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.

**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\textsuperscript{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\textsuperscript{1,4}.

In Brazil, VL is considered an endemic disease, though outbreaks occur more or less often due to poor immune response of hosts\textsuperscript{4} and to an eco-epidemiological scenario that favors the proliferation of infected vector populations\textsuperscript{4}. In 2003, the State of Tocantins, Northern Brazil, recorded the highest prevalence of leishmaniasis in the country, with 20 cases per 100,000 inhabitants\textsuperscript{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\textsuperscript{1}. Also, non-specific cross-reactions and delay between infection and seroconversion occur often\textsuperscript{1}.

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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. 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.

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 (10°12’46”S; 48°21’37”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...
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 27 mM 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 (saccarose 5mM; Tris-HCl 10mM pH 7.5; MgCl₂ 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 37°C for 3h. Immediately after incubation, DNA was extracted according to the phenol-chloroform method¹³, precipitated with ethanol 100% and NaCl SM, resuspended in 50μL TE + RNAse (10mg/mL), and freeze-stored at -20°C.

**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.²⁶ 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), MgCl₂ 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: 94°C for 5min, 35 denaturation cycles at 94°C, primer hybridization at 58°C, extension at 72°C, 72°C for 10min, and 4°C for 10min. All reactions were conducted in triplicate, and the DNA of a *Leishmania*-negative individual, in triplicate.

**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 oligosymptomatic, and 65 (31.9%) were polysymptomatic, according to the criteria previously described.¹⁸,¹⁹ The most frequent symptoms were oncroyphosis, 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).

### 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.

<table>
<thead>
<tr>
<th>Regions</th>
<th>Sample size (number of dogs)</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-1</td>
<td>16</td>
<td>75.0</td>
</tr>
<tr>
<td>R-2</td>
<td>32</td>
<td>75.0</td>
</tr>
<tr>
<td>R-3</td>
<td>10</td>
<td>50.0</td>
</tr>
<tr>
<td>R-4</td>
<td>13</td>
<td>46.2</td>
</tr>
<tr>
<td>R-5</td>
<td>27</td>
<td>66.7</td>
</tr>
<tr>
<td>R-6</td>
<td>38</td>
<td>39.5</td>
</tr>
<tr>
<td>R-7</td>
<td>60</td>
<td>61.7</td>
</tr>
<tr>
<td>R-8</td>
<td>8</td>
<td>50.0</td>
</tr>
<tr>
<td>Total</td>
<td>204</td>
<td>59.3</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Aspects evaluated</th>
<th>Regions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R-1</td>
</tr>
<tr>
<td>Urbanization level</td>
<td>low</td>
</tr>
<tr>
<td>Garbage collection system</td>
<td>≈ 80%</td>
</tr>
<tr>
<td>Open sewers</td>
<td>≈ 25%</td>
</tr>
<tr>
<td>Vacant lots</td>
<td>+</td>
</tr>
<tr>
<td>Stray dogs</td>
<td>+</td>
</tr>
<tr>
<td>Henhouses</td>
<td>+</td>
</tr>
</tbody>
</table>

As pointed out by Fallah et al., 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 infecting 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.

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 VL.

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 and the high prevalence of L. (L.) chagasi is in loco. The 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 and the high prevalence 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. Fallah et al. and Chargui et al. 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.

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. 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. Fallah et al. and Chargui et al. 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.

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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.

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.
Females of *Lutzomyia longipalpis* lay their eggs preferably on a humid substrate presenting high content of organic matter on soil1. 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 homes80. 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 with the disease in zones visited in Palmas. This precariousness of homes favors the accumulation of properties and vacant lots also deserves special attention, as it was regularly observed in the zones visited in Palmas.

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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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