HISTOPATHOLOGICAL, IMMUNOHISTOCHEMICAL AND MOLECULAR CHARACTERIZATION OF OCULAR AND ADNEXAL INVOLVEMENT IN CANINE LEISHMANIOSIS

Tesi Doctoral

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CERTIFIQUEN:

Que la memòria de la Tesi doctoral titulada "**Histopathological, immunohistochemical and molecular characterization of ocular and adnexal involvement in canine leishmaniosis**", presentada per la Sra. CAROLINA NARANJO I FREIXA per optar al Grau de Doctor en Veterinària per la Universitat Autònoma de Barcelona, ha estat realitzada sota la nostra direcció i supervisió i, considerant-la finalitzada, autoritzem la seva presentació per a que pugui ser jutjada pel tribunal corresponent.

I per a que així consti, signem aquest certificat.

Bellaterra, a 29 de Març de 2011

Sig. Mª TERESA PEÑA GIMÉNEZ Sig. XAVIER ROURA LÓPEZ

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ABBREVIATIONS

ACAID	Anterior chamber-associated immune deviation
AUC	Area under the curve
CD	Cluster of differentiation
CL	Canine leishmaniosis
Ct	Cycle threshold
HE	Hematoxylin and eosin
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
KCS	Keratoconjunctivitis sicca
LR+	Likelihood ratio of positives
LST	Leishmanin skin test
MG	Meibomian gland
MLG	Main lacrimal gland
NMG	Nictitating membrane gland
PCR	Polymerase chain reaction
ROC curve	Receiver operating characteristic curve
Th	T cell helper
TNF	Tumor necrosis factor

INTRODUCTION

1. CANINE LEISHMANIOSIS

Canine Leishmaniosis (CL) is a potentially fatal systemic infectious disease of dogs of great economic and zoonotic importance in endemic areas. The unique features of this disease, including the high prevalence of infection with much lower incidence of disease and the role of the immune system in the development of the disease, have resulted in a great deal of interest and research over many years.

1. 1. ETIOLOGY

CL is caused by the diphasic protozoan *Leishmania infantum* in the Old World, or its New World synonym, *Leishmania chagasi*, that belongs to the class Kinetoplastida and the family Trypanosomatidae (Baneth, 2006; Paltrinieri *et al.*, 2010).

1. 2. EPIDEMILOGY

CL is endemic in the Mediterranean shore and Central and South America and dogs are the main reservoir of *L. infantum* for humans in these areas (Ferrer, 1992; Gramiccia and Gradoni, 2005; Miró *et al.*, 2008).

L. infantum is transmitted by hematophagous sand flies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World (Baneth, 2006; Paltrinieri *et al.*, 2010). Female sand flies containing the flagellated extracellular promastigotes feed on dogs in scarcely haired skin areas and the parasite gains access to the dog's dermis, where phagocytosis by macrophages ensues. The parasites lose the flagellum in the phagolysosomal compartment and become amastigotes, which are able to evade the oxidative burst, survive and replicate within the macrophage. Amastigotes distribute throughout the organism with preference for the hematopoietic organs and later migrate to other tissues, particularly the skin, liver, pancreas, kidneys, adrenal glands, digestive tract, eyes, testes, bone and joints (Ferrer, 1992; Baneth, 2006). When other phlebotomine sand flies ingest the parasites, these are released from macrophages and transformed into promastigotes, completing the cycle (Figure 1).

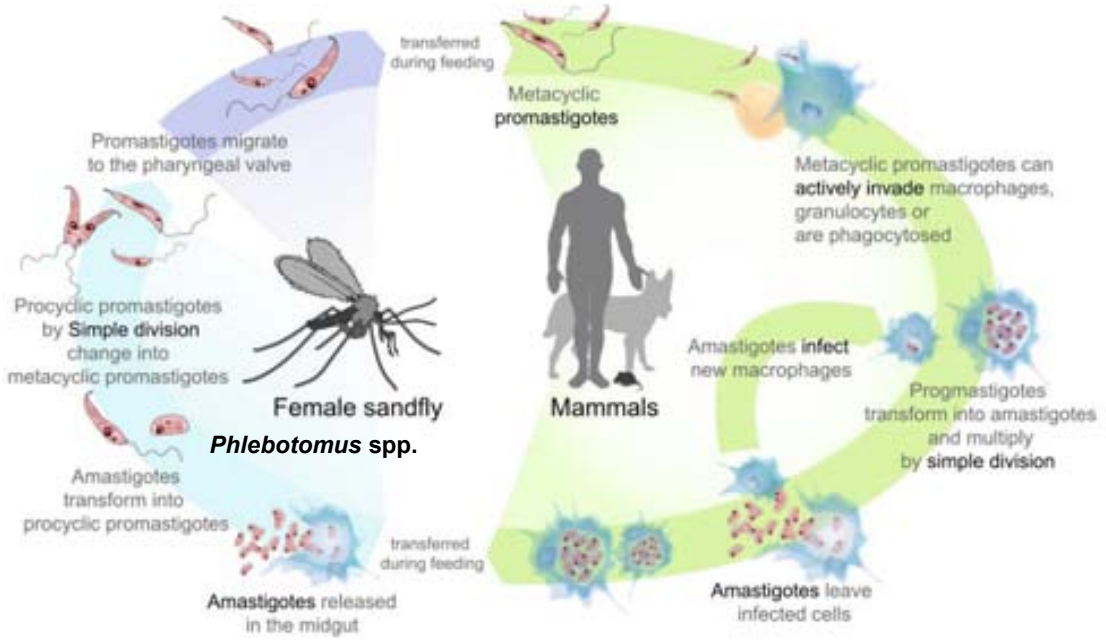

Figure 1. Life cycle of *Leishmania* spp. Modified from: http://commons.wikimedia.org

Transmission of *Leishmania* spp. in endemic areas occurs focally, so broad variations in the prevalence of infection are found in neighbouring regions, depending mainly on relative vector densities (Paltrinieri *et al.*, 2010). The seroprevalence in the Mediterranean basin ranges from 10.2% to 50%, depending on the area sampled (Cabral *et al.*, 1998; Sideris *et al.*, 1999; Solano-Gallego *et al.*, 2001) but prevalence of infection was estimated to be 67% in one study (Solano-Gallego *et al.*, 2001), suggesting that there is a population of dogs that harbor the parasite but do not develop antibodies. Similarly, the prevalence of disease in the same study was 13%, indicating that a proportion of infected dogs (wheter they are seropositive or not) are either resistant to the development of CL at a given point of time (without clinical signs) or will subsequently develop the disease (Solano-Gallego *et al.*, 2001). For this reason, CL is considered a disease in which infection does not equal clinical illness (Solano-Gallego *et al.*, 2001; Baneth *et al.*, 2008).

1. 3. PATHOGENESIS

These differences between rates of infection, seroconversion and development of the disease in CL have been attributed to the interactions between the parasite and the host's immune response, and the nature of this immune response (Pinelli *et al.*, 1994; Pinelli *et al.*, 1995; Solano-Gallego *et al.*, 2001).

In the experimental murine model of cutaneous leishmaniosis, infection of susceptible strains with *Leishmania major* results in development of the disease. Balb/c mice develop a T-helper type 2 (Th2) response, characterized by the secretion of interleukin (IL)-4 and IL-10 and prominent antibody production. Resistant mouse strains (C57BL/6, CBA) respond with the production of interferon (IFN)-γ and IL-12, which are Th-1 cytokines. INF-γ secreted from T cells is the major macrophage-activating cytokine, which results in the elimination of the parasite (Scott *et al.*, 1988).

A similar, but less dichotomized, response has been observed in experimental models of CL, and the actual balance between Th1 and Th2 responses is believed to be important in controlling parasite replication, disease progression, or cure. Resistant dogs respond to natural and experimental infection with *L. infantum* with proliferation of peripheral blood lymphocytes, which produce IL-2, tumour necrosis factor (TNF) and IFN-γ (Pinelli *et al.*, 1994; Pinelli *et al.*, 1995; Manna *et al.*, 2006). The latter is the major macrophage-activating cytokine, which results in the production of nitric oxide and killing of intracellular parasites (Vouldoukis *et al.*, 1996). Conversely, in susceptible dogs these processes are suppressed and a strong but non-protective humoral immune response, characterized by the production of abundant anti-*Leishmani*a antibodies, results (Pinelli *et al.*, 1994; Martinez-Moreno *et al.*, 1995; Pinelli *et al.*, 1995; Rodríguez-Cortés *et al.*, 2007).

In any case, the absence of cell-mediated immunity is a key aspect in the pathogenesis of the disease, with infected dogs having significantly lower percentage of CD4+ lymphocytes and a lower CD4+/CD8+ ratio than healthy dogs (Bourdoiseau *et al.*, 1997a; Moreno *et al.*, 1999).

After inoculation by the sandfly, the parasites are distributed to the hematopoietic organs first and from there they further spread to the liver and kidney, reproductive organs, skin, bladder, digestive and respiratory system (Alvar *et al.*, 2004). The presence of the parasite in these tissues causes inflammatory cell infiltration with ensuing progressive alteration and functional derangement of the affected organs (Alvar *et al.*, 2004). A humoral response, with high concentrations of specific and non-specific γ-globulins, is also produced. Circulating immune complexes, composed of IgG and complement fractions C1, C2 and C4, deposit in blood vessels, causing vasculitis, polyarthritis, uveitis and glomerulonephritis (Poli *et al.*, 1991;

Pumarola *et al.*, 1991; García-Alonso *et al.*, 1996; Vamvakidis *et al.*, 2000; Torrent *et al.*, 2005; Baneth, 2006).

1.4. CLINICAL FEATURES

CL is usually chronic and sometimes subclinical, later evolving toward overt clinical disease (Paltrinieri *et al.*, 2010). The systemic distribution of the parasite determines the wide variety of clinical signs observed in CL.

Skin lesions, including alopecia, dry exfoliative dermatitis, ulcerative dermatitis, sterile pustular dermatitis, footpad hyperkeratosis and nodular disease, are amongst the most commonly observed clinical manifestations of CL (Figure 2), occurring in 56% to 90% of dogs with patent leishmaniosis (Slappendel, 1988; Ferrer *et al.*, 1988b; Koutinas *et al.*, 1992; Ciaramella *et al.*, 1997; Koutinas *et al.*, 1999; Baneth *et al.*, 2008; Paltrinieri *et al.*, 2010).

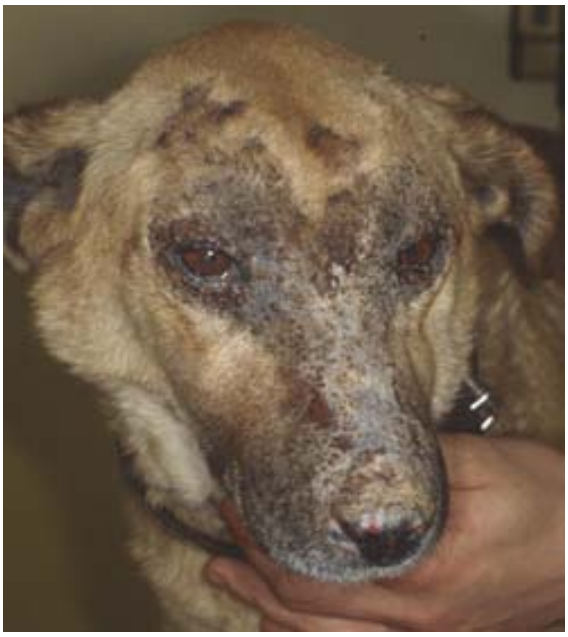

Figure 2. Dog with leishmaniosis showing facial exfoliative dermatitis and alopecia.

Lymphadenomegaly, especially of the prescapular and popliteal lymph nodes, is also frequently found in CL, with a prevalence that ranges from 62% to 90% of cases (Ciaramella *et al.*, 1997; Koutinas *et al.*, 1999).

Other reported clinical signs with variable prevalence include: weight loss, cachexia, pale mucous membranes, splenomegaly, onycogryphosis, epistaxis, fever, polyuria/polydipsia, polyarthritis, masticatory muscle atrophy, diarrhea and ocular manifestations (Slappendel, 1988; Ciaramella *et al.*, 1997; Koutinas *et al.*, 1999).

1.5. DIAGNOSIS

Diagnosis of CL in an endemic area is complicated by the wide variety of clinical signs associated with the disease and the fact that the prevalence of infection is much higher than the prevalence of disease (Ferrer, 1997; Solano-Gallego *et al.*, 2001). Furthermore, there is no single test that is 100% specific and sensitive; hence, a combination of diagnostic techniques is usually applied to dogs that have clinical and clinical-pathological signs compatible with the disease (Ferrer, 1997; Solano-Gallego *et al.*, 2009; Paltrinieri *et al.*, 2010).

1.5.1. DIRECT METHODS

Direct methods allow the observation of the parasite (microscopic examination) or detect its DNA (molecular methods).

Microscopic examination

A. CYTOLOGY

Cytologic examination of bone marrow or peripheral blood smears, lymph node or splenic aspirates, or skin impression smears, can be useful in the diagnosis of CL, although the sensitivity is usually low, ranging from 40-50% for lymph node to 60-75% for bone marrow (Alvar *et al.*, 2004; Moreira *et al.*, 2007; Maia and Campino, 2008). However, sensitivities of over 90% have been obtained in some studies, when using bone marrow or lymph node aspirates (Mylonakis *et al.*, 2005; Rosypal *et al.*, 2005; Saridomichelakis *et al.*, 2005). These methods are, in general, not useful for the detection of the parasite in dogs without clinical signs (Alvar *et al.*, 2004, Baneth, 2006).

B. HISTOPATHOLOGY

Histopathology of CL is not specific and consists of granulomatous to pyogranulomatous infiltrates with macrophage predominance and variable numbers of lymphocytes, plasma cells and neutrophils in the skin (Figures 3 and 4), spleen, lymph nodes, liver, kidneys, bone marrow, intestine and conjunctiva (Keenan *et al.*, 1984; Ferrer *et al.*, 1988b; Tafuri *et al.*, 2001; Solano-Gallego *et al.*, 2004; Baneth, 2006). Lymphoid hyperplasia of hematopoietic tissues is responsible for the clinically detected lymphadenopathy and splenomegaly (Lima *et al.*, 2004; Giunchetti *et al.*, 2008; Moreira *et al.*, 2010). Systemic vasculitis has been described (Pumarola et al., 1991; Paltrinieri et al., 2010). Kidney lesions include glomerulonephritis, interstitial nephritis and occasionally amyloidosis (Nieto *et al.*, 1992; Zatelli *et al.*, 2003).

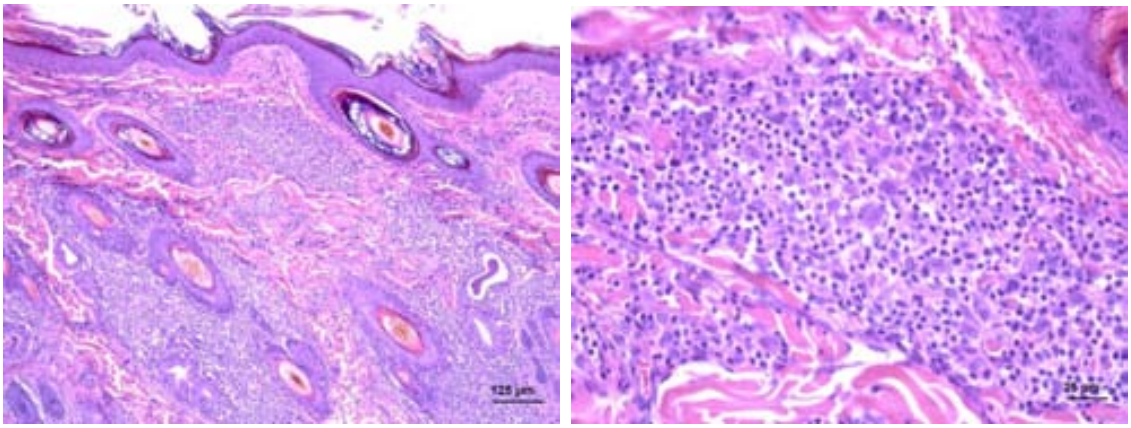

Figure 3 (left). Skin, dog with leishmaniosis. Low power image showing severe perifollicular and perivascular to interstitial granulomatous dermatitis and sebaceous adenitis. Hematoxylin and Eosin. Photo courtesy of Dr. Dolors Fondevila.

Figure 4 (right). Skin, dog with leishmaniosis. Higher magnification picture, in which macrophages, lymphocytes and plasma cells are noted within dermal infiltrates. Hematoxylin and Eosin. Photo courtesy of Dr. Dolors Fondevila.

If the number of organisms is high, these can be easily detected in routine Hematoxylin and Eosin (HE) sections, but in many cases the number of amastigotes in tissues is small and the diagnosis is difficult (Ferrer, 1997), so the sensitivity of this method is usually regarded as low, ranging between 12% and 44.8% depending on the study and the tissue evaluated (Bourdoiseau *et al.*, 1997b; Xavier *et al.*, 2006; Moreira *et al.*, 2007).

C. IMMUNOHISTOCHEMISTRY

In tissues where the parasite load is low, immunohistochemistry (IHC) (immunoperoxidase or direct immunofluorescence) can be used as a supplementary tool (Maia and Campino 2008). Various techniques, using canine hyperimmune serum as the primary antibody or by the use of polyclonal or monoclonal anti-*Leishmania* antibodies, have been described (Ferrer *et al.*, 1988a; Bourdoiseau *et al.*, 1997b; Tafuri *et al.*, 2004; Moreira *et al.*, 2007). IHC is a simple technique with a reported sensitivity ranging between 62.1% and 92.68 % and a specificity of 100% (Ferrer *et al.*, 1988a; Xavier *et al.*, 2006; Moreira *et al.*, 2007). In one particular study (Xavier *et al.*, 2006), IHC on skin biopsy samples resulted in a higher sensitivity (62.1%) than HE alone (44.8%). This technique can be applied to many tissues, although the ones showing *Leishmania* amastigotes more frequently are skin (Figure 5), liver, spleen, lymph nodes and bone marrow (Ferrer *et al.*, 1988a; Moreira *et al.*, 2007). IHC may also highlight amastigotes in tissues with a low parasite load, including the kidney, lung, intestines, central nervous system and testis (Ferrer *et al.*, 1988a; Tafuri *et al.*, 2004). Parasites are most commonly noted intracellularly within macrophages, although other cells may contain *Leishmania* parasites, including fibroblasts (Hervás-Rodríguez *et al.*, 1996) and hepatocytes (Tafuri *et al.*, 2001). Amastigotes may also be found extracellularly (Baneth and Aroch, 2008).

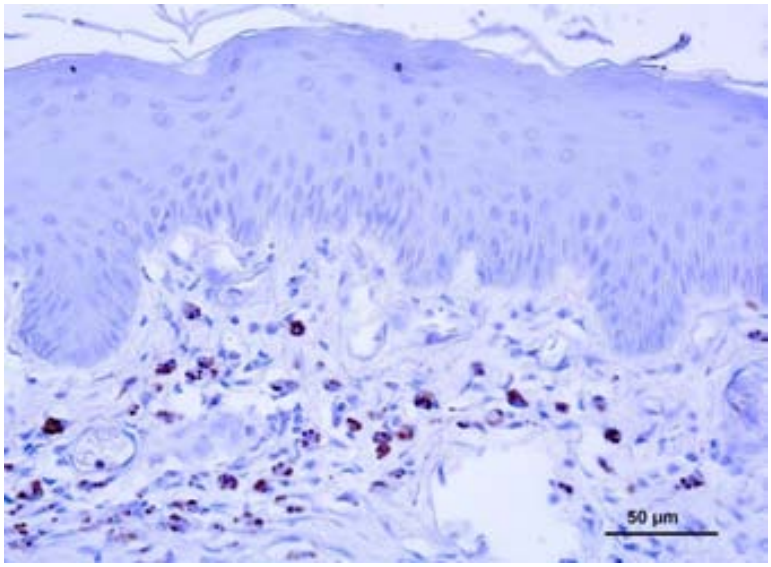

Figure 5. Skin, dog with leishmaniosis. Labeled *Leishmania* amastigotes within macrophages in the superficial dermis. Immunohistochemistry for *Leishmania* spp. Photo courtesy of Dr. Dolors Fondevila.

Molecular techniques

A. END-POINT PCR

End-Point or conventional PCR and nested PCR have proved to be sensitive methods for diagnosing CL. PCR can also be used in epidemiologic surveys and when screening for blood donors (Ashford *et al.*, 1995; Tabar *et al.*, 2008). Fresh or formalin-fixed tissues or blood have been used to assess the presence of the parasite (Roura *et al.*, 1999a and b; Fisa *et al.*, 2001; Solano-Gallego *et al.*, 2001; Lachaud *et al.*, 2002). Blood is easy to sample and handle, but sensitivity of PCR using this sample ranges from 17% to 100% depending on the study, the type of sample (whole blood vs. buffy coat) and the clinical status of the dog (with vs. without clinical signs) (Reale *et al.*, 1999; Fisa *et al.*, 2001; Lachaud *et al.*, 2002; Strauss-Ayali *et al.*, 2004). A wide variety of more or less invasively obtained tissues have been used to overcome this limitation, including bone marrow, spleen, lymph nodes, skin and conjunctiva (Solano-Gallego *et al.*, 2001; Ikonomopoulos *et al.*, 2003; Manna *et al.*, 2004; Strauss-Ayali *et al.*, 2004; Xavier *et al.*, 2006), with similarly diverse results. Part of the variation in sensitivity observed can be explained by the heterogeneous distribution of the parasite, the tropism of *Leishmania* strain and the local immune response (Maia and Campino, 2008), as well as by the fact that PCR sensitivity can vary depending on the biological sample used (Leite *et al.*, 2010). Other factors influencing the efficacy of PCR include primers used, number of copies of the target, method of DNA extraction and PCR protocol (Alvar *et al.*, 2004).

Because in an endemic area approximately two-thirds of dogs can be infected with *Leishmania infantum* but only 13% of them develop the disease (Solano-Gallego *et al.*, 2001), a positive result has to be interpreted with caution, especially in clinically healthy dogs (Solano-Gallego *et al.*, 2009). These dogs can be seronegative for *Leishmania* while being PCR positive (Oliva *et al.*, 2006) or PCR negative while seropositive (Solano-Gallego *et al.*, 2001; Ikonomopoulos *et al.*, 2003).

B. REAL-TIME PCR

Real-Time PCR allows quantification of the number of DNA molecules of the target sequence, based on the continuous monitoring of PCR product formation throughout the reaction. This technique relies on the fluorescence of a reporter molecule that increases as product accumulates during the amplification process (Bell and Ranford-Cartwright, 2002).

Two methods of analyzing data obtained from real-time PCR studies exist: absolute quantification and relative quantification (Livak and Schmittgen, 2001). Absolute quantification determines the input copy number of the transcript of interest by relating the PCR signal to a standard curve. Relative quantification describes the change in expression of the target gene relative to a reference or calibrator sample, which could be an untreated control or a sample obtained at time zero in a longitudinal study (Livak and Schmittgen, 2001). Raw results are obtained as Ct (cycle threshold), which represents the point at which the fluorescence exceeds the threshold limit (Figure 6); therefore, higher Ct values correspond to lower amounts of target molecules, as these require more amplification cycles to produce a detectable quantity of reporter molecules (Rolão *et al.*, 2004).

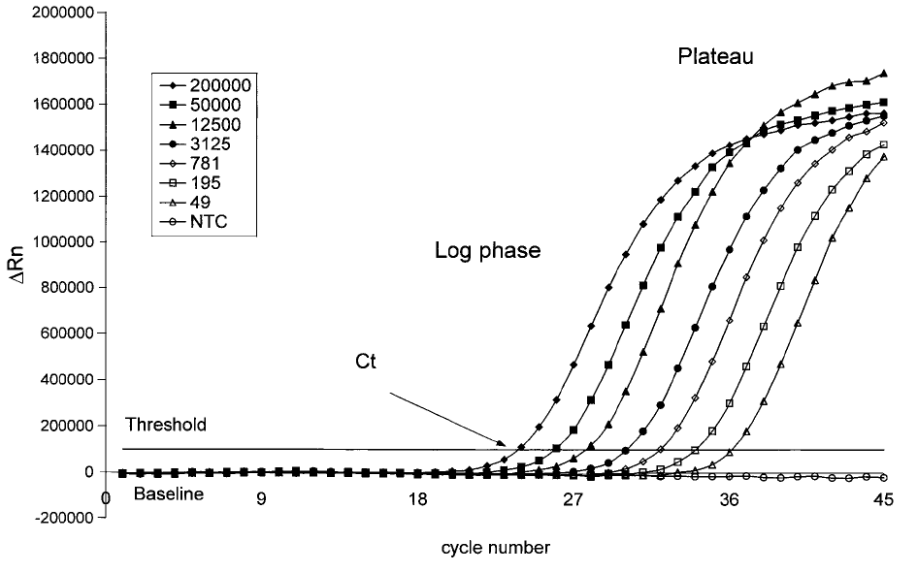

Figure 6. Example of real-time PCR graph in which fluorescence emission (ΔRn) is plotted against cycle number. Cycle threshold (Ct) represents the cycle at which the fluorescence exceeds the threshold limit. Higher Ct values correspond to lower amounts of target molecule. Modified from: Giulietti *et al.*, 2001.

Advantages of real-time PCR over conventional end-point PCR techniques are a reduction in assay time, reduced risk of contamination and hence reduction in false-positive results, and improved sensitivity (Nicolas *et al.*, 2002; Rolão *et al.*, 2004; Paltrinieri *et al.*, 2010).

Real-Time PCR technology has recently been applied to the diagnosis and monitoring of CL (Francino *et al.*, 2006; Manna *et al.*, 2008a and b; Manna *et al.*, 2009a). Quantification of the parasite load can assist the clinician in the diagnosis of CL, especially in those dogs that have compatible clinical and pathologic findings, but medium- or low-positive serology titers (Solano-Gallego *et al.*, 2009; Paltrinieri *et al.*, 2010). A quantitative approach may also aid the clinician during the post-treatment follow-up; a decrease in parasite load seems to parallel clinical resolution of signs whereas an increase in the number of parasites is associated with clinical recurrence (Francino *et al.*, 2006; Manna *et al.*, 2008b; Manna *et al.*, 2009a). This is especially important because parasitological cure is rarely achieved (Miró *et al.*, 2008; Solano-Gallego *et al.*, 2009).

Various samples, including blood, bone marrow, lymph node aspirates, urine and fresh skin biopsies, have been used to diagnose, monitor and study *Leishmania* spp. infection in dogs (Vitale *et al.*, 2004; Francino *et al.*, 2006; Manna *et al.*, 2006; Solano-Gallego *et al.*, 2007; Manna *et al.*, 2008b).

Other direct methods

Culture in several media and inoculation to mice or hamsters are time-consuming and expensive methods usually restricted to experimental conditions (Ferrer, 1997; Alvar *et al.*, 2004; Maia and Campino, 2008; Paltrinieri *et al.*, 2010).

Xenodiagnosis consists of allowing laboratory-bred sand flies to feed on a dog suspected of having leishmaniosis and then the insects are examined for the presence of promastigotes in the gut (Alvar *et al.*, 2004; Paltrinieri *et al.*, 2010). Similar to culture and inoculation, this method is not applicable for routine clinical practice.

1.5.2. INDIRECT METHODS

Indirect techniques measure the responses of the immune system of the host against *Leishmania*, and can be divided into those that evaluate the humoral immunity and those that examine the cellular immunity.

Humoral immunity

Serological diagnosis of CL is a widely used technique to diagnose CL. Several serological tests are available, including enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence antibody test (IFAT), rapid immunochromatographic strip test, counter-immunoelectrophoresis (CIE), direct agglutination test (DAT) and Western blotting (WB) (Maia and Campino, 2008; Paltrinieri *et al.*, 2010). The first three are the most commonly used in clinical settings (Ferrer, 1997; Alvar *et al.*, 2004).

Due to the fact that susceptible dogs that succumb to the disease develop a mainly a humoral response, these tests are especially useful in dogs with clinical signs (Alvar *et al.*, 2004). A high level of antibodies is conclusive of a diagnosis of CL (Solano-Gallego *et al.*, 2009; Paltrinieri *et al.*, 2010). However, in an endemic area, the results of these tests must be interpreted with caution, as various scenarios are possible. First, having a positive titer does not mean having the disease, as some dogs will seroconvert after infection but will never develop the disease (Solano-Gallego *et al.*, 2001). Also, cross-reactions can occur, especially with *Trypanosoma cruzi* in endemic areas (Alvar *et al.*, 2004). The opposite is also true, as some dogs, especially during the early stages of infection, are seronegative (Ferrer *et al.*, 1995). Second, anti-*Leishmania* titers remain positive for long periods of time, even after the clinical cure, so serologic methods are not always adequate for follow-up of treated dogs (Ferrer *et al.*, 1995; Baneth, 2006; Solano-Gallego *et al.*, 2009). Finally, when a dog with clinical signs compatible with CL has a low to medium serology titer, additional diagnostic tests such as cytology, histopathology or PCR must be undertaken to confirm or exclude the disease (Alvar *et al.*, 2004; Solano-Gallego *et al.*, 2009, Paltrinieri *et al.*, 2010).

Cellular immunity

The Montenegro or leishmanin skin test (LST) is a delayed hypersensitivity test that evaluates the cellular immune response to *Leishmania* antigen. In this test, inactivated promastigotes are injected intradermally and 48 to 72 hours later the inoculated area is examined for the presence and the diameter of the induration (Cardoso *et al.*, 1998; Fernández-Bellón *et al.*, 2005). The LST is negative in dogs with clinical signs, indicating a lack of cellular response in these dogs; conversely, dogs without clinical signs show a positive LST, indicating the presence of a protective cellular immune response (Cardoso *et al.*, 1998).

Other tests that evaluate the cellular immunity, including the lymphocyte proliferation assay and IFN-γ cytopathic effect inhibition bioassay, are mainly used in research settings or epidemiologic studies (Pinelli *et al.*, 1994; Martínez-Moreno *et al.*, 1995; Cabral *et al.*, 1998; Fernández-Bellón *et al.*, 2005; Rodríguez-Cortés *et al.*, 2007).

1.6. TREATMENT

Treatment of CL is very complex. Various drugs and treatment protocols have been used for treatment of CL, but recurrence is not uncommon (Baneth, 2006). In addition, although clinical cure can be achieved, available treatments do not reliably eliminate the infection and these dogs are still infectious to sand flies (Baneth and Shaw, 2002; Ribeiro *et al.*, 2008).

Meglumine antimoniate in combination with allopurinol is the most commonly used regime and considered the first-line protocol and most effective therapy in the treatment of CL (Miró *et al.*, 2008; Solano-Gallego *et al.*, 2009; Oliva *et al.*, 2010). Several protocols have been used regarding the dosage, dose interval and duration of treatment (Noli and Auxilia, 2005; Oliva *et al.*, 2010).

Other proposed drugs include amphotericin B (Oliva *et al.*, 1995; Cortadellas, 2003), miltefosine (Manna *et al.*, 2009b), aminosidine (Poli *et al.*, 1997; Oliva *et al.*, 1998), pentamidine (Rhalem *et al.*, 1999), metronidazole in combination with enrofloxacin or spiramycin (Bianciardi *et al.*, 2004; Pennisi *et al.*, 2005), antimicrobial peptides (Alberola *et al.*, 2004), domperidone (Gómez-Ochoa *et al.*, 2009) and ketoconazole (Noli and Auxilia,

2005), although these are considered second-line drugs and their effectiveness needs to be confirmed with more extensive clinical trials (Miró *et al.*, 2008, Solano-Gallego *et al.*, 2009, Oliva *et al.*, 2010).

1.7. ZOONOTIC POTENTIAL

Dogs are considered the main domestic reservoir of *L. infantum* infection to humans (Ferrer, 1992; Gramiccia and Gradoni, 2005; Miró *et al.*, 2008). Human visceral leishmaniosis and cutaneous leishmaniosis are both associated with infection with *L. infantum* (Gramiccia and Gradoni, 2005). As human susceptibility to *L. infantum* is low, asymptomatic infections are common in healthy populations, and clinical disease is most often found in infants below 2 years of age, malnourished populations and immunosuppressed individuals (i.e., Human Immunodeficiency Virus (HIV) coinfection) (Gramiccia and Gradoni, 2005).

Despite the fact that there is an established linkage between human and canine infection at the population level, dog ownership in Southern Europe is not believed to be associated with an increased risk of leishmaniosis for humans (Miró *et al.*, 2008; Solano-Gallego *et al.*, 2009).

2. OCULAR DISEASE IN LEISHMANIOSIS

2.1. OPHTHALMOLOGIC CLINICAL SIGNS IN HUMAN LEISHMANIOSIS

Studies reporting ocular disease in human leishmaniosis are sparse and mostly reduced to single case reports and small case-series. Most of the reported cases describe a blepharitis with or without conjunctivitis that occurs in association with and as an extension of facial cutaneous and mucocutaneous human leishmaniosis (Chu *et al.*, 1983; Oliveira-Neto *et al.*, 2000; Abrishami *et al.*, 2002; Yaghoobi *et al.*, 2010). In these cases, granulomatous inflammation with intralesional parasites is reported (Oliveira-Neto *et al.*, 2000; Abrishami *et al.*, 2002).

Intraocular disease is rarely reported in human cases, and mostly refers to post-kala azar uveitis, which is usually seen in association with post-kala-azar dermal leishmaniosis

(Dechant *et al.*, 1980; el-Hassan *et al.*, 1991; el-Hassan *et al.*, 1998; Ramos *et al.*, 2008). These patients develop an anterior uveitis, which can be nodular, and only rarely has posterior uveitis been reported (Ramos *et al.*, 2008). Post-kala azar uveitis occurs after treatment for visceral leishmaniosis caused by *L. donovani* or *L. infantum*. In these cases, involvement of intraocular tissues and skin is believed to occur when the host develops immune-competence against residual parasites and a granulomatous infiltrate results (Dechant *et al.*, 1980; Ramos *et al.*, 2008). Conjunctivitis and blepharitis have also been described in cases of post-kala-azar dermal leishmaniosis (Nandy *et al.*, 1991).

Less frequently described findings include intraretinal hemorrhages (Montero *et al.*, 2003), scleromalacia (Reinecke *et al.*, 2001) and dacryocystitis resulting from nasolacrimal duct stenosis in patients with mucocutaneous leishmaniosis (Baddini-Caramelli *et al.*, 2001).

2.2. OPHTHALMOLOGIC CLINICAL SIGNS IN CANINE LEISHMANIOSIS

As opposed to human leishmaniosis, ocular manifestations are amongst the most commonly reported manifestations of CL, with a relative prevalence ranging between 16% and 80.49% depending on the source consulted (Slappendel, 1988; Molleda *et al.*, 1993; Ciaramella *et al.*, 1997; Koutinas *et al.*, 1999; Peña *et al.*, 2000; Cortadellas *et al.*, 2006). Ocular problems may represent between 7% and 14% of presenting complaints in dogs with CL (Koutinas *et al.*, 1997; Peña *et al.*, 2000) and generally occur concurrently with other systemic signs, but may occasionally be the sole clinical abnormality identified in a percentage of cases that ranges between 3.48% and 15.2% (McConnell *et al.*, 1970; Roze, 1986; Slappendel, 1988; Molleda *et al.*, 1993; Roze, 1994; Ciaramella *et al.*, 1997; Peña *et al.*, 2000).

Blepharitis and conjunctivitis (Figure 7) have been described as the most frequent ocular signs in some reports (Roze, 1986; Slappendel, 1988; Molleda *et al.*, 1993; Ciaramella *et al.*, 1997), but anterior uveitis is most frequent in other studies (Peña *et al.*, 2000). Other reported ocular clinical signs include cyclitis, chorioretinitis, retinal detachment, keratoconjunctivitis sicca (KCS), nodular granulomatous episcleritis (NGE), cataract, glaucoma and orbital cellulitis (McConnell *et al.*, 1970, Giles *et al.*, 1975, Roze 1986, García-Alonso *et al.*, 1996, Ciaramella *et al.*, 1997, Peña *et al.*, 2000, Leiva *et al.*, 2010).

Retinal hemorrhages, retinal detachment and hyphema have been associated to systemic hypertension in a small percentage of hypertensive dogs affected with CL (5.7%), although these findings could also be associated to the direct action of the parasite on ocular tissues (Cortadellas *et al.*, 2006).

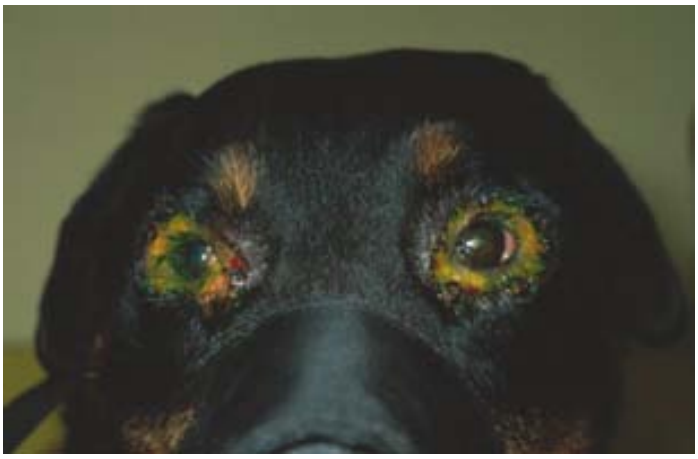

Figure 7. Dog with leishmaniosis showing severe blepharitis and conjunctivitis.

Conjunctiva has become an increasingly popular tissue for the molecular diagnosis of CL (Solano-Gallego *et al.*, 2001, Strauss-Ayali *et al.*, 2004; Ferreira *et al.*, 2008; Pilatti *et al.*, 2009; Leite *et al.*, 2010). End-point PCR performed on conjunctival biopsies showed higher sensitivity (32%) than bone marrow aspirates (17.8%), but lower than that of skin biopsies (51%) in an epidemiological study (Solano-Gallego *et al.*, 2001). Non-invasively obtained conjunctival swabs have demonstrated increased end-point PCR sensitivity when compared to skin scrapings, blood, buffy coat and other more invasively obtained samples, such as fine-needle aspirates of lymph node and spleen (Strauss-Ayali *et al.*, 2004, Ferreira *et al.*, 2008). The sensitivity was further increased when both eyes from the same dog were swabbed, with values over 90% (Strauss-Ayali *et al.*, 2004, Ferreira *et al.*, 2008). Recently, similar sensitivity rates have been obtained in conjunctival swabs obtained from dogs without clinical signs (Leite *et al.*, 2010).

2.2.1. Keratoconjunctivitis sicca

KCS has been reported to occur in 2.8 to 26.83% of dogs with CL (Figure 8) (Molleda *et al.*, 1993; Ciaramella *et al.*, 1997; Koutinas *et al.*, 1999; Peña *et al.*, 2000).

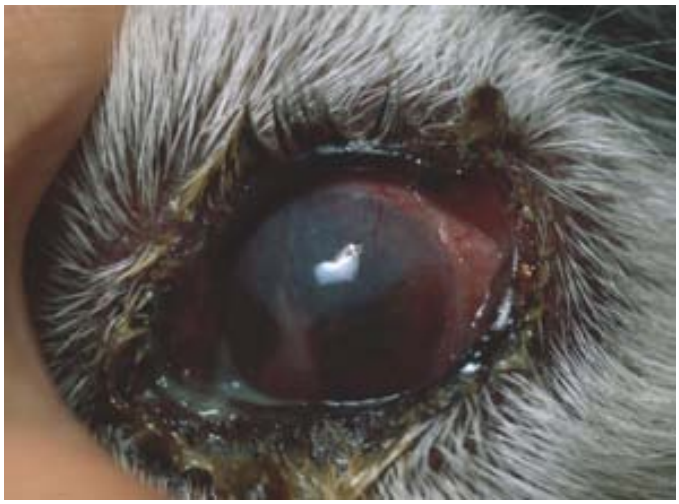

Figure 8. Eye, dog with KCS caused by leishmaniosis. Muco-purulent discharge, conjunctival hyperemia, corneal neovascularization and corneal pigment are noted.

KCS is a potentially blinding disease characterized by aqueous tear deficiency resulting in desiccation and inflammation of the conjunctiva and cornea (Giuliano and Moore, 2007; Williams, 2008). If untreated, KCS can progress to complete pigmentation of the cornea with resulting blindness. Similar signs may result from qualitative tear abnormalities, in which the lipid or mucin layer of the lacrimal film is altered (Giuliano and Moore, 2007).

Various causes of KCS are reported in dogs, the most common being a breed-related, local autoimmune process in which the lacrimal glands are targeted and destroyed (Giuliano and Moore, 2007). Other causes include: infectious diseases (canine distemper, CL), metabolic diseases (hypothyroidism, diabetes mellitus), drug-related (topical or general anesthesia, sulfadiazines), neurogenic and idiopathic (Giuliano and Moore, 2007).

2.3. Pathogenesis of ocular disease in canine leishmaniosis

Studies investigating the pathogenesis of ocular disease in CL are sparse and mostly consist of histopathologic analysis of individual cases (McConnell *et al.*, 1970; Giles *et al.*, 1975; García-Alonso *et al.*, 1996) and a single case-series of 23 dogs (Molleda *et al.*, 1993).

García-Alonso *et al.* (1996) hypothesized that inflammation in the eye could be either a consequence of the leukocyte infiltration secondary to the presence of the parasite, or the result of an immunemediated process, mainly type III hypersensitivity with immune-complex

deposition at the blood-aqueous barrier, similar to what is described for the glomerulus. Few studies published to date support both hypotheses. A granulomatous and lymphoplasmacytic infiltrate, similar to what is described for other organs, occurs in the conjunctiva, cornea, eyelids and uvea of dogs with CL, with variable presence of *Leishmania* amastigotes (Molleda *et al.*, 1993; García-Alonso *et al.*, 1996). Deposits of IgG were also noted in the ciliary body and ciliary processes in one of these studies (García-Alonso *et al.*, 1996), supporting the hypothesis of immune-complex deposition.

Pathogenesis of KCS in CL has not been elucidated and various hypotheses have been cited in the literature that could explain tear deficiency in dogs with leishmaniosis. KCS could be the result of a direct destructive action of the parasite accompanied with intense inflammation of the lacrimal glands (Roze, 1986). Alternatively, reduced reflex secretion following hypoaesthesia of the damaged cornea could eventually result in KCS (Roze, 1986). Finally, KCS could be secondary to obstruction of the secretory ducts due to inflammation of the adjacent structures (Molleda *et al.*, 1993). None of these hypotheses has been proved or disproved to date.

OBJECTIVES

Canine leishmaniosis is a severe systemic and highly prevalent disease in the Mediterranean basin. Ocular manifestations are relatively common in dogs with leishmaniosis, some of which are potentially blinding, including uveitis and keratoconjunctivitis sicca. Considering the paucity of studies investigating the effects of *Leishmania infantum* on the eye, the objectives of this doctoral thesis were the following:

1. To characterize the inflammatory infiltrate and to detect *Leishmania* amastigotes by immunohistochemistry in globes from dogs with leishmaniosis, and associate these findings to the presence of clinical signs.

2. To characterize the inflammatory infiltrate and detect *Leishmania* amastigotes by immunohistochemistry in intraocular, extraocular and adnexal muscles from dogs with leishmaniosis.

3. To evaluate the glands implicated in the production of the lacrimal film (Meibomian glands, main lacrimal gland and nictitating membrane gland) in order to gain a better understanding of the mechanisms that result in keratoconjunctivitis sicca in dogs with leishmaniosis. In particular:

    a. To characterize the inflammatory infiltrate and to assess of the presence of *Leishmania* amastigotes with immunohistochemistry.

    b. To assess the parasite load with real-time PCR.

    c. To associate real-time PCR results to the presence of clinical disease compatible with keratoconjunctivitis sicca, in order to establish possible cut-off values that could be used by clinicians in the distinction between keratoconjunctivitis sicca caused by *Leishmania* spp. from other causes of keratoconjunctivitis sicca.

    d. To compare real-time PCR results to immunohistochemistry.

STUDY 1

Histopathological Features of Ocular Leishmaniosis in the Dog. M.T. Peña, C. Naranjo, G. Klauss, D. Fondevila, M. Leiva, X. Roura, M.G. Davidson, R.R. Dubielzig. Journal of Comparative Pathology 2008;138:32-39.

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Histopathological Features of Ocular Leishmaniosis in the Dog

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Summary

Canine leishmaniosis (CL) can present with multiple clinical signs and ocular disease is reported to occur in almost 25% of affected dogs. The purpose of the present study was to characterize the nature of inflammation within the eyes of dogs with leishmaniosis and to determine whether parasites were present in these lesions. Eyes from 60 dogs with confirmed leishmaniosis that died or were humanely destroyed over a 4 year period were included in the study. Sections of formalin-fixed globes were stained with haematoxylin and eosin (HE) and subjected to immunohistochemistry using a *Leishmania*-specific antibody. Clinically evident ocular signs were present in 15 of 60 dogs (13 bilaterally and 2 unilaterally). Thirty-five of 60 dogs received some form of anti-protozoal treatment. In 36 of 120 eyes (30%) a granulomatous inflammatory infiltrate was found and in 32 of 120 eyes (26.6%) the parasite was identified immunohistochemically within the globe. Ocular tissues affected, in order of frequency, were conjunctiva and limbus, ciliary body, iris, cornea, sclera and iridocorneal angle, choroid and the optic nerve sheath. Different microscopical patterns were defined in each of these structures. *Leishmania* organisms and associated inflammation can be found in different ocular tissues, accounting for some of the ocular clinical signs described for this disease.

Keywords: dog; eye; leishmaniosis; histopathology; immunohistochemistry

Introduction

Canine leishmaniosis (CL) is a chronic and sometimes fatal systemic disease that is endemic along the Mediterranean basin, parts of east Africa, India, and Central and South America. In the Mediterranean area, *Leishmania infantum* is the causative species and sand flies of the genus *Phlebotomus* are the vectors (Slappendel, 1988; Ferrer, 1992; Slappendel and Ferrer, 1998).

Clinical signs associated with leishmaniosis are highly variable as a consequence of the numerous different pathogenic mechanisms involved in this disease and because of the diversity of immune responses of individual hosts (Cabral *et al.*, 1992; Ferrer, 1992; Pinelli *et al.*, 1994). Ocular manifestations are frequently described and generally occur concurrently with other systemic signs, but may rarely be the sole clinical abnormality identified (McConnell *et al.*, 1970; Roze, 1986, 1988; Slappendel, 1988; Molleda *et al.*, 1993; Ciaramella *et al.*, 1997; Peña *et al.*, 2000). The relative prevalence of ocular signs in dogs with systemic leishmaniosis was reported to range between 16% and 80% in five clinical studies (Slappendel, 1988; Molleda *et al.*, 1993; Ciaramella *et al.*, 1997; Koutinas *et al.*, 1999; Peña *et al.*, 2000).

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Blepharitis and keratoconjunctivitis are described as the most frequent signs in some reports (Roze, 1986; Slappendel, 1988; Molleda *et al.*, 1993; Ciaramella *et al.*, 1997), and anterior uveitis is most frequent in other studies (Peña *et al.*, 2000). Other reported ocular manifestations include cyclitis, chorioretinitis, retinal detachment, keratoconjunctivitis sicca (KCS), cataract, glaucoma, and orbital cellulitis (McConnell *et al.*, 1970; Giles *et al.*, 1975; Roze, 1986; Ciaramella *et al.*, 1997; Peña *et al.*, 2000).

Two principal pathogenic mechanisms have been hypothesized as the cause of both ocular and non-ocular changes in CL: local granulomatous inflammation and the production of immune complexes that deposit in the tissues (Roze, 1986; Ferrer, 1992; García-Alonso *et al.*, 1996). Previous pathological studies of the ocular lesions in CL involve individual cases (McConnell *et al.*, 1970; Giles *et al.*, 1975; García-Alonso *et al.*, 1996) and a single-selected series of 23 cases in which only the anterior part of the globe was evaluated (Molleda *et al.*, 1993). The purpose of the present study was to characterize the nature and distribution of ocular lesions in dogs with leishmaniosis, to determine their frequency of presentation and to compare these lesions with those reported in other organ systems of affected dogs.

Materials and Methods

Animals

Dogs with a diagnosis of leishmaniosis that died or were humanely destroyed at the Veterinary Teaching Hospital (HCV) of the Universitat Autònoma de Barcelona (UAB) over a 4-year period (2000–2004) were studied. Medical records of these dogs were reviewed and information was extracted regarding the sex, age, breed, systemic and ocular clinical signs and treatment.

All dogs had systemic clinical and clinicopathological findings compatible with leishmaniosis. An *ante mortem* ophthalmological evaluation, including inspection of the eyelids and globe with a focal light source and a Schirmer tear test (STT), was possible in 26 cases. Only in 9 cases was the examination completed by a veterinary ophthalmologist using slit lamp biomicroscopy, tonometry and indirect ophthalmoscopy. In all cases, the general physical examination performed by the internist included a basic ophthalmological assessment.

The diagnosis was confirmed by serology (Riera *et al.*, 1999) and by direct observation of the parasite and/or polymerase chain reaction (PCR) testing performed on bone marrow samples, lymph nodes or skin biopsies (Roura *et al.*, 1999). Serological testing involved performance of an enzyme-linked immunosorbent assay (ELISA). Briefly, microtitre plates were coated with *L. infantum* antigen (20 µg/ml in 0.1 ml of carbonate-bicarbonate buffer; pH 9.6, 0.1 M) and incubated overnight at 4 °C. Dog serum samples were diluted at 1 in 400 in phosphate-buffered saline (PBS) incorporating 0.05% Tween 20 (PBST) and 1% dried skim milk. Diluted sera (100 µl volumes) were added to antigen-coated wells of the plate which was subsequently incubated for 1 h at 37 °C. Plates were then washed three times with PBST and once with PBS. Following washing, anti-dog immunoglobulin G (IgG) conjugated to horseradish peroxidase was added to each well (100 µl of a 1 in 20,000 dilution) for 1 h at 37 °C. The plates were rewashed after this incubation. Finally, a 200 µl volume of substrate solution (orthophenylene-diamine; 0.4 mg/ml) and H_2O_2 (0.4 mg/ml) in phosphate-citrate buffer (pH 5.0; 0.1 M) was added to each well and incubated for 20 min at 24 °C. The reaction was stopped with 50 µl of H_2SO_4 (3.0 M). Absorbance was read at 490 nm with an automatic ELISA plate reader. ELISA results were reported relative to a standard serum and a positive test was defined by an absorbance within the range of 80 −> 150% of this standard. This ELISA is known to have good sensitivity and specificity, and when used to test dogs in an endemic area, the combination of compatible clinical signs and high serum antibody concentration ($\geqslant$100%) is considered to support the diagnosis of leishmaniosis.

PCR was performed as previously described by Roura *et al.* (1999). Briefly, *Leishmania*-specific oligonucleotide primers SP176 (5′-TCTTGCGGGGAGGGGGTG-3′) and SP177 (5′-TTGACCCCCAACCACATTTTA-3′) were used to amplify a 120-base-pair fragment of *Leishmania* kinetoplast DNA minicircles. The thermal cycling profile was 31 cycles at 94 °C over 30 min and 50 °C for 2 min. Amplified fragments were analyzed by agarose gel electrophoresis. A 2.5% agarose gel was run and then stained in an ethidium bromide solution (0.5 µg/ml) for 20 min.

Sample Collection and Processing

Owners gave informed consent for collection of both eyes after death. These were collected within 10 h of death and were fixed in 10% neutral-buffered formalin. After fixation, globes were sectioned and embedded in paraffin wax. Sections were cut from these tissues and were stained with haematoxylin and eosin (HE). Immunohistochemical (IHC) detection of *Leishmania* was accomplished using a standard Avidin–Biotin Complex (ABC) protocol with a rabbit polyclonal anti-*Leishmania* antibody provided by Dr Dominguez (CSIC, Madrid, Spain) (Ferrer *et al.*, 1988).

Statistical Analysis

For statistical analysis SPSS® v12 (2003) software for Windows was used. In order to establish relationships between variables, contingency table analysis was performed and statistical significance was set at $P < 0.05$.

Results

Clinical Information

Sixty dogs were included in the study, including 37 males (61.6%), 18 females (30%) and 5 with no recorded gender. Only 1 male and 3 females were neutered. The mean age at death was 6.6 years (standard deviation 3.51; range 2–16 years). Forty-one dogs (68.3%) were large breed (>20 kg body weight) and the most common breeds were mixed breed ($n = 11$) and German shepherd dog ($n = 10$), with 22 other breeds also represented.

A wide variety of clinical signs were present, and 33/60 dogs (55%) had renal failure as defined by the presence of stage 2–4 renal disease according to the IRIS (International Renal Interest Society) classification. In these cases the serum creatinine concentration was ⩾ 1.4 mg/dl (normal range 0.5–1.4 mg/dl).

Clinically evident ocular or periocular signs compatible with leishmaniosis were present in 15/60 dogs (25%). Signs were bilateral in 13/15 cases and unilateral in 2 cases. Clinical signs included conjunctivitis (20 eyes from 10 dogs), blepharitis (10 eyes from 6 dogs), periocular alopecia (4 eyes from 2 dogs), exophthalmia (1 eye), keratitis (3 eyes from 2 dogs), KCS (4 eyes from 2 dogs), anterior uveitis (6 eyes from 3 dogs), glaucoma (1 eye) and retinal detachment (2 eyes from 1 dog).

Thirty-five dogs received systemic anti-*Leishmania* medication (meglumine antimonate 75 mg/kg q 24 h, and allopurinol 10 mg/kg q 12 h), and 13 of these dogs also received short-term treatment with anti-inflammatory doses of prednisone. Three dogs received only systemic prednisone. Treatment was administered from the time of diagnosis to the time of death for a mean period of 15.2 months (standard deviation 26.1; range 1 day–10 years). Six dogs received some form of topical treatment 1 month prior to sample collection, which included antibiotic (tobramycin or chloramphenicol), dexamethasone and mydriatics (tropicamide or atropine). Twenty-two dogs received no treatment at all. The relationship between treatment and the presence of ophthalmic clinical signs at the time of sample collection was not statistically significant ($P = 0.806$).

Histological Findings

One hundred and twenty globes from 60 dogs were studied and the results of this analysis are presented in Table 1. After evaluating all the samples, inflammatory patterns were defined for the different structures of the eye, based on the distribution and types of inflammatory cells. A tissue was defined as positive for *Leishmania* when parasites were identified by IHC or within the HE-stained tissue.

Inflammatory infiltration and *Leishmania* organisms (as defined by IHC) were found in 36 (30%) and 32 (26.6%) of globes, respectively. Organisms consistent with *Leishmania* were observed in the HE sections in 18 of the 32 eyes in which identification was confirmed by IHC. No statistically significant differences were found between the left and right eye regarding the presence of inflammation ($P = 0.590$) or parasites ($P = 0.588$). No statistically significant association was found between treatment and the presence of inflammatory infiltration ($P = 0.741$) or parasites ($P = 0.909$), not even when only dogs that had received systemic corticosteroids were considered ($P = 0.787$ and 0.474 for inflammatory infiltration and parasite presence, respectively).

In the subconjunctival tissue at the limbus, three kinds of inflammatory patterns were found: perivascular

Table 1
Histopathological findings in the eyes of 60 dogs with leishmaniosis

		Infiltrate presence		*Parasite presence*	
Tissue	*Pattern*	*(n =)*	*%*	*(n =)*	*%*
Conjunctiva	Perivascular LP	26	21.7	1	0.8
	Perivascular LP + Mo	12	10.0	12	10.0
	Diffuse Mo + LP	17	14.2	15	12.5
	Pyogranuloma	2	1.7	2	1.7
Limbus	Perivascular LP + Mo	12	10.0	12	10.0
	Diffuse Mo + LP	17	14.2	15	12.5
	Pyogranuloma	2	1.7	2	1.7
Sclera	Diffuse Mo + LP	7	5.8	7	5.8
Cornea	Diffuse Mo + LP	7	5.8	7	5.8
Iris	Diffuse Mo + LP	10	8.3	17	14.2
	Granulomata	2	1.7	1	0.8
Ciliary body	Diffuse LP	8	6.7	0	0.0
	Diffuse Mo + LP	18	15.0	18	15.0
Choroid	Diffuse Mo + LP	7	5.8	4	3.3

LP, lymphocytes and plasma cells; Mo, macrophages.

lymphoplasmacytic infiltration ranging from few cells to mild infiltration (26 eyes from 16 dogs; 21.6%) with parasites only seen in one of these eyes; perivascular lymphoplasmacytic infiltrate with scattered macrophages (12 eyes from 7 dogs; 10%), all of which were positive by IHC; and diffuse inflammatory infiltration composed predominantly of macrophages with few lymphocytes and plasma cells (17 eyes from 9 animals; 14.1%), with only 2 eyes from the same animal not containing the parasite (Fig. 1). Besides these patterns, pyogranulomatous aggregates were found in 2 eyes from the same dog, with *Leishmania* organisms seen by IHC. In 15/30 eyes in which the parasite was detected in the subconjunctival tissue at the limbus by IHC, organisms consistent with *Leishmania* were also observed in the HE-stained section (the 15 eyes were from 8 dogs).

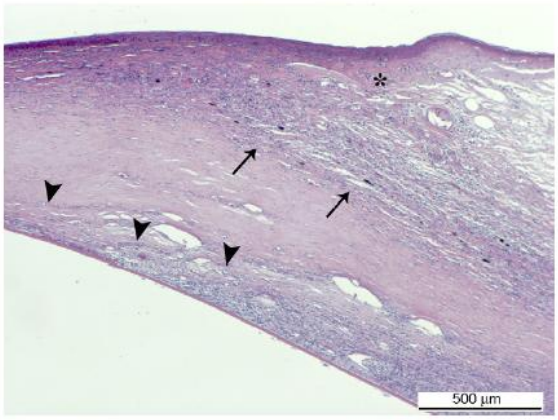

Fig. 1. Limbal area. Diffuse inflammatory infiltration of the subconjunctival tissue (asterisk) extending into the limbus (arrows) and cornea (arrowheads), maintaining the same pattern and intensity of inflammation. HE. Bar, 500 µm.

In all of the eyes in which the inflammatory infiltration of the subconjunctival tissue contained macrophages, this infiltrate extended into the limbus, maintaining the same pattern and intensity of inflammation (Fig. 1). The parasite was seen by IHC in all of these eyes except the two already mentioned.

This same infiltrate extended into the cornea in 7 eyes from 4 animals (5.8%). The sclera was infiltrated by inflammatory cells in 7 eyes from 4 animals (5.8%). In all of these eyes parasites were detected by IHC and, in the sclera of two of them, the parasite could be observed in the HE stained sections. Multiple cross sections of microfilaria within the peripheral superficial corneal stroma were seen in 5 eyes from 3 of the 60 dogs included in the study. The specific species of filarid was not identified.

In the iris, two inflammatory patterns were identified. Diffuse infiltration with macrophages, sparse lymphocytes and plasma cells was detected in 10 eyes from 6 animals (8.3%) and in all of these eyes the parasite was identified by IHC (in one of them *Leishmania* organisms were observed in the HE stained section). In 7 eyes from 5 animals, melanin precluded visualization of the inflammatory cells with the HE stain, but parasites were seen by IHC. The second pattern consisted of small granulomas within the stroma of the iris and this was recorded in 2 eyes from 2 different dogs (Fig. 2). Organisms were identified by IHC in one of these 2 eyes.

In the ciliary body, 2 inflammatory patterns were defined. Diffuse lymphoplasmacytic infiltration was seen in 8 eyes from 4 animals (6.6%) and none of these contained the parasite. Diffuse inflammatory infiltration by macrophages and scattered lymphocytes and plasma cells was detected in 18 eyes from 11 dogs (15%). All of these eyes were positive for the presence of *Leishmania* by IHC.

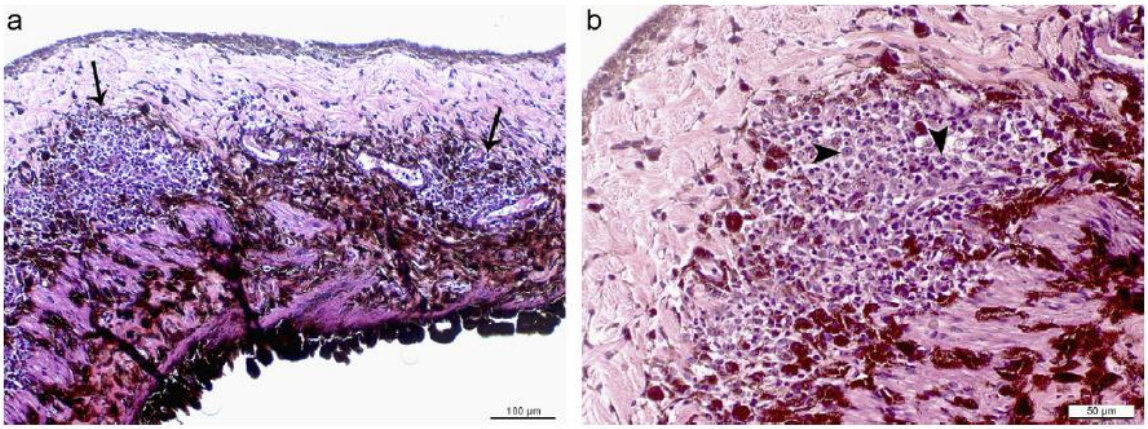

Fig. 2. (a, b). Iris. (a) Focal granulomas within the stroma (arrows). HE. Bar, 100 µm. (b) Higher magnification of one of the granulomas shown in (a). Arrowheads indicate macrophages that predominate in this inflammatory infiltrate. HE. Bar, 50 µm.

The iridocorneal angle was diffusely infiltrated by macrophages, lymphocytes and plasma cells in 7 eyes from 4 dogs (5.8%) (Fig. 3a). In all of these eyes, the infiltrate extended from the ciliary body and/or iris root, and in all cases contained the parasite (Fig. 3b).

Choroidal tissue was infiltrated by macrophages in 7 eyes from 4 animals (5.8%), all of these being positive by IHC (Fig. 4). Finally, the optic nerve sheath contained macrophages in 3 eyes, 2 of them containing the parasite as seen with IHC. Other lesions recorded were serous retinal detachment in 6 eyes from 3 animals and peripheral end-stage retinal atrophy in 3 eyes from 2 dogs.

There was an association between samples obtained from dogs with clinical signs and the presence of granulomatous infiltration in the corresponding structures of the eye ($P<0.001$) and with the presence of parasites within those structures ($P<0.001$). The presence of an inflammatory infiltration of macrophages, lymphocytes and plasma cells was related to the presence of parasites in the corresponding structures, and this relationship was statistically significant ($P<0.001$).

Discussion

Ocular lesions in the current study were similar to those previously reported for ocular leishmaniosis (McConnell *et al.*, 1970; Giles *et al.*, 1975; Molleda *et al.*, 1993; García-Alonso *et al.*, 1996). In the present study, an association was found between the presence of ocular clinical signs and the presence of a characteristic inflammatory infiltration and parasites. Despite this relationship, the percentage of eyes with some form of inflammation (30%) or *Leishmania* parasites (26%) was slightly higher than the number of eyes with clinical signs (25%). This underestimation could be due to the fact that most of the ocular signs were described by the attendant clinician and only 9 cases were examined and treated by a board-certified ophthalmologist. Otherwise, this prevalence of ocular findings is in agreement with a previous study (Peña *et al.*, 2000), in which 24.4% of dogs with leishmaniosis had some form of ocular clinical signs.

The histopathological features of CL have been described for various organs in many reports and fundamentally consist of a granulomatous or pyogranulomatous inflammatory reaction (Keenan *et al.*, 1984; Slappendel and Ferrer, 1998). In the present study, inflammation related to the presence of *Leishmania* parasites consisted of macrophage infiltration with variable numbers of lymphocytes, plasma cells and scattered neutrophils. This pattern of infiltration has been described for many tissues taken from dogs with

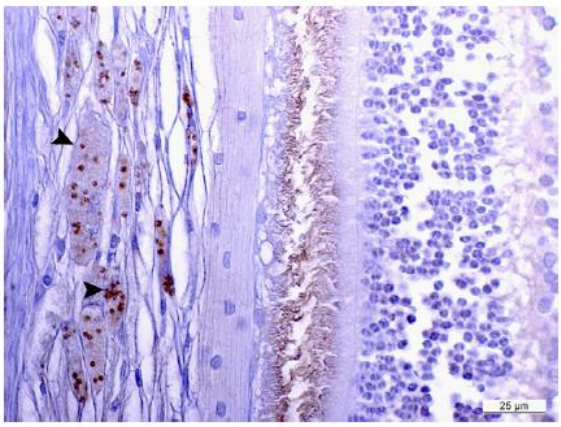

Fig. 4. Choroid. Inflammatory infiltration of macrophages with immunolabelled *Leishmania* parasites (arrowheads). IHC. Bar, 25 µm.

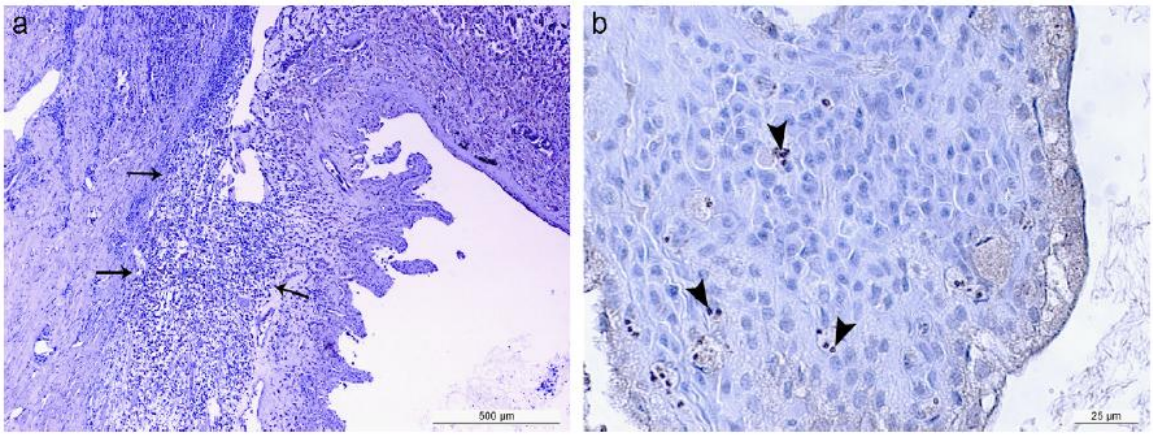

Fig. 3. (a, b). Iridocorneal angle and ciliary body. (a) Iridocorneal angle. Diffuse inflammatory infiltration of the trabecular meshwork, extending from the ciliary body (arrows). HE. Bar, 500 µm. (b) Higher magnification of the ciliary body showing multiple *Leishmania* organisms (arrowheads). IHC. Bar, 25 µm.

leishmaniosis, including the eye (McConnell *et al.*, 1970; Giles *et al.*, 1975; Molleda *et al.*, 1993; García-Alonso *et al.*, 1996). Therefore, minor lymphoplasmacytic infiltration without the presence of macrophages or parasites may not be indicative of ocular leishmaniosis.

However, inflammatory cell infiltration which includes at least some macrophages is not specific for leishmaniosis as such a reaction pattern may occur in many other infections or immune-mediated conditions (Ferrer, 1997). For this reason, identification of the parasite by morphology or immunohistochemical labelling (Ferrer *et al.*, 1988; Bourdoiseau *et al.*, 1997) is necessary for a specific diagnosis. In the present study, parasites were observed in HE-stained sections of 56.2% of eyes that contained the parasite as confirmed by IHC. In addition, 88.9% of cases in which granulomatous infiltration was present concurrently had *Leishmania* organisms identified by IHC. These results contrast with those of Molleda *et al.* (1993) in which the parasite was seen by light microscopy in only 5 cases of 22 (22.7%) that had microscopical evidence of inflammation. This emphasizes the importance of performing IHC to detect the parasite, as HE staining alone is insufficiently sensitive.

In the current study, 30% of eyes had microscopical lesions related to leishmaniosis. A previous study found that 96.6% of dogs ($n = 23$) had microscopical lesions in the anterior segment of the eye attributable to the infection (Molleda *et al.*, 1993); however, these authors studied only cases with clinical evidence of ocular lesions. In the current study, we sampled all dogs with a diagnosis of systemic leishmaniosis, regardless of the clinical involvement of the eye or eyelids.

Some degree of inflammation was present within different ocular and periocular structures in the present study, including, in order of frequency: conjunctiva and limbus, ciliary body, iris, cornea, sclera and iridocorneal angle, choroid and optic nerve sheath. These sites have already been described as being involved in CL (McConnell *et al.*, 1970; Giles *et al.*, 1975; Gallego *et al.*, 1986; Henriquez and Prats-Esteve, 1988; Molleda *et al.*, 1993; García-Alonso *et al.*, 1996), and are in agreement with clinical studies in which conjunctivitis with or without keratitis was more commonly detected than anterior uveitis (Roze, 1986; Slappendel, 1988; Molleda *et al.*, 1993; Ciaramella *et al.*, 1997).

The conjunctiva was the most commonly involved ocular structure microscopically (25% of eyes). Samples obtained from the conjunctiva (by biopsy or scraping) have been used in many studies to diagnose leishmaniosis cytologically (Peña *et al.*, 2000) or by PCR (Berrahal *et al.*, 1996; Solano-Gallego *et al.*, 2001; Strauss-Ayali *et al.*, 2004). The conjunctiva is one of the tissues in which the sensitivity of PCR for diagnosis is highest (Strauss-Ayali *et al.*, 2004). Clinically evident conjunctivitis was detected in 20 eyes (16.6%) in the present study. The differences between clinical and histopathological results may be due to the fact that not all of the dogs underwent an ophthalmological examination. Conjunctivitis in dogs is usually secondary to other causes (Hendrix, 1999), but results of our study suggest that leishmaniosis is one of the few diseases in which canine conjunctivitis can be primary. However, secondary conjunctivitis may also occur in dogs with leishmaniosis, as eyelid or lachrimal involvement can provoke surface disease, triggering secondary conjunctival hyperaemia and inflammation (Naranjo *et al.*, 2005). This may account for those cases of the present series in which we found that there was mild lymphoplasmacytic conjunctivitis without macrophages or parasites.

In those cases in which the limbus, cornea and/or sclera were affected, the parasite and accompanying inflammatory infiltration arose from the conjunctiva, and further extension occurred in more severe cases. This observation may explain why the cornea and sclera were only affected in 7 eyes. These 7 eyes included the 3 eyes reported to have keratitis clinically. In other clinical studies, keratitis has been more commonly detected (for example in 31.4% of eyes in the study by Peña *et al.*, 2000). It is possible that milder forms of keratitis (with oedema or mild hyperkeratosis) are secondary to external complications due to palpebral or lachrimal involvement rather than direct action of the parasite (Naranjo *et al.*, 2005).

Anterior uveal tract involvement was noted in 24 eyes (20% of eyes when data from the iris and ciliary body were combined). In another study, the percentage of anterior uveal involvement was as high as 45% of dogs, but as stated previously, in that study it was unclear whether a sequential sample of dogs with systemic leishmaniosis was included (Molleda *et al.*, 1993). Two forms of uveitis were detected in our study, matching the two clinical presentations described for *Leishmania* uveitis (Roze, 1986; Peña *et al.*, 2000); the non-granulomatous form, that was associated with the diffuse granulomatous pattern described for the iris and ciliary body in our study, and the granulomatous or nodular form, related to the presence of granulomas within the iris seen in 2 eyes. There were 7 eyes in which parasites were detected by IHC in the iris where no inflammatory infiltration was recognized. We attribute this to the presence of melanophages obscuring the ability to detect macrophages containing parasites.

In this study, more eyes had microscopical changes in the uveal tract than had clinically evident anterior uveitis. When compared with other clinical studies, the anterior uveal involvement recorded here (20%) is lower than that recorded by Peña *et al.* (2000) (42.8%),

but higher than that reported by Molleda *et al.* (1993), Ciaramella *et al.* (1997), and Koutinas *et al.* (1999) (14.6%, 4% and 8.2%, respectively). Discordance between clinical and histopathological identification of uveitis is recognized, as mild forms of anterior uveitis with clinical findings including aqueous flare or ocular hypotension can go unnoticed microscopically (Moore *et al.*, 2003).

The choroid was affected in 7 eyes (5.8%), which is in keeping with the findings of previous clinical studies in which the prevalence of chorioretinitis was reported to be 9.8% and 3.8% (Molleda *et al.*, 1993; Peña *et al.*, 2000). Choroidal involvement was always associated with lesions of the anterior uveal tract, which is in agreement with previous descriptions (Peña *et al.*, 2000). This may indicate that posterior uveitis may only occur in more severe cases of ocular leishmaniosis. In general, posterior segment lesions are thought to occur infrequently in CL (Roze, 1986; Molleda *et al.*, 1993; Peña *et al.*, 2000).

Glaucoma has been sporadically associated with leishmaniosis (Roze, 1986; Peña *et al.*, 2000) and is always considered to be secondary to uveal inflammation. In the present study, no histopathological signs of glaucoma were detected in any of the eyes examined (not even in the single eye in which glaucoma was diagnosed clinically), but 7 eyes had inflammatory infiltration and parasites in the iridocorneal angle, suggesting that inflammatory cells invading this structure may contribute to the development of glaucoma in leishmaniosis. Inflammatory infiltration was found in the optic nerve sheath in 3 eyes. No previous study has reported optic neuritis in CL, although parasites have been noted in the optic nerve sheath area (Gallego *et al.*, 1986). Two of these 3 eyes with optic neuritis were from a dog that was examined by an ophthalmologist, but the optic disc was considered normal. In these dogs the inflammation was in the body of the nerve distant from the optic disc.

Retinal detachment was associated with choroidal lesions in 4 eyes, but no lesions were seen throughout the ocular structures in 2 eyes from 1 dog. This dog had systemic hypertension (systolic blood pressure 220 mmHg; normal range $\leqslant$160 mmHg) which was the likely cause of the retinal detachment.

In conclusion, granulomatous inflammatory infiltration with macrophages, lymphocytes and plasma cells, and *Leishmania* parasites were found in different structures of the eyes of dogs with leishmaniosis, accounting for some of the clinical signs seen in this disease. The structures affected, in order of frequency, were the conjunctiva and limbus, ciliary body, iris, cornea, sclera and iridocorneal angle, choroid and peri-neural area of the optic nerve.

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STUDY 2

Detection of *Leishmania* spp. and associated inflammation in ocular-associated smooth and striated muscles in dogs with patent leishmaniosis.

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Detection of *Leishmania* spp. and associated inflammation in ocular-associated smooth and striated muscles in dogs with patent leishmaniosis

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Abstract

Objective Canine leishmaniosis is a disease characterized by the wide distribution of the parasite throughout the tissues of the host. The purpose of this study was to describe the presence of *Leishmania* spp. and associated inflammation in ocular-associated muscles of dogs with patent leishmaniosis.

Procedures Smooth muscles (iris dilator muscle, iris sphincter muscle, ciliary muscle, Müller muscle, smooth muscle of the periorbita and smooth muscle of the nictitating membrane) and striated muscles (orbicularis oculi muscle, obliquus dorsalis muscle and dorsal rectus muscle) were evaluated. Routine staining with hematoxylin and eosin and immunohistochemistry to detect *Leishmania* spp. were performed on tissue sections.

Results Granulomatous inflammation was seen surrounding muscular fibers and was composed mainly of macrophages with scattered lymphocytes and plasma cells. This infiltrate could be seen in 52/473 (10.99%) samples of smooth muscle and 36/142 (25.35%) samples of striated muscle. Parasites were detected in 43/473 (9.09%) samples of smooth muscle and in 28/142 (19.71%) samples of striated muscle.

Conclusions To the authors' knowledge, this is the first report assessing the presence of *Leishmania* spp. and associated infiltrate in intraocular, extraocular and adnexal smooth and striated muscles. The inflammation present in those muscles could contribute to clinical signs already described, such as blepharitis, uveitis, and orbital cellulitis.

Key Words: dog, extraocular muscles, eye, immunohistochemistry, intraocular muscles, *Leishmania* spp.

INTRODUCTION

Canine leishmaniosis is a protozoan disease endemic in the Mediterranean area caused by *Leishmania infantum*.[1] It is always a systemic disease and, as a result, the parasite has been found in many tissues. Due to this wide distribution in the host, clinical signs are variable.[1,2] Masticatory muscle atrophy has been reported to occur in 24.7% of dogs affected by leishmaniosis.[3] Muscle atrophy was first attributed to the catabolic nature of the disease but recent studies have confirmed the presence of granulomatous inflammation with intrahistiocytic *Leishmania* amastigotes in skeletal muscle, including temporal, cranial tibial, and biceps femoris muscles.[4,5]

To the authors' knowledge, no study has reported either clinical signs or histopathologic lesions related to intraocular, extraocular or adnexal smooth and striated muscles involvement in dogs with leishmaniosis. This is the first description of granulomatous myositis of eye-associated muscles due to the presence of *Leishmania* organisms.

MATERIAL AND METHODS

Samples from 77 dogs (154 eyes and/or adnexal structures) were obtained for this study. Dogs were enrolled in a study on ocular and periocular leishmaniosis that has been carried out during 4 years.[6,7] Dogs that died or were euthanized at

the Veterinary Teaching Hospital from Universitat Autònoma de Barcelona with a diagnosis of leishmaniosis were included in the study. Diagnosis was made when both clinical signs and clinicopathological abnormalities compatible with leishmaniosis were present, and only if dogs were serologically positive by a previously described method.[8] In addition, in some of the dogs, the parasite was detected by means of cytology or PCR on either lymph node or bone marrow samples.

Twelve of the dogs were examined by a board-certified ophthalmologist; eight of those dogs had bilateral blepharitis, five had bilateral anterior uveitis and one dog had a history of bilateral exophthalmos 6 months prior to sampling. No neuromuscular signs were present.

Globes and adnexal structures (eyelids, main lacrimal gland, and nictitating membrane) from a total of 77 dogs (154 eyes and/or adnexal structures) were collected. Not every structure was collected from every dog, so percentages will refer to the total number of samples examined of each muscle (Table 1). Smooth muscles evaluated included iridal sphincter and dilator muscles, ciliary body muscle (118 samples of each), Müller muscle (12 samples), periorbital smooth muscle (50 samples) and smooth muscle of the nictitating membrane (57 samples). A total of 473 samples of smooth muscle were examined. Striated muscles included orbicularis oculi (98 samples), dorsal rectus and dorsal oblique (the last two, as seen in association with the lacrimal gland; 44 samples). A total of 142 samples of striated muscles were examined.

All samples were formalin fixed and routinely processed for histopathological examination. Sections of all samples were stained immunohistochemically for *Leishmania* detection, as previously described.[9]

Table 1. Samples of each muscle in which inflammation and *Leishmania* amastigotes were detected

Muscle	Samples with inflammation (%)	Samples with parasite (%)	Total	Total (striated vs. smooth muscle)
Iridal dilator	2 (1.69)	2 (1.69)	118	473
Iridal sphincter	7 (5.93)	7 (5.93)	118	
Ciliary body muscle	17 (14.40)	16 (13.55)	118	
Müller muscle	4 (33.33)	4 (33.33)	12	
Smooth muscle of NM	12 (21.05)	9 (15.78)	57	
Periorbital smooth muscle	10 (20)	5 (10)	50	
Dorsal oblique and rectus	9 (20.45)	7 (15.9)	44	142
Orbicularis oculi muscle	27 (27.55)	21 (21.42)	98	
Total	88 (14.30)	71 (11.54)	615	

Data expressed as number of affected samples (percentage of total examined of each type).
NM = nictitating membrane.

RESULTS

Histopathologic results are shown in Table 1. In all muscles examined, the inflammation consisted predominantly of macrophages with variable amounts of lymphocytes and plasma cells and occasional scattered neutrophils. Usually, it was diffusely located around muscle fibers throughout the endomysium of smooth and striated muscles (Fig. 1). The parasites were typically found in the cytoplasm of macrophages. Occasionally, parasites were seen within the myofibers (Fig. 2). Myofiber necrosis, degeneration or regeneration were not seen in any of the samples examined, although atrophy of the muscle fibers was occasionally found and interpreted to be secondary to the associated inflammatory process.

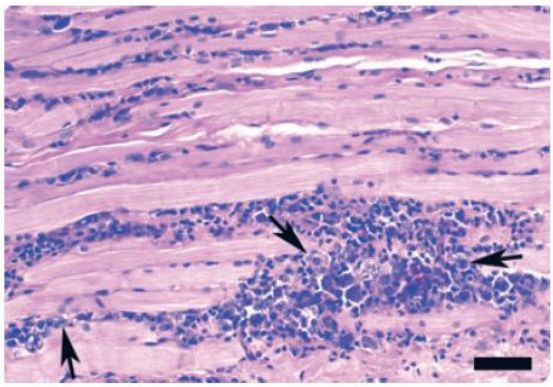

Figure 1. Extraocular striated muscle. Inflammatory infiltrate composed predominantly of macrophages located around muscular fibers. Parasites can be seen inside macrophages (arrows). Hematoxylin and eosin. Bar = 100 µm.

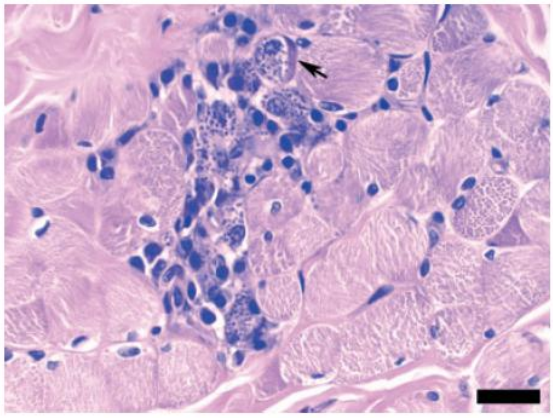

Figure 2. Orbicularis oculi muscle. Occasionally, parasites could be seen inside the muscular fiber (arrow). Hematoxylin and eosin. Bar = 25 µm.

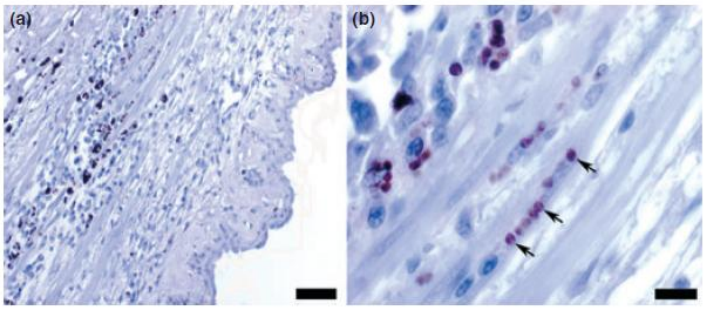

Figure 3. Ciliary muscle. Immunohistochemistry for *Leishmania*. (a) Inflammatory infiltrate located around muscular fibers. Bar = 50 µm. (b) Higher magnification of inset in Fig. 3a. Parasites are rowing inside a muscular fiber (arrow). Bar = 10 µm.

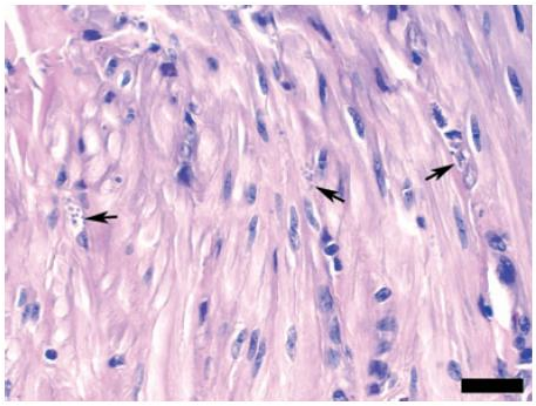

Figure 4. Müller muscle. Scattered parasites interspersed with muscular fibers (arrows). Hematoxylin and eosin. Bar = 25 µm.

In 37/154 (24.03%) eyes and adnexal structures, inflammation with or without parasites was detected in at least one of the muscles evaluated. When considering the types of muscle evaluated, inflammation was detected in 52/473 (10.99%) samples of smooth muscle and in 36/142 (25.35%) samples of striated muscle. Parasites were detected by means of immunohistochemistry in 43/473 (9.09%) samples of smooth muscle and in 28/142 (19.71%) samples of striated muscle.

The above-described granulomatous myositis was found in 2/118 (1.69%) samples of iridal dilator muscle, in 7/118 (5.93%) samples of iridal sphincter muscle, 17/118 (14.40%) samples of ciliary body muscle (Fig. 3a,b), 4/12 (33.33%) samples of Müller muscle (Fig. 4), 27/98 (27.55%) samples of orbicularis oculi muscle (Fig. 2), 10/50 (20%) samples of periorbital smooth muscle, 9/44 (20.45%) samples of orbital striated muscle (dorsal rectus and dorsal oblique), and 12/57 (21.05%) samples of smooth muscle of the nictitating membrane.

Immunohistochemistry revealed *Leishmania* amastigotes in 2/118 (1.69%) samples of iridal dilator muscle, 7/118 (5.93%) samples of iridal sphincter muscle, 16/118 (13.55%) samples of ciliary body muscle (Fig. 3a,b), 4/12 (33.33%) samples of Müller muscle, 21/98 (21.42%) samples of orbicularis oculi muscle, 5/50 (10%) samples of periorbital smooth muscle, 7/44 (15.90%) samples of orbital striated muscles (dorsal rectus and dorsal oblique), and 9/57 (15.78%) samples of smooth muscle of the nictitating membrane (Fig. 5a,b).

Twelve of the 16 eyes with clinically diagnosed blepharitis showed lesions in the examined palpebral muscles (including orbicularis oculi and/or Müller muscle), two of these did not show any lesions, and the remaining two eyelids from the same dog were not collected. Of the five dogs (10 eyes) with clinical uveitis, two dogs (4 eyes) had lesions in the intraocular muscles. In the only dog in which exophthalmos was diagnosed 6 months prior to sample

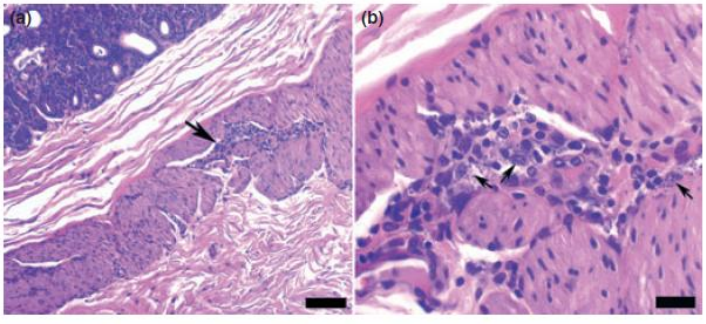

Figure 5. Smooth muscle of the nictitating membrane. Hematoxylin and eosin. (a) Nictitating membrane gland can be seen at the left top left. Inflammatory infiltrates located between muscular fibers (arrow). Bar = 100 µm. (b) Higher magnification of Fig. 5a. Macrophages filled with parasites can be seen (arrows). Bar = 25 µm.

collection, no lesions were detected in the examined orbital muscles.

DISCUSSION

Canine leishmaniosis is a protozoan disease characterized by the widespread distribution of the parasite throughout the organism, explaining the wide variety of clinical signs seen.[1,2] To the best of the authors' knowledge, this is the first report to describe *Leishmania* spp. amastigotes in intraocular, extraocular and periocular striated and smooth muscles. Although these muscles are probably not a specific target of the parasite, these findings further highlight the potential to find lesions of leishmaniosis in every organ and tissue.

The granulomatous inflammation noted in the muscles examined in this study, composed mainly of macrophages and variable amounts of lymphocytes, plasma cells and neutrophils, is similar to what has been previously described in other tissues,[2,10] including ocular and periocular structures.[6,7,11–14]

Other muscles have been reported to be affected in canine leishmaniosis. Amongst striated muscles, myositis of the masticatory muscles (temporalis and masseter muscles) and cranial tibial muscle has been described.[4] A recent report used immunohistochemistry to evaluate the inflammatory cell types in biopsies from the biceps femoris muscle from dogs with leishmaniosis showing signs of muscle weakness and atrophy.[5] While in these studies granulomatous myositis was noted, both reports described necrosis, degeneration and regeneration of muscle fibers, which was not present in the samples examined in this study. This discrepancy might represent a variation in the muscles studied or ranges of disease progression and severity.

Cardiac muscle has been recently reported to be involved in canine leishmaniosis, where a severe granulomatous myocarditis was noted and detection of the parasite within macrophages was described.[15,16]

Other protozoa have been described to involve canine striated muscle, such as *Hepatozoon americanum*,[17] *Sarcocystis neurona*,[18] *Neospora caninum*,[19] and *Toxoplasma gondii*.[20] Only *N. caninum* has been reported to affect the eye and extraocular muscles, although affected structures within the globe were not specified.[19]

Smooth muscle inflammation has been previously reported with leishmaniosis, in a previous report on two dogs with colitis, intense inflammation in the mucosa, including the muscularis mucosae, and submucosa of the large intestine was described.[21] To the authors' knowledge, no other smooth muscles have been described to be involved in canine leishmaniosis.

Myoinhibitory peptides synthesized by *Leishmania major* have been demonstrated to inhibit or decrease force of contraction of smooth and striated muscles of vertebrates *in vitro*.[22] Production of these peptides by *Leishmania infantum* has not been reported so, at this point, it is not possible to determine if similar myoinhibitory effects could contribute to the pathogenesis of clinical signs such as weakness in canine leishmaniosis. Presence of granulomatous inflammation distorting the architecture of muscle bundles suggests that at least mechanical and inflammatory actions may be implicated in muscle pathology and associated clinical signs in canine leishmaniosis. Occasionally, amastigotes were detected within myofibers, a finding that has been described by other authors.[4] In a recent study, laser capture microdissection of muscle fibers was performed and parasite DNA was not detected in the sarcoplasm of isolated muscle fibers.[5] These authors concluded that *Leishmania* does not penetrate into myofibers, but rather stays within the macrophages present in the endomysium. In this study isolation of the myofibers was not attempted, and results are based on examination of routinely stained histopathology slides and immunohistochemistry results.

In this study, striated muscles were more commonly affected than smooth muscles, both by granulomatous inflammation (25.35% vs. 10.99%) and parasites (19.79% vs. 9.01%). These differences are not significant if we exclude intraocular muscles, which were the least affected and contributed to the overall low percentage of smooth muscle myositis. When excluding intraocular muscles, percentages of granulomatous myositis are 25.35% vs. 21.48% for striated and smooth muscles respectively, and 15.12% vs. 19.71% for parasite presence in striated and smooth muscles respectively. These percentages are similar to those described for other ocular structures,[7] in which inflammation was found in 30% of the eyes and parasites were detected in 26.6% of the eyes. When comparing tissues anatomically related, it is also possible to find similarities. In particular, in a previous study, granulomatous iritis and cyclitis was described in 8.3% and 15.3% of eyes respectively,[7] which compares to the percentage of granulomatous myositis of iridal sphincter muscle (5.93%) and ciliary body muscle (14.40%) in this study.

In this study, the number of dogs with clinical signs potentially attributable to periocular and intraocular myositis, such as blepharitis, exophthalmos or uveitis, was too low to establish a clinical-histopathological correlation, but it is possible that the lesions detected could contribute to some of the previously described ophthalmological signs to a certain degree.[7,11–14,23,24] Myositis of the various eyelid muscles could contribute to blepharitis seen in some dogs with leishmaniosis,[13,23,24] although inflammation in adjacent structures, such as conjunctiva and meibomian glands, has been described and is probably also implicated in the development of clinical blepharitis.[6,7] Only 16 eyelid samples in this study came from dogs with clinically diagnosed blepharitis, 12 of them showing myositis of Müller muscle and/or orbicularis oculi muscle. Myositis of orbital muscles (extraocular muscles and smooth muscle of the periorbita) could contribute to orbital cellulitis described in dogs with leishmaniosis,[24] although none of the dogs included in this study had clinical signs compatible with

orbital cellulitis at the time of sample collection. The only dog with reported exophthalmos 6 months prior to sampling had no microscopic lesions.

Intraocular myositis, along with the previously reported granulomatous iritis/cyclitis,[7] may in some cases contribute to the clinical signs of anterior uveitis described with canine leishmaniosis,[11–14,23,24] Ten eyes had clinical uveitis, and only four of them had microscopic lesions in any of the intraocular muscles evaluated. This finding has to be evaluated with caution, as not every dog underwent a systematic ophthalmic examination, so ocular signs such as blepharitis, uveitis, and orbital cellulitis may have been underestimated in this study.

In conclusion, this is the first report to describe myositis of adnexal, extraocular and intraocular smooth and striated muscles in canine leishmaniosis, as well as the presence of *Leishmania* spp. amastigotes in these structures. It is not possible to conclude from the current study if periocular, extraocular and intraocular myositis are clinically relevant, but these findings underscore the importance of always considering canine leishmaniosis as a systemic disease, potentially affecting every tissue and organ. In an endemic area, leishmaniosis has to be included in the differential diagnosis of dogs with ocular disease of undetermined etiology.

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STUDY 3

Characterization of lacrimal gland lesions and possible pathogenic mechanisms of keratoconjunctivitis sicca in dogs with leishmaniosis. C. Naranjo, D. Fondevila, M. Leiva, X. Roura, T. Peña. Veterinary Parasitology 2005;133:37-47.

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Characterization of lacrimal gland lesions and possible pathogenic mechanisms of keratoconjunctivitis sicca in dogs with leishmaniosis

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Abstract

In a previous study, it was found that 2.8% of dogs with leishmaniosis had keratoconjunctivitis sicca (KCS). The aim of this study was to characterize the lesions present in the lacrimal glands of dogs with leishmaniosis and to determine the presence of the parasite by means of immunohistochemistry. The inflammatory infiltrate was described as granulomatouos or pyogranulomatous and was located around the ductal component of the glands. Immunoperoxidase staining localized the parasites following the same pattern. Samples from eyes that had clinical signs compatible with KCS presented inflammatory infiltrate and parasite more commonly than those from eyes without clinical signs. One of the mechanisms of KCS in dogs with leishmaniosis may be the inflammatory infiltrate located around the ducts of lacrimal glands, producing retrograde accumulation and retention of secretion. Meibomian gland was the most commonly affected by the infiltrate, highlighting the possibility of a qualitative KCS in these dogs.

Keywords: Keratoconjunctivitis sicca; Lacrimal glands; Immunohistochemistry; *Leishmania* spp.; Dog

1. Introduction

Canine leishmaniosis (CL) is endemic in the Mediterranean basin. In this region, it is caused by the parasite *Leishmania infantum* and it is transmitted by sand flies of the genus *Phlebotomus* (Slappendel, 1988; Ferrer, 1992; Ciaramella et al., 1997; Slappendel and Ferrer, 1998; Fisa et al., 1999; Koutinas et al., 1999; Solano-Gallego et al., 2001). CL is a systemic disease with widely variable clinical signs including skin disorders, generalized lymphadenomegaly, weight loss, lameness, ocular lesions, renal failure, epistaxis and diarrhea (Slappendel, 1988; Ciaramella et al., 1997; Koutinas et al.,

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1999). Ocular manifestations are reported to range between 16 and 80.49% of the affected animals (Slappendel, 1988; Molleda et al., 1993; Ciaramella et al., 1997; Koutinas et al., 1999; Peña et al., 2000). These signs can represent between 3.48 and 7% of presenting complaints in dogs with CL and they can be the only clinical manifestation in a percentage of cases that varies from 3.72 to 16%. The prevalence of keratoconjunctivitis sicca (KCS) in dogs suffering from leishmaniosis with ocular signs varies between 2.8 and 26.83% (Molleda et al., 1993; Ciaramella et al., 1997; Koutinas et al., 1999; Peña et al., 2000; Ciaramella and Corona, 2003).

The pathogenic mechanisms by which *Leishmania* parasites cause lesions in different organs include non-suppurative inflammatory infiltrate and the production of circulating immune complexes that deposit in various tissues (Ferrer, 1992). Often, very few amastigotes are present within the inflammatory infiltrate and, in these cases, immunoperoxidase staining has proved a sensitive and specific method to detect the parasite in tissue samples (Ferrer et al., 1988; Bourdoiseau et al., 1997). Roze (1986) cited three theories that could explain tear deficiency in dogs with leishmaniosis. Firstly, it could result from the direct destructive action of the parasite accompanied with intense inflammation of the lacrimal glands. Secondly, it could also be due to obstruction of the secretory ducts due to inflammation of the adjacent structures or finally, it could derive from reduced reflex secretion following hypoaesthesia of the damaged cornea.

Tear film is a trilaminar fluid consisting of lipid, aqueous and mucin components. The outer lipid layer is secreted by Meibomian glands (MG), the intermediate aqueous fluid is produced by main lacrimal gland (MLG) and nictitating membrane gland (NMG), and the innermost mucus layer is secreted by goblet cells of the conjunctiva. When there is a deficit of the aqueous component, quantitative KCS develops, whereas qualitative tear film disease occurs when there is a deficiency of one of the other two components of tears (Moore, 1990, 1999).

To the best of the authors' knowledge, no histopathological or parasitological studies have tried to describe the pathogenic mechanisms of KCS in dogs with leishmaniosis. The purpose of this study was to describe the histopathological findings present in lacrimal glands of dogs affected with leishmaniosis and to assess the presence of the parasite in these tissues by means of immunohistochemical techniques, in order to determine the pathogenic mechanism of keratoconjunctivitis sicca in CL.

2. Materials and methods

2.1. Animals

Twenty-eight dogs were included in the study, 25 of which were clinical cases affected with leishmaniosis and the remaining 3 were used as controls. Twenty-two of the ill dogs suffered from natural infection and three had been inoculated with *Leishmania infantum* promastigotes. Dogs with experimental infection and control dogs belonged to a research group of the Universitat Autonoma de Barcelona (UAB).

The diseased dogs were diagnosed at the Hospital Clínic Veterinari (HCV) from UAB. They all had clinical signs, complete blood count and serum biochemistry results compatible with CL. Diagnosis was confirmed with serology (Riera et al., 1999) and in some of them the parasite was detected by direct observation or amplification of DNA in bone marrow, lymph nodes or biopsies.

The three control dogs were from an endemic area and had no signs of CL. They had negative serology titers and negative delayed hypersensitivity test to leishmanin. Eighteen of the dogs with CL had been treated against *Leishmania* during a range of time that varied from 3 days to 10 years. The remaining seven dogs had not received any specific treatment.

Ophthalmic signs (Table 1) were detected in 11 of the cases by the attending clinician, four of which were evaluated by a certified ophthalmologist. Six dogs (12 eyes) had blepharitis, four dogs (8 eyes) had periocular alopecia, three dogs (5 eyes) had conjunctivitis, six dogs (11 eyes) had keratoconjunctivitis and two dogs (4 eyes) had anterior uveitis. The 22 dogs that suffered from natural infection died or were euthanized at the HCV from UAB after progressive worsening of the dogs' condition, due to non-responsiveness to treatment or because of the owner decision. The three inoculated dogs and the three control dogs were euthanized at the end of their study period.

Table 1
Clinical signs, histological and immunohistochemical results of lacrimal glands lesions from dogs with leishmaniosis

Case	Eye	Clinical signs	Meibomian glands		Main lacrimal gland		Nictitating membrane gland	
			HE	IH	HE	IH	HE	IH
1	OD	C, K	G	+	0	+	G	+
	OS	C, K	G	+	0	+	G	+
2	OD	A, C, K	G	+	0	+	G	+
	OS	A	G	+	0	+	G	+
3	OD		0	–	0	–	0	–
	OS		LP	–	0	–	LP	–
4	OD	B, C, K	G	–	0	–	0	–
	OS	B, C, K	G	–	0	–	0	–
5	OD		0	–	0	–	LP	–
	OS		0	–	0	–	LP	–
6	OD		G	–	0	–	LP	–
	OS		G	–	0	–	G	–
7	OD		0	–	0	–	G	+
	OS		0	+	/	/	G	+
8	OD	B	G	–	0	–	LP	–
	OS	B	G	–	0	–	LP	–
9	OD		LP	–	0	–	LP	–
	OS		0	–	0	–	G	–
10	OD	A, C	G	–	LP	–	0	+
	OS	A, C	G	–	LP	–	G	–
11	OD		LP	–	0	–	LP	–
	OS		0	–	0	–	LP	–
12	OD	C, K	G	+	LP	+	G	+
	OS	C, K	G	+	LP	+	G	+
13	OD		0	–	0	–	0	–
	OS		0	–	0	–	G	–
14	OD		0	–	0	–	LP	–
	OS		G	–	0	–	0	–
15	OD		0	–	0	–	G	–
	OS		0	–	0	–	0	–
16	OD		G	–	LP	–	G	–
	OS		G	+	0	–	LP	–
17	OD	A, B, C	G	–	0	–	G	–
	OS	A, B	G	–	LP	–	G	–
18	OD		G	–	/	/	G	–
	OS		G	–	0	–	G	–
19	OD	C, K	0	–	0	–	LP	–
	OS	C, K	0	–	LP	–	LP	–
20	OD		G	–	0	–	0	–
	OS		0	–	0	–	0	–
21	OD	B	G	+	LP	–	G	–

Table 1 (*Continued*)

Case	Eye	Clinical signs	Meibomian glands		Main lacrimal gland		Nictitating membrane gland	
			HE	IH	HE	IH	HE	IH
	OS	B	G	+	LP	–	G	–
22	OD		G	+	LP	+	0	–
	OS		0	+	LP	–	G	–
23	OD	B, C	G	+	0	+	G	+
	OS	B, C	G	+	LP	–	G	+
24	OD	A, B, C, K	G	+	G	+	G	+
	OS	A, B, C, K	G	+	G	+	G	+
25	OD		G	–	0	–	G	–
	OS		G	–	0	–	G	–

OD, right eye; OS, left eye; HE, hematoxilin and eosin; IH, immunohistochemistry; A, alopecia; B, blepharitis; C, conjunctivitis; K, keratoconjunctivitis; G, granulomatous to pyogranulomatous infiltrate; LP, lymphoplasmacytic infiltrate; 0, absence of infiltrate; +, presence of parasite; –, absence of parasite; /, sample that cannot be evaluated.

2.2. Collection and processing of samples

For each animal, samples of MG (eyelids), MLG and NMG of both eyes were collected. That is, three samples from each eye (six samples per animal) were obtained. MLG could not be retrieved from two eyes (left eye from case 7 and right eye from case 18) due to problems with the dissection (Table 1).

These samples were fixed in 10% neutral buffered formalin and paraffin embedded. Sections of the tissue were cut and stained for routine histological and immunohistochemical examination as described by Ferrer et al. (1988).

2.3. Statistical analysis

For statistical analysis, SPSS® software was used. In order to establish relationships between variables, contingence table analysis was performed and statistical significance was set at $P < 0.05$.

3. Results

3.1. Histopathology

No histopathologic alteration was found in the samples from control dogs (Figs. 1A and 2A) (Table 1). Occasional infiltration of lymphocytes and plasma cells were seen in the interstitium of the glands of control dogs (Figs. 1B and 2B).

Inflammatory infiltrate seen in samples from ill dogs was classified as granulomatous or pyogranulomatous. It was located mainly around the ductal component (Figs. 1C and 2C) of the MG, MLG and NMG, and occasionally around the acinar component. Only in one case this infiltrate was also seen destroying the acinar component in the three glands of both eyes (case 24). Macrophages were predominant (Figs. 1D and 2D), with scattered lymphocytes and plasma cells. Isolated neutrophils could also be seen in some sections. This periductal infiltrate, that in some cases was even destroying duct walls, provoked retrograde dilation of the ducts and retention of secretion (Figs. 2C and 5A).

In sections of the nictitating membrane in which conjunctiva could be seen, the same kind of infiltrate could also be detected in the subepithelial region of the bulbar surface, surrounding lymphoid follicles and gland secretory ducts.

This infiltrate was present in 60 out of 148 total samples (40.54%) that belonged to 37 eyes (74%) from 21 dogs (84%). No significant differences between left and right eye were found ($P = 0.738$).

Inflammatory infiltrate presence in each of the glands is represented in Fig. 3. MG was the most commonly affected gland, but there was no statistical significance in infiltrate presence between this structure and NMG ($P = 0.418$). Only two MLG had this infiltrate, and they were from the same dog (case 24). Differences were statistically significant between MG and MLG ($P < 0.001$) and between

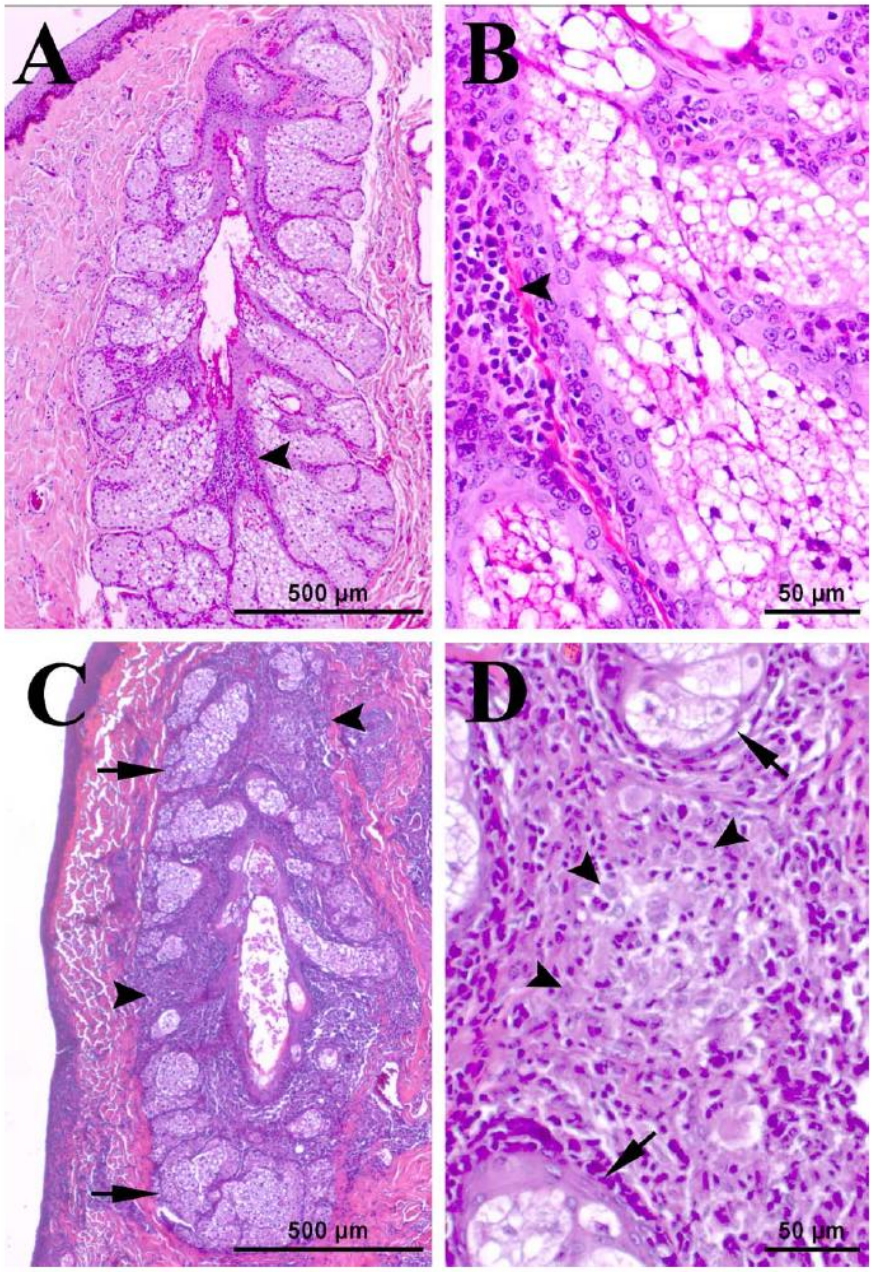

Fig. 1. Meibomian gland. (A and B) Control dog. (A) Focal periductal infiltrate (arrowhead). (B) Scant periductal infiltrate composed of lymphocytes and plasma cells (arrowhead). (C and D) Dog with leishmaniosis. (C) Granulomatous to pyogranulomatorus periductal inflammatory infiltrate (arrowheads). Acinar component is preserved (arrows). (D) Macrophages (arrowheads) were predominant, with scattered lymphocytes, plasma cells and neutrophils. H&E.

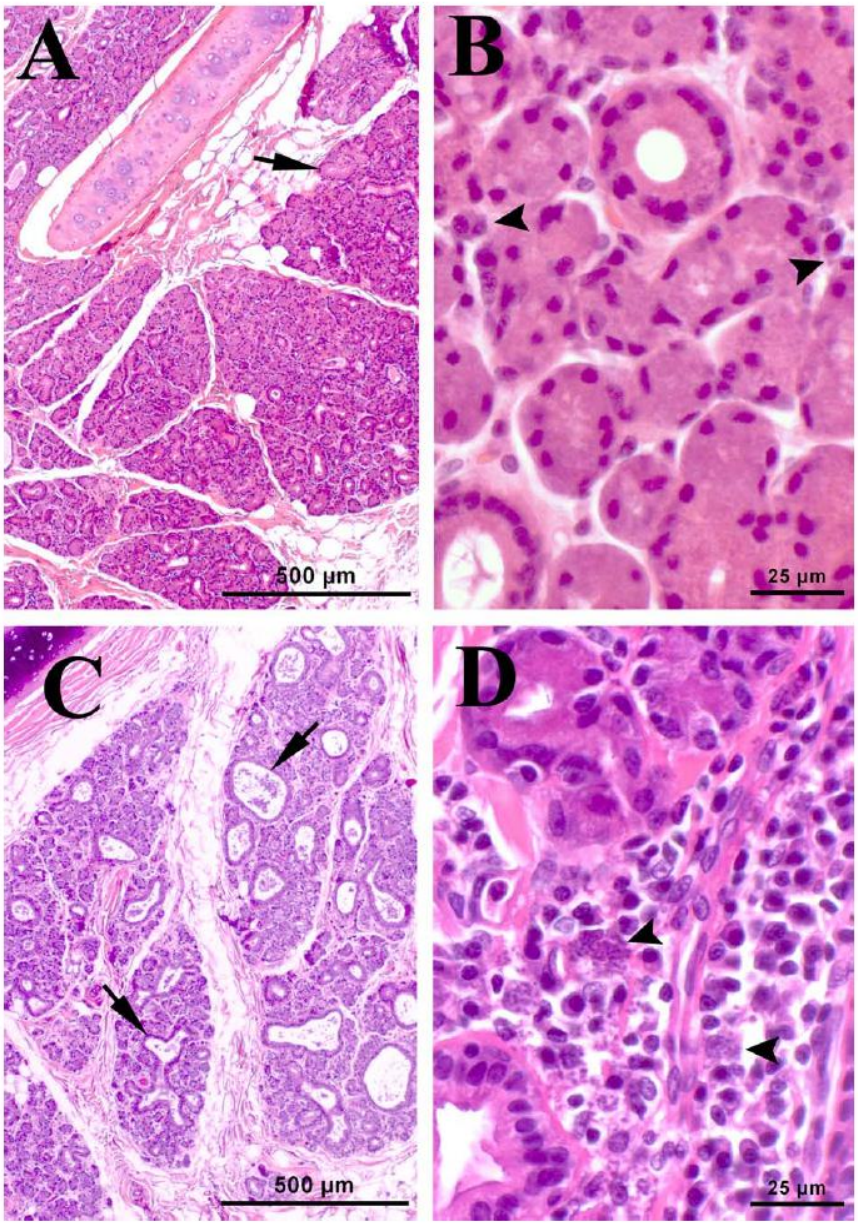

Fig. 2. Nictitating membrane. (A and B) Control dog. (A) Normal gland architecture with non-dilated ducts (arrow). (B) Acinar component with scarce interstitial lymphocytes and plasma cells (arrowhead). (C and D) Dogs with leishmaniosis. (C) Dilation of ducts with retention of secretion (arrows) and granulomatous to pyogranulomatous periductal inflammatory infiltrate. (D) Macrophages with intracytoplasmic amastigotes (arrowheads), lymphocytes and plasma cells. H&E.

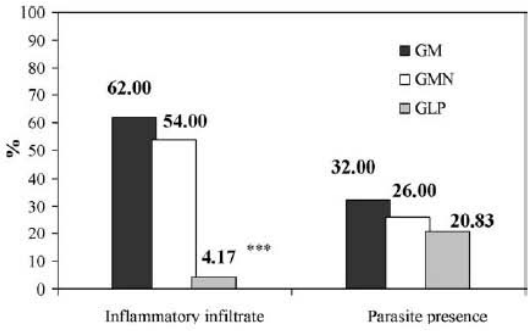

Fig. 3. Percentage of granulomatous/pyogranulomatous inflammatory infiltrate and parasite presence in lacrimal glands of dogs with leishmaniosis. Number above bar is the percentage of cases represented by the bar (50 MG and NMG and 48 MLG were examined). MG, Meibomian gland; MLG, main lacrimal gland; NMG, nictitating membrane gland. ***Significant differences between MG and MLG ($P < 0.001$) and between NMG and MLG ($P < 0.001$).

NMG and MLG ($P < 0.001$). Of the 37 eyes affected, only in 2 of them (5.4%) the infiltrate was present in the three glands. These two eyes were from the same dog (case 24), which suffered from natural infection. Nineteen eyes (51.35%) had two of the three glands affected, and these glands were always MG and NMG. Sixteen eyes (43.24%) had only one gland affected. In 10 of these eyes, this structure was MG and, in the remaining 6 eyes, NMG was the only one affected.

Samples belonging to eyes with clinical signs compatible with KCS were more commonly affected by the inflammatory infiltrate described than those from eyes without clinical signs ($P < 0.001$) (Fig. 4). When evaluating ocular clinical signs separately, MG

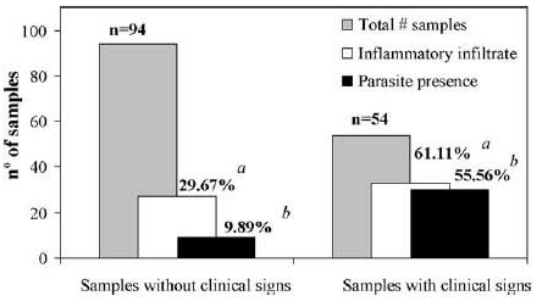

Fig. 4. Inflammatory infiltrate and parasite presence in samples from dogs without clinical signs related to KCS (left) or with clinical signs related to KCS (right). [a]Significant differences in inflammatory infiltrate $P < 0.001$. [b]Significant differences in parasite presence $P < 0.001$.

from eyes with periocular alopecia, blepharitis and conjunctivitis were more commonly affected by the inflammatory infiltrate ($P = 0.018$, 0.002 and 0.011). There was also relationship between MLG infiltration and occurrence of keratoconjunctivis ($P = 0.049$). There was no association between MG affection and keratoconjunctivitis ($P = 0.125$), MLG and conjunctivitis ($P = 0.106$), NMG and conjunctivitis ($P = 0.151$) and NMG and keratoconjuntvitis ($P = 0.468$).

Clinical signs compatible with KCS were more commonly related to those eyes that had two or three glands affected by the infiltrate than to those eyes in which no lacrimal gland or only one of the three glands was affected by the inflammatory infiltrate ($P = 0.001$).

3.2. Immunohistochemistry

Parasite was not observed in any of the samples from the control dogs (Table 1).

In the samples from ill dogs, macrophages with immunolabeled parasites (Fig. 5A and B) were located around the ductal component of the glands studied, following the same pattern as the inflammatory infiltrate.

Parasite was visualized in 39 out of 148 samples (26.35%) that belonged to 18 eyes (36%) from 10 animals (40%). Differences between left and right eyes were not statistically significant ($P = 0.351$).

Parasite presence in the different types of glands is represented in Fig. 3. No statistical significance was found between the different tissues ($P = 0.454$), but in the 10 MLG in which parasite was found, only one or two organisms were detected in each sample.

Within the 18 eyes that had the parasite detected, it was present in the three glands in 9 of them (50%). In three eyes (16.6%) parasite was seen in two of the three glands. One of these two glands was MG in the three eyes, and the other gland that contained the parasite was the NMG in two eyes and MLG in one eye. Six eyes (33.3%) had the parasite in only one gland. This gland was MG in four eyes and NMG in two eyes.

Parasite was more frequently detected in samples obtained from eyes that had clinical signs than in those from eyes that had not ($P < 0.001$) (Fig. 4). Those eyes in which more than one gland contained the parasite had clinical signs more commonly than those

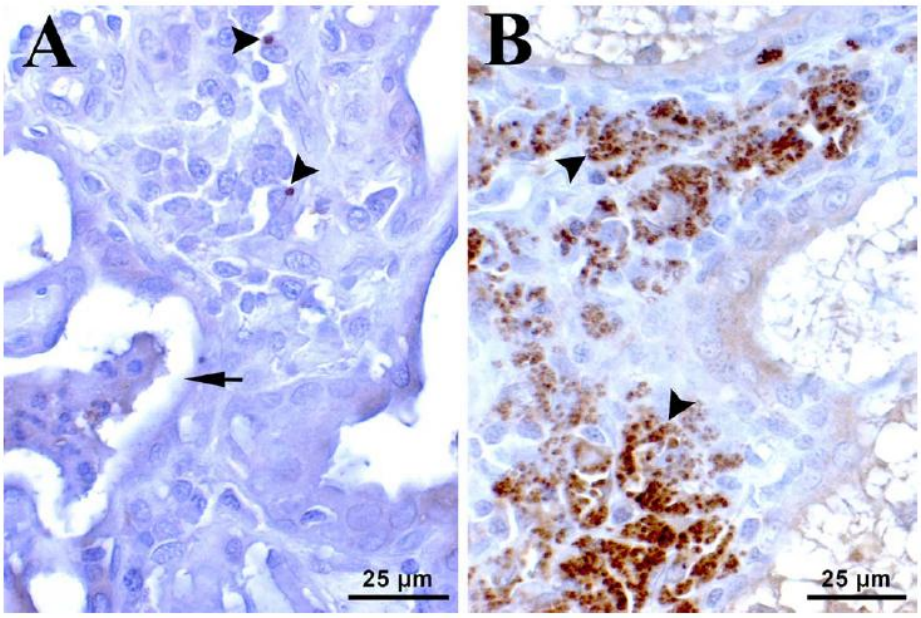

Fig. 5. Immunohistochemistry to *Leishmania*. (A) Nictitating membrane. Dilation of ducts with retention of secretion (arrow). Macrophages with immunolabeled parasites within the cytoplasm (arrowheads). (B) Meibomian gland. Granulomatous periductal inflammatory infiltrate composed of macrophages packed with immunolabeled amastigotes (arrowheads).

in which only one gland or no gland had the parasite detected ($P = 0.002$). There was an association between eyes that had conjunctivitis and parasite presence in the three glands ($P = 0.012$ for MG and $P < 0.001$ for MLG and NMG) and eyes that had keratoconjunctivitis and parasite presence in the three glands ($P = 0.011$ for MG, $P < 0.001$ for MLG and $P = 0.001$ for NMG). There was no relationship between parasite presence in MG and alopecia ($P = 0.234$) or blepharitis ($P = 0.125$).

Presence of granulomatous infiltrate was related to presence of parasite when all samples were considered together, regardless of the tissue considered ($P < 0.001$), and also when every gland was considered separately ($P = 0.012$, 0.005 and 0.001 for the MG, MLG and NMG, respectively).

4. Discussion

Although KCS is a potentially blinding disease, no attempt had been made to determine the etiology of this disease in dogs suffering from leishmaniosis. Results obtained in this study allow us to propose a pathogenic mechanism by which KCS in dogs with leishmaniosis may develop. Granulomatous infiltrate that accumulates around lacrimal glands ducts provokes retrograde dilation with accumulation of secretion. These findings are in agreement with the second theory postulated by Roze (1986). We have not seen destruction of the acinar component of the glands in any of the samples, not even in the case in which we detected the inflammatory cells and the parasite around the acinis.

In our study we have not evaluated the contribution of corneal hypoaesthesia to the decrease in tear secretion. Inflammation in the cornea, specially when it becomes edematous and hyperpigmented, can diminish corneal sensation and thus decrease reflex component of tears (Xu et al., 1996; Mathers, 2000). Due to the design of our study, in which we obtained the samples just after death or euthanasia of the dogs, we could not determine neither corneal sensitivity nor Schirmer Tear Tests (I and II). Anyway, if a dog would have been examined for evaluation of corneal disease, it would have been impossible to determine what had

occurred first: primary keratitis due to leishmaniosis with subsequent decrease in Schirmer Tear Test or corneal alteration following the development of KCS.

In another study (García-Alonso et al., 1996), histopathological findings in two dogs affected with CL revealed intense inflammatory reaction with granulation around lacrimal ducts, composed of lymphocytes, plasma cells and macrophages. These authors also described epithelial necrosis and partial loss of epithelium, but they did not discuss about the possible implications on tear deficiency. We have not seen granulation tissue or necrosis in our samples.

Inflammatory infiltrate detected in samples from control dogs, composed of scattered lymphocytes and plasma cells, is the one described in normal lacrimal glands from healthy dogs (Martin et al., 1988). These plasma cells are responsible for the production of IgA that tears contain (Martin et al., 1988; Schlegel et al., 2003). So, we have not considered it as pathologic when we have seen it either in the samples from control or diseased dogs.

MG was the most commonly affected by the inflammatory infiltrate in diseased dogs, although differences with NMG were not statistically significant. MG produce the most external layer of the lacrimal film. When these glands are damaged, lipid secretion gains polarity and therefore early evaporation of tears occurs. These altered lipids may also be directly toxic to corneal surface. This combination of insults leave the cornea unprotected and they can lead to surface disease. Qualitative tear film disease can evolve with a Schirmer Tear Test of over 15 mm, due to reflex tearing that tries to compensate for the early evaporation and dilute abnormal lipids secreted (Moore, 1990, 1999). So, it is possible that dogs with leishmaniosis might suffer from a qualitative tear film disease, although it has not been clinically evaluated in this study nor cited in other publications.

NMG was more frequently affected than MLG. In addition, we have seen inflammatory infiltrate not only in the ductal component of the gland, but also subepithelially in the bulbar surface of nictitating membrane, where secretory ducts exit (Moore et al., 1996). This can create even more retention of secretion in these glands. NMG can produce between 30 and 50% of aqueous component of tears, depending on the individual dog (Helper et al., 1974; Gelatt et al., 1975). So it is possible that, when this gland is massively infiltrated in dogs whose NMG synthesizes a high proportion of the tears, KCS can develop, even when MLG remains unaffected. Conversely, in those dogs whose aqueous component of tears is mainly secreted by MLG, lesions in NMG can have no clinical consequences. This also occurs in animals in which NMG is removed, as in some of them MLG can compensate increasing its production (Helper et al., 1974; Gelatt et al., 1975; Dugan et al., 1992).

In addition, although we have not evaluated goblet cells systematically, subepithelial infiltration of macrophages and parasites in nictitating membrane sections in which bulbar surface of conjunctiva could be seen suggests that mucous secretion can be also affected in these dogs.

The fact that MLG has been the least frequently affected may be due to its location. Whereas MG and NMG are located near tissues presumed to have high concentrations of parasites, MLG is situated within orbital tissue and it can be more difficult to reach for the parasite. MG are located in the eyelid, the external part of which is a prolongation of the skin, and they are modified sebaceous glands (Moore, 1990, 1999). Eyelids are highly vascularized tissues, and in other studies on leishmaniosis (Koutinas et al., 1992; Solano-Gallego et al., 2004), skin sebaceous glands have been observed totally obliterated by perifollicular granulomatous inflammation. NMG is located in the nictitating membrane, which is a fold of the conjunctiva. Conjunctival biopsies or scrapings have been used in many studies to diagnose leishmaniosis (Berrahal et al., 1996; Solano-Gallego et al., 2001; Strauss-Ayali et al., 2004), as it is a tissue that usually harbors high amounts of parasites.

Samples with inflammatory infiltrate and parasite presence were associated to eyes with clinical signs compatible with KCS. This is in disagreement with what has been reported in another study about KCS from other causes, in which histopathologic lesions in lacrimal glands were not correlated to severity of clinical signs (Kaswan et al., 1984), although we have not graded clinical nor microscopical severity. Despite this relationship we have found, there were samples with infiltrate and parasite that belonged to eyes without clinical signs. This discordance may be due to an underestimation of clinical signs in our study, given the fact that not all the dogs were submitted to a systematic ophthalmic examination. It is also possible

that a certain degree of inflammation or parasite presence is required in order to achieve the retention of secretion necessary to produce KCS. That means that minimal inflammation or parasite presence can be associated with subclinical degrees of KCS.

Another possible explanation is that the affection of more than one gland may be necessary in order to produce KCS. This hypothesis is supported by the association that we have found between eyes in which there are two or three glands affected, either by the inflammatory infiltrate and/or the parasite, and incidence of clinical signs. This is in agreement with the fact that in autoimmune KCS, it is believed that MLG and NMG are affected simultaneously when they produce the disease (Helper, 1976; Kaswan et al., 1984).

Although statistical relationships have been found between some specific ocular signs and each of the glands, numbers are too low to interpret these results. Many of the dogs included in the study had been treated for leishmaniosis during varying amounts of time. This fact could also have influenced the disagreements between microscopic findings and presence of clinical signs. The treatment could have improved some of the ophthalmologic signs and it could also have diminished inflammatory infiltrate or even make the parasite disappear from the tissue. It can be discussed that lesions we have seen in these lacrimal glands can be due to immune mediated KCS, as many dogs included in the study were from breeds predisposed to this condition (ACVO, 1999). Histopathologic lesions in spontaneous KCS have been described elsewhere (Kaswan et al., 1984; Izci et al., 2002), and they include massive lymphoplasmacytic infiltrate with acinar atrophy and fibrosis in advanced cases. We have not seen any of these alterations, and inflammatory infiltrate observed in our study is typical of leishmaniosis and is related to parasite presence. In conclusion, one mechanism by which KCS in dogs with leishmaniosis occurs is the dilation of the ducts of lacrimal glands due to pyogranulomatous inflammation surrounding these ducts, induced by the presence of amastigotes leading to accumulation and retention of secretion.

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STUDY 4

Evaluation of the presence of *Leishmania* spp. by real-time PCR in lacrimal glands of dogs with leishmaniosis. C. Naranjo, D. Fondevila, L. Altet, O. Francino, J. Ríos, X. Roura, T. Peña.

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Evaluation of the presence of *Leishmania* spp. by real-time PCR in lacrimal glands of dogs with leishmaniosis

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Abstract

Leishmania infantum infection is highly prevalent in endemic areas. Dogs with leishmaniosis may develop keratoconjunctivitis sicca (KCS). The goals of this study were: (1) to quantify *Leishmania* amastigotes in Meibomian glands (MG), main lacrimal gland (MLG) and gland of nictitating membrane (NMG) from dogs with leishmaniosis, (2) compare these results to immunohistochemistry (IHC); and (3) to explore the association between the amount of *Leishmania* parasites and the presence of ocular clinical signs. Twenty-five dogs diagnosed with leishmaniosis were included. MG, MLG and NMG from both eyes were collected. Histopathology, immunohistochemistry and real-time PCR were performed. All samples from all dogs yielded positive real-time PCR results. For all 3 glands, samples from dogs with ocular clinical signs had mean ΔCt (cycle threshold) values significantly lower (higher parasite loads) than those from dogs without signs. Cut-off values of $\Delta Ct < 0$, $\Delta Ct < 4$ and $\Delta Ct < 4.9$ for MG, MLG and NMG, resulted in a likelihood ratio of positives of 5.9, 6.38 and 6.38, respectively. Samples with ΔCt values below the reported cut-off were significantly more likely to display clinical signs related to KCS than those with results above the cut-off, for all 3 glands. Similarly, ΔCt values below the cut-off were significantly associated with positive IHC. In conclusion, real-time PCR has been standardized for use in MG, MLG and NMG. A cut-off value established for each of these tissues may be of great help to the clinician in the discrimination between ocular signs related to *Leishmania* from those associated with other causes of KCS.

Introduction

Canine leishmaniosis (CL) is a chronic and severe systemic disease caused by the protozoan parasite *Leishmania infantum*. It is endemic in the Mediterranean basin, and in this area the vectors involved in the transmission of the parasite are blood-sucking sand flies of the genus *Phlebotomus*. The importance of leishmaniosis lies on the fact that it is a zoonotic disease for which dogs are considered the main reservoir of the parasite (Slappendel, 1988; Ferrer, 1992; Ciaramella *et al.*, 1997; Fisa *et al.*, 1999; Koutinas *et al.*, 1999; Solano-Gallego *et al.*, 2001; Baneth, 2006; Solano-Gallego *et al.*, 2009; Paltrinieri *et al.*, 2010).

Clinical manifestations of CL are very variable and include dermatological signs, lymphadenomegaly, cachexia and muscle atrophy, epistaxis and splenomegaly, amongst others. Ocular signs are reported to occur in 16-80% of affected dogs (Slappendel, 1988; Molleda *et al.*, 1993; Ciaramella *et al.*, 1997; Koutinas *et al.*, 1999; Peña *et al.*, 2000). The incidence of keratoconjunctivitis sicca (KCS) in dogs with leishmaniosis and ocular manifestations varies from 2.8 to 26.43% (Molleda *et al.*, 1993; Ciaramella *et al.*, 1997; Koutinas *et al.*, 1999; Peña *et al.*, 2000).

A variety of techniques have been used to diagnose and study CL, including those that allow direct observation of parasite (cytology, histopathology and immunohistochemistry (IHC)), those that detect the immune response of the host (serology, leishmanin skin test) and molecular techniques that detect DNA of the parasite (polymerase chain reaction (PCR)) (Baneth, 2006; Maia and Campino, 2008; Miró *et al.*, 2008; Solano-Gallego *et al.*, 2009). Since it was first used for the study of leishmaniosis in murine models (Bretagne *et al.*, 2001; Bell and Cartwright, 2002; Nicolas *et al.*, 2002; Rolão *et al.*, 2004), real-time PCR has increasingly become a popular tool for the study and diagnosis of CL (Vitale *et al.*, 2004; Francino *et al.*, 2006; Manna *et al.*, 2008; de Paiva Cavalcanti *et al.*, 2009; Manna *et al.*, 2009). This technique allows not only to detect the parasite, but also to quantify the parasite load, making it a very useful tool for the clinician when deciding the therapeutic options in a particular patient and monitoring the response to treatment (Pennisi *et al.*, 2005; Francino *et al.*, 2006; Manna *et al.*, 2008).

While end-point PCR has been applied to conjunctival biopsies and swabs (Roze, 1995; Solano-Gallego, *et al.*, 2001; Strauss-Ayali *et al.*, 2004), to the authors' knowledge, no attempts to apply real-Time PCR to ocular tissues have been made to date.

In a previous study (Naranjo *et al*., 2005), we observed *Leishmania* amastigotes by IHC and the associated inflammatory infiltrate surrounding secretory ducts of lacrimal glands, which could contribute to the accumulation and retention of the lacrimal fluid and result in KCS.

Because *Leishmania* parasites have been observed in lacrimal glands of dogs with leishmaniosis (Naranjo *et al.*, 2005), we wanted to further study the role of the parasite in KCS and assess the use of real-time PCR as a diagnostic tool. The goals of this study were (1) to evaluate the presence of *Leishmania infantum* DNA in lacrimal glands of dogs with leishmaniosis by means of real-time PCR, (2) to compare the results to a well-established IHC technique and (3) to associate these results with the occurrence of ocular signs associated with KCS, with the aim to assess the role of real-time PCR as a potential diagnostic technique in these tissues.

Materials and Methods

Animals

Twenty-five dogs diagnosed with leishmaniosis were included in the study (Table 1). Twenty-two of these dogs suffered from natural infection and 3 had been inoculated with *Leishmania infantum* promastigotes and belonged to a research colony from the Universitat Autònoma de Barcelona (UAB). All protocols had been approved by the Comitè d'Ètica en Experimentació Animal (CEEA) of the UAB (Ethics Committee in Animal Experimentation of the UAB).

The 22 dogs with spontaneous disease were diagnosed at the Hospital Clínic Veterinari (HCV) of the UAB. They all had clinical signs, complete blood count, serum biochemistry and urinalysis results compatible with CL. Diagnosis was confirmed with serology in all cases (Riera *et al.*, 99) and in some of them the parasite was detected by direct cytological observation (4 dogs) or amplification of DNA by means of PCR in either bone marrow or lymph node aspirates or skin biopsies (4 dogs).

Ocular signs were detected in 11 of the cases by the attending clinician, 4 of which were further evaluated by a certified ophthalmologist. Six dogs (12 eyes) had blepharitis, 4 dogs (8 eyes) had periocular alopecia, 3 dogs (5 eyes) had conjunctivitis, 6 dogs (11 eyes) had keratoconjunctivitis and 2 dogs (4 eyes) had anterior uveitis.

Eighteen of the dogs with CL had been treated against *Leishmania* with antimonials and allopurinol during a range of time that varied from 3 days to 10 years. The remaining 7 dogs had not received any specific treatment.

The 22 dogs that suffered from natural infection died or were euthanized at the HCV of the UAB after progressive worsening of the dogs' condition or due to non-responsiveness to treatment, always after the owner's consent. Owners also signed an informed consent before death or euthanasia, allowing collection of the samples. The 3 inoculated dogs (dogs # 16, 17, 18) were euthanized at the end of their study period.

A 6-month-old dog that was euthanized for unrelated reasons was used as control, and the same samples were obtained from this dog.

Collection of Samples

For each dog, samples of eyelids containing Meibomian glands (MG), main lacrimal gland (MLG) and nictitating membrane gland (NMG) of both eyes were collected. That is, 3 samples from each eye (6 samples per dog) were obtained, except for dog # 9, from which Meibomian glands were not obtained.

Histopathology and IHC

Half of each sample was formalin fixed and paraffin embedded. Sections of the tissue were stained routinely stains (Hematoxylin and Eosin-HE) and the IHC technique described by Ferrer *et al*., (1988) to detect *Leishmania* spp. parasites. Results of this part of the study have been extensively reported in Naranjo *et al.*, (2005).

Real-time PCR

DNA isolation. The other half of the sample was frozen at -80ºC until DNA was extracted. First, every sample of tissue was lysed overnight with 1 ml of buffer (TRIS 50mM at pH 8.0, EDTA 20 mM and SDS at 2%), and 10 µl of proteinase K at 10 mg/ml, maintained at 56ºC of temperature. Deproteinization was performed on 300 µl of the lysis product with 150 µl of NaCl 5M. After agitation and 15 minutes of centrifugation, supernatant was transferred to another eppendorf and 1 ml of absolute ethanol was added. After 15 minutes of centrifugation, supernatant was discarded and 500 µl of 70% ethanol was added. After 5 minutes of centrifugation, pellet was dried, 50 µl of water mQ and 2 µl of RNAsa (10 mg/ml) were added and the mixture was incubated at 37ºC for 1 hour. Finally, water mQ was added until a final volume of 500 µl and was frozen at -20ºC until real-time PCR was performed.

Real-time PCR. TaqMan® technology was used to detect *Leishmania infantum* as described by Francino et al. (2006). Briefly, forward primer (5'-AACTTTCTGGTCCTCCGGGTAG-3') and reverse primer (5'-ACCCCCAGTTTCCCGCC-3') were added at 900 nM, and TaqMan-MGB® probe (FAM-5'-AAAAATGGGTGCAGAAAT-3'-non-fluorescent-quencher-MGB) was added at 200 nM. The eukaryotic 18S RNA pre-Developed assay reagent was used as an internal reference of canine genomic DNA. Each amplification run was performed in duplicate in 25-µl reaction mixture (TaqMan Universal PCR Master Mix; Applied Biosystems). Thermal cycling profile was 50ºC for 2 min, 95ºC for 10 min, 40 cycles at 95ºC for 15 s and 60ºC for 60 s. Each amplification run contained positive and negative controls. If the standard deviation of the two samples was higher than 0.38, the sample was analyzed again.

Standardization

In order to adjust real-time PCR for use in our tissues (MG, MLG, NMG) standard curves were performed. Four serial dilutions of one sample from each tissue (1, 10^{-1}, 10^{-2} and 10^{-3}) were prepared. This allowed us to calculate the efficiency of the *Leishmania* PCR and 18S RNA PCR to validate the relative quantification method. We also verified that ΔCt (Cycle threshold; ΔCt = Ct *Leishmania* – Ct 18S RNA) was constant through the dilutions,

confirming that the PCR efficiency obtained was not dependent on the initial DNA amount of each reaction (Livak and Schmittgen, 2001).

Statistical analysis

For descriptive purposes, for qualitative variables, absolute frequencies and percentages were used, and mean (or median) with standard deviation (SD) or Percentiles 25^{th} and 75^{th} were used for quantitative variables. Fisher's Exact Test or U-Mann Whitney test for qualitative or quantitative variables respectively were performed when necessary. The discriminative ability of ΔCt for positive in ocular disease, histopathological lesions or IHC, in each tissue, were assessed by means receiver operating characteristic (ROC) curves and the area under curve (AUC) was calculated as a measure of validity of ΔCt as a possible prognostic factor of 'positive'. As a first approach, cut-off values of ΔCt for each type of tissue were performed by means of the evaluation of Likelihood Ratio (LR), defined as the ratio between Sensitivity and (1-Especificity); the highest value of LR was used as ΔCt cut-off. The ability of clinical use of these cut-offs in the studied tissues for prognostic of 'positive' were assessed by means of risk estimations with Odds Ratios (OR) calculations and their 95% Confidence Intervals (95% CI) were performed with Logistic Regression analyses.

SPSS (Chicago IL) v15 for Windows was used for all statistical analyses. All reported P-values are two-sided. Only P values ≤ 0.05 were considered statistically significant.

Results

Histopathology and IHC

Detailed histopathological and IHC results have been reported in a previous study (Naranjo *et al.*, 2005) and they are summarized in Table 1. Briefly, 31 MG (64.6%) samples had histopathologic lesions compatible with leishmaniosis, consisting of a granulomatous to pyogranulomatous infiltrates with variable amounts of lymphocyte and plasma cells. Two (4%) of the MLG samples and 27 (54%) of the NMG samples had histopathologic lesions compatible with leishmaniosis. IHC revealed presence of parasite in 16 samples of MG (33.3%), 10 samples of MLG (20%) and 13 samples of NMG (26%).

Standardization

Standard curves were constructed for the two genes (*Leishmania* and 18S RNA) in the 3 glands studied. The slope and coefficient of determination of the curves are represented in Table 2. Slopes near -3.3 indicate a PCR efficiency of 100%.

ΔCt was maintained constant through the dilutions of the 3 types of glands, as evidenced by an absolute value of the slope of less than 0.1 in the curves.

Real-time PCR

Samples from the control dog yielded negative results. All samples from the 25 infected dogs yielded positive results with real-time PCR (Table 1). Mean ΔCt values and SD for MG were 4.9±7.3, for MLG were 7±6.2 and for NMG were 7.7±6.5.

ROC curves were constructed for all 3 tissues (Figures 1, 2 and 3). For MG, the area under the curve (AUC) was 0.766±0.068, with a confidence interval (CI) of 0.633-0.898 (P = 0.002). A cut-off value of ΔCt < 0 resulted in a likelihood ratio of positives (LR+) of 5.9 (Figure 1). For MLG, the AUC was 0.768±0.077, with a CI of 0.618-0.919 (P = 0.002). A cut-off value of ΔCt < 4 resulted in a LR+ of 6.38 (Figure 2). For the NMG, the AUC was 0.765±0.082, with a CI of 0.306-0.925 (P = 0.003). A cut-off value of ΔCt < 4.9 resulted in a LR+ of 6.38 (Figure 3).

Relationship between real-time PCR results and clinical signs associated to KCS

Real-time PCR results were contrasted to the presence of ocular clinical signs related to KCS in the various glands studied (Tables 3 and 4).

For MG, the mean ΔCt values and SD for eyes with clinical signs was 1.1±6.2, whereas ΔCt of MG samples from eyes without clinical signs was 8.1±6.7. There was a significant difference in ΔCt values (P = 0.001), with samples from dogs with clinical signs harboring a higher amount of parasites (lower ΔCt) than samples from dogs without clinical signs (Table 3). Logistic regression analysis revealed that samples with results below the cut-off value

(ΔCt < 0) were 10 times more likely to display ocular clinical signs than those with results above the cut-off value (Odds Ratio [OR] = 10, 95% CI 1.9-53.1, *P* = 0.007) (Table 4).

For MLG, ΔCt for eyes with clinical signs was 2.4±7, while ΔCt of MLG samples from eyes without clinical signs was 9.1±4.5. These differences were statistically significant (*P* = 0.002) (Table 3). Logistic regression analysis revealed that a cut-off value of ΔCt < 4 was significantly associated with the presence of clinical signs (OR = 13.3, 95% CI 2.8-62.1, *P* = 0.001) (Table 4).

For NMG, ΔCt for eyes with clinical signs was 2.6±8, while ΔCt of NMG samples from eyes without clinical signs was 10.1±4. These differences were statistically significant (*P* = 0.002) (Table 3). Logistic regression analysis showed that a cut-off value of ΔCt < 4.9 was significantly associated with the presence of clinical signs (OR = 13.3, 95% CI 2.8-62.1, *P* = 0.001) (Table 4).

Comparison of real-time PCR results with histopathological lesions

The mean ΔCt for MG that had histopathologic lesions compatible with CL was 1.8±5.9, whereas the mean ΔCt for MG that did not have histopathological lesions was 10.5±6.4. There was a significant difference in ΔCt values between samples from dogs with histopathological lesions and those from dogs without histopathological lesions (*P* < 0.001), so that samples from dogs with histopathological lesions consistent with leishmaniosis had higher parasite counts (lower ΔCt) than those from dogs without histopathological lesions (Table 3). Logistic regression analysis revealed that samples with real-time PCR below the cut-off value (ΔCt < 0) were 8.8 times more likely to display histopathological features consistent with *Leishmania* infection than those with results above the cut-off value (OR = 8.8, 95% CI 1.02-75.6, *P* = 0.04) (Table 4).

For MLG, the mean ΔCt from samples with histopathologic lesions was -9.7±0.2, while the mean ΔCt from samples without histopathologic lesions was 7.7±5.3. These means were significantly different (*P* = 0.002) (Table 3). None of the samples of MLG had both real-time PCR results above the cut-off value and granulomatous lesions on histopathology, so logistic regression analysis could not be performed on this tissue (Table 4).

For NMG, the mean ΔCt from samples with histopathologic lesions was 4.8±7, while the mean ΔCt from samples without histopathologic lesions was 11.1±3.8. These means were significantly different ($P < 0.001$) (Table 3). Logistic regression analysis revealed that a cut-off value of ΔCt < 4.9 was significantly associated with presence of histopathological features consistent with *Leishmania* infection (OR = 15.1, 95% CI 1.8-129.3, $P = 0.013$) (Table 4).

Comparison of real-time PCR results with detection of the parasite by IHC

The mean ΔCt for MG that had positive IHC results was -1.5±5.1, whereas the mean ΔCt for MG that had negative IHC was 8.1±6.1. These means were significantly different ($P < 0.001$), implying that samples from dogs with positive IHC had higher parasite burdens (lower ΔCt) than those from dogs with negative IHC (Table 3). Logistic regression analysis revealed that MG glands with real-time PCR results below the cut-off value (ΔCt < 0) were 25 times more likely to yield a positive IHC result than did samples with results above this cut-off (OR = 25, 95% CI 4.3-144-3, $P < 0.001$) (Table 4).

For MLG, the mean ΔCt from samples with positive IHC was -0.1±5.7, while the mean ΔCt from samples negative IHC was 8.7±5. These means were significantly different ($P < 0.001$) (Table 3). Logistic regression analysis revealed that real-time PCR results below the cut-off value (ΔCt < 4) were significantly associated with positive IHC results (OR = 16.3, 95% CI 3.2-84.7, $P = 0.001$) (Table 4).

For NMG, the mean ΔCt from samples with positive IHC was -0.6±6.1, while the mean ΔCt from samples with negative IHC was 10.6±3.5. These means were significantly different ($P < 0.001$) (Table 3). Logistic regression showed that real-time PCR results below the cut-off value (ΔCt < 4.9) were significantly associated with IHC results (OR = 58.3, 95% CI 8.5-398.8, $P < 0.001$) (Table 4).

Discussion

Previous clinical studies have reported the occurrence of KCS in dogs with leishmaniosis (Molleda *et al.*, 1993; Ciaramella *et al.*, 1997; Koutinas *et al.*, 1999; Peña *et al.*, 2000), but the

pathogenetic mechanism of this ocular affection in CL is still unclear. Because CL is a very common disease in the Mediterranean area (Solano-Gallego *et al.*, 2001) and KCS is a potentially blinding and complex ophthalmic disease, knowing the mechanism of KCS in dogs with leishmaniosis would help in the design of better diagnostic and treatment options for those dogs. In a previous study (Naranjo *et al*., 2005) we pointed out the existence of a granulomatous to pyogranulomatous infiltrate mainly surrounding the lacrimal ducts, and we noticed that this infiltrate and the parasite, as detected by IHC, were more often present in samples from dogs with ocular signs when compared to dogs without ocular clinical disease. In the present study this relationship has been confirmed by a quantitative technique, real-time PCR, by which samples from dogs with clinical signs had significantly lower ΔCt (and hence higher parasite loads) than those from dogs without ocular manifestations. This finding suggests that the presence of the parasite itself may play an important role in the pathogenic mechanism of KCS in CL.

To the authors' knowledge, this is the first study in which real-time PCR has been used to detect *Leishmania infantum* in canine lacrimal glands. This technique allowed us to increase the sensitivity of IHC, as the parasite was detected in every sample obtained from this group of infected dogs. However, IHC allows for direct visualization of the parasite in the context of the tissue, making possible the distinction between parasites that are intimately associated with the glandular tissues from those that are within surrounding connective tissue. Furthermore, because these glands are grossly poorly defined and they are interspersed within other tissues such as the eyelid (in the case of MG), orbital fat and musculature (in the case of MLG) and conjunctiva (in the case of NMG), it could be assumed that the presence of *Leishmania* parasites in the periglandular connective tissue could account for at least some of the low-positive samples by real-time PCR with negative IHC results. In any case, because our sample population was composed of infected dogs that died or were euthanized as a consequence of the disease, it is not surprising to find a sensitivity of 100% with real-time PCR, regardless of the microscopical location of the parasite.

Despite the differences in sensitivity between both techniques, statistical analysis revealed a relationship between them, so that samples with positive IHC results had higher parasite loads (and lower ΔCt) when compared to samples with negative IHC. Furthermore, with logistic regression analysis it was shown that samples with results below established cut-off values

were 25, 16 and 58 times (for MG, MLG and NMG, respectively) more likely to yield positive IHC results. Although few studies have compared IHC to end-point PCR for the diagnosis of CL (Xavier *et al.*, 2006; Moreira *et al.*, 2007), no reports have previously evaluated the association between IHC and real-time PCR in CL. A previous study on human cutaneous leishmaniosis (Martín-Ezquerra *et al.*, 2009) found a high correlation between the two techniques.

Real-time PCR detected the parasite in all the samples studied, emphasizing the need to consider CL as a systemic disease in which the parasite can spread to any tissue (Baneth, 2006; Solano-Gallego *et al.*, 2009). This technique has become a valued tool in the diagnosis and monitoring of CL in the past years and has been applied to a variety of biological samples, including bone marrow, blood, tissue biopsies and aspirates, and urine (Vitale *et al.*, 2004; Francino *et al.*, 2006; Manna *et al.*, 2006; Solano-Gallego *et al.*, 2007; Manna *et al.*, 2008; Manna *et al.*, 2009)

Previous studies have demonstrated that within an endemic area for CL, prevalence of infection is much higher than prevalence of disease (Solano-Gallego *et al.*, 2001). This means that a dog in which parasite has been detected by molecular techniques such as end-point PCR is not necessarily suffering from CL. Real-time PCR may be helpful in these cases in which clinical signs may be compatible with CL and serology results are doubtful (Solano-Gallego *et al.*, 2009), as well as in monitoring treated dogs (Francino *et al.*, 2006). Calculating a cut-off value for the parasite load in various tissues would allow discrimination between dogs that harbor the parasite and whose clinical signs are not associated with leishmaniosis and dogs that are infected and suffer from leishmaniosis at a given point in time. In this study, cut-off values have been established for all 3 lacrimal glands evaluated, so that real-time PCR results below these values are 10 to 13 times more likely to display clinical signs associated with KCS.

Canine KCS has multiple causes, including infectious (distemper virus, *Leishmania infantum*) and endocrine (hypothyroidism, diabetes mellitus) diseases, neurogenic (damage to cranial nerve VII), drug-induced (sulfonamides), immune-mediated in breeds such as Cocker Spaniels, English Bulldogs or West Highland White Terriers, iatrogenic (removal of the nictitating membrane) and idiopathic (Giuliano and Moore, 2007). Of these, immune-

mediated is the most commonly recognized cause (Giuliano and Moore, 2007). A dog from an endemic area that has been diagnosed with leishmaniosis and shows ocular signs compatible with KCS (conjunctival hyperemia, corneal neovascularization and/or pigment, corneal ulceration, and a low Shirmer Tear Test), could be suffering from immune-mediated KCS, especially if it is of one of the previously mentioned breeds, or, alternatively, from KCS associated with CL. The treatment regimes would change in either case, so it is important to make this distinction. Although no particular study has evaluated the treatment of KCS in dogs with leishmaniosis, the multiorganic nature of the disease would warrant systemic treatment, whereas dogs with immune-mediated KCS are typically treated with topical cyclosporine (Giuliano and Moore, 2007). Establishing cut-off values for parasite load in the glands associated with the production of lacrimal film can aid the clinician in this distinction, as values below the cut-off would indicate that the parasite is likely responsible for the observed ocular signs.

The results of the present study show that the LR+, that is, the proportion of dogs truly suffering from ocular signs associated to CL in relation to false positive results, is similar between MLG and NMG (6.38 for both), and these in turn are similar to the LR+ for MG. This translates into the fact that, in a clinical case, samples from any one of these 3 glands would be enough to establish a diagnosis of KCS caused by *Leishmania infantum*. Because MLG is hard to access and not routinely biopsied in a clinical setting, fresh or snap-frozen samples of NMG or MG would be more appropriate for real-time PCR testing.

The heterogeneity of the dogs enrolled in this study may have influenced the obtained results. All these dogs were diagnosed with CL, but the duration and type of treatment, if any, were very variable between the cases. We did not attempt to categorize the dogs according to the type and length of treatment received, as the number of animals would have been too low in each group. This diversity could have influenced our results, giving erroneously lower parasite numbers in cases with ocular clinical signs or, on the contrary, masking those signs in dogs with high parasitic amounts. Moreover, dogs included in the present study did not undergo a systematic ophthalmologic examination, so ocular signs may have been underestimated.

In conclusion, *L.infantum* is widely disseminated in dogs with leishmaniosis and, in the present study, has been detected by means of real-time PCR in Meibomian glands, main lacrimal glands and nictitating membrane glands. Real-time PCR is highly sensitive and shows good concordance to previously used immunohistochemical techniques. This technique could be helpful to establish cut-off values to determine in individual dogs if ocular signs present are due to leishmaniosis or not.

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FIGURES AND TABLES

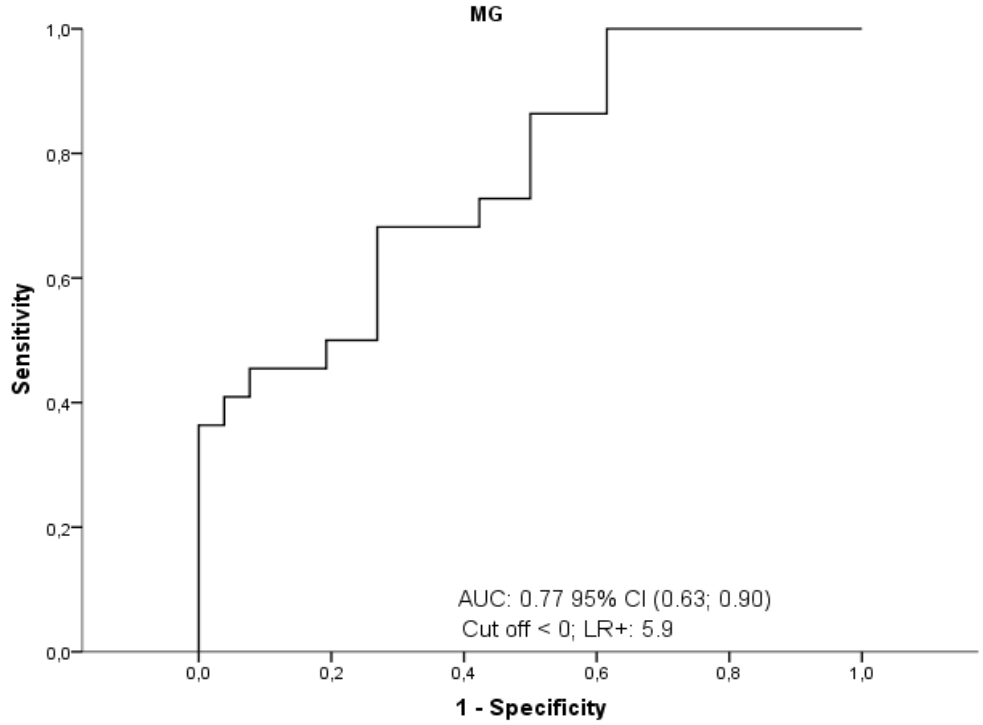

Figure 1. Receiver Operating Characteristics curve for Meibomian gland.

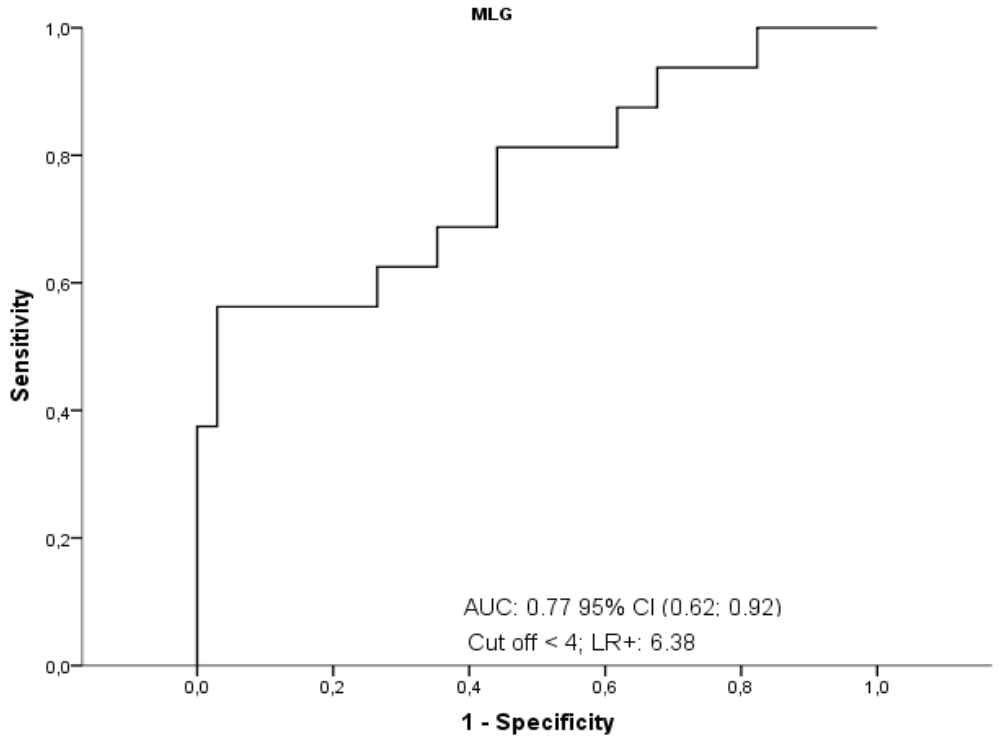

Figure 2. Receiver Operating Characteristics curve for main lacrimal gland.

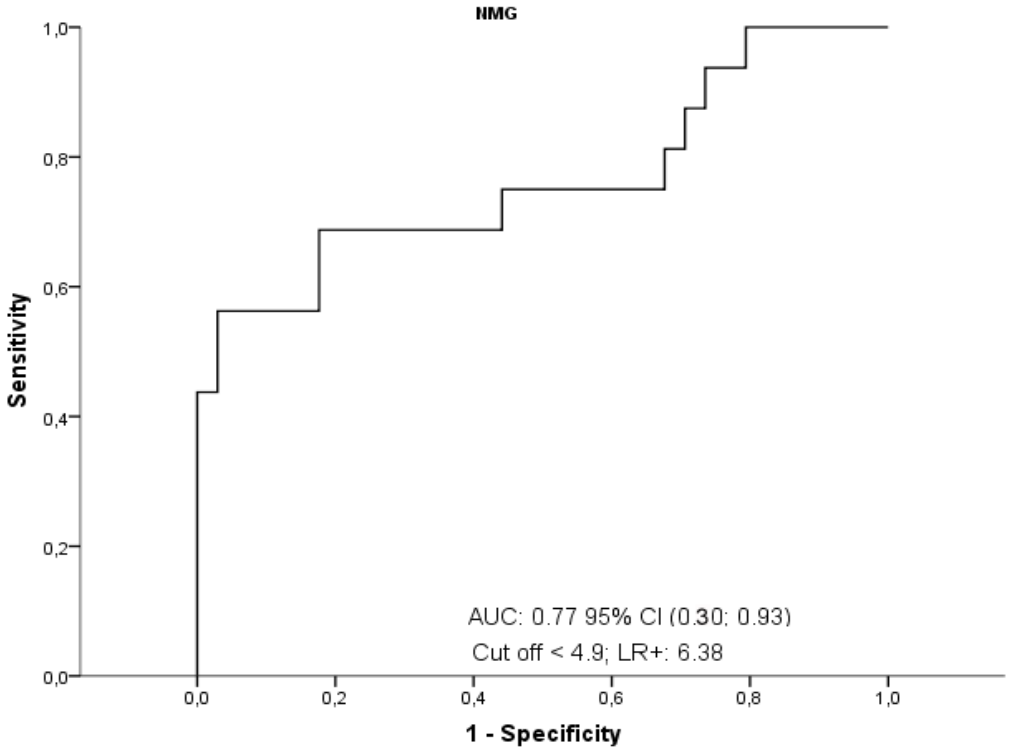

Figure 3. Receiver Operating Characteristics curve for nictitating membrane gland.

Table 1: Clinical signs, histopathology, immunohistochemistry and real-time PCR results

Case	Eye	Clinical signs	Meibomian Glands			Main Lacrimal Gland			Nictitating membrane gland		
			PCR	HE	IHC	PCR	HE	IHC	PCR	HE	IHC
1	OD	+	-1,82	+	+	-0,15	-	+	-4,89	+	+
	OS	+	-0,48	+	+	6,46	-	+	0,08	+	+
2	OD	+	-3,51	+	+	-0,28	-	+	-0,57	+	+
	OS	+	-3,22	+	+	6,62	-	+	1,21	+	+
3	OD	-	9,35	-	-	0,14	-	-	13,27	-	-
	OS	-	9,26	-	-	11,99	-	-	13,17	-	-
4	OD	+	5	+	-	13,07	-	-	13,23	-	-
	OS	+	8,68	+	-	3,14	-	-	11,47	-	-
5	OD	-	16,85	-	-	18,83	-	-	11,62	-	-
	OS	-	16,86	-	-	14,56	-	-	10,08	-	-
6	OD	-	9,74	+	-	13,03	-	-	9,37	-	-
	OS	-	12,17	+	-	10,98	-	-	10,37	+	-
7	OD	-	1,77	-	-	8,03	-	-	8,37	+	+
	OS	-	6,28	-	+	9,52	/	/	5,99	+	+
8	OD	+	6,62	+	-	8,82	-	-	8,12	-	-
	OS	+	6,63	+	-	8,45	-	-	8,06	-	-
9	OD	-	/	/	/	9,38	-	-	10,77	-	-
	OS	-	/	/	/	8,36	-	-	7,83	+	-
10	OD	+	9,86	+	-	10,74	-	-	9,71	-	-
	OS	+	10,19	+	-	6,9	-	-	7,66	+	+
11	OD	-	15,81	-	-	12,57	-	-	14,97	-	-
	OS	-	20,73	-	-	17,76	-	-	18,55	-	-
12	OD	+	-4,32	+	+	0,66	-	+	1,91	+	+
	OS	+	-2,31	+	+	0	-	+	2,61	+	+
13	OD	-	12,42	-	-	7,29	-	-	5,07	-	-
	OS	-	4,45	-	-	3,32	-	-	4,81	+	-
14	OD	-	10,62	-	-	13,85	-	-	13,6	-	-
	OS	-	10,75	+	-	12,18	-	-	13,99	-	-
15	OD	-	17,05	-	-	16,61	-	-	16,8	+	-
	OS	-	17,8	-	-	17,99	-	-	18,24	-	-
16	OD	-	0,11	+	-	5,84	-	-	8,79	+	-
	OS	-	0,12	+	+	5,95	-	-	9,96	-	-
17	OD	+	1,64	+	-	7,53	-	-	7,22	+	-
	OS	+	2,86	+	-	5,8	-	-	8,38	+	-
18	OD	-	2,05	+	-	5,69	/	/	9,34	+	-
	OS	-	0,54	+	-	4,76	-	-	10,76	+	-
19	OD	+	10,27	-	-	8,83	-	-	11,66	-	-
	OS	+	3,75	-	-	7,6	-	-	11,87	-	-
20	OD	-	-2,14	+	-	7,29	-	-	5	-	-
	OS	-	-1,3	-	-	7,19	-	-	2,96	-	-
21	OD	+	2,93	+	+	8,03	-	-	10,12	+	-
	OS	+	3,61	+	+	7,96	-	-	12,54	+	-

Case	Eye	Clinical signs	Meibomian Glands			Main Lacrimal Gland			Nictitating membrane gland		
			PCR	HE	IHC	PCR	HE	IHC	PCR	HE	IHC
22	OD	-	3,79	+	+	3,37	-	-	9,9	-	-
	OS	-	6,5	-	+	4,05	-	+	11,86	+	-
23	OD	+	-6,1	+	+	-7,52	-	-	-3,9	+	+
	OS	+	-6,39	+	+	0,62	-	+	-5,68	+	+
24	OD	+	-9,05	+	+	-9,83	+	+	-8,56	+	+
	OS	+	-9,78	+	+	-9,49	+	+	-11,58	+	+
25	OD	-	4,6	+	-	6,69	-	-	8,1	+	-
	OS	-	4,47	+	-	6,8	-	-	10,57	+	-

OD: Right eye; OS: Left eye; Clinical signs: + (presence), - (absence); PCR: real-time PCR result, expressed as ΔCt; HE (histopathology): + (presence of granulomatous infiltrate), - (absence of granulomatous infiltrate); IHC (immunohistochemistry): + (presence of *Leishmania*), - (absence of *Leishmania*).

Table 2. Slope and coefficient of determination of standard curves

Tissue	*Leishmania*		18S	
	slope	R^2	slope	R^2
Meibomian gland	-3,38	0,999	-3,41	0,999
Main lacrimal gland	-3,35	0,997	-3,24	0,999
Nictitating membrane gland	-3,32	0,999	-3,38	0,995

R2: coefficient of determination

Table 3. Real-time PCR results and comparison with clinical signs, histopathology and immunohistochemistry results

Tissue	Ocular signs			HE			IHC		
	Yes	No	*P*-value	Positive	Negative	*P*-value	Positive	Negative	*P*-value
Meibomian gland									
Mean (standard deviation)	1.1 (6.2)	8.1 (6.7)	0.001	1.8 (5.9)	10.5 (6.4)	<0.001	-1.5 (5.1)	8.1 (6.1)	<0.001
Median [interquartile range]	2.3 [-3.5; 6.6]	7.9 [2.1; 12.4]		2.1 [-2.3; 6.6]	10.3 [6.3; 16.9]		-2.1 [-5.2; 3.3]	9 [3.3; 11.5]	
Main lacrimal gland									
Mean (standard deviation)	2.4 (7)	9.1 (4.5)	0.002	-9.7 (0.2)	7.7 (5.3)	0.002	-0.1 (5.7)	8.7 (5)	<0.001
Median [interquartile range]	1.9 [-0.2; 7.6]	8 [6; 12.2]		-9.7 [-9.8; -9.5]	7.4 [5.2; 10.9]		0.3 [-0.3; 4.1]	8 [6.3; 12.1]	
Nictitating membrane gland									
Mean (standard deviation)	2.6 (8)	10.1 (4)	0.002	4.8 (7)	11.1 (3.8)	0.002	-0.6 (6.1)	10.6 (3.5)	<0.001
Median [interquartile range]	2.3 [-4.4; 10.6]	10 [8.1; 12.5]		7.7 [0.1; 10.1]	11.5 [9.4; 13.3]		0.1 [-4.9; 2.6]	10.4 [8.4; 12.5]	

HE: Histopathology; IHC: immunohistochemistry.

Table 4. Logistic regression analysis

Tissue	Ocular signs		HE		IHC	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Meibomian gland						
ΔCt ≥ 0	1		1		1	
ΔCt < 0	10 (1,9-53.1)	0.007	8.8 (1.02-75.6)	0.047	25 (4.3-144.3)	< 0.001
Main lacrimal gland						
ΔCt ≥ 4	1		1		1	
ΔCt < 4	13.3 (2.8-62.1)	0.001	- *	- *	16.3 (3.2-84.7)	0.001
Nictitating membrane gland						
ΔCt ≥ 4.9	1		1		1	
ΔCt < 4.9	13.3 (2.8-62.1)	0.001	15.1 (1.8-129.3)		58.3 (8.5-398.8)	< 0.001

ΔCt (Cycle threshold) = Ct Leishmania - Ct 18S RNA; OR: Odds Ratio; HE: histopathology; IHC: immunohistochemistry.
*: OR could not be calculated (see text).

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DISCUSSION

Canine leishmaniosis (CL) is an important zoonotic disease endemic in the Mediterranean area. The importance of this disease lies on the fact that dogs are the main reservoir of *Leishmania infantum*, which can infect humans and cause disease in immunocompromised individuals (Ferrer, 1992; Baneth, 2006; Baneth *et al.*, 2008; Solano-Gallego *et al.*, 2009; Paltrinieri *et al.*, 2010). Once *Leishmania* amastigotes are inoculated, these spread throughout the organism with involvement of virtually every tissue and organ. This wide dissemination explains the wide variety of clinical signs that can be seen in dogs with CL and lies below the notion that CL always has to be considered a systemic disease (Ferrer, 1992; Baneth, 2006).

Ocular involvement in CL is well-recognized (Slappendel, 1988; Molleda *et al.*, 1993; Ciaramella *et al.*, 1997; Koutinas *et al.*, 1999; Peña *et al.*, 2000; Cortadellas *et al.*, 2006), although reports that study the characterization and pathogenesis of the ophthalmic clinical signs are sparse (Molleda *et al.*, 1993; García-Alonso *et al.*, 1996). Establishing the particular sites of involvement in the ocular and periocular structures, as well as quantification of parasite loads may aid in the postulation of possible pathogenic mechanisms and hence, design better diagnostic and therapeutic options.

In the studies here presented, virtually all structures within the globe and ocular adnexa have been noticed to be variably involved in dogs with CL. Thirty per cent of the examined globes displayed inflammatory infiltrates related to *Leishmania* in one or multiple tissues, which is consistent with reported prevalence of ocular disease (Peña *et al.*, 2000). The typical granulomatous to pyogranulomatous infiltrate with fewer lymphocytes and plasma cells, described in other organs and tissues (Keenan *et al.*, 1984; Ferrer *et al.*, 1988b; Ferrer, 1992; Tafuri *et al.*, 2001; Baneth, 2006), has been noted to also affect the globe and its adnexa in the current and in previous studies (Molleda *et al.*, 1993; García-Alonso *et al.*, 1996). Similar to our findings, the few reports on human leishmaniosis involving ocular tissues in which histopathology was performed also describe a granulomatous infiltrate (Dechant *et al.*, 1980; Oliveira-Neto *et al.*, 2000; Yaghoobi *et al.*, 2010). Other types of infiltrate, most commonly lymphoplasmacytic in nature, have been noted during the evaluation of our samples, but these were not accompanied by the presence of *Leishmania* amastigotes in any case, so this type of infiltrate was not considered to be caused by the parasite. This is especially important when evaluating structures like the conjunctiva, where lymphocytic or lymphoplasmacytic

aggregates, occasionally forming follicles, are frequently noted as part of the local mucosal mechanisms of adaptive immunity, known as the conjunctiva-associated lymphoid tissue or CALT (Gilger, 2008).

Despite the finding of an infiltrate consistent with previous reports, it is important to remember that a granulomatous or pyogranulomatous infiltrate is not specific for leishmaniosis (Ferrer, 1997), as many other infectious (fungal, parasitic, certain bacteria) and non-infectious (foreign bodies, sterile granulomatous inflammation) etiologies are associated with this type of infiltrate (Ackerman, 2007). In all our cases, the diagnosis of leishmaniosis had been confirmed prior to death or euthanasia, and the detection of amastigotes in routine sections or by immunohistochemistry (IHC) supported the notion that the inflammation present was due to the parasite.

The conjunctiva and adjacent corneoscleral limbus were the most frequently involved structures in the globe and immediate vicinity. The ocular surface, with exception of the central cornea, is not considered an immune-privileged site, and as such, is not subject to the Anterior Chamber-Associated Immune Deviation (ACAID) typical of the intraocular structures (Biros, 2007). This is important because many recent studies have focused on studying the value of the conjunctiva as a good sample for diagnosis of CL (Solano-Gallego *et al.*, 2001, Strauss-Ayali *et al.*, 2004; Ferreira *et al.*, 2008; Pilatti *et al.*, 2009; Leite *et al.*, 2010). Our results would support the use of this tissue for diagnosis, although it was not our objective to evaluate the diagnostic value of the conjunctiva, but rather to evaluate the various ocular and periocular structures in order to describe the contribution of the parasite and its associated inflammation to the observed clinical signs in dogs previously diagnosed with leishmaniosis.

It is also important to note that involvement of the cornea and sclera was always associated to infiltration of the conjunctiva, and these structures were never infiltrated without conjunctival disease. Furthermore, the degree of involvement of these structures was related to the severity of infiltration. This could indicate that the parasite may reach the ocular surface via conjunctival vessels and may disseminate from there into adjacent structures, although this hypothesis cannot be inferred from our studies and further research would be necessary to prove it.

Uveal involvement was not as frequently found as ocular surface involvement and, when present, it was mostly located in the anterior uvea, more commonly in the ciliary body than in the iris. This was in agreement with clinical studies in which posterior uveal involvement was infrequent (Roze, 1986; Molleda *et al.*, 1993; Peña *et al.*, 2000), and generally associated to more severe disease. In contrast, other systemic infections such as toxoplasmosis, protothecosis or systemic mycosis, preferentially involve the posterior segment (Cullen and Webb, 2007). Regardless of the location, finding inflammation and/or parasites within uveal structures is indicative of blood-ocular barrier breakdown, with overcoming of the ACAID response (Biros, 2007), which again is in agreement with the notion of CL being a systemic disease.

Of note is the correlation between clinical and histopathological observations regarding the presence of two patterns of involvement of the anterior uvea: the diffuse granulomatous and the nodular granulomatous form that had been previously described in clinical studies (Roze, 1986; Peña *et al.*, 2000). Although we did not attempt to characterize the lymphocyte subsets present or cytokine production in these two forms, it is possible that these two forms may be equivalent to the two types of granulomas associated with the corresponding T cell helper (Th) response: the nodular form, which is associated with a Th1 response and low parasite numbers and the diffuse form, which is related to a Th2 response and high parasite burden (Ackerman, 2007). Various studies have examined the various populations of macrophages in human and murine leishmaniosis and the array of cytokines and other inflammatory mediators produced (Modlin *et al.*, 1985; Lemos de Souza *et al.*, 2000; Murray, 2001; Murray *et al.*, 2006). Similarly, various patterns of histiocytic inflammation of the skin are noted and described in CL (Ferrer *et al.*, 1988b, Fondevila *et al.*, 1997, dos-Santos *et al.*, 2004). At this point, the significance and prognostic value, if any, of these two clinical and histologic patterns in the anterior uvea is not known, although some authors have hypothesized that immune-mediated mechanisms may play a role in the anterior uveitis seen in CL (Slappendel, 1998; García-Alonso *et al.*, 1996). García-Alonso *et al.* (1996) found deposits of IgG in the ciliary processes, together with *Leishmania* amastigotes, and hence hypothesized that the presence of immune complexes in combination with the granulomatous infiltrate could contribute to ocular disease. Nodular iridocyclitis has also been described in humans with post-kala-azar uveitis (Dechant *et al.*, 1980; el-Hassan *et al.*, 1991), which is considered to

occur only after treatment of visceral leishmaniosis upon gaining of immune function against the parasite (Dechant *et al.*, 1980). Of the two globes of our study that contained discrete granulomas in the iris, only one of them belonged to a dog that had been treated, so numbers are too low to draw any conclusions.

Myositis of the various intra-, extra- and periocular smooth and striated muscles was a surprising finding of the current research project that once more highlights the importance of considering CL a widely spread systemic infection. These tissues are likely not a specific target of the parasite, and their involvement was chiefly associated to inflammation of adjacent structures, such as Meibomian glands (MG) and conjunctiva for muscles of the eyelids (including nictitating membrane), main lacrimal gland (MLG) for muscles in the orbit and uveal stroma for intraocular muscles. The finding of a similar and typical granulomatous to pyogranulomatous infiltrate with associated *Leishmania* amastigotes further supports the involvement of these muscles as being part of the systemic picture.

The proportion of affected eye-associated muscles in CL was similar to the percentage of affected globes (24.03% of eyes contained inflammatory infiltrates in at least one of the muscles examined versus 30% of globes that had some degree of inflammation in any of the structures evaluated), further supporting the notion that these muscles are not preferentially involved but rather part of the overall inflammatory status in the area.

Myositis in CL has become more important as the pathogenic mechanisms are being elucidated. Initially, masticatory muscle myositis was attributed to the catabolic nature of any chronic disease, but subsequent research demonstrated that temporal and masseter muscles are affected by the same granulomatous infiltrate seen in other tissues, and parasites were noted within infiltrating macrophages (Vamvakidis *et al.*, 2000). Other skeletal muscles have been reported to be affected, including the cranial tibial (Vamvakidis *et al.*, 2000) and biceps femoris muscle (Paciello *et al.*, 2009), but the main difference with the muscles here reported is that we did not see myofiber necrosis, degeneration and regeneration, as described in these reports. We did see occasional myofiber atrophy, although this was presumed to be secondary to the associated inflammation. A potential explanation is the differences in severity of the disease, as the dogs in these reports were selected because they were already suffering from muscle atrophy or weakness, whereas most of the dogs included in our project had no

reported signs related to muscular disease in the globe, eyelids or orbit. Both studies (Vamvakidis *et al.*, 2000; Paciello *et al.*, 2009) reported an increase in severity of microscopical findings with increased severity of clinical signs, further supporting this hypothesis.

Another important observation is that we detected parasites within the sarcoplasm of myofibers, a finding that has been reported in previous histopathological studies (Vamvakidis *et al.*, 2000; Silva-Almeida *et al.*, 2010). Although macrophages are the main cell infected by *Leishmania* amastigotes, these can also be seen free in the tissues and within other cells, including hepatocytes (Tafuri *et al.*, 2001) and fibroblasts (Hervás-Rodríguez *et al.*, 1996). This is in disagreement with a previous study on skeletal myositis in CL, which reported that parasites did not penetrate into myofibers but rather stayed within epimyseal macrophages (Paciello *et al.*, 2009). These authors did laser capture microdissection of myofibers and failed to detect *Leishmania* DNA within them, a protocol that was not included in our study.

Canine keratoconjunctivitis sicca (KCS) is a common and severe ocular condition characterized by the reduction in the aqueous portion of the tear film with resultant inflammation of the ocular surface (Giuliano and Moore, 2007; Williams, 2008). The prevalence of KCS in the canine population ranges from 0.4% to 35% depending on the source consulted (Helper *et al.*, 1976; Kaswan *et al.*, 1984; Williams *et al.*, 2008). Characteristic clinical signs include conjunctival hyperemia, erosion, ulceration, corneal edema and neovascularization, with pigmentation occurring in chronically diseased eyes (Giuliano and Moore, 2007). Corneal pigmentation, if extensive, may lead to blindness. The most commonly reported cause is immune-mediated (Giuliano and Moore, 2007), which occurs at higher risk in certain breeds like West Highland White Terriers, Cockers, Cavalier King Charles Spaniel and others (Williams, 2008). Various other etiologies are described; infectious disease (canine distemper virus, *Leishmania* spp.), endocrine disease (diabetes mellitus, hypothyroidism), neurogenic KCS, traumatic, iatrogenic (treatment with sulfamides, topical or general anesthesia) or idiopathic (Giuliano and Moore, 2007). Because the percentage of dogs with leishmaniosis that develop KCS is significant, ranging from 2.8% to 26.83% in various published studies (Molleda *et al.*, 1993; Ciaramella *et al.*, 1997; Koutinas *et al.*, 1999; Peña *et al.* 2000), in an endemic area the distinction between immune-mediated KCS and KCS caused by *L. infantum* is important in a dog with compatible clinical signs.

We examined the glands responsible for the production of the aqueous (MLG and nictitating membrane gland (NMG)) and lipid (MG) portions of the lacrimal film and found that these glands are affected by the typical granulomatous to pyogranulomatous infiltrate described for dogs with leishmaniosis. This infiltrate was mostly located around the ductal component of the glands, and less frequently around the acinar elements, with no evidence of glandular necrosis, destruction or fibrosis. These findings allowed us to propose a possible pathogenic mechanism for *Leishmania*-induced KCS; infiltration around lacrimal gland ducts would lead to chronic obstruction with retention of secretions and eventual tear film deficiency.

In dogs that suffer from immune-mediated KCS, a lymphocytic to lymphoplasmacytic infiltrate in the lacrimal glands predominates (Kaswan *et al.*, 1984; Iczy *et al.*, 2002), which is consistent with an immune-mediated etiology. The different nature of the infiltrates and the different etiologies (infectious vs. immune-mediated) support the hypothesis of differing pathogenic mechanisms for the two conditions. Furthermore, Kaswan *et al.* (1984) described acinar atrophy with replacement by the infiltrate and later on in the disease, by fibrous connective tissue (fibrosis). We did not see these features in any of the glands examined, further distinguishing the two entities. Although we did not perform studies in this direction, it is tempting to hypothesize that these apparently different pathogeneses and the systemic vs. local nature of CL and immune-mediated KCS, respectively, warrant different treatments.

The fact that MG were one of the most commonly affected glands in terms of inflammatory infiltrate is relevant because it brings in the possibility of a qualitative tear film deficiency in dogs with leishmaniosis that could contribute to ocular surface disease. To the best of our knowledge, this has not been previously reported nor has it been clinically evaluated, but certainly warrants future studies. Qualitative tear film deficiencies are less well characterized in veterinary medicine, which is in part complicated by the difficulty in assessing MG functionality, as Shirmer Tear Test can be within normal limits in these patients (Giuliano and Moore, 2007). Recent studies, in which meibometry has been applied to canine patients, have opened up new possibilities in this direction (Ofri *et al.*, 2007, Benz *et al.*, 2008).

Throughout the past decade, real-time PCR has become an important tool in the study of CL. Because parasitic cure is rarely achieved in endemic areas (Solano-Gallego *et al.*, 2009), monitoring the parasite load, mainly in bone marrow and blood, is becoming increasingly

important in managing these cases (Francino *et al.*, 2006; Manna *et al.*, 2008a and b; Manna *et al.*, 2009a). In our project, in which we did not perform a longitudinal study, real-time PCR was useful in further characterizing the role of *Leishmania* parasites in the pathogenesis of KCS and, moreover, allowed us to establish cut-off values for further reference. These cut-off values could be useful to the clinician when trying to determine in a particular dog that has been diagnosed with leishmaniosis and shows ocular surface disease consistent with KCS if these signs are due to *Leishmania* spp. or, conversely, it suffers from concurrent immune-mediated KCS. This would be especially important in dogs from breeds predisposed to immune-mediated KCS, due to the relatively high prevalence of both diseases in endemic regions of CL. Differentiating KCS caused by *Leishmania* spp. from other types of KCS would likely have an important impact on the treatment regime of a particular dog. Immune-mediated KCS is usually treated with topical cyclosporine or other immune-modulators like tacrolimus (Kaswan *et al.*, 1990; Giuliano and Moore, 2007) and supportive therapy in the form of lacrimomimetics (Giuliano and Moore, 2007). These immune-modulators inhibit IL-2 production by CD4+ T helper lymphocytes, which results in impaired T-helper and T-cytotoxic proliferation (Moore, 2004). Because the outcome of *Leishmania* spp. infection is so dependent on the dog's immune system (Pinelli *et al.*, 1994; Pinelli *et al.*, 1995; Manna *et al.*, 2006), it is tempting to hypothesize that treatment with these drugs could cause an imbalance between the various populations of lymphocytes that could potentially be detrimental to the local ocular surface immunity. As previously mentioned, our project was not designed to evaluate the efficacy of various treatments against KCS caused by *Leishmania* spp., but this is an intriguing hypothesis that opens new avenues for future research. In any case, in a dog suffering from *Leishmania*-induced KCS, standard systemic treatment with antimonials and allopurinol would be advised.

Of importance is that we did not find statistical differences between the three glands evaluated by real-time PCR, in terms of Likelihood Ratio of positives (LR+). LR+ is defined as the proportion of dogs that are truly positive (that is, dogs with real-time PCR results below the established cut-off and whose ocular signs are indeed caused by leishmaniosis) with respect to false positives (dogs that yield results below the cut-off but whose clinical signs are not actually caused by leishmaniosis). The implication is that any of the three glands would be equally good for the purpose we intended. Bearing this in mind, we propose MG and NMG as

samples of choice, because they are more accessible to the clinician, especially when compared to MLG.

We also wanted to compare the use of real-time PCR with the well-established IHC technique that is routinely used for the diagnosis of CL in tissue biopsies (Ferrer *et al.*, 1988a, Tafuri *et al.*, 2004). Both techniques have their strengths and may be used for different purposes or depending on the type of sample available. With IHC, the tissue is directly visualized and the parasite is localized to specific structures and, importantly, it can be performed on archived paraffin-embedded samples (Ramos-Vara, 2005). Real-time PCR has shown a much higher sensitivity in our tissues when compared to IHC and, more notably, allowed quantification of the parasite load with respect to a housekeeping dog gene (18S RNA gene), which enabled normalization regarding the baseline DNA amount (Livak and Schmittgen, 2001). Regardless of these differences, the results of both techniques were associated, so that positive samples for IHC yielded significantly higher parasite loads when compared to negative IHC results.

We were able to establish statistical relationships between ocular clinical signs and the involvement of corresponding ocular and periocular structures histopathologically, immunohistochemically and molecularly by real-time PCR, so that inflammatory infiltrates, positive IHC and higher numbers of parasites were more likely present in dogs with clinically detected disease. This relationship is relevant; firstly, because it links our studies to clinical disease and, secondly, because it could imply that these signs are the result of the involvement of the tissues where they originate. In the case of real-time PCR, the association between the number of parasites and clinical signs has allowed us to propose the aforementioned cut-off values that could have a diagnostic value.

Despite this association, a common feature to all the studies performed in this project was the relative mismatch, to variable extent depending on the study, between the clinically described ocular signs and the histopathological or parasitological (either by IHC or by real-time PCR in the case of lacrimal glands) involvement in particular cases. In instances in which the microscopical or parasitologic involvement was more frequent than clinically observed disease, possible causes include subtle clinical disease or posterior segment involvement not noticed by the attending clinician, who usually does not perform a thorough ophthalmic examination. In occasions in which the clinical signs were noted more frequently than was

microscopical or parasitological disease, a possible cause could be the presence of mild clinical disease not adequately represented histologically, as occurs in mild corneal edema or minimal aqueous flare (Moore *et al.*, 2003). In both cases, various treatment regimes administered during variable amounts of time to the dogs here included, or the lack thereof, may have strongly influenced either the presence of clinical signs (masking them) or the amount of inflammation and/or numbers of parasites detected by either method. Ideally, future studies should involve dogs that have undergone complete ophthalmic examinations, including Shirmer Tear Test and funduscopy, in order minimize the discrepancies.

The studies here exposed highlight the importance of ocular involvement in CL and stress the need for further research evaluating parasite dynamics in ocular and periocular structures, as well as the associated local inflammatory reaction, in the context of a systemic immune response, and the resultant clinical disease. Potential specific, in addition to supportive therapies to prevent serious sequelae such as blindness should also be investigated.

CONCLUSIONS

1. Microscopic involvement of ocular structures in canine leishmaniosis occurs in approximately one third of dogs and consists of a granulomatous infiltrate chiefly composed of macrophages with variable numbers of lymphocytes, plasma cells and neutrophils, with approximately one fourth of the globes containing *Leishmania* amastigotes.

2. Ocular and periocular structures microscopically affected by canine leishmaniosis include, in order of frequency: conjunctiva and limbus, ciliary body, iris, cornea, sclera and iridocorneal angle, choroid and optic nerve sheath.

3. There is a positive correlation between the presence of granulomatous infiltrate and *Leshmania* amastigotes with the occurrence of ophthalmic clinical signs in the corresponding structures.

4. In dogs with leishmaniosis, granulomatous myositis of adnexal, extraocular and intraocular smooth and striated muscles occurs in association with the presence of *Leishmania* amastigotes and in the absence of myofiber necrosis, degeneration or regeneration.

5. One mechanism by which keratoconjunctivitis sicca occurs in dogs with leishmaniosis is the periductal granulomatous inflammation of lacrimal glands, induced by the presence of amastigotes, leading to accumulation and retention of secretion.

6. Real-time PCR allows for establishment of cut-off values for parasite load in lacrimal glands from dogs with leishmaniosis, which may aid in the distinction between keratoconjunctivitis sicca caused by *Leishmania* from other causes of this ocular disease.

7. Samples with parasite burdens in lacrimal glands below the cut-off values are more likely to be from animals showing ocular surface disease compatible with keratoconjunctivitis sicca than those with parasite burdens above the cut-offs.

8. Although real-time PCR is more sensitive than immunohistochemistry, an association exists between these two techniques, so that samples with parasite loads below the cut-off values are more likely to yield positive immunohistochemical results than samples with parasite loads above the established cut-offs.

SUMMARY

Canine leishmaniosis (CL) is endemic and highly prevalent in the Mediterranean shore, where it is caused by *Leishmania infantum* and transmitted by sand flies of the genus *Phlebotomus*. CL is one of the major global zoonoses and dogs are thought to be the main reservoir of *L. infantum* for humans. In endemic areas, the prevalence of infection of dogs is much higher than the prevalence of disease; many dogs can harbor the parasite without ever showing clinical disease.

CL is considered a systemic disease and hence dogs can present with a wide variety of clinical signs, including a range of dermatological manifestations, pale mucous membranes, cachexia, muscle atrophy, lymphadenopathy, splenomegaly or renal disease. Ocular disease has been reported to occur in 16% to 80% of dogs with leishmaniosis and can be the sole manifestation of the disease in 3% to 15% of cases. Keratoconjunctivitis sicca (KCS) is diagnosed in 2% to 27% of dogs with leishmaniosis.

Although the pathogenesis of CL has been and still is extensively researched, studies investigating the pathogenic mechanisms underlying ocular disease are sparse. Because ocular manifestations are relatively common in this disease, the main objective of this project was to gain further knowledge about the events occurring in the eyes and adnexal structures from dogs affected by leishmaniosis.

The project started by collecting eyes and ocular adnexal structures from dogs that suffered from leishmaniosis, in order to characterize the inflammatory infiltrate present, where this inflammation was located within the globe and adnexa and to evaluate the presence of *Leishmania* amastigotes in these lesions by immunohistochemistry. Thirty percent of globes had some degree of inflammation, which consisted of a granulomatous to pyogranulomatous infiltrate with variable presence of lymphocytes and plasma cells. The parasite was detected in 26.6% of globes. There was a significant association between presence of granulomatous infiltrate and detection of the parasite. Most commonly affected structures were conjunctiva and limbus, followed by ciliary body, iris, cornea, sclera and iridocorneal angle, choroid and optic nerve sheath. Furthermore, both the described granulomatous infiltrate and detection of the parasite were significantly associated with clinically diagnosed ocular disease.

Next, intraocular, extraocular and adnexal smooth and striated muscles were similarly evaluated, and granulomatous infiltrate with associated *Leishmania* parasites were noted. Inflammatory infiltrate was noted around muscle fibers, mostly within the endomysium. Myofiber necrosis, degeneration or regeneration was not noted, although myofiber atrophy was rarely seen. Occasionally, parasites were noted within myofibers. Inflammation with or without parasites was noted in at least one of the muscles in 24.03% of the evaluated eyes and adnexa. Involvement of these muscles had not been previously reported in CL and may contribute to some of the reported clinical signs.

Because KCS is a potentially blinding disease, adnexal glands that synthesize the aqueous (main lacrimal gland (MLG) and nictitating membrane gland (NMG)) and lipid (Meibomian glands (MG)) components of the tear film were investigated next. A similar granulomatous infiltrate was noted in these glands, mainly surrounding the ductal component, and occasionally surrounding the acini. MG and NMG were more commonly affected by the infiltrate than MLG. No significant differences in detection of *Leishmania* parasites were noted between glands. Samples from eyes with clinical signs compatible with KCS were more commonly affected by the granulomatous infiltrate than those from eyes without clinical signs. A similar relationship was found for parasite presence. Clinical signs were more commonly related to those eyes that had two or three glands affected by the infiltrate or contained the parasite than to those eyes in which no lacrimal gland or only one of the three glands was affected. A possible pathogenic mechanism of KCS in CL that was derived from this study is granulomatous infiltration around ducts of lacrimal glands, induced by the parasite, with subsequent retention of secretion.

Finally, the project focused on investigating the amount of parasites in MG, MLG and NMG by real-time PCR, in order to elucidate if the number of parasites was related to development of KCS associated to CL. After standardization of this technique for use in our tissues, *Leishmania* parasites were found in various amounts in all samples analyzed. For all three glands, samples from dogs with clinical signs contained significantly more parasites (lower ΔCt (Cycle threshold)) than samples from dogs without clinical signs. Optimal cut-off values of ΔCt were established for all three glands, so that samples with ΔCt below these cut-offs were more likely to be from dogs that displayed clinical signs related to KCS than samples with ΔCt above the cut-off. These findings indicate that cut-off values could aid the clinician

in determining, in a dog infected with *Leishmania* spp., if compatible ocular clinical signs are due to leishmaniosis or to other causes of KCS.

RESUMEN

La leishmaniosis canina (LC) es endémica en la costa Mediterránea, donde ocurre con una alta prevalencia. En esta área, la LC está causada por *Leishmania infantum* y se transmite por *Phlebotomus*. La LC es una de las zoonosis más importantes y el perro se considera el principal reservorio de *L. infantum*. En áreas endémicas, la prevalencia de la infección es muy superior a la prevalencia de la enfermedad, indicando que hay muchos más perros que son portadores del parásito de los que desarrollarán la enfermedad.

La LC se considera una enfermedad sistémica y, por lo tanto, los perros se pueden presentar con una gran variedad de signos clínicos, incluyendo manifestaciones dermatológicas, mucosas pálidas, caquexia, atrofia muscular, linfadenopatía, esplenomegalia o enfermedad renal, entre muchas otras. Los signos clínicos oculares aparecen en un porcentaje que varía del 16% al 80% de los perros con leishmaniosis, y éstos pueden ser la única manifestación de la enfermedad en el 3-15% de los casos. La queratoconjuntivitis seca (KCS) se diagnostica en un 2- 17% de los perros con leishmaniosis.

Aunque la patogenia de la LC es foco de amplias investigaciones, hay pocos estudios que investiguen los mecanismos patogénicos que causan los signos clínicos oculares. Como estos signos clínicos son relativamente frecuentes en esta enfermedad, el principal objetivo de este proyecto fue adquirir un mayor conocimiento de las alteraciones que ocurren en los ojos y en los anejos oculares de los perros con leishmaniosis.

El proyecto empezó por la recogida de muestras de ojos y anejos oculares de perros que habían sido diagnosticados de leishmaniosis, con el fin de caracterizar el tipo de infiltrado inflamatorio, en qué estructuras oculares y anejas estaba localizado este infiltrado y evaluar la presencia de amastigotes de *Leishmania* en esas lesiones mediante inmunohistoquímica. El 30% de los globos oculares presentaban inflamación, que consistía en un infiltrado granulomatoso a piogranulomatoso con presencia variable de linfocitos y células plasmáticas. El parásito se detectó en el 26,6% de los ojos. La presencia del infiltrado granulomatoso y la detección del parásito estaban asociadas significativamente. Las estructuras más frecuentemente afectadas fueron: conjuntiva y limbo, seguidas del cuerpo ciliar, iris, córnea, esclera y ángulo iridocorneal, coroides y nervio óptico. Además, tanto el infiltrado inflamatorio como la detección del parásito estaban asociados estadísticamente con los signos clínicos oculares descritos.

A continuación se evaluaron los músculos lisos y estriados intraoculares, extraoculares y de los anejos y se describió un infiltrado granulomatoso con presencia de parásitos de *Leishmania*. El infiltrado inflamatorio se detectó alrededor de las fibras musculares, especialmente en el endomisio. No se detectaron necrosis, degeneración o regeneración de las fibras musculares, aunque atrofia de las fibras se vio esporádicamente. En algunos casos, los parásitos se vieron dentro de las fibras musculares. La inflamación, con o sin parásitos, se detectó en al menos uno de los músculos examinados en 24,03% de los ojos y anejos evaluados. La implicación de estos músculos en la LC no se había descrito previamente, y puede que contribuya a algunos de los signos clínicos descritos.

Como la KCS es una enfermedad que puede llevar a la ceguera, se procedió a la investigación de las glándulas encargadas de la síntesis del componente acuoso (glándula lacrimal principal (MLG) y glándula de la membrana nictitante (NMG)) y lipídico (glándulas de Meibomio (MG)) de la película lacrimal. Se encontró un infiltrado granulomatoso similar al descrito en los ojos, que principalmente rodeaba los conductos de dichas glándulas, y ocasionalmente rodeando los acinos. La MG y la NMG estaban más frecuentemente afectadas que la MLG. No hubo diferencias significativas en la detección de parásitos entre las distintas glándulas. Las muestras de ojos con signos clínicos compatibles con KCS mostraban el infiltrado granulomatoso y la presencia del parásito más frecuentemente que las muestras de ojos sin signos clínicos. La presencia de signos clínicos estaba relacionada con aquellos ojos en los que dos o tres de las glándulas evaluadas estaban afectadas por el infiltrado o contenían el parásito. Un posible mecanismo por el que los perros con leishmaniosis desarrollan KCS sería la infiltración granulomatosa alrededor de los conductos de las glándulas lacrimales, lo que causaría retención de la secreción.

Finalmente, el proyecto se concentró en investigar la cantidad de parásitos en las MG, MLG y NMG mediante PCR cuantitativa, para así determinar si el número de parásitos está relacionado con la KCS asociada a la LC. Previa estandarización de la técnica para uso en estos tejidos, parásitos de *Leishmania* se detectaron en todas las muestras analizadas en cantidades variables. En las tres glándulas, las muestras obtenidas de perros con signos clínicos tenían más parásitos (es decir, un ΔCt o ciclo umbral más bajo) que las muestras de perros sin signos clínicos. Se establecieron puntos de corte óptimos para las tres glándulas, de

manera que las muestras con resultados por debajo del punto de corte tenían más probabilidad de pertenecer a perros con signos clínicos asociados a KCS que las muestras con resultados por encima del punto de corte. Estos resultados indican que establecer puntos de corte puede ayudar al clínico a determinar, en perros infectados con *Leishmania* spp., si los signos oculares se deben a leishmaniosis o a otras causas de KCS.

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