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

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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.

1. Introduction

Canine leishmaniasis (CanL), caused by *Leishmania infantum*, is a worldwide zoonosis transmitted by the bite of infected female phlebotomine sandflies (Maia and Campino, 2018). The presence of domestic dogs in endemic areas is a risk factor for the transmission of infection to humans, as dogs are considered the main reservoirs of *L. infantum* (Baneth et al., 2008).

Infected dogs can remain asymptomatic or present a wide spectrum of non-specific clinical signs derived from the infection of any organ. The most frequent manifestation is skins lesions, while chronic renal failure due to immune complexes deposition is the main cause of death (Solano-Gallego et al., 2011). In Mediterranean countries, the prevalence of *L. infantum* infection in the canine population (63–80%) is much higher than the proportion of seropositive dogs (10–30%) and, in addition, than the percentage of dogs with clinical disease (2–5%) (Berrahal et al., 1996; Solano-Gallego et al., 2001a,b; Maia and Campino, 2008).

Leishmania infantum induces a combination of Th1 (cellular) and Th2 (humoral) immune responses in the infected dog, and the progression of infection and disease is determined by the balance between each response. The Th1 activity increases dog's resistance to the disease. On the other hand, a predominant Th2 response leads to an increase in anti-*Leishmania* antibody production, parasite spread, and clinical illness. This humoral response is more predominant in symptomatic dogs than in asymptomatic dogs (Baneth et al., 2008). The total levels of anti-*Leishmania* IgG, IgG1 and IgG2 are reported to be elevated during the active phase of *Leishmania* infection. However, several studies have reported that the predominant immune response in infected dogs with clinical disease may be due to the IgG2 subclass (Solano-Gallego et al., 2001a,b; Cardoso et al., 2007; Rodríguez-Cortés et al., 2007a,b; Santarém et al., 2010; Chaabouni et al., 2018). IgA is considered the main class of immunoglobulin involved in mucosal immunology. This immunoglobulin blocks the interaction between the antigens and the epithelium overlying the mucosal surface (Macpherson et al., 2008). In leishmaniasis, the presence of parasite antigens stimulates the active export of IgA to the mucosal surface and, consequently, an increase in anti-*Leishmania* IgA levels can be detected in saliva (Brandtzaeg, 2009).

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In vertebrates, the prevalence and intensity of parasitic infections are higher in males than in females. Although the cause of sex differences in response to certain parasites is not well established, it may be due to differences in exposure and the endocrine-immune interactions (Morales-Montor et al., 2004). Experimental studies of *Leishmania* infection in mice reveal that males are more susceptible to infection than females, influenced by the immunosuppressive presence of testosterone or the effects of estrogens on the induction of a protective Th1 response. Several studies of *Plasmodium* spp. infection show that the castration of mice decreases the intensity of infection (Klein, 2004).

The ELISA and the immunofluorescence antibody test (IFAT) (Solano-Gallego et al., 2011) are the most frequently used techniques for the quantification of anti-*Leishmania* antibodies. A time-resolved immunofluorometric assay (TR-IFMA) is able to quantify biomolecules with high sensitivity, even in samples with a low concentration and high background, such as saliva (Escribano et al., 2013; Contreras-Aguilar et al., 2017). In recent studies, we reported that TR-IFMA is useful for the diagnosis and treatment monitoring of symptomatic *Leishmania*-infected dogs in serum (Cantos-Barreda et al., 2017a, 2018a) and potentially in saliva samples (Cantos-Barreda et al., 2017b, 2018b).

Due to the promising nature of TR-IFMA in the quantification of anti-*Leishmania* antibodies, we planned to evaluate the kinetics of anti-*Leishmania* IgG2 and IgA in both serum and saliva using TR-IFMA during the course of experimental infection, allowing us to follow the kinetics of infection from basal levels (Rodríguez-Cortés et al., 2010). To date, no long-term studies involving the quantification of anti-*Leishmania* antibodies in paired samples of serum and saliva have been reported. Therefore, the main objective was to evaluate and compare the kinetics of anti-*Leishmania* IgG2 and IgA measured by TR-IFMA in serum and saliva from dogs experimentally infected with *L. infantum* during a 1-year p.i. follow-up period.

2. Materials and methods

2.1. Animals

The present study was carried out with 11 experimental Beagle dogs purchased from a commercial dog breeder (Isoquimen S.L., Barcelona, Spain). These dogs participated in a *L. infantum* vaccine trial and were included in the control group, so they did not receive *Leishmania* vaccination. Throughout the study, the dogs were housed in open-air, partially-covered kennels in a research colony of the Animal Resources Center of the University of Murcia, located in the peri-urban area of Murcia, Spain. A microchip number was used for the identification of the dogs and a number between 1 and 11 was arbitrarily assigned to each one. All dogs showed a seronegative status for *Leishmania* spp. as no detectable concentrations of anti-*Leishmania* antibodies were observed by IFAT performed by the breeder. In addition, *Leishmania* DNA in bone marrow aspirates was not detected by real-time quantitative *Leishmania* kinetoplast-specific PCR (RT-qPCR) (Ledesma et al., 2017).

Dogs were vaccinated against distemper virus, canine adenovirus type 2, canine parvovirus, canine parainfluenza virus type 2, *Leptospira interrogans*, and rabies virus (Eurican® DAPPi-LR; Merial Laboratorios S.A., Barcelona, Spain), and against *Bordetella bronchiseptica* (Eurican® Pneumo; Merial Laboratorios S.A.). Because the study was conducted in an area in which CanL is endemic, and where dogs could be in contact with infected phlebotomine sandflies, an external deworming was performed every 3 weeks with an anti-sandfly activity insecticide containing 500 mg/mL of permethrin and 100 mg/mL of imidacloprid (Advantix® Spot-on; Bayer Hispania S.L., Barcelona, Spain). In addition, dogs were internally dewormed based on their body weight with tablets containing 50 mg of pyrantel, 50 mg of praziquantel, and 150 mg of febantel (Prazitel® Plus Worming tablets; Ecuphar S.A., Barcelona, Spain). Dogs were kept under surveillance, received veterinary attendance, and were fed ad libitum on a balanced chicken-based diet (LIBRA®; Affinity Petcare S.A., Barcelona, Spain) and water. Each dog was submitted, monthly, to a thorough physical examination during which clinical signs compatible with CanL were recorded.

2.2. Ethics statement

The study received approval of the Regional Government of Murcia (identification code number: A13151002). All procedures were carried out in accordance with the current Spanish (RD 53/2013) and European (Directive 2010/63/EU) legislation on animal protection.

2.3. Leishmania parasites and experimental infection

Leishmania infantum (MCAN/BR/00/BA262) parasites isolated from the spleen of an experimentally infected hamster were cultured in Schneider's *Drosophila* medium (Biowest, Nuaillé, France) supplemented with 10% heat-inactivated fetal bovine serum (iFBS) and 50 μg/mL of gentamicin at 26 °C. Promastigotes transformed from spleen-derived amastigotes were cultured in a modified RPMI-1640 medium supplemented with 20% iFBS, and 50 μg/mL of gentamicin at 26 °C. The dogs were inoculated i.v. with 1×10^6 stationary-phase promastigotes of *L. infantum*. After the infectious challenge, the success of the infection was confirmed by serological tests against *Leishmania* antigens and by RT-qPCR using DNA from bone marrow aspirates as the template, as previously described (Ledesma et al., 2017).

2.4. Sampling and experimental design

Serum and saliva samples were used for the quantification of anti-*Leishmania* antibodies. For this, pre-infection samples were obtained immediately before the experimental infection, and p.i. samples were collected monthly over the course of a year. A total of 13 samplings per dog was performed. The study was conducted between April 2015 and April 2016.

In order to obtain serum, blood samples (1 mL) were collected by jugular venipuncture in tubes without anticoagulant and centrifuged at 1166*g* for 5 min to separate the serum. Saliva samples were collected using Salivette® saliva collection devices (Sarstedt, Nümbrecht, Germany) using sponges instead of the original cotton swabs in order to maximise saliva recovery. The sponge was clipped with tweezers and placed in the dog's mouth for 1 min. Afterwards, the sponge was inserted into the Salivette® and centrifuged at 1303*g* for 10 min to obtain the saliva. All samples were preserved at −80 °C until the time of analysis.

2.5. Assessment of serological response

Concentrations of anti-*Leishmania* IgG2 and IgA antibodies in both serum and saliva samples were measured in accordance with previous reports (Cantos-Barreda et al., 2017a,b). These assays are based on the principle of detecting biomolecules using lanthanide chelates-labelled reagents. The biotinylated rK39 antigen was used as the capture reagent, and the Europium-labelled anti-dog IgG2 polyclonal antibody (Sheep anti-Dog IgG2, Bio-Rad, Hercules, CA, USA) and anti-dog IgA polyclonal antibody (Goat anti-Dog IgA, Bethyl, Montgomery, TX, USA) were employed as detector reagents. The results of the assay were expressed as units of fluorometry for *Leishmania* (UFL) (1 UFL = 10^3 counts per second (cps)) by means of the WorkOut program (WorkOut Plus MMD

software program for data analysis, Perkin-Elmer Life and Analytical Sciences, Wallac Oy, Turku, Finland). For the purpose of assessing the non-detection of anti-*Leishmania* IgG2 by the TR-IFMA assay while detecting anti-*Leishmania* IgA, anti-dog IgG2 polyclonal antibody (Sheep anti-Dog IgG2, Bio-Rad, Hercules, CA, USA) was measured as standard. Pools of five *Leishmania*-seropositive and five *Leishmania*-seronegative serum or saliva samples were used as controls. In addition, a pool of five serum or saliva samples from *Leishmania*-seropositive dogs with the previously established upper values for each assay, was used as a calibrator. The controls and the calibrator were included in all plates.

The cut-offs (mean + 4 S.D. of 12 *Leishmania*-negative dogs by qPCR after their acquisition from Isoquimen S.L. (Barcelona, Spain)) were set at 22 and 28 UFL for anti-*Leishmania* IgG2 and IgA antibodies in serum, respectively, and at 32 and 100 UFL for anti-*Leishmania* IgG2 and IgA antibodies in saliva, respectively. Seroconversion was considered as the period of time during which anti-*Leishmania* antibodies were developed and became detectable, overcoming the cut-off value.

2.6. Statistical analysis

All the dogs were included in the statistical analysis. Data did not follow a normal distribution, as assessed by a Kolmogorov-Smirnov normality test. The changes in anti-*Leishmania* IgG2 and IgA concentrations, either in serum or in saliva over time, were assessed using a linear mixed model in which repeated measures and symptoms were used as factors, and the sex and age of each animal were considered as covariables. To normalise the distribution of anti-*Leishmania* IgG2, the inverse transformation of data had previously been performed. Relationships between anti-*Leishmania* antibody concentrations in saliva and serum samples throughout the study period, as well as between these specific antibody concentrations at the time of first clinical signs, were evaluated using the non-parametric Spearman correlation. The statistical analyses were performed using the SPSS statistics package (SPSS UK, Inc., Woking, UK). Figures were represented using the GraphPad Prism 6 statistics package (GraphPad Software, La Jolla, CA, USA) involving crude data. Significance was set for $P < 0.05$, and the symbols used were $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

3. Results

3.1. Assessment of serological response

A total of 11 Beagle dogs were included in the study. Four of the dogs were spayed females (36.4%) and seven were castrated males (63.6%). All dogs were castrated at the same time, 1 month before the experimental infection. The ages of the dogs varied between 6 months and 6 years at the time of infection, with a mean ± S.D. of 3.3 ± 2.3 years. No influence of sex and age in the anti-*Leishmania* antibody levels was observed.

Anti-*Leishmania* IgG2 concentrations pre-infection and monthly for a 1-year p.i. follow-up period, from the saliva and serum of all the dogs, are expressed as UFL in Table 1. In addition, anti-*Leishmania* IgA concentrations from both saliva and serum are expressed as UFL in Table 2. The kinetics of anti-*Leishmania* antibodies throughout the study period is graphically represented in Fig. 1.

Significant differences ($P < 0.01$) in the concentration of anti-*Leishmania* IgG2 in the serum between pre-infection and the p.i. samplings were observed from 3 months p.i. (Fig. 1A). Median values were above the cut-off at 4 months p.i. All dogs seroconverted between 3 and 7 months p.i. Dogs 1 and 6 (18.2%) seroconverted at 3 months p.i.; six out of the total of 11 dogs (numbers 2, 3, 7, 8, 9, and 11; 54.5%) showed seroconversion at 4 months p.i.; dog 10 (9.1%) seroconverted at 5 months p.i.; dog 5 (9.1%) at 6 months

Table 1
Anti-*Leishmania* IgG2 concentrations in both serum and saliva at pre-infection (pre-i.) and monthly over a 1-year p.i. follow-up period.

Sample	Dog	Sex[a]	Age (years)[b]	Anti-*Leishmania* IgG2 (UFL)[c]											
				Pre-i.	Months p.i.										
					1	2	3	4	5	6	7	8	9	10	11	12
Serum	1	Female	6	7.4	7.5	8	32.4	269	437	418	363	268	252	292	181	116
	2	Male	2	9	9.4	9.1	12.4	416	482	3236	10,204	8716	14,175	7772	4922	5006
	3	Male	2	5.7	7	6.9	19.2	1250	13,968	15,305	20,585	19,562	18,938	16,929	13,983	
	4	Female	1.5	12.2	11.9	17	18.4	13.7	18.8	21.8	26	35	69.6	46.4	43	62.2
	5	Male	0.5	5.3	8.4	8.2	17.7	9.8	14.2	42.6	87.4	82	49.6	33.7	28	30.2
	6	Male	0.5	9.8	6.9	19.1	22.1	15.3	54.6	63.1	130	213	282	433	710	1553
	7	Male	0.5	11.7	16.6	9.7	9.9	209	13,015	37,330	34,802	34,886	37,705	‡	‡	‡
	8	Male	0.5	9.8	6.3	11.2	8.1	11,121	226	3979	17,445	16,960	‡	‡	‡	‡
	9	Female	0.5	5.4	8.2	11.2	4.5	689	1210	5746	15,971	18,913	19,950	18,823	18,543	19,214
	10	Male	0.5	8.2	14.8	7.3	10.5	9.6	41.5	629	1190	1571	2009	1950	2337	1635
	11	Female	0.5	9.6	14.4	6.7	11.6	397	2221	16,765	15,436	7217	5065	3413	3027	3085
Saliva	1	Female	6	6.7	9.2	8	21	21.8	11.9	18	11.6	12.8	20.8	25.1	46	14.4
	2	Male	2	4.9	8.3	5.4	9.3	19.4	34.9	48.4	242	223	228	135	241	219
	3	Male	2	6.4	7.6	4.9	7.8	75.5	288	197	387	371	635	790	1588	‡
	4	Female	1.5	10.3	8	3.3	8.9	14.8	14.1	11.8	13.7	52.3	59.9	18.8	–[d]	–[d]
	5	Male	0.5	22.1	18.4	9.4	7	30.2	21.4	32.6	35.9	30	64.6	44.8	17.1	16.4
	6	Male	0.5	5.9	27.8	18.9	20.5	8.3	7.6	10.5	20.1	33.4	30.1	47.1	30.2	752
	7	Male	0.5	5	4.8	12.9	8.3	16.2	296	3285	1362	7872	9448	‡	‡	‡
	8	Male	0.5	5	6	4.9	7.7	8.1	196	611	647	1852	‡	‡	‡	‡
	9	Female	0.5	18.3	14.9	7.8	14.5	13.3	57.2	150	218	184	439	464	323	224
	10	Male	0.5	9.1	5.9	4.6	4.9	7	8.6	35	53.5	107	461	96.5	94.7	53.4
	11	Female	0.5	4.9	5.5	4.9	4.9	27.7	314	917	165	278	316	612	161	66.7

[a] All the males and females were castrated.
[b] Age in years at the time of infection with *Leishmania infantum*.
[c] Units of fluorometry for *Leishmania*.
‡ Found dead or euthanised.
[d] Insufficient sample.

Table 2
Anti-*Leishmania* IgA concentrations in serum and saliva at pre-infection (pre-i.) and monthly over a 1-year p.i. follow-up period.

Sample	Dog	Sex[a]	Age (years)[b]	Anti-*Leishmania* IgA (UFL)[c]											
				Pre-i.	Months p.i.										
					1	2	3	4	5	6	7	8	9	10	11	12
Serum	1	Female	6	16.2	14.7	18.9	16.7	37.5	51.5	67.6	68.2	51.2	49.3	53	39.2	37.8
	2	Male	2	14.9	18.2	16.2	16.7	49.2	204	396	489	530	542	478	429	407
	3	Male	2	14.1	16.9	15.9	18	290	965	1193	1186	1205	1121	1050	992	‡
	4	Female	1.5	17.1	16.5	15.4	18.1	18	19.7	16.1	21.4	19.1	27.3	24.4	23.4	31
	5	Male	0.5	10.3	10.3	10.5	11.4	10.4	12.9	13.5	14.5	13.7	13	15.7	14.9	14.6
	6	Male	0.5	20.5	16.2	21.9	7.2	14.7	21.6	22.6	28.6	28.8	34.7	48.5	66	137
	7	Male	0.5	25.1	25.4	18.1	42.6	495.8	689	648	629	581	574	‡	‡	‡
	8	Male	0.5	17.6	15.3	15.7	16.3	15.4	29	287	472	449	‡	‡	‡	‡
	9	Female	0.5	9.7	12.8	13.6	10.9	32.8	276	445	464	481	498	519	461	498
	10	Male	0.5	8.9	10.1	10.2	9.2	10.6	14.7	78.9	132	195	174	181	177	158
	11	Female	0.5	7.5	15.4	7.4	9.8	52.5	323	418	393	324	305	277	249	243
Saliva	1	Female	6	27	32.6	33.9	63.4	30.8	19.2	23.8	34.3	31.3	14.2	32.7	84.7	42.6
	2	Male	2	15.2	28.9	27	44.5	39.8	86.4	38.6	136	85.5	62.9	33.3	80.6	46.7
	3	Male	2	26.2	27.7	25	28.8	29.6	39.9	46.1	74	46	61.6	84.1	85.7	‡
	4	Female	1.5	14.6	17.3	26.8	37.6	62.4	67.7	48.8	46.8	189	249	50.5	–[d]	–[d]
	5	Male	0.5	129	73.2	48.3	36.1	130.7	107	110	143	56.7	252	164.7	72.1	58.9
	6	Male	0.5	28.1	81.7	66.5	82.4	39.1	29.8	25.4	41.4	39.5	39	148	38.2	79.7
	7	Male	0.5	24.3	22.2	54.4	33.5	52.7	138	211	473	2348	2667	‡	‡	‡
	8	Male	0.5	25.4	30.9	21.9	40	27.9	40.7	75.4	104	182	‡	‡	‡	‡
	9	Female	0.5	78	43.2	57.5	57	32.3	88	57.6	96	91	88.3	113	71	62.3
	10	Male	0.5	35.7	38.9	22.1	20.6	22.4	31.6	21.4	30.5	30	39.8	61.6	26.7	27.1
	11	Female	0.5	14.4	15.7	14.3	21.8	24.9	24.3	67.5	27.5	35.8	50	49.4	36.2	18.8

[a] All the males and females were castrated.
[b] Age in years at the time of infection with *Leishmania infantum*.
[c] Units of fluorometry for *Leishmania*.
‡ Found dead or euthanised.
[d] Insufficient sample.

p.i.; and finally, dog 4 (9.1%) at 7 months p.i. (Table 1). The highest median concentration of anti-*Leishmania* IgG2 in serum (10,204 UFL; 25th-75th percentiles: 130–17,445 UFL) was observed at 7 months p.i. (Fig. 1A).

Regarding anti-*Leishmania* IgA concentrations in the serum, significant differences ($P < 0.001$) between pre-infection and p.i. were observed from 4 months p.i. (Fig. 1B). In addition, the median anti-*Leishmania* IgA value was above the cut-off at 4 months p.i. However, dog 7 (9.1%) showed seroconversion at 3 months p.i.; five out of the total 11 dogs (1, 2, 3, 9, and 11; 45.5%) seroconverted at 4 months p.i.; dog 8 (9.1%) at 5 months p.i.; dog 10 (9.1%) at 6 months p.i.; and dog 6 (9.1%) at 7 months p.i. Dog 5 (9.1%) did not show seroconversion, and dog 4 (9.1%) only had values of anti-*Leishmania* IgA above the cut-off at 12 months p.i., which were very close to the cut-off value (Table 2). The highest median concentration of anti-*Leishmania* IgA in serum (393 UFL; 25th-75th percentiles: 28.6–489 UFL) was observed at 7 months p.i., in accordance with anti-*Leishmania* IgG2 (Fig. 1B).

In the saliva samples, significant increases ($P < 0.0001$) in the concentration of anti-*Leishmania* IgG2 compared with pre-infection were observed at 4 months p.i. (Fig. 1C). However, the median concentration of anti-*Leishmania* IgG2 was above the cut-off at 5 months p.i. Dog 3 (9.1%) seroconverted at 4 months p.i.; five out of the total of 11 dogs (2, 7, 8, 9 and 11; 45.5%) showed detectable amounts of anti-*Leishmania* IgG2 at 5 months p.i.; dogs 5 and 10 (18.2%) seroconverted at 6 months p.i.; dogs 4 and 6 (18.2%) seroconverted at 8 months p.i.; and finally, dog 1 (9.1%) only showed punctual seroconversion at 11 months p.i. (Table 1). The highest median concentration of anti-*Leishmania* IgG2 (272 UFL; 25th-75th percentiles: 52.4–505 UFL) was observed at 9 months p.i. (Fig. 1C).

Once the dogs seroconverted, until the end of the study the concentrations of the anti-*Leishmania* IgG2 in serum were higher than the cut-off used by our laboratory to consider a dog positive for CanL. However, in saliva samples there were three cases (dogs 1, 4, and 5) in which the anti-*Leishmania* IgG2 concentrations were lower than the cut-off before the end of the study.

Regarding anti-*Leishmania* IgA concentrations in saliva, significant increases ($P < 0.05$) compared with pre-infection were observed at 3 months p.i. (Fig. 1D). However, median values of these specific antibodies did not surpass the cut-off throughout the study. Seven out of the total of 11 dogs (63.6%) developed detectable concentrations of anti-*Leishmania* IgA as follows: dog 2 only showed seroconversion at 7 months p.i., dog 4 at 8 and 9 months p.i., dog 5 between 4 and 10 months p.i. but not at 8 months p.i., dogs 6 and 9 only at 10 months p.i., dog 7 from 5 months p.i., and dog 8 from 7 months p.i. Concentrations of anti-*Leishmania* IgA did not surpass the cut-off in 4 cases (dogs 1, 3, 10, and 11; 36.4%) (Table 2). The highest median concentration of anti-*Leishmania* IgA in saliva (73.9 UFL; 25th-75th percentiles: 34.3–136 UFL) was observed at 7 months p.i. (Fig. 1D).

3.2. Clinical evaluation

No dog included in the study presented clinical signs compatible with CanL at the time of infection. Seven out of the 11 dogs (1, 2, 3, 7, 8, 9, and 11; 63.6%) showed the first clinical signs associated with CanL at 6 months p.i., while dog 6 (9.1%) presented the first clinical signs at 8 months p.i. The clinical signs that could be observed in dogs were lymphadenomegaly ($n = 7$), ulcerative dermatitis ($n = 5$), erosive dermatitis ($n = 6$), and ocular signs such as blepharitis and/or conjunctivitis ($n = 6$). However, three dogs (4, 5, and 10; 27.3%) did not progress to clinical disease and remained asymptomatic.

Dogs 3, 7, and 8 progressed to a severe clinical state of leishmaniasis and were classified as Stage III according to the LeishVet guidelines (Solano-Gallego et al., 2011). Consequently,

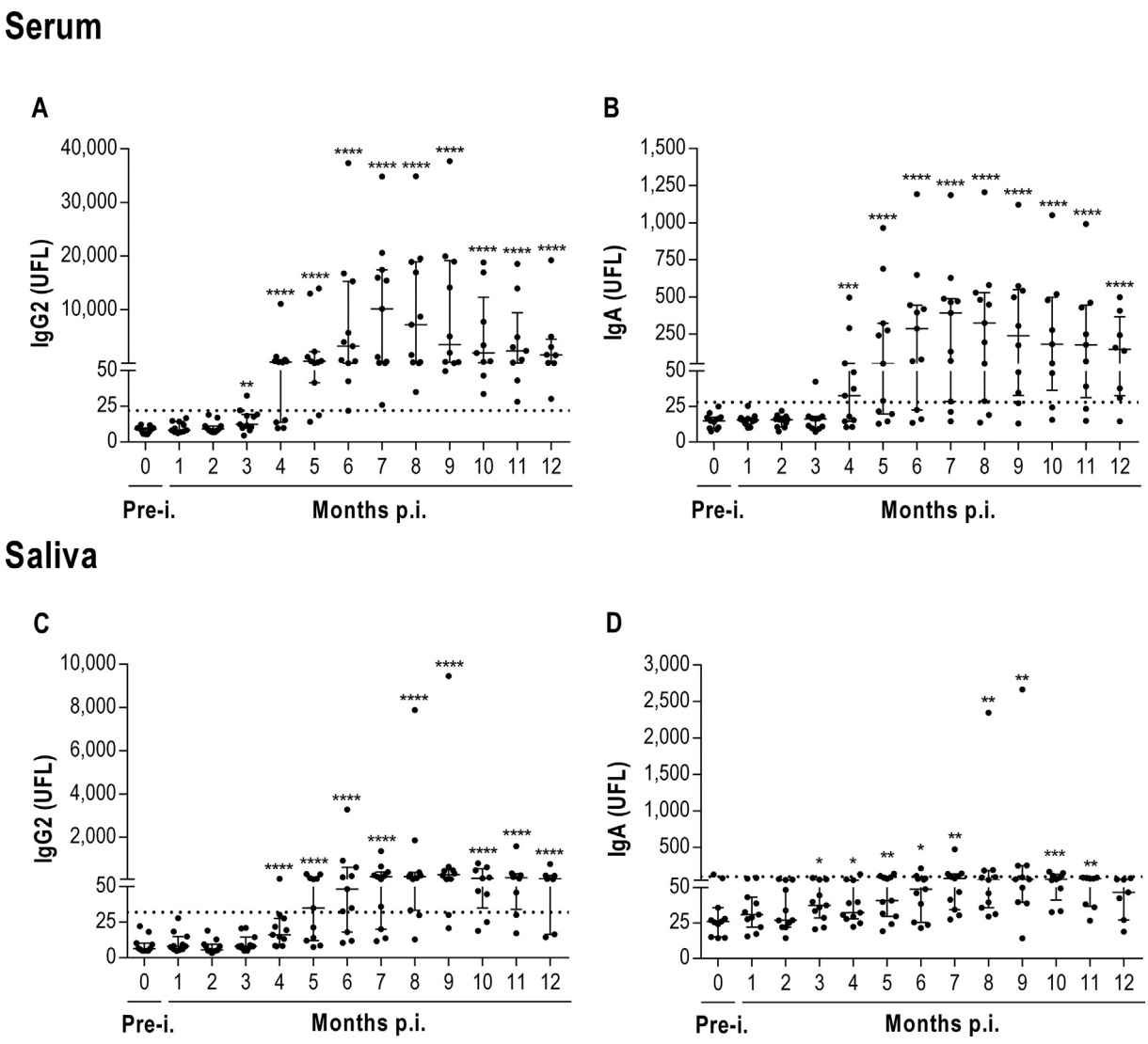

Fig 1. Anti-*Leishmania* IgG2 and IgA concentrations in dog serum (A, B) and saliva (C, D) detected by a time-resolved immunofluorometric assay (TR-IFMA) pre-infection (pre-i.) and over a 1-year p.i. follow-up period. The boxes (25th-75th percentiles) show the median concentrations of anti-*Leishmania* antibodies (line within the box) and the whisker plots represent the minimum (5th percentiles) and maximum values (95th percentiles). Statistically significant differences are indicated by asterisks (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$). UFL, units of fluorometry for *Leishmania*. A horizontal dashed line represents the cut-off for each antibody and sample type (22 and 28 UFL for anti-*Leishmania* IgG2 and IgA in serum, respectively, and 32 and 100 UFL for anti-*Leishmania* IgG2 and IgA in saliva, respectively).

and according to ethical procedure, these dogs were euthanised at 12, 10, and 9 months p.i., respectively.

3.3. Correlation study

A high and significant correlation was found between serum and saliva samples for anti-*Leishmania* IgG2 concentrations ($r = 0.853$; $P < 0.0001$) (Fig. 2A). However, a lower correlation was found between serum and saliva samples for anti-*Leishmania* IgA concentrations ($r = 0.289$; $P < 0.001$) (Fig. 2B). Significant correlations between concentrations of anti-*Leishmania* IgG2 and IgA in serum ($r = 0.889$; $P < 0.0001$) (Fig. 2C) and in saliva ($r = 0.640$; $P < 0.0001$) (Fig. 2D) were observed.

In addition, significant correlations were observed at the time of the first clinical signs between the anti-*Leishmania* IgG2 concentrations 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 ($r = 0.762$; $P < 0.05$).

4. Discussion

The kinetics of anti-*Leishmania* IgG2 and IgA in the serum and saliva of *L. infantum* experimentally infected dogs were evaluated during a year after experimental infection. The novelty of this study lies in the fact that this is, to our knowledge, the first time that the kinetics of anti-*Leishmania* antibodies has been evaluated in saliva derived from an experimental infection. This report provides data that can contribute to a better clinical interpretation when measuring specific antibodies against *Leishmania* spp. in saliva samples in order to diagnose CanL.

Overall, in our experimental study, there was variability in the appearance of clinical signs with some dogs remaining asymptomatic. This has been previously reported in other experimental infections (Rodríguez-Cortés et al., 2007a,b) and our experimental design allowed the inclusion in our study of dogs with different responses to the infection. These variations are reported to be due to genetic differences in activation of the immune responses

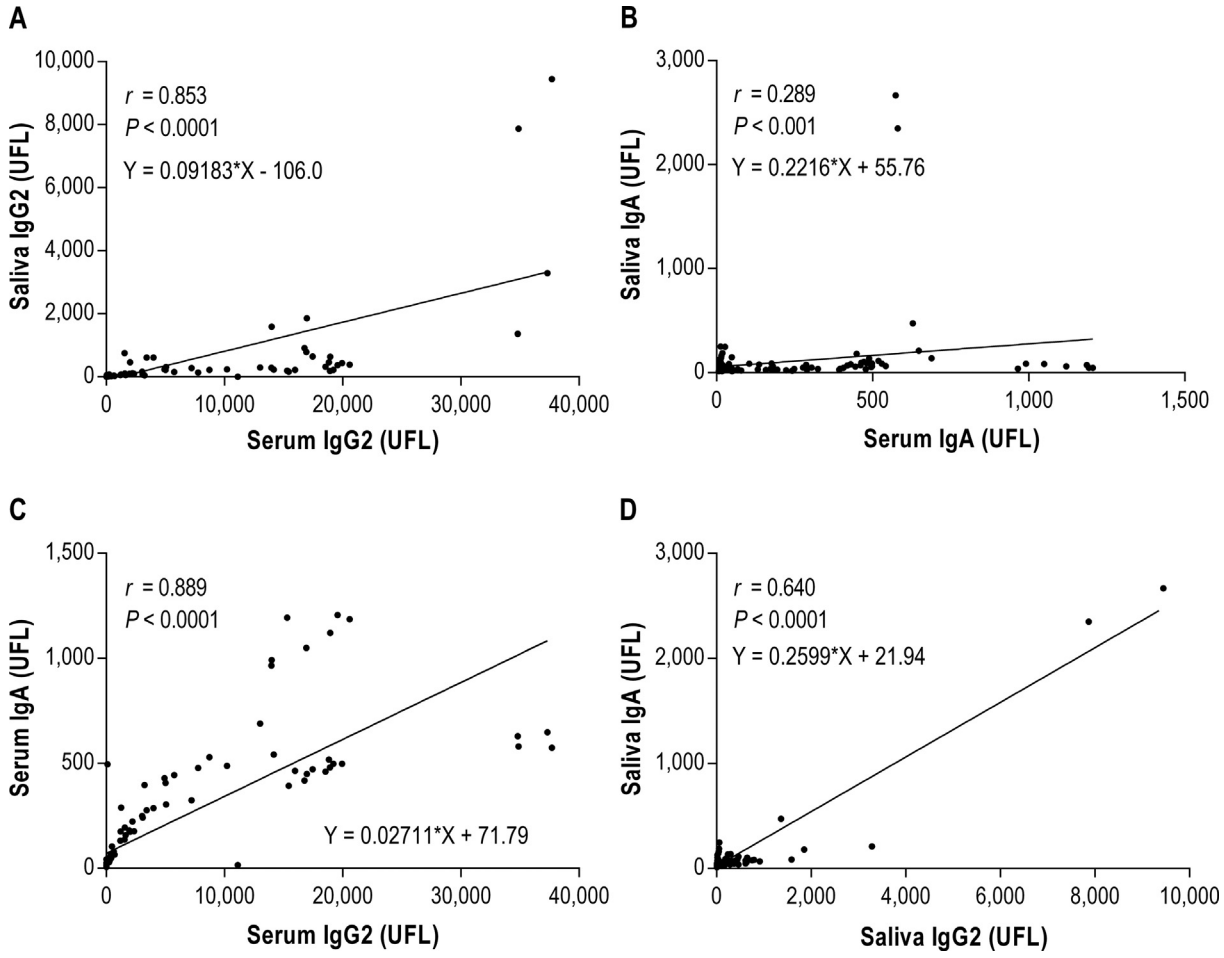

Fig 2. Linear regression and correlation plots. Four correlation studies were performed, evaluating: (A) the relationship between anti-*Leishmania* IgG2 concentrations measured in both dog serum and saliva samples, (B) the relationship between anti-*Leishmania* IgA concentrations measured in both dog serum and saliva samples, (C) the relationship between anti-*Leishmania* IgG2 and anti-*Leishmania* IgA measured in serum samples, and (D) the relationship between anti-*Leishmania* IgG2 and anti-*Leishmania* IgA measured in saliva samples. *r* = Spearman's rank correlation coefficient; *P* = significance level.

(Solano-Gallego et al., 2000). Thus, the progression of the disease seems to be associated with the type of immune response that the infected dogs develop (Solano-Gallego et al., 2016). Dogs presenting with a clinical disease had high concentrations of *Leishmania*-specific IgG (mainly), IgA and IgM antibodies and a decreased cellular immune response (Rodríguez-Cortés et al., 2007a,b). In this sense, in our study, the amount of anti-*Leishmania* IgG2 in serum and saliva was higher in those dogs with more evident clinical signs, as previously reported in serum (Proverbio et al., 2014; Hernández et al., 2015). In cases of dogs with very mild or absent clinical signs, the amounts of anti-*Leishmania* IgG2 in serum and saliva were low, and in one case (dog 1), detectable amounts of anti-*Leishmania* antibodies in serum but not in saliva were observed. This could indicate a threshold for the passing of anti-*Leishmania* IgG2 from serum to saliva, and could represent a limitation of saliva for detecting situations in which there is a very low amount of this antibody in serum, as previously reported (Cantos-Barreda et al., 2018b).

In our study, most dogs (>72%) showed detectable concentrations of anti-*Leishmania* IgG2 in serum between 3 and 4 months p.i., similar to findings in other studies in which an experimental infection was performed and concentrations of specific antibodies were measured using an in-house ELISA (Rodríguez-Cortés et al., 2007a,b), and IFAT (Hernández et al., 2015). The main difference in terms of the kinetics of anti-*Leishmania* IgG2 between serum and saliva was that the appearance of these antibodies in serum occurred approximately 1 month earlier that those in saliva. This data could indicate that the anti-*Leishmania* IgG2 in saliva might come from the diffusion from serum (Brandtzaeg, 2013). It would be important to consider this fact from the clinical point of view, since this can lead in the early stages of *Leishmania* infection to the presence of dogs positive to leishmaniasis in serum, but negative in saliva. Therefore, the use of serum would be highly recommended for diagnostic purposes in early stages of *Leishmania* infection.

Greater variability in the concentration of anti-*Leishmania* IgG2 antibodies was found in saliva than in serum. This fact leads to antibody values that vary in terms of sampling times and, as a result, could affect interpretation of the results (German et al., 1998). In three cases (dogs 1, 4, and 5) the anti-*Leishmania* IgG2 concentrations in the last saliva samplings were lower than the cut-off. This was accompanied with the absence of clinical signs and a decrease in the anti-*Leishmania* IgG2 antibodies in serum, but in this case they were still above than the cut-off previously established (Cantos-Barreda et al., 2018c). This could be due to the threshold previously indicated for passing antibodies from serum to saliva, and could indicate that the use of saliva for treatment monitoring could be more sensitive with regard to detecting reductions in anti-*Leishmania* IgG2 antibodies than serum (Cantos-Barreda et al., 2018b).

Regarding the concentrations of anti-*Leishmania* IgA in serum, it was noted that in some cases they appeared later than anti-*Leishmania* IgG2 and, in other cases, seroconversion was not observed. In addition, in many cases, anti-*Leishmania* IgA concentrations above the cut-off were not detected in saliva. This finding reinforces the fact that measuring anti-*Leishmania* IgA in saliva has no diagnostic value for CanL, as reported previously (Cantos-Barreda et al., 2017b). However, in the present study, dog 10 seroconverted at 6 months p.i., and dogs 4 and 5 did not show seroconversion. Interestingly, these dogs were all asymptomatic.

Clinical signs appeared between 6 and 8 months p.i., later than the appearance of detectable amounts of anti-*Leishmania* IgG2 antibodies in both saliva and serum. This fact leads to the conclusion that the presence of anti-*Leishmania* IgG2 in serum above the cut-off pre-dates clinical signs.

In our study, higher correlations were found between anti-*Leishmania* IgG2 in saliva and serum samples ($r = 0.853$; $P < 0.0001$) than in a previous report in which concentrations of anti-*Leishmania* antibodies in saliva and serum were evaluated in dogs with CanL after 1 month of treatment (IgG2: $r = 0.528$, $P < 0.01$) (Cantos-Barreda et al., 2018b).

Saliva has several advantages over serum for diagnosis since saliva collection is non-invasive, and saliva is cheaper and easier to collect and store than serum (Lazutka et al., 2015). However, it has to be considered that detectable amounts of anti-*Leishmania* antibodies may be observed later in saliva than in serum, and it is possible that no detectable concentrations of anti-*Leishmania* antibodies in saliva might be observed in seropositive dogs with low concentrations of antibodies in serum. So, when used for diagnostic purposes, negative results in saliva have to be viewed with caution. However, for treatment monitoring, we hypothesised that the quantification of antibodies in saliva may be more sensitive in terms of detecting a positive response to treatment, since they show higher decreases and disappear while they are found in serum. Therefore, a decrease or disappearance of *Leishmania*-specific antibodies in saliva after treatment could be a sign of a positive response to treatment although the values in serum do not change. Future studies that evaluate this possibility would be desirable.

It is important to point out that one limitation of this study is the low number of animals studied, and that no a priori sample size calculation was done to prove the hypothesis under evaluation. The inclusion of only Beagle dogs in the present study could also be considered as a limitation since the observations reported herein could have limited generalisation to other canine breeds.

Here we report, to our knowledge, the first long-term study in which the kinetics of anti-*Leishmania* antibodies in paired serum and saliva samples from dogs experimentally infected with *L. infantum* was evaluated. In conclusion, (i) the experimental infection of dogs with *L. infantum* lead to an increase in anti-*Leishmania* IgG2 and IgA concentrations in both serum and saliva samples; (ii) the presence of detectable anti-*Leishmania* antibody concentrations above the cut-off was observed earlier in serum than in saliva; (iii) at the end of our experimental period, the anti-*Leishmania* IgG2 concentrations in saliva of some cases decreased below the cut-off, while they were still elevated in serum; and (iv) anti-*Leishmania* IgG2 showed better diagnostic value than anti-*Leishmania* IgA. These conclusions can help the clinical interpretation of the measurements of anti-*Leishmania* antibodies in saliva for the diagnosis of CanL.

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