IMMUNOFLUORESCENT ANTIBODY TEST (IFAT) FOR TRYPANOSOMA CRUZI IN DOGS FROM URBAN AND RURAL AREAS OF PELOTAS, RS

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ABSTRACT: Chagas disease (CD) is a zoonosis with the protozoan Trypanosoma cruzi as the causative agent. Dogs are considered the main domestic reservoir for T. cruzi in most Latin American countries and in some areas of the United States. In southern Brazil, despite being an endemic area of the disease, the prevalence in dogs is still unknown. This study aimed to evaluate the frequency of T. cruzi antibodies in dogs from urban and rural areas of Pelotas, RS, Brazil. A total of 227 canine sera were used for serological tests, of which 99 were from urban areas and 128 were from rural areas of Pelotas. Information regarding the environment and the possible risk factors (origin, rural contact, age, breed, confinement and gender) to which the dogs were exposed were recorded. Indirect immunofluorescence was used to assess the presence of specific immunoglobulins (IgG) anti-T. cruzi in the serum of dogs. Of the 227 sera analyzed, 81 (35.7%) exhibited anti-T. cruzi, which represented 34.3% of the dogs from the urban area and 36.7% of the dogs from the rural area. Among the variables analyzed, rural contact and male gender showed an association with seropositivity for T. cruzi. The detection of T. cruzi antibodies in the serum of these dogs emphasizes the need to study trypanosomiasis in this important domestic reservoir of Chagas disease.

Keywords: Chagas disease, domestic reservoir, indirect immunofluorescence.

IMUNOFLUORESCÊNCIA INDIRETA (IFI) PARA TRYPANOSOMA CRUZI EM CÃES DAS ÁREAS URBANA E RURAL DE PELOTAS, RS

RESUMO: A Doença de Chagas (DC) é uma zoonose que tem o protozoário Trypanosoma cruzi como agente etiológico. Os cães são considerados como o principal reservatório doméstico de T. cruzi na maioria dos países da América Latina e em algumas áreas dos Estados Unidos. No sul do Brasil, apesar de ser uma área endêmica para a doença, a prevalência em cães ainda é desconhecida. Este estudo teve como objetivo avaliar a frequência de anticorpos anti-T. cruzi em cães das áreas urbana e rural de Pelotas, RS, Brasil. Um total de 227 soros caninos foi usado para os testes sorológicos, dos quais 99 eram da zona urbana e 128 da zona rural de Pelotas. Foram registradas informações sobre o ambiente e os possíveis fatores de risco (origem, contato com meio rural, idade, raça, confinamento e gênero) aos quais os cães foram expostos. A técnica de imunofluorescência indireta foi usada para avaliar a presença de imunoglobulinas específicas (IgG) anti-T. cruzi no soro dos cães. De 227 soros analisados, 81 (35,7%) apresentaram anticorpos anti-T. cruzi, representando 34,3% dos cães da área urbana e 36,7% da área rural. Dentre as variáveis analisadas, o contato com o meio rural e o gênero masculino apresentaram associação com a soropositividade para T. cruzi. A detecção de anticorpos anti-T. cruzi nos soros desses cães enfatiza a necessidade de estudar a tripanossomíase nestes importantes reservatórios domésticos da Doença de Chagas.

Palavras-chave: Doença de Chagas, reservatório doméstico, imunofluorescência indireta.
INTRODUCTION

Chagas Disease (CD) is a neglected tropical parasitic disease caused by the protozoan Trypanosoma cruzi, which is nevertheless considered a public health problem in Latin America. According to the World Health Organization (WHO, 2010), between 8 and 10 million people worldwide are infected by T. cruzi and another 90 million are at risk of contracting the infection with 11,000 deaths annually. The parasite can be transmitted to humans by insect vectors, organ transplants, congenital transmission, oral transmission, laboratory accidents or blood transfusions (Esch and Petersen, 2013). In Brazil, because of the favorable climate, there are large number of triatomine species that can transmit the disease to humans and other animals (Pereira et al., 2013).

Dogs can maintain high T. cruzi parasitaemia, which makes them important sources of infection for triatomine bugs (Enriquez et al., 2014). According to Ramírez et al., (2013) dogs serve as a transmission bridge between domestic and sylvatic hosts, and this type of transmission has resulted in the emergence of the TcId genotype of T. cruzi among humans, which can cause a severe manifestation of the disease.

Data on the prevalence and geographical distribution of CD in dogs are important for the formulation of control strategies related to combating the role of canines as reservoirs of the disease. In southern Brazil, although it is an endemic area for the occurrence of CD in humans (Araújo et al., 2008), little is known about its prevalence in dogs. Thus, this study aimed to evaluate the seropositivity for T. cruzi in domestic dogs from urban and rural areas of Pelotas by IFAT and its association with possible risk factors.

MATERIALS AND METHODS

Geo-population data

The dog blood samples used in this study were collected from urban and rural areas of Pelotas. Pelotas is located in southern Brazil and occupies an area of 1,610.084 km², with a population of 328,275 inhabitants (IBGE, 2014), of which approximately 92% reside in the urban municipality. The city’s climate is temperate humid (subtropical) with an average annual temperature of 17.5 °C.

Collection of the samples

Based on the data from the Pelotas Municipality Health Department, it was estimated that there were approximately 63,453 urban and 1,115 rural dogs. The sample size was estimated using a prevalence of 50% (corresponding to the prevalence of unknown diseases in the determined area), a minimal precision of 10%, and confidence interval of 95% with the statistic program Epi-Info version 3.3.2. A total of 227 canine sera were used for the serological tests, of which 99 were from the urban and 128 were from the rural area of Pelotas. During the collection of blood samples, data from each dog were recorded in a questionnaire which contained information about the animal’s environment and the possible risk factors to which they were exposed (origin, rural contact, age, breed, confinement and gender). The blood collection was performed from the cephalic vein with Vacutainer® tubes without anticoagulant, identified and kept at room temperature for clot retraction and posterior centrifugation. The obtained sera were identified and kept at -20 °C until the serological test was performed.

Immunofluorescent Antibody Test (IFAT)

Detection of IgG anti-T. cruzi was conducted by IFAT coated with the T. cruzi suspension and using the Immuno-CON (WAMA® Diagnostics, Campinas, SP, Brazil) commercial kit. The manufacturer’s protocol was followed with minor changes, and a Nikon Eclipse E400 microscope was used. Slides containing T. cruzi were incubated with serum diluted from 1:40 to 1:160. To establish a cutoff point (1:40), a standardization with known T. cruzi positive or negative serum, kindly provided by Dr. Marcelo B. Labruna (University of São Paulo), was performed (Beard et al., 2003; Gürtler et al., 2007). Because Trypanosoma and Leishmania have certain similar antigens, which allow the existence of cross reactions (Alves et al., 2012), all sera used in this study were tested initially for Leishmania spp. by an enzyme-linked immunosorbent assay - ELISA (in house assay, using lysed promastigotes, Lima et al., 2003), and they were all negative.

Following the IFAT standardization, the anti-T. cruzi quantitative testing was performed. The sera were assayed in duplicate and diluted in phosphate-buffered saline (PBS). The results were considered positive by the highest dilution showing a yellow-green fluorescence throughout the outlines of the parasite.
**Statistical analysis**

Data from the epidemiological variables were analyzed using a chi-square test and Epi Info version 6.04. The OR (odds ratio) was determined with a confidence interval (CI) of 95%. A logistic regression model was used to simultaneously study the multiple effects that may be involved in the prevalence of seropositive dogs. The epidemiological variables with $P<0.25$ in the univariate analysis were included in the multivariate model. The data were analyzed using the Statistix 9.0. This study was approved by the Ethics Committee of the Federal University of Pelotas (number 8681).

**RESULTS**

This study concluded that from a total of 227 dogs, 81 (35.7%) were seropositive for *T. cruzi*. The antibody titer detected by the IFAT technique varied between 1:40 and 1:160, and most *T. cruzi* positive dogs, 64 of 81, showed a titration of 1:40 (Table 1). With respect to the origin of the animals, we observed a high percentage of positive dogs in both urban and rural areas with no significant difference. From the 99 urban area dogs, 34 (34.3%) were seropositive for *T. cruzi*; whereas in the rural area, from the 128 analyzed, 47 (36.7%) presented seropositivity ($P>0.05$). Although there was no significant difference between the urban and rural areas, half of the dogs seropositive for *T. cruzi* in the urban area had previous contact with the rural area ($P=0.03$) (Table 2).

The variables age, breed, and confinement were not significant in the univariate analysis ($P>0.05$). In regard to gender, the percentage of seropositive males to *T. cruzi* was significantly higher than that of females, 43.6% and 30.1%, respectively ($P=0.04$). In the multivariate analysis (logistic regression), only the rural contact variable had a result with statistical significance (Table 2).

**DISCUSSION**

Studies previously conducted in Brazil (LuChes et al., 2005; Souza et al., 2009; Lima et al., 2012; Bezerra et al., 2014) and in several American countries (Garcia-Vazquez et al., 1995; Montenegro et al., 2002; Beard et al., 2003; LuChes et al., 2005; Gürtler et al., 2007; Ramírez et al., 2013) have demonstrated the important role that dogs play in the epidemiology of CD and their importance as *T. cruzi* reservoirs. In the present study, 35.7% of the dogs were seropositive for *T. cruzi*, suggesting that these animals were exposed to protozoa and thus responded by producing specific antibodies. Similar results were observed in dogs from other regions of Brazil (LuChes et al., 2005; Souza et al., 2009). In Venezuela, a study concluded that 22.1% of 363 analyzed dogs had *T. cruzi* antibodies (Berrizbeitia et al., 2013). In Argentina, this rate was higher and showed a 60% rate of seropositive dogs for *T. cruzi* (Gürtler et al., 2007). It is important to note that variations from one region to another are common, and they are probably due to different ecosystems and the availability of vectors for *T. cruzi* transmission.

In this study, the titration of anti-*T. cruzi* varied between 1:40 and 1:160, and the dilution most frequently detected was 1:40 in both urban and rural areas. In the US, although the percentage of dogs seropositive for *T. cruzi* is lower, ranging between 1 and 7.5%, the antibody titers were higher than those observed in this study, ranging from 1:32 to 1:512 (Beard et al., 2003; Rosyspal et al., 2010). Our observations agreed with the findings of these authors, showing a similar range of titers among the analyzed animals and suggesting that the rate of infection and immune response is comparable to our findings.

Another study with blood donors from Mexico found that living in a rural area was a risk factor for CD (Hernández-Romano et al., 2014). In the present study, there was no significant difference

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### Table 1. Distribution of anti-*T. cruzi* detected by IFAT$^1$ in dog sera from urban and rural areas of Pelotas, Brazil

<table>
<thead>
<tr>
<th>Antibody titer</th>
<th>Number of positive dogs (%)</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urban area (n = 99)</td>
<td>Rural area (n = 128)</td>
</tr>
<tr>
<td>1:40</td>
<td>23 (23.2)</td>
<td>41 (32.0)</td>
</tr>
<tr>
<td>1:80</td>
<td>8 (8.1)</td>
<td>4 (3.1)</td>
</tr>
<tr>
<td>1:160</td>
<td>3 (3.0)</td>
<td>2 (1.6)</td>
</tr>
<tr>
<td>Total</td>
<td>34 (34.3)</td>
<td>47 (36.7)</td>
</tr>
</tbody>
</table>

$^1$IFAT: Immunofluorescent Antibody Test.
between the frequency of antibodies in dogs from urban and rural areas. Interestingly, half of the seropositive dogs from urban areas had contact with the rural area; this may suggest a risk factor for *T. cruzi* infection (Gürtler et al., 1991; Gürtler et al., 1993; Bustamante et al., 2014). However, further studies are necessary to confirm this hypothesis. In a study conducted in Botucatu, state of São Paulo (southeastern region of Brazil), no significant correlations were observed regarding the origin of the dogs, but those from urban areas had higher rates of infection than those from rural areas, 90.9 and 76.4%, respectively (Lucheis et al., 2005).

Regarding the gender of the animals, males had a significantly higher frequency of anti-*T. cruzi* than females. Similar data were observed in Costa Rica (Reyes et al., 2002) and Mexico (García-Vázquez et al., 1995). The higher positivity in males is probably because they are used in fieldwork, which makes them more exposed the disease, whereas females are unavailable during the fertile and pregnancy periods. There was no significant difference in regards to the variable confinement of animals, although a higher seropositivity in dogs bred unconfined was shown. A study conducted in Mexico, using the ELISA technique, showed a higher positivity in stray dogs (24.2%) than in those from households (8.8%) (García-Vázquez et al., 1995). According to this study, dogs that spent most of their time in the home environment were less likely to be exposed to the vector, which might explain the lower prevalence of antibodies. The breed and age variables were also not associated with the seropositivity of dogs, which is consistent with the findings of other studies conducted in Brazil (Lucheis et al., 2005) and México (García-Vázquez et al., 1995). In Argentina, the prevalence of *T. cruzi* infection was found to increase with a dog’s age (Gürtler et al., 2007).

It is important to note that differences in the occurrence of dog seropositivity can be assigned to differences in the serological tests that are used (Beard et al., 2003; García-Vázquez et al., 1995; Gutiérrez et al., 2004; Lucheis et al., 2005). The IFAT technique used in this study has been employed in various serological studies with canines and has an estimated sensitivity of 94% (Beard et al., 2003) and a specificity of 100% (Gürtler et al., 2007).

### Table 2. Characterization of the dog population and associations between the variables and seropositivity for *T. cruzi*

<table>
<thead>
<tr>
<th>Variables</th>
<th>Number of dogs</th>
<th>Positive dogs (%)</th>
<th>Univariate analysis</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Odds ratio (95% CI)</td>
<td></td>
</tr>
<tr>
<td><strong>Origin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urban</td>
<td>99</td>
<td>34 (34.3)</td>
<td>1.11 (0.62-2.00)</td>
<td>0.71</td>
</tr>
<tr>
<td>Rural</td>
<td>128</td>
<td>47 (36.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rural contact*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>69</td>
<td>19 (27.5)</td>
<td>2.63 (0.99-7.06)</td>
<td>0.03</td>
</tr>
<tr>
<td>Yes</td>
<td>30</td>
<td>15 (50.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-3</td>
<td>94</td>
<td>33 (35.1)</td>
<td>1.04 (0.58-1.89)</td>
<td>0.87</td>
</tr>
<tr>
<td>&gt;3</td>
<td>133</td>
<td>48 (36.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Breed</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Purebred</td>
<td>40</td>
<td>14 (35.0)</td>
<td>1.04 (0.48-2.30)</td>
<td>0.92</td>
</tr>
<tr>
<td>Mixed-breed</td>
<td>187</td>
<td>67 (35.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Confinement</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temporary</td>
<td>61</td>
<td>19 (31.1)</td>
<td>1.32 (0.68-2.62)</td>
<td>0.38</td>
</tr>
<tr>
<td>Unconfined</td>
<td>166</td>
<td>62 (37.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>133</td>
<td>40 (30.1)</td>
<td>1.80 (1.00-3.24)</td>
<td>0.04</td>
</tr>
<tr>
<td>Male</td>
<td>94</td>
<td>41 (43.6)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Only dogs from urban area. In the multivariate analysis, only the variable rural contact (P=0.03) showed significant results.*
Although the cross-reactivity with Leishmania spp. was excluded by testing, a limitation of this study was the possibility of cross-reaction with other species of the Trypanosoma, such as T. evansi and T. caninum, which can also infect dogs (Disquesnes et al., 2007; Alves et al., 2012). However, this fact was not investigated because there were no available tools.

In conclusion, the detection of T. cruzi antibodies in the serum of dogs from urban and rural areas showed an estimated prevalence of 35.7%, evidencing the exposure of both the rural and urban area to T. cruzi. Additionally, our findings suggest that rural contact and male gender might represent risk factors for T. cruzi infection in this population. Therefore, these results emphasize the need for constant surveillance of T. cruzi in this domestic reservoir.

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