibilidade ao *T. gondii*. A provável influência do quadro de imunossupressão em diferentes formas clínicas da leishmaniose canina é abordada.

TERMOS DE INDEXAÇÃO: Leishmaniose visceral canina, *Leishmania chagasi*, *Toxoplasma gondii*, *Neospora caninum*, co-infecção, formas clínicas.

INTRODUCTION

Leishmaniasis is a group of illnesses transmitted by sand flies that affects domestic and wild animals, as well as humans. Dogs are considered the main reservoir of the etiological agent of visceral leishmaniasis (VL) in the urban context and one of the targets in the control strategy. The clinical characteristics of canine visceral leishmaniasis (CVL) vary from animals that are apparently healthy to animals with severe disease (Blavier et al. 2001), with involvement of multiple organs and a wide variety of cutaneous lesions (Slappendel 1988, Ferrer 1992).

With the emergence of foci of the disease in urban areas, VL has assumed an important role in public health in many regions around the world (Marzochi et al. 1994). In the last several years, the number of human and canine cases of VL in the metropolitan region of Belo Horizonte, Minas Gerais, Brazil, has increased. In addition to the great lethality of disease if left untreated, *Leishmania infantum* (= *L.chagasi*), the agent of VL, is capable of modulating lymphocyte function, causing severe immunosuppression in the host (Carvalho et al. 1981, Sacks et al. 1987, Pinelli et al. 1994, Reis et al. 2006a). The compromised immune system can facilitate the infection, proliferation and spread of the pathogens after the initial infection and can allow the emergence of latent infection by opportunistic agents. Since the first case of co-infection of human immunodeficiency virus (HIV) and leishmaniasis, studies have demonstrated that this association increases the replication of HIV, as much *in vitro* as *in vivo* (Olivier et al. 2003), and consequently accelerates the course of infection by HIV (WHO 1997, Wolday et al. 1999, Cruz et al. 2006).

In VL, as well as in all parasitic diseases, specific factors found in the host and the parasite determines the development of pathogenic lesions and, consequently, the clinical signs of infection (Noli 1999, Handman 2001). Mancianti et al. (1988) evaluated dogs naturally infected with *L. infantum* in Italy and suggested that these animals could be classified as asymptomatic, oligosymptomatic or symptomatic.

Neospora caninum and the related parasite *Toxoplasma gondii* are cosmopolitan coccidians that cause neurological disease in dogs, using the dog (McAllister et al. 1998) and the cat (Dubey & Beattie 1988) as the definitive host, respectively; these animals excrete oocysts in their feces, contaminating the environment and infecting a wide range of intermediate hosts.

Co-infection of *L. infantum* and *N. caninum* (Cringoli et al. 1996, Tarantino et al. 2001, Cringoli et al. 2002, Andreotti et al. 2006) and between these parasites and *T. gondii* (Gennari et al. 2006) has been investigated. Some studies have suggested that the immunosuppression caused by *Leishmania* sp. could enhance the susceptibility of dogs to *N. caninum* (Cringoli et al. 2002, Gennari et al. 2006).

The clinical signs of parasitic diseases reflect the interactions of the species or strain with the immune response of the host (Pearson 1993). In VL, the dogs manifest classically distinct clinical forms (Mancianti et al. 1988). The objective of this study was to analyze the humoral immune response against *N. caninum* and *T. gondii* in dogs naturally infected with *L. chagasi* presenting different clinical conditions, and to compare this with healthy dogs' immune response to the same agents.

MATERIALS AND METHODS

Experimental animals

The urban dogs used in this study were captured by the Zoonosis Control Center in the county of Santa Luzia, in the metropolitan region of Belo Horizonte, Minas Gerais, Brazil. Sixty dogs of different breeds, of unknown age and naturally infected with *L. chagasi,* were identified during an epidemiological survey of CVL. The infection and serology diagnoses were confirmed by indirect immunofluorescence antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA). In addition, parasitological diagnosis was reached by observations of parasite forms in a cytological examination of bone marrow aspirates. All infected dogs were clinically classified according to Mancianti et al. (1988) as follows: *asymptomatic* dogs - apparently healthy animals; *oligosymptomatic* dogs - animals exhibiting lymphoid adenopathy, moderate weight loss and/or dull brittle fur and *symptomatic* dogs - animals that exhibited the classical signs of the disease such as cutaneous alterations (alopecia, exfoliative dermatitis or ulcers), onicogriphosis, keratoconjunctivitis, cachexia and anemia. Other 50 healthy dogs with negative serological and parasitological diagnosis tests for *L. chagasi*, from the same municipality, also participated of the study as the VL-uninfected control group.

Serological analysis

Blood samples were obtained by jugular vein puncture utilizing vacutainer-type tubes, and the serum samples were stored at -20ºC prior to testing. The diagnosis of VL was conducted through IFAT with a cut-off of 1:40 as described by Camargo (1974) with modifications. The ELISA was carried out according to a technique modified from Voller et al. (1979), using antigen from lysed cultured promastigotes of *L. chagasi* strain MHOM/BR/1967/BH46, and rabbit IgG anti-dog antibody conjugated with horseradish peroxidase. Optical density (OD) readings were done at 490 nm using a microplate reader (Bio-Rad 2550, Bio-Rad). The serum cut-off corresponded to the average OD of eight samples from *L. chagasi*-negative dogs from a nonendemic leishmaniasis area added to three standard deviations.

Anti-*N. caninum* and anti-*T. gondii* antibodies were detected by IFAT based on the studies of Dubey et al. (1988) and

Camargo (1974), respectively, with cut-off titers of 50 for *N. caninum* and 16 for *T. gondii*. Positive and negative control sera were included in each slide. Positive sera were serially diluted two-fold and tested to establish the maximum reaction titer.

Statistical analysis

Statistical analyses were conducted using the SPSS software version 9.0 for Windows. Possible associations between samples seropositive and seronegative for *Leishmania* and the presence of anti-*T. gondii* and anti-*N. caninum* antibodies were analyzed using the Fischer test or the Chi-square test ($P \leq 0.05$). The comparisons between *T. gondii* and *N. caninum* occurrence in the different VL dog groups were analyzed by Chi-square test ($P \leq 0.05$).

The comparison of *Leishmania* titer ranks from asymptomatic, oligosymptomatic and symptomatic dogs was performed using the Kruskal-Wallis test, with the Mann-Whitney test as a post-roc test.

RESULTS

The antibody titer values and the clinical category of the dogs naturally infected with *L. chagasi* are shown in Table 1. Among the 60 dogs naturally infected with *L. chagasi*, 10 (16.7%) were clinically classified as asymptomatic, 22 (36.7%) as oligosymptomatic and 28 (46.7%) as symptomatic. The titers ranged from 80 to 81,920 and the most frequent titer was 20,480 (14/60). There was a significant difference between *Leishmania* titer ranks among the three clinical forms ($P<0.05$). Titer ranks from asymptomatic animals were lower than oligosymptomatic titer ranks ($P=0.03$), which were in turn lower than symptomatic titer ranks ($P=0.03$).

Table 2 displays the frequency of different clinical signs of VL in naturally infected dogs (oligosymptomatic and symptomatic). Lymphoadenopathy (44/50, 88%) was the most frequent sign, followed by skin disorders, which included in decreasing order of frequency, dull fur (40/50, 80%), exfoliative dermatitis (38/50, 76%), hipotricosis/ alopecia (30/50, 60%) and skin ulcers (16/50, 32%).

Table 1. IgG antibody titers for *Leishmania chagasi* as evaluated by immunofluorescent antibody test in dogs (*n*=60) from the municipality of Santa Luzia, Minas Gerais, Brazil

Leishmania chagasi			Clinical forms of Leishmaniasis		
Titer	Number of positive dogs	%	Number of dogs		
			Asympto-matic	Oligosympto-matic	Sympto-matic
40	0	0	0	0	0
80	1	1.7	1	0	0
160	5	8.3	2	3	0
320	6	10.0	2	3	1
640	3	5.0	2	1	0
1280	3	5.0	1	1	1
2560	4	6.7	1	2	1
5120	3	5.0	0	0	3
10,240	7	11.7	0	3	4
20,480	14	23.3	1	4	9
40,960	10	16.7	0	5	5
81,920	4	6.7	0	0	4
Total (%)	60	100	10 (16.6)	22 (36.6)	28 (46.6)

Table 2. Frequency of different clinical signs in dogs (*n*=50) naturally infected with *Leishmania chagasi* from Santa Luzia, Minas Gerais, Brazil

Clinical signs	Number of dogs	Percent with symptom
Lymphoadenopathy	44	88
Dull fur	40	80
Exfoliative dermatitis	38	76
Hypotrichosis / Alopecia	30	60
Skin ulcers	16	32
Emaciation	16	32
Nasal hyperkeratosis	12	24
Ophthalmopathies	12	24
Onicogriphosis	4	8

Table 3. IgG antibody titers for *Neospora caninum* and *Toxoplasma gondii* as evaluated by immunofluorescent antibody test in dogs (*n*=110) from the municipality of Santa Luzia, Minas Gerais, Brazil

N. caninum			*T. gondii*		
Titer	Number of positive dogs	Occurrence (%)	Titer	Number of positive dogs	Occurrence (%)
50	5	100.0	16	4	11.1
			32	3	8.3
			64	5	13.9
			128	9	25.0
			256	5	13.9
			512	3	8.3
			1,024	5	13.9
			4,096	2	5.6
Total	5	4.5		36	32.7

Table 4. Occurrence of antibodies against *Neospora caninum* and *Toxoplasma gondii* in dogs positive (*n*=60) and negative (*n*=50) for *Leishmania chagasi* from the municipality of Santa Luzia, Minas Gerais, Brazil

	L. chagasi		
	Positive(%)	Negative(%)	*P* value
T. gondii			
Positive	15 (25.0)	21 (42.0)	0.058[a]
Negative	45 (75.0)	29 (58.0)	
N. caninum			
Positive	2 (3.3)	3 (6.0)	0.657[b]
Negative	58 (96.7)	47 (94.0)	

[a] Chi-square Test.
[b] Fisher Test.

Ophthalmopathies were observed in 12 of 50 (24%) dogs and onicogriphosis was observed in 8% (4/50) of the animals.

Table 3 illustrates the antibody titer values found for *N. caninum* and *T. gondii*. The occurrence of anti-*N. caninum* antibodies was 4.5% (1.5%<95% CI<10.3%) with 5 positive out of 110 dogs, all with titers of 50. For *T. gondii*, 36 out of 110 dogs (32.7%) had positive results (24.1%<CI 95%<42.3%) and titers ranged from 16 to 4,096, with 128 being the most frequent titer (9/36).

Table 4 presents the occurrence of anti-*T. gondii* and *anti-N. caninum* antibodies in dogs positive and negative for *L.*

Table 5. Occurrence of antibodies against *Neospora caninum* and *Toxoplasma gondii* in dogs infected with *Leishmania chagasi* with different clinical forms of canine leishmaniasis (*n*=60) and in non-infected control dogs (*n*=50) from the municipality of Santa Luzia, Minas Gerais, Brazil

Clinical Signs	Nº of Dogs (%)	Nº of Positive Dogs(%)	
		N. caninum	*T. gondii*
Asymptomatic	10 (16.7)	0(0.0)[a]	0(0.0)[a]
Oligosymptomatic	22 (36.7)	1(4.5)[a]	7(31.8)[a]
Symptomatic	28 (46.6)	1(3.6)[a]	8(28.6)[a]
Absence	10 (16.7)	0(0.0)[a]	0(0.0)[a]
Presence	50 (83.3)	2(2.5)[a]	15(30.0)[b]
Total	60 (100.0)	2(3.3)[c]	15(25.0)[d]
Control Group	50 (100.0)	3 (6.0)[c]	21(42.0)[d]
Total	110 (100,0)	5(4,5)	36(32,7)

Comparisons are between categories in the same column.
[a] $P \geq 0.05$ (Chi-square Test).
[b] $P=0.053$ (Fisher Test).
[c] $P=0.657$ (Fisher Test).
[d] $P=0.058$ (Chi-square Test).

chagasi infection. The occurrence of anti-*N. caninum* antibodies was 6.0% (3/50) in the *L. chagasi*-negative group, whereas in the *L. chagasi* seropositive group, the occurrence was 3.3% (2/60). No association between *Leishmania* infection and presence of anti-*N. caninum* antibodies was observed ($P=0.657$). An occurrence of 42.0% (21/50) and 25.0% (15/60) of anti-*T. gondii* antibodies in the seronegative and seropositive *L. chagasi* groups, respectively, was observed. The statistical analyses showed no significant association between canine leishmaniasis and *T. gondii* antibodies. However, when the presence (oligosymptomatic and symptomatic) or absence (asymptomatic) of clinical signs in VL dogs were evaluated (Table 5), a tendency of association was observed with a *P* value in the limit of significance ($P=0.053$, Fisher Test), with higher occurrence of infection of *T. gondii* in animals with symptoms of VL. No significant difference was observed among *T. gondii* titer ranks between the oligosymptomatic and symptomatic groups ($P>0.05$).

In the VL-infected group, 3.3% (2/60) of the animals were simultaneously positive for *N. caninum* and *T. gondii,* and in the *Leishmania* seronegative group, co-infection was observed in 2.0% (1/50) of the dogs. The proportion test revealed no significant difference in *T. gondii* and *N. caninum* co-infections in the *L. chagasi* positive or negative groups ($P>0.05$).

DISCUSSION

Studies with dogs infected with *Leishmania* spp. and associations with other coccidian parasites have been conducted; however, different results were observed. Co-infection among these three parasites have been studied in dogs from Brazil by Gennari et al. (2006) in Araçatuba, SP, however, the clinical status of the animals relative to VL was not reported. In the study conducted in Araçatuba, the prevalence of *N. caninum* (17.6%) and *T. gondii* (36.8%) was higher than the values found in the present study, and an association between the presence of *L. chagasi* and *N. caninum* antibodies was observed.

Two other studies conducted in Mato Grosso do Sul, Brazil (Andreotti et al. 2006) and in South of Italy (Cringoli et al. 2002) observed, respectively, a lack of association and an association between *L. chagasi* and *N. caninum*-positive dogs. Also in Italy, a description of a single case of a simultaneous infection with *L. infantum* and *N. caninum* in a dog with a skin infection was reported. The authors concluded that the immunosuppressive effect of *L. infantum* may have contributed to the development of the *N. caninum* infection; however, based on histological features, the skin lesions observed were most likely due to *Leishmania* (Tarantino et al. 2001).

In the present study, *N. caninum* seroprevalence was 4.5%, and its occurrence in *Leishmania* positive (3.3%) and negative (6.0%) dogs was lower than the values observed in dogs from other regions of Brazil (Gennari et al. 2002, Cañón-Franco et al. 2003, Azevedo et al. 2005, Andreotti et al. 2006, Gennari et al. 2006), indicating a low rate of environmental exposure of the dogs in the region to this agent. Another explanation for the relatively low occurrence of anti-*N. caninum* antibodies is the feeding habits of the dogs in urban areas, where the diet is mainly based on commercial food and contact with other sources of animal products is reduced. Studies have demonstrated significant differences in *N. caninum* occurrence in rural and urban dogs (Wouda et al. 1999, Fernandes et al. 2004, Wanha et al. 2005), and between stray and owned animals (Gennari et al. 2002), with low values for the owned and urban dogs. The dogs from the present study were captured by the Zoonosis Control Center, so the majority was stray dogs with a higher opportunity to acquire the oocysts contaminating the environment.

All dogs with anti-*N. caninum* antibodies had titers of 50, and against *T. gondii,* the most frequent titer was 128. In a cross-section study conducted in apparently healthy dogs from the state of Paraíba, in the northeast region of Brazil, 50% of the seropositive dogs presented low titers; however, very high titers were also observed for both coccidian parasites (Azevedo et al. 2005).

Toxoplasma gondii occurrence was significantly higher than that of *N. caninum* in tested dogs from both groups, which is expected based on observations from other regions from Brazil (Souza et al. 2002, Souza et al. 2003, Azevedo et al. 2005, Dubey et al. 2007), reflecting the high exposure of this host to *T. gondii* in Brazil and the fact that dogs are considered good sentinels for *T. gondii* environmental contamination (Dubey & Beattie 1988).

The co-infection between coccidians in the dogs was 2.0% (1/50), and 3.3% (2/60) of animals were simultaneously positive for all three parasites. These data were similar with previous studies, in which co-infection of *N. caninum* and *T. gondii* in dogs varied between 1% and 3% (Lindsay et al. 1990, Mineo et al. 2001), and was different from the 17.3% observed by Gennari et al. (2006).

The number of dogs positive for *N. caninum* in each *L.*

chagasi clinical group was not significantly different, suggesting that VL, independently of the clinical form of disease, does not influence *N. caninum* infection. When VL dogs were analyzed as a single group, no association between *Leishmania* infection and the presence of antibodies against *N. caninum* was observed. Similar results were found in asymptomatic dogs from Campo Grande (Andreotti et al. 2006). However, Gennari et al. (2006) verified a significant association between dogs naturally infected with *L. chagasi* and high occurrence of anti-*N. caninum* antibodies (8.6 times greater than VL negative dogs). Cringoli et al. (2002), studying 1,058 asymptomatic dogs from Italy, also reported the same association and suggested that immunosuppression is likely the cause of the greater prevalence of *N. caninum* in *Leishmania* seropositive dogs.

Toxoplasmosis is a disease usually associated with immunosuppressive disorders (Innes 1997). In the present survey, among dogs infected with *L. chagasi*, a tendency of association between infection with *T. gondii* and presence of symptoms was verified, suggesting that the clinical form of canine leishmaniasis seems to enhance susceptibility to *T. gondii*. It is expected that the immunodeficiency caused by *Leishmania* helped underlying agents and subsequently, an immune response to these agents was mounted. In humans, mice (Liew & O'Donnell 1993) and dogs (Cabral et al. 1992, Pinelli et al. 1994, Pinelli et al. 1995), it was demonstrated that protective immunity against leishmaniasis is mediated by T cells and is associated with the production of IFN-γ. The ineffectiveness of cellular immunity is a basic aspect of pathogenesis of the illness and its progression (Alvar et al. 2004). Due to the ineffectiveness of cellular immunity in infected dogs, it is likely that dogs with VL that are manifesting symptoms facilitate the multiplication and spread of parasites that are primarily controlled by cellular defenses, such as *T. gondii* (Denkers & Gazzinelli 1998), when compared to asymptomatic dogs, in which protective cellular immunity is present (Cabral et al. 1992, Pinelli et al. 1994, Pinelli et al. 1995).

Some authors have shown that detection of antibodies indicates presence of the parasite, but antibodies titers are not correlated with the severity of clinical signs or presentation of the illness in canine VL (Abranches et al. 1991, Ferrer et al. 1995, Campino 2002). However, in this study, significant levels of anti-*Leishmania* antibodies were observed in different clinical groups, with higher titers in symptomatic dogs. Other publications have also noted a correlation between the levels of immunoglobulin isotypes and different clinical forms of canine VL (Almeida et al. 2005, Reis et al. 2006b, Costa-Val et al. 2007).

The clinical signs seen in the dogs in this experiment corroborated the results of the majority of authors (Slappendel 1988, Ciaramella et al. 1997, Koutinas et al. 2001, Amusategui et al. 2003, Costa-Val et al. 2007), and demonstrated lymphoadenopathy as the most common clinical manifestation of the disease.

Further studies involving different immunological aspects of the infection should be developed with dogs infected with *Leishmania,* including all clinical forms, to determine the role of the immune system in co-infection by other parasites.

Acknowledgements.- To Mr. Ricardo A. Dias for guidance in statistical analysis, and Mrs. Elza Moreira and Mr. Weverton M. Sampaio for technical support. This research was supported by the Laboratório de Sorologia/ICB/UFMG and the Laboratório de Doenças Parasitárias/ FMVZ/USP.

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