TITLE: The impact of canine leishmaniosis vaccination with CaniLeish® in *Leishmania infantum* infection seroprevalence studies

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ABSTRACT

Effective vaccines against *Leishmania* parasites are a goal for the scientific community working with both canine and human leishmaniosis. However, possible side effects of vaccination should also be considered and evaluated, preferably before vaccine licensing and marketing. One of these possible effects is the cross-reaction of vaccine-induced antibodies with standard serological tests for detection of *Leishmania infantum* infection. Longitudinal studies were performed on the type of humoral profile induced by Brazilian marketed canine leishmaniosis vaccines, but little is known regarding the European situation. In this study, an annual follow-up of 85 CaniLeish® vaccinated dogs and 83 non-vaccinated control dogs was performed. Blood samples were taken for all animals at pre-determined time points: before vaccination; immediately before each one of the two following vaccine doses (at 21 days intervals); and then one, four, six, nine and 12 months after finishing the vaccination course. All samples were tested by an in-house ELISA, using a whole promastigote antigen, for the presence of anti-*L. infantum* antibodies. Humoral response detectable by the used serological diagnostic method was significantly higher in the vaccine group when compared with the control group ($p<0.01$) until one-month post-vaccination. Results show that CaniLeish® vaccine-induced antibodies cross-react with a commonly used serological test for diagnosis of *L. infantum* natural infection. Implications of this interference are discussed, with special emphasis on a possible negative impact on canine leishmaniosis surveillance studies.

KEYWORDS: canine leishmaniosis; vaccine; serological diagnostic tests; *Leishmania infantum* epidemiological surveillance.

1. INTRODUCTION

Vaccination is considered one of the most effective methods of controlling canine leishmaniosis (CanL) and, indirectly, human leishmaniosis (HL) (Palatnik-de-Sousa et al., 2009). Mathematical models have shown that this method could be more effective than treatment or culling of infected dogs (Dye, 1996). A vaccine for CanL should induce a strong, parasite-specific and long-lasting cellular mediated immunity to control infection progression, as well as block *Leishmania infantum* transmission to sand fly vectors by significantly reducing parasite burden at the vertebrate host level (Gradoni, 2015). It should also be equally effective in protecting against infection or disease (Alvar et al., 2013). A possible side effect of most vaccines is the stimulation of humoral immunity and the consequent induction of antibody production (Solano-Gallego et al., 2017a). These can be vaccine-specific and undetectable by common serological tests for *L. infantum* infection diagnosis. However, vaccines can also elicit the production of non-specific antibodies that cross-react with standard diagnostic tests (Marcondes et al., 2013). In these cases, vaccinated individuals cannot be differentiated from naturally infected animals (Marcondes et al., 2011).

Cross-sectional seroprevalence surveys are the most common type of assessment of *L. infantum* infection in canine populations from endemic areas. These methods are simple and straightforward to perform and interpret, being particularly useful in field study settings (Maia and Campino, 2008). Furthermore, quantitative serological techniques can provide a reasonably accurate characterization of *L. infantum* infection in a population, as diseased dogs tend to present significantly higher anti-*Leishmania* antibody readings than exposed or asymptomatic individuals (Oliva et al., 2006). Whole antigen enzyme-linked immunosorbent assay (ELISA) and immunofluorescence antibody test (IFAT) are two of the most commonly used quantitative

serological techniques, being the recommended screening tests by the World Organization for Animal Health (OIE) for CanL prevalence and surveillance studies (OIE, 2018).

CaniLeish® vaccine (Virbac) was licensed in Europe in 2011 (EMA, 2011). It is a second-generation vaccine, composed of purified excreted-secreted proteins of *L. infantum* (LiESP) and a saponin adjuvant (QA-21) from a purified fraction of *Quillaja saponaria* (Moreno et al., 2012). Pre-licensing CaniLeish® field trial (Oliva et al., 2014) showed that vaccine efficacy in the prevention of CanL clinical signs is of 68.4% and the risk of developing active disease is reduced by 3.6 times in vaccinated dogs (EMA, 2016). This field trial also reported that vaccine-induced antibodies were detected by a diagnostic IFAT, which was confirmed by two later vaccine follow-up reports (Sagols et al., 2013; Montoya et al., 2017).

Speed Leish K™, a qualitative immunocromatographic test (ICT), is the pre-vaccination screening method recommended by CaniLeish® vaccine manufacturer (Virbac, no date). The test antigen is composed of a complex of recombinant *L. infantum* kinesins (Ferroglio et al., 2013). During vaccine follow-up studies, it showed no (Sagols et al., 2013) or low (Montoya et al., 2017) cross-reactivity with vaccine-induced antibodies and its use as a diagnostic tool able to discriminate between vaccinated and infected individuals has been proposed. However, reports of this ICT performance are not consistent (Ferroglio et al., 2013; Solano-Gallego et al., 2014) and its sensitivity in *L. infantum* detection has been questioned (Solano-Gallego et al., 2017a).

In the present study, a one-year follow-up of CaniLeish® vaccinated dogs was performed and an in-house ELISA test using whole antigen was used to measure anti-*L. infantum* antibodies at pre-determined time points. The results reported are expected to provide information on the possible impact of CaniLeish® vaccination on *L. infantum* seroprevalence studies and to motivate the need to rethink CanL surveillance and control measures in endemic areas where vaccination has been implemented.

2. MATERIALS AND METHODS

2.1. Study population and study design

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, 168 dogs, assessed by an in-house ELISA to be seronegative for specific *L. infantum*-antibodies, were selected. Additional inclusion criteria were: normal clinical exam, minimum age of 6 months, non-gestating or lactating females (either at the beginning of the study or expected to be so during the trial), and no previous vaccination against CanL. These were all owned dogs, used mainly for hunting, but some breeding and racing individuals were also included. All animals were kept in large packs in open-air facilities, mostly in rural and periurban areas. Selected animals were distributed across 12 locations, with the number of individuals per location ranging from 4 to 23. No insecticide treatments were applied to the dogs at any time throughout the course of the study.

Dogs were randomly assigned to either vaccine (n=85) or control (n=83) groups. Individuals from the vaccine group received a three dose CaniLeish® vaccine course, 21 days apart, following the protocol recommended by the vaccine's manufacturer. Dogs in the control group did not receive any vaccine dose. Individuals were sampled at eight pre-determined time points, three of which corresponding to the immunization period [T1, prior to the first vaccine dose (n=168); T2, prior to the second vaccine dose (n=165); and T3, prior to the third vaccine dose (n=168)], and to five time points after completion of the vaccination protocol [T4, one month (n=167); T5, four months (n=33); T6, six months (n=161); T7, nine months (n=152); and T8, twelve months (n=145) after the third

144 vaccine dose]. Blood was collected from the cephalic or jugular veins to EDTA tubes, centrifuged for
145 plasma isolation and frozen at -40°C until processing. Samples from the same individual were
146 analysed in parallel.
147
148 *2.2. Serological technique*
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150 Plasma samples were analysed by an in-house ELISA, using whole sonicated promastigote antigen,
151 for the presence of anti-*L. infantum* antibodies, as previously described (Riera et al., 1999; Velez et
152 al., 2019). Briefly, dog plasma samples diluted at 1:400 were incubated in titration plates (Costar®)
153 previously coated with sonicated whole promastigotes at a protein concentration of 20 µg/ml in
154 0.05 M carbonate buffer at pH 9.6. Protein A peroxidase ((1:30,000, Sigma®) was used as conjugate
155 and reactions were stopped with H_2SO_4 3M when a pre-determined positive control serum reached
156 an optical density of 450 read at 450 nm. Sample optical densities were then read at 492 nm. All
157 samples were run in duplicate and calibrator, positive and negative serums were included in all
158 plates. Results were expressed in standard units (U) compared to a calibrator control sample set
159 arbitrarily at 100U. The cut-off was established at 24U.
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161 *2.3. Statistical analysis*
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163 All statistical analyses were performed using Stata 15 software (StataCorp LP, College Station, TX,
164 USA). ELISA OD results did not present a normal distribution and normality could not be achieved
165 by data transformation. Therefore, comparisons between groups at each time point were
166 performed by the non-parametric Mann-Whitney *U* test. Statistical significance of difference in
167 proportions between groups was tested by the Pearson Chi-square test.

3. RESULTS

Humoral response to whole *L. infantum* antigen in the trial groups is summarized in Figure 1. During the immunization course (T1, T2 and T3), vaccinated dogs showed a progressive increase in anti-*L. infantum* antibody levels, which peaked at one-month post-vaccination (T4). Differences between groups at T2 [median (vaccine group)=16.2; median (control group)=14.0; z=-3.120; p=0.002], T3 [median (vaccine)=24.1; median (control)=14.1; z=-7.149; p<0.001] and T4 [median (vaccine)=32.3; median (control)=16.6; z=-7.052; p<0.001] were considered statistically significant. At T2, 27.1% (23/85) of vaccinated dogs would be considered seropositive [in comparison with 8.8% (7/80) in the control group], at T3 seropositivity would be of 50.6% (43/85) amongst vaccinated dogs [against 10.8% (9/83) in controls], and at T4 the proportion of seropositive vaccinated dogs would be of 74.1% (63/85) [20.7% (17/82) in controls]. Differences in proportions of seropositive individuals between vaccine and control groups at these sampling points (T2 to T4) were considered statistically significant (p<0.01).

After this (T5-T8; four to twelve months after vaccination completion), antibody levels between groups followed a similar trend, with no statistically significant differences detected between groups, except for T7 (in January 2017, 9 months post-vaccination) [median (vaccine)=16.7; median (control)=14.2; z=-2.010; p=0.044].

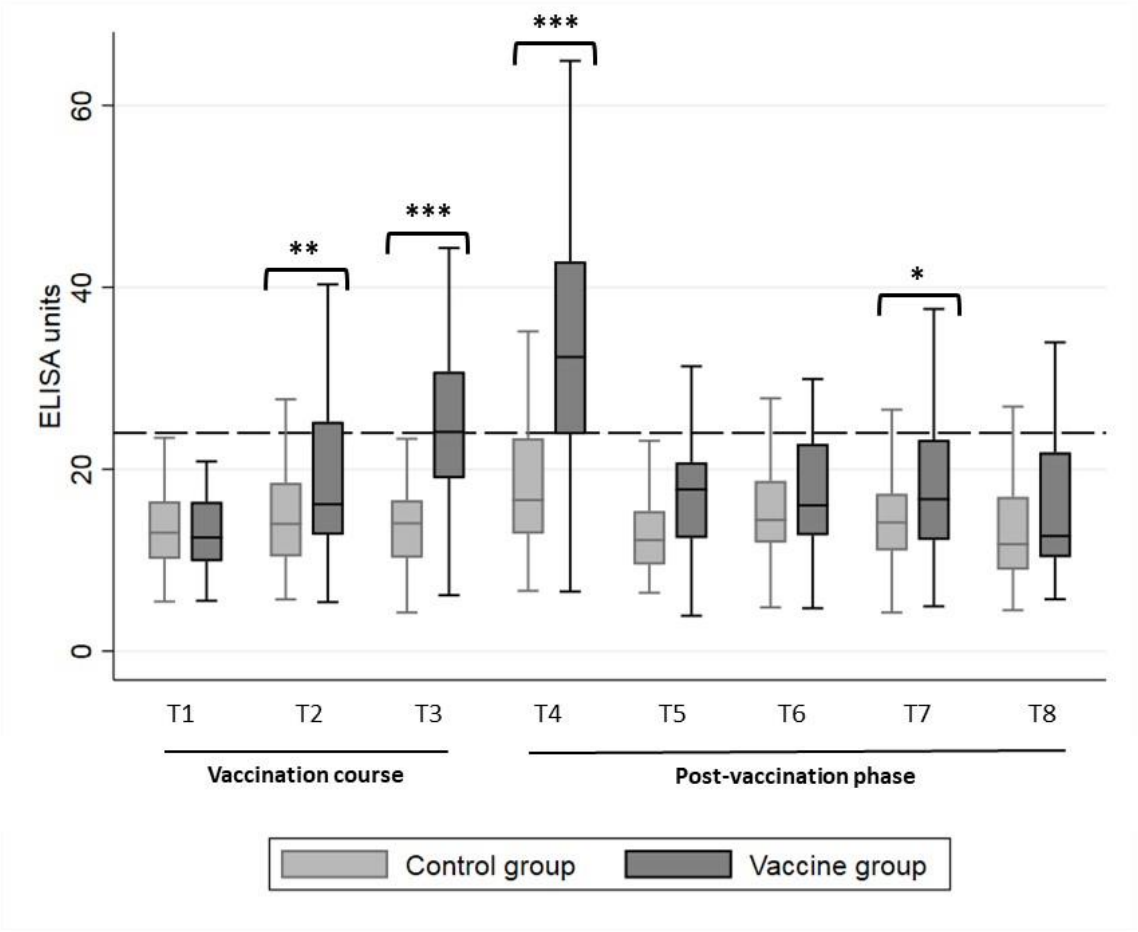

Figure 1. Median and interquartile range ELISA units observed in control and vaccine groups at each sampling point. The time points under "Vaccination course" refer to samplings performed during the immunization period (prior to the first (T1), second (T2) and third (T3) vaccine doses); the "Post-vaccination phase" corresponds to the period after completion of the vaccination protocol (one (T4), four (T5), six (T6), nine (T7) and twelve (T8) months after the third vaccine dose). Statistically significant differences between groups assessed by a Mann-Whitney *U* test are marked with asterisks: *p <0.05; **p<0.01; ***p<0.001. The horizontal dashed line marks the ELISA cut-off, set at 24U. Outlier results are not represented in the figure, but were considered for the statistical analysis.

4. DISCUSSION

The development of effective vaccines for CanL and HL should be the ultimate goal for disease control (Dye, 1996; Gramiccia and Gradoni, 2005; Palatnik-de-Sousa, 2012). However, despite the obvious advantages of vaccination, possible drawbacks of its implementation should also be considered. Vaccines which are unable to block parasite transmission have the detrimental effect of "masking" vaccinated asymptomatic carriers (Miró et al., 2017). These animals, although possibly showing a lower susceptibility to developing active *L. infantum* infection or clinical disease, can still harbour the parasite and play a potential role in maintaining its life cycle in endemic areas (Solano-Gallego et al., 2001). Furthermore, vaccines with low or only moderate efficacy do not prevent disease in all vaccinated dogs, and these represent a potential diagnostic challenge (Solano-Gallego et al., 2017a).

"Differentiating between infected and vaccinated animals" (DIVA) is a well-known concept in veterinary vaccinology (Liu et al., 2013; Solano-Gallego et al., 2017a). According to DIVA principle, veterinary vaccines should be produced in such a way to allow serological differentiation between vaccinated and infected animals. This differentiation can be achieved by the non-interference with standard serological techniques or through the development of specific diagnostic tests, which should present high specificity and sensitivity (Schmitt, 2005). In any case, this aspect must be considered during the development of any new vaccine.

The impossibility of distinguishing between vaccinated and naturally infected dogs can introduce considerable restrictions to disease or infection diagnosis and surveillance, especially in endemic areas (Solano-Gallego et al., 2017a). CanL serves as a good example of this problem. The diversity of possible infection outcomes and the high proportion of asymptomatic infected animals (Baneth et al., 2008), make CanL a diagnostic challenge that often requires the use of multiple diagnostic

methods (Otranto et al., 2009; Morales-Yuste et al., 2012). After the introduction of Leishmune® in Brazil, a second-generation vaccine composed of the fucose-mannose ligand (FML) glycoprotein complex of *L. donovani*, several studies suggested the possibility of vaccine-induced antibodies cross-reacting with CanL official diagnostic tests (Marcondes et al., 2011, 2013). The CanL Brazilian control programme consists of individual screening with an immunocromatography assay, composed of a recombinant rK26/rK39 fusion protein of *L. infantum* (DPP®-CVL rapid test, Bio-Manguinhos/Fiocruz), followed by a confirmatory commercial ELISA kit, which uses soluble antigens of *L. major*-like parasites (EIE®-CVL, Bio-Manguinhos/Fiocruz). Despite the differences observed between both tests in the detection of vaccine-induced antibodies, with the ELISA showing a higher seropositivity rate when compared to the DPP® test, both assays presented false-positive results in vaccinated dogs (Marcondes et al., 2013). In a country where detection and culling of infected dogs is the control measure established by the Ministry of Health (Ministério da Saúde Brasileiro, 2014), this would pose a risk to healthy vaccinated dogs, which could be mistakenly identified as naturally infected individuals and removed (Marcondes et al., 2013). A more recent study demonstrated that Leishmune® vaccinated dogs did not test positive with the fast agglutination screening test (FAST) or the direct agglutination test (DAT) (Ribeiro et al., 2015), both based on whole *L. donovani* promastigote antigen, which could eventually be used as confirmatory diagnostic methods for seropositive vaccinated dogs. Meanwhile, Leishmune® vaccine was withdrawn from the market by the Brazilian Ministry of Agriculture due to lack of effectiveness evidence in phase III trials (MAPA, 2014) and no further cross-reactivity studies were performed.

CaniLeish® vaccine was licensed in Europe in 2011. Its multi-antigenic, non-recombinant composition makes distinction of vaccine and infection-induced antibodies particularly difficult and hampers the development of differentiating diagnostic tests. Results of preliminary studies on vaccine's safety and efficacy showed that vaccine-induced antibodies could be detected by

commonly used IFAT, which consisted of an in-house (Oliva et al., 2014) or commercial technique (Martin et al., 2014) using whole promastigote antigen. The use of IFAT to test for *L. infantum* infection in CaniLeish® vaccinated dogs was not recommended, as these animals consistently presented positive titres due to vaccine-induced antibodies (Oliva et al., 2014). This has also been confirmed by a long-term follow-up of owned CaniLeish® vaccinated dogs, in which 31.9-40.3% and 3.2% of individuals tested positive on IFAT one month and one year after vaccination, respectively (Montoya et al., 2017), while another study reported 80% seropositivity with IFAT one month after the first annual vaccine booster (Sagols et al., 2013).

Results from the longitudinal study presented here show that a similar situation occurs when an in-house ELISA with whole promastigote antigen is used. This assay, developed for the detection of *L. infantum* infection in the canine host, as well as in other animal species, has been widely used in CanL research (see Velez et al., 2019 for a comprehensive list of references), in the study of other possible reservoirs of *L. infantum* (Portús et al., 2002; Solano-Gallego et al., 2003), as well as in studies of human leishmaniosis (Fisa et al., 2002; Riera et al., 2004). In this study, vaccine-induced antibodies were detected by this commonly used *L. infantum* diagnostic ELISA technique three weeks after the first vaccine dose and continued to rise until they peaked one month after vaccination completion (T4). At this time point, 74.1% of vaccinated dogs would be classified as seropositive to *L. infantum*. Three months later (four months after the third vaccine dose, T5), antibody levels in the vaccine and control groups did not show a statistically significant difference and the same non-significant results were observed at six- and 12-months post-vaccination. These results show that CaniLeish® vaccinated dogs have a high probability of testing positive by this ELISA test until one-month post-vaccination (T4). Unfortunately, the absence of data between this time point and the following one (T5, four months post-vaccination), which showed a marked decrease in vaccine-induced humoral immunity (in comparison with the control group), did not allow

detection of an antibody inflexion point. It should also be mentioned that the reduced number of animals tested four months after vaccination (n=33), due to the constraint of dog owners' availability, could be a possible reason for the failure in detecting a significant difference between groups. Statistically significant differences in ELISA results between groups were again detected nine months after vaccination (T7). However, because this assessment was preceded by two sampling points where no significant differences between groups were observed and since it corresponds to a post-transmission season sampling, the observed difference is more likely due to natural contact with the parasite than to a vaccine effect. The rise in anti-*L. infantum* antibody levels after the transmission season (when compared to T1) was only discrete, which can be explained by the observed incidence of seropositivity and infection at the end of the trial (17.6% and 5.4%, respectively, in the control group). All infected dogs presented clinical signs and/or laboratory findings compatible with CanL. Dogs included in this trial were not tested for other vector-borne agents, which could cross-react with a whole *Leishmania* antigen assay inducing false-seropositive results, however, the potential bias introduced would affect both trial groups in a similar way and would not be expected to significantly influence the overall study results.

The impact of serological diagnostic tests failure in differentiating between vaccinated and naturally infected dogs at the individual level is well documented. Several reports describing CanL cases in CaniLeish® vaccinated dogs highlight the added complexity on the diagnosis of these animals (Ceccarelli et al., 2016; Gavazza et al., 2016; Solano-Gallego et al., 2017b). Unfortunately, no information exists on the impact of vaccination on *L. infantum* infection serological surveillance. Diagnostic techniques for CanL large-scale surveys should be simple to perform and interpret, low-cost and highly sensitive and specific. Due to the variable clinical presentation of *L. infantum* infection, quantitative tests, which can provide an assessment of infection stages across the community, should be preferred to qualitative ones. Furthermore, techniques based on whole crude

297 *Leishmania* antigen are usually associated with a higher diagnostic sensitivity than single antigen-
298 based assays, mainly due to a greater capacity in detecting the heterogeneous range of individual
299 immune responses expected to be present in endemic settings (Morales-Yuste et al., 2012). Finally,
300 survey techniques must be applicable to the whole population studied so that results can be
301 comparable and conclusions can be drawn. Considering these points, quantitative whole antigen-
302 based serological methods still remain the best tools for *L. infantum* infection mass-screening
303 surveys (OIE, 2018) and have been used for many years in epidemiological studies on CanL in
304 endemic Mediterranean countries (Acedo-Sanchez et al., 1986; Amela et al., 1995; Fisa et al., 1999;
305 Dereure et al., 2009; Maroli et al., 2008; Gálvez et al., 2010; Ballart et al., 2013; Maia et al., 2013;
306 Piantedosi et al., 2016). Rapid qualitative serological techniques, aside from only providing
307 dichotomous results, can also show lower sensitivity in infection detection (Maia and Campino,
308 2018). In the case of Speed Leish K™, the CaniLeish® recommended pre-vaccination screening and
309 post-vaccination diagnostic test, reported performance results were inconsistent. Although a
310 preliminary comparative study of this ICT with IFAT and Western blot (WB) showed very high test
311 sensitivity and specificity (Ferroglio et al., 2013), a later study did not confirm these results,
312 considering Speed Leish K™ inferior to all the quantitative serological tests evaluated (Solano-
313 Gallego et al., 2014). The use of a less sensitive screening test in epidemiological studies, even if it
314 holds the advantage of not reacting with vaccine-induced antibodies, compromises infection
315 detection and yields false lower prevalence and incidence rates. Likewise, the use of this test in pre-
316 vaccination screening may produce apparent vaccine failure due to vaccination of previously
317 infected dogs (Solano-Gallego et al., 2017a).
318 Considering the results presented here and others previously obtained, a critical appraisal of the
319 methods currently used for CanL epidemiological surveillance must be performed. The need to
320 either change or complement the currently used diagnostic techniques, mostly based in whole

antigen assays, and/or to develop new, more efficient, diagnostic methods capable of differentiating between vaccinated and naturally infected individuals is urgently needed.

5. CONCLUSIONS

CaniLeish® vaccination induces the production of antibodies which cross-react with a whole crude antigen in-house ELISA, a commonly used serological method for the detection of *L. infantum* infection. According to the results presented here, antibody levels start increasing after the first vaccine dose and peak one month after vaccination completion, when 74.1% of vaccinated dogs would be classified as seropositive by the ELISA test. The growing number of vaccinated dogs in endemic countries raises the need to rethink current CanL diagnosis and surveillance methodologies.

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. The project was also submitted to and approved by ISGlobal Internal Scientific Committee (ISC). 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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