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Experimental model for reproduction of canine visceral leishmaniosis by *Leishmania infantum*

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ABSTRACT

In this report an experimental model of *Leishmania infantum* (*L. infantum*) infection in dogs is described. The data presented are derived from an overall and comparative analysis of the clinical outcomes of three groups of dogs intravenously infected with 500,000 promastigotes on different dates (2003, 2006 and 2008). The parasites used for challenge were isolated from a dog having a patent form of leishmaniosis, classified as MCAN/ES/1996/BCN150 zymodeme MON-1. Late-log-phase promastigote forms derived from cultured amastigotes obtained from the spleen of the heavily infected hamsters were used for infection. Only one single infective dose was administered to each dog. After challenge, the animals were monitored for 12 months. To analyze the disease outcome, several biopathological, immunological and parasitological end-points were considered. The analysis of the infected dogs indicated that the development of the clinical disease was very similar in the three experimental challenges, as shown by the immune response, the parasite load and the clinical and histopathological lesions detected at necropsy. A high similarity was also observed between the disease development after the experimental challenge and the one reported to occur in endemic natural infection areas, as various degrees of susceptibility to the disease and even resistance were observed in the experimentally infected animals. We believe that this challenge model faithfully reproduces and mimics the course of a natural infection and that it could be used as a suitable tool for analyzing the efficacy of anti-*Leishmania* drugs and vaccines.

1. Introduction

Visceral leishmaniosis (VL) has not received the attention it deserved because until late 1940s the disease was considered to be mainly a local problem, and the world's attention was engaged in tackling highly fatal and epidemic bacterial and viral infections (Garg and Dube, 2006). Attention was given to VL after its recognition as re-emerging zoonosis. For the understanding of the role that the host immune response has in the onset and the maintenance of the susceptibility and resistance to leishmaniosis, the murine model of *Leishmania* infection has been particularly useful and largely described (Garg and Dube, 2006; Pereira and Alves, 2008; Sharma and Singh, 2009; Gupta and Nishi, 2011). Dogs, however, may considered to be a more appropriate model to study the potential effects of drugs and vaccines than mice, since the disease pattern in dogs is

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rather similar to that observed in humans (Campino et al., 2000; Leandro et al., 2001; Prianti et al., 2007; Gupta and Nishi, 2011): a spectrum of clinical manifestations going from a self-controlled infection to a progressive disease (Garg and Dube, 2006). Moreover, dogs are the main target reservoirs of the zoonotic visceral leishmaniosis in the Mediterranean area and Latin America, due to *L. infantum* and *Leishmania chagasi*, respectively, considered now to be the same species (Oliva et al., 2006).

One of the problems of the dog models of experimental challenge is the unpredictable nature and the variability of the pattern of the disease induced after administration of infective parasites (Garg and Dube, 2006). The use of genetically homogeneous animals can lead to reduce the variation of clinical signs and the immune response. However, differences in the pattern of the clinical signs were observed after infection even when inbred dogs were used (Killick-Kendrick et al., 1994; Leandro et al., 2001). The standardization and comparison of experimental infections seems also to be difficult due to the use of different routes of administration (Leandro et al., 2001; Paranhos-Silva et al., 2003; Poot et al., 2005; Rodríguez-Cortés et al., 2007; Travi et al., 2009), the inoculum size, ranging from a few thousand to several millions of parasites (Moreno and Alvar, 2002; Maia et al., 2010), and the infective stage of those infecting parasites (Abranches et al., 1991; Campino et al., 2000; Leandro et al., 2001). All these parameters are likely to induce clinical variability. The use of promastigotes instead of amastigotes seems to mimic what it can be seen in natural conditions, but it has the disadvantage of their low infectivity when they are inoculated into skin (Poot et al., 2005) or after successive passages in culture media (Bhaumik et al., 2008). It should be taken into consideration, moreover, that under natural infection sandfly saliva, that is known to enhance the course of infection (Norsworthy et al., 2004), is inoculated to host together with promastigotes.

Thus, there seems to be no consensus on whether models in which an experimental infection is followed by rapid parasite proliferation and clinical signs are preferable to those in which an experimental infection is not immediately followed by disease. In principle, a long-term study to examine parasite proliferation control together with a type of infection that is able to induce a consistent and reproducible disease is going to be the preferable model in accordance with Rodríguez-Cortés et al. (2007).

In the present paper we have compared the outcomes of three independent 12 months-long experimental challenges of *L. infantum* infection in dogs. The type of experimental challenge described has been successfully used for the last decade (Molano et al., 2003; Carcelén et al., 2009). The data presented indicate that in all cases and under the conditions described, similar infection and disease patterns were generated in all animals that closely reproduces the typical course of the canine leishmaniosis observed in endemic areas. Thus, in view of the data presented, we believe that the experimental challenge reported could be considered as a model system that consistently induces an infection and clinical disease pattern useful to test the effectiveness of drugs and vaccines.

2. Materials and methods

2.1. Dogs

25 healthy beagle dogs of both genders, 7–34 months old, had been distributed in three groups: 1 (7 animals, infected in 2003), 2 (10 animals, infected in 2006) and 3 (8 animals, infected in 2008). They were purchased from ISOQUIMEN S.L. (Spain), bred under vector-free conditions, maintained in quarantine before challenge and housed also under vector-free conditions at the Animal Service Facilities of Extremadura University (according to the European and National Guidelines for the Care and Use of Experimental Animals) under constant veterinary supervision.

2.2. Parasites and challenge

L. infantum was isolated from a naturally infected dog (strain code: MCAN/ES/1996/BCN150) and typed at the reference WHO laboratory of Barcelona University (Spain) as *L. infantum* zymodeme MON-1, responsible of most of cases of visceral leishmaniosis in humans and dogs (Bulle et al., 2002; Alvar et al., 2004; Martin-Sánchez et al., 2004). To ensure the virulence of the parasites used to infect the dogs, golden hamsters were intracardially infected with 10^7 promastigotes derived from frozen amastigotes that had been isolated from the spleen of 4 months heavily infected hamsters. To obtain the amastigotes, the spleens of the heavily infected hamsters were homogenized in Schneider's culture medium (Sigma–Aldrich, containing 10% fetal calf serum, 200 U/ml of penicillin/200 μg/ml of streptomycin/200 U/ml of gentamycin), filtered through a nylon filter and centrifuged at 2000 rpm for 10 min. The pellet was suspended in fetal bovine serum containing DMSO to a final concentration of 10% in 1 ml samples and kept in liquid nitrogen.

For the dog challenge, a sample of the frozen amastigotes was thawed at 37 °C and transferred to a Falcon tube containing 10 ml of the Schneider's medium indicated above. The sample was centrifuged for 10 min at 2500 rpm, and the pellet was suspended in 10 ml Schneider's medium. This procedure was repeated twice. The final pellet was suspended in 10 ml of Schneider's medium, distributed into two 25 cm^2 flasks and incubated in a vertical position at 26 °C. After 5 days, the amastigotes derived to promastigotes, adopting a flagellated and elongated shape. At that point, 1 ml of the supernatant was suspended in 4 ml of fresh Schneider's medium. After 2 days in culture at 26 °C the promastigotes reached the late-log-phase, before the beginning of the stationary phase (the stationary phase is reached when the parasite density is 10^7/ml). The parasite culture was, then, centrifuged at 2500 rpm for 10 min and the pellet was suspended in sterile phosphate buffered saline (PBS). The procedure was repeated twice. Finally, parasites were counted and homogenized in PBS to prepare the infective inoculum. Each dog was intravenously challenged with 500,000 parasites in 0.5 ml of PBS in the cephalic vein.

2.3. Experimental design

The experiments were performed in compliance with Good Laboratory Principles (OCDE). The immunological, parasitological and biopathological evaluations of the animals were carried out according to LeishmanCeres Laboratory GLP Standard Operating Procedures. Each one of the experimental designs lasted 12 months (365 days approximately). Clinical examinations, blood and urine samples and lymph node (LN) biopsies of each dog were periodically performed after challenge. At necropsy, performed 365 days post infection (dpi), several tissue samples were obtained to detect the presence of parasites by tissue culture, parasite counts in smears samples, real-time PCR and for histopathological analysis. The results of the three experimental challenges were compared at the final time point.

2.4. Immunological analyses

ELISA technique was performed as described previously (Molano et al., 2003; Carcelén et al., 2009) and used for the detection of reactivity against *L. infantum* total soluble antigen (SLA). Sera were tested in duplicate. A 1/8000 dilution of horse radish peroxidase-conjugated sheep anti-dog IgG2 (Bethyl Labs, TX, USA) was used as secondary antibody. Negative and positive control sera were included in each plate. The reaction was developed with OPD substrate (Sigma–Aldrich, MO, USA) and the optical density (OD) was read at 492 nm. Results were expressed as normalized OD respect to the positive control (considered as OD = 1 in each case).

IFAT technique was performed as described (Carcelén et al., 2009). *L. infantum* promastigotes were immobilized on microscope slides, sera were assayed in two-fold serial dilutions from 1/40 to 1/640 in PBS and immunofluorescence was developed using a rabbit fluorescein-labeled anti-dog IgG conjugate (Nordic Immunological Laboratories; Tillburg, Netherlands). IFAT was considered positive at a sera dilution $\geq$1/80 (Pedras et al., 2008).

Delayed type hypersensitivity test (DTH) was performed before necropsy (Carcelén et al., 2009) injecting intradermally an inactivated suspension of *L. infantum* promastigotes in PBS–0.5% phenol (10^6/dog) in the right shaved groin. Skin reactions were recorded 48 and 72 h after inoculation, measuring the largest diameter with a digital caliper. Any measure $\geq$5 mm was considered positive.

2.5. Parasitological evaluation

To detect the presence of *L. infantum* with higher reliability three different techniques were used. LN biopsies from 60 to 270 days post infection and LN and spleen samples collected during necropsy were sterile homogenized in Schneider's medium, cultured in duplicate and incubated at $25 \pm 2\,^{\circ}C$ for 10 days to observe the presence/absence of differentiated *Leishmania* promastigote forms. Samples were considered positive when parasites were observed in any of the duplicates.

Spleen and LN smears obtained *post-mortem* were stained with Diff-Quick (Panreac BCN, Spain) and observed at a magnification of 1000×. Results were expressed at the estimated number of parasites per 10,000 nucleated cells.

Real-time PCR was employed for the absolute quantification of parasites in paraffin-embedded LN and spleen samples. Paraffin was removed following the modified protocol of Müller et al. (2003). DNA was extracted with UltraClean® BloodSpin® DNA Isolation kit (MoBio Labs, CA, USA) according to the manufacturer's instructions. A standard curve experiment (Rutledge and Côté, 2003) using a *Leishmania* TaqMan® assay was performed. PCR Primers, TaqMan® probe (Applied Biosystems, CA, USA) and thermal cycling profile were designed by Francino et al. (2006). Triplicates with 5 μl of DNA sample were amplified in 96-well plates using the Maxima® Probe/ROX qPCR Master Mix (Fermentas-Thermo Scientific Laboratories, ON, Canada). All plates contained positive and negative controls. The analyses were performed in a StepOne Plus® real-time PCR System (Applied Biosystems). Results were considered positive when the C_t (threshold cycle) was <35 (Applied Biosystems Application Note, 2006; Dantas-Torres et al., 2011) and the amplification was detected in all triplicates.

2.6. Assessment of clinical and lesional outcomes after challenge

Physical examinations were performed monthly, classifying dogs by the presence and number of clinical signs. Hematological evaluations were carried out using an automatic ABCvet® (Scil, Viernhein, Germany). Biochemical profiles of urea, creatinine and alanine aminotransferase (ALT) were also determined by using Reflotron® (Roche Diagnostic Ltd., UK). Serum protein electrophoresis was performed using an automatic SAS-3 system (Platinum software Helena®, Beaumont, UK). Urine samples were analyzed using test strips (Medi-Test Combi 9®, Macherey-Nagel, Düren, Germany).

Specimens from liver, kidney and spleen obtained *post mortem* were fixed in 4% phosphate-buffered formalin, embedded in low-fusion paraffin, cut in 4-μm-thick sections, stained with hematoxylin–eosin (Isokit®, Bio-Optica, Italy) and examined by light microscopy (Nikon®, Tokyo, Japan).

2.7. Statistical analysis

The comparative analysis between groups was performed with the R statistic software version 2.14.0 (R Development Core Team, 2011). Appropriate parametric or non-parametric statistical test for unpaired samples were used for quantitative data (analysis of variance or Kruskal–Wallis test) and categorical variables (Fisher exact probability test). *p*-Values >0.05 were not considered statistically significant.

Table 1
Immunological results at final timepoint (12 months after experimental infection).

Group	ELISA SLA IgG2	IFAT	DTH evaluation
Group 1 (no. 1–7)	Neg: 0 dogs (0%) Pos: 7 dogs (100%) +: (0%) ++: 6 (14.29%) +++: 1, 2, 3, 4, 5, 7 (85.71%)	Neg: 1 dog (14.29%) 6 Pos: 6 dogs (85.71%) +: 1, 4 (28.58%) ++: (0%) +++: 2, 3, 5, 7 (57.14%)	Neg: 6 dogs (85.71%) 1, 3, 4, 5, 6, 7 Pos: 1 dog (14.29%) 2
Group 2 (no. 8–17)	Neg: 0 dogs (0%) Pos: 10 dogs (100%) +: 8, 9, 14, 17 (40%) ++: 10, 11, 13 (30%) +++: 12, 15, 16 (30%)	Neg: 0 dogs (0%) Pos: 10 dogs (100%) +: 8, 9, 11, 14, 17 (50%) ++: 10, 12, 13 (30%) +++: 15, 16 (20%)	Neg: 5 dogs (50%) 8, 10, 12, 16, 17 Pos: 5 dogs (50%) 9, 11, 13, 14, 15
Group 3 (no. 18–25)	Neg: 0 dogs (0%) Pos: 8 dogs (100%) +: 21, 22, 24 (37.5%) ++: 20, 23 (25%) +++: 18, 19, 25 (37.5%)	Neg: 0 dogs (0%) Pos: 8 dogs (100%) +: 21, 22, 23, 24 (50%) ++: (0%) +++: 18, 19, 20, 25 (50%)	Neg: 5 dogs (62.5%) 18, 19, 20, 21, 25 Pos: 3 dogs (37.5%) 22, 23, 24
Total 25 dogs	Neg: 0 dogs (0%) Pos: 25 dogs (100%) +: 7 dogs (28%) ++: 6 dogs (24%) +++: 12 dogs (48%)	Neg: 1 dog (4%) Pos: 24 dogs (96%) +: 11 (44%) ++: 3 (12%) +++: 10 (40%)	Neg: 16 dogs (64%) Pos: 9 dogs (36%)

Neg: negative; Pos: positive. ELISA: neg: ≤0.4 of normalized OD; + (low): 0.4–0.6 of normalized OD; ++ (moderate): 0.6–0.8 of normalized OD; +++ (high): ≥0.8 of normalized OD. IFAT: Neg: ≤1/40; + (low): 1/80–1/160; ++ (moderate): 1/320; +++ (high): ≥1/640. DTH: Neg: <5 mm; Pos: ≥5 mm.

3. Results

3.1. Immunological response after infection

The monitoring of humoral response confirmed the presence of active infection following the experimental challenge, as 100% of dogs generated specific antibodies against the parasite 12 months after infection (Table 1). Fig. 1 is just an example of the course of the anti-SLA reactivity of dogs from Group 3. As observed, at day 90 post-infection all dogs developed antibodies. Thus, the pre-patent period could be considered to happen before this time point. Certain variability in the immune response was observed, since high (dogs 18, 19, 25), moderate (dogs 20, 23) and low responders (dogs 21, 22, 24) were detected. At the end of the study (Table 1) all dogs were SLA positive

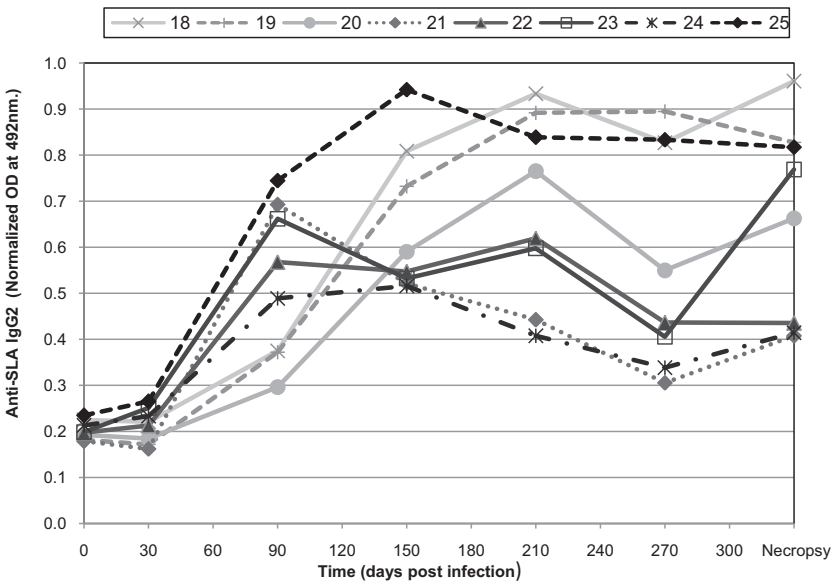

Fig. 1. Changes in the IgG2 anti-SLA antibody normalized OD values by ELISA in sera samples of dogs which belonged to Group 3, during the infection period until the end of the study. Sera samples were used at a 1/100 dilution and OD readings were measured at 492 nm. Positive and negative control sera were included in each assay. Neg.: ≤0.4 of normalized OD; + (low): 0.4–0.6 of normalized OD; ++ (moderate): 0.6–0.8 of normalized OD; +++ (high): ≥0.8 of normalized OD.

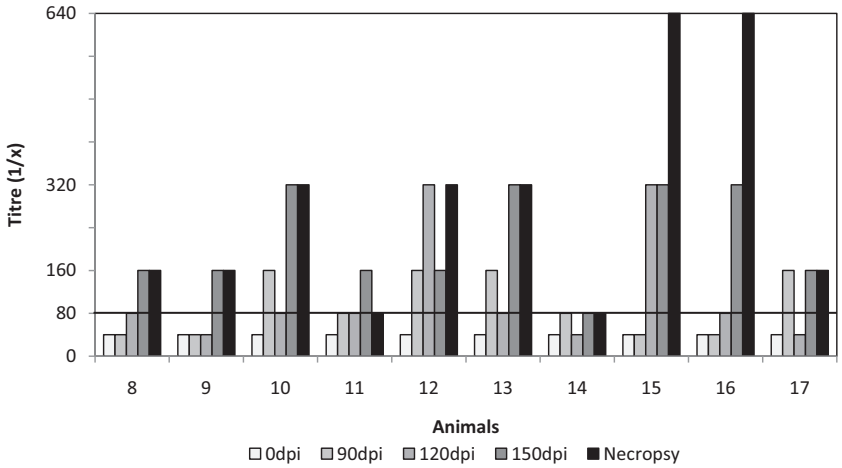

Fig. 2. Antibody response analyzed by IFAT in sera samples of dogs belonging to Group 2, during the infection period and until the end of the study (Necropsy = 365 dpi). Sera were assayed in two-fold serial dilutions from 1/40 to 1/640 to determine total IgG levels. Conjugate was 1/80 diluted. Positive and negative control sera were included in each assay. Neg.: ≤1/40; + (low): 1/80–1/160; ++ (moderate): 1/320; +++ (high): ≥1/640.

(28% were low responders, 24% had an intermediate response, and 48% were high responders). However, when the variability among the three groups was compared, no significant differences in responses by SLA ELISA were observed ($p = 0.125$).

Since IFAT technique is less sensitive than ELISA, the pre-patent period was established to occur between day 90 and 150 after infection. As shown in Fig. 2 (Group 2 example), 6 out of 10 dogs were positive at day 90 post-infection, whereas all dogs were positive at day 150 and at necropsy, except dog 6 (Group 1) which was negative by IFAT at the end of the study but was previously positive (data not shown). Variability in the magnitude of the immune response among dogs (Table 1 and Fig. 2) was also observed. At the end of the study (Table 1) 96% of dogs were IFAT positive. It was observed that 44% ($n = 11$) were low, 12% ($n = 3$) moderate and 40% ($n = 10$) high responders. As before, no significant differences among groups ($p = 0.949$) were detected.

This variability was also observed by Western-Blotting. The number of reactive bands was the largest in the blots developed with the sera from the dogs highly reactive by ELISA and IFAT techniques (data not shown).

Table 1 summarizes the DTH classification of dogs according to their skin reaction. In 16 out of the 25 infected animals (64%) the DTH was negative. No significant differences were observed among groups ($p = 0.342$).

3.2. Parasitological diagnosis

L. infantum was detected in all dogs, although variability in parasite load among individuals was observed (Table 2). The pre-patent period was established to occur up to 90 days after challenge, when live parasites in LN biopsies were detected in 10 out of 25 animals (40%). At the end of the study, 80% of dogs were positive in LN and 68% in spleen, by culture. At necropsy, amastigote forms were also detected in 76% (LN) and 80% (spleen) of animals by tissue smears observation.

Real-time PCR confirmed these results. Parasite DNA was present in 96% of dogs analyzed (*versus* only 88% by direct microscopic observation); just one animal (no. 22, Group 3) was consistently PCR negative in both samples. Spleen seemed to be more parasitized than LN (92% *versus* 80%). Variability in parasite burden (high, medium, low or even negative) was observed after 1 year of infection (Table 2). There is a relationship between high parasite load and intense antibody response as determined by ELISA and IFAT. In some dogs this high response is related to negative DTH results (Table 1). PCR negative animals were associated with low humoral response.

No significant differences among groups were found when the three diagnostic techniques were employed to detect parasites in samples of spleen ($p = 1$) or LN ($p = 0.163$).

3.3. Clinical and pathological features

Typical clinical signs of leishmaniosis (weight loss, dermatological alterations, inflammation of lymphoid organs, *etc.*) were detected in most dogs from the three groups. The incubation period (until the appearance of the first alterations or clinical signs) could be established to occur approximately between 4 and 6 months post challenge, depending on the animal. The first clinical signs observed refer to lymph node enlargement, ocular manifestations such as episcleritis or conjunctivitis, dermatitis/slight alopecia, and hyperthermia in some dogs. The clinical expression varied among individuals: while some animals did not show any or very limited clinical expression of the disease, others progressed toward a symptomatic form. At necropsy (Table 3), 92% of animals showed clinical signs with a mean of 1–3 signs in 36% of dogs and more than 4 signs in 56% of them. The percentage of dogs showing signs of the disease was similar in all groups, without statistical differences ($p = 0.997$).

Hematological, biochemical and urine analysis showed that the infected dogs manifested the classic alterations

Table 2
Parasitological results during the study (lymph node culture) and at necropsy (rest of parameters): analysis of lymph node and spleen samples by culture, tissue smears and real-time PCR.

Group	Culture		Tissue Smears		Real-time PCR	
	Lymph node and 1st dpi of detection	Spleen (necropsy)	Lymph node (necropsy)	Spleen (necropsy)	Lymph node (necropsy)	Spleen (necropsy)
Group 1 (no. 1–7)	Neg: 0 dogs (0%) Pos: 7 dogs (100%) 60 dpi: 4, 5, 6 150 dpi: 1, 2, 7 210 dpi: 3	Neg: 0 dogs (0%) Pos: 7 dogs (100%) 1, 2, 3, 4, 5, 6, 7	Neg: 2 dogs (28.57%) 4, 6 Pos: 5 dogs (71.46%) 1, 2, 3, 5, 7	Neg: 1 dog (14.28%) 6 Pos: 6 dogs (85.72%) 1, 2, 3, 4, 5, 7	Neg: 0 dogs (0%) Pos: 7 dogs (100%) Low/medium: 1, 2, 4, 5, 6 High: 3, 7	Neg: 0 dogs (0%) Pos: 7 dogs (100%) Low/medium: 2, 4, 6 High: 1, 3, 5, 7
Group 2 (no. 8–17)	Neg: 2 dogs (20%) 10, 17 Pos: 8 dogs (80%) 90 dpi: 9, 11, 12, 14 120 dpi: 8, 13, 16 210 dpi: 15	Neg: 6 dogs (60%) 10, 11, 12, 13, 14, 17 Pos: 4 dogs (40%) 8, 9, 15, 16	Neg: 0 dogs (0%) Pos: 10 dogs (100%) 8, 9, 10, 11, 12, 13, 14, 15, 16, 17	Neg: 2 dogs (20%) 10, 12 Pos: 8 dogs (80%) 8, 9, 11, 13, 14, 15, 16, 17	Neg: 1 dog (10%) 11 Pos: 9 dogs (90%) Low/medium: 8, 9, 10, 12, 13, 14, 15, 17 High: 16	Neg: 0 dogs (0%) Pos: 10 dogs (100%) Low/medium: 8, 9, 10, 11, 12, 13, 14 High: 15, 16, 17
Group 3 (no. 18–25)	Neg: 3 dogs (37.5%) 22, 23, 24 Pos: 5 dogs (62.5%) 90 dpi: 19, 21, 25 270 dpi: 20 NECROP; 18	Neg: 2 dogs (25%) 23, 24 Pos: 6 dogs (75%) 18, 19, 20, 21, 22, 25	Neg: 4 dogs (50%) 22, 23, 24, 25 Pos: 4 dogs (50%) 18, 19, 20, 21	Neg: 2 dogs (25%) 22, 23 Pos: 6 dogs (75%) 18, 19, 20, 21, 24, 25	Neg: 4 dogs (50%) 20, 21, 22, 24 Pos: 4 dogs (50%) Low/medium: 18, 19, 23, 25 High: 0	Neg: 2 dogs (20%) 22, 25 Pos: 6 dogs (60%) Low/medium: 18, 19, 20, 21, 23, 24 High: 0
Total 25 dogs	Neg: 5 dogs (20%) Pos: 20 dogs (80%)	Neg: 8 dogs (32%) Pos: 17 dogs (68%)	Neg 6 dogs (24%) Pos: 19 dogs (76%)	Neg: 5 dogs (20%) Pos: 20 dogs (80%)	Neg: 5 dogs (20%) Pos: 20 dogs (80%)	Neg: 2 dogs (8%) Pos: 23 dogs (92%)

Neg: negative; Pos: positive.
Real-time PCR parasite quantity range: $C_t \geq 35$: negative result; $25 \leq C_t < 35$: low/medium parasite load; $C_t < 25$: high parasite load.

Table 3
Biopathological results at final timepoint (12 months after experimental infection).

Group	No. clinical signs	Hematology	Proteinuria	Serum electrophoresis
Group 1[a] (no. 1–7)	0 signs: 1 dog (14.28%) 6 1–3 signs: 3 dogs (42.85%) 1, 2, 4 >3 signs: 3 dogs (42.85%) 3, 5, 7	Normal: 0 dogs (0%) Altered: 7 dogs (100%) RBC: 1, 2, 3, 5, 7 WBC: 1–7 PT: 1	Normal: 0 dogs (0%) Altered: 7 dogs (100%) +: 6 ++: 1,2,4,5,7 +++: 3	Normal: 3 dogs (42.85%) 1, 2, 6 Altered: 4 dogs (57.14%) 3, 4, 5, 7
Group 2[b] (no. 8–17)	0 signs: 1 dog (10%) 15 1–3 signs: 3 dogs (30%) 8, 16, 17 >3 signs: 6 dogs (60%) 9,10,11, 12, 13, 14	Normal: 2 dogs (20%) 9, 15 Altered WBC: 8 dogs (80%): 8, 10, 11, 12, 13, 14, 16, 17	Normal: 3 dogs (30%) 12, 13, 14 Altered: 7 dogs (70%) +: 8, 10, 11, 15, 16, 17 +++: 9	Normal: 6 dogs (60%) 8, 9, 10, 14, 15, 17 Altered: 4 dogs (40%) 11, 12, 13, 16
Group 3[c] (no. 18–25)	0 signs: none (0%) 1–3 signs: 3 dogs (37.5%) 18, 21, 22 >3 signs: 5 dogs (62.5%) 19, 20, 23, 24, 25	Normal: 0 dogs (0%) Altered: 8 dogs (100%) RBC: 18, 19, 20, 21, 22, 25 WBC: all (18–25) PT: 25	Normal: 0 dogs (0%) Altered: 8 dogs (100%) +: 19, 20, 22, 23, 24, 25 ++: 21 +++: 18	Normal: 3 dogs (37.5%) 23,24, 25 Altered: 5 dogs (62.5%) 18, 19, 20, 21, 22
Total 25 dogs	0 signs: 2 dogs (8%) 1–3 signs: 9 dogs (36%) >3 signs: 14 dogs (56%)	Normal: 2 dogs (8%) Altered: 23 dogs (92%) RBC: 11 dogs (44%) WBC: 23 dogs (92%) PT: 2 dogs (8%)	Normal: 3 dogs (12%) Altered: 22 dogs (88%) +: 13 dogs (52%) ++: 6 dogs (24%) +++: 3 dogs (12%)	Normal: 12 dogs (48%) Altered: 13 dogs (52%)

Type of clinical signs observed at final timepoint:
RBC: red blood cells; WBC: white blood cells; PT: platelets. Proteinuria range: + (low): 30 mg/dl; ++ (moderate): 100 mg/dl; +++ (high): 500 mg/dl.
Normal: parameters in the physiological range. Altered: parameters out of the physiological range.

[a] Lymph node enlargement (dogs 1, 3, 4, 5, 7), episcleritis (dogs 3, 4, 5, 7), blepharitis and keratoconjunctivitis (dog 3), dermatitis (dogs 1, 2, 3), alopecia (dog 3, 5), onychogryphosis (dogs 5, 7), anorexia/apathy (dog 2).

[b] Lymph node enlargement (dogs 9, 10, 11, 12, 13, 14), episcleritis (dogs 8, 9, 10, 12, 14), conjunctivitis (dogs 12, 14), dermatitis/erythema (dogs 9, 10, 11, 12, 13, 14, 17), exfoliation (dogs 9, 10, 12), alopecia (dogs 10, 11, 13, 14, 17), dull coat (dogs 10, 12), body fat loss (dogs 8, 9, 10, 11, 12, 16).

[c] Lymph node enlargement (dogs 20, 23), episcleritis (dogs 19, 22, 23, 25), iridociclitis (dog 25), dermatitis (dogs 19, 23, 24, 25), exfoliation (dogs 19, 20, 21, 23, 24), alopecia (dogs 20, 21, 23, 24, 25), dull coat (dogs 18, 19, 20, 24, 25), body fat loss (dogs 20, 21), pale mucous membranes (dog 23), yellowish mucosal membranes (dogs 23, 25).

observed in naturally infected dogs (Table 3). Infection induced slight alteration in cell counts in 92% of animals, particularly in white blood cells. We observed that 44% of dogs developed anemia and an 8% developed thrombocytopenia. Globally, no statistical differences could be observed in the frequency of hematological alterations among groups ($p = 0.313$). In the sera levels of urea, creatinine and ALT in serum were in the physiological range in all animals. Dysproteinemia was present in 52% of dogs (most of them also showed clinical signs of the disease) and proteinuria was detected in 88% at the end of the study. No significant differences were observed in the frequency of altered values of these two parameters among groups ($p = 0.687$ and $p = 0.091$, respectively).

At the anatomopathological level various alterations were observed in all groups for spleen, liver and kidney (Fig. 3). Hyperplasia with follicular hypertrophy and cellular depletion with lymphocytolysis were observed in spleen (Fig. 3A and B1) as well as splenomegaly together with prominent nodules on the surface (Fig. 3B2) and focal areas of necrosis. In liver, a granulomatous hepatitis process was detected, being variable among individuals. Small (Fig. 3C) or large size (Fig. 3D1) granulomas were observed. Hepatocyte destruction, vacuolar degeneration (Fig. 3C) and necrosis together with disruptions of the normal tissue architecture in the portal area and parenchyma were observed. An abundant diffuse portal infiltration of lymphocytes, plasmocytes and macrophages was also detected in this organ (Fig. 3D1). Hepatomegaly was also present (Fig. 3D2). Animals also showed signs of renal failure with serose glomerulonephritis (Fig. 3E) and congestion together with interstitial nephritis (Fig. 3F) and tubular system necrosis.

4. Discussion

The interest to develop a canine model of *Leishmania* infection is due to the fact that dogs are not only the main target hosts of *Leishmania*, but also because their disease pattern is characterized by a spectrum of clinical manifestations similar to that observed in humans. One of the main efforts that should be made to develop a consistent model of experimental challenge is to induce a type of disease that mimics as much as possible the natural infection.

Nowadays there is still some controversy on which would be the most useful model for the analysis of the visceral course of the disease. There seems to be no doubt that the effectiveness of leishmanicidal drugs or vaccines must be evaluated in an infection model that mostly resembles the disease occurring in natural conditions.

The primary requirement that must be met by any experimental challenge is the induction of active infection in all animals. Several experimental models have been previously described after administration of amastigote

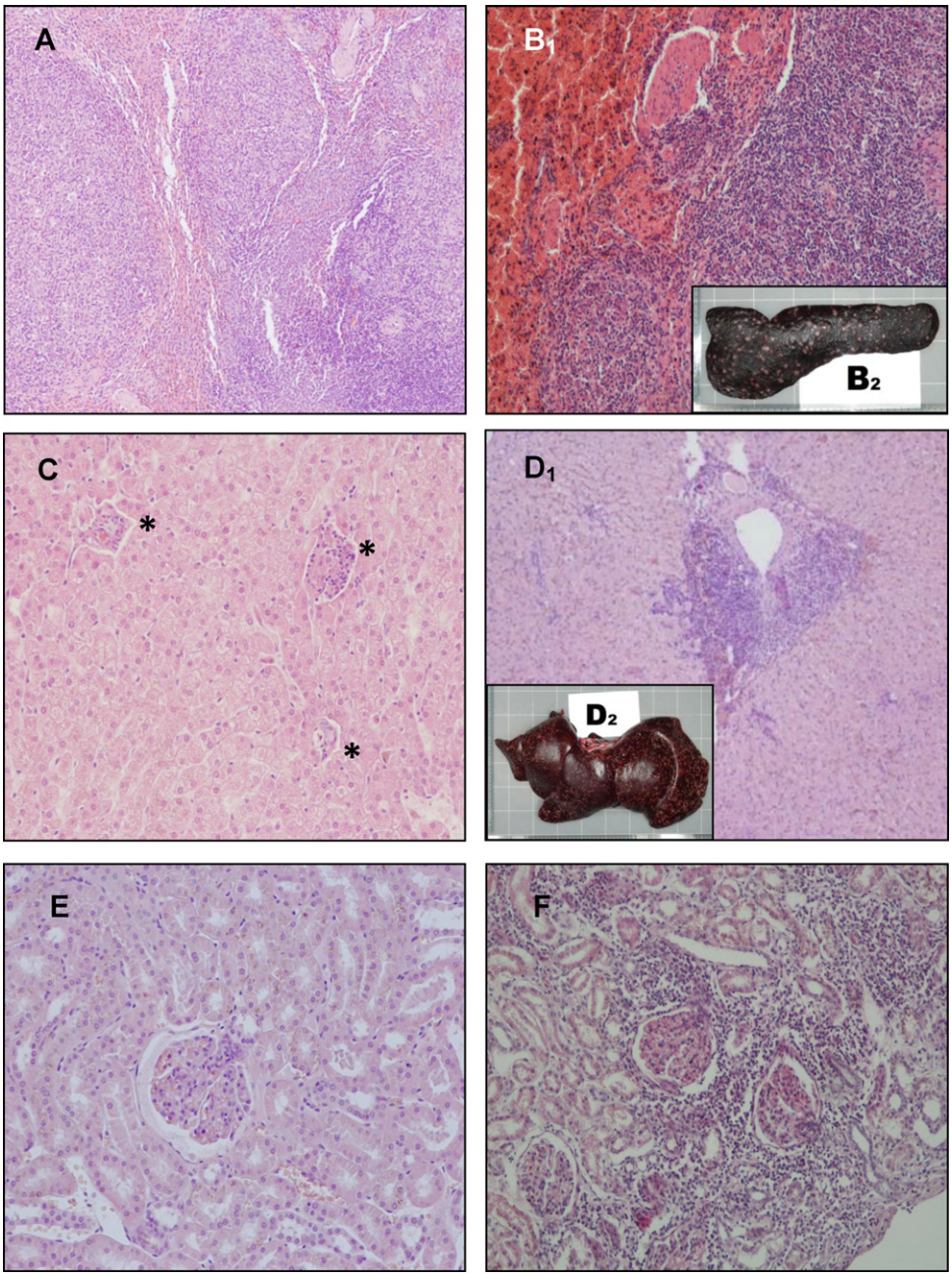

Fig. 3. Images with different degrees of alteration in spleen, liver and kidney. (A) Animal no. 15 (Group 2). Spleen – Increase of white pulp. Hematoxylin–eosin (H–E) 200×. (B) Animal no. 25 (Group 3). Spleen – (B1) Lymphoid hyperplasia. H–E 400×. (B2) Corresponding macroscopic image. (C) Animal no. 14 (Group 2). Liver – Granulomas of small size (*). Vacuolar degeneration. H–E 400×. (D) Animal no.1 (Group 1). Liver – (D1) Granuloma of big size located around the portal area. H–E 200×. (D2) Corresponding macroscopic image. (E) Animal no. 9 (Group 2). Kidney – Serose glomerulonephritis. H–E 400×. (F) Animal no. 20 (Group 3). Kidney – Interstitial nephritis. H–E 200×.

or promastigote forms and various inoculation sites, with different outcomes and results (Abranches et al., 1991; Campino et al., 2000; Leandro et al., 2001; Travi et al., 2009). It is known that the infectivity of promastigotes declines progressively with successive laboratory passes (Bhaumik et al., 2008). In this paper we have shown that a single intravenous administration to dogs of a primary culture of amastigote-derived promastigotes in late-log-phase guarantees a 100% successful active infection. Our model mimics the pathological and external clinical signs observed in diseased dogs after natural infection.

The results obtained from the comparison of the three independent experimental challenges and the use of several methods to ensure high accuracy of diagnosis indicate that the challenge model described induces a consistent type of disease similar in all groups. As shown by the combination of the three techniques employed parasites were detected in tissue samples of all animals at necropsy.

The highest sensitivity was obtained when the real-time PCR methodology was used, as it has been also previously described (Oliva et al., 2006; Singh, 2006; Miró et al., 2008). Spleen was the most parasitized tissue when compared to LN, as reported (Sánchez et al., 2004). We observed that after challenge all dogs were actively infected and that the infection remained for 12 months. The PCR negative results obtained in one dog (no. 22) could be due to the low parasite load present in this animal, the type of sample (not fresh) and its conservation (Gilbert et al., 2007). Besides lymph node PCR negative results in dogs 11, 20 and 21 when formalin-fixation paraffin-embedded samples were used could be probably due to the crosslinking of tissue components, as a consequence of the formalin-fixation process (Lehmann and Kreipe, 2001), which may cause partial DNA-destruction (Müller et al., 2003).

The percentage of positivity obtained in our study both by parasitological methods and the IFAT serological technique is similar to that observed in studies performed under natural infection conditions (Oliva et al., 2006).

The pre-patent period in this model happened to occur between day 90 and 120 post infection as shown by the presence of parasites in LN and anti-*Leishmania* antibodies in sera. These results are in agreement with previous data regarding the antibody appearance (Nieto et al., 1999; Molano et al., 2003; Carcelén et al., 2009; Travi et al., 2009) and parasite detection (Poot et al., 2005) in experimental challenges, but slightly different from natural disease, where pre-patent period has been observed to happen approximately 6 months post infection (Oliva et al., 2006). This could be due to the lower number of parasites present in the infective inoculum injected by sandfly, compared to the inoculum injected in the experimental challenge described.

The differences observed in the magnitude of the antibody response among dogs most likely reflect the pathochronic courses of the infection. Although the same breed of animals was used, the genetic background of each dog may influence the degree of resistance/susceptibility to the infection and to the disease development (Handman, 2001; Altet et al., 2002). The existence of resistant phenotypes characterized by the presence of low antibody titers against parasite antigens, and susceptible phenotypes in which high titers of antibodies are detected is also common in groups of animals living in endemic areas (Pinelli et al., 1994), confirming that the model described mimics the variability observed in nature. DTH positivity response, was detected in about 1/3 of animals, similar to that observed in naturally infected dogs by skin administration of leishmanin (Cardoso et al., 1998). Since DTH is a clinic-based diagnostic test it is quite variable depending on the presence of a cell based preserved response. In our case, the DTH test was positive mainly in animals that developed a moderate antibody levels together with a low parasite load as measured by PCR. This cellular response, as previously reported, is associated to early stages of canine leishmaniosis (Rodríguez-Cortés et al., 2007) or subclinical infections (Solano-Gallego et al., 2000) and also to certain parasite dissemination control (Pinelli et al., 1994).

We believe that the most striking similarity between the natural disease outcome and the disease development after experimental challenge is the individual variability in the expression of the disease since some animals presented an earlier course of infection *versus* others in which there was a low or absence of clinical disease expression. The duration of the incubation period and the onset and type of clinical signs observed in our study is in accordance to those previously reported for other experimental infections (Poot et al., 2005; Rodríguez-Cortés et al., 2007). The presence of subacute and chronic courses of the disease and even the regressive forms observed in the three groups of dogs showed close similarity with natural infection conditions (Lanotte et al., 1979; Pozio et al., 1981), where the dog population could be distributed in susceptible (80–90%) and resistant (10–20%) phenotypes (Pozio et al., 1981; Bettini and Gradoni, 1986). Analytical results were in accordance with natural findings, such us anemia, thrombocytopenia or alteration in white blood cell counts, as well as dysproteinemia (Martinez-Subiela and Ceron, 2005) or proteinuria (Saridomichelakis, 2009). Histopathological lesions were also those typically described in canine leishmaniosis (Saridomichelakis, 2009). Thus, we believe that from a clinical point of view the experimental conditions described mimic the features of the disease development classically reported in nature (Lanotte et al., 1979; Ciaramella et al., 1997; Saridomichelakis, 2009). It was observed the degree of infectivity reached with the model proposed is very high (100% effective) and that the course of the clinical disease progress is quite similar to what happens in nature in terms of timing and disease severity. One of the main points to be highlighted is that the disease outcomes were very similar in all groups as an indication of the consistency of the model.

5. Conclusion

The described experimental model of *L. infantum* infection in dogs faithfully reproduces and mimics the course of the natural disease, providing a potent tool for the screening of new potential treatments and immunization tests for canine leishmaniosis.

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