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Research article

Anatomomopathological and immunohistochemical analyses of the spleen and lymph node of dogs seropositives for leishmaniasis in serological tests

Análises anatomopatológica e imuno-histoquímica do baço e linfonodo de cães soropositivos para leishmaniose em testes sorológicos

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Abstract
Canine leishmaniasis (CanL) is a zoonosis caused by the protozoan of the species *Leishmania infantum*. The spleen and lymph nodes undergo morphological changes during CanL. This research aimed to perform an anatomopathological and immunohistochemical study of these organs in dogs reactive to leishmaniasis in the Dual-path Platform chromatographic immunoassay (DPP®) and Enzyme Immunoabsorption Assay (ELISA). Twenty-seven dogs were evaluated for anatomopathological examination with 92.6% showing changes at gross evaluation, specially splenomegaly and lymphadenomegaly. All dogs showed changes in the spleen unrelated to the parasitic load, with granulomatous splenitis being the most severe change. Diffuse cortical and paracortical hyperplasia, and hyperplasia and hypertrophy of the medullary cords were observed in the lymph node. Amastigote forms of *Leishmania* spp. were found in the spleen and lymph node at histopathological and immunohistochemical evaluations, with good agreement between these evaluations (k = 0.55, p = 0.00124), but no difference was observed in the parasitic intensity of these organs at immunohistochemistry (p = 0.23). It was concluded that spleen and lymph node from dogs reactive to leishmaniasis on the DPP® and ELISA tests show histomorphological changes resulting from the disease, independent to the parasitic load, as well as these organs show similar parasitic load at immunohistochemical test.
Keywords: amastigote; histopathology; immunostaining; *Leishmania* spp.; lymphoid tissue

Resumo
A leishmaniose canina (CanL) é uma zoonose causada pelo protozoário da espécie *Leishmania infantum*. O baço e os

linfonodos sofrem alterações morfológicas durante o CanL. Esta pesquisa teve como objetivo realizar um estudo anatomopatológico e imuno-histoquímico desses órgãos em cães reativos para leishmaniose aos testes de Imunoensaio Cromatográfico "Dual Path Platform" (DPP®) e Ensaio de Imunoabsorção Enzimática (ELISA). Vinte e sete cães foram avaliados ao exame anatomopatológico, com 92,6% exibindo alterações à avaliação macroscópica, especialmente esplenomegalia e linfadenomegalia. Todos os cães apresentaram alterações no baço não relacionadas à carga parasitária, sendo a esplenite granulomatosa a alteração mais grave. Hiperplasia cortical e paracortical difusa e hiperplasia e hipertrofia dos cordões medulares foram observadas nos linfonodos. Formas amastigotas de *Leishmania* spp. foram encontradas no baço e linfonodo às avaliações histopatológica e imuno-histoquímica, com boa concordância entre os métodos (k = 0,55, p = 0,00124), mas não foi observada diferença na intensidade parasitária entre esses órgãos à imuno-histoquímica (p = 0,23). Conclui-se que baço e linfonodo de cães reativos para leishmaniose aos testes DPP® e ELISA apresentam alterações histomorfológicas decorrentes da doença, independente da carga parasitária, assim como esses órgãos apresentam carga parasitária semelhante ao método imuno-histoquímico.
Palavras-chave: amastigota; histopatologia; imunocoloração; *Leishmania* spp.; tecido linfoide

Introduction

Canine leishmaniasis (CanL) is a systemic and zoonotic disease[1,2]. The parasite that causes the disease is transmitted specially by the bite of infected phlebotomines, which is represented in Brazil by the species *Lutzomyia longipalpis*[3] and *Lutzomyia cruzi*[1] transmitting the protozoan of the species *Leishmania infantum*[4]. The dog (*Canis familiaris*) plays an important role in maintaining the disease, as it acts as a domestic reservoir for visceral leishmaniasis (VL) in urban areas [1,4,5]).

These protozoa are intracellular parasites that infect cells of the mononuclear phagocytic system in the vertebrate host[6], being spread mainly to lymph nodes, spleen, bone marrow, and liver[7]. The clinical signs of the disease result from the parasite interaction with the host's immune system, and dogs susceptible to infection develop clinical signs due to a marked cellular and humoral immune response against the parasite[8,9].

CanL is a progressive disease that causes a chronic inflammatory reaction consisting of macrophages, plasma cells, and lymphocytes[10,11], with tissue remodeling and re-

pair(11). Affected dogs commonly develop lymphadenomegaly[10,12], splenomegaly, hepatomegaly, anemia, and cutaneous lesions[12]. Spleen and lymph nodes are among the most affected organs in CanL, participating in specific immune responses[13,14] and where the presentation of antigens occurs[14,15].

The spleen is a lymphoid organ that directly influences the outcome of the infection[16]. The white pulp is divided into a periarteriolar lymphatic sheath (PALS), nodular lymphoid follicles (NLF), and marginal zone (MZ). T lymphocytes predominate in PALS and B lymphocytes in NLF[17]. The structure of the splenic lymphoid tissue allows the movement and differentiation of lymphocytes involved in immune responses and enables the ideal location of memory cells to respond to antigens. Follicular atrophy, reduction of lymphoid tissue boundaries[18], cellular hyperplasia and hypertrophy, granulomatous inflammatory reaction, high parasitic load, and fibrosis[14] are among the splenic changes in CanL.

Lymph nodes are also affected by CanL[14] and lymphadenomegaly is a common clinical sign[11]. Among its three portions, the cortical region has lymphoid follicles with a predominance of B cells; the paracortical region has no follicles and is composed of T lymphocytes; and the medullary region contains medullary cords and B lymphocytes. Furthermore, all regions may have reticular cells, rare plasma cells, and macrophages[19]. Lymph nodes of dogs with CanL exhibit hyperplasia and hypertrophy of the cortical and medullary regions, granulomatous inflammation[7,20]), and fibrosis[21].

Considering the participation of the spleen and lymph nodes in the immune response of CanL, this study aimed to perform a histomorphological and immunohistochemical study of the spleen and lymph node of dogs reactive for leishmaniasis to the Dual Path Platform (DPP®) and enzyme-linked immunosorbent assay (ELISA) tests.

Material and methods

This research was approved by the Animal Ethics Committee of the Federal University of Goiás (CEUA/UFG), Goiânia, GO, Brazil, under protocol number 061/19. Were used 27 leishmaniasis reagent dogs to the rapid immunochromatographic tests Dual Path Platform (DDP® – Bio-Manguinhos, Rio de Janeiro, Brazil) and enzyme-linked immunosorbent assay (ELISA), from the routine of epidemiological surveillance for CanL performed by the Directorate of Zoonosis Surveillance of Goiânia (DVZ), Goiás, Brazil. As recommended by the Ministry of Health, the dogs were euthanized after positivity to DPP® and ELISA tests and the consent of their respective tutors, being sent to the Animal Pathology Service of the School of Veterinary and Animal Science of UFG (SPA/EVZ/UFG), Goiânia, GO, Brazil, for anatomopathological examination.

The variables sex, age, and breed were considered for the epidemiological data tabulation. The animals were classified as young (up to two years old), adults (three to seven years old), or elderly (over eight years old) and divided into mongrel (MB) and purebred (PB) dogs, according to information provided by DVZ. Variables related to clinical signs of CanL were also considered at gross evaluation, including onychogryphosis, alopecia,

desquamation, oral ulcer, nasal ulcer[22,23], and body score (scale from 1 to 5 points: 1- cachectic, visible ribs, without fat cover, showing palpable bony prominences, evident abdominal indentation with loss of muscle mass; 2 – low body score, lean animals, with easily palpable ribs showing minimal fat coverage, bony prominences easily palpable, abdominal indentation easily visible in the flank region and minimal abdominal fat; 3 – medium body, ideal score, palpable ribs with small fat cover, well-proportioned abdominal recess and minimal layer of abdominal fat; 4 – overweight, ribs difficult to be palpated with moderate fat coverage, minimal or absent abdominal indentation, rounded abdomen with moderate fat coverage, 5- obesity, difficult rib palpation, marked fat deposits, distended abdomen with exaggerated fat deposits, and fat deposits in the lumbar region, on the face and limbs[24]. According to the external and internal lesions at gross evaluation, the dogs were classified as asymptomatic or symptomatic[17].

Changes related to splenomegaly and lymphadenomegaly (regional or generalized) were also evaluated on necroscopic examination. Histomorphological and immunohistochemical evaluations were performed in the spleen and axillary lymph node samples fixed in 10% neutral buffered formalin for 48 hours[22] and, sequentially, maintained in 70% alcohol until the histological processing and paraffin embedding.

Histopathological evaluation

From the paraffinized blocks containing spleen and lymph node samples were prepared 5 µm thick histological sections, which were stretched over histological slides and stained with hematoxylin and eosin (HE)[22].

The structural organization of the lymphoid tissue and granuloma formation were considered in the spleen, as adapted from Silva et al.[25]. For this, the following scores were attributed: zero (0 – organized), lymphoid follicles showing all the structural regions developed and outlined, including the distinct periarteriolar lymphatic sheath, germinal center, mantle zone, and marginal zone; one (1 – slightly disorganized), lymphoid follicles with a reduction in a defined region or discrete disorganization of any structure of the lymph node, and discrete hyperplasia or rarefaction of the white pulp; two (2 – moderately disorganized), evident white pulp but with poorly defined or indistinct regions and moderate lymphoid hyperplasia; three (3 – extensively disorganized), indistinct or poorly differentiated lymphoid tissue of the red pulp, with fibroplasia and marked follicular hyperplasia; and four (4 – well-defined granulomas), samples with lymphoid tissue markedly disorganized, with boundaries established by the proliferation of fibrosis and epithelioid cells.

Splenic samples classified with scores three and four were subjected to Masson trichrome staining to identify and delimitate fibrosis formation in the splenic parenchyma. Also, splenic capsule thickness, perisplenitis according to the type of inflammatory infiltrate, hemosiderosis, extramedullary hematopoiesis, and macrophages parasitized by amastigote forms of *Leishmania* spp. were considered as spleen variables.

The criteria adapted from Toplu and Aydogan[26] were adopted in the lymph node evaluation, considering the variables diffuse cortical and paracortical hyperplasia; follicular

hyperplasia with reactive germinal center in the cortical region; hyperplasia and hypertrophy of macrophages in subcapsular and medullary sinuses; hyperplasia and hypertrophy of medullary cords; capsular thickening; type of capsular inflammatory infiltrate; presence or absence of macrophages with amastigote forms of *Leishmania* spp.; and edema in cords and sinuses.

Immunohistochemical (IHC) evaluation

For immunohistochemical analysis, 4 µm histological sections were placed on silanized histological slides (StarFrost®) and subjected to deparaffinization in xylol and rehydration in decreasing ethanol concentrations. Antigenic recovery was performed in sodium citrate buffer (pH 6.0) at 95 °C for 30 minutes in a water bath. Endogenous peroxidase was blocked by immersing the sections in 30% hydrogen peroxide and 1:10 distilled water for 10 minutes. The sections were incubated in a background block reagent (Cell Marque, Rocklin, CA) for 12 minutes at room temperature in order to block nonspecific protein binding. Subsequently, the sections were incubated for 18 h at a temperature from 2-8 °C with polyclonal rabbit anti-*Leishmania* antibody diluted to a concentration of 1:1000 in antibody diluent (Diamond; Cell Marque, Rocklin, CA). The amplification of signals was done with the HiDef Detection HRP Polymer System kit in two stages, with sequential incubation of HiDef Detection™ Amplifier (Mouse and Rabbit), followed by HiDef Detection™ HRP Polymer Detector (HiDef Detection HRP Polymer System; Cell Marque, Rocklin, HERE). The amastigote forms of *Leishmania* spp. were visualized using DAB chromogen diluted in distilled water and urea, according to the manufacturer's recommendations. The samples were counterstained in Harris' hematoxylin, dehydrated in absolute ethanol, clarified in xylol, and covered with synthetic resin and coverslips. Two samples of canine tissue markedly parasitized with the amastigote forms of *Leishmania* spp. were used as a positive control of the reaction.

Macrophages with amastigote forms of *Leishmania* spp. were counted in five fields with a higher density of parasitized cells at 40x and using an optical grid of 1 mm2 and a manual cell counter for the evaluation of parasitic intensity. Subsequently, the average of parasitized macrophages in the five fields was obtained for each animal and the scores of parasite intensity were assigned as follows: absent (when amastigote forms were not observed), slightly to moderate (0.2 to 10 parasitized macrophages), and accentuated (more than 10 parasitized macrophages)[22].

Statistical analyses

Descriptive statistics were used for the variables sex, age, breed, clinical classification, and histomorphological changes in the spleen and lymph node. The Kappa non-parametric test at the 5% significance level was used for the analysis of agreement regarding the number of positive cases in the histopathological and immunohistochemical examinations, followed by the application of the scores very good ($0.8<k\leq1$), good ($0.6<k\leq0.8$), moderate ($0.4<k\leq 0.6$), fair ($0.2<k\leq0.4$) or poor ($k\leq0.2$) to determine the degree of agreement between examinations. The Mann-Whitney test was applied to compare the parasite intensity between the spleen and lymph node, considering the 5% significance level. For this, Excel 2016 spreadsheets and the software R were used, including the libraries "*irr*" and "*stats*".

Results

Among the 27 dogs reactive for leishmaniasis in the DPP® and ELISA tests, 51.9% (n=14) of the animals were young, 25.9% (n=7) adults, and 22.2% (n=6) elderly. Regarding sex, 66.7% (n=18) of the animals were females and 33.3% (n=9) males. Additionally, 51.9% (n=14) consisted of purebred and 48.1% (n=13) mongrel animals. At gross evaluation 77.8% (n=21) of the dogs presented skin desquamation, 55.5% (n=15) onychogryphosis, and 48.1% (n=13) alopecia. Moreover, one dog had nasal ulcer and another oral ulcer, which represents 3.7% for each of these changes. According to the variable body score, 14.8% (n=4) of the animals had a low body score, 51.8% (n=14) a mean body score, and 11.1% (n=3) a slight overweight. The body score was not established in six animals. According to the lesions at gross evaluation, 7.4% (n=2) of the dogs were asymptomatic and 92.6% (n=25) symptomatic.

At macroscopic evaluation 81.5% (n=22) of the animals had splenomegaly and 22.2% (n=6) some degree of splenic nodulation (Figure 1). Also, 63.0% (n=17) of the dogs exhibited generalized lymphadenomegaly, one dog had axillary lymphadenomegaly, and another one presented axillary and mandibular lymphadenomegaly.

Histopathological evaluation

Histomorphological evaluation of splenic samples showed 100% (n=27) of the dogs with some degree of disorganization of the spleen architecture (Table 1) and, among them, 18.5% (n=5) developed granulomas with evidence of fibrosis confirmed by Masson trichrome staining (Figure 1).

Moreover, 55.6% (n=15) of the animals showed perisplenitis with varied cellularity (Table 1), 66.6% (n=18) splenic capsule thickening (Figure 2), and 88.8% (n=24) extramedullary hematopoiesis, characterized by the evidence of megakaryocytes in the splenic parenchyma. Also, 92.6% (n=25) of the dogs had some degree of hemosiderosis, and 14.8% (n=4) splenic macrophages were parasitized by *Leishmania* spp. (Figure 2). Two out of the five samples classified as score four exhibited amastigote forms of *Leishmania* spp. in the macrophages cytoplasm.

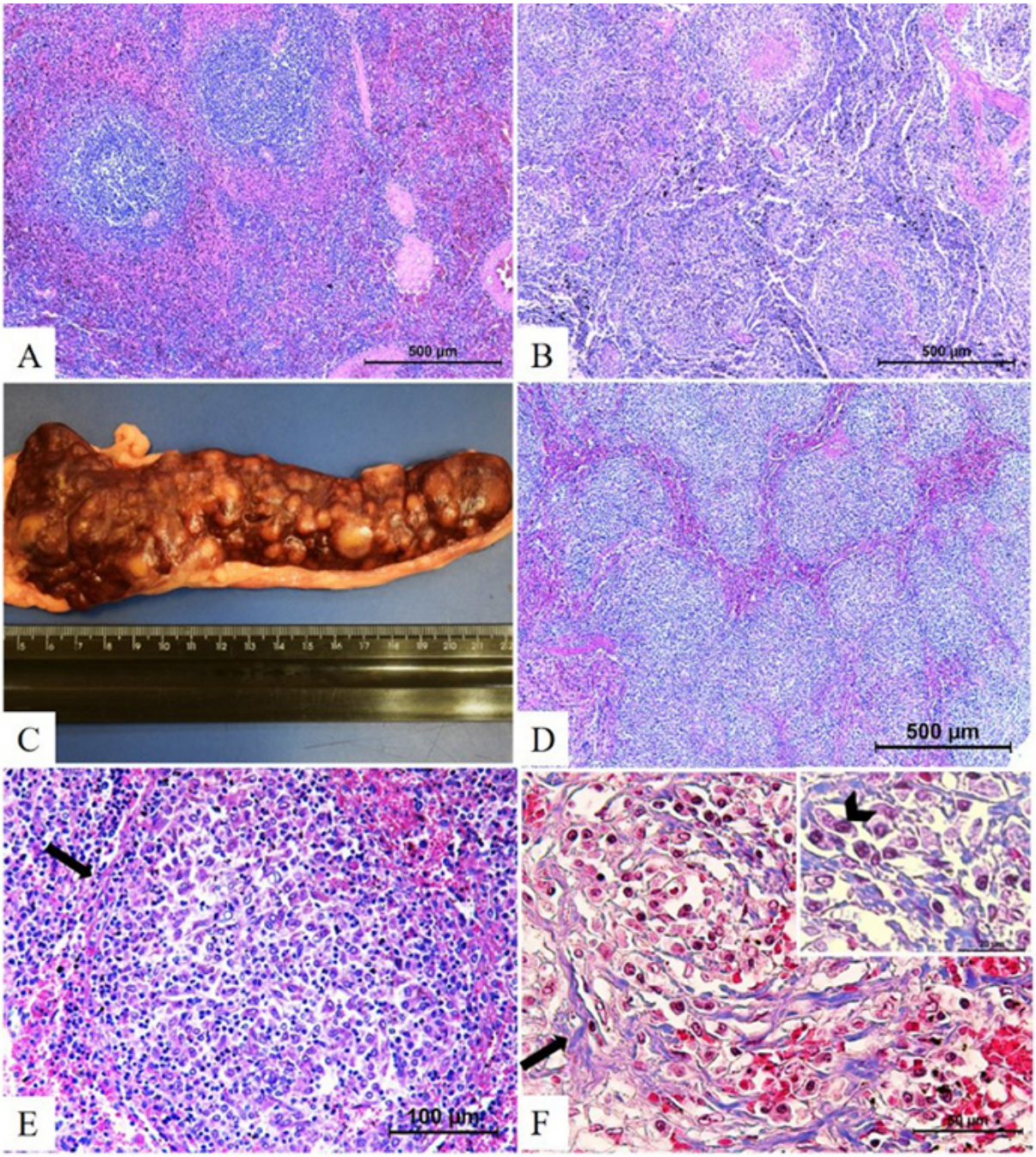

Figure 1. Spleen of dogs reactive for CanL to DPP® and ELISA tests. A) Lymphoid tissue with a score 2 of disorganization. Slightly disorganized follicular regions with little evidence and delimitation of lymphoid regions. HE. B) Lymphoid tissue with a score 3 of disorganization. Splenic microarchitecture diffusely disorganized, with indistinct lymphoid follicle structures from the red pulp. HE. C) Macroscopic aspect of a spleen showing splenomegaly and a diffuse nodular surface. D) Lymphoid tissue with a score 4 of disorganization. Splenic parenchyma exhibits well-defined granulomas. HE. E) Higher magnification of splenic granuloma, showing an infiltration of lymphocytes, plasma cells, and epithelioid cells delimited by fibrosis (arrow). HE. F) Splenic granuloma with evidence of epithelioid cells (arrowhead) and collagen fibers (arrow). Masson trichrome.

Table 1 - Scores of disorganization of splenic lymphoid tissue and perisplenitis in the spleen of dogs reactive for leishmaniasis to DPP® and ELISA tests

Parameters	Frequency	%
Scores of disorganization of splenic lymphoid tissue	27	100.0
Scores 0	0/27	0.0
Scores 1	fev.-27	7.4
Scores 2	14/27	51.9
Scores 3	jun.-27	22.2
Scores 4	mai.-27	18.5
Perisplenitis	15/27	55.6
Lymphocytes, plasm cells and macrophages	dez.-27	44.4
Lymphocytes, plasma cells and macrophages with amastigotes	fev.-27	7.4
Lymphocytes and plasma cells	jan.-27	3.7

Histomorphological changes in the lymph node included diffuse cortical and paracortical hyperplasia (96.3%, n=26), follicular hyperplasia with a reactive germinal center in the cortical region (55.6%, n=15), hyperplasia and hypertrophy of macrophages in the subcapsular sinus (70.3%, n=19), hyperplasia and hypertrophy of spinal cord macrophages (88.9%, n=24), and hyperplasia and hypertrophy of medullar cord (100%, n=27). Also, 22.2% (n=6) of the lymph nodes presented capsular thickening due to fibrosis, and 85.2% (n=23) thickening due to fibrosis and capsular inflammatory infiltrate. Among those with capsular infiltrate, 59.3% (n=16) consisted of the lymphoplasmocytic type and 25.9% (n=7) of the lymphoplasmocytic and macrophage type. Moreover, 18.5% (n=5) of the lymph nodes presented macrophages with amastigote forms of *Leishmania* spp. and 40.7% (n=11) had edema of cords and sinuses. Figure 3 illustrates the main histomorphological changes in the lymph node of dogs reactive for leishmaniasis to DPP® and ELISA tests.

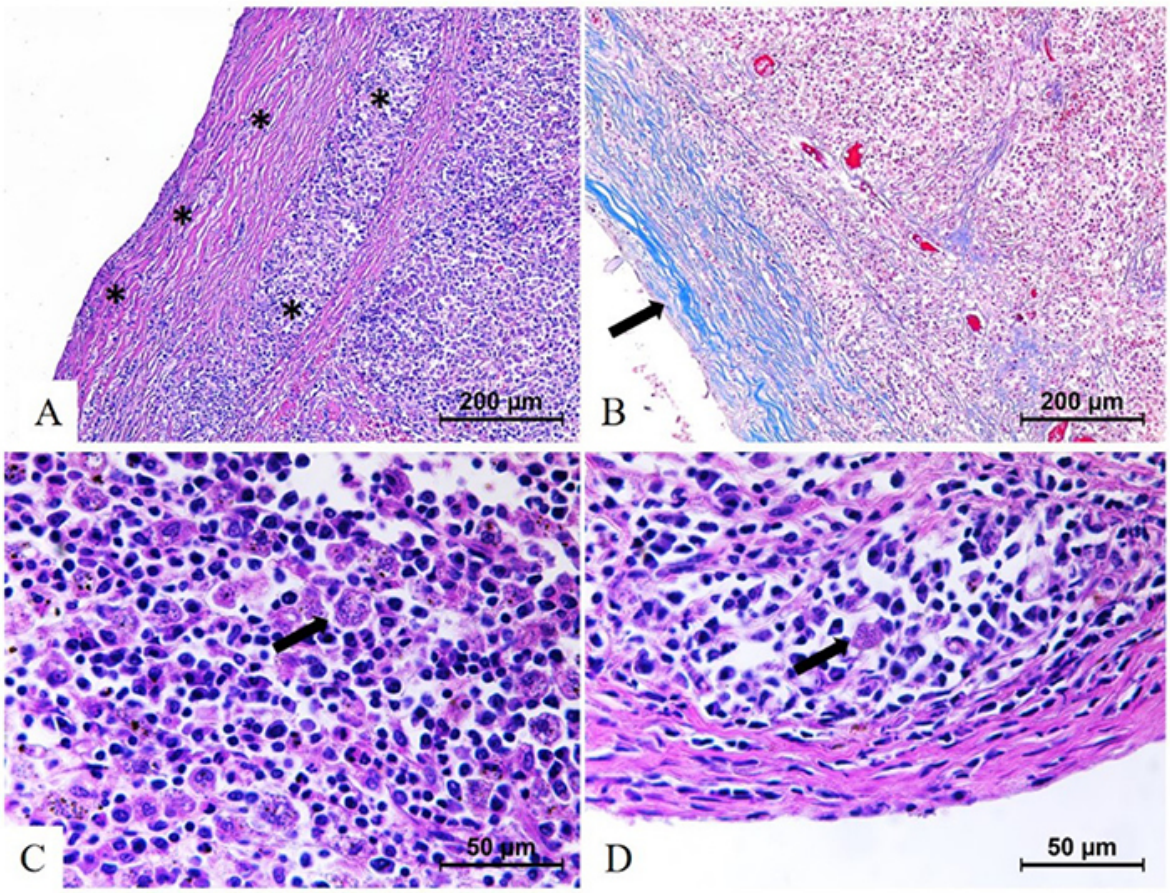

Figure 2. Photomicrographs of the spleen of dogs reactive for leishmaniasis to DPP® and ELISA tests. A) Perisplenitis. Splenic capsule thickened and interspersed with lymphoplasmocytic and macrophagic inflammatory infiltrate (asterisks). HE. B) Marked collagen deposition (fibrosis) in the splenic capsule (arrow). Masson trichrome. C) Amastigote forms of Leishmania spp. in the cytoplasm of macrophages of the splenic parenchyma (arrow). HE. D) Macrophages next to the capsule, showing amastigote forms of Leishmania spp. HE.

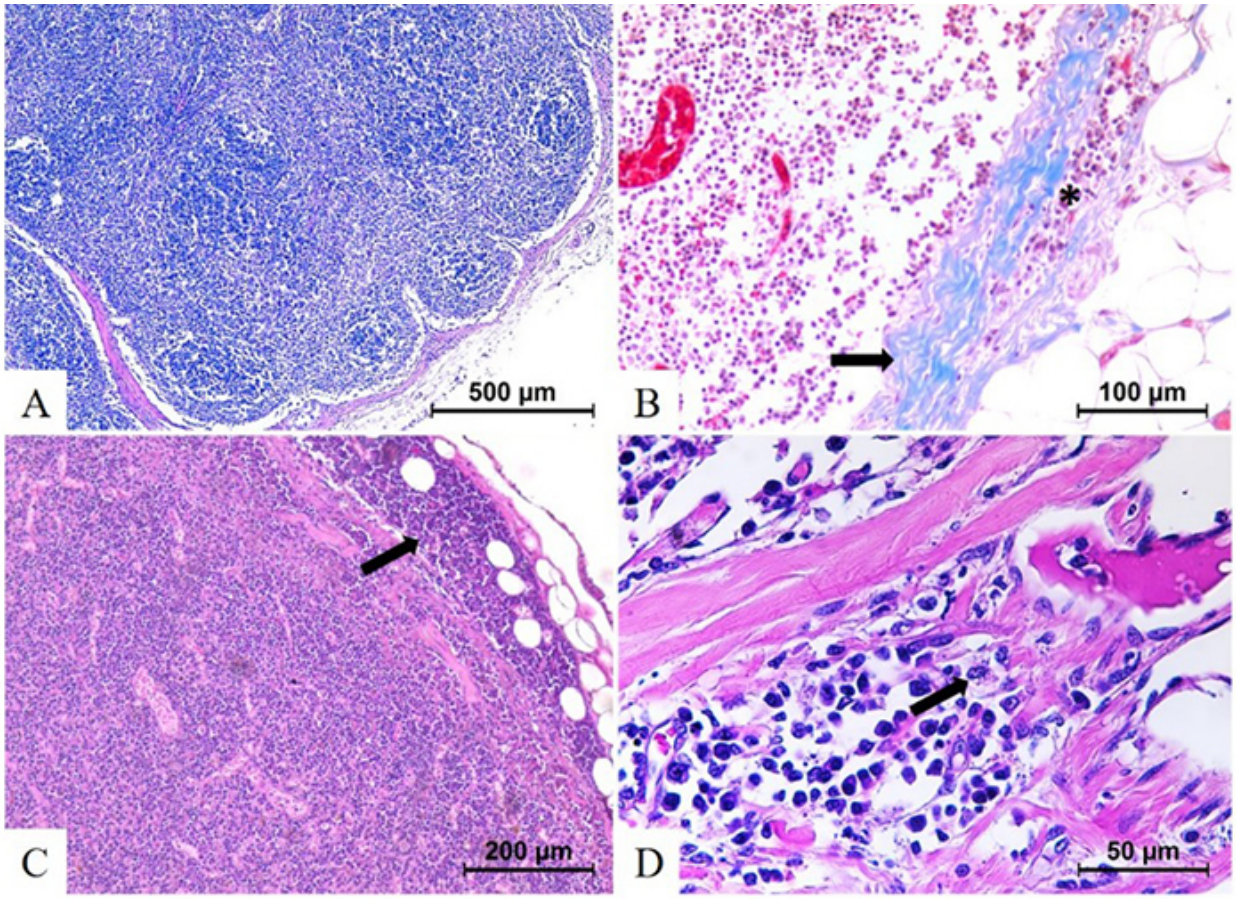

Figure 3. Photomicrograph of the lymph node of dogs reactive for CanL to DPP® and ELISA tests. A) Diffuse cortical and paracortical hyperplasia. HE. B) Capsular thickening due to fibrosis (arrow) and inflammatory infiltrate (asterisk). Masson trichrome. C) Capsular inflammatory infiltrate (arrow). HE. D) Amastigote forms of Leishmania spp. in the cytoplasm of macrophages (arrow) in the cortical region of interfolicular septum. HE.

Immunohistochemical evaluation

The IHC technique detected amastigotes of *Leishmania* spp. in the spleen and lymph node samples from dogs reactive for leishmaniasis to DPP® and ELISA tests (Figure 4), with 22.2% (n=6) of splenic immunostaining and 37.0% (n= 10) in the lymph node.

The analysis of agreement of the results regarding the number of positive cases on histopathological (n=5, higher number of positive samples between the spleen and lymph node) and immunohistochemical examinations (n=10, higher number of samples between the spleen and lymph node) showed a good agreement (k=0.55; p=0.00124). The evaluation of parasite intensity in the spleen and lymph node using the IHC technique showed animals with absent, slightly to moderate, and accentuated scores. Also, the comparison of the parasite intensity had no difference between the spleen and lymph node to the immunohistochemical evaluation (p= 0.23) (Table 2).

Data regarding the classification of lymphoid tissue disorganization scores and parasitic intensity in the immunohistochemistry technique are described in Table 3.

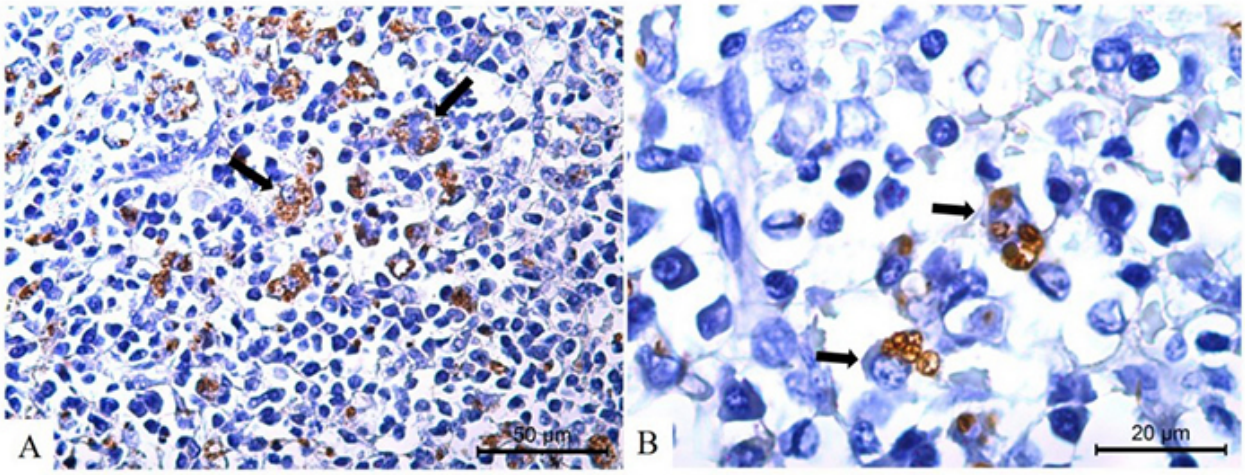

Figure 4. Photomicrographs of the spleen and lymph node of dogs reactive for leishmaniasis to DPP® and ELISA tests. Immunostaining of amastigote forms of Leishmania spp. in the cytoplasm of macrophages of the spleen (arrows) (A) and lymph node (arrows) (B). IHC, anti-Leishmania.

Table 2 - Distribution of scores and comparison of means of the parasite intensity in the spleen and lymph node using the IHC technique

Organ	Parasite intensity	n	%	Average (p=0,23)
Spleen	Absent	21/27	77.8	1.44[a]
	Discreet to moderate	abr.-27	14.8	
	Accentuated	fev.-27	7.4	
Lymph node	Absent	17/27	63.0	3.55[a]
	Discreet to moderate	ago.-27	29.6	
	Accentuated	fev.-27	7.4	

n – number of samples; % - percentage; Average - of parasite intensity; The averages followed by the same letter do not differ statistically from each other

Table 3 - Disorganization scores of lymphoid tissue and frequency of parasitic intensity

Score	Frequency of positive cases (%)	Discreet to moderate (%)	Sharp (%)
0	0/27 (0)	0 (0)	0 (0)
1	0/27 (0)	0 (0)	0 (0)
2	3/27 (11.1)	2 (7.4)	1 (3.7)
3	1/27 (3.7)	1 (3.7)	0 (0)
4	2/27 (7.4)	1 (3.7)	1 (3.7)

Discussion

Twenty-four out of 27 dogs reactive for CanL to DDP® and ELISA tests were symptomatic, which showed mainly exfoliative desquamation, onychogryphosis, and alopecia. Studies have also shown that the common findings in CanL[22,23] include onychogryphosis[12] and skin lesions such as exfoliative desquamation, ulcers, and alopecia[27], besides a low body score[7,10], the latter not observed in most animals in this study. Thus, factors such as individual response to the disease and the degree of development of systemic lesions may directly mediate the progression of CanL and reflect the weight loss of affected animals. Splenomegaly[12] and lymphadenomegaly[7,10] are also described in most dogs of the present research.

Changes in lymphoid tissues are frequent in infections due to *Leishmania*, such as the spleen, which plays important role in CanL, as it has components of the immune response and acts in the interaction with the parasite, which reflects morphological changes[16], as observed in the spleen of animals in this research. These changes include mainly some degree of disorganization of the lymphoid tissue microstructure due to immunological reactions against the parasite[25].

All animals in this study exhibited some degree of splenic structural disorganization, showing moderate disorganization of the lymphoid tissue in most cases, with little evident follicular regions or hyperplasia of lymphoid follicles. At the same time, cases with the formation of well-defined granulomas in association or not with amastigotes of *Leishmania* spp. were observed, with no relationship between these changes and the parasite presence or intensity. In this context, Santana et al.[16] and Silva et al.[25] also described splenic changes in dogs with CanL, including atrophy or hyperplasia of the lymphoid follicle, resulting in loss of definition of follicular regions. On the other hand, some authors have described the formation of granulomas frequently associated with parasitic load[12,16].

The disorganized condition of the splenic architecture has been described in animals positive for serological tests and negative for parasitological tests for CanL, suggesting

that the cause of this finding may be related to the prolonged inflammatory response in the spleen, with a consequent reduction in the parasite load[11,28]. Furthermore, a study that evaluated rats infected with *Leishmania donovani* showed splenic changes stimulated by high levels of tumor necrosis factor (TNF), without association with the parasite load[29]. Although TNF was not evaluated in this research, this hypothesis may justify the fact that all dogs in this research showed splenic changes, regardless of the parasite presence or intensity.

Despite the disorganization of the splenic architecture is an important finding in this study, samples classified at scores three and four showed marked disorganization of the white pulp and formation of fibrosis associated or not with the parasite presence. In this sense, Santana et al.[16] described the rupture of the splenic architecture and fibrosis formation as a result of the production of a reticular web, seeking to prevent or slow the progression of the parasite to other tissues[16,30]. Moreover, many samples from this study presented perisplenitis, possibly due to the extension of the parenchymal splenic inflammatory reaction, associated or not with the parasite presence in these cases, which is also often described in cases of CanL, but normally related to the parasite load[16,31,32].

A relevant number of animals with lymphadenomegaly was observed in this study, which is a frequent change in cases of CanL[7,10]. The histopathological evaluation of the axillary lymph node showed hyperplasia and hypertrophy of the medullary and cortical regions, with no association with clinical signs, corroborating the findings of Lima et al.[10]. Moreover, hypertrophy and hyperplasia of macrophages in capsular and/or medullary sinuses are among the most frequent changes in the lymph nodes of dogs infected with *Leishmania* spp.[25], as also observed in this study. Also, Lima et al.[10] suggested that hyperplasia and hypertrophy in the cortical and medullary zones plus chronic and diffuse capsular inflammation are frequent and justify the occurrence of lymphadenomegaly in cases of CanL.

In addition, most lymph node samples from this study exhibited capsular thickening due to the lymphoplasmocytic and macrophagic inflammatory infiltrate aggregated to the proliferation of fibrosis, a frequent data in cases of CanL[10]. In this context, Giunchetti et al.[33] described that the capsular infiltrate is often composed of macrophages, including the presence of parasites, but these changes were not associated with the presence of amastigote forms of *Leishmania* spp. in this study.

Histopathological evaluation was used to observe morphological changes and identify amastigote forms of the parasite, and this identification was also carried out by IHC. The results of the identification for the two evaluations were compared and, in the qualitative analysis, the IHC of the spleen and lymph node expressed the best numerical result, but with no statistical difference. This data corroborates that described by authors who pointed out the IHC technique as a supplementary tool for CanL diagnosis, considering it has higher sensitivity and specificity(21,26,27,33). Tafuri et al.(27) also reported that IHC increases the effectiveness of diagnostic tests by 50% compared to histopathological evaluation using hematoxylin-eosin staining. On the other hand, the good agreement between the histopathological and immunohistochemical evaluations observed in this study, inferring similarity between the tests, may have occurred due to the small number of samples, a fact also mentioned by Xavier et al.(34), who compared

the same diagnostic methods for evaluation of parasite intensity in dogs with CanL.

In addition, no difference was observed between the spleen and lymph node in terms of parasite intensity, indicating that both organs are useful for CanL diagnosis. However, no data were found in the literature to ratify or rectify the result relative to these organs. Nevertheless, Paparcone et al.(35) compared different bone marrow collection sites for CanL diagnosis and also observed no difference regarding parasite intensity. Despite this, the authors mentioned that although the lymph node is equally rich in parasites, especially in symptomatic dogs, the access to the lymphoid tissue in this organ can be limited, particularly when there is no increase in volume, which commonly occurs in animals at early stage of the disease or in chronic asymptomatic animals.

Conclusions

Spleen and lymph node of dogs reactive for leishmaniasis to DPP® and ELISA tests develop histomorphological changes resulting from the immunological reactions caused by the disease, regardless of the presence and intensity of the parasite. Disorganization of lymphoid tissue in different degrees in the spleen comprises the main change, with granulomatous splenitis being the most serious lesion, as well as hyperplasia and hypertrophy of the medullary cords and hyperplasia of the cortical and paracortical regions prevail in the lymph node. In addition, the spleen and lymph node show similar parasitic load by immunohistochemical test.

Conflict of interests

The authors declare no conflict of interest

Acknowledgments

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