Available online at www.sciencedirect.com
SCIENCE DIRECT®
Vaccine 23 (2005) 4805–4810
Vaccine
www.elsevier.com/locate/vaccine

Short communication

Leishmune® vaccine blocks the transmission of canine visceral leishmaniasis Absence of *Leishmania* parasites in blood, skin and lymph nodes of vaccinated exposed dogs

F.S. Nogueira [a], M.A.B. Moreira [b], G.P. Borja-Cabrera [c], F.N. Santos [c], I. Menz [d], L.E. Parra [d], Z. Xu [e], H.J. Chu [e], C.B. Palatnik-de-Sousa [c,*], M.C.R. Luvizotto [f]

[a] *Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista "Júlio de Mesquita Filho", UNESP—Botucatu Distrito Rubião Jr. s/n Botucatu SP CEP 18600-000, Brazil*
[b] *Universidade Anhembi-Morumbi, Rua Conselheiro Lafaiete, 64 Bairro Brás São Paulo, SP CEP 03164-000, Brazil*
[c] *Instituto de Microbiologia, "Professor Paulo de Góes", Universidade Federal do Rio de Janeiro (UFRJ), CCS, Cidade Universitária, Ilha do Fundão, CP 68040, CEP 21941-590, Rio de Janeiro, Brazil*
[d] *Fort Dodge Saúde Animal Ltda, Rua Luiz Fernando Rodrigues 1701, Vila Boa Vista, Campinas, CEP 13065-858, SP, Brazil*
[e] *Bio Research and Development, Fort Dodge Animal Health, 800 5th Street NW, Fort Dodge, IA 50501, USA*
[f] *Departamento de Patologia da Faculdade de Medicina Veterinária e Zootecnia UNESP-Araçatuba, Rua Clóvis Pestana, 793 São Paulo CEP 16050-680, Brazil*

Received 17 January 2005; accepted 16 May 2005
Available online 8 June 2005

Abstract

Leishmune® vaccine is the first licensed vaccine against canine visceral leishmaniasis. It contains the Fucose–Mannose-ligand (FML) antigen of *Leishmania donovani*. The potential Leishmune® vaccine effect on the interruption of the transmission of the disease, was assayed by monitoring, in untreated ($n = 40$) and vaccinated dogs ($n = 32$) of a Brazilian epidemic area: the kala-azar clinical signs, the FML-seropositivity and the *Leishmania* parasite evidence by immunohistochemistry of skin and PCR for Leishmanial DNA of lymph node and blood samples. On month 11 after vaccination, untreated controls showed: 25% of symptomatic cases, 50% of FML-seropositivity, 56.7% of lymph node PCR, 15.7% of blood PCR and 25% of immunohistochemical positive reactions. The Leishmune®-vaccinated dogs showed 100% of seropositivity to FML and a complete absence of clinical signs and of parasites (0%) in skin, lymph node and blood PCR samples ($p < 0.01$). The positivity in FML-ELISA in untreated dogs significantly correlates with the PCR in lymph node samples ($p < 0.001$) and with the increase in number of symptoms ($p = 0.006$) being strong markers of infectiousness. The absence of symptoms and of evidence of *Leishmania* DNA and parasites in Leishmune®-vaccinated animals indicates the non-infectious condition of the Leishmune®-vaccinated dogs.
© 2005 Elsevier Ltd. All rights reserved.

Keywords: Canine visceral leishmaniasis; FML-vaccine; Transmission blocking vaccines

1. Introduction

The etiological agents of visceral leishmaniasis, *Leishmania chagasi* (America) and *Leishmania infantum* (Europe and Mediterranean), are exposed on the skin of foxes, wild canids and dogs, and transmitted to humans through the sandflyĭs bite [1]. Zoonotic visceral leishmaniasis (ZVL) is thus a re-emergent canid zoonosis, the epidemiological control of which involves in Brazil: the elimination of seropositive infected dogs, insecticide treatment within domestic and peridomestic habitations and the systematic treatment of human cases [1]. The development of a protective vaccine

* Corresponding author. Tel.: +55 21 25626742; fax: +55 21 2560 8344/2560 8028.
E-mail address: clarisaps@infolink.com.br (C.B. Palatnik-de-Sousa).

0264-410X/$ – see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2005.05.011

against canine visceral leishmaniasis has been recommended [2–4] as a possible tool for effective eradication of the disease, reducing the offer of parasites to sandfly vectors and consequently the number of human kala-azar cases. We described the protective effect of the FML-vaccine on canine visceral leishmaniasis [5,6]. The fucose- and mannose-containing glycoprotein-enriched fraction was isolated from *Leishmania donovani* promastigotes [7,8], being a potent immunogen [8–10] and a sensitive, predictive and specific serodiagnostic antigen [11,12]. The FML formulation was safe, immunogenic and protective in Phase I–IIa trials in mice and hamsters [9,10,13,14]. In a Brazilian area endemic for both human and dog visceral leishmaniasis, recent Phase III trials of efficacy using the FML-vaccine in dogs induced 92% [5] and 95% [6] of long-lasting protection in exposed vaccinees (76% and 80% of vaccine efficacy, respectively). Also, the FML-vaccine with increased adjuvant concentration, showed its potential in immunotherapy of the canine disease [15]. The reduction of the number of deaths and symptomatic cases of canine kala-azar in the field assays and the concomitant reduction in human cases of kala-azar in the same district [5,6], suggested the interruption of the transmission of the disease.

Recently, the FML-vaccine was industrialized and licensed for commercialization in Brazil under the name of Leishmune®. To determine if Leishmune® interferes with or blocks the transmission of parasites from dogs to phlebotomines, we assayed the infectious condition of vaccinated and untreated dogs, by PCR analysis for leishmanial DNA of blood and lymph node samples and by immunohistochemistry with anti-*Leishmania* antibody of skin samples, which were shown to be rapid, sensitive and definitive diagnostic tools for canine visceral leishmaniasis [16–21].

2. Materials and methods

2.1. Animals, vaccination and follow-up

A severe epidemy of canine visceral leishmaniasis has recently spread out in Brazil, from the Mato Grosso to the São Paulo state. Andradina was one of the first towns to disclose the canine disease in 1999, showing a 13% annual prevalence of infected dogs (2000/15,000 animals analyzed each year by immunofluorescency of sera samples). To assay the Leishmune® vaccine potential in blocking the transmission of canine visceral leishmaniasis, we used dogs chosen among usual patients of two veterinary clinics of Andradina, according to the geographical distribution of their residences. The city can be divided in four different regions (North, South, East and West), which show equivalent incidence of canine disease. Seventy-two adult asymptomatic healthy dogs, showing no symptoms neither previous history of visceral leishmaniasis, nor anti-*Leishmania* antibodies in blood (day 0) when analyzed by *L. chagasi* immunofluorescence and/or FML-ELISA assay [12], were then considered eligible for the assay. Thirty-two dogs were treated with Leishmune® vaccine (Fort Dodge Saúde Animal Ltda, Brazil) against canine visceral leishmaniasis. The animals received three subcutaneous doses of Leishmune® vaccine on the flank with a 21 days interval. Each Leishmune® vaccine dose is reconstituted in 1ml NaCl 0.9% sterile saline solution, before use on each vaccination day [5]. Forty dogs remained as the untreated controls. The final distribution determined that 12 vaccinated animals and 11 controls remained in the North; 6 vaccinees and 4 controls in the South; 17 vaccinees and 15 controls in the East and 5 vaccinees and 10 controls in the West area of the city. Whenever it was possible, vaccinees and controls co-habitated in the same residence. Consent was obtained from the dog's owners who were informed about the risk of the procedures and the requirement for a 1-year follow-up. On month 11, all the animals were analyzed according to their anti-FML antibodies in ELISA assay and their clinical signs for visceral leishmaniasis (loss of weight, cachexia, alopecia, onycogryphosis, apathy, ulcerative skin lesions, dermatitis, discrete muscular atrophy, exfoliation, epistaxis, hyperkeratosis, skin thickness). Furthermore, the presence of *Leishmania* parasites in dogs was evaluated in samples of blood and lymph node puncture by the PCR amplification technique and in skin by anti-*Leishmania* immunohistochemistry. The domestic or peri-domestic habits of the dogs were recorded as well as the use of sand fly repellent deltamethrine collar necklace (Scallibur®, Intervet, Holland) were recorded and compared. The insecticide collar is distributed each semester by the control campaign to be used in the dogs that remain healthy and seronegative in the immunofluorescence assay. 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 minimize animal suffering.

The Leishmune® vaccine formulation is the industrialized and registered FML-vaccine [5], composed of the Fucose–Mannose-ligand antigen isolated from the aqueous extract of stationary phase promastigotes of *Leishmania (L.) donovani Sudan* (LD 1S/MHOM/SD/00-strain 1S) antigen [14] (Patent: INPI number: PI1100173-9, 18.3.97, Federal University of Rio de Janeiro, Brazil). The first concern in the development of a vaccine against visceral leishmaniasis was to use the most characterized and defined strain of *L. donovani* known as a kala-azar agent. The *Leishmania (L.) donovani* (LD-1S/MHOM/SD/00-strain 1S) strain, used for FML isolation, was isolated from a Sudanese patient in the late 1960s by Dr. Stauber and kindly forwarded to us by Dr. Dennis Dwyer (NIH, Bethesda, USA), who cloned it and denominated it *L. donovani* 1-S strain [22]. Later on, the 1-S strain received the denomination MHOM/SD/00-strain 1S, from the WHO *Leishmania* bank collection. It was used for isolation of Lypophosphoglycan (LPG) and was the object of intensive biochemical characterization [23,24]. Another goal was to use an FML complex glycoproteic antigen that could give cross-protection against infections due to *L. chagasi* or *L.*

infantum, agents of canine disease in America and Europe. In fact, although isolated from the *L. donovani* species, agent of kala-azar in Africa and Asia, the FML proved to have impressive antigenic and diagnostic potential on American human [11] and canine [12] visceral leishmaniasis caused by *L. chagasi* infection (100% sensitivity and 96–100% specificity, respectively). The FML-ELISA assay achieved a similar performance when tested against sera of humans or dogs infected with *L. infantum* in Spain (unpublished results). Coincidently, canine vaccination with FML induced a highly protective effect both in prophylaxis or immunotherapy of dogs experimentally infected with the *L. donovani* species [25,15] as well as in dogs naturally infected with *L. chagasi* [5,6,15].

2.2. FML-ELISA assay

The FML-ELISA assay for the presence of anti-*L. chagasi* antibodies was performed as described elsewhere [12]. The cut-off of the FML-ELISA assay [12] is Abs 492 nm: 0.450 (mean average of absorbance values of normal healthy serum plus 2 standard deviations).

2.3. PCR for Leishmanial DNA

Five milliliters of peripheral blood from the jugular vein were collected into EDTA tubes and fine needle lymph node biopsies [26], from popliteal lymph nodes were added to 0.5 ml of PBS and stored at −20 °C. For DNA extraction, 0.7 ml samples were thawed, washed with 0.5 ml of TE buffer (10 mM Tris, 1 mM EDTA), centrifuged at 14,000 × *g* and treated with lysis buffer (10% sodium dodecyl sulphate—SDS in 0.2 M sodium acetate and 20 μg/ml proteinase K) at 56 °C for one hour. The lysates were further treated with 400 μl of phenol/chloroform/iso-amilic alcohol, and the DNA precipitated with ethanol, dried, and ressuspended in 50 μl of TE buffer. PCR analysis was performed using primers 13A (5′-GTG GGG GAG GGG CGT TCT-3′) and 13B (5′-ATT TTA CAC CAA CCC CCA GTT-3′) that amplify the conserved region of the kinetoplast minicircle DNA of the *Leishmania* genus (120 bp), as previously described by Rodgers et al. [27]. This PCR assay is able to detect a minimum of 25 *Leishmania* parasites. The amplified products were analyzed on a 2% agarose gel containing 0.5 μg/ml ethidium bromide (Sigma Co.), and a 100 bp DNA ladder (Invitrogen™) was used as a marker. The gels were visualized under UV light with a transilluminator.

2.4. Immunohistochemical staining of skin

Skin biopsies (0.5 mm punch) were obtained from the prescapular region of all dogs, after local asepsis, trichotomy and local anesthesia, stored in buffered 10% formaldehyde, embedded in paraffin, cut (5 μM) and plated on to 3-aminopropyltriethoxy-silane (Sigma Co., St Louis, USA) treated microscope slides. Paraffin was removed at 58 °C. Rehydration was performed with decreasing ethanol concentrations, tap and distilled water. Antigen recovery was done with citric acid. The endogenous peroxidase was blocked with hydrogen peroxide rinsing with top and distilled water. Slides were incubated with polyclonal mouse anti-*Leishmania* serum diluted in 1% BSA–PBS, with a biotin conjugated secondary antibody (kit LSAB, DAKO®, CA, USA) and with streptavidine-peroxidase (kit LSAB, DAKO®) followed by diamenobenzidine (SIGMA Co. SL, USA), hematoxylin, increasing alcohol solutions and xylol, and were mounted and microscopically analyzed.

2.5. Statistical analysis

Chi square and Fisher's exact tests were used in comparing proportions [28]. Correlation coefficient analysis was determined on a Pearson bivariate, two-tailed test of significance (SPSS).

3. Results

All vaccinated dogs ($n = 32$) were seronegative to FML at day 0 (Abs 492 nm, average ± S.D. = 0.278 ± 0.089) and seropositive on day 70 (Abs 492 nm, average ± S.D. = 1.021 ± 0.214), after complete vaccination. On month 11, however, the FML-ELISA assay positivity disclosed the induction of a specific anti-*Leishmania* humoral response in vaccinated dogs (100%) and the *L. chagasi* infection in untreated controls (50%) (Table 1). Also, striking differences in positivity were noted between the two groups in all the other diagnostic variables. While clear evidence of *Leishmania* infection was disclosed in untreated animals by lymph node PCR (56.7%), number of kala-azar symptoms (25%), immunohistochemistry (25%) and blood PCR (15.8%), complete absence of parasites was indicated by these analyses in Leishmune® vaccine treated animals. The difference between the two groups in all these variables was highly significant (Table 1). The complete absence of parasite evidence in Leishmune®-vaccinated dogs strongly supports its prophylactic effect on the development of *L. chagasi* infection in endemic areas. In the untreated dogs, the PCR of lymph node punctures was shown to be the most sensitive method, followed by symptomatology or immunohistochemistry and blood PCR. The difference between PCR of lymph node or blood specimens was significant ($p < 0.005$) while the differences between the positivities in immunohistochemistry and blood PCR were not ($p > 0.05$). This means that although more invasive, the PCR analysis of lymph node samples is a more accurate test for *Leishmania* infection. Although a trend to a higher exposure of the vaccinated dogs to phlebotomine bite was noted in the higher number of dogs living peridomestically (67.5%), the differences between vaccinated and control groups in this variable were however, not significant. Also, no differences were found in the proportion of dogs bearing the delta-methrin insecticide necklace that is used as a tool for epidemiological

Table 1
Serological, parasitological and clinical follow-up of exposed untreated and Leishmune®-vaccinated dogs

Results at month 11	Untreated		Leishmune®		Significance (p)
	Positive/total	(%)	Positive/total	(%)	
FMLELISA[a]	20/40	50	24/24	100	<0.005
PCR lymphnode	17/30	56.7	0/18	0	<0.005
Kala-azar symptoms	10/40	25	0/32	0	<0.005
Immunohistochemistry	10/40	25	0/26	0	<0.005
PCR blood	6/38	15.8	0/29	0	<0.01
Peridomiciliary habits	18/40	45	20/32	62.5	>0.05
Insecticide necklace	19/40	47.5	18/32	56.2	>0.05

[a] The cut-off value for the FML-ELISA assay is 0.450 (absorbance at 492 nm).

Table 2
Comparison of results obtained by FMLELISA, PCR and immunohistochemistry and follow-up of exposed and Leishmune®-vaccinated dogs

Dogs	FML-ELISA	Abs 492 nm[a]	PCR		Immunohisto-chemistry	Peridomiciliar habit	Insecticide necklace	Kala-azar symptoms[b]
			Blood	Lymph node				
Leishmune®-vaccinated								
23	+	0.943	–	–	–	+	–	–
24	+	0.904	–	–	–	–	–	–
25	+	0.993	–	–	–	+	–	–
26	+	0.769	–	–	–	+	–	–
28	+	0.605	–	–	–	–	+	–
29	+	0.878	–	–	–	–	–	–
30	+	0.806	–	–	–	–	+	–
32	+	1.407	–	–	–	+	–	–
33	+	0.925	–	–	–	–	–	–
34	+	1.343	–	–	–	+	+	–
38	+	0.458	–	–	–	+	+	–
39	+	1.351	–	–	–	–	+	–
40	+	0.733	–	–	–	–	+	–
Controls								
28	+	1.342	–	+	–	–	–	–
38	+	1.218	+	+	+	–	–	On
22	+	1.179	–	+	+	–	+	Al, on, ex, atr
25	+	1.174	–	+	–	–	–	–
1	+	1.171	–	+	–	+	–	Al, hk, st, on
23	+	1.166	–	+	+	–	–	Lw, al, der
35	+	1.139	+	+	+	+	+	Al, on, lw
24	+	1.104	–	+	–	–	+	–
2	+	1.035	–	+	–	–	–	Der, al, lw
12	+	0.924	–	+	–	–	+	Lw, al, ex
39	+	0.747	–	+	–	+	+	–
18	+	0.721	+	+	–	+	+	Ep, ex, on
40	+	0.661	–	+	+	–	+	On, lw
17	+	0.579	–	+	–	–	+	–
29	+	0.558	+	–	–	–	–	–
27	+	0.552	–	+	–	–	+	–
26	+	0.536	–	–	–	–	–	–
30	+	0.492	–	+	–	+	–	–
31	–	0.346	–	+	–	+	–	–
11	–	0.204	–	–	+	+	–	–
36	–	0.175	–	–	+	–	+	–
3	–	0.154	–	–	+	+	–	–

[a] Results of the FML-ELISA assay are expressed as the mean values of triplicates.
[b] Symptoms: loss of weight (lw), allopecia (al), onycogryphosis (on), dermatitis (der), discrete muscular atrophy (atr), exfoliation (ex), epistaxis (ep), hyperkeratosis (hk) and skin thickness (st).

control in this area (Table 1), indicating that protection against the sandfly bite was homogeneous in both groups.

A correlation analysis of all variables was performed (Table 2) among all individuals that showed complete results in all variables at month 11 (22 untreated and 13 Leishmune®-vaccinated). In the control group, the positivity in FML-ELISA significantly correlates with the PCR in lymph node samples ($p < 0.001$). Also, increasing absorbencies in the FML-ELISA assay correlate with the increase in number of symptoms ($p = 0.006$) being strong markers of infectiousness. On the other hand, in the group of Leishmune® treated dogs, the negative reactions in lymph node PCR correlate with those of immunohistochemical analysis ($p < 0.0001$), PCR of blood samples ($p < 0.0001$) and absence of symptoms ($p < 0.0001$), confirming the absence of parasites and indicating the non-infectious condition of the vaccinated dogs.

4. Discussion

In previous Phase III field assays, the FML-vaccine protected 92% and 95% of the vaccinated dogs [5,6], which remained healthy, and asymtpomatic, reducing the reservoir of the *L. chagasi* parasite in the endemic area until 3.5 years after vaccination [6]. At this point, control animals showed positive reactions in bone marrow (3/3) and blood PCR (3/4) and in bone marrow smears for amastigotes (1/4), indicating their infectious condition while FML-vaccinees, showed no reactivity in bone marrow (0/4) or blood PCR (0/4), or bone marrow smears (0/4), suggesting their long-lasting non-infectious condition [6].

Recent studies showed that low levels of CD4 and CD21 lymphocytes in infected dogs correlate with their infectivity to sandflies demonstrated by xenodiagnosis [29–31]. Of note, normal proportions of CD4 and CD21 lymphocytes were detected in PBMC by FACS analysis, of *L. chagasi* and *L. donovani* infected dogs, after one and 2 years, respectively, of immunotherapy with FML-vaccine, indicating their non-infectious condition [15].

In this investigation and in previous work [25], the untreated dogs showed an increase in absorbency values of the anti-FML IgG assay that strongly correlates with the increase in number of symptoms. The Leishmune® vaccine treated dogs remained healthy and asymptomatic. Courtenay et al. [32], working with Brazilian dogs disclosed that the best predictors of infectiousness, as evidenced by xenodiagnosis, were IgG antibody titers and clinical disease, finding also positive correlations between anti-*Leishmania* IgG, parasite detection by PCR, clinical disease and infectiousness to sandflies [32]. Correlation between symptomatology and infectiousness to sandflies has been confirmed [32,33,21]. The work of Travi et al. [33] indicated that polysymptomatic dogs are more infectious to sandflies than oligosymptomatic and asymptomatic dogs. Also, parasites were found in skin biopsies of symptomatic and highly seropositive dogs, considered the *Leishmania* reservoir in endemic areas, while no parasites were found in the skin of mildly seropositive dogs [21].

In this investigation, increasing serology strongly correlates with PCR of lymph nodes that was more sensitive than PCR of blood specimens. Manna et al. [16] concluded that in symptomatic dogs, PCR was positive in 99% of lymph node samples, 95% of skin samples and 94% of blood samples. After chemotherapy, PCR analysis was still positive on skin and lymph node samples but not always on blood samples [16]. As described for humans [34], PCR in blood is less sensitive. Only the high sensitivity of real-time PCR could allow the use of blood sampling that is less invasive and easily performed, for monitoring the status of the dogs [35]. Diagnosis using PCR from lymph node was also shown to be more sensitive than in vitro culture [36]. Either axillary, cervical and popliteal nodes showed the same *Leishmania* parasitic load [37].

We conclude that, in this investigation, the untreated dogs showed the clinical and laboratory parameters of infectious dogs, while the Leishmune®-vaccinated, symptomless animals, remain healthy and with a non-infectious condition which makes the Leishmune® vaccine eligible for prophylactic use against canine visceral leishmaniasis in endemic and epidemic areas.

Acknowledgements

This study received financial support from: National Council for Scientific and Technological Development (CNPq); FAPERJ/PRONEX and FAPERJ/CNE (Rio de Janeiro State Research Foundation); RHAE-CNPQ (Human sources in strategic areas program), Brazil, and Fort Dodge Animal Health, Brazil and USA. English language editing was by Dr. Jack Woodall, Universidade Federal do Rio de Janeiro.

References

[1] Tesh R. Control of zoonotic visceral leishmaniasis. Is it time to change strategies? Am J Trop Med Hyg 1995;52:287–92.
[2] World Health Organization-TDR. The TDR fifteenth programm report. Research progress 1999–2000. New and Improved Tools, 2003. http://www.who.int/tdr/research/progress9900/tools/vdr.htm.
[3] Modabber F, Leishmaniasis. *Tropical disease research progress*. 1991–1992. Geneva: World Health Organization; 1993. p. 77–87.
[4] Dye C. The logic of visceral leishmaniasis control. Am J Trop Med Hyg 1996;55:125–30.
[5] da Silva VO, Borja-Cabrera GP, Correia Pontes NN, Paraguai de Souza E, Luz KG, Palatnik M, et al. A Phase III trial of Efficacy of the FML-vaccine against canine kala-azar in an endemic area of Brazil (São Gonçalo do Amarante, RN). Vaccine 2001;19:1082–92.
[6] Borja-Cabrera GP, Correia Pontes NN, da Silva VO, Paraguai de Souza E, Santos WR, Gomes EM, et al. Long lasting protection against canine kala-azar using the FML-QuilA saponin vaccine in an endemic area of Brazil (São Gonçalo do Amarante). Vaccine 2002;20:3277–84.

[7] Palatnik CB, Borojevic R, Previato JO, Mendonça-Previato L. Inhibition of *Leishmania donovani* promastigote internalization into murine macrophages by chemically defined parasite glycoconjugate. Infect Immun 1989;57:754–63.
[8] Palatnik de Sousa CB, Dutra HS, Borojevic R. *Leishmania donovani* surface glycoconjugate GP36 is the major immunogen component of the Fucose–Mannose Ligand (FML). Acta Trop 1993;53:59–72.
[9] Santos WR, Paraguai de Souza E, Palatnik M, Palatnik de Sousa CB. Vaccination with the FML antigen (Fucose Mannose Ligand) of *Leishmania donovani* in the Swiss Albino model. Vaccine 1999;17:2554–61.
[10] Santos WR, de Lima VMF, Paraguai de Souza E, Bernardo RR, Palatnik M, Palatnik de Sousa CB. Saponins, IL12 and BCG adjuvant in the FML-vaccine formulation against murine visceral leishmaniasis. Vaccine 2002;21:30–43.
[11] Palatnik de Sousa CB, Gomes E, Paraguai de Souza E, Palatnik M, Luz K, Borojevic R. *Leishmania donovani*: titration of antibodies to the fucose–mannose-ligand as an aid in diagnosis and prognosis of visceral leishmaniasis. Trans R Soc Trop Med Hyg 1995;89:390–3.
[12] Borja Cabrera GP, da Silva VO, da Costa RT, Barbosa Reis A, Mayrink W, Genaro O, et al. The FML-ELISA assay in diagnosis and prognosis of canine visceral leishmaniasis. Am J Trop Med 1999;61:296–301.
[13] Palatnik de Sousa CB, Paraguai de Souza E, Gomes EM, Borojevic R. Experimental murine *Leishmania donovani* infection: immunoprotection by the Fucose Mannose Ligand (FML). Braz J Med Biol Res 1994;27:547–51.
[14] Palatnik de Sousa CB, Moreno MB, Paraguai de Souza E, Borojevic R. The FML-vaccine (Fucose–Mannose Ligand) protects hamsters from experimental Kala-Azar. Ciência e Cultura (J Braz Assoc Adv Sci) 1994;46:290–6.
[15] Borja-Cabrera GP, Cruz Mendes A, Paraguai de Souza W, Okada LYH, Trivellato FAA, Kawasaki JKA, et al. Effective immunotherapy against canine visceral leishmaniasis with the FML-vaccine. Vaccine 2004;22:2234–43.
[16] Manna L, Vitale F, Reale S, Caracappa S, Pavone LM, Della Morte R, et al. Comparison of different tissue sampling for PCR-Based diagnosis and follow-up of canine visceral leishmaniosis. Vet Parasitol 2004;125:251–62.
[17] Berrahal F, Mary C, Roze M, Berenger A, Escoffier K, Lamouroux D, et al. Canine leishmaniasis: identification of asymptomatic carriers by polymerase chain reaction and immunoblotting. Am J Trop Med Hyg 1996;55:273–7.
[18] Leontides LS, Saridomichelakis MN, Billinis C, Kontos V, Koutinas AF, Galatos AD, et al. A cross-sectional study of Leishmania spp. infection in clinically healthy dogs with polymerase chain reaction and serology in Greece. Vet Parasitol 2002;109:19–27.
[19] Muller N, Zimmermann V, Forster U, Bienz M, Gottstein B, Welle M. PCR-based detection of canine *Leishmania* infections in formalin-fixed and paraffin-embedded skin biopsies: elaboration of a protocol for quality assessment of the diagnostic amplification reaction. Vet Parasitol 2003;114:223–9.
[20] Lachaud L, Marchergui-Hammami S, Chabbert E, Dereure J, Dedet JP, Bastien P. Comparison of six PCR methods using peripheral blood for detection of canine visceral leishmaniasis. J Clin Microbiol 2002;40:210–5.
[21] Solano–Gallego L, Fernández-Bellon H, Morell P, Fondevilla D, Alberola J, Ramis A, et al. Histological and immmunohistochemical study of clinically normal skin of Leishmania infantum-infected dogs. J Comp Path 2004;130:7–12.
[22] Dwyer DM. *Leishmania donovani*: surface membrane carbohydrates of promastigotes. Exp Parasitol 1977;41:341–58.
[23] Turco SJ, Wilkerson MA, Clawson DR. Expression of an unusual acidic glycoconjugate in *Leishmania donovani*. J Biol Chem 1984;259:3883–9.
[24] Sacks DL, Modi G, Rowton E, Spath G, Epstein L, Turco SJ, et al. The role of phosphoglycans in Leishmania-sand fly interactions. Proc Natl Acad Sci USA 2000;97:406–11.
[25] Borja-Cabrera GP. Análise do potencial diagnóstico, prognóstico e imunoprotetor do antígeno FML (Ligante de Fucose Manose) de *Leishmania (L.) donovani*, no calazar canino experimental e de área endêmica, Ph.D. Thesis. Department of Experimental Pathology, Federal Fluminense University; 2000. p. 79.
[26] Cowell RL, Tyler RD. Cytology of cutaneous lesions. Vet Clin North Am Small Anim Pract 1989;19:769–94.
[27] Rodgers MR, Popper SJ, Wirth DF. Amplification of kinetoplast DNA as a tool in the detection and diagnosis of *Leishmania*. Exp Parasitol 1990;71:267–75.
[28] Fisher RA, Yates F. Student's *t*-test of significance. In: *Statistical Tables for Agricultural, Biological and Other Research Workers*. Edinburgh: Oliver and Boyd; 1957. p. 157.
[29] Guarga JL, Moreno J, Lucientes J, Gracia MJ, Peribanez MA, Alvar J, et al. Canine leishmaniasis transmission: higher infectivity among naturally infected dogs to sandflies is associated with lower proportions of T helper cells. Res Vet Sci 2000;69:249–53.
[30] Bourdoiseau G, Bonnefont C, Hoareau E, Boehringer C, Stolle T, Chabanne L. Specific IgG1 and IgG2 antibody and lymphocyte subset levels in naturally *Leishmania infantum*-infected treated and untreated dogs. Vet Immunopathol 1997;59:21–30.
[31] Moreno J, Nieto J, Chamizo C, Gonzalez F, Blanco F, Barker F, et al. The immune response and PBMC subsets in canine visceral leishmaniasis before and after chemoterapy. Vet Immunopathol 1999;30:181–95.
[32] Courtenay O, Quinnell RJ, Garcez LM, Shaw JJ, Dye C. Infectiousness in a Cohort of Brazilian dogs: why culling fails to control visceral leishmaniasis in areas of high transmission. J Inf Dis 2002;186:1314–20.
[33] Travi BL, Tabares CJ, Cadena H, Ferro C, Osorio Y. Canine visceral leishmaniasis in Colombia: relationship between clinical and parasitologic status and infectivity for sand flies. Am J Trop Med Hyg 2001;64:119–24.
[34] Otero ACS, da Silva VO, Luz KG, Palatnik M, Pirmez C, Fernandes O, et al. Occurrence of *Leishmania donovani* DNA in seroreactive Brazilian blood donors. Am J Trop Med 2000;62:128–31.
[35] Mortarino M, Franceschi A, Mancianti F, Bazzocchi C, Genchi C, Bandi C. Quantitative PCR in the diagnosis of Leishmania. Parassitologia 2004;46:163–7.
[36] Strauss-Ayali D, Jaffe CL, Burshtain O, Gonen L. Baneth G.Polymerase chain reaction using noninvasively obtained samples, for the detection of Leishmania infantum DNA in dogs. J Infect Dis 2004;189:1729–33.
[37] Lima WG, Michalick MS, de Melo MN, Luiz Tafuri W, Luiz Tafuri W. Canine visceral leishmaniasis: a histopathological study of lymph nodes. Acta Trop 2004;92:43–53.