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Research paper

Relationship between serum anti-*Leishmania* antibody levels and acute phase proteins in dogs with canine leishmaniosis

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ABSTRACT

This study examined the relationship between two serologic assays which quantify anti-*Leishmania* antibodies (a commercial enzyme-linked immunosorbent assay (ELISA) and a time-resolved immunofluorometric assay (TR-IFMA)) and selected acute phase proteins (APPs) and analytes related to protein concentration. Data were obtained from 205 canine serum samples from different veterinary clinics located in an area in which canine leishmaniosis (CanL) is endemic. The samples were submitted to the Interdisciplinary Laboratory of Clinical Analysis (Interlab-UMU), University of Murcia, Spain, for analysis. The biochemical analytes evaluated were serum ferritin, C-reactive protein (CRP), haptoglobin, paraoxonase-1 (PON-1) and albumin as APPs and total proteins and globulins as indicative analytes of protein concentration. Samples were submitted for the initial diagnosis of CanL, or to monitor the response to treatment in patients with CanL. The evaluation of the biochemical analytes did not show differences between *Leishmania*-seronegative and *Leishmania*-seropositive dogs. However, dogs with high antibody titers showed more pronounced clinicopathological abnormalities. Both serological assays had correlations of different significance with the biochemical analytes, showing higher significant correlations with total proteins and globulins than with the rest of the analytes. When the samples submitted for diagnosis and treatment monitoring were analyzed separately, serological assays showed lower correlation in samples for treatment monitoring ($r = 0.531$, $p < 0.0001$) than in samples for diagnosis ($r = 0.769$, $p < 0.0001$). In addition, higher correlations were found between TR-IFMA and analytes such as serum ferritin and CRP in the treatment monitoring group than with the ELISA. These results may help to clarify the relationship between anti-*Leishmania* antibody levels and selected biochemical analytes related to inflammation and protein concentration in CanL.

1. Introduction

Canine leishmaniosis (CanL) is an infectious disease in the Mediterranean area caused by *Leishmania infantum*. It is marked by its zoonotic potential and endemic character (Mettler et al., 2005; Baneth and Aroch, 2008). The spread of CanL depends on the distribution of the vectors and reservoir animals (Kato et al., 2005). Dogs play an important role in the transmission of the disease to humans and other mammals since they are the main reservoirs for sandfly infection (Ready, 2010).

CanL ranges from subclinical disease to fatality depending on the host's immune response (Barbosa et al., 2009). When the immune response is mainly mediated by Th2 lymphocytes, the antibody production is high, leading to the deposition of soluble immune-complexes in organs and tissues (Solano-Gallego et al., 2001; Proverbio et al., 2014). This pathogenic reaction is associated with the presence of a great variety of clinical signs, the presence of skin lesions being the most common manifestation in dogs with clinical leishmaniosis. Other clinical signs that may be present are generalized lymphadenomegaly, weight loss, blepharitis, conjunctivitis, epistaxis or polyarthritis, among others (Solano-Gallego et al., 2011).

The estimated seroprevalence of CanL in endemic areas is typically 10–30%, depending on where the dogs live and their exposure to infection, as well as on the sensitivity and specificity of the diagnostic tests (Maia and Campino, 2008). A variety of laboratory techniques to detect anti-*Leishmania* antibodies using quantitative serological techniques have been developed (Wolf et al., 2014). The most commonly-used techniques are the enzyme-linked immunosorbent assay (ELISA)

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and the immunofluorescence antibody test (IFAT) (Solano-Gallego et al., 2011). Recently, a highly-sensitive time-resolved immunofluorometric assay (TR-IFMA) for the quantification of anti-*Leishmania* antibodies in canine serum has been developed and validated (Cantos-Barreda et al., 2017). This TR-IFMA was based on the recombinant antigen K39 (rK39), a repetitive and highly conserved protein among viscerotropic *Leishmania* spp. (Burns et al., 1993). In addition, this TR-IFMA showed a wider difference in anti-*Leishmania* antibody levels between *Leishmania*-seropositive and *Leishmania*-seronegative dogs and between clinical stages, than did a commercially-available ELISA (Cantos-Barreda et al., 2017).

Acute phase proteins (APPs) are proteins present in plasma whose concentrations are modified in response to tissue injury, infection or inflammation, and which allow the assessing of the innate immune system of the host. Their quantification provides relevant information in support of clinical diagnosis, prognosis and monitoring response to treatment (Murata et al., 2004; Eckersall and Bell, 2010). APPs can be classified as positive or negative if their levels increase or decrease, respectively, after inflammation. Serum ferritin, C-reactive protein (CRP) and haptoglobin are considered positive APPs, while albumin and paraoxonase-1 (PON-1) are considered negative APPs (Cerón et al., 2005; Martínez-Subiela et al., 2014). Previous studies have reported an acute phase response in dogs with CanL, characterized by variations of APPs with increases in CRP, serum ferritin and haptoglobin (Martínez-Subiela et al., 2011; Cantos-Barreda et al., 2018a). Furthermore, the progression of the disease through the determination of the APPs at the time of diagnosis and following treatment, showed an improvement in the concentration of APPs as the clinical manifestations disappeared (Martínez-Subiela et al., 2016).

To the authors' knowledge, there are no studies that correlate antibody levels and APPs in dogs with CanL. The objective of this study was to assess the possible relationship between serum anti-*Leishmania* antibody levels measured by two different assays and selected biochemical analytes related to inflammation and protein concentration in dogs naturally infected with L. *infantum*. For this purpose, we evaluated: (1) the possible differences in the concentration of APPs and analytes related to proteins between *Leishmania*-seronegative and *Leishmania*-seropositive dogs; (2) the values of these analytes in the different quartiles of anti-*Leishmania* antibodies; and, finally, (3) the possible differences in the correlations between antibody levels and the biochemical analytes evaluated, depending on whether they are measured at the time of diagnosis or during treatment monitoring.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Local Ethical Committee of the University of Murcia under protocol no. 36/2014. All procedures were conducted in accordance with law RD 53/2013 regarding animal experimentation in Spain and with the European Directive 2010/63/EU concerning the protection of animals used for scientific purposes.

2.2. Animals and study design

Samples from 205 dogs were included in the study. These samples were submitted to our laboratory from different veterinary clinics throughout Spain in order to measure anti-*Leishmania* antibody levels and APPs. A set of criteria were taken into consideration when it came to accepting the samples for this study: (1) they had to have undergone the quantification of anti-*Leishmania* antibodies by a commercially-available ELISA (Leiscan® Leishmania ELISA Test, Esteve Veterinaria, Laboratorios Dr. Esteve SA, Barcelona, Spain) and the TR-IFMA between January 2016 and January 2018; (2) the serological measurement had to be accompanied by the analysis of a panel of inflammatory analytes including CRP, haptoglobin, serum ferritin, PON-1, albumin, total proteins and globulins; (3) in the case of samples submitted for initial diagnosis of CanL there should be no history of suffering a previous episode of CanL; (4) there should be no history of *Leishmania* vaccination; and (5) the dogs involved should be free from other diseases.

Dogs were allocated into two groups: (1) animals whose samples were submitted to our laboratory for the initial serological diagnosis of CanL and (2) animals whose samples were submitted for treatment monitoring of CanL.

2.3. Sampling

Blood samples were obtained at different clinics using venipuncture of the cephalic or jugular vein, and were collected in tubes containing a coagulation activator and a gel separator. Tubes were allowed to clot at room temperature and centrifuged at 3500 rpm for 5 min. The serum was separated and sent the same day to the Interdisciplinary Laboratory of Clinical Analysis (Interlab-UMU, University of Murcia, Spain). Biochemical analytes included in the study were analyzed on arrival. Samples were stored at -80 °C until analyzed for antibody titers. Such an analysis was undertaken in the lab once a week.

2.4. Anti-Leishmania antibodies

In order to determine the level of anti-*Leishmania* antibodies in the serum samples two different quantitative methods, a commercially-available ELISA test and a TR-IFMA, were employed. The ELISA was performed following manufacturer's instructions using the recommended 1/20 dilution for serum samples and results were expressed as sample-to-positive (S/P) ratio calculated as optical density (OD) sample/OD low positive control. TR-IFMA was performed as previously described (Cantos-Barreda et al., 2017) and samples were diluted 1/4000 in an assay buffer. The results were expressed as Units of Fluorometry for *Leishmania* (UFL).

2.5. Biochemical analytes

CRP concentration was measured using a human immunoturbidimetric test (CRP OSR 6147 Olympus Life and Material Science Europe GmbH, Lismeehan, O'Callaghan Mills, Co. Clare, Ireland) used previously in dogs (Muñoz-Prieto et al., 2017). Haptoglobin was determined using a hemoglobin-binding method (Tridelta Phase, Tridelta Development Ltd., Bray, Ireland) in a biochemistry autoanalyzer (Cobas Mira Plus, ABX Diagnostics, Montpellier, France). PON-1 activity was analyzed following a previously described method (Tvarijonaviciute et al., 2012). Ferritin concentrations were measured using an immunoturbidimetric assay with polyclonal anti-human ferritin antibodies (Tina-quant Ferritin, Boehringer Mannheim, Germany) previously validated for use in dogs (Martínez-Subiela et al., 2014). Serum total protein and albumin concentrations were measured using a human colorimetric assay (Total protein OSR 6132, Albumin OSR 6102, Olympus Life and Material Science Europe GmbH, Hamburg, Germany). Globulin concentrations were calculated by the difference between total proteins and albumin concentrations as previously described in dogs with CanL (Silvestrini et al., 2012). Determinations of CRP, total proteins and albumin concentrations were performed using an automated biochemistry analyzer (Olympus AU600; Olympus Diagnostica GmbH, Hamburg, Germany).

2.6. Statistical analysis

All data were assessed for normality of distribution using the Kolmogorov-Smirnov normality test. Descriptive statistics for the different groups were determined using the non-parametric Kruskal-Wallis test. To compare the TR-IFMA and the ELISA results, and to compare the serological results and the biochemical analytes values, a