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Advances in Canine Leishmaniosis with Emphasis on the Use of Saliva Samples

Avances en la Leishmaniosis Canina con Énfasis en el Uso de Muestras de Saliva

Dña. Ana Cantos Barreda

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"Advances in canine leishmaniosis with emphasis on the use of saliva samples"

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Memoria presentada por la graduada en veterinaria

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Para optar al grado de Doctora en Ciencias Veterinarias con Mención Internacional

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DOCTORAL THESIS AS COMPENDIUM OF PUBLICATIONS

DOCTORAL THESIS AS COMPENDIUM OF PUBLICATIONS

The present PhD thesis, in accordance with the authorization of the directors of the PhD thesis and the Academic Commission responsible of the Veterinary Sciences PhD Program, is presented as a compendium of six experiments previously published or accepted for publication. This PhD thesis is composed by the following **article** references:

I. **Cantos-Barreda, A.**, Escribano, D., Bernal, L.J., Pardo-Marín, L., Cerón, J.J., Martínez-Subiela, S., 2017. New wide dynamic range assays for quantification of anti-*Leishmania* IgG2 and IgA antibodies in canine serum. Vet. Immunol. Immunopathol. 189, 11-16.

II. **Cantos-Barreda, A.**, Escribano, D., Bernal, L.J., Cerón, J.J., Martínez-Subiela, S., 2017. Quantification of anti-*Leishmania* antibodies in saliva of dogs. Vet. Parasitol. 242, 54-58.

III. **Cantos-Barreda, A.**, Escribano, D., Martínez-Subiela, S., Pardo-Marín, L., Segarra, S., Cerón, J.J., 2018. Changes in serum anti-*Leishmania* antibody concentrations measured by time-resolved immunofluorometric assays in dogs with leishmaniosis after treatment. Vet. Immunol. Immunopathol. 198, 65-69.

IV. **Cantos-Barreda, A.**, Escribano, D., Cerón, J.J., Tecles, F., Bernal, L.J., Martínez-Subiela, S., 2018. Changes in the concentration of anti-*Leishmania* antibodies in saliva of dogs with clinical leishmaniosis after short-term treatment. Vet. Parasitol. 254, 135-141.

V. **Cantos-Barreda, A.**, Escribano, D., Cerón, J.J., Bernal, L.J., Furlanello, T., Tecles, F., Pardo-Marín, L., Martínez-Subiela, S., 2018. Relationship between serum anti-*Leishmania* antibody levels and acute phase proteins in dogs with canine leishmaniosis. Vet. Parasitol. 260, 63-68.

VI. **Cantos-Barreda, A.**, Escribano, D., Egui, A., Thomas, M.C., López, M.C., Tecles, F., Bernal, L.J., Cerón, J.J., Martínez-Subiela, S., 2019. One-year follow-up of anti-*Leishmania* antibody concentrations in serum and saliva from experimentally-infected dogs. Int. J. Parasitol. (Accepted for publication).

In addition, it is considered appropriate to include in **Appendix** 1 and 2 of this PhD thesis data from experiments related to the work carried out during the thesis. This data is planned to be submitted for possible publication in the future.

I. **Cantos-Barreda, A.**, Escribano, D., Egui, A., López, M.C., Cerón, J.J., Bernal, J.J., Martínez-Subiela, S. Evaluation of the circadian rhythm of anti-*Leishmania* IgG2 and IgA antibodies in serum and saliva of dogs with clinical leishmaniosis.

II. **Cantos-Barreda, A.**, Escribano, D., Siriyasatien, P., Cerón, J.J., Thomas, M.C., Afonso-Lehmann, R.N., López, M.C., Bernal, L.J., Phumee, A., Martínez-Subiela, S. Detection of *Leishmania infantum* kinetoplast minicircle DNA by real-time PCR in saliva of experimentally-infected dogs.

ABBREVIATIONS

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APPs: acute phase proteins

CanL: canine leishmaniosis

CBC: complete blood cell count

CRP: C-reactive protein

CV: coefficient of variation

DNA: deoxyribonucleic acid

EDTA: ethylenediaminetetraacetic acid

ELISA: enzyme-linked immunosorbent assay

Eu: europium

Hp: haptoglobin

IFAT: immunofluorescence antibody test

IgA: immunoglobulin A

IgG2: immunoglobulin G subtype 2

LAMP: loop-mediated isothermal assay

PCR : polymerase chain reaction

PON-1: paraoxonase-1

qPCR: quantitative polymerase chain reaction

r: coefficient of correlation

SD: standard deviation

Th: T helper cell

TR-IFMA: time-resolved immunofluorometric assay

INTRODUCTION

INTRODUCTION

Canine leishmaniosis (CanL) is a zoonotic disease caused by the protozoan parasite *Leishmania* spp. and transmitted between hosts by the bite of infected phlebotomine sandflies (Kaszak *et al*., 2015).

This disease affects millions of dogs all over the world including the Mediterranean areas of Europe, North Africa, Asia, and Central and South America (Baneth et al., 2008). The distribution of the vectors and reservoir animals influences the spread of CanL (Kato *et al*., 2005). It is estimated that approximately 5-30% of dogs in southern Europe are seropositive for CanL (Solano-Gallego *et al*., 2001). The main causative species of CanL in south-western Europe is *Leishmania infantum* and the main vector is *Phlebotomus perniciosus* (Vlkova *et al*., 2011).

The CanL is a result of complex pathogenesis that leads to unspecific clinical symptoms (Kaszak *et al*., 2015). Clinical manifestations are related to the immune response mediated mainly by T helper (Th) 2 lymphocytes, with an increase of specific anti-*Leishmania* antibody levels. As a result, deposition of immune-complexes in organs and tissues are responsible for most of the clinical signs (Solano-Gallego *et al*., 2001 Proverbio *et al*., 2014). Skin lesions are the most common manifestation in dogs that develop the clinical disease but many additional clinical signs that may be present, such as weight loss, generalized lymphadenomegaly, blepharitis, conjunctivitis, polyarthritis or epistaxis, among others (Solano-Gallego *et al*., 2011).

Diagnosis of leishmaniosis can be performed by direct identification of the parasite, by indirect identification through detecting *Leishmania*-specific antibodies or by molecular detection of its deoxyribonucleic acid (DNA). The immunochromatographic qualitative tests (Otranto *et al*., 2004), the immunofluorescence antibody tests (IFAT) (Mettler *et al*., 2005) and the enzyme-linked immunosorbent assays (ELISA) (Santarém *et al*., 2010) are the most commonly used techniques to perform the serological diagnosis of CanL. However, these conventional serological tests are limited by the low sensitivity of the rapid tests, the subjective interpretation of the results and the narrow optical density margin between the positive and negative results, respectively (Swets *et al*., 1988; Solano-Gallego *et al*., 2014; Wolf *et al*., 2014;). In this current scenario, the need for the

development of a highly-sensitive technique for quantifying the specific antibodies against *Leishmania* spp. was detected. Regarding diagnosis by molecular methods, the use of quantitative polymerase chain reaction (qPCR) provides reproducible results with good accuracy and sensitivity, which depends on the type of target gene and samples selected. The kinetoplast DNA (kDNA) is the main target used for diagnostic screening because of its high sensitivity as a result of the high number of copies (~10,000) per parasite (Rodgers *et al*., 1990). Detection of *L. infantum* DNA by PCR in saliva samples has been reported in human medicine (Siriyasatien *et al*., 2016; Pandey *et al*., 2018). However, *L. infantum* DNA in saliva of dogs has not yet been reported. In addition, the loop-mediated isothermal amplification assay (LAMP) has been reported useful for diagnosis of CanL in blood (Chaouch *et al*., 2013) and conjunctival swab samples (Gao *et al*., 2015) and it could be considered as an alternative for its diagnosis.

Saliva is a sample obtained through non-invasive procedures, being painless and non-stressful for the animals. Moreover, saliva collection is simple, cheap, and the repeated sampling at short-time intervals following modest training levels can be performed (Escribano *et al*., 2012; Yoshizawa *et al*., 2013).

The lack of sensitive assays for the quantification of *Leishmania*-specific antibodies and the molecular detection of this parasite using non-invasive samples motivated the performance of the present PhD thesis.

OBJECTIVES

OBJECTIVES

This PhD thesis was conceived to go in-depth on the study of the development of assays that could allow the use of saliva samples for diagnosing leishmaniosis in dogs. For this purpose, the following objectives were contemplated for the development of the present thesis:

1. To develop and validate an ultrasensitive assay for anti-*Leishmania* antibodies quantification that could allow their measurement in both serum and saliva samples of dogs (**Article 1** and **Article 2**).

2. To evaluate the usefulness of the assay for anti-*Leishmania* antibodies measurement in both serum and saliva samples as a tool for monitoring the treatment of CanL (**Article 3** and **Article 4**).

3. To assess the possible relationship between serum anti-*Leishmania* antibody levels measured by the new assay and by a commercially-available ELISA kit and the concentration of acute phase proteins (APPs) (**Article 5**).

4. To evaluate and compare the kinetics of anti-*Leishmania* immunoglobulin G subtype 2 (IgG2) and immunoglobulin A (IgA) by the new assay in serum and saliva from experimentally-infected dogs with *L. infantum* during one-year follow-up (**Article 6**).

5. To assess the possible circadian rhythm of anti-*Leishmania* IgG2 and IgA levels in both serum and saliva samples from experimentally-infected dogs (**Appendix 1**).

6. To develop and validate a qPCR for quantification of *Leishmania* spp. DNA in canine saliva (**Appendix 2**).

EXTENDED SUMMARY

EXTENDED SUMMARY

1. MATERIALS AND METHODS

1.1. BIOMEDICAL ETHICS

The experimental studies included in this thesis were approved by the Local Ethical Committee of the University of Murcia under the protocol no. 36/2014 and 276/2016. All procedures were conducted in accordance with the current Spanish and European legislation: *Real Decreto 53/2013, de 1 de febrero, por el que se establecen las normas básicas aplicables para la protección de los animals utilizados en experimentación y otros fines científicos, incluyendo la docencia*; and Directive 2010/63/EU of the European Parliament and of the Council, of 22 September 2010, on the protection of animals used for scientific purposes.

1.2. SAMPLING PROCEDURE

Samples of saliva and serum from dogs were used in order to perform the different experiments included in the present thesis.

On one hand, saliva samples were obtained using Salivette® saliva collection devices (Sarstedt, Nümbrecht, Germany). The cotton rod commercially provided into the Salivette® tubes was replaced by a sponge in order to maximize the total volume of saliva recovered. Each sponge was clipped with tweezers and rubbed against the buccal mucosa on each side of the mouth, above and below the tongue and through every nook of the oral cavity for approximately 1 min. Afterward, the sponge was inserted into the Salivette® and centrifuged at 3,700 rpm for 10 min. Saliva samples were maintained at −80ºC until analyzed. These samples were used to quantify the anti-*Leishmania* antibody levels and *Leishmania* spp. DNA.

On the other hand, serum was obtained from whole blood collected from the jugular or cephalic vein into tubes containing a coagulation activator and a gel separator, allowed to clot at room temperature and centrifuged at 3,500 rpm for 5 min. Serum samples were stored at −80°C until analysis. These samples were used to perform the biochemical

analyses and the measurement of the anti-*Leishmania* antibody levels. When a complete blood cell count (CBC) was included in the study, and additional milliliter of whole blood was collected in tubes containing ethylenediaminetetraacetic acid (EDTA).

1.3. ANTI-*LEISHMANIA* ANTIBODIES DETERMINATION IN SERUM AND SALIVA SAMPLES

1.3.1 Development of a time-resolved immunofluorometric assay

A TR-IFMA was developed for the measurement of specific IgG2 and IgA antibodies against *Leishmania* spp. proteins in serum and saliva samples. For that, the biotinylated recombinant antigen K39 (RAG0061BIOT; Rekom Biotech S.L., Granada, Spain) was used as capture reagent. The polyclonal antibodies Sheep anti-Dog IgG2 (AHP948; Bio-Rad, Hercules, California, USA) and Goat anti-Dog IgA (A40-121A; Bethyl, Montgomery, Texas, USA) commercially-obtained were labeled with europium (Eu) chelate (DELFIA® Eu-Labeling kit (Eu-N1-ITC chelate) 0.2 mg (1244-302; PerkinElmer Life and Analytical Sciences, Turku, Finland) and used as detection reagents.

The TR-IFMA procedure after optimization was represented as follows:

1. Add 200 µL of biotinylated K39 recombinant antigen into streptavidin-coated microtitration wells (DELFIA® Streptavidin Microtitration Strips, PerkinElmer Life and Analytical Sciences, Turku, Finland).
2. Incubate for 1 h at 20-25ºC with continuous shaking.
3. Wash 4 times with 300 µL/well of wash buffer (DELFIA® Wash Concentrate, PerkinElmer Life and Analytical Sciences, Turku, Finland).
4. Add 200 µL/well of diluted standard or diluted serum (1:4,000 for IgG2 or 1:500 for IgA) or saliva (1:100 both IgG2 and IgA) samples with assay buffer (DELFIA® Assay Buffer, PerkinElmer Life and Analytical Sciences, Turku, Finland).
5. Incubate for 1 h at 20-25ºC with continuous shaking.
6. Wash 4 times with 300 µL/well of wash concentrate.
7. Add 200 µL/well of Eu-labelled antibody anti-IgG2 or anti-IgA.
8. Incubate for 1 h at 20-25ºC with continuous shaking.
9. Wash 4 times with 300 µL/well of wash concentrate.
10. Add 200 µL/well of enhancement solution (DELFIA® Enhancement Solution, PerkinElmer Life and Analytical Sciences, Turku, Finland).
11. Incubate for 5 min at 20-25ºC with continuous shaking.

12. Measurement of the emitted fluorescence using a multilabel counter (VICTOR^2 1420, PerkinElmer Life and Analytical Sciences, Turku, Finland). Anti-*Leishmania* antibody levels were calculated by means of the WorkOut Program (WorkOut Plus MMD software program for data analysis, PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland). The results were expressed as Units of Fluorometry for *Leishmania* (UFL), 1 UFL being equivalent to 10^3 cps.

1.4. ANALYTICAL VALIDATION

1.4.1 Assessment of the assay precision

The intra- and inter-assay precisions were performed. The intra-assay precision was expressed as the coefficient of variation (CV) calculated after five analyses of two pools of five serum samples, one of them with high levels of anti-*Leishmania* antibodies and the other one with low levels of these specific antibodies, in a single analytical execution. The inter-assay precision was expressed as the CV of the measurement of the same pools of samples on five different days within the same week.

1.4.2 Assessment of assay accuracy

Accuracy was evaluated thorough linearity under dilution, recovery procedure and correlation study as follows:

Linearity under dilution was calculated using serial dilutions of two serum samples with high levels of anti-*Leishmania* antibodies. The coefficient of determination (R^2) was calculated from curves representing the anti-*Leishmania* antibody levels measured vs. the anti-*Leishmania* antibody levels expected.

For the recovery experiment, a sample of serum with high levels of anti-*Leishmania* antibodies was diluted two-fold (50%), four-fold (25%), and ten-fold (10%) with a serum sample with low levels of anti-*Leishmania* antibodies. The detected levels of anti-*Leishmania* antibodies were compared with the expected values and the percentages of recovery were calculated.

For the correlation study, a comparison between TR-IFMAs and a commercial ELISA results were performed by calculation of the Spearman correlation coefficients.

1.4.3 Assessment of the assay sensitivity

Assay sensitivity was evaluated by calculation of the analytical limit of detection and the lower limit of quantification.

The analytical limit of detection was calculated as the mean value of ten replicates of the assay buffer + 3 SD. For its part, the lower limit of quantification was estimate by analyzing a serum sample with high anti-*Leishmania* antibody levels serially diluted in assay buffer five times in the same analytical run.

2. EXPERIMENTAL DESIGN, RESULTS, AND DISCUSSION

2.1. OBJECTIVE 1

Objective 1 was covered by two experiments corresponding to Article 1 (Experiment 1) and Article 2 (Experiment 2).

2.1.1. EXPERIMENT 1. ***New wide dynamic range assays for quantification of anti-Leishmania IgG2 and IgA antibodies in canine serum.***

Experimental design

In order to achieve the aim of the experiment, a total of 94 serum samples were used. These serum samples proceed from dogs with different breeds and ages from the Southern of Spain, which is considered an endemic area for canine leishmaniosis (CanL). All samples were analyzed by a sensitive and specific commercially-available ELISA test (Leiscan® Leishmania ELISA Test, Esteve Veterinaria, Laboratorios Dr. Esteve SA, Barcelona, Spain) (Rodríguez-Cortés *et al.*, 2010). Serum samples from 25 *Leishmania*-seronegative dogs belonging to the Animal Resources Center of University of Murcia and from 69 naturally-infected dogs with different stages of CanL (Solano-Gallego *et al.*, 2011) were included in the study. These samples were used to evaluate the correlation between TR-IFMA and ELISA and to assess the overlap performance. In addition, sera from 16 *Leishmania*-seronegative dogs but suffering other diseases were used to evaluate cross-reactions.

Results and discussion

Analytical validation: The intra-assay variation showed CVs ranging from 7.29% to 3.05% and from 5.37% to 1.95% for pools with low and high levels of anti-*Leishmania* IgG2 and IgA, respectively. The inter-assay variation provided CVs of the pools with low and high levels of antibodies ranging between 9.80% and 8.51% and between 7.63% and 7.96% for anti-*Leishmania* IgG2 and IgA, respectively. All the intra- and inter-assays CVs were < 20%, the percentage accepted for immunological assays (Guidance for Industry, 2001). The dilution of two canine serum samples with high anti-*Leishmania* antibody levels resulted in linear regression equations where $r = 0.995$ and $r = 0.996$ for IgG2 and IgA, respectively, with $R^2 = 0.99$. The recovery results ranged from 93.14% to 99.54%, and from 85.40% to 94.90% for IgG2 and IgA, respectively, all of them inside the stablished range for immunological assays (80-120%) (Andreasson *et al*., 2015). TR-IFMA and Leiscan® were positively correlated for IgG2 ($r = 0.792$, $p < 0.0001$) and for IgA ($r = 0.743$, $p < 0.0001$). Limits of detection for anti-*Leishmania* IgG2 and IgA were 4.41 and 7.16 UFL, respectively, and the lower limits of quantification were 4.84 and 7.45 UFL for anti-*Leishmania* IgG2 and IgA, respectively. In addition, the anti-*Leishmania* IgG2 and IgA levels for the group of *Leishmania*-negative dogs but with other infectious disease were < 4.84 UFL and < 17.9 UFL, respectively.

Assessment of the overlap performance: The UFL values for anti-*Leishmania* IgG2 and IgA were significantly higher in *Leishmania*-seropositive dogs compared with seronegative ($p < 0.0001$). The median value for anti-*Leishmania* IgG2 was 818 and 4.84 UFL in the seropositive and the seronegative group, respectively. Whereas, the median value for anti-*Leishmania* IgA was 208 and 15.53 UFL in seropositive and the seronegative group, respectively. In addition, some dogs showed negative results for anti-*Leishmania* IgA, which could lead to false-negative results, previously reported in human medicine (Da Silva *et al*., 2015). The Leiscan® S/P ratios for IgG were significantly higher in seropositive (median 3.69 S/P ratio) compared with seronegative dogs (median 0.25 S/P ratio) ($p < 0.0001$). Our TR-IFMA for quantification of anti-*Leishmania* IgG2 showed greater differences in the levels of antibodies between seropositive and seronegative dogs than Leiscan® and a previous reported ELISA (Solano-Gallego *et al*., 2001).

The median values for anti-*Leishmania* IgG2 were 198, 1275, 2400, and 891 UFL and for anti-*Leishmania* IgA were 100, 554, 640 and 132 UFL for the LeishVet stage I, II, III and IV, respectively. The UFL results for anti-*Leishmania* IgG2 and IgA were significantly higher for LeishVet stage II ($p < 0.05$) and III ($p < 0.01$) in comparison with

dogs of stage I. Leiscan® yielded median values of 2.75, 4.58, 3.99, and 2.68 S/P ratio in LeishVet stages I, II, III, and IV, respectively. In addition, no statistical differences between LeishVet stages were found using Leiscan®.

2.1.2. EXPERIMENT 2. ***Quantification of anti-Leishmania antibodies in saliva of dogs.***

Experimental design

For experiment 2, 48 dogs of different ages, sexes, and breeds living in Southeastern Spain and tested negative to other parasitic diseases were used. Dogs were classified as *Leishmania*-seronegative or *Leishmania*-seropositive using Leiscan®. The *Leishmania*-seronegative dogs (n = 27) were clinically and analytically healthy, whereas the *Leishmania*-seropositive dogs (n = 21) showed clinical signs compatible with CanL, were diagnoses with CanL for the first time, and were classified as stage III based on the LeishVet guidelines (Solano-Gallego *et al*., 2011).

Results and discussion

Analytical validation: The intra-assay CVs of the pools with low and high levels of antibodies ranged from 5.08% to 5.17% and from 9.42% to 10.44%, for anti-*Leishmania* IgG2 and IgA, respectively. The inter-assay CVs of the pools with low and high levels of antibodies ranged from 8.09% to 5.79% and from 10.43% to 10.87% for anti-*Leishmania* IgG2 and IgA, respectively. All the intra- and inter-assays CVs were < 20%, the percentage accepted for immunological assays (Guidance for Industry, 2001). The dilution of two canine saliva samples with high anti-*Leishmania* antibody levels resulted in linear regression equations with $R^2 = 0.99$. Results of recovery ranged from 93.76% to 97.00 % and from 88.60% to 98.00% for anti-*Leishmania* IgG2 and IgA, respectively, all of them inside the stablished range for immunological assays (80-120%) (Andreasson *et al*., 2015). The limits of detection for IgG2 and IgA were 4.41 UFL and 7.16 UFL, respectively. The lower limit of quantification for IgG2 and IgA were 4.91 UFL and 9.28 UFL, respectively.

Assessment of the overlap performance: The UFL values for anti-*Leishmania* IgG2 UFL measured in saliva were significantly high ($p < 0.0001$) in the seropositive group than in the seronegative group. In this case, no overlap between both groups was observed, contrary to that reported in studies for immunochromatographic tests in human

medicine (Vaish *et al.*, 2012; Ribeiro-Dias *et al.*, 2015). No significant differences between these groups were observed for anti-*Leishmania* IgA and an overlap was observed (Vaish *et al.*, 2012; Ribeiro-Dias *et al.*, 2015). The median value for anti-*Leishmania* IgG2 in the seronegative group was 4.91 UFL (4.91-14.00 UFL; 25th-75th percentiles), whereas in the seropositive group was 328.70 UFL (105.30-21704 UFL; 25th-75th percentiles). The median values for anti-*Leishmania* IgA were 45.86 UFL (40.76-58.55UFL; 25th-75th percentiles) and 50.41 UFL (30.98-168.10 UFL; 25th-75th percentiles) for the seronegative and the seropositive groups, respectively. For Leiscan®, the median value of antibodies in serum of the seronegative group was 0.25 S/P ratio (0.20-0.43 S/P ratio; 25th-75th percentiles), whereas in the seropositive group was 3.43 S/P ratio (3.11-4.25 S/P ratio; 25th-75th percentiles). Moreover, the anti-*Leishmania* IgG2 levels in saliva by TR-IFMA were significantly correlated with the Leiscan® results in serum ($r = 0.768$, $p < 0.0001$). Nevertheless, no correlation between anti-*Leishmania* IgG2 and IgA UFLs from saliva samples was observed.

2.2. OBJECTIVE 2

Objective 2 was covered by two experiments corresponding to Article 3 (Experiment 1) and Article 4 (Experiment 2).

2.2.1. EXPERIMENT 1. ***Changes in serum anti-Leishmania antibody concentrations measured by time-resolved immunofluorometric assays in dogs with leishmaniosis after treatment.***

<u>Experimental design</u>

A total of 16 dogs attending veterinary clinics were included in the study and serum samples were collected. These dogs presented clinical signs and laboratory abnormalities compatible with CanL, were *Leishmania*-seropositive by TR-IFMA in serum and positive to *Leishmania* infection by cytology or PCR, were not been vaccinated against *Leishmania*, were free from other diseases, and did not receive any drug that can interfere in the study results. Dogs were treated with a combination of N-methylglucamine antimoniate (Glucantime®, Merial Laboratorios S.A., Barcelona, Spain; 50 mg/kg twice a day, subcutaneously) for one month and allopurinol (generic formulation; 10 mg/kg every 12 h, orally) for six months. The clinical follow-up of the dogs was performed at

the time of enrollment (day 0) and after one month (day 30) and 6 months (day 180) of treatment. Dogs were physically examined and a clinical score according to Segarra *et al.* (2017) was assigned to each one. Moreover, a biochemical analysis including UPC ratio, total proteins, globulins and albumin, and the quantification of anti-*Leishmania* IgG2 and IgA levels by TR-IFMA was performed at each time point follow-up.

Results and discussion

Changes in the clinical score: The median with 25th–75th percentiles of the clinical score was 4.5 (1.75-8) at the time of enrollment, which significantly reduced after 30 (0.5; 0–1.25; $p < 0.01$) and 180 (0; 0–0; $p < 0.0001$) days of treatment. However, two dogs maintained a high clinical score on day 180 of treatment.

Changes in the concentration of anti-Leishmania antibodies: Significant decreases in the level of anti-*Leishmania* IgG2 and IgA were found after 30 ($p < 0.05$; 1.66-fold of decrease for IgG2 and 1.3-fold for IgA) and 180 ($p > 0.0001$; 20.4-fold of decrease for IgG2 and 11.43-fold for IgA) days of treatment regarding pre-treatment values, in accordance with clinical improvement. This study suggested that measuring of specific IgG2 against *Leishmania* spp. by TR-IFMA is useful for treatment monitoring of CanL, in accordance with previous studies (Solano-Gallego *et al*., 2011; 2016). However, Leiscan® S/P ratios for IgGs showed significant differences after 180 days of treatment ($p < 0.0001$; 2.06-fold of decrease). Nevertheless, two dogs did not reduce their anti-*Leishmania* antibody levels after treatment. One of them presented higher titers after 180 days of treatment than at day 0 and IgA progressively decreased. The other one, showed decreases in the anti-*Leishmania* IgG2 and IgA levels at day 30 but increases at day 180 of treatment.

Other analytes: total proteins and globulins showed significant differences in their concentration at day 30 ($p < 0.05$) and 180 ($p < 0.0001$) of treatment; albumin only showed significant differences in its concentration at day 180 of treatment ($p < 0.001$) and no differences among time points were observed for UPC ratio concentrations.

Correlation between antibodies measured by TR-IFMA, clinical score, analytes related to protein concentration, and S/P ratio: The anti-*Leishmania* IgG2 levels were significantly correlated with the clinical score, total proteins, globulins, albumin, and the S/P ratio. For its part, anti-*Leishmania* IgA levels were significantly correlated with total proteins, globulins, and albumin concentrations, and with the S/P ratio.

2.2.2. *EXPERIMENT 2.* ***Changes in the concentration of anti-Leishmania antibodies in saliva of dogs with clinical leishmaniosis after short-term treatment.***

Experimental design

Saliva and serum samples were collected from dogs 20 dogs attending veterinary clinics. To be included in the study, dogs have to show at least one clinical sign and one laboratory finding compatible with CanL, be *Leishmania*-seropositive by TR-IFMA in serum and positive to *Leishmania* infection by cytology or PCR, not been vaccinated against *Leishmania*, be free from other diseases, and not have receive any drug that can interfere in the study results. Immediately after receiving a positive diagnosis for CanL by TR-IFMA dogs were treated with a combination of N-methylglucamine antimoniate (Glucantime®, Merial Laboratorios S.A., Barcelona, Spain; 50mg/kg twice a day, subcutaneously) and allopurinol (generic formulation; 10mg/kg every 12h, orally) for one month. Follow-up of the dogs was performed at the time of the diagnosis (day 0) and after one month of treatment (day 30). This monitoring consisted of physical examination with the assignation of a clinical score according to Segarra *et al*. (2017), a CBC, a biochemical analysis, and a serology detection anti-*Leishmania* antibodies using TR-IFMA.

Results and discussion

Changes in the clinical score: The median with 25th–75th percentiles of the clinical score was 6.0 (4.5–10) at the time of diagnosis, which was significantly reduced ($p < 0.0001$) after 30 days of treatment (0; 0–2). In addition, several dogs showed clinical scores below 5 (85%), of which 65% achieved total cure with a clinical score of 0. Nevertheless, three dogs did not clinically improve and maintained high clinical scores at day 30. From these, dogs 12 and 18 had clinical scores above 9 but decreases in anti-*Leishmania* antibody levels in saliva and serum, and also decreases in the concentration of C-reactive protein (CRP) and ferritin. For its part, dog 19 showed a clinical score of 10 at day 30 and increases in serum and saliva anti-*Leishmania* antibody levels and concentration of CRP and ferritin. In these three cases, treatment with allopurinol was maintained and dogs 12 and 18 achieved the clinical cure after the second month of treatment, but not dog 19, which still showed clinical signs.

Changes in anti-Leishmania antibody concentrations: Dogs that responded adequately to treatment showed significant decreases in the concentration of anti-

Leishmania IgG2 ($p < 0.0001$) and IgA ($p < 0.01$) in saliva after 30 days of treatment, with folds of 3.4 (70.3%) and 1.5 (34.1%), respectively. In serum samples, the anti-*Leishmania* IgG2 and IgA levels also significantly decreased after 30 days of treatment ($p < 0.0001$), with folds of 3.3 (70.1%) and 2.5 (60.1%), respectively. Two dogs that clinically improved after treatment (dogs 14 and 17) did not show decreases in anti-*Leishmania* IgG2 and IgA in saliva. The anti-*Leishmania* IgA levels in saliva increased for dog 14 and the clinical signs did not completely disappear. Dog 17 showed low values at presentation and a minor increase in both anti-*Leishmania* antibodies in saliva but achieved the clinical cure. In this case, anti-*Leishmania* antibodies in serum were also low concerning the other sick dogs of the study. This fact could suggest that low antibody levels in serum could influence the levels of antibodies in the saliva (Brandtzaeg, 2013). In addition, 3 dogs didn't clinically improve after treatment, in accordance with a previous report (Solano-Gallego *et al.*, 2016).

Changes in hematological and biochemical analytes: significant increases in hematocrit ($p < 0.01$), leukocytes ($p < 0.05$) and lymphocytes ($p < 0.01$) were observed after treatment. In addition, significant differences between days 0 and 30 for total proteins ($p < 0.0001$), globulins ($p < 0.0001$), albumin ($p < 0.001$), CRP ($p < 0.05$), ferritin ($p < 0.0001$) and paraoxonase-1 (PON-1) ($p < 0.05$) concentrations were detected. The biochemical analytes that showed greater folds of decrease after one month of treatment were CRP and ferritin, with 4.5 (77.6%) and 4.0 (74.9%) folds of decrease, respectively. All the hematological and biochemical analytes evaluated improved after 30 days of treatment, in accordance with previous studies (Rubio *et al.*, 2016; Segarra *et al.*, 2017; Solano-Gallego *et al.*, 2016).

Correlation study: Significant correlations were observed between serum and saliva anti-*Leishmania* IgG2 ($r = 0.528$; $p < 0.01$) and anti-*Leishmania* IgA ($r = 0.361$; $p < 0.05$) levels. Moreover, significant correlations between both anti-*Leishmania* antibodies were observed in saliva ($r = 0.709$; $p < 0.0001$) and in serum ($r = 0.900$; $p < 0.0001$). Significant negative correlations between anti-*Leishmania* IgG2 antibodies in saliva and PON-1 ($r = -0.347$; $p < 0.05$), hematocrit ($r = -0.404$; $p < 0.05$) and lymphocytes ($r = -0.382$; $p < 0.05$) were found. However, anti-*Leishmania* IgA levels in saliva only were correlated with hematocrit ($r = -0.459$; $p < 0.05$). Absolute levels of anti-*Leishmania* antibodies were not correlated with the magnitude of clinical score.

2.3. OBJECTIVE 3

Objective 3 was covered by the following experiment corresponding to the Article 5.

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

Experimental design

A total of 205 dogs suspicious of CanL (a group of samples submitted for initial diagnosis of CanL) or under anti-*Leishmania* treatment monitoring (a group of samples submitted for treatment monitoring of CanL) were included in the study. Serum samples from these dogs were submitted from veterinary clinics to our laboratory between January 2016 and January 2018. To be included in the retrospective study, each clinical case had to fulfil the following conditions: (1) undergone the anti-*Leishmania* antibodies quantification by both the ELISA and the TR-IFMA; (2) undergo the analysis of the acute phase proteins (APPs) panel including CRP, haptoglobin (Hp), PON-1, ferritin, total proteins, albumin, and globulins; (3) no having previous history of CanL in dogs for initial diagnosis; (4) no having a history of vaccination against *Leishmania*; (5) and be free from other diseases.

Results and discussion

Animals: From the total of 205 dogs, 61 (78.5%) were classified in the group of samples for initial diagnosis of CanL, whereas 44 (21.5%) were included in the group of samples submitted for treatment monitoring of CanL. Regarding the anti-*Leishmania* antibody level, dogs were allocated in quartiles for each serological assay.

Relationship between Leishmania-seronegative and Leishmania-seropositive dogs and the selected biochemical analytes: Dogs were divided in *Leishmania*-seropositive and *Leishmania*-seronegative. Significant differences ($p < 0.0001$) between the seropositive and the seronegative groups were found. Nevertheless, no differences in the concentration of the selected biochemical analytes between seronegative and seropositive dogs were observed.

Relationship between the magnitude of serology values and the selected biochemical analytes: Significant differences were observed among quartiles of anti-

Leishmania antibody levels as follows. For the TR-IFMA quartiles 1 and 2 ($p < 0.05$), 1 and 3 (p < 0.0001), 2 and 3 ($p < 0.0001$), 1 and 4 ($p < 0.0001$), 2 and 4 ($p < 0.0001$) and 3 and 4 ($p < 0.0001$) for TR-IFMA. For the ELISA, between quartiles 1 and 3 ($p < 0.001$), 1 and 4 ($p < 0.0001$) and 2 and 4 ($p < 0.0001$). Moreover, dogs from the quartile 4 showed more pronounced changes in the biochemical analytes compared with other quartiles, which is associated with active disease, and, as a result, with abnormalities in the concentration of APPs, as previously reported (Martínez-Subiela *et al.*, 2016; Cantos-Barreda *et al.*, 2018a). Especially, CRP, PON-1, ferritin and albumin showed greater significant differences between quartiles when TR-IFMA, not ELISA, was used.

Relationship between serology at diagnosis or during treatment monitoring and the selected biochemical analytes: When all the data were correlated, a significant correlation between the anti-*Leishmania* IgG2 UFL and the S/P ratio ($r = 0.768$; $p < 0.0001$) was observed. Both TR-IFMA and ELISA were significantly correlated with total proteins, globulins, ferritin, and albumin. However, greater significant correlations were observed for TR-IFMA. In addition, TR-IFMA and ELISA results were more correlated in the diagnostic group ($r = 0.769$; $p < 0.0001$) than in the treatment monitoring group ($r = 0.531$; $p < 0.0001$). When results from both serological assays were correlated with the concentration of the biochemical analytes, the TR-IFMA showed higher correlations with ferritin and CRP in the treatment monitoring group than the ELISA, which reinforced the utility of the TR-IFMA for treatment monitoring of CanL (Cantos-Barreda *et al.*, 2018b).

2.4. OBJECTIVE 4

Objective 4 was covered by the experiment corresponding to the Article 6.

2.4.1. EXPERIMENT. ***One-year follow-up of anti-Leishmania antibody concentrations in serum and saliva from experimentall- infected dogs.***

Experimental design

In order to achieve the aim of this experiment, 11 Beagle dogs were infected intravenously with 1×10^6 stationary-phase promastigotes of *L. infantum*. The success of the infection was confirmed by serological tests against *Leishmania* antigens and by qPCR from bone marrow aspirates, as previously described (Ledesma *et al.*, 2017). Serum

and saliva samples were used for the quantification of anti-*Leishmania* antibodies by TR-IFMA. Pre-infection samples were obtained immediately before the experimental infection, and post-infection samples were collected monthly for one year.

Results and discussion

Assessment of the serological response: Significant differences ($p < 0.01$) in the concentration of anti-*Leishmania* IgG2 in the serum between the pre-infection time and the p.i. samplings were observed from month 3 p.i. Median values were above the cut-off at month 4 p.i. All dogs seroconverted between 3 and 7 months p.i., similar to that reported in previous studies (Hernández *et al*., 2015; Rodríguez-Cortés *et al*., 2017). Regarding anti-*Leishmania* IgA concentrations in the serum, significant differences ($p < 0.001$) between the pre-infection time and the p.i. months were observed from month 4 p.i. In addition, the median anti-*Leishmania* IgA value was above the cut-off in month 4 p.i.

In the saliva samples, significant increases ($p < 0.0001$) in the concentration of anti-*Leishmania* IgG2 compared to the pre-infection time were observed at 4 months p.i. However, the median concentration of anti-*Leishmania* IgG2 was above the cut-off at 5 months p.i. Regarding anti-*Leishmania* IgA concentrations in saliva, significant increases ($p < 0.05$) compared to the pre-infection time were observed at 3 months p.i. However, the median values of these specific antibodies did not surpass the cut-off throughout the study. Seven (63.6%) out of the total of eleven dogs developed detectable concentrations of anti-*Leishmania* IgA, reinforcing the finding that measuring anti-*Leishmania* IgA in saliva has no diagnostic value for CanL (Cantos-Barreda *et al*., 2017). In addition, greater intra-individual variability in the levels of anti-*Leishmania* antibodies was observed in saliva than in serum, in accordance with a previous report regarding total antibody levels (German *et al*., 1998). Our results suggested the pass of antibodies from serum to saliva (Brandtzaeg, 2013).

Clinical evaluation: All dogs included in the study were asymptomatic at the time of infection. First clinical signs compatible with CanL appeared in the majority of dogs (63.6%) at 6 months p.i. The most frequent clinical signs were lymphadenomegaly (n = 7), erosive dermatitis (n = 6), blepharitis and/or conjunctivitis (n = 6), and ulcerative dermatitis (n = 5). Three out of the eleven dogs (27.3%) were asymptomatic along the study period.

Correlation study: The level of anti-*Leishmania* IgG2 between serum and saliva samples had a good correlation ($r = 0.853$; $p < 0.0001$), but lower correlation between the level of anti-*Leishmania* IgA in both serum and saliva samples was observed ($r = 0.289$; $p < 0.001$). When the first clinical signs appeared, high significant correlations were observed between the anti-*Leishmania* IgG2 levels in serum and saliva ($r = 0.905$; $p < 0.01$), IgG2 and IgA in serum ($r = 0.857$; $p < 0.05$), IgG2 and IgA in saliva ($r = 0.929$; $p < 0.01$), and IgG2 in serum and IgA in saliva ($r = 0.762$; $p < 0.05$).

2.5. OBJECTIVE 5

In order to achieve the objective 5 an experiment was performed, which corresponds to the Appendix 1.

2.5.1. EXPERIMENT. ***Evaluation of the circadian rhythm of anti-Leishmania IgG2 and IgA antibodies in serum and saliva of dogs with clinical leishmaniosis.***

Experimental design

A total of 6 healthy Beagle dogs were experimentally-infected with *L. infantum*. Paired serum and saliva samples were obtained from each dog at 67-weeks post-infection at 4-h intervals for a 16-h period on two consecutive days. A complete hemogram, a biochemical analysis, and a qPCR on bone marrow aspirates were performed before starting the experiment. Levels of anti-*Leishmania* IgG2 and IgA antibodies in serum and saliva samples were determined by TR-IFMA. Serological results derived from saliva samples were expressed in three different ways: without any correction, corrected by the protein content, and corrected by the salivary flow rate.

Results and discussion

No significant differences in anti-*Leishmania* IgG2 levels in both serum and saliva samples were observed between the different sampling times. Regarding anti-*Leishmania* IgA levels, significant differences among sampling times were observed when saliva was corrected by the salivary flow rate. Results from our study suggested that the way of reporting the anti-*Leishmania* antibody levels, mainly the anti-*Leishmania* IgA corrected by the salivary flow rate, can influence the interpretation of the results. Moreover, Greater intra-individual variations in the anti-*Leishmania* antibody levels were found in saliva

than in serum, in accordance with a previous study from German *et al*. (1998), which reported high intra-individual variability in the secreted antibody levels. Nevertheless, the intra-individual variations that we found didn't follow a circadian pattern and the serological status of each dog was not modified. The absence of a circadian pattern in the anti-*Leishmania* antibody levels is in line with previous results reported by German *et al*. (1998). In that study, no circadian pattern was observed in the total level of antibodies canine saliva and tears.

2.6. OBJECTIVE 6

Objective 6 was achieved by the experiment corresponding to Appendix 2.

2.6.1. *EXPERIMENT.* ***Detection of Leishmania infantum kinetoplast minicircle DNA by real-time PCR in saliva of experimentally-infected dogs.***

Experimental design

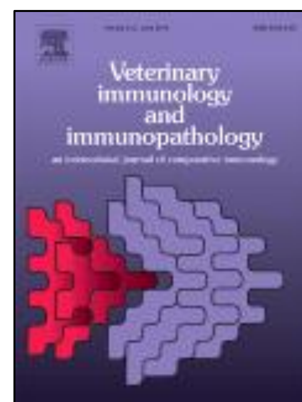
To achieve the aim of the study, a total of 16 healthy Beagle dogs were experimentally-infected with *L. infantum*. Saliva samples and bone marrow aspirates were obtained before the infection and at 16 weeks post-infection. DNA was extracted from the samples using the phenol/chloroform precipitation method. Quality and quantity of DNA were measured using the spectrophotometer NanoDrop® 2000. The extracted DNA was analyzed for possible detection of *Leishmania* kinetoplast minicircle DNA using the qPCR developed and validated in this study.

Results and discussion

The qPCR developed and validated in our study was sensible, with a detection limit of 0.25 fg/reaction (0.0025 parasites) using DNA from *L. infantum* derived from cell culture. These results were in accordance with those previously reported for detection of DNA from *Leishmania* kinetoplast minicircle (Mary *et al*., 2004; Francino *et al*., 2006; Ortega *et al*., 2017). This study reported a PCR efficiency between 87.6%-93.8%, R^2 between 0.99 and 0.996, and repeatability of 0.67. The *Leishmania* kinetoplast was chosen as the molecular target due to the high sensitivity reported (Mary *et al*., 2004). The *Leishmania* kinetoplast DNA was detected by our qPCR in 14 out of 16 (87.5%) bone marrow samples and in 8 of 16 (50%) saliva samples. However, our results were in

line with those previously reported using non-invasive samples, in which the qPCR sensitivity was lower than in samples obtained thorough invasive procedures (Aschar *et al*., 2016; Pandey *et al*., 2018). Moreover, 7 out of the 14 *Leishmania*-positive bone marrow dogs (50%) were also positive in saliva, with a concordance of 8 results between saliva and bone marrow.

ARTICLES



ARTICLE 1 (Published)

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

Journal: Veterinary Immunology and Immunopathology

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.

URL: https://doi.org/10.1016/j.vetimm.2017.05.007

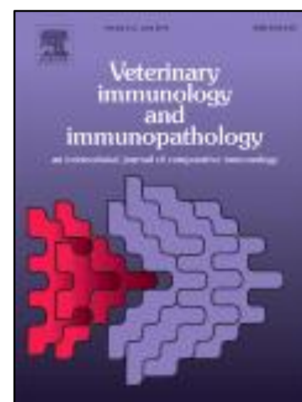
ARTICLE 2 (Published)

Quantification of anti-Leishmania antibodies in saliva of dogs

Journal: Veterinary Parasitology

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.

URL: https://doi.org/10.1016/j.vetpar.2017.05.017



ARTICLE 3 (Published)

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

Journal: Veterinary Immunology and Immunopathology

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.

URL: https://doi.org/10.1016/j.vetimm.2018.03.003

ARTICLE 4 (Published)

Changes in the concentration of anti-Leishmania antibodies in saliva of dogs with clinical leishmaniosis after short-term treatment

Journal: Veterinary Parasitology

Abstract: The aim of this study was to evaluate the possible changes in the concentration of anti-*Leishmania* antibodies in saliva samples from dogs with clinical leishmaniosis after short-term treatment. Twenty dogs with clinical signs and laboratory abnormalities compatible with canine leishmaniosis (CanL) were diagnosed and treated with a standard antimonial plus allopurinol therapy. The concentration of anti-*Leishmania* IgG2 and IgA antibodies in saliva was measured at the time of diagnosis (day 0) and after treatment (day 30) by time-resolved immunofluorometric assays (TR-IFMAs) and results were compared with those of serum. In addition, correlations between antibody concentrations in saliva and serum, clinical scores and selected laboratory analytes were calculated. TR-IFMA results were expressed as Units of Fluorometry for *Leishmania* (UFL). Most dogs that adequately responded to treatment (n=17) showed a reduction of anti-*Leishmania* antibodies in saliva [median IgG2: from 678.0 (day 0) to 201.1 UFL (day 30), $p < 0.0001$; median IgA: from 91.3 (day 0) to 60.2 UFL (day 30), $p < 0.01$] in accordance with clinical improvement ($p < 0.0001$). However, two of these dogs showed an increase of anti-*Leishmania* antibodies in saliva. Among dogs that did not improve after one month of treatment (n=3), two showed a reduction in serum and saliva antibodies. In these two dogs, clinical recovery was achieved after one additional month of treatment with allopurinol. The other dog that did not respond to treatment showed increases in the concentration of anti-*Leishmania* antibodies, both in saliva and serum, and did not adequately respond to an additional month of treatment with allopurinol. From this pilot study, it could be concluded that, despite the low number of dogs used, the measurement of anti-*Leishmania* IgG2 and IgA antibodies in saliva could have a potential use for treatment monitoring of CanL, provided that a sufficient amount of specific antibodies is present at diagnosis. This is because, especially in the case of IgG2, there is a high correlation between the saliva and serum concentrations, and the reduction of antibodies is generally in accordance with the clinical improvement. Further long-term studies with a larger population should be undertaken to confirm this potential.

URL: https://doi.org/10.1016/j.vetpar.2018.03.014

ARTICLE 5 (Published)

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

Journal: Veterinary Parasitology

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.

URL: https://doi.org/10.1016/j.vetpar.2018.08.010

ARTICLE 6 (Accepted for publication)

One-year follow-up of anti-Leishmania antibody concentrations in serum and saliva from experimentally-infected dogs

Journal: International Journal for Parasitology

Abstract: The quantification of anti-*Leishmania* antibodies in serum and saliva by a time-resolved immunofluorometric assay is useful for the diagnosis and treatment monitoring of dogs with clinical leishmaniasis. We compared the kinetics of anti-*Leishmania* IgG2 and IgA antibodies in serum and saliva from 11 Beagle dogs experimentally-infected with *Leishmania infantum*. Most dogs showed detectable concentrations of anti-*Leishmania* IgG2 earlier in serum (between 3 and 4 months p.i.) than in saliva (between 4 and 6 months p.i.). Overall, a high correlation between concentrations of anti-*Leishmania* IgG2 in serum and saliva ($r = 0.853$; $P < 0.0001$) was observed. The quantification of anti-*Leishmania* IgA showed less diagnostic value than IgG2, since detectable amounts of IgA were not observed in the saliva of four dogs and in the serum of one dog. In addition, a very low correlation between anti-*Leishmania* IgA in serum and saliva ($r = 0.289$; $P < 0.001$) was observed. Our results indicate that the antibodies against *L. infantum* in saliva appear approximately 1 month later than in serum, and suggest that there is a threshold for the passing of immunoglobulins from serum to saliva in dogs. These facts should be taken into consideration for a proper interpretation of saliva assays for quantification of antibodies.

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Date: 26 Jun 2019
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Subject: IJPara19_060R1

Ms. Ref. No.: IJPara19_060R1

Title: One-year follow-up of anti-Leishmania antibody concentrations in serum and saliva from experimentally-infected dogs

International Journal for Parasitology

Dear Dr. Escribano,

I am pleased to confirm that your paper "One-year follow-up of anti-Leishmania antibody concentrations in serum and saliva from experimentally-infected dogs" has been accepted for publication in the International Journal for Parasitology.

For every issue of the IJP the Editor will endeavour to feature an image on the cover from, or relating to, an article in that issue. We invite all authors to submit images that would be suitable.

To maximize their aesthetic qualities, these images may be stylized/modified versions of pictures or diagrams from the author's article.

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Thank you for submitting your work to this journal.

Yours sincerely,

Jan Slapeta
Deputy Editor
International Journal for Parasitology

Reviewers' comments:

Reviewer #1: The manuscript titled "One-year follow-up of anti-Leishmania antibody concentrations in serum and saliva from experimentally-infected dogs" by Cantos-Barreda and collaborators was revised according to the suggestions and comments of the editor and reviewers.

I believe that the majority of the suggestions were incorporated into the manuscript, and the questions were answered properly.

In addition, as already mentioned, the results obtained in the study add new values to the area of knowledge.
So, in my point of view, the revised manuscript is eligible for publication in the International Journal for Parasitology.

Reviewer #2: None
****

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CONCLUSIONS

CONCLUSIONS

1. The immunofluorometric assays developed in this PhD thesis allow the quantification of anti-*Leishmania* IgG2 and IgA in canine serum and saliva samples in a reliable way. An overlap between seropositive and seronegative dogs was observed for IgA.

2. The assays developed detect decreases in anti-*Leishmania* IgG2 and IgA levels in serum and saliva from dogs with clinical leishmaniosis after treatment, which is associated with clinical improvement.

3. The assay developed for anti-*Leishmania* IgG2 is more correlated with CRP and ferritin concentrations than the ELISA results in treated dogs.

4. Levels of anti-*Leishmania* IgG2 and IgA in canine serum and saliva increase after experimental infection, surpassing the cut-off approximately one month earlier in serum than in saliva. Anti-*Leishmania* IgG2 shows better diagnostic value than IgA.

5. No circadian rhythm is observed for anti-*Leishmania* IgG2 and IgA levels in both serum and saliva samples of dogs with CanL during a 16 h-period. Additionally, higher intra-individual variations in the specific antibody levels are observed in saliva than in serum.

6. The qPCR assay developed allows the quantification of *Leishmania* kDNA in saliva samples of experimentally-infected dogs with *L. infantum*. However, the sensitivity of this assay in the saliva is lower than in bone marrow.

RESUMEN

RESUMEN

La leishmaniosis canina (LCan) es una enfermedad zoonótica producida por el parásito protozoo *Leishmania* spp., el cual se transmite entre hospedadores, principalmente, por la picadura de flebótomos infectados (Kaszak *et al*., 2015).

La distribución geográfica de la LCan depende de la presencia de vectores y de reservorios (Kato *et al*., 2005), siendo una enfermedad endémica en la Europa mediterránea, norte de África, Asia, Centroamérica y Sudamérica (Baneth *et al*., 2008). Así mismo, la seroprevalencia de LCan en el sur de Europa se estima en un 5-30% (Solano-Gallego *et al*., 2001), siendo *Leishmania infantum* el principal agente causal y *Phlebotomus perniciosus* el principal vector (Vlkova *et al*., 2011).

El curso de la LCan es impredecible debido a la compleja patogénesis y a las manifestaciones clínicas, que pueden ser muy diferentes e inespecíficas (Kaszak *et al*., 2015). Las manifestaciones clínicas están relacionadas con una respuesta immune mediada por los linfocitos "*T helper*" 2 (Th2), lo que conlleva un incremento en la producción de anticuerpos frente a *Leishmania* spp. Como resultado, se produce el depósito de inmuno-complejos en órganos y tejidos, siendo este hecho responsable de la mayoría de los signos clínicos (Solano-Gallego *et al*., 2001; Proverbio *et al*., 2014). Las lesiones cutáneas los síntomas más comunes, aunque también pueden aparecer linfoadenopatía generalizada, pérdida de peso, blefaritis, conjuntivitis, epistaxis o poliartritis, entre otros (Solano-Gallego *et al*., 2011).

El diagnóstico de LCan puede llevarse a cabo mediante identificación directa del parásito o por identificación indirecta mediante la detección de anticuerpos específicos o de su ácido desoxirribonucleico (ADN). Los tests inmunocromatográficos (Otranto *et al*., 2004), los tests de inmunofluorescencia indirecta (IFI) (Mettler *et al*., 2005) y los ensayos por immunoabsorción ligados a enzimas (ELISA) (Santarém *et al*., 2010) son comúnmente utilizados para el diagnóstico serológico de LCan. No obstante, estos ensayos serológicos están limitados por la baja sensibilidad de los tests rápidos, la interpretación subjetiva del IFI o el estrecho margen de densidad óptica entre resultados positivos y negativos del ELISA (Swets *et al*., 1988; Solano-Gallego *et al*., 2014; Wolf

et al., 2014). Frente a este escenario, se detectó la necesidad de desarrollar técnicas ultrasensibles para la cuantificación de anticuerpos específicos frente a *Leishmania* spp. En cuanto al diagnóstico por métodos moleculares, el uso de la reacción en cadena de la polimerasa (PCR) cuantitativa aporta resultados reproducibles y con buena sensibilidad y precisión dependiendo de la secuencia diana seleccionada. El ADN del cinetoplasto de *Leishmania* spp. es la principal secuencia diana para el diagnóstico de infección por *Leishmania* spp. debido a su elevada sensibilidad como resultados del gran número de copias por parásito que produce (~10,000) (Rodgers *et al*., 1990). La detección de ADN de *Leishmania* spp. en saliva por PCR ha sido reportada en medicina humana (Siriyasatien *et al*., 2016; Pandey *et al*., 2018). No obstante, la detección de ADN de *L. infantum* en saliva de perro todavía no ha sido publicada. También se ha publicado que los ensayos de amplificación isotérmica mediada por bucle (LAMP) son útiles para la detección de perros infectados con *Leishmania* spp., usando tanto muestras de sangre (Chaouch *et al*., 2013) como hisopos conjuntivales (Gao *et al*., 2015). Esta técnica podría considerarse como una alternativa para el diagnóstico de LCan.

En cuanto a la saliva, es una muestra que puede ser obtenida mediante procedimientos no invasivos, siendo, por lo tanto, un proceso indoloro y sin estrés para el animal. Además, la recogida de saliva es económica y simple. Además, la obtención de saliva puede hacerse de forma repetida y separada por cortos periodos de tiempo que, además, podría realizar cualquier persona tras un pequeño entrenamiento (Escribano *et al*., 2012; Yoshizawa *et al*., 2013).

Con todo ello, la ausencia de ensayos suficientemente sensibles para la cuantificación de anticuerpos específicos frente a *Leishmania* spp. y el interés por el desarrollo de métodos serológicos y moleculares que permitan el uso de muestras no invasivas han motivado la realización de la presente tesis doctoral.

Así pues, los **objetivos** de esta tesis doctoral son los siguientes:

1. Desarrollar y validar un ensayo ultrasensible para la cuantificación de anticuerpos anti-*Leishmania* que permitiera su medición tanto en suero como en saliva de perro (**Artículo 1** y **Artículo 2**).
2. Evaluar la utilidad del ensayo para la medición de anticuerpos anti-*Leishmania* en suero y saliva como herramienta para la monitorización del tratamiento de la LCan (**Artículo 3** y **Artículo 4**).

3. Evaluar la posible relación entre los anticuerpos anti-*Leishmania* en suero y saliva medidos por el nuevo ensayo desarrollado y por un ELISA comercial y la concentración de proteínas de fase aguda (**Artículo 5**).
4. Evaluar y comparar la cinética de las inmunoglobulinas G subtipo 2 (IgG2) y A (IgA) anti-*Leishmania* medidas por el nuevo ensayo en suero y saliva de perros infectados experimentalmente durante un año de seguimiento (**Artículo 6**).
5. Evaluar un posible ritmo circadiano en los niveles de anticuerpos anti-*Leishmania* tanto en suero como en saliva de perros infectados experimentalmente con *L. infantum* (**Apéndice 1**).
6. Desarrollar y validar una PCR cuantitativa para la cuantificación de ADN de *Leishmania* spp. en saliva canina (**Apéndice 2**).

Los estudios experimentales incluidos en esta tesis doctoral recibieron **aprobación** por parte del **Comité de Ética** de la Universidad de Murcia bajo los números de protocolo 36/2014 y 276/2016. Todos los procedimientos fueron llevados a cabo de acuerdo con la vigente legislación española (RD 53/2013) y europea (Directiva 2010/63/EU) relativas a la protección de los animales usados para fines científicos.

Para la realización de los experimentos se usaron **muestras** de suero y saliva. El suero se obtuvo a partir de sangre entera recogida por venoclisis de la vena yugular o cefálica en tubos que contienen activador de la coagulación. Estos tubos se dejaron coagular a temperatura ambiente y se centrifugaron a 3,500 rpm 5 min para obtener el suero. Por otro lado, para la obtención de saliva se usaron dispositivos colectores de la marca Salivette® (Sarstedt, Nümbrecht, Alemania). El algodón incluido en estos dispositivos por defecto es sustituido por una esponja con el objetivo de maximizar el volumen de saliva obtenido. Cada esponja se sujeta con la ayuda de unas pinzas y se introduce en la boca del perro, frotándola sobre la mucosa de todas las partes de la cavidad bucal durante aproximadamente 1 min. A continuación, la esponja se inserta en el dispositivo colector y éste se centrifuga a 3,700 rpm durante 10 minutos. Las muestras de suero y saliva se almacenaron a -80ºC hasta su análisis.

Para la **determinación de los niveles de anticuerpos IgG2 e IgA anti-*Leishmania*** en suero y saliva se desarrollaron y validaron ensayos TR-IFMA. Para ello, se usó el antígeno recombinante K39 (RAG0061BIOT; Rekom Biotech S.L., Granada, España) como reactivo de captura y los anticuerpos policlonales anti IgG2 de perro producido en oveja (AHP948; Bio-Rad, Hércules, California, EE.UU.) y anti-IgA de perro producido

en cabra (A40-121A; Bethyl, Montgomery, Texas, EE.UU.) unidos al quelato de europio (Eu) (DELFIA Eu-Labeling kit, PerkinElmer Life and Analytical Sciences, Turku, Finlandia) como reactivos de detección.

Tras la optimización de la técnica, el procedimiento quedó de la siguiente forma: se añaden 200 µL del antígeno recombinante K39 en los pocillos de la placa, los cuales están recubiertos con estreptavidina, y se incuba durante 1 hora a temperatura ambiente y en agitación continua. Se lava cada pocillo 4 veces con 300 µL de solución de lavado (se usan estas mismas condiciones de lavado para cada vez) y se añaden 200 µL de los estándares o las muestras diluidas en tampón de ensayo. Se incuba 1 hora a temperatura ambiente en agitación continua, se lava, y se añaden 200 µL del anticuerpo de captura diluido en tampón de ensayo. Se vuelve a incubar 1 hora a temperatura ambiente en agitación continua, se lava y se añaden 200 µL de solución intensificadora. Se deja incubar 5 minutos a temperatura ambiente y agitación continua. Finalmente, se mide la fluorescencia emitida, la cual es proporcional a la cantidad de anticuerpo anti-*Leishmania* detectado en concreto por cada ensayo. Los resultados se cuantifican en cuentas por segundo (cps) y fueron expresados en Unidades de Fluorometría para *Leishmania* (UFL), equivalente 1 UFL a 1×10^3 cps. Los tampones de ensayo y lavado, así como la solución intensificadora son productos DELFIA® (PerkinElmer Life and Analytical Sciences, Turku, Finlandia).

Los ensayos TR-IFMA se validaron analítica y clínicamente. A continuación, se resume la **validación analítica**. La validación clínica se comentará en el resumen de los artículos.

La validación analítica se llevó a cabo mediante la evaluación de la precisión, la exactitud y la sensibilidad. Para la evaluación de la precisión se estudiaron las precisiones intra e inter ensayo, expresadas como coeficiente de variación (CV) y calculadas mediante el análisis de un pool de sueros con niveles altos de anticuerpos frente *Leishmania* spp. y un pool con niveles bajos 5 veces en un mismo ensayo o en 5 ensayos distintos. Por su parte, para evaluar la exactitud del ensayo, se calcularon la linealidad bajo dilución, la recuperación y la correlación del ensayo. Para la linealidad bajo dilución se usaron diluciones seriadas de dos muestras de suero con altos niveles de anticuerpos, se midieron los anticuerpos anti-*Leishmania* por TR-IFMA, se representaron los resultados medidos frente a los esperados y se calculó el coeficiente de determinación (R^2). Para el experimento de recuperación se diluyó una muestra de suero con altos niveles de

anticuerpos con otra muestra de suero con bajos niveles de anticuerpos a una proporción de 50, 25 y 10%. Se compararon los resultados obtenidos con los esperados. Para evaluar la correlación, los niveles de anticuerpos obtenidos por TR-IFMA se correlacionaron con los obtenidos por un ELISA comercial (Leiscan®) usando la correlación de Spearman. Finalmente, para evaluar la sensibilidad del ensayo se calculó el límite de detección analítico y el límite inferior de cuantificación. El primero se expresó como la media de 10 replicados del tampón de ensayo + 3 SD. El segundo se expresó como la menor cantidad de anticuerpo que puede ser medida en la parte linear de la curva de calibración con un CV < 20% y por encima del límite de detección, para lo cual se usaron diluciones de un suero con altos niveles de anticuerpos medidas 5 veces en el mismo ensayo.

La presente tesis por compendio de publicaciones está compuesta por los siguientes **artículos científicos** publicados o aceptados para publicación en revistas científicas de prestigio internacional:

Artículo 1: Se usaron 94 muestras de suero, de las cuales 25 procedían de perros sanos *Leishmania*-negativos del animalario de la Universidad de Murcia y las otras 69 procedían de perros enfermos *Leishmania*-positivos atendidos en diferentes clínicas del sureste español. Todas las muestras se analizaron por ELISA (Leiscan®). Además, 16 muestras de suero procedente de perros *Leishmania*-negativos pero afectados por otras enfermedades fueron usados para evaluar posibles reacciones cruzadas.

La validación analítica mostró que los ensayos tuvieron buena precisión con CVs intra- e inter-ensayos inferiores al 10%, por debajo del límite máximo aceptado para ensayos inmunológicos (20%) (Guidance for Industry, 2001). Además, los ensayos permitieron la cuantificación de concentraciones muy bajas de anticuerpos, al mismo tiempo que demostraron un alto nivel de precisión, determinado por linealidad bajo dilución ($R^2 = 0.99$) y con un porcentaje de recuperación > 85 %, situado dentro del rango establecido para inmunoensayos (80-120%) (Andreasson *et al*., 2015). Además, no se apreciaron reacciones cruzadas en muestras positivas a otros patógenos o enfermedades comúnmente presentes en la zona. Los ensayos fueron capaces de detector valores más altos de anticuerpos IgG2 e IgA anti-*Leishmania* en el grupo de perros seropositivos con respecto al grupo de perros seronegativos ($p < 0.0001$). No obstante, se observó solapamiento en los valores de anti-*Leishmania* IgA entre los grupos seropositivo y seronegativo. Este hecho podría dar lugar a falsos negativos, como ha sido reportado en medicina humana (Da Silvia *et al*., 2015). Además, se detectaron niveles de anticuerpos

más altos en los grupos LeishVet II ($p < 0.05$) y III ($p < 0.01$) con respecto al I. Cabe destacar que el ensayo TR-IFMA para cuantificación de IgG2 anti-*Leishmania* mostró mayores diferencias en los niveles de anticuerpos entre grupos seropositivo y seronegativo que el ELISA testado (Leiscan®), así como entre los diferentes estadios de la clasificación LeishVet que el Leiscan® y un ELISA previamente publicado (Solano-Gallego *et al*., 2001).

Artículo 2: Para alcanzar el objetivo del estudio se usaron muestras pareadas de suero y saliva procedentes de 48 perros. Los sueros se analizaron por ELISA (Leiscan®) y las muestras se clasificaron en *Leishmania*-seronegativas (n = 27) y *Leishmania*-seropositivas (n = 21). Los perros seropositivos mostraron síntomas compatibles con LCan en el momento del diagnóstico.

La validación analítica mostró que los ensayos desarrollados fueron precisos, con CVs intra- e inter-ensayo < 11%, por debajo del límite máximo aceptado para ensayos inmunológicos (20%) (Guidance for Industry, 2001). Además, los ensayos mostraron un alto nivel de exactitud, determinado por un alto coeficiente de determinación en la linealidad bajo dilución ($R^2 = 0.99$) y por un alto porcentaje de recuperación (> 88.60%), situado dentro del rango establecido para inmunoensayos (80-120%) (Andreasson *et al*., 2015). Además, los niveles de IgG2 anti-*Leishmania* en saliva fueron significativamente mayores en el grupo de perros seropositivos que en el de seronegativos ($p < 0.0001$), no apreciándose solapamiento entre valores de ambos grupos como ocurre en algunos tests rápidos en humana (Vaish *et al*., 2012; Ribeiro-Dias *et al*., 2015). Por el contrario, se observó solapamiento, como se ha descrito anteriormente en humana (Vaish *et al*., 2012; Ribeiro-Dias *et al*., 2015), y ausencia de diferencias significativas en los niveles de IgA anti-*Leishmania* entre estos dos grupos, por lo que se concluyó que la IgA anti-*Leishmania* en saliva no tenía valor diagnóstico para LCan. Además, los niveles de IgG2 anti-*Leishmania* en saliva medidos por TR-IFMA mostraron mayores diferencias entre perros seropositivos y seronegativos que los obtenidos por Leiscan® en suero.

Artículo 3: Se usaron sueros procedentes de 16 perros *Leishmania*-positivos y con síntomas compatibles con LCan atendidos en clínicas veterinarias. Los perros fueron tratados con 50 mg/kg de antimoniato de meglumine dos veces al día por vía subcutánea durante un mes y con 10 mg/kg de alopurinol dos veces al día por vía oral durante 6 meses. Los perros fueron monitorizados clínica y analíticamente (serología, ratio proteína-creatinina, proteínas totales, globulinas y albumina) en el momento del

diagnóstico (día 0) y al mes (día 30) y a los 6 meses (día 180) de tratamiento. Además, estos cambios serológicos fueron comparados con los síntomas y la concentración de varios analitos seleccionados, incluyendo proteínas totales, albúmina, globulinas y ratio proteína-creatinina en orina).

Los niveles de IgG2 e IgA anti-*Leishmania* fueron significativamente menores a los 30 ($p < 0.05$) y 180 ($p < 0.0001$) días de tratamiento con respecto a los valores previos a empezar con la terapia, relacionado con la mejora de la sintomatología (día 30: $p < 0.01$; día 180: $p < 0.0001$). Por lo tanto, los resultados del TR-IFMA sugieren que la medición de las variaciones en los niveles de anticuerpos anti-*Leishmania* son útiles para monitorizar el tratamiento, en línea con estudios publicados anteriormente por Solano-Gallego *et al*. (2011, 2016). A su vez, las magnitudes de descenso en los niveles de IgG2 e IgA anti-*Leishmania* en los días 30 y 180 de tratamiento fueron mayores que aquellos valores obtenidos por Leiscan® (1.29 y 1.74-veces), y que las concentraciones de algunos analitos (proteínas totales: 1.11 y 1.25-veces; globulinas 1.22 y 1.74-veces; y albúmina: 0.93 y 0.8-veces).

<u>Artículo 4</u>: Se usaron muestras pareadas de suero y saliva procedentes de 20 perros *Leishmania*-positivos con síntomas y anormalidades laboratoriales compatibles con LCan. Los perros fueron tratados con 50 mg/kg de antimoniato de meglumine dos veces al día por vía subcutánea y con 10 mg/kg de alopurinol dos veces al día por vía oral durante 1 mes. La monitorización del estado de los animales se llevó a cabo de forma clínica y analítica (cuantificación de IgG2 e IgA anti-*Leishmania* por TR-IFMA y hemograma y bioquímica completas) en el momento del diagnóstico (día 0) y al mes (día 30) de tratamiento. Los resultados obtenidos por TR-IFMA en saliva se compararon con los obtenidos en suero. Además, se estudió la correlación entre los niveles de anticuerpos, los síntomas y la concentración de los analitos laboratoriales seleccionados.

La mayoría de los perros ($n = 17$) mostró una reducción en los niveles de IgG2 ($p < 0.0001$) e IgA ($p < 0.01$) anti-*Leishmania* acompañada por una mejoría clínica ($p < 0.0001$) a los 30 días de tratamiento. No obstante, dos de estos perros mostraron un incremento en los niveles de anticuerpos anti-*Leishmania* en saliva. En uno de estos casos el incremento fue menor y los niveles eran menores que en otros perros enfermos, de acuerdo con sus valores en suero. Este hecho puede sugerir que bajas concentraciones de anticuerpo en suero pueden influir en los niveles de estos anticuerpos en saliva (Brandtzaeg, 2013). Hubo 3 perros que no mejoraron clínicamente tras un mes de

tratamiento. La existencia de perros que no responden al tratamiento ha sido reportada anteriormente (Solano-Gallego *et al*., 2016). Dos de estos perros mostraron descensos en los niveles de anticuerpos anti-*Leishmania* tanto en suero como en saliva y la mejoría clínica se consiguió tras un mes adicional de tratamiento con alopurinol. El otro perro que no respondió al tratamiento mostró aumentos en los niveles de anticuerpos anti-*Leishmania* tanto en suero como en saliva y no mejoró clínicamente tras un mes adicional de tratamiento con alopurinol. En cuanto a los parámetros evaluados en el hemograma y la bioquímica, todos ellos mejoraron a los 30 días de tratamiento, de acuerdo con previos estudios (Rubio *et al*., 2016; Segarra *et al*., 2017; Solano-Gallego *et al*., 2016).

Artículo 5: Con el fin de alcanzar el objetivo propuesto para este estudio, se evaluaron (1) las posibles diferencias en la concentración de proteínas de fase aguda y analitos relacionados con la concentración proteica entre perros *Leishmania*-seropositivos y seronegativos, (2) la concentración de los analitos bioquímicos seleccionados en los diferentes cuartiles de anticuerpos y, (3) las correlaciones entre los niveles de anticuerpos y los analitos estudiados tanto en el grupo de muestras para diagnóstico inicial de LCan como en el grupo de muestras para monitorización del tratamiento. Para ello, se analizaron un total de 205 muestras de suero remitidas al laboratorio Interlab-UMU entre enero de 2016 y enero de 2018. Los casos debían incluir la cuantificación de anticuerpos anti-*Leishmania* por TR-IFMA y la determinación de proteínas de fase aguda.

No se observaron diferencias significativas en la concentración de los analitos bioquímicos entre perros *Leishmania*-seronegativos y *Leishmania*-seropositivos. No obstante, los perros con niveles de anticuerpos altos mostraron anormalidades clinicopatológicas más marcadas, lo cual está asociado con enfermedad activa y, por lo tanto, anormalidades en la concentración de proteínas de fase aguda, como se ha reportado previamente (Martínez-Subiela *et al*., 2016; Cantos-Barreda *et al*., 2018a). Ambos ensayos serológicos tuvieron correlaciones de diferente significancia con los analitos bioquímicos, mostrando correlaciones de mayor significancia con proteínas totales y globulinas que con el resto de analitos. Cuando las muestras se remitieron para diagnóstico inicial de LCan y monitorización del tratamiento en perros ya diagnosticados de LCan, los ensayos serológicos mostraron menor correlación en muestras para monitorización del tratamiento ($r = 0.531$, $p < 0.0001$) que en muestras para diagnóstico inicial ($r = 0.769$, $p < 0.0001$). Además, mayores correlaciones se observaron entre TR-IFMA y analitos como la proteína C reactiva (CRP) y ferritina en muestras remitidas para

monitorización del tratamiento que con el Leiscan®, remarcando la utilidad del TR-IFMA para la monitorización del tratamiento frente a LCan (Cantos-Barreda *et al*., 2018b).

Artículo 6: Para alcanzar el objetivo fijado para este estudio, 11 perros sanos se infectaron experimentalmente con *L. infantum*. Se tomaron muestras antes de la infección y mensualmente durante 1 año y se midieron los niveles de anticuerpos IgG2 e IgA anti-*Leishmania* en cada toma.

La mayoría de los perros mostraron niveles detectables de IgG2 anti-*Leishmania* en antes en suero (a los 3-4 meses post-infección) que en saliva (a los 4-6 meses post-infección). Estos niveles detectables de IgG2 anti-*Leishmania* aparecen a unos tiempos similares a los reportados previamente (Hernández *et al*., 2015; Rodríguez-Cortés *et al*, 2007). Además, se observó una buena correlación entre los niveles de IgG2 anti-*Leishmania* en suero y saliva ($r = 0.853$; $p < 0.0001$). Por otro lado, la cuantificación de IgA anti-*Leishmania* mostró menor valor diagnóstico que la cuantificación de IgG2, como se ha reportado anteriormente (Cantos-Barreda *et al*., 2017), ya que no se observaron valores detectables de IgA anti-*Leishmania* en saliva de 4 perros y en suero de 1 perro. Además, se observó una correlación muy baja ($r = 0.289$; $p < 0.001$) entre los niveles de IgA anti-*Leishmania* en suero y saliva. Además, se observó mayor variabilidad en los niveles de anticuerpos en saliva que en suero, de acuerdo con lo publicado previamente por German *et al*. (1998). Nuestros resultados indicaron que los anticuerpos anti-*Leishmania* aparecen aproximadamente un mes después en saliva con respecto al suero, y sugieren la existencia del paso de anticuerpos desde el suero a la saliva (Brandtzaeg, 2013).

A su vez, también se considera oportuno incluir en la presente tesis doctoral **datos derivados de experimentos llevados a cabo durante la misma** y que se pretenden enviar a revistas internacionales **para su posible publicación**. Estos datos se han recogido en los Apéndices 1 y 2:

Apéndice 1: Para lograr el objetivo propuesto, 6 perros sanos se infectaron experimentalmente con *L. infantum.* Se obtuvieron muestras de suero y saliva pareadas a las 67 semanas post-infección a intervalos de 4 horas durante 16 horas y en dos días consecutivos. Además del análisis serológico por TR-IFMA, se realizaron hemogramas y bioquímicas completas a cada perro antes de empezar el estudio. Los niveles de

anticuerpos se expresaron de tres formas distintas: sin ninguna corrección, corregido por la concentración proteica y corregido por el flujo salival.

No se observaron diferencias significativas entre tomas en los niveles de IgG2 anti-*Leishmania* en suero y saliva sin o con correcciones. En cuanto a los niveles de IgA anti-*Leishmania*, sólo se observaron diferencias significativas en saliva cuando los valores de anticuerpos se corregían por el flujo salival. Estos resultados sugieren que la forma de expresar los niveles de anticuerpos anti-*Leishmania* en saliva, principalmente en el caso de IgA anti-*Leishmania* corregido por el flujo salival, pueden influir en la interpretación de los resultados. Además, se han observado mayores variaciones intraindividuales en los niveles de anticuerpos en saliva que en suero, en consonancia con el artículo publicado por German *et al*. (1998), que reportó gran variabilidad intraindividual en los niveles de anticuerpos secretados. No obstante, esta variación no siguió un patrón de ritmo circadiano y no modificó el estatus serológico de los perros. La ausencia de un patrón de ritmo circadiano en los niveles de anticuerpos anti-*Leishmania* está en línea con los resultados del estudio de German *et al*. (1998), en el que no se observa un patron circadiano en el nivel de anticuerpos totales en saliva y lágrima de perro.

Apéndice 2: Con el fin de alcanzar el objetivo previsto, 16 perros sanos se infectaron experimentalmente con *L. infantum.* Muestras de saliva y de médula ósea fueron obtenidas antes de la infección experimental y a las 16 semanas post-infección. El ADN de estas muestras se extrajo mediante precipitación con fenol/cloroformo y la calidad y cantidad de ADN se evaluó con NanoDrop® 2000. Finalmente, el ADN extraído se analizó para el cinetoplasto de *Leishmania* spp. mediante la PCR a tiempo real desarrollada y validada.

La PCR a tiempo real desarrollada fue sensible, con un límite de detección de 0.25 fg/reacción (0.0025 parásitos) para ADN de *L. infantum* derivado de cultivo celular, similar a aquéllos obtenidos previamente para la detección de ADN del cinetoplasto de *Leishmania* spp. (Mary *et al*., 2004; Francino *et al*., 2006; Ortega *et al*., 2017). La PCR a tiempo real desarrollada en este estudio ha tenido una eficiencia de PCR entre 87.6%-93.8%, R^2 entre 0.99 y 0.996, y una repetibilidad del 0.67. El ADN del cinetoplasto de *Leishmania* ha sido utilizado como secuencia diana extensamente para diagnosticar la infección por *Leishmania* debido a su alta sensibilidad (Mary *et al*., 2004). Este ADN fue detectado por nuestra PCR a tiempo real en 14 de las 16 (87.5%) médulas óseas y en 8 de las 16 (50%) muestras de saliva. No obstante, estos resultados están en línea con otros

resultados publicados en muestras no invasivas, en las que la sensibilidad de la PCR a tiempo real ha sido menor que en muestras obtenidas invasivamente (Aschar *et al*., 2016; Pandey *et al*., 2018). Además, 7 de los 14 perros *Leishmania*-positivos en médula ósea (50%) fueron también positivos en saliva, con una concordancia de 8 resultados entre saliva y médula ósea.

Con todo lo expuesto, de la presente tesis doctoral se pueden derivar las siguientes **conclusiones**:

1. Los ensayos inmunofluorométricos desarrollados en esta tesis doctoral permiten la cuantificación de anticuerpos IgG2 e IgA frente a *Leishmania* en suero y saliva de perro, siendo los niveles más elevados en perros seropositivos. Se observó solapamiento en los niveles de anti-*Leishmania* IgA entre seropositivos y seronegativos.
2. Los ensayos desarrollados detectan disminuciones en los niveles de anti-*Leishmania* IgG2 e IgA en suero y saliva de perros después del tratamiento asociado con mejoría clínica.
3. El ensayo desarrollado para detectar anti-*Leishmania* IgG2 mostró mayor correlación con los resultados de CRP y ferritina en perros tratados que el ELISA.
4. Los niveles de IgG2 e IgA anti-*Leishmania* en suero y saliva de perros aumentan por encima del punto de corte del ensayo TR-IFMA tras la infección experimental. Además, IgG2 anti-*Leishmania* ofrece mejor valor diagnóstico que el desarrollado para IgA anti-*Leishmania*.
5. No se ha observado ritmo circadiano en los niveles de IgG2 e IgA anti-*Leishmania* tanto en suero como en saliva de perros con LCan en un periodo de 16 horas en dos días consecutivos. Además, se han observado mayores variaciones en los niveles de estos anticuerpos en saliva que en suero.
6. La PCR a tiempo real desarrollada permite la cuantificación de ADN del cinetoplasto de *Leishmania* spp. en saliva de perros infectados experimentalmente con *L. infantum*. No obstante, hay que tener en cuenta que la sensibilidad del ensayo en saliva es menor que en médula ósea.

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APPENDIX

APPENDIX 1

Evaluation of the circadian rhythm of anti-Leishmania IgG2 and IgA antibodies in serum and saliva of dogs with clinical leishmaniosis

Planned to be submitted for possible publication.

Abstract: In this study, the circadian rhythm of IgG2 and IgA specific antibodies in serum and saliva samples of 6 dogs experimentally-infected with *Leishmania infantum* was assessed. Sampling was performed at 8.00, 12.00, 16.00, 20.00, and 00.00h on two consecutive days. Anti-*Leishmania* antibody levels in saliva were shown in different ways: without any correction, adjusted by protein concentration and corrected by the salivary flow rate. No significant differences in anti-*Leishmania* IgG2 antibody levels with or without correction were found, but significant differences were found corrected by the flow rate in anti-*Leishmania* IgA. In addition, a greater intra-individual variation of antibody levels was observed in saliva than in serum. However, this variation did not modify the serological status of the dogs. Therefore, it could be concluded that there is no circadian rhythm in serum and saliva samples and sampling can be performed at any time of the day.

APPENDIX 2

Detection of Leishmania infantum kinetoplast minicircle DNA by real-time PCR in saliva of experimentally-infected dogs

Planned to be submitted for possible publication.

Abstract: This study aimed to develop and validate a quantitative real-time PCR (qPCR) assay to amplify the kinetoplast DNA (kDNA) sequence of *Leishmania infantum* in saliva samples from dogs and to evaluate its diagnostic utility. For this, a qPCR assay was analytically validated by the evaluating of the PCR characteristics including slope, efficiency, and correlation coefficient, and by evaluating precision, accuracy and analytical sensitivity. This method was applied to saliva samples in 16 Beagle dogs experimentally-infected with *L. infantum* prior to infection and at 16 weeks post-infection. The qPCR showed to be sensitive, with a detection limit of 0.25 fg/reaction (0.0025 parasites) for *L. infantum* genomic DNA derived from cell culture. *Leishmania* kDNA was detected by qPCR in 14 out of 16 (87.5 %) bone marrow samples and in 8 out of 16 (50 %) saliva samples. Additionally, 7 out of 14 *Leishmania*-positive dogs on bone marrow (50%) were also positive on saliva, with an overall concordance of 8 qPCR results between saliva and bone marrow. Results reported herein demonstrate that the qPCR used in this study is adequate for detecting *Leishmania* kDNA in saliva samples. Our findings suggest that saliva could be an adequate sample to diagnose *L. infantum* infection in dogs by non-invasive procedures.

APPENDIX 3

Impact factors and categories of the journals

Journal	Impact factor	Quartile	Category
Veterinary Immunology and Immunopathology	1.632 (2017) 1.846 (2018)	Q1	Veterinary Sciences
Veterinary Parasitology	2.422 (2017) 2.009 (2018)	Q1	Veterinary Sciences
International Journal for Parasitology	3.478 (2018)	Q1	Parasitology