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Review

Commercially approved vaccines for canine leishmaniosis: a review of available data on their safety and efficacy

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Abstract Canine leishmaniosis is an important vector-borne zoonosis caused mainly by *Leishmania infantum*. Diagnosis and treatment of affected individuals can be particularly complex, hindering infection control in endemic areas. Methods to prevent canine leishmaniosis include the use of topical insecticides, prophylactic immunotherapy and vaccination. Four vaccines against canine leishmaniosis have been licensed since 2004, two in Brazil (Leishmune®, the production and marketing licence of which was withdrawn in 2014, and Leish-Tec®) and two in Europe (CaniLeish® and LetiFend®). After several years of marketing, doubts remain regarding vaccine efficacy and effectiveness, potential infectiousness of vaccinated and infected animals or the interference of vaccine-induced antibodies in *L. infantum* serological diagnosis. This review summarises the scientific evidence for each of the vaccines commercially approved for canine leishmaniosis, while discussing possible weaknesses of these studies. Furthermore, it raises the need to address important questions related to vaccination impact in *Leishmania*-endemic countries and the importance of post-marketing pharmacological surveillance.

keywords canine leishmaniosis, post-marketing pharmacological surveillance, vaccine effectiveness

Sustainable Development Goals (SDGs): SDG 3 (good health and well-being)

Introduction

Leishmaniosis is a vector-borne zoonosis caused by *Leishmania* spp. (Kinetoplastida: Trypanosomatidae) protozoan parasites. The disease is distributed worldwide and is considered endemic in tropical and sub-tropical regions [1,2]. *Leishmania infantum* (syn. *L. chagasi*) is the species responsible for a zoonotic form of the disease where the domestic dog is the main reservoir host and which is widespread in the Mediterranean region, Middle East, Central Asia and in some countries of Central and South America [3]. Vectors implicated in disease transmission belong to the genus *Phlebotomus* in the Old World and *Lutzomyia* 'sensu Young and Duncan, 1994 [4]' in the New World (both Diptera: Psychodidae: Phlebotominae) [5,6]. Canine leishmaniosis (CanL) is a potentially severe and fatal disease, although the infection outcome in individual dogs is highly variable and dependent on each animal's immune response [7]. Available CanL treatments are not totally effective and rely on the detection of infected animals, many of which are asymptomatic carriers [8]. Topical application of repellent and insecticide treatments on dogs living in endemic areas is one of the most frequently used methods of disease prevention [9,10]. However, even when correctly used, these products cannot protect against all infectious bites and there is still a need for further control measures [11]. Insecticide environmental and indoor spraying, early detection and treatment of infected dogs, preventive administration of immunomodulators, and owners' awareness and compliance can also play an important role in infection control [12,13]. Dog culling has also been recommended in some endemic countries to control human and CanL, but its effectiveness and ethical implications are under debate [14]. According to the WHO [15], vaccination is probably the best way of controlling a vector-borne disease such as leishmaniosis and research aimed at developing vaccines for HL has been ongoing [16]. Vaccines against CanL have been trialled

and licensed in Brazil and Europe, but an effective vaccine available worldwide is still lacking [17].

Four vaccines are or have been commercialised for prevention of CanL: Leishmune® and Leish-Tec® in Brazil and CaniLeish® and LetiFend® in Europe. In this review, published studies on each of the vaccines marketed for CanL are summarised and the information available for each vaccine is presented and discussed.

Commercially Approved Vaccines for CanL

Leishmune®

Leishmune® (Fort Dodge Wyeth, later Zoetis, Brazil) was the first licensed vaccine for CanL, registered in Brazil in 2004. It is a second-generation vaccine, composed of the fucose-mannose ligand (FML) of *L. donovani*, and a saponin adjuvant [18]. Vaccination protocol consisted of three vaccine doses administered subcutaneously every 21 days to dogs four months old or older, followed by annual boosters [19]. The Leishmune® vaccine production and marketing licence was withdrawn in 2014 by the Brazilian Ministry of Agriculture due to lack of effectiveness evidence in phase III trials [20].

FML-vaccine immunogenicity was first tested in murine models, where it was able to induce specific seroconversion, mainly of the IgG2 subtype, enhanced lymphoproliferative response to GP36, which is considered to be the main antigen of the FML complex [21], and a positive delayed-type hypersensitivity (DTH) reaction to promastigote lysate of *L. donovani* [22–25]. Vaccination of Balb/c mice with FML plus saponin [23,24], or with purified GP36 plus saponin [25], was considered to induce significant protection against experimental infection with *L. donovani* by significantly reducing the parasite burden in the liver of previously immunised animals, when compared with the control groups. The same criteria were used to prove cross-protective efficacy of the FML-vaccine against *L. chagasi* infection [26].

FML-vaccine efficacy in preventing CanL was tested in two phase III field trials conducted in a zoonotic visceral leishmaniosis (ZVL) endemic area (São Gonçalo do Amaranto, Rio Grande do Norte, Brazil). FML antigen (1.5 mg) plus Riedel de Haën saponin (R) [27] was used in the first study, which included 117 owned dogs followed for a two-year period [28]. Vaccinated dogs showed vaccine-specific seroconversion and positive DTH reaction, which lasted until the end of the trial. Based on the number of dog obits due to CanL observed during the study (4 in the control group versus no deaths in the vaccine group), and the incidence of confirmed *L. infantum* infections (characterised by presence of clinical signs, seropositivity to FML-Enzyme-Linked Immunosorbent Assay (ELISA) and DTH response, followed by parasite detection in post-mortem samples), a vaccine efficacy (VE) of 76% [27] and a protection against disease of 92% were obtained. However, lack of sample randomisation or blinded evaluation of trial individuals [29], and other methodological shortcomings, such as an unclear use of criteria to signal infected or diseased dogs in both study groups, did not allow fully validation of such results. A vaccine formulation composed of FML antigen (1.5 mg) plus QuilA saponin adjuvant (1 mg) was used in the second field trial, performed in the same ZVL endemic area [18]. This study included 85 owned dogs and followed a similar CanL case detection methodology to the one presented by da Silva et al. [28]. Again, vaccinated dogs presented specific seroconversion detected by an FML-ELISA and positive DTH. After a 3.5-year follow-up, 8/41 of control dogs and 1/44 of vaccinated dogs were diagnosed with CanL, yielding an 80% VE and 95% vaccine protection against CanL.

The phase III trials were followed by a large-scale field study, which included 600 owned dogs living in two CanL endemic areas (Araçatuba, São Paulo State; and Belo Horizonte, Minas Gerais state; both in Brazil) [30,31]. For the first time, the licensed formulation of the FML-vaccine (Leishmune®), composed of 1.5 mg of FML antigen and 0.5 mg of saponin adjuvant, was used in a field trial. Leishmune® vaccine proved to be safe and well-tolerated, as no severe adverse reactions were observed during the vaccination course. Mild adverse reactions detected after the first vaccine dose were transient and dissipated before the following vaccine administration [30]. A subgroup of 550 vaccinated dogs was further followed for a two-year period in an immunogenicity trial [31]. Apart from the vaccine-specific seroconversion and positive DTH to *L. donovani* antigen previously demonstrated by other studies, peripheral blood lymphocyte phenotypes were characterised by flow cytometry in a subsample of 15 randomly selected vaccinated dogs. Samples were taken 18 months post-vaccination, showing sustained CD4+ lymphocytes and a rise in CD8+ and CD21+ populations when compared with a group of unvaccinated healthy controls ($n = 9$) from a different CanL endemic area in Brazil. For this analysis, no pre-vaccination results were provided for the vaccinated individuals. Furthermore, due to ethical reasons, a control group ($n = 588$) living in another CanL endemic area (Jardim Progresso, Natal, Brazil) was used to compare *L. chagasi*-induced morbidity and mortality between vaccinated and non-vaccinated dogs. Study results revealed 98.8% asymptomatic dogs (at the end of the first year) and 99% healthy survivors (at the end of the

second year) amongst vaccinated dogs, while the untreated exposed cohort presented 79.4% asymptomatic and 61% survivor dogs. However, any statistical comparisons made between vaccine and control groups were hindered by possible differences in location infection pressures, as well as for the distinct criteria used to diagnose infection in dogs from the vaccine group (clinical signs, polymerase chain reaction (PCR) and parasitological assays) and the control group (seropositivity and euthanasia). Authors claimed a 66.1% ($P < 0.005$) and an 80.2% ($P < 0.005$) reduction in the incidence of CanL amongst vaccinated dogs in the two trial locations, when compared with the global incidence of the disease in the same regions [31].

Subsequent studies confirmed the selective T-cell dependent profile promoted by Leishmune®, particularly associated with the up-regulation of CD8+ lymphocytes [32,33]. Immunisation with Leishmune® induced an immunological pattern characterised by enhanced levels of IFN-γ, NO and anti-*L. chagasi* IgG2 [33–36]. Cross-sectional evaluation of dogs at different time points after Leishmune® immunisation allowed for the confirmation of increased levels of IFN-γ and IL-8 at one and six months post-vaccination, which returned to basal values at 12 months post-vaccination and before the annual booster. A higher production of IL-17 and TNF-α by T-lymphocytes was also observed [36,37]. Likewise, regulatory cytokines, such as IL-4 and IL-10, suffered suppression during the first six months after immunisation [36,37], while another study did not find changes in IL-4 levels 10 days after the last dose of Leishmune® [35]. Both CD4+ and CD8+ T-cell subsets were implicated in the production of pro-inflammatory cytokines [37]. Vaccination with Leishmune® was also able to elicit a protective innate immune response profile, through the stimulation of neutrophils and monocytes [33,36].

A Leishmune® formulation with double saponin adjuvant concentration (1 mg) was investigated as a possible immunotherapeutic vaccine both in experimentally and naturally infected dogs [38–40]. A three-dose course vaccination of seropositive asymptomatic dogs was able to confer positive DTH results to the majority of vaccinated individuals, to extend the asymptomatic state and to reduce CanL-induced mortality, when compared with untreated infected controls [38,39]. Another study, with experimentally infected dogs, could not find any differences between DTH responses, deaths attributable to CanL or parasite detection by PCR on bone marrow samples between immunised and untreated controls [40]. Lymphocyte phenotyping revealed an increment of CD8+ T cells in FML-treated dogs [38], while no changes in lymphocyte subgroups were detected in a later study [40]. The comparison between immunotherapy with enriched-Leishmune® alone or in association with allopurinol or allopurinol + amphotericin B treatment resulted in a discrete advantage of the immunochemotherapeutic protocol [39].

Leishmune® was considered to be a transmission-blocking vaccine based on the assumption that vaccinated dogs could not be infectious to sandflies as no CanL clinical signs or *Leishmania* DNA could be detected in these animals [41]. For the same purpose, Saraiva et al. [42] demonstrated that FML-induced antibodies present in dog sera were capable of inhibiting *L. donovani* and *L. chagasi* procyclic promastigote-binding to dissected *L. longipalpis* midgut. In a comparative study of Leishmune® and Leish-Tec® vaccines, 5.1% (2/39) of Leishmune®-vaccinated dogs were infectious to sandflies by xenodiagnosis, against a 36.6% (11/30) infectiousness in the control group [43]. These proportions were based on the total sample of Leishmune®-vaccinated dogs ($n = 39$) and not on the number of vaccinated and infected animals ($n = 4$), which would be the only ones capable of parasite transmission.

Vaccination of dogs with Leishmune® was claimed to reduce CanL and human leishmaniosis (HL) incidence in Brazilian endemic areas [44] which, in the case of an increased vaccine coverage, could prove to be more effective in controlling *Leishmania* infection than dog culling, the method currently adopted by the Brazilian Ministry of Health [45]. The study reported results on the detection of canine and human infection cases before and after Leishmune® introduction in regions subject to different vaccination coverage rates. Official reports from the Ministry of Health's Centre for Zoonosis Control and pharmacovigilance data from the vaccine manufacturer and local veterinarians were used. After two years of vaccine use, a correlation was found between the number of vaccinated dogs and a decrease in CanL and HL cases [44].

This study also included the results of a canine serological screening for *L. chagasi* with the official tests used at that time (commercial ELISA and Indirect Fluorescent Antibody Technique (IFAT), both from Biomanguinhos, Fiocruz, Rio de Janeiro, Brazil), showing that from a population of 5860 vaccinated dogs, only 1.3% were considered seropositive. From these, none was positive in a confirmatory test (an ELISA test based on a recombinant anti-heat shock protein (HSP) of *L. chagasi*, Biogen) or presented visible parasites in lymph node or bone marrow smears [44]. In contrast, Marcondes et al. [46,47] detected sustained seropositivity up to six months post-vaccination in Leishmune®-immunised dogs, which could not be differentiated by the official diagnostic tests in 11.1–72.2% of cases if the ELISA kit was used, while

5.5–33.3% of vaccinated dogs would be detected by the DPP® test (Dual Path Platform® CVL rapid test, Biomanguinhos, Fiocruz, Rio de Janeiro, Brazil). In samples collected from 71 dogs 45 days after the first Leishmune® annual booster, seropositivity was detected in 5.8% and 1.4% with the official ELISA kit and the DPP® test, respectively [48]. De Amorim et al. [49] described different humoral immunological profiles for Leishmune®-vaccinated and naturally infected dogs depending on the antigen used [FML vs *Leishmania* soluble antigen (LSA)], showing that the type of antigen employed interfered with immunoglobulin detection, but without allowing a clear distinction between the two groups of dogs.

Leish-Tec®

Leish-Tec® (Hertape Calier Saúde Animal, later Ceva, Brazil) is formulated with a recombinant protein A2 from *L. donovani* amastigotes and saponin as vaccine adjuvant. It was licensed in Brazil in 2007 and is currently the only authorised CanL vaccine in that country. It should be administered to dogs four months or older, and the primary vaccination course consists of three doses, administered subcutaneously at 21-day intervals, followed by annual boosters [50].

Experiments in murine models showed that immunisation with the recombinant A2 protein conferred a high degree of protection to experimentally challenged BALB/c mice, evaluated by levels of parasite burden in the liver of vaccinated and control animals [51]. The humoral immune response elicited by the vaccine was highly specific, and vaccine-induced cell-mediated immunity was classified as mixed Th1-Th2. Splenocytes of vaccinated individuals produced significantly increased levels of IFN-γ in the presence of A2 antigen when compared with the control group, while no difference in the production of IL-4 was detected between groups.

During subsequent research with beagle dogs (n = 21), Leish-Tec® was shown to induce protective immunity against a high dose intravenous infection of *L. chagasi*, but only partial protection against the parasite [52]. Immunised dogs produced increased levels of anti-A2 IgG2 shortly after vaccination, and a significantly higher production of IFN-γ was detected in peripheral blood mononuclear cells (PBMC) of vaccinated dogs when stimulated with A2 antigen or *L. chagasi* total protein extract, while IL-10 levels did not differ from the control group. The appearance of clinical signs was delayed in the vaccine group (one-year post-infection) when compared with the control group (3–6 months), but the parasite was isolated in culture of bone marrow samples from 4 out of 7 vaccinated dogs [52].

Side effects after Leish-Tec® administration were not found to be severe in a safety analysis, which registered a 3.09% rate of mild, site-specific, adverse reactions in vaccinated dogs, against a 0.68% rate in placebo animals [53]. Leish-Tec® did not induce unspecific seroconversion in the large majority of vaccinated animals (69/70), showing no cross-reactions with the Brazilian official diagnostic tests, either the *Leishmania* Promastigote Antigen (LPA)-ELISA or the DPP® test [54]. Vaccination with Leish-Tec® was also considered to significantly reduce the infectiousness of dogs to sandflies, as demonstrated by xenodiagnosis [43]. The same comparative study between Leishmune® and Leish-Tec® found no significant differences between vaccines in elicited humoral response or infection and transmission rates to the sandfly vector; the only difference detected was a higher rate of adverse reactions in the Leish-Tec® group [43].

The first Leish-Tec® field trial included more than 500 dogs, evenly allocated to vaccine and control groups [55]. Vaccine immunogenicity was evaluated by comparing anti-A2 humoral responses, while *L. chagasi* infection was detected by serology (crude antigen ELISA and IFAT) and confirmed by parasite detection in smears, culture or histopathology of dog tissues collected at necropsy. Xenodiagnosis was also performed in a subsample of dogs (n = 154; 77 in each group). According to the criteria used, a significant reduction in the number of CanL cases was observed in the vaccine group. Calculated VE varied according to the criteria applied: results of parasitological tests alone (VE = 71.4%), parasitological tests associated to xenodiagnosis (VE = 58.1%) or seroconversion to A2 (80.8%). The study was unable to demonstrate a reduction in infectiousness in vaccinated dogs, as no statistically significant differences were found in the prevalence of positive sandfly pools feeding on each of the trial groups [55]. In a more recent Leish-Tec® efficacy trial, in which vaccine and control groups consisted of very distinct dog populations (a natural dog population from a VL endemic area for the vaccine group and naïve beagles or mongrel dogs recruited from a VL-free area for the control group), a significant difference in incidence of infection between vaccine (27%; 40/151) and control (42%; 33/78) animals was reported. However, a two-fold higher proportion of diseased dogs amongst the immunised seropositive animals (44%; 18/40), when compared with the placebo group (21.2%; 7/33), was also observed, and no significant differences in histopathological changes at necropsy of seropositive dogs from both groups were detected [56]. The study concluded that Leish-Tec® was not effective in dogs under field conditions and that its use, in combination with the official dog culling programme, would not have

an impact on the incidence of CanL in areas of high transmission. A similar conclusion had been previously reported in a systematic review of the efficacy of prophylactic control measures for CanL, which found an apparent lack of efficacy evidence for Leish-Tec® vaccine [57].

The effectiveness of Leish-Tec® as an immunotherapeutic vaccine was assessed in a recent randomised, double-blinded field trial [58]. A sample of 557 *L. infantum*-seropositive and asymptomatic owned hunting dogs from the United States were enrolled (n = 282 in the vaccine group) and followed over nine months. The risk of clinical progression (RR = 1.33, 95%CI: 1.009-1.786, P = 0.0450) and of all-cause mortality (RR = 3.19, 95% CI: 1.185–8.502, P = 0.0245) were considered higher in placebo-treated dogs vs. vaccinated ones in six-year-old or younger animals.

CaniLeish®

CaniLeish® vaccine (Virbac, France) was released in Europe in 2011 [59]. It is composed of purified excreted–secreted proteins of *L. infantum* (LiESP) and adjuvanted with a purified fraction of the *Quilaja saponaria* saponin (QA-21) [60]. The vaccination protocol consists of one vaccine dose administered subcutaneously to dogs older than six months every 21 days for a total of three doses, followed by single dose annual boosters. According to the pharmacovigilance data reported by Virbac in October 2015, more than 1.8 million doses of CaniLeish® have been sold during the first 3.5 years of marketing in the European Economic Area, Switzerland and Tunisia [61].

Several studies focusing on the purified excreted–secreted antigens of *L. infantum* promastigote (LiESAp) antigens associated with muramyl dipeptide (MDP) as adjuvant have been published prior to CaniLeish® release [62–65]. In these studies, humoral and cellular markers of *L. infantum* immune response were assessed, as well as parasite establishment after experimental IV challenge [62,64] or natural infection [63]. Results from these studies showed that vaccination elicited a specific IgG2 humoral response to LiESAp and a predominantly Th1-type cellular immune response. Infection protection rates when using LiESAp concentrations of 100 μg were of 100% in the laboratory study [62,64] and of 99.4% in the field study [63]. The LiESAp-MDP vaccine proved to be safe, as no adverse effects, apart from mild local reactions, were reported in either experiment. However, this vaccine formulation was never licensed for dog immunisation against CanL, and the same antigenic preparation was associated to a different adjuvant to formulate CaniLeish®.

The first study performed on CaniLeish® measured the impact of a primary course of the vaccine in beagle dogs on selected humoral and cellular markers of immunity [60]. Twenty beagles aged six months and previously dewormed and vaccinated against conventional canine diseases were kept indoors in controlled conditions throughout the clinical randomised study. Post-vaccination levels of IgG1 and IgG2 antibodies to both LiESP and parasite surface antigen (PSA) were measured by an ELISA assay. Cellular markers of immunity were assessed through the lymphoblastic transformation test (LTT), interferon-γ enzyme-linked immunospot assay (IFN-γ ELISpot) and canine macrophage leishmanicidal assay (CMLA). Results showed that only vaccinated dogs produced antibodies to both LiESP and PSA, with a bias towards an IgG2 profile, particularly in response to PSA. Vaccination also induced a proper cellular immunity profile, with PBMC from vaccinated animals showing a specific T-cell response, with IFN-γ production, when exposed to soluble *Leishmania* antigens (SLA). Monocyte-derived macrophages from the vaccinated group, when infected with *L. infantum* promastigotes and exposed to autologous lymphocytes, presented an increased parasite killing capacity, inducible nitric oxide synthase (iNOS) expression and nitrogen dioxide (NO_2) production.

The same immunity markers were evaluated at different time points during the first year after vaccination [66], showing that a similar immune profile persisted during this period of time. One year after completing the vaccine primary course and before the annual booster, study dogs were challenged intravenously with $10^{8.5}$ infectious *L. infantum* promastigotes [67]. Animals were then clinically followed for nearly one year, and parasite detection techniques, including quantitative PCR (qPCR) and culture of bone marrow samples, were regularly performed. As in the previous studies, the same humoral and cellular assays were used to assess immunity patterns in both groups. Additionally, a glutathione redox balance test was also performed. Significantly higher results were observed for all the three CMLA parameters (CMLA index, percentage of iNOS positive macrophages and NO_2 production) and IFN-γ production in the vaccinated group. Seroconversion after exposure to total *L. infantum* antigens was of 100% and unrelated to the infectious status in the vaccinated group, while in the control group only actively infected animals presented positive titres. Redox ratio was significantly higher in control dogs than in vaccinated individuals. During the study, some animals from both groups developed mild clinical signs compatible with CanL, although no severe signs were observed. No significant differences between study groups were

detected for changes in biochemical or haematological parameters. At the end of the trial (approximately 11 months post-artificial challenge) and based on the results of the last parasitological tests, seven dogs in the control group and three dogs in the vaccine group were considered actively infected, while one case of subpatent infection was detected in the control group. Two vaccinated dogs, which had shown positive *L. infantum* culture results in previous parasitological assessments, were considered to have reverted to a parasite-free status at the end of the study.

The only pre-licensing randomised efficacy field trial of CaniLeish® included 90 beagle dogs introduced in two CanL endemic areas in Italy and Spain [68]. From these, 46 animals were randomly assigned to the vaccinated group and 44 were kept as controls. The same pre-vaccination criteria were adopted as in previous studies (*Leishmania* seronegative, previously dewormed and routinely vaccinated, aged 5–7.5 months) [60,66,67], as well as the manufacturer's recommended vaccination protocol [59]. The vaccination phase was held in controlled conditions, during which vaccine safety was assessed by regular clinical examinations and serological responses to vaccination were quantified. Animals were then transferred to the study sites and naturally exposed to *L. infantum* vectors bites for two transmission seasons. Every three months, dogs were examined for symptoms attributable to CanL and specific haematological and biochemical parameters were measured. Parasitological follow-up by nested-PCR and culture on bone marrow or lymph node samples was also performed on a regular basis. Humoral immune response to *L. infantum* was assessed by IFAT. Observed vaccine adverse effects were local oedema after injection and crusting followed by local alopecia, all resolving spontaneously. Humoral profile in response to vaccination followed the same trends as observed in a previous study [60]. During the two-year study, 10 dogs died of causes unrelated to leishmaniosis and were not included in the vaccine efficacy analysis. The results of this study showed a significant difference between the number of dogs demonstrating active infection (33.3% in the control group vs. 12.2% in the vaccinated group; $P = 0.025$) and the number of symptomatic cases (23.1% in the control group vs. 7.3% in the vaccinated group; $P = 0.046$). However, no significant difference was observed in the proportion of dogs presenting a PCR-positive result on at least one occasion throughout the trial, confirming that the vaccine does not prevent the entry and migration of the parasite to 'deep' tissues [67]. From these, some dogs reverted to a *Leishmania*-free status during the observation period, and this was considered to be more frequent in the vaccinated group ($P = 0.0396$). The reported progression to fatal stages, in which animals either died or were euthanised due to severe CanL, was five in the control group and zero in the vaccinated group ($P < 0.0001$), though one vaccinated and infected dog had to be euthanised a few days after the conclusion of the study. Based on the results obtained in this field trial, the efficacy of CaniLeish® in preventing clinical signs was considered to be 68.4% and the vaccine protection level, defined as the percentage of non-symptomatic vaccinated animals, was 92.7%. An odds ratio of 3.8 expressed the difference in the prevention of clinical disease between the groups. An important additional conclusion of this study is that IFAT alone cannot be used to test vaccinated dogs for *Leishmania* infection, as animals from this group consistently presented positive titres due to vaccine-induced antibodies. This was later confirmed by two follow-up studies 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 [69], and 80% seropositivity with IFAT one month after the first annual vaccine booster was observed [70].

A more recent study, which evaluated the individual efficacy of two insecticide dog collars and CaniLeish® vaccine in the prevention of CanL in highly endemic areas, found no statistically significant differences in the number of animals positive at bone marrow PCR and/or cytology in the vaccinated (15.4%; 8/52) or control (10%; 5/50) groups at one-year post-vaccination ($P = 0.417$) [71]. This trial enrolled mixed breed dogs that were kept in four dog kennels in CanL endemic regions of Italy. Similarly, no differences were observed in the development of active symptomatic infections, characterised by positive PCR and cytology results, high IFAT titres and lymph node enlargement, between CaniLeish® and control groups ($P = 0.495$).

Similar results were reported in a field trial performed in Girona province, an endemic area for CanL in northeast Catalonia, Spain [72]. This trial included a mixed population of 177 native, privately owned dogs, which were followed during one-year post-vaccination. At the end of the trial, the number of active *L. infantum* infections was the same in the vaccine (5.6%; 4/71) and control (5.4%; 4/74) groups. Furthermore, vaccine-induced cellular-mediated immunity (CMI), evaluated by the production of IFN-γ by stimulated PBMC, disclosed a possible short-lived CMI, which could be an explanation for the apparent lack of CaniLeish® efficacy in protecting against *L. infantum* infection during the first-year post-vaccination [73].

The infectiousness potential of *Leishmania* infected dogs previously vaccinated with CaniLeish® was assessed by a preliminary xenodiagnosis study by Bongiorno et al.

[74]. Ten three-year-old beagle dogs at different stages of *L. infantum* infection were enrolled in the study (six vaccinated animals and four controls), which was performed in an endemic area of Italy. The results showed no difference in the rate of sandfly infection in symptomatic dogs between groups, but the infectiousness burden was considered lower in the vaccinated cohort.

A work published in 2016 focused on the impact of CaniLeish® vaccination in several haematological, biochemical and serological parameters of healthy canine blood donors [75]. Twenty-seven client-owned dogs were divided into three groups, according to their CanL vaccination status, and were subject to regular blood assessments. Slight hyperproteinaemia and a rise in some globulin fractions were the only haematological and biochemical changes detected. Once again, CanL serological diagnosis of vaccinated dogs with IFAT proved unreliable, as the assay could not distinguish between vaccine and infection-induced antibodies, confirming the results reported in previous studies [67–70].

Likewise, the use of CTLA-ELISA (crude total *L. infantum* antigen ELISA) in the diagnosis of *Leishmania* infection in CaniLeish® vaccinated dogs should not be recommended [76]. According to this recent study, which evaluated the possible impact of vaccination with CaniLeish® in *L. infantum* seroprevalence studies in endemic areas, vaccine administration induces a rise in IgG levels which is detectable by a common diagnostic ELISA and persists during one to four months post-vaccination. This was also confirmed by Lima et al. [77], who suggested the use of a ratio between the seroreactivity to soluble promastigote *Leishmania* antigens (SPLA) and recombinant protein K39 (rK39) to identify vaccinated and non-infected dogs.

LetiFend®

LetiFend® (Laboratorios LETI, Spain) was licensed in Europe in February 2016 [78]. It is a recombinant vaccine containing a chimerical protein (protein Q) formed by five antigenic fragments from four different *L. infantum* proteins (ribosomal proteins LiP2a, LiP2b and LiP0 and the histone H2A), to which no adjuvant has been added. Vaccination protocol consists of one vaccine dose, followed by annual boosters, and should only be administered to dogs aged six months or older.

Preliminary studies in mice have demonstrated the potential of protein Q in the immunisation against *L. infantum* [79,80]. The association of protein Q with live bacillus Calmette-Guérin (BCG) adjuvant administered i.p. in a three-dose protocol, followed by *L. infantum* experimental infection, prevented parasite establishment in both mice and dogs [79]. Because BCG is frequently found to induce local pain, skin irritation, abscesses, ulcers and, occasionally, hypersensitivity reactions and is not considered a safe adjuvant in dogs [81,82], a protein Q-like protein was then tested with six different adjuvant combinations [81]. As in the protocol adopted by Molano et al. [79], LSA-ELISA serology, culture in bone marrow or lymph node samples, DTH test and necropsy at the end of the study were performed to assess the level of infection in study animals. Each experimental group was composed of five or seven dogs, which received two subcutaneous immunisations with a 21-day interval. No differences were observed between the vaccinated groups and the control animals, concluding that none of the candidate vaccines prevented either parasite establishment or the development of clinical signs, and suggesting that live BCG could have been responsible for the protective effect against *L. infantum* infection previously observed. In a parallel study, protein Q immunisation with no adjuvants (which corresponds to the commercial LetiFend® formulation) was able to demonstrate a protective effect in vaccinated dogs [83].

The LetiFend® pre-licensing phase III trial included 549 dogs (275 vaccinated and 274 controls) exposed to natural infection in two CanL endemic areas in France and Spain during a two-year period [84]. These were privately owned dogs of different breeds and ages and kept outdoors in 19 dog kennels. Humoral response to protein Q antigen and SLA, parasite detection in lymphoid organs and clinical assessment of all animals were performed at pre-determined time points. A case of confirmed CanL was defined as any individual presenting clinical signs compatible with CanL, positive serology to *L. infantum* and parasite detection in bone marrow or lymph node samples. At the end of the study, 4.7% of vaccinated dogs ($n = 8$) and 10.2% of control dogs ($n = 19$) developed CanL, and this difference was considered statistically significant ($P = 0.048$). Only two study sites were selected to perform the analysis of vaccine efficacy due to an unexpectedly low incidence of infection in some dog kennels. According to the results of this field study, LetiFend® showed a 72% VE in the prevention of CanL clinical signs and reduced the likelihood of confirmed CanL cases or development of clinical signs in vaccinated dogs versus placebo dogs in five and 9.8 times, respectively [84]. No general or local adverse effects were observed after LetiFend® administration during laboratory or field studies [83,84]. Furthermore, vaccination with LetiFend® in this field trial did not seem to elicit false-positive results in *L. infantum* serological diagnostic tests, confirming previously reported results [85].

Discussion

The development of effective vaccines against CanL (as well as HL) should be considered an important step towards leishmaniosis control. Not only is immunisation important because other prophylactic measures fail to prevent all infections, but also due to the growing reports of non-vectorial transmission of *Leishmania* parasites [86,87].

There are currently three vaccines commercially available for immunisation against CanL (Leish-Tec®, CaniLeish® and LetiFend®). Their reported efficacy in the prevention of active infection ranges from 68.4% to 80%, and the protection against clinical disease varies between 92.7% and 95%. Field trials performed on commercially approved CanL vaccines are summarised in Table 1, demonstrating that any attempt in comparing the efficacy of these vaccines will be hindered by several aspects, such as their different compositions (both antigens and adjuvants), the variable number and type of studies performed on each one of them, as well as the differing methodology used to assess vaccine immunogenicity and protection against infection or disease [57]. Additionally, the completely different conditions observed in CanL endemic areas in Brazil and in European countries add complexity to any comparative evaluation of vaccines used under each scenario.

When evaluating CanL vaccine efficacy trials, Wylie et al. [57] found substantial within-study variations in baseline characteristics of the study population, significant differences in study design and several potential methodological shortcomings, which precluded the conduction of a meta-analysis. Standardisation of field models for testing *Leishmania* vaccines in dogs have been previously suggested as a means to facilitate the interpretation of efficacy results [88]. The characteristics of the dog population used should also be considered an important aspect when evaluating different vaccine field trials. Although laboratory-based phase II trials must be performed with dogs bred for experimental purposes, the same is not applicable to field trials, and the use of native, heterogeneous dog populations should be pursued [89]. This procedure would avoid the bias produced by an expected genetic similarity amongst study individuals and is more likely to provide more representative results of the general canine population of a country or area. However, although the majority of published field trials reported the use of heterogeneous dog populations, CaniLeish® vaccine licensing in Europe was based solely on efficacy studies performed on five to 7.5-month-old beagle dogs introduced in endemic areas [68].

All commercialised vaccines against CanL recommend the simultaneous application of topical insecticides on vaccinated individuals, as the levels of protection conferred by immunisation alone are not considered satisfactory in the prevention of *L. infantum* infection. Furthermore, any vaccinated and infected dogs represent potential sources of the parasite for other dogs and humans [43,74]. If this is the case, the use of vaccines which only reduce the appearance or severity of clinical signs, 'masking' infected individuals, would actually prove to be detrimental in the global control of CanL and, in areas where the zoonotic risk is high, of HL [90]. Field evidence of CanL vaccines' effectiveness in reducing both CanL and HL is essential to truly assess the usefulness of such control measures [91]. Furthermore, studies of vaccine efficacy designed to demonstrate the advantages of vaccination at the individual level do not provide clear information on the impact of such interventions in *Leishmania* infection epidemiology in endemic areas. The only published example of population-level evaluation of vaccine impact was performed for Leishmune® in Brazil, showing a decrease in the number of seropositive dogs and in the incidence of HL in areas where vaccination had been adopted [44]. Nevertheless, comparative data from other Brazilian endemic areas for the same period, as well as an extended follow-up in the regions reported in this study, would have been important to clearly understand the impact of Leishmune® usage in *L. chagasi* epidemiology.

A retrospective study which performed a comparison of efficacy and safety of preventive measures against CanL in southern Europe and included 1,647 client-owned dogs found that the only preventive method which showed no statistically significant reduction in the number of CanL-diseased dogs when compared with the control group was vaccination alone [92]. Furthermore, the vaccine group was showing a higher incidence of adverse effects. The study only included individuals immunised with CaniLeish® (LetiFend® was not yet being commercialised at the time of data collection), as well as dogs treated with insecticide repellents (both collars and spot-on), domperidone and all possible combinations of the three prophylactic methods. This is a good example of how retrospective data collected from daily veterinary practice can be used to assess the effectiveness of CanL preventive methods in a much larger and representative dog sample.

Another essential aspect of CanL control is the possible interference of vaccination in *Leishmania* infection diagnosis [89]. Although the subunit vaccines (Leishmune® and CaniLeish®) are expected to be of greater concern in

Table 1 Summary of field trials performed on commercially approved CanL vaccines

Vaccine	Vaccinal formulation	Canine population	Measured outcome	Laboratory techniques	Trial duration	Vaccine efficacy	Vaccine safety	References
Leishmune®	FML antigen (1.5 mg) plus R adjuvant (500 µg)	Mixed population of native privately owned dogs (n = 117)	Protection against clinical CanL	Serological: • FML-ELISA • *L. chagasi* IFAT Cellular: • DTH test Molecular: • Parasite detection on blood and BM samples Parasitological: • Giemsa-stained tissue smears	2 years	76% (92% protection against disease)	N.D.	[27]
	FML antigen (1.5 mg) plus saponin adjuvant (1 mg)	Mixed population of native privately owned dogs (n = 85)	*Leishmania* infection Protection against clinical CanL	Serological: • FML-ELISA Cellular: • DTH test Molecular: • Parasite detection on blood and BM samples Parasitological: • Giemsa-stained tissue smears	3.5 years	80% (95% protection against disease)	N.D.	[17]
	FML antigen (1.5 mg) plus saponin adjuvant (0.5 mg)	Mixed population of native privately owned dogs (n = 600)	Vaccine safety and toxicity	N.D. (only physical examination was performed)	14 days after each vaccine dose	N.D.	Mostly mild and transient reactions were observed	[29]
	FML antigen (1.5 mg) plus saponin adjuvant (0.5 mg)	Mixed population of native privately owned dogs (n = 550)*	Protection against clinical CanL Survival rate Changes in the incidence of CanL	Serological: • FML-ELISA (only vaccine group) • Seroconversion to *L. chagasi* (only control group) Cellular: • DTH test (only vaccine group) • PBMC		immunophenotype analysis by flow cytometry Molecular: • Parasite detection on blood and LN samples (only vaccine group) Parasitological: • Giemsa-stained LN smears (only vaccine group)	2 years	N.D.
N.D.	[30]							

Table 1 (*Continued*)

Vaccine	Vaccinal formulation	Canine population	Measured outcome	Laboratory techniques	Trial duration	Vaccine efficacy	Vaccine safety	References
Leish-Tec®	rA2 antigen (100 μg) plus saponin adjuvant (250 μg)	Mixed population of native privately owned dogs (n = 140)	Seroconversion in vaccinated dogs: specificity, levels, and duration of antibody responses	Serological: • LPA-IFAT • LPA-ELISA • DPP-CVL test • rA2-ELISA • Immunoblot analysis	14 months	N.D.	No significant adverse reactions were observed	[53]
	rA2 antigen (100 μg) plus saponin adjuvant (500 μg)	Mixed population of native privately owned dogs (n = 847)	CanL cases (defined as serology (+) and parasite detection by parasitological methods) Dog infectiousness to sandflies	Serological: • cELISA • IFAT test • KD test Parasitological: • Giemsa-stained tissue smears • Culture on BM samples Xenodiagnosis	18 months	71.4%† 58.1%‡ 80.8%§	N.D.	[54]
	rA2 antigen (100 μg) plus saponin adjuvant (500 μg)	Vaccine group: mixed population of native privately owned dogs (n = N.S.) Control group: introduced sentinel beagle dogs and newly recruited healthy dogs (n = N.S.)	Cases of *L. infantum* infection Cases of clinical CanL	Serological: • A2-ELISA • LPA-ELISA Histopathology: • Liver, spleen, LN and ear skin	2 years	No significant differences were observed between vaccine and control groups	No significant adverse reactions were observed	[55]
Leishmune® and Leish-Tec®	Leishmune®: FML antigen (1.5 mg) plus saponin adjuvant (0.5 mg) Leish-Tec®: rA2 antigen (100 μg) plus saponin adjuvant (500 μg)	Mixed population of native privately owned dogs (n = 180) No seronegative control group	Vaccine comparison regarding vaccine reactogenicity, seroconversion, parasitism, CanL clinical signs, and dog infectiousness to sandflies	Serological: • LPA-ELISA Parasitological: • Culture on spleen samples Xenodiagnosis Molecular: • Parasite detection on spleen samples and on phlebotomine sandflies after xenodiagnosis	11 months	No significant differences were observed between vaccine groups with respect to CanL clinical signs, parasitism, seropositivity, or dog infectiousness	Observed adverse reactions in the Leish-Tec® group were more frequent and severe	[42]

Table 1 (*Continued*)

Vaccine	Vaccinal formulation	Canine population	Measured outcome	Laboratory techniques	Trial duration	Vaccine efficacy	Vaccine safety	References
CaniLeish®	LiESAP antigen (100 μg) plus MDP adjuvant (200 μg)	Mixed population of native privately owned dogs (*n* = 414)	Protection against *L. infantum* infection Prevention of disease development	Serological: • *Leishmania* IFAT • LiESAP-ELISA Cellular: • Macrophage killing ability • NO production • IFN-γ and IL-4 production on stimulated PBMC Molecular: • Parasite detection on BM aspirates Parasitological: • Culture on BM aspirates	2 years	92%	No significant adverse reactions were observed	[62]
	LiESP antigen (100 μg) plus QA-21 adjuvant (60 μg)	Naïve beagle dogs, aged 5–7.5 months and kept in kennels (*n* = 90)	Protection against *L. infantum* infection Prevention of disease development	Serological: • *Leishmania* IFAT • ESP-ELISA • PSA-ELISA Molecular: • Parasite detection on BM aspirates Parasitological: • Culture on BM and LN aspirates Others: • Haematological and biochemical parameters	2 years	68.4% (92.7% protection level, i.e. % of non-symptomatic vaccinated dogs)	No significant adverse reactions were observed	[67]

Table 1 (*Continued*)

Vaccine	Vaccinal formulation	Canine population	Measured outcome	Laboratory techniques	Trial duration	Vaccine efficacy	Vaccine safety	References
	LiESP antigen (100 μg) plus QA-21 adjuvant (60 μg)	Mixed population of shelter dogs,	aged < 18 months (n = 224)	Active symptomatic *Leishmania* infection Detection of *Leishmania* parasites in BM samples		Serological: • *Leishmania* IFAT Molecular: • Parasite detection on BM and skin samples Parasitological: • BM smears stained with MGG Quick Stain	1 year	No
significant differences were observed between vaccine and control groups	N.D.	[70]						
	LiESP antigen (100 μg) plus QA-21 adjuvant (60 μg)	Mixed population of native privately owned dogs (n = 177)	Active *Leishmania* infection Evaluation of vaccine-induced CMI	Serological: • CTLA-ELISA Molecular: • Parasite detection on LN aspirates Cellular: • IFN-γ production in stimulated PBMC Others: • Haematological and biochemical parameters	1 year	No significant differences were observed between vaccine and control groups	No significant adverse reactions were observed	[73]

Table 1 (*Continued*)

Vaccine	Vaccinal formulation	Canine population	Measured outcome	Laboratory techniques	Trial duration	Vaccine efficacy	Vaccine safety	References
LetiFend®	Recombinant protein Q from *L. infantum* MON-1 (≥36.7 ELISA units)	Mixed population of native privately owned dogs (n = 549)	Confirmed CanL cases No. of dogs with positive *Leishmania* serology No. of dogs with presence of parasites on BM or LN at the last time point No. of dogs presenting clinical signs at the last time point	Serological: • PQ-ELISA • SLA-ELISA • *Leishmania* IFAT Molecular: • Parasite detection on LN and BM aspirates Parasitological: • LN and BM smears Others: Haematological and biochemical parameters	2 years	72%	No local or systemic side effects were observed	[82]

N.D., not determined; N.S., not stated; R, Riedel de Haën saponin; FML, fucose-mannose ligand; FML-ELISA, fucose-mannose ligand enzyme-linked immunosorbent assay; IFAT, indirect fluorescent antibody test; DTH, delayed-type hypersensitivity; BM, bone marrow; PBMC, peripheral blood mononuclear cells; LN, lymph node; rA2, A2 recombinant protein; LPA, *Leishmania* promastigote antigen; DPP-CVL, Dual-Path Platform® Canine Visceral Leishmaniasis Rapid Test (BioManguinhos); cELISA, crude antigen ELISA; KD, Kalazar Detect™ Rapid Test for Visceral Leishmaniasis (InBios); LiESAP, excreted/secreted antigens of *L. infantum* promastigotes; MDP, muramyl dipeptide; NO, nitric oxide; IFN-γ, interferon gamma; IL-4, interleukin 4; LiESP, *Leishmania infantum* excreted/secreted proteins; QA-21, Purified extract of *Quillaja saponaria*; PSA, parasite surface antigen; MGG, May-Grünwald Giemsa; CMI, cellular-mediated immunity; CTLA, crude total *L. infantum* antigen; PQ, protein Q; SLA, soluble *L. infantum* antigen.

*Control group (n = 588) belonged to a different CanL area in Brazil, with a similar CanL incidence; different criteria were used to detect *Leishmania* infection in dogs from vaccine and control groups.

†According to parasitological examinations, imprinting, culture and/or histopathology of dog tissues (skin, lymph nodes, spleen and bone marrow).

‡According to parasitological examinations plus xenodiagnosis.

§According to anti-A2 serology.

this respect than the recombinant formulations (Leish-Tec® and LetiFend®), the impact of vaccination on the infection detection ability of common diagnostic methods should be assessed prior to any CanL vaccine licensing. The added difficulty in CanL diagnosis has been reported for dogs vaccinated with CaniLeish® [93–95], and the possible interference of Leishmune® and CaniLeish® vaccines in the serological detection of infected dogs in Brazil and Europe has also been described [44,47,76]. The negative impact of CanL vaccination on *Leishmania* infection diagnosis and control is expected to be higher whenever vaccines with only low to moderate efficacy are widely implemented in endemic areas. In such cases, a significant proportion of vaccinated and potentially infected dogs would be expected, which, if left undetected, could represent an important reservoir of the parasite, indirectly inducing a rise in the incidence of infection (both in vaccinated and non-vaccinated dogs).

Pharmacological surveillance and phase IV clinical trials should be considered essential procedures after any veterinary vaccine licensing to confirm safety and efficacy rates reported in phase II and III trials, thus avoiding long-term commercialisation of suboptimal or ineffective products. Importantly, these results would also provide reliable information to the general public, who would then be able to make informed decisions on whether to adopt these vaccines.

Conclusion

Currently available studies on licensed vaccines for CanL are considered insufficient. A lack of study design standardisation, methodological shortcomings and substantial differences in the characteristics of study populations are some of the issues precluding comparative studies between available vaccines. Furthermore, research is needed in other aspects of vaccination. Xenodiagnosis studies to assess vaccinated and infected dogs' infectiousness and a proper evaluation of potential vaccination interference in the diagnosis of *Leishmania* infection are some examples. Also, long-term pharmacological surveillance should be maintained after any vaccine licensing to provide reliable information to relevant organisations and the general public.

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