IMMUNOFLUORESCENT ANTIBODY TEST (IFAT) FOR *TRYPANOSOMA CRUZI* IN DOGS FROM URBAN AND RURAL AREAS OF PELOTAS, RS[1]

Charlene Nascimento dos Santos Trindade[2], Nilton Azevedo da Cunha Filho[2], Luciana Farias da Costa de Avila[2], Marcos Marreiro Villela[2], Nara Amélia da Rosa Farias[2], Fábio Pereira Leivas Leite[3]*

[1]Received: 26/01/15. Accepted: 01/04/15.
[2]Universidade Federal de Pelotas, Departamento de Microbiologia e Parasitologia, Pelotas, RS, Brazil.
[3]Universidade Federal de Pelotas, Centro de Desenvolvimento Tecnológico, Núcleo de Biotecnologia, Pelotas, RS, Brazil.
*Corresponding author: fabio@leivasleite.com.br

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 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 a 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 Ramirez *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 (Lucheis *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; Lucheis *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 (Lucheis *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; Rosypal *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

Table 1. Distribution of anti–*T. cruzi* detected by IFAT[1] in dog sera from urban and rural areas of Pelotas, Brazil

Antibody titer	Number of positive dogs (%)		Total (%)
	Urban area (n = 99)	Rural area (n = 128)	(n = 227)
1:40	23 (23.2)	41 (32.0)	64 (28.2)
1:80	8 (8.1)	4 (3.1)	12 (5.3)
1:160	3 (3.0)	2 (1.6)	5 (2.2)
Total	34 (34.3)	47 (36.7)	81 (35.7)

[1]IFAT: Immunofluorescent Antibody Test.

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

Variables	Number of dogs	Positive dogs (%)	Univariate analysis	
			Odds ratio (95% CI)	P-value
Origin				
Urban	99	34 (34.3)	1.11 (0.62-2.00)	0.71
Rural	128	47 (36.7)		
Rural contact[1]				
No	69	19 (27.5)	2.63 (0.99-7.06)	0.03[2]
Yes	30	15 (50.0)		
Age (years)				
0-3	94	33 (35.1)	1.04 (0.58-1.89)	0.87
>3	133	48 (36.1)		
Breed				
Purebred	40	14 (35.0)	1.04 (0.48-2.30)	0.92
Mixed-breed	187	67 (35.8)		
Confinement				
Temporary	61	19 (31.1)	1.32 (0.68-2.62)	0.38
Unconfined	166	62 (37.3)		
Gender				
Female	133	40 (30.1)	1.80 (1.00-3.24)	0.04
Male	94	41 (43.6)		

[1]Only dogs from urban area. [2]In the multivariate analysis, only the variable rural contact (P=0.03) showed significant results.

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 (Garcia-Vazquez *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%) (Garcia-Vazquez *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 (Garcia-Vazquez *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; Garcia-Vazquez *et al*., 1995; Gutierrez *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).

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 (Desquesnes *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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REFERENCES

ALVES, A.S.; MOUTA-CONFORT, E.; FIGUEIREDO, F.B.; OLIVEIRA, R.V.C.; SCHUBACH, A.O.; MADEIRA, M.F. Evaluation of serological cross-reactivity between canine visceral leishmaniasis and natural infection by *Trypanosoma caninum*. **Research in Veterinary Science**, v.93, p.1329–1333, 2012.

ARAÚJO, A.B.; VIANNA, E.E.S.; BERNE M.E.A. Anti-*Trypanosoma cruzi* antibody detection in blood donors in the Southern Brazil. **Brazilian Journal of Infection Diseases**, v.12, p.480-482, 2008.

BEARD, C.B.; PYE, G.; STEURER, F.J.; RODRIGUEZ, R.; CAMPMAN, R.; PETERSON, A.T.; RAMSEY J.; WIRTZ, R.A..; ROBINSON, L.E. Chagas Disease in a domestic transmission cycle in southern Texas, USA. **Emerging Infection Diseases**, v.9, p.103-105, 2003.

BERRIZBEITIA, M.; CONCEPCIÓN, J.L.; CARZOLA, V.; RODRÍGUEZ, J.; CÁCERES, A.; QUIÑONES, W. Seroprevalence of *T. cruzi* infection in *Canis familiaris*, state of Sucre, Venezuela. **Biomedica,** v.33, p.214-25, 2013.

BEZERRA, C.M.; CAVALCANTI, L.P.G.; SOUZA, R.C.M.; BARBOSA, S.E.; XAVIER, S.C.C.; JANSEN, A.M.; RAMALHO, R.D.; DIOTAIUTI, L. Domestic, peridomestic and wild hosts in the transmission of *Trypanosoma cruzi* in the Caatinga area colonised by *Triatoma brasiliensis*. **Mem**órias do **Instituto Oswaldo Cruz**, v.109, p.887-898, 2014.

BUSTAMANTE, D.M.; DE URIOSTE-STONE, S.M.; CRUZ, J.G.; PENNINGTON, P.M. Ecological, social and biological risk factors for continued Trypanosoma cruzi transmission by Triatoma dimidiata in Guatemala. **PLoS One**, v.9, e1045992014, 2014.

DESQUESNES, M.; BOSSENO, M.F.; BRENIÉRE, S.F. Detection of Chagas infections using *Trypanosoma evansi* crude antigen demonstrates high cross-reactions with *Trypanosoma cruzi*. **Infection Genetics and Evolution**, v.7, p.457–462, 2007.

ENRIQUEZ, G.F.; BUA, J.; OROZCO, M.M.; WIRTH, S.; SCHIJMAN, A.G.; GÜRTLER, R.; CARDINAL, M.V. High levels of *Trypanosoma cruzi* DNA determined by qPCR and infectiousness to *Triatoma infestans* support dogs and cats are major sources of parasites for domestic transmission. **Infection, Genetics and Evolution**, v.25, p.36–43, 2014.

ESCH, K.J.; PETERSEN, C.A. Transmission and epidemiology of zoonotic protozoal diseases of companion animals. **Clinical Microbiology Reviews,** v.26, p.58–85, 2013.

GARCIA-VAZQUEZ, Z.; ROSARIO-CRUZ, R.; MIRANDA-MIRANDA, E.; DOMINGUEZ-MARQUEZ, A. A serological survey of *Trypanosoma cruzi* infection in dogs of two urban areas of Mexico. **Preventive Veterinary Medicine,** v.25, p.l-6, 1995.

GÜRTLER, R.E.; CÉCERE, M.C.; RUBEL, D.N.; PETERSEN, R.M.; SCHWEIGMANN, N.J.; LAURICELLA, M.A.; BUJAS, M.A.; SEGURA, E.L.; WISNIVESKY-COLLI, C. Chagas disease in north-west Argentina: infected dogs as a risk factor for the domestic transmission of Trypanosoma cruzi. **Transactions of the Royal Society of Tropical Medicine & Hygiene,** v.6, p.741-745, 1991.

GÜRTLER, R.E.; CÉCERE, M.C.; PETERSEN, R.M.; RUBEL, D.N.; SCHWEIGMANN, N.J. Chagas disease in north-west Argentina: association between Trypanosoma cruzi parasitaemia in dogs and cats and infection rates in domestic Triatoma infestans. **Transactions of the Royal Society of Tropical Medicine & Hygiene,** v.1, p.12-15, 1993.

GÜRTLER, R.E.; CECERE, M.C.; LAURICELLA, M.A.; CARDINAL, M.V.; KITRON, U.; COHEN, J.E. Domestic dogs and cats as sources of *Trypanosoma cruzi* infection in rural northwestern Argentina. **Parasitology**, v.134, p.69-82, 2007.

GUTIERREZ, R.; ANGULO, V.M.; TARAZONA, Z.; BRITTO, C.; FERNANDES, O. Comparison of four

serological tests for the diagnosis of Chagas disease in a Colombian endemic area. **Parasitology,** v.129, p.439-444, 2004.

IBGE – INSTITUTO BRASILEIRO GEOGRAFIA E ESTATÍSTICA. **Cidades@**. Disponível em: < http://cidades.ibge.gov.br/painel/painel.php?codmun=431440>. Acesso em: 20 dez. 2014.

LIMA, M.M.; SARQUISA, O.; OLIVEIRA, T.G.; GOMES, T.F.; COUTINHO, C.; DAFLON-TEIXEIRA, N.F.; TOMA, H.K.; BRITTO, C.; TEIXEIRA, B.R.; D'ANDREA, P.S.; JANSEN, A.M.; BÓIA, M.N.; CARVALHO-COSTA, F.A. Investigation of Chagas disease in four periurban areas in northeastern Brazil: epidemiologic survey in man, vectors, non-human hosts and reservoirs. **Transactions of the Royal Society of Tropical Medicine and Hygiene,** v.106, p. 143– 149, 2012.

LIMA, V.M.F.; GONÇALVES, M.E.; IKEDA, F.A.; LUVIZOTTO, M.C.R.; FEITOSA, M.M. Anti-leishmania antibodies in cerebrospinal fluid from dogs with visceral leishmaniasis. **Brazilian Journal of Medical and Biological Research,** v.36, p.485-489, 2003.

LUCHEIS, S.B.; SILVA, A.V.; ARAÚJO JR., J.P.; LANGONI, H.; MEIRA, D.A.; MARCONDES-MACHADO, J. Trypanosomatids in dogs belonging to individuals with chronic Chagas Disease living in Botucatu town and surrounding region, São Paulo state, Brazil. **Journal of Venomous Animals and Toxins including Tropical Diseases**, v.11, p.492-509, 2005.

MONTENEGRO, V.M.; JIMENEZ, M.; DIAS, J.C.; ZELEDON, R. Chagas disease in dogs from endemic areas of Costa Rica. **Memórias do Instituto Oswaldo Cruz,** v.97, p.491-494, 2002.

PEREIRA, J.M.; ALMEIDA, P.S.; SOUSA, A.V.; SOUSA, A.V.; PAULA, A.M.; MACHADO, R.B.; GURGEL-GONÇALVES, R. Climatic factors influencing triatomine occurrence in Central-West Brazil. **Memórias do Instituto Oswaldo Cruz**, v.108, p.335-341, 2013.

RAMÍREZ, J.D.; TURRIAGO, B.; TAPIA-CALLE, G.; GUHL, F. Understanding the role of dogs (*Canis lupus familiaris*) in the transmission dynamics of *Trypanosoma cruzi* genotypes in Colombia. **Veterinary Parasitology,** v.196, p.216–219, 2013.

REYES, L.; SILESKY, R.; CERDAS, C.; CHINCHILLA, M.; GUERRERO, O. Presencia de anticuerpos contra *Trypanosoma cruzi* em perros de Costa Rica. **Parasitologia Latinoamericana,** v.57, p.66-68, 2002.

ROSYPAL, A.C.; HILL, R.; LEWIS, S.; BRAXTON, K.;. ZAJAC, A.M.; LINDSAY, D.S. *Toxoplasma gondii* and *Trypanosoma cruzi* Antibodies in Dogs from Virginia. **Zoonoses and Public Health,** v.57, p.e76–e80, 2010.

SOUZA, A.I.; OLIVEIRA, T.M.F.S.; MACHADO, R.Z.; CAMACHO, A.A. Soroprevalência da infecção por *Trypanosoma cruzi* em cães de uma área rural do Estado de Mato Grosso do Sul. **Pesquisa Veterinária Brasileira,** v.29, p.150-152, 2009.

WHO - WORLD HEALTH ORGANIZATION. **Chagas disease:** control and elimination. Geneva: World Health Organization, 2010. p.1–4. Disponível em: < http://apps.who.int/gb/ebwha/pdf_files/WHA63/A63_17-en.pdf>. Acesso em: 20 dez.2014.