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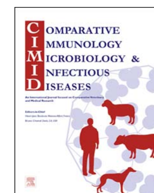

Parasitic load and histological aspects in different regions of the spleen of dogs with visceral leishmaniasis

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ABSTRACT

Leishmania infantum causes from subclinical infection to severe disease in humans and dogs. The spleen is one of the organs most affected by the infection. Although evidence exists that the parasitic load distribution and histological alterations may not be homogeneous in the affected organs of naturally infected individuals, it has not been formally demonstrated using the current techniques used for studying the disease.

In six dogs naturally infected with *Leishmania*, parasitic load and histological changes were compared in samples collected from the lower, middle and upper third of the spleen.

Parasitic load in the spleen of the group of dogs was variable, revealing a difference of 61 times between animals with the lowest and the highest parasitism. The set of parasitic load values of each dog showed a cluster trend, when compared to the other animals. Nevertheless, the parasitic load values of each dog showed a variation ranging from 3.2 to 34.7 times between lowest and highest value. Histological changes showed recognizable variation in frequency (granulomas) or intensity (perisplenitis) in the spleen of 2 out of the 6 dogs. The agreement of histological findings between samples collected from the different thirds of the spleen was good (kappa coeficient, 0.61–0.80) very good (0.81–0.99) or perfect (1.00), for most of the parameters analyzed.

Variability of parasitic load and, to a lesser extent, histological changes in spleen of dogs with visceral leishmaniasis is observed. Such variability may be taken in account in the design of studies on pathogenesis, vaccine and therapeutic drug development.

1. Introduction

The zoonotic form of visceral leishmaniasis (VL) is found mainly in Brazil, in the Mediterranean Basin and in the Middle East [1]. The disease affects predominantly human beings and the domestic dog, being the latter the main reservoir of *Leishmania infantum* (syn. *L. chagasi*) [2,3], the causal agent. In endemic areas, the infection rate may exceed 60% of the canine population [4–7]. Unfortunately, a vaccine demonstrated to be effective to control canine VL is still unavailable [8]. In addition, dogs treated with the currently available drugs tend to develop only transient clinical cure, without parasitological clearance, and, after treatment, relapses are frequent even in the absence of reinfection [9,10]. For the reasons mentioned above, canine VL poses a major public health and veterinary medicine concern.

After dermal inoculation by the phlebotomine vector, *L. infantum* spread to organs of the mononuclear phagocytic system [11], especially the spleen, bone marrow, liver and lymph nodes. In these organs, the innate and adaptive immune system cells will determine the extent of parasitic survival and multiplication, development of host tissue damage, and fate of the infection [12].

In the infected individuals, the spleen tends to be parasitized during the whole period of the infection [11–14], and very often develops functional and architectural changes [15–18]. These changes are associated to a state of immunosuppression that compromises the host capacity to control the protozoan infection and infections caused by other microorganisms [19]. For these reasons, splenic evaluation is used to

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detect *Leishmania* infection and determine parasitic load as well as host immunological state, and response to treatment in dogs and humans with VL [16,17,20,21]. Perisplenitis, granuloma and white pulp disorganization has been associated with susceptibility and with severe forms of the disease and the spleen size regression is used as clinical evidence treatment response [16,22].

There is evidence that parasitic distribution and histological changes in organs of individuals (e.g. humans and dogs) naturally infected with *Leishmania* may be heterogeneous [16]. Yet, there are no reports formally demonstrating a heterogeneous distribution of these findings in the affected organs.

Further knowledge on distribution of *Leishmania* load and histological changes in different organs may contribute to improve diagnosis of infection/disease and understanding of compartmentalization of host response to the parasite. This will allow for designing more rational studies on pathogenesis and evaluation of outcome of immunization or treatment protocols.

In the present work, we systematically studied distribution of parasitic load and morphological changes in samples collected from the upper, middle and lower third of the spleen of dogs with VL. The parasitic load was assessed by real-time PCR using TaqMan system and morphological changes were assessed by conventional histology.

2. Methods

2.1. Animals

Blood samples and splenic aspirates were obtained from six mongrel dogs from Camaçari, Brazil, an area endemic for VL [23]. Spleen aspirate punctures were carried out by the method previously described by Barrouin-Melo et al. [24], followed by culturing the aspirate in NNN biphasic medium supplemented with 20% fetal bovine serum (FBS) [24]. All six animals showed: a) *Leishmania*-specific antibodies, detected by indirect immunofluorescence assay (IFAT, carried out with a BioManguinhos leishmaniasis detection kit, Rio de Janeiro, Brazil), b) *Leishmania* promastigote growth in cultures of splenic aspirate, and c) clinical signs compatible with VL (described in Table 1). Four months after the initial clinical evaluation, the dogs were clinically re-examined and then euthanized, in accordance with Brazilian Ministry of Health guidelines, under deep anesthesia (1 mg/kg of xylazine, 15 mg/kg ketamine and 25 mg/kg thiopental) by intravenous injection of saturated potassium chloride solution.

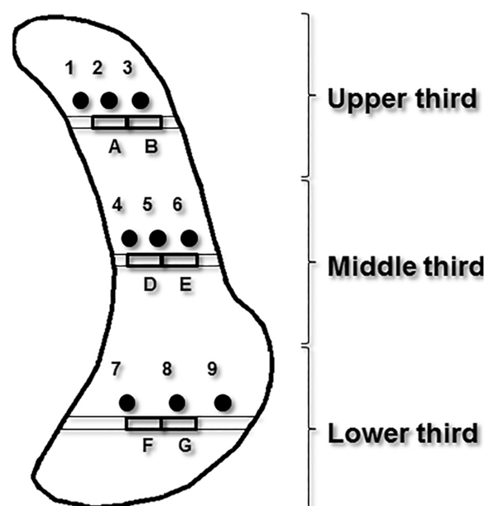

Fig 1. Schematic drawing of spleen showing sampling areas.
Schematic drawing of canine spleen indicating the spots (numbered 1–9) where separate aspirate punctures were carried out for determination of parasitic burden and splenic fragments were taken out (labelled A to G) for histological analysis. The brackets show each third section of the organ.

2.2. Collection of splenic aspirates for real-time PCR and histological study

Following euthanasia procedures, each animal's spleen was removed from its abdominal cavity and subjected to needle puncture aspiration at nine different sites (three in each third of the organ: upper, middle and lower; Fig. 1). Sample collection was performed using a separate 42 × 1.2 mm needle attached to a 20 mL syringe. Each splenic aspirate was then transferred to previously weighed Eppendorf tubes, immediately placed under dry ice and stored at −70 °C until use. Later, the weight of each sample was determined. In addition, cross sections of the spleens were performed in each third (Fig. 1) and two fragments approximately 4 mm in thickness from each third were collected and fixed in phosphate-buffered 10% formalin. Splenic sampling was carried out uniformly without representing anatomical segments of the organ.

2.3. Real-time PCR

DNA was extracted from the splenic aspirate samples using a DNAeasy Blood & Tissue kit (Qiagen, Hilden, Germany) in accordance with manufacturer recommendations, and concentrations were

Table 1
Clinical characterization of dogs with visceral leishmaniasis.

Dog #	Sex[a]	Age[b] (years)	Body[c] weight (kg)	Clinical evaluation[d]	
				First	Second
320	M	5–7	9.4	Hyperkeratosis on the nose and popliteal lymphadenopathy	Hyperkeratosis on the nose and submandibular lymphadenopathy
325	M	5–7	11	Hyperkeratosis on the nose and submadibular lymphodenopathy	Hyperkeratosis on the nose, popliteal and submandibular lymphadenopathy, alopecia, periocular dermatitis, conjunctivitis and onychogryphosis
332	F	3–4	19	Hyperkeratosis on the nose, periocular dermatitis and onychogryphosis	Hyperkeratosis on the nose, periocular dermatitis, onychogryphosis, alopecia, popliteal and submandibular lymphadenopathy
335	F	3–4	11.2	Hyperkeratosis on the nose, popliteal and submandibular lymphadenopathy	Hyperkeratosis on the nose, popliteal and submandibular lymphadenopathy, alopecia and onychogryphosis
343	F	5–7	25	Popliteal lymphadenopathy	Alopecia and onychogryphosis
353	F	3–4	23	Hyperkeratosis on the nose, alopecia, and popliteal lymphadenopathy	Dermatitis, conjunctivitis and submandibular lymphadenopathy

[a] Sex: male (M), female (F).
[b] Age estimated by physical examination.
[c] Body weight measured on the day of euthanasia.
[d] The two clinical evaluations were carried out 4 months apart.

determined on a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). Tissue samples were digested with proteinase K overnight [25]. Real-time PCR assays were performed according to a slightly modified protocol described by Francino et al. [26]. Briefly, triplicates of 25 μL reactions were performed using a 150 ng sample of DNA, primers LEISH-1 (5′-AACTTTTCTGGTCCTCCG GGTAG-3′) and LEISH-2 (5′-ACCCCCAGTTTCCCGCC-3′), in addition to a FAM-MGB probe (FAM-5′-AAAAATGGGTGCAGAAAT-3′-MGB) for the conserved DNA region of the *Leishmania* minicircle, or a specific set of primers and probe for the detection of mammalian 18S rDNA (assay positive control, Applied Biosystems, Carlsbad, USA), as well as 0.2 mg/μL of bovine serum albumin and TaqMan master mix (Applied Biosystems). To construct a calibration curve, amounts ranging from 0.1 to 10^5 of *Leishmania infantum* prosmatigote DNA were used. Reactions were carried out and read on a 7500 Fast real-time PCR system (Applied Biosystems, Foster City, USA). The parasitic load of each sample was expressed as the number of *Leishmania* per gram of splenic tissue in accordance with the formula below:

$$\text{Number of parasites per gram of spleen} = \frac{\text{Number of parasites per 150ng of DNA sample} \times \frac{\text{Weight of DNA purified from aspirate sample in ng}}{\text{Weight of aspirate sample in mg}} \times 1,000}{150}$$

2.4. Tissue processing and analysis

Histological sections measuring 3 μm in thickness were obtained from the splenic fragments and stained with hematoxylin and eosin, then examined under an optical microscope. Tissue section analysis was performed by a trained pathologist, one of the authors of the present report, with extensive experience in the histological evaluation of dogs with VL, who had no prior knowledge regarding the clinical manifestations or splenic parasitic load of each animal under analysis. The criteria used for analyzing the tissue sections were essentially those previously described by Santana and collaborators [16]. The intensity of each histological finding was semi-quantified as mild (1), moderate (2) or intense (3).

2.5. Ethical statements

All experimentation was carried out in accordance with the Brazilian Federal Law on Animal Experimentation (Law 11794) (http://www.planalto.gov.br/ccivil_03/_ato2007-2010/2008/lei/l11794.htm). The present protocol received approval from the Institutional Review Board for Animal Experimentation of the Goncalo Moniz Research Center, Oswaldo Cruz Foundation (license number: 017/2010). A group of dogs not infected with *Leishmania* were not collected from the endemic area for use as negative controls, due to ethical concerns regarding subjecting these animals to euthanasia.

2.6. Statistical analysis

The parasitic burdens the splenic aspirate samples were expressed as the arithmetic means of real-time PCR reactions performed in triplicate. Statistical analyses were carried out using GraphPad Software version 6 (La Jolla, USA), or GraphPad software obtainable online at http://www.graphpad.com/quickcalcs/kappa1/ Agreement between the categorical results of testing was evaluated by k (calculated according to Landis and Koch) [27]. The k statistic, a measure of the agreement between two observers or tests, ranges from −1 to +1, with 1 indicating perfect agreement and 0 indicating the expected agreement based on chance alone. The following proposed standards for strength of agreement for the kappa coefficient were used: 0.00–0.20, poor; 0.21–0.40, fair; 0.41–0.60, moderate; 0.61–0.80, good; 0.81–0.99 very good; 1.00 perfect.

3. Results

3.1. Immunological, parasitological and clinical characterization of the studied dogs

The experimental animals consisted of two males and four females with an estimated age range of 3–7 years. All six dogs showed positive results for IFAT (> 1:50). In addition, *Leishmania* promastigotes were detected in each dog's semi-solid splenic aspirate culture at the time of initial evaluation (Table 1). Table 1 delineates the clinical signs compatible with VL observed in animals during two clinical examinations carried out four months apart. The most frequent clinical manifestations were skin lesions, including dermatitis with hyperkeratosis, alopecia, onychogryphosis and popliteal lymphadenopathy (Table 1). No animals exhibited weight loss. Most animals (5/6) exhibited a greater number of clinical signs compatible with VL at the second clinical evaluation as compared to the first; however, these differences were not statistically significant (Wilcoxon test, $p > 0.09$, Table 1).

3.2. Evaluation of parasitic burden in the spleens of dogs with VL

Five out of six animals had parasitic DNA in all nine splenic samples evaluated (Fig. 2). In these five dogs, a large variation in parasitic load was observed, with median values ranging from 15,863 (dog #325) to 978,694 (dog #320) parasites per gram of spleen, corresponding to a 62-fold difference between animals with the lowest and highest degree of parasitism. Comparisons of parasitic load among the animals revealed that dogs #320 (978,694) exhibited median significantly higher than dogs #353 (23,505), #335 (20,169) and #325 (15,863) (Kruskall-Wallis, followed by Dunn's test, $p < 0.05$). Parasitic load also varied among the nine splenic tissue samples from each animal, with the following ratios between the highest and the lowest values: 34.7 (dog # 335), 25.8 (dog # 320), 5.6 (dog # 353), 5.4 (dog # 332) and 3.2 (dog # 325). No statistically significant differences were found among the parasitic loads estimated in the upper, medial or lower thirds of the spleens (Friedman test, $p > 0.69$). Dog #343 showed no detectable *Leishmania* DNA four months after the initial detection of parasites in its

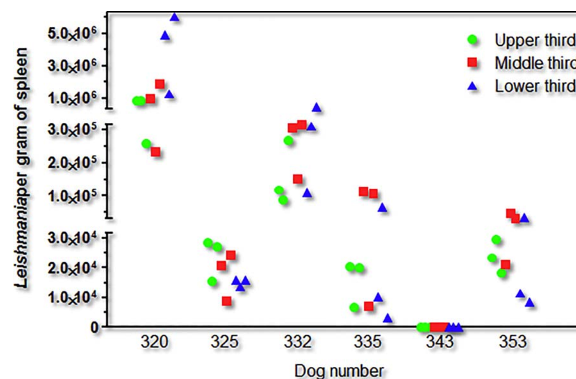

Fig. 2. Parasitic load in spleens of dogs with visceral leishmaniasis.
Parasitic load measured in tissue from 9 different spots of the spleen (3 from each third section) in 6 dogs by real time PCR using TaqMan system as described in the Material and Methods section. These dogs showed clinical manifestations compatible with visceral leishmaniasis, positive indirect immunofluorescence assay test (IFAT) and positive culture for *Leishmania* 4 months prior to parasitic load determination. PCR efficiency and coefficient of determination (R^2) were 91% and 0.998, respectively. The difference between the highest and the lowest value of CT (ΔCT) obtained for each animal (which correlates with the higher and lower parasitic load in the spleen, respectively) and the highest amplitude value of CT of the 9 triplicates (RA) of each animal, given in number of cycles of PCR, were the following (ΔCT and RA): dog # 320 (4.001 and 0.182), dog # 325 (1.975 and 0.309), dog # 332 (2.943 and 0.173), dog # 335 (3.811 and 0.513), dog # 343 (not applicable), and dog # 353 (2.179 and 0.350).

Table 2
Histological findings in the spleen of dogs with visceral leishmaniasis.

PARAMETERS	HISTOLOGICAL CHANGES (SEMIQUANTITATIVE ANALYSIS)[b]																	
	White pulp type			Lymphoid follicle size			Granuloma			Plasma cell density			Neutrophil density			Perisplenitis		
Spleen segment[a]	U	M	L	U	M	L	U	M	L	U	M	L	U	M	L	U	M	L
Dog identification:																		
320	2	2	2	1	1	1	1	1	1	0	0	1	1	1	1	0	0	0
325	1	1	1	2	2	2	0	0	0	1	1	1	2	3	2	0	0	0
332	1	2	1	3	3	3	3	1	1	0	1	1	1	2	1	1	1	1
335	3	3	2	3	3	2	1	1	1	1	2	2	1	1	1	3	3	2
343	1	1	1	2	2	2	0	0	0	2	2	2	2	2	1	0	0	0
353	1	1	1	2	2	2	1	1	1	1	1	1	1	1	1	1	1	3
Kappa coefficient:																		
U vs. M		0.71			1.00			0.71			0.48			0.40			1.00	
U vs. L		0.67			1.00			0.71			0.18			0.74			0.24	
M vs. L		0.40			0.71			1.00			0.70			0.57			0.50	

[a] Spleen segments: U = upper third, M = middle third, L = lower third.
[b] Attributed scores for the histological spleen changes: White pulp type: 1 = well organized, 2 = slightly disorganized, 3 = moderately to extensively disorganized. The size of lymphoid follicles was graded as follows: 0 = absent, 1 = small, 2 = average, 3 = large. Granuloma, plasma cell density or neutrophil density: 0 = absent, 1 = a few dispersed cells, 2 = small clusters, 3 coalescent aggregates. Perisplenitis: 0 = absent, 1 = focal, 2 = multifocal, 3. = continuous.

spleen, despite successful amplification of 18 s rDNA, confirming that the DNA samples were preserved and devoid of inhibitory PCR factors.

3.3. Histological analysis of the spleen

The major histological findings are shown in Table 2. The most common histological findings were: a) plasma cell infiltration (in 6 out of 6 dogs), b) polymorphonuclear neutrophil infiltration (in 6 out of 6), c) granulomas (in 4 out of 6), d) chronic perisplenitis (in 3 out of 6), e) disorganized white pulp (in 3 out of 6), and f) large lymphoid follicles (in 2 out of 6) (Table 2 and Fig. 3). Agreement (k) between the histological features found in two different regions of the spleen in a given animal was good, very good or perfect with respect to most of the parameters analyzed (Table 2), while a few pairs of splenic regions showed poor agreement (upper vs. lower section, plasma cell infiltration) or fair (middle vs. lower section, white pulp organization; upper vs. middle section, neutrophilic infiltration; upper vs. lower section, perisplenitis).

4. Discussion

This work represents the first attempt to systematically study the distribution of *Leishmania* load and histological alterations in the spleens of dogs with VL. *Leishmania* DNA was detected by real-time PCR in all nine samples from five out of the six animals evaluated. In the dogs in which *Leishmania* DNA was detected, variation in parasitic load was observed as evidenced by the median number (M = 978,694) of parasites per gram of tissue in the most parasitized dog being 61.7 times greater than that of the least parasitized animal (M = 15,863). This is expected, since variation in parasitic load among dogs naturally infected with *Leishmania infantum* is quite common [23,28]. In addition, variation between parasitic load in samples from a single dog was also seen, as the ratio between the largest and the smallest values ranged from 3.2 (dog # 325) to 34.7 (dog # 335). Despite the great degree of variation in parasitic load, parasitic load values among the nine samples from each dog exhibited a clustering tendency (Fig. 2) and it was possible, in some cases, to statistically distinguish animals by *Leishmania* load. For instance, dog #320 displayed a median parasitic load significantly higher than that of dogs #325, #335, and #353 (Kuskall-Wallis, followed by Dunn's test, $p < 0.05$). Therefore, as expected, the evaluation of several samples of the spleen can be used for the definition of parasitic load and, consequently, of the outcome in studies on VL in dogs, including studies on pathogenesis and development of treatment or vaccine for the disease. In addition, once there was no statistically significant differences in the parasitism in any of the regions of the spleen evaluated (Friedman, $p > 0.69$), informative parasitic load may be obtained by puncture in any region of the organ. However, as distribution of *Leishmania* in the spleen of naturally infected dogs is not homogeneous (Fig. 2), during the planning of experiments with small numbers of animals, researchers should consider the number of animals per group and/or the possibility of carrying out repeated punctures to obtain accurate information. One animal (dog # 343) displayed positive splenic aspirate culture for *Leishmania* in the initial evaluation and four months later *Leishmania* DNA was not detected in any of the 9 spleen samples evaluated, even though the 18S rDNA was amplified by real-time PCR in the same samples. One possibility is that dog #343, which showed only few signs compatible with VL (Table 1) and minor histological changes in the spleen (organized structure with only plasma cell and neutrophil infiltration, but no granulomas), has controlled the infection [16,17]. Another possibility is that the infection in dog #343 was caused by another species of *Leishmania*, such as *L. amazonensis* [29,30], which would grow in culture but be poorly detected by the PCR in real time protocol used in this study [23].

In the current study, in 5 dogs in which *Leishmania* DNA was detected by real-time PCR, 4 showed at least one histological change (white pulp disorganization, granulomas, or perisplenitis) frequently found in potentially susceptible animals [16,17] and all of them displayed plasma cell infiltration in leastwise one section of the spleen. Curiously, every single one of these 5 animals showed some degree of polymorphonuclear neutrophil infiltration. One could speculate that this finding could be related to secondary bacterial infections predisposed by VL [19,31].

The agreement of histological findings in sections of lower, middle or upper third of the spleen was moderate, good, very good or perfect most of the time (in 14 out of 18). This comparison revealed only a poor or fair concordance in the minority of times (in 4 out of 18), with poor agreement only in plasma cell infiltration between lower and upper third of the spleen. Once there is increasing pressure, due to ethical concerns, for carrying out studies with small numbers of animals, the number of sections and representation of different regions of the spleen should be taken into account when planning sampling the organ.

5. Conclusions

Parasitic load and histological changes show some degree of variability in different segments of the spleen of dogs with VL. Such variability does not impair comparisons among different groups of

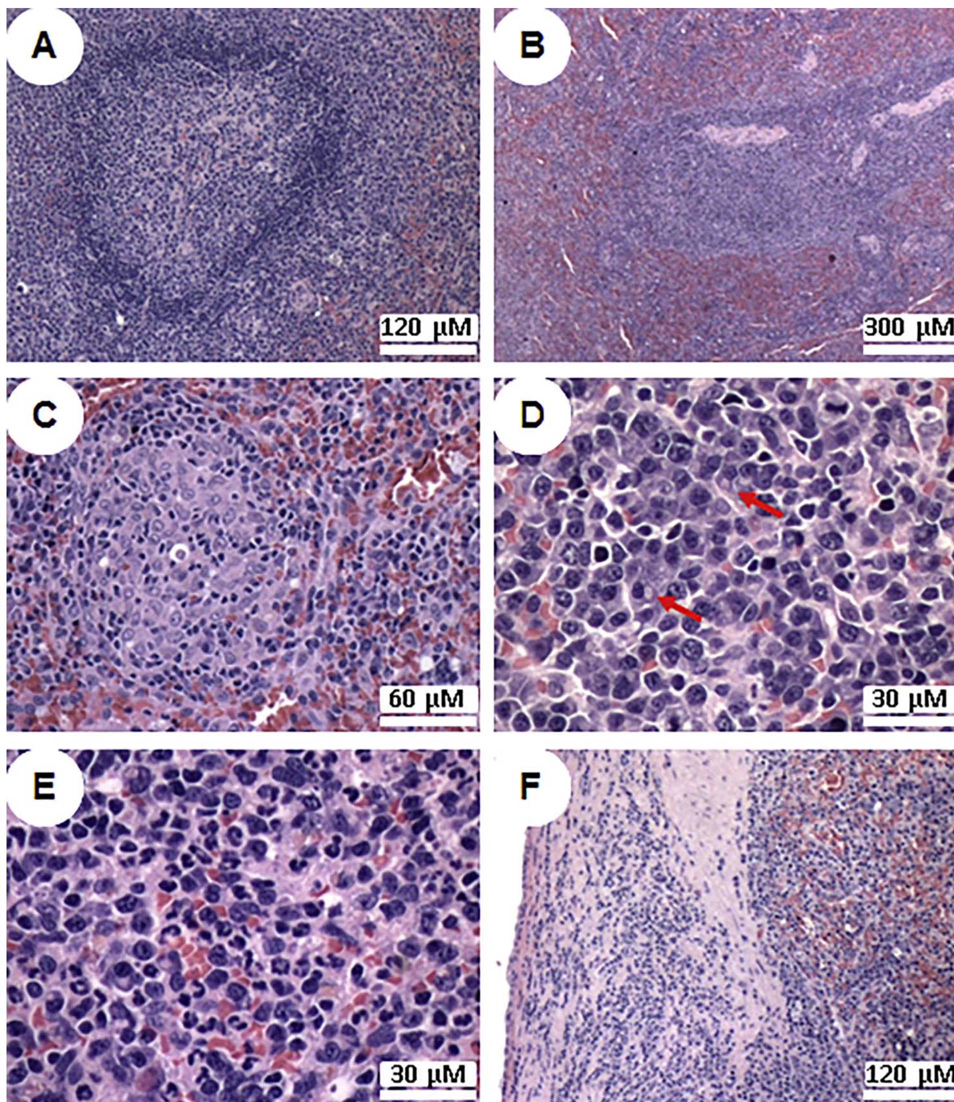

Fig. 3. Histological findings in spleens of dogs with visceral leishmaniasis.
Major histological findings: A) Organized lymphoid follicle (type 1 follicle, x40), B) Hightly disorganized lymphoid follicle (type 3 follicle, x40), C) Well organized granuloma, x200, D) Plasma cell infiltration (arrows indicate plasma cell characteristic Golgi apparatus) x200, E) Polymorphnuclear neutrophil infiltration, x400, F) Chronic perisplenitis, x200.

animals and should be considered during the planning of experiments with small number of dogs.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NCTB was involved in carrying out all of the experiments and manuscript writing, CGMP was involved in carrying out some of the experiments and manuscript writing, LAB was involved in carrying out some of the experiments, DBMF was involved with field experiments, PSTV contributed with reagents, materials and analysis tools, LCPC was involved in planning the study and manuscript writing, WLCS was involved with histological and data analysis and GGSO was involved in planning the study, histological analysis and manuscript writing.

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