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A large-scale field randomized trial demonstrates safety and efficacy of the vaccine LetiFend® against canine leishmaniosis

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ABSTRACT

Canine leishmaniosis is a zoonotic disease caused by *Leishmania infantum*. Extensive research is currently ongoing to develop safe and effective vaccines to protect from disease development. The European Commission has granted a marketing authorization for LetiFend®, a new vaccine containing recombinant Protein Q. The efficacy of LetiFend® vaccination in a large-scale dog population of both sexes, different breeds and ages in endemic areas is reported in this multicenter, randomized, double-blind, placebo-controlled field trial.

Dogs (n = 549) living in France and Spain were randomly selected to receive a single subcutaneous dose of LetiFend® or placebo per year, and were naturally exposed to two *L. infantum* transmission seasons. Clinical examinations, blood and lymphoid organ sampling to evaluate serological, parasitological and disease status of the dogs were performed at different time points during the study.

LetiFend® was very well tolerated and clearly reduced the incidence of clinical signs related to leishmaniosis. The number of confirmed cases of leishmaniosis was statistically significantly lower in the vaccine group. The number of dogs with parasites was close to be significantly reduced in the vaccine group ($p = 0.0564$). Re-vaccination of seropositive dogs demonstrated to be safe and not to worsen the course of the disease. The likelihood that a dog vaccinated with LetiFend® develops a confirmed case or clinical signs of leishmaniosis in areas with high pressure is, respectively, 5 and 9.8 time less than that for an unvaccinated dog. Thus, the overall efficacy of the LetiFend® vaccine in the prevention of confirmed cases of leishmaniosis in endemic areas with high disease pressure was shown to be 72%.

In conclusion, this field trial demonstrates that LetiFend® is a novel, safe and effective vaccine for the active immunization of non-infected dogs from 6 months of age in reducing the risk of developing clinical leishmaniosis after natural infection with *Leishmania infantum*.

1. Introduction

Canine leishmaniosis (CanL) is a serious zoonotic disease which is endemic in the Mediterranean basin, the Middle East, Central Asia and Latin America [1]. In these areas, domestic dogs are the main reservoir host; the parasite (*Leishmania infantum*) is efficiently transmitted to other dogs or humans by the bite of sand fly species of the subgenus *Phlebotomus* sp. in most of the Old World, whereas members of the *Lutzomyia* sp. are the main vectors in Latin America [2].

There is a growing consensus that an ideal control program for CanL is likely to involve combined use of vaccines with repellent products to maximize the protection of dogs and humans [3–5].

For this reason, extensive research is ongoing to develop safe and effective vaccines to prevent this devastating disease from spreading [6]. As a consequence of these vaccine development

Abbreviations: CanL, Canine leishmaniosis; PQ, Protein Q; SLA, Soluble *Leishmania* Antigen; IFAT, Immunofluorescence Antibody Testing.

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programs, two CanL vaccines were registered in Brazil in the past years. Both have a primary course consisting of three injections at 3-week intervals, followed by annual booster injections. Leishmune® (Zoetis, Brazil), based on the Fucose-Mannose Ligand of *L. donovani* with QuilA adjuvant, which demonstrated 80% efficacy against disease or death after natural infection in a field study [7]. However, Leishmune® is not currently recommended by the Brazilian Ministry of Health. The vaccine currently available in Brazil is Leish-Tec® (Hertape Calier, Brazil), composed of the A2 antigen (a recombinant protein of different *Leishmania* species containing saponin as adjuvant) which demonstrated 43% protection against a culture-positive state in an artificial challenge model [8]. An efficacy of 71.4% based on parasitological results was observed in a randomized field trial in an endemic area of Brazil [9].

CaniLeish® (Virbac, France) was the first vaccine to be launched in Europe. The vaccine is composed of *L. infantum* Excreted-Secreted Proteins (LiESP) and a purified extract of *Quillaja saponaria* (QA-21). It has a primary vaccination course of three injections at three-week intervals, followed by annual booster injections. The efficacy of CaniLeish® was assessed in a randomized, double-blind, controlled trial by exposing Beagle dogs to natural *L. infantum* infection in endemic areas of the Mediterranean basin. The vaccine showed an efficacy of 68.4% and decreased the risk of an individual dog progressing to symptomatic active infection 3.8-fold [10].

Published studies have proven that the immune response against internal antigens of *Leishmania* may play a role in the control of the disease [11]. Moreover, extracts of ribosomal proteins from *L. infantum* or *L. major* have shown to induce protection against experimental leishmaniosis in mice [12–14]. These proteins were highly recognized by murine [15], canine [16] and human [17] sera with visceral leishmaniosis. Nucleosomal histones from *Leishmania* have also shown disease protection in mice [18] and the antigenic determinants from the *L. infantum* histone H2A were recognized by sera from dogs with leishmaniosis [19,20].

Thus sera from dogs with active leishmaniosis were employed to identify 4 highly antigenic proteins of *L. infantum*: the acidic ribosomal proteins LiP2A, LiP2B, LiP0 and the histone H2A. LiP2A, LiP2B were recognized by more than 80% of dog sera [20,21], and LiP0 and H2A were also recognized by a 78% of sera from dogs with leishmaniosis [19,22,23]. Several studies in mice and dogs with these ribosomal and histone proteins have shown their high immunogenicity, safety and efficacy against the development of the disease [12,24,25].

At the light of these results, a recombinant chimeric protein (Protein Q) formed by the genetic fusion of 5 antigenic fragments of these proteins was constructed [26]. Vaccination studies with Protein Q in mice [27] and dogs [28,29] have also shown its high immunogenicity, as well as their safety profile, protective capability and efficacy against the development of the disease. A single dose of Protein Q has demonstrated to be immunogenic and to protect dogs against experimental *L. infantum* infection in the absence of an adjuvant [28].

Recently, the European Commission granted a marketing authorization for LetiFend® valid throughout the European Union. LetiFend® (which contains Protein Q as active ingredient) is indicated for the immunization of non-infected dogs from 6 months of age to reduce the risk of developing an active infection and/or clinical disease after exposure to *L. infantum* [30].

In this article, we report the results of a multicenter, randomized, double-blind, placebo-controlled efficacy field trial of LetiFend®, which was performed in a large-scale dog population of different breeds and ages in endemic areas of Southern Europe. Dogs were naturally exposed to two *L. infantum* transmission seasons in Spain and in France, two countries where the vector and parasite are present and where the disease is endemic.

The objective of this pivotal study was to evaluate the efficacy and safety of the LetiFend® vaccine against CanL under real field conditions in a large representative population of dogs of both sexes, and a large variety of breeds, sizes and ages.

2. Methods

2.1. Ethics statement

All study procedures were approved by the national authorities in France and Spain. The study design and study protocol were approved by the *Agence Nationale du Médicament Vétérinaire* (with reference ENR/DD EC-00123-0), and by the *Agencia Española de Medicamentos y Productos Sanitarios* (with number 221/ECV) in accordance with French and Spanish legislation, respectively, for the protection of animals used for experimentation and other scientific purposes.

2.2. Study area

Study areas were selected based on published data on local phlebotomine vector distribution [31,32], *L. infantum* presence [33], and prevalence of the disease [34–37].

A total of 19 kennels of dogs were selected. These kennels were located in areas with endemic CanL, in order to represent the canine population exposed to the parasite. The study was conducted at four veterinary practices in France (Sites A01, A02, A03 and A04, B01, B02 and B03, C01, C02 and C03, D01), corresponding to the regions of Auvergne-Rhône-Alpes, Corsica and Provence-Alpes-Côte d'Azur and at one veterinary practice in Spain (Sites K01, K02, K03, K04, K05, K06, K07, K08) in the region of Extremadura. The average seroprevalence in French kennels just before vaccination was 12.2% with a maximum of 42.1%, while in Spain the seroprevalence was 20.8% with a maximum of 30.4% (screening data before inclusion) (see Table 1).

2.3. Dog population

The intended sample size of each group was estimated based on the following assumptions: (a) a 1:1 ratio between vaccine and placebo groups; (b) a 5% incidence of leishmaniosis confirmed cases over the two year observation period in the placebo group; (c) 2% of expected difference in cases of leishmaniosis between the two groups (vaccine and placebo); and (d) an 80% chance to detect a one-sided difference between the placebo and the vaccine group if 225 animals were analyzed per group (alpha = 5%).

Five hundred and forty-nine (549) seronegative dogs to ELISA SLA (Soluble *Leishmania* Antigen) were included in the study (309 dogs in Spain and 240 dogs in France) in 2008. Each dog was identified prior to study initiation by tattoo, microchip, or by individual pictures and description. All dogs included in the study lived outdoor in kennels of at least 20 dogs, with open exposure to *Leishmania* infection, and were mainly used by their owners for hunting. No changes were implemented in their normal housing conditions for the purpose of the study. Animals were fed and watered as usual by their owners throughout the study. As animals were recruited, they were randomly assigned to one treatment group (placebo or vaccine). At study Day 0, 275 dogs were vaccinated with LetiFend® and 274 dogs were treated with placebo.

The age of the total population enrolled in the study ranged from 6 months to 14 years. The mean age of the dogs was 43.8 ± 26.8 months (mean ± SD) in the vaccine group, and 44.4 ± 28.1 months in the placebo group (no statistically significant differences between both groups, Pearson chi square test).

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Table 1
Screening data before inclusion.

Site	Region	Dpt/provence	Location	Dogs screened (n)	Eligible dogs (n)	Seropositives ELISA SLA (n)	Seropositives ELISA SLA (%)
A01	Auvergne-Rhône-Alpes	Drôme	La Begude De Mazenc	20	19	1	5.0%
A02			La Roche St Secret	19	17	2	10.5%
A03			Lepegue	31	30	1	3.2%
A04			Dieulefit	24	23	1	4.2%
B01	Corse	Corse du Sud	Alata	51	48	3	5.9%
B02			Cuttoli	22	22	0	0.0%
B03			Porticcio	22	20	2	9.1%
C01	Corse	Haute Corse	Ghisonaccia	23	21	2	8.7%
C02			Ghisonaccia	19	11	8	42.1%
C03			Ghisonaccia	17	10	7	41.2%
D01	Provence-Alpes-Côte d'Azur.	Bouche-du Rhône	Roquefort La Bedoule	25	24	1	4.0%
K01	Extremadura	Cáceres	Jaraiz de la Vera	60	48	9	15.0%
K02			Cáceres	30	23	6	20.0%
K03			Cilleros	58	47	11	19.0%
K04			Segura de Toro	43	27	12	27.9%
K05			Losar de la Vera	43	33	9	20.9%
K06			Navaconcejo	46	27	14	30.4%
K07			Valencia de Alcántara	44	26	13	29.5%
K08			Navalmoral de la Mata	83	78	3	3.6%

Thirty-five (35) different pure breeds (361 dogs) plus cross breeds (188 dogs) were included in the study. In both groups, approximately 66% of dogs were pure breeds and approximately 34% were cross breeds (no statistically significant differences).

In the placebo group, 70% of the dogs were male and 30% were female, whereas in the vaccine group 65% were male and 35% were female. No significant differences were found in the sex distribution between both groups.

At study Day 365, 215 dogs of the vaccine group were revaccinated with LetiFend® and 218 dogs of the placebo group were treated with the corresponding placebo. Three hundred and forty-eight (348) dogs survived until the last study day (Day 730): 168 vaccinated dogs and 180 dogs in the placebo group. Mortality (unrelated to leishmaniosis) was 35% and was mainly related to hunting activities.

2.4. Inclusion/exclusion criteria

Only healthy dogs older than 6 months that were negative for antibodies against *Leishmania* by ELISA SLA, and that had never been vaccinated against CanL were included. Dogs that had received any treatment that could affect immunity following vaccination, that had received long-acting corticosteroids within 30 days prior to study Day 0, or that had received short-acting systemic corticosteroids within 14 days prior to study Day 0 were excluded. Dogs that had received any other vaccination within 15 days prior to study Day 0 or that would receive another vaccination within the next 15 days were also excluded.

Dogs were removed from the study at any time if illness, injury, complication, or adverse reaction prohibited the animal from completing the study. In addition, any dog that was confirmed with leishmaniosis as per the study protocol criteria was removed and treated after owner consent.

Concomitant treatments for conditions other than leishmaniosis including antimicrobials, steroidal and non-steroidal anti-inflammatory drugs, anesthetics, and deworming treatment were authorized throughout the study. Insecticide treatment including topical, oral, collars and environmental sprays was forbidden, since insecticide treatment may interfere with the sand fly vector and affect the natural infection of dogs by *Leishmania*.

2.5. General experimental design

This multicenter, randomized, placebo-controlled field study was conducted in endemic CanL areas. The study was conducted by Triveritas Ltd (UK) according to the Guidelines on Good Clinical Practices CVMP/VICH/595/98 VICH Topic GL9 Step 7. All analyses and clinical examinations were performed in a blinded manner by professionals who had access only to the dog identification codes. Treatment group codes were only unblinded after all data were entered into the data management system and all decisions regarding the status of each dog were taken.

The owners were informed about the details of the study and signed the Informed Consent prior to any manipulation of the animals for the purpose of the study. Dogs met all inclusion criteria and none of the exclusion criteria. At pre-inclusion, dogs were seronegative for *Leishmania infantum*.

Dogs were assigned randomly to one of the two treatment groups: vaccine or placebo. Dogs were randomized in blocks of two on the basis of the order of entry into the study, and the two treatments (vaccine or placebo) were randomly assigned within blocks. Blocking was used to maintain balance during enrolment of animals at each site and was not included in the statistical analyses as a design variable. As this study was designed to evaluate dogs in a clinical setting, there was no grouping or restriction to randomization to equalize for gender, weight, or age.

The study procedures included clinical examinations, blood and lymphoid organ sampling at different time points during the period of the study. Fig. 1 shows a flow chart of the canine population during the study.

2.6. Vaccine and treatment regimen

The Protein Q vaccine is authorized for commercialization in the European Union under the trade name LetiFend® (Laboratorios LETI, Spain). It is composed of a recombinant protein (Protein Q) obtained through the genetic fusion of five antigenic determinants from four *Leishmania infantum* proteins. The vaccine does not contain an adjuvant. The doses used in this study were formulated as for the commercial product. Each dose (0.5 mL) of the vaccine is

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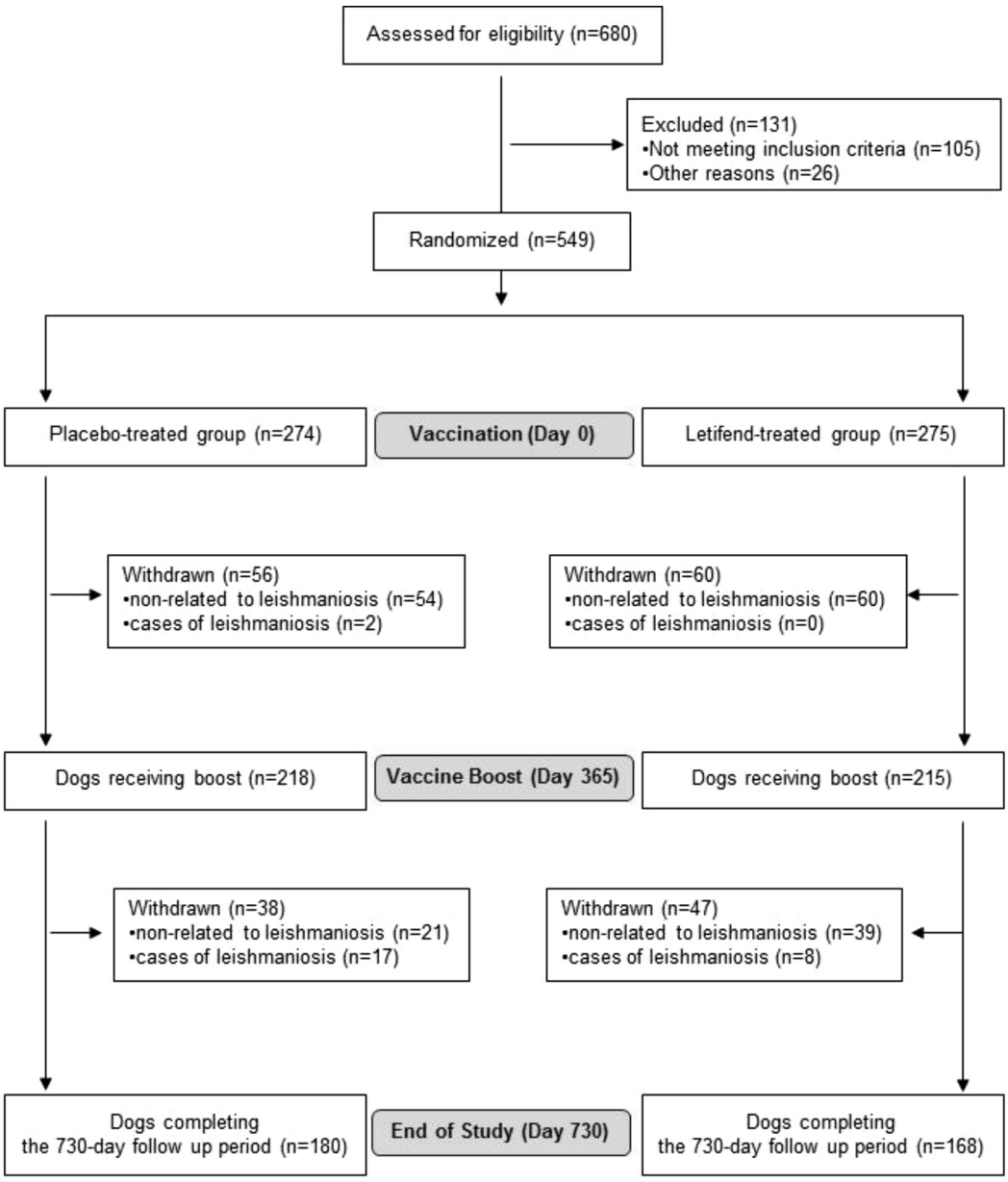

Fig. 1. Flow chart showing screening, treatments and losses of dogs included in the field vaccine trial.

presented as a lyophilisate containing recombinant Protein Q from *L. infantum* MON-1 (≥36.7 ELISA Units -antigen content determined against an internal standard), in a buffered medium containing arginine hydrochloride, boric acid and sodium chloride, to be reconstituted in water for injection.

At the start of the study (Day 0) the dogs assigned to the vaccine group (n = 275) received a single dose of vaccine (0.5 mL, batch 171,412) subcutaneously at the interscapular area, and one annual booster during the natural exposure period (Day 365). Placebo-treated dogs (n = 274) received the same injection as vaccinated animals, with the only exception that the Protein Q was absent from the formulation.

2.7. Vaccine safety

Physical examinations to detect local and general side-effects were performed at Days 0, 14 and 28 after the primary dose and after the booster vaccination on Day 365. Assessment of local tolerance included presence or absence of oedema, pain, inflammation, and induration at the injection site. Assessment of general tolerance included observations of general appearance, the integumentary, musculoskeletal, circulatory, respiratory, digestive, genitourinary and nervous systems, as well as eyes, ears, lymph nodes and mucous membranes. Rectal temperature was taken and recorded at each time point.

2.8. Serology testing of the humoral immune response

In previous studies, it was found that vaccination with Protein Q (PQ) induced a specific IgG2 dominant response in vaccinated dogs [28]. For this reason, humoral response to vaccination was evaluated by ELISA PQ prior to vaccination at Day 0 and at Days 14, 28, 180, 300, 379, 393, 545, 665 and 730 to determine the level of IgG2 antibodies to PQ, which is the antigenic component of

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the LetiFend® vaccine. The technique was performed on serum samples as previously described [28] using a 1/200 sera and 1/20.000 secondary antibody dilution

The presence and level of IgG2 antibodies to total soluble *Leishmania infantum* antigen (SLA) in plasma was evaluated with an ELISA as reported in [28] prior to vaccination at Day 0, and at Days 180, 300, 545, 665 and 730. Sera and secondary antibody were diluted 1/200, 1/20.000-, respectively. Seropositivity (measured as optical density, OD) was considered as follows: negative: OD < 0.48; low/medium: 0.48 ≤ OD ≤ 0.64; high: OD > 0.64.

Immunofluorescence Antibody Testing (IFAT) was also performed on serum samples at Days 300, 545, 665 and 730 to determine the level of total anti-*Leishmania* IgG antibodies as described previously [28]. Secondary antibody was diluted 1/80. Serological status against *Leishmania* was considered as follows: seronegative: titre < 1/80; low/medium seropositive: 1/80 ≤ titre ≤ 1/320; high seropositive: titre ≥ 1/640.

2.9. Parasitological follow-up

Parasite detection in lymphoid organs was performed in aspirated biopsies of bone marrow and lymph nodes using both microscopic examination of smears (smear test) and qualitative real-time PCR (PCR).

The microscopic observation of lymph node and bone marrow smears has been widely used due to its high specificity [38]. In order to ensure the highest sensitivity the minimum number of microscopic fields that were inspected per preparation was 25 [39,40]. On the other hand, the PCR technique has also shown to be accurate and sensitive in the diagnosis of leishmaniosis [39,41].

Along the study, sampling of lymph nodes and bone marrow was done in dogs that were either clinically suspect or serologically suspect of developing the disease. On Day 730, samples of bone marrow and lymph nodes were obtained from all dogs for microscopic identification of parasites in smears and for PCR detection of *Leishmania* DNA.

Smear tests were performed in lymph nodes and bone marrow samples as described elsewhere [28]. Briefly, aspirated biopsies were fixed and stained on a microscope slide for observation of amastigote forms under the microscope. A total of 25 different fields (lymph node) or 300 nucleated cellular elements (bone marrow) were observed. Results were expressed as positive or negative observation of *Leishmania* amastigote forms. A positive result in any of the parasitological techniques defined a dog as infected.

The PCR analysis was performed in bone marrow aspiration samples to detect the *gp63* gene [42], and in lymph node samples to detect parasitic kinetoplast DNA (kDNA) [28].

2.10. Circulation of Leishmania in the kennels

Circulation of the parasite in kennels was expressed as the percentage of animals in the placebo group showing infection with *L. infantum*. This was done by means of PCR assays or smear tests, or showing antibody seroconversion by ELISA SLA or IFAT during the course of the study.

2.11. Clinical follow-up

A physical examination was performed on each study dog at the following times: at the pre-inclusion visit between Day -21 and Day 0; before inclusion at Day 0; at Days 180 and 300; prior to treatment administration at Day 365; at Days 545 and 665; at study completion at Day 730, and for each dog that terminated or was withdrawn from the study before Day 730 completion.

Physical examination included general condition, the integumentary, musculoskeletal, circulatory, respiratory, digestive, genitourinary and nervous systems, as well as eyes, ears, lymph nodes and mucous membranes. Rectal temperature was taken and recorded at each time point. Any abnormal signs were recorded (using a pre-established list of clinical signs), paying with special attention to those signs that might be attributed to *Leishmania* infection (e.g. body condition, lymph node enlargement, exfoliative-desquamative dermatitis, cutaneous ulcers, skin nodules, onychogryphosis, blepharitis, conjunctivitis, keratitis, uveitis and arthritis).

Abnormal health observations were recorded as adverse events at any time outside the scheduled time points by the Investigator. The dogs received appropriate treatment if needed. Dogs with confirmed leishmaniosis were withdrawn from the study.

2.12. Definition of confirmed leishmaniosis case

The classification of the dogs' *Leishmania* status was determined based on the presence of clinical signs, the results of serological tests and the parasitological tests at each clinical assessment and at the completion of the study. A confirmed case of leishmaniosis was defined as:

- Presence of clinical signs compatible with leishmaniosis and,
- Positive ELISA SLA or IFAT measurement and,
- Presence of parasites in either bone marrow or lymph nodes.

In addition, animals without clinical signs, but with high positive IFAT (≥1/640) and presence of *Leishmania* in bone marrow or lymph nodes at the last time point were also considered as confirmed asymptomatic cases of leishmaniosis.

2.13. Euthanasia endpoint

According to the ethical requirements of the study protocol, euthanasia was performed on sick dogs that showed severe clinical signs such as emaciation, severe sensorial depression and dehydration due to renal involvement. All euthanized dogs were submitted to necropsy, provided permission was granted by the owner.

2.14. Statistical analysis

The primary variable for determining effectiveness of vaccination with LetiFend® vaccine was the percentage of dogs presenting a confirmed case of leishmaniosis, as defined previously. Secondary criteria for determining effectiveness of vaccination with LetiFend® included: (1) the number of dogs with positive serology for *Leishmania* as measured by ELISA SLA or IFAT at each time point; (2) the number of dogs with presence of parasites in the lymphoid organs at the last observation time point (Day 730); (3) the number of dogs presenting clinical signs at the last examination time point.

The efficacy of the vaccine (percentage of efficacy and odds ratio) was calculated in selected kennels using standard efficacy calculations [43].

The following statistical methods were used for evaluating the statistical significance of the results: for categorical variables, the Pearson Chi-square (with or without Yates' correction) or Fisher's exact test were used when comparing between groups. Continuous variables were compared by Student's *t*-test. In all comparisons, a significance level of $\alpha < 0.05$ was used. Statistical analyses were performed using the StatGraphics Centurion XV.II (2006) software package.

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3. Results

3.1. Vaccine-related adverse effects

All dogs were observed for general and local reactions on Day 0 immediately after vaccination, and two and four weeks after each vaccination. They were subjected to a thorough physical examination, with special focus on the presence of lesions at the site of the injection. None of the dogs (vaccine and placebo) showed any local reactions or systemic clinical signs that could be attributed to the vaccination.

3.2. Anti-protein Q IgG2 antibodies

The serological response of dogs vaccinated with LetiFend® was evaluated with an ELISA for Protein Q (ELISA PQ). Antibodies against Protein Q, the antigenic component of LetiFend® were determined at Days 14, 28, 180, 300, 379, 393, 545, 665 and 730 after the first vaccination (Fig. 2).

Antibody increased significantly in the vaccine group 14 and 28 days after the first vaccine dose (Day 0) ($p < 0.001$) and after the booster dose on Day 365 ($p < 0.001$). The antibody levels peaked 14 days after each vaccination and declined to basal levels thereafter.

3.3. Serum anti Leishmania infantum antibodies

The serological response to infection by *Leishmania* was evaluated at different time points with ELISA SLA and IFAT. The number of dogs with positive serological response for ELISA SLA in both groups was compared at each study time point. Dogs that became seropositive increased gradually during the study in both groups. The number of dogs that seroconverted was similar in the placebo group than in the vaccinated group at most time points. At the last time point (Day 730) 18.3% and 16.9% of the animals in the placebo and vaccine group, respectively, were seropositive (data not shown).

The number of *Leishmania* seropositive dogs as measured by IFAT, was counted on Days 300, 545, 665 and 730 and compared between both groups. In the placebo group, the number of seropositive dogs increased gradually during the study (Fig. 3A). At the end of the study (Day 730), 18/186 (9.7%) and 12/171 (7.0%) animals had seroconverted in the placebo and vaccine group, respectively.

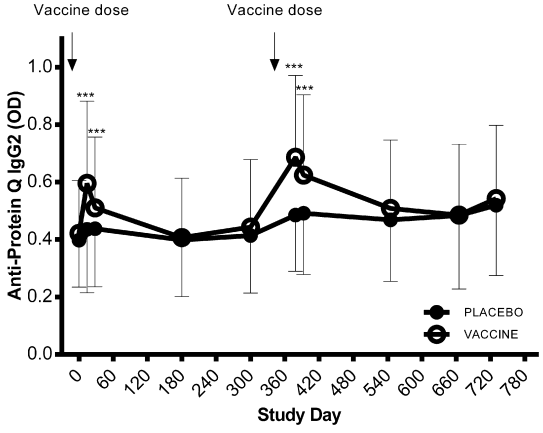

Fig. 2. Progression of anti-Protein Q IgG2 antibodies in vaccinated and placebo dogs. Vaccination was performed at study Day 0 and revaccination at study Day 365. Data is expressed as mean ± SD of the individual values of all animals present at each time point of the study. *** $p < 0.001$ (Student's *t*-test).

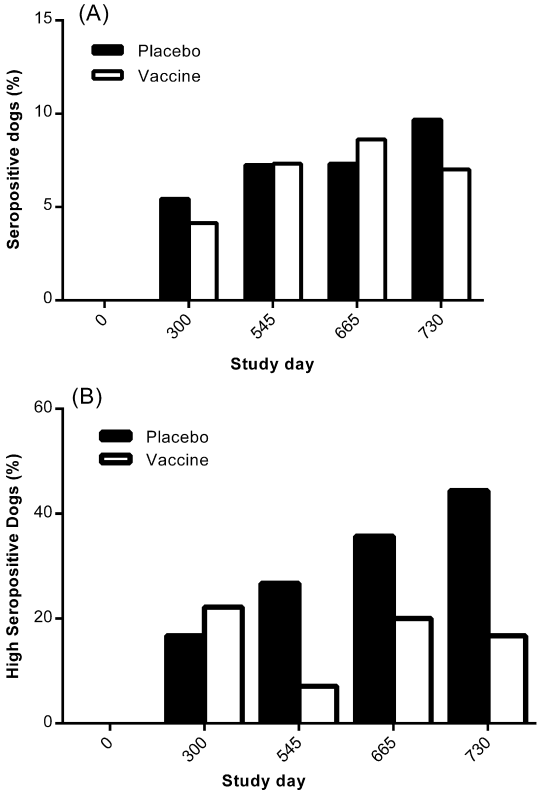

Fig. 3. Seropositive dogs against Leishmania antigen as measured by IFAT. (A) Percentage of total seropositive dogs, (B) percentage of seropositive dogs presenting high positive serology (IFAT titres ≥ 1/640), among all seropositive animals. (Test: Chi-square test with Yates' correction).

The number of dogs with low/moderate (1/320 ≥ titre ≥ 1/80) and high titres (≥1/640) in the IFAT were also recorded. Although not statistically significant, 4.3% of the animals were high seropositive (titre ≥ 1/640) in the placebo group at the last time point, whereas in the vaccine group only 1.2% of dogs had high titres (≥1/640). Among seropositive animals, 44.4% presented high IFAT titres (≥1/640) at the last time point, whereas in the vaccine group only 16.6% presented high titres (Fig. 3B).

3.4. Prevalence of infection

The prevalence of infection at the end of the study was evaluated by PCR and smear test in lymphoid organs (Table 2).

The total number of dogs with presence of *Leishmania* parasites at Day 730 was higher in the placebo group than in the vaccine group; this difference was very close to being statistically significant (16.1% vs 9.4%, $p = 0.0564$).

3.5. Development of clinical signs of leishmaniosis

The number of dogs showing clinical signs attributable to leishmaniosis increased gradually over time in the placebo group (Fig. 4). At the end of the study, the number of dogs with clinical signs attributed to leishmaniosis was significantly lower ($p < 0.001$) in the vaccine group when compared to the placebo group. In the placebo group, 61/186 dogs (32.8%) presented a total of 168 clinical signs ranging from 1 to 14 clinical signs per dog, while in the vaccine group only 22/171 dogs (12.9%) presented 44 clinical signs ranging from 1 to 8 clinical signs per dog (Fig. 4). Typically, the clinical signs observed included (but were not limited to): general (asthenia, anorexia, weight loss,

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Table 2
Dogs with presence of Leishmania in lymph nodes or bone marrow at Day 730.

Group	Positive PCR (n)	Positive smear (n)	Total parasite positive (n)	Total parasite negative (n)	Total parasite positive (%)
Placebo	29	25	30	156	16.1%
Vaccine	12	9	16	155	9.4%

Data is expressed as the number (n, %) of positive dogs for *Leishmania* spp. in lymph nodes and/or bone marrow at the last time point of the study (Day 730) measured by PCR and/or smear test. p = 0.0564 (Chi-square test with Yate's correction).

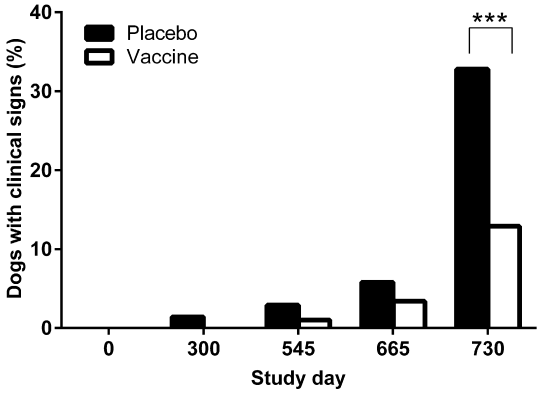

Fig. 4. Proportion of dogs with clinical signs throughout the study period. Data is expressed as the percentage of dogs showing clinical signs related to leishmaniosis. ***p < 0.001 (Chi-Square Test).

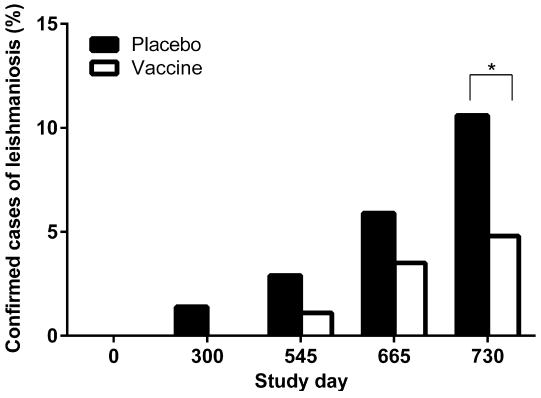

Fig. 5. Proportion (%) of dogs that progressed to leishmaniosis cases throughout the study period. Data is expressed as the percentage of dogs diagnosed as case of leishmaniosis. *p < 0.05 (Chi-Square test).

lymphadenomegaly, onychogryphosis, lameness); skin (alopecia, dull hair coat, seborrhea and exfoliation, hyperkeratosis, dermatitis, ulcers); eyes and ears (conjunctivitis, episcleritis, blepharitis); and mucosa (mucosal secretion, paleness of mucous membranes).

The mean number of clinical signs related to leishmaniosis per affected dog was statistically significantly reduced (p = 0.026) in the vaccinated group (2.00 signs per dog) when compared to the placebo group (2.75 signs per dog).

3.6. Prevention of canine leishmaniosis

An individual dog was considered a confirmed case of canine leishmaniosis when it presented (a) clinical signs compatible with the disease; (b) a positive ELISA SLA (≥0.48) or IFAT (≥1/80); and (c) parasites in either bone marrow or lymph nodes detected by smear test or PCR. In addition, dogs were considered asymptomatic cases in the absence of clinical signs, high positive IFAT (≥1/640) and presence of *Leishmania* in bone marrow or lymph nodes at the last time point; however, no dog fell in these conditions during the study.

The development of confirmed cases of the disease was observed during the course of the study in the placebo group (Fig. 5). A statistically significantly (p = 0.048) lower number of cases of leishmaniosis was observed in the vaccine group (8 cases, 4.7%) compared to the placebo group (19 cases, 10.2%). The profiles of these cases are detailed in Table 3.

In the placebo group, 17 dogs out of those 19 cases (89%) showed signs of the disease in more than one organ system at the time of diagnosis as case. In the vaccine group, clinical signs were limited to one organ system in 4 of the 8 cases; the four other dogs (50%) presented general signs in more than one organ system.

At the revaccination time (on Day 365), 13 animals in each group were found to be seropositive. Of these, 10 dogs in each group survived until the last time point (Day 730). Among these 10 dogs, 8 dogs (80%) were classified as cases of leishmaniosis in the placebo group one year later at the last time point of the study, whereas only 3 cases (30%) were detected in the vaccinated group (Day 730).

4. Discussion

This multi-site, randomized GCP field trial demonstrates the efficacy and safety of the LetiFend® vaccine in preventing clinical leishmaniosis in a large dog population of different breeds, ages and weights exposed to two *Leishmania infantum* transmission seasons in two endemic areas of Europe.

A total of 19 kennels of dogs located in endemic areas of Spain and France were studied in this clinical trial. The circulation of *L. infantum* was proven in these kennels either by seroconversion of dogs (IFAT titres ≥ 1/80 or SLA > 0.480 OD) and/or positive PCR for *L. infantum* DNA in the lymphoid tissues of the placebo group (24.8%), on at least in one occasion. However, prevalence of infection in some kennels was lower than expected, and at the end of the study the overall percentage of infected dogs in the placebo group was only 16%. The causes of this low prevalence of infection are difficult to determine.

Protein Q (active component of LetiFend® vaccine) was shown to be very safe in previous GLP and GCP studies [28,30]. This safety profile has been confirmed in this Phase III GCP-field trial, since none of the dogs presented adverse events attributed to the vaccination. This could be linked to the fact that the vaccine is only one single dose and does not contain external adjuvants or immune enhancers, as apposite as other commercial veterinary vaccines, that may induce adverse events [44,45]. The vaccine is therefore considered to have a very good safety profile in a wide range of dog breeds, ages and weights. In addition, vaccine administration to seropositive dogs was followed up for one year after the revaccination, demonstrating that LetiFend® was also safe in these dogs and did not worsen the course of the disease.

Previous studies have demonstrated that this recombinant protein is able to elicit a cellular and humoral immune response in the dog [29], even in the absence of an adjuvant [28]. The results of the current study are in agreement with these previous data: dogs of

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Table 3
Profile of dogs diagnosed as confirmed cases of leishmaniosis.

Treatment group	Animal ID	Antibodies anti-Leish (ELISA SLA, OD)	Antibodies anti-Leish (IFAT, 1/X)	Parasite in BM and/or LN (PCR, Smear)	Clinical findings (suggestive of leishmaniosis)
Placebo	A-01-018	0.752	40	Positive	Otitis, bilateral conjunctivitis
	B-01-021	0.820	640	Positive	Weight loss, lymph node enlargement, conjunctivitis, facial, auricular and peri-ocular alopecia
	B-03-001	1.035	640	Positive	Weight loss, dull hair/coat, lymph node enlargement
	B-03-006	1.012	320	Positive	Weight loss, dull hair coat, lymph node enlargement
	C-01-006	0.956	640	Positive	Peri-ocular seborrhea and desquamation, lymph node enlargement
	C-01-013	1.070	320	Positive	Asthenia
	K-01-006	0.535	0	Positive	Moderate enlargement of pre-scapular lymph nodes, bilateral mild epiescleritis
	K-01-024	0.444	80	Positive	Weight loss, lymph node enlargement (left pre-scapular and right popliteal), bilateral epiescleritis and blepharitis
	K-01-029	0.966	640	Positive	Lymph node enlargement, epiescleritis
	K-01-039	1.007	640	Positive	Weight loss, asthenia, marked lymph node enlargement, nasal hyperkeratosis and desquamation, nasal mucosal secretion, bilateral blepharitis and conjunctivitis, exfoliative dermatitis with alopecia, pale mucous membranes
	K-01-041	0.982	640	Positive	Marked lymph node enlargement, multiple cutaneous ulcers, bilateral, epiescleritis
	K-01-043	0.574	160	Positive	Lymph node enlargement, epiescleritis
	K-03-017	0.859	320	Positive	Weight loss, dull coat, epiescleritis, lymph node enlargement, bilateral blepharitis and conjunctivitis
	K-04-027	0.906	640	Positive	Weight loss, lymph node enlargement, nasal hyperkeratosis, multiple cutaneous ulcers, onychogryphosis
	K-04-014	0.729	40	Positive	Lymph nodes enlargement
	K-04-020	0.500	80	Positive	Lymph node enlargement, dermatitis on pectoral region, mild bilateral epiescleritis
	K-04-024	0.500	80	Positive	Lymph node enlargement
	K-05-002	0.886	640	Positive	Lymph node enlargement, multiple cutaneous ulcers, areas of alopecia with desquamation
	K-07-010	0.741	0	Positive	Lymph node enlargement, areas of alopecia
Vaccine	B-03-016	0.981	640	Positive	Clinical signs of Leishmaniosis (not specified)
	C-01-022	0.708	40	Positive	Ulcer on the nose
	K-01-013	0.906	160	Positive	Lymph node enlargement
	K-01-040	0.926	320	Positive	Weight loss, lymph node enlargement, nasal hyperkeratosis, pale mucous membranes
	K-05-003	0.770	80	Positive	Nasal hyperkeratosis, moderate mucous nasal secretion
	K-05-024	0.889	320	Positive	Weight loss, lymph node enlargement, nasal hyperkeratosis, areas of alopecia, multiple cutaneous ulcers, bilateral blepharitis
	K-06-005	0.881	640	Positive	Pale mucous membranes, onychogryphosis
	K-07-004	0.696	0	Positive	Mild bilateral epiescleritis, mucous conjunctivitis

Data are referred to the respective day of disease diagnosis. LN: lymph node; BM: bone marrow.

the vaccinated group consistently showed an early and statistically significant increase of IgG2 antibodies against Protein Q, the antigenic component of LetiFend®, 14 days after vaccination. This response was significantly different to the control group, where no antibody production was detected. The increase in IgG2 antibodies in vaccinated animals was also observed when the dogs were revaccinated one year later.

Regarding the immune response elicited against the natural *L. infantum* infection, it is remarkable that the anti-SLA antibody response was higher in the control group, whereas it showed a tendency to decrease in vaccinated animals. This result was more evident with the IFAT data, where among seropositive animals, 44.4% of dogs of the placebo group presented high IFAT titres (≥1/640) at the last time point, whereas, only 17.7% of vaccinated dogs presented high titres. These differences were not statistically significant, probably due to the low number of seropositive dogs. However, these data are relevant because it has been widely described that high and unbalanced antibody responses are related to pathological forms of the disease [23,46–48], as well as the development of clinical signs [49], whereas, a less pronounced humoral response against total parasite antigens may be a sign of resistance and good prognosis [48]. A reduction in both IFAT titres and the number of IFAT positive animals in the vaccinated group indicates that the intensity of the antigenic stimulus due to infection was lower in these animals when compared to placebo. This reduction in IFAT is described to be associated with disease resistance [46].

Tissue parasite load has also been related to the development of clinical signs characteristic of canine leishmaniosis [50,51]. In this field trial, the percentage of infected dogs measured by PCR and/or smear test was lower in the vaccine group than in the placebo group (9.4% vs 16.1%, very close to statistical significance). These data can be considered as an indication of the efficacy of LetiFend®, and are in agreement with previous results with either Protein Q in experimentally infected dogs [28,29] or other vaccine candidates [10,52–55].

The appearance of clinical signs in canine leishmaniosis is characterized by presenting a chronic course with a progressive worsening of the general condition [56–59]. The results of this study demonstrate that LetiFend® vaccination clearly reduces the incidence of clinical signs potentially related to leishmaniosis. In the placebo group 32.8% of dogs, while in the vaccine group only 12.9% of presented clinical signs attributed to leishmaniosis dogs showed clinical signs ($p < 0.001$). Within the group of dogs presenting clinical signs, a significantly higher number of individual clinical signs were seen in the placebo than in the vaccine group, indicating a reduction of the severity of the disease. Due to the heterogeneity of the clinical expression of CanL, it is difficult to assure that each clinical sign is a direct consequence of the *Leishmania* infection. However, dogs from both groups showed a different clinical progression of the disease and the health condition of the vaccinated dogs was significantly better than that of the dogs that only received the placebo. This reduction in the clinical manifestation of the disease is in agreement with that described by several authors with other canine vaccine candidates [10,52,60].

Since CanL is a disease that is difficult to diagnose, a multidiagnostic approach has been recommended in the literature [39,56,60–62]. In the current study, a dog was considered a confirmed case of leishmaniosis if the animal showed compatible clinical signs of the disease, a positive serology and presence of *Leishmania* in lymphoid organs. Anti-*Leishmania* antibody serology could be used for the leishmaniosis case classification and disease diagnosis since the vaccination with LetiFend® does not interfere with leishmaniosis serological diagnostic tests [28,63].

According to this definition, the number of cases of leishmaniosis was statistically significant ($p = 0.048$) lower in the vaccine group than in the placebo group, which provides strong evidence of the efficacy of the vaccine. At the end of the study, 10.2% of placebo dogs developed canine leishmaniosis, whereas only 4.7% of vaccinated animals did.

The infection pressure and the incidence of the disease varied markedly in the placebo group among the 19 different kennels during the study. The highest incidence of leishmaniosis were shown to be at sites K01 (30%) and K04 (40%) (Cáceres, Spain), and this was comparable to the prevalence levels expected in endemic areas [37]. These two sites also presented a high number of surviving animals at the end of the study ($n = 50$), as well as the highest parasite circulation in the placebo group (36–58%, as measured by serology and PCR). In consequence, these two sites were selected for analysis of vaccine efficacy as representatives of an endemic area of CanL with high disease pressure. The data showed a 34.5% of disease in the placebo group, whereas only 9.5% of clinical cases were found in the LetiFend group. Thus LetiFend® vaccine was found to be 72% efficacious in the prevention of clinical cases of leishmaniosis in areas with high disease pressure. The likelihood that a dog vaccinated with LetiFend® developed a confirmed case of leishmaniosis or developed clinical signs of the disease was 5 and 9.8 time less, respectively, than that for an unvaccinated dog.

Clinical studies of CanL are difficult to perform due to the complexity of the disease: this implies, among other factors, high number of study dogs, long duration of the trial and the unpredictable natural challenge during the study [64]. In our study, animal losses not related to leishmaniosis were numerous due to the inherent complexity of large field trials involving privately owned dogs, and also due to the hunting activity of the majority of these dogs. Probably these facts had a relevant impact on reducing the power of some of the statistical results obtained.

Although there are two other vaccines available in the Brazilian and EU market, it is very difficult to compare their relative effectiveness with that of LetiFend®, mainly due to the major differences in the study designs of the clinical trials, such as breeds, study areas, case definition, analytical techniques used and differences in parasite pressure [9,10,65].

As a summary, the administration of LetiFend led to the development of an immune response against *Leishmania* in vaccinated animals which could control the development of the disease.

5. Conclusion

Overall, the data of this field trial demonstrate that LetiFend® is a novel, safe and effective vaccine for the active immunization of non-infected dogs from 6 months of age in reducing the risk of developing leishmaniosis after natural infection with *Leishmania infantum*. The vaccine constitutes a significant step forward in the control of CanL.

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Conflict of interest statement

The authors of this manuscript have read the journal's policy and some of them have the following competing interests: MF and FM are employees of Laboratorios LETI S.L.U. but they did not participate in the conduction of the study, data collection or analysis. This project was supported by Laboratorios LETI S.L.U.

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