



CD45⁺, CD68⁺ and E-cadherin⁺ Expressions in Skin Dogs Naturally Infected by *Leishmania infantum*

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ABSTRACT

Background: In canine leishmaniasis (CanL), infection occurs through phlebotomine vectors that inoculate the protozoan *Leishmania infantum* into the skin that infected macrophages and activated dendritic cells (CD). Dogs with CanL present variable clinical manifestations, being common the presence of cutaneous lesions. The aim of this study was to evaluate the expression of CD45⁺, CD68⁺ and E-cadherin⁺ associating the skin sentinels cells and to compare the clinical-dermatological manifestations in the skin of dogs naturally infected by *L. infantum*.

Materials, Methods & Results: Dogs infected (n = 22) by *L. infantum* were divided into asymptomatic group (AD, n = 9), and symptomatic group (SD, n = 13), according criteria based on the presence or absence of skin changes. Dogs non-infected (CD, n = 5) were included as control group. Samples of skin biopsies collected from scapular region were processed by routine histology and labeled by immunohistochemistry with monoclonal antibodies against CD45⁺, CD68⁺ and E-cadherin⁺, and were described as none, mild, moderate and intense. SD presented keratoconjunctivitis, onychogryphose, lichenification, depigmentation, alopecia, hypotrichosis, erythematous dermatitis, exfoliative dermatitis, ulcerative dermatitis and crusted dermatitis, and the frequency these alterations was expressed as percentage. The results of hematological and biochemical parameters were analyzed by Kruskal-Wallis test followed by the Dunn's test and expressed as mean \pm standard deviation, with values $P < 0.05$. Leukocytosis (not significant), red blood cells, hematocrit and hemoglobin ($P < 0.05$), total protein serum ($P < 0.05$), globulins ($P < 0.05$), albumin and A/G ratio ($P < 0.01$) were altered in SD in relation to CD. Cutaneous cellular infiltration, composed by macrophages, plasma cells, lymphocytes and neutrophils, was observed in CD. There was an increase of expression of the markers in SD when compared to the other groups, as moderate CD68⁺ expression and *L. infantum*, and intense CD45⁺ and E-cadherin⁺ expressions.

Discussion: Cutaneous involvement is very important in CanL, as it corresponds to where is the first interaction between the parasite and the immune system. Dermatological clinical signs, leukocytosis, anemia, globulins levels have been reported for dogs naturally infected by *L. infantum*. Inflammatory infiltrate was distributed at superficial and deep dermis, which was composed by mononuclear cells as macrophages, plasma cells, lymphocytes and neutrophils. To characterize the immune sentinels cells in the skin it was evaluated CD45⁺, CD68⁺ and E-cadherin⁺ expressions. In symptomatic dogs, our results revealed an increase of expression of these markers. CD45⁺ is one of the most abundant molecules expressed on the white blood cell surface in various mammals, while CD68⁺ is a myelomonocytic marker that seems to be retained during monocyte differentiation. In the skin, increased numbers of CD68⁺ are related to dendritic epidermal cells, which can be expressed as CD45⁺/CD1a⁺/HLA-DR⁺. DCs of the skin, particularly epidermal Langerhans cells (LCs), form networks anchored to neighboring keratinocytes via E-cadherin. Thus, CD45⁺, CD68⁺ and E-cadherin⁺ expressions may be related to activation of skin sentinels cells in dogs naturally infected by *L. infantum*. Our results indicated that CanL modify the CD45⁺, CD68⁺ and E-cadherin⁺ expressions, which characterize the immune sentinels cells activation that promote the recruitment the cellular infiltrate, which was composed by macrophages, plasma cells, lymphocytes and neutrophils. Thus, these informations may contribute to the follow-up of CanL progression in skin.

Keywords: leishmaniasis, *Leishmania infantum*, cutaneous immune system, skin alterations, immune sentinels cells, dogs.

INTRODUCTION

The skin corresponds to the outer covering of body, appearing as a mechanical barrier to environmental and pathogenic insults, consisting of epidermis, dermis and hypodermis. The epidermis and dermis participate in the maintenance of skin homeostasis and have in their composition a variety of pro-inflammatory cells and mediators that initiate and regulate the innate and acquired immune response [11,14]. Among the immune cells present in the skin are keratinocytes, T lymphocytes and sentinel cells, as dendritic cells (DCs), macrophages, and mast cells [2,14,23]. DCs make a link between the innate and adaptive immune response. They have the ability to capture the pathogen *Leishmania* spp., and migrate to the lymphoid organs where they present the processed antigens to naive T cells, so that they regulate the cellular response [3,17,23].

Canine leishmaniasis (CanL) is a severe disease, usually subacute or chronic in dogs, transmitted through the bite of female sandfly vectors in the skin, which inoculate in dermis promastigotes forms corresponding to the infective stage of the parasite *Leishmania infantum* [23]. Clinical manifestations of CanL vary widely as a result of numerous pathogenic mechanisms of the immune response and different affected organs [1,23]. Cutaneous involvement is very important in CanL, as it corresponds to where is the first interaction between the parasite and the immune system [21]. Changes in skin of dogs with CanL are closely related to the progress of infection and present themselves in variable forms such as alopecia, hypotrichosis, hyperkeratosis, lichenification, erythema, exfoliative dermatitis, crusty dermatitis, ulcerative lesion, nodular dermatitis, papular dermatitis or pustular dermatitis [22].

The aim of this study was to evaluate the CD45⁺, CD68⁺ and E-cadherin⁺ expressions associating the skin immune sentinels cells and to compare the clinical-dermatological manifestations in the skin of dogs naturally infected by *Leishmania infantum*.

MATERIALS AND METHODS

Study population

Twenty-seven mixed-breed adult dogs of both genders (ages ranging from 2 to 6 years) were selected. They were provided by the Zoonosis Control Center, in Fortaleza (Ceará, Brazil), a region with a high prevalence of CanL. The experimental animals were selected based on the results of DPP rapid test Canine Visceral

Leishmaniasis¹, serologic ELISA test¹ and bone marrow parasitological test. Negative animals in serological and parasitological tests were considered not infected and used as control animals (CD, n = 5). Dogs infected (n = 22) by *L. infantum* were divided into asymptomatic group (AD, n = 9), and symptomatic group (SD, n = 13), according criteria based on the presence or absence of skin changes. SD presents the characteristic cutaneous manifestations of CanL, including skin lesions, onychogryphosis and keratoconjunctivitis. Blood samples were collected by jugular venipuncture for determination of hematological (Mindray BC-200 Vet[®]; Mindray BA 88A[®])² and biochemical (Labtest[®])³ parameters.

Skin sample

The collection of skin specimens was carried out after euthanasia (Potassium chloride 10%) of dogs with a barbituric anesthesia (Thiopentax[®])⁴. Skin sections of 5 mm were collected with the help of a punch from the scapular region.

Histological analysis

Skin samples were fixed in 10% buffered neutral formalin and were embedded in paraffin for routine histological processing. Five-micrometer sections were cut and stained with hematoxylin and eosin (HE). The analysis of histological sections was performed under a light microscope (Nikon - Eclipse E200[®])⁵ at a 200x magnification. We assessed the degree (none, mild, moderate and intense) of cellular inflammatory infiltrate in dermis, according to the average subjective perception of two observers.

CD45⁺, CD68⁺ and E-cadherin⁺ immunohistochemistry

Sections (5 µm) were mounted on silanized glass slides and subsequently subjected to an incubator at 36°C. Antigen retrieval was performed using citrate buffer pH 9.0 for 30 min at 97°C. The activity of endogenous peroxidase was inhibited by Hydrogen Peroxide 3%⁶ for 10 min and the slides were subjected to monoclonal antibody mouse anti-human CD45⁺ (clone 2B11 + PD7/26)⁶, CD68⁺ (Clone KP1)⁶ monoclonal antibody and mouse anti-human E-cadherin⁺ (UMR-38 clone)⁶ Flex incubated for 1 h at room temperature. The slides were then washed twice in PBS and then incubated with En-Vision polymer reagent (EnVision TMBDual connection System/HRP)⁶ for 30 min at room temperature. Finally, diamine benzidine (DAB)⁶ was applied for 10 min. Sections were counterstained with Mayer's hematoxylin⁶ for

5 min. The intensity of the staining was analyzed by light microscopy (Leica DM2000®)⁷ at 200x magnification. The expression of the markers was classified as none (-), mild (+), moderate (++) and intense (+++), according to the average subjective perception of two observers [16].

L. infantum immunohistochemistry

Cutaneous sections prepared from paraffin-embedded biopsies were cut into 5 µm sections and mounted on silanized slides. Slides were deparaffinised in xylene, and the tissue was rehydrated using graded alcohols. The slides were incubated in a 4% hydrogen peroxide solution and PBS (0.01 M, pH 7.2), followed by incubation in normal goat serum (1:100 dilution). Antibody from rabbits experimentally infected with *L. infantum* (1:200 dilution in 0.01 M PBS), donated by the Institute of Tropical Medicine of São Paulo, was used as the primary antibody. The slides were then incubated for 22 h at 4°C in a humid chamber. Then, the PBS lavage was performed and the slides were incubated again in a biotin solution, and then washed again with PBS and incubated with the streptavidin-peroxidase complex for 20 min at room temperature. The reaction was carried out with the addition of 0.024% diamino-benzidine⁸ and 0.16% hydrogen peroxide⁸. Analysis of amastigote forms of *L. infantum* was performed by light microscopy⁷ at 200x magnification. The intensity of staining was classified as none, mild, moderate and intense, according to the average subjective perception of two observers [8].

Statistical analysis

Analyses were performed using the statistical software⁹. Results were expressed as mean ± standard deviation (SD). Prior, data were submitted to Grubbs and Kolmogorov test to verify the presence of outliers and to determine data homoscedasticity, respectively. Data of clinical changes observed were expressed as a percentage. For hematological and biochemical analyzes it was used Kruskal-Wallis test followed by the Dunn's test. In all cases, significance was defined at $P < 0.05$.

RESULTS

Clinical dermatological findings are shown in Figures 1 and 2. Symptomatic dogs (SD) have been identified keratoconjunctivitis, onychogryphosis, lichenification, depigmentation, alopecia, hypotrichosis, erythematous dermatitis, exfoliative dermatitis, ulcerative dermatitis and crusted dermatitis (Figure 1). Among these cutaneous lesions, there were observed

a higher incidence of alopecia/hypotrichosis (92.3%) and ulcerative dermatitis (53.8%) [Figure 2].

Hematological and serum biochemical parameters are shown in Table 1. Regarding the white blood cell (WBC) count, AD and SD differed significantly ($P < 0.05$). It's worth highlighting the presence of leukocytosis in AD (16.89 ± 3.74) when compared to the CD (12.60 ± 2.29), although it is not statistically significant. Red blood cells (RBC), hematocrit (HCT) and hemoglobin (HGB) were reduced in SD when compared to CD ($P < 0.05$). There was no significant difference between the groups regarding to platelets (PLT) count. Total protein serum (TPS) levels differ ($P < 0.05$) between AD and SD. Globulins levels were elevated ($P < 0.05$) but the albumin and A/G ratio were decreased ($P < 0.01$) in SD in relation to CD.

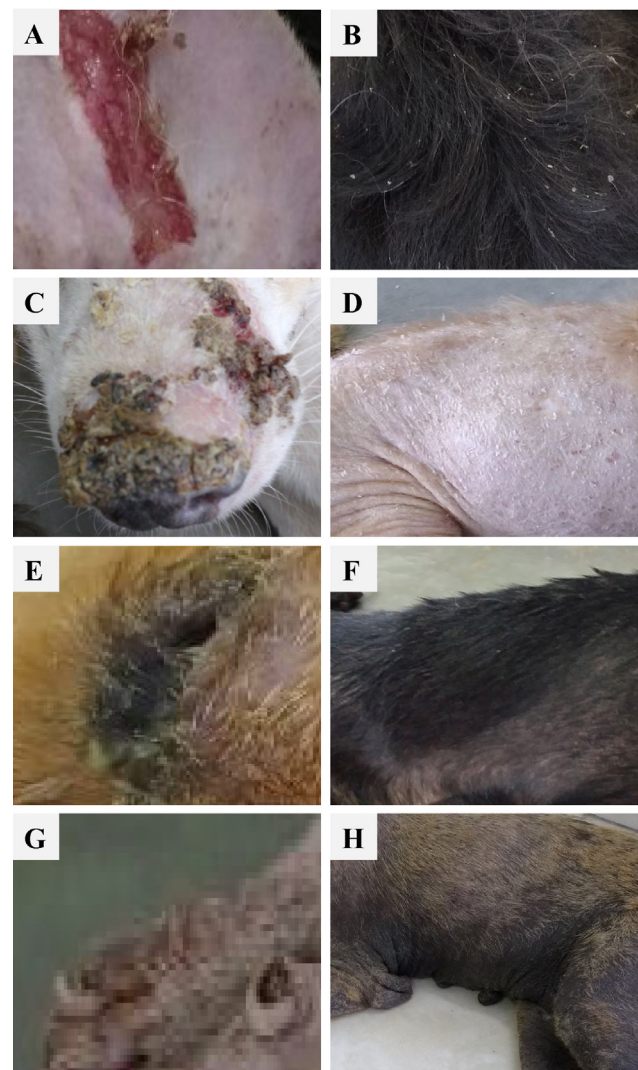


Figure 1. Skin macroscopic lesions of dogs diagnosed with CanL. A, dermatitis ulcerative in ear; B and D, dry exfoliative dermatitis areas diffused by the animal body; C, dermatitis crusted nasal region; D, diffuse alopecia; E, keratoconjunctivitis and hypotrichosis periorcular; F, diffuse hypotrichosis; G, onychogryphosis; H, lichenification and hyperpigmentation diffuse the animal's body.

The skin histopathological findings were shown in Figure 3. Cutaneous cellular infiltration was identified in 54.0% (12/22) of the naturally infected dogs, being 83.3% (10/12) in SD and 16.7% (2/12) in AD. Inflammatory infiltrate was present in AD and SD as a mild (Figure 3B) and mild to moderate (Figure 3C), respectively, which was composed by mononuclear cells as macrophages, plasma cells, lymphocytes and neutrophils. The cellular infiltrate

shown a distribution multifocal in the superficial and deep dermis.

The main immunohistochemical findings were shown in Table 2 and Figure 3. There was a greater expression of the markers in SD when compared to the other groups, with moderate CD68⁺ expression and *L. infantum*, and intense CD45⁺ and E-cadherin⁺ expressions. It is worth noting that in AD there was a greater expression of CD45⁺, while in CD it was highlighted CD68⁺ and E-cadherin⁺.

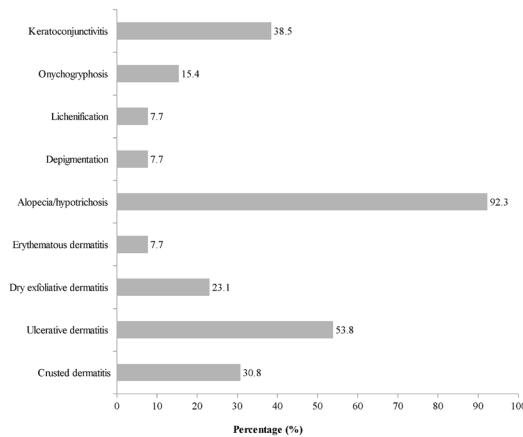


Figure 2. Dermatological clinical findings of dogs naturally infected by *Leishmania infantum*.

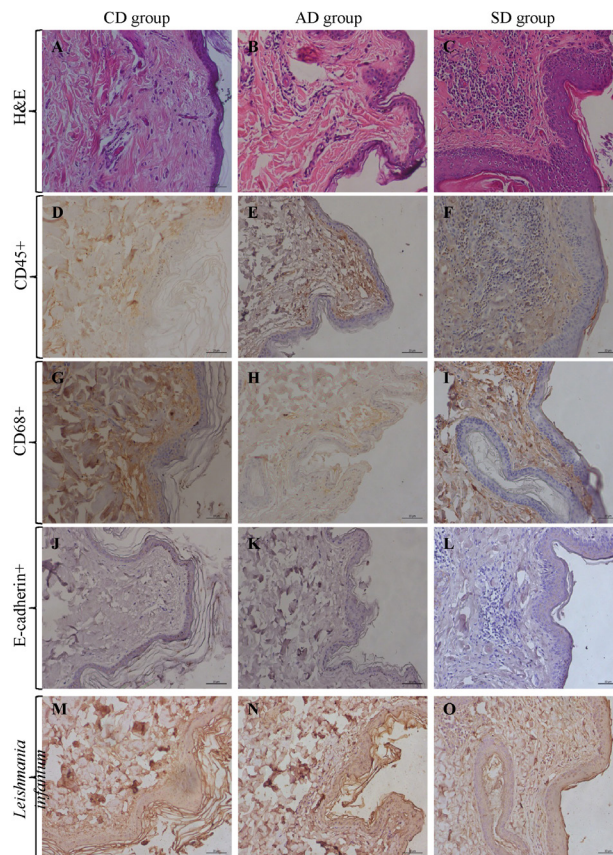


Figure 3. Histopathological and immunohistochemistry of skin dogs naturally infected by *Leishmania infantum*. Animals were categorized according to their dermatological clinical status into asymptomatic (AD) or symptomatic (SD). The control group is represented by CD. Representative cutaneous cellular infiltrates of groups were classified as none (3A), mild (3B) and moderate (3C). CD45⁺ expression was classified as mild (3D), moderate (3E) and intense (3F). CD68⁺ was classified as mild (3H) and moderate (3G; 3I) expressions. E-cadherin⁺ was classified as none (3K), moderate (3J) and intense (3L) expressions. *L. infantum* detection was classified as none (3M), mild (3N) and moderate (3O). 200x. [Bars = 20 µm].

Table 1. Hematological and biochemical analyses parameters in asymptomatic and symptomatic dogs naturally infected by *Leishmania infantum*.

Parameter	Seronegative dogs (CD, n = 5)	Serum reagent dogs	
		Asymptomatic (AD, n = 9)	Symptomatic (SD, n = 13)
WBC (x10 ⁹ /L)	12.60 ± 2.29 ^{ab}	16.89 ± 3.74 ^a	11.44 ± 3.20 ^b
RBC (x10 ¹² /L)	6.87 ± 0.59 ^a	6.36 ± 1.04 ^{ab}	5.31 ± 1.25 ^b
HGB (mmol/L)	18.2 ± 2.08 ^a	14.13 ± 2.39 ^{ab}	11.72 ± 2.64 ^b
HCT (L/L)	52.59 ± 4.73 ^a	42.00 ± 6.60 ^{ab}	35.23 ± 7.83 ^b
MCV (fL)	76.57 ± 2.71 ^a	66.54 ± 4.18 ^b	66.97 ± 5.54 ^b
MCHC (mmol/L)	34.58 ± 1.75 ^a	33.36 ± 0.89 ^b	33.13 ± 0.74 ^b
PLT (x10 ⁹ /L)	234.00 ± 60.73	177.22 ± 67.34	254.31 ± 197.15
TPS (g/dL)	7.31 ± 0.50 ^{ab}	7.29 ± 1.31 ^b	8.54 ± 0.93 ^a
Albumin(g/dL)	2.51 ± 0.15 ^a	1.83 ± 0.47 ^{ab}	1.37 ± 0.33 ^b
Globulin (g/dL)	4.79 ± 0.45 ^b	5.47 ± 1.64 ^b	7.17 ± 1.06 ^a
A/G	0.53 ± 0.05 ^a	0.37 ± 0.17 ^{ab}	0.20 ± 0.06 ^b

WBC: White blood cells; RBC: red blood cells; HGB: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCHC: mean corpuscular hemoglobin concentration; PLT: platelet. TPS: total protein serum; A/G: albumin-globulin ratio. Different letters in the same row mean significant difference among treatments ($P < 0.05$).

Table 2. Expressions of CD45⁺, CD68⁺, E-cadherin⁺ and *Leishmania infantum* on skin dogs naturally infected by *L. infantum*.

Marker	CD group	AD group	SD group
CD45+	+	++	+++
CD68+	++	+	++
E-cadherin+	++	-	+++
<i>Leishmania infantum</i>	-	+	++

DISCUSSION

Cutaneous involvement is very important in CanL, as it corresponds to where is the first interaction between the parasite and the immune system [21]. All dermatological clinical signs observed for the SD have been reported for infected by *Leishmania infantum* [21,22]. The presence of leukocytosis in AD was described by Reis *et al.* [20]. RBC, HCT and HGB reduced in SD were also reported in previous studies [5,15,20]. Regarding PLT count, previously described data, also did not observe significant difference between the groups [15]. High levels of TPS and globulins, and decreased albumin and A/G ratio in SD were reported by other authors [7].

Inflammatory infiltrate present as a mild to moderate, composed by mononuclear cells as macrophages, plasma cells, lymphocytes and neutrophils, is

a characteristic of CanL [6]. In addition, the cellular infiltrate shown in the superficial and deep dermis with multifocal distribution, consists of findings described by Reis *et al.* [21].

A greater expression of the markers CD45⁺, CD68⁺ and E-cadherin⁺ in SD when compared to the other groups, may be related to activation of skin sentinels cells in dogs naturally infected by *L. infantum*. CD45⁺ is one of the most abundant molecules expressed on the white blood cell surface in various mammals, while CD68⁺ is a myelomonocytic marker that seems to be retained during monocyte differentiation. The differential expression of CD45⁺ isoforms in canine leukocytes were shown to vary among leukocyte phenotype and by the activation status [9]. In dogs, it was demonstrate that leukocytes and CD45⁺ epithelial cells was characteristic of an immature stage [4]. In

the skin, increased numbers of CD68⁺ are related to dendritic epidermal cells, which can be expressed as CD45⁺/CD1a⁺/HLA-DR⁺ [13]. DCs of the skin, particularly epidermal Langerhans cells (LCs), form networks anchored to neighboring keratinocytes via E-cadherin, a component of epithelial cell junctions that is also expressed by LCs. It has been demonstrated that E-Cadherin-mediated contacts induce DC maturation [10]. On the other hand, the expression of cadherins have been observed during tumor progression [18]. E-cadherin⁺ is expressed in not only canine cutaneous histiocytomas and mastocytomas, but also multiple other canine tumor [12,19].

CONCLUSION

Our results indicated that CanL modify the CD45⁺, CD68⁺ and E-cadherin⁺ expressions, which characterize the immune sentinels cells activation that promote the recruitment of cellular infiltrate composed by macrophages, plasma cells, lymphocytes and neutrophils. Thus, these informations may contribute to the follow-up of CanL progression in skin.

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Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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