Dogs infection by *Trypanosoma cruzi* in São Domingos do Capim, State of Pará, Brazil*

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ABSTRACT. Almeida V.T., Kobayashi Y.T. da S., Roque A.L.R., Barros J.H.S., de Castro L.R.S., Madeira E.A.O., Uzcategui R.A.R. & Fernandes J.I. **Dogs infection by *Trypanosoma cruzi* in São Domingos do Capim, State of Pará, Brazil.** [Infecção por *Trypanosoma cruzi* em cães em São Domingos do Capim, Estado do Pará, Brasil.] *Revista Brasileira de Medicina Veterinária, 37(supl. 1):106-112, 2015.* Programa de Pós-Graduação em Saúde Animal na Amazônia, Universidade Federal do Pará, *Campus* II, BR 316 Km 62, Castanhal, PA 68743-970, Brasil. E-mail: vitalmeida21@hotmail.com

The objective of this study was to determine the presence of *Trypanosoma cruzi* among dogs naturally infected by it inside four rural communities at the Municipality of São Domingos do Capim located in the Northeastern Pará, Brazil. Blood samples were collected from 113 dogs and 85.7% (30/35) of the serologically positive dogs had their blood re-collected after three months. The diagnosis of *T. cruzi* infection was performed by: fresh blood examination, hemoconcentration, hemoculture, as well as the serological assays Indirect Immunofluorescence Essay (IFAT) and Imunoenzimatic essay (ELISA). The presence of positive dogs in both serologic tests (IFAT + ELISA) was 31% (35/113), distributed among the four communities as follows: (12/44) Uricuriteua, (19/40) Cezaréia, (1/16) Aliança and (3/13) Catita. None of the samples was positive in the fresh blood examination or hemoconcentration, although it was possible to isolate *T. cruzi*, DTU TcI in one dog sample during its blood re-collection. These results show how dogs are exposed to the *T. cruzi* transmission cycle, revealing their importance as sentinels for the presence of this parasite in the studied area.

KEY WORDS. Occurrence, Chagas disease, diagnosis, dog, natural infection.

RESUMO. O objetivo deste estudo foi determinar a presença de *Trypanosoma cruzi* entre os cães naturalmente infectados por ela dentro de quatro comunidades rurais no município de São Domingos do Capim localizadas na região nordeste do Pará, Brasil. Amostras de sangue foram coletadas de

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113 cães e 85,7% (30/35) dos cães sorologicamente positivos tiveram seu sangue recolhidas após três meses. O diagnóstico da infecção por *T. cruzi* foi realizada através do exame de sangue fresco, hemoconcentração, hemocultura, bem como a ensaios serológicos como a Técnica de Imunofluorescência Indireta (IFAT) e ensaio imunoenzimático (ELISA). A presença de cães positivos em ambos os testes sorológicos (IFAT + ELISA) foi de 31% (35/113), distribuídos entre as quatro comunidades da seguinte forma: (12/44) Uricuriteua, (19/40) Cezaréia, (1 / 16) Aliança e (3/13) Catita. Nenhuma das amostras foi positiva no exame de sangue fresco ou hemoconcentração, embora fosse possível isolar *T. cruzi*, DTU TcI na amostra um cão durante a sua re-coleta de sangue. Os resultados indicaram que os cães são expostos ao ciclo de transmissão de *T. cruzi*, assinalando a sua importância como sentinelas para a presença deste protozoário na região estudada.

PALAVRAS-CHAVE. Ocorrência, doença cardíaca, diagnóstico, cão, infecção natural.

INTRODUÇÃO

Trypanosoma cruzi (Kinetoplastida: Trypanosomatidae) is a protozoan parasite highly capable of infecting any mammal species (Zingales et al. 2012) Many species of triatomine bugs can act as vectors, and the infection of mammals can occur orally through ingestion of infected triatomine (or its fecal matter) or predation of other infected mammals; as well as contamination of skin lesions by infected triatomine feces (Roque et al. 2008, Jansen & Roque 2010).

There are at least 11 triatominae species already found infected by *T. cruzi* in Amazon. The ones usually involved in human cases are from the genus *Rhodnius*, since they are the most abundant in the region and their main ecotopes include palm trees that can be found very close to human dwellings. Sometimes, the foliage is used as coverage of human settings, the fibers in domestic tools and the fruits are consumed in natura by humans and domestic animals (Abad-Franch & Monteiro 2007).

Human infection by *T. cruzi* is known by Chagas disease, considered one of the most important parasitological infections in human beings in Latin America (Gürtler et al. 2007, Barr 2009). In Brazil, it is estimated that 4.6 million people are infected (Martins-Melo et al. 2014).

Dogs from this are very close to humans and often treated like family members. Their proximity to wild environments exposes themselves to the *T. cruzi* transmission cycle, increasing their chance of becoming infected. Their role as reservoirs is still up for debate, but it have been known that they playing an important role in the Argentinian Chaco region (Gürtler et al. 2007). Reservoirs may be considered as a complex ecological system consisting of one or more species responsible for maintaining a given parasite species in nature. This system should always be considered in a single space-time scale (Jansen et al. 2015). Sentinels are those mammals that display high rates of infection detected only by serological methods, and do not display positive parasitological diagnose. In this situation, these mammals show that, although infected, are not important for parasite transmission and can signalize the presence of the parasite in surveillance programs (Roque et al. 2008). In Brazil, their importance has been connected to an epidemiological surveillance system due to the possibility of dogs playing an important role as parasite sentinels in certain regions (Souza et al. 2008, Rocha et al. 2013).

The town of São Domingos do Capim, located only 146.8 kilometers away from the state capital (Belém-Pará) belongs to the Amazon Basin. Communities within this town have been developed across the forest borders. Because of the exploitation characteristics of the local economical trade, which is based on subsistence agriculture and consequently deforestation, the population has been migrating deeper into rainforest areas (IBGE 2015). According with data available from municipal and state health department, registered in the SINAN (information system of notification of diseases) five cases of Chagas Disease, in humans, were diagnosed between 2006 and 2011 and another eight between 2012 and 2013.

It is known that Amazon region display *T. cruzi* wild transmission cycles, involving both mammals and vectors. It is also known that most of the current Brazilian new cases come from this region, especially Para State (Coura & Junqueira 2015). Triatomine bugs are often reported by the local population in this town, invading the domestic and peri domestic environment where humans and dogs are found together. In the selected communities mentioned above, dogs are part of the families' daily lives, as pets or working dogs, which shows their closer relationship to the family members. The objective of this study was to evaluate the presence of *T. cruzi* infections among dogs in four areas of the town, define the infection pattern and discuss the parasite's role inside a parasite zoonotic context as a way to bring practical information to health surveillance and control programs.

METHODS

Local of the study

The study was done at four rural communities in the Municipality of São Domingos do Capim (S 01º40'270" e W 47º46"160") located in the northeastern meso-region of Pará and Guamá micro region. The town sits alongside Guamá River bay and its main vegetation consists of tropical plants that are part of the Amazon forest. It is 1,677.249 km² wide and its population is estimated to be 30,847 habitants (IBGE 2015). Four rural communities that have had cases of Chagas Disease were selected to be part of the study. These communities were classified by area: A1 (Cezaréia), A2 (Aliança), A3 (Catita) and A4 (Urucuriteua), (Figure 1) all of them showing the same landscaping profile with remaining vegetation from the Amazon forest.

Laboratory investigation

Blood samples from dogs older than six months old were collected. Their respective owners signed the informed consent form authorizing their participation in the research study. All of the examined dogs are autochthonous, and all owners answered negative when asked about the possibility of any dislocation of their dogs to other areas. The method used to diagnose dogs' infection by *T. cruzi* was based on four techniques, two of them parasitological: direct fresh examination and blood culture; and two serological ones: indirect immunofluorescence antibody test (IFAT) and ELISA (Enzyme-linked Immunoabsorbent Assay).

Two different techniques were used for the direct fresh examination. The direct examination was done by placing a drop of blood between the slide and the micro slide on a 400x magnification optic microscope. After that, through the parasite concentration method using microhematocrit tubes (Sambrook 1989), the direct presence of the parasite on the plasma was investigated, having the buffy coat as a reference, after concentration by centrifugation.

As for the blood culture, 300 µL of blood were inoculated in a tube with NNN (Nicolle, Novyand Mc Neal) and LIT (Liver Infusion Tryptosea) biphasic culture media. For this test, the same samples were examined every 15 days over four months.

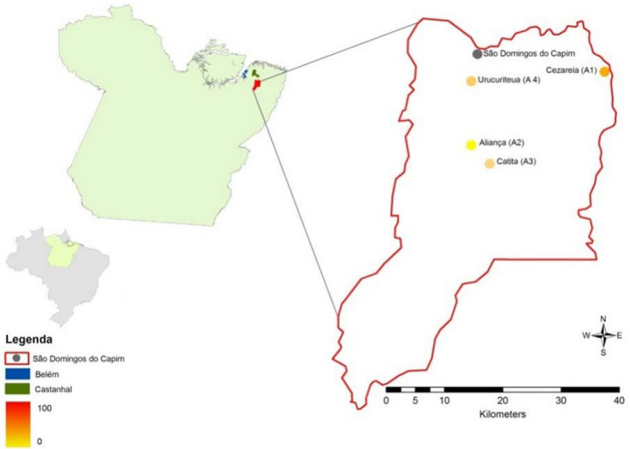

Figure 1. Location in the Municipality of São Domingos do Capim, Pará, highlighting the four communities where blood collection was done.

The molecular characterization was done by the phenol-chloroform (Fernandes et al. 2001) DNA extraction technique with subsequent magnification by PCR-Multiplex of the partial region of the non-transcribed spacer of the mini-exon gene, utilizing the following primers and a reverse initiator located in the conserved region of the mini-exon gene 5´TACCAATATAGTACAGAAACTG 3´ and specific TcI primers 5´ACACTTTCTGTGGCGCTGATCG3´,TcII 5´TTGCTCGCACACTCGGCTGATCG3´, Z3 5´CCGCGWACAACCCCTMATAAAAATG3´, *Trypanosoma rangeli* 5´ CCTATTGTGATCCCCATCTTCG3´ (Camargo 1966). The magnified PCR products were analyzed by ethidium bromide-stained agarose gel electrophoresis (2%) and visualized under UV light.

IFAT was performed using serological samples from dogs tested with anti-dog IgG conjugate fluorescein isothiocyanate (Sigma) for diagnosing antibodies against *T. cruzi* (Y and F90 strains) (Fernandes et al. 2001, Xavier et al. 2012). The cut-off values adopted were 1:40, as recommended by The Health Surveillance Department for trypanosomatidae.

ELISA test was performed using cytosol antigen for the *T. cruzi* Y strain. All the samples with an optic absorbance cut off value ≥0.200 (Xavier et al. 2012) were considered positive. The reaction's quantification was determined by spectrophometer reading wavelength at 450nm.

All the dogs were considered as having tested positive if their parasitological tests were positive or, in the absence of those, they demonstrated two positive serological tests, as recommended by the Health Ministry to diagnose trypanosomiasis. Statistical analysis was done with the specialized software R-Project®. All the different area proportions, age group and gender, were compared by the Chi-square method and the titers, considered non-parametric data, by the Kruskal-Wallis test, both with a significance level of 95%.

Ethical considerations

All methods for collecting samples were approved by the ethical committee from the Paulista State University Júlio de Mesquita Filho/FMVZ/UNESP/Botucatu Campus-SP, number 216/12, in accordance with Brazilian law and ethical principles.

RESULTS

Were collected 113 blood samples from dogs belonging to four different communities and sent to a laboratory facility for serological and parasitological evaluation. Parasites flagellated were not visualized through the parasitological tests of hemoconcentration, direct fresh examination and hemoculture. This way, the diagnosis of *T. cruzi* infection on dogs was performed only through the

serological tests, in a total of 31% ± 9.9% (35/113) of seroreactive dogs, and among those, 47.5% ± 19.2% (19/40) in Cezaréia (A1), 6.3% ± 12.2 (1/16) in Aliança (A2), 23.1% ± 25.3%, (3/13) in Catita (A3) and 27.3% ± 15% (12/44) in Uricuriteua (A4) (Table 1). Prevalence of infection in A1 was significantly greater than in A2 (p=0.009), and prevalence among the other areas was similar (p=0.21).

There was no statistical difference between male and female dogs, 32.2% and 31.3% (p=1) respectively. It was also similar among the different age groups (p=0.54), and infection was detected in 22.2% of young dogs (up to two years old), 25% of young adults (between two and five years old) and 14.3% of adults (animals older than 5 years old). Anti-*T.cruzi* antibody titers were statistically similar (p=0.08) and there was no difference among the age groups (p=0.3).

After three months of the first blood sample collection, dogs that had a positive serological test had another blood sample collected in order to have new serological and parasitological test done. Out of the initial 35 dogs, only 30 could be found again. The other ones were not in the same place and one had died, however all of those who have had their blood re-collected and evaluated have showed positive serological tests, and the IFAT titers were the same or greater than the first test.

In this second analysis, the only dog from A4 that had shown high IFAT titers (1/160), besides positive ELISA, shown detectable parasitemia on hemoculture. The parasite population was magnified in NNN with LIT media and characterized as *T. cruzi,* DTU TcI. This sample is cryopreservated in the Collection of *Trypanosoma* from Sylvatic and Domestic Mammals and Vectors, at Oswaldo Cruz Foundation (ColTryp) under the file number Coltryp490.

In the first blood collection, this animal was apparently healthy in clinical examination. After three months, in a new assessment, the animal had become apathetic, anorexic and emaciated. Physical examination revealed inspiratory dyspnea, slightly pale mucosa, lymphadenopathy. Auscultation showed normofonese of sounds, respiratory sinus arrhythmia. The blood count showed regenerative anemia, thrombocytosis, leukocytosis and lymphocytosis. Enzymatic tests for renal and hepatic assessment were normal.

Table 1. Serological tests of 113 dogs from four different locations in the Municipality of São Domingos do Capim, Pará.

Serological tests	Communities Studied			
	Cezaréia (A1) 47%(19/40)	Aliança (A2) 6%(1/16)	Catita (A3) 23%(3/13)	Urucuriteua (A4) 27%(13/44)
ELISA				
Positive	19	1	3	13
RIFI				
1:40	11	-	3	18
1:80	1	1	1	6
Above 1:80	8	-	1	1
RIFI+ELISA	19	1	3	13
All animals	40	16	13	44

DISCUSSION

Since the first records (Shaw et al. 1969) Chagas Disease has been continuously reported in the Brazilian Amazon Region (Coura et al. 2002, Aguilar et al. 2007, Valente et al. 2009). In 2011 there were a total 162 cases of Chagas Disease documented in Brazil, 155 of those were in the Amazon region and 125 cases in the state of Pará. And besides oral transmission has been reported as the main cause of Chagas disease outbreaks in many Brazilian states, mostly in the North region in predominantly rural areas, epidemiological surveys with human beings and animals report that domestic mammals are infected by *T. cruzi* (Nóbrega et al. 2009, Roque et al. 2013).

The study was done at the Municipality of São Domingos do Capim in the State of Pará, where native flora originally belongs to the Amazonian forest. There, the presence of serological positive dogs naturally infected by *T. cruzi* was 31% (35/113), which is below the number found in other countries, like in the west coast of Argentina, reported by Gürtler et al. (2007) that showed 60% (n=68) of seropositivity in dogs —superior to the one reported by Gómez et al. (2008) in Colombia, where 10.7% in a total of 261 dogs were positive when tested by IFAT. When compared to studies done in Brazil, the prevalence of infected dogs in our studied region is above the one documented in rural areas in the state of Mato Grosso do Sul by Souza et al. (2009), with 22.7% (n=17) of dogs being positive on ELISA and IFAT tests; the one found at Barra do Pojuca/BA with 11.1% of dogs infected according to Aguiar et al. (2009); and in Abaetetuba/PA and towns of the northern region of Tocantins showing prevalences of 23% and 25% respectively, according to Xavier et al. (2012). It is only inferior to the prevalence of infected dogs found in the town of Curralinho (89% on ELISA and IFAT tests), also in the Amazonian region of Pará state and immediately after an outbreak that resulted in more than 20 people infected in 2009 (Xavier et al. 2012).

It is also worth mentioning that the fluctuation

of the number of infected dogs inside one same state or municipality is influenced by epidemiological characteristics belonging to each specific area, including factors like sanitary conditions, housing, triatomine density and the occurrence of synanthropic animals and infected humans in the area (Barr et al. 1991). Moreover, the prevalence index previously described, even though important when comparing different areas, should be carefully analyzed due to the difference among diagnostic tests and methodological differences in different studies.

Cesaréia (A1) and Urucuriteua (A4) communities had shown the highest prevalences of infection by *T. cruzi* among canines (47% and 27%, respectively) and also a larger number of dogs, with approximately three to five dogs per household. These two areas had in common the fact that the houses were located far away from each other, inside a forest with mainly palm trees and large açaí fruit plantations. The dogs had free access to the surrounding vegetation and the houses did not have any type of fencing structure that prevented the dogs from being in touch with the sylvatic animals, nor prevented the latter from having access to the houses. In some houses there was not any type of screen on windows or doors to prevent insects from coming into the house. The lack of any physical barrier, together with light spots at dusk, ends up attracting insects. A triatomine invasion was often mentioned by residents, which explains the results found in those two areas.

Aliança (A2) and Catita (A3) communities had the lowest prevalence of infection (6% and 23% respectively) and a lower number of dogs, ranging from one to three per household. These communities were organized in villages with less rural features, with schools, park and churches right next to each other, and even though they were surrounded by forest they were not inserted in the middle of it, like the other two communities mentioned above. The dogs also had free access to the surrounding flora, mainly because there was no fencing structure whatsoever, but it was not a frequent habit. There was a larger area free of vegetation between houses and the sylvatic environment, therefore making its access less frequent. The beginning of an urban development and a less intense contact between dogs and the forest seem to explain the lower infection rates when compared to the communities where the domestic and sylvatic environments do not have a well defined boundary. Almost all the animals were involved in hunting wildlife species, but there is no way to assert that this activity was responsible for the infection of the animals.

Dogs seroreactive to *T. cruzi* were found in all areas, showing that the parasite is present in areas where these animals live. In our case the infection might have happened in the sylvatic environment, during hunting or harvesting activities with their owners, since some of them have reported those activities. However, infected young females (younger than 1 year old) without any records of being in touch with the sylvatic environment show that the parasite is also being transmitted in areas surrounding the house, in the peri-domestic environment. Under these conditions dogs act like important sentinels of the parasite's presence in the area, like it has been previously shown by (Roque & Jansen 2008, Xavier et al. 2012).

The only isolate case happened in A4 (Urucuriteua community), in one of the thirteen serologically positive dogs in this area that had its blood collected again. This dog was a young female less than one year old and did not have any record of hunting activity. Molecular characterization confirmed infection by *T. cruzi* subpopulation TcI, predominant in mammals and humans in the Amazon Basin (Fernandes 1999, Marcili 2009). This dog only had a positive hemoculture in a new blood collection three months after the first one, probably when it was already at the chronic phase of infection (Machado 2001). This finding might be interpreted in three different ways: (i) this is one more isolate case; (ii) dogs can have parasitemia peaks during a supposedly chronic phase potentially becoming transmission agents; (iii) dogs can act like *T. cruzi* reservoir hosts.

The problem in detecting parasitemia in dogs that are not going through the acute phase of infection, which has been previously reported in different studies, supports our first interpretation. Find circulating parasite on blood is much more frequent in acute phases of the disease, but this not implies that it does not occur in chronic phases. There is a report of *T. cruzi* isolation on chronically infected dog from Abaetetuba (also Pará State), seven months after the first isolation (Roque et al. 2013). It´s supposed that this dog was in chronic phase of infection because it was already found infected by us three months before, presenting a high IgG serological titer (1/160). Two reasons led us to consider this dog in chronic phase of infection: (i) the elevated rates of IgG titers already observed three months before parasite isolation; (ii) the classical studies on experimental *T. cruzi* infection in

dogs (Machado et al. 2001) report no more than 60 days until dogs reach the chronic phase of the infection.

With regard to our second interpretation, it is well known that the longer the infection time, the lower the parasitemia is, but that does not exempt the possibility of finding positive hemocultures according to Castro et al. (1999) and like it is shown in this research study. Among the factors that can be involved in maintaining the circulating forms in the blood are the size of the initial inoculum, inoculation route, and the parasite subpopulation according to Krettli et al. (1984), in addition to factors related to the host, like the animal's immunological status and associated infections. In both situations, it becomes evident the dog's role as an important sentinel, but not as a competent transmission agent, i.e. not playing a relevant role as a reservoir (Xavier et al. 2012).

Its importante to emphasize, that *Trypanosoma rangeli* a multihost parasite transmitted by *Rhodnius* spp. and dispersed in the Amazon region was not observed in blood cultures in the present study, and the only *Trypanosoma* detected was characterized as *T. cruzi* by molecular methods that distinguishes beteween *T. cruzi* and *T. rangeli*. The infection of this parasite in mammals is reported to be non-pathogenic and also do not result in expressive immune response (Guhl & Vallejo 2003). For this reason, although recognizing that this parasite is present in Amazon, we do not believe that this would result in high serological titers, at least over than 1:40, since this was the cut-off adopted here.

It is also important to emphasize that this is a common profile of infected dogs in Brazil, which is completely different from the profile seen in the Argentine Chaco dogs that consistently reported having both high infection prevalence and infectivity potential, as proved by xenodiagnoses (Gürtler et al. 2007). That shows that factors affiliated with the host (in this case, the dog) are important to the course of infection, but are also influenced by parasite factors and the environment itself and it is from this dynamic and complex interaction that the course of an infection appears.

Along those lines, the Municipality of São Domingo do Capim demonstrated to have its dogs exposed to the transmission cycle of *T. cruzi* (and, therefore, infected), however they did not reveal a high parasitemia infection profile and, consequently were not involved in the amplification of the parasite population. These dogs cannot be considered as *T. cruzi* reservoirs, but play an important role indicating the presence of the parasite thus supporting the idea of monitoring these animals as sentinels in identifying areas with a high risk of transmission.

The transmission cycle of *T. cruzi* in dwelling areas of the Amazon Basin is far from being defined according to the peculiar features of each biome and the hosts involved in it, making it crucial to study all the different hosts responsible for sustaining transmission in the different areas.

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