TITLE: Evaluation of canine leishmaniosis vaccine CaniLeish® under field conditions in native

dog populations from an endemic Mediterranean area – a randomized controlled trial

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ABSTRACT

Dog vaccination is considered an effective way of reducing *Leishmania infantum* infection incidence in the canine population, as well as its transmission to humans. However, the use of partially effective vaccines can have the detrimental effect of “masking” vaccinated asymptomatic carriers, capable of harbouring the parasite and transmitting it to naïve individuals. After eight years on the European market, few studies have been released on CaniLeish® vaccine safety and efficacy. The present study, a one-year randomized CaniLeish® vaccine field trial, was performed in a canine leishmaniosis endemic area and included animals selected from a native dog population (n=168). No severe adverse reactions were observed in vaccinated dogs (n=85). Cases of active *L. infantum* infection were detected by serological, molecular and clinical follow-up of dogs. One-year post-vaccination, no differences in number or severity of *L. infantum* active infections were observed between study groups (n=4 in each group). Vaccine-induced cellular immunity, assessed through interferon-γ quantification, showed significantly higher levels of this cytokine one-month post-vaccination in the vaccine group (p<0.001), but no differences were observed after nine months between trial groups (p=0.078). These results fail to support the reported CaniLeish® efficacy in the prevention of active *L. infantum* infection in dogs from endemic areas and naturally exposed to the parasite.

KEYWORDS: canine leishmaniosis, CaniLeish® vaccine, longitudinal field trial, serology, qPCR, IFN-γ
1. INTRODUCTION

Canine leishmaniosis (CanL) is a severe vector-borne disease which affects the domestic dog and is caused by *Leishmania infantum* (Gállego, 2004). The disease is endemic in the Mediterranean basin, where it is estimated to affect more than 2.5 million dogs and present an overall CanL seroprevalence of 23.2%, showing variation within micro-foci (Moreno and Alvar, 2002; Gálvez et al., 2010; Franco et al., 2011; Morales-Yuste et al., 2012). It is transmitted by the bite of phlebotomine sand flies and, in the Mediterranean region, eight *Phlebotomus* species have been identified as vectors of the parasite (Alten et al., 2016). Detection of infected dogs is hindered by the array of possible clinical presentations, as well as by the high prevalence of asymptomatic individuals (Baneth et al., 2008). The impact of this zoonosis also extends to human health, with dogs being the main domestic reservoir for the parasite (Alvar et al., 2004). Therefore, controlling infection at the reservoir level is essential for reducing transmission amongst canids and to humans.

Vaccination is seen as one of the best methods for controlling the infection (Dye, 1996) and the development of effective vaccines against both CanL and human leishmaniosis has been a goal for the scientific community. A vaccine for CanL should induce a strong and long-lasting Th1-dominated cellular immunity to control infection progression, while simultaneously reducing parasite burden in dogs to decrease their infectiousness to sand flies (Gradoni, 2015). Furthermore, it should be equally effective in protecting against infection or disease (Alvar et al., 2013).

CaniLeish® (Virbac, France) was the first CanL vaccine to be licensed in Europe, in 2011 (European Medicines Agency, 2011). It is a second-generation vaccine composed of purified excreted-secreted proteins (LiESP) of *L. infantum* and a saponin adjuvant (Moreno et al., 2012). According to pharmacovigilance data reported by Virbac in October 2015, more than 1.8 million doses of CaniLeish® had been sold during the first 3.5 years of marketing in the
European Economic Area, Switzerland and Tunisia (Breton et al., 2015). However, few studies have been published since the preliminary phase II research (Moreno et al., 2012, 2014; Martin et al., 2014) and the only phase III trial performed before licensing was granted (Oliva et al., 2014). After eight years on the European market, very little is known about the vaccine safety and efficacy in heterogeneous dog populations from endemic areas. Cases of CanL in vaccinated dogs have been reported (Ceccarelli et al., 2016; Gavazza et al., 2016), and the performance of the recommended pre-vaccination screening method has presented inconsistent results (Solano-Gallego et al., 2017).

The present study consists of a one-year randomized controlled CaniLeish® vaccine field trial performed in a CanL Mediterranean endemic area with a heterogeneous and autochthonous canine population. Dogs of both sexes, different ages and breeds have been included. Inclusion criteria were the same as recommended by the vaccine’s manufacturer for dog vaccination and were followed for both experimental groups. The objective of this study was to provide preliminary data on CaniLeish® vaccine performance under real field conditions in a heterogeneous population of native dogs from a CanL endemic area.

2. MATERIALS AND METHODS

2.1. Study design and vaccination protocol

The study took place in Girona province, in north-east Catalonia (Spain), an endemic area for CanL (Velez et al., 2019). At the beginning of the trial, in March 2016, 177 dogs were selected from a population of 406 dogs previously tested for the presence of anti-L. infantum antibodies by the same method described in the subsection “Serological follow-up”. All animals were kept in large packs in open-air facilities, mostly in rural and periurban areas. Dog owners were previously informed of all details of the study and signed an informed consent before the start of the trial.
Inclusion criteria for the vaccine trial followed those recommended by the CaniLeish® manufacturer and are described in Figure 1.

According to the CaniLeish® vaccine manufacturer, the risk of developing *L. infantum* active infection is reduced by 3.6 times in vaccinated dogs (European Medicines Agency, 2011), and this was the parameter used to compare both groups. Sample size was calculated assuming a 1:1 ratio between the two experimental groups, an expected 17.6% incidence of *L. infantum* infection in the control group (Velez and Gállego, unpublished data), 3.6 times fewer cases of active infection in the vaccine group, 10% estimated losses during one year trial, a power of 0.8 and a significance level of 0.05 in a two-sided test. Final sample size of 192 dogs (96 per study group) was constrained by the number of animals available and the limitations of the research team to follow a larger group of dogs during the one-year trial.

Selected animals were distributed over 12 locations, with the number of dogs per location ranging from four to 23 (Figure 2). Dogs were randomly assigned to either vaccine (n=90) or control (n=87) groups by a blinded operator using a statistical analysis software (Stata 15; StataCorp LP, College Station, TX, USA). As different locations had shown distinct infection levels, animals’ allocation to study groups was first stratified per dog kennel and then randomized. This way, an even proportion of dogs were assigned to each study group in each location, avoiding possible result bias introduced by distinct infection pressures.

As recommended by the CaniLeish® vaccine manufacturer, all dogs from both groups were dewormed with a mixture of febantel, pyrantel pamoate and praziquantel prior to vaccination. From the initial selected sample of 177 individuals, only 168 dogs (85 in the vaccine group and 83 in the control group) completed the vaccination course and were considered for the vaccine study (Figure 1).

Both groups were followed for one year and samples were taken at different pre-determined time points, according to the study design (serological follow-up, parasitological assessment and evaluation of vaccine-induced cellular mediated immunity). Blood was collected from the
cephalic or jugular veins and transferred to EDTA tubes (for serology and clinical blood analysis) or heparin tubes (for peripheral blood mononuclear cells (PBMC) isolation and cellular mediated immunity tests). Lymph node samples were collected by fine needle aspiration and placed in 100 µL of sterile 0.9% sodium chloride solution. Plasma and lymph node samples were frozen at -40°C, and PBMC were preserved in liquid nitrogen until processing. Follow-up samples from the same dog were analysed in parallel.

2.2. Clinical follow-up

All dogs were clinically assessed before the beginning of the vaccine trial. This included a physical exam, complete blood count (CBC), renal and hepatic function assessment, and serum protein electrophoretogram. These results were kept as a baseline (T0) to compare with subsequent exams throughout the study.

The physical exam included inspection of general body condition, hydration status, skin, hair and nail condition, mucosae, external lymph nodes and ocular lesions. Owners were asked about any recent disease, visible weight loss, anorexia, exercise intolerance, polyuria/polydipsia, vomiting or diarrhoea. Clinical assessments were repeated throughout the field trial whenever there was a suspicion of CanL, either detected by the veterinarian researchers (RV and ED) during follow-up visits or by the dog owners. At the end of the trial, a thorough physical exam was performed on all dogs. Likewise, blood analyses were repeated whenever needed to confirm a CanL case and at the end of the study for all seropositive dogs. Due to the nonspecific clinical presentation of CanL, dogs were considered symptomatic only if two or more clinical signs compatible with the disease were observed. The same criterion was followed for any detected laboratory changes.

2.3. Vaccine safety assessment
After each vaccine dose, dog owners were asked to monitor their dogs and to report any adverse clinical signs observed to the researchers. Periodic revisions by the veterinarians of the team were performed.

2.4. Serological follow-up

A crude total *L. infantum* antigen in house enzyme linked immunosorbent assay (CTLA-ELISA) was used to detect IgG antibodies to *L. infantum* in trial dogs. The technique used has been previously described (Riera et al., 1999; Velez et al., 2019). Briefly, dog plasma samples diluted at 1:400 were incubated in titration plates (Costar®) previously coated with sonicated whole promastigotes at a protein concentration of 20 µg/mL in 0.05 M carbonate buffer at pH 9.6. Protein A peroxidase (1:30,000, Sigma®) was used as conjugate and reactions were stopped with H₂SO₄ 3M. Results were expressed in standard units (U) compared to a calibrator control sample set arbitrarily at 100U. The cut-off was established at 24U (mean + 4 standard deviations of U of sera of dogs from non-endemic areas).

Serological assessments were performed at eight time points throughout the study: before each vaccine dose (T0, Vac2, Vac3) and at one (1M), four (4M), six (6M), nine (9M) and 12 months (12M) after vaccination completion. An increase of four-fold ELISA units when compared with the same dog’s basal values (ELISA units measured at T0) was considered evidence of seroconversion to *L. infantum* (Solano-Gallego et al., 2009).

2.5. Parasitological assessment

*L. infantum* qPCR on lymph node samples was performed in suspected cases of CanL and at the last sampling time point for seropositive dogs (12M). DNA was extracted from lymph node aspirates using the High Pure PCR Template Preparation Kit (Roche®), following the manufacturer’s instructions. A quantitative PCR was performed in all samples as described elsewhere (Martín-Ezquerra et al., 2009) with minor modifications. Briefly, qPCR mix reaction
was prepared with 5 µL of DNA, 10 µL of master mix (FastStart Universal Probe Master (ROX), Roche®), 10 µM of *Leishmania* primers (Leim 1 and Leim 2) and 5 µM of probe designed to target a kinetoplast DNA (kDNA) sequence, and 1 µL of H₂O. Eukariotic 18S rRNA was used as endogenous control (VIC™/MGB probe, primer limited, Thermo Fisher Scientific®). Amplifications and detection were performed in an ABI7900 device (Applied Biosystems) (Genomics Service, CCITUB) and the thermal cycling profile was 50°C for 2 min, 95°C for 10 min, 45 cycles at 95°C for 15 sec, and 60°C for 1 min. All samples were analysed in triplicate and positive (DNA from *L. infantum* MHOM/FR/95/LEM3141 strain) and negative controls were included in all qPCR reactions. Parasite quantification was performed by extrapolation from a standard curve generated with *L. infantum* DNA extracted from 1 x 10⁶ parasites/mL serially diluted from 10⁵ to 1 parasites/mL.

2.6. Evaluation of vaccine-induced cellular mediated immunity (CMI)

PBMC were obtained from each animal at three time points: before the first vaccine dose (T0), four weeks after the third vaccine dose (1M) and nine months after vaccination completion (9M). Only dogs with samples from the three time points were included in the CMI assessment (a total of 152 animals, 75 in the vaccine group and 77 in the control group).

Heparinized whole blood samples were processed no later than 4h after collection. PBMC were isolated by centrifugation with a density gradient medium (Lymphoprep™; Stemcell Technologies), frozen in foetal bovine serum (FBS) supplemented with 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen until processing.

For the assessment of antigen-specific cytokine responses, samples from the same dog were processed together. PBMC were slowly thawed, washed and left to rest overnight at 37°C in 5% CO₂. The following day, cells were counted on a TC20™ Automated Cell Counter (Bio-Rad Laboratories, Inc.) and incubated in 96-well culture plates at a density of 10⁶ cells/mL as described elsewhere (Rodríguez-Cortés et al., 2017). Briefly, PBMC were incubated with 10
µg/mL soluble *L. infantum* antigen (SLA), or 2.5 µg/mL concanavalin A (ConA) (positive control), or culture media (unstimulated, negative control) for a period of five days at 37°C in 5% CO₂. On the fifth day, plates were centrifuged, and supernatants were collected and stored at -40°C. Interferon-γ (IFN-γ) concentration on PBMC supernatants was determined using the Canine IFN-γ DuoSet ELISA kit (R&D Systems), following manufacturer’s instructions. All samples were processed in duplicate and a standard curve was included in all plates, with a range of IFN-γ concentrations from 0 to 2000 pg/mL. Optical densities were determined at 450 nm, with wavelength correction set to 570 nm. IFN-γ concentrations were calculated using a four-parameter logistic standard curve produced in GraphPad Prism® version 5.3 (GraphPad Software, San Diego, California, USA). To obtain the specific IFN-γ concentration for each sample, readings from the unstimulated cell supernatant were subtracted from the SLA-stimulated cell supernatant. All plates presented a coefficient of determination (R²) above 0.99.

### 2.7. Definition of active *L. infantum* infection case

Screening of trial dogs’ infection status was based on the results of serological tests, presence of clinical signs, and detection of CBC or plasma biochemical abnormalities compatible with CanL. Any suspicion of *L. infantum* infection detected by the researchers during sample collection or the dog owners throughout the trial period was further evaluated. Apart from these reported cases, and because all analyses were performed in parallel at the end of the trial, identification of CanL cases was mainly performed in April 2017.

A confirmed case of active *L. infantum* infection was defined as:

- Seroconversion to *L. infantum*, defined as a four-fold increase in ELISA units when compared with basal values (ELISA units measured at T0) for the same individual and,

- Detection of *L. infantum* DNA in lymph node samples.

Only animals presenting both criteria were classified as positive.
2.8. Study endpoint

Cases of CanL confirmed during the field study were submitted to treatment and follow-up or euthanasia, according to the dog owner’s decision.

2.9. Statistical analysis

All statistical analyses were performed using Stata 15 software (StataCorp LP, College Station, TX, USA). Continuous variables included in this study did not present a normal distribution and normality could not be achieved by data transformation. Therefore, non-parametric statistical tests were used to compare between and within groups. Comparisons between groups at each time point were performed by Mann-Whitney U test. Longitudinal comparisons within groups were performed by Wilcoxon signed-rank test. Statistical significance of difference in proportions between groups was tested by the Pearson Chi-square test. Graphs were built in GraphPad Prism® 5.3 (GraphPad Software, San Diego, California, USA).

3. RESULTS

3.1. Characteristics of the study population

The majority of the study dog population was composed of hunting dogs (87.1% of the vaccine group and 83.9% of the control group), but breeding (8.9% of the total dog population), racing (6.5%) or pet dogs (0.6%) were also represented; no statistically significant differences in dog purpose between trial groups were observed ($\chi^2=3.66$, $p=0.3$). Crossbred dogs represented 55.3% of the vaccine group and 45.8% of the control group ($\chi^2=1.52$, $p=0.218$), and 55.3% and 65.1% of the vaccine and control groups, respectively, were males ($\chi^2=1.67$, $p=0.196$). Mean dog age in the vaccine group was 3.3 years (SD=2.9) and 3.4 years in the control group (SD=3.0), ranging from six months to 11 years ($\chi^2=6.58$, $p=0.832$).
3.2. Vaccine safety

No severe adverse reactions were observed in vaccinated dogs. One case of transient anorexia and apathy following first vaccine dose administration was reported, which was not observed again in the same animal following the second or third vaccination dose. No other adverse reactions were reported.

3.3. Humoral and molecular detection of L. infantum

In April 2017 (12M post-vaccination), 35 animals were seropositive for L. infantum and were further tested by qPCR on lymph node samples (21 in the vaccine group and 14 in the control group). From these, 19 (54.3%) presented a positive qPCR result [nine vaccine (42.9%) and ten control (71.4%)], with parasite loads ranging from 0.39 to 1.24x10^7 parasites/mL (Table 1). No statistically significant differences were detected in the incidence of positive results (χ²=2.76, p=0.096) or in lymph node parasite loads (z=1.31, p=0.1903) between groups.

3.4. Vaccine-induced CMI

At the pre-vaccination sampling point, 28.3% of the trial dogs (43/152) presented L. infantum-specific IFN-γ production (20 dogs in the vaccine group and 23 in the control group). Measurable IFN-γ concentrations at this time point ranged from 2.50 to 7317.25 pg/mL. Levels of IFN-γ in vaccine and control groups throughout the study are presented in Figure 3. Median IFN-γ levels for the control group were equal to zero (range: 0 to 7317.25 pg/mL) in the three sampling points tested and no differences were detected in this group between time points (p>0.05). Dogs in the vaccine group showed a statistically significant increase in IFN-γ levels 1M after vaccination completion (median=38.95 pg/mL; range: 0 to 5136.58 pg/mL) compared to pre-vaccination (T0) levels (z=-6.624, p<0.001). At 9M after vaccination, IFN-γ levels had dropped considerably (median=12.74 pg/mL; range: 0 to 6235.92 pg/mL), being...
significantly lower when compared to the 1M time point \((z=3.149, p=0.002)\), but still significantly higher than pre-vaccination levels \((z=-2.931, p=0.003)\). Differences between vaccine and control groups were only significant at the 1M time point \((z=-3.297, p=0.001)\). No statistically significant differences in IFN-γ levels were detected between groups at the pre-vaccination (T0) \((p=0.730)\) or 9M time points \((p=0.078)\).

The levels of IFN-γ presented by healthy and diseased dogs are presented as supplementary material (Figure S1). IFN-γ levels tended to be lower in diseased dogs from both groups, although no statistically significant differences were observed.

### 3.5. Clinical assessment of trial dogs

At the end of the vaccine trial, 87.6% of dogs \((127/145)\) were considered asymptomatic for CanL \([62\text{ dogs in the vaccine group (87.3%) and 65 in the control group (87.8%)}]\). The remaining 18 animals \((12.4\%)\) showed two or more clinical signs compatible with CanL \([nine\text{ in the vaccine group (12.7%) and nine in the control group (12.2%)}]\). These were mainly characterized by localized or multifocal lymphadenomegaly (detected in 100% of symptomatic dogs) and pale mucous membranes (50% of symptomatic dogs). Other observed clinical signs were dermatological lesions \((38.9\%)\), poor body condition \((27.8\%)\) and ocular alterations \((22.2\%)\).

Laboratory exams after T0 were only performed in dogs suspected of CanL during the trial and in seropositive dogs at the end of the trial. At 12M, 37.1% of the analysed dogs \((13/35)\) were considered healthy \([none\text{ or one laboratory change compatible with CanL (11 in the vaccine group and two in the control group), while 42.9% presented two or three analytical alterations (seven dogs in the vaccine group and eight dogs in the control group), and 20% showed four to six laboratory abnormalities (three vaccine and four control)}]\). Table 1 describes clinical and laboratory alterations found in confirmed cases of active *L. infantum* infection.
3.6. **Confirmed cases of active L. infantum infection in the vaccine and control groups**

Dogs were evaluated one year after vaccination completion for seropositivity against *L. infantum*. From these, 35 dogs showed positive anti-*L. infantum* antibody levels (21 in the vaccine (29.6%) and 14 in the control (18.9%) groups) in one of the two post-transmission season serological assessments (January and April 2017). These 35 dogs were further assessed by *L. infantum* DNA detection in lymph node samples by qPCR and clinical-laboratory evaluation. Only dogs that met the parameters previously defined for *L. infantum* active infection (seroconversion to *L. infantum* and parasite DNA detection in lymph node aspirate) were considered to be confirmed infection cases. From these, four cases were observed in both vaccinated (5.6%; 4/71) and control dogs (5.4%; 4/74). Results showed no difference in the development of active *L. infantum* infection between the two study groups (Table 1).

**4. DISCUSSION**

The objective of the present study, a multi-site randomized vaccine field trial, was to obtain a preliminary and independent evaluation of CaniLeish® vaccine efficacy in field conditions in a native heterogeneous population of dogs living in a *L. infantum* endemic region. From the 177 dogs initially enrolled in the vaccine study, 168 completed the vaccination phase (95%) [85 dogs in the vaccine group (94.4%) and 83 in the control group (95.4%)]. Similarly, the expected loss to follow-up in this study was 10%, based on preliminary assessments performed on the same dog population. However, at the end of the study, 18% of the initial dog sample had been lost, mainly due to deaths related to hunting activities and animal movement to other dog kennels.

Canine seropositivity to *L. infantum* at the end of the trial was detected in 75% (9/12) of the trial locations, demonstrating the presence of infection in most dog kennels. Accordingly, a
homogeneous vector presence has been shown in the study area together with a high
incidence of dog exposure to sand fly saliva (Velez et al., 2018).

The studied outcome was active *L. infantum* infection and not clinical CanL as detection of
CanL clinical cases was not expected due to the short duration of the present clinical trial,
which included only one *L. infantum* transmission season. Nevertheless, CanL clinical cases
were identified during this field trial in both study groups. The mean period between infection
and development of clinical disease was reported to be seven months, ranging from three to
14 months (Oliva et al., 2006), but it can extend to years in resistant dogs (Baneth et al., 2008).
CaniLeish® vaccine proved to be safe in the dog population studied. Apart from one single case
of transient apathy and anorexia, no other adverse effects were reported by dog owners or
observed by the researchers, which is in accordance with previous vaccine safety reports
(Breton et al., 2015; Marino et al., 2017). However, it should be noted that the study
population was mainly composed of robust crossbred or purebred hunting dogs weighing
between 15 and 25 kg, which may be less likely to show discomfort than dogs of smaller
breeds. In a questionnaire-based survey of veterinary practitioners working in Girona region,
82% of vaccine appliers reported adverse reactions, ranging from the most commonly
observed local swelling and pain, to cases of anaphylactic shock (Lladró et al., 2017). However,
as also pointed out by the study authors, the attribution of these adverse effects to vaccine
administration was based on veterinarians’ criteria and confirmation of the cause of clinical
signs may not have been pursued in all occasions.

In the present study, a CTLA-ELISA that measures the humoral immune response to *L. infantum*
was used as a diagnostic test for infection. Quantitative serological tests are considered
reliable indicators of active infection and good predictors of the onset of clinical signs (Oliva et
al., 2006). Seroconversion has been defined as a four-fold increase in sequential samples from
the same dog (Paltrinieri et al., 2010) or a three-fold increase in the cut-off value of a well-
standardized diagnostic test (Solano-Gallego et al., 2009). In endemic areas, the median time
between the establishment of progressive infection and seroconversion was estimated to be 10.5 months (ranging from four to 22 months) (Oliva et al., 2006). The dynamic of antibody levels during this study corresponded to the one described in previous studies for IFAT (Martin et al., 2014; Oliva et al., 2014), and indicates that vaccine-induced antibodies can interfere with *L. infantum* screening by a CTLA-ELISA (Velez et al., 2020).

Molecular detection of the parasite was performed in lymph node samples at the end of the trial to confirm the diagnosis of active *L. infantum* infection in seropositive dogs. Although the levels of seropositivity considered for infection diagnosis in the study were very conservative and clear indicators of progressive infection, the detection of the parasite in a target organ validated the serological results. In addition, the detection of parasite DNA in lymph nodes in the absence of seroconversion would not have been considered as a definitive confirmation of infection.

IFN-γ is considered a high-quality biomarker of immunogenicity and protection against *Leishmania* infection (Reis et al., 2010). It is considered the key cytokine involved in the activation of macrophages and the killing of intracellular *L. infantum* amastigotes, in collaboration with other immune mechanisms (Carrillo and Moreno, 2009). High levels of IFN-γ are associated with host resistance to *L. infantum* infection (Chamizo et al., 2005; Solano-Gallego et al., 2016) and this has been used as a marker of response to CaniL therapy (Manna et al., 2008; Martínez-Orellana et al., 2017), including in the evaluation of new drugs (Corpas-López et al., 2018). It has also been quantified as a marker of protection in previous vaccine studies, both for CaniLeish® (Moreno et al., 2012, 2014; Martin et al., 2014), and for other vaccines (Fernandes et al., 2008; De Lima et al., 2010). According to the results obtained in this study, IFN-γ levels tended to be lower in diseased dogs (presented as supplementary material S1). Although not statistically significant, possibly due to the reduced number of infected dogs, the observed difference between healthy and diseased animals supports a protective effect of IFN-γ. Apart from providing an indication of vaccine-induced CMI, the quantification of IFN-γ in
this study also allowed the assessment of previous exposure to *L. infantum* in the trial population. According to the results obtained in the pre-vaccination assessment, almost 30% of dogs presented a measurable IFN-γ response when exposed to SLA, which indicates *L. infantum* recognition and possible pre-established natural immunity to the parasite. Some degree of resistance to infection is expected in canine populations from endemic areas (Baneth et al., 2008), although its impact may be difficult to quantify and account for when setting a field trial.

Levels of IFN-γ measured in the vaccine group one month after vaccination completion showed a marked increase when compared to the pre-vaccination time point or to parallel levels in the control group, in accordance with the results obtained in a previous CaniLeish® study (Moreno et al., 2012). This corresponds to the point when vaccine-induced immunity should be established (European Medicines Agency, 2011), and illustrates the stimulation of CMI response in vaccinated dogs. IFN-γ concentrations were measured again 9M after vaccination, showing a marked decrease in this cytokine levels in the vaccine group. Results from previous CaniLeish® studies, performed with a sample of 20 beagle dogs under laboratory conditions, have shown a statistically significant difference in the proportion of IFN-γ producing cells between vaccine and control dogs at 6M post-vaccination (Moreno et al., 2014), but no difference between groups was reported at one year post-vaccination (Martin et al., 2014; Moreno et al., 2014). In these studies, the 9M post-vaccination time point was not assessed. Unlike the two studies mentioned, the present study was performed in field conditions and animals were naturally exposed to one *L. infantum* transmission season, therefore exposure-induced IFN-γ may have interfered with vaccine-induced cytokine levels. Nevertheless, three months after the end of the transmission season, vaccinated dogs did not show differences in IFN-γ production when compared to the control group. A short-lived vaccine induced CMI which fails to be protective during the whole period of expected vaccine coverage could explain the lack of difference in detected active *L. infantum* infection cases between vaccine
and control groups observed at the end of this study. Nevertheless, care should be taken in the over-interpretation of a single parameter as it is known that IFN-γ is only part of a complex network of regulatory and counter-regulatory interactions involving multiple cells and cytokines (Reis et al., 2010; Hosein et al., 2017). Further studies on the immune response developed by trial dogs would be needed to fully characterize vaccine induced CMI.

The combined information provided by humoral and molecular assays allowed the identification of eight active *L. infantum* infection cases. Two dogs, one in each trial group, were identified as diseased during the study. The remaining six (three in each group) were detected at the end of the trial. According to previous vaccine studies in natural conditions, where a continued parasite challenge is present, it is unlikely that these animals may revert to a negative state (Oliva et al., 2014). The CaniLeish® vaccine reports an efficacy of 68.4% in the prevention of clinical signs of CanL and a protection level, defined as the percentage of vaccinated animals which do not develop clinical signs, of 92.7%. These results were obtained during the only vaccine pre-licensing field study in a homogeneous population of 90 naïve beagle dogs, five to 7.5 months old (Oliva et al., 2014). In the study by Oliva et al. (2014), four cases of active *Leishmania* infection were recorded at 12M post-vaccination, one in the vaccine group (2.4%) and three in the control group (7.7%); all these dogs progressed to symptomatic active infection in the following months. In the present trial, no differences in number or severity of active infection cases were detected between vaccine and control groups one-year post-vaccination. Although the reduced number of observed positive cases demands caution in the interpretation of the results of this study, these are supported by a recent field study, which compared the efficacy of CaniLeish® vaccine and two insecticide dog collars in the prevention of CanL (Brianti *et al.*, 2016). After one year, although different protection efficacies could be determined for each insecticide collar, no difference was detected in the number of CanL cases between CaniLeish® vaccinated dogs and the control group. Again, the total
The number of CanL cases detected in the aforementioned trial presented by Brianti et al. was low, which may have impaired the detection of a difference between groups. The ultimate step to assess the efficacy of a vaccine against CanL is a phase III field trial with native canine populations from endemic areas, where vaccinated and control dogs are exposed to natural infection by sand fly bites (Reis et al., 2010). However, in contrast to laboratory experimental challenge, natural infection depends on many variable factors related to the canine host, the vector and the parasite. According to Solano-Gallego et al. (2009), only an estimated one third of dogs living in CanL endemic areas will be susceptible to infection during the course of their lives. This implies that, at the time of enrolment for a vaccine field trial, a high proportion of animals testing negative for *L. infantum* are already resistant to the parasite and will be “useless” in terms of vaccine effect assessment. Another important factor of variability in field trials is vector related. Sand fly populations are highly influenced by biotic and abiotic factors (Barón et al., 2011; Hartemink et al., 2011; Ballart et al., 2014), which change annually. Some of these factors, such as temperature, are also known to influence *L. infantum* development inside the vector (Rioux et al., 1985). Likewise, it is impossible to guarantee the success of natural parasite transmission in a given area and year. For these reasons, field trials with privately owned dogs are challenging and their success difficult to predict. Nevertheless, they represent the closest situation to a “real life” scenario, allowing for a more realistic assessment of vaccine performance.

**5. CONCLUSION**

The CaniLeish® vaccine proved to be safe in the studied population of dogs from a CanL endemic area. However, no difference in number or severity of active *L. infantum* infection cases between vaccine and control groups was observed during the first-year post-vaccination. The vaccine induced *L. infantum*-specific IFN-γ production one month after vaccination.
completion, but levels were not maintained at nine months post-vaccination. The results obtained in this study do not support the previously reported CaniLeish® efficacy in the prevention of active *L. infantum* infection in dogs.

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ETHICS APPROVAL

The research protocol was submitted to the Ethics Committee on Animal Experimentation (CEEA) of University of Barcelona, which considered that an ethical approval was not required.
for this study. All dog owners were informed about the research protocol and signed an informed consent allowing for sample and data collection.

**AVAILABILITY OF DATA AND MATERIAL**

The datasets used and/or analysed during the current study are available from the corresponding authors upon reasonable request.

**COMPETING INTERESTS**

The authors declare no competing interests.

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**FIGURE LEGENDS:**

Figure 1. Flow chart of pre-vaccination procedures and vaccine field trial.

Figure 2. Map of Girona province. Field trial locations are marked in black circles; the number of study dogs per location (n) is presented.

Figure 3. Median and interquartile ranges of IFN-γ levels observed in the vaccine and control groups at three time points: before vaccination (T0), one month after vaccination completion (1M) and nine months after vaccination completion (9M). (a) Within group comparison with T0; (b) within group comparison with 1M; (c) between group comparison. (***) indicates statistical significance of p≤0.001.

Supplementary figure S1. Levels of IFN-γ observed in infected and non-infected dogs at three time points: before vaccination (T0), one month after vaccination completion (1M) and nine months after vaccination completion (9M). Panel A: includes all dogs from both vaccine and control groups. Panel B: includes only dogs from vaccine group.
February 2016  
Assessment for enrolment (n=406)

Stratified randomization (n=177)

Vaccine group  N=90  
Control group  N=87

March-May 2016  
Vaccination course (3 vaccine dose, 21 days apart)

Vaccine group  N=85  
Control group  N=83

Vaccine field trial (n=168)

June-October 2016  
Natural *L. infantum* transmission season

May 2017  
End of study (n=145)

Vaccine group  N=71  
Control group  N=74

Inclusion criteria:
- Seronegative to *L. infantum*
- Healthy
- Age > 6 months
- Females: non-gestating or lactating
- Not previously vaccinated for CanL

Samples collected:
- Whole blood
- Plasma
- PBMC
- LN aspirate

PBMC: peripheral blood mononuclear cells; LN: lymph node