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Research paper

Changes in serum anti-*Leishmania* antibody concentrations measured by time-resolved immunofluorometric assays in dogs with leishmaniosis after treatment

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ABSTRACT

The aim of this study was to evaluate the changes in anti-*Leishmania* IgG2 and IgA antibodies measured by two time-resolved immunofluorometric assays (TR-IFMAs) recently validated and by means of a commercially available ELISA test in dogs with leishmaniosis after treatment. Serum samples from 16 dogs with clinical leishmaniosis were obtained on days 0, 30 and 180 of treatment. In addition, these serological changes were compared with the clinical signs and selected analytes (total proteins, albumin, globulins and urinary protein:creatinine ratio). Concentrations of IgG2 and IgA by TR-IFMA were significantly lower on days 30 ($p < 0.05$) and 180 of treatment ($p < 0.0001$) compared to day 0 in dogs that showed a positive response to treatment. Magnitudes of decrease of IgG2 (1.66 and 20.4-fold) and IgA (1.3 and 11.43-fold) concentrations on days 30 and 180 were greater than those of the commercially available ELISA test (1.29 and 2.06-fold), and that of other analytes (total proteins: 1.11 and 1.25-fold; globulins: 1.22 and 1.74-fold; and albumin: 0.93 and 0.8-fold). This study shows that serum IgG2 and IgA anti-*Leishmania* antibodies measured by TR-IFMAs were useful for treatment monitoring in dogs with leishmaniosis, showing a significant reduction in antibody concentrations earlier than the commercial ELISA assay. Results suggest that the method used for antibody measurements greatly influences the results and, consequently, the usefulness for measuring anti-*Leishmania* antibodies to monitor the treatment of canine leishmaniosis.

1. Introduction

Canine leishmaniosis (CanL) is a zoonotic disease in regions of Europe, America, Asia and Africa caused by the protozoan parasite *Leishmania infantum* (syn. *Leishmania chagasi* in the New World). The parasite is transmitted between hosts by the bite of infected phlebotomine sand flies. The domestic dog is considered the main reservoir for human infection (Yasur-Landau et al., 2017).

The course of CanL can be unpredictable due to its complex pathogenesis and clinical manifestations, which can be very different and non-specific (Kaszak et al., 2015). Clinical manifestations have been reported to be related to an increase in the concentrations of specific anti-*Leishmania* antibodies, indicating a failure of the immune response associated with increased production and deposition of immune complexes, which are responsible for most of the clinical signs. The most common clinical signs are poor body condition, generalized muscular atrophy, lymphadenomegaly and excessive skin scaling (Solano-Gallego et al., 2011).

Changes in anti-*Leishmania* antibody concentrations after treatment have been investigated in previous reports (Solano-Gallego et al., 2001, 2016; Torres et al., 2011). However, the usefulness of antibody measurement for treatment monitoring is not clearly established and controversial results have been reported. Previous studies described that the concentration of antibodies did not decrease within the first months of treatment (Ferrer et al., 1995). Nevertheless, more recent studies reported a progressive decrease in the concentration of specific antibodies associated with clinical improvement (Solano-Gallego et al., 2001; Rodríguez et al., 2006). In addition, a report by Solano-Gallego et al. (2016) described a marked reduction of *Leishmania*-specific antibodies one month after treatment.

Quantitative serological techniques, such as the immunofluorescence antibody test (IFAT) and enzyme-linked immunosorbent

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assay (ELISA) have traditionally been used to detect specific serum antibodies (Solano-Gallego et al., 2009). In a recent research paper, we developed and validated two time-resolved immunofluorometric assays (TR-IFMAs) that showed wider differences for the quantification of anti-*Leishmania* IgG2 and IgA antibodies between seronegative and seropositive dogs and between different clinical stages of CanL than a current commercial ELISA kit (Cantos-Barreda et al., 2017). Nevertheless, these assays have never been used for treatment monitoring of CanL.

The main objective of this study was to evaluate the usefulness of TR-IFMAs to measure serum anti-*Leishmania* IgG2 and IgA antibodies as tools for treatment monitoring of CanL. A secondary objective was to compare the results of TR-IFMAs with those obtained using a commercially available ELISA kit and with other laboratory analytes used to evaluate serum proteins.

2. Materials and methods

2.1. Dogs and sampling procedures

A total of sixteen dogs from different Spanish veterinary clinics were enrolled in the study at the time of diagnosis. The inclusion criteria for the dogs were: (1) presence of clinical signs and laboratory findings compatible with CanL; (2) being *Leishmania*-positive by quantitative serology (*in house* TR-IFMAs and Leiscan® Leishmania ELISA Test, Esteve Veterinaria, Laboratorios Dr. Esteve SA, Barcelona, Spain) and also by PCR of bone marrow or lymph node aspirates obtained at the first visit; (3) having no history of *Leishmania* vaccination; (4) not having received treatment with meglumine antimoniate, miltefosine, domperidone or any glucocorticoid in the last 2 months or treatment with allopurinol in the last 3 weeks; and (5) no presence of another disease. Dogs were evaluated at initial diagnosis and immediately treated with N-methylglucamine antimoniate (50 mg/kg SC, twice daily) for one month and allopurinol (10 mg/kg PO two times daily) for six months.

Clinical follow-up evaluations were conducted by each corresponding veterinarian on days 0 (day of enrollment), 30 and 180 of treatment. Each follow-up session consisted of a general physical examination and scoring for clinical signs associated with CanL using a previously reported scoring system (Segarra et al., 2017). In addition, hemogram and biochemical analysis, urinalysis and quantitative serology were performed at each follow-up visit. Apart from these follow-up visits, owners were contacted by phone at days 60 and 120 of treatment so that they could report any clinical sign that might require an additional visit to the veterinary practice. Owner's consent was obtained in all cases.

Blood samples were collected from the cephalic vein of each dog at the time of diagnosis (day 0), and after 30 and 180 days of treatment. Blood samples were placed in tubes containing EDTA and in tubes containing a clotting activator and a gel separator. The latter tubes were allowed to clot at room temperature and were centrifuged at 3500 rpm for 5 min to obtain serum. Samples were stored at − 80 °C until analysis.

The study was approved by the Local Ethical Committee of the University of Murcia (number 36/2014). All procedures involving animals were performed in accordance with the law RD53/2013 regarding animal experimentation in Spain and with the European Union legislation (2010/63/EU) concerning the protection of animals used for scientific purposes.

2.2. Determination of anti-Leishmania antibody concentrations

To determine the serological status of dogs, two different quantitative methods were used: a commercially available ELISA test (Leiscan® Leishmania ELISA Test, Esteve Veterinaria, Laboratorios Dr. Esteve SA, Barcelona, Spain) and two recently validated assays to detect anti-*Leishmania* IgG2 and IgA antibodies in canine serum by TR-IFMA (Cantos-Barreda et al., 2017).

Leiscan® Leishmania ELISA Test was performed following manufacturer's instructions using the recommended dilution for serum samples of 1/20. Results were expressed as sample-to-positive (S/P) ratio calculated by optical density (OD) sample/OD low positive control. Serum TR-IFMAs were performed as described in a previous study (Cantos-Barreda et al., 2017). Results were expressed as Units of Fluorometry for *Leishmania* (UFL) and were calculated by the WorkOut program (WorkOut Plus MMD software program for data analysis, Perkin-Elmer Life and Analytical Sciences, Wallac Oy, Turku, Finland), being 1 UFL equivalent to 10^3 counts per second (cps). A calibrator consisting in a pool of canine serum from seropositive dogs with an upper value of 1000 UFL (1000×10^3 cps) and 250 UFL (250×10^3 cps) for anti-*Leishmania* IgG2 and IgA antibodies, respectively, were used. Higher values than the upper range of the calibrator were diluted for including them in range and values. This calibrator serum, always the same, was included in all plates. In addition, a pool of positive samples and a pool of negative samples based on the commercial ELISA test (Leiscan® Leishmania ELISA Test, Esteve Veterinaria, Laboratorios Dr. Esteve SA, Barcelona, Spain) were used as controls in all the assays performed. To perform the TR-IFMAs serum samples were diluted 1/4000 and 1/500 in assay buffer for IgG2 and IgA antibodies determination, respectively.

2.3. Determination of laboratorial analytes

Serum total protein and albumin concentrations were measured using a commercially available colorimetric assay (Total protein OSR 6132, Albumin OSR 6102 and Olympus Life and Material Science Europe GmbH, Hamburg, Germany) and globulins concentration was calculated by the difference between total proteins and albumin concentrations as previously described in dogs with leishmaniosis (Silvestrini et al., 2012). Urinary protein and creatinine analytes to obtain urinary protein:creatinine ratio (UPC) were measured by methods previously described (García-Martínez et al., 2015). All analytes were measured using an automated biochemistry analyzer (Olympus AU600 Automatic Chemistry Analyzer, Olympus Europe GmbH, Hamburg, Germany). All assays showed an intra- and inter-assay imprecision lower than 10% and were linear after serial sample dilutions.

2.4. Statistical analysis

Statistical analysis was performed with the GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA). Two dogs of the sixteen did not show a marked clinical and serological improvement and were not included in the statistical analysis. Therefore, the statistical analysis was performed in the fourteen dogs that showed clinical and serological improvement. Concentrations of the different analytes studied were evaluated for normality of distribution by using the Shapiro-Wilk normality test statistics. As data did not follow a normal distribution, a non-parametric test of repeated measures with a Friedman's test and a Dunn's multiple comparisons post-test were used to compare the concentration of the different parameters at each time point with that of day 0. The correlation between IgG2 and IgA antibody concentrations by TR-IFMA, the Leiscan® S/P ratio, the clinical score and all analytes evaluated were studied by the calculation of Spearman's correlation coefficient. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Changes in the clinical score

Initially, all dogs showed clinical signs compatible with CanL (median: 4.5; 25th–75th percentiles: 1.75–8), which were significantly