Presence of anti- *Neospora caninum* and *Toxoplasma gondii* antibodies in dogs with visceral leishmaniosis from the region of Araçatuba, São Paulo, Brazil

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Abstract

Dogs that had positive and negative sera to *Leishmania chagasi* from the region of Araçatuba, São Paulo, Brazil, were evaluated for the presence of anti-*Neospora caninum* and anti-*Toxoplasma gondii* antibodies as potential co-infecting agents. Blood samples were collected from 204 dogs and out of them 98 were carriers of leishmaniosis. Sera were tested for the presence of anti-*L. chagasi* antibodies by ELISA, and anti-*T. gondii* and anti-*N. caninum* by an indirect fluorescent antibody test (IFAT). Age, gender, and association between the presences of anti-*L. chagasi* antibodies and seroprevalence to *N. caninum* and *T. gondii* were analyzed by chi-square test. Out of the 204 sera investigated, 36 (17.6%) were positive for *N. caninum* (IFAT=50) and 75 (36.8%) to *T. gondii* (IFAT=16) with titers that varied from 50 to 6400 for *N. caninum*, and from 16 to 16384 for *T. gondii*. The co-presence of anti-*L. chagasi*, *N. caninum* and *T. gondii* antibodies was observed in 17 (8.3%) dogs. Antibodies to *N. caninum* were observed in four (3.8%) out of 106 dogs that were negative for *L. chagasi*, and in 32 (32.6%) out of the 98 dogs that were positive for *L. chagasi*. Anti-*T. gondii* antibodies were found in 40 (41.0%) and in 35 (33.0%) of the 98 positive dogs and in 106 negative dogs for *L. chagasi*, respectively. An association between the presence of antibodies against *L. chagasi* and a positive response to *N. caninum* ($p<0.001$) was observed. The gender and age of the dogs did not show an association between the presence of antibodies and any of the agents studied ($p>0.05$), with the exception of age and presence of anti-*L. chagasi* antibodies, in which only a slight association was observed ($p=0.038$). Within this interaction, a higher number of dogs, older than four years, were positive for this agent when compared to other age groups.

Key-words:
Leishmania chagasi.
Toxoplasma gondii.
Neospora caninum.
Dogs.
Epidemiology.
Brazil.

Introduction

Visceral leishmaniosis is an antropozoonosis caused by a protozoan from the genus *Leishmania,* with *Leishmania chagasi* being responsible for most of the cases reported in Brazil. While this agent have been found in wild dogs, cats, marsupials and rodents, domestic dogs are considered to be the main epidemiological source of this microorganism [1, 2].

The first case of visceral leishmaniosis reported in the State of São Paulo was diagnosed in the region of Araçatuba in 1998, and since then, the number of cases has increased in the region (a total of 40 municipals) through canine transmission [3]. From a total number of 27,465 dogs, approximately 15,000 were found to be infected with visceral leishmaniosis and all were sacrificed. Moreover, in the same region a human case was reported in 1999, followed

by 187 occurrences and 19 deaths in 2002[4].

The clinical disease in dogs may present complications due to the simultaneously occurrence of opportunistic infections such as demodicosis, scabieis and dermatophytosis, in addition to associated infections with *Ehrlichia* sp, *Babesia* sp, *Dirofilaria imitis*, *Hepatozoon canis* and *Cryptococcus* sp[5,6,7,8,9].

Neospora caninum is a widespread parasite in the world and causes abortion and problems in the central nervous system of many animals including dogs[10]. Canids (dogs and coyotes) are considered as definitive hosts of *N. caninum*[11,12] and this species has strongly influenced the epidemiology of this disease. In Brazil, this agent has been reported to be highly prevalent in dogs[13,14,15]. Likewise, *Toxoplasma gondii,* the agent of toxoplasmosis, is highly common in Brazilian dogs and is a structurally related *N. caninum* protozoan[16,17]. Its establishment and dissemination depend upon the host's immune response, and in many cases, toxoplasmosis is associated with immunosuppression diseases[18].

Studies conducted by Tarantino et al.[19] and Cringoli et al.[20], in Italy, showed a co-presence of anti-*N. caninum* and anti-*Leishmania infantum* antibodies in dogs. These authors reported that immunosuppresion is the most likely cause of greater prevalence of *N. caninum* antibodies in dogs that were seropositive for *Leishmania*. Furthermore, Cringoli et al.[20] found that the presence of one of the agents is a risk factor for the incidence of the other.

The objectives of this study were to determine the presence of anti- *Toxoplasma gondii* and anti-*Neospora caninum* antibodies in dogs that were or were not carriers of *Leishmania chagasi*, and evaluate the data for possible associations between these pathogenic agents.

Materials and Methods

Experimental animals and blood samples

The dogs used to conduct this study were examined at the "Hospital Veterinário da Universidade Estadual Paulista", located in the region of Araçatuba (latitude: 21.20889°S and longitude 50.43278°W), São Paulo, Brazil, from September 2000 to September 2001. Two hundred and four urban dogs were examined, and out of them, 98 were carriers of visceral leishmaniosis, while 106 were serologically negative to this agent.

The total group had 98 females (48.0%) and 106 males (52.0%), and they were divided into three age categories: 47.5% were younger than two years (97/204); 29.4% were from two to four years (60/204), and 23.1% were older than four years (47/204). The dogs examined were from a variety of breeds (17 breeds), but 70.6% were of a non-defined mix breed (144/204).

Blood samples were drawn by jugular vein puncture utilizing vacutainer type tubes, and the serum samples were stored at $^{-}$20 ºC prior testing.

Serological analysis

The diagnosis of the visceral leishmaniosis was conducted through ELISA utilizing lyzed antigen from *L. chagasi* promastigotes in culture, which were obtained from animals from an endemic area, and rabbit IgG anti-dog as conjugated with horseradish peroxidase (Sigma, St. Louis, MO, USA). Optical density (OD) readings were done at 492nm using a microplate reader (Labsystems Multiskan EX), according to Lima et al.[21] The cut-off (serum) was determined using the mean + 3 SD of the readings obtained for serum of healthy dogs (n=20) from nonendemic leishmaniasis areas. Results were expressed by the means of sera ODs from three replicates.

Anti-*N. caninum* and anti-*T. gondii* antibodies were detected by indirect immunofluorescence antibody test (IFAT) based on Dubey et al.[22] and Camargo [23], respectively, with cut-off point titers at 1:50 for *N. caninum* and 1:16 for *T. gondii*.

Positive sera were serial diluted and tested to establish the maximum reaction titer.

Positive and negative control sera were included in each reaction.

Statistical analysis

Statistical analysis were conducted utilizing the SPSS software version 10.0 for Windows. Possible associations between seropositive and seronegative samples for *Leishmania*, the presence of anti-*T. gondii* and anti-*N. caninum* antibodies and the sex and age of dogs were analyzed through chi-square test (P=0.05). Due to the large number of animals without a defined breed and to the low number of dogs of a known breed, this variable was not analyzed.

The mean values of the anti-*T. gondii* and anti-*N. caninum* antibody titers in dogs serologically positive and negative for *L. chagasi* were compared by the non-parametric Mann-Whitney test.

Results

The occurrence of leishmaniosis in the group of samples was 48.0% (I.C. 95%: 41.0% to 55.0%). The presence of anti-*N. caninum* antibodies was 17.6% (I.C. 95%: 12.7% to 23.6%) with 36 out of 204 dogs positive and titers of 50 to 6400. For *T. gondii*, 36.8%, or 75 out of 204 dogs, had positive results (I.C. 95%: 30.1% to 43.8%) and titers that ranged from 16 to 16384. Table 1 illustrates the antibody titer values found for *N. caninum* and *T. gondii*.

The occurrence of antibodies against each one of the agents as well as the associations are given in Table 2. Out of the 204 animals examined, 17 (8.3%) had antibodies to the three agents *(L. chagasi, T. gondii* and *N. caninum*) and 69 (33.8%) dogs were seronegative to the agents studied.

Anti-*N. caninum* antibodies were found in 33.8% (4/106) dogs seronegative for *L. chagasi* and in 3,8% (32/98) of the dogs seropositive for this agent. The most frequent titer was 100 (13/204), and a titer of 6400 was established in two seronegative and in one seropositive sample for *L. chagasi*.

Anti-*T. gondii* antibodies in the seronegative group for *L. chagasi* showed an occurrence of 33.0% (35/106), while in the seropositive group for *L. chagasi* the presence of anti-*T. gondii* antibodies was 40.8% (40/98). Titers equal to 16384 were found in four of the 98 animals that were seropositive for *L. chagasi*, whereas a maximum titer of 1024 was found among the seronegative samples for *L. chagasi*.

The chi-square test showed a non-association between the positive condition for *Leishmania* and the presence of antibodies anti-*T. gondii* (p=0.248), but it revealed an interaction between the positive condition for *Leishmania* and the presence of antibodies anti-*N. caninum* (p<0.001).

Results on the serology for each one of the agents by sex and age are found in table 3. Females had a higher prevalence of antibodies for *N. caninum,* 20.4% (20/98), than that observed for males, which was 15.1% (16/106). However, the chi-square test did not show an association between sex and the presence of anti-*N. caninum* antibodies in this population (p=0.320).

The prevalence of anti-*T. gondii* antibodies in females and males was very similar, 37.8% (37/98) and 35.9% (38/106), respectively, and no significant difference was observed between these values (p=0.778).

The studied group was divided into three age ranges and showed a uniform distribution in relationship to the occurrence of anti-*T. gondii* and *N. caninum* antibodies. The highest number of *N. caninum* seropositive samples (18.6%) was found in dogs younger than two-year-old (18/97), followed by those older than four-year-old (17.0%, 8/47), and by those between two- and four-year-old (16.7%, 10/60). No association between age and presence of anti-*N. caninum* antibodies was established (p=0.948).

Regarding *T. gondii*, dogs older than four years had a prevalence of 44.7% (21/47), while those younger than two and between two and four years old showed similar frequencies, 35.1% (34/97) and 33.3% (20/60), respectively. An association between

age and *T. gondii* was not observed (p=0.43). With respect to *L. chagasi*, animals younger than two years and between two and four years old had the lowest frequency, with 45.4% (44/97) and 40.0% (24/60), respectively, when compared to the group of dogs that was older than four years (63.8%, 30/47). A slight interaction (p=0.038) was found between age and the presence of anti-*L. chagasi* antibodies.

No significant difference was observed between the mean titer values of anti-*T. gondii* (p=0.1622) and anti-*N. caninum* (p=0.1079) antibodies in dogs serologically positive and negative for *L. chagasi*.

Discussion

The prevalence of *L. chagasi* as a causative agent of visceral leishmaniosis in dogs occurs endemically in several municipalities from all regions of Brazil, with the exception of the South. In Araçatuba, located in the Northwest of São Paulo state, there is a high number of annual cases of this disease [4].

Studies conducted in Italy showed the occurrence of simultaneous infections with seropositive dogs for *L. infantum* also being positive for *N. caninum* [19, 20]. Cringoli et al. [20] concluded from their studies that the main risk factor for the infection caused by *N. caninum* in dogs found in the Southern Italy was the presence of anti-*L. infantum* antibodies and this interaction showed non-correlation between sex and age of the animals.

Similar results were found in this study regarding the infection caused by *N. caninum*. It showed that dogs naturally infected with *L. chagasi* had occurrence of anti-*N. caninum* antibodies (32.6%) 8.6 times greater than dogs that were negative for *L. chagasi* (3.8%), and these were significantly different. This result corroborates those reported by Tarantino et al.[19] and Cringoli et al.[20], who stated that immunosupression is likely the cause of the greater prevalence of *N. caninum* in *Leishmania* seropositive dogs.

This study also showed that the presence or absence of infection caused by *L. chagasi* did not have any influence on anti-*T.gondii* antibodies. Data showed occurrence values similar to those found for dogs from *L. chagasi* positive and negative groups, even though toxoplasmosis is a disease that usually is associated with immunosuppressive disorders[18]. However, we observed that

Table 1 - IgG antibody titers evaluated by IFAT for *Neospora caninum* and *Toxoplasma gondii* in dogs (n=204) from the municipality of Araçatuba, São Paulo, Brazil

Neospora caninum			*Toxoplasma gondii*		
Titer	Number of positive dogs	Occurrence%	Titer	Number of positive dogs	Occurrence %
50	6	2.9	16	8	3.9
100	13	6.4	32	5	2.5
200	4	2.0	64	14	6.9
400	6	2.9	128	12	5.9
800	1	0.5	256	10	4.9
3200	3	1.5	512	8	3.9
6400	3	1.5	1024	7	3.4
			2048	4	2.0
			4096	3	1.5
			16384	4	2.0
Total	36	17.6		75	36.8

Table 2 - Occurrence of antibodies against Leishmania chagasi, Toxoplasma gondii and/or Neospora caninum in dogs (n = 204) from the municipality of Araçatuba, São Paulo, Brazil

Condition	Number of Dogs	Occurrence (%)	
		Condition	Population
Negative *L. chagasi*, negative *T. gondii* and negative *N. caninum*	69	65.1	33.82
Negative *L. chagasi*, positive *T. gondii* and negative *N. caninum*	33	31.1	16.18
Negative *L. chagasi*, negative *T. gondii* and positive *N. caninum*	2[a]	1.9	1.0
Negative *L. chagasi*, positive *T. gondii* and positive *N. caninum*	2[a]	1.9	1.0
TOTAL	106	100	
Positive *L. chagasi*, negative *T. gondii* and negative *N. caninum*	43	43.9	21.1
Positive *L. chagasi*, positive *T. gondii* and negative *N. caninum*	23	23.5	11.3
Positive *L. chagasi*, negative *T. gondii* and positive *N. caninum*	15[b]	15.3	7.3
Positive *L. chagasi*, positive *T. gondii* and positive *N. caninum*	17[b]	17.3	8.3
TOTAL	98	100	

[a, b] Different letters at the same column - P=0,05

Table 3 - Composition of the sample groups and serological data from the 204 dogs tested for *Leishmania chagasi*, *Neospora caninum* and *Toxoplasma gondii*, from the municipality of Araçatuba, São Paulo, Brazil

Characteristics	Number of Dogs (%)	Number of positive dogs (%)		
		L. chagasi	*N. caninum*	*T. gondii*
SEX				
Male	106 (52.0)	50 (47.2)	16 (15.1)	38 (35.9)
Female	98 (48.0)	48 (49.0)	20 (20.4)	37 (37.8)
AGE				
≤ 2-year-old	97 (47.5)	44 (45.4[a])	18 (18.6)	34 (35.1)
> 2- to 4-year-old	60 (29.4)	24 (40.0[a])	10 (16.7)	20 (33.3)
≥ 4-year-old	47 (23.1)	30 (63.8[b])	8 (17.0)	21 (44.7)

[a, b] Different letters at the same column - P=0,05

56% (55/98) of the dogs with leishmaniosis were positive for either *T. gondii* or *N. caninum* or for both, in contrast with 35% (37/106) that was found in the leishmaniosis negative group.

This study did not show an association between animal age and gender, and the presence of *N. caninum* and *T. gondii*. However, there was a positive association between the age and *L. chagasi* infection, which was more frequently found in dogs older than four years. Ferrer [24] stated that the majority of dogs younger than three years that showed symptoms of visceral leishmaniosis were genetically predisposed animals. Nevertheless, when this disease occurred in animals older than three years, the most likely causes were related to immunosuppressive factors, such as neoplasia, use of medication or the presence of other infections.

While studies conducted in Italy showed that male dogs were more likely to be infected with *Leishmania* than females [20, 25] no such results were found in our study.

The present study supports the need for further studies with dogs presenting visceral leishmaniosis and anti-*T. gondii* and anti-*N. caninum* antibodies in endemic areas to better understand the interaction between these agents and the role of each in the epidemiology of these diseases.

Presença de anticorpos anti-*Neospora caninum* e *Toxoplasma gondii* em cães com leishmaniose visceral da região de Araçatuba, São Paulo, Brasil

Palavras-chave:
Leishmania chagasi.
Toxoplasma gondii.
Neospora caninum.
Cães.
Epidemiologia.
Brasil.

Resumo

Cães soropositivos e soronegativos à *Leishmania chagasi* da região de Araçatuba, São Paulo, Brasil, foram avaliados quanto à presença de possível co-infecção por *Neospora caninum* e *Toxoplasma gondii*. Amostras de sangue de 204 cães, sendo 98 portadores de leishmaniose, foram coletadas e os soros testados quanto à presença de anticorpos anti-*L. chagasi* pelo método de ELISA e anti-*T. gondii* e *N. caninum* pela reação de imunofluorescência indireta (RIFI). Idade, sexo e possíveis associações entre a presença de anticorpos anti-*L. chagasi* e a soroprevalência de *N. caninum* e *T. gondii* foram analisados pelo teste do Qui-quadrado. Dos 204 soros examinados, 36 (17,6%) foram positivos para *N. caninum* (RIFI ^{3}50) e 75 (36,8%) para *T. gondii* (RIFI ^{3}16) com títulos que variaram de 50 a 6.400 para *N. caninum*, e de 16 a 16.384 para *T. gondii*. A co-presença de anticorpos anti-*L. chagasi, N. caninum* e *T. gondii* foi observada em 17 (8,3%) cães. Anticorpos anti-*N. caninum* foram observados em quatro (3,8%) dos 106 cães negativos e em 32 (32,6%) dos 98 cães positivos à *L. chagasi*. Anticorpos anti-*T. gondii* foram encontrados em 40 (41%) e em 35 (33%) dos 98 cães positivos e dos 106 cães negativos à *L. chagasi*, respectivamente. Foi observada associação entre a presença de anticorpos anti-*L.chagasi* e a presença de anticorpos anti-*N. caninum* ($p<0,001$). O sexo e a idade dos cães não apresentaram associação com a presença de anticorpos para nenhum dos agentes estudados ($p>0,05$), com exceção da idade e presença de anticorpos anti-*L. chagasi*, que apresentou uma associação fraca ($p=0,038$), com maior número de cães com mais de quatro anos de idade positivos a esse agente quando comparado às outras idades.

References

1 RIBEIRO, V. M. Leishmanioses. **Revista do Conselho Federal de Medicina Veterinária**, v. 3, n. 11, p. 13-14, 1997.

2 SANTA ROSA, I. C. A., OLIVEIRA, I. C. S. Leishmaniose visceral: breve revisão sobre uma zoonose reemergente. **Clinica Veterinária**, v. 2, n. 11, p. 24-28, 1997.

3 FEITOSA, M. M. **Leishamaniose visceral:** um desafio crescente. São Paulo: Intervet Pet, 2001. 15 p.

4 MIRACELLY, K. Experiência pode mudar o quadro da doença no país. **Folha da Região,** Araçatuba, 26 jan. 2003. Cidades, p. B4.

5 SCHAER, M., MEYER, D. J., YOUNG, D. G. A dual infection of *Leishmania donovani* and *Ehrlichia canis* in a dog. **Compendium on Continuing Education**, v. 7, n. 7, p. 531-534, 1995.

6 SLAPPENDEL, R. J., FERRER, L. Leishmaniosis. In: GREENE, C. E. **Clinical microbiology and infectious diseases of the dog and cat.** Philadelphia: W. B. Saunders, 1990, p.450-458.

7 CIARAMELLA, P., et al. A retrospective clinical study of canine leishmaniasis in 150 dogs naturally infected by *Leishmania infantum*. **Veterinary Record**, v. 141, p. 539-543, 1997.

8 NOLI, C. *Leishmaniosis canina* . **Waltham Focus,** v. 9, n. 2, p. 16-24, 1999.

9 BURACCO, P.; GALLO, M. G. Bone lesions caused by *Cryptococcus laurentii* associated with subclinical visceral leishmaniasis in a dog. **Canine Practice**, v. 15, n. 2, p.5-11, 1990.

10 DUBEY, J. P., et al. Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent and experimental transmission. **Journal of the American Veterinary Medical Association**, v. 193, p. 1259-1263, 1988.

11 McALLISTER, M. M., et al. Dogs are definitive hosts of *Neospora caninum*. **International Journal Parasitology**, v. 28, p. 1473-1478, 1998.

12 GONDIM, L. F. P., et al. Coyotes (*Canis latrans*) are definitive hosts of *Neospora caninum*. **International Journal for Parasitology**, v. 34, p. 159-161, 2004.

13 GENNARI, S. M., et al. Occurrence of *Neospora*

caninum antibodies in sera from dogs of the city of São Paulo, Brazil. **Veterinary Parasitology**, v. 106, n. 2, p. 177-179, 2002.

14 SOUZA, S. L. P.;et al Prevalence of *Neospora caninum* antibodies in dogs from dairy farms in Paraná, **Brazil. Journal of Parasitology**, v. 88, n. 2, p. 408-409, 2002.

15 CAÑÓN-FRANCO, E. A., et al. Prevalence of antibodies anti-*Neospora caninum* in dogs from Amazon, Brazil. **Veterinary Parasitology**, v. 115, p. 71-74, 2003.

16 DUBEY, J. P., LINDSAY, D. S. A review of *Neospora caninum* and neosporosis. **Veterinary Parasitology**, v. 67, p. 1-59, 1996.

17 SOUZA, S. L. P., et al. Occurrence of *Toxoplasma gondii* antibodies in sera from dogs of the urban and rural areas from Brazil. **Brazilian Journal of Veterinary Parasitology,** v. 12, n. 1, p. 1-3, 2003.

18 INNES, E.A. Toxoplasmosis: comparative species susceptibility and host immune response. **Comparative immunology, microbiology and infectious diseases**, v. 20, n. 2, p. 131-138, 1997.

19 TARANTINO, C., et al. *Leishmania infantum* and *Neospora caninum* simultaneous skin infection in a young dog in Italy. **Veterinary Parasitology**, v. 102, p. 77-83, 2001.

20 CRINGOLI, G., et al. Serological survey of *Neospora caninum* and *Leishmania infantum* co-infection in dogs. **Veterinary Parasitology**, v. 106, p. 307-313, 2002.

21 LIMA, V.M.F.; et al. Anti-*Leishmania* antibodies in cerebrospinal fluid from dogs with visceral leishmaniasis. Brazilian **Journal of Medical and Biological Research**, v. 36, n. 4, p. 485-489, 2003.

22 DUBEY, J. P., et al. Newly recognized fatal protozoan disease of dogs. **Journal of the American Veterinary Medical Association**, v. 192, n. 9, p. 1269-1285, 1988.

23 CAMARGO, M. E. Introdução às técnicas de imunofluorência. 1974. **Revista Brasileira de Patologia Clínica**, v. 10, n. 4, p. 143-171, 1974.

24 FERRER, L.M. Canine leishmaniosis: evaluation of the immunocompromised patient. In: WSAVA CONGRESS CHOOSES, 8., 2002, Granada. ***Proceedings***. Disponível em: http://www.vin.com/proceedings/Proceedings.plx?CID=WSAVA2002&PID+2653>. Acesso em: 17 March 2004.

25 ZAFFARONI, E.; et al. Epidemiological patterns of canine leishmaniosis in western Ligúria (Italy). **Veterinary Parasitology**, v. 81, p. 11-19, 1999.