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**Original Full Paper**

# Comparative study of parasite load in the spleen, lymph node, and skin of dogs with visceral leishmaniasis

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**Abstract**

Canine visceral leishmaniasis (VL) is a zoonosis caused by the protozoan *Leishmania infantum*. The lymph nodes, spleen, and skin are essential organs in the immunopathogenesis of the disease. This study aimed to investigate the histomorphological alterations and parasite load in the popliteal lymph node, spleen, and skin of eleven VL-positive dogs in the fine needle aspiration (FNA), Dual-path Platform chromatographic immunoassay (DPP® CVL rapid test) and Enzyme-linked immunosorbent assay (ELISA). Histopathological and immunohistochemical methods were used to evaluate the samples, and the results showed variable histopathological changes and parasite load. The popliteal lymph nodes and spleen exhibited granulomatous reaction, lymphoid atrophy, presence of plasma cells, and disorganization of the architecture was marked. The skin showed multifocal to diffuse inflammation in the superficial dermis, composed of lymphoplasmacytic infiltrate and granulomatous reaction. Immunodetection of the parasite *Leishmania* sp. was observed in all organs. The intensity of histological changes was not associated with the higher number of parasitized macrophages. The popliteal lymph node had the highest median parasite load (11.2) compared to the skin and spleen. Statistically, the Pearson correlation test revealed a highly significant correlation in the parasite load between the popliteal lymph node and spleen (r=0.89081, p=0.0002) and between the popliteal lymph node and skin (r=0.71185, p=0.0140). The study concludes that VL-positive dogs' lymph nodes, spleen, and skin suffer histomorphological alterations that could be one of the aspects that favor the maintenance of the infection.

**Keywords:** zoonosis, immunohistochemistry, histopathology, *Leishmania* sp., immune response, correlation, dog.

## Introduction

Visceral leishmaniasis (VL) is a vector-borne zoonosis caused by the protozoan *Leishmania infantum* (5,4,23). This parasite is intracellular and infects cells of the mononuclear phagocytic system, such as macrophages (21). Dogs are the primary source of infection in urban environments and play a crucial role in transmitting and maintaining the disease (29).

The clinical progression of canine VL depends on the host's immune response against the parasite (17). Symptomatic animals exhibit a more significant proportion of Th2 cells, resulting in a humoral immune response with immunosuppression of the cellular response (8,28). In contrast, asymptomatic animals exhibit a specific cellular response and weak humoral response (8,2,16).

The lymph nodes, spleen, and skin are essential organs in the immunopathogenesis of the disease. Following the

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inoculation by the blood-feeding vector, the female *Lutzomyia longipalpis* (24), the protozoan initially multiplies in the skin (4) and subsequently affects the lymph nodes and spleen due to their abundance of mononuclear phagocytic system cells (1,26). Researchers have observed that symptomatic dogs with canine VL exhibit high parasite loads, specifically in the spleen, skin, and bone marrow, indicating a compromised immune response. Parasite load in the spleen and bone marrow is a reliable parasitological marker for assessing the clinical progression of the disease in dogs (15).

Therefore, assessing the parasite load of VL-positive dogs' lymph nodes, spleen, and skin and relating it to histopathological findings is crucial to understanding the disease's pathogenesis, progression, and impact on the immune response. This study explores the potential relationship between the heightened occurrence of parasitized macrophages and the severity of lesions within these organs of VL-positive dogs.

## Material and Methods

### *Samples*

In the study, we used paraffin-embedded (FFPE) blocks of tissue from the archives of the Department of Pathology, Theriogenology and One Health, School of Agricultural and Veterinarian Studies (FCAV), São Paulo State University (Unesp), Jaboticabal, SP, Brazil, prepared from samples of 11 VL-positive dogs.

The animals included in this study had no prior clinical history and were sourced from the Zoonosis Control Center of the municipal district of Araçatuba (21º 12'32" south and 50º 25'58" west), SP, Brazil, located in an endemic area for VL (12). The available information indicated that all animals were adults, with no definition of breed or sex. It exhibited clinical symptoms, including onychogryphosis, lymphadenomegaly, splenomegaly, and skin lesions such as alopecia, crusting, and scaling. They had been previously diagnosed as VL-positive through Dual-path Platform chromatographic immunoassay (DPP® CVL rapid test, Bio-Manguinhos/Fiocruz, Rio de Janeiro, Brazil), fine-needle aspiration biopsy (FNAB) of the popliteal lymph node, and enzyme-linked immunosorbent assay (ELISA). The original project that used these samples was approved by the Ethics Committee on Animal Use (CEUA, FCAV - UNESP, Jaboticabal, SP, Brazil, protocol No. 024686/10).

### *Histopathological evaluation*

Paraffin-embedded (FFPE) blocks of spleen, lymph node, and snout skin fixed in 10% formalin buffered with phosphates (0.15 M, pH 7.2) for 48 hours were sliced at 3 µm thickness and stained with Hematoxylin and Eosin (HE) (27). Histological sections were examined under light microscopy, and histopathological changes were graded on a scale of absent (0), mild (1), moderate (2), and marked (3).

### *Immunohistochemical evaluation*

To identify amastigotes forms of *Leishmania* sp., hyperimmune serum from VL-positive dogs was used with the immunohistochemistry technique (adapted from Tafuri et al., 2004). The immunohistochemistry protocol included dewaxing the slides in an oven at 60 °C for 60 minutes. Subsequently, the sections were immersed in xylene for 20 minutes and then hydrated in solutions of decreasing alcohol concentrations until they were washed in distilled water.

The spleen and lymph node were heated for antigen retrieval (Pascal pressure chamber, Dako, with citrate buffer pH 6.0). At the same time, skin samples were not heat recovered to minimize slide section loss. Aiming to block the endogenous peroxidase, a solution of methanol and oxygenated water (Synth; 30 volumes) at 12% was used for 30 minutes at room temperature, protected from light. Nonspecific proteins were blocked with a commercial product (Protein Block, Dako, cod. X0909, Glostrup, Denmark) for 30 minutes in a dark, humid chamber at room temperature.

The primary antibody (VL-positive dog hyperimmune serum, dilution 1:500) was incubated for two hours in a humid and dark chamber at room temperature. The secondary antibody (Envision Flex, Dako) was set for one hour under the same conditions. The chromogen used was diaminobenzidine (*3*,*3*-diaminobenzidine, Dako), for 3 minutes. Harris hematoxylin was used for counter-staining for eight minutes. Between each step of the protocol, the sections were washed with a wash buffer solution of TRIS HCl (pH 7.4) for 5 minutes.

To create our positive control, we used animal tissues where amastigotes were previously observed in macrophages. For our negative control, we replaced the primary antibody with the antibody diluent (Antibody diluent with background-reducing components, cod. S3022, Dako).

The density of immunolabeled cells for parasite load was determined by counting the cells in the spleen, lymph node, and skin in ten high magnification fields (obj. 40x, area=0.19625 µm$^2$), in a light microscope, as described by Moreira et al. (27). The obtained values from these areas were used to calculate the average number of immunolabeled cells per organ and animal. These averages were then subjected to statistical analysis to establish precise and dependable data correlations between the different organs.

### *Statistical analysis*

Our data analysis from the spleen, popliteal lymph node, and skin was conducted using GraphPad Prism software (v. 8.4.3). The software allowed the performance of several

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statistical tests, such as the non-parametric Kruskal-Wallis and Dunn's non-parametric multiple comparison tests. Moreover, we conducted a Pearson correlation analysis to determine the coefficient (r) for pairwise comparisons between the organs. A p-value below 0.05 was deemed significant, and the correlation coefficient (r) values were considered strong, with values closer to -1 or 1, suggesting a linear solid connection.

## Results

### *Histopathology*

Histopathological evaluation (Fig. 1 and Table 1) indicated that the popliteal lymph nodes exhibited marked disorganization of the architecture and absence of evidence between the cortical and medullary layers, as well as foci of mild to moderate fibrosis around the medullary vessels, except in one animal (dog five). All lymph nodes exhibited variable intensities of inflammation in the capsule and surrounding tissues (Fig. 2A), composed of macrophages, lymphocytes, plasma cells, and sometimes granulomatous reactions, often with parasitized macrophages (Fig. 2D).

Granulomatous inflammation (100%; n=11) corresponded to multifocal to diffuse epithelioid macrophage aggregates distributed in all layers of the lymphoid parenchyma. This reaction was pronounced in 81.8% (n=9) of the animals. In some cases, the macrophages even infiltrated the cortical lymphoid follicles (Fig. 2C). This resulted in a reduction in the number of lymphoid cells present in the cortical lymphoid follicles and the paracortical region (atrophy, Fig. 2B), ranging from moderate (9.1%; n=1) to marked (90.9%; n=10). The granulomatous reaction was also associated with plasma cell infiltrate in 100% (n=11) of the samples, and Mott cells were observed in the lymph nodes of three animals (dogs three, six, and nine).

Perisplenitis of varying intensity was observed in 81.8% (n=9) of the spleen samples (Fig. 3A). Inflammation of the capsule and surrounding tissue was present in the form of epithelioid macrophages (63.6%; n=7), lymphocytes, and many plasma cells (Fig. 3C). Lymphoid reactivity, architecture distortion with apoptosis and mitosis in the white pulp, and poor definition between the marginal and mantle zones were seen in dogs one, two, and three (Fig. 3B). On the other hand, in the remaining animals (72.7%; n=8), different intensities of lymphoid atrophy were observed in the periarteriolar sheath, mantle zone, and marginal zone in the germinal center. The granulomatous reaction (81.8%; n=9) was characterized by multifocal to diffuse aggregates of epithelioid macrophages with ill-defined borders distributed in the red pulp, sometimes invading the white pulp, many of which had intracytoplasmic parasites (Fig. 3D). Myeloid metaplasia with varying intensities, plasma cells, and Mott cells were present in 100% of the samples in the red pulp (Table 2 and Fig. 1).

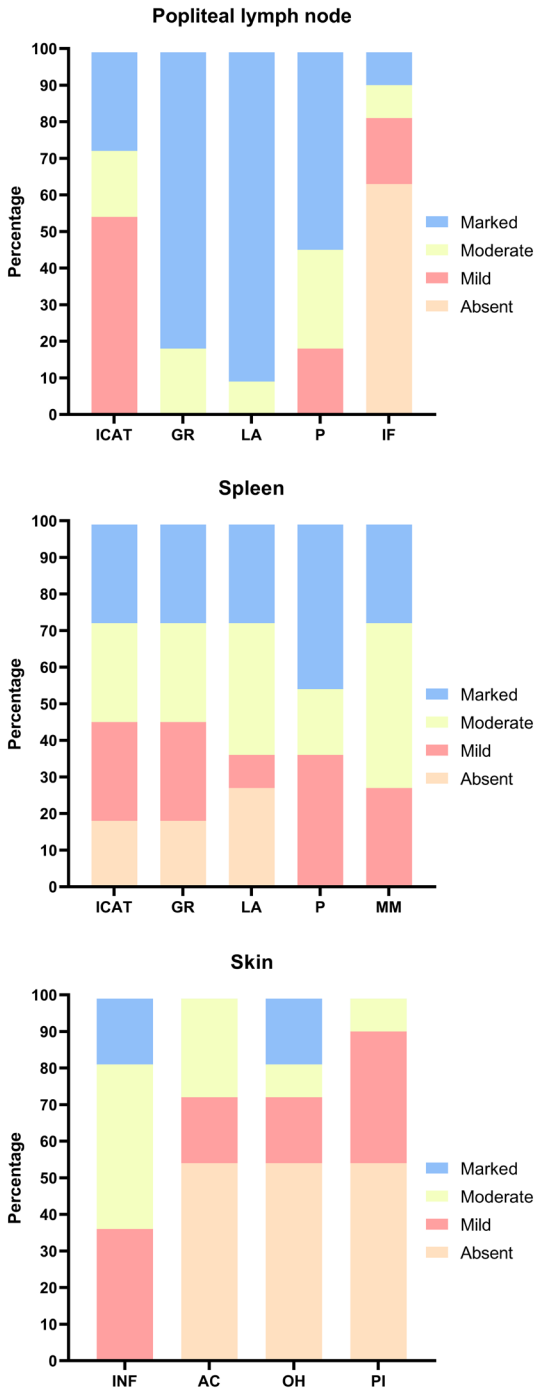


**Figure 1**. Proportion of cell score in the popliteal lymph node, spleen, and skin of dogs with Visceral Leishmaniasis. Lymph node (ICAT = inflammation in capsule and adjacent tissue, GR = granulomatous reaction, LA = lymphoid atrophy, P = plasma cells, IF = interstitial fibrosis); Spleen (ICAT = inflammation in capsule and adjacent tissue, GR = granulomatous reaction, LA = lymphoid atrophy, P = plasma cells, MM = myeloid metaplasia); Skin (INF = inflammation, AC = acanthosis, OH = orthokeratotic hyperkeratosis, PI = pigmentary incontinence). Lesion intensity scores (0) Absent; (1) Mild; (2) Moderate; (3) Marked.

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**Table 1**. Lesion scores in the popliteal lymph node of dogs with Visceral Leishmaniasis.

| Dog | ICAT | GR | LA | P | FI |
|---|---|---|---|---|---|
| D1 | 1 | 2 | 2 | 1 | 0 |
| D2 | 3 | 3 | 3 | 2 | 2 |
| D3 | 2 | 3 | 3 | 3 | 3 |
| D4 | 3 | 3 | 3 | 1 | 1 |
| D5 | 1 | 3 | 3 | 3 | 0 |
| D6 | 2 | 3 | 3 | 3 | 1 |
| D7 | 1 | 2 | 3 | 3 | 0 |
| D8 | 1 | 3 | 3 | 3 | 0 |
| D9 | 3 | 3 | 3 | 3 | 0 |
| D10 | 1 | 3 | 3 | 2 | 0 |
| D11 | 1 | 3 | 3 | 2 | 0 |

*ICAT = inflammation in capsule and adjacent tissue, GR = granulomatous reaction, LA = lymphoid atrophy, P = plasma cells, IF = interstitial fibrosis); (0) Absent; (1) Mild; (2) Moderate; (3) Marked.

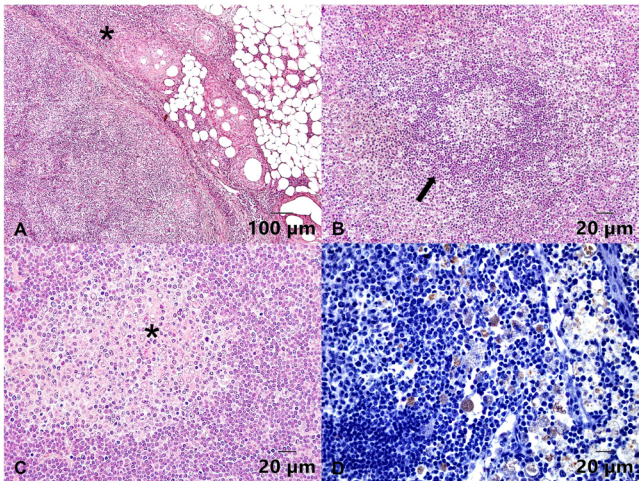


**Figure 2**. Photomicrographs of the popliteal lymph node of a dog with VL. A- Note the capsule and adjacent adipose tissue with marked thickening (*) due to inflammation (HE, bar = 100 µm). B- In the cortical layer, note marked lymphoid atrophy (arrow, HE, bar = 20 µm). C- Detail of the lymphoid node with epithelioid macrophages (*) forming a granulomatous reaction (HE, bar = 20 µm). D- Positive immunolabeling (brown) for amastigote forms of *Leishmania* sp. in the cytoplasm of macrophages (Peroxidase-linked Polymer Complex, Bar = 20 µm).

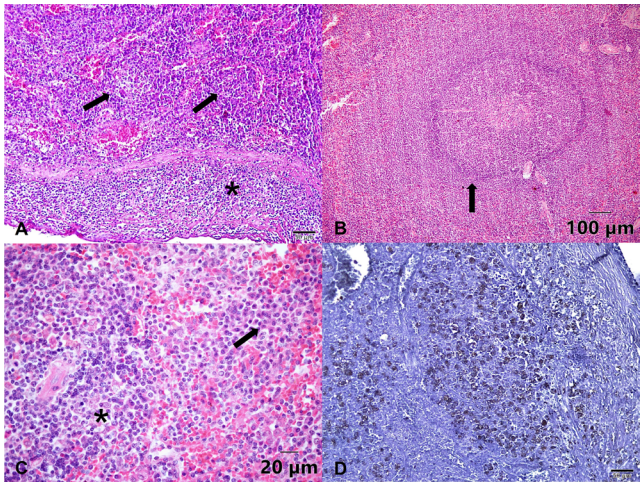


**Figure 3**. Photomicrographs of the spleen of a dog with VL. A- Note the markedly thickened capsule (*) due to inflammation. Also note subcapsular granulomatous formations (arrows, HE, bar = 50 µm). B- The white pulp shows marked lymphoid atrophy and poor definition between the central portion and the mantle and marginal zones of the germinal center (arrow, HE, bar = 100 µm). C- Note epithelioid macrophages (*) forming granulomatous reaction in the mantle zone and plasma cell infiltrate in the red pulp (arrow, HE, bar = 20 µm). D- Positive immunolabeling (brown) for amastigote forms of *Leishmania* sp. in the cytoplasm of macrophages (Peroxidase-linked Polymer Complex, Bar = 50 µm).

**Table 2**. Lesion scores in the spleen of dogs with Visceral Leishmaniasis.

| Dog | ICAT | GR | LA | P | MM |
|---|---|---|---|---|---|
| D1 | 1 | 3 | 0 | 1 | 3 |
| D2 | 1 | 0 | 0 | 3 | 2 |
| D3 | 2 | 2 | 0 | 2 | 2 |
| D4 | 3 | 1 | 1 | 3 | 3 |
| D5 | 2 | 1 | 2 | 3 | 1 |
| D6 | 1 | 1 | 2 | 3 | 1 |
| D7 | 3 | 3 | 2 | 3 | 1 |
| D8 | 0 | 3 | 3 | 1 | 2 |
| D9 | 2 | 2 | 2 | 1 | 3 |
| D10 | 3 | 2 | 3 | 2 | 2 |
| D11 | 0 | 0 | 3 | 1 | 2 |

*ICAT = inflammation in capsule and adjacent tissue, GR = granulomatous reaction, LA = lymphoid atrophy, P = plasma cells, MM = (myeloid metaplasia); (0) Absent; (1) Mild; (2) Moderate; (3) Marked.

All skin samples taken from the snout region (Table 3 and Fig. 1) showed different levels of inflammation. In 81.8% of cases (n=9), the intensity of inflammation was mild to moderate. The inflammation pattern was multifocal in the superficial dermis and peri adnexal region (Figs. 4A and 4B). It was composed of lymphocytes and plasma cells that were associated with epithelioid macrophages (granulomatous reaction). Parasitized macrophages were abundant in the affected area (Figs. 4C and 4D).

This inflammation was accompanied by thickening of the skin (acanthosis; 45.5%; n=5) and overgrowth of keratin cells (orthokeratotic hyperkeratosis; 45.5%; n=5).

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**Table 3**. Lesion scores in the skin of dogs with Visceral Leishmaniasis.

| Dog | INF | AC | OH | PI |
|---|---|---|---|---|
| D1 | 1 | 0 | 0 | 0 |
| D2 | 2 | 1 | 0 | 1 |
| D3 | 2 | 0 | 0 | 1 |
| D4 | 1 | 0 | 0 | 1 |
| D5 | 1 | 2 | 2 | 2 |
| D6 | 3 | 1 | 1 | 0 |
| D7 | 2 | 2 | 3 | 1 |
| D8 | 2 | 0 | 1 | 0 |
| D9 | 1 | 2 | 3 | 0 |
| D10 | 2 | 0 | 0 | 0 |
| D11 | 3 | 0 | 0 | 0 |

*INF = inflammation, AC = acanthosis, OH = orthokeratotic hyperkeratosis, PI = pigmentary incontinence); (0) Absent; (1) Mild; (2) Moderate; (3) Marked.

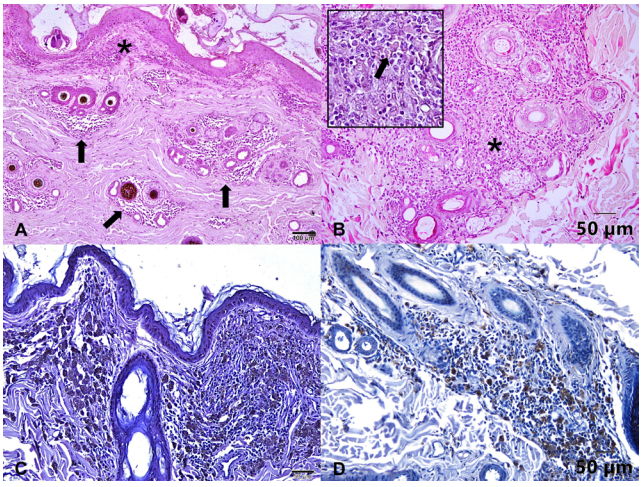


**Figure 4**. Photomicrographs of the skin of a dog with VL. A- Note multifocal areas of inflammation in the superficial dermis (*) and around appendages (arrows, HE, bar = 100 µm). B- Periadnexal inflammation (*) with detail of epithelioid macrophages (arrow, HE, bar = 50 µm). C- Note macrophages immunolabeled for the parasite in the superficial dermis (Peroxidase Bound Polymer Complex, bar = 50 µm). D- Detail of positive immunolabeling of amastigote forms of *Leishmania* sp. (Peroxidase-linked Polymer Complex, bar = 50 µm).

Pigment incontinence was also observed in the superficial dermis (45.5%; n=5). The inflammation also caused significant damage to the collagen in the skin. In dogs two and five, a crusted ulceration on the epidermis was associated with a neutrophilic infiltrate in the dermis and ballooning degeneration of the surrounding keratinocytes. Dog seven presented coinfection by the *Sarcoptes scabiei* mite, which caused the formation of galleries in the epidermis (horny layer), accompanied by a discrete eosinophilic inflammatory infiltrate.

*Immunohistochemistry*

Immunohistochemistry revealed the presence of *Leishmania* sp. in all the popliteal lymph nodes and spleens (n=11), while in skin samples, the parasite was detected in only 72.7% (n=8) of cases. The parasite was observed in the cytoplasm of macrophages from granulomatous inflammation (Figs. 2D, 3D, 4C, and 4D).

The immunolabeling results for parasite load showed variations, with dogs nine and eleven exhibiting notably high levels (Table 4). Intriguingly, even in animals with low parasite load, the lymph nodes presented lesions consistent with granulomatous lymphadenitis, while the spleens displayed perisplenitis and splenitis. Both conditions were linked to organ architecture distortion caused by a chronic inflammatory response to the parasite (Tables 1 and 2).

*Statistical Analysis*

Median values of parasite load immunolabeling in the popliteal lymph node, spleen, and skin were 11.2, 3.6, and 7.2, respectively (Table 4). These findings suggest that the spleen had the least parasitized macrophages, while the popliteal lymph node had the highest number of parasitized macrophages.

The Kruskal-Wallis test results indicated no significant difference (p=0.4273) in the parasite load counts between the organs (Fig. 5). Further analysis of the parasite load using the Pearson correlation test revealed a highly significant correlation between the popliteal lymph node and spleen (p=0.0002) and a significance between the popliteal lymph node and skin (p=0.0140). However, no significant correlation was found between the spleen and skin (p=0.0734). The observed correlation coefficient values in this study were as follows: popliteal lymph node and spleen (r=0.89081), popliteal lymph node and skin (r=0.71185), and spleen and skin (r=0.55971) (Fig. 6). These results suggest a robust linear correlation between the lymph node and spleen and between the lymph node and skin. These findings indicate that the parasite load in these organs is directly proportional, making them dependent variables.

## Discussion

Our study has shown that even with a low parasite load, animals with VL have significant changes in the morphology of their lymphoid organs. These changes indicate that disorganizing the lymphoid organ's architecture can negatively impact the host's ability to mount an effective immune response against the infection (6).

A previous study revealed that asymptomatic dogs have hypertrophy or hyperplasia of the cortical zone in their lymph nodes, while symptomatic dogs display atrophy of

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**Table 4**. Average parasite load immunolabeled cells in dogs' popliteal lymph node, spleen, and skin with Visceral Leishmaniasis.

| Dog | Popliteal lymph node | Spleen | Skin |
|---|---|---|---|
| D1 | 12,8 | 3,1 | 0 |
| D2 | 9,7 | 0,8 | 30,3 |
| D3 | 4,2 | 3,6 | 42,1 |
| D4 | 4,9 | 0,8 | 0 |
| D5 | 5,7 | 34,4 | 6,6 |
| D6 | 11,2 | 5,7 | 26,9 |
| D7 | 11,9 | 4,3 | 50,6 |
| D8 | 0,8 | 3,2 | 0 |
| D9 | 123,3 | 50,2 | 7,2 |
| D10 | 16,9 | 2,6 | 3,7 |
| D11 | 183,4 | 61,2 | 154,1 |

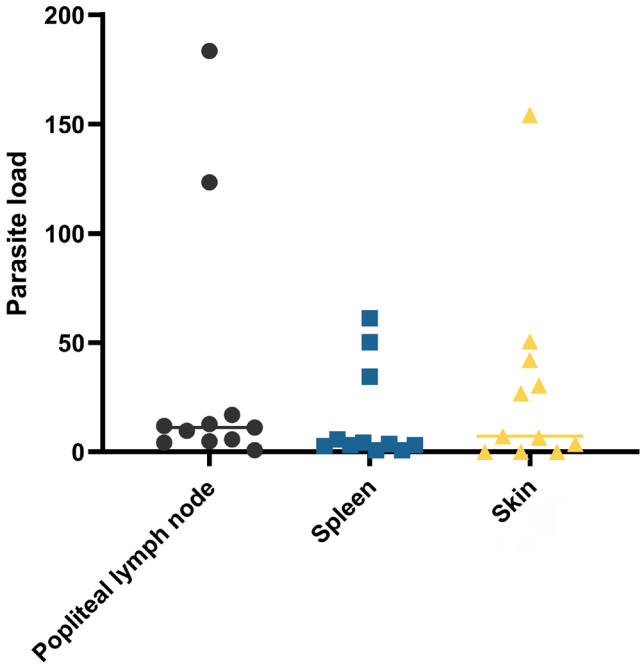


**Figure 5**. Medians of cells immunolabeled for the parasite in the popliteal lymph node (red), spleen (blue), and skin (green) of VL-positive dogs. The Kruskal-Wallis non-parametric test observed no organ differences (p>0.05).

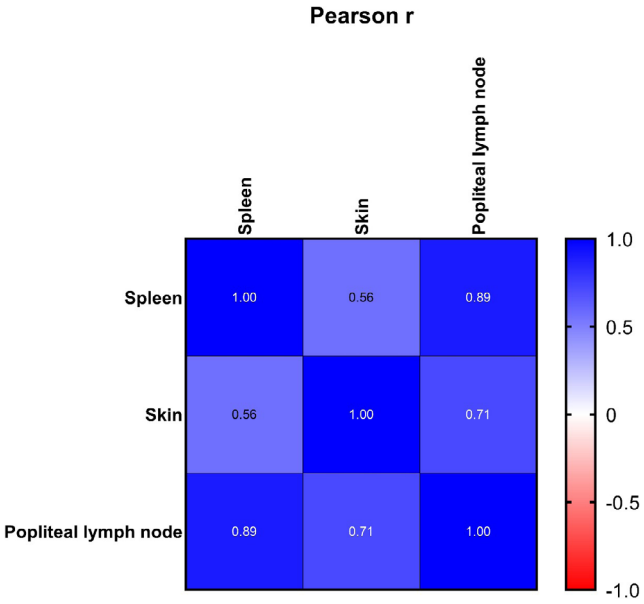


**Figure 6**. Correlation matrix with correlation coefficients corresponding to popliteal lymph node, spleen, and skin of dogs with Visceral Leishmaniasis.

the cortical zone (7). In the present study, it was observed that all dogs had varying degrees of lymphoid atrophy in the popliteal lymph node, and the disorganization of the lymph node architecture was caused by granulomatous inflammation that affected all layers of the lymph node. Lima et al. (9) found in a related study that the increased number and size of lymphoid follicles in the lymph nodes of VL-positive dogs was not associated with an increase in lymphocytes but with their replacement by the significant presence of macrophages, inflammation, and fibrosis of the capsule. Moreira et al. (12) also noted lymphoid atrophy and more parasites in symptomatic dogs' lymph nodes compared to asymptomatic dogs. They also found that the atrophy was linked to severe apoptosis in T lymphocytes (11).

VL significantly impacts the spleen's structure and function, which plays a critical role in the immunopathogenesis of the disease. Disorganization and alteration of the white pulp microenvironment in the spleen are linked to an elevated risk of coinfections and patient mortality (6). Dogs with white pulp disorganization exhibit more severe clinical symptoms (22).

This study found that a reduction in the lymphoid population within the white pulp, particularly in the mantle zone, and the presence of macrophages and plasma cells in the marginal zone were clear indicators of splenic lymphoid atrophy. Research has shown that dogs with disorganized white pulp have lower CD4+ T lymphocyte density, even with low parasite load (22,10). In addition, perisplenitis, which is associated with a granulomatous reaction and parasitized macrophages, is common in VL-positive dogs (6).

Likely, the infiltration of plasma cells in the popliteal lymph node and spleen is linked to the movement of B lymphocytes from the bloodstream, which is stimulated by the humoral response in VL (2,7). The presence of plasma cells may be related to the polyclonal activation of B cells and the production of cytokines (IFN-γ, IL10, IL6) and chemokines (CXCL12) that contribute to the differentiation and retention of plasma cells in the red pulp (19). In VL-positive dogs, most plasma cells in the spleen produce IgG (25), leading to

polyclonal hypergammaglobulinemia (16), which is unable to control parasite multiplication and ultimately results in the persistence of the disease (8,28).

*Leishmania* sp. gains entry to the body via a vector and initially contacts the skin (13). While animals may not exhibit symptoms, the skin still contains inflammation and parasites (20). In the case of VL, affected animals display a multifocal or diffuse dermatitis characterized by lymphoplasmacytic infiltrate and epithelioid macrophages (7). The inflammation primarily affects the superficial dermis, with either an interstitial and diffuse or perivascular and peri adnexal pattern (20). Additionally, some cases exhibit hyperplasia of the epidermis, orthokeratotic hyperkeratosis, pigmentary incontinence, and ulceration (13,3). The study found that all 11 dogs showed these changes.

Porcellato et al. (14) did not detect parasites in VL-positive dogs' skin samples. However, our study revealed that 72.7% of the animals had evidence of parasite presence through immunostaining. Even dogs with low parasite counts in their skin had parasitized lymphoid organs and depleted lymphocytes. Santana et al. (18) found that dogs with diffuse dermatitis, spleen inflammation, and germinal center lymphoid atrophy had an inadequate immune response to canine VL. This observation implies a relationship between these conditions and poor immune response in dogs with VL.

According to our research, one dog (D11) exhibited the most parasitized macrophages among all the dogs observed. This dog displayed a diffuse dermal inflammatory infiltrate in the skin and significant lymphoid atrophy scores in both the spleen and lymph node. These results indicate that a disruption in the lymphoid organization may result in an insufficient immune response, impair the ability to fight the parasite, and result in the maintenance and evolution of the disease (6). Additionally, previous studies have established that the severity of dermatitis is directly linked to the existence of amastigote forms of *Leishmania* sp. (20).

The animals generally exhibited chronic dermatitis, granulomatous splenitis, and lymphadenitis. Although not associated with a higher parasite load, these changes were connected to a distortion of lymphoid organ architecture and atrophy. The compromised function of these organs may result in an inadequate cellular response to antigenic presentation and T lymphocyte activation. Silva et al. (22) and Magalhães et al. (10) have reported similar changes in lymphoid organs at low parasite loads.

VL is a widespread disease in Brazil, affecting over 1,600 municipalities across 19 states with autochthonous transmission (4). The vulnerability of the host's immune system to infection may be responsible for the disease's expansion and spread.

This study reveals significant morphological alterations in crucial organs that contribute to VL immunopathogenesis and progression, such as lymph nodes, spleen, and skin. Interestingly, these changes were not linked to the parasite load. The inability of lymphoid organs to mount an effective immune response against the *Leishmania infantum* parasite probably allows the infection to persist in animals with VL.

In conclusion, this study highlights the importance of assessing parasite load and histological alterations in VL-positive dogs' organs. According to the findings of this study, a robust association exists between the parasite load values in the spleen, lymph nodes, and skin of VL-positive dogs. The parasite loads can significantly differ within infected dogs' lymph nodes, spleen, and skin, and significant histomorphological alterations compromise the immune response. Further studies are needed to investigate the correlation between parasite load, histological changes, and clinical progression of the disease.

## Conflict of Interest

The authors declare no competing interests.

## Acknowledgments

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