Protective vaccination against experimental canine visceral leishmaniasis using a combination of DNA and protein immunization with cysteine proteinases type I and II of *L. infantum*

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Abstract

*Leishmania infantum* is known to be associated with visceral leishmaniasis in Iran and canids are natural reservoirs. Control of disease in dogs appears to be one of the most effective approaches for interrupting the domestic cycle of the disease. In search for successful vaccine strategies, we evaluated the cysteine proteinases (CPs) type I and II using a heterologous prime–boost regime for vaccination against experimental visceral leishmaniasis in dogs. Following vaccination and challenge, dogs were followed for 12 months. Ten dogs vaccinated by prime/boost with DNA/recombinant CPs (in combination with CpG ODN and Montanide 720) remained free of infection in their bone marrow. In contrast, three out of four dogs in the control groups had infection in their bone marrow. The peripheral lymphocytes from protected animals had generally higher proliferation responses to F/T antigen, recombinant CPA (rCPA) and recombinant CPB (rCPB) than controls. During post-challenge period, the difference in stimulation index is significant (∗p < 0.05) on months 11 and 12 to F/T antigens, all months for rCPA and 5, 7, 9, 11 and 12 months for rCPB. Analysis of cytokine mRNA level suggested that vaccinated dogs had elevated IFN-γ mRNA in peripheral blood mononuclear cells (PBMC), whereas there was a consistent increase in the level of IL-10 in the control groups and some vaccinated dogs. The level of total IgG and IgG2, but not IgG1, to rCPA and rCPB was significantly higher in the vaccinated group (∗p < 0.05) than the control groups. We also showed that with the exception of one dog, all dogs in the vaccinated group in comparison to control dogs had strong DTH responses. We propose that the combination of DNA and recombinant protein vaccination using CPs could be instrumental to control (VL) in dogs.

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Keywords: Dog DNA vaccination; Canine visceral leishmaniasis; Cysteine proteinases type I and II; CpG ODN

1. Introduction

The World Health Organization has recently declared leishmaniasis as one of the world’s most serious parasitic diseases [1]. This disease is endemic in developing countries, ranges from self-limiting cutaneous leishmaniasis to visceral leishmaniasis, also known as Kala-azar, which is a fatal infection if not treated successfully [2]. *Leishmania infantum* is responsible for visceral leishmaniasis (VL) in the Mediterranean basin, extending to several Middle East and Asian countries [3]. This form of disease appears as an opportunistic disease associated with HIV infection and in other immunosuppressed patients [4]. An estimated 200 million people are at risk of VL and 500,000 of new human kala-azar cases are registered annually [1]. Dogs are the principal...
reservoir of these parasites and play a central role in the transmission cycle to man by phlebotomine sand flies [5]. Control of disease in dogs is difficult. Attempts to treat infected dogs with antimonial drugs have given poor results and relapse following treatment is common. Furthermore, chemotherapy (with amphotericin B) for infected dogs is impractical because of the high cost of treatment [6]. Vector control has been largely unsuccessful [6], but recently, it has been shown that deltamethrin-impregnated dog collars had protective effect for domestic dogs against L. infantum infection [7]. As control of disease in dogs appears to be one of the most effective approaches for interrupting the domestic cycle of the disease, a vaccine that prevents parasite establishment in dogs would be highly desirable. Lasti et al. [8] demonstrated that dogs vaccinated with autoclaved Leishmania major promastigote lysate (ALM) and BCG showed an in vitro lymphocyte proliferative response, while dogs immunized with ALM and saponin expressed humoral antibody response against L. infantum. Mayrink et al. [9] by using Leishmania obtained 90% protection against experimental canine kala-azar in the kennel while failed to detect any significant differences between vaccines and placebo in field phase III assay [10]. Protection against canine kala-azar was also investigated in naturally exposed dogs vaccinated with the FML vaccine [11] and showed 95% seropositivity to FML and 100% intradermal reaction to Leishmania donovani lysate 7 months after vaccination. In addition, the FML vaccine induced a significant long lasting protective effect against canine kala-azar in the field [12]. In the search for successful vaccine strategies against Leishmania infection, we have examined the effectiveness of heterologous prime-boost vaccination using L. infantum cysteine type I (CPB) and II (CPA). The importance of Leishmania cysteine proteinases (CPs) in host-parasite interaction and particularly in immune evasion [13] highlights their potential both as drug targets and as vaccine candidates. In our previous studies, we have shown that the native form of L. major cysteine proteinases was recognized by lymphocytes taken from humans and mice infected with Leishmania [14,15]. Additionally, it has been reported that sera from either cured or active cases of cutaneous and visceral leishmaniasis patients recognize the recombinant CPA (rCPA) and recombinant CPB (rCPB) L. major and L. infantum [16,17]. Peripheral blood mononuclear cells (PBMC) as well as sera from asymptomatic dogs also respond to rCPA and rCPB. Here, we now report our results obtained from pilot study after vaccination with CPs. We assayed the efficacy of the vaccine during 12 months follow-up using both humoral and cellular responses.

2. Materials and methods

2.1. Parasites

L. infantum strain (MCAN/ES/98/LLM-877) was kept virulent by continuous passage in hamsters. After 2–3 months, the spleen was isolated, homogenized and cultured in NNN media in the presence of 100 μg/ml of gentamicin. Metacyclic infective promastigotes were collected by centrifugation (270 × g, 10 min, 4 °C), washed three times in PBS (8 mM Na₂HPO₄, 1.75 mM KH₂PO₄, 0.25 mM KCl and 137 mM NaCl) and resuspended at a concentration of 2 × 10⁵ cells/ml. Hamsters were infected intraperitoneally with 500 ml of this preparation.

2.2. Plasmid construction and purification

The complete open reading frames of L. infantum cp/a and cp/b genes were inserted into the multiple cloning sites of the eukaryotic expression vector pCB6 (gift from Dr. Nicolas Fasel). Primers were designed on the basis of published data [17] and contained Kozak sequence (cpa primers: upstream 5′-GAC GGA TCC ACC A TG GCC CCC A TG GGT-3′, downstream 5′-GGG AAG CTT CTA-3′; cpb primers: upstream 5′-GGG GAT CCA CCA GTG TGT CGT CGG CAC-3′; cpb primers: downstream 5′-GGG CTC TAG ACT GCA GCT ACA CGT ACT GCC AAA TG-3′). This resulted in two constructs, pCB6-cpa and pCB6-cpb, containing the cpa and cpb reading frames, respectively, under the control of CMV promoter, inserted downstream of a kozak consensus sequence and in frame with an initiation codon. Each plasmid was purified with endotoxin-free plasmid Mega DNA kit (QIAGEN).

2.3. Expression and purification of recombinant CPA and CPB

The rCPA and rCPB of L. infantum were expressed in pET23a (Novagen) as previously described [17]. Then recombinant proteins were purified from inclusion bodies by two rounds of separation on preparative SDS–PAGE gels by imidazole–SDS–Zn reverse staining method [18]. The purified proteins were concentrated by ultrafiltration using an Amicon Filter (MWCO: 10kDa) and dialyzed extensively against PBS. Protein concentration was determined with BCA reagent and analyzed by SDS–PAGE to assess the purity of preparation.

2.4. Animals, immunization and infection

Four groups of dogs were used for this pilot study. Fifteen healthy mixed breed dogs were selected from non-endemic part of Iran. Dogs were between 12 months and 4 years old and were physically examined by the Veterinary Faculty of the University of Tehran. All 15 dogs received the routine vaccination including distemper (DHP produced by NOBITVAC, Intervet), Canine adenovirus type 2 (CAV 2 strain Manhattan LPV3), canine parvovirus (CPV strain 154) and rabies (BHK, produced by Pasteur Institute of Iran). Dogs were also treated with anti-helminthic drugs. All of them were negative for the presence of L. infantum antibodies, as confirmed by ELISA, immunoblotting and DAT. We also randomly selected 5 out of 15 dogs for lymphoproliferation assay with freeze/thawed
The reaction was stopped by adding 4 M H$_2$SO$_4$ and 0.1 M citrate–phosphate buffer (pH 4.5) containing 0.03% L. infantum 3718 visualized with gate concentration were determined previously). The plates 1:5000 and 1:50,000, respectively (optimal serum and conjugation was collected every month and assayed by ELISA. Briefly, to compare the level of parasite (F/T antigen) and rCPs with the freezed/thawed was determined by trypane blue dye exclusion that was more than 95%. Then, PBMC cultured in flat-bottom 96-well microtiter plates (Costar) at a density 3 × 10$^5$ cells per well in the presence or absence of rCP (5 μg/ml) or rCP (20 μg/ml) or parasite crude lysate (5 μg/ml) or PHA (20 μg/ml) in total volume 200 μl in complete medium (RPMI 1640 medium supplemented with 15% heat-inactivated normal dog serum, 2 mM l-glutamine, 10 μM HEPES, 100 μg/ml gentamicine and 0.1% 2-mercaptoethanol (all from Sigma, USA)). The plates were incubated for 5 days at 37°C in 5% CO$_2$ humidified atmosphere. The concentration of antigens and time of incubation were optimized previously. Cells were pulsed during the last 16 h of culture with 0.5 μCi [³H] thymidine (Amersham, UK) and were harvested on glass fibre filters (Amersham, UK) and were harvested on glassfibre filters (WALLAC, Turku, Finland). [³H] thymidine incorporation was determined by liquid scintillation counting in a β liquid scintillation counter (Pharmacia). Data are expressed as stimulation index representing the ratio of cpm of the stimulated culture to the cpm of control culture. An SI greater than 2.5 was taken as a positive response.

2.7. Quantification of cytokine gene expression using real time PCR

Lymphocytes isolated before challenge and during post-challenge follow-up were kept in “RNA Later” solution (Ambion) at −20°C for RNA isolation. No in vitro stimulation was performed. RNA was isolated by RNeasy mini kit with on-column DNase digestion (QIAGEN), according to the manufacturer’s instructions. mRNA was reverse transcribed using a superscript preamplification system for the first strand cDNA synthesis (invitrogen). Five micrograms of total RNA was used for total reverse transcription in the presence of 1 μg of oligo-dt, 1 μl dNTP (10 mM). The mixture was incubated at 65°C for 5 min. Then, 7 μl of reaction mix (5× first strand buffer, 10 mM DTT, 40 U RNase out) and 200 U superscript II RT was added and incubated at 42°C for 60 min. The reaction was inactivated by heating at 70°C for 15 min. Finally, 2 U RNase H was added and incubated at 37°C for 20 min. The prepared cDNAs were used for RT-PCR using the following primers, according to the published data [19]. IFN-γ:  forward primer 5′-CATTCAAAGAGACATGGATACC-3′, reverse primer 5′-GACTCCCTTTCCGCTCTTAG-3′, annealing temperature 62°C with 30 cycles); IL-10:  forward primer 5′-GAGGCTCC-
GAGCTGCTCTC-3'; R: 5'-GATGAAGATGCTAAACTCT-3' annealing temperature 56 °C, 50 cycles with an extra step at 75 °C). GAPDH: (F: 5'-GTGATGCTGTGTCGTAAGATG-3'; R: 5'-GTGATGGAAGGACTAAGTG-3'); annealing temperature 61 °C, 30 cycles). The number of IL-10, IFN-γ and GAPDH cDNA molecules in each sample was calculated by real time reverse transcription (RT-PCR) using quantitect SYBR green master mix (QIAGEN) in a Micro AMP 96-well plates (Applied Biosystem), according to the manufacturer's instructor. Standard curves were constructed with known amount of IFN-γ, IL-10 and GAPDH cDNA, and the number of IFN-γ and IL-10 per 1000 GAPDH molecules in addition to their ratio in each sample were calculated.

2.8. Leishmanin skin test

Dogs were tested for DTH reaction to leishmanin. Leishmanin reagent was an inactivated suspension of L. infantum LEM 75 which was kindly provided by Dr. Jorge Alvar (Institute de Salud Carlos III, Madrid, Spain). This leishmanin 3×10^8 promastigotes/ml in 0.4% phenol-saline. Control of the test was the diluent. The solution was injected interadermally for 0.1 ml in the left shaved groin and the diluent (0.1 ml) in the right shaved groin. The largest diameter of the induced induration and its perpendicular diameter were measured and averaged at 72 h. The number of IL-10, IFN-γ and GAPDH cDNA molecules in addition to their ratio in each sample were calculated.

2.9. DNA purification and amplification by PCR

DNA was extracted from blood of all dogs each month. The genomic DNA extraction from blood was performed using QiAamp blood kit (QIAGEN). The kinetoplast DNA was the target sequence and the used primers formed using QIAamp blood kit (QIAGEN). The kinetoplast DNA was extracted from blood and tissue kit (QIAGEN) in a Micro AMP 96-well plates (Applied Biosystem), according to the manufacturer’s protocol. Similar primers as in the case of DNA purification were used.

2.10. Statistical analysis

Means were compared by a standard t-test and ANOVA analysis, simple fractional test (SPSS). The χ² and Fisher’s exact tests were used in comparing proportions.

3. Results

3.1. Determination of total IgG, IgG1 and IgG2 subtypes in dog sera

To compare the level of (F/T) parasites and rCPs-specific antibody production in all groups, sera from each dog was collected every month and measured by ELISA. The level of total IgG, IgG1 and IgG2 before challenge is shown in Fig. 1. In the vaccinated group, no significant increase in antibody responses was observed after either the first or second DNA immunization. However, following the third immunization, specific Ab responses to both rCPA and rCPB were readily observed and were significantly higher than in the control groups (p < 0.05), stimulating equally the production of IgG1 and IgG2 (Fig. 1B and C). These data indicated the efficiency of the prime-boost strategy for inducing immune responses to rCPs. In the case of PBS control group, there was no specific Ab production against rCPs at any time interval. In contrast, in the vector control group, there is an increase in the level of IgG2 against rCPB after receiving the third injection (CpG + Montanide, Fig. 1C) although it is significantly lower than the vaccinated group (p < 0.05). This non-specific response may due to administration of CpG ODN as shown in the mouse model [21]. During the 12 months post-challenge, the animals were also monitored for Ab production to F/T (Fig. 2A), rCPA (Fig. 2B) and rCPB (Fig. 2C). Administration of leishmanin at 11 months (in order to evaluate the DTH response) had a mild influence on the humoral immune response to F/T antigen, but there are no significant differences (p > 0.05) in the level of Ab response (total IgG, IgG1 and IgG2) to F/T antigen between groups (V: vaccinated group; C: control groups). Each serum sample was then subsequently analyzed for anti CPs IgG antibody. In this case, significant differences between groups were found. The level of total IgG and IgG2, but not IgG1, to rCPA and rCPB was significantly higher in the vaccinated group (p < 0.05) compared to the control groups (Fig. 2B and C).
3.2. Cell proliferation of PBMC during 12 months follow-up

The comparison of lymphoproliferation responses in two different time intervals, pre and 1 month post-challenge, is indicated as stimulation index for each antigen and is shown in Table 1. In the vaccinated group, the SI for all three antigens was significantly higher post-challenge than pre-challenge ($p < 0.05$). In the vector control group, there was no response to rCPA in pre- and post-challenge, but in regard to rCPB and F/T antigen, there is some stimulation in post-challenge (Table 1). In PBS control group, there is no stimulation for rCPs at these two time intervals (Table 1). Comparing the vaccinated and vector control groups, the SI in the vaccinated group for rCPB and F/T antigen was higher post-challenge, but this difference is not significant ($p > 0.05$). It is interesting to note that the vector control group in comparison to the PBS control group also showed a higher level of IgG2 to rCPB but not to rCPA after receiving CpG/Montanide. The observed discrepancy in the vector control group may be due to the CpG ODN and/or Montanide which has been used.

The stimulation index to F/T (Fig. 3A), rCPA (Fig. 3B) and rCPB (Fig. 3C) during the 12 months follow-up showed heterogeneity likely due to the use of mixed breed dogs in addition to age and sex. The SI for the normal dogs ranged between 1.5 and 2.3 against all three antigens. In the case of the PBS and the vector control groups, there was no significant proliferation against rCPA (Fig. 3B) and rCPB (Fig. 3C) during 12 months after challenge. In the case of the F/T antigen (Fig. 3A), there was some stimulation for vector control

![Fig. 1](3720). Specific antibody production (total IgG, IgG1 and IgG2) in sera of different groups detected by ELISA at different time periods before challenge. Data are presented as the absorbance at 490 nm and are mean ± S.D.

<table>
<thead>
<tr>
<th></th>
<th>Vaccinated group</th>
<th>Control-vector group</th>
<th>Control-PBS group</th>
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<tr>
<td></td>
<td>Pre-challenge</td>
<td>Post-challenge</td>
<td>Pre-challenge</td>
</tr>
<tr>
<td>rCPA</td>
<td>3.04 ± 2.60</td>
<td>13.94 ± 11.3</td>
<td>1.00 ± 0.33</td>
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<tr>
<td>rCPB</td>
<td>2.81 ± 3.2</td>
<td>17.61 ± 21.54</td>
<td>3.30 ± 1.20</td>
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<tr>
<td>F/T</td>
<td>0.97 ± 0.6</td>
<td>21.10 ± 15.6</td>
<td>0.00 ± 0.41</td>
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<tr>
<td>PHA</td>
<td>155.21 ± 120</td>
<td>88.7 ± 53</td>
<td>92.0 ± 45</td>
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Data represented as mean ± S.D.
Cells from the vaccinated group stimulated with F/T antigen had high stimulation indices except for two dogs (ID 969 and 989). These two dogs did not have any significant response during the 12 months follow-up.
up. The remaining vaccinated dogs gave a high to moderate level of lymphocyte response. It is interesting to note that PBMC from dogs with ID 969 and 989 also had a very low response to rCPA and rCPB suggesting a failure to respond to vaccination. In contrast, dogs with ID 970, 979, 983, 984 and 987 are among the high responders to all three antigens. Comparing the SI during the 12 months follow-up of the vaccinated and control groups, there are significant differences (p < 0.05) in months 11 and 12 after challenge to F/T antigens, all months to rCPA and months 5, 7, 9, 11 and 12 to rCPB.

3.3. Measurement of IFN-γ and IL-10 mRNA accumulation in PBMC

We have analyzed the accumulation of IFN-γ and IL-10 mRNA in PBMC from all dogs at pre-challenge and 1, 5 and 12 months after challenge (Fig. 4A and B). Pre-challenge, 6 out of 10 vaccinated dogs had detectable IFN-γ mRNA although the level was very heterogeneous. Only one dog of the control group had detectable IFN-γ mRNA (ID 965). After 5 and 12 months post-challenge, 7 out of 10 vaccinated dogs had moderate to high levels of IFN-γ mRNA accumulation. As it is shown in Fig. 4B, some of the vaccinated dogs also had high levels of IL-10 mRNA (ID 969, 970 and 989). Control groups also had variable levels of IL-10 mRNA. The secretion of IFN-γ and IL-10 is also expressed as a ratio for each dog along the time as demonstrated in Fig. 4C. At 12 months, the mean ratio of IFN-γ:IL-10 for all control dogs was 0.19, compared to 2.0 in vaccinated dogs. Therefore, the difference is significant (p < 0.5) and this ratio is 10 times higher in vaccinated group than the control groups.

3.4. Measurement of delayed type hypersensitivity (DTH) by Leishmanin

Eleven months after infection, a delayed type hypersensitivity assay was performed. The size of the indurations was determined 72 h after administration of leishmanin (Fig. 5). All vaccinated dogs except one (ID 969) had the indurations higher than the cut-off value. The induration size of the control groups were similar and below the cut-off value.

3.5. Diagnosis of Leishmania infection in blood and bone marrow by PCR

During the 12 months follow-up, the blood genomic DNA of all dogs were prepared and evaluated using K13A and K13B as primers. During the first 8 months, there was no amplification in any dogs. In all PCRs, we have used a positive genomic DNA of infected dogs from Meshkin shahr area.
Fig. 5. Induration size in millimeter after administration of leishmanin 11 months after challenge. Skin reactions of each dog were recorded 72 h after administration of the leishmanin. The cut-off values were defined as three standard deviations above the mean absorbance of skin indurations of control groups.

Nine months after infection, some of the dogs became positive (Table 2). As indicated, in the control groups, dogs with ID 962 and 963 were always positive. At 12 months after infection, another dog in the PBS control group (ID 961) also became positive. In the vaccinated group, dog with ID 969 was positive for the last 2 months, as was dog ID 970 which became positive at month 12, however, these differences were not significant ($p > 0.05$).

Bone marrow aspirates were divided into two tubes for culture and PCR. As indicated in Table 3, only three of the dogs have positive culture after 4 weeks, which included dogs with ID 962, 963 and 965. The rest of the cultures which were kept for more than 8 weeks were negative for parasites. These differences were significant ($p < 0.001$). The PCR were also positive for these dogs and in addition, for the dogs with ID 969.

### Table 2

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dog ID</th>
<th>Ach6</th>
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4. Discussion

Immunization with plasmid DNA has been shown to induce protective immunity in a number of experimental models of infection including leishmaniasis [22,23]. Some of the cloned Leishmania antigens have been investigated for protection against *L. infantum* through immunization of mice and hamster with their encoding DNAs, such as LACK [24], papLe22 [25], respectively. In this pilot study, we have shown the protective effect of cocktail CPA and CPB antigens from *L. infantum* using a heterologous prime–boost regime for vaccination against experimental visceral leishmaniasis in dogs. We have monitored the course of the infection by analysis of the immune response against the F/T lysate as well as rCPs. Although we observed response heterogeneity among dogs, the control groups (PBS and vector control groups) had significantly lower proliferation against F/T lysate and almost no proliferation against rCPs in comparison. Among the vaccinated dogs, two were low responders (ID 969 and 989) when treated with F/T, rCPA and rCPB, and interestingly these animals expressed the highest level of IL-10 mRNA at 12 months post-challenge. In some other dogs, like dog ID 970, both IFN-γ and IL-10 mRNA were detected simultaneously 5 months after infection. In control dogs, IL-10 mRNA was similar to vaccinated dogs at 12 months after infection but with low level of IFN-γ. It has been shown in many studies that in human cutaneous and visceral leishmaniasis, IFN-γ and IL-10 can co-exist both at the protein and mRNA [26] levels. Therefore, IL-10 does not seem to have an immunoregulatory role in preventing the expression of IFN-γ. We also did not observe any significant differences in lymphoblastic proliferation between vaccinated and control dogs when treated with PHA. Similar studies by Abranches et al. [27] also indicated that although there was no significant differ-
It has been shown by Lachaud et al. [32] that PCR is more sensitive in comparison to bone marrow aspirates 12 months after infection. It was observed that three out of four control dogs had a positive blood PCR versus 81%, respectively. The dog with ID 969, which belongs to vaccinated group, also had a positive blood PCR after 10 months. The DTH to leishmanin has proven to be a useful tool to detect animals with a strong cellular immune response that protects the animals from infection so that most of them would be resistant [33]. In our pilot study by using this surrogate marker of protection, only one dog (ID 969) from vaccinated group has skin reactivity lower than the cut-off value and which was similar to the control dogs also. In other studies, it has been reported that there is a 98% correlation between positive DTH and cellular immune response as shown by in vitro proliferative assay [34]. The PCR of bone marrow could confirm that one of the dogs in the vaccinated group (ID 969) is not protected and the rest of them in the group during the 12 months follow-up were able to resist infection. The only clinical sign, which was noticeable, was the weight loss and was only observed in control groups as well as dog ID 969.

Although there are not too many studies using the DNA vaccination in dog model especially against visceral leishmaniasis [35], our data support use of the prime-boost vaccination regime with DNA and recombinant vaccinia virus vector expressing LACK also showed the effectiveness of this strategy [38]. They observed a 60% protection against infection in dogs immunized by DNA-LACK prime/rVV-LACK boost while two dose of DNA-LACK did not elicit protection against this disease. The IL-4, IFN-γ and IL-12 cytokine mRNA expression profile in PBMC as well as lymphocyte proliferative response and IgG2/IgG1 ratio specific for LACK confirmed the cellular immune response in this study.

Although studies in the dog are more complicated in comparison to the mouse model and show greater heterogeneity, dogs represent the main reservoir for visceral leishmaniasis in many areas and it is necessary to find a feasible strategy to control this fatal disease. Different control strategies based on detection and destruction or treatment of infected dogs have proved inconsistent [6], therefore, it is an urgent task to confirm by field trials whether vaccination now represent the best strategy to control this disease in endemic countries.

Acknowledgements

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