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Epidemiology of canine leishmaniasis in southern Bahia, Brazil

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ABSTRACT

Leishmaniosis is a zoonosis caused by protozoa of the genus *Leishmania*. American cutaneous leishmaniosis (ACL) is mainly caused by the species *L. amazonensis* and *L. braziliensis*, and American visceral leishmaniosis (AVL) is caused by *L. infantum chagasi*. In addition to their proven roles as reservoirs of AVL, dogs are also suspected by researchers to be reservoirs of ACL due to reports of this infection in domestic environments and of infected dogs in endemic areas. The aim of this study was to detect *Leishmania* sp. infection in dogs from Vila Operária, Buerarema, Bahia, using parasitological tests, indirect immunofluorescent assay (IFA) and polymerase chain reaction (PCR). Furthermore, this study also aimed to identify risk factors associated with illness in dogs in this locality by conducting an epidemiological survey. For this purpose, 292 dogs were clinically evaluated for the presence of skin lesions, and the dogs that showed these changes were submitted to scarification injury to enable preparation of slides for microscopic study of amastigotes. Subsequently, the dogs underwent blood sampling for serological (IFA) and molecular (PCR) tests. Additionally, the owners of the dogs answered an epidemiological questionnaire to facilitate the identification of risk factors for exposure of dogs to pathogens of ACL. Of the 292 dogs studied, 13 (4.5%) had lesions suggestive of ACL, but with a negative parasitological examination and 147 (50.3%) were seropositive according to the IFA. Of the 273 dogs studied using PCR test, 10 (3.66%) were positive for *L. braziliensis*, and all samples were negative for *L. infantum chagasi*. Wastelands in the peridomicile and the presence of light in the household were risk factors associated with ACL. The results show that Vila Operária has asymptomatic dogs with ACL and that the detection sensitivity of the IFA was higher than that of PCR for the infected dogs.

1. Introduction

The leishmaniases are zoonoses caused by digenetic protozoa of the genus *Leishmania* (Dawit et al., 2013), that are divided into two categories: American visceral leishmaniosis (AVL) and American cutaneous leishmaniosis (ACL) (Ashford, 2000; Brasil, 2010; Chappuis et al., 2007; Heusser Júnior et al., 2010).

The cutaneous form of the disease is described from the southern tip of the United States to northern Argentina, except for Chile and Uruguay. In Brazil, seven disease causing species are recognized, with the most important being *Leishmania* (*Vianna*) *braziliensis* and *Leishmania* (*Leishmania*) *amazonensis* (Brasil, 2010). Data published by the Secretaria de Vigilância em Saúde (2011) revealed that the North and Northeast regions were responsible for the vast majority of diagnoses recorded between the years 2000 and 2010, highlighting the state of Bahia, which recorded 10.2% of the human cases diagnosed in the country during this period. The high number of human cases of ACL that were associated with the diagnosis of this infection in dogs led some authors to suspect that such animals may be acting as a natural source of infection (Dantas-Torres, 2007; Falqueto et al., 1986; Heusser Júnior et al., 2010; Pittner et al., 2009).

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The AVL, caused by *L. infantum chagasi*, in turn, is a disease of chronic evolution and, in Latin America, has been reported in at least 12 countries, with 90% of the cases of the disease on the continent concentrated in Brazil (Brasil, 2013; Chappuis et al., 2007; Maia-Elkhoury et al., 2008; Melo, 2004; Rondon et al., 2008; Tolezano et al., 2007). Infected dogs are considered reservoirs of the disease in rural and urban areas, having great ability to transmit the agent to the vector (Rodrigues et al., 2007).

Thus, considering the role of dogs as reservoirs of AVL and the possibility to also act as a reservoir of ACL, thereby influencing the transmission and maintenance of the disease, this study aimed to detect the presence of *Leishmania* sp. in dogs by parasitological, serological and molecular tests, as well as to identify factors associated with infection in an endemic area for human ACL in northeastern Brazil.

2. Materials and methods

2.1. Local search

The study was conducted between the months of March and August 2010 in the District of Vila Operária, City of Buerarema (Latitude: 14°56′ South, Longitude: 39°18′ west), southern Bahia, which is considered an endemic area for human ACL (Castellano et al., 2009). The Vila Operária is a rural district, approximately 7 km away from the town center of Buerarema, consisting of a human settlement of predominantly rural character, popularly known by residents as "Sururu," and other areas composed of farms. Vila Operária has a humid tropical climate, an average annual temperature of 26 °C and annual rainfall ranging from 1500 mm to 2000 mm. This locality is within the Atlantic forest, and the main livelihood is cocoa farming.

2.2. Animals

All canine populations residing in the locality were included in this study, excluding dogs less than six months of age, for a total of 292 dogs. After authorization of the owners, the dogs were initially assessed clinically for the presence of skin lesions, and subsequently, blood samples (approximately 5 ml) were collected by venipuncture of the cephalic or jugular veins. The samples were separated into two tubes without anticoagulant for conducting serological tests and into one tube with anticoagulant (EDTA), for molecular biology procedures. The methodology used in this research was judged and approved by the Comitê de Ética no Uso de Animais (CEUA) from the Universidade Estadual de Santa Cruz—UESC, under protocol no. 002/10.

2.3. Epidemiological data

A semistructured interview was conducted with the owners of the dogs with the aim of identifying factors associated with infection and covered issues such as review of the dog, characteristics of the environment in which the dog lives and contact with other animals (other dogs, cats, donkeys and rodents) (Almeida et al., 2009).

2.4. Parasitological exam

Dogs that presented with skin changes consistent with ACL during the clinical examination underwent scarification of lesions with the aid of scalpel blades. The collected material was distributed to glass slides, which were stained with fast Panoptic for microscopic observation of *Leishmania* sp. amastigotes (Dantas-Torres, 2006).

2.5. Serology

The tubes without EDTA were centrifuged at 1292 × *g* for 15 min to obtain serum. The serological technique used was immunofluorescence assay (IFA), and a kit for the diagnosis of Canine Leishmaniasis-Bio-Manguinhos® (Ribeiro et al., 2007) was used, following the manufacturer's recommended protocol. Titration of the conjugate was performed according to the guidelines of FUNED-Fundação Ezequiel Dias. Serologic titers ≥1:40 were considered positive.

2.6. Molecular biology

The blood samples with anticoagulant were centrifuged at 1292 × *g* for 15 min to separate the buffy coat. DNA was extracted from the buffy coat using the phenol-chloroform method and stored at −20 °C. After extraction, the DNA of all samples was measured using a spectrophotometer.

PCR was performed to detect the DNA of the etiological agent involved in the infection because there are reports in the literature of possible cross-reactivity in serological tests among agents of dermotropic leishmaniosis, visceral leishmaniosis and Chagas disease (Camargo and Rebonato, 1969; Luciano et al., 2009). For this, specific primers were used for the detection of DNA from *L. braziliensis* and *L. infantum chagasi*.

For detection of DNA from *L. braziliensis*, the sequences of the primers used were B1 (5′GGGGTTGGTGTAATATAGTGG 3′) and B2 (5′CTAATTGTGCACGGGGAGG 3′), which amplify a fragment of 750 bp of DNA from *Leishmania* (*Viana*) *braziliensis* (Bruijn and Barker, 1992). The conditions for the PCR reaction, including the total number of cycles; the denaturation, annealing and extension temperatures; and the concentrations of the MgCl2 and Taq DNA polymerase, were adapted from those previously described by Reithinger et al. (2000). The reaction was initiated in a final volume of 25 μl, composed of 17 μl Supermix® (Invitrogen), 1 U Taq DNA polymerase (Invitrogen), 0.6 mM MgCl (Invitrogen), 20 pmol of each primer and 100 ng of DNA. DNA amplification was performed using a thermocycler (*Biocycler* MJ96G) programmed for initial denaturation for 5 min at 94 °C; followed by 35 cycles of 94 °C for 1 min, 59 °C for 1 min, and 72 °C for 1 min; and final extension at 72 °C for 7 min. The PCR products were subjected to electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and photodocumented. A pure culture strain of *L. braziliensis* (MHOM/BR/3456) was used as a positive control and ultrapure water as a negative control.

For detection of DNA from *Leishmania infantum chagasi*, the sequences of the primers used were RV1 (5′CTTTTCTGGTCCCGCGGGTAGG 3′), and RV2 (5′CCACCTGGCCTATTTTACACCA 3′), which amplify a fragment of 145 bp of DNA from *L. infantum chagasi* (Ravel et al., 1995). The conditions for the PCR reaction, such as the total number of cycles; the denaturation, annealing and extension temperatures; and the concentrations of the MgCl2 and Taq DNA polymerase, were adapted from those previously described by Lachaud et al. (2002). The reaction was conducted in a final volume of 25 μl, which was composed of 17 μl Supermix® (Invitrogen) 20 pmole of each primer and 100 ng of DNA. DNA amplification was performed according to the program used by Reithinger et al. (2000) with adaptations, with an initial denaturation of 5 min at 94 °C; followed by 35 cycles of 94 °C for 45 s, 59 °C for 45 s, and 72 °C for 45 s; and a final extension of 72 °C for 7 min. The PCR products were subjected to electrophoresis on a 2% agarose gel, stained with ethidium bromide and photodocumented. A pure culture strain of *L. chagasi* (MHOM/BR2000/Merivaldo) was used as a positive control and ultrapure water as the negative control.

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Table 1
Factors associated with infection by *Leishmania* sp. in dogs from the Vila Operária, Buerarema, Bahia.

Variable		Positive	Negative	*P*-value	OR	CI-95%
Sex	Male	95	93	0.97	1.02	0.63–1.64
	Female	52	52			
Age	≤4 years	104	85	0.05	1.79	1.02–3.13
	>4 years	28	41			
Presence of banana tree	Yes	132	135	0.42	0.65	0.28–1.50
	No	15	10			
Presence of septic tank at the dwelling	Yes	46	29	0.03	1.82	1.06–3.11
	No	101	116			
Presence of electric light in the dwelling	Yes	142	132	0.08	2.79	0.97–8.05
	No	05	13			
Presence of vacant land around the home	Yes	126	112	0.08	1.76	0.96–3.23
	No	21	33			
Contact with donkeys	Yes	69	68	0.91	1.00	0.63–1.58
	No	78	77			
Presence of chicken coop	Yes	90	88	0.97	1.02	0.63–1.63
	No	57	57			
Contact with stray dogs	Yes	73	75	0.81	0.92	0.58–1.45
	No	74	70			
Contact with marsupials	Yes	53	44	0.36	1.29	0.79–2.10
	No	94	101			
Contact with rodents	Yes	62	58	0.79	1.09	0.68–1.74
	No	85	87			
Contact with foxes	Yes	3	2	0.98	1.48	0.24–9.04
	No	144	143			

2.7. Statistical analysis

Statistical analyses were performed using Epi Info 7.1.3.0 (CDC, USA), taking into account the exposure variables, on the results obtained using a serological technique. Data were compared using Fisher's exact test and chi-square (χ^2). The odds ratio (OR) of the bivariate analysis was calculated using measures of association and a confidence interval of 95%. Variables with a *p* value less than or equal to 20%, which were obtained using bivariate analysis that demonstrated biological plausibility, were selected and subjected to multivariate analysis using unconditional logistic regression, with the final model created through the output variables of the system.

3. Results

During the clinical evaluation, only 13 (4.45%) dogs showed skin changes consistent with ACL, and these were characterized by circular, ulcerated lesions and humid aspect, located in the inner and outer ear canal, nasal mirror, scrotum and tissue skin; however, no amastigote forms of protozoa of the genus *Leishmania* sp. were observed in the samples obtained by scraping the lesions.

On serological evaluation, 147 dogs (50.3%) showed positive results, with titers ≥40. Of the 13 dogs that had skin lesions consistent with cutaneous leishmaniosis on clinical examination, six were serologically positive.

Of the 292 samples collected, 19 had become nonviable for PCR because the recommended time between collection and processing of the material for DNA extraction was exceeded. Thus, only 273 samples were analyzed using PCR. Of the 273 dogs tested for DNA of *L. braziliensis*, 10 (3.7%) had positive results. Of these 10 dogs, only 4 had positive results according to IFA. There was no detection of *L. infantum chagasi* DNA in the samples evaluated using PCR.

The analysis of epidemiological data revealed that basic services and structures such as health networks, septic tanks, garbage collection, residential plumbing and electricity were present, respectively, at 7.5%, 25.7%, 50.7%, 72.3% and 93.8% of the households visited. In addition, in the epidemiological questionnaire, dog owners reported the presence of wild animals around the homes, mainly rodents, marsupials (opossum) and foxes. The risk factors associated with canine leishmaniosis identified in this study were the presence of vacant land around the homes and electric light in the homes (Tables 1 and 2).

4. Discussion

The absence of amastigotes in samples of skin lesions observed using light microscopy demonstrated that cytological examination for the diagnosis of leishmaniosis was less sensitive than other techniques. According to Andrade et al. (2005), the sensitivity of direct detection of parasites in blood smears varies from 50 to 70%; however, sensitivity tends to decrease with disease progression, reaching only 20% after a year of infection. Possibly, the dogs of this study, whose injuries were subjected to direct detection of parasites, were in advanced stages of infection, when the possibility of visualization of amastigotes at the site of skin injury was reduced. In addition to the parasitic load in lesions being reduced in most cases, the bacterial contamination that occurs makes it difficult to view the samples of protozoa (Andrade et al., 2005; Schubach et al., 2001). Additionally, the cutaneous lesions observed in dogs of this study, although consistent with those developed in ACL, were nonspecific and may have been symptomatic of other skin disorders. This suggests that the lesions found may not be related to the ACL, and therefore, a parasitic load would not be expected.

Prevalence data from this study corroborate the results published by Dantas-Torres et al. (2006), who reported a high prevalence (40.3%) of anti-*Leishmania* sp. antibodies in a canine population resident in an endemic area for leishmaniosis. These results take into account that IFA is a technique with high sensitivity (90% to 100%) and specificity (80%) in the detection of anti-*Leishmania* antibodies (Luciano et al., 2009). From the IFA-positive

Table 2
Association between positive dogs and factors: the presence of vacant land around the home, living in urban areas and the presence of a septic tank at homes in the Vila Operária District.

Variable	Odds ratio	Confidence interval 95%	*P*-value
Presence of vacant land around the home	1.92	1.04–3.52	0.0354
Presence of electric light in the dwelling	3.17	1.09–9.21	0.0333

$p = 0.0085$; likehood = 0.0138.

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dogs, only six (4.1%) had suggestive ACL lesions, which leads us to infer that in the locality studied, a large number of dogs were infected and asymptomatic (95.9%).

Although IFA can cross-react with antigens of *T. cruzi* and *L. infantum chagasi*, thereby allowing false positives (Luciano et al., 2009), in this study there was no detection of DNA of *L. infantum chagasi* in the samples evaluated using PCR. Additionally, in a previous study in the same area by Leça Júnior et al. (2013), DNA of *T. cruzi* was detected using PCR in only two of the 273 (0.7%) dogs evaluated. These results minimize the possibility of cross-reactions that may have occurred in the serological tests, confirming the high prevalence of cutaneous leishmaniosis in dogs in the study location. Additionally, studies carried out by Azevedo et al. (1996) and Carvalho et al. (2010) didnot observe the presence of *Lutzomyia longipalpis*, the biological vector incriminated in the transmission of American visceral leishmaniosis, which would minimize the emergence of autochthonous outbreaks in the region under study. The results published in a previous study in humans in Ilhéus, southern Bahia, suggested that *L. braziliensis* was the etiological agent involved in cases of cutaneous leishmaniosis in the region (Carvalho et al., 2010).

The low detection of *L. braziliensis* DNA in seropositive dogs can be explained by the fact that the DNA was taken from samples of blood (leukocyte layer), which reduces the sensitivity of PCR for *Leishmania* according to Reithinger et al. (2000). These authors also claim that the protozoa of the genus *Leishmania* subgenus *Viannia* are located initially at the site of infection in the dermis and that dissemination by the hematogenous pathway occurs only after an indefinite period of time. In the case of ACL, the parasites are located predominantly at the site of infection in the dermis, with reduced spread, which occurs only when the parasites are phagocytosed. Moreover, although the research data from this study corroborate the results obtained by Reithinger et al. (2000), they contradict the data obtained by Velasquez et al. (2006) and Dantas-Torres et al. (2010), who concluded that the origin of the sample, whether from blood or tissue, does not influence the sensitivity of PCR for the detection of DNA from *L. braziliensis*.

Regarding factors associated with infection, the presence of vacant land around the home proved to be a risk factor for exposure of the dog to leishmaniosis, increasing the chances of infection by 1.92 times. This is due to the possibility for these lands to serve as garbage dumps or dumps for organic matter, mainly because the on-site garbage collection is inefficient, and these encourage the approach of infected animals, such as small rodents and wild animals, and the vector of the disease to the peridomestic environment. The presence of small rodents infected with *L. braziliensis* has been reported by Brandão-Filho et al. (2003) in an endemic area for ACL in the state of Pernambuco, and these have proven roles as wild reservoirs of the parasite (Brasil, 2010). Furthermore, Lainson (1985) concluded that the deposition of organic matter close to home would also act as an attractant to marsupials, wild reservoirs of ACL (Brasil, 2010) that feed on household waste. This would facilitate the transmission link between the sylvatic and peridomiciliary environment because these animals would act both as a reservoir of the disease and as a food source for the vector in the environment.

Additionally, the presence of electricity in the households included in the study was also revealed as a risk factor for leishmaniosis, and increased the possibility of exposure of dogs to infection by 3.17 times. *Lutzomyia whitmani*, one of the species of vector incriminated in the transmission of leishmaniosis in Brazil, has already been identified in the study area, in the city of Ilhéus, Bahia (Azevedo et al., 1990a,b; Carvalho et al., 2010), which is limited to the southwest with Buerarema (Faria Filho and Araújo, 2003). Thus, given the proximity of the two municipalities, it can be suggested that the vector species found in Ilhéus can also be found in Buerarema. *Lutzomyia whitmani* differs from other sand flies because it is characterized by daytime activity (Santos et al., 2009). Furthermore, the results of work undertaken by Teodoro et al. (2007) in the state of Paraná proved that this sand fly species can be attracted by the presence of light sources in the peridomiciliary environment. Taken together, these data may explain the influence of the electric light on the establishment of ACL in the study area.

The results obtained in this work suggest that the region has a high prevalence of canine cutaneous leishmaniosis and a large number of asymptomatic infected dogs. Although the role of dogs as reservoirs of ACL is not yet proven, these results indicate that asymptomatic dogs may be important in maintaining the agent of ATL in the study area because the area is considered an endemic area for human ACL.

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