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Research paper

New wide dynamic range assays for quantification of anti-*Leishmania* IgG2 and IgA antibodies in canine serum

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ABSTRACT

The aims of this study were (1) to develop and validate time resolved-immunofluorometric assays for the detection of anti-*Leishmania* IgG2 and IgA antibodies in canine serum and (2) to evaluate the ability of these assays to quantify different amounts of anti-*Leishmania* antibodies in *Leishmania*-seronegative and seropositive dogs, determined by a commercial ELISA assay, and between different clinical stages according to LeishVet guidelines. The analytical validation showed that the assays had a good precision with intra- and inter-assay coefficients of variation lower than 10%. In addition, the assays allowed the quantification of very low concentration of antibodies as well as demonstrated a high level of accuracy, as determined by linearity under dilution ($R^2 = 0.99$) and recovery tests (> 85%). Moreover, no cross-reactions with *Ehrlichia canis,* Canine Parvovirus Type 2, *Anaplasma phagocytophilum*, *Babesia canis, Dirofilaria immitis* and pyometra were found. The assays were able to detect higher values of anti-*Leishmania* IgG2 and IgA antibodies in seropositive dogs compared with seronegative dogs ($p < 0.0001$), although an overlap between groups existed in the case of IgA. In addition, significantly higher values for both antibodies were detected in LeishVet groups II ($p < 0.05$) and III ($p < 0.01$) when compared with LeishVet group I. From our study, it could be concluded that the immunofluorometric assays developed would be suitable for determination of anti-*Leishmania* IgG2 and IgA antibodies in serum samples with an adequate precision, analytical sensitivity and accuracy. In addition, these assays showed a wider difference in the concentration of both IgG2 and IgA antibodies between seronegative and seropositive dogs and between different clinical stages of CanL than a current commercial ELISA kit. Further studies would be recommended to evaluate the diagnostic sensitivity and specificity of these new assays as well as their application in monitoring CanL.

1. Introduction

Canine Leishmaniosis (CanL) caused by *Leishmania* spp. is a disease transmitted between hosts by phlebotomine sandflies that affects millions of dogs in more than 70 countries all over the world from Mediterranean Europe, Asia, North Africa, Central and South America to North America or Northern Europe, where it is an emergent disease (Duprey et al., 2006; Martínez-Subiela et al., 2011; Kaszak et al., 2015). Leishmaniosis represents a public health problem since it is one of the most prevalent zoonotic diseases worldwide, dogs being the main reservoir of this infection. Seroprevalence of this disease in dogs of southern Europe is estimated to be around 5–30%, but real prevalence is higher (Solano-Gallego et al., 2001).

The dogs' response to the infective sandfly bite can result in an asymptomatic period that may develop to symptomatic infection or remain cryptic. Both clinical symptomatic and asymptomatic dogs harbor the parasite and are infective to sandfly vectors (Gradoni et al., 1987). The clinical manifestations of the disease may be very different and non-specific (Kaszak et al., 2015). Therefore, the clinical diagnosis of CanL is difficult due to the variable symptomatology. However, reliable identification of infected dogs is of paramount importance to determine the risk and the ways to control this zoonosis (Rodríguez-Cortés et al., 2013).

Serological assays based on the detection of specific anti-*Leishmania* antibodies in serum along with molecular analysis are considered the main diagnostic techniques to detect infection in sick and sub-clinically infected dogs. In addition, they are among the criteria used to divide the disease into four clinical stages ranging from mild to very severe disease based on LeishVet clinical staging (Solano-Gallego et al., 2009).

K39 antigen is widely used in serological assays because it contains

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a repetitive and immunodominant epitope of a kinesin-related protein that is highly conserved among viscerotropic *Leishmania* spp. (Burns et al., 1993), allowing an increase in the sensitivity of the tests (Mettler et al., 2005). In addition, IgG2 is usually measured because is the main contributor to the humoral response of this disease (Santarém et al., 2010) and it is considered as the IgG subtype with highest potential to detect *Leishmania* infection (Rodríguez-Cortés et al., 2010). This is due to the fact that dogs with clinical leishmaniosis initially present high levels of both IgG subclasses (IgG1 and IgG2), but after time, the level of IgG1 decreases and that of IgG2 remains constant (Solano-Gallego et al., 2001). In addition, *Leishmania*-infected dogs produce not only specific IgG, but also *Leishmania*-specific IgA (Rodríguez-Cortés et al., 2007), which is the main antibody isotype of the mucosal immune system. Therefore, detection of this immunoglobulin in CanL might reflect *L. infantum* dissemination to mucosal surfaces, becoming useful as a means of supporting the clinical assessment in clinical practice (Rodríguez et al., 2006; Rodríguez-Cortés et al., 2007).

Qualitative tests based on immunochromatography (Otranto et al., 2004) or quantitative tests such as the immunofluorescence antibody test (IFAT) (Mettler et al., 2005) and the enzyme-linked immunosorbent assay (ELISA) (Santarém et al., 2010; Solano-Gallego et al., 2014) can be used to perform serological assays. Nevertheless, one of the main limitations of the conventional serological tests is the relatively small optical density (OD) margin between the positive and negative results, which makes it difficult to accurately distinguish between seronegative and seropositive results (Wolf et al., 2014). Time resolved-immunofluorometry is a technique based on the quantification of an emitted fluorescence by a lanthanide chelate label attached to antigens or antibodies (Martínez-Subiela et al., 2011) that shows a higher analytical sensitivity and broader detection range for serum proteins quantification than ELISA (Hu et al., 2015).

Although the use of time resolved-immunofluorometric assays (TR-IFMAs) might be a promising approach for the determination of *Leishmania* antibodies, this technique has never been reported in literature applied to CanL. The aims of this study were to develop and analytically validate two novel TR-IFMAs for the detection of anti-*Leishmania* IgG2 and IgA antibodies in canine serum and to evaluate the ability of these assays to quantify different amounts of these antibodies in: (1) *Leishmania*-seronegative and seropositive dogs determined by a validated commercial ELISA and (2) different clinical stages of the disease according to LeishVet guidelines.

2. Materials and methods

2.1. Animal and sampling procedures

A total of 125 serum samples from dogs of different breeds and ages from southern Spain, which is an endemic area, were used in this study. The samples were analysed by a commercial ELISA test (Leiscan® Leishmania ELISA Test, Esteve Veterinaria, Laboratorios Dr. Esteve SA, Barcelona, Spain), which has been demonstrated to be the most sensitive and specific test of the currently commercially available (Rodríguez-Cortés et al., 2010). From these samples, 15 samples with different degree of seropositivity were used to perform the analytical precision, lower limit of quantification, linearity under dilution and recovery test of the assays; 94 samples from *Leishmania*-seronegative (n = 25) and seropositive (n = 69) dogs were used to evaluate the correlation between TR-IFMAs and ELISA and for the overlap performance of the study, which tested the ability of the assays to produce different values between seronegative and seropositive dogs; and 16 samples from *Leishmania*-seronegative dogs affected by other diseases were also included to evaluate possible cross-reactions of our assays with other pathogens.

Blood samples were collected from all dogs by venipuncture into tubes containing a coagulation activator and a gel separator, allowed to clot at room temperature, and centrifuged at 3500 rpm for 5 min. Serum samples obtained were stored at −80°C until analysis. All procedures involving animals were approved by the Murcia University Ethical Committee in compliance with laws RD32/2007 and RD1201/2005 relating to animal experimentation in Spain.

2.2. Assays

The two TR-IFMAs for measurement of anti-*Leishmania* IgG2 and IgA antibodies in canine serum had the same basis.

In brief, 200 µL of biotinylated K39 recombinant antigen were pipetted into streptavidin-coated microtitration wells (DELFIA streptavidin microtitration strips, PerkinElmer Life and Analytical Sciences, Turku, Finland) and incubated for 1 h at room temperature with continuous shaking. Then, the strips were washed four times with 200 µL/well of wash buffer (DELFIA wash concentrate, PerkinElmer Life and Analytical Sciences, Turku, Finland), and 200 µL/well of diluted samples or calibrator were added followed by 1 h of incubation. After a second wash cycle, 200 µL of europium-labelled antibody anti-IgG2 (Sheep anti-Dog IgG2, Bio-Rad, Hercules, California, USA) or anti-IgA (Goat anti-Dog IgA, Bethyl, Montgomery, TX, USA) were added to each well. The strips were incubated for 1 h. A third wash was done and finally, 200 µL/well of enhancement solution (DELFIA enhancement solution, PerkinElmer Life and Analytical Sciences, Turku, Finland) were added and plates were shaken for 5 min. The emitted fluorescence, proportional to the quantity of *Leishmania* antibodies in the sample, was measured as cps (counts per second) using a multilabel counter (VICTOR^2 1420, PerkinElmer Life and Analytical Sciences, Turku, Finland).

The results were expressed as Units of Fluorometry for *Leishmania* (UFL), calculated by the Wallac MultiCalc program (MultiCalc function software, PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland), being 1 UFL equivalent to 10^3 cps. A calibrator consisting of 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, respectively, was used. Higher values than the upper range of the calibrator were diluted for including them in range and values below the lower limit of quantification were expressed as the latter. This calibrator serum, always the same, was included in all plates. Moreover, 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 assays.

To perform the assays, serum samples were diluted 1/4000 and 1/500 in the assay buffer for IgG2 and IgA antibodies determination, respectively.

The commercial ELISA (Leiscan® Leishmania ELISA Test, Esteve Veterinaria, Laboratorios Dr. Esteve SA, Barcelona, Spain) for *Leishmania* IgGs antibodies determination was performed following the manufacturer's instructions (where the serum samples were diluted 1/20) and the results were expressed as sample-to-positive (S/P) ratio (calculated by OD sample/OD Low Positive Control).

2.3. Analytical validation

2.3.1. Assay precision

For evaluation of the assay precision, intra- and inter-assay precision were performed. The intra-assay precision, expressed by the coefficient of variation (CV), were calculated after five analyses in a single analytical run of one pool of five serum samples containing high quantities and one pool of five serum samples with low quantities of *Leishmania* antibodies. The same sample pools were analysed to determine the inter-assay precision by performing a single measurement in each of the different pools on five different days within one week. The samples were frozen in aliquots, and vials were only thawed as required for each analytical run in order to prevent any possible variation as a result of repeated freeze-thaw cycles.