Veterinary Parasitology 242 (2017) 54–58

Contents lists available at ScienceDirect

Veterinary Parasitology

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

Research paper

Quantification of anti-*Leishmania* antibodies in saliva of dogs

Ana Cantos-Barreda, Damián Escribano, Luis J. Bernal, José J. Cerón*, Silvia Martínez-Subiela

Interdisciplinary Laboratory of Clinical Analysis, Interlab-UMU, Regional Campus of International Excellence "Campus Mare Nostrum", University of Murcia, Espinardo, Murcia, Spain

ARTICLE INFO

Keywords:
Canine leishmaniosis
Diagnosis
Saliva
IgG2
IgA
TR-IFMA

ABSTRACT

Detection of serum anti-*Leishmania* antibodies by quantitative or qualitative techniques has been the most used method to diagnose Canine Leishmaniosis (CanL). Nevertheless, saliva may represent an alternative to blood because it is easy to collect, painless and non-invasive in comparison with serum. In this study, two time-resolved immunofluorometric assays (TR-IFMAs) for quantification of anti-*Leishmania* IgG2 and IgA antibodies in saliva were developed and validated and their ability to distinguish *Leishmania*-seronegative from seropositive dogs was evaluated. The analytical study was performed by evaluation of assay precision, sensitivity and accuracy. In addition, serum from 48 dogs (21 *Leishmania*-seropositive and 27 *Leishmania*-seronegative) were analyzed by TR-IFMAs. The assays were precise, with an intra- and inter-assay coefficients of variation lower than 11%, and showed high level of accuracy, as determined by linearity under dilution ($R^2 = 0.99$) and recovery tests (> 88.60%). Anti-*Leishmania* IgG2 antibodies in saliva were significantly higher in the seropositive group compared with the seronegative ($p < 0.0001$), whereas no significant differences for anti-*Leishmania* IgA antibodies between both groups were observed. Furthermore, TR-IFMA for quantification of anti-*Leishmania* IgG2 antibodies in saliva showed higher differences between seropositive and seronegative dogs than the commercial assay used in serum. In conclusion, TR-IFMAs developed may be used to quantify anti-*Leishmania* IgG2 and IgA antibodies in canine saliva with an adequate precision, analytical sensitivity and accuracy. Quantification of anti-*Leishmania* IgG2 antibodies in saliva could be potentially used to evaluate the humoral response in CanL. However, IgA in saliva seemed not to have diagnostic value for this disease. For future studies, it would be desirable to evaluate the ability of the IgG2 assay to detect dogs with subclinical disease or with low antibody titers in serum and also to study the antibodies behaviour in saliva during the treatment of CanL.

1. Introduction

Canine Leishmaniosis (CanL) is a zoonotic disease caused by *Leishmania spp*. parasites and transmitted between hosts by the bite of an infected phlebotomine sand fly (Kaszak et al., 2015). CanL affects dogs in Europe, Africa, Asia and America, being domestic dog the main reservoir for human infection. In south-western Europe, it has been estimated that at least 2.5 million dogs are infected (Baneth and Aroch, 2008), being *Leishmania infantum* the main causative species and *Phlebotomus perniciosus* the main vector (Vlkova et al., 2011).

Traditionally, immunodiagnosis of infectious diseases has relied on the analyses of blood products (Debattista et al., 2007). Detection of specific serum anti-*Leishmania* antibodies by quantitative or qualitative serological techniques has been the most commonly used method in routine for the diagnosis of CanL (Solano-Gallego et al., 2014). Nevertheless, some studies have revealed that saliva may be used as an alternative to blood for the detection of antibodies against infectious diseases (Chang et al., 2009). Saliva is a sample easy to collect (dog owners might do it), painless and non-invasive (Yoshizawa et al., 2013) compared with blood sampling (Parra et al., 2005). In particular, saliva-based tests have the potential to make a big impact in screening programs, especially in areas where laboratory infrastructure is poor or unavailable (Pant Pai et al., 2007). Currently, oral fluid-based diagnostics are not being used to the fullest capacity; nonetheless, it is envisioned that saliva will play an increasingly important role in the early diagnosis and monitoring of disease progression (Tabak, 2001).

Saliva samples have been used to detect anti-*Leishmania* antibodies in human medicine (Vaish et al., 2012; Ribeiro-dias, 2015; Mohapatra et al., 2016). However, to the author's knowledge, there are no reports in dogs, despite the high prevalence of this disease in endemic areas as well as the importance of this species as a reservoir of *Leishmania spp*. for humans (Kaszak et al., 2015).

The present study aims to evaluate if saliva could be a suitable sample to detect anti-*Leishmania* antibodies in dog. For this purpose,

* Corresponding author.
E-mail address: jjceron@um.es (J.J. Cerón).

http://dx.doi.org/10.1016/j.vetpar.2017.05.017
Received 17 February 2017; Received in revised form 5 April 2017; Accepted 19 May 2017

two highly sensitive TR-IFMAs for quantification of anti-*Leishmania* IgG2 and IgA antibodies in canine saliva were developed and validated. In addition, the ability of these assays to distinguish between *Leishmania*-seronegative and seropositive dogs by using saliva samples were assessed. K39 antigen, a 39-amino acid epitope highly conserved among Leishmania spp. (Burns et al., 1993) which has been demonstrated to increase the sensitivity (Mettler et al., 2005), was used in the assays developed.

2. Materials and methods

2.1. Sample collection

Saliva samples were collected by using Salivette tubes (Sarstedt, Nümbrecht, Germany) and sponges. Each dog was allowed to gently chew on a sponge, until it was completely moistened. Saliva samples were obtained after its centrifugation at 3700 rpm for 10 min and stored at −80 °C until analysis.

Blood samples were collected from the cephalic vein 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 were stored at −80 °C until analysis.

All procedures involving animals were approved by the University of Murcia's Ethical Committee and were performed in compliance with laws RD32/2007 and RD1201/2005 concerning animal experimentation in Spain.

2.2. Assays

TR-IFMAs for measurement of anti-*Leishmania* IgG2 and IgA antibodies in saliva were developed as explained as follows. 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 with an upper value of 200 UFL and 85 UFL for anti-*Leishmania* IgG2 and IgA antibodies, respectively, consisting in a pool of saliva from *Leishmania*-seropositive dogs that had high serum titer of antibodies by ELISA test (Leiscan® Leishmania ELISA Test, Esteve Veterinaria, Laboratorios Dr. Esteve SA, Barcelona, Spain) was used. Values higher than the upper range of the calibrator were additionally diluted for including them in range and retested again and values below the lower limit of quantitation were expressed as the latter. The calibrator, always the same, were included in all plates. In addition, a pool of positive and a pool of negative serum samples based on serological results by a commercial ELISA test (Leiscan® Leishmania ELISA Test, Esteve Veterinaria, Laboratorios Dr. Esteve SA, Barcelona, Spain) were used as controls in all assays performed. Saliva samples were diluted 1/100 in assay buffer for both IgG2 and IgA antibodies determination.

Leiscan® was the quantitative assay used for the serum analysis since it showed the best sensitivity, specificity and accuracy when compared with other ELISA and immunochromatographic tests (Rodríguez-Cortés et al., 2013). This assay was carried out according to the manufacturer's instructions and the results were expressed as sample-to-positive (S/P) ratio (calculated by OD sample/OD Low Positive Control).

2.3. Analytical study

2.3.1. Evaluation of the assay precision

The intra-assay precision, expressed by the coefficient of variation (CV), was calculated after analysis of five saliva samples containing high quantities and five saliva samples with low quantities of *Leishmania* antibodies in a single analytical run.

The same samples were used to determinate the inter-assay precision by analyzing them 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.

2.3.2. Evaluation of the assay sensitivity

The detection limit was defined as the lowest amount of *Leishmania* antibodies that could be distinguished from a specimen of zero value. This parameter was expressed as the mean value of 10 replicate determinations of the zero standard (assay buffer) plus three standard deviations.

The lower limit of quantification was calculated based on the lowest UFL that could be measured in the linear part of the calibration curve with a CV below 20% and above the limit of detection. To estimate this parameter, a saliva sample with a high antibody quantity was serially diluted in assay buffer and each dilution was analyzed in five replicates in the same run.

2.3.3. Evaluation of the assay accuracy

No reference assay is available to quantify anti-*Leishmania* antibodies in canine saliva, so the accuracy was indirectly calculated by linearity under dilution and recovery procedure.

Linearity under dilution was determined by using two saliva samples with high quantities of *Leishmania* antibodies serially diluted with assay buffer. Amount of antibodies in diluted samples were measured with the TR-IFMAs and, afterward, curves representing UFL of *Leishmania* antibodies measured vs. UFL of *Leishmania* antibodies expected and the coefficient of determination (R^2) were calculated.

For the recovery procedure, one saliva sample containing a high quantity of *Leishmania* antibodies and one containing a low amount of these were used. The sample with high quantity of antibodies was diluted two-fold (50%), four-fold (25%) and ten-fold (10%) with the sample with low antibody amount. Besides, the low antibody amount saliva sample was diluted four-fold (25%) with the high antibody quantity saliva sample (75%). Detected and expected antibody amounts for each diluted saliva sample were compared and the percentages of recovery were calculated.

2.4. Ability to differentiate between seronegative and seropositive dogs

To test the ability of the method to differentiate *Leishmania*-seronegative from seropositive dogs, 48 dogs of different ages, sexes and breeds with negative results when tested for presence of *Anaplasma phagocytophylum, Borrelia Burgdorferi, Dirofilaria immitis* and *Ehrlichia canis* antibodies using SNAP test (Canine SNAP 4Dx, IDEXX laboratories) were used. They were client-owned dogs from different clinics of south-eastern Spain. Animals were classified into two groups:

2.4.1. Seronegative dogs

Saliva and serum samples from 27 dogs were used. Dogs were