Parasite load in intact and ulcerative skin of dogs with leishmaniais

Carga parasitária em fragmentos de pele intacta e ulcerada em cães com leishmaniose

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Abstract

The skin is the site of inoculation of *Leishmania* spp. in susceptible hosts, and consequently dermatopathies, especially ulcerative dermatitis, are the main clinical signs observed. The aim of this study was to assess parasitism of the skin (intact and ulcerated) among dogs that were naturally infected by *Leishmania* spp., through immunohistochemical analysis. Skin fragments (intact and ulcerated) were collected from 13 dogs with positive parasitological (bone marrow aspiration and exfoliative skin) and serological examinations (ELISA S7® Biogene) for *Leishmania* spp. These samples were processed using the immunohistochemical technique, involving the streptavidin-peroxidase complex. Ulcerative lesions were mainly observed on the elbows (53.84%; 7/13), nostrils (15.38%; 2/13), ears (23.07%; 3/13) and wings of the ilium (7.69%; 1/13). A severe parasite load was detected in 46.15% and 76.92% of the intact and ulcerated skin samples tested, respectively. The parasite load on ulcerated skin was statistically higher than on intact skin (p = 0.0221). These results indicate that the intact and ulcerated skin may host a high parasite load of amastigote forms of *Leishmania* spp., which can favor the transmission of the parasite.

Keywords: Skin, leishmaniasis, immunohistochemistry, reservoir, dog.

Resumo

A pele é o local de inoculação da *Leishmania* spp. nos hospedeiros suscetíveis e dessa forma, as dermatopatias, principalmente as dermatites ulcerativas são os principais sinais clínicos observados. O objetivo deste estudo foi avaliar o parasitismo na pele (íntegra e ulcerada) em cães naturalmente infectados por *Leishmania* spp. através da técnica de imunohistoquímica. Fragmentos de pele (íntegra e ulcerada) foram coletados de 13 cães com diagnóstico parasitológico (aspirado de medula óssea e esfoliação cutânea) e sorológico positivos (ELISA S7® Biogene) para *Leishmania* spp. Amostras foram processadas por imunohistoquímica pelo complexo estreptoavidina-peroxidase. As lesões ulcerativas foram observadas principalmente nas regiões do cotovelo 53,84% (7/13), narina 15,38% (2/13), orelha 23,07% (3/13) e sobre a asa do ílio 7,69% (1/13). Uma intensa carga parasitária foi detectada 46,15% e 76,92% das amostras de pele íntegra e ulcerada, respectivamente. A carga parasitária na pele ulcerada foi estatisticamente superior à pele íntegra (p = 0,0221). Esses resultados indicam que a pele intacta e ulcerada pode albergar uma intensa carga parasitária de formas amastigotas de *Leishmania* spp., o que pode favorecer a transmissão do parasita.

Palavras-chave: Pele, leishmaniose, imunohistoquímica, reservatório, cão.

Visceral leishmaniasis (VL) is an important disease caused by *Leishmania infantum* parasites that may affect several species of animals, including dogs (GRAMICCIA, 2011). Positive dogs may present VL in forms ranging from asymptomatic infection to severe disease, which may present systemic involvement and have a wide variety of clinical signs, such as lymphadenopathy, weight loss, ocular lesions, circulatory disorders, chronic kidney disease and dermatopathies (CHAMIZO et al., 2005).

The main cutaneous alterations in canine visceral leishmaniasis (CVL) include alopecia, onychogryphosis, desquamation, ulcerative lesions, hyperkeratosis, hypotrichosis and presence of localized or generalized crusts (REIS et al., 2006; QUEIROZ et al., 2010). In CVL, the dermatological patterns presented by dogs are characterized by desquamative and ulcerative patterns, along with pustular and nodular lesions (FERRER et al., 1988). These ulcerative lesions may be observed on the nostrils, lips, face, periocular region, ears and areas of bone projections, especially in the humerus-radioulnar

region, calcaneus and ischial tuberosity (CAMINHA & SOTO-BLANCO, 2008; TORRES-NETO et al., 2008).

In this context, the skin represent an excellent biological sample for detection of amastigote forms in parasitological tests (e.g. exfoliative cytology), immunostaining (TAFURI et al., 2004; QUEIROZ et al., 2011) or parasite DNA detection (QUEIROZ et al., 2011; REIS et al., 2013, RAMOS et al., 2013). Indeed, assessment of the parasite load in both healthy and ulcerated skin fragments from dogs could contribute towards better understanding of their role as parasite reservoirs for *L. infantum* transmission to susceptible hosts (SOLANO-GALLEGO et al., 2004). Therefore, the aim of this study was to assess the parasite load in the intact and ulcerated skin of dogs positive for *Leishmania* spp..

This study was conducted using 13 positive dogs for *Leishmnania* spp. of different ages, breeds and sex. The animals were being kept at the Zoonotic Disease Control Centers of the municipalities of Petrolina (9° 23' 39" S, 40° 30' 35" W) and Goiana (7° 34' 19" S, 35° 0' 7"), both located in the state of Pernambuco, Brazil. All procedures performed in this study were approved by the Ethics Committee for Animal Use (ECAU) of the Federal Rural University of Pernambuco (protocol number 010/2011).

All the animals were clinically examined and the dermatological alterations presented were classified as previously proposed (FERRER et al., 1988). All dogs were diagnosed positive at enzyme-linked immunosorbent assay (ELISA S7® Biogene) and microscopic cytological examination of exfoliative skin tissue and bone marrow biopsy material. Intact skin fragments were collected from the scapular region, while ulcerated skin was obtained from any part of the body at the periphery of the skin lesion. All the fragments were collected using a biopsy punch (4 mm) and were then fixed in 10% buffered formalin for 48 hours. Following this, they were transferred to glass vials containing a solution of 70% ethanol until the time of immunohistochemical processing.

For the immunohistochemical examination, immunolabeling of the amastigote forms of *Leishmania* spp. was performed using the streptavidin-peroxidase technique (TAFURI et al., 2004). Skin from a dog with intense cutaneous parasitism due to *Leishmania* spp. was used as a positive control. In addition, skin from a negative dog confirmed by PCR examination of bone marrow and skin was used as negative control.

The intensity of parasitism was reported as the number of immunolabeled amastigote forms and was expressed as the mean number observed in five microscope fields at 400X magnification. The parasite load was defined as follows: (-) absent, (+) low, (+ +) moderate and (+ + +) high, corresponding to 0, 1-100, 101-300 and > 300 amastigote forms of *Leishmania* spp., respectively (GIUNCHETTI et al., 2006).

Differences among the parasite loads in the skin fragments were statistically analyzed through the Mann-Whitney test using the Biostat 5.0 software (AYRES et al., 2007). Differences were considered statistically significant when P ≤ 0.05.

All the animals examined here presented at least one cutaneous clinical sign. These dermatological alterations ranged from ulcerative lesions to scaly alopecia. Ulcerative lesions were mainly observed on the elbows (53.84%; 7/13), nostrils (15.38%; 2/13), ears (23.07%; 3/13) and wings of the ilium (7.69%; 1/13).

Severe parasitism was observed in 46.15% (6/13) of the intact skin samples and in 76.92% (10/13) of the ulcerated skin samples (Figure 1). The overall results regarding the parasite load are reported in Table 1. Interestingly, the parasite load detected in ulcerated skin samples was higher than that recorded for intact skin samples (p = 0.0238).

In this study, the cutaneous parasitism of 13 dogs that were naturally infected by *Leishmania* spp. was assessed. The presence of skin lesions observed here is a common clinical finding in CVL cases and may occur in 45% of the infected dogs (GIUNCHETTI et al., 2006; COSTA et al., 2008). In the animals studied, the ulcerative lesions were observed mainly on the elbows. It is known that the presence of ulcerative dermatitis is normally associated with areas of bone projections (FERRER et al., 1988).

All the skin fragments (both the intact and the ulcerated skin samples) analyzed here through immunohistochemical examination scored positive. In fact, the immunohistochemical technique enables high contrast between amastigote forms of *Leishmania* spp. and the host tissue, thus enabling a more accurate diagnosis (ORDEIX et al., 2005; TAFURI et al., 2004; FIGUEIREDO et al., 2010). The parasite load in ulcerated samples was statistically higher than that in intact skin samples. These results differ from
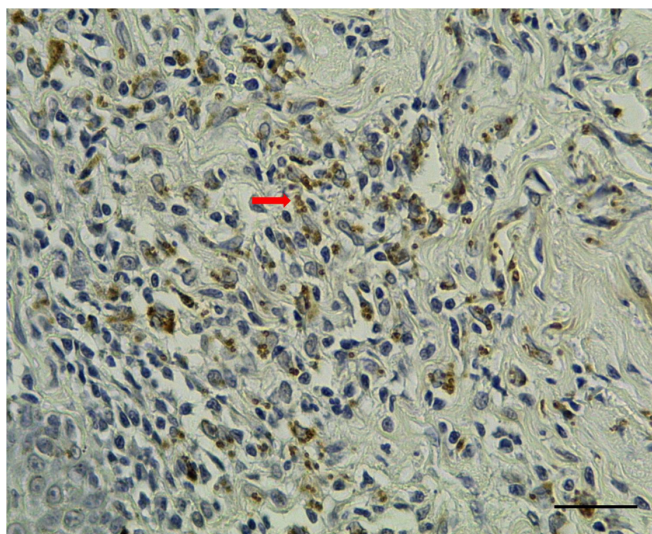
Figure 1. Photomicrograph of the skin of a dog that was naturally infected by *Leishmania* spp.. Lesioned dermis with an ulcerative dermatological pattern in a dog with a severe parasite load (immunohistochemical evaluation by means of the streptavidin-peroxidase technique); Note the presence of numerous intracytoplasmic amastigotes of *Leishmania* spp. in the macrophage revealed by immunohistochemistry (red arrow) (scale bar = 50 μm).

Table 1. Semi-quantitative assessment of parasite load by means of immunohistochemistry on the intact and ulcerated skin samples from dogs that were naturally infected by *Leishmania* spp.

Skin	Parasite load		
	Mild	**Moderate**	**Severe**
Intact	4/13 (30.76%)	3/13 (23.07%)	6/13 (46.15%)
Ulcerated	2/13 (15.38%)	1/13 (7.69%)	10/13 (76.92%)

those reported by Papadogiannakis et al. (2005), who did not detect any significant difference between the parasitism in the two skin samples.

Our findings suggest that the mild parasitic load on intact skin samples (30.76%) reported here most likely occurred due to an early immune response with participation by macrophages and by IL-4, TNF-α and IFN-γ, which play an important role in parasite control (CALABRESE et al., 2010; MENEZES-SOUZA et al., 2012; VERAS et al., 2010). It is important to highlight that 46.15% (6/13) of the intact skin samples exhibited a severe parasite load. Failure of the local cellular immune response probably did not allow efficient parasite control (CALABRESE et al., 2010; MENEZES-SOUZA et al., 2012). In addition, this high parasite load may have been associated with the chronic phase of the disease (REIS et al., 2006).

The presence of moderate and severe parasite loads in intact skin samples suggests that, even without macroscopic lesions, the infected animal can exhibit a large number of parasites, thereby playing an important role as a source of infection for phlebotomine vectors (TAFURI et al., 2004; LAURENTI et al., 2013). In intact skin fragments, a higher number of amastigote forms may be detected around the hair follicle and the dermal vascular plexus, thus suggesting that spreading of *Leishmania* spp. may occur via blood (SOLANO-GALLEGO et al., 2004).

Predominantly intense parasite load, rather than moderate and mild parasitism, has previously been reported as a difference between symptomatic and asymptomatic dogs (GIUNCHETTI et al., 2006). By contrast, Saridomichelakis et al. (2007) did not find any association between parasite load and the severity of skin lesions.

The present study demonstrates that intact and ulcerated skin may host a high number of amastigote forms of *Leishmania* spp., which can favor transmission of the parasite. Over the last years, the role of asymptomatic dogs in the life cycle of leishmaniasis has been extensively disputed. Recently, a study suggested that both symptomatic and asymptomatic animals are potentially infective to sand flies (LAURENTI et al., 2013). In addition, it has been demonstrated that only the cutaneous parasitism is not pivotal for successful transmissibility (TRAVI et al., 2001). Therefore, animals where cutaneous lesions are absent, may act as important source of infection by phlebotomine sand flies.

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