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Histopathological and parasitological analysis of skin tissues biopsies from two distinct anatomical areas of the ears of dogs naturally infected with *Leishmania (Leishmania) chagasi*.

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Abstract

Canine visceral leishmaniasis is an endemic disease in Latin America caused by *Leishmania (Leishmania) chagasi* and transmitted to man and animals by infected blood-sucking sandflies) of the genus Lutzomyia. Dogs are considered to be the primary domestic reservoir of disease because they present an intense cutaneous parasitism. The aim of this study was to evaluate the intensity of the inflammatory process and to compare it to the parasite load of tissue from two different sites of the ear skin of dogs naturally infected with *Leishmania chagasi*. We think that exist a specific anatomical region that exhibits a relatively higher rate of parasitism. For diagnostic analysis, serological tests were carried out using the indirect fluorescence antibody test (IFAT) and the enzyme-linked immunosorbent assay (ELISA). Twelve animals naturally infected with *Leishmania chagasi* were euthanatized with a lethal dose of Sodium Thiopental™ and T61™. During the necropsy, fragments of the extremity and middlę anatomical regions of the ear were collected. All tissues were fixed in a 10% formalin solution and then paraffin-embedded for histopathological (HE) and immunohistochemical analysis. The streptoavidin-peroxidase immunohistochemistry method was used to detect tissue amastigotes using optical microscopy. Our results indicated a chronic inflammatory reaction, ranging from discrete to an intense magnitude. The inflammatory process was more frequently observed in the extremity of the ear than in the middle portion of the ear ($p<0.05$). The presence of parasites in the ear extremity was higher than in other evaluated regions. A positive correlation between the tissue inflammation, parasitism, and serological data was confirmed at both ear positions ($p<0.05$). Skin biopsies are an important tool for CVL diagnosis and the ear extremity represents an appropriated area to perform the assays.

Keywords: *Leishmania chagasi*, dogs, skin biopsies, diagnosis, ear extremity, ear middle.

Introduction

Visceral leishmaniasis (VL) is an important public health problem for which 0.5 million cases has been reported a year worldwide (21). In the New World, the disease is caused by *Leishmania (Leishmania) chagasi* (sin. L. infantum) species (13) and it is endemic in several Latin American countries. Brazil accounts for 90% of the

human cases disease. The parasite is transmitted to man and animals by infected blood-sucking sandflies of the genus Lutzomyia (9). The dog is the main reservoir of VL and, in Brazil, some authors have reported a strict correlation between the canine infection to the human disease (4,6,12).

Canine Visceral Leishmaniasis (CVL) is a severe systemic disease of dogs and symptomatic CVL due to *Leishmania chagasi* in America presents clinically with anemia, emaciation, splenomegaly, local or generalized lymphadenopathy, cutaneous lesions, ocular lesions, weight loss, and cachexia (2,7,17). Several authors have been demonstrated that phlebotomine sandflies that fed in symptomatic dogs exhibit a higher infection rate than of flies feeding on asymptomatic animals (5,8,14). Nevertheless even the lower infectivity rates of asymptomatic animals must be taken into account in the epidemiology of the disease (8,15). Moreover, upon clinical examination it is possible for both asymptomatic and symptomatic animals to not exhibit macroscopic skin lesions, but these animals can harbor parasites in the skin tissue, especially in the ears (2,6,22,23). Furthermore, about half of all infected dogs lack clinical signs of leishmaniasis, but these asymptomatic dogs could be as infective to the vector as symptomatic dogs (1,2,16).

Accurate and rapid diagnosis of *Leishmania* infection in dogs is of great importance for epidemiology surveys and veterinary practice. The methods employed for the diagnosis of CVL include: (1) microscopic detection of the parasite in bone marrow and lymph node aspirates stained with Giemsa, (2) demonstration of specific anti-*Leishmania* antibodies in the serum of infected animals by the indirect fluorescence antibody test (IFAT), (3) the direct agglutination test (DAT), (4) the enzyme-linked immunosorbent assay (ELISA), and (4) isolation of the parasite by "in vitro" culture or by hamster inoculation (2,20). Thus, definitive diagnosis is based on detection of the parasite. Direct parasite detection in skin biopsies, which can be obtained through an extremely simple surgical procedure, is a good tool for the definitive diagnosis. Moreover, PCR of an ear skin biopsy sample was the best method to diagnose canine *Leishmania* infection in comparison to immunohistochemical and histological methods (23).

We believe should exist an anatomical area that exhibits a higher rate of parasitism than others; histopathological determination of these sites could improve tissue parasitism detection. The aim of this study was therefore to evaluate the intensity of inflammatory process and to compare it to the parasite tissue load in different sites of the ears of naturally infected dogs with a defined clinical status of the infection by *L. chagasi*.

Materials and Method

Animals and Defined clinical status

Animal care and experimentation followed the current strategy for zoonotic leishmaniasis as proposed by the WHO (20). Twelve mongrel dogs of unknown age were obtained from the City of Santa Luzia, Belo Horizonte metropolitan area, Minas Gerais (MG), Brazil. All dogs were positive for *Leishmania* as tested by an indirect fluorescence antibody titers (IFAT), (Titers > 1:40; Camargo et al.(3) and enzyme-linked immunosorbent assay (ELISA) (19). Tissue pouch preparations of bone marrow were stained in a 10% solution Giemsa to detect Leishmania parasites; all animals were positive based on this assay.

All infected dogs were clinically classified into the following groups: (1) symptomatic dogs – animals that exhibited the classical signs of the disease such as cutaneous alterations (alopecia, dry exfoliative dermatitis or ulcers), onychogryphosis, keratoconjunctivitis, cachexia and anemia, and (2) asymptomatic dogs – animals that appeared to be healthy without any clinical sign of the disease (10,11).

Histopathology

Dogs were euthanatized with a lethal dose [2,5% (1,0 ml/Kg)] of Sodium Thiopental, administered intravenously and T61™ (0,3 ml/Kg). During the necropsy, two anatomical regions of the external ear (pinna) and another one from the middle of the ear were collected. These two samples were collected from both ears of each dog resulting in four tissue samples of each animal. In addition, the skin of the area from which the biopsies were taken was macroscopically normal.

All tissues samples were fixed in 10% formalin solution. After 72h of fixation, the samples were dehydrated, cleared, embedded in paraffin, cut (4- to 5mm thick), and stained with hematoxylin and eosin (HE) for histological study. This study was carried by light microscopy and the inflammatory process classified in absence, discrete, moderate and intense inflammatory reaction.

Immunohistochemical method for labeling amastigote forms of Leishmania

The streptoavidin-peroxidase immuno histochemistry method was carried out for tissue amastigotes detection by optical microscopy (18). Deparaffinized slides were hydrated and incubated in 4% hydrogen peroxide (30 v/v) in 0.01 M PBS, pH 7.2, followed by incubation with normal goat serum (diluted 1:100). A heterologous immune serum from dogs naturally infected with *L. chagasi* (diluted 1:100 in 0.01 M PBS) was used as primary antibody. Slides were incubated for 18–22 h at 4°C in a humid chamber. After washing in PBS, the slides were incubated with goat anti-mouse and anti-rabbit biotinylated (Link-DAKO, LSAB2 kit, California, USA), washed again in PBS and incubated with

streptoavidin-peroxidase complex (Link-DAKO, LSAB2 kit, California, USA) for 20 min at room temperature. The reaction was developed with 0.024% diaminobenzidine (DAB; Sigma, St Louis, USA) and 0.16% hydrogen peroxide (40 v/v). Finally, the slides were dehydrated, cleared, counter-stained with Harris's Hematoxylin and mounted with coverslips.

Statistical analysis

To compare the two anatomical sites of the ear, the Wilcoxon test was used. The Spearman test was carried out to detect correlations between the parasite load and the inflammatory response.

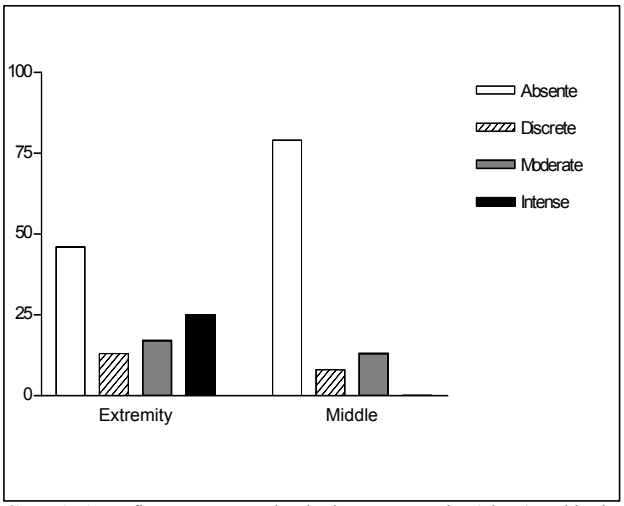
Results

Histologically, skin samples showed a chronic inflammatory reaction irrespective of anatomical region; however, the intensity of this reaction varied with the animal's clinical status. In general, the reaction ranged in intensity from discrete to moderate. However, an intense inflammatory process was more frequent in some cases of symptomatic animals. In general, the chronic inflammatory reaction was characterized by a diffuse mononuclear infiltrate in the upper dermis and focal around vessels, hair follicles and glands of the deep dermis (Figs. 1A-B). Immunolabelled amastigotes were detected in both upper and deep dermis (Figs 1C-E).

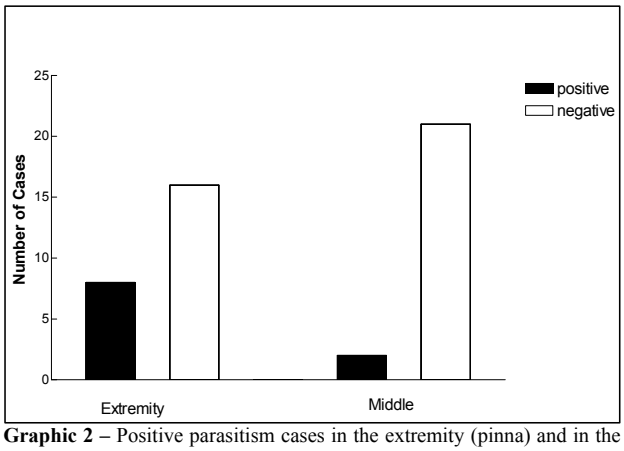
In general, the inflammatory reaction was more intense in the ear extremity (pinna) then the middle of the pinna ($p<0.05$) (Graphic 1). Moreover, parasites were more readily identified in biopsies of the ear extremity than biopsies of the middle of the pinna. Indeed, immunolabelled amastigotes were more frequently observed in the ear extremity ($p<0.05$) (Graphic 2).

A positive correlation was observed between the intensity of the inflammatory process and the presence of amastigotes forms of *Leishmania* ($p<0.05$), but it occurs undependably of the anatomical site (Graphic 3).

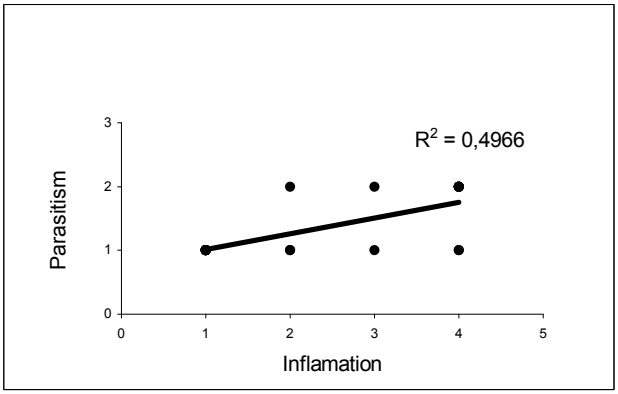
Correlation testing between the serology (ELISA) and inflammatory reaction was preformed for all cases. Our data indicate a positive correlation of the intensity of the inflammatory reaction in the extremity and middle portions of the ear ($p<0.05$) with positive ELISA results titers (Graphic 4). The same results were observed between the parasite load and ELISA data ($p<0.05$) (Graphic 5). However, we did not observe any correlation with all parameters with nose skin sections (data not shown).



Graphic 1 – Inflammatory reaction in the ear extremity (pinna) and in the middle pinna of dogs naturally infected with *L. chagasi*



Graphic 2 – Positive parasitism cases in the extremity (pinna) and in the middle pinna of dogs naturally infected with *L. chagasi*



Graphic 3 – Positive correlation between the intensity of the inflammatory response and the parasitism in the ear extremity of pinna of dogs naturally infected with *L. chagasi*

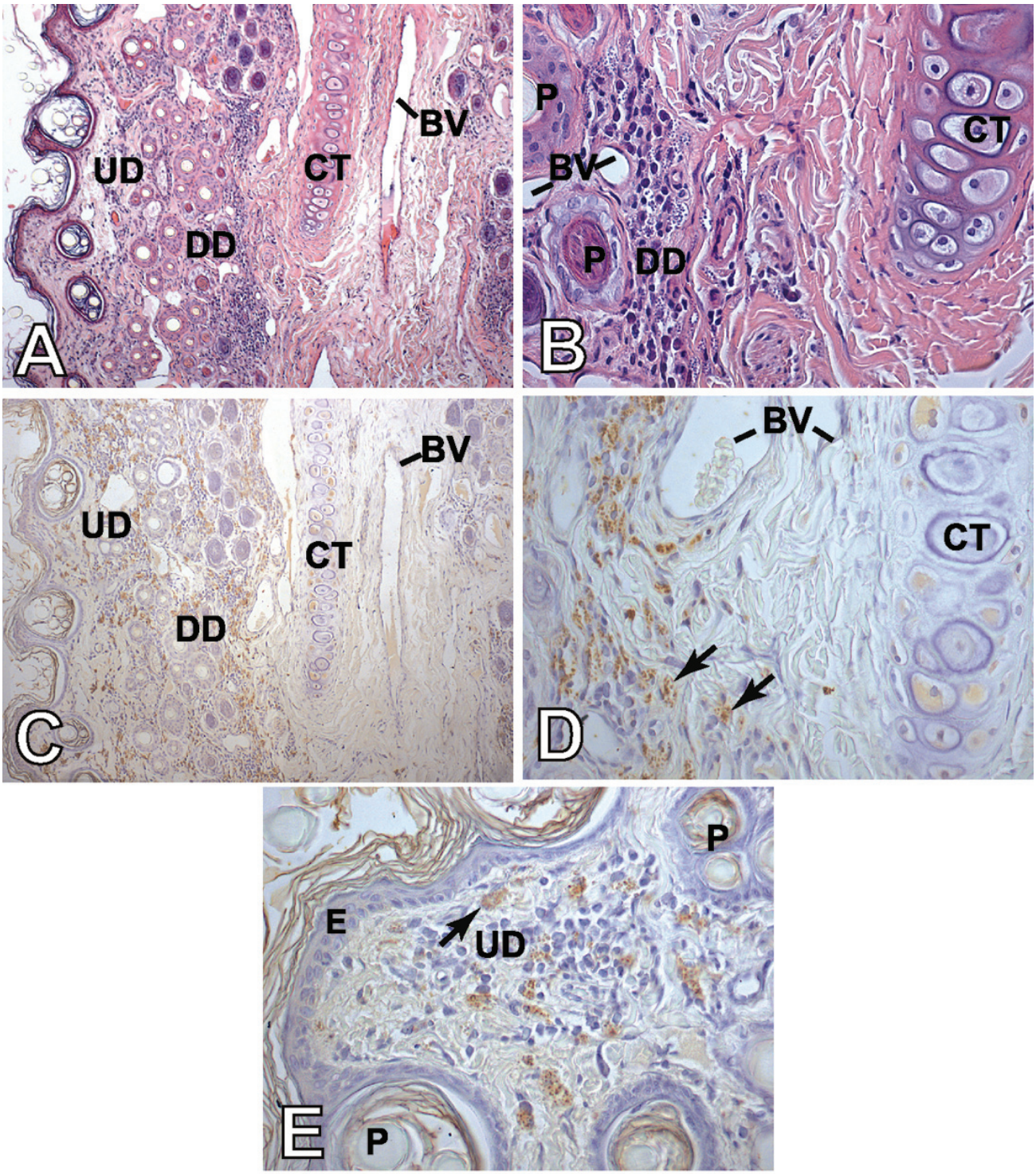
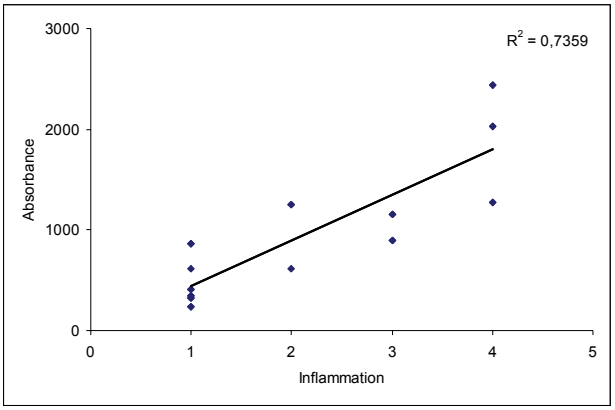
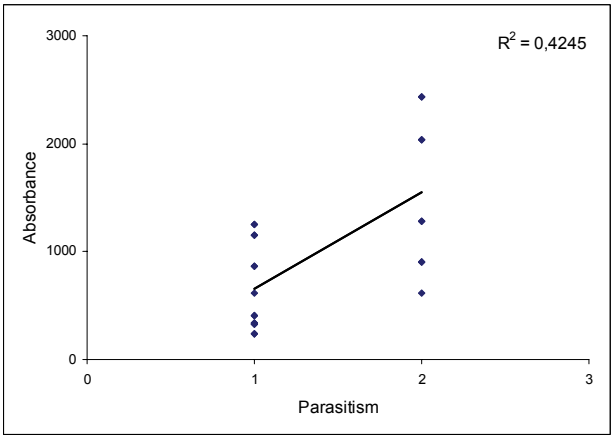


Figure 1: Skin ear section (extremity of pinna) of a dog naturally infected with *L. chagasi:* (A) Observe a presence of inflammatory reaction diffuse in the upper dermis (UD) and focal in the deep dermis (DD) HE 40x. CT means cartilage tissue and BV means Blood Vessel. (B) High magnification showing a chronic cellular exudate around the blood vessels (BV) and pyli (P) in the deep dermis (DD) HE 440x; (C) Note a presence of immunolabeled amastigotes (brown color) in upper dermis (UD) and deep dermis (DD) Streptoavidin-peroxidase counter-staining with Harris Hematoxylin 40x.; (D) High magnification showing immunolabeled amastigotes inside macrophages in the deep dermis (arrows). Streptoavidin-peroxidase counter-staining with Harris Hematoxylin 440x; (E) Observe details of parasitized cells nearest to the epithelial cells layers (arrow). Streptoavidin-peroxidase counter-staining with Harris Hematoxylin 440x.



Graphic 4: Positive correlation between the intensity of the inflammatory response in the ear extremity of pinna and the ELISA absorbance of serum of dogs naturally infected with *L. chagasi*



Graphic 5: Positive correlation between the positive cases of parasitism in the ear extremity of pinna and the ELISA absorbance of serum of dogs naturally infected with *L. chagasi*

Discussion

Direct parasite detection on histological skin biopsies, which can be obtained by an extremely simple surgical procedure, is a good tool for a definitive diagnosis (22). In this work, we aimed to determine whether the extremity of the external ear (pinna) is more parasitized than the middle area. Inflammatory reaction was more intense in the ear extremity than the middle of pinna, presenting a strict correlation between the parasitism data and the inflammatory response.

Xavier et al. (23), for example, demonstrated that skin samples from ears, nose and abdomen, though mainly the ears and nose, are potentially useful for diagnosing CVL independently of the clinical status of the dogs. In the present work, in agreement to these authors, the differences between the two distinct areas of the ear were observed to be independent of a definitive clinical animal status. Also, in our study, nose samples biopsies showed less inflammatory alterations and parasite tissue load when compared to the ears, with the extremity of the pinna showing the greatest inflammation (data not shown).

The extremity of the ears, independent of the animal clinical status, was found to represent the best area to carry out ears skin biopsies, considering both the parasitism data and inflammatory processes. In general, during veterinarian practice, biopsies are obtained from the middle of the ears because this procedure is less harmful and because it avoids cicatrization response (7). According to this study, biopsy site interferes with sensitivity in diagnosis. Using samples from middle canine ear tissue instead of the extremity of the ear (pinna) displays a lower sensitivity. This finding could also be considered for use in parasitological surveys of canine visceral disease, xenodiagnosis practice studies and vaccine strategies research.

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