

Long lasting protection against canine kala-azar using the FML-QuilA saponin vaccine in an endemic area of Brazil (São Gonçalo do Amarante, RN)

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Abstract

Naturally exposed dogs of an endemic area were vaccinated with the fucose mannose ligand (FML) antigen of *Leishmania donovani* in formulation with QuilA saponin. The 100% of vaccinees were seropositive to FML and showed intradermal reaction to *L. donovani* lysate, 2 months after vaccination. The absorbency values and size of intradermal reaction were both significantly higher in vaccinees than in controls along a 3.5 years period (ANOVA, $P < 0.0001$). The 25% of the control animals (two dogs on the first year and six dogs on the fourth year, respectively) and 5% of the vaccinees (one dog during the fourth year) developed clinical and fatal disease until the end of experiment. This difference was significant ($\chi^2 = 3.93$, $P < 0.05$). This means that 95% protection against kala-azar was achieved in vaccinees, after FML-QuilA vaccination (80% of vaccine efficacy (VE)). *Leishmania* infection was also confirmed, 3.5 years after vaccination, in saline controls that showed positive polymerase chain reaction (PCR) for *Leishmania* DNA and FML-serology with no intradermal reaction. Higher seropositivities and intradermal reactions with no Leishmanial DNA were detected in vaccinees. The FML-QuilA vaccine induced a significant, long lasting and strong protective effect against canine kala-azar in the field.

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1. Introduction

Human kala-azar is a canid zoonosis whose etiological agents, (*Leishmania chagasi* or *L. infantum*) are acquired by peridomestic sandflies by feeding on skin of infected foxes and dogs. Their subsequent transmission to humans causes kala-azar or human visceral leishmaniasis. About 500,000 human cases of the disease are registered annually; 90% of them in Bangladesh, Brazil, India and Sudan. The current strategy for control of zoonotic visceral leishmaniasis (ZVL), as recommended by the World Health Organization (WHO), is based on detection and destruction of seropositive infected dogs and vector control [1]. In Brazil, the impact of canine control performed by removal of seropositive infected dogs was supported by some authors and contested

by others since it is very laborious, expensive and of doubtful efficacy, most probably due to the low sensitive serological methods used for diagnosis, delay in dog removal from endemic areas and resistance of the dog owners to the control campaign (reviewed in [2]). Dog removal had a positive impact in the maintenance of a low ratio of increase of kala-azar human incidence [2]. However, the canine incidence and seroprevalence do not negate, maintaining a residual reservoir of parasites in the field [3]. Mathematical models [4] and experimental results [5] suggest that the use of a canine or human protective vaccine, rather than dog removal to sacrifice, would represent an efficient control tool, of easier use, reducing the offer of parasites to sandfly vectors and consequently the number of human kala-azar cases.

An effective vaccine against human kala-azar is not yet available and only few reports in literature deal with a vaccine against canine visceral leishmaniasis. Lasri et al. [6] demonstrated that dogs vaccinated with autoclaved *L. major* promastigote lysate (ALM) and BCG showed an in vitro

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lymphocyte proliferative response, while dogs immunized with ALM and saponin expressed a main humoral antibody response against *L. infantum*. In this study, neither experimental nor natural challenge was included. Dunan et al. [7] using a *L. infantum* semi-purified lyophilized protein preparation (94–67 kDa) in naturally exposed dogs, achieved significantly higher ($P < 0.05$) rate of infection in vaccinees than in control dogs, during the first year of experiment. This vaccine then, while effective in murine models, developed no protection against canine kala-azar in the field [7]. Mayrink and co-workers [9], using a *L. brasiliensis* lysate and BCG vaccine, in a formulation related to the *Leishvacin*, previously shown to be about 50% protective against tegumentar leishmaniasis in humans [8], obtained 90% protection against experimental canine kala-azar in the kennel [9] while failed to detect any significant difference between vaccinees and placebos in a well designed field phase III assay [10].

In previous report, the protective potential of a *L. donovani* promastigote glycoproteic complex was assayed [5,11–13]. The fucose and mannose containing glycoprotein-enriched fraction was named fucose mannose ligand (FML), since it strongly inhibits the in vitro infection of murine macrophages by promastigotes and amastigotes of *L. donovani* [14,15]. The FML antigen is present on the surface of the parasite throughout the life cycle [15] being a potent immunogen in mice and rabbits [11,12,15] and a sensitive, predictive and specific antigen in serodiagnosis of human [16] and canine kala-azar [17]. The FML vaccine was assayed in phase I–IIa trials in Balb/c, Swiss albino mice and CB hamsters [11–13]. An 87.7% ($P < 0.01$), 84% ($P < 0.001$) and 85% ($P < 0.01$) average of significant and specific protection was achieved in the Balb/c, Swiss Albino outbred and CB hamster models, respectively [11–13]. Control animals showed significantly different levels of anti-FML antibodies, in vitro proliferative splenocyte response and liver LDU ($P < 0.01$ and < 0.001 , respectively) [11–13]. Recently, a phase III trial of efficacy of the FML vaccine was performed in São Gonçalo do Amaranto, RN, Brazil, an endemic area both, for human and canine kala-azar [5,16,17]. Protection against canine kala-azar was investigated in naturally exposed dogs vaccinated with the FML vaccine [5]. Ninety-seven percent of vaccinees were seropositive to FML and 100% showed intradermal reaction to *L. donovani* lysate, 7 months after vaccination. The absorbency values and size of intradermal reaction were both significantly higher in vaccinees than in controls (ANOVA, $P < 0.0001$). After 2 years, 92% ($\chi^2 = 6.996$; $P < 0.0025$) protection was achieved: only 8% of vaccinees showed mild signs of kala-azar with no deaths while 33% of controls developed clinical or fatal disease. The FML vaccine induced a significant, long lasting and strong protective effect against canine kala-azar in the field [5].

The QuilA saponin, isolated from the bark of *Quillaja saponaria* Molina, consists of a mixture of saponins with

their carbohydrate moiety in glycosidic linkage to the triterpenoid quillaic acid. It is the adjuvant of experimental vaccines against canine babesioses [18], HIV [19,20], foot and mouth disease [21], Erlichiose [22], and Lyme disease [23]. Cumulative references point out their outstanding and specific adjuvant potential [18–23] although with slight deleterious effects such as: local pain; listless; anorexia for the first hours after injection [18].

In this work, we report our results obtained after vaccination of seronegative uninfected dogs of São Gonçalo do Amaranto, with the FML-QuilA vaccine. We assayed the efficacy of the vaccine during 41 months follow up. We used the FML-ELISA assay and the IDR as tools for evaluation of the humoral and cellular dog immune responses.

2. Materials and methods

2.1. Animals and study design

Sample size calculations were based on human protection against *Leishmania* achieved after vaccination with a first generation vaccine called *Leishvacin*, in a previous study done in Brazil [8]. In that report, 50% of protection was achieved. In the present study we calculated that on an 85% power to detect 85% of vaccine efficacy (VE) with an α error of 5%, using a two-tailed test, the study required 41 animals per group. The 179 domiciliary dogs in good physical condition were included in this study. The initial serological screening excluded 34 seropositive animals. The 145 animals were seronegative both by *L. chagasi* immunofluorescence and FML-ELISA assay and considered eligible. The 60 animals could not be vaccinated since they were absent on vaccination days or their owners did not allow to complete the vaccination schedule. Therefore, 85 dogs were candidates for vaccination distributed in two groups. Forty-four received the FML-QuilA vaccine while 41 remained as placebo control treated only with saline. Consent was obtained from the dog's owners who were informed about the risk of the procedures and the requirement for a 4 years follow-up.

A pre-immunization census with the FML-ELISA assay disclosed seropositivity oscillating from 0 to 60% in different quarters. We stratified our sample before randomization by a variable that affect the exposure and susceptibility: the presence of seropositive dogs in the quarter. In each quarter, independently of its seroprevalence, half of the dogs received vaccine and half of them, placebo. This happened both, in the prevalent and in the non-prevalent quarters ensuring equal risk of infection. The intention of this design was to rule out the possibility of vaccinated dogs being in the low incidence area and control dogs being in high prevalent area. However, after the determination of the number of dogs that composed the saline and vaccine group in each quarter, the decision about which dog will be included in each group was indeed performed by randomization by an operator in

Rio de Janeiro (2000 km distance to São Gonçalo, RN), that did not know the area nor had any contact with the dogs. Furthermore, personnel involved in vaccination was not aware about the formulation of the vaccine they were distributing.

2.2. Vaccine, vaccination and follow-up

Vaccine doses were composed of lyophilized FML antigen (1.5 mg) and QuilA saponin (1 mg), reconstituted in 1 ml NaCl 0.9% sterile saline solution, on each vaccination day, and administered as three subcutaneous doses in the right flank of the animals within 21 days interval. Placebo control was treated with 1 ml sterile saline. Twelve months after vaccination a fourth dose was injected. The FML vaccine is registered as a patent: *INPI number*: PI1100173-9 (18.3.97). Federal University of Rio de Janeiro, Brazil. Trained field workers and our team, made follow-up visits at 2, 7 and 19, 33 and 41 months after the complete vaccination. Clinical signs or obits due to kala-azar were recorded. Liver and spleen were weighted after autopsy of symptomatic dogs in order to determine possible hepato-splenomegaly. The presence of parasites was assayed in their spleen, liver, kidneys, lymphnodes and bone marrow (aspiration of sternal bone) by optical microscopy analysis of Giemsa-stained smears. The first available pre-vaccination and post-dose serum samples from 85 dogs were tested for the presence of anti-*L. donovani* antibodies by the FML–ELISA assay [5,17]. The immunofluorescent assay was also performed only in the pre-immunization census [17]. Blood and bone-marrow samples were collected in EDTA-tubes for DNA isolation and polymerase chain reaction (PCR) analysis using primers that amplify the conserved region of the minicircle molecule of *Leishmania* genus, as previously described [5]. The delayed type of hypersensitivity-DTH was determined by injecting dogs intradermally, in the internal part of the right hind leg, with 0.1 ml of *L. donovani* freeze and thawed antigen containing 200 µg protein in NaCl 0.9% sterile saline solution (10^8 stationary phase promastigotes per milliliter) while the left hind received only 0.1 ml saline. Measure of the increase of intradermal reaction was performed 24–72 h after antigen injection as described [5]. Reactions ≥ 5 mm diameters were considered positive [5].

All the animals included in this investigation were treated following the guidelines for animal experimentation of the USA National Institute of Health, and experiments were done in accordance with the institutional guidelines in order to keep the animal suffering the minimum as possible.

2.3. Statistical analysis

Means were compared by a standard *t*-test and ANOVA analysis, simple factorial test (SPSS). The χ^2 - and Fisher's exact tests were used in comparing proportions [24]. Correlation coefficient analysis was determined on a Pearson bivariate, two-tailed test of significance (SPSS).

3. Results

3.1. Antibody and IDR response in vaccinated and control dogs

Eighty-five healthy seronegative dogs were included in the assay. The 22/85 dogs (26%) corresponded to the non-prevalent (0%) while the 63/85 dogs (74%) corresponded to the prevalent quarters (14.2–60% of seropositivity). The 22 dogs in the non-prevalent quarters were divided into 9 animals treated with saline and 13 animals that received the FMLQuilA vaccine. On the other hand, in the prevalent quarters: 32 dogs were distributed for saline and 31 for FMLQuilA vaccination, respectively. Therefore, most of the dogs corresponded to the prevalent quarters.

Antibody rises against FML antigen were detected in FML–QuilA vaccinated animals, 2 months after vaccination (Table 1) when seropositivity reached 100% of individuals, maintaining this saturating value along the first and second year of the experiment with no apparent decay. Seropositivity was significantly higher in vaccinees than in saline control until 19 months after vaccination. Likewise, a 100% of vaccinated animals were IDR positive by the end of the month 2 (Table 1). Control animals treated with saline developed significant lower proportions of IDR reaction along the whole assay and different from what detected in serology, the IDR positivity showed a decrease starting from month

Table 1
Positivity, absorbencies and size of skin tests of vaccinated and control dogs

	2 months			7 months			19 months			33 months			41 months		
	Sal	FML	<i>P</i>	Sal	FML	<i>P</i>	Sal	FML	<i>P</i>	Sal	FML	<i>P</i>	Sal	FML	<i>P</i>
ELISA (%) ^a	45	100	<0.005	34	96	<0.005	55	100	<0.005	91	100	>0.05	100	100	<0.05
DTH (%) ^a	32	100	<0.005	37.5	100	<0.005	29	100	<0.005	ND			0	100	<0.005
Abs ^b	0.476	1.309		0.411	1.260		0.454	1.113		0.690	1.300		0.610	0.990	<0.001
DHT ^b	1.716	9.090		1.765	9.160		1.428	10		ND	ND		0	8.750	<0.001

^a Values represent percent of positive reactions in samples collected 2, 7, 19, 33 and 41 months after vaccination. Significance of proportions compared by χ^2 - and Fisher's exact tests.

^b Abs = mean average of the absorbencies at 492 nm values of sera in the FML–ELISA assay. DTH = delayed type of hypersensitivity (mm): mean values of the size of the intradermal reaction to *Leishmania donovani* promastigote lysate. Significance of differences compared by ANOVA.

19 as expected for kala-azar cellular immunosuppression. The surviving control animals showed no IDR response at month 41. The mean of absorbency readings at 492 nm, for total anti-FML immunoglobulins, in 1:100 diluted serum of each seropositive animal are also represented in Table 1. The humoral response was significantly higher in the FML-QuilA group than in controls at all tested times (ANOVA analysis, $P < 0.0001$ differences for treatment, $F = 264.229$ and for time, $F = 106.088$). Absorbency values were maximal by the month 2 with no decline detected until the 3.5 years after vaccination. This long lasting effect is impressive considering that no vaccine booster was giving after the month 13. Also, the increase in the average size of the IDR to promastigote lysate (Table 1) was significantly higher in the FML-QuilA group than in saline controls (ANOVA analysis, $P < 0.0001$ differences for treatment, $F = 206,414$) at all tested times, but no significant differences between times were recorded since reactions were maximal and stable soon after vaccination ($P = 0.511$, $F = 0.711$). Correlation coefficient analysis between of the anti-FML humoral and IDR response disclosed that the two variables were highly correlated ($P = 0.003$).

3.2. Prevention of kala-azar and *Leishmania* infection

Eight fatal canine kala-azar cases occurred in the saline control with only one case in vaccinated group. Among the 8, 3 of the obits belonged to the non-prevalent area while 5 to the prevalent area. Therefore, 25% of the control animals and only 5% of vaccinated dogs developed clinical and fatal disease until the end of experiment. This difference was significant ($\chi^2 = 3.93$, $P < 0.05$). This means that 95% protection against kala-azar was achieved

after FML-QuilA vaccination. The χ^2 analysis disclosed significant differences between the total number of dogs in quarters where the obits were detected and in quarters with no incidence ($\chi^2 = 12.6$; $P < 0.005$). Also, the number of kala-azar cases was significantly higher in quarters with previous seropositive dogs than in quarters with no previous prevalence ($\chi^2 = 10.77$; $P < 0.005$). Therefore, total dog population and previous dog seroprevalence were risk factors for the development of canine kala-azar. As expected for reservoirs, the infective pressure increases with the number of dogs and with their previous seroprevalence. The detection of the unique case of disease among vaccinated dogs in one of the most prevalent quarters (quarter 24; 50% seropositivity) strongly supports this hypothesis.

Also, vaccinated and placebo injected dogs were included in each house whenever was possible. The 13 pairs of animals met this condition. One of this pair included the unique vaccinated dog that developed canine kala-azar and its control. Another two pairs showed obits only in the placebo treated animal and the remaining 11 pairs showed no signal of disease.

We were able to examine four vaccinees and four control dogs, 41 months after vaccination. While most control animals were seronegative until month 19 (Fig. 1A), the seropositivity increased and reached a peak at month 33. These four individuals were part of the control dog population not reactive to FML (Table 1), and their increase in antibodies, after month 19 is coincident with the increase in incidence of kala-azar fatal cases (33–41 months) indicating that they were probably infected during this period. Three out of four control animals showed no IDR response (Fig. 1C) and the only dog showing a positive IDR at month 2 and 19, suppressed its weak response until month 41. On

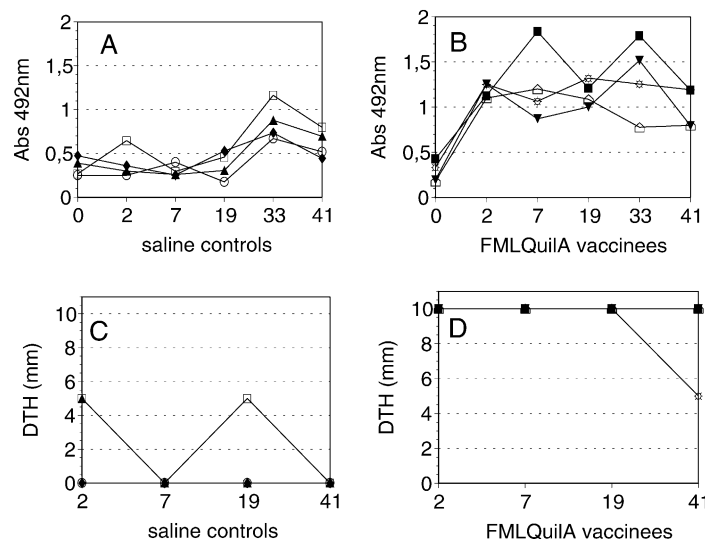


Fig. 1. Evolution of the anti-FML antibody absorbency values and size of intradermal skin reaction to *L. donovani* lysate with time in naturally exposed vaccinated dogs and saline treated controls. Four vaccinees and four control dogs were individually analyzed for a 41 month period after vaccination. (A) and (B) Evolution of seropositivity to FML in saline controls and FML-QuilA vaccinees, respectively. (C) and (D) Evolution of size of intradermal reaction in saline controls and FML-QuilA vaccinees, respectively.

Table 2
Parasite assessment in vaccinated and control dogs 41 months after vaccination

Groups	FML–ELISA ^a IgG total	FML–ELISA IgG1	FML–ELISA IgG2	Symptoms 41 months ^a	PCR blood	PCR bone marrow	Ama ^b
Sal	0.443	0.448	0.315	As	+	+	–
Sal	0.799	1.127	0.701	Al	–	ND	ND
Sal	0.695	0.428	0.368	As	+	+	–
Sal	0.518	0.552	0.252	As	+	+	+
FML	0.798	0.418	0.535	As	–	–	–
FML	1.192	0.848	0.903	O, Al, Lw	–	–	–
FML	0.795	0.485	0.416	As	–	–	–
FML	1.187	0.796	0.585	As	–	–	–

^a These are the absorbency values of sera in the FML–ELISA assay 41 months after vaccination. Absorbency values higher than 0.435 were considered positive results. Animals were clinically evaluated at the moment of serum collection: Al, alopecia; O, onychogryphosis; C, cachexia; An, anorexia; I, isolation; A, apathy; U, disseminated ulcer; Lw, loss of weight. PCR, polymerase chain reaction performed on genomic DNA extracted from 500 µl of whole blood or bone marrow and amplified using a pair of oligonucleotides that anneal to the origin of replication of both strands of the mini circle molecules which are one of the components of the genus *Leishmania* mitochondrial DNA (kDNA).

^b The presence of parasites was assayed by microscopical examination of Giemsa-stained bone-marrow smears.

the other hand, the FML–QuilA vaccinees showed strong seropositivity from the beginning of the assay (Fig. 1B) and impressive maximal skin reaction to promastigote lysate all over the period (Fig. 1D). All dogs were seropositive on the FML–ELISA assay performed with protein A peroxidase (Table 2). A main IgG1 anti-FML response while a dominance of either IgG2 or IgG1 subtype was seen in each half of the vaccinated animals. The presence of Leishmanial infection was proved in saline controls (3 animals) by means of the PCR analysis of blood and bone-marrow samples and by the finding of parasites in bone-marrow puncture (1 animals). On the other hand, FML vaccinees showed no evidence of *Leishmania* infection (Table 2).

Taken together, the effect on humoral and cellular immune responses, and the absence of parasitological and clinical signs of disease, the FML–QuilA vaccine induced a significant, long lasting and strong protective effect against canine visceral leishmaniasis in the field.

4. Discussion

The 95% of significant and long lasting protection against canine kala-azar obtained in this phase III trial of the FML–QuilA vaccine is probably accountable for the reduction of human disease in this area: from 15 cases in 1996 to 6 cases up to July 1997 and to zero until May 1998 [5]. Previous results [5,17,25] demonstrated that the distribution of seropositive dogs in quarters was highly heterogeneous, indicating the presence of localized phlebotomine foci. Paranhos-Silva et al. [25] divided the urban and inhabited peri-urban areas of Jequié, Bahia, Brazil, into 140 clusters of 0.25 km², describing an overall prevalence of *Leishmania* antibodies in dog population (ELISA assay) of 23.5%, with intra-cluster prevalences ranging from 0 to 67%. In our previous work, we also described a canine seroprevalence of 23 [17] and 18.9% [5] with a non-homogeneous distribution [5]. Knowing this, in the present work, we distributed

equivalent number of control and vaccinated dogs both, in the prevalent and non-prevalent quarters in order to guarantee equal risk of infection. The finding of more kala-azar obits in the quarters with higher number of dogs and higher seropositivity to *Leishmania*, justified the design used in this assay. Supporting our hypothesis, WHO guidelines states that although fully adequate design of phase III trials requires a double blind, randomized, controlled comparison between vaccinated and unvaccinated, individuals may be stratified, before randomization, by variables likely to affect exposure or susceptibility, e.g. residence, age, sex, etc. Such stratified randomization will yield balanced groups, with a high probability. The probability of obtaining balanced groups through randomization of a necessarily small number of communities will not be high [26].

Maximal positivities were detected in vaccinees FML-serology and IDR, at month 7 in previous work [5] and at month 2, in this investigation. However, no significant differences in VE and protection to kala-azar were detected between the two compositions. Indeed, 92% of significant protection with 76% of VE and 95% of protection and 80% of VE were achieved in the previous [5] and present investigations, respectively. The number of kala-azar cases in vaccinees or controls was equivalent in both experiments ($\chi^2 = 0.4$ and 0.25, respectively).

We previously showed that the repeated intradermal *Leishmania* antigen injections could be partially responsible for the increase in seropositivity (68%) of the placebo group over the expected incidence for this endemic area (23%) [5]. In the present work, however, seropositivity in control group increased to 91% (month 33). By this time, also, 19% of fatal kala-azar were detected in controls while the incidence expected for this area was 3% ($\chi^2 = 12.38$; $P < 0.0001$) meaning that, the increase in seropositivity of the control group was not only due to the successive intradermal tests but to the increase in risk of infection, as well. This temporary increase in kala-azar incidence did not induce however, any significant change in VE index. Two

hundred micrograms of *Leishmania* protein in 0.1 ml (10^8 promastigotes per milliliter), and 0.1 ml of a 3×10^8 promastigotes per milliliter suspension were the recommended dosage for skin test of dogs infected with *L. brasiliensis* [27] and *L. infantum* [28,29], respectively. The dose skin tested used in this work: 200 μg protein in 0.1 ml sterile saline solution (10^8 stationary phase promastigotes per milliliter) is 50 times higher than that used in humans in field studies for tegumentar leishmaniasis (4 μg) [8]. This might be the reason for skin test conversion in placebo injected dogs. However, even the use of 4 μg in skin test selection of human candidates for tegumentar leishmaniasis has been reported to induce sensitization and a significant increase of the in vitro PBMC production of IL2 and γ Interferon in placebo treated individuals [30]. In order to clarify this question we skin tested a cohort of dogs vaccinated with three doses of the FML vaccine ($n = 60$) or with saline. Each group of dogs ($n = 20$) was skin tested with either 200, 100 or 4 μg of *Leishmania* protein. IDR was positive in 79, 50 and 17% of the vaccinated animals and in 18, 17 and 0% of saline controls, treated with 200, 100 or 4 μg , respectively. The use of 4 μg of antigen was then inadequate for dogs and while no significant difference was found between false positive reactions in controls using either 200 or 100 μg of antigen a strong increase in positivity was detected using 200 μg for FML-vaccinated dogs ($\chi^2 = 4.07$; $P < 0.05$).

In *L. donovani* experimentally infected mongrel dogs, the skin test started to positivitate coincidentally with the rise in anti-FML antibody titers [31,32] and start to be eventual and negative as the symptomatology and gravity increase, just before death [31,32,28] and this investigation). On the other hand, the skin test is present in naturally infected dogs resistant to disease [33]. In the present work, the percent of IDR positivity of saline control dogs starts to decline after 19 months being completely negative in the few surviving animals at month 41. The intradermal reaction is then a useful and simple tool for the monitoring of the acquisition of a protective status after a kala-azar vaccine protocol in field assays. Noteworthy, protection against tegumentar (50%) or visceral human leishmaniasis (43%) due to first generation human vaccines was only found among individuals that achieved a positive intradermal reaction [8,34]. The achievement of 100% skin test conversion in dogs vaccinated with FML vaccines in this and previous assays [5], point out the remarkable potential of these formulations in prophylaxis against canine kala-azar.

Previous to this investigation mongrel dogs were treated with the FML-QuilA vaccine and challenged with 10^8 amastigotes of *L. donovani*, in our kennel. A significant increase in antibody response ($P < 0.0001$) was observed for vaccinees, along a 2 years period and starting from a week after the first vaccine doses. After the complete vaccination, absorbency values in serum reached similar values to those obtained in this field assay (1.109 ± 0.029 and 1.309 ± 0.27 , respectively, $P > 0.05$). The sizes of skin tests were also similar ($P > 0.05$) in the kennel (7.5 ± 3.54 mm) and the

field assay (9.09 ± 1.97 mm). A second trial in the kennel disclosed again, similar values (Abs 492 nm = 1.210 ± 0.07 and skin test (mm) = 9.20 ± 5.54) confirming the high reproducibility of the results obtained with the FMLQuilA vaccine. Significant protection in comparison to saline control was obtained in: anti-FML antibodies, IDR, maintenance of normal platelets levels and reduction of the number of clinical signs of disease as well as in the delay in onset of the disease [31]. In the Swiss Albino murine model, treated with three sc vaccine injections (1/10 of the doses used for dog assays) and further challenged with 10^7 amastigotes, the antibody IgG and IgG2a response after vaccination (mean of two assays: Abs 492 nm = 0.57 ± 0.03 and 1.00 ± 0.17 , respectively) as well as the IDR response (0.53 ± 0.14 mm) were also reproducible. In comparison to saline control: a significant and specific reduction in liver LDU ($92.50 \pm 0.7\%$) and a slight non-specific effect due to QuilA treatment ($38.50 \pm 26.16\%$) was observed in both assays (Santos et al., unpublished results).

In dogs infected with *L. infantum* increasing or higher levels of IgG1 were associated with disease (symptomatic dogs, non- or low-responsive to chemotherapy) [35,36] and this to infectivity to phlebotomine [37]. On the other hand, IgG2 increase would be related to asymptomatic infections or natural resistance [35]. In the present work, a main IgG1 anti-FML response was found in *L. chagasi* naturally infected control animals while a dominance of either IgG2 and IgG1 subtype was seen in each half of the protected vaccinated animals.

Considering that only few reports in literature deal with a vaccine against canine visceral leishmaniasis [5–10] and most of them showed efficacy only in murine models [7] or in dogs with experimental infection [6,9], the protective effect demonstrated by the FML vaccine in the field, in the present and previous investigation [5] strongly supports their potential use in large scale assays of vaccination against canine visceral leishmaniasis.

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