Immunogenicity of HSP-70, KMP-11 and PFR-2 leishmanial antigens in the experimental model of canine visceral leishmaniasis

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Summary Zoonotic visceral leishmaniasis (ZVL) is a parasitic disease caused by Leishmania infantum/L. chagasi that is emerging as an important medical and veterinary problem. Dogs are the domestic reservoir for this parasite and, therefore, the main target for controlling the transmission to humans. In the present work, we have evaluated the immunogenicity of the Leishmania infantum heat shock protein (HSP)-70, paraflagellar rod protein (PFR)-2 and kine-toplastida membrane protein (KMP)-11 recombinant proteins in dogs experimentally infected with the parasite. We have shown that peripheral blood mononuclear cells (PBMC) from experimentally infected dogs proliferated in response to these recombinant antigens and against the soluble leishmanial antigen (SLA). We have also quantified the mRNA expression level of the cytokines induced in PBMC upon stimulation with the HSP-70, PFR-2 and KMP-11 proteins. These recombinant proteins induced an up-regulation of IFN-γ. HSP-70 and PFR-2 also produced an increase of the TNF-α transcripts abundance. No measurable induction of IL-10 was observed and low levels of IL-4 mRNA were produced in response to the three mentioned recombinant antigens. Serum levels of specific antibodies against HSP-70, PFR-2 and KMP-11 recombinant proteins were also determined in these animals. Our study showed that HSP-70, KMP-11 and PFR-2 proteins are recognized by infected canines. Furthermore, these antigens produce a Th1-type immune response, suggesting that they may be involved in protection. The identification...
as vaccine candidates of *Leishmania* antigens that elicit appropriate immune responses in the canine model is a key step in the rational approach to generate a vaccine for canine visceral leishmaniasis.

**Introduction**

Zoonotic visceral leishmaniasis (ZVL) caused by *Leishmania infantum/Leishmania chagasi* is an endemic disease in the Mediterranean basin, Asia, Central and South America, representing a serious public health problem [1]. The parasite is transmitted by the bite of phlebotomine sandflies. Dogs as the domestic reservoirs for the parasite play a key role in the transmission cycle to human. The prevalence of infected dogs in endemic areas is high [2], but the outcome of the disease after infection is variable and only part of the animals develop symptoms while the rest remain asymptomatic; nevertheless, in addition to symptomatic animals, the capability of subclinically infected dogs to infect sandflies has been demonstrated [3,4]. Current control strategy to interrupt transmission by culling seropositive animals is ineffective and although chemotherapy reduces or eliminates clinical symptoms [5–8] it does not eliminate the parasite, implying that treated dogs could still represent an epidemiological risk [9]. A vaccine against canine visceral leishmaniasis would constitute an important tool and a cost-effective strategy for controlling canine visceral leishmaniasis [10]. Furthermore, an effective immunization of dogs could significantly reduce the incidence of human visceral leishmaniasis caused by *L. infantum* [11].

Previous studies have used experimentally infected animals to study the immunogenic capability of defined leishmanial antigens [12,13]. The canine model of leishmaniasis has demonstrated its utility to evaluate both infection treatment and vaccine efficacy. However, there is a low number of immunization/efficacy assays performed in the canine model of visceral leishmaniasis in comparison to that reported in the murine model [14]. *Leishmania* antigens such as the fucose-mannose ligand [15], protein Q [16], purified excreted/secreted antigens from *L. infantum* [17], Histone 1 and HASPB1 proteins [18], TSA-LmsT11-LeIF trifusion protein [19], *Leishmania* homologue of receptors for activated C kinase (LACK) [20] and cysteine proteinases [21,22] have been used in vaccine trials with variable success in providing protection to dogs against a parasite challenge. Therefore, identification of new vaccine candidates with immunogenic and protective capacity in the canine reservoir is needed for the development of an effective vaccine to prevent canine visceral leishmaniasis and parasite transmission.

In this work, we evaluated the antigenicity of *Leishmania* recombinant proteins HSP-70, PFR-2 and KMP-11 in the experimental canine model of visceral leishmaniasis. HSP-70 is a major heat shock protein, a group of evolutionarily conserved proteins that have been described as antigens in infectious diseases caused by bacteria, protozoa, fungi and nematodes [23]. It has been shown that HSP-70 from *Leishmania braziliensis* induced the secretion of IFN-γ and IL-2 in absence of IL-10 and IL-4 in patients with mucocutaneous leishmaniasis [24]. Further, the immunization of mice with HSP-70 produced a Th1-type immune response [25,26]. PFR is a potent immunogen able to induce a protective Th1 response against the experimental infection with *Trypanosoma cruzi* [27]. In cutaneous leishmaniasis, PFR-2 from *Leishmania mexicana* induced protection against the experimental infection with *L. panamensis*, showing that PFR-2 is a conserved immunogen between *Leishmania* species [28]. KMP-11 is a surface membrane protein associated to lipophosphoglycan (LPG), which is expressed differentially in amastigote and promastigote stages of *Leishmania* [29—31]. Patients cured from visceral leishmaniasis caused by *L. donovani* showed cellular proliferation against KMP-11 from *L. donovani* and also production of IFN-γ and IL-4 [32,33]. It has been described that the DNA vaccine of KMP-11 protected hamsters experimentally infected with *L. donovani* [34].

We examined the immunogenicity of HSP-70, PFR-2 and KMP-11 in dogs experimentally infected with *Leishmania infantum* evaluating the lymphoproliferative responses of PBMCs and their cytokine mRNA gene expression elicited upon stimulation with these proteins. In addition, we determined the serum antibody levels against HSP-70, PFR-2 and KMP-11 in these animals.

**Materials and methods**

**Animals**

Beagle dogs purchased to a local breeder, ranging in age from 24 to 30 months were used in this study. The animals were kept at the facilities of the National Centre for Microbiology in Majadahonda (Madrid). All dogs received routine vaccinations and both the anti-leishmanial antibody and cellular immune responses to soluble leishmanial antigen (SLA) were negative in all animals. After quarantine, these dogs were experimentally infected by intravenous (iv) inoculation with 10⁸ *L. infantum* promastigotes (MCAN/ES/98/LLM-724). Dogs were monitored for 1.5 years after infection, and a complete characterization of the animals was carried out, including haematological, parasitological and immunological aspects. These animals were kept and handled in the facilities under veterinary care, following ethical guidelines in accordance with national and European Union regulations. The various analyses employed a total of 13 dogs representative of each clinical group: 4 asymptomatic, 5 oligosymptomatic and 4 polysymptomatic dogs [35,36]. In addition, two non-infected dogs were used as controls; these animals were negative for anti-leishmanial antibody, lymphoproliferative response to SLA and parasitological studies performed.
Purification of leishmanial recombinant antigen

*L. infantum* HSP-70 was purified as described previously [37]. Briefly, *L. infantum* HSP-70 was produced in *Escherichia coli* transformed with pQE/HSP-70 clone. The protein was purified by affinity chromatography on a nitrilotriacetic acid-Ni²⁺ column (Qiagen). Finally, proteins were passed through a polyminix-agarose column (Sigma) to eliminate endotoxins. Residual endotoxin was measured with Quantitative Chromogenic Limulus amebocyte assay (QCL-1000, BioWhittaker), showing that preparations were essentially endotoxin-free (less than 30 ng/mg of recombinant protein).

The KMP-11 and PFR-2 genes were isolated by PCR amplification of genomic DNA of *L. infantum* using oligonucleotides that correspond with the 5' and 3' ends, respectively, of each gene. The amplified DNA fragments were cloned, respectively, into the pQE30 and pQE32 vector (Qiagen) using BamHI and SphI sites for KMP-11 and BamHI XbaI sites for PFR-2. The restriction sites were generated ad hoc in the amplified fragments. The *E. coli* M15 strain was chosen as host bacteria and the expressed proteins were purified by Ni²⁺ affinity chromatography. KMP-11 recombinant protein was overexpressed in *E. coli* after induction for 3 h at 37 °C with 0.1 mM IPTG. Thus, the protein was solubilized in phosphate buffer (50 mM NaHPO₄, 300 mM NaCl) pH 8 and bound to the Ni²⁺-NTA resin for 2 h. The resin was washed with the same buffer at pH 8 followed by buffer at pH 7.5, pH 6 and pH 5.5. The recombinant protein was finally eluted with phosphate buffer at pH 4.

The PFR-2 recombinant protein was expressed in *E. coli* cells (M15 strain) after addition of IPTG at 1 mM concentration and grown for 3 h. The cells were pelleted and resuspended in buffer (0.1 M Na₂HPO₄, 0.01 M Tris—HCl, 1 mM PMSF and 8 M urea) at pH 8, broken by sonication, and centrifuged at 10,000 × g for 10 min to pellet cellular debris. The soluble fraction was used for protein purification by affinity chromatography after incubation with Ni²⁺-NTA columns for 2 h at room temperature. The resin was subsequently washed twice with the previously described buffer at pH 8 and at pH 7.5. The recombinant PFR-2 protein was eluted with resuspension buffer at pH 6. The purified recombinant protein was extensively dialysed against PBS.

The purity degree of recombinant proteins was evaluated by SDS-PAGE and Coomassie blue staining. Moreover, all purified recombinant protein preparations were tested by the E-Toxate reaction kit (Limulus amebocyte lysate (LAL), Sigma, St. Louis, MO) showing that the endotoxin levels were below the detection limit of the kit (<0.1 endotoxin units per ml). Protein concentration was measured using the Bradford method.

ELISA tests

Maxisorp microtiter plates (Nunc, Roskilde, Denmark) were coated overnight with 200 ng of HSP-70, 750 ng of KMP-11; 400 ng of PFR-2 or 1000 ng of SLA per well in carbonate buffer (15 mM Na₂CO₃, 28 mM NaHCO₃, pH 9.6) and then blocked with 200 μl of blocking buffer (PBS containing 1% BSA and 0.1% Tween 20, pH 7.4) for 1 h at room temperature. After washing the plates three times with PBS (0.15 M NaCl, 0.05 M Na₂PO₄, pH 7.4) containing 0.01% Tween 20, 100 μl of sera diluted 1:100 in dilution buffer (PBS containing 0.1% BSA and 0.1% Tween 20) were added to each well and incubated for 30 min. Plates were washed and then incubated for 30 min with horseradish peroxidase (HRP)-conjugated sheep anti-canine IgG (Bethyl Laboratories, Montgomery, TX; 1:2500). After intensive washing, 50 μl of a substrate solution containing 0.8 mg/ml 2,2'-azino-bis(3-ethylbenzthioliolene-6-sulfonic acid) diluted in phosphate-citrate buffer (ABTS; Sigma—Aldrich, St. Louis MO, USA) were added to each well. The absorbance at 405 nm was measured using a microELISA reader (Benchmark, Biorad, USA). Results were expressed as mean values of duplicate samples.

Cell isolation

PBMC were prepared by density gradient centrifugation of heparinized blood samples obtained from dogs (Lympocyte Isolation Solution, RAFER, Spain). Erythrocytes were removed after treatment with ACK erythrocyte lysis buffer (0.15 M NH₄Cl, 1.0 mM KHCO₃, 0.1 mM Na₂ EDTA, pH 7.4). PBMC were washed twice in PBS (pH 7.4), counted and adjusted up to 2.5 × 10⁶ cells/ml in complete medium (RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 25 mM HEPE and 10% heat-inactivated foetal calf serum).

Cell-proliferation assay

Cells were cultured in 96-well plates at a density of 2 × 10⁵ PBMC per well for 6 days with either the recombinant antigens (5 μg/ml), SLA (10 μg/ml) or 10 μg/ml of concanavalin A (ConA) in a final volume of 200 μl per well. Plates were pulsed during the last 18 h with BrdU and cell proliferation was determined using a non-radioactive ELISA technique, according to manufacturer’s instruction (BrdU Cell Proliferation Assay Kit, Biotrak, Amersham, UK). All tests were determined in triplicate and results were expressed as the mean increase of optical density regarding to the blank.

PBMC stimulation and RNA extraction

PBMC (5 × 10⁶ cells/well) from infected animals (four asymptomatic, five oligosymptomatic and four polysymptomatic dogs) and controls (two non-infected) were cultured in a 6-well plate (Nunc, Roskilde, Denmark) with either complete media (unstimulated), 5 μg/ml recombinant antigens, 10 μg/ml SLA or 10 μg/ml ConA. Cells were incubated at 37 °C for 24 h in 5% CO₂ atmosphere. Total RNA was extracted using a RNA extraction kit according to the manufacturer’s recommendations (SV-total RNA isolation system, Promega, Madison, USA). The concentration of total RNA was determined spectrophotometrically (ND-1000 UV-V Spectrophotometer, NanoDrop Technology, USA) and each sample was adjusted to a final working concentration of 5 ng/μl with nuclease-free H₂O and stored at −80 °C until use. RNA was free of genomic DNA as determined by PCR.
Cytokine mRNA quantification by real-time-PCR

Real-time-PCR (qRT-PCR) was performed using the ABI Prism 7000 DNA sequence detection system (PE Applied Biosystems, Foster City, CA, USA). Reverse transcription (RT) and PCR amplifications were carried out in a single well by using TaqMan PCR Core Reagent Kit (PE Applied Biosystems). The amplification conditions, the gene-specific primers and probes are as described previously [12]. Briefly, a final volume of 25 μl reaction mixture was used containing 50 ng of template RNA, 5 mM MgCl₂, 2.5 mM dNTPs, 0.625 U of AmpliTaq Gold, 6.25 U of MultiScribe reverse transcriptase, 10 U of RNase inhibitor, each primer at 200 nM and 100 nM of TaqMan probe. Parallel reactions were performed for the detection of canine IL-10, TNF-α, IFN-γ, IL-4 and IL-18 transcripts from PBMC.

The response to SLA in different groups of infected dogs is shown as the amount of the target RNA relative to the control gene β-actin, expressed as 2⁻ΔΔCt value [38]. Data presented are the averaged mRNA levels of PBMC from four asymptomatic, five oligosymptomatic, four polysymptomatic and two control non-infected dogs. The effect of specific antigen stimulation in canines is shown as the cytokine expression of individual animals: asymptomatic (A-1, A-2 and A-3) and oligosymptomatic (O-1, O-2 and O-3) infected dogs and a non-infected control animal (C-1). For these results, the β-actin gene was used as the control gene (for calculation of ΔCt) and unstimulated samples for each animal were used as the calibrator (for calculation of ΔΔCt) [38]. Differences in gene transcription between stimulated and unstimulated cells are expressed as n-fold difference relative to the calibrator. Basal expression of IL-4 was not detected and a Ct value of 35 was assigned to the undetected unstimulated PBMCs for calculation of ΔΔCt in IL-4 gene expression.

Statistical analysis

The effect of antigen stimulation on cytokine gene expression of PBMC from different groups of infected dogs was analyzed using the Mann-Whitney U-test. Logarithmic transformation was performed for all mRNA levels before the data were analyzed. Significance was set at P ≤ 0.05.

Results

Antibody response to leishmanial antigens

The sera from five dogs experimentally infected with Leishmania were collected every 2 months post-infection. IgG antibody levels measured by ELISA are shown in Fig. 1. Two control non-infected dogs were also examined and these animals were negative for anti-leishmanial antibody, as well as in their lymphoproliferative responses to leishmanial antigens (data not shown).

The pattern of HSP-70-specific IgG antibody response observed post-infection was similar to that against SLA and showed to be consistently high throughout the observation period (Fig. 1). The levels of IgG antibodies against KMP-11 were lower than those against HSP-70 and were detected later. In general, IgG antibodies against PFR-2 were undetectable.

Lymphoproliferative response to leishmanial antigens

The proliferative responses to HSP-70, PFR-2 and KMP-11 as well as SLA were examined in asymptomatic and oligosymptomatic dogs at 20 month post-infection. As shown in Fig. 2, PBMC proliferated upon exposure to HSP-70, PFR-2 and KMP-11, even though it was lower than the proliferation induced by SLA. Stimulation with ConA induced high lymphoproliferative responses in PBMC from both asymptomatic and oligosymptomatic infected dogs.
Expression of cytokine transcripts in SLA-stimulated PBMC from dogs experimentally infected with L. infantum

We have analyzed the cytokine pattern elicited in PBMC stimulated for 24 h with SLA from infected dogs with different clinical status. Both asymptomatic and oligosymptomatic dogs significantly (P = 0.016) up-regulated IFN-γ expression in comparison to unstimulated PBMC (Fig. 3); however polysymptomatic dogs showed no changes after SLA stimulation. Furthermore, SLA stimulation produced different IFN-γ expression in PBMC from asymptomatic and oligosymptomatic animals in comparison to polysymptomatic (P = 0.032 and P = 0.029, respectively).

Up-regulation of TNF-α mRNA expression in the SLA-stimulated PBMC also occurred in asymptomatic and oligosymptomatic dogs, but reached significant levels in the latter group (P = 0.016). We have also found significant differences in the TNF-α expression of PBMC from oligosymptomatic animals in comparison to polysymptomatic (P = 0.029).

Asymptomatic and oligosymptomatic animals showed a tendency to express the IL-10 transcripts below the basal levels of the control unstimulated cells; this decrease was statistically significant in the oligosymptomatic group (P = 0.016). While the expression of IL-4 by unstimulated PBMC was below the detection limit, SLA stimulation produced detectable IL-4 in infected animals. However, these levels of IL-4 did not differ significantly among clinical groups. We have found that polysymptomatic animals had a tendency (not significant) to produce higher expression levels of IL-18 (Fig. 3).

Discussion

It has been proposed that the development of a successful vaccine could be the most effective approach for the control of canine leishmaniasis [10]. For this purpose, it is necessary to evaluate the immunogenicity of vaccine candidates. The induction of a Th1-type cytokine response (IFN-γ, TNF-α) would indicate the vaccine potential to confer protection against Leishmania infection. For example, in vitro assays of candidate vaccine antigens like P-8 using PBMC from Leishmania infantum infected dogs have shown to induce mainly antigen specific Th1-like responses during an ongoing infection [12].

In the present work, we have studied the potential as vaccine candidates for canine leishmaniasis of the leishmanial antigens HSP-70, KMP-11 and PFR-2. We have determined the cell proliferation and cytokine pattern elicited by these proteins in PBMC from dogs infected with Leishmania infantum and compared them with that obtained after stimulation with SLA. Our results showed that the stimulation with SLA from L. infantum produced a strong proliferation and IFN-γ mRNA expression in PBMC from asymptomatic and oligosymptomatic animals, as previously described [12,40]. SLA stimulation also elicited a high expression of TNF-α and low IL-4 transcript abundance in PBMC from asymptomatic dogs without changes in the expression of IL-10 or IL-18. These data confirm that a preferential Th1-like response is associated with disease resistance and protection in canine visceral leishmaniasis [12,17,22,41–43].

Sera from dogs infected with Leishmania infantum showed a strong specific reaction against the recombinant L. infantum HSP-70 throughout the study period. The recognition of HSP-70 isolated from L. infantum and L. donovani by sera from dogs with visceral leishmaniasis has been previously described [44], as well as the recognition of HSP-70 isolated from L. braziliensis by sera from cutaneous, mucocutaneous and visceral leishmaniasis patients [45–47]. The mitogenic capability of HSP-70 from L. infantum for murine
B cells has also been described [48]. Further, HSP-70 isolated from *L. braziliensis* induced cell proliferation and production of cytokines in PBMC from *L. chagasi*, *L. guayanensis* and *L. amazonensis* infected patients [24]. We have shown that HSP-70 from *L. infantum* produced a moderate lymphoproliferative response in the asymptomatic and oligosymptomatic dogs studied. Consequently, the results point to the presence of T-cell immunodominant epitope(s) in the HSP-70 antigen and the conservation of the T-cell epitope(s) between some species. *In vitro* exposure to HSP-70 of cultured PBMC from cured patients of mucocutaneous leishmaniasis, resulted in increased levels of mRNA for IFN-γ and IL-2 in the absence of IL-10 and IL-4 [24]. Our study in the canine model of visceral leishmaniasis, the stimulation of PBMC with HSP-70 from *L. infantum* produced an increase in the IFN-γ and TNF-α expression in all animals but not changes in IL-10 mRNA. Therefore, *L. infantum* HSP-70 induced a moderate Th1-like response. The B and T epitope(s) of HSP-70 and the Th1-like expression pattern elicited *in vitro* by this molecule in canine PBMC suggest that HSP-70 is immunogenic under the conditions used in this study. The conservation of epitope(s) among *Leishmania* species and the capacity of HSP-70 to generate a specific response against *Leishmania* in distinct hosts (human patients and dogs) and different clinical manifestations (cutaneous, mucocutaneous and visceral) show the potential of HSP-70 as a candidate for vaccine trials.

Paraflagellar rod proteins (PFR) are strong immunogens against infection with *Trypanosoma cruzi* [49,50] able to induce a Th1-like immune response [27]. Despite PFR proteins are highly conserved among kinetoplastid parasites, there are few studies about the immunogenicity of this antigen against *Leishmania*. In fact, this is the first study of the PFR-2 immunogenic profile in visceral leishmaniasis. In cutaneous leishmaniasis, it has been described that PFR-2 from *L. mexicana* induced protection against the experimental infection with *L. panamensis*. Hence, it seems that PFR-2 is conserved among *Leishmania* species [28]. In our study in the canine model of visceral leishmaniasis, PFR-2 isolated from *L. infantum* was slightly recognized by sera...
Figure 4  Cytokine gene expression in PBMC from asymptomatic and oligosymptomatic *L. infantum* infected dogs and a healthy control dog after stimulation with the leishmanial antigens. Asymptomatic and oligosymptomatic dogs showed antigen specific proliferation to SLA. qRT-PCR data were analyzed by using the $2^{-\Delta\Delta C_T}$ method. Differences in gene transcription after stimulation are expressed as n-fold difference relative to the calibrator (unstimulated cells). Shown are the cytokine expression (IFN-γ, TNF-α, IL-10 and IL-18) for three asymptomatic (A-1, A-2 and A-3) and three oligosymptomatic (O-4, O-5 and O-6) infected animals as well as one control (C-1). Light grey bars represent results from HSP-70 stimulation, stripped bars represent PFR-2, dark grey bars represent KMP-11, black bars represent SLA stimulation and open bars represent ConA stimulation.

from *L. infantum* infected animals, however, it was able to induce a low but specific lymphoproliferative response in *L. infantum* infected dogs. The cell stimulation with PFR-2 showed a Th1-like cytokine expression pattern, with modest IL-4 mRNA levels. The protective immune response showed against different manifestations of leishmaniasis (cutaneous and visceral) suggests that PFR-2 has potential as a leishmanial vaccine candidate. Due to the moderate Th1-like response elicited in infected dogs, we propose the inclusion of this antigen in a multicomponent leishmanial vaccine with other potent immunogens able to induce a strong Th1-like response.

Kinetoplastid membrane protein-11 (KMP-11) is an LPG-associated surface membrane protein present in all members of the family Kinetoplastidae, differentially expressed both in amastigote and promastigote forms of *Leishmania* [29,30,51]. The present study showed the presence of B and T epitope(s) in KMP-11 isolated from *L. infantum* after the humoral and cellular assays performed in dogs infected with *L. infantum*. The immunogenic properties
of this protein were previously described in dogs and hamsters infected with *L. infantum* [31,52], as well as in cured patients of visceral leishmaniasis caused by *L. donovani* who showed an important lymphoproliferative response to the antigen, with a specific production of IFN-γ and IL-4 [33,53]. It has also been described that the *L. donovani* KMP-11 DNA vaccine conferred protection against the experimental infection of hamsters with *L. donovani*, inducing increased expression of IFN-γ, TNF-α and IL-12 and the diminution of IL-10 response, promoting the elimination of *Leishmania* in the spleen and liver of these animals [34]. In the experimental model of canine leishmaniasis, the stimulation with *L. infantum* KMP-11 induced a modest increase in the IFN-γ gene expression in three animals; however, we did not find changes in the expression of TNF-α and IL-4 mRNA. The marked conservation among *Leishmania* species of KMP-11 [54] and the recognition of the B and T epitope(s) by different host of *L. infantum* (human, dogs) show the potential of KMP-11 as a vaccine candidate against visceral leishmaniasis. Despite the expression pattern induced by *L. infantum* KMP-11 in canine PBMC was preferentially Th1-like, the IFN-γ transcript abundance was only moderate. In consequence, it will be necessary to assess the protective capability of the KMP-11 as a component of a multiantigen vaccine against visceral leishmaniasis constituted by some antigens and/or adjuvant able to induce a potent Th1 response including the production of IFN-γ.

In the present work, we have described the cytokine pattern involved in a specific cellular response against *Leishmania* in canine visceral leishmaniasis. The SLA-stimulation of PBMC from dogs infected with *L. infantum* revealed the cytokines involved in the defence against the parasite. This also allowed the evaluation of the immunogenicity of several leishmanial antigens as potential vaccine candidates for canine leishmaniasis, previously in *in vivo* immunization assays. The higher lymphoproliferative response and the IFN-γ mRNA abundance induced by HSP-70, KMP-11 and PFR-2 in asymptomatic dogs suggest that these antigens may be implicated in protection and consequently are potential vaccine candidates against visceral leishmaniasis caused by *L. infantum*.

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**References**


