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Disorganization of spleen compartments and dermatitis in canine visceral leishmaniasis

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Abstract

Canine visceral leishmaniasis is associated with splenic changes that may interfere with the surveillance of blood borne antigens. Dogs with terminal visceral leishmaniasis present with a variety of skin lesions that may reflect a failure of the immune system to cope with infection. In this study, we compare the frequency of dermatitis in dogs from an endemic area of visceral leishmaniasis and take account of the following parameters: presence/absence of laboratory markers of infection and susceptibility to visceral leishmaniasis, and presence/absence of splenic structural changes associated with severe forms of the disease. Dermatitis was present in 48 of 64 (75%) of the animals. Dermatitis was more frequent in animals with positive splenic culture and negative leishmanin skin test (14/15, 93%) than in non-infected controls ($P = 0.01$). Diffuse dermatitis was present only in animals with evidence of *Leishmania* infection. Diffuse dermatitis was also more frequent in animals with positive (9/27, 33%) as opposed to negative (3/34, 9%) serology against *Leishmania* ($P = 0.01$). Presence of dermatitis correlated with both perisplenitis ($P = 0.03$) and with an increase in plasma cell density in the splenic tissue ($P = 0.02$). Diffuse dermatitis also correlated with splenic lymphoid tissue disorganization ($P = 0.03$) and germinal center atrophy ($P < 0.01$). The relationship between chronic dermatitis and spleen disorganization suggests an impairment of immune response to deal with skin aggressions in dogs with severe forms of visceral leishmaniasis. It also suggest that skin biopsy can be used in a model to define severity of canine visceral leishmaniasis.

Keywords: *Leishmania chagasi*, Canine visceral leishmaniasis, Spleen, Dermatitis, Dog, Leishmanin skin test

Introuction

Canine visceral leishmaniasis (CVL) is a re-emergent zoonotic disease caused by *Leishmania infantum* that is largely found in tropical and subtropical countries (Steverding 2017). This disease is characterized in both humans and dogs by emaciation, hepatosplenomegaly, fever, anemia, and a predisposition to bacterial infection (Costa et al. 2010)(Barrouin-Melo et al. 2006) (Lima et al. 2014). Furthermore, dogs present with a remarkable pattern of parasite dissemination to the skin with associated lesions that are uncommon in human beings (Lima et al. 2014). In fact, the high parasite burden in the skin of dogs with CVL support the idea that dogs play an important role as a reservoir in the *L. infantum* transmission cycle in urban centers. The most common dermatological manifestations in CVL include alopecia, desquamation, and nodular, ulcerative or papular dermatitis (Lima et al. 2014) (Perego et al. 2014). Hence, skin sampling from the ear and nose has been recommended for diagnosing CVL using conventional histology, immunohistochemistry, or PCR in symptomatic and asymptomatic dogs (Leontides et al. 2002) (Manna et al. 2004) (Xavier et al. 2006). A spectrum of inflammatory infiltrates varying from a focal (periadnexal) to a diffuse or granulomatous has been associated with CVL (Brachelente et al. 2005)(Dos-Santos et al. 2004)(Solano-Gallego et al. 2004). The predominant inflammatory cell types present in these inflammatory infiltrates are macrophages and plasma cells. Some patterns of inflammatory infiltrate have been associated with *L. infantum* infection and parasite burden (Dos-Santos et al. 2004). The factors

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Santana *et al. Surgical and Experimental Pathology*   (2019) 2:14

Page 2 of 8

that contribute to the presence and characteristics of the inflammatory infiltrate and the parasite burden in the skin are unknown. In tegumentar forms of leishmaniasis and other infectious diseases, such as leprosy, the pattern of inflammatory infiltrate reflects the immunological status of the host and the host's capability to control parasite proliferation (Lemos de Souza et al. 2000) (Lastoria and de Abreu 2014). A similar association between the characteristics of the inflammatory infiltrate and the host immunological response may also exist in CVL.

The spleen is affected in the whole course of visceral leishmaniasis (VL). It increases in size and develop white pulp hyperplasia followed by atrophy, red pulp plasmacytosis and structural disorganization (Veress et al. 1983) (Santana et al. 2008)(Silva-O'Hare et al. 2016). These changes interfere with important spleen functions. Spleen enlargement leads to hypersplenism, with enhanced erythrocyte destruction and blood cells and platelets retention leading to susceptibility to infection and bleeding (Costa et al. 2010)(dos-Santos et al. 2014). The structural disorganization of the spleen may impair spleen function of surveillance against pathogens circulating in the blood (Mebius and Kraal 2005) (Hermida et al. 2018). In CVL white pulp disruption is associated with severity of the disease (Lima et al. 2014). The spleen disorganization in CVL is associated with parasite burden and with changes in the normal leukocyte populations and in the normal pattern of cytokine expression (Lima et al. 2014) (Cavalcanti et al. 2015) (Silva et al. 2012).

In this work, we study the potential associations between dermatitis and disorganization of spleen compartments in dogs naturally infected with *Leishmania*. Our hypothesis is that the disruption of spleen structure may interfere with skin response to aggression by *Leishmania* and other pathogens. The skin is the first line of defense against infection. It is also the most accessible organ to clinical investigation for signs of disease. Hence, the study of skin lesions may help to design strategies of treatment of dogs with CVL and to predict spleen changes associated with severe forms of the disease.

Materials and methods
Animal and specimens
This is a retrospective study based on histological specimens and experimental records archived in the Gonçalo Moniz Institute-FIOCRUZ, Salvador, BA, Brazil. The histological specimens consisted of spleen and ear skin fragments obtained from stray dogs of different breeds and estimated ages that were collected in collaboration with the Endemic Diseases Surveillance Program of the State Health Service from the streets of Jequié (Bahia, Brazil, an endemic area for VL) from 1997 to 2001. The tissue samples had been fixed in 10% phosphate-buffered

formalin and embedded in paraffin. In this study, we included all animals subjected to serology, splenic culture for *Leishmania* detection, and leishmanin skin test (LST) for which spleen and ear skin samples were available. Details about the tests performed in these animals were presented previously (Baleeiro et al. 2006)(dos-Santos et al. 2008), and the results of these tests are summarized in Table 1.

Histological analysis
Four to five-micron skin and splenic sections were stained with hematoxylin-eosin (H&E) and Giemsa stain and were independently examined by at least two observers. Each animal was included in a single category of normal skin or dermatitis or of splenic alteration using the following criteria of histological analysis.

Skin
Skin inflammatory infiltrate was studied using previously defined categories (Dos-Santos et al. 2004) with the following modifications: (1) normal skin: without inflammatory infiltrate; (2) skin with mixed focal dermatitis: containing a variety of inflammatory cells including lymphocytes, macrophages, and plasma cells, present as single or multiple small clusters in the upper dermis and around the skin annexa; and (3) skin with mixed diffuse dermatitis: similar to the previous category but with extensive coalescent spreading of the inflammatory infiltrate through the dermis. Focal or diffuse dermatitis could be further classified as (a) with granuloma: focal or diffuse dermatitis containing clusters of epithelioid macrophages or (b) with monomorphic macrophage

Table 1 General characteristics of the stray dogs found in an endemic area of visceral leishmaniasis that were used in this study

Parameter	N	(%)
Total number of animals	64	(100)
With emaciation	26/60	(43)
With positive spleen culture	32/64	(50)
With positive serology (ELISA)	27/61	(44)
With positive leishmanin skin test (*L. chagasi* antigen)	29/47	(51)
Spleen histology:		
With normal white pulp (type I)	32/64	(50)
With white pulp disorganization:	32/64	(50)
Slight disorganization (type II)	19/64	(30)
Intense disorganization (type III)	13/64	(20)
Skin histology:		
With normal histology	16/64	(25)
With dermatitis:	48/64	(75)
Focal dermatitis	36/64	(56)
Diffuse dermatitis	12/64	(19)
With *Leishmania* amastigotes	8/64	(13)

Santana *et al. Surgical and Experimental Pathology*   (2019) 2:14

Page 3 of 8

inflammatory infiltrate: focal or diffuse dermatitis containing large clusters of non-epithelioid, vacuolated macrophages (Vichowian-like inflammatory infiltrate). Representative aspects of each histological pattern were previously presented (Dos-Santos et al. 2004).

Spleen
A detailed description of splenic lesions is presented elsewhere and reviewed in Hermida et al. 2018 (Santana et al. 2008) (Hermida et al. 2018). Briefly, the presence of chronic perisplenitis and granuloma were recorded, and the following characteristics of the white pulp were semi-quantitatively estimated: lymphoid follicle and marginal zone frequency and size were classified as very small, small, average and large. The degree of structural organization of the white pulp was classified into four categories: Well organized spleen (spleen type 1) has a distinct periarteriolar lymphocyte sheath, lymphoid follicles and a marginal zone. Present a varied number of secondary lymphoid follicles, containing a germinal center clearly delimited by a rim of small lymphocytes (the mantle zone); slightly disorganized (spleen type 2) has either hyperplastic or hypoplastic changes blurring the boundaries between regions of the white pulp; and moderately to extensively disorganized (spleen type 3) the white pulp regions are poorly individualized or indistinct lymphoid follicles are barely distinct from the red pulp and T-cell areas and secondary lymphoid follicles are absent..

Leukocyte populations in the skin and spleen
The frequency of lymphocytes, plasma cells, macrophages, granulocytes, and mast cells present in the inflammatory foci of the skin and the spleen was estimated using conventional microscopy as either absent, rare (scattered cells present in discontinuous areas), few (small cell aggregates present in many areas of the inflammatory infiltrate), average (readily found in the inflammatory infiltrate), or high (representing one of the main cell populations in the inflammatory infiltrate). Details of the morphological identification of the cells are provided elsewhere (Silva et al. 2012).

Histological parasitism in the skin and spleen
The density of *Leishmania* amastigotes in the skin and spleen was estimated using conventional microscopy as described elsewhere (Santana et al. 2008). Briefly, the number of infected macrophages observed in a minimum of 40 and a maximum of 100 non-overlapping high power (1000x) fields per section was recorded. In the spleen, the examined fields were equally distributed between the subcapsular and internal red pulp.

Statistical analysis
The results are expressed in tables as absolute numbers and percentages. The significance of the differences observed between groups was tested using the Chi-square test with Yates' correction or Fisher's exact test when recommended. The strength of association between variables was tested using a standardized Pearson's contingency coefficient (C*). This coefficient ranges from − 1 to + 1, indicating perfect association, where 0 (zero) indicates no association (Blaikie 2003) (Akoglu 2018). The following are proposed standards for association strength for Pearson's contingency coefficients: 0 = poor, 0.01–0.20 = slight, 0.21–0.39 = fair, 0.40–0.60 = moderate, 0.61–0.80 = substantial, and 0.81–1 = almost perfect to perfect. The critical level of significance was established at $P < 0.05$.

Results
Skin inflammation and *Leishmania* to infection
The data on the inflammatory infiltrate in the skin of the animals are summarized in Table 1. Dermatitis was observed in 48 (75%) out of 64 dogs. The inflammatory infiltrate was focal in 36 (56%) animals (Fig. 1a) and diffuse in 12 (19%) animals (Fig. 1b). The most frequent cells in the infiltrate were plasma cells and macrophages (Fig. 1c). Three of the 12 animals with diffuse dermatitis also had granulomas (Fig. 1d and e). *Leishmania* amastigotes were seen in the dermis of eight animals, five with diffuse dermatitis and three with focal dermatitis (Fig. 1f).

Positive markers of infection, susceptibility to VL, and presence of dermatitis
Dermatitis was associated with evidence of *Leishmania* infection: among the 51 animals with positive splenic culture, positive serology, or positive LST, 41 (80%) had dermatitis. Among the nine animals with all negative tests for *Leishmania* infection, only four (44%) animals had dermatitis (Fisher exact test, $P < 0.04$, Table 2). Dermatitis was focal in all animals without evidence of *Leishmania* infection.

Among the immunological and parasitological tests used in this study, positive anti-*Leishmania* serology and positive splenic culture but not LST were associated with presence of dermatitis (Table 2). Diffuse dermatitis was more frequent in animals with positive (9 out of 27 animals, 33%) than negative serology (3 out of 34 animals, 9%; Chi-square, $P = 0.01$). Dermatitis was also more frequent in animals with positive (28 out of 32 animals, 88%) than negative splenic culture (20 out of 32 animals, 63%; Chi-square, $P = 0.04$). Dermatitis was present in 24 (92%) out of 26 emaciated animals and 23 (66%) out of 35 animals that were not emaciated (Chi-square, $P = 0.03$). Moreover, among the eight animals with histological parasitism of the skin, seven (87%) were emaciated; among the 53 animals without histological parasitism of the skin, only 19 (36%) were undernourished (Chi-square test, $P = 0.02$).
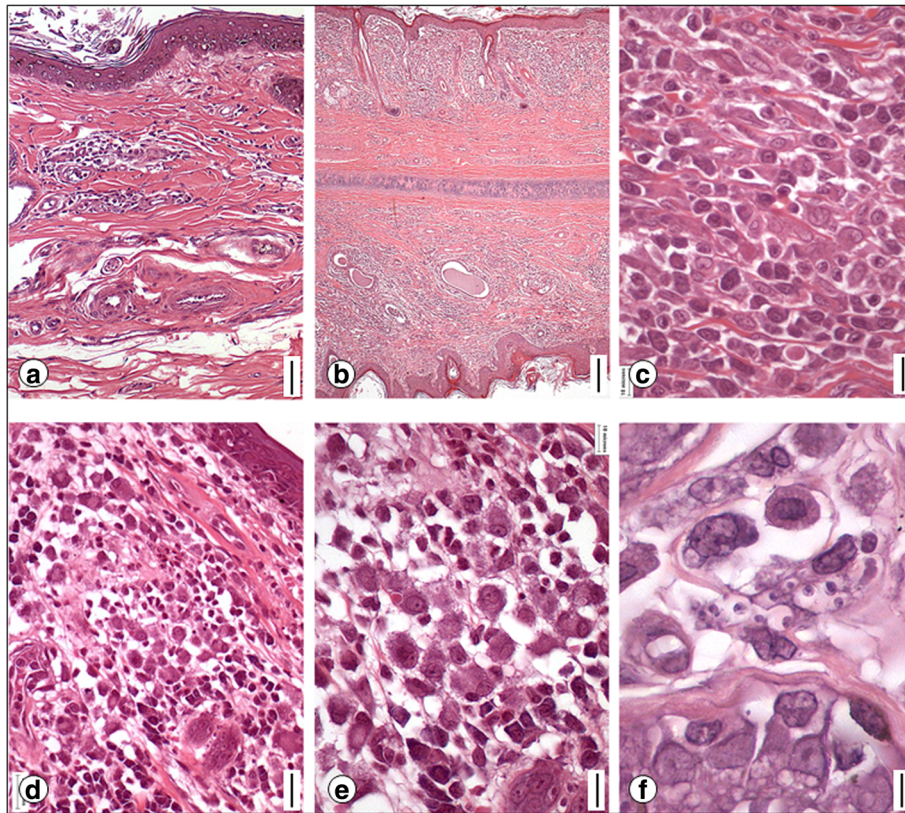
Fig. 1 Histological patterns of dermatitis present in dogs from an endemic area of visceral leishmaniasis: **a** focal dermatitis; **b** diffuse dermatitis; **c** inflammatory infiltrate with a predominance of plasma cell and macrophages; **d** inflammatory infiltrate with granulomatoid area; **e** a granuloma in detail; and **f** – amastigote-loaded macrophage in the dermis. Lengths of the black bars on the right hand side at the bottom of the figures: **a**, 40 μm; **b**, 100 μm; **c** and **e**, 10 μm; **d**, 20 μm; **f**, 4 μm

Table 2 Frequency of dermatitis in stray dogs from an endemic area of visceral leishmaniasis according to laboratory tests for infection and clinical signs of disease

Parameter	N	(%)	Skin							
			Normal	(%)	With dermatitis					
					Focal	(%)	Diffuse	(%)	Total	(%)
Serology:										
Negative	34	(100)	12	(35)	19	(56)	3	(9)	22	(65)
Positive	27	(100)	3	(11)	15	(56)	9	(33)[a]	24	(89)[b]
Leishmanine skin test										
Negative	28	(100)	7	(25)	16	(57)	5	(18)	21	(75)
Positive	29	(100)	8	(28)	17	(59)	4	(14)	21	(72)
Splenic culture										
Negative	32	(100)	12	(38)	17	(53)	3	(9)	20	(63)
Positive	32	(100)	4	(13)	19	(59)	9	(28)[c]	28	(88)[d]
Emaciation:										
Absent	35	(100)	12	(34)	19	(54)	4	(11)	23	(66)
Present	26	(100)	2	(8)	16	(62)	8	(31)[e]	24	(92)[f]

[a-f] Statistically different from the group with normal skin, Chi-square test: [a] *P* = 0.01; [b] *P* = 0.06, [c] *P* = 0.02; [d] *P* = 0.04; [e] *P* = 0.01; and [f] *P* = 0.03

Dermatitis was more frequent in the animals with positive culture or positive serology and negative LST (14 out of 15 animals, 93%) than in *non-infected* animals (4 out of 9 animals, 44%; Fisher exact test, $P = 0.02$, Table 3).

Associations between inflammatory changes in the skin and in the spleen in canine visceral leishmaniasis

Dermatitis was moderately associated with splenic lymphoid tissue disorganization, increased splenic plasma cell frequency, and perisplenitis. Dermatitis was also strongly associated with splenic lymphoid follicle atrophy. Among 32 animals with disorganized splenic lymphoid tissue, 28 (88%) had dermatitis, while 20 of 32 (63%) animals with organized splenic lymphoid tissue had dermatitis (Chi-square, $P = 0.02$; C* = 0.39;). Such associations were even stronger when only animals with diffuse dermatitis were considered (Chi-square, $P = 0.03$; C* = 0.54, Table 4). Among the 36 animals with lymphoid follicles with small or no germinal centers, 32 (89%) had dermatitis. Among the 28 animals with follicles containing average or large germinal centers, 16 (57%) had dermatitis (Chi-square, $P < 0.01$; C* = 0.48, Table 4). This association was stronger when only animals with diffuse dermatitis were considered (Chi-square, $P = 0.01$; C* = 0.63, Table 4). Among the 28 animals with a high frequency of splenic plasma cells, 25 (89%) had dermatitis. Among the 36 animals with average or low concentrations of splenic plasma cells, 23 (64%) had dermatitis (Chi-square, $P = 0.02$; C* = 0.39, Table 4). Among the 19 animals with perispelenitis, 11 (58%) had focal dermatitis, and seven (37%) had diffuse dermatitis. Among the 42 animals without perisplenitis, 22 (52%) had focal dermatitis, and 5 (12%) had diffuse dermatitis (Chi-square, $P = 0.03$; C* = 0.40, Table 4).

Table 3 Response to *L. chagasi* infection and frequency of dermatitis in stray dogs from an endemic area of visceral leishmaniasis

Parameters	Uninfected		Infected					
Serology	–		–		+		–/+	
Leishmanin skin test	–		+		–		+	
Splenic culture	–		–		+		+	
N	9	(100)[a]	12	(100)	15	(100)	10	(100)
Skin histology:								
Normal	5	(56)	3	(25)	1	(7)	2	(20)
With dermatitis:	4	(44)[b]	9	(75)	14	(93)[c]	8	(80)
Focal dermatitis	4	(44)	7	(58)	10	(67)	7	(70)
Diffuse dermatitis	0	(0)	2	(17)	4	(27)	1	(10)

[a] Values in parentheses are percentage from the total. [b] Statistically different from the other groups, Chi-square test, $P = 0.0408$; [c] Statistically different from the control uninfected group, Fisher's exact probability test, $P = 0.02$

Leishmania amastigotes were seen both in the skin and spleen of five dogs in only the skin of three animals, and in only the spleen of four animals. Such concomitance of high parasite burden in the skin and spleen was statistically significant (Fisher exact test, $P < 0.01$). All the animals with histological spleen parasitism also had dermatitis. The dermatitis was focal in five and diffuse in four animals. The difference in the frequency of dermatitis between animals with and without histological spelenic parasitism was not, however, statistically significant.

Discussion

Together with a previous study from our group, performed in another endemic area (Jacobina) 300 km north of Jequie, suggests that dermatitis is a frequent finding in stray dogs from endemic areas of VL in Brazil (Dos-Santos et al. 2004). In this series, 75% of the animals had inflammation in the skin. These data agree with Giunchetti and collaborators, who found dermatitis in 66% of dogs examined in the endemic area of Belo Horizonte (770 km south of Jequie) (Giunchetti et al. 2006). In our study, even in the nine animals that had all three tests (splenic culture, LST and serology) negative for *Leishmania* infection, four of these animals had dermatitis. The causes of dermatitis in these animals are unknown. A variety of ordinary conditions, such as insect bites, trauma, and other parasitic infections, may be involved (Kim et al. 2011) (Mozos et al. 1999). In spite of this high dermatitis frequency, the dermatitis present in dogs without positive markers of infection with *Leishmania* was focal. On the other hand, the frequency of dermatitis was higher in animals with than without evidence of infection, and diffuse dermatitis was observed only in this group of *Leishmania*-infected dogs. In our previous study, a trend to association between dermatitis and infection was also observed (Dos-Santos et al. 2004).

In this work, we showed that dermatitis is associated with splenic lymphoid tissue disorganization, germinal center atrophy, and increased plasma cell frequency in the red pulp in CVL. These associations were of average to strong intensity (Pierson C coefficient). A variety of alterations of splenic histological architecture have been described in humans, mouse, and dogs throughout the course of VL (Veress et al. 1977) (Santana et al. 2008) (Cavalcanti et al. 2015) (Engwerda et al. 2002). Spleen enlargement leads to hypersplenism and pancytopenia, impairing host response to infection. Splenic white pulp disorganization in VL may disturb B-cell differentiation, affecting the immune response to blood-borne antigens (Mebius and Kraal 2005). In fact, an increased prevalence of bacterial infections is observed in the late stages of VL (Costa et al. 2010) (Endris et al. 2014). The clinical characteristics of some skin lesions in dogs with advanced VL are suggestive of bacterial infection, which

Table 4 Associations between dermatitis and splenic changes in stray dogs from an endemic area of visceral leishmaniasis

Spleen	N	(%)	Skin								Contingency Coefficient
			Normal (%)		With dermatitis						
					Focal (%)		Diffuse (%)		Total (%)		
White pulp organization:											
Organized	32	(100)	12	(38)	16	(50)	4	(13)	20	(63)	[a] C* = 0.38, P = 0.04
Disorganized	32	(100)	4	(13)	20	(63)[a]	8	(25)[b]	28	(88)[c]	[b] C* = 0.54, P = 0.03
											[c] C* = 0.39, P = 0.02
Lymphoid follicle germinal center:											
Average or large	28	(100)	12	(43)	13	(46)	3	(11)	16	(57)	[d] C* = 0.48, P = 0.01
											[e] C* = 0.63, P = 0.01
Small or absent	36	(100)	4	(11)	23	(64)[d]	9	(25)[e]	32	(89)[f]	[f] C* = 0.48, P > 0.01
Marginal zone:											
Average or large	41	(100)	13	(32)	23	(56)	5	(12)	28	(68)	NS
Small or absent	23	(100)	3	(13)	13	(57)	7	(30)	20	(87)	
Plasma cells:											
Average to absent	36	(100)	13	(36)	16	(44)	7	(19)	23	(64)	[g] C* = 0.46, P = 0.01
High	28	(100)	3	(11)	20	(71)[g]	5	(18)	25	(89)[h]	[h] C* = 0.39, P = 0.02

C* = Standardized Pearson's contingency coefficient, followed by the significance levels estimated using Chi-square test with Yates' correction. [a, b, c, d, e, f] Represent the groups tested against the normal (without dermatitis) group and their respective association and significance tests

may reflect an impaired ability to fight these pathogens (Barrouin-Melo et al. 2006) (Lima et al. 2014). Dogs with severe VL also present an increased skin parasitism by Leishmania. Therefore, the disruption of spleen compartments observed in CVL may contribute to an impairment of skin immune response to different pathogens. Further studies are necessary in order to investigate the pathways involved in lymphoid tissue changes and dermatitis in CVL.

This work also shows that positive serology and active parasitism, both markers of susceptibility to VL, are associated with dermatitis. Conversely, LST alone did not show a clear association with dermatitis. It is interesting to note, however, that skin inflammation tended to be more frequent and severe in infected animals with negative LST (see Table 3). In tegumentary leishmaniasis, the inflammation pattern reflects the host immunological response to the parasite. In our previous study, we observed an association between granulomatous and monomorphic inflammatory infiltrate and high parasite burden in the skin (Dos-Santos et al. 2004). In this study, we only noticed a trend of increasing diffuse dermatitis frequency in the animals with positive culture or positive serology and negative LST. Unfortunately, we only had three animals with granulomatous infiltrate in this series, and animals with the monomorphic macrophage skin infiltrate was not observed in this sample, which impaired further stratification for analysis.

Finally, the data presented here suggest that the distribution pattern of dermatitis in CVL can be used in a model to predict susceptibility and progression of the disease.

Conclusions

1) Dermatitis is frequent in infected and uninfected dogs from endemic areas of VL in Brazil;
2) Dermatitis is, however, more frequent in dogs with than in dogs without infection with *Leishmania*, mostly in dogs with infection and a sign of susceptibility to VL;
3) In *L. infantum*-infected dogs, dermatitis is associated with spleen disorganization and susceptibility markers to VL.

analysis and wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate
This study was performed using spleen and skin samples previously collected and stored in the histological records of the Gonçalo Moniz Institute in the period between 1997 to 2001. No new animal manipulations were performed. All the procedures involving animals were conducted in accordance with the Oswaldo Cruz Foundation guidelines for animal experimentation and with the manual for the surveillance and control of visceral leishmaniasis (http://bvsms.saude.gov.br/bvs/publicacoes/manual_vigilancia_controle_leishmaniose_visceral.pdf) (Baleeiro et al. 2006).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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