Ciência Animal Brasileira
Ciência Animal Brasileira
Brazilian Animal Science
e-ISSN 1809-6891
DOI: 10.1590/1809-6891v23e-73104E

Section: Veterinary medicine
Research article

Histological and immunohistochemical evaluations of the bone marrow from femur and sternal manubrium of dogs reactive for leishmaniasis by DPP® and ELISA tests

Avaliações histológica e imunoistoquímica da medula óssea do fêmur e do manúbrio esternal de cães reagentes para leishmaniose aos testes DPP® e ELISA

Aline Oliveira de Magalhães[1], Lucilandia Maria Bezerra[1], Diego Pereira Araújo[1], Bruna Siqueira Gomes de Lima[1], Leandro do Prado Assunção[1], Rodrigo Caldas Menezes[2], Veridiana Maria Brianezi Dignani de Moura[1]

[1]Universidade Federal de Goiás (UFG), Goiânia, Goiás, Brazil
[2]Fundação Oswaldo Cruz (FIOCRUZ), Rio de Janeiro, Rio de Janeiro, Brazil
*correspondent: vdmoura@ufg.br

Abstract
As the bone marrow is one of the most organs affected by canine visceral leishmaniasis (CVL), samples from this are frequently taken for parasitological tests, with occurrence of myelodysplastic changes, with consequent anemia, leukopenia, and thrombocytopenia. Thus, this study aimed to investigate the histological and immunohistochemical changes in the bone marrow of the femur and sternal manubrium of dogs reactive for leishmaniasis by DPP® and ELISA tests. For this, thirteen canines from the epidemiological routine for CVL carried out by the Directorate of Zoonosis Surveillance of Goiânia (DVZ), GO, Brazil, were subjected to anatomopathological examination. 46.2% of bone marrow samples from the femur showed a higher proportion of the red series, and 53.9% of bone marrow of the sternal manubrium evidenced a higher proportion of the red series. Also, there were varied macrophage hyperplasia, hemosiderosis, and megakaryocytic emperipolesis. Amastigote forms of *Leishmania* spp. in the bone marrow of the femur and sternal manubrium to histopathological and immunohistochemical evaluations were observed, with good agreement them, but without difference in the parasite intensity between the bone marrow of these anatomical sites. It was concluded that bone marrow of the femur and sternal manubrium of dogs reactive for leishmaniasis by DPP® and ELISA tests has histological changes resulting from the disease, regardless of the parasite presence or intensity, with macrophage hyperplasia, hemosiderosis, and emperipolesis being the main medullary changes in these animals. Also, the bone marrow of the femur and sternal manubrium are useful anatomical sites for the diagnosis of CVL by direct methods.
Keywords: amastigotes; canine visceral leishmaniasis; histopathology; immunostaining; medullary changes

Resumo
Como a medula óssea é um dos órgãos mais acometidos pela leishmaniose visceral canina (LVC), amostras desta são frequentemente colhidas para exames parasitológicos, sendo possível a ocorrência de alterações mielodisplásicas, com consequente anemia, leucopenia e trombocitopenia. Assim, este estudo teve como objetivo investigar alterações histológicas e imunoistoquímicas na medula óssea do fêmur e manúbrio esternal de cães reativos para leishmaniose aos testes DPP® e ELISA. Para isso, 13 caninos da rotina epidemiológica para LVC realizada pela Diretoria de Vigilância de Zoonoses de Goiânia (DVZ), GO, Brasil, foram submetidos ao exame anatomopatológico. 46,2% e 53,9% das amostras de medula óssea do fêmur e do manúbrio esternal apresentaram maior proporção da série vermelha, respectivamente. Além disso, havia variados graus de hiperplasia macrofágica, hemossiderose e emperipolese megacariocítica. Formas amastigotas de *Leishmania* spp. na medula óssea do fêmur e do manúbrio esternal às avaliações histopatológica e imunoistoquímica foram observadas, com boa concordância entre essas, mas sem diferença na intensidade parasitária entre a medula óssea desses sítios anatômicos. Conclui-se que a medula óssea do fêmur e do manúbrio esternal de cães reativos para leishmaniose aos testes DPP® e ELISA apresenta alterações histológicas decorrentes da doença, independente da presença ou intensidade do parasito, sendo hiperplasia de macrófagos, hemossiderose e emperipolese as principais alterações medulares nesses animais. Além disso, a medula óssea do fêmur e do manúbrio esternal são úteis ao diagnóstico de LVC por métodos diretos.
Palavras-chave: alterações medulares; amastigotas; histopatologia; imunocoloração; leishmaniose visceral canina

Received: June 13, 2022. Accepted: September 5, 2022. Published: September 21, 2022.

Introduction

Visceral leishmaniasis (VL) is caused by the protozoa *Leishmania infantum,* occurs worldwide, commonly in tropical and subtropical regions, and affects animals and humans[1]. A total of 91,055 cases of VL were confirmed in humans in Brazil from 1990 to 2018, with an average of 3,140 cases per year[2]. In the state of Goiás, 646 cases were reported during the same period, with an average of 22.28 cases per year[2]. The expansion of urbanization has led to an increase in the number of cases of VL in Brazil and, at the same time, an increase in the incidence of the disease in dogs of large and medium cities[3,4]. The dog is the main reservoir of the parasite, and the transmission occurs by the bite of an infected phlebotomine[5].

Bone marrow and lymph nodes are the most used sites for collecting samples[5] aimed at direct parasitological diagnosis of canine visceral leishmaniasis (CVL), especially in asymptomatic animals[6,7]. The most common clinical signs of the disease include onychogryphosis, skin lesions, lymphadenomegaly, splenomegaly, low body score[8-11], and anemia[7,10,12-14]. Signs such as epistaxis, increased blood clotting time, hematuria, medullary aplasia, and thrombocytopenia are also observed[7,15,16].

Bone marrow presents high parasitemia in CVL, with no changes in the production of the white and red series at the beginning of the infection, but a reduction in cell production and, consequently, hematological disorders are observed as the disease progress, resulting from changes such as macrophage hyperplasia, erythrocyte hypoplasia, and medullary aplasia[16]. Myelodysplastic alterations have been described in humans with VL, including anemia[16-17], triggered by the sequestration of red cells in the spleen, pro-inflammatory cytokines that inhibit erythropoietin synthesis, and immunological mechanisms[16].

Most studies of CVL use organs such as the spleen, lymph node, and bone marrow for the identification and quantification of the parasite. However, there is one study addressing histopathological changes in the bone marrow of dogs with the disease[16], and comparative studies regarding the parasite intensity at different anatomical sites of bone marrow in these animals were not found. Thus, this study aimed to perform histopathological and immunohistochemical analysis of the bone marrow of the femur and sternal manubrium of dogs reactive for leishmaniasis by Dual Path Platform (DPP®) and enzyme-linked immunosorbent assay (ELISA) tests.

Material and methods

This research was approved by the Ethics Committee on the Use of Animals of the Federal University of Goiás (CEUA/UFG), Goiânia, GO, Brazil, under protocol number 061/19. Thirteen leishmaniasis reagent dogs to the rapid immunochromatographic tests Dual Path Platform (DDP® - Bio-Manguinhos, Rio de Janeiro, Brazil) and ELISA, from the routine of epidemiological surveillance for CVL, performed by the Directorate of Zoonosis Surveillance of the Municipality of Goiânia (DVZ), GO, Brazil, were used. 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, and submitted to anatomopathological examination.

The variables sex, age, and breed were considered for investigating the epidemiological data. Regarding age, the animals were classified as young (up to two years old), adults (three to seven years old), or elderly (over eight years old). Regarding the breed, the animals were divided into purebred and mixed-breed dogs. Variables related to clinical signs of CVL were also considered, including onychogryphosis, alopecia, desquamation, oral ulcer, staining of the oral mucosa[18,19] 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 palpable ribs showing minimal fat coverage, bony prominences palpable, abdominal indentation visible in the flank region and minimal abdominal fat; 3 - medium body score, 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)[20].

The animals were also classified as asymptomatic (without clinical signs) or symptomatic (with clinical signs compatible with CVL) according to their clinical signs[21]. Changes related to splenomegaly and lymphadenomegaly (regional or generalized) were evaluated on the macroscopic examination. Bone marrow samples from the femur and sternal manubrium were collected on the necroscopic examination and fixed in 10% neutral buffered formalin for 48 hours[22], and, sequentially, transferred to 70% alcoholic solution. The sternal manubrium samples were decalcified with EDTA solution, distilled water, and sodium hydroxide, which was not necessary for marrow samples from the femur, which were collected immediately after the mechanical breakage of the bone in its extension and exposure of the marrow[23]. Subsequently, all bone marrow samples were submitted to histological processing and inclusion in paraffin[22,24].

Histopathological evaluation

Paraffinized blocks with bone marrow samples from the femur and sternal manubrium allowed preparing 5-µm thick histological sections, which were stretched on histological slides and stained with hematoxylin and eosin (HE)[22]. Histomorphological changes in the bone marrow of the femur and sternal manubrium were analyzed according to descriptions adapted from Momo et al.[16], including the variables proportion of the red and yellow series, macrophage hyperplasia (discrete, moderate, or accentuated), macrophages with amastigote forms of *Leishmania infantum* (discrete, moderate, or accentuated), hemosiderosis, and megakaryocytic emperipolesis.

Immunohistochemical evaluation

The immunohistochemical analysis was carried out using 4 µm histological sections placed on silanized histological slides (StarFrost®), which were 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 to block nonspecific protein binding. Then, the sections were incubated for 18 h at a temperature between 2 and 8 °C, with polyclonal anti-*Leishmania* antibody diluted to a concentration of 1: 1000 in antibody diluent (Diamond; Cell Marque, Rocklin, CA). The amplification of signals used 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, CA). The reaction was visualized using the DAB chromogen, according to the manufacturer's recommendations. The slides were counterstained with Harris' hematoxylin, dehydrated in absolute ethanol, clarified in xylol, and mounted with coverslips and synthetic resin. Two samples of canine tissue markedly parasitized with amastigotes of *Leishmania* spp. were used as positive control of the reaction, and two samples not infected by *Leishmania* spp. amastigotes were used as negative controls.

Macrophages with amastigote forms of *Leishmania* spp. were counted in five fields with a higher density of parasitized cells at 40x magnification and using a 1-mm^2 optical grid and a manual cell counter for the evaluation of parasite intensity. Subsequently, the average number of parasitized macrophages was obtained in the five fields for each animal, and parasite intensity scores were assigned as follows: absent (when amastigote forms were not observed), discrete to moderate (0.2 to 10 parasitized macrophages), and accentuated (more than ten parasitized macrophages)[5].

Statistical analysis

Descriptive statistics were used for the variables sex, age, breed, clinical classification, and histomorphological changes in the bone marrow of the femur and sternal manubrium. The Kappa non-parametric test at the 5% significance level was used for the analysis of agreement regarding the number of positive cases in histopathological and immunohistochemical examinations, followed by the application of the scores very good ($0.8 < k \leq 1$), good ($0.6 < k \leq 0.8$), moderate ($0.4 < k \leq 0.6$), fair ($0.2 < k \leq 0.4$) or poor ($k \leq 0.2$) to determine the degree of agreement between examinations. The Mann-Whitney test at the 5% significance level was applied to compare the parasite intensity between the bone marrow of the femur and the sternal manubrium. For this, Excel 2016 spreadsheets and the R® software, version R 4.0.0, were used, including the libraries *irr* (version 0.84) and *stats* (version 3.4.4).

Results

Among the 13 dogs reactive for leishmaniasis by DDP® and ELISA tests included in this study, 61.5% (n=8) were young, 23.1% (n=3) adults, and 15.4% (n=2) elderly. Regarding sex, 69.2% (n=9) were females and 30.8% (n=4) males. Moreover, 76.9% (n=10) consisted of purebred animals, and 23.1% (n=3) mixed-breed animals.

The evaluation of clinical changes showed that 92.3% (n=12) of the animals had a hypocolored oral mucosa, 76.9% (n=10) alopecia and skin desquamation, 53.9% (n=7) onychogryphosis, and 7.7% (n=1) oral ulcer. The evaluation of body score showed that 76.9% (n=10) of the dogs had a medium body score, 15.4% (n=2) low body score, and 7.7% (n=1) overweight. Among the 13 animals in this study, 92.3% (n=12) were symptomatic and 7.7% (n=1) asymptomatic. The macroscopic evaluation showed that 100% (n=13) of the animals had splenomegaly, 92.3% (n=12) generalized lymphadenomegaly, and 7.7% (n=1) dog exhibited localized lymphadenomegaly.

Histopathological evaluation

The histomorphological evaluation of bone marrow samples from the femur showed that 46.2% (n=6) of the dogs had a higher proportion of the red series, 46.2% (n=6) presented a similar proportion of the red and yellow series, and 7.7% (n=1) a higher proportion of the yellow series (Figure 1). The evaluation of the bone marrow of the sternal manubrium evidenced that 53.9% (n=7) of the animals had a higher proportion of the red

series and 46.2% (n=6) the same proportion of the red and yellow series. The result of the evaluation of the variables macrophage hyperplasia in the bone marrow (Figure 1) and macrophages with amastigote forms of *Leishmania* spp. (Figure 2) is shown in Table 1.

Table 1. Distribution of macrophage hyperplasia in the bone marrow and macrophages with amastigote forms of *Leishmania* spp. in the bone marrow of the femur and the sternal manubrium, according to the intensity

Sample	n	D%	n	M%	n	A%
Femur – macrophage hyperplasia (n=13)	4	30.8	3	23.1	6	46.1
Femur – macrophages with amastigotes forms of *Leishmania* spp. (n=8)	3	23.1	2	15.4	3	23.1
Sternal manubrium – macrophages hyperplasia (n=13)	5	38.5	6	46.1	2	15.4
Sternal manubrium – macrophages with amastigotes (n=4)	2	15.4	0	0	2	15.4

n – number of cases. Intensity: D – discrete, M – moderate, A – accentuated.

Among the bone marrow samples, 84.6% (n=11) had some degree of hemosiderosis in the femur and 15.4% (n=2) in the sternal manubrium. Megakaryocytic emperipolesis was observed in 92.3% (n=12) of the bone marrow samples from the sternal manubrium and 53.85% (n=7) of the bone marrow samples from the femur (Figure 1).

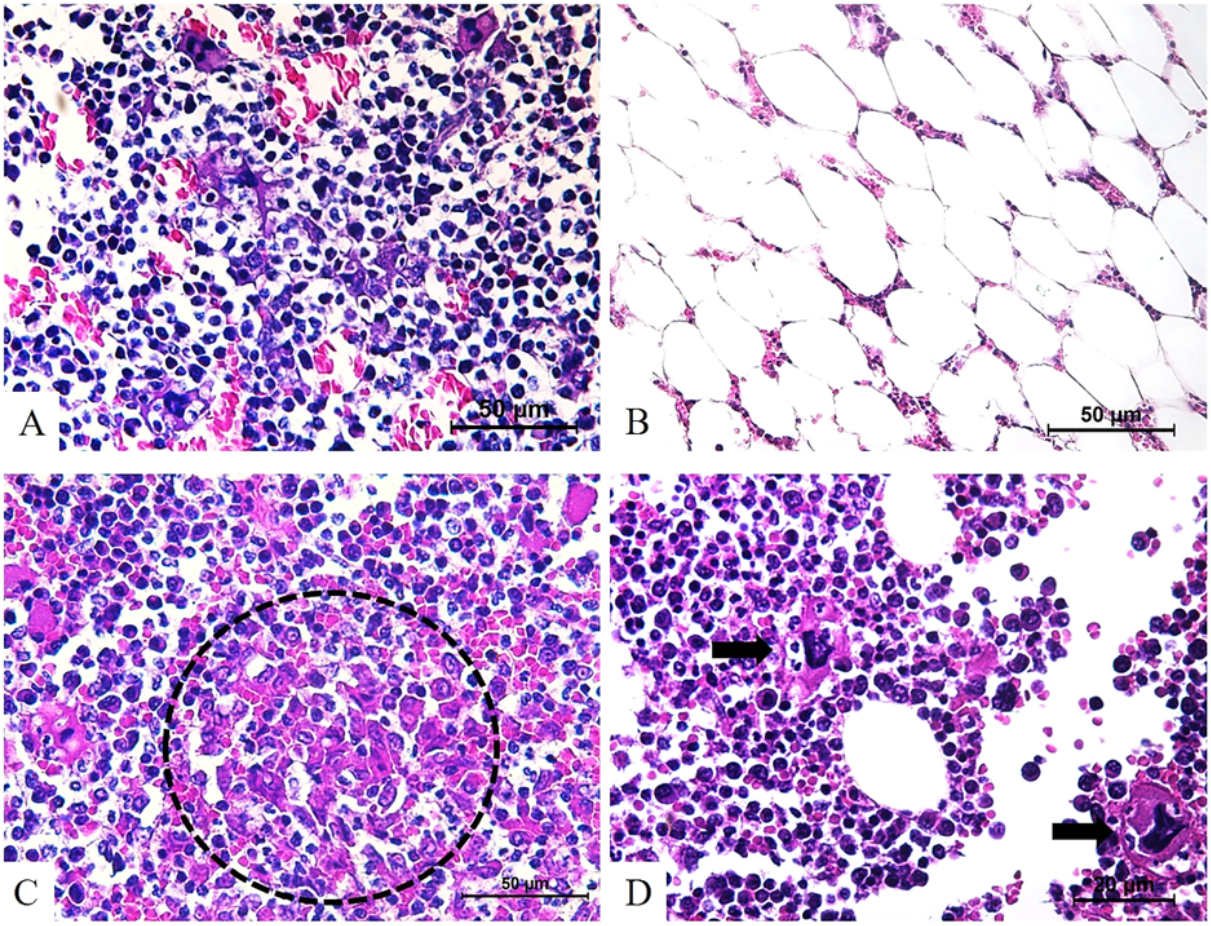

Figure 1. Photomicrographs of the bone marrow of dogs reactive for leishmaniasis by DPP® and ELISA tests. A) Higher proportion of red and B) Higher proportion of yellow, HE. C) Accentuated macrophage infiltrate (dotted area), HE. D) Megakaryocytic emperipolesis (arrows), HE.

Immunohistochemical evaluation

As in HE (Figure 2A), the IHC (Figure 2B) technique presented a variety of immunostaining of the amastigote forms of *Leishmania* spp. in bone marrow samples from the femur and sternal manubrium of dogs reactive for leishmaniasis by DPP® and ELISA tests, with 76.9% (n=10) of immunostaining in the bone marrow of the femur and 38.5% (n=5) in the bone marrow of the sternal manubrium.

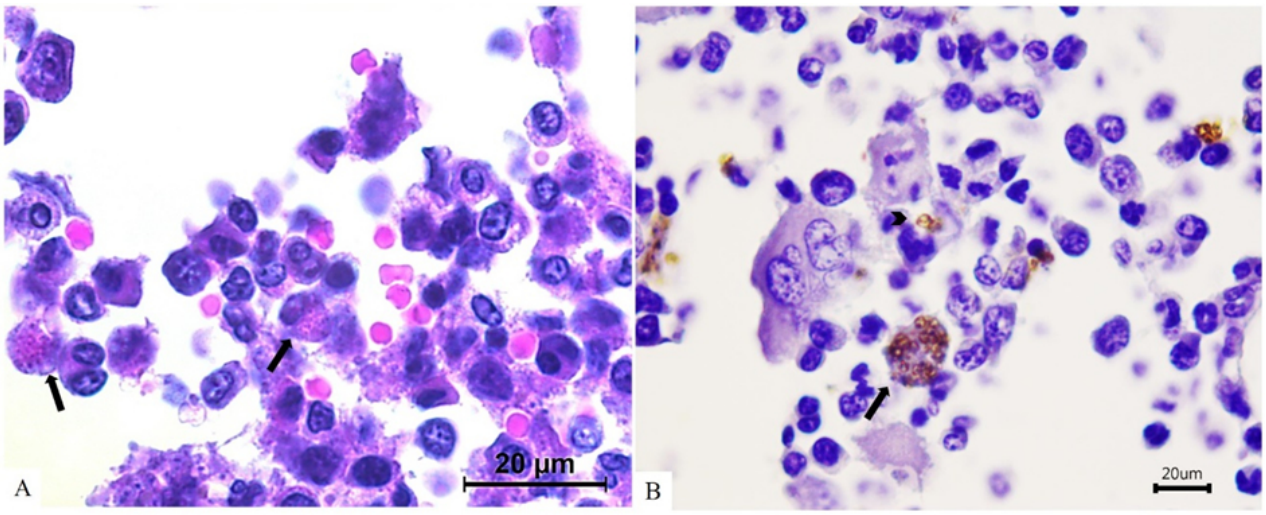

Figure 2. Photomicrographs of the bone marrow of the femur of dogs reactive for leishmaniasis by DPP® and ELISA tests. A) Macrophages with amastigote forms of *Leishmania* spp. (arrows), HE. B) Immunostaining of amastigote forms of *Leishmania* spp. in the cytoplasm of macrophages (arrows), IHC, anti-*Leishmania*.

The analysis of agreement of results regarding the number of positive cases on histopathological (n=8, a higher number of positive samples between the bone marrow of the femur and sternal manubrium in HE) and immunohistochemical examinations (n=10, a higher number of samples among the bone marrow of the femur and sternal manubrium in IHC) of the bone marrow showed a good agreement (k=0.60; p=0.00125). The evaluation of parasite intensity in the femur and sternal manubrium using the IHC technique showed animals with the scores absent, discrete to moderate, and accentuated. The comparison of parasite intensity between the femur and sternal manubrium presented no difference by the immunohistochemical evaluation (p=0.18) (Table 2).

Table 2. Distribution of scores and comparison of means of parasite intensity in the bone marrow of the femur and sternal manubrium

Sample	n	A %	n	DM %	n	I%	Mean (p=0.18)
Femur	3	23.1	8	61.5	2	15.4	4.44[a]
Sternal manubrium	8	61.5	3	23.1	2	15.4	3.41[a]

n – number of cases; A% – percentage of absent; DM% – percentage of discrete to moderate; I% – percentage of intense. Values followed by the same letters in the same column do not differ from each other.

Discussion

Most of the dogs in this study were symptomatic, with skin changes, onychogryphosis, hypocolored mucosa, and generalized lymphadenomegaly, which are commonly described in CVL[9-11,18,19].

All animals in this study exhibited macrophage hyperplasia in the bone marrow of the femur and sternal manubrium to varying degrees of intensity. This finding was also described by Momo et al.[16], which considered these lesions as diffuse granulomas without typical nodule formation. However, in this study these findings were considered hyperplastic, since the bone marrow did not have epithelioid cells and lymphoplasmacytic infiltrate delimited by fibrous tissue as morphological changes that support the classification of granulomas, and as has been widely described and characterized in the spleen[25,26] and liver[27].

Most of the dogs in this study presented hypocolored mucosa and hemosiderosis in the bone marrow. These findings have been described in cases of CVL and related to immune-mediated anemia and hypergammaglobulinemia, resulting from disease progression[15,16,28]. Anemia in humans with VL is justified by several factors, including splenic enlargement, increased plasma volume, reticuloendothelial hyperplasia, hemolysis, irregular erythropoiesis, and autoimmune anemia[29]. In this context, it is considered that similar mechanisms may occur in cases of CVL and end up triggering alterations such as pale mucous membranes and hemosiderosis, as observed in this study.

Megakaryocytic emperipolesis was also observed in most of the bone marrow samples, characterized by the active insertion of one cell in the cytoplasm of another, but of non-phagocytic origin. In this case, neutrophils were observed in the megakaryocyte cytoplasm, mainly in the bone marrow of the sternal manubrium. Megakaryocytic emperipolesis has been described in cases of CVL[16,30], but the cause for its occurrence is not known for certain, and additional studies are needed to confirm its relationship with infection by *Leishmania* spp.[16]. On the other hand, this phenomenon is common in hematolymphoid and myeloproliferative disorders[31], which have been described in cases of VL in humans, and in other intracellular parasites such as *Trypanosoma* spp., however, there is not a defined mecanism[32,33].

Histopathological evaluation allows examining morphological changes in the bone marrow of dogs infected with *Leishmania* spp. and, similar to immunohistochemistry, it allows the visualization and counting of the amastigote forms of the parasite[34]. The results of this study showed that in the qualitative analysis by immunohistochemistry of the bone marrow of the femur and sternal manubrium, there is a higher number of positive cases relative to histopathology, but with no difference. This data contrasts with has been described in the literature on the relevance of the immunohistochemical test in relation to the histopathology technique to identify the amastigote forms of *Leishmania* spp.[12,34-36]. In this sense, Toplu et al.[18] reported that the immunohistochemical technique is more sensitive and specific to detect amastigotes than cytopathology and histopathology and describe the immunostaining of amastigotes in macrophages in the bone marrow in cases of CVL[8]. Also, the good agreement of CVL positivity to the histopathology and IHC techniques observed in this study directs the use of both techniques for the direct diagnosis of the disease.

As no difference was observed between the bone marrow of the femur and sternal manubrium in terms of parasite intensity, both organs are useful for CVL diagnosis. In this context, Paparcone et al.[7] also found no significant differences in the cellularity and the degree of parasitism in the bone marrow collected from different sites for direct diagnosis of CVL, which was further ratified by Xavier et al.[12] in a comparative study of diagnostic techniques for the disease.

Conclusions

The bone marrow of the femur and sternal manubrium of dogs seropositive for leishmaniasis by DPP® and ELISA tests develop histomorphological changes as result of the disease, which do not depend on the presence or intensity of the parasite, with macrophage hyperplasia, hemosiderosis and emperipolesis being the main medullary changes. In addition, the bone marrow of the femur and the sternal manubrium comprise useful anatomical sites for the diagnosis of CVL by direct methods.

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgment

To the Directorate of Surveillance in Zoonoses of Goiânia (DVZ), GO, Brazil, for providing animals to the study and assistance during the development of the research. To the Laboratory of Clinical Research on Dermatozoonosis in Domestic Animals and the Pathological Anatomy Service, both from the National Institute of Infectious Diseases Evandro Chagas, Fundação Oswaldo Cruz, Rio de Janeiro, Rio de Janeiro, Brazil, for the technical support.

Author contributions

Conceptualization: A. O. de Magalhães, L. M. Bezerra and V. M. B. D. de Moura. *Investigation*: A. O. de Magalhães, L. M. Bezerra, D. P. Araújo, B. S. G. de Lima and R. C. Menezes. *Methodology*: A. O. de Magalhães, V. M. B. D. de Moura, L. do P. Assunção and R. C. Menezes. *Formal analysis:* L. do P. Assunção. *Validation*: L. do P. Assunção. *Writing (original draft):* Aline Oliveira de Magalhães. *Supervisão*: V.M. B. D. de Moura. *Writing (review & editing):* V. M. B.D. de Moura and R. C. Menezes.

References

1. World Health Organization. Control of the leishmaniases; 2016. 375p.

2. Ministério da Saúde. Leishmaniose visceral: o que é, causas, sintomas, tratamento, diagnóstico e prevenção. Situação epidemiológica da leishmaniose visceral. 2020. [cited 2020 jul 20]. Available from: http://www.saude.gov.br/saude-de-a-z/leishmaniose-visceral. Portuguese.

3. Coura-Vital W, Marques MJ, Veloso VM, Roatt BM, Aguiar-Soares RDO, Reis LES, et al. Prevalence and factors associated with *Leishmania infantum* infection of dogs from an urban area of Brazil as identified by molecular methods. Plos Neglected Tropical Diseases. [Internet]. 2011 aug [cited 2020 jun 25],

5(8):e1291. Available from: https://doi.org/10.1371/journal.pntd.0001291

4. Castro-Júnior JG, Freire ML, Campos SPS, Scopel KKG, Porrozzi R, Silva ED, et al. Evidence of *Leishmania (Leishmania) infantum* infection in dogs from Juiz de Fora, Minas Gerais State, Brazil, based on immunochromatographic dual-path platform (DPP®) and PCR assays. Revista do Instituto de Medicina Tropical de São Paulo. [Internet]. 2014 may [cited 2020 jun 28]; 56(3): 225-229. Available from: doi: https://doi.org/10.1590/S0036-46652014000300008

5. Ministério da Saúde. Secretaria de Vigilância em Saúde. Departamento de Vigilância Epidemiológica. Manual de vigilância e controle da leishmaniose visceral; 2014.[cited 2020 jul 20];120 p. Available from: https://bvsms.saude.gov.br/bvs/publicacoes/manual_vigilancia_controle_leishmaniose_visceral_1edicao.pdf. Portuguese

6. Menezes RC, Madeira MF, Ferreira LC, Barbosa CJLF, Miranda LHM, Figueiredo FB. Cell-block immunohistochemistry of bone marrow aspirates: a novel tool to improve the diagnosis of *Leishmania* infection in dogs. Journal of comparative pathology. [Internet]. 2016. Feb-Apr [cited 2020 jul 28]; 154(2-3):157-60. Available from: doi: https://doi.org/10.1016/j.jcpa.2015.12.005

7. Paparcone R, Fiorentino E, Cappiello S, Gizzarelli M, Gradoni L, Oliva G, Manzillo VF. Sternal aspiration of bone marrow in dogs: a practical approach for canine leishmaniasis diagnosis and monitoring. Journal of Veterinary Medicine. [Internet]. 2013.[cited 2020 jul 29]; 2013: 217314. Available from: https://doi.org/10.1155/2013/217314

8. Tafuri WL, Santos RL, Arantes RME, Gonçalves R, Melo MN, Michalik MSM, et al An alternative immunohistochemical method for detecting *Leishmania* amastigotes in paraffin-embedded canine tissues. Journal of immunological methods. [Internet]. 2004, Sep [cited 2020 jul 15]; 292(1-2):17-23. Available from: doi: https://doi.org/10.1016/j.jim.2004.05.009

9. Keenan CM, Hendricks LD, Lightner L, Johnson AJ. Visceral leishmaniasis in the German Shepherd dog. II. Pathology. Veterinary pathology. [Internet]. 1984 Jan [cited 2020 jul 19]; 21(1):80-6. Available from: doi: https://doi.org/10.1177/030098588402100114

10.Lima WG, Michalick MSM, Melo MN, Tafuri WL, Tafuri WL. Canine visceral leishmaniasis: a histopathological study of lymph nodes. Acta Tropica. [Internet]. 2004. Sep [cited 2020 jul 26]; 92(1):43-53. doi: https://doi.org/10.1016/j.actatropica.2004.04.007.

11.Cavalcanti AS, Ribeiro-Alves M, Pereira LOR, Mestre GL, Ferreira ABR, Morgado FN, et al. Parasite load induces progressive spleen architecture breakage and impairs cytokine mRNA expression in *Leishmania infantum*-naturally infected dogs. Plos one. [Internet]. 2015, apr [cited 2020 jul 26]; 10(4):e0123009. Available from: doi: https://doi.org/10.1371/journal.pone.0123009

12.Xavier SC, Andrade HM, Monte SJH, Chiarelli IM, LimaWG, Michalick MSM, et al. Comparison of paraffin-embedded skin biopsies from different anatomical regions as sampling methods for detection of *Leishmania* infection in dogs using histological, immunohistochemical and PCR methods. BMC Veterinary Research. [Internet]. 2006. 2:17. [cited 2020 de jul de 20]; 8;2:17. Availables from: doi: https://doi.org/10.1186/1746-6148-2-17

13.Tryphonas L, Zawidzka Z, Bernard MA, Janzen EA. Visceral Leishmaniasis in a dog: clinical, hematological and pathological observations. Canadian Journal of Comparative Medicine. [Internet]. 1977 jan [cited 2020 jul 30]; 41(1):1-12. Available from: Disponível em: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1277686/

14.Reis AB, Martins OAF, Teixeira-Carvalho A, Giunchetti RC, Carneiro CM, Mayrink W, et al. Systemic and compartmentalized immune response in canine visceral leishmaniasis. Veterinary Immunology and Immunopathology. [Internet]. 2009 mar [cited 2020 jul 28]; 128(1-3):87-95. Available from: https://doi.org/10.1016/j.vetimm.2008.10.307

15.Mahajan V, Marwaha RK. Case report immune mediated hemolysis in visceral leishmaniasis. Journal of Tropical Pediatrics. [Internet]. 2007 aug [cited 2020 jul 30]; 53(4):284–286. Available from: https://doi.org/10.1093/tropej/fmm018

16.Momo C, Jacintinho APP, Moreira PRR, Munari DP, Machado GF, Vasconcelos RO. Morphological changes in the bone marrow of the dogs with visceral leishmaniasis. Veterinary Medicine International. [Internet]. 2014 [cited 2020 jul 12] 2014: 5p. Available from: http://dx.doi.org/10.1155/2014/150582

17.Solimando AG, Coniglio G, Desantis V, Lauletta G, Bavaro DF, Diella L, et al. A Challenging Case of Visceral Leishmaniasis. Reports. [Internet]. 2002 [cited 2020 aug 30]; 5(2):23. Available from: https://doi.org/10.3390/reports5020023

18.Toplu N, Aydogan A. An immunohistochemical study in cases with usual and unusual clinicopathological findings of canine visceral leishmaniosis. Parasitology research. [Internet]. 2011 oct [cited 2020 jul 29]; 109(4):1051-7. Available from: doi: https://doi.org/10.1007/s00436-011-2345-0

19.Oliveira VC, Boechat VC, Mendes AAVJ, Madeira MF, Ferreira LC, Figueiredo FB, et al. Occurrence of *Leishmania infantum* in the central nervous system of naturally infected dogs: Parasite load, viability, co-infections and histological alterations. Plos one. [Internet]. 2017 apr [cited 2020 jul 20]; 12(4):e0175588. Available from: doi: https://doi.org/10.1371/journal.pone.0175588

20.Castro MC, Vieira AB, Santos MCS, Gershony LC, Soares AMB, Ferreira AMR. Escore de condição corporal como indicador do prognóstico de gatos com doença renal crônica. Ciência Rural [Internet], 2010 fev [cited 2020 jul 20]; 40(2):365-370. Available from: https://doi.org/10.1590/S0103-84782010005000010 .Portuguese

21.Lima IS, Silva JS, Almeida VA, Leal GLJ, Souza PAN, Larangeira DF, Moura-Neto JP, et al. Severe clinical presentation of visceral leishmaniasis in naturally infected dogs with disruption of the splenic White pulp. Plos One. [Internet]. 2014.fev [cited 2020 jul 28]; 3;9(2):e87742. Available from: doi: https://doi.org/10.1371/journal.pone.0087742

22.Boechat VC, Mendes AAVJ, Madeira MF, Ferreira LC, Figueiredo FB, Rodrigues FCC, et al. Occurrence of Leishmania infantum and associated histological alterations in the genital tract and mammary glands of naturally infected dogs. Parasitology research. [Internet]. 2016.jun [cited 2020 jul 20]; 115(6):2371-9. Available from: doi: https://doi.org/10.1007/s00436-016-4987-4.

23.Caputo LFG, Gitirana LB, Manso PPA. Capítulo 3. Técnicas histológicas. IN: Molinaro EM, Caputo LFG, Amendoeira MRR. Conceitos e métodos para a formação de profissionais em laboratórios de saúde: volume 2 / Rio de Janeiro: EPSJV; IOC, 2010, p.89-188. Portuguese. (https://www.epsjv.fiocruz.br/publicacao/livro/conceitos-e-metodos-para-formacao-de-profissionais-em-laboratorios-de-saude-volum-2) . Portuguese

24.Nunes CS, Cinsa LA. Princípios Do Processamento Histológico De Rotina. Revista Interdisciplinar de Estudos Experimentais, v. 8, n. único, p. 31-40, 2016. Portuguese (https://docs.bv-

salud.org/biblioref/2018/11/964830/2884-8890-1-sm.pdf)

25.Santana CC, Vassallo J, Freitas LAR, Oliveira GGS, Pontes-de-Carvalho LC, Dos-Santos WLC. Inflammation and structural changes of splenic lymphoid tissue in visceral leishmaniasis: A study on naturally infected dogs. Parasite Immunology. [Internet]. 2008 oct [cited 28 jul 2020]; 30(10): 515–524. Available from: doi: https://doi.org/10.1111/j.1365-3024.2008.01051.x

26.Silva AVA, Figueiredo FB, Menezes RC, Mendes-Junior AA, Miranda LHM, Cupolillo E. Morphophysiological changes in the splenic extracellular matrix of Leishmania infantum-naturally infected dogs is associated with alterations in lymphoid niches and the CD4+T cell frequency in spleens. PLoS neglected tropical diseases [Internet]. 2018 apr [cited 2020 jul 20]; 12(4):e0006445. Available from: https://doi.org/10.1371/journal.pntd.0006445

27.Tafuri WL, Tafuri WL, Barbosa AJA, Michalick MSM, Genaro O, França-Silva JC. Histopathology and immunocytochemical study of type 3 and type 4 complement receptors in the liver and spleen of dogs naturally and experimentally infected with *Leishmania (Leishmania) chagasi*. Revista do Instituto de Medicina Tropical de São Paulo [Internet]. 1996 apr [cited 2020 jul 25]; 38(2):81-89. [acesso 25 de julho de 2020]. Available from: http://dx.doi.org/10.1590/S0036-46651996000200001

28.Reis AB, Martins-Filho AO, Teixeira-Carvalho A, Carvalho MG, Mayrink W, França-Silva JC. Parasite density and impaired biochemical/hematological status are associated with severe clinical aspects of canine visceral leishmaniasis Research in Veterinary Science. [Internet]. 2006 aug [cited 2020 jul 25]; 81:68-75. Available from: https://doi.org/10.1016/j.rvsc.2005.09.011

29.Yarali N, Fişgin T, Duru F, Kara A. Myelodysplastic features in visceral leishmaniasis. American Journal of Hematology. [Internet]. 2002 oct [cited 2020 jul 26]; 71:191-195. Available from: doi: https://doi.org/10.1002/ajh.10200

30.Manzillo VF, Restucci B, Pagano A, Gradoni, Oliva G. Pathological changes in the bone marrow of dogs with leishmaniosis. The Veterinary Record. [Internet]. 2006, may [cited 2020 jul 20]; 158:690-694. Available from: http://dx-doi-org.ez49.periodicos.capes.gov.br/10.1136/vr.158.20.690

31.Sable MN, Sehgal K, Gadage VS, Subramanian PG, Gujral S. Megakaryocytic emperipolesis: A histological finding in myelodysplastic syndrome. Indian journal of pathology & microbiology. [Internet]. 2009 oct-dec [cited 2020 jul 26]; 52(4):599-600. Available from: https://doi.org/10.4103/0377-4929.56153

32.Anosa VO, Logan-Henfrey LL, Shaw MK. A light and electron microscopic study of changes in blood and bone marrow in acute hemorrhagic Trypanosoma vivax infection in calves. Veterinary pathology. [Internet] 1992 jan [cited 2020 aug 31]; 29(1):33-45. Available from: doi: https://doi.org/10.1177/030098589202900105

33.Cunin P, Nigrovic PA. Megakaryocyte emperipolesis: a new frontier in cell-in-cell interaction. Platelets.[Internet] 2020 aug [cited 2020 aug 30]; 31(6):700-706. Available from: doi:https://doi.org/10.1080/09537104.2019.1693035

34.Menezes RC, Figueiredo FB, Wise AG, Madeira MF, Oliveira RVC, Schubach TMP, et al. Sensitivity and specificity of in situ hybridization for diagnosis of cutaneous infection by Leishmania infantum in dogs. Journal of Clinical Microbiology. [Internet]. 2013 jane [cited 2020 jul 28]; 51:206-211. Available from: doi: https://doi.org/10.1128/JCM.02123-12

35.Queiroz NMGP, Silveira RCV, Noronha ACFJ, Oliveira TMFS, Machado RZ, Starke-Buzetti WA. Detection of *Leishmania (L.) chagasi* in skin. Veterinary Parasitology. [Internet]. 2011 may [cited 2020 jul 28]; 178:1-8. Available from: doi:https://doi.org/10.1016/j.vetpar.2010.12.033

36.Quintella LP, Cuzzi T, Madeira MF, Okamoto T, Schubach AO. Immunoperoxidase technique using an anti-*Leishmania (L.) chagasi* hyperimmune serum in the diagnosis of culture-confirmed american tegumentary leishmaniasis. Revista do Instituto de Medicina Tropical de São Paulo. [Internet]. 2009 apr [cited 2020 jul 28] 51(2): 83-86. Available from: doi:https://doi.org/10. 1590/S0036-46652009000200005