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Parasitological diagnosis of canine visceral leishmaniasis: Is intact skin a good target?

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ABSTRACT

The objective of this study was to evaluate intact skin of seroreactive dogs as a possible target for the parasitological confirmation of canine visceral leishmaniasis (CVL). For this purpose, 394 dogs identified in serological surveys carried out in the metropolitan region of Belo Horizonte were studied. Blood was collected from all animals for serology and a tissue sample was obtained from two sites for parasitological diagnosis. Skin obtained from the ear and scapular region was simultaneously analyzed in 247 animals and lesion samples and ear skin were analyzed in 147 dogs. *Leishmania* parasites were isolated from 310 (78.7%) animals, and all isolates were identified as *Leishmania chagasi*. Simultaneous isolation from two sites was possible in 240 of the 310 animals, including ear and scapular skin in 151/247 (61.1%) and ear skin and skin lesions in 89/147 (60.5%). Ours results suggest that intact skin is one of the main target sites for the parasitological confirmation of CVL in seroreactive dogs.

Visceral leishmaniasis (VL) is a zoonosis of worldwide importance for public health. Dogs represent one of the main reservoirs, especially in urban centers where establishment of the disease has been observed as a consequence of several factors that influence the natural epidemiological scenario (Ministério da Saúde, 2006). In Brazil, the Program for the Control of VL has adopted measures recommended by the Ministry of Health, such as the identification and culling of seropositive dogs.

Infected dogs exhibit a broad spectrum of clinical manifestations, including an asymptomatic state. The diagnosis of visceral leishmaniasis is usually made by serological tests because of the expressive humoral immune response, one of the characteristics of infection with *Leishmania (Leishmania) chagasi* in these animals (Alvar et al., 2004). However, these diagnostic tools have a limited value in areas where visceral leishmaniasis overlaps with the cutaneous form of the disease (Madeira et al., 2006), or even in areas where other trypanosomatids circulate (Montenegro et al., 2002).

Parasitism of intact skin by *L. chagasi*, first described by Deane and Deane (1954), is one of the characteristics causing infected dogs to be an important link in the epidemiological cycles of VL. Thus, this site has been investigated in an attempt to correlate parasitism of this organ with the clinical status of the animal and to determine its importance for vector infection (Travi et al., 2001; Solano-Gallego et al., 2004; dos-Santos et al., 2004; Giunchetti et al., 2006; Madeira et al., 2004).

The objective of the present study was to evaluate cultured intact skin fragments as a target site for the parasitological and etiological confirmation of canine visceral leishmaniasis (CVL) in 394 seroreactive dogs euthanized due to a suspicion of visceral leishmaniasis.

The dogs included in this study originated from the metropolitan region of Belo Horizonte and all of them presented IFAT titers of 1:40. Euthanasia was performed by an overdose of thiopental according to the Ethics Committee on Animal Research of the Oswaldo Cruz Foundation (CEUA/FIOCRUZ, process P-0286/06).

About 10 mL of blood was collected from each animal for confirmation of the serological diagnosis at the time of sacrifice. Intact skin specimens showing no macroscopic alterations were collected from the inner surface of the auricle and the scapular region. In the case of animals that presented skin lesions, a biopsy was obtained from one of these lesions rather than from the scapular skin sample. The biopsies were cultured in NNN plus Schneider's supplemented with 10% fetal bovine serum. Anti-leishmania IgG antibodies were evaluated in serum of all animals by IFAT using the CVL kit produced by Biomanguinhos/FIOCRUZ/MS according to manufacturer instructions.

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The *Leishmania* strains isolated were identified by multilocus enzyme electrophoresis using the following four enzymatic systems: NH, G6PDH, GPI, and 6PGDH.

The frequency of parasite isolation according to the intact skin site investigated (ear and scapula) and the median and interquartile range of the IFAT titers were compared. Cohen's kappa value and its respective 95% confidence interval were used to assess the agreement between the two sites of isolation. The correlation between IFAT titers and positive parasite isolation was evaluated using Spearman's coefficient. Median titers were compared between the two groups by the nonparametric Mann–Whitney test. Odds Ratio (OR) with 95% confidence interval was calculated to IFAT titers considering parasite isolation like reference.

Leishmania parasites were isolated from 310 (78.7%) of the 394 dogs studied, irrespective of the site examined. The parasitological test was negative in 83 dogs (21.1%). Among the 247 dogs submitted to the simultaneous evaluation of cultured skin fragments (ear and scapula), parasites were isolated from both sites in 151 (61.1%) animals. Parasites were only isolated from the scapular region in 12 (4.9%) dogs and only from the ear region in 27 (10.9%). However, 10 of the latter 27 scapular samples were contaminated. Agreement between the results obtained for the two intact skin sites was very good, with a kappa value of 0.71 (0.61–0.81) ($p < 0.001$).

Skin lesions were observed in 147 (37.3%) animals and were mainly located in regions with bone prominences. In most cases (89 of 147 animals, 60.5%) parasites were isolated both from skin lesions and from intact ear skin. Exclusive isolation of parasites either from skin lesions or from ear skin was observed in 18 (12.2%) and 13 (8.9%) of the 147 animals, respectively. In five animals the skin lesion samples were contaminated and both samples were negative in 27 cases. The kappa value of agreement between the two procedures was regular (0.54; $p < 0.001$), and the correlation was direct and low but significant ($p < 0.001$).

The frequency of serological titers obtained by IFAT is shown in Table 1. Positivity in the parasitological test was directly and positively correlated with increasing IFAT titers (rho = 0.43, $p < 0.001$). Higher percentages of positive parasitological tests were observed in animals with higher IFAT titers, with more than two-thirds of positive tests in the case of titers of 1:1280 or higher. The median (interquartile range) IFAT titers were lower in animals with a negative parasitological test (1:1280; 1:640–1:2560) compared to animals that tested positive (1:2560; 1:1280–1:5120).

In the case of animals in which parasites were isolated from both skin sites (ear and scapula), only one of the isolates was processed for identification. In contrast, in the group of dogs with positive simultaneous isolation from ear skin and skin lesions, both isolates were analyzed. A total of 400 samples, including 267 ear skin samples, 27 scapular samples and 106 skin lesion samples, were processed for isoenzyme electrophoresis and all isolates were identified as *L. chagasi*.

Table 1
Prevalence rates of IFAT titers with *Leishmania chagasi* infection in dogs (394) referred for euthanasia as a control for visceral leishmaniasis in Belo Horizonte, Brazil.

IFAT titers	Frequency *n* (%)	Dogs with *L. chagasi* isolation	Odds Ratios(CI 95%)
Negative	1 (0.3)	1	
1:40	4 (1.0)	0	0.03 (0.02–0.05)
1:80	5 (1.3)	0	0.03 (0.02–0.04)
1:160	11 (2.8)	3	0.10 (0.01–0.42)
1:320	9 (2.3)	5	0.34 (0.07–1.78)
1:640	45 (11.4)	24	0.27 (0.14–0.51)
1:1280	63 (16.0)	52	1.22 (0.60–2.48)
1: 2560	109 (27.7)	88	1.12 (0.65–1.91)
1:5120	81 (20.6)	72	2.66 (1.27–5.56)
1:10240	57 (14.5)	55	9.28 (2.21–38.84)
1:20480	8 (2.0)	7	20.1 (2.75–146.6)
1:40960	1 (0.3)	1	0.56 (0.48–0.64)

95% Confidence interval.

Possible markers of infection have been widely investigated in CVL, with serology being one of the parameters most frequently used for this purpose (Dye et al., 1993; Ashford et al., 1995; Reis et al., 2006b; dos-Santos et al., 2008). In the present study, 394 seroreactive dogs were euthanized in order to fulfill one of the control measures of VL, and the presence of the parasite was confirmed in 78.7% of the animals regardless of the site investigated. A wide variation in these percentages of parasite identification in seroreactive dogs is observed in the literature, a fact that might be related to the diagnostic method used, to the site investigated or even to the course of the disease in the animal. In this study, although all animals presented serological reactivity (IFAT = 1:40), IFAT titers at the time of euthanasia ranged from 1:40 to 1:40,960, demonstrating that the group of animals studied might have been in different phases of the disease. This fact may thus explain the negative parasitological results observed in 83 dogs, although infection of these animals cannot be ruled out. Additionally, studies have demonstrated that the frequency of parasitologically positive animals is directly correlated with the serum antibody levels of infected dogs (Dye et al., 1993; dos-Santos et al., 2008), in agreement with the present results.

Serological reactivity was not confirmed in only one of the dogs studied, in which *L. chagasi* was isolated. Other studies have also demonstrated the presence of parasites in seronegative animals (Barrouin-Melo et al., 2006; dos-Santos et al., 2008), a fact indicating the need for the evaluation of different parameters.

Although the presence of skin lesions is one of the most frequent dermatological manifestations of CVL (Silva et al., 2001; Alvar et al., 2004; Lima et al., 2004), *L. chagasi* is not always investigated at this site. In this study, skin lesions were observed in 37.3% ($n = 147$) of the animals, with *L. chagasi* being demonstrated in 100% of these cases. Although visceral and cutaneous leishmaniasis have also been documented in the metropolitan region of Belo Horizonte, the area of origin of all animals studied, *L. braziliensis* was not detected in any of the animals. In contrast, in areas where cutaneous and visceral leishmaniasis overlap, *L. braziliensis* has been isolated from lesions of seroreactive dogs euthanized due to a suspicion of CVL (Madeira et al., 2006).

Parasitism of intact skin, described in a pioneering study in the 1950s (Deane and Deane, 1954), is one of the characteristics that causes dogs to be an important element in the transmission cycle of VL. The present results demonstrating the presence of this species in the skin of 78.7% of the dogs studied emphasize this potential. Although skin is a site of parasitological investigation, the distribution of parasites in this organ is unknown in dogs. The detection of parasites might be related to areas of increased blood irrigation, as suggested by Travi et al. (2001) who reported a greater susceptibility of ear skin to sandfly infection compared to abdominal skin. Other authors investigating different skin sites in infected dogs found no difference in parasite density in this organ (Saridomichelakis et al., 2007). In this study, we simultaneously investigated skin from the ear and scapular region in a group of 247 dogs and *Leishmania* was isolated from both regions in 61.1% of the animals, with no significant difference between the two sites.

Numerous factors are responsible for the dissemination of *L. chagasi* to different sites in the host (Colmenares et al., 2002). However, skin parasitism has been indicated as a late event during the course of canine infection (Tafuri et al., 2001; Travi et al., 2001) and is strongly associated with common clinical manifestations of CVL (Reis et al., 2006a; Solano-Gallego et al., 2004; Giunchetti et al., 2006). In this study, although *L. chagasi* was isolated from the skin of 292 dogs, only 21.9% of these animals were classified as symp-

tomatic. This observation is very important and supports the notion that asymptomatic dogs are potential sources of infection for sandfly vectors.

The present results suggest that *L. chagasi* might be uniformly distributed in the skin of infected dogs and indicate this site as a good target for the parasitological confirmation of CVL, irrespective of the clinical condition of the animal.

Conflict of interest statement

The authors have no conflict of interest concerning the work reported in this paper.

Author's contributions

MFM, FBF and SRLP were responsible for the conception and design of the study; FBF and MF were responsible for the collection of the biological samples and clinical examination of the animals; MCAG and AMSB were responsible for the initial diagnosis of the dogs; EMC and LDN were responsible for the serological diagnosis; MFM, AGSP, CCP, and AB carried out the parasitological diagnosis and characterization of the parasites; MFM, FBF and SRLP drafted the manuscript and were responsible for the analysis and interpretation of the data; MFM and SRLP are guarantors of the study. All authors read and approved the final manuscript.

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