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%) 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.

Introduction
Visceral leishmaniosis is an anthropozoonosis 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.

Key-words:
Leishmania chagasi.
Toxoplasma gondii.
Neospora caninum.
Dogs.
Epidemiology.
Brazil.
by 187 occurrences and 19 deaths in 20024.

The clinical disease in dogs may present complications due to the simultaneously occurrence of opportunistic infections such as demodicosis, scabies and dermatophytosis, in addition to associated infections with *Ehrlichia* sp, *Babesia* sp, *Dirofilaria imitis*, *Hepatozoon canis* and *Cryptococcus* sp5,7,8,9.

*Neospora caninum* is a widespread parasite in the world and causes abortion and problems in the central nervous system of many animals including dogs10. 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 dogs13,14,15. Likewise, *Toxoplasma gondii*, the agent of toxoplasmosis, is highly common in Brazilian dogs and is a structurally related *N. caninum* protozoan16,17. Its establishment and dissemination depend upon the host's immune response, and in many cases, toxoplasmosis is associated with immunosuppression diseases18.

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 immunosuppression 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.

Serological analysis

The diagnosis of the visceral leishmaniosis was conducted through ELISA utilizing lysed 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 leishmanioses 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 disorders18. However, we observed that

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

<table>
<thead>
<tr>
<th></th>
<th>Neospora caninum</th>
<th>Toxoplasma gondii</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Titer</strong></td>
<td><strong>Number of positive dogs</strong></td>
<td><strong>Occurrence %</strong></td>
</tr>
<tr>
<td>50</td>
<td>6</td>
<td>2.9</td>
</tr>
<tr>
<td>100</td>
<td>13</td>
<td>6.4</td>
</tr>
<tr>
<td>200</td>
<td>4</td>
<td>2.0</td>
</tr>
<tr>
<td>400</td>
<td>6</td>
<td>2.9</td>
</tr>
<tr>
<td>800</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>3200</td>
<td>3</td>
<td>1.5</td>
</tr>
<tr>
<td>6400</td>
<td>3</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>36</strong></td>
<td><strong>17.6</strong></td>
</tr>
</tbody>
</table>

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 (2004) 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 (Dondi et al., 2002; Nardi et al., 2002), 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.

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**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**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Number of Dogs</th>
<th>Occurrence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative <em>L. chagasi</em>, negative <em>T. gondii</em> and negative <em>N. caninum</em></td>
<td>69</td>
<td>65.1</td>
</tr>
<tr>
<td>Negative <em>L. chagasi</em>, positive <em>T. gondii</em> and negative <em>N. caninum</em></td>
<td>33</td>
<td>31.1</td>
</tr>
<tr>
<td>Negative <em>L. chagasi</em>, negative <em>T. gondii</em> and positive <em>N. caninum</em></td>
<td>2*</td>
<td>1.9</td>
</tr>
<tr>
<td>Negative <em>L. chagasi</em>, positive <em>T. gondii</em> and positive <em>N. caninum</em></td>
<td>2*</td>
<td>1.9</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>106</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>

**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**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number of Dogs (%)</th>
<th>Number of positive dogs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SEX</strong></td>
<td></td>
<td><em>L. chagasi</em></td>
</tr>
<tr>
<td>Male</td>
<td>106 (52.0)</td>
<td>50 (47.2)</td>
</tr>
<tr>
<td>Female</td>
<td>98 (48.0)</td>
<td>48 (49.0)</td>
</tr>
<tr>
<td><strong>AGE</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 2-year-old</td>
<td>97 (47.5)</td>
<td>44 (45.4)</td>
</tr>
<tr>
<td>&gt; 2- to 4-year-old</td>
<td>60 (29.4)</td>
<td>24 (40.0)</td>
</tr>
<tr>
<td>≥ 4-year-old</td>
<td>47 (23.1)</td>
<td>30 (63.8)</td>
</tr>
</tbody>
</table>

* Different letters at the same column - *p* = 0.05

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

Resumo

Cães soropositivos e soronegativos à Leishmania chagasi da região de Araçatuba, São Paulo, Brasil, foram avaliados quanto à presença de possível co-infeção 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 ≥50) e 75 (36,8%) para T. gondii (RIFI ≥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.

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

References

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