ANA CLARA BASTOS RODRIGUES

Morphological changes affecting the eye bulb of dogs naturally infected with *Leishmania infantum/chagasi*

São Paulo

2019

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Dissertation presented to the Postgraduate Program in Anatomy of Domestic and Wild Animals of the Faculty of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the title of Master in Science.

Departament:

Surgery

Concentration Area:

Anatomy

Advisor:

Profa. Dra. Maria Angélica Miglino

São Paulo

2019

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DADOS INTERNACIONAIS DE CATALOGAÇÃO NA PUBLICAÇÃO

(Biblioteca Virginie Buff D'Ápice da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo)

T. 3828
FMVZ

Rodrigues, Ana Clara Bastos

Morphological changes affecting the eye bulb of dogs naturally infected with *Leishmania infantum/chagasi* / Ana Clara Bastos Rodrigues. – 2019.
43 f. : il.

Título traduzido: Alterações morfológicas que acometem o bulbo do olho de cães infectados naturalmente por *Leishmania infantum/chagasi*.

Dissertação (Mestrado) – Universidade de São Paulo. Faculdade de Medicina Veterinária e Zootecnia. Departamento de Cirurgia, São Paulo, 2019.

Programa de Pós-Graduação: Anatomia dos Animais Domésticos e Silvestres.
Área de concentração: Anatomia dos Animais Domésticos e Silvestres.
Orientadora: Profa. Dra. Maria Angélica Miglino.

1. Leishmaniose ocular canina. 2. Fibroblastos. 3. Córnea. 4. Bulbo do olho. 5. Microscopia eletrônica de varredura. I. Título.

Certificate of Ethics Committee

UNIVERSIDADE DE SÃO PAULO

FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

Comissão de Ética no Uso de Animais

São Paulo, 19 de junho de 2019
CEUA N [8545080817](#)

Ilmo(a). Sr(a).

Responsável: Maria Angélica Miglino

Área: Anatomia Dos Animais Domésticos E Silvestres

Título da proposta: "Alterações morfológicas que acometem o bulbo do olho de cães infectados naturalmente por *Leishmania infantum*/chagasi".

Parecer Consubstanciado da Comissão de Ética no Uso de Animais FMVZ (ID 004789)

A Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, no cumprimento das suas atribuições, analisou e **APROVOU** a Alteração do cadastro (versão de 16/abril/2019) da proposta acima referenciada.

Resumo apresentado pelo pesquisador: "Alteração do título da proposta para se ajustar à nomina anatômica atualizada.".

Comentário da CEUA: "".

Profa. Dra. Anneliese de Souza Traldi

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Faculdade de Medicina Veterinária e Zootecnia da Universidade
de São Paulo

Roseli da Costa Gomes

Secretária

Faculdade de Medicina Veterinária e Zootecnia da Universidade
de São Paulo

PERFORMANCE EVALUATION

Author: RODRIGUES, Ana Clara Bastos

Title: **Morphological changes affecting the eye bulb of dogs naturally infected with *Leishmania infantum/chagasi***

Dissertation presented to the Postgraduate Program in Anatomy of Domestic and Wild Animals of the Faculty of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the title of Master in Science.

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DEDICATION

Dedico todo o esforço que depositei neste trabalho ao meu pai Eduardo e à minha mãe Ana Letícia que nunca mediram esforços para me proporcionar a melhor educação e as melhores oportunidades; à eles que me ajudam com calma, força e esperança durante toda essa jornada.

ACKNOWLEDGMENT

Agradeço a Deus pela força e infinita misericórdia que recebi d'Ele durante esses anos de estudo. Agradeço imensamente à minha família e amigos por me darem apoio e calma nas horas difíceis, em especial ao meu namorado Daniel por todo o companheirismo e amizade durante esses dois anos.

À Anaelise, Ana Lúcia, Carla Maria, Luana e Marisol que foram essenciais na concretização desse projeto. Agradeço também à Celina pela ajuda com as descrições patológicas bem como a Profa. Dra. Claudia Momo;

Agradeço a Profa. Dra. Maria Angélica Miglino pela orientação e por abrir as portas da pós-graduação para mim; e a Profa. Dra. Ana Lúcia pelas suas instruções e por dividir um pouco de seus conhecimentos comigo abrindo as portas da vida acadêmica desde a graduação;

Aos técnicos dos laboratórios Diogo, Ronaldo e Rose Eli e à secretária Fabiana pelo apoio e prestação de serviço;

À Faculdade de Medicina Veterinária e Zootecnia da USP - Departamento de Cirurgia e ao Programa de Anatomia dos Animais Domésticos e Silvestres pela formação e instrução acadêmica.

À Universidade Estadual do Maranhão e ao laboratório de patologia do curso de medicina veterinária por ser uma base de apoio durante às coletas realizadas;

À CAPES: “O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001;

Ao Centro Avançado em Diagnóstico por Imagem – CADI da Central de Facilidades à Pesquisa da Faculdade de Medicina Veterinária e Zootecnia da USP;

Por fim, a todos aqueles que direta e indiretamente contribuíram para a minha formação acadêmica e pessoal.

“...porque existe uma grande verdade neste planeta: seja você quem for, quando quer com vontade alguma coisa, é porque este desejo nasceu na alma do Universo. É sua missão na Terra.

(O Alquimista)

RESUMO

RODRIGUES, A.C.B. **Alterações morfológicas que acometem o bulbo do olho de cães infectados naturalmente por *Leishmania infantum/chagasi***. 2019. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2019.

A leishmaniose visceral é uma zoonose transmitida de forma vetorial, que sucede a picada de flebotomíneos infectados, e possui o agente etiológico protozoário do gênero *Leishmania*. O objetivo proposto é descrever as alterações oculares que podem afetar o bulbo do olho de cães infectados naturalmente pela leishmaniose e descrever as alterações morfológicas que ocorrem através da interação entre o parasito e as estruturas oculares. Foram realizadas duas coletas no município de São Luís – MA do bulbo do olho de 12 cães ao todo. Os animais foram submetidos a uma avaliação clínica oftalmológica e individualizados quanto à presença de doenças oculares. Os cães foram doados com o consentimento do proprietário, de acordo com as regras do Conselho Nacional de Controle de Experimentação Animal (CONCEA). Todos os animais foram classificados como sintomáticos por avaliação clínica e as manifestações oculares observadas foram: uveíte, ceratoconjuntivite seca, úlcera de córnea, conjuntivite e secreção ocular purulenta, principalmente. De acordo com o tipo de infiltrado inflamatório, o mais prevalente foi o linfoplasmático. A conjuntiva bulbar, limbo e córnea foram a região do olho mais afetada por infiltrados. Pela IHC observamos a marcação de amastigotas pelo anticorpo anti-*Leishmania* nas túnicas bulbares e o anticorpo Vimentina para fibroblastos associados com as formas amastigotas. A marcação dos fibroblastos foi positiva na córnea e limbo do olho. Através da microscopia eletrônica de varredura (MEV), observamos a ultraestrutura do que apresentou hiperplasia do epitélio da córnea, desorganização do estroma e reação inflamatória exacerbada na túnica vascular e fibrosa. Diante os resultados obtidos, as manifestações são caracterizadas pela alta frequência de infiltrados inflamatórios, principalmente do tipo linfoplasmático. Os fibroblastos presentes na córnea podem atuar como células hospedeiras importantes frente à doença sistêmica, o que traz uma nova perspectiva sobre o modo de ação do parasito nas túnicas oculares.

Palavras-chave: Leishmaniose ocular canina. Fibroblastos. Córnea. Bulbo do olho. Microscopia eletrônica de varredura.

ABSTRACT

RODRIGUES, A.C.B. **Morphological changes affecting the eye bulb of naturally infected dogs by *Leishmania infantum/chagasi***. 2019. Dissertation (Master in Science) – Faculty of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, 2019.

Visceral leishmaniasis is a vector-borne zoonosis that succeeds the bite of infected sandflies and has the protozoan etiological agent of the genus *Leishmania*. The main idea of this study is to describe the ocular changes that can affect the eye bulb of dogs naturally infected by leishmaniasis and to describe the morphological changes that occur through the interaction between the parasite and the ocular structures. Two collections were carried out in the city of São Luís - MA from the eye bulb of 12 animals. The animals were submitted to a clinical ophthalmologic evaluation and later individualized for the presence of ocular diseases. The animals were donated with consent of the owner, according to the rules of National Council for Control of Animal Experimentation (CONCEA). All animals were classified as symptomatic by clinical evaluation. Ocular manifestations were: uveitis, keratoconjunctivitis, corneal ulcer, conjunctivitis and purulent ocular secretion mainly. The bulbar conjunctiva, limbus and cornea were the most affected region by infiltrates. By IHC we observed amastigote labeling by anti-*Leishmania* antibody on bulbar tunics and Vimentin antibody for fibroblasts associated with amastigote forms. Fibroblast labeling was positive in the cornea and limbus of the eye. Through scanning electron microscopy (SEM), we observed the ultrastructure of corneal epithelium hyperplasia, stromal disorganization and exacerbated inflammatory reaction in fibrous tunic. Given the results obtained, the manifestations are characterized by the high frequency of inflammatory infiltrates, mainly lymphoplasmocytic type. Fibroblasts present in cornea may act as important host cells in face of systemic disease, which brings a new perspective on the action of the parasite in eye tunics.

Keywords: Canine ocular leishmaniasis. Fibroblast. Cornea. Eye bulb. Scanning Electron Microscopy.

L IST OF FIGURES

Figure 1 - Photomicrograph of inflammatory infiltrates in the eye bulb tunics	26
Figure 2 - Photomicrograph of discrete inflammatory infiltrate with amastigous forms compatible with <i>Leishmania sp.</i>	27
Figure 3 – Photomicrograph of the eye bulb tunics	28
Figure 4 – Scanning electron microscopy photomicrograph of the cornea	28
Figure 5 – Photomicrograph of scanning electron microscopy of the vascular bulb of the eye bulb	29
Figure 6 – Photomicrograph of the corneal immunohistochemistry of Animal 1	29
Figure 7 – Photomicrograph of the immunohistochemistry of the fibrous tunic represented by the transition area between the cornea and the sclera (limbus)	30
Figure 8 – Photomicrograph of the immunohistochemistry of the fibrous tunic represented by corneal region with Vimentin marker. A) Control with H-E staininig demostrating amastigotes - 100X magnification. B) IHC for vimentin marker demonstrating fibroblast as host cells for amastigostes - 100X magnification	31

LIST OF TABLES

Table 1 – Clinical findings observed in <i>Leishmania sp.</i>	23
Table 2 – Ophthalmic diseases in dogs naturally infected by <i>Leishmania sp.</i>	23

LIST OF GRAPHICS

Graphic 1 – Ophtalmic changes observed in dogs positives for leishmaniasis	24
Graphic 2 - Frequency of inflammatory infiltrate based on the number of studied animals.....	25
Graphic 3 – Frequency of inflammatory infiltrate based on the affected area of the eye.....	25

SUMMARY

1.INTRODUCTION	15
2. PURPOSE.....	16
2.1 General purpose.....	16
2.2 Specific purpose	16
3. LITERATURE REVIEW.....	16
Leishmaniasis	16
Historic	16
Etiology.....	17
Transmission.....	17
Diagnostic	18
Clinical manifestations.....	18
Eye structures and injuries.....	18
4. MATERIAL AND METHOD.....	19
Animals and region of study.....	19
Animals and Clinical evaluation.....	20
Histological processing	21
Scanning Electron Microscopy Technique	21
Immunohistochemistry (IHC) technique	21
IHC for anti-Leishmania antibody	21
IHC for fibroblast with vimentin antibody.....	22
Statistical Analysis.....	22
5. RESULTS.....	22
Animals and ophthalmic clinical sings	22
Histopathological Analysis.....	26
Scanning Electron Microscopy (SEM).....	28
Immunohistochemistry (IHC)	29
6. DISCUSSION	31
7. CONCLUSION	34
ANNEX.....	Erro! Indicador não definido.

1.INTRODUCTION

Visceral leishmaniasis is a vector-borne zoonotic disease that follows a bite from infected sand flies. It has as protozoan etiological agent of the genus *Leishmania* (WHO, 2010). In Brazil, the disease remains as a major challenge in public health issues, especially due to its endemic potential in many states. Dogs are considered the main domestic reservoirs and should be monitored through serological surveys recommended by the Ministry of Agriculture, Livestock and Supply (MAPA, 2016).

Researchs linked to the world health organization (WHO) found that leishmaniasis has an impact in about 90 countries and hamper in their productivity and socioeconomic progress. They occur due to mainly environmental risk factors, as many of these countries suffer from serious economic, social and public health problems such as Syria, India, Bangladesh, Brazil, Bolivia and Peru (OMS, 2014).

Leishmaniasis is a chronic disease with an incubation period ranging from 3 months to 7 years in reservoir animal. Clinical manifestations of visceral leishmaniasis are extremely variable and represent an association of visceral and cutaneous disease. Intracellular microorganism induces an extreme immune response and parasitized dogs have a wide and varied clinical symptomatology involving various organs, including eyes and its attachments (SOLANO-GALLEGO et al., 2009) that may be the first or only apparent alteration of this disease (FULGÊNCIO 2006; ROZE, 2014).

Eye damage occurs as a result of direct parasitism and immunomediated mechanisms through the deposition of immunocomplexes caused by the agent, it can be unilateral or bilateral and present more than one alteration in the same eye (PEÑA et al., 2000; BRITO et al., 2006). Anterior ocular segment (cornea, anterior chamber, iris, posterior and crystalline chamber) and posterior ocular segment (vitreous chamber) may be affected, but research reports of ophthalmopathies prevalence are more related to anterior segment (MOLLEDA et al., 1993; BRITO et al., 2006).

2. PURPOSE

2.1 General purpose

To describe the main morphological changes that affect the eye bulb of dogs infected with leishmaniasis.

2.2 Specific purpose

- Describe macroscopically morphological changes that affect the eye bulb of infected dogs and the ophthalmic diseases that dogs with leishmaniasis may present;
- Describe microscopically by H-E staining the histopathological changes that affect the ocular structures;
- Perform immunohistochemistry (IHC) labeling of amastigotes with anti-*Leishmania* antibody in order to determine the mechanisms of action of the parasite in the eye.
- Perform fibroblast labeling using Vimentin antibody, on the eye tunics to characterize this cell type as amastigote host cell.
- Evaluate changes in bulb ultrastructure (scanning electron microscopy) of infected dogs.

3. LITERATURE REVIEW

Leishmaniasis

Historic

Human Visceral Leishmaniasis (HVL) was only described in 1835 in Greece, being called “ponos” or “hapoplinakon”, in which a case of infantile splenomegaly was reported. Subsequent records occurred mainly in India in 1869, where the HVL was named "kala-jwar", meaning black fever or "kala-azar" (MARZOCHI et al., 1981).

The first autochthonous case in Americas was registered in Paraguay by Migone in 1913 of a man from Brazil. After 20 years, Penna first identified the parasite on histological slides from livers of 41 patients out of a universe of 40,000 biopsies of individuals who died with suspected yellow fever (CAVALCANTI, 2012). The parasite was classified as *Leishmania chagasi* (Chagas et al., 1938). In 1956, dogs and foxes were then classified as a natural reservoir of parasite in areas of greatest endemic expression, defining the disease as a zoonosis (DEANE, 1956).

Etiology

Parasites of genus *Leishmania* are flagellated from *Trypanosomatidae* family of the order *Kinetoplastida* and phylum *Protozoa*. They have heteroxene biological behavior, that is, they need more than one host to complete their life cycle, one vertebrate and one invertebrate represented by the vector (phlebotomine) (SCHLEIN, 1993).

They have two evolutionary forms: promastigote, which is flagellate and extracellular found in the gut of sandflies, and obligate intracellular amastigote, found inside macrophages of vertebrate hosts (URQUHART et al., 1998). Promastigote forms have elongated body, measuring between 14 and 20 mm and free flagella. Amastigotes have an ovoid body, measuring between 2.1 and 3.2 mm and internal flagella (CUNNINGHAM, 1885).

Vectors are Diptera of the family *Psychodidae*, hematophagous belonging to the genera *Phlebotomus* (Old World) and *Lutzomyia* (New World), with wide distribution in warm and temperate climates. They are generally small insects that have a body covered with long and numerous bristles, often mixed with scales, and have long wings resembling a tiny moth. They have twilight and post-twilight activity, shelter during the day in humid, dark and well protected from the winds. Eggs are laid in moss-covered places and evolution to adulthood takes about a month (FIOCRUZ, 2014).

Transmission

Transmission of parasite occurs predominantly through vector insects that, when performing a blood repast on the vertebrate host, ingest the amastigote form and, after numerous changes in their digestive tract, evolve to promastigote phase. After morphological and physiological modulations, some of these forms differ in a metacyclic promastigotes about 48 hours after ingestion of the amastigote forms. Infective promastigotes replicate in the insect's gut and then migrate to the alimentary canal region, where adhering to the stomode valve epithelium, damages it. Thus, when performing a new repast, female sandfly regurgitates na infecting form of the parasite, infecting a new host, in which case it may be a human being (BATES, 2007; PACE, 2014).

After being inoculated, it remains in extracellular space, promoting complement activation and consequent leukotaxis. During this process, some promastigote forms are destroyed by polymorphonuclear cells, while others are incorporated by macrophages and surrounded by phagosomal membrane, resulting in a parasitophagus vacuole where they multiply (HANDMAN & BULLEN, 2002).

Diagnostic

The main serological methods used to detect circulating antibodies in dogs are the Indirect Immunofluorescence Reaction (RIFI), the enzyme linked immunosorbent or enzyme linked immunosorbent assay (ELISA) and the TRDPP® immunochromatographic rapid test produced by Biomanguinhos (MAPA, 2016).

RIFI has sensitivity and specificity ranging from 60% to 100% (SIDDIG et al., 1988; ALMEIDA et al., 2005; LIMA et al., 2015), so it may present cross-reactivity with other diseases such as: trypanosomiasis, erlichiosis, babesiosis, heartworm and borreliosis (ZANETTE et al., 2014). Therefore, TR DPP® is used as a screening method by field teams and, within laboratories, ELISA as a confirmatory test for seroreactive animals (FUNED, 2013).

PCR technique is the most widely used molecular diagnostic technique as a counterproof against negative results in samples where it is not possible to isolate parasites in serological tests, as it allows the identification of the DNA in samples, which makes this method most expensive (IKEDA-GARCIA & MARCONDES, 2007).

Studies in dogs have shown 100% sensitivity in symptomatic animals, 96% in oligosymptomatic animals and 95.65% in asymptomatic animals. However, this test requires equipment and well-trained laboratory technicians to avoid sample contamination as this technique is capable of detecting small amounts of parasite DNA (MOREIRA et al., 2007).

Clinical manifestations

Clinical manifestations of Canine Visceral Leishmaniasis (CVL) are extremely variable and represent an association of visceral and cutaneous disease. Intracellular microorganism induces an extreme immune response and parasitized dogs have a wide clinical symptomatology involving various organs, including the eyes and their attachments (KOUTINAS et al., 1999) that may constitute the first or only apparent alteration of the disease. (FULGÊNCIO, 2006; ROZE, 2004).

The most commonly found alterations are in the skin barrier resulting in skin diseases, erythema, ulcers, onychogryphosis, localized or generalized lymphadenopathy, ophthalmopathies, anorexia, weight loss, cough, hyperthermia, epistaxis, gastroenteropathies, nephropathy, polyuria, polydipsia, haematuria and hepatopathy (CIARAMELLA et al., 1997).

Eye structures and injuries

Eye damage occurs as a result of direct parasitism and/or immunomediated mechanisms through the deposition of immunocomplexes caused by the agent, can be unilateral

or bilateral and present more than one alteration in the same eye (PEÑA et al., 2000; BRITO et al., 2006). Anterior ocular segment (cornea, anterior chamber, iris, posterior and crystalline chamber) and posterior ocular segment (vitreous chamber) may be affected, but research reports of ophthalmopathies prevalence are more related to anterior segment (MOLLEDA et al., 1993; BRITO et al., 2006).

Eyelids have three types of well-differentiated lesions, which are determined by time, degree of involvement and chronicity of disease. Such lesions differ histologically in the amount of inflammatory infiltrate and number of parasitized macrophages; they are called blepharitis that can be ulcerative, nodular and diffuse type (VILLAGRASA et al., 2002).

Conjunctiva is one of the main sites of inflammation due to its lymphoid activity, which justifies in histological sections, presence of amastigote forms in this tissue (BRITO et al. 2006). Due to these characteristics it can cause intense inflammation at the site, characterized by conjunctivitis (FULGÊNCIO et al. 2004).

In the ocular tunics, the sclera and cornea are observed externally, the vascular portion comprising the uveal tract (ciliary body, iris, choroid) and the nerve portion of the eye corresponding to the retina and optic nerve. (GELATT, 2006).

In cornea evaluation of infected animals, a disorganization of stroma, neovascularization and corneal edema can be observed, causing loss of transparency and pigmentation, especially regarding the junction between the sclera and the cornea, a region called limbus. (BRITO et al., 2010).

In some studies, uveitis is the most common ocular manifestation in leishmaniasis due to its high lymphoid activity (MOLLEDA et al., 1993). Therefore, when amastigote form reaches uveal tract, there is widespread formation of immunocomplexes and deposition of these in vascular wall associated with an immune response, hyperglobulinemia and antinuclear antibodies, inducing uveitis in infected dogs, being the most prevalent, granulomatous uveitis (GARCIA-ALONSO et al., 1996).

4. MATERIAL AND METHOD

Animals and region of study

The Study was partially carried out in São Luís county, located in Maranhão state in northeastern Brazil. The region occupies an area of 834,785 km² at the geographic coordinates, latitude 2 ° 35'37 "S, longitude 44 ° 12'0" W, at 24 meters above sea level and has

a tropical, hot and humid climate (IBGE, 2010), a predisposing factor for leishmaniasis expansion in this region.

Eye bulbs were collected from dogs naturally affected by the disease. The gathering was made in the service of Francisco Uchoa Lopes Veterinary Hospital of State University of Maranhão (UEMA) campus and in the Zoonoses Surveillance Unit (UVZ) of the municipality.

The research was conducted at Surgery Department (VCI) of Faculty of Veterinary Medicine and Animal Science at the laboratories of the Post-Graduate Program in Anatomy of Domestic and Wild Animals of State University of São Paulo, Butantã campus, where it was possible to perform all analyzes, macroscopic, microscopic and scanning electron microscopy analysis. These laboratories are part of the “facilitie” CADI - Advanced Diagnostic Imaging Center of FMVZ-USP.

Animals and Clinical evaluation

In all, 12 animals were euthanized according to leishmaniasis positivity by ELISA tests and leishmaniasis rapid test. All animals tested positive for *Leishmania spp.* towards the rapid test, which performs the qualitative detection of anti-*Leishmania* antibody in whole blood samples.

Subsequently, blood was collected from these animals to perform ELISA test, complete blood count and tests for differential diagnosis of other infectious and parasitic diseases that may cause eye damage, such as: babesiosis, erlichiosis and distemper.

The animals were clinically evaluated by semiological inspection and palpation techniques to observe clinical signs compatible with CVL, such as lymphadenopathy, cachexia, skin lesions, onychogryphosis and ophthalmopathies.

Eye bulb and its attachments were initially examined in a bright environment, and presence of more evident alterations was observed, such as secretion, hyperemia, edema, periocular alopecia, injuries and asymmetries. Subsequently, the examination was performed in a dark room with na aid of a spotlight, allowing a visualization of structures such as cornea, iris and lens, as well as the eye attachments.

After clinical examination, animals were contained and submitted to peripheral blood collection for serological analysis. Blood collection was performed by puncture of the cephalic or jugular vein after adequate aseptic site, about 4.0mL of blood was collected with sterile and disposable syringe and needle (25 x 7mm).

Blood samples were collected from animals, placed in tubes without EDTA and kept refrigerated until further centrifugation at 1500 rpm for 10 minutes to separate the serum.

Once separated, the serum were removed from the tubes by means of tips and transferred to eppendorfs and frozen at -20°C until biochemical and serological tests were performed to confirm the disease.

Histological processing

Samples were fixed in 10% formaldehyde solution for 48 hours. It were then dehydrated at serial alcohol concentrations (70% to 100%) over a period of 24h in each alcohol, later diaphanized in Xylol (4h) and embedded in paraffin (4h). Bocks of paraffin were obtained and sectioned with 5µm in automatic microtome (Leica, RM2165). Sections were adhered to histological slides and stained with Hematoxylin and Eosin (HE). Slides were then photographed under a light microscope (Nikon Eclipse E-80i) at the Advanced Center for Diagnostic Imaging - CADI-FMVZ-USP.

Scanning Electron Microscopy Technique

Eye fragments related to the bulb tunic were analyzed in order to observe the microstructure through cross sections. All samples, after fixation in 10% buffered formaldehyde solution, were washed with 1% PBS in three baths for 5 minutes and washed with distilled water in the ultrasound machine for 3 minutes. They were then dehydrated in increasing series of alcohols at concentrations of 70%, 80%, 90% and 100%, passed to LEICA EM CPD300 critical point apparatus (FMVZ-USP) and bonded with carbon glue on metal bases aluminum and gold plated on the EMITECH K550 (FMVZ-USP). They were then analyzed and photographed in a LEO 435VP scanning electron microscope (FMVZ-USP).

Immunohistochemistry (IHC) technique

The purpose of the IHC was to observe the marking of amastigote forms of *Leishmania* sp. using anti-Leishmania antibody and fibroblast using vimentin antibody in histological sections of the eye bulb of dogs naturally infected with canine visceral leishmaniasis. This technique was performed in two laboratories, the first was the Pathology Laboratory from UEMA and also the Histology lab from Surgery Department of FMVZ.

IHC for anti-Leishmania antibody

Immunohistochemistry was performed by the streptavidine peroxidase technique. Tissue was fixed in 4% paraformol (PFA), and histological processing was performed as previously described. The slides were deparaffinized in Xylol I and II (15 minutes each), incubated in alcohol (absolute I and II, 90%, 80% and 70% alcohol for 3 minutes each) and

water and submitted to a *Phosphate buffer bath* 1x saline (PBS) for 5 minutes. Next, 3% endogenous peroxidase blockade, diluted in 1x PBS, was performed for 30 minutes at room temperature and a PBS bath for 5 minutes. Nonspecific protein blockade was performed with skimmed milk powder (Molico® 0.06g to 20ml of 1X PBS) and then incubated in a humid chamber for 45 minutes and washed with 1X PBS for 5 minutes. Then, primary antibody (hyperimmune dog serum naturally infected with *L. infantum chagasi* at 1: 1000 dilution in 1X PBS) was added. Then, it was incubated in a humid chamber at 4°C for 24 hours.

After this time, biotinylated secondary antibody (goat anti-mouse serum) at 1/100 dilution (Dako Envision + Dual Link System-HRP) was added. Again the slides were incubated in a humid chamber for 45 minutes at room temperature and then washed in 1X PBS. The reaction was visualized using a Diaminobenzidine substrate (DAB) incubated for 5 min and the reaction stopped in distilled water. The sections were counterstained with Harris Hematoxylin for 60 seconds and washed in water for 10 minutes. Subsequently, increasing dehydration of ethanol (70 °, 80 °, 90 °), incubated in Xylol I and II, and permount mounting were performed. The sections were analyzed under light microscope in the 40x and 100x objective.

IHC for fibroblast with vimentin antibody

For this protocol, we follow all producer recommendations (Kit DAKO System K800 for imunohistochemistry), with 1:50 antibody (Vimentin) concentration.

Statistical Analysis

A descriptive frequency analysis was performed for nominal qualitative variables by the R program. Two frequency analysis for pathological lesions (macroscopy and microscopy) ophthalmic diseases and inflammatory infiltrates in the eye according to the total number of animals.

5. RESULTS

Animals and ophthalmic clinical sings

All animals included in the study were positive for leishmaniasis by the indirect ELISA test and the TRDPP Biomanguinhos® and Alere® AC leishmaniosis Test. Both are recommended by the Ministry of Agriculture, Livestock and Supply (MAPA). All animals (100%) were classified as symptomatic by clinical evaluation and showed at least two or more characteristic clinical signs of canine visceral leishmaniasis (CVL) (Table 1).

Table 1: Clinical findings observed in *Leishmania sp.* seropositive antibodies.

CLINICAL SIGNS	% OF ANIMALS
Lymphadenomegaly	91,66%
Eye Manifestations	83,33%
Skin lesions	66,66%
Onychogryphosis	50%
Hepatomegaly	33,33%
Cachexia	41,66%
Total	Nº of animals = 12 (100%)

Source: Rodrigues (2019).

Legend: Percentage related to clinical signs observed in dogs with CVL.

Regarding ophthalmic lesions (Table 2), changes related more to the anterior compartment of the eye than to the posterior were observed, where purulent ocular secretion was the most frequent alteration, represented in 66.67% of the animals, followed by changes in the córnea and blepharitis.

Table 2: Ophthalmic diseases in dogs naturally infected with *Leishmania sp.*

Animal	Macroscopic Ophthalmic Pathologies
Animal 1 (A1)	No apparent macroscopic injury
Animal 2 (A2)	Conjunctivitis and Purulent Ocular Secretion
Animal 3 (A3)	Uveitis and Lens Opacity
Animal 4 (A4)	Corneal Ulcer
Animal 5 (A5)	Dry Keratoconjunctivitis and Purulent Ocular Secretion
Animal 6 (A6)	Purulent Eye Secretion / Conjunctivitis / Blepharitis / Chemosis
Animal 7 (A7)	Purulent Secretion / Tropical Keratopathy
Animal 8 (A8)	Corneal Edema / Blepharitis / Corneal Ulcer Scar
Animal 9 (A9)	Lens Opacity/Corneal Ulcer Scar
Animal 10 (A10)	Purulent Ocular Secretion / Conjunctivitis
Animal 11 (A11)	Lens Opacity / Corneal Edema / Purulent Eye Secretion
Animal 12 (A12)	No apparent macroscopic injury

Source: Rodrigues (2019)

Legend: Ophthalmic clinical signs observed in dogs positive for leishmaniasis by the Biomanguinhos® and ELISA rapid test.

Other prominent eye changes are blepharitis, conjunctivitis and corneal changes, representing about 25% to 34% of animals with ophthalmic pathological lesions (Graphic 1). Graphic 1 shows the frequency with which ophthalmic changes appear, thus highlighting purulent eye discharge and changes in the cornea as the most significant changes.

Graphic 1: Ophthalmic changes observed in dogs positive for leishmaniasis.

Source: Rodrigues (2019).

Regarding inflammatory infiltrates, a frequency analysis was performed based on the number of animals and not the amount of infiltrate, since there were animals that had different types of infiltrates in different regions of the eye (Graph 2). It was observed that the most frequent type of infiltrate was the lymphoplasmocytic type shown in the limbo (66.66%), conjunctiva (66.66%), ciliary process (41.66%), cornea (16.66%), and retina (8.33%). The lowest frequency was the lymphocytic type identified only in the bulbar conjunctiva (8.33%).

Graphic 2: Frequency of inflammatory infiltrate based on the number of studied animals.

Source: Rodrigues (2019).

A frequency analysis was also performed according to the intensity of infiltrates in each region of the eye (graphic 3), showing a higher prevalence of infiltrates in the limb and conjunctival bulbar region.

Graphic 3: Frequency of inflammatory infiltrate based on the affected area of the eye.

Source: Rodrigues (2019).

Legend: **LPI** - lymphoplasmocytic; **LCI** – lymphocytic; **PI** – plasmocytic; **HI** – histiocytic; **NI** – neutrophilic.

Histopathological Analysis

Regarding histopathological examination, several areas of infiltrate and lesions could be observed, especially in anterior segment of the eye. According to the type of inflammatory infiltrate, the most prevalent was lymphoplasmic cell in fibrous tunic of the eye and transition junction between cornea and sclera. Bulbar conjunctiva, limb and cornea was the region of the eye most affected by infiltrates, where it was possible to observe a wide involvement ranging from the most discreet to the most intense.

In fibrous tunic, region corresponding to cornea and sclera, inflammatory infiltrates ranging from mild to intense were observed. In anterior epithelium of the cornea, neutrophilic and plasmacytic infiltrates stand out, whereas in stroma, neutrophilic, lymphoplasmic and histiocytic prevail associated with presence of intense intracytoplasmic amastigotes compatible with *Leishmania sp* (Figure 1). In one animal, an area of discrete hyperplasia of the focally extended corneal anterior epithelium could also be observed.

Figure 1: Photomicrograph of inflammatory infiltrates in the eye bulb tunics. A) Iris edema (arrow) - 40X magnification. B) Inflammatory lymphoplasmic cell infiltrate - 20X magnification. C) Inflammatory histiocytic infiltrate with compatible forms of amastigostas - 40X magnification. D) Amastigote forms compatible with *leishmania* spp. - sp (arrow) - 100X magnification.

Source: Rodrigues (2019).

In limbus, a discrete lymphoplasmocytic infiltrate with histiocytes associated with edema and a discrete focal plasmocytic infiltrate with lymphocytes and histiocytes associated with perivascularitis and edema were observed. In this region, amastigote forms compatible with *Leishmania sp.* in animals A7 and A11 in table 2 can be seen. Regarding the bulbar conjunctiva, it was possible to observe focal lymphocytic infiltrates with mild eosinophils and mild focal lymphoplasmocytic. In animals A7 and A11 (Figure 2), it can observe presence of amastigote forms compatible with *Leishmania sp.* in stroma of cornea.

Figure 2: Photomicrograph of discrete inflammatory infiltrate with amastigous forms compatible with *Leishmania sp.* A) A6 corneal stroma with moderate inflammatory infiltrate. 40X magnification. B) Amastigote forms compatible with *Leishmania sp.* (*). 100X magnification.

Source: Rodrigues (2019).

In vascular tunic, a structure comprising the iris, ciliary process and choroid, histopathological lesions could be observed. In iris, a structure that has a central opening to the pupil, pupillary occlusion was observed in two animals, where one of them had an association of this condition with hyperplasia of the iris pigmented superficial cells. In the ciliary process, discrete focal plasmocytic inflammatory infiltrate with a slight amount of lymphocytes and lymphoplasmic and histiocytic infiltrate associated with edema were observed. As for the retina, corresponding to nervous tunic, there was multifocal and focal dislocation with hypertrophy of the adjacent cells of retinal pigmented epithelium and the outer nuclear layer, respectively, observed in animals A2, A3 and A5 (Figure 3). In animal 10, there was discrete lymphoplasmic cell infiltrate.

Figure 3: Photomicrograph of eye bulb tunics. A) Retinal region with focal displacement (*). 40X magnification.

Source: Rodrigues (2019)

Attached is a table summarizing all histopathological changes related to inflammatory infiltrates and individualized lesions observed in the different regions of the eye bulb of this study (Annex 1).

Scanning Electron Microscopy (SEM)

As for the electronic scanning analysis, the ultrastructure of the eye bulb tunics was verified, in order to investigate the corneal alterations, bulbar structure disorders and destructive eye lesions. In cornea (Figure 4), epithelial hyperplasia, structural stromal disorganization, and inflammatory cell infiltration regions could be observed.

Figure 4: Photomicrograph of corneal scanning electron microscopy. A) Stromal disorganization. B) Disruption in the extracellular matrix of the cornea.

Source: Rodrigues (2019).

In vascular tunic (Figure 5), destructive lesions could be observed in addition to the infiltration of cells of the immune system, demonstrated by an exacerbated inflammatory reaction.

Figure 5: Photomicrograph of scanning electron microscopy of the vascular bulb of the eye bulb. A) Destructive lesion between the surface and central region of the choroid associated with immune system cells (arrow). B) Exacerbated inflammatory reaction (arrow) associated with uveitis.

Source: Rodrigues (2019).

Immunohistochemistry (IHC)

Amastigote forms from *Leishmania sp.* were identified inside macrophages and associated with fibroblasts in cornea of 03/12 (16.6%) samples of the eye tunics (Figure 6). One dog (A1) did not have macroscopic lesions associated with ophthalmic clinical signs; however, it presented discrete lymphoplasmic inflammatory infiltrate containing a slight amount of histiocytes associated with edema in the limbus region and positive staining of amastigotes to IHC in cornea.

Figure 6: Photomicrograph of corneal immunohistochemistry of animal 1 (A1). A) Control for amastigote marker. B) Positive-labeled control in the corneal epithelium region (arrows) 20X magnification

Source: Rodrigues (2019).

In the limbus, there was also positive staining for amastigotes (Figure 7); This area was associated with a higher frequency of lymphoplasmic and histiocytic inflammatory infiltrates.

Figure 7: Photomicrograph of the immunohistochemistry of the fibrous tunic represented by the transition area between the cornea and the sclera (limbus) A) Negative control for amastigote marking. B) Control with positive marking in the limbus region. 40X magnification.

Source: Rodrigues (2019).

IHC was also performed to observe corneal fibroblast labeling associated with amastigous forms was performed using the vimentin antibody. Labeling for fibroblasts was positive in the cornea and limbus of animals that had inflammatory infiltrates associated with the presence of amastigotes in this tissue, binding fibroblasts as a host cell for amastigoste.

Figure 8: Photomicrograph of the immunohistochemistry of the fibrous tunic represented by corneal region with Vimentin marker. A) Control with H-E stainning demonstrating amastigotes - 100X magnification. B) IHC for vimentin marker demonstrating fibroblast as host cells for amastigostes - 100X magnification.

Source: Rodrigues (2019).

6. DISCUSSION

Among the clinical signs observed in dogs with leishmaniasis, the most prevalent was lymphadenopathy, observed in 91.66% of the animals included in this study, according to Fulgêncio et al. (2006) who observed lymphadenopathy as the most frequent sign. Eye injuries were observed as the second most frequent alteration, represented by 83.33% of dogs included in the study. According to Peña et al. (2000), this characteristic may vary between 24.1% to 80.5% of dogs with leishmaniasis.

Several studies have linked the prevalence of eye lesions with canine visceral leishmaniasis, Slappendel et al., (1988) in the Netherlands, evaluated 95 dogs with leishmaniasis and found 40 animals with ophthalmopathies. Among these changes, conjunctivitis (27.4%), keratitis (7.4%), uveitis and pan-ophthalmitis (1.0%) stood out.

In the present study, the most frequent alteration was the presence of purulent eye secretions, affecting 66.67% of the animals, followed by blepharitis in 33.33% and corneal ulcers in 25%. This finding agrees with Cunha et al. (2015) who observed the The most constant ocular clinical manifestations were: purulent ocular discharge and conjunctivitis, followed by blepharitis. On the other hand, Penna et al. (2010) described that keratitis was the most frequent clinical sign.

Conjunctivitis was found in 25% diverging from the findings of Garcia-Alonso et al. (1998) where they recognized it as the most frequent ocular sign, with acute or chronic evolution, in animals with systemic clinical signs. Brito et al. (2004) found a high frequency of conjunctivitis, characterized by conjunctival hyperemia and purulent exudate.

Corneal alterations were also frequently observed, this finding is in agreement with an experimental research developed by Ribeiro et al. (2007) in which corneal opacity was observed in 100% of the animals from a inoculated group, which presented in various forms mainly as focal opacities which corroborates with Brito et al. (2004) who observed corneal alterations in 12% of the dogs studied in his research.

Dry keratoconjunctivitis (KCS) has been observed in 8.33% of dogs and, according to Roze et al. (1986a), this condition can occur in three ways: through direct destruction of the third eyelid gland by the parasite, by obstruction of the gland ducts in the adjacent inflammatory process and, finally, by the reduction of the lacrimal secretion reflex due to decreased corneal sensitivity. In addition, it has concluded that isolated corneal involvement is rarely seen in CVL, usually occurring in association with other ophthalmopathies. Fulgêncio et al. (2004) reported CCS in 10% of the animals, supporting the present research.

Other more uncommon ophthalmopathies in leishmaniasis are chorioretinitis, retinal detachment, cataract, and glaucoma according to Peña et al. (2008) and although no correlation is established between cataract formation and leishmaniasis, it is prudent to assume that disorders aqueous humor may envelop the lens, opacifying it.

Regarding the presence of inflammatory infiltrates, Fulgêncio et al. (2004) states that the presence of infiltrates occurs frequently in the eye tunics, and highlights predominant lymphocytes and plasma cells in the uveal tract, arranged focal and diffuse and with discrete to intense intensity. In the choroid the authors observed focal to diffuse lymphoplasmocytic inflammatory infiltrate with slight and intense variation. In the present study, the uveal tract is one of the most affected regions, and we highlight the lymphoplasmocytic cell infiltrate intensely.

Cunha et al. (2015) observed changes in the cornea in 16% of the study group and highlights intense plasmacytic inflammatory infiltrate, while Fulgêncio et al. (2004) highlights microscopic changes in the cornea of animals, one of them as an extension of limbal inflammation and the other with diffuse and marked loss of the anterior epithelium. He also highlighted moderate and multifocal inflammatory infiltrate, consisting mainly of lymphocytes and histiocytes, and emphasizes that the limbus had a moderate and multifocal inflammatory infiltrate with great variety in relation to the cell type.

In the present study, we observed that the cornea has a great variety frequency with relation to cell type, but what prevailed was the mildly neutrophilic type. We also observed that lymphoplasmocytic cell infiltrate is frequent in the transition region, represented by the limbus, but other cell types were seen less frequently.

Regarding the region with the highest lymphoid activity of the eye, Brito et al. (2010) states that the bulbar and eyelid conjunctiva are the ocular structures in which parasitism is most observed and justify the presence of lymphoplasmocytic inflammatory infiltrate and parasitic activity in this area. This corroborates the findings found in this study, also associated with the presence of lymphocytic and histiocytic infiltrates.

Regarding the mechanism of action of the parasite in the eye, several authors associate eye diseases with leishmaniasis in a systemic way. Peña et al. (2000) and Brito et al. (2006) associate the presence of eye lesions with the deposition of immunocomplexes in the eye tunics or even the presence of amastigote forms in the tunics. According to Bogdan et al. (2000) *Leishmania* may persist in the hosts after the clinical cure of the disease, which was demonstrated in this work is the presence of fibroblast associated amastigotes in the cornea and

fibrous tunic transition region of infected dogs. Bogdan et al. (2000) states that this cell type acts as a host cell for *Leishmania* during the chronic phase of the disease, suggesting that these fibroblasts may serve as targets for parasites in the latent phase of the disease.

7. CONCLUSION

Ocular changes are commonly found in dogs with visceral leishmaniasis as 83.3% of the study population had systemically correlated ophthalmic lesions and the most frequent clinical signs were: ocular discharge and changes in the cornea. Such manifestations are characterized by the high frequency of inflammatory infiltrates, mainly of the lymphoplasmic type in the uveal tract and bulbar conjunctiva.

Therefore, we found that the fibroblasts present in the cornea may act as important host cells in the face of systemic disease, which brings a new perspective on the mode of action of the parasite in the eye tunics.

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ANNEX A – Representation of all inflammatory infiltrates and eye changes found in dogs in this research.

	Cornea	Pupil	Ciliary Process	Ciliary Body	Limbo	Bulbar Conjunctiva	Retina
Animal 1	-	-	-	-	Moderate lymphoplasmocytic inflammatory infiltrate containing slight amount of histiocytes associated with edema	-	-
Animal 2	Moderate hyperplasia of focally extensive anterior epithelium	Pupillary occlusion associated with pigmented superficial cell hyperplasia	-	-	-	Discrete focal lymphocytic inflammatory infiltrate containing slight amount of eosinophils	Multifocal retinal detachment with hypertrophy of adjacent retinal pigment epithelium and focal hyperplasia cells of the outer nuclear layer
Animal 3	Discrete neutrophilic infiltrate in stroma	-	Focal discrete plasmocytic inflammatory infiltrate containing slight amount of lymphocytes	Discrete focal plasmocytic inflammatory infiltrate containing slight amount of lymphocytes and histiocytes associated with edema	-	-	Multifocal retinal detachment with hypertrophy of adjacent retinal pigment epithelium and focal hyperplasia cells of the outer nuclear layer
Animal 4	Moderate neutrophilic inflammatory infiltrate in anterior epithelium	Pupillary Occlusion	-	-	-	Moderate discrete lymphoplasmic inflammatory infiltrate	-
Animal 5	-	-	-	-	Focal Moderate lymphoplasmic inflammatory infiltrate containing slight amount of histiocytes	-	Multifocal retinal detachment with focal hyperplasia of the outer nuclear layer
Animal 6	-	-	Discrete lymphoplasmic inflammatory infiltrate	-	Moderate lymphoplasmic and histiocytic inflammatory infiltrate containing Moderate amount of neutrophils associated with perivasculitis and edema	-	-
Animal 7	Discrete neutrophilic infiltrate in stroma	-	Discrete lymphoplasmic inflammatory infiltrate	-	Intense lymphoplasmic and histiocytic inflammatory infiltrate containing Moderate amount of neutrophils associated with Moderate	-	-

Animal 8	Moderate plasmacytic inflammatory infiltrate with few stromal lymphocytes and neutrophils and few anterior epithelium neutrophils	-	Intense lymphoplasmacytic inflammatory infiltrate with few neutrophils associated with edema. Moderate lymphoplasmacytic perivasculitis	-	lymphoplasmic cell perivasculitis and edema, with large amount of amastigotes - compatible with intracytoplasmic histiocyte Leishmania sp.-	-
Animal 9	Moderate lymphoplasmacytic inflammatory infiltrate containing slight amount of stromal neutrophils	-	Moderate lymphoplasmacytic inflammatory infiltrate and Moderate lymphoplasmacytic perivasculitis	-	Moderate lymphoplasmacytic inflammatory infiltrate and Moderate lymphoplasmacytic perivasculitis	-
Animal 10	-	-	-	-	-	Discrete lymphoplasmic cell inflammation
Animal 11	Intense lymphoplasmic and histiocytic inflammatory infiltrate containing a slight amount of neutrophils, with a large amount of intracytoplasmic amastigotes compatible with Leishmania sp. in stroma	-	Intense lymphoplasmic and histiocytic inflammatory infiltrate containing a slight amount of neutrophils, with a large amount of intracytoplasmic amastigotes compatible with Leishmania sp. associated with edema	-	Intense lymphoplasmic and histiocytic inflammatory infiltrate containing a slight amount of neutrophils, with a large amount of intracytoplasmic amastigotes compatible with Leishmania sp. associated with edema	-
Animal 12	-	-	-	-	Moderate lymphoplasmic and histiocytic inflammatory infiltrate containing Moderate amount of neutrophils associated with perivasculitis and edema	-

¹ Amastigotes with ovoid shape (2 ~4) µm with a nucleus measuring 1.0 µm surrounded by a kinetoplast perpendicular to the nucleus measuring 1.0 to 2.0 cm.