

# A phase III trial of efficacy of the FML-vaccine against canine kala-azar in an endemic area of Brazil (São Gonçalo do Amaranto, RN)

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## Abstract

Protection against canine kala-azar was investigated in naturally exposed dogs of an endemic area, vaccinated with the fucose mannose ligand (FML)-vaccine of *Leishmania donovani*. A total of 97% 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. © 2000 Elsevier Science Ltd. All rights reserved.

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## 1. Introduction

Zoonotic visceral leishmaniasis (ZVL) is one of the most important emerging diseases. Its etiological agent, (*Leishmania chagasi* or *Leishmania infantum*) infects canids. Peridomestic sandflies acquire the parasite by feeding on the skin of infected foxes and transfer it to wild or domestic dogs. The subsequent transmission to humans, also through sandfly bites, causes human visceral leishmaniasis or kala-azar: a severe disease, lethal if not treated soon after the onset of symptoms. Clinical signs in humans include: malaise, anemia, hepatosplenomegaly, hypergammaglobulinemia, fever, cachexia and progressive suppression of the cellular

immune response [1]. About 500 000 human cases of kala-azar are registered annually. A total of 90% of them are in Bangladesh, Brazil, India and Sudan [2]. In Brazil, the highest incidence of disease is found in North-East of the country.

Being a zoonotic disease, the control of canine kala-azar should have an impact by reducing the parasite available to sandflies and therefore reducing the human incidence of disease [1]. The current strategy for control of ZVL, as recommended by the World Health Organization, is based on detection and destruction or treatment of infected dogs, treatment of human cases and vector control [1]. Since the efficacy of this control has shown to be inconsistent, the development of vaccines has been given priority and is considered to be urgent by the World Health Organization.

Immunization against leishmaniasis was achieved in the past by inoculating humans with living parasites that induced localized self-healing cutaneous lesions

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(leishmanization). Since then, a first generation of vaccines against leishmaniasis was composed of formulations including killed parasites (reviewed in Ref. [3]). These formulations were developed against cutaneous but not visceral leishmaniasis and were used in large clinical trials on human populations of endemic areas. Most second-generation vaccines can be divided into three categories according to their composition: live vaccines, defined subunits and crude fractions. Among the recombinant and native antigens tested in murine models, the LACK protein [4], the LPGAP peptides [5], the LeIF protein [6] and the DP72 glycoprotein of *L. donovani* [7] were protective immunogens for mice. Finally, protection results obtained with the third-generation vaccines composed of cDNA encoding leishmanial antigens cloned into an eukaryotic expression vector are still preliminary (reviewed in Ref. [3]). Although a great number of antigens have been tested for protection against the cutaneous disease with in-vitro cell or mouse models, no vaccine against human or canine visceral leishmaniasis is yet available.

In previous reports, the protective potential of a glycoproteic complex purified from promastigotes of *L. donovani* was analyzed [8–12]. The protocol for isolation of this antigen involves aqueous extraction followed by denaturing conditions that determine the further stability of the vaccine [8]. This glycoprotein-enriched fraction was named FML (Fucose mannose ligand) [8]. It is composed of 29% neutral sugar, 44% protein 11% carbohydrate and hexosamines in trace amounts. Among its neutral sugars: fucose (10%), mannose (47%), glucose (30%) and galactose (12%) were identified [8]. The SDS-PAGE analysis of FML disclosed major proteic components with molecular weight ranging from 9 to 95 kDa. Bands of 36 and 55 kDa also showed a positive staining reaction for carbohydrates. The FML aminoacid analysis disclosed the presence of aspartic acid (10%), glutamic acid (16%), alanine (12%), and glycine (10%) as major components [8]. No cross reactivity was observed between FML and the gp63 antigen of *Leishmania* by monoclonal antibodies in ELISA and Western blots [13]. The Western Blot analysis of the FML-reactivity disclosed the GP36 glycoprotein band as the only component reacting with sera of kala-azar patients' sera [14]. No labeling was detected with sera of patients with tegumentar leishmaniasis, Chagas' Disease, or normal sera. The GP36 antigen of FML was shown to be a marker of human kala-azar [14]. A total of 23 hybridomas secreting IgG were obtained from FML immunized Balb/c mice [13]. Twenty-two IgG clones recognized the 36 kDa band of FML. Periodate oxidation of the FML, blotted onto nitrocellulose, prior to reaction with one of monoclonal antibodies raised against the 36 kDa band, abolished the reaction completely [13]. The FML antigen is a potent immunogen to rabbits and mice, present on the

parasite surface throughout the life cycle [13] that strongly inhibits the in-vitro infection of murine macrophages by promastigotes and amastigotes of *L. donovani* [8,13] in a species-specific manner [15].

With the FML-vaccine of *Leishmania donovani*, an average protection of 87.7% ( $P < 0.01$ ) and 84% ( $P < 0.001$ ) against kala-azar was achieved in the isogenic CB hamster and Balb/c mouse models, respectively [10,9]. Control animals showed significantly lower levels of anti-FML antibodies, in vitro proliferative splenocyte response and liver LDU ( $P < 0.01$  and  $P < 0.001$ , respectively) with no protective effect. Protection criteria included maintenance of the delayed type of hypersensitivity, the reduction of splenomegaly [10] and parasite load, increase of the in vitro splenocyte proliferation against leishmanial antigen, and increase in anti-FML specific antibody response [9,10]. Also, outbred mice (Swiss Albino) immunized with the FML-vaccine developed higher antibody titers and a significant and specific reduction of parasite load in relation to controls (85%,  $P < 0.025$ ) [12].

In canine kala-azar a strong antibody response is frequent. Therefore, in the field, an anti-FML specific antibody response could indicate the natural infection by *L. chagasi* or the protective response induced by the FML-vaccine formulation. Intradermal reaction (IDR), on the other hand, is known to appear soon after infection in humans and dogs, and turn negative in the advanced kala-azar, indicating the immunosuppressive status of the individual. Therefore, one should expect a transient IDR in naturally infected dogs of saline control and a long-lasting positive IDR in animals that achieved protection to kala-azar after FML-vaccine treatment [16]. In the present study we evaluate the efficacy of the FML-vaccine in the field, in a randomized controlled trial. We used a medium scale population of an endemic area, divided it into vaccine and placebo treated control groups, under conditions that equalized their chance of exposure to natural infection. We used the FML-ELISA assay and the IDR as tools for evaluation of the humoral and cellular dog immune responses.

São Gonçalo do Amaranto is an endemic area for both human and canine visceral leishmaniasis. The overall prevalence of anti-*Leishmania* antibodies disclosed by the FML-ELISA assay in canine sera of this area was 23% (79/343) [17]. The seroreactivity disclosed by an *L. chagasi* immunofluorescent assay (IF) was much lower: 2.9% (10/343), and closely related to the percent of kala-azar symptomatic dogs 2.6% (9/343). All 21 asymptomatic, FML-seropositive animals died from kala-azar in a period ranging from 0 to 6 months after diagnosis, disclosing for the FML-ELISA assay a highly predictive value for the development of both canine and human kala-azar [17,18].

For these reasons we vaccinated uninfected seronegative dogs of São Gonçalo do Amaranto with the FML-vaccine and assayed the efficacy of the vaccine during a 2 year follow up.

## 2. Material and methods

### 2.1. Animals and study design

The study was conducted in São Gonçalo do Amaranto, Rio Grande do Norte, Brazil, a populated peri-urban area of Natal, of low socioeconomic status where human and canine kala-azar are highly endemic [17,18]. Since December 1996, we have been studying the domestic dog population there. More than 400 animals are being followed serologically and clinically in a longitudinal study.

Since no data was available about vaccination against canine kala-azar in the field, neither with FML-vaccine nor with any other anti-*Leishmania* vaccine, 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 Brazilian Army conscripts during their training in the Amazon jungle, Brazil [19]. In that report, 67.3% of significant reduction on the incidence of cutaneous leishmaniasis was achieved [19]. In the present study we calculated that on a 95% power to detect 85% of vaccine efficacy (VE) [20] with an  $\alpha$  error of 5%, using a two-tailed test, the study required 54 animals per group. 148 domestic dogs in good physical condition were included in this study. The initial serological screening excluded 28 seropositive animals. 120 animals were seronegative both by *L. chagasi* immunofluorescence and FML-ELISA assay and considered eligible. Three animals couldn't be vaccinated since they were absent on vaccination days. The 117 dogs were distributed in two groups: 58 received the FML-vaccine while 59 remained as placebo control treated only with saline. Since previous distribution of seropositive dogs by city blocks was highly heterogeneous, indicating the presence of localized phlebotomine foci, we included vaccinated and placebo control individuals in each house, whenever possible, in order to equalize their degree of exposure to the risk of natural infection. Consent was obtained from the dogs' owners who were informed about the risk of the procedures and the requirement for a 2-year follow-up. In this investigation the collection of biological samples from dogs was performed following the animal experimentation guidelines of the US National Institutes of Health, and removal and sacrifice of animals was done in accordance with the institutional guidelines for the humane use of laboratory animals in order to reduce animal suffering to a minimum.

### 2.2. Vaccine and vaccination

Vaccine doses, prepared as has been previously described for vaccination of mice [9,12], included the lyophilized FML antigen (1.5 mg), reconstituted in 1 ml NaCl 0.9% sterile saline solution, on each vaccination day. The necessary number of vials were transported to the field with cold packs. Vaccines were administered as three subcutaneous doses in the right flank of the animals at 21 day intervals. The placebo control group was treated with 1 ml sterile saline. Twelve months after vaccination a fourth dose of the vaccine was injected. The FML-vaccine is registered as a Patent: *INPI number*: PI1100173-9 (18.3.97). Federal University of Rio de Janeiro, Brazil.

### 2.3. Follow-up

Trained field workers, together with our team, made follow-up visits to the vaccinated animals at 2, 7, 13 and 24 months after the complete vaccination. The seroreactivity to FML, IDR response to *L. donovani* promastigote lysate, clinical signs or deaths due to kala-azar were recorded. By the end of the assay (24 months), FML-seropositive animals considered symptomatic were removed from the area, sacrificed by total anesthesia (intravenous Thionembutal, Abbot, São Paulo, Brazil), submitted to autopsy and to parasitological evaluation. We considered as clinical signs of kala-azar: loss of weight, cachexia, alopecia, onychogryphosis, apathy, anorexia, increase of popliteal lymphnode size and ulcerative skin lesions. Liver and spleen were weighed 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 [9]. Also the possible presence of *Leishmania* DNA was assayed in peripheral blood by polymerase chain reaction (PCR) analysis [21].

### 2.4. Laboratory procedures

The first available prevaccination and post-dose 2, 7, 13 and 24 months serum samples from 117 dogs were tested for the presence of anti-*Leishmania donovani* antibodies by the FML-ELISA assay [17,18]. All dog serum samples were collected from the cephalic vein and conserved in glycerol (1:1, v:v) at  $-20^{\circ}\text{C}$ . Isolation and chemical characterization of the Fucose Mannose Ligand antigen, obtained from stationary-growth phase promastigotes of *L. donovani* Sudan (*LD 1S/MHOM/SD/00-strain 1S*) were performed as previously described [8]. The FML (2  $\mu\text{g}/\text{well}$ ) was solubilized in carbonate buffer (pH 9.6), and used to coat flat-bottom 96-well plates (Haemobag, Ribeirão Preto, SP, Brazil).

Antibodies were detected by peroxidase-labeled protein-A (Sigma, St. Louis, MO) in a 1:1000 dilution; the reaction was developed as described elsewhere [17,18]. The absorbency values at 492 nm were compared using a 1:100 dilution of the serum samples. Results are expressed as mean values of triplicates. The cut-off of the method for the analysis of canine sera was determined according to the Youden test [18]. Briefly, sera from healthy dogs were assayed and the mean absorbency values  $+1$ ,  $2$  or  $3$  SD were determined. Using these values as cut-off, all samples were analyzed and classified into the following groups:  $a$  = disease seropositive;  $b$  = disease seronegative;  $c$  = healthy seronegative; and  $d$  = healthy seropositive. The Youden index was then calculated by means of the formula  $j = [a/(a + b) + c/(c + d)] - 1$ . The Youden test circumvents the use of an arbitrary cut-off by taking into consideration the possible errors that could lead to negative results for diseased individuals, and positive results for healthy individuals. All serological determinations in this report were done by double-blind tests.

For the indirect immunofluorescent assay (IF), drops of dog blood from the cephalic vein were collected on squares of filter paper, dried and stored at  $-4^{\circ}\text{C}$  until analysis. Antibodies were eluted from filter paper with PBS. Dilutions of eluates were incubated with *L. chagasi* promastigotes (Biomanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil) plated on slides. The immunofluorescent assay was performed as described elsewhere [17]. Reactions were considered positive if fluorescent at a 1:40 dilution.

Blood and bone-marrow samples were collected in EDTA-tubes for DNA isolation and PCR analysis [21]. Genomic DNA was extracted from 500  $\mu\text{l}$  of whole blood using the RapidPrep™ Kit (Pharmacia Biotech-Upsala, Sweden) following the manufacturer's instructions. The DNA eluted from the chromatographic procedure was precipitated with sodium acetate and ethanol, and resuspended in 10  $\mu\text{l}$  of TE (10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0). The hot start PCR was performed with 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). Primer A was 5'(G/C)(G/C)(C/G)CC(A/C)CTAT(A/T)TTACACCAACCCC and primer B was 5'GGGGAGGGCGTTCTGCGAA [18]. These oligonucleotides amplify the conserved region of the minicircle molecule, using 35 cycles of 94/50/72°C. The reactions were performed using 200 ng of each nucleotide, 200  $\mu\text{M}$  of a dNTPs mixture, 1.5 mM  $\text{MgCl}_2$ , 50 mM KCl, 10 mM Tris-HCl pH 8.3 and 2.5 U of Taq polymerase (Perkin-Elmer, Branchburg, NJ, USA). This assay detects kDNA from less than one organism by amplifying a product of a conserved region, and thus is specific for the genus *Leishmania*. The 120 bp

amplification products were analyzed by electrophoresis on 2% agarose gels followed by ethidium bromide staining and visualization under UV light. All reactions (every eight samples) were performed with two extra tubes: one negative control where no DNA was added to the mixture and one positive control, using DNA isolated from cultured promastigote parasites of *Leishmania donovani* Sudan (LD 1S/MHOM/SD/00-strain 1S). Irrelevant DNA from other infectious and non-infectious human diseases was included as negative controls [21].

### 2.5. Delayed type hypersensitivity (intra-dermal reaction to promastigote lysate)

This was determined by injecting dogs intradermally, in the inner aspect of the right hind leg, with 0.1 ml of *L. donovani* freeze-thawed antigen containing 200  $\mu\text{g}$  protein in NaCl 0.9% sterile saline solution ( $10^8$  stationary phase promastigotes/ml). The left hind leg received only 0.1 ml saline. Measure of the increase of intra-dermal reaction was performed 24, 48 and 72 h after antigen injection. Indurated areas were marked and each time, the values of the saline control were subtracted from the reaction due to the *Leishmania* antigen. Reactions showing diameters  $\geq 5$  mm were considered positive.

### 2.6. Statistical analysis

Means were compared by a standard  $t$  test and ANOVA analysis, simple factorial test (SPSS). Chi-square and Fisher's exact tests were used in comparing proportions [22]. Correlation coefficient analysis was determined by a Pearson bivariate, two-tailed test of significance (SPSS).

## 3. Results

### 3.1. Antibody and IDR response in vaccinated and control dogs

A pre-immunization census performed in São Gonçalo do Amaranto, RN, excluded 8/148 dogs that showed a positive reaction in the *L. chagasi* IF and 28/148 dogs seropositive in FML-ELISA indicating a 5.4 and 18.9% seroprevalence, respectively in both assays. Among the last 28 were the 8-IF-positive specimens already mentioned.

Thus, prior to vaccination, all dogs included in the experiment were seronegative by both IF and FML-ELISA assays. Between January and February 1997, 117 dogs received their three vaccine or placebo injections. Antibody rises against FML antigen were detected in FML-vaccinated animals, 2 months after

vaccination (Table 1). The increase was significantly higher than that detected in saline controls which was probably due to exposure to natural infection by *L. chagasi*. 7 months after vaccination 97% of vaccinated animals already showed seropositivity, maintaining this saturating value throughout the first year of the experiment with no apparent decay. A booster was given at the thirteenth month (after sera collection) and 100% of the animals were seropositive 24 months after complete vaccination. The differences with saline control were highly significant along the whole period (Table 1), even though the proportion of seropositive animals also increased in the controls, indicating natural infection by *L. chagasi*.

Likewise, the proportion of positive IDR responses to the f/t lysate of *L. donovani* was also significantly increased in vaccinated animals over saline controls, starting from 2 months after complete vaccination (Table 1). A total of 100% of animals were IDR positive by the end of the first year of the experiment. Control animals treated with saline developed lower proportions of IDR reactions, during the first 7 months of the experiment, probably related to the presence of active leishmanial natural infection. In contrast to what was detected in serology, the IDR positivity showed a slight decrease, 13 months after vaccination, indicating the expected initiation of suppressive cellular immune response in the exposed animals.

The anti-FML humoral response induced by the FML-vaccine could be analyzed not only by means of proportion of seroconversion but also by the increase in absorbency values. Fig. 1 shows the evolution of the anti-FML total antibody levels achieved in the vaccinated and control animals. The results represent the mean  $\pm$  SE values of absorbency readings at 492 nm, for total anti-FML immunoglobulins, in 1:100 diluted serum of each seropositive animal. The humoral response was significantly higher in the FML-vaccine group than in controls treated with only saline at all tested times (ANOVA analysis,  $P < 0.0001$  differences for treatment,  $F: 244,782$  and  $P < 0.0001$  in differences for time,  $F: 35,386$ ). A plateau in absorbency values was achieved soon after the booster dose (13 months

after vaccination) with no decline detected until the end of the second year of the experiment.

Also, the increase in the average size of the IDR to promastigote lysate was evaluated (Fig. 2) in the vaccinated and control animals. The results represent the mean  $\pm$  SE values of skin reaction diameters (mm) of all reactive animals, 24 h after antigen injection. The IDR response was significantly higher in the FML-vaccine group than in saline treated controls at all tested times (ANOVA analysis,  $P < 0.0001$  differences for treatment,  $F: 234,109$  and  $P < 0.0001$  in differences for time,  $F: 12,708$ ).

The mean value  $\pm$  SE of serum absorbencies at the different time points (2, 7, 13 and 24 months after vaccination) was  $0.432 \pm 0.125$  (Fig. 1). The corresponding mean value  $\pm$  SE of skin reaction sizes was  $5.083 \pm 0.167$  mm (Fig. 2). These results demonstrate the exposure of animals in this endemic area to a constant natural infection pressure. The homogeneity of positive response in serology to FML and IDR to promastigote lysate demonstrate the strong potential of FML vaccine that induced both, humoral and cellular immune responses in a highly heterogeneous vaccinated dog population composed of outbred animals of different ages, sexes and races. Correlation coefficient analysis between the anti-FML humoral and IDR response was determined on a Pearson bivariate, two-tailed test of significance. The two variables were shown to be highly correlated ( $n = 657$ ;  $P < 0.001$ ).

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

Four placebo-treated control dogs died of serologically, clinically and parasitologically confirmed kala-azar in the control group, by the end of the first year of the experiment, while no death was recorded in vaccinated animals during the first or second year period.

By the end of the assay (24 months) FML-seropositive animals considered symptomatic were removed from the area, analyzed for anti-FML antibodies and IDR response, sacrificed, submitted to autopsy and to parasitological evaluation (Table 2). The presence of parasites was assayed microscopically in Giemsa-

Table 1  
Seropositivity in the FML-ELISA assay of vaccinated and control dogs<sup>a</sup>

Protective response	2 months			7 months			13 months			24 months		
	Sal	FMLv	* $P <$	Sal	FMLv	$P <$	Sal	FMLv	$P <$	Sal	FMLv	$P <$
FML-ELISA +	14	62	0.005	45	97	0.005	40	97	0.005	68	100	0.0025
IDR + (24 h)	15	58	0.005	37.5	97	0.005	25	100	0.005	32	94	0.005
IDR + (48 h)	6	12	0.05	15	82.3	0.005	7.7	91	0.005	14	94	0.005

<sup>a</sup> NOTE. Values represent percent of positive reactions in samples collected 2, 7, 13 and 24 months after vaccination. Sal: percent of saline-treated controls; FMLv: percent of FML-vaccine treated animals; IDR: intradermal reaction to *L. (L.) donovani* promastigote lysate (200  $\mu$ g of protein). Significance of proportions compared by Chi square and Fisher's exact tests.

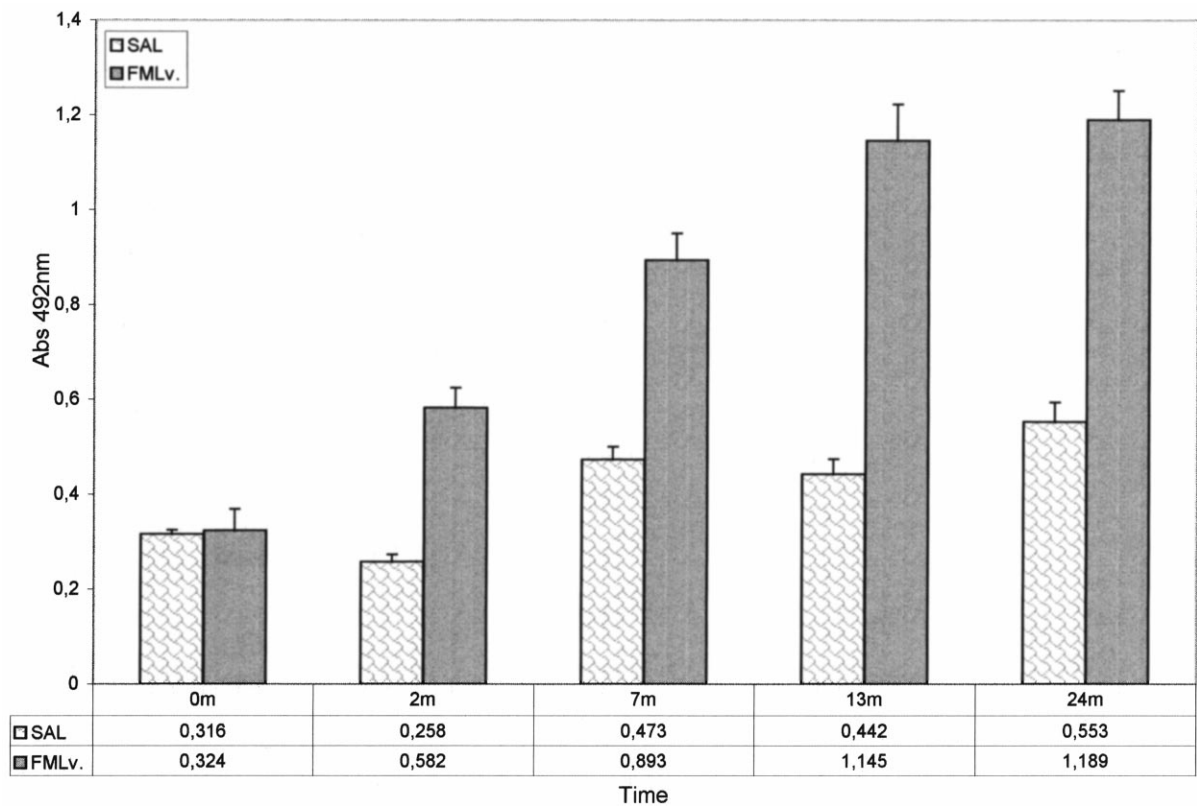


Fig. 1. Evolution of the anti-FML antibody absorbency values with time in naturally exposed vaccinated dogs and saline treated controls. The results represent the mean  $\pm$  SE values of absorbency readings at 492 nm, for total anti-FML immunoglobulins, in 1:100 diluted serum of each seropositive animal. Sal: saline-treated controls; FMLv: FML-vaccine treated animals.

stained smears and in peripheral blood by PCR analysis. In the saline controls, five/six animals were FML-seropositive already. While the weakly positive IDR in two animals indicated recent infection by *Leishmania*, the skin reaction was absent, suppressed in four/six dogs. Although amastigotes were absent from all smears, the presence of infection was confirmed by the detection of leishmanial DNA in peripheral blood of all the six oligosymptomatic animals (Table 2).

Conversely, all FML-vaccinated individuals were positive by serology and IDR, indicating the long-lasting protective status against the disease. Indeed, amastigotes were absent from all smears and infection by *Leishmania* was only confirmed in three/five symptomatic subjects. The anorexia and alopecia detected in the 2 PCR negative dogs was, therefore, not related to kala-azar infection (Table 2).

Fig. 3 shows the proportion of protection against kala-azar achieved in the FML-vaccinated dogs. The rate of infection was significantly higher in saline controls. Indeed, 33% of the animals developed either clinical or fatal disease while only 8% of vaccinated dogs showed mild signs of kala-azar with no deaths. This means that 92% protection against kala-azar was achieved after FML vaccination. The difference between the groups was highly significant ( $\chi^2 = 6.446$ ;

$P < 0.0025$ ).

Taken together, the effect on humoral and cellular immune responses, survival after kala-azar infection and decline in parasitological and clinical signs of disease, the FML-vaccine induced a significant, long-lasting and strong protective effect against canine visceral leishmaniasis in the field.

#### 4. Discussion

Vaccine efficacy is measured by calculating the incidence rates of disease among vaccinated and unvaccinated subjects and determining the percentage reduction in the incidence rate of disease between the two populations [20]. In practice, vaccines are neither perfectly effective nor totally ineffective. Human measles vaccine and veterinary rabies vaccines, for instance, are 80–95% [20] and 80–90% effective, respectively, when appropriately administered.

The ideal vaccine efficacy study is a clinical trial starting with subjects susceptible to the disease. In a double blind placebo control trial (Phase III), half of the subjects receive vaccine and half receive placebo. Both groups are prospectively followed [20]. Regarding the FML-vaccine, the trials performed in CB hamsters,

Balb/c and Swiss albino mice correspond to Phase I-IIa trials [9–12]. It was possible to gather information about safety, immunogenicity and efficacy of the vaccine, both before and after artificial challenge, using a small scale population (10–100). An 84–87.7% average of significant and specific protection ( $0.01 < P < 0.001$ ) was achieved in these models [9,10,12]. Furthermore, the standardization trial performed on vaccinated and experimentally infected dogs, also represents a Phase IIa trial in a more appropriate experimental model [16].

In the present work, a Phase III trial of the FML-vaccine was developed, on a large scale dog population of an endemic area composed of vaccinees and placebo treated animals that were prospectively studied for a 2 year period. A total of 92% significant and long-lasting protection against canine kala-azar was obtained with this formulation. This means that the next step in FML-vaccine development could be the development of either a new Phase III or Phase IV trials. Protection against canine kala-azar due to FML-vaccine is also probably related to the reduction of human disease in this area. Indeed, São Gonçalo district represented until 1996 the origin of 6% of total human kala-azar cases in Rio Grande do Norte. This dog vaccination trial started at December 1996. The number of human

cases in this area decreased from 15 cases in 1996 to 6 cases up to July 1997 and to zero until May 1998.

The control of visceral leishmaniasis in North-East Brazil in the past 30 years has centered on the detection of infected dogs by serological enquires and elimination of seropositive dogs [23]. Usually 50% of seropositive dogs had amastigote-like organisms [23]. Some problems, however, still need to be solved in order to improve the control program efficacy. The commonly used method for control programs is immunofluorescence (IF). It usually shows high correlation with parasitologic confirmation of infection. Due to its low sensitivity, this method underestimates the true prevalence of canine infection, missing some cases. It is known that, in endemic areas, some dogs are seropositive and infected but asymptomatic or oligosymptomatic [24–26], and others, also infected, spontaneously recover [27]. It is important to note that in the endemic area, the FML-vaccine not only reduced the number of deaths due to kala-azar but the number of poly and oligosymptomatic dogs as well. This fact is relevant since naturally infected dogs, both asymptomatic and oligosymptomatic, proved to be infective for sandflies [24].

The seropositivity in IF and FML-ELISA disclosed in the pre-immunization census showed correlation with

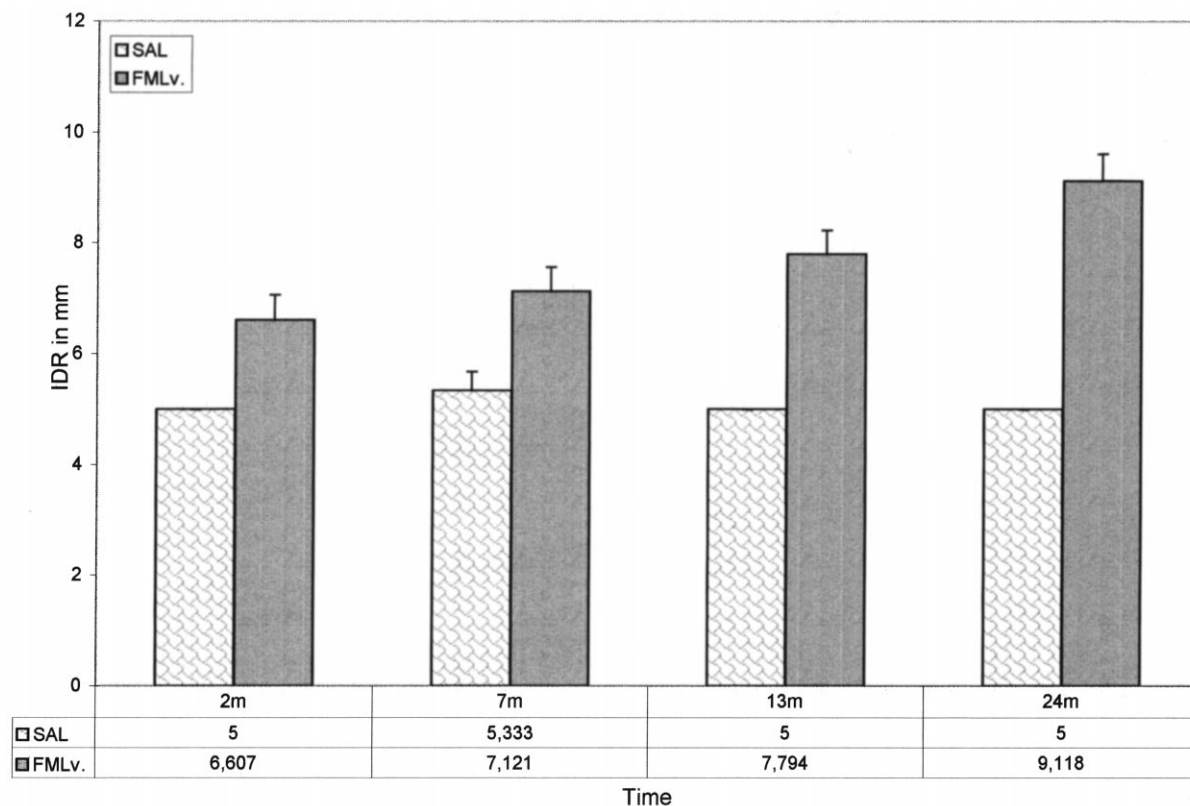


Fig. 2. Evolution of the size of intradermal skin reaction to *L. donovani* lysate with time in naturally exposed vaccinated dogs and saline treated controls. The results represent the mean  $\pm$  SE values of skin reaction diameters (mm) of all reactive animals, 24 h after antigen injection. Sal: saline-treated controls; FMLv: FML-vaccine treated animals.

Table 2  
Parasite confirmation in symptomatic dogs 24 months after vaccination with the FML-vaccine<sup>a</sup>

Group	FML-ELISA* (Abs 492 nm)	IDR** (mm)	Symptoms	PCR	Amastigotes
Saline	0.564	–	Disseminated ulcer	+	–
Saline	0.936	–	Onycogryphosis	+	–
Saline	0.484	–	Isolation	+	–
Saline	0.386	5	Alopecia	+	–
Saline	0.766	–	Onycogryphosis	+	–
Saline	1.088	5	Alopecia	+	–
FML-v	1.618	10	Onycogryphosis	+	–
FML-v	1.760	10	Anorexia	–	–
FML-v	1.177	10	Alopecia	+	–
FML-v	0.659	10	Onycogryphosis	+	–
FML-v	1.439	5	Alopecia	–	–

<sup>a</sup> NOTE. \* These are the absorbency values of sera in the FML-ELISA assay. Sera of symptomatic dogs were collected before sacrifice. Absorbency values higher than 0.435 were considered positive results. \*\* These are the diameters of intradermal reactions after 24 h injection of promastigote lysate antigen. Reactions were considered positive if  $\geq 5$  mm. Saline: percent of saline-treated controls; FMLv: percent of FML-vaccine treated animals; IDR: intradermal reaction to *L. donovani* promastigote lysate (200  $\mu$ g of protein); PCR: polymerase chain reaction performed on genomic DNA extracted from 500  $\mu$ l of whole blood 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).

results previously obtained in the same area (2.6 and 23%, respectively) [17] and in Jequié, Bahia, another Brazilian kala-azar endemic region (23.5%) [28]. However, two years after vaccination, seropositivity to FML in saline treated controls was 68%. The proportion of deaths due to confirmed kala-azar in the control group was 6.8%. This value is not different from the usual expected in this area is 2–3% ( $\chi^2 = 0.0003$ ;  $P > 0.05$ ). Therefore, the increase in seropositivity was not related to any change in risk of infection. Regarding human tegumentar leishmaniasis, it was already proved that the amount of antigen utilized in the MST (Montenegro skin test), used for diagnostic purposes, or as a criterion for inclusion into vaccination or immunological assays, is in fact capable of inducing a new or modifying a pre-existing, immune response to *Leishmania*. Indeed, a first skin test in human naive individuals induced positivity in a second one (4/5) and an increase in anti-*Leishmania* antibody titers on ELISA assay (2/6) [29]. The skin test is used for the screening of individual candidates to participate in a vaccination assay against tegumentar leishmaniasis. Only subjects that show negative reactions, meaning no previous contact with *Leishmania*, would be included in the experiment. This means that the relatively small amount of antigen (4  $\mu$ g of protein) injected for the skin test leads to a specific sensitization. In our dog vaccination experiment, initial screening was performed with a non-invasive tool: the FML-ELISA assay. The skin test was only used for monitoring the protective cellular immune response. As we previously stated, intradermal reaction is a clear marker of protection in human or canine kala-azar. Seropositivity, on the other hand, would characterize both the presence of *Leishmania* infection and a hu-

moral post-vaccination response. In the present investigation, the proportion of positivity and the size of the skin reaction was significantly higher in vaccinees than in placebo controls throughout the two year period. However, the successive *L. donovani* antigen injections performed for skin testing could be partially responsible for the increase in seropositivity of the placebo group (68%) over the expected prevalence for this endemic area (2–3%). The maintenance of the same incidence of the disease in the placebo group showed that these injections, while inducing an antibody response, did not elicit any effective protective response against kala-azar.

In the present study, the characterization of an in-vitro lymphoproliferative response to leishmanial antigen was not achieved. The endemic area of São Gonçalo do Amaranto is more than 2000 km away from our laboratory and according to the sanitary control policy, the removal of *Leishmania chagasi* infected dogs from endemic to non-endemic areas is forbidden, in order to avoid the possibility of dissemination of disease. However, in our experimental kennel, we were able to analyze the lymphoproliferative response of mongrel dogs immunized with three doses of the FML-vaccine, and experimentally challenged with  $10^8$  amastigotes of *L. donovani* through the i.v. route. The protective response was monitored monthly, up to 540 days after infection. The proliferative in vitro response of blood mononuclear cells to FML, GP36 and promastigote lysate antigens was evaluated. The differences between the in vitro proliferative response of vaccinees and controls were not significant. Both groups developed a proliferation peak between 180 and 300 days after infection. The IDR response was positive and significantly higher in vaccinees than in saline

controls ( $P < 0.01$ ) while clinical signs of disease were more evident in saline controls ( $P < 0.001$ ) [16]. In agreement with our results, Binhazim et al. (1993), demonstrated a similar proliferative response in normal controls and infected dogs [30]. Abranches et al. (1991) did not show differences in lymphoblastic proliferation between infected and uninfected dogs, when treated with ConcanavalinA e Phytohaemagglutinin mitogens. However, the proliferative response against total antigens of *L. infantum* e *L. donovani* was suppressed in six/seven infected individuals [31]. Pinelli et al. (1994) showed that asymptomatic or resistant dogs responded to *L. infantum* antigen both by lymphocyte proliferation assays and by the IDR reaction [32]. In contrast, symptomatic dogs failed to respond to parasite antigen both in-vitro and in-vivo. Furthermore, higher levels of IL-2 and tumor necrosis factor were found in supernatants from stimulated mononuclear cells from asymptomatic dogs compared with those of symptomatic and control dogs [32]. The characterization of the cytokine response of cells derived from dogs treated with FML-vaccine is under progress.

Costa et al. (1996) during a field trial in Montes Claros, Brazil, described a higher incidence of *L. chagasi* infections in Dobermans, followed by Fox Terrier and Pinscher dogs [33]. In the metropolitan region of Lisbon, Portugal, Abranches et al. (1991) pointed out the Doberman, followed by German Shepherd, as the most affected breeds [34]. Therefore, different susceptibilities to disease are expected in relation to the genetic background. The dogs included in our assay, however, correspond mostly to the mongrel kind (97.5%), being then a genetically highly heterogeneous population. Indeed, only one Doberman and two German Shepherd dogs were included among the 120 animals considered eligible for the assay, making up 2.5% of the whole population. Therefore, the analysis of the impact of the FML-vaccine protection in relation to any genetic basis of susceptibility to visceral leishmania-

sis, is not possible. All animals included in this study were healthy and well nourished, since they were domestic pets. Also, they were at least 4 months old, by the beginning of the experiment, in order to guarantee that any potential seroreactivity to leishmanial antigens would not be related to maternal antibodies acquired during breast feeding. The initial number of dogs submitted to serological screening for anti-*Leishmania* antibodies included a majority of males, (95/148) representing 64% of the whole population. One of the possible reasons for this significant difference ( $P < 0.001$ ), found in the endemic area, could be a sex related susceptibility to the disease, e.g. male dogs being more resistant to kala-azar. Two pieces of evidence supported this hypothesis: (1) the first serological screening performed in the area disclosed higher proportions of seropositivities to FML among females, indicating previous infection, and (2) by the end of the assay, higher proportions of kala-azar confirmed cases were found in females. However, the statistical analysis of these differences showed no significance (seropositivity: 12/54 females; 17/95 males; kala-azar cases: 6/37 females; 7/67 males). Future studies with larger samples could clarify this point.

To our knowledge, there is only one reference in literature about canine vaccination against visceral leishmaniasis. Dunan et al. (1989) used a *L. infantum* vaccine composed of semi-purified lyophilized protein preparation of a mol. wt. ranging from 94 to 67 kDa [35]. The authors achieved, during the first year of experiment, a rate of infection significantly higher ( $P < 0.05$ ) in vaccines (17) than in control dogs (5), but this difference disappeared during the second year ( $P = 0.44$ ). These results led the authors to the conclusion that this vaccine, while effective in murine models, developed no protection against canine kala-azar in the field. Hence, the present investigation constitutes the first report of a canine vaccine for visceral leishmaniasis that elicits a long-lasting and significant protective effect.

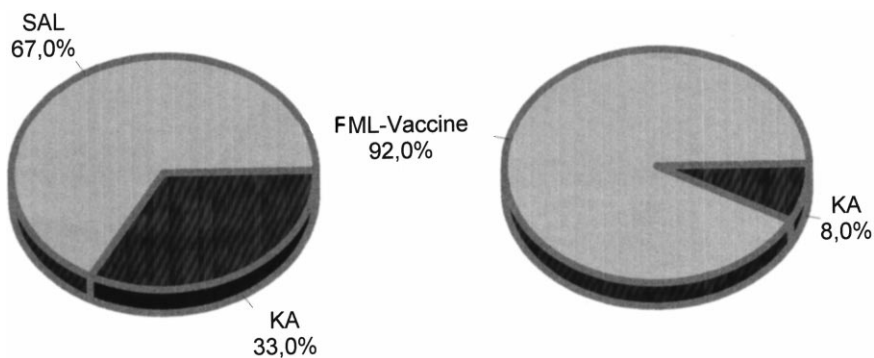


Fig. 3. Proportion of protection against kala-azar achieved in the FML-vaccinated dogs. Sal: saline-treated controls; FMLv: FML-vaccine treated animals.

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