MORPHOLOGICAL, IMMUNOHISTOCHEMICAL AND MOLECULAR STUDY OF RENAL LESIONS IN CANINE VISCERAL LEISHMANIASIS (CVL)

Heitor Flávio Ferrari[1]
Vinícius Soares Carreira[2]
Marcio Antonio Batistela Moreira[3]
Vania Ribeiro da Matta[4]
Cláudio Hiroyassu Haga[5]
Maria Cecília Rui Luvizotto[6]

ABSTRACT

Canine visceral leishmaniasis (CVL) is an anthropozoonosis characterized by a clinically chronic progressive disease. Non lymphoid organs are also affected, especially the kidneys. Dogs with leishmaniasis usually die with renal failure despite treatment. Haematoxylin-eosin (HE) staining in kidney tissue sections has low sensitivity for parasite identification. Immunohistochemistry (IHC) and polymerase chain reaction (PCR) are efficient methods for *Leishmania* sp. antigen and DNA detection in cases of low parasite burden. The present study aims to identify renal lesions of CVL and correlate them with microscopic findings determined by histochemistry, IHC and PCR. Both IHC and PCR provided similar positivity for amastigote identification, 3/20 animals (15%), thus increasing detection of the parasite in renal tissues when compared with histopathologic examination. The lesion most commonly observed with visceral leishmaniasis-positive canine kidney tissue was membranoproliferative glomerulonephritis, followed by interstitial nephritis without correlation to the number of amastigotes.

Key words: dog, visceral leishmaniasis, kidney, immunohistochemistry, Polymerase Chain Reaction (PCR).

ESTUDIO MORFOLÓGICO, INMUNOISTOQUÍMICO Y MOLECULAR EN RIÑONES DE PERROS PORTADORES DE LEISHMANIOSIS VISCERAL.

RESUMEN

La leishmaniosis visceral canina (CVL) es una antropozoonosis caracterizada por evolución crónica y progresiva. Órganos no pertenecientes al sistema linfático también son afectados, destacándose los riñones. Perros con leishmaniosis desarrolan lesión renal crónica, que evoluye al óbito, aún después del tratamiento. La identificación del parásito en la tejido renal, corado con hematoxilina y eosina (HE), presenta baja sensibilidad. Las técnicas de

[1] Médico Veterinário Docente da Faculdade de Medicina Veterinária de Andradina/SP – Rua Amazonas 571. heitorferrari15@gmail.com
[2] Médico Veterinário Residente da Universidade de Michigan - Michigan State University 4125, Beaumont Road, Lasing. MI 48910 USA: vinicius.carreira@gmail.com
[3] Professor da Faculdade Anhembi Morumbi Rua Dr. Almeida Lima, 1134, Brás - São Paulo, Brazil. E-mail address: mabmob@terra.com.br
[4] Laboratório de Investigação Médica/Patologia das Moléstias Infecciosas (LIM-50), do Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo - Av. Dr. Enéas de Carvalho Aguiar, 255 –Cerqueira César 05403-000, São Paulo, Brasil- mattav@usp.br
[5] Médico Veterinário – hagavet@hotmail.com
[6] Professora Assistente Doutora, Departamento de Clínica, Cirurgia e Reprodução Animal – Curso de Medicina Veterinária – UNESP- Araçatuba; Rua Clovis Pestana - 793, Dona Amélia, Araçatuba-SP - 16050-680. ruimcl@fmva.unesp.br – autor para correspondência

inmunoistoquímica (IHC) y de Reacción en Cadena por la Polimerase (PCR) son eficientes en la detección del antígeno y del DNA de la *Leishmania* sp en los casos de bajo parasitismo. El presente trabajo tuvo como objetivos identificar las alteraciones renales decurrentes de la CVL y asociar con lesiones histológicas observadas en las coloraciones de histoquímica, IHC y PCR. El análisis de la IHC y PCR revelaron positividad semejante en la detección amastigotas, 3/20 animales (15%), lo que muestra el parásito en el tejido renal, cuando comparado con el examen histopatológico HE. En los casos de leishmaniosis visceral con exhibición en de amastigotas en el tejido renal, las lesiones microscópicas predominantes fueron glomerulonefritis membranoproliferativa y nefritis intersticial, no correlacionada con número de amastigotas.

Palabras-claves: perro, leishmaniosis visceral, riñones, inmunoistoquímica, Reacción en Cadena por la Polimerase (PCR).

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INTRODUCTION

Visceral Leishmaniasis (VL), also known as Calazar, is an anthropozoonosis caused by a protozoal organism belonging to the genus *Leishmania* (1). The worldwide prevalence of VL is 12 million people infected, with 500,000 new cases each year, especially among immune-compromised individuals (2). Once inoculated in the host skin, the parasites multiply inside the cytoplasm of macrophages, causing alterations especially in liver and lymphoid organs such as lymph node, spleen and bone marrow. Infection compromises other organs from the gastrointestinal, central nervous, genital and urinary systems (3). Depending on the host immune status, the onset of clinical signs occurs in a period of one month to few years post infection. Once clinically evident, the disease inevitably results in the host's death (4). Some of the classical disease symptoms include lymphadenomegaly, splenomegaly, anemia,

skin lesions, ocular lesions, ulcerative stomatitis, cachexia, ascites, epistaxis, polyarthritis, muscular atrophy, onychogryphosis (5). The disease also affects nonlymphoid organs. Kidneys are especially involved, even though only low numbers of parasites can be found within renal tissue, even in humans or dogs (6, 7). Renal failure is considered the main cause of death in canine leishmaniasis, occurring even though in treated individuals (8). The most common microscopic renal lesion is glomerulonephritis, with or without nephrotic syndrome (9). According to Benderitter et al. (10) the two most important glomerular lesions observed in canine visceral leishmaniasis are acute glomerulonephritis and membranous glomerulonephritis, and no correlation or transition between them can be observed.

Histopathological lesions described by Soares et al. (11) in symptomatic and asymptomatic dogs, when compared, display a higher incidence of membranoproliferative and mesangioproliferative glomerulonephritis, with the former more common in symptomatic and the latter more common in asymptomatic dogs. Also, in symptomatic dogs *Leishmania* sp amastigote forms can be observed within macrophages and mixed with an interstitial mononuclear inflammatory infiltrate. An increase in immune complex production is associated with the pathological process (12). It can result in immune complex deposition in target organs, potentially causing disturbances such as the gomerulopathies described in canine leishmaniasis (13). Histochemical analysis of canine kidney positive for Canine Visceral Leishmaniasis (CVL) revealed focal and diffuse segmental glomerulosclerosis with deposition of Periodic Acid Schiff (PAS) positive material on Bowman´s capsule. Mesangioproliferative and membranoproliferative glomerulonephritis was characterized by thickening and duplication of the glomerular capillaries, lobulation and marked mesangial cell proliferation (9, 11).

Identification of amastigotes with HE-stained sections has low sensitivity and is therefore usually unrewarding, especially in kidney, lung, central nervous system, and testicle. Immunohistochemistry can successfully detects *Leishmania* antigen in paraffin embedded tissue (14). Previous studies identified particulate *Leishmania* antigen within the interstitial inflammatory infiltrate, mainly within glomerular phagocytic cells and macrophages. Whole amastigotes, however, could not be detected (9). The parasite detection is the ultimate goal in diagnosing CVL; therefore, molecular approaches such as polymerase chain reaction (PCR) are vital in tissues where the parasite does not occur in high numbers (15). The detection of *Leishmania* sp. DNA in kidneys by PCR has been previously reported in symptomatic dogs (16); however it has not been performed in combination with localization of the organism by immunohistochemistry. Morphologic characterization of renal lesions, in addition to parasite detection by immunohistochemistry (IHC) and molecular study, are the main goals of the present study.

MATERIAL AND METHODS

Local

Samples tissues analyzed in this study were from the northwest area of São Paulo State, Brazil. This area is considered endemic for CVL caused by *Leishmania* sp.

Samples

A total of 20 adult mongrel dogs, including males and females, were selected for this study. They were from the Veterinary Teaching Hospital or the Zoonotic Disease Control Center (CCZ). All individuals were positives for CVL by direct microscopic examination of lymph node aspirate in cytologic preparations stained with Giemsa. Dogs were anesthetized

with Sodium Thiopental and euthanasia was performed by intravenous injection of a 19.1% KCl solution. Immediately after euthanasia, both kidneys were removed. After gross examination of both kidneys, one half of each kidney was stored in a -80ºC freezer for PCR and the other half in buffered 10% formalin solution for histopathological and immunohistochemical analysis.

Histopathology and histochemistry

Cortico-medullary fragments of right and left kidneys were formalin fixed and routinely processed. Paraffin-embbeded 5 μm sections were stained with Hematoxylin-Eosin, Periodic Acid Shiff (PAS), and Masson's trichrome. Lesions were graded according to intensity in "Neg" in absence of lesion, discrete (+), moderate (++) and severe (+++) inflammatory reation, following the criterious described by Costa (9).

Immunohistochemistry

Slides were deparaffinized and hydrated followed by antigen retrieved in 10mM citrate buffer, pH 6.0 for 3 minutes. Endogenous peroxidase was blocked by 3 baths (10 minutes each) in 3% hydrogen peroxide (H_20_2) followed by 3 baths (10 minutes each) in methanol and 5% H_20_2 solution. Nonspecific binding was blocked with 1.5% swine normal serum in 0.01M PBS incubated for 1 hour in a humid chamber at 37°C. Nonspecific ionic interactions were blocked with 60mg/L powder milk diluted in distilled water for 30 minutes at room temperature. Immunolabelling was performed with a 0.01M phosphate buffered saline (PBS) 1/2000 dilution mouse primary anti-*Leishmania* polyclonal antibody with 1% bovine albumin serum (BAS). Incubation was performed in a humid chamber at 37ºC for 30 minutes, followed by overnight incubation at 4ºC. After washing, the slides were incubated with a biotinylated secondary antibody (Link-DAKO, LSAB2 Kit, catalog # KO675-1 Carpinteria, California, USA) (at 37ºC for 30 minutes) and then with the Streptavidin-peroxidase complex at 37ºC for 30min (Link-DAKO, LSAB2 Kit, catalog # KO675-1 Carpinteria, California, USA). The substrate used was 60mg/100ml of 3.3-diaminobenzidine (DAB; Sigma, St. Louis, USA) with 0.01M PBS and 1mL hydrogen peroxide. Slides were then counterstained with Harris haematoxilin, dehydrated, and mounted.

Polymerase Chain Reaction (PCR)
DNA extraction

Aproximattely 25 mg of frozen kidney (-80ºC) was smashed individually in porcelain bowls containing liquid nitrogen and sodium citrate solution (SCS). Samples were centrifuged at 12000g for 10 minutes, followed by SCS washing for 5 minutes at 14000g. Lysis buffer (375 μL of 0.2 M sodium acetate, 25 μL of 10% sodium dodecylsulfate and 5 μL of 20 mg/μL K proteinase) was added to the pellets, followed by incubation at 37.8° C overnight. The lysates were treated with phenol/chloroform/isoamyl alcohol (25:24:1); DNA was precipitated with ethanol, dried and resuspended in 200 μL of TE buffer (15).

Primers

The pair of primers was prepared according to Rodgers et al. (17), with initiators (13A and 13B - A (5'- TCT TGC GGG GAG GGG GTG - 3') e B (5'- TTG ACC CCC AAC CAC ATT TTA - 3') that amplify the DNA fragments of kinetoplast minicircles; this region is preserved in different species of *Leishmania* genus.

DNA amplification

The PCR consisted of: Taq buffer (50 mM KCl, 10 mM Tris pH 8.4), MgCl2 standardized concentration of 1.5 mM, dNTPs (dATP, dCTP, dGTP and dTTP), initiators 13A and 13B, Taq DNA polymerase and DNA, in a final volume of 50 µl. The samples were amplified in a thermocycler (PTC-100 MJ-Research) using an initial denaturation step of 95 ºC for 5 min, followed by 30 cycles of 94ºC for 30 seconds, 54ºC for 45 seconds, and 72ºC for 30 seconds, with a final extension at 72ºC for 5 minutes; the samples were then kept at 4ºC until the next step. Amplification with primers 13A and 13B resulted in products of 120 base pairs (bp). Negative (tube without DNA) and positive (DNA from cultured promastigotes) controls were performed for each experiment. After amplification, the PCR products were subjected to 2% agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet light.

RESULTS

Kidney gross lesions consisted of 1mm white foci multifocally distributed on the cortex natural surface. Small irregular depressions, diffuse paleness of the parenchyma, focal adherence of the renal capsule, and fine diffuse cortical granulation on cut surface were noted. HE stained sections revealed a chronic interstitial nephritis in 75% of the dogs, either focal or diffuse, with variable intensity (Table 1). Tubulointerstitial nephritis was observed in 55% of the dogs. Interstitial inflammatory infiltrates exhibited predominantly periglomerular and perivascular deposition. Hyaline cylinders were also observed in tubular lumens. Characteristic proliferative and membranoproliferative glomerulonephritis lesions, with Bowman´s capsule and basement membrane thickening, synechia and Bowman´s capsule epithelization were observed in 15% of the dogs (Figure 1A).

Interstitial and glomerular inflammatory changes were associated in 60% (12/20) of the kidneys. Of all 20 dogs, only 2 did not display microscopic lesions in HE sections. PAS-stained sections revealed that 75% (n=15) of the kidneys had glomerular basement membrane thickness (Figure1B). Masson's trichrome-stained sections revealed that 65% (n=13) displayed a marked fibroblastic reaction with collagen proliferation in the glomerular capsule and in tubular interstitium, presenting in both cortex and medulla (Figure 1C). Immunohistochemistry detected *Leishmania* sp. amastigotes in 15% of the kidney, located within the interstitial mononuclear inflammatory infiltrate and in the glomerular mesangial region (Figure 1D). PCR detected *Leishmania* sp 120 bp amplicon in 3 kidneys (15%) (Figure 2).

DISCUSSION

Intense and variable renal glomerular alterations were observed among the dogs in this study. In agreement with previous studies, membranoproliferative glomerulonephritis was the most common lesion observed (10, 11). Despite of the lower glomerular involvement in human leishmaniasis (6), glomerulonephritis is emphasized as a typical sequela of the disease in dogs (7). The pathogenesis of glomerulonephritis is immune-mediated, strictly related to either the cell type or glomerular structure involved in the immune response, or by the immunogen localization within glomeruli (18). Histopathological analysis of the renal tissue revealed glomerulonephritis characterized by Bowman´s capsule thickening as a result of a thick glomerular basement membrane, parietal epithelial cell hyperplasia, and periglomerular fibrosis, in agreement with Tafuri et al. (14). Severe interstitial nephritis and fibrosis was a

frequent finding in naturally infected animals, symptomatic or asymptomatic, and at different development stages of the disease (9).

The intensity of interstitial nephritis, hydropic tubular degeneration, cylindruria, tubular vacuolization, interstitial fibrosis, and tubular necrosis was not considered statistically different between the symptomatic or asymptomatic dogs (11). Although in three kidneys amastigotes forms were detected by IHC and with membranaproliferative glomerulonephritis, the relationship between the presence of the amastigote and the lesion pattern could not be established. No amastigotes forms could be identified in HE sections. This finding corroborates with the higher sensitivity and specificity of the PCR and IHC, which were invaluable for parasite detection (16). Our findings support that renal lesions are frequently present in CVL. The most common lesions observed are membranoproliferative glomerulonephritis and interstitial nephritis, with a predominance of lymphoplasmacytic inflammatory infiltrate that was rarely associated with amastigote presence as evidenced by IHC and confirmed by PCR.

Table 1. Renal histopathological lesion pattern findings in canine visceral leishmaniasis symptomatic and asymptomatic positive dogs.

Dogs	**Lesions**					
	PGN	MPGN	IN	TIN	GBMT	GCT
1	Neg.	+++	+++	+++	+++	+++
2	++	Neg.	+++	++	Neg.	Neg.
3	Neg.	Neg.	++	+	Neg.	Neg.
4	Neg.	+++	++	Neg.	++	+++
5	Neg.	Neg.	++	++	Neg.	Neg.
6	Neg.	+++	++	+	++	+
7	Neg.	++	Neg.	Neg.	+	++
8	Neg.	++	+	Neg.	++	++
9	++	Neg.	Neg.	Neg.	+	+++
10	Neg.	Neg.	Neg.	Neg.	+	Neg.
11	++	Neg.	+++	++	+	++
12	Neg.	+	+++	++	+	+
13	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
14	Neg.	+	+++	+++	++	+
15	Neg.	Neg.	+	+	Neg.	Neg.
16	++	Neg.	Neg.	Neg.	++	+
17	Neg.	+	+	Neg.	+	Neg.
18	Neg.	+++	+++	++	+++	+
19	Neg.	+++	+++	+	++	+
20	Neg.	+	+	Neg.	++	++

PGN- proliferative glomerulonephritis; MPGN- membranoproliferative glomerulonephritis; IN – interstitial nephritis; TIN – tubular-interstitial nephritis; GBMT- Glomerular basement membrane thickening; GCT- glomerular capsule thickening. Intensity : + discrete; ++ moderate; +++ severe, Neg. – no lesion.

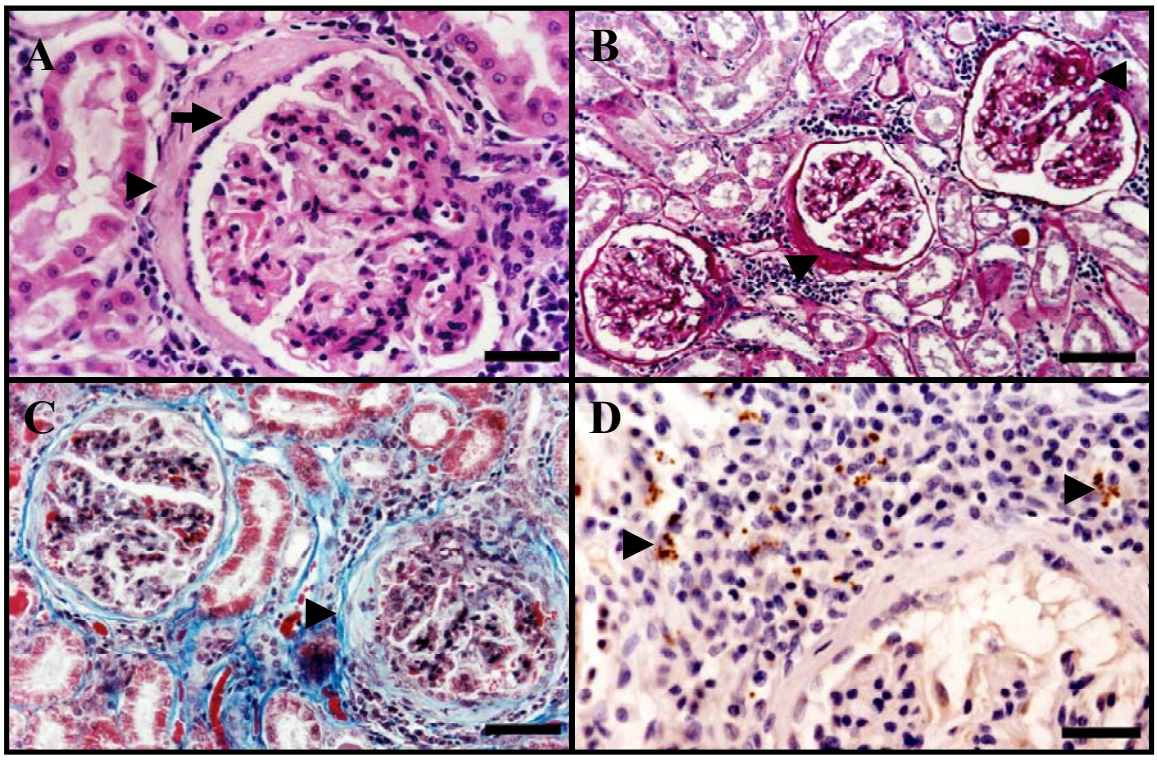

Figure 1. A) Canine Visceral Leishmaniasis in *Leishmania* sp. naturally infected dog, kidney. Interstitial and periglomerular lymphoplasmacytic inflammatory infiltrate. Basement membrane and Bowman´s capsule thickening (arrowhead) and epithelization (arrow) (H&E – 40x, bar=30µm). B) PAS positive material deposition on glomerular basement membrane (arrowheads) and periglomerular mononuclear infiltrate (PAS - 20x, bar=30µm). C) Bowman´s capsule fibrosis (arrowhead) associated with periglomerular mononuclear infiltrate (Masson trichrome – 40x, bar=30µm). D) Immunolabelled *Leishmania* sp. amastigotes (arrowheads) mild lymphoplasmacytic periglomerular inflammatory infiltrate (LSAB, 40x, bar=30µm).

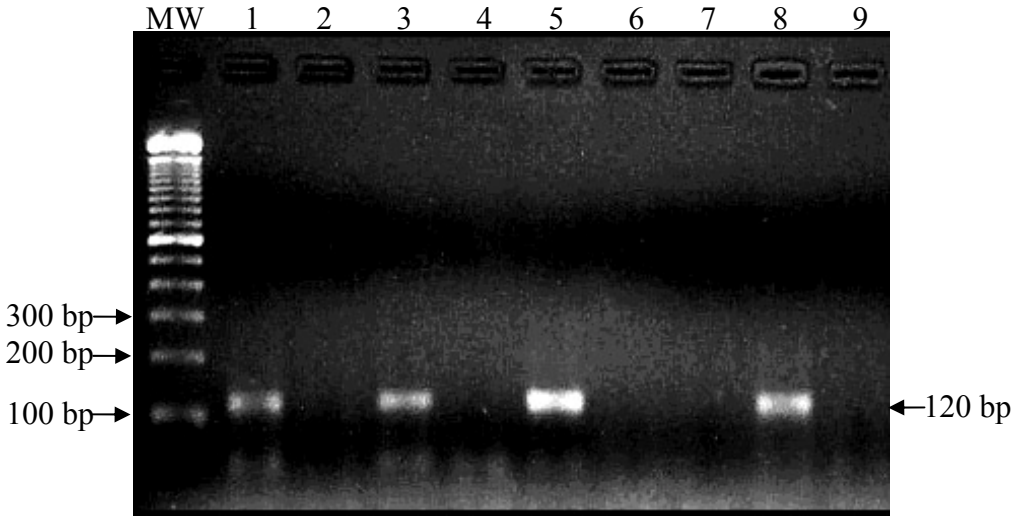

Figure 2. Agarose gel electrophoresis analyzed under UV light. MW – molecular weight; Lines 1, 3 and 5: kidney samples presenting amplification of a 120 bp amplicon consistent with *Leishmania* sp.; Lines 2, 4, 6 and 7: negative kidney samples; Line 8: positive control (cultured *Leishmania* DNA); Line 9: negative control.

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REFERENCES

1. Castro AG. Controle, diagnóstico e tratamento da leishmaniose visceral. Brasília: Fundação Nacional de Saúde, 1996. p. 86.

2. Botilde Y, Laurent T, Quispe TW, Chicharro C, Cañavate C, Cruz I, et al. Comparison of molecular markers for strain typing of Leishmania infantum. Infect Genet Evol. 2006; 6: 440-6.

3. Blavier A, Keroack S, Denerolle PH, Goy-Thollot I, Chabanne L, Cadoré JL, et al. Atypical forms of canine leishmaniosis. Vet J. 2001; 162: 108- 20.

4. Ferrer L, Aisa MJ, Roura X, Portu M. Serological diagnosis and tratament canine leishmaniasis. Vet Rec. 1995; 136: 514- 6.

5. Abranches P, Silva-Pereira MCD, Conceição-Silva FM, Santos GGM, Janz JG. Canine leishmaniasis: pathological and ecological factors influencing transmission of infection. J Parasitol. 1991; 77: 557- 61.

6. Duarte MIS, Silva MR, Goto H, Nicodemo EL, Amato NV. Intersticial nephritis in human Kala-azar. Trans R Soc Trop Med Hyg. 1983; 77: 531- 7.

7. Poli A, Abramo F, Manciante F, Nigro M, Pieri S, Bionda A. Renal involvement in canine leishmaniasis: a light-microscopic, immunohistochemical and electron-microscopic study. Nephron. 1991; 57: 444-52.

8. Slappendel RJ, Ferrer L. Leishmaniasis. In: Greene CE. Infectious disease of the dog and cat. 2th ed. Philadelphia: Saunders, 1998. p. 450-8.

9. Costa FAL. Patologia e imunopatogenia da nefropatia da Leishmaniose visceral canina [dissertação]. São Paulo: Universidade de São Paulo, Faculdade de Medicina Veterinária e Zootecnia; 2001.

10. Benderitter TH, Casanova P, Nashkidachvili L, Quilici M. Glomerulonephritis in dogs with canine leishmaniasis. Ann Trop Med Parasitol. 1988; 82: 335- 41.

11. Soares MJV, Moraes JRE, Palmeira Borgues V, Miyazato LG, Moraes FR. Renal Involviment in Visceral Leishmaniasis Dogs. J Venom Anim Toxins Incl Trop Dis. 2005; 11: 579- 93.

12. Yancey KB, Lawley TJ. Circulating immune complexes: Their immunochemistry, biology and detection in selected dermatologic and systemic diseases. J Am Acad Dermatol. 1984; 10: 711-31.

13. Mancianti F, Bionda A. Analysis of renal immune-deposits in canine leishmaniasis. Preliminary results. Parasitology. 1989; 31: 213-30.

14. Tafuri WL, Olivera MR, Melo MN. Canine visceral leishmaniosis: a remarkable histopathological picture of one case reported from Brazil. Vet Parasitol. 2001; 96: 203-12.

15. Moreira MAB, Luvizotto MCR, Garcia JF, Corbett CE, Laurenti MD. Comparison of parasitological, immunological and molecular methods for the diagnosis of leishmaniasis in dogs with different clinical signs. Vet Parasitol. 2007; 145: 245-52.

16. Moraes JRE, Soares MJV, Roselino AMF. Polymerase chain reaction in detecting *Leishmania sp* in symptomatic and asymptomatic seropositive dogs. J Venom Anim Toxins Incl Trop Dis. 2005; 11: 523- 39.

17. Rodgers MR, Popper SJ, Wirth DF. Amplification of kinetoplast DNA as a tool in the detection diagnosis of Leishmania. Exp Parasitol. 1990; 71: 267-75.

18. Blantz RC, Wilson CB, Gabbai FB. Pathophysiology of acute immune injury. In*:* Neilson EG, Couser WG, editors. Immunologic renal diseases. Philadelphia: Lippincott-Raven Publishers, 1997. p. 237-50.

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