1   **TITLE:** Evaluation of canine leishmaniosis vaccine CaniLeish[®] under field conditions in native

2   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

5

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

154 European Economic Area, Switzerland and Tunisia (Breton et al., 2015). However, few studies

155 have been published since the preliminary phase II research (Moreno et al., 2012, 2014; Martin

156 et al., 2014) and the only phase III trial performed before licensing was granted (Oliva et al.,

157 2014). After eight years on the European market, very little is known about the vaccine safety

158 and efficacy in heterogeneous dog populations from endemic areas. Cases of CanL in

159 vaccinated dogs have been reported (Ceccarelli et al., 2016; Gavazza et al., 2016), and the

160 performance of the recommended pre-vaccination screening method has presented

161 inconsistent results (Solano-Gallego et al., 2017).

162 The present study consists of a one-year randomized controlled CaniLeish[®] vaccine field trial

163 performed in a CanL Mediterranean endemic area with a heterogeneous and autochthonous

164 canine population. Dogs of both sexes, different ages and breeds have been included. Inclusion

165 criteria were the same as recommended by the vaccine's manufacturer for dog vaccination

166 and were followed for both experimental groups. The objective of this study was to provide

167 preliminary data on CaniLeish[®] vaccine performance under real field conditions in a

168 heterogeneous population of native dogs from a CanL endemic area.

169

170 **2. MATERIALS AND METHODS**

171

172 *2.1. Study design and vaccination protocol*

173 The study took place in Girona province, in north-east Catalonia (Spain), an endemic area for

174 CanL (Velez et al., 2019). At the beginning of the trial, in March 2016, 177 dogs were selected

175 from a population of 406 dogs previously tested for the presence of anti-*L. infantum*

176 antibodies by the same method described in the subsection "Serological follow-up". All

177 animals were kept in large packs in open-air facilities, mostly in rural and periurban areas. Dog

178 owners were previously informed of all details of the study and signed an informed consent

179 before the start of the trial.

180   Inclusion criteria for the vaccine trial followed those recommended by the CaniLeish[®]

181   manufacturer and are described in Figure 1.

182   According to the CaniLeish[®] vaccine manufacturer, the risk of developing *L. infantum* active

183   infection is reduced by 3.6 times in vaccinated dogs (European Medicines Agency, 2011), and

184   this was the parameter used to compare both groups. Sample size was calculated assuming a

185   1:1 ratio between the two experimental groups, an expected 17.6% incidence of *L. infantum*

186   infection in the control group (Velez and Gállego, unpublished data), 3.6 times fewer cases of

187   active infection in the vaccine group, 10% estimated losses during one year trial, a power of

188   0.8 and a significance level of 0.05 in a two-sided test. Final sample size of 192 dogs (96 per

189   study group) was constrained by the number of animals available and the limitations of the

190   research team to follow a larger group of dogs during the one-year trial.

191   Selected animals were distributed over 12 locations, with the number of dogs per location

192   ranging from four to 23 (Figure 2). Dogs were randomly assigned to either vaccine (n=90) or

193   control (n=87) groups by a blinded operator using a statistical analysis software (Stata 15;

194   StataCorp LP, College Station, TX, USA). As different locations had shown distinct infection

195   levels, animals' allocation to study groups was first stratified per dog kennel and then

196   randomized. This way, an even proportion of dogs were assigned to each study group in each

197   location, avoiding possible result bias introduced by distinct infection pressures.

198   As recommended by the CaniLeish[®] vaccine manufacturer, all dogs from both groups were

199   dewormed with a mixture of febantel, pyrantel pamoate and praziquantel prior to vaccination.

200   From the initial selected sample of 177 individuals, only 168 dogs (85 in the vaccine group and

201   83 in the control group) completed the vaccination course and were considered for the vaccine

202   study (Figure 1).

203   Both groups were followed for one year and samples were taken at different pre-determined

204   time points, according to the study design (serological follow-up, parasitological assessment

205   and evaluation of vaccine-induced cellular mediated immunity). Blood was collected from the

206 cephalic or jugular veins and transferred to EDTA tubes (for serology and clinical blood

207 analysis) or heparin tubes (for peripheral blood mononuclear cells (PBMC) isolation and

208 cellular mediated immunity tests). Lymph node samples were collected by fine needle

209 aspiration and placed in 100 µL of sterile 0.9% sodium chloride solution. Plasma and lymph

210 node samples were frozen at -40°C, and PBMC were preserved in liquid nitrogen until

211 processing. Follow-up samples from the same dog were analysed in parallel.

212

213 *2.2. Clinical follow-up*

214 All dogs were clinically assessed before the beginning of the vaccine trial. This included a

215 physical exam, complete blood count (CBC), renal and hepatic function assessment, and serum

216 protein electrophoretogram. These results were kept as a baseline (T0) to compare with

217 subsequent exams throughout the study.

218 The physical exam included inspection of general body condition, hydration status, skin, hair

219 and nail condition, mucosae, external lymph nodes and ocular lesions. Owners were asked

220 about any recent disease, visible weight loss, anorexia, exercise intolerance,

221 polyuria/polydipsia, vomiting or diarrhoea. Clinical assessments were repeated throughout the

222 field trial whenever there was a suspicion of CanL, either detected by the veterinarian

223 researchers (RV and ED) during follow-up visits or by the dog owners. At the end of the trial, a

224 thorough physical exam was performed on all dogs. Likewise, blood analyses were repeated

225 whenever needed to confirm a CanL case and at the end of the study for all seropositive dogs.

226 Due to the nonspecific clinical presentation of CanL, dogs were considered symptomatic only if

227 two or more clinical signs compatible with the disease were observed. The same criterion was

228 followed for any detected laboratory changes.

229

230 *2.3. Vaccine safety assessment*

231 After each vaccine dose, dog owners were asked to monitor their dogs and to report any

232 adverse clinical signs observed to the researchers. Periodic revisions by the veterinarians of the

233 team were performed.

234

235 *2.4. Serological follow-up*

236 A crude total *L. infantum* antigen in house enzyme linked immunosorbent assay (CTLA-ELISA)

237 was used to detect IgG antibodies to *L. infantum* in trial dogs. The technique used has been

238 previously described (Riera et al., 1999; Velez et al., 2019). Briefly, dog plasma samples diluted

239 at 1:400 were incubated in titration plates (Costar®) previously coated with sonicated whole

240 promastigotes at a protein concentration of 20 μg/mL in 0.05 M carbonate buffer at pH 9.6.

241 Protein A peroxidase (1:30,000, Sigma®) was used as conjugate and reactions were stopped

242 with H_2SO_4 3M. Results were expressed in standard units (U) compared to a calibrator control

243 sample set arbitrarily at 100U. The cut-off was established at 24U (mean + 4 standard

244 deviations of U of sera of dogs from non-endemic areas).

245 Serological assessments were performed at eight time points throughout the study: before

246 each vaccine dose (T0, Vac2, Vac3) and at one (1M), four (4M), six (6M), nine (9M) and 12

247 months (12M) after vaccination completion. An increase of four-fold ELISA units when

248 compared with the same dog's basal values (ELISA units measured at T0) was considered

249 evidence of seroconversion to *L. infantum* (Solano-Gallego et al., 2009).

250

251 *2.5. Parasitological assessment*

252 *L. infantum* qPCR on lymph node samples was performed in suspected cases of CanL and at the

253 last sampling time point for seropositive dogs (12M). DNA was extracted from lymph node

254 aspirates using the High Pure PCR Template Preparation Kit (Roche®), following the

255 manufacturer's instructions. A quantitative PCR was performed in all samples as described

256 elsewhere (Martín-Ezquerra et al., 2009) with minor modifications. Briefly, qPCR mix reaction

257 was prepared with 5 μL of DNA, 10 μL of master mix (FastStart Universal Probe Master (ROX),

258 Roche®), 10 μM of *Leishmania* primers (Leim 1 and Leim 2) and 5 μM of probe designed to

259 target a kinetoplast DNA (kDNA) sequence, and 1 μL of H_2O. Eukariotic 18S rRNA was used as

260 endogenous control (VIC^{TM}/MGB probe, primer limited, Thermo Fisher Scientific®).

261 Amplifications and detection were performed in an ABI7900 device (Applied Biosystems)

262 (Genomics Service, CCITUB) and the thermal cycling profile was 50°C for 2 min, 95°C for 10

263 min, 45 cycles at 95°C for 15 sec, and 60°C for 1 min. All samples were analysed in triplicate

264 and positive (DNA from *L. infantum* MHOM/FR/95/LEM3141 strain) and negative controls

265 were included in all qPCR reactions. Parasite quantification was performed by extrapolation

266 from a standard curve generated with *L. infantum* DNA extracted from 1 x 10^6 parasites/mL

267 serially diluted from 10^5 to 1 parasites/mL.

268

269 *2.6. Evaluation of vaccine-induced cellular mediated immunity (CMI)*

270 PBMC were obtained from each animal at three time points: before the first vaccine dose (T0),

271 four weeks after the third vaccine dose (1M) and nine months after vaccination completion

272 (9M). Only dogs with samples from the three time points were included in the CMI assessment

273 (a total of 152 animals, 75 in the vaccine group and 77 in the control group).

274 Heparinized whole blood samples were processed no later than 4h after collection. PBMC were

275 isolated by centrifugation with a density gradient medium (Lymphoprep^{TM}; Stemcell

276 Technologies), frozen in foetal bovine serum (FBS) supplemented with 10% dimethyl sulfoxide

277 (DMSO) and stored in liquid nitrogen until processing.

278 For the assessment of antigen-specific cytokine responses, samples from the same dog were

279 processed together. PBMC were slowly thawed, washed and left to rest overnight at 37°C in

280 5% CO_2. The following day, cells were counted on a TC20^{TM} Automated Cell Counter (Bio-Rad

281 Laboratories, Inc.) and incubated in 96-well culture plates at a density of 10^6 cells/mL as

282 described elsewhere (Rodríguez-Cortés et al., 2017). Briefly, PBMC were incubated with 10

283 μg/mL soluble *L. infantum* antigen (SLA), or 2.5 μg/mL concanavalin A (ConA) (positive control),

284 or culture media (unstimulated, negative control) for a period of five days at 37°C in 5% CO_2.

285 On the fifth day, plates were centrifuged, and supernatants were collected and stored at -40°C.

286 Interferon-γ (IFN-γ) concentration on PBMC supernatants was determined using the Canine

287 IFN-γ DuoSet ELISA kit (R&D Systems), following manufacturer's instructions. All samples were

288 processed in duplicate and a standard curve was included in all plates, with a range of IFN-γ

289 concentrations from 0 to 2000 pg/mL. Optical densities were determined at 450 nm, with

290 wavelength correction set to 570 nm. IFN-γ concentrations were calculated using a four-

291 parameter logistic standard curve produced in GraphPad Prism® version 5.3 (GraphPad

292 Software, San Diego, California, USA). To obtain the specific IFN-γ concentration for each

293 sample, readings from the unstimulated cell supernatant were subtracted from the SLA-

294 stimulated cell supernatant. All plates presented a coefficient of determination (R^2) above

295 0.99.

296

297 *2.7. Definition of active* L. infantum *infection case*

298 Screening of trial dogs' infection status was based on the results of serological tests, presence

299 of clinical signs, and detection of CBC or plasma biochemical abnormalities compatible with

300 CanL. Any suspicion of *L. infantum* infection detected by the researchers during sample

301 collection or the dog owners throughout the trial period was further evaluated. Apart from

302 these reported cases, and because all analyses were performed in parallel at the end of the

303 trial, identification of CanL cases was mainly performed in April 2017.

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

305 - Seroconversion to *L. infantum*, defined as a four-fold increase in ELISA units when compared

306 with basal values (ELISA units measured at T0) for the same individual and,

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

308 Only animals presenting both criteria were classified as positive.

309

310 *2.8. Study endpoint*

311 Cases of CanL confirmed during the field study were submitted to treatment and follow-up or

312 euthanasia, according to the dog owner's decision.

313

314 *2.9. Statistical analysis*

315 All statistical analyses were performed using Stata 15 software (StataCorp LP, College Station,

316 TX, USA). Continuous variables included in this study did not present a normal distribution and

317 normality could not be achieved by data transformation. Therefore, non-parametric statistical

318 tests were used to compare between and within groups. Comparisons between groups at each

319 time point were performed by Mann-Whitney *U* test. Longitudinal comparisons within groups

320 were performed by Wilcoxon signed-rank test. Statistical significance of difference in

321 proportions between groups was tested by the Pearson Chi-square test. Graphs were built in

322 GraphPad Prism® 5.3 (GraphPad Software, San Diego, California, USA).

323

324 **3. RESULTS**

325

326 *3.1. Characteristics of the study population*

327 The majority of the study dog population was composed of hunting dogs (87.1% of the vaccine

328 group and 83.9% of the control group), but breeding (8.9% of the total dog population), racing

329 (6.5%) or pet dogs (0.6%) were also represented; no statistically significant differences in dog

330 purpose between trial groups were observed ($\chi 2$=3.66, p=0.3). Crossbred dogs represented

331 55.3% of the vaccine group and 45.8% of the control group ($\chi 2$=1.52, p=0.218), and 55.3% and

332 65.1% of the vaccine and control groups, respectively, were males ($\chi 2$=1.67, p=0.196). Mean

333 dog age in the vaccine group was 3.3 years (SD=2.9) and 3.4 years in the control group

334 (SD=3.0), ranging from six months to 11 years ($\chi 2$=6.58, p=0.832).

335

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.

341

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 1.24×10^7 parasites/mL (Table 1). No statistically significant differences were detected in the incidence of positive results ($\chi 2=2.76$, $p=0.096$) or in lymph node parasite loads ($z=1.31$, $p=0.1903$) between groups.

349

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

14

361 significantly lower when compared to the 1M time point (z=3.149, p=0.002), but still

362 significantly higher than pre-vaccination levels (z=-2.931, p=0.003). Differences between

363 vaccine and control groups were only significant at the 1M time point (z=-3.297, p=0.001). No

364 statistically significant differences in IFN-γ levels were detected between groups at the pre-

365 vaccination (T0) (p=0.730) or 9M time points (p=0.078).

366 The levels of IFN-γ presented by healthy and diseased dogs are presented as supplementary

367 material (Figure S1). IFN-γ levels tended to be lower in diseased dogs from both groups,

368 although no statistically significant differences were observed.

369

370 *3.5. Clinical assessment of trial dogs*

371 At the end of the vaccine trial, 87.6% of dogs (127/145) were considered asymptomatic for

372 CanL [62 dogs in the vaccine group (87.3%) and 65 in the control group (87.8%)]. The

373 remaining 18 animals (12.4%) showed two or more clinical signs compatible with CanL [nine in

374 the vaccine group (12.7%) and nine in the control group (12.2%)]. These were mainly

375 characterized by localized or multifocal lymphadenomegaly (detected in 100% of symptomatic

376 dogs) and pale mucous membranes (50% of symptomatic dogs). Other observed clinical signs

377 were dermatological lesions (38.9%), poor body condition (27.8%) and ocular alterations

378 (22.2%).

379 Laboratory exams after T0 were only performed in dogs suspected of CanL during the trial and

380 in seropositive dogs at the end of the trial. At 12M, 37.1% of the analysed dogs (13/35) were

381 considered healthy (none or one laboratory change compatible with CanL) (11 in the vaccine

382 group and two in the control group), while 42.9% presented two or three analytical alterations

383 (seven dogs in the vaccine group and eight dogs in the control group), and 20% showed four to

384 six laboratory abnormalities (three vaccine and four control).

385 Table 1 describes clinical and laboratory alterations found in confirmed cases of active *L.*

386 *infantum* infection.

387

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).

399

4. DISCUSSION

401

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

413 homogeneous vector presence has been shown in the study area together with a high

414 incidence of dog exposure to sand fly saliva (Velez et al., 2018).

415 The studied outcome was active *L. infantum* infection and not clinical CanL as detection of

416 CanL clinical cases was not expected due to the short duration of the present clinical trial,

417 which included only one *L. infantum* transmission season. Nevertheless, CanL clinical cases

418 were identified during this field trial in both study groups. The mean period between infection

419 and development of clinical disease was reported to be seven months, ranging from three to

420 14 months (Oliva et al., 2006), but it can extend to years in resistant dogs (Baneth et al., 2008).

421 CaniLeish® vaccine proved to be safe in the dog population studied. Apart from one single case

422 of transient apathy and anorexia, no other adverse effects were reported by dog owners or

423 observed by the researchers, which is in accordance with previous vaccine safety reports

424 (Breton et al., 2015; Marino et al., 2017). However, it should be noted that the study

425 population was mainly composed of robust crossbred or purebred hunting dogs weighing

426 between 15 and 25 kg, which may be less likely to show discomfort than dogs of smaller

427 breeds. In a questionnaire-based survey of veterinary practitioners working in Girona region,

428 82% of vaccine appliers reported adverse reactions, ranging from the most commonly

429 observed local swelling and pain, to cases of anaphylactic shock (Lladró et al., 2017). However,

430 as also pointed out by the study authors, the attribution of these adverse effects to vaccine

431 administration was based on veterinarians' criteria and confirmation of the cause of clinical

432 signs may not have been pursued in all occasions.

433 In the present study, a CTLA-ELISA that measures the humoral immune response to *L. infantum*

434 was used as a diagnostic test for infection. Quantitative serological tests are considered

435 reliable indicators of active infection and good predictors of the onset of clinical signs (Oliva et

436 al., 2006). Seroconversion has been defined as a four-fold increase in sequential samples from

437 the same dog (Paltrinieri et al., 2010) or a three-fold increase in the cut-off value of a well-

438 standardized diagnostic test (Solano-Gallego et al., 2009). In endemic areas, the median time

439   between the establishment of progressive infection and seroconversion was estimated to be

440   10.5 months (ranging from four to 22 months) (Oliva et al., 2006). The dynamic of antibody

441   levels during this study corresponded to the one described in previous studies for IFAT (Martin

442   et al., 2014; Oliva et al., 2014), and indicates that vaccine-induced antibodies can interfere

443   with *L. infantum* screening by a CTLA-ELISA (Velez et al., 2020).

444   Molecular detection of the parasite was performed in lymph node samples at the end of the

445   trial to confirm the diagnosis of active *L. infantum* infection in seropositive dogs. Although the

446   levels of seropositivity considered for infection diagnosis in the study were very conservative

447   and clear indicators of progressive infection, the detection of the parasite in a target organ

448   validated the serological results. In addition, the detection of parasite DNA in lymph nodes in

449   the absence of seroconversion would not have been considered as a definitive confirmation of

450   infection.

451   IFN-γ is considered a high-quality biomarker of immunogenicity and protection against

452   *Leishmania* infection (Reis et al., 2010). It is considered the key cytokine involved in the

453   activation of macrophages and the killing of intracellular *L. infantum* amastigotes, in

454   collaboration with other immune mechanisms (Carrillo and Moreno, 2009). High levels of IFN-γ

455   are associated with host resistance to *L. infantum* infection (Chamizo et al., 2005; Solano-

456   Gallego et al., 2016) and this has been used as a marker of response to CanL therapy (Manna

457   et al., 2008; Martínez-Orellana et al., 2017), including in the evaluation of new drugs (Corpas-

458   López et al., 2018). It has also been quantified as a marker of protection in previous vaccine

459   studies, both for CaniLeish® (Moreno et al., 2012, 2014; Martin et al., 2014), and for other

460   vaccines (Fernandes et al., 2008; De Lima et al., 2010). According to the results obtained in this

461   study, IFN-γ levels tended to be lower in diseased dogs (presented as supplementary material

462   S1). Although not statistically significant, possibly due to the reduced number of infected dogs,

463   the observed difference between healthy and diseased animals supports a protective effect of

464   IFN-γ. Apart from providing an indication of vaccine-induced CMI, the quantification of IFN-γ in

465   this study also allowed the assessment of previous exposure to *L. infantum* in the trial

466   population. According to the results obtained in the pre-vaccination assessment, almost 30%

467   of dogs presented a measurable IFN-γ response when exposed to SLA, which indicates *L.*

468   *infantum* recognition and possible pre-established natural immunity to the parasite. Some

469   degree of resistance to infection is expected in canine populations from endemic areas

470   (Baneth et al., 2008), although its impact may be difficult to quantify and account for when

471   setting a field trial.

472   Levels of IFN-γ measured in the vaccine group one month after vaccination completion showed

473   a marked increase when compared to the pre-vaccination time point or to parallel levels in the

474   control group, in accordance with the results obtained in a previous CaniLeish® study (Moreno

475   et al., 2012). This corresponds to the point when vaccine-induced immunity should be

476   established (European Medicines Agency, 2011), and illustrates the stimulation of CMI

477   response in vaccinated dogs. IFN-γ concentrations were measured again 9M after vaccination,

478   showing a marked decrease in this cytokine levels in the vaccine group. Results from previous

479   CaniLeish® studies, performed with a sample of 20 beagle dogs under laboratory conditions,

480   have shown a statistically significant difference in the proportion of IFN-γ producing cells

481   between vaccine and control dogs at 6M post-vaccination (Moreno et al., 2014), but no

482   difference between groups was reported at one year post-vaccination (Martin et al., 2014;

483   Moreno et al., 2014). In these studies, the 9M post-vaccination time point was not assessed.

484   Unlike the two studies mentioned, the present study was performed in field conditions and

485   animals were naturally exposed to one *L. infantum* transmission season, therefore exposure-

486   induced IFN-γ may have interfered with vaccine-induced cytokine levels. Nevertheless, three

487   months after the end of the transmission season, vaccinated dogs did not show differences in

488   IFN-γ production when compared to the control group. A short-lived vaccine induced CMI

489   which fails to be protective during the whole period of expected vaccine coverage could

490   explain the lack of difference in detected active *L. infantum* infection cases between vaccine

491 and control groups observed at the end of this study. Nevertheless, care should be taken in the

492 over-interpretation of a single parameter as it is known that IFN-γ is only part of a complex

493 network of regulatory and counter-regulatory interactions involving multiple cells and

494 cytokines (Reis et al., 2010; Hosein et al., 2017). Further studies on the immune response

495 developed by trial dogs would be needed to fully characterize vaccine induced CMI.

496 The combined information provided by humoral and molecular assays allowed the

497 identification of eight active *L. infantum* infection cases. Two dogs, one in each trial group,

498 were identified as diseased during the study. The remaining six (three in each group) were

499 detected at the end of the trial. According to previous vaccine studies in natural conditions,

500 where a continued parasite challenge is present, it is unlikely that these animals may revert to

501 a negative state (Oliva et al., 2014). The CaniLeish® vaccine reports an efficacy of 68.4% in the

502 prevention of clinical signs of CanL and a protection level, defined as the percentage of

503 vaccinated animals which do not develop clinical signs, of 92.7%. These results were obtained

504 during the only vaccine pre-licensing field study in a homogeneous population of 90 naïve

505 beagle dogs, five to 7.5 months old (Oliva et al., 2014). In the study by Oliva et al. (2014), four

506 cases of active *Leishmania* infection were recorded at 12M post-vaccination, one in the vaccine

507 group (2.4%) and three in the control group (7.7%); all these dogs progressed to symptomatic

508 active infection in the following months. In the present trial, no differences in number or

509 severity of active infection cases were detected between vaccine and control groups one-year

510 post-vaccination. Although the reduced number of observed positive cases demands caution in

511 the interpretation of the results of this study, these are supported by a recent field study,

512 which compared the efficacy of CaniLeish® vaccine and two insecticide dog collars in the

513 prevention of CanL (Brianti *el al*., 2016). After one year, although different protection efficacies

514 could be determined for each insecticide collar, no difference was detected in the number of

515 CanL cases between CaniLeish® vaccinated dogs and the control group. Again, the total

516 number of CanL cases detected in the aforementioned trial presented by Brianti et al. was low,

517 which may have impaired the detection of a difference between groups.

518 The ultimate step to assess the efficacy of a vaccine against CanL is a phase III field trial with

519 native canine populations from endemic areas, where vaccinated and control dogs are

520 exposed to natural infection by sand fly bites (Reis et al., 2010). However, in contrast to

521 laboratory experimental challenge, natural infection depends on many variable factors related

522 to the canine host, the vector and the parasite. According to Solano-Gallego et al. (2009), only

523 an estimated one third of dogs living in CanL endemic areas will be susceptible to infection

524 during the course of their lives. This implies that, at the time of enrolment for a vaccine field

525 trial, a high proportion of animals testing negative for *L. infantum* are already resistant to the

526 parasite and will be "useless" in terms of vaccine effect assessment. Another important factor

527 of variability in field trials is vector related. Sand fly populations are highly influenced by biotic

528 and abiotic factors (Barón et al., 2011; Hartemink et al., 2011; Ballart et al., 2014), which

529 change annually. Some of these factors, such as temperature, are also known to influence *L.*

530 *infantum* development inside the vector (Rioux et al., 1985). Likewise, it is impossible to

531 guarantee the success of natural parasite transmission in a given area and year. For these

532 reasons, field trials with privately owned dogs are challenging and their success difficult to

533 predict. Nevertheless, they represent the closest situation to a "real life" scenario, allowing for

534 a more realistic assessment of vaccine performance.

535

536 **5. CONCLUSION**

537

538 The CaniLeish® vaccine proved to be safe in the studied population of dogs from a CanL

539 endemic area. However, no difference in number or severity of active *L. infantum* infection

540 cases between vaccine and control groups was observed during the first-year post-vaccination.

541 The vaccine induced *L. infantum*-specific IFN-γ production one month after vaccination

542 completion, but levels were not maintained at nine months post-vaccination. The results

543 obtained in this study do not support the previously reported CaniLeish® efficacy in the

544 prevention of active *L. infantum* infection in dogs.

545

546

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556

557

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563

564 **ETHICS APPROVAL**

565 The research protocol was submitted to the Ethics Committee on Animal Experimentation

566 (CEEA) of University of Barcelona, which considered that an ethical approval was not required

567   for this study. All dog owners were informed about the research protocol and signed an

568   informed consent allowing for sample and data collection.

569

570   **AVAILABILITY OF DATA AND MATERIAL**

571   The datasets used and/or analysed during the current study are available from the

572   corresponding authors upon reasonable request.

573

574   **COMPETING INTERESTS**

575   The authors declare no competing interests.

576

577

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757

758 **FIGURE LEGENDS:**

759

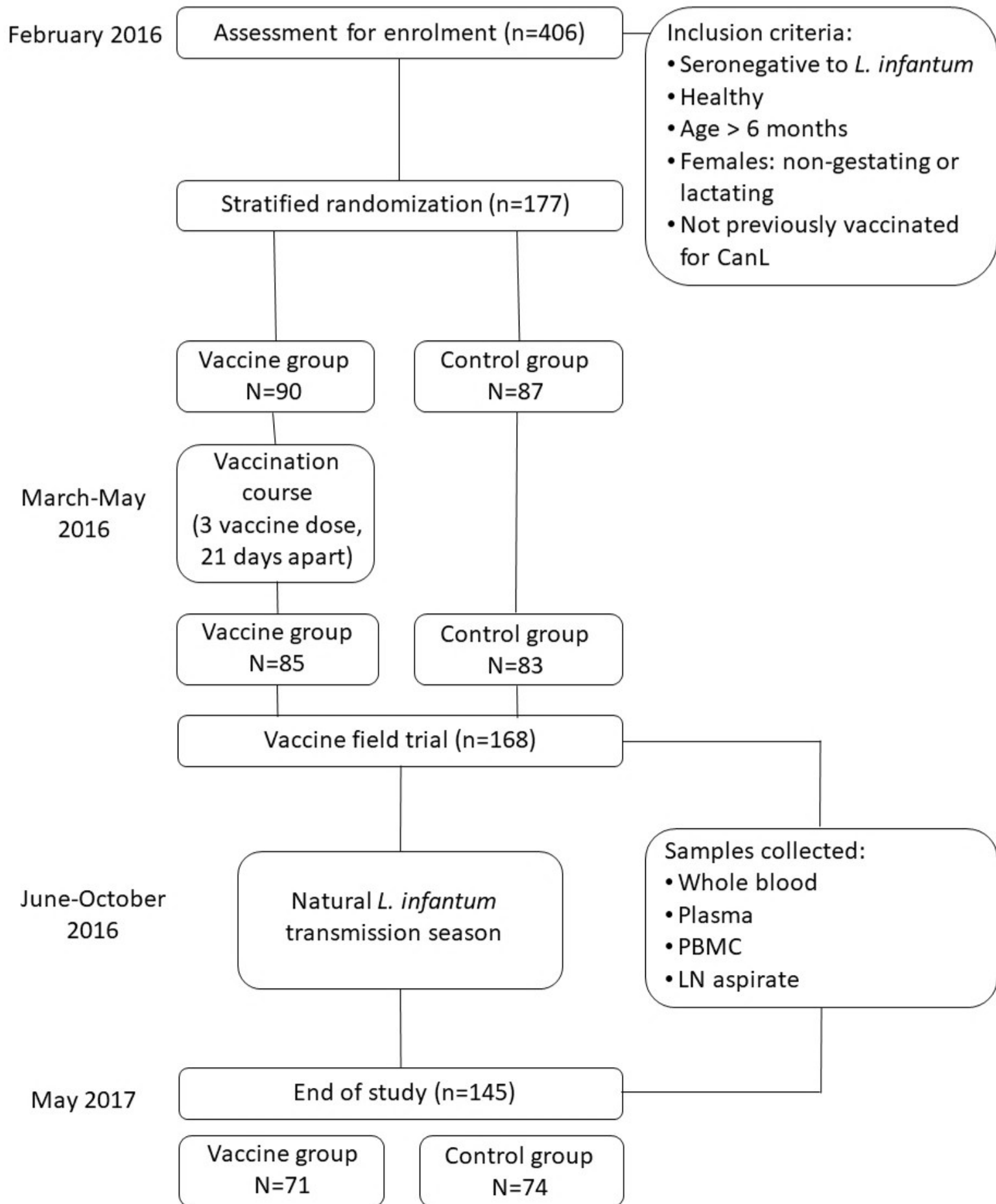760 Figure 1. Flow chart of pre-vaccination procedures and vaccine field trial.

761

762 Figure 2. Map of Girona province. Field trial locations are marked in black circles; the number

763 of study dogs per location (n) is presented.

764

765 Figure 3. Median and interquartile ranges of IFN-γ levels observed in the vaccine and control

766 groups at three time points: before vaccination (T0), one month after vaccination completion

767 (1M) and nine months after vaccination completion (9M). (a) Within group comparison with

768 T0; (b) within group comparison with 1M; (c) between group comparison. (**) indicates

769 statistical significance of p≤0.01; (***) indicates statistical significance of p≤0.001.

770

771 Supplementary figure S1. Levels of IFN-γ observed in infected and non-infected dogs at three

772 time points: before vaccination (T0), one month after vaccination completion (1M) and nine

773 months after vaccination completion (9M). Panel A: includes all dogs from both vaccine and

774 control groups. Panel B: includes only dogs from vaccine group.

February 2016 — Assessment for enrolment (n=406)

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

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

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

May 2017 — End of study (n=145)

Vaccine group
N=71

Control group
N=74

PBMC: peripheral blood mononuclear cells; LN: lymph node