Article/Artigo

Diagnosis of *Leishmania* (*Leishmania*) *chagasi* infection in dogs and the relationship with environmental and sanitary aspects in the municipality of Palmas, State of Tocantins, Brazil

Identificação de cães infectados por *Leishmania (Leishmania) chagasi* e sua relação com aspectos ambientais e sanitários no município de Palmas, Estado do Tocantins, Brasil

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ABSTRACT

Introduction: The aim of the present study was to identify the presence of *Leishmania (Leishmania) chagasi* infection in dogs in the City of Palmas, Tocantins, Brazil, using the PCR technique to list the hot spots of infected dogs in the city and associate their occurrence to significant environmental changes at capture sites. **Methods:** DNA was extracted from blood of dogs, and the PCR were performed with primers RV1/RV2. After screening the population studied, the regions of the city that had the highest occurrence of canine infection were detected. These sites were visited, and ecological parameters denoting anthropogenic disturbance were evaluated. **Results:** Some important features were listed in the regions visited, such as low urbanization, lack of public collection of sewage, limited garbage collection, vacant lots with tall vegetation, decaying organic matter, and, most importantly, the occurrence of stray dogs and poultry in homes. **Conclusions:** The methodology for screening the population was very efficient, especially in evaluating a large number of individuals in a short time, with a high degree of automation. The results indicate an association between the observed parameters and the occurrence of infection in dogs. The model presented in the city is ideal for studies of disease progression and expansion and for the evaluation of control measures adopted for canine VL.

Keywords: Canine visceral leishmaniasis. PCR. Risk conditions.

RESUMO

Introdução: O estudo foi realizado com o objetivo de identificar, através da PCR, a presença da infecção por *Leishmania (Leishmania) chagasi* em cães no município de Palmas, no Estado do Tocantins, Brasil, de modo a elencar os *hot spots* de cães infectados no município e associar sua ocorrência a alterações ambientais marcantes nos locais de captura. **Métodos:** O DNA foi extraído do sangue dos cães e as reações de PCR foram realizadas com os *primers* RV1/RV2. Após o *screening* da população estudada, foram detectadas as regiões do município que apresentavam as maiores ocorrências da infecção canina. Esses locais foram visitados, e parâmetros de distúrbio ecológico com origem antrópica foram avaliados. **Resultados:** Algumas características importantes foram constantes entre as regiões visitadas, entre elas a baixa urbanização, inexistência de coleta pública de esgoto, coleta publica de lixo pouco abrangente, lotes vagos com vegetação alta, e matéria orgânica em decomposição, com destaque para criação de cães soltos, e aves nas residências. **Conclusões:** A metodologia adotada para *screening* da população se mostrou bastante eficiente, sobretudo na avaliação de um grande número de indivíduos em tempo reduzido, com alto grau de automatização. Os resultados apresentados indicam associação entre os parâmetros observados e a ocorrência da infecção em cães. O modelo apresentado no município é ideal para estudos do desenvolvimento da doença, bem como sua expansão, além da avaliação das medidas de controle adotadas para a leishmaniose visceral canina.

Palavras-chaves: Leishmaniose visceral canina. PCR. Condições de risco.

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INTRODUCTION

In Latin America, visceral leishmaniasis (VL) is caused by *Leishmania (Leishmania) chagasi* (Cunha & Chagas, 1937), a kinetoplastid protozoan member of the *Leishmania donovani* complex. The disease is transmitted mainly by the bite of the phlebotomine *Lutzomyia longipalpis*. VL is a consumptive debilitating disease whose clinical manifestations are intrinsically dependent on the type of immune response expressed by the infected animal[1,2]. Dogs are considered the main reservoir of the disease in urban environments, which have afforded these animals a central role in this disease's transmission cycle[3,4].

In Brazil, VL is considered an endemic disease, though outbreaks occur more or less often due to poor immune response of hosts[4] and to an eco-epidemiological scenario that favors the proliferation of infected vector populations[5]. In 2003, the State of Tocantins, Northern Brazil, recorded the highest prevalence of leishmaniasis in the country, with 20 cases per 100,000 inhabitants[6,7]. In 2008 and 2009, the recorded prevalence was 36.8 and 33 cases per 100,000 inhabitants, respectively. In 2010, 21 municipalities in Tocantins were considered a priority region in efforts of VL surveillance and control (*Secretaria de Saúde do Estado do Tocantins* - SESAU/TO: unpublished data).

The diagnosis of leishmaniasis poses one of the most significant problems concerning the disease, which in most cases may render ineffective VL surveillance and control measures. Despite the difficulties associated to the interpretation of seroprevalence data, the Health Ministry of Brazil recommends that serological diagnosis be performed based on sampling and population surveys[1]. Also, non-specific cross-reactions and delay between infection and seroconversion occur often[8].

Therefore, several polymerase chain reaction (PCR) protocols have been developed to detect *Leishmania*. The technique has been consistently validated as the fastest and most sensitive and specific one as compared to other diagnosis protocols — apart from its suitability when used in leishmaniasis surveillance programs[2,8-11]. Concerning molecular diagnosis, kinetoplast DNA minicircles (KDNA) are good targets in the *Leishmania* genome, as these present up to 10,000 copies per cell, which increases sensitivity of detection procedures[2,12].

The present study identifies the presence of *L.* (*L.*) *chagasi* in dogs living in the City of Palmas, State of Tocantins, Brazil, using the PCR protocol. The investigation surveyed hot spots of infected dogs in the city to establish a link between the canine infection by *L.* (*L.*) *chagasi* and marked environmental changes in capture sites.

METHODS

Study area

Collections were carried out in the City of Palmas (10º12'46"S; 48º21'37"W), located in central State of Tocantins, Brazil. The municipality area was divided in 8 zones according to geographic position and urbanization level to allow the pooling of samples and respective results (**Figure 1**).

Collection of biological material

In this study, 204 dogs were analyzed, including animals kept by owners as well as stray dogs captured by the *Centro de Controle de Zoonoses*, City of Palmas, State of Tocantins, Brazil, by request of

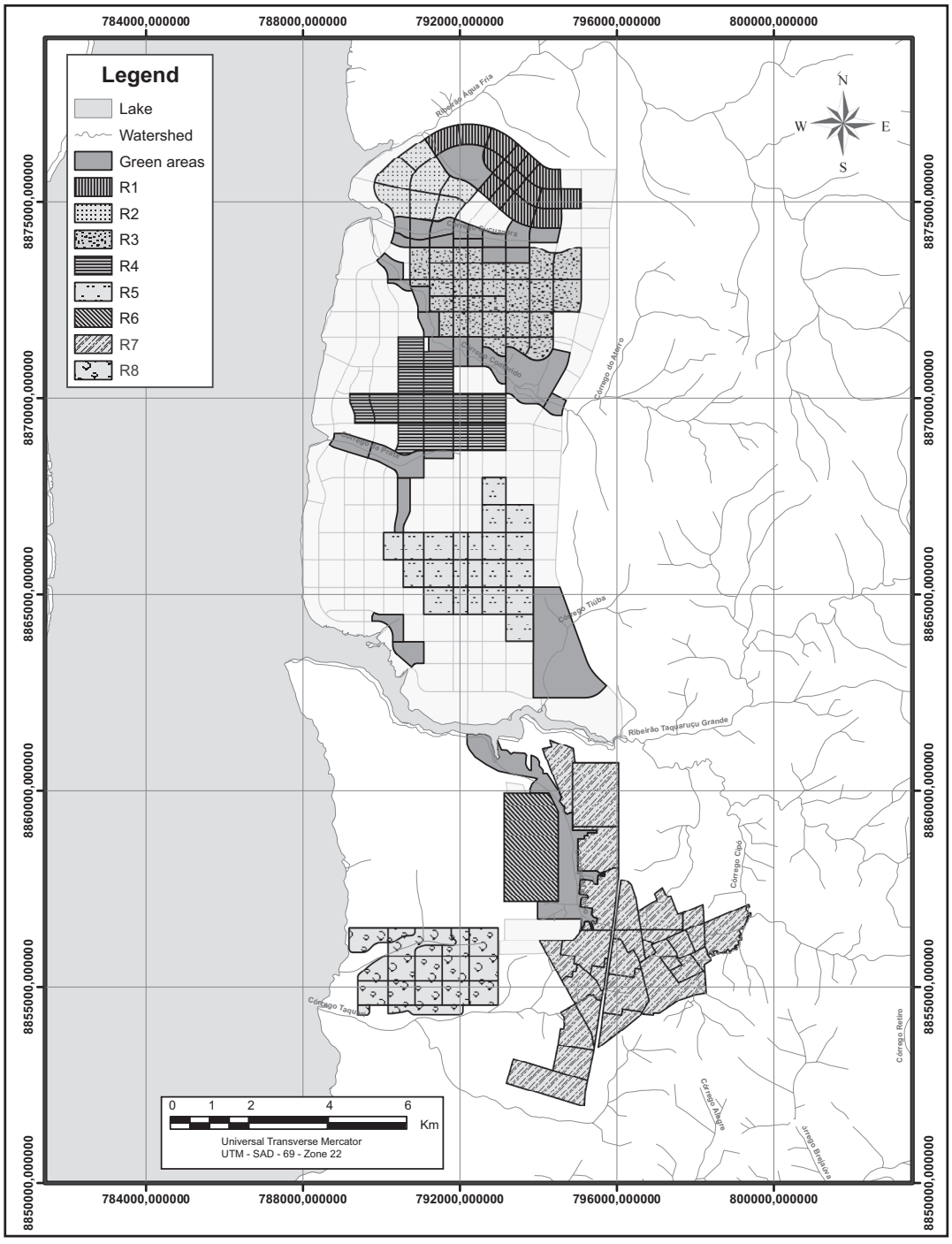

FIGURE 1 - Zoning of the City of Palmas, State of Tocantins, Brazil.

the city's inhabitants, between July 2007 and January 2009. DNA extraction was carried out using 3- to 5-mL blood samples collected from these dogs using sterile tubes containing EDTA 27mM as anticoagulant.

Total DNA extraction and isolation

Blood samples were resuspended in 1:1 (v/v) saline 0.9% (m/v) and centrifuged at 285g for 10min. After that, samples were washed three times in 1:1 (v/v) red blood cells (RBC) lysis buffer (saccharose 5mM; Tris-HCl 10M pH 7.5; $MgCl_2$ 5 mM) and then centrifuged as above. Then, nuclear lysis buffer (Tris 10mM pH 8.0; EDTA 5mM; NaCl 10mM) and 24µL K proteinase (10mg/mL) were added to the cell pellets. Samples were then incubated at 37ºC for 3h. Immediately after incubation, DNA was extracted according to the phenol-chloroform method[13], precipitated with ethanol 100% and NaCl 5M, resuspended in 50µL TE + RNAse (10mg/mL), and freeze-stored at -20ºC.

Primer-specific PCR

The PCR was conducted using the pair of primers RV1 (5' - CTT TTC TGG TCC CGC GGG TAG G - 3') and RV2 (5' - CCA CCT GGG CTA TTT TAC ACC A - 3')[14,15] to detect the 145-bp target sequence in the LT1 fragment, located in the kinetoplast DNA minicircle of the *L. donovani* complex[2,16]. The PCR were conducted in 1.5U/µL Taq DNA polymerase (LGC™ Biotecnologia), Taq reaction buffer (Tris-HCl 100mM pH 8.5 and KCl 500mM), $MgCl_2$ 1.5mM, deoxyribonucleotide triphosphate (dNTP) 0.2mM, 10pmol each primer, and 300ng DNA of each individual and complemented to a final 20µL volume with water. The reactions were carried out in a thermal cycler (PxE0.2, Thermo Electron Corporation™, Milford, MA, USA) according to the following steps: 94ºC for 5min, 35 denaturation cycles at 94ºC, primer hybridization at 58ºC, extension at 72ºC, 72ºC for 10min, and 4ºC for 10min. All reactions were conducted in triplicate, and the DNA of a *L.* (*L.*) *chagasi* culture (Merivaldo strain, IOC-LC2455, isolated from a VL patient in an endemic area in Jequié)[17] was used as positive control. Finally, the amplicons were analyzed by horizontal electrophoresis on agarose gels 2% (m/v) stained with ethidium bromide 0.2µg/mL at 90V for 90min. Gels were photographed using a video documentation system (Vilber Lourmat™, France). Amplicon visualization was conducted in triplicate, and individuals were assigned a PCR positive or negative status. The molecular analysis results obtained did not influence CCZ standard conduct, as the seropositive animals, evaluated in compliance with the standard methodology defined by the Health Ministry of Brazil and conducted in a laboratory certified by the health authorities, were euthanized in accordance with the Health Ministry guidelines.

Statistical analysis

The statistical analysis of data was conducted using the software BioEstat version 4.0. The Chi-square test was employed to observe the occurrence of statistically significant differences between subpopulations, as defined according to the zoning of the city area, as to the occurrence of canine infection by *L. (L.) chagasi*. Significance level was 5%.

In loco visits: identification of hot spots

After the PCR analyses were finished, results were analyzed to identify the areas presenting the highest prevalence of dogs infected with *L.* (*L.*) *chagasi* in Palmas, Tocantins. Then, *in loco* visits were undertaken to determine anthropogenic parameters of ecological disturbance. The parameters assessed were: urbanization level, type of construction, garbage and sewage collection systems, vacant lots, vegetation, presence of dogs and breeding practices, and presence of hens and other animals. The geographic data about the sites were collected using a GPS eTrex H device (Garmin™, Chicago, IL, USA). The parameters observed were written down on spreadsheets and then photodocumented using a digital camera.

Ethical considerations

All experimental procedures were approved by the Project and Research Assessment Committee, Palmas Health Authority, protocol no. 52-03/19.

RESULTS

Clinical evaluation

Of the 204 dogs assessed in this study, 41 (20.1%) were classified as asymptomatic, 98 (48%) were considered oligtosymptomatic, and 65 (31.9%) were polysymptomatic, according to the criteria previously described[18,19]. The most frequent symptoms were onicogryphosis, alopecia, emaciation, and crusty lesions.

Polymerase chain reaction

The analysis of the 204 samples collected using the PCR protocol indicated the occurrence of 121 (59.3%) of dogs positive for *L.* (*L.*) *chagasi* (**Figure 2**).

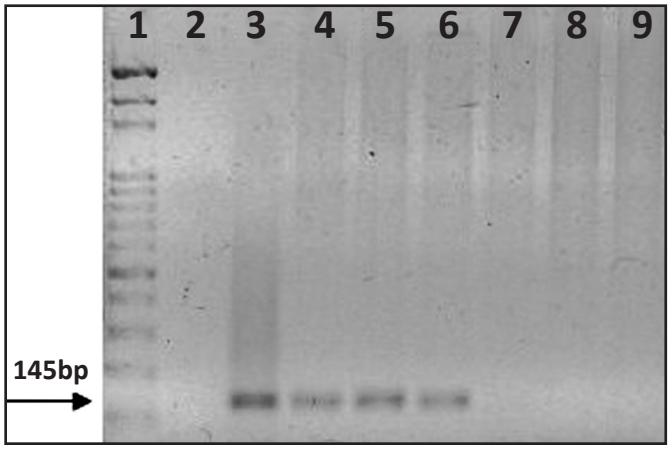

FIGURE 2 - Electrophoretic profile on agarose gel of 145-bp amplicons obtained by PCR using the primers RV1/RV2.
Column 1: 100-bp molecular ladder**; Column 2:** Negative control, without DNA**; Column 3:** Positive control using DNA of *Leishmania (Leishmania) chagasi* culture Merivaldo strain – IOC-LC2455)**; Columns 4, 5, and 6:** Sample from a *Leishmania* (*Leishmania) chagasi*-positive individual, in triplicate**; Columns 7, 8, and 9:** Sample from a *Leishmania* (*Leishmania*) *chagasi*-negative individual, in triplicate.

Prevalence of *Leishmania* (*Leishmania*) *chagasi* in Palmas, Tocantins region

The data on canine infection prevalence and geographical distribution across the different zones of Palmas are shown in **Table 1**. The Chi-square test revealed statistically significant difference at 5% level of significance (Chi-square = 52.4; $p < 0.0001$), indicating that there are zones with comparatively higher prevalence values of canine infection by *L.* (*L.*) *chagasi* in the city of Palmas, Tocantins.

TABLE 1 - Prevalence of *Leishmania (Leishmania) chagasi* infection in dogs for different regions of the City of Palmas, State of Tocantins, Brazil.

Regions	Sample size (number of dogs)	Prevalence (%)
R-1	16	75.0
R-2	32	75.0
R-3	10	50.0
R-4	13	46.2
R-5	27	66.7
R-6	38	39.5
R-7	60	61.7
R-8	8	50.0
Total	**204**	**59.3**

TABLE 2 - Parameters evaluated in the regions with the highest prevalence of canine infection by *Leishmania (Leishmania) chagasi*.

Aspects evaluated	Regions		
	R-1	R-2	R-5
Urbanization level	low	high	average
Garbage collection system[a]	≈ 80%	≈ 85%	≈ 60%
Open sewers[b]	≈ 25%	≈ 20%	≈ 15%
Vacant lots[c]	+	+	+
Stray dogs[c]	+	+	+
Henhouses[c]	+	+	+

[a] Percentage of the area covered by the public garbage collection system; [b] percentage of privately owned homes. The regions evaluated did not present sewage collection system; [c]+: represents presence of the aspect.

Anthropogenic parameters of ecological disturbance

After the zones with the highest prevalence of *L.* (*L.*) *chagasi* infection were established for the population sampled, *in loco* visits were undertaken in the blocks corresponding to the regions designated as R-1, R-2, and R-5. Among the main aspects observed during the visits were (**Table 2**): I) low urbanization level, with, on average, 50% of blocks presenting streets paved with asphalt; II) absence of a public sewage collection system, with some homes having their own cesspools (around 80%) and many still discharging sewage into open-air ditches; III) poor public garbage collection that fails to cover all the area, including the zones studied (only 75% of the area); IV) large number of vacant lots presenting tall vegetation, which are used by the population as a landfill for demolition debris, domestic garbage, and organic waste (a very common characteristic in the zones evaluated); V) high number of stray dogs on the streets; and VI) considerable number of homes where dwellers raised poultry, another aspect commonly observed in the geographic evaluation.

DISCUSSION

The inclusion of PCR in surveillance and control measures against leishmaniasis is a tool that may afford faster and more efficient answers in the fight against the propagation of the disease, especially when specifically applied to evaluate asymptomatic reservoirs (data not shown) and also in scenarios of hosts with poor immune response. The adoption of the PCR protocol in these measures is a practical reality, as the PCR technique affords to detect the causal parasite of VL, independently from the quality of the immune response produced by the infected organism.

PCR is a fast-response technique, as it affords to analyze numerous individuals at once, apart from being subject to little interference from the operator due to its high automation. Mohebali et al.[20] state that the detection of *L. (L.) chagasi* in samples of infected organs is the gold-standard method to diagnose canine infection by the parasite. However, these samples are obtained using invasive procedures, like aspiration of bone marrow, lymph nodes, and splenic puncture. Serum tests are not appropriate for immunocompromised patients, apart from the fact that false-negative results may occur. PCR has been proven to be as appropriate, or even more so, as the diagnostic methods mentioned above, with the added benefit of producing more timely results. Falah et al.[21] and Chargui et al.[22] conducted a study in Kairouan, Tunisia, and observed that PCR was more efficient than immunofluorescence and *in vitro* culture. Other studies also suggest that PCR offers more potential for a direct, efficient, sensitive, and species-specific diagnosis[8,20].

As pointed out by Fallah et al.[21], apart from being less invasive, collection of blood samples affords good reproducibility and is better accepted by dog owners, as compared to aspiration puncture of bone marrow, spleen, or lymph nodes. As *L. (L.) chagasi* are intracellular parasites that infect cells of the mononuclear phagocytic system, like monocytes and macrophages, the use of the leukocyte layer instead of whole blood may increase sensitivity, reducing the interference of potential reaction inhibitors. PCR has gradually become the technique indicated to diagnose leishmaniasis, as conventional parasitology methods are not sufficiently sensitive[23,24].

In accordance with other papers, peripheral blood samples were analyzed by PCR in the present study, producing particularly motivating results and confirming its use in routine diagnosis of canine VL[21,25].

The results obtained using PCR suggest that dogs infected with *L. (L.) chagasi* are widely distributed in the City of Palmas, State of Tocantins, Brazil (**Table 1**). High prevalence is observed in all regions of the municipality, varying between 39.5% in region R-6 and 75% in regions R-1 and R-2. These data characterize the region as an important endemic area in the country. This endemic status is explained by recent urbanization process the region has undergone in the past 20 years, in a transformation triggered by intense anthropic activities in the period. This characteristic underscores the importance of the City of Palmas in studies on VL progression and on the efficiency of control strategies against the expansion of leishmaniasis.

Here, it was also possible to observe the association between the high prevalence of *L.* (*L.*) *chagasi* in dogs in the area studied and poultry rearing in the homes located therein. These observations are an important finding to take into account, as the presence of a dog in the domestic environment and in the property as a whole does not represent a primordial risk factor in terms of VL infection, as opposed to the presence of a vector organism. The attraction of phlebotomines to hens has been fully established. *Lutzomyia longipalpis* promptly fed on hens and the abundance of these organisms in henhouses have epidemiologic importance considering VL occurrence[26,27].

Several authors have previously indicated that the presence of trees, vegetable gardens, plant pots, heaps of wood, leaf and debris build-ups, domestic animals, fowl-breeding activities, animal feces, high contents of organic matter and garbage in soils, and inappropriate wood storage are factors associated to the risk of acquiring leishmaniasis[28,29].

Females of *Lutzomyia longipalpis* lay their eggs preferably on a humid substrate presenting high content of organic matter on soil[1]. This shows that the presence of henhouses promotes the best conditions for greater reproductive success of the vector. The accumulation of organic matter as well as of domestic waste in properties and vacant lots also deserves special attention, as it was regularly observed in the zones visited in Palmas.

The occurrence of a high number of homes in poor maintenance conditions is also noteworthy because it is in endemic areas that socioeconomic and environmental conditions, side by side with life habits of the population, become important factors in the VL epidemiology. These variables also play a defining role in the progression of the disease in rural areas and in zones surrounding cities, which are places inhabited by low-income populations in makeshift homes[30]. This precariousness of homes favors the accumulation of organic matter (waste from rearing of animals like swine and poultry) in the roundabouts, which promotes the development of some species that are able to adapt to this new environment, as observed for vectors like *L. longipalpis* and some native mammals (rodents, sloths, etc.) that may play a role as hosts and reservoirs in VL cycle. In these locations, the disease follows an endemic behavior and may, eventually, even acquire epidemiologic status (Costa: unpublished data). Several studies have pointed, in detail, to a series of risk factors associated mainly to local socioeconomic conditions, which may determine the occurrence of *L. (L.) chagasi* in canine and human population, like precarious housing conditions that promote the development of the vector[31-35], apart from the lack of elementary sanitation and the sporadic garbage collection, which furnish the ideal microenvironments for phlebotomine reproduction[5,36]. These risk factors were observed in all regions evaluated in the present study.

PCR has been proven to be a fast and efficient methodology in investigations on diseases affecting canine populations, representing a good technical alternative in epidemiological actions by public authorities, especially in situations where a large number of samples has to be analyzed. This affords fast diagnosis in support for surveillance and control of VL.

Considering VL, the City of Palmas is an endemic region, and the present study demonstrates the existence of zones where the prevalence of *L.* (*L.*) *chagasi* in dogs is comparatively higher. The existence of these hot spots may be related to the presence of debris, domestic garbage, and organic waste accumulated in properties and vacant lots, in association to the high canine population — mainly of stray dogs. Additionally, poultry-rearing activities, together with the large canine population observed in these properties, are associated to the increased prevalence of the infection by *L.* (*L.*) *chagasi* in dogs.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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