

Inflammatory response, parasite load and AgNOR expression in ear skin of symptomatic and asymptomatic *Leishmania (Leishmania) chagasi* infected dogs

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Abstract: The skin has an important role in the transmission of visceral leishmaniasis (VL) as the infection pathway in dogs. To better characterize the inflammatory response of intact skin in VL, sixty infected dogs (30 symptomatic and 30 asymptomatic) and six non-infected controls were studied. Diagnosis of visceral leishmaniasis was confirmed by RIFI and ELISA; direct visualization of the parasite in bone marrow aspirate; imprints of popliteal lymph nodes, spleen, liver and skin; culture in NNN-phase liquid Schneider's medium; and PCR (performed only in the ear skin). Amastigote forms of the parasite in intact skin were found only in symptomatic dogs. Inflammatory infiltrates were observed in all groups, varying from intense and/or moderate in symptomatic to discrete and/or negligible in asymptomatic and control animals. Parasite load was associated with the intensity of the inflammatory response and with clinical manifestations in canine visceral leishmaniasis. AgNOR as active transcription markers were expressed in inflammatory cells and within apoptotic bodies in all groups, including controls, with no statistical difference. Therefore, cell activation and transcription do occur in both symptomatic and asymptomatic canine visceral leishmaniasis and may result in more necrosis and inflammation or in apoptosis and less symptoms, depending on the parasite load.

Key words: visceral leishmaniasis, inflammation, parasites, *Leishmania chagasi*.

INTRODUCTION

Visceral leishmaniasis (VL), an endemic zoonosis, is caused by the parasite *Leishmania (Leishmania) chagasi*, which is transmitted by bites of *Lutzomyia longipalpis* sand flies (1, 2). Clinically, the infection may be asymptomatic, oligosymptomatic (with nonspecific signs of infection) or symptomatic with manifestations including keratitis, onychogryphosis and evident splenomegaly (3).

During its life cycle, *Leishmania* multiply within cells of the mononuclear phagocytic system of infected humans and dogs (4-6). The vector, a sand fly, bites an affected animal and the amastigote forms of the parasite turn into

promastigotes in the gut of the invertebrate host. Transmission occurs during the blood meal of an infected vector, which inoculates promastigotes into the skin of the vertebrate host. Promastigotes engulfed by macrophages are transformed into amastigotes that proliferate and spread, triggering the disease and immunosuppression (7).

Dogs comprise the most important domestic reservoir of visceral leishmaniasis because they show intense cutaneous parasitism, which allows an easy infection of the sand fly (1, 8, 9). The indiscriminate killing of infected dogs has not diminished the incidence of the disease in endemic areas, probably due to the presence of other infected synanthropic animals or due to a likely human to human transmission (10-

16). Parasitism in skin does not occur at the same intensity in all stages of the infection and xenodiagnosis has pointed out that a higher percentage of infected vectors is observed only when sand flies feed on symptomatic dogs in advanced stages of the disease (17). The parasite load and severity of skin lesions in symptomatic animals differ significantly from those observed in asymptomatic animals (6). Thus, the intensity of the parasite load and severity of the skin lesions seems to reflect the immune status of the host (18).

Argyrophilic nucleolar organizer regions (AgNORs) are nuclear proteins involved in active transcription during interphase and mitosis. They are good markers for cell activation and/or proliferation and may be detected by silver staining. Under light microscopy, AgNORs appear as well defined black brownish clusters, from 0.5 to 1.0 μm in diameter, with variable number and forms, primarily in and around the nucleolus. Each black dot corresponds, at the ultra-structural level, to the fibrillar center and dense fibrillar component adjacent to the nucleolus and can be considered as a transcriptionally active unit (19).

In this context, the objective of this study was to evaluate the active transcription by AgNORs expression within the inflammatory infiltrates and correlate these findings with the intensity of the inflammatory response, the parasite load and clinical manifestations in dogs naturally infected with *Leishmania (Leishmania) chagasi*.

MATERIAL AND METHODS

Animals

Sixty naturally infected dogs that had tested positive for serological and parasitological tests for VL were divided into two groups: symptomatic (n = 30) – that showed at least one classic clinical manifestation of the disease (except skin involvement), and asymptomatic (n = 30), according to the method proposed by Pozio *et al.* (3). Six animals were used as non-infected controls, selected from an initial group of 12. They varied in breed, weight, age and gender. All dogs were from the Zoonosis Control Center of Timon, in the State of Maranhão, Brazil. Efforts were made to avoid unnecessary distress to the animals. Housing, anesthesia and euthanasia concurred with the guidelines established by the Ethics Committee in Animal Experimentation

of the Federal University of Minas Gerais that also reviewed and approved this work (UFMG-CETEA, process number 198/2007, approved in March 27, 2008).

Diagnosis of Visceral Leishmaniasis

Diagnosis of visceral leishmaniasis was confirmed by the detection of anti-*Leishmania* antibodies in sera by indirect immunofluorescence assay and enzyme-linked immunosorbent assay. After clinical examination, sternal bone marrow aspirates and imprints of popliteal lymph nodes, spleen, liver and skin were fixed, stained with Giemsa, and examined for direct visualization of the parasite (20). Aspirates from the spleen, liver, bone marrow and lymph nodes were also cultured for promastigotes in NNN-phase liquid Schneider's medium. Polymerase chain reaction was performed to detect parasites' nucleic acids in the ear skin, using a target DNA sequence of the *Leishmania donovani* complex. Animals regarded as non-infected controls presented negative results in all tests, including PCR.

Collection and Processing of Ear Skin Samples

The animals were anesthetized and fragments of ear skin were harvested for histology and immunohistochemistry. The fragments were processed routinely and stained with HE, Shorr's Trichrome (for conjunctive tissue), Giemsa-Lemert (for *Leishmania* and mast cells), silver impregnation was also used (for AgNOR) whereas Goodpasture's stain and Grocott's stain were employed to rule out bacterial or fungal infection, respectively (21).

Immunohistochemistry for the Detection of Amastigotes of *Leishmania (L.) chagasi* in the Skin, Liver, Popliteal Lymph Nodes and Spleen

Hyperimmune serum of dogs naturally infected with *Leishmania (L.) chagasi* was used as a primary antibody for the detection of amastigotes. The reaction was then amplified with LSAB[®] System-HRP (Biotinylated Link for Streptavidin HRP, cat. K0675, DAKO Corporation, USA) and revealed with a solution of 3,3'-diaminobenzidine (DAB, Sigma Chemical, USA). Slides with skin sections were incubated with hyperimmune serum of infected dog and PBS only, as positive and negative controls, respectively (22).

Analysis of Cell Activation and Transcription – AgNORs

Cell activation and transcription was evaluated through the quantification of AgNORs in 100 nuclei of inflammatory cells per animal, within the inflammatory foci, according to Crocker *et al.* (19). The total number was divided by 100 to obtain the mean of AgNORs per animal.

Polymerase Chain Reaction (PCR) in Ear Skin Fragments

DNA was extracted from samples of all animals with a genomic DNA tissue kit (NucleoSpin® Tissue, Macherey-Nagel, Germany). The polymerase chain reaction was performed with the GoTaq® Green Master Mix kit (Promega Corporation, cat. M7122, USA). *L. donovani* complex-specific sequence was used as described by Piarroux *et al.* (23). DNA from an embedded paraffin skin sample of dogs naturally infected with *Leishmania (L.) chagasi* (with intense cutaneous parasite load) and DNA of *Leishmania (L.) chagasi* culture (strain MHOM/BR/BH46) were used as positive controls. Skin DNA of a confirmed non-infected dog and a solution with all reaction components (master mix), except DNA, were used as negative controls. The DNA amplification protocol was described by Laskay *et al.* (24). PCR amplicons were analyzed in polyacrylamide gel electrophoresis in 5% non-denaturing TBE (89 mM Tris-borate, 2 mM EDTA pH 8.0). Subsequently, the gels were fixed and photographed with a digital camera.

Descriptive Evaluation of the Parasite Load and Inflammatory Response of the Skin

Intensity, composition and distribution of the inflammatory response and parasite load in ear skin were evaluated on Shorr stained sections and immunohistochemistry. Inflammatory infiltrates were classified, as described by Solano-Gallego *et al.* (25), into discrete and focal, moderate and multifocal, and severe and diffuse. The parasite load and intensity of the inflammatory infiltrate were graded according to Verçosa *et al.* (6) as follows: 0 – absent, 1 – minimal, 2 – mild and 3 – severe.

Statistical Analysis

Data collection and statistical tests were performed using blind analysis (26). Data were analyzed by nonparametric Kruskal-Wallis test

and Dunn post-test for multiple comparisons. The chi-square was also employed to test the association of variables.

RESULTS

The main clinical manifestations of symptomatic animals with intact skin were: lymphadenopathy (23 cases; 76.7%), conjunctivitis (12 cases; 40.0%), splenomegaly (8 cases; 26.7%) and onychogryphosis (8 cases; 26.7%), as displayed in Table 1. All other clinical manifestations of VL were less frequent than 10%.

Initially, 12 dogs were assigned into the control group (with negative results on serological and parasitological tests), but two of them presented positive results in PCR, other two animals presented at least one clinical sign of VL (lymphadenopathy and/or conjunctivitis, despite the negative result in serological and parasitological tests) and two others had bacterial and/or fungal skin infections. All these animals were excluded from the samples analyzed in this study, remaining six animals as controls. Anti-*Leishmania* antibodies were detected in all infected animals, with titers ranging from 1:40 through 1:640. All symptomatic and asymptomatic animals were positive in at least three of the four parasitological tests (Giemsa, culture, PCR and immunohistochemistry) in different organs, according to Table 2.

Both infected and control animals showed inflammatory infiltrates in ear skin. The intensity of inflammation was directly associated with clinical manifestations and parasite load ($p < 0.01$, chi-square test). Thus, more intense inflammation and a higher parasite load were found in the symptomatic group (Figure 1, $p < 0.05$, Kruskal Wallis and Dunn).

Fourteen out of 30 symptomatic animals showed moderate and multifocal inflammatory infiltrates, located around hair follicles, sebaceous glands and blood vessels (Figure 2 – A). Infiltrates were classified as discrete and focal occurring in the subepidermal region and rarely deep in the dermis in 11 of the 30 symptomatic dogs. Severe and diffuse inflammatory infiltrates were observed in only five of the 30 symptomatic animals. Macrophages were the predominant cells, besides lymphocytes and plasma cells. Neutrophils were seen in the dermis in seven out of 30 symptomatic animals (Figure 2 – B). Only one symptomatic

Table 1. Association among inflammatory infiltrate, parasite load and clinical signs in canine leishmaniasis (n = 30)

Dogs	Clinical manifestations				Inflammatory infiltrate*	Parasite load [#]	PCR [#]
	Lymphadenopathy	Conjunctivitis	Splenomegaly	Onychophosis	Shorr's trichrome	Immunohistochemistry	
01	+	+	+	-	2	1	+
02	+	+	-	-	1	1	+
03	+	-	+	-	1	0	+
04	-	+	-	+	2	1	+
05	+	+	-	-	2	1	+
06	+	-	-	-	1	0	+
07	+	+	+	+	2	1	+
08	+	+	-	-	2	0	+
09	-	-	-	+	3	2	+
10	+	-	-	+	1	0	+
11	+	-	-	-	1	0	+
12	+	+	-	-	2	2	+
13	-	-	+	-	2	1	+
14	+	-	-	-	3	2	+
15	-	-	+	-	1	0	+
16	+	-	-	-	1	0	-
17	+	-	+	-	2	1	+
18	+	-	+	-	1	0	+
19	-	-	-	+	1	0	+
20	+	+	-	-	3	3	+
21	+	-	-	-	3	2	+
22	+	+	-	+	1	0	+
23	+	-	+	-	2	1	+
24	-	+	-	-	1	0	-
25	-	+	-	+	3	3	+
26	+	-	-	-	1	0	+
27	+	-	-	-	2	2	+
28	+	+	-	+	2	1	+
29	+	-	-	-	1	0	+
30	+	-	-	-	1	0	+

*[#]For grading method, see "Materials and Methods".

Table 2. Parasitological tests (Giemsa, culture, PCR and immunohistochemistry) performed in different organ samples according to clinical status of dogs naturally infected with *Leishmania (L.) chagasi* (n = 60)

Dogs	Clinical status	Parasitological tests													
		Liver			Popliteal lymph node			Spleen			Bone marrow		Skin		
		Gi- emsa	Cul- ture	IHC	Gi- emsa	Cul- ture	IHC	Gi- emsa	Cul- ture	IHC	Gi- emsa	Cul- ture	Gi- emsa	PCR	IHC
01	S	-	+	+	+	-	+	+	+	+	+	+	+	+	+
02	S	-	+	-	+	-	+	-	+	+	+	+	+	+	+
03	S	-	-	+	+	-	+	-	+	+	+	+	+	+	-
04	S	+	+	-	+	-	+	-	+	+	+	+	+	+	+
05	S	-	-	+	+	-	+	+	+	-	+	+	+	+	+
06	S	-	+	-	+	-	+	+	+	-	+	+	+	+	-
07	S	-	-	+	-	+	+	+	+	-	+	+	+	+	+
08	S	+	-	+	-	+	+	+	+	+	-	+	-	+	-
09	S	+	-	+	+	+	+	+	+	+	-	+	-	+	+
10	S	+	-	+	+	-	+	+	-	+	-	+	+	+	-
11	S	+	+	+	-	+	+	+	-	+	+	+	+	+	-
12	S	+	+	+	-	+	+	+	-	+	+	+	+	+	+
13	S	+	+	+	-	+	+	+	-	+	+	+	+	+	+
14	S	+	+	+	+	+	-	+	-	+	+	-	+	+	+
15	S	-	+	+	+	+	-	+	+	-	+	-	+	+	-
16	S	-	+	+	+	+	+	+	+	-	+	+	+	-	-
17	S	-	+	+	+	+	+	+	+	-	+	+	+	+	+
18	S	-	-	+	+	-	+	+	+	+	-	+	-	+	-
19	S	+	-	+	+	-	+	+	-	+	-	+	+	+	-
20	S	+	-	+	+	-	+	+	-	+	-	+	+	+	+
21	S	+	+	-	+	+	+	+	+	+	-	+	+	+	+
22	S	-	+	+	+	+	+	+	-	+	+	-	+	+	-
23	S	-	+	+	+	+	+	+	-	+	+	-	+	+	+
24	S	-	+	+	+	+	+	+	-	-	+	+	-	-	-
25	S	+	+	+	+	+	+	+	-	-	+	+	+	+	+
26	S	+	+	+	+	+	+	+	-	-	+	+	+	+	-
27	S	+	+	+	+	-	+	-	+	+	+	+	+	+	+
28	S	+	-	-	+	-	+	-	+	+	-	+	+	+	+
29	S	+	-	-	+	+	+	-	+	+	-	+	-	+	-
30	S	+	-	-	+	+	+	-	+	+	-	+	-	+	-
31	A	+	+	+	-	+	-	+	-	+	+	-	-	+	-
32	A	+	+	+	-	+	+	+	+	+	+	-	-	+	-
33	A	+	+	+	-	+	+	+	+	+	+	+	-	+	-
34	A	+	+	+	-	+	-	+	+	+	+	+	-	-	-
35	A	-	+	+	-	+	-	+	+	+	+	+	-	+	-
36	A	-	+	+	-	+	+	-	+	+	-	+	-	+	-
37	A	+	+	+	+	+	+	-	+	+	+	+	-	+	-
38	A	-	+	-	+	+	+	-	+	+	+	+	-	+	-
39	A	-	+	+	+	+	+	-	+	+	+	+	-	+	-
40	A	-	+	+	+	+	+	+	+	+	-	+	-	-	-
41	A	-	+	+	-	+	+	+	+	+	-	+	-	+	-
42	A	-	+	+	-	+	+	-	+	+	-	+	-	+	-
43	A	+	+	+	-	+	+	+	+	-	+	+	-	+	-
44	A	+	-	+	-	+	+	+	+	-	+	+	-	+	-
45	A	+	+	+	+	+	+	+	+	+	+	+	-	-	-
46	A	+	+	-	+	+	-	+	+	+	+	+	-	+	-
47	A	-	+	-	+	+	-	-	+	+	+	+	-	+	-
48	A	-	+	-	-	+	-	-	+	+	+	+	-	+	-
49	A	-	+	-	-	-	+	+	+	+	+	+	-	+	-
50	A	-	+	-	-	-	+	+	-	+	+	+	-	+	-
51	A	+	+	+	-	+	+	+	-	+	+	+	-	-	-
52	A	+	+	+	+	+	+	+	+	+	+	+	-	+	-
53	A	+	+	+	+	+	+	+	+	-	+	+	-	+	-
54	A	-	+	+	+	+	+	-	+	+	-	+	-	+	-
55	A	-	+	+	+	+	+	+	+	+	-	+	-	+	-
56	A	-	+	+	+	+	+	+	+	+	-	+	-	+	-
57	A	+	+	+	+	+	+	+	+	+	+	+	-	+	-
58	A	+	+	+	+	-	+	+	+	+	+	+	-	+	-
59	A	+	+	+	+	-	+	-	+	-	-	+	-	+	-
60	A	+	+	+	+	-	+	-	+	-	-	+	-	+	-

S: symptomatic dog; A: asymptomatic dog; IHC: immunohistochemistry.

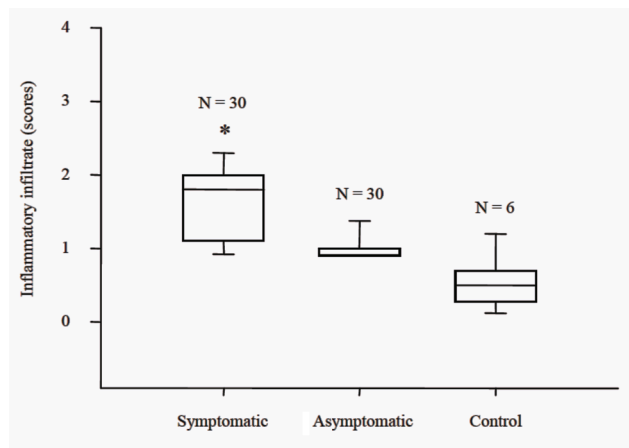


Figure 1. Graph showing inflammatory areas in the ear skin of dogs infected with visceral leishmaniasis. Symptomatic dogs (n = 30) presented more inflammation than asymptomatic ones (n = 30) and controls (n = 6) (Kruskal Wallis and Dunn, *p < 0.05).

animal had diffuse infiltrates composed mainly of neutrophils and a few macrophages.

Most of the asymptomatic animals (28 of 30) showed discrete and focal inflammation (perifollicular, perivascular and rarely subepidermal), composed predominantly of macrophages, lymphocytes and plasma cells (Figure 2 – C). In these animals, moderate and multifocal infiltrates were observed in only two out of 30. Four of the six non-infected controls had discrete and focal inflammation. In such cases, the inflammatory infiltrate was composed of few lymphocytes, plasma cells and a few macrophages.

Skin biopsies from symptomatic dogs, besides showing more intense inflammatory infiltrate were the only ones that presented parasite load. Sixteen out of 30 symptomatic animals had *Leishmania* amastigotes in the skin identified by immunohistochemistry (Figure 2 – D). Intense presence of *Leishmania* parasites was observed only in one dog (Table 1). Six dogs showed moderate infection and in nine of them, the parasite load was graded as discrete to minimal. By contrast, the asymptomatic group had only discrete and/or negligible focal inflammation, and immunohistochemical examination failed to reveal *Leishmania* amastigotes (Figures 2 – E). The PCR showed that 28 of 30 symptomatic dogs had *Leishmania* DNA. On the other hand, 26 of 30 asymptomatic dogs showed positive results by PCR.

AgNORs were diffusely expressed in inflammatory cells and within apoptotic bodies in all infected animals (Figure 2 – F). Symptomatic dogs showed 3.364 ± 0.3954 AgNORs per animal. On the other hand, asymptomatic animals and controls expressed 2.978 ± 0.2567 and 3.283 ± 0.3883 AgNORs per animal, respectively. There was no statistical difference between groups (p > 0.05, Kruskal Wallis and Dunn).

DISCUSSION

Clinical diagnosis of canine VL in endemic areas is quite difficult because the symptoms may vary and frequently overlap the ones found in other diseases (27-28). The three most frequent clinical manifestations found in this study were lymphadenopathy, conjunctivitis and splenomegaly, which were also observed by Almeida *et al.* (29). However, two out of 12 animals with negative parasitological and serological tests (candidates to our control group) presented one or more clinical signs of VL and because of that were excluded from the control group. Therefore, clinical diagnosis should not rely on the number of clinical manifestations, since only one or two clinical signs are insufficient to be used as clinical parameters of VL. However, if the three most common clinical signs observed in this study (lymphadenopathy, conjunctivitis and splenomegaly) occur in one animal in an endemic area, it strongly suggests of *Leishmania (L.) chagasi* infection. Amastigotes were found only in intact ear skin of symptomatic animals. The intensity of parasite load in these animals ranged from minimal to severe. No evidence of *Leishmania* in asymptomatic dogs was also observed in another study that analyzed ear skin samples of 35 animals, examined by immunohistochemistry and PCR (25). Such results indicate that amastigotes in the skin of infected dogs are frequent only in symptomatic animals. Accordingly, intact skin can be used as a good site to search for parasites in endemic areas and in symptomatic animals, as established in this study (6, 30).

Our results indicate that asymptomatic dogs do not appear to harbor parasites in the skin, which corroborates the reports by Verçosa *et al.* (6), Travi *et al.* (31) and Guarga *et al.* (32) that stated that asymptomatic dogs are not able to infect the vector and therefore have low epidemiological

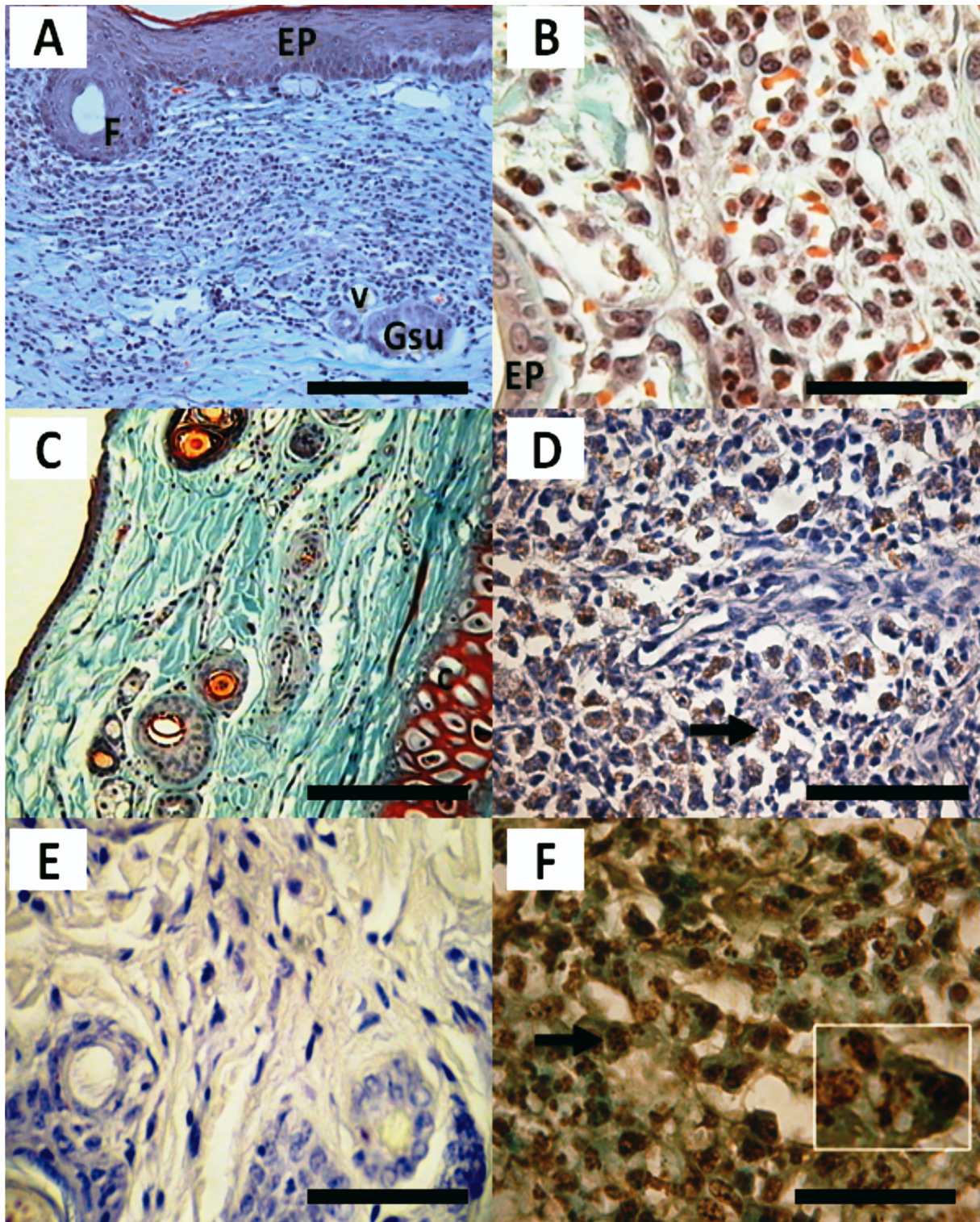


Figure 2. Representative histological sections (5 μ m) of ear skin samples from dogs affected by canine leishmaniasis. (A) Symptomatic dogs: moderate, multifocal, perivascular, subepidermal and periglandular inflammatory infiltrates. Shorr's trichrome stain. Bar: 100 μ m. (B) Symptomatic dogs: focal and subepidermal inflammatory infiltrates with neutrophils and macrophages. Shorr's trichromic stain. Bar: 25 μ m. (C) Asymptomatic dogs: mild, multifocal perivascular and perifollicular inflammatory infiltrates. Shorr's trichrome stain. Bar: 100 μ m. (D) Symptomatic dogs: amastigotes within macrophages (black arrow), immunohistochemical test for *Leishmania chagasi* detection. Immunoperoxidase staining. Bar: 25 μ m. (E) Asymptomatic dogs: absence of amastigotes forms of the parasite, immunohistochemical test for *Leishmania chagasi* detection. Immunoperoxidase staining. Bar: 25 μ m. (F) Symptomatic dogs: AgNORs within nuclei of inflammatory cells (black arrows, small brown circular or oval structures) and apoptotic bodies (seen in detail below left). AgNOR staining. Bar: 25 μ m. EP: keratinized stratified squamous epithelium, F: follicle, V: blood vessel (arteriole), Gsu: sweat gland, C: hyaline cartilage, Gse: sebaceous gland.

risk. On the other hand, asymptomatic dogs of an endemic area in Spain were capable of transmitting *Leishmania infantum* (9). These differences may be associated with *Leishmania* strain and vector species, which are different in Mediterranean region and South America. It is well known that *Phlebotomus perniciosus* is more efficient than *Lutzomyia longipalpis* as vector to transmit viscerotropic *Leishmania* (31).

Inflammation was observed in all groups, even in controls. The ear of dogs is a common target for insect bites, trauma and other parasitic infections (33). That may be an acceptable explanation for the inflammation in the ear skin of controls. Although it was not possible to have a prior knowledge of health and nutritional conditions of the dogs used in this study, one could not rule out previous infections. The present work studied only homeless dogs, captured as part of a program for the control of visceral leishmaniasis and urban rabies. Because we excluded animals presenting macroscopical skin lesions and bacterial/fungal infections, it seems to be acceptable that the inflammatory lesions found in symptomatic and asymptomatic dogs were provoked by *Leishmania (L.) chagasi*. Moreover, in all infected dogs, leishmaniasis was confirmed by cytological, immunological and molecular approaches. Besides, clinical examination and autopsy showed no evidences of other diseases.

Our results clearly show that *Leishmania* can induce inflammatory responses in different intensities and patterns in the ear skin of affected dogs. This fact, combined with the absence of amastigotes in asymptomatic dogs, suggests that the animals are immunocompetent and capable of controlling the infection over time as observed in a mouse model infected with *Leishmania major* (18). The overall results corroborate earlier previous study by Verçosa *et al.* (6), in which the skin of symptomatic dogs exhibited amastigotes and inflammatory infiltrates, whereas asymptomatic animals had an inflammatory profile similar to non-infected controls.

In this context, the type of inflammatory infiltrate corresponds to the immune response profile against the parasite and the degree of susceptibility of animals to the disease (19). As observed in this study, inflammatory infiltrates in the perifollicular, perivascular and periglandular regions of the skin of resistant hosts (controls and asymptomatic dogs) is composed of

lymphocytes, plasma cells and macrophages (6, 25, 34-36). Whereas inflammatory infiltrates in susceptible individuals (symptomatic dogs) is composed frequently of macrophages containing cytoplasmic vacuoles filled with parasites (37).

Neutrophils were observed only in symptomatic dogs that had increased parasite load associated with intense inflammatory infiltrate. In early infection, neutrophils seem to play a role in controlling the parasite load in the spleen, but not in the liver (38). It has been observed also that dead neutrophils isolated from BALBc mice exacerbated the growth and death of *Leishmania (L.) major*, respectively (39). Therefore, the role of neutrophils appears to be dependent on the target organ, the time of exposure to infection and the genotype of the individual. Moreover, *in vitro* infection of neutrophils with *Leishmania major* protects these cells from apoptosis, which results in intracellular survival of the parasite. Infected neutrophils were then engulfed by activated macrophages, which in advanced stages of infection become resistant to apoptosis, contributing to maintenance of the infection (40). It is likely that ear skin lesions in this study are involved in interactions among neutrophils, macrophages and *Leishmania*, leading to maintenance and progression of the parasitic infection by mechanisms not well defined yet.

Quantification of nucleolus organizing regions (NOR) as transcription markers to evaluate cell activation could not discriminate symptomatic, asymptomatic and controls in the current study. Although *Leishmania (L.) chagasi* induces inflammation, it is quite possible that it inhibits cell activation in dogs, which could deregulate the microbicidal mechanism of the host cells and therefore facilitate the parasite survival (41). Besides that, it is likely that cell activation and transcription do occur in both symptomatic and asymptomatic canine visceral leishmaniasis and may result in more necrosis and inflammation or in apoptosis and less symptoms, depending on the parasite load.

In conclusion, there is an association among the parasite load in the skin, inflammatory response and clinical manifestations in dogs naturally infected with *Leishmania chagasi*. Symptomatic animals presented more intense inflammatory response associated with higher parasite load. Additionally, AgNOR as transcription markers that evaluate cell activation could not differentiate symptomatic and asymptomatic dogs. This

finding suggests that cell activation may occur in both situations, but with different outputs, resulting in more necrosis and inflammation in symptomatic infection or in apoptosis and less symptoms in asymptomatic infections, depending on the parasite load.

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CONFLICTS OF INTEREST

There is no conflict.

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ETHICS COMMITTEE APPROVAL

The present study was approved by the Ethics Committee in Animal Experimentation of the Federal University of Minas Gerais (UFMG-CETEA, process number 198/2007, approved in March 27, 2008).

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