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Research paper

Hair parasite load as a new biomarker for monitoring treatment response in canine leishmaniasis

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ABSTRACT

Canine leishmaniasis treatment focuses on the reduction of parasite load, the clinical improvement of the animal, and the avoidance of relapses, in a scenario where the definitive parasite clearance is not achievable. Therefore, monitoring is crucial during the treatment of this disease. Quantitative PCR has been shown as an ideal tool for the treatment monitoring when quantifying parasite load in target organs such as lymph node or bone marrow, tissues that are too invasive for regular evaluation. This study aims to prove the potential of hair parasite load in the treatment monitoring of canine leishmaniasis. Six dogs were treated with meglumine antimoniate and monitored up to four months after the end of the treatment. Parasite loads in bone marrow, blood, lymph node and hair were quantified by real-time quantitative PCR. Total IgG, IgG1, and IgG2 antibody titres were analysed by immunofluorescent assay and a clinical assessment was carried out. Treatment consisted of two 28-day courses of meglumine antimoniate (100 mg/kg/day) separated by an one-month interval. Analyses were performed before (day 0), during (day 60) and after treatment (day 120), and at the end of a follow-up period (day 210, four months after the end of treatment). Hair parasite load turned out to be strongly correlated with bone marrow, lymph node and blood parasite loads and with the clinical score and the IgG1 antibody titre. The evolution of this biomarker reflects the evolution of the parasitological, immunological and clinical state of the dog, highlighting its potential as a non-invasive marker for the treatment monitoring in canine leishmaniasis.

1. Introduction

Canine leishmaniasis (CanL) is a multisystemic disease which affects the main reservoir of leishmaniasis due to *Leishmania infantum*, the dog. Treatment of this disease is focused on: improving clinical signs given that a parasitological cure is not achievable (Baneth and Shaw, 2002; Ginel et al., 1998), avoiding relapses, which are frequent after the treatment, improving the cell-mediated immunity profile of the dog and reducing the competence to transmit the parasite to sand flies (Alvar et al., 1994; Miró et al., 2008).

The monitoring through serological techniques, such as IFAT or ELISA, is not completely satisfactory given that the antibody levels vary very slowly after treatment and it might remain high for several months, not correlating with the clinical evolution of the dog (Ferrer et al., 1995; Manna et al., 2015). However, some authors have successfully employed serological techniques such as ELISA or Western blot for the follow-up of dogs under antileishmanial treatment (Fernández-Pérez et al., 1999; Vercammen et al., 2002) highlighting their usefulness in the prognosis and the detection of relapses. The quantitation of parasite kDNA through quantitative polymerase chain reaction (qPCR) in target tissues has been shown to be useful in the treatment monitoring, disease prognosis and for the prediction of relapses before clinical signs are shown (Francino et al., 2006; Martínez et al., 2011; Molina et al., 2013; Roura et al., 2013) besides the diagnosis of this challenging disease (Miró et al., 2008). Samples used for the molecular diagnosis range from highly to slightly invasive (bone marrow and popliteal lymph node aspirations, skin biopsy and peripheral blood).

Non-invasive techniques are ideal for the diagnosis and evaluation of CanL progression. Recently, a set of non-invasive samples, such as oral swab, conjunctival swab, vulvar swab and urine, were

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used for the diagnosis and follow-up of a CanL experimental infection through qPCR (Hernández et al., 2015). Also in the last few years, attention has been drawn on the detection of *Leishmania* kDNA in dog hair with diagnostic purposes (Belinchón-Lorenzo et al., 2013), in comparison to its detection in peripheral blood. This approach had been used previously in calves for the detection of bovine viral diarrhoea virus RNA (Singh et al., 2011) and rabies virus RNA (Wacharapluesadee et al., 2012) obtaining good results in dogs.

The aim of this study is to demonstrate the usefulness of the quantitation of *L.infantum* kDNA in the hair of dogs with naturally acquired CanL for the monitoring of therapy and to provide an insight into the relationship of the parasite load in this material with other biomarkers such as parasite load of other tissues, the antibody titres and the clinical picture of the animals in order to facilitate data interpretation.

2. Material and methods

2.1. Animals and housing

Six dogs from southern Spain, an endemic area of CanL (Martín-Sánchez et al., 2009), were selected, with the consent of their owners, upon the following inclusion criteria: two signs compatible with CanL, IFAT titre ≥40 and presence of *L. infantum* kDNA in blood detected by qPCR. The owners were informed of the possible toxicity of the treatment and regimen employed. Exclusion criteria were established as well: dogs with severe hepatic or renal disease or stage IV of CanL (Solano-Gallego et al., 2009), pregnant or lactating dogs, dogs with concomitant infections and dogs under leishmanicidal treatment in the last two years were excluded from the assay if they fulfilled any of those criteria, before or during the treatment.

The dogs were housed in adequate facilities for the course of the clinical trial. These facilities were protected with mosquito nets of small grid size and the animals were provided with deltamethrin-impregnated collars Scalibor® (MSD Animal Health, United States) and a repellent-insecticide (Advantix®, Bayer, Germany) was also applied monthly during the high-intensity sandfly activity period.

2.2. Treatment and follow-up

Two treatment courses were administered, separated by a month interval. For every course, the dogs were treated subcutaneously with a daily dose of 100 mg/kg of meglumine antimoniate (Glucantime®, Sanofi, France) for 28 days. The follow-up period lasted for four months since the end of the treatment. The dogs were treated under the supervision of a veterinary practitioner.

2.3. Sample collection, processing and clinical assessment

Sampling was carried out before the start of the treatment (day 0), between treatment courses (day 60) and after the two treatment courses (day 120 and day 210).

Peripheral blood (PB) was collected by cephalic vein puncture and empty eppendorf tubes or vacutainer tubes containing either heparin or EDTA were filled in order to carry out indirect immunofluorescence antibody test (IFAT), haematological, biochemical and enzymatic tests (safety assessment) and qPCR analysis. Bone marrow (BM) and popliteal lymph node (LN) aspirates were also taken and each of them were divided into two aliquots: one for parasite culture and another one for qPCR analysis. Ear hair samples (H) were extracted carefully with forceps in order to take complete hairs including the root. Samples for molecular analysis were stored at −80 °C.

At each sampling time point, a clinical assessment was made and all dogs were scored for each clinical parameter on a scale from 0 (absence of clinical sign) to 3 (severe clinical sign) as previously reported (Proverbio et al., 2014).

2.4. Control parasite strain

A locally isolated strain, MCAN/ES/2007/DP534, characterized through isoenzyme electrophoresis and identified as *L. infantum* MON-1 was used for IFAT analyses and as a control for qPCR assays.

2.5. Parasite culture

Bone marrow and popliteal lymph node aspirates aliquots were cultured in EMTM solid phase and RPMI-1640 supplemented with 20% fetal bovine serum and 5% human urine as the liquid phase. Cultures were subinoculated and checked weekly for the presence of *L. infantum* promastigotes for two months.

2.6. DNA extraction

This process was carried out in a room exclusively designed for this purpose. DNA was obtained from 200 μL of EDTA anticoagulated blood, 200 μL of EDTA anticoagulated bone marrow aspirate or popliteal lymph node aspirate using the MasterPure® Extraction Kit (Epicentre, United States) according to the manufacturer's instructions. For each hair sample, 10 hairs were cut into small pieces with a sterile scalpel, placed in a sterile tube and grinded in liquid nitrogen using a pestle in order to facilitate DNA extraction, which was performed thereafter. Each DNA extract was rehydrated in a final volume of 20 μL of sterile water. In order to discard DNA contamination, an extraction control was included in every extraction set, which consisted of an empty sterile tube to which the whole extraction process was applied in parallel. The quality and quantity of the DNA extracts was assessed using a Nanodrop® 2000D device (Thermo Scientific, United States).

2.7. Quantitative PCR

The parasite load was quantified using the real-time PCR technique that was described elsewhere (Corpas-López et al., 2015). For every amplification reaction, 1 μL of extraction product or 100 ng of DNA (for lymph node aspirate) were added to the PCR tubes and every biological sample was analysed in triplicate. In addition, a negative control (containing only PCR reagents), an extraction control and a positive control (DNA corresponding to 1000 *L. infantum* promastigotes of the control strain) were employed in every amplification run to assess the quality of the results.

The number of parasites in every qPCR reaction was calculated through the interpolation of the cycle threshold (Ct) value in a standard curve. A negative value was assigned when no amplification occurred or when Ct value was higher than 36.00. The parasite load was estimated in parasites per microliter for bone marrow; parasites per 100 μL for peripheral blood; in parasites per 1000 ng of DNA for popliteal lymph node aspirates, as well as in parasites per one hair.

2.8. Immunofluorescence antibody test

Antibodies specific to *L. infantum* in the dog sera were measured by IFAT against a suspension of 2×10^6 acetone-fixed *L. infantum* promastigotes from the control strain. The antibody titre was determined in geometric dilutions from the sera obtained from dogs, using a starting dilution of 1/20, as reported previously (Acedo Sánchez et al., 1996). Rabbit anti-dog IgG (ICN Biomedical, United