



Field randomized trial to evaluate the efficacy of the Leish-Tec[®] vaccine against canine visceral leishmaniasis in an endemic area of Brazil



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ARTICLE INFO

Article history:

Received 22 December 2015

Received in revised form 1 March 2016

Accepted 8 March 2016

Available online 18 March 2016

Keywords:

Randomized field trial

Canine visceral leishmaniasis

Vaccine

A2 antigen

Leish-Tec[®]

Efficacy

ABSTRACT

Background: A canine vaccine remains a promising approach for effective control of visceral leishmaniasis (VL), given its complex epidemiology in areas where zoonotic VL is prevalent. Leish-Tec[®] is a recombinant vaccine, based on the *Leishmania* A2 antigen, against canine VL (CVL). It is, since 2014, the single commercial vaccine licensed in Brazil. Here, Leish-Tec[®] efficacy was estimated through a randomized field trial (RFT), in a highly VL endemic area.

Methods: The RFT was conducted from 2008 to 2010 in an endemic area of southeastern Brazil, presenting a CVL seroprevalence of 41.9%. Eight hundred forty-seven seronegative dogs were randomly selected to receive Leish-Tec[®] ($n = 429$) or placebo ($n = 418$). Animals were followed up by clinical, serological, and parasitological exams for 18 months. The CVL incidence in both groups was compared through proportion analysis.

Results: A significant reduction in the number of cases of CVL was observed in the vaccine group, as compared with the placebo group, whether efficacy was estimated according to parasitological results (71.4%; 95% CI: 34.9–87.3%; $p = 0.001$; risk ratio = 0.287), by adding results of xenodiagnosis and parasitological exams (58.1%; 95% CI: 26.0–76.3%; $p = 0.002$; risk ratio = 0.419). Among the animals that converted to a positive anti-A2 serology, efficacy reached 80.8% (95% CI: 37.6–94.1%, $p = 0.001$; risk ratio = 0.192). Xenodiagnosis has detected a reduction of 46.6% ($p = 0.05$) in transmission to sand flies from vaccinated animals presenting anti-A2 positive serology.

Conclusion: The Leish-Tec[®] vaccine proved significantly effective for prophylaxis of CVL, after natural challenge assured by transmission of *Leishmania* parasites, in a highly endemic area. Noteworthy, this report has unveiled the complexity of performing a RFT for anti-CVL vaccines in Brazil, which may be helpful for designing of future studies.

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1. Introduction

Visceral leishmaniasis (VL) is a protozoan parasitic disease that, if untreated, leads to high mortality rates. VL, similarly to other leishmaniasis forms, is a neglected disease, resulting in 20,000–40,000 deaths [1]. In zoonotic VL transmission areas, domestic dogs are reservoirs of *L. (L.) infantum* (Mediterranean basin) and *L. (L.) infantum chagasi* (South America) parasites for human infection [2–4].

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Dogs are also very susceptible to infection and may develop severe symptoms, ending to death [3,5]. The available chemotherapeutic treatment is limited [6] and once clinically cured, asymptomatic animals may still transmit the infection. Therefore, treatment is not recommended as a mass control measure in Brazil [4]. Prophylactic control programs in Brazil have focused on euthanizing seropositive dogs [7]. However, control campaigns are very expensive and their relatively poor success rate is aggravated by the lack of highly sensitive, specific biomarkers for the infectious status in dogs [7–9]. Consequently, VL has expanded to areas where its occurrence had not been previously reported [7,9–10]. Therefore, vaccination emerges as a potential strategy to protect dogs from being infectious to the sand fly vector, thereby reducing transmission rates.

Currently, Leish-Tec[®], a vaccine formulation containing the recombinant protein A2 of *L. (L.) donovani* and saponin, is the unique commercial CVL vaccine licensed in Brazil. Several pre-clinical trials in mice have provided evidence of the protective responses induced by vaccination with the A2 antigen, against VL [11–16]. In *Rhesus* monkeys, an impressive protective effect was induced by prime-boosting vaccination protocols using recombinant A2 protein and adenovirus expressing A2 [17]. Leish-Tec[®] was shown to induce protective immunity in beagle dogs against a high dose intravenous infection with *L. (L.) infantum chagasi* [18]. Moreover, Leish-Tec[®] does not induce seroconversion in vaccinated animals, an important requirement for CVL vaccines when euthanasia of seropositive dogs is recommended [19]. Vaccination also reduced significantly the infectiousness of dogs to sand flies, as demonstrated by xenodiagnosis [20].

Here, we report the results of a randomized field trial (RFT) carried out in an endemic area for VL, to evaluate the efficacy of Leish-Tec[®].

2. Methods

2.1. Ethics statement

The Research Ethics Committee of the Federal University of Ouro Preto (CEP/UFOP, Brazil) approved this RFT (document No. 58/2008). This RFT was also registered in the Brazilian Ministry of Agriculture (MAPA, Brazil), under the official document 028/2013-CPV/DFIP/MAPA.

2.2. Vaccine

The doses of the Leish-Tec[®] vaccine were produced by Hertape Saúde Animal (Juatuba, Minas Gerais, Brazil), and standardized to contain 100 µg/mL of recombinant A2 protein and 500 µg/mL of saponin, as adjuvant. The vaccine doses had the same formulation of the commercially available vaccine. Dogs received three 1.0 mL doses administered through subcutaneous injection at 21-day intervals [18,19]. Placebo and vaccine doses were coded at Hertape Saúde Animal; had the same appearance and were given to the animals by field veterinarians, blindly.

2.3. Sample size estimation

A sample size of 568 dogs in each group was estimated, according to Fleiss [21], using the following assumptions: (a) a 1/1 ratio between vaccinated and placebo groups; (b) 50% vaccine effectiveness; (c) 10% annual CVL incidence in Porteirinha [22,23]; (d) power (0.90); (e) significant difference 5%; and (f) 20% estimated losses. Sensitivity of 96% was considered when applying diagnosis tests in parallel [24].

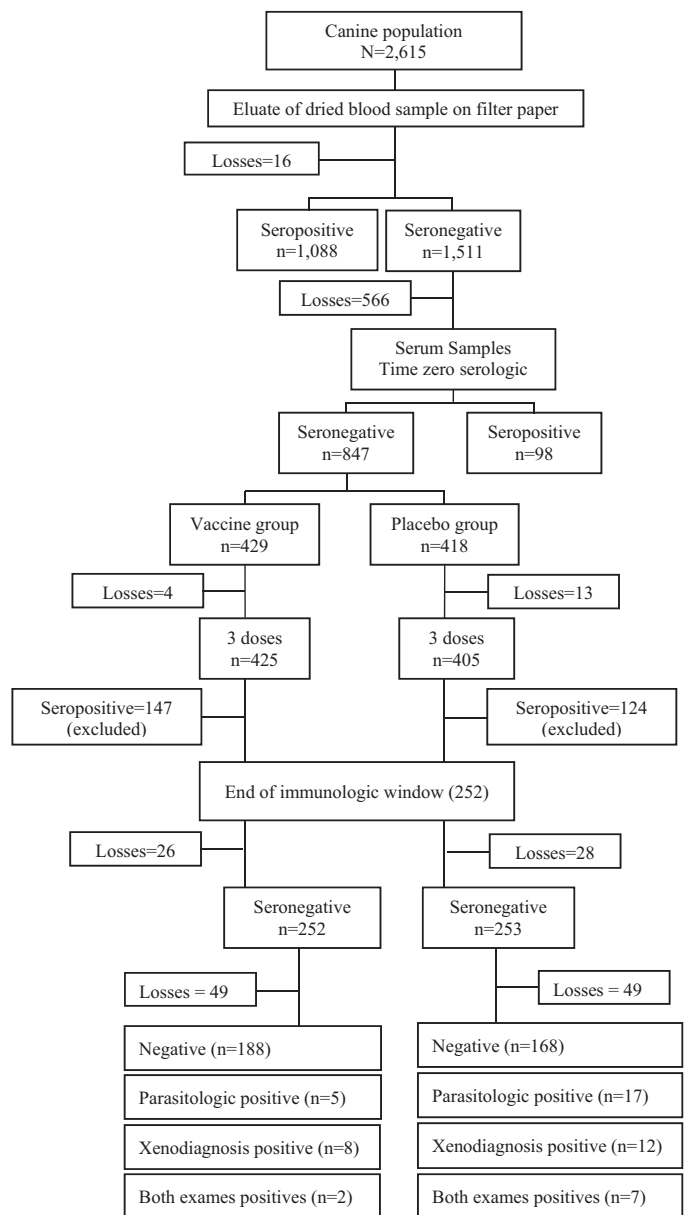


Fig. 1. Flow chart showing screening, sampling and losses of dogs included in the randomized field vaccine trial, in Porteirinha, Minas Gerais, Brazil, 2008.

2.4. Study design and population

The RFT was conducted from 2008 to 2010, in urban and rural areas of the municipality of Porteirinha, Minas Gerais, Brazil. Porteirinha is a VL endemic area and no control measures, including euthanasia of seropositive dogs, insecticide spraying, or use of impregnated collars, had been applied in the eight years prior to this study [22,23].

Fig. 1 shows a flow chart of the screening and selection of the canine population. Initially, a canine serological census ($N=2615$) was performed in the urban neighborhoods and rural outskirts of Porteirinha. The number of VL seropositive dogs per city block was determined, with an overall CVL prevalence of 41.9%, corresponding to 31.6% within the city limits and 80.1% in the rural areas. Reactive dogs in at least one serological exam crude ELISA (cELISA) or indirect fluorescence antibody test (IFAT) were excluded from the vaccination trial, corresponding to 1088 animals. A total of 1511 seronegative dogs were pre-selected to be included in the trial.

Four months later, before vaccination (time zero), serological testing (cELISA, IFAT and Kalazar detect™ (KD)) was repeated in these 1511 dogs, to finally determine eligibility of these animals. Of those, 98 animals were excluded due to positive serology. Other 566 seronegative dogs were also excluded, due to the owners' refusal to participate, animal's death, escape or closed houses. The final sample of 847 dogs, negative by serological analysis, were randomly allocated into two groups: vaccinated ($n = 429$) and placebo ($n = 418$). For randomization a simple method was used, consisting of blocks or sets of four codes (two letters for vaccine and two for placebo). Thus, four animals, two allocated in the placebo and two in the vaccinated group, composed each block. Veterinarians actively monitored the occurrence of any adverse effects such as fever, pain, or edema at the site of vaccine administration, over a period of 72 h after vaccination.

2.5. Inclusion and exclusion criteria

Only healthy dogs were eligible for the study, as clinically determined by veterinarians, whether a purebred or mongrel, regardless of gender, at least four months old and seronegative for CVL. Clinical examination included evaluation for clinical signs of CVL [25]. Exclusion criteria were the presence of other pathologies or positive serology for CVL, during the immunological window. Animals were considered as seropositive if both cELISA and IFAT resulted positive. CVL negative animals, in clinical and serological evaluations, were included in the RFT, only after their owners had agreed to take part in the study, following an interview to explain the purpose of the study and after they had signed a Statement of Informed Consent.

Eligible dogs were dewormed and received two doses of Multi-Dog® (Hertape Saúde Animal) a polyvalent vaccine, administered 42 and 21 days, before Leish-Tec® doses. Each animal was microchipped (Animal TAG®, Korth RFID Ltd., São Carlos, São Paulo, Brazil), identified by a four-digit code, and then registered in the project database.

All dogs were maintained in their original households and naturally exposed to *Leishmania* spp. infection (natural challenge). Veterinarians conducted monthly clinical examinations in all dogs. Blood samples were collected every three months for CVL serological testing. A period of 252 days was considered as immunological window [24,25], i.e., a period in which animals may manifest clinical and laboratory results positive for CVL due to previous infection. This window period was also based on the report by Courtenay et al. [8], which described a cohort of naive dogs exposed to natural infection in an endemic area. In this cohort, dogs became infectious to sand flies in average 333 days (105 days after seroconversion).

Accordingly, 271 dogs were excluded due to positive results in serological tests during the immunological window. These animals were not submitted to a complete follow up, since some were euthanized by public health authorities and other were lost due to natural interurrences. The outcome among the remaining dogs varied according to the diagnostic method: 137 were positive in parasitological tests, and 14 in the xenodiagnosis. Of those, only 17 animals presented the typical symptoms of CVL. All the other had only positive serology ($n = 120$). The remaining seronegative animals were followed up for 557 days, including the period of vaccination (time 0) and the immunological window (252 days).

2.6. Outcome variables and case definition

After the immunological window, dogs presenting positive serology (cELISA and IFAT) were considered as potential VL cases. They were euthanized and submitted to parasitological examination. Bone marrow smears and imprints of tissue fragments (skin, mesenteric lymph nodes, and spleen biopsy) on glass slides were

stained with Giemsa and examined microscopically. Potential VL cases were considered positive VL cases only after the detection of parasites in at least one dog's tissue, in any of the parasitological tests, including bone marrow cultures. Direct parasitological exams and histopathology were performed according to standard procedures [23,26].

2.7. Bone marrow culture and identification of *Leishmania* species

Bone marrow culture was performed as previously described [19]. Genomic DNA extracted from parasites isolated from bone marrow aspirates was submitted to kDNA PCR-RFLP analysis for identification of *Leishmania* species, as previously described, with modifications [27]. Typing of *L. (L.) amazonensis* isolates were confirmed by *Ava-I* digestion.

2.8. Serological tests

cELISA (Biomanguinhos Institute, Rio de Janeiro, Brazil) was used for serological screening and IFAT (Biomanguinhos Institute, Rio de Janeiro, Brazil), as a confirmatory test. KD (Inbios NDI) was also used as a confirmatory test. All tests were carried out in accordance with manufacturers' instructions. Nine serological evaluations were performed, for longitudinal follow up of all animals. Measuring of serological anti-A2 responses were performed by ELISA using purified A2 recombinant protein, as previously described [19,28].

2.9. Xenodiagnosis

Xenodiagnosis was performed after the immunological window period, starting at day 497. The number of selected dogs ($n = 154$, 77 in each group) was based on the following assumptions: (a) statistical significance (0.05); (b) power (0.90); (c) the proof of bidirectional (or two-tailed) hypothesis; (d) an estimated proportion of 50% of vector infection by *Leishmania* in non-vaccinated infected dogs [29]; (e) an expected difference of 50% between groups; (f) 10% sample loss. All dogs allocated in randomized blocks lodging at least two animals with serological positive results, were selected to ensure that xenodiagnosis was performed in a blinded manner.

The phlebotomine sand flies were F1 off springs from a *Lutzomyia longipalpis* colony of insects provided by CpqRR/Fiocruz (Brazil). They were transported to the Porteirinha (640 km), in appropriated containers under controlled humidity and temperature. Xenodiagnosis was performed as previously described [30]. The presence of *Leishmania* spp. DNA was investigated by PCR amplification of a 120 bp segment from the conserved minicircle of kDNA for the *Leishmania* genus [31].

2.10. Statistical analysis

An external clinical monitor (ECM) reviewed data files and locked them after the vaccination procedures. Initially, each dog was identified as a confirmed or negative case for *Leishmania* spp. infection, according to parasitological diagnosis. Data management, descriptive analyses of baseline characteristics (frequencies and means \pm SD), for each trial group and bivariate analyses of differences between proportions (Fisher exact test) and means (t -tests) were performed with SPSS 20 software (SPSS Inc., Chicago, IL, USA). Vaccine efficacy was estimated according to either parasitological definition of cases or by adding xenodiagnosis and parasitological results. Analyses were also stratified according to anti-A2 serological conversion to estimate efficacy. RR is the ratio of CVL incidence in the vaccine group as compared to the placebo group and used to calculated efficacy as $100 \times (1 - \text{relative risk (RR)})$. Only those subjects who completed all vaccination doses were included in

the efficacy analyses. Differences between groups were considered significant when $p \leq 0.05$.

3. Results

3.1. Demographic and geographic distribution of dogs

Table 1 shows the number of dogs allocated in the vaccine and placebo groups by city location. There were no significant differences in ratios between the vaccine and placebo groups, or the distribution in rural and urban areas, indicating that successful randomization of vaccine doses was achieved.

After the immunological window, 559 animals remained included in the RFT. Table 2 describes their serological and parasitological results in all tests, during the challenge period of the RFT. The majority of the animals was asymptomatic and remained seronegative ($n = 356$, 63.7%), throughout the study. In contrast, only 5.6% ($n = 31$) of animals presented suggestive clinical signs of CVL. Parasitological exams were performed in 65 seropositive animals. Of those, 34 dogs resulted parasitological negative and were not included in the estimation of efficacy, due to the uncertainty in their infectious status. Confirmed cases, i.e., animals presenting positive parasitological results in at least one parasitological test, corresponding to 5.6% ($n = 31$) of the final sample, were included for efficacy estimation. Thus, the final sample for efficacy estimation corresponded to 356 negative animals and 31 cases. A high degree of discordance was observed in serological analysis, since only 6.6% ($n = 37/559$) of the animals were concomitantly positive in two serological tests (cELISA + IFAT), while 13.5% (44/325) were positive only in KD, 19.3% (108/559) in cELISA and 20.9% (117/559) in IFAT.

Table 1
Randomization of dogs receiving vaccine or placebo doses, in rural and urban areas in Porteirinha, Minas Gerais, Brazil.

Group	Rural area N (%)	Urban area N (%)	Total N (%)	p value
Vaccine	50 (50.5)	380 (50.8)	430 (50.8)	0.955
Placebo	49 (49.5)	368 (49.2)	417 (49.2)	
Total	99	748	847	

Table 2
Parasitological and serological results in dogs, after the immunological window.

Final classification	Diagnostic test	No. of positive/No. of evaluated dogs (%)
Confirmed cases ^a	Parallel Parasitological Exams	31/65 (47.7%) ^b
	Xenodiagnosis	20/71 (28.1%)
Non-confirmed cases ^c	Serology (ELISA and IFAT)	37/559 (6.6%)
	ELISA	108/559 (19.3%)
	IFAT	117/559 (20.9%)
	Rapid test-Kalazar Detect TM	44/325 (13.5%)
Negative ^d		356/559 (63.7%)
Total		559

^a Parasites were detected in at least one of the parallel parasitological exams, including bone marrow culture, direct parasitological exam (imprint or smear in microscopic slides) of skin, lymph nodes, spleen and bone marrow or histopathological analysis.

^b Nine animals were also positive in xenodiagnosis.

^c Non-confirmed cases were not considered for efficacy estimation.

^d Asymptomatic animals, presenting negative results in all serological diagnostic tests, applied longitudinally throughout the study period.

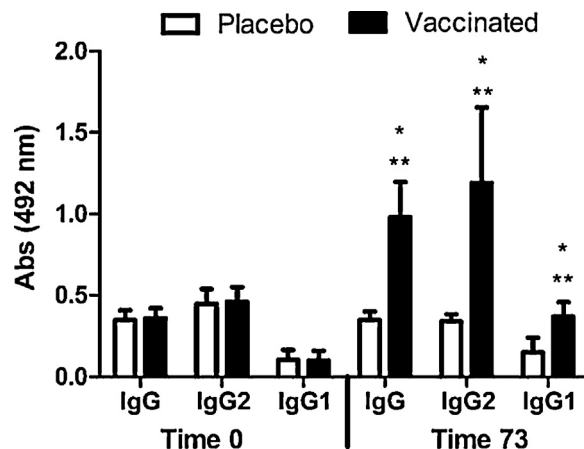


Fig. 2. Anti-A2 specific serological responses in animals vaccinated with Leish-Tec[®]. Levels of anti-A2 IgG, IgG1, and IgG2 antibodies in pre-immune sera (time 0) and after vaccination (time 73) were detected by ELISA. Each bar represents the average \pm standard deviation for the optical density in each group. One asterisk indicates statistically significant differences between placebo and vaccinated animals at the same time point, and two asterisks, the differences between antibody levels at different time points for the same group of animals.

A sample of 71 animals was also tested in xenodiagnosis, resulting in 40.8% (29/71) positive dogs. Of those, nine were also positive in parasitological tests. As expected for a sample composed predominantly of asymptomatic animals, a large degree of discordant results was observed among the CVL diagnostic tools used, mainly xenodiagnosis or serological tests. Given the uncertainty in the CVL diagnosis among animals classified as non-confirmed cases, they were not included in the sample used to estimate efficacy.

Immunogenicity was evaluated by comparing anti-A2 humoral responses, measured in sera samples collected immediately before (time 0) and after the priming vaccination protocol (time 73), from both vaccinated and placebo animals. As shown in Fig. 2, after vaccination, anti-A2 total IgG, IgG2, and IgG1 antibody levels increased significantly in vaccinated dogs, as compared to the antibody levels detected at time zero or to the levels presented by placebo animals at time 73.

Table 3 shows the proportion of CVL cases within the vaccine and placebo groups. A significant reduction in the number of cases of CVL was observed in the vaccine group, as compared to placebo, reaching an overall efficacy, based on the parasitological definition of cases of 71.4% (95% CI: 34.9–87.3%; $p = 0.001$; RR = 0.287). By adding results of xenodiagnosis to parasitological exams, efficacy remained high and significant (58.1%; 95% CI: 26.0–76.3%; $p = 0.002$; RR = 0.419). Among the animals that converted to a positive anti-A2 serology, efficacy was 80.8% (95% CI: 37.6–94.1%, $p = 0.001$; RR = 0.192).

Table 4 describes the distribution of *Leishmania* species, identified from 41 bone marrow cultures, between vaccinated and placebo groups. As expected, *L. (L.) infantum chagasi* was the most prevalent species (85.4%; $n = 35$), whereas *L. (L.) amazonensis* was identified in 14.6% ($n = 6$) of the cultures. No significant differences were observed in the distribution of these parasite species within each group.

Results from xenodiagnoses are presented in Table 5. *Leishmania* spp. kDNA was detected in 29 out of the 71 sand flies' samples, corresponding to an overall prevalence 40.8%. The prevalence of positive sand fly pools that fed in animals of the placebo and vaccinated groups was 44.2% (19 out of 43 pools) and 35.7% (10 out of 28 pools), respectively ($p = 0.48$). However, a significant ($p = 0.05$) of reduction in infectivity to sand flies was observed should the animals be stratified according to anti-A2 serological responses. While similar proportions of negative ($n = 4$) and positive ($n = 4$) sand

Table 3
Leish-Tec® efficacy, as determined by parasitological exams, xenodiagnosis and anti-A2 serology, in Porteirinha, Minas Gerais, Brazil.

Group	CVL criteria ^a			CVL criteria ^b			CVL criteria ^c		
	Positive	Negative	Total	Positive	Negative	Total	Positive	Negative	Total
Vaccine	7	188	195	15	188	203	3	122	125
n (%)	(3.6)	(96.4)	(100.0)	(7.4)	(92.6)	(100.0)	(2.4)	(97.6)	(100.0)
Placebo	24	168	192	36	168	204	24	168	192
n (%)	(12.5)	(87.5)	(100.0)	(17.7)	(82.3)	(100.0)	(12.5)	(87.5)	(100.0)
Total	31	356	387	51	356	407	27	290	317
n (%)	(8.0)	(92.0)	(100.0)	(12.5)	(87.5)	(100.0)	(8.5)	(91.5)	(100.0)

^a According to parasitological exams: imprinting, culture, or histopathology of dog tissues (skin, lymph nodes, spleen and bone marrow), $p=0.001$; risk ratio = 0.287; efficacy = 71.4% (95% CI: 34.9–87.3%).

^b According to parasitological exams plus xenodiagnosis, $p=0.002$; risk ratio = 0.419; efficacy = 58.1% (95% CI: 26.0–76.3%).

^c According to anti-A2 serology, $p=0.001$; risk ratio = 0.192; efficacy = 80.8% (95% CI: 37.6–94.1%).

Table 4
Distribution of *Leishmania* species isolated from bone marrow of infected dogs as determined by kDNA PCR-RFLP, in Porteirinha, Minas Gerais, Brazil.

Group	<i>L. (L.) infantum chagasi</i> n (%)	<i>L. (L.) amazonensis</i> n (%)
Placebo	20 (87.0)	3 (13.0)
Vaccine	15 (83.3)	3 (16.7)
Total	35 (85.4)	6 (14.6)
Asymptomatic animals	24 (68.6)	6 (100)

p value = 0.74.

Table 5
Frequencies of *Leishmania* spp. DNA, as detected by PCR, in *Lutzomyia longipalpis* phlebotomine sand flies' pools feeding (xenodiagnosis) on placebo and vaccinated dogs, in Porteirinha, Minas Gerais, Brazil, 2010.

Group	A2 classification	kDNA PCR		Total
		Negative	Positive	
Placebo		24 (55.8)	19 (44.2)	43
Vaccinated	Positive	11 (73.3)	4 (26.7)	15
	Negative	4 (50.0)	4 (50.0)	8
	No information	3 (60.0)	2 (40.0)	5
	Total	18 (64.3)	10 (35.7)	28
Total		42 (59.2)	29 (40.8)	71

p value = 0.48 (not stratified – placebo × vaccinated).

p value = 0.05 (stratified according to positive serology for A2 – placebo × vaccinated).

fly' pools were observed, after feeding in anti-A2 negative dogs, only four out of 15 pools (26.7%) feeding on vaccinated animals were positive. Therefore, anti-A2 seropositive vaccinated animals (73.3%) were less infectious to sand flies, corresponding to 46.6% reduction in transmission to sand flies.

4. Discussion

This RFT was carried out in a highly endemic area of VL [22,32–33]. Most probably due to the lack of application control measures for a long period (from 2004 to 2008), the canine seroprevalence in Porteirinha was higher than that used to estimate sample size, reaching 41.9%. Nonetheless, the high CVL prevalence in Porteirinha allowed a significant challenge for demonstrating Leish-Tec® efficacy. Moreover, Leish-Tec® efficacy was tested in a native heterogeneous population, previously exposed to infection, an extreme condition and challenging scenario, that badly needs control interventions.

Different study designs have been used to test efficacy of anti-CVL vaccines, including testing on native animals or through the introduction of naïve animals, in endemic areas [34–36]. Both conditions may significantly shape the response to vaccination.

Application of an immunological window is required, when testing efficacy in native animals, allowing exclusion of previously exposed animals or those infected during screening and vaccination. In agreement, this study included an immunological window. The criteria applied in Brazil for VL epidemiologic control (serological tests for screening of and euthanasia of seropositive animals) was adopted, in order to test Leish-Tec® in the conditions it might be used, in endemic areas [23].

By assuming an improved sensitivity of parallel parasitological tests, in this RFT, estimation of efficacy was based on results of parasitological tests, mostly due to the predominance of asymptomatic animals in the final sample, and to the low sensitivity and specificity of serological tests. According to these criteria, and despite the high transmission pressure, the results of this RFT showed that vaccination with Leish-Tec® resulted in significant efficacy (71.4%, CI 34.9–87.3%). Moreover, considering only vaccinated animals, protection levels reached 96.4%, according to parasitological criteria.

It is largely known that CVL diagnosis in asymptomatic animals poses one of the main challenges in the veterinary routine, given the limitations of serological and other non-invasive diagnostic tests and the lack of pathognomonic CVL signs [25]. In endemic areas, the low positive predictive value of serological diagnostic tests impairs diagnosis of dogs as true positive, due to cross-reactions with other pathogens [37]. In agreement, a wide discordance among results of all serological tests was observed for animals during the challenge period, contributing for the uncertainty regarding the use of serological results for efficacy estimation. In contrast, there was a high concordance between the serological and the parasitological analyses performed. Among the 220 seropositive dogs submitted to euthanasia, 214 were positive in parasitological analysis (data not shown). On the other hand, the negative animals were clinically healthy and presented negative serological results, throughout the longitudinal serological follow up.

Leish-Tec® does not induce anti-promastigote cross-reactive antibodies, but raises specific cellular and humoral immune responses in dogs [18–19,38]. Hence, anti-A2 antibody responses were used here as biomarkers of immunogenicity of Leish-Tec® and as an auxiliary tool for interpreting vaccine efficacy. In agreement, vaccine efficacy among dogs that responded to vaccination with increased anti-A2 antibody levels was higher (80.8%), as compared to the overall efficacy (71.4%). It is noteworthy that, in another study, seropositivity for anti-A2 total IgG antibodies was found in 98% of vaccinated animals shortly, after vaccination [19]. This value decreased to 81.13% six months later, before rising again (98%) after the vaccination boost, as expected for a protein vaccine. Antibody responses induced by vaccination may be also affected several factors, including concomitant infections, and poor nutrition status of animals. In addition, in natural populations, not all individuals respond equally to antigenic stimulation, due to MHC restriction. For instance, trials that evaluated the first vaccine against human American cutaneous leishmaniasis (Leishvacin)

showed that regardless of the population, 10–30% of individuals do not convert the test of Montenegro to positive, 45 days after vaccination [39,40]. Nonetheless, the adjuvant used is another aspect to be considered in order to improve cellular and immune responses induced by vaccination.

The anti-A2 IgG2/IgG1 ratios were higher than 1, suggesting the predominance of Th1 immune responses in Leish-Tec[®] vaccinated dogs, as similarly observed in the phase II trial in beagle dogs [18], and in an heterogeneous dog population kept in kennels [19]. Saponin was the adjuvant selected to formulate all the three commercially available CVL vaccines, since it induces Type I immune responses when added to vaccine formulations [18,41–42]. As previously reported, the levels of anti-A2 IgG1 and IgG2 antibodies and the IgG2/IgG1 ratio were correlated with specific high IFN- γ and low IL-10 levels, in Leish-Tec[®] vaccinated beagle dogs [18] or in other studies with different vaccine formulations [41,43]. IgG2 antibodies have been associated with opsonization and complement activation in vaccinated animals [44].

L. (L.) amazonensis was identified in 14.6% of the cultures, in agreement with other reports in Brazil [45–48]. Still, the presence of two distinct *Leishmania* infecting species seemed not to have influenced the results of Leish-Tec[®] vaccine efficacy. *L. (L.) amazonensis* also contains and express A2 gene sequences [28] and mice vaccination with the A2 antigen resulted in significant protection, providing pre-clinical evidence that the protective effect of Leish-Tec[®] may be extended to *L. (L.) amazonensis* infections in dogs [11,15]. Therefore, we believe that the difference in the percentage of infecting species observed in our assay is in line with the rate of prevalence of these species in the area, and not by a change in vaccine protection. Although there are no population basis' studies to estimate the prevalence of *L. amazonensis* in dogs and humans, it is noteworthy that Barral et al. [49] found that, among 144 human VL patients in Bahia, 35% were infected by *L. amazonensis*.

Xenodiagnosis an important strategy for accessing the potential for transmission of a given animal, though limited by low sensitivity and specificity in field conditions [20,29–30,50–53]. Nonetheless, by adding xenodiagnoses and parasitological findings, Leish-Tec[®] efficacy remained significantly high (58.1%; 95% CI: 26.0–76.3%; $p = 0.002$). Moreover, vaccination also induced a significant reduction (46.6%) in *Leishmania* spp. transmission to sand flies that fed in anti-A2 seropositive vaccinated dogs. In agreement, in another study, only 5.4% of animals vaccinated with Leish-Tec[®] were infectious to sand flies, in an endemic area, as compared to a positive rate of 36.6% among control dogs [20]. Thereby, Leish-Tec[®] seems to induce an appreciable reduction in *Leishmania* transmission to sand flies.

The poor nutritional and immunological status of animals, besides exposure to other infections may have affected significantly efficacy. Losses exceeding 50% and much higher than expected (20%), also constituted an important drawback. Losses were mainly due to the population refusal to deliver the dogs for testing. However, there were no differences in losses between the vaccinated and placebo groups.

Finally, this report has unveiled the complexity of performing a RFT for anti-CVL vaccines, in Brazil, given the epidemiological, diagnostic, logistic, and other issues, which may be helpful for designing future studies.

5. Conclusion

The Leish-Tec[®] vaccine was significantly effective for prophylaxis CVL, after natural challenge assured by a high transmission exposure to parasites, notwithstanding the RFT operating and logistical limitations.

Acknowledgements

We would like to thank Hertape Saúde Animal SA for financial support, the local government of Porteirinha, Ms. Vivian Walter dos Reis for your technical help during this study, and the Porteirinha Zoonosis Center team for their collaboration in collecting data and samples. We also want to express our thanks to the owners who allowed their dogs to participate in this trial.

Conflict of interest: None.

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