The Veterinary Journal 193 (2012) 168–173

Contents lists available at SciVerse ScienceDirect

The Veterinary Journal

journal homepage: www.elsevier.com/locate/tvjl

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

Carolina Naranjo [a,*], Dolors Fondevila [a], Laura Altet [b], Olga Francino [b], José Ríos [c], Xavier Roura [d], Teresa Peña [a]

[a] *Departament de Medicina i Cirurgia Animals, Universitat Autònoma de Barcelona, Barcelona, Spain*
[b] *Departament de Ciència Animal i dels Aliments, Universitat Autònoma de Barcelona, Barcelona, Spain*
[c] *Laboratory of Biostatistics and Epidemiology (Universitat Autònoma de Barcelona), Clinical Pharmacology Service, IDIBAPS, (Hospital Clinic), Barcelona, Spain*
[d] *Hospital Clínic Veterinari, Universitat Autònoma de Barcelona, Barcelona, Spain*

ARTICLE INFO

Article history:
Accepted 4 October 2011

Keywords:
Leishmania spp.
Real-time PCR
Eye
Keratoconjuntivitis sicca
Dog

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 the Meibomian glands (MG), main lacrimal gland (MLG) and nictitating membrane gland (NMG) from dogs with leishmaniosis; (2) to compare these results to immunohistochemistry (IHC), and (3) to explore the association between the *Leishmania* parasite load 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, IHC and real-time PCR were performed.

All specimens yielded positive real-time PCR results. For all three 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 ΔCt < 0, ΔCt < 4 and ΔCt < 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 three glands. Similarly, ΔCt values below the cut-off were significantly associated with positive IHC. In this study real-time PCR has been standardised for use in MG, MLG and NMG. A cut-off value established for each of these tissues may aid the clinician in the discrimination between ocular signs related to *Leishmania* from those associated with other causes of KCS.

Introduction

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

Clinical manifestations of CanL are very variable and include dermatological signs, lymphadenomegaly, cachexia and muscle atrophy, epistaxis and splenomegaly. Ocular signs 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 prevalence 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 CanL, 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 Ranford-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 CanL (Vitale et al., 2004; Francino et al., 2006; Manna et al., 2008, 2009; de Paiva Cavalcanti et al., 2009). This technique allows detection and quantification of the parasite load, and it is a very useful tool for the clinician when deciding therapeutic options

* Corresponding author. Tel.: +34 5811387.
E-mail address: Carolina.Naranjo@uab.es (C. Naranjo).

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doi:10.1016/j.tvjl.2011.10.001

and in 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 best of our knowledge, there have been no attempts to apply real-time PCR to ocular tissues. In a previous study (Naranjo et al., 2005), we observed *Leishmania* amastigotes by IHC and the associated inflammatory infiltrate surrounding the secretory ducts of the lacrimal glands, which could contribute to the accumulation and retention of the lacrimal fluid resulting in KCS.

The goals of the present 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, 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 were naturally infected and three 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 on 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 as well as serum biochemistry and urinalysis results that were compatible with CanL. Diagnosis was confirmed in all cases by serology using a previously described enzyme-linked immunosorbent assay (ELISA) (Riera et al., 1999), and in some cases the parasite was detected by direct cytological observation (four dogs) or amplification of DNA by means of PCR in either bone marrow or lymph node aspirates or skin biopsies (four dogs).

Ocular signs were detected in 11 of the cases by the attending clinician and 4/11 were further evaluated by a certified ophthalmologist. Six dogs (12 eyes) had blepharitis, four (eight eyes) had periocular alopecia, three (five eyes) had conjunctivitis, six dogs (11 eyes) had keratoconjunctivitis and two (four eyes) had anterior uveitis.

Eighteen of the dogs with CanL had been treated for *Leishmania* over periods varying from 3 days to 10 years. The standard regimen includes a 4 week treatment with SC meglumine antimoniate (Glucantime, Merial Laboratorios SA) at a dose of 50 mg/kg twice daily, and allopurinol (Zyloric, Glaxo Wellcome SA) PO at a dose of 10 mg/kg twice a day over a period of 6 months to 1 year (until the recheck). Table 1 shows which animals were receiving any or both of these drugs at the time of euthanasia.

The 22 dogs with natural infections died or were euthanased with the owners' consent after progressive worsening of the dogs' condition or non-responsiveness to treatment. Owners also signed an informed consent before death or euthanasia, allowing collection of the samples. The three inoculated dogs (dogs 16, 17, 18) were euthanased at the end of their study period. A 6-month-old dog that was euthanased for unrelated reasons was used as control, and the same samples were obtained from this dog.

Collection of samples

For each dog, bilateral eyelid specimens containing the Meibomian glands (MG), the main lacrimal gland (MLG) and the nictitating membrane gland (NMG) were collected, except for dog 9, from which MGs were not obtained.

Histopathology and IHC

Half of each sample was formalin fixed and paraffin embedded. Sections of the tissue were stained routinely (haematoxylin and eosin, HE) and the IHC technique described by Ferrer et al., (1988) to detect *Leishmania* spp. parasites was used. 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 −80 °C until DNA extraction. First, each tissue specimen was lysed overnight with 1 mL of buffer (TRIS 50 mM at pH 8.0, EDTA 20 mM and SDS at 2%), and 10 μL of proteinase K at 10 mg/mL, maintained at 56 °C. Deproteinisation was performed on 300 μL of the lysis product with 150 μL of 5 M NaCl. After agitation and 15 min centrifugation, the supernatant was transferred to another Eppendorf tube and 1 mL of absolute ethanol was added. After 15 min centrifugation, the supernatant was discarded and 500 μL of 70% ethanol was added. After a further 5 min centrifugation, the pellet was dried, 50 μL of water mQ and 2 μL of RNAsa (10 mg/mL) were added and the mixture was incubated at 37 °C for 1 h. Finally, mQ water was added until the final volume was 500 μL. This was frozen at −20 °C 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. These primers and probe were designed to target conserved DNA regions of the kinetoplast minicircle DNA of *L. infantum*. The eukaryotic 18S RNA pre-Developed TaqMan assay reagent (Applied Biosystems) was used as an internal reference of canine genomic DNA. This assay targets 18S RNA genes in mammals but does not amplify *Leishmania* genomic DNA.

Each amplification was performed in duplicate in 25 μL of reaction mixture (TaqMan Universal PCR Master Mix; Applied Biosystems). Thermal cycling profile was 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 60 s. Each amplification run contained positive and negative controls. If the standard deviation of the two samples was >0.38, the sample was reanalysed.

Standardisation

In order to adjust real-time PCR for use in our tissues (MG, MLG, NMG) standard curves were created. 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 eukaryotic 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 amount of DNA in 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 25th and 75th percentiles were used for quantitative variables. Fisher's exact test or the Mann–Whitney *U* test for qualitative or quantitative variables, respectively, was performed when necessary.

The discriminative ability of ΔCt for positive (presence of ocular disease, histopathological lesions or positive IHC), in each tissue, was assessed by means of receiver operating characteristic (ROC) curves and the area under the 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 obtained 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' was assessed by means of risk estimations with odds ratios (OR) calculations and their 95% confidence intervals (95% CI), which were obtained 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 summarised in Table 1. Briefly, 31 MG (64.6%) samples had histopathological lesions compatible with leishmaniosis, consisting of a granulomatous to pyogranulomatous infiltrates with variable numbers of lymphocyte and plasma cells. Two (4%) of the MLG samples and 27 (54%) of the NMG samples had histopathological 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%).