

**American cutaneous leishmaniasis in dogs from an endemic urban area in
Cianorte municipality, Paraná State, Brazil**

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ABSTRACT: American cutaneous leishmaniasis (ACL) was investigated in dogs from an urban endemic area in Cianorte, Paraná state, Brazil. Of 169 studied dogs, none presented suspected ACL lesions. Eleven animals (6.6%) had anti-*Leishmania braziliensis* antibodies (titers ≥ 40) detected by the immunofluorescent antibody test (IFAT) while four (2.4%) showed *L. braziliensis*-complex DNA by the polymerase chain reaction (PCR). Although no associations were found between IFAT or PCR results and age, sex, origin, free-roaming animals or length of residence at the address, the majority of IFAT- or PCR-positive dogs were from the urban area of the city and were allowed to roam freely beyond their neighborhood. The presence of anti-*Leishmania braziliensis* antibodies and *L. braziliensis*-complex DNA in dogs from this urban area near a native-forest park indicates the importance of following up on these dogs to confirm the ACL diagnosis.

KEY WORDS: *Leishmania braziliensis*, fluorescent antibody technique, polymerase chain reaction, urban park.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

American cutaneous leishmaniasis (ACL) is a widespread zoonosis from the southern United States to northern Argentina (1). In Brazil, it comprises a serious public-health problem due to its high incidence rate and broad geographic distribution (2). From 1980 to 2005, 98.7% of all ACL cases in the southern region of Brazil occurred in the state of Paraná (3). In northwest Paraná, ACL cases are mostly provoked by *L. (V.) braziliensis* (4-7). Images from orbital remote sensing of this region reveal a close relationship between ACL and areas of modified native forest, small river or lakeside forests or their remnants (8).

The life cycle of parasites of the genus *Leishmania* depends on vertebrate (wild mammals) and invertebrate (sandflies) hosts (9, 10). Until the 1940s, ACL was considered a wild animal zoonosis that occasionally affected humans in contact with forests. In recent decades, the literature has reported changes in the epidemiology of the disease (8). Recently, this infection has been occurring in highly populated rural areas and in urban peripheries. Normally, remnants of native forests surround such areas, where enzootic foci persist (8). The adaptation of sandflies and wild reservoirs, such as synanthropic rodents, to densely populated areas is evident and favors the transmission of this infection in peridomestic and domestic areas of rural and urban areas (11, 12). In endemic areas, besides humans, domestic animals – especially dogs and horses – are frequently affected by the disease (4, 13-16). The concomitance of dog and human infection cases in the same environment suggests that dogs may play a role in maintaining ACL transmission in domiciliary environments (17, 18).

Cianorte city, in northwest Paraná, presents one of the highest incidences in the state. In 2005, it was 35.4 per 100,000 inhabitants, with a large concentration of human ACL cases in the urban area (8). The Cianorte urban area is surrounded by a modified native forest reserve (about 7 km long, called the Green Belt City Park) that was labeled as “the site where the infection probability is higher”.

The presence of human ACL cases in the urban area, the proximity of houses to the forest and the possibility that dogs may play a role in the ACL transmission cycle were the motives for the present study, which aims to investigate *Leishmania* sp. infection in dogs from the urban area of Cianorte.

MATERIALS AND METHODS

Area of Study

Cianorte is situated in the Northwest Mesoregion (23°39' S; 52°38' W) of Paraná state in southern Brazil, at an altitude of 530 meters. The city presents 812 km² total area, of which the urban perimeter occupies approximately 35 km². Its urban population is 53,735 inhabitants, while the rural is 8,401 (19).

The study was carried out in the urban area of the city, in nine blocks (# 73, 74, 77, 78, 79, 80, 96, 97 and 98) of the district Zone 3, in the vicinity of the Manduí part of the Green Belt Park, which presents altered native forest vegetation.

Study Design

The study was carried out from February to August 2006. The blocks were chosen according to their proximity to the forest reserve and because they showed a high concentration of human ACL cases within the Cianorte urban area from 1993 to 1998 (8).

All residences on these blocks were inspected and all dog owners were informed about the disease and the study procedures. Those who agreed to participate in the study gave written authorization, through a free and informed consent form.

Each dog was clinically examined for the presence of suspected ACL lesions and provided blood samples. Data were collected – including name and address of the owner; name, age and sex of the dog; length of residence at the current address, origin, and whether the animal could roam freely beyond the domiciliary area – and recorded on epidemiological index cards.

The study was submitted to the Committee for Ethical Conduct in the Use of Animals in Experiments (CEAE) of the State University of Maringá.

Biological Material

An aliquot (1-1.5 mL) of blood was added to an equal volume of ACD solution (25 mM citric acid; 50 mM sodium citrate; 81 mM glucose). The leukocyte layer was obtained after centrifugation (1,200 g for ten minutes) and stored at –20°C until DNA extraction. From another aliquot of blood, serum was obtained and stored at –18°C until use.

Immunofluorescent Antibody Test (IFAT)

IFAT for *Leishmania* was carried out according to Silveira *et al.* (20), using *Leishmania (Viannia) braziliensis* promastigotes as antigens and anti-dog immunoglobulin G conjugated with fluorescein (Sigma, Germany). Titers of at least 40 were considered positive results (5). The Imunocruzi® antigen (Biolab, Brazil) was employed to reveal anti-*Trypanosoma cruzi* antibodies. Serum obtained from dogs with ACL (positive microscopy of lesions) was used as positive control, whereas serum of dogs from non-endemic areas served as the negative control; all samples were previously analyzed by IFAT.

Polymerase Chain Reaction (PCR)

The phenol-guanidine isothiocyanate method was utilized to extract the DNA from leukocyte layer samples (21). In brief, the samples were thawed and centrifuged (3,500 g for 15 minutes) and the sediment washed with PBS. Three-hundred microliters of phenol-guanidine isothiocyanate solution and 50 µL of chloroform were added to the sediment. After centrifugation (9,200 g for ten minutes), the supernatant was added to 300 µL of absolute ethanol and centrifuged (9,200 g for 15 minutes). The sediment was washed twice with 300 µL of absolute ethanol, dried in a drying bath (Bioplus IT-2002®, Brazil) at 95°C, rehydrated in 50 µL of TE buffer (10 mM TRIS; 1 mM Na₂EDTA.H₂O, pH 8.0) and stored at 4°C until use. Negative and positive controls were, respectively, uninfected dog blood and dog blood with *L. (V.) braziliensis* (MHOM/BR/1987/M11272) promastigotes. For each set of samples submitted to DNA extraction, one positive and one negative extraction control were employed.

The primers LU-5A (5'-TTT ATT GGT ATG CGA AAC TTC-3') and LB-3C (5'-CGT (C/G)CC GAA CCC CGT GTC-3') were utilized for DNA amplification, as described by Harris *et al.* (22), in which a fragment of 146 to 149 base pairs (bp) of RNA gene multicopy leader sequence of the *L. braziliensis* complex is amplified. The reaction medium (25 µL) consisted of 1 µM of each primer (Invitrogen, Brazil), 0.2 mM dNTP (Invitrogen, USA), 1 U Taq DNA polymerase (Invitrogen, Brazil), 1.5 mM MgCl₂, enzyme buffer and 5 µL of the DNA sample. A positive [2 pg DNA obtained by boiling *L. (V.) braziliensis* (MHOM/BR/1987/M11272) promastigotes] and a negative (ultrapure water) amplification control were used.

The amplification was carried out in a Personal Thermocycler® (Biometra, Germany) at a constant temperature of 95°C for five minutes. Then, 30 cycles were carried out comprising denaturation (95°C, 1.5 minute), annealing (57°C, 1.5 minute) and polymerization (72°C, 2 minutes) processes. Subsequently, microtubes were kept at 72°C for ten minutes and then stored at 4°C until use. The amplified samples were submitted to electrophoresis in 2% agarose gel, containing 0.1 µg/mL of ethyl bromide, at 10-15 V/cm. The presence of bands was verified in a transilluminator (MacroVue UV-20®, Hoefer, USA). For every five amplified samples, one positive and one negative amplification control were amplified.

Data Analysis

The software EpiInfo® (Centers for Disease Control and Prevention, USA), version 3.32, was employed to analyze data, at a significance level of 5%. The associations between positive IFAT or PCR results and age, sex, length of residence in the area, ability to roam freely beyond the domiciliary area and the animal origin were assessed by Fisher's exact test.

RESULTS

A total of 169 dogs – 106 (62.7%) males and 63 (37.3%) females – from 117 houses were examined. Their ages ranged from two months to 13 years (mean: 3 years ±1 month); the owners of 13 animals did not know the ages of their dogs.

The lengths of residence of dogs at their current addresses ranged from one month to 13 years (mean: 2 years ±9 months). Regarding the origin, 91 (53.8%) were from the urban area of the city, 29 (17.2%) were stray dogs, 17 (10.1%) were from a rural area, 16 (9.5%) were born in their current house, 11 (6.5%) were from urban areas of other municipalities and five (2.9%) were of origins unknown to their owners. With regard to the ability to roam freely beyond the domiciliary area, 94 dogs (55.6%) were able to leave their yards and 75 (44.4%) were not.

None of the animals had suspected ACL lesions. IFAT, carried out in 166 dogs, was positive in 11 (6.6%), all from different houses. Seven of these dogs (63.6%) had titers of 40, and four (36.4%) had titers of 80. Examination for anti-*T. cruzi* antibodies in these 11 dogs showed negative results (titers ≤ 20). PCR, carried out in 167 dogs, was positive in four (2.4%), all from different houses and had titers inferior to 40

through IFAT. The PCR sensitivity was 0.5 pg. Figure 1 shows residential locations of dogs with positive serology and PCR.

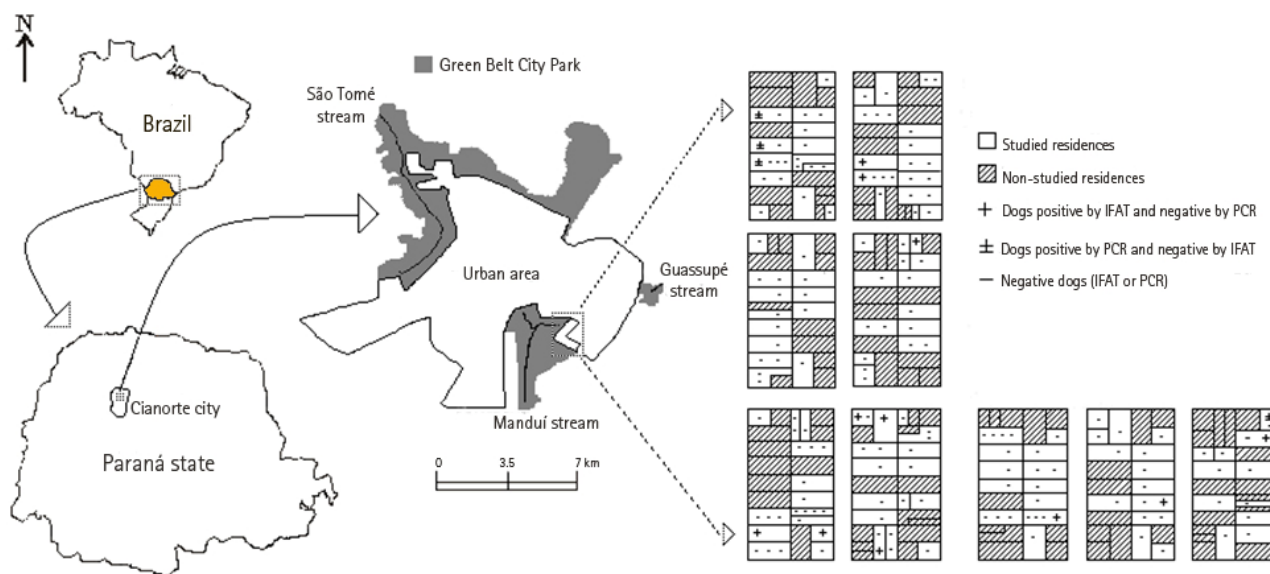


Figure 1. Location of Cianorte city, Paraná, Brazil. The study area and the analyzed dogs are indicated according to the results of the immunofluorescent antibody test (IFAT) and polymerase chain reaction (PCR).

No association between the IFAT and PCR results was found (Fisher; $p = 0.7554$). Additionally, no relation between IFAT or PCR results and other parameters (age, sex, origin, habit of roaming freely and length of residence at the address) was found (Table 1).

Table 1. Epidemiological data and results of IFAT and PCR from dogs studied in Cianorte city, Paraná, Brazil

	IFAT (n = 166)			PCR (n = 167)		
	Positive (11 – 6.6%)	Negative	<i>p</i> *	Positive (4 – 2.4%)	Negative	<i>p</i> *
Age						
≤ 3 years	8	93	0.3095	1	100	0.1709
> 3 years	3	62		3	63	
Sex						
Male	8	95	0.3403	2	102	0.4858
Female	3	60		2	61	
Origin						
Urban area	8	109	0.5842	1	106	0.0955
Unknown place	3	46		3	47	
Habit of roaming freely						
Yes	9	2	0.0675	3	91	0.4106
No	84	71		1	72	
Length of residence at the address						
≤ 3 years	9	113	0.4035	2	120	0.2939
> 3 years	2	42		2	43	

DISCUSSION

The study region in the urban area of Cianorte was considered by Lima *et al.* (8) a place where *Leishmania* infection is highly probable due to the elevated concentration of human ACL cases diagnosed from 1993 to 1998. In northwest Paraná, *Leishmania (Viannia) braziliensis* has been isolated from both human and canine ACL cases (6, 14). The role of dogs in ACL transmission cycle is still controversial. It is not completely understood whether the animals participate or if they are only accidentally infected with the parasite, as are humans (23).

In the present study, no dog showed suspected ACL lesions, but IFAT was positive in 6.6% of them. This test has been used in several studies to detect *Leishmania* infection in dogs (24-27). Although serological cross-reactivity in visceral leishmaniasis is well known, autochthonous cases of visceral leishmaniasis have never been reported in Paraná (3, 28). Passos *et al.* (18) found 3.2% positivity in dogs without lesions in the urban periphery of Sabará city, Minas Gerais state. In

Paraná state, several studies have demonstrated higher percentages of IFAT positivity in dogs without lesions that lived in rural areas of endemic regions (5, 7, 15, 29). In these localities, dogs presenting ACL lesions have also been found (14, 15, 29, 30).

The PCR test using LU-5A and LB-3C primers had a sensitivity of 0.5 pg for DNA from the *L. braziliensis* complex, close to that found by Harris *et al.* (22). DNA from the *L. braziliensis* complex was detected in 2.4% of analyzed dogs. In other studies of animals from endemic areas, higher percentages have been found in dogs without suspected lesions. However, using both different protocols to obtain the DNA and diverse targets for PCR, Reithinger *et al.* (31) found 7.1% positivity and Velásquez *et al.* (29) 15.4% positivity, after an outbreak of human ACL. In both studies, dogs with lesions were also discovered. The lower positivity for IFAT and PCR in the present study may be related to the location from which these dogs originated, an urban area, and to the fact that none of them had suspected lesions. Follow-up of positive animals is essential to confirm the ACL diagnosis, as suggested by Madeira *et al.* (32).

No association was found between positive IFAT and PCR results. Furthermore, in other ACL endemic areas, no connection between serology and the presence of *Leishmania* DNA has been reported (29, 33). An explanation for this finding is that dogs had not yet developed an immune response to this infection (33). There was also no relationship between positive results from these techniques and age, sex, origin, length of residence or habit of roaming freely beyond the canine domiciliary area. However, most animals that revealed positive serology (63.6%) and other four positive PCR dogs (100%) were from either the urban area of the city or had been found in its streets (strays); nine dogs (81.8%) with positive serology and three (75.0%) with positive PCR were allowed to roam freely in streets and could enter the edges of the forest. These data suggest that forest may constitute a possible natural focus of ACL transmission in the urban area, where these dogs could have contact with *Leishmania* (34, 35).

A similar work in Rio de Janeiro showed 74.6% seropositivity in dogs born either in houses or somewhere else in the study area, and that all serologically positive animals were allowed to leave their yards, suggesting that they participated in the ACL transmission chain (27).

The present study demonstrated the presence of anti-*Leishmania braziliensis* antibodies and of *L. braziliensis*-complex DNA in dogs from an urban area of Cianorte, which indicates the importance of subsequently monitoring the dogs to confirm the ACL diagnosis. Further studies should be carried out in order to better understand ACL transmission in the urban area and the role of domestic dogs in its cycle.

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