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Immune response to inactivated bacterial vector carrying the recombinant K39 antigen of *Leishmania infantum* in mice

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ABSTRACT

Objective: To evaluate the immunological response elicited by an inactivated bacterial vector carrying the K39 antigen of *Leishmania infantum*, and a purified antigen.

Methods: Mice were subjected to the following treatments: (1) Purified recombinant K39 (rK39) protein at a 20 µg dose with complete Freund's adjuvant; (2) Inactivated *Escherichia coli* (BL21 DE3) carrying the K39 protein at an equivalent total protein content of 200 µg; (3) Inactivated bacteria lacking the K39 protein; (4) Non-immunized control animals. Serological monitoring was performed. All groups were challenged by intraperitoneal injection of 10⁷ *Leishmania infantum* promastigotes. After euthanasia, the liver and spleen were collected to analyze the levels of TNF, IFN-γ, IL-12, IL-4, and IL-10.

Results: Mice immunized with purified rK39 or the inactivated bacterial vector carrying the K39 antigen of *Leishmania infantum* showed a long-lasting immune response with high levels of polyclonal antibodies specifically recognizing the recombinant proteins. The IgG1 subclass was the predominant immunoglobulin; however, the induction of IgG2a and the profile of cytokines produced were indicative of the induction of a mixed-type response.

Conclusions: The inactivated bacterial vector carrying the K39 antigen, as well as the purified antigen can induce a long-lasting immune response in immunized mice, predominantly favouring a Th2 profile response.

KEYWORDS: Visceral leishmaniasis; K39; Inactivated bacterial vector; Vaccine; Immune response; Th1; Th2; *Leishmania infantum*

1. Introduction

Visceral leishmaniasis (VL) is a chronic and systemic neglected

tropical disease caused by intracellular protozoa of the genus *Leishmania*, such as *Leishmania infantum* (*L. infantum*). It is transmitted by the bite of infected insects[1,2].

L. infantum can cause disease in several species, with the domestic dog (*Canis familiaris*) as the main reservoir of this zoonosis[3,4]. The World Health Organization aims to eliminate VL as a public health problem by 2030. However, this will be a significant challenge due to the complexity of the transmission cycle of the disease[5–7].

The production of recombinant antigens has enabled potential new vaccines against VL. Research using promastigote proteins such as A2, K26, HASPB, gp63, LACK, Ldcys1 and LiESA has shown promising results in preclinical and clinical trials. However, there is still a need for new antigens for the development of highly protective

Significance

Bacterial vectors expressing heterologous proteins allow rapid, cost-effective and scalable production of vaccines with enhanced immunogenicity. This study shows that the use of an inactivated bacterial vector containing recombinant K39 protein contributed to the induction of a long-lasting and durable immune response against this antigen, equivalent to the use of complete Freund's adjuvant, but biased towards a Th2-type response.

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vaccines[8,9].

The K39 antigen is a 37 kDa protein conserved among *Leishmania* species and characterized by tandem repeats of a 39 amino acid sequence. These repeats provide the protein with high-density epitopes and specific identity with viscerotropic species of *Leishmania*, primarily *Leishmania donovani* and *L. infantum*[10]. Bacterial vectors expressing heterologous proteins allow rapid, cost-effective, and scalable production of vaccines with high immunogenicity. Even when attenuated or inactivated, the presence of inherent molecular patterns in these microorganisms promotes the activation of dendritic cells by binding of pattern recognition receptors, eliciting an intense and sustained adaptive immune response to the produced heterologous protein[11,12].

This study investigated the immunogenicity of the recombinant K39 (rK39) antigen derived from *L. infantum*. It compared the immunological response of an inactivated bacterial vector carrying the K39 antigen and its purified counterpart as potential vaccine candidates.

2. Materials and methods

2.1. Production of rK39 in *Escherichia coli* (*E. coli*)

For the production of the rK39 antigen, the coding sequence under accession number DQ831678.1 (Genbank) was synthesized (BioBasic Inc., Ontario, Canada) and cloned into the pET-28a plasmid (Novagen, Darmstadt, Germany). The recombinant plasmid was transformed into *E. coli* BL21 DE3 (Invitrogen) grown in Luria-Bertani (LB) medium with antibiotic (kanamycin, 50 µg/mL). Transformed colonies were confirmed by capillary sequencing (Helixxa Genomic Services).

For rK39 expression, a pre-inoculum culture was diluted (1:25) in LB kanamycin broth and incubated at 37 °C with shaking (250 rpm). After reaching an optical density (OD: 600 nm) between 0.6 and 0.8, the induction process was started using the isopropyl-β-D-1-thiogalactopyranoside inducer (Sigma-Aldrich) at three different concentrations (0.1 mM, 0.5 mM, and 1 mM). After three hours of incubation, the bacteria were centrifuged at 8000 ×g, 4 °C, for 15 min, and the resulting pellet was resuspended in lysis buffer (200 mM NaCl (Vetec); 20 mM Tris-HCl (USB/Dinâmica) pH 8; 200 µg lysozyme; 2 mM phenylmethanesulphonyl fluoride (Sigma-Aldrich) with 1% Triton X-100 (USB/Dinâmica). Samples were sonicated (12 cycles of 15 s) to promote cell lysis, followed by centrifugation (8000 ×g, 4 °C, 15 min) to separate soluble and insoluble protein fractions for further analysis by SDS-PAGE and Western blotting using monoclonal anti-His6 antibody (GE Healthcare Life Sciences) at 1:6000 dilution. A bacterial culture carrying the pET-28a plasmid without the insert was used as a negative control, but induced under the same conditions.

2.2. Purification of rK39 by Nickel Affinity Chromatography

The rK39 protein was purified from the supernatant of bacterial lysis using a Nickel-NTA (Ni-NTA) affinity column (GE Healthcare, USA) pre-equilibrated with a buffer (20 mM Tris pH 8.0, 500 mM NaCl, 10% glycerol, 20 mM imidazole). After washing with the equilibration buffer, the retained recombinant proteins were eluted with a buffer containing 500 mM imidazole. Purification efficiency was assessed by SDS-PAGE and Western blotting and quantified using the Bradford assay and measuring absorbance at 260 nm using a Nanodrop (Thermo Scientific™) spectrophotometer.

2.3. Production of the inactivated bacterial vector containing the rK39 protein

The preparation of the inactivated bacterial vector (BL21-rK39) followed the same expression protocol described above up to the induction step. After culturing, the vector was inactivated with a formalin solution (neutral buffered, 10%, Sigma-Aldrich) for 18 h at 30 °C. After this period, the cultures were centrifuged at 4000 ×g, 4 °C, for 30 min, and the bacterial pellet was resuspended in phosphate-buffered saline (PBS) to prepare serial dilutions (up to a maximum of 10⁸). The suspension was then plated on LB agar plates without antibiotics and incubated at 37 °C for 24 h. The effectiveness of inactivation was determined by counting colony-forming units (CFU)/mL according to a protocol adapted from Da Silva *et al.*[13].

2.4. Immunization assay with purified rK39 and inactivated bacterial vector (BL21-rK39)

Female BALB/c mice, aged between 6 and 8 weeks and weighing 25–30 g, were housed under controlled temperature and humidity conditions with a 12-hour light/dark cycle. Food and water were provided *ad libitum* throughout the experiment. After an acclimatization period, the mice were divided into four groups (*n*=6) and received the following treatments:

Group 1 (rK39): Immunization with purified rK39 protein at 20 µg associated with complete Freund's adjuvant (Sigma-Aldrich) in the first dose and incomplete adjuvant in subsequent boosters;

Group 2 (BL21-rK39): Immunization with inactivated bacteria (BL21 DE3) containing the pET28a bacterial vector with the K39 protein at a total protein equivalent of 200 µg;

Group 3 (BL21-Mock): Immunization with inactivated bacteria (BL21 DE3) containing the pET28a bacterial vector without the K39 protein at a total protein equivalent of 200 µg;

Group 4 (Control): Animals not immunized but infected with *L. infantum*.

Mice were immunized subcutaneously and received booster doses on days 21 and 35 after the start of immunization. The booster dose consisted of an equivalent amount of immunogen for the rK39, BL21-rK39, and BL21-Mock groups. Only the booster for the rK39

group was associated with incomplete Freund's adjuvant (Sigma-Aldrich).

The blood was collected from the mice *via* the retro-orbital plexus on day 0 (pre-immune) and days 7, 14, 21, 28, 35, 42, 77, and 187 after the start of immunization. Blood was centrifuged at 4000 $\times g$ for 10 min post-clot formation. The resulting sera were individually collected into labeled microtubes and stored at -20°C for subsequent analysis.

After 187 days of the first immunization, all groups were intraperitoneally infected with 10^7 promastigotes of *L. infantum* (strain MHOM/BR/74/PP75, ATCC[®] 50133[™]) obtained from *in vitro* culture. After the infection challenge, the mice were bled *via* the retro-orbital plexus on the 7th, 14th, 22nd, and 32nd days (namely days 194, 201, 209 and 219). Following the protocol of Carrion *et al.*[14], on the 22nd day, half of the animals from each group were euthanized by cervical dislocation, and the remaining animals were euthanized on the 32nd day. After euthanasia, liver and spleen samples were collected to analyze cytokine expression involved in the response to the parasite.

2.5. Evaluation of the immune kinetics by enzyme-linked immunosorbent assay (ELISA)

The profiles of total immunoglobulins IgG and its subclasses (IgG1 and IgG2a) were quantified using an ELISA. In brief, 96-well plates (Nunc-Immuno[™] MicroWell[™] 96 well solid plates, Sigma-Aldrich) were sensitized overnight at 4°C with 0.1 μg of purified rK39 protein per well in 0.05 M carbonate-bicarbonate buffer (pH 9.6). After washing with PBS-Tween 20 (0.05%), the wells were blocked with 1% gelatine (Sigma Aldrich, USA) at a volume of 200 μL per well and incubated at 37°C for 1 h. After another wash, the serum from each animal was diluted at 1:1 000 in PBS-Tween 20 and applied at a total volume of 100 μL per well. The plates were incubated for 1 h at 37°C , then washed again to add the secondary antibody (goat anti-IgG, anti-IgG1, or anti-IgG2a conjugated to peroxidase, Invitrogen). The plates were revealed by adding 100 μL of 3,3',5,5'-tetramethylbenzidine (Life Technology) to each well, protected from light. The reaction was stopped after 15 min by adding 50 μL of 3 M sulfuric acid solution to each well, and the absorbance was read at 450 nm using an automated microplate reader (Synergy 2, Biotrak). The assay for all serum samples was performed in duplicate.

2.6. Analysis of cytokine gene expression by RT-qPCR

Immediately after euthanasia, the liver and spleen of the animals from each group were collected and divided into 100 mg fragments for storage in RNA preservation solution (RNA later, Ambion) and subsequent total RNA extraction (Purelink RNA mini kit, Ambion), following the manufacturer's instructions, including an on-column DNase treatment step (PureLink[™] DNase Set, Invitrogen). At the end of the process, the yields and qualities of the extracted total RNA were analyzed using spectrophotometry (Synergy 2, Biotek). Two mg of total RNA from each sample were used for cDNA synthesis (High-Capacity RNA-to-cDNA[™], Applied Biosystems).

The analysis of the mRNA expression of the cytokines including TNF, IFN- γ , IL-12, IL-4, and IL-10 was performed by relative quantification using qPCR with an intercalating dye, and the genes *Ubc* and *Hprt* were used for result normalization. The primers are described in Table 1.

Each qPCR reaction was performed in triplicate, as described below: 10 μL of GoTaq[®] qPCR Master Mix (Promega), 10 pmol of each primer, and an amount of cDNA equivalent to 20 ng of the total extracted RNA, totalling 20 μL per reaction. The qPCR was carried out using a real-time thermocycler (Esco Swift, Esco) with the following program: 10 min at 94°C (1 cycle); 20 s at 94°C , 20 s at 60°C , and 20 s at 72°C , with data acquisition at the end of this last step (40 cycles); and 2 min at 72°C (1 cycle). After the PCR step, the amplified products were analyzed using a melting curve. A triplicate of no-template controls and no-reverse transcriptase controls were used for each primer. From the average Cq values of each group, the relative expression of each gene was calculated using the comparative Cq method ($\Delta\Delta\text{Cq}$), with the geometric mean of the housekeeping genes as a reference for normalizing the expressions.

2.7. Statistical analysis

The homogeneity of variance was verified using Bartlett's test, and normality was checked using the Kolmogorov-Smirnov test. When the assumptions were not met, data transformation was applied. The statistical software SAS (Statistical Analysis System, version 6.12) was used, and the PROC MIXED procedure was employed. In this analysis, the fixed effects included the days (0, 7, 14, 21, 28, 35, 42, 77, and 187 for immunization; 0, 7, 14, 22, and 32 for post-infection), groups (rK39, BL21-rK39, BL21-Mock), and immunoglobulins (IgG, IgG1, and IgG2a). The random effect was

Table 1. Primers for cytokines and reference genes.

Gene	Forward (5'-3')	Reverse (5'-3')	RT-PrimerDB ID
<i>TNF</i>	AGGGATGAGAAGTTCCCAAATG	CACTTGGTGGTTTGCTACGAC	8751
<i>IFN-γ</i>	TCAAGTGGCATAGATGTGGAAGAA	TGGCTCTGCAGGATTTTCATG	146
<i>IL-12</i>	GGAAGCACGGCAGCAGAATA	AACTTGAGGGAGAAAGTAGGAATGG	141
<i>IL-4</i>	ACAGGAGAAGGGACGCCAT	GAAGCCCTACAGACGAGCTCA	136
<i>IL-10</i>	CTATGCTGCCTGCTCTTACTG	AACCCAAGTAACCCCTTAAAGTC	8298
<i>Ubc</i>	AGGTCAAACAGGAAGACAGACGTA	TCACACCCAAGAACAGCACA	42
<i>Hprt</i>	AGCTACTGTAATGATCAGTCAACG	AGAGGTCCTTTTCACCAGCA	3583

the repetitions of BALB/c mouse sera at a dilution of 1:1 000. The restricted maximum likelihood method was used to estimate the variance components. Means were compared using LSMEANS with the PDIF option adjusted by the Tukey-Kramer test, considering a significance level of 5%.

Regarding cytokine data, the SAS GLM procedure was applied with the effect of the groups rK39, BL21-rK39, BL21-Mock, and control. One-way ANOVA was performed for cytokine analysis, followed by the Bonferroni *post-hoc* test ($P < 0.05$).

2.8. Ethical statement

The project was approved under protocol 3630450/2015 and complied with the ethical principles of animal experimentation adopted by the Ethics Committee for Animal Use of the State University of Ceará (CEUA-UECE).

3. Results

3.1. Cloning, expression, and purification of the rK39 protein in a prokaryotic system

The subcloning of the *K39* gene from *L. infantum* into the pET28a expression vector was confirmed by treatment with specific restriction enzymes (Figure 1A). Different concentrations of the inducer agent isopropyl- β -D-1-thiogalactopyranoside did not prove to be a significant variable during the optimization process of protein production, as the protein remained predominantly in the soluble fraction of the bacterial lysate extract. SDS-PAGE (Figure 1B) and Western blotting analyses (Figure 1C), using an anti-His6 monoclonal antibody, showed a single predominant band with a molecular mass consistent with the target protein (37 kDa).

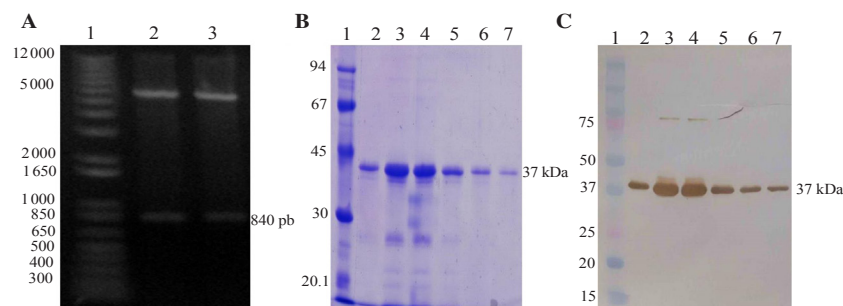


Figure 1. Molecular cloning and recovery of the recombinant K39 (rK39) protein from *Leishmania infantum*. (A) Confirmation of the *K39* gene in the pET28a vector transformed into *Escherichia coli*. Lane 1: 1 kb Plus DNA Ladder (Invitrogen); Lanes 2 and 3: recombinant plasmids treated with restriction enzymes. (B) 15% SDS-PAGE and (C) Western blotting with monoclonal anti-His6 antibody, stained with 3,3'-diaminobenzidine. Lane 1: Protein ladder; Lanes 2 to 7: fractions eluted during rK39 purification.

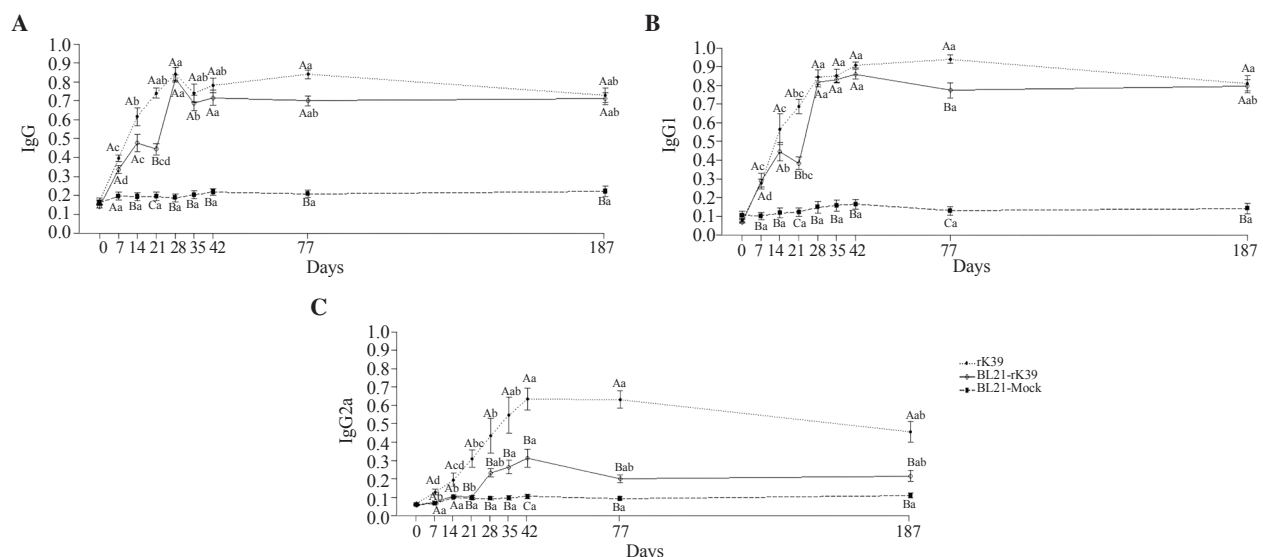


Figure 2. Specific humoral response of mice vaccinated with rK39 combined with an adjuvant or in an inactivated bacterial vector. Titers of (A) IgG, (B) IgG1, and (C) IgG2a were determined by ELISA. Absorbance was read at 450 nm. Each sample was individually analyzed in duplicate. Capital letters indicate a significant difference between groups within a day and small letters indicate a significant difference between days within the same group, as determined by the Tukey-Kramer test at a 5% significance level.

3.2. Humoral response in mice after immunization with rK39 and infection with *L. infantum*

The results showed that immunization of mice generated a specific humoral immune response, demonstrating the immunogenicity of the protein in the two formulations. The rK39 and BL21-rK39 groups induced a long-lasting immune response with high levels of polyclonal antibodies that specifically recognized the recombinant proteins. The rK39 associated with Freund's adjuvant showed higher IgG (Figure 2A), IgG1 (Figure 2B), and IgG2a (Figure 2C) at 14 and 21 days after immunization ($P < 0.05$) when compared to the response induced in the BL21-rK39 and BL21-Mock groups. The levels of IgG and IgG1 in the rK39 and BL21-rK39 groups remained elevated throughout the 187 days of serological monitoring.

After the challenge with *L. infantum*, the BL21-Mock group showed

low levels of all classes of IgGs throughout the 187 days of post-immunization monitoring and remained stable until the animals were euthanized (Figure 3). The BL21-Mock and control groups showed no significant difference in all classes of IgGs (Figure 3). This result indicates that *E. coli* proteins were not able to elicit a potent immunological response when compared to the groups containing the K39 protein ($P < 0.01$). The IgG (Figure 3A) and IgG1 (Figure 3B) titers were similar between the BL21-rK39 and the rK39 groups, but only the rK39 group showed increased IgG2a level (Figure 3C).

3.3. Cytokine level after immunization with rK39 and infection with *L. infantum*

The analysis of cytokine gene expression in the liver tissue revealed slight variation between the groups except IL-4 ($P > 0.05$). There was

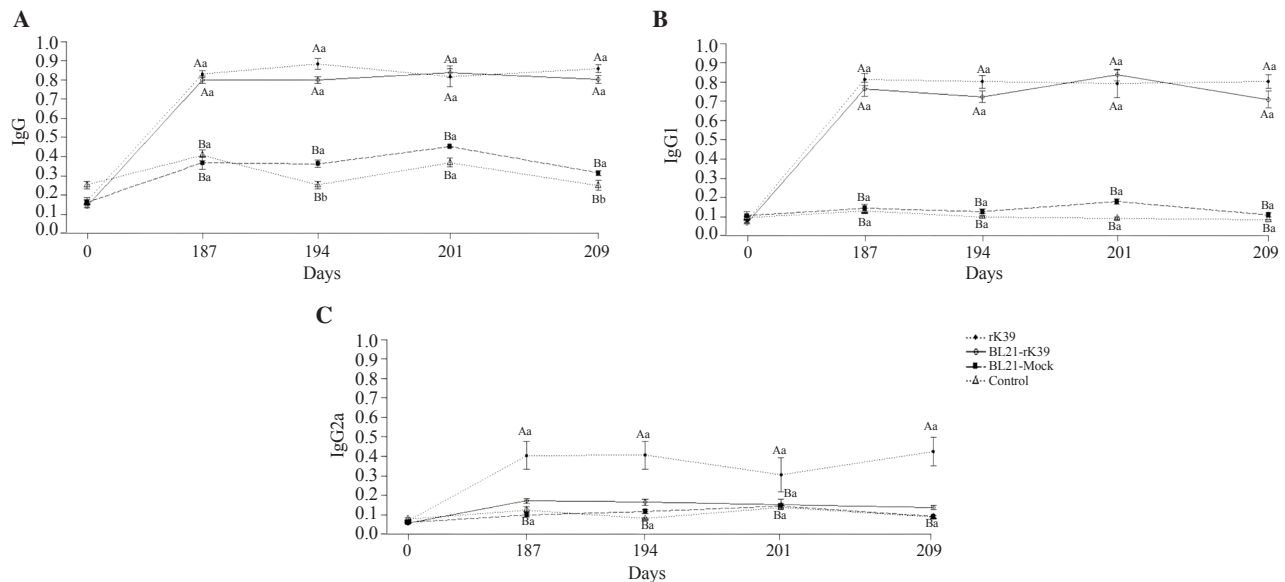


Figure 3. Specific humoral response of mice challenged by intraperitoneal injection with 10^7 promastigotes of *Leishmania infantum*. Titers of (A) IgG, (B) IgG1, and (C) IgG2a after challenge were determined by ELISA. Absorbance was read at 450 nm. Each sample was individually analyzed in duplicate. Capital letters indicate a significant difference between groups within a day and small letters indicate a significant difference between days within the same group, as determined by the Tukey-Kramer test at a 5% significance level.

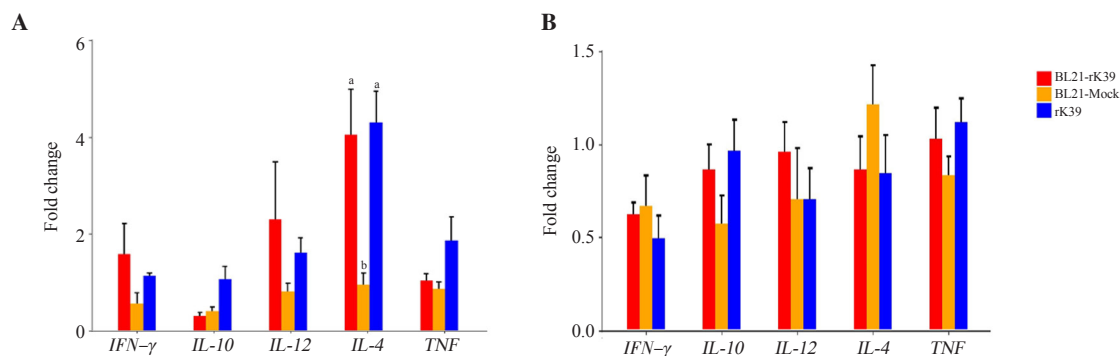


Figure 4. Cytokine gene expression levels in the liver (A) and spleen (B) of immunized mice challenged with 10^7 *Leishmania infantum* promastigotes. Levels of mRNA expression are normalized ratios between target gene expression and the geometric mean of *Ubc* and *Hprt* genes. One-way ANOVA followed by Bonferroni *post-hoc* test was used to analyze the data.

a significant increase in the expression of *IL-4* in mice immunized with the vaccine antigens containing the rK39 protein ($P < 0.01$) (Figure 4A). In contrast, although no statistically significant differences were observed for the cytokines in the spleen (Figure 4B), slightly upregulated expression of TNF was noticed in the purified rK39 and BL21-rK39 groups.

4. Discussion

In this study, we evaluated the ability of the rK39 to elicit a specific humoral response in mice against *L. infantum* infection. The results show that compared to the BL21-Mock group, both purified immunogen formulations and inactivated bacterial vectors induced high levels of IgG, with a predominance of the IgG1 subclass. The purified recombinant antigen rK39 induced an early and significant production of IgG2a until day 42. After that, the levels gradually decreased. The group immunized with the inactivated bacterial vector (BL21-rK39) induced specific IgG and IgG1 production at levels equivalent to the group with purified protein, while IgG2a production occurred later, after booster administrations.

The challenges faced in the development of prophylactic vaccines against *L. infantum* open the way for antigens present in the amastigote forms, such as the K39 protein, to be explored as potential therapeutic vaccines. Unlike promastigote forms, amastigotes reside within host cells and become targets of CD8⁺ T cells, which play a crucial role in stimulating components of the immune response against *Leishmania* spp.[1].

Pathogen-associated molecular patterns inherent to the use of inactivated bacterial vectors allow for the activation of innate immune responses, followed by the development of acquired immune responses[15]. Using an equivalent formalin inactivation protocol, Gohar *et al.*[16] reported the development of an experimental vaccine against diarrheagenic strains of *E. coli* administered *via* the subcutaneous route, demonstrating the production of a specific and protective immune response induced against the inactivated pathogen's proteins. Similarly, recombinant *E. coli* carrying the rK39 antigen showed the capacity to generate high titres of specific antibodies.

The immunogenicity of both formulations indicates a switch of isotypes due to the modulation of B lymphocytes, resulting from the activation of primarily Th1 and Th2 types of CD4⁺ T lymphocytes. The LeishTec[™] vaccine, licensed in Brazil and based on the A2 amastigote antigen, also confers immunity with a mixed Th1/Th2 profile[17,18]. Ghosh *et al.* demonstrated that a vaccine based on recombinant A2 antigen induced a specific protective humoral response. The authors associated this response with the activation of the classical pathway of the complement system, which protected macrophages from infection by the amastigote *Leishmania donovani* *in vitro*[17]. The robust IgG1 response elicited by the formulations in this study may not have protective properties, as the IgG2 subclass is mainly responsible for complement fixation[19]. However, the debate

on the role of IgG1 and IgG2 antibodies in the protection against VL is still ongoing[18,20].

This response profile is also supported by the findings of Bhowmick and Ali[21], who immunized BALB/c mice with different liposomal formulations of *Leishmania* antigens (LAg), resulting in elevated levels of both immunoglobulins (IgG1 and IgG2a). Their results highlight that although cell-mediated immune response plays a crucial role in controlling leishmaniasis, the ability to induce a Th1-type response should not be the sole criterion for evaluating potential vaccines.

Despite the mechanisms of the immune response to VL not being fully elucidated, evidence indicates that a Th1-type response profile is beneficial for effective disease control. This type of response begins with the activation and maturation of dendritic cells, which induce a more significant proliferation of T cells. Moreover, it promotes increased levels of IL-12, IFN- γ , and TNF- α through the activation of M1-type macrophages, which respond with an enhanced killer activity by increasing the production of reactive oxygen species and nitric oxide[22,23].

The immunization with the rK39-containing vaccine antigens induced a specific increase in the expression of IL-4 in the liver tissue. Significantly elevated levels of IL-4 were observed only in the rK39 and BL21-K39 groups, demonstrating the specificity of this response to the presence of the recombinant protein in the formulation. Although classically associated with susceptibility to infection by *L. infantum* and *Leishmania major*, IL-4 has recently been shown to play an essential role in developing a protective immune response against these microorganisms. IL-4, like the structurally related cytokine IL-13, can activate IL-4R α -dependent and -independent signaling mechanisms in macrophages and neutrophils, thereby promoting granuloma maturation and enhancing the host's response against the parasite[24,25].

Further investigations may be required to understand the cytokine profile and its significance in the immune response to *L. infantum* infection, however, it is important to note that the production of IgG2a after booster administration suggests a mixed Th1/Th2 response, which could be advantageous for combating *L. infantum* infection effectively. According to Gradoni[26], despite the protective response against VL being mediated by cellular immunity, developing a Th1/Th2 response is necessary. Moreover, Khadem and Uzonna[27] state that there is a fine line between immune responses that effectively control parasite growth and induce long-term immunity. They also highlight reports suggesting that IL-4 is necessary to resolve hepatic infection, influencing the activation of CD8⁺ T cells capable of conferring long-term protection.

Basu *et al.*[28], when immunizing hamsters with kinetoplastid membrane protein-11 DNA, observed that the formulation elicited a mixed response profile similar to that observed in this study. The study linked the presence of IL-4 with protection against VL. Additionally, the authors suggested that IL-4 may play a role in an alternative pathway for inducing inducible nitric oxide synthase (iNOS), thereby complementing the signaling by IFN- γ in the

leishmania killing activity exerted by macrophages. It is important to recognize the specificity of the immune response mechanisms of each host. Therefore, these findings may represent a unique aspect of the immune response in hamsters, which may be absent in other species.

Singh and Sundar[29] conducted a combined neutralization assay of IFN- γ and TNF- α in splenic biopsy cultures with viable *Leishmania* parasites. They found that IL-4 production can lead to protective immunity by increasing the expression of MHC class II in macrophages, consequently enhancing parasite elimination.

Even though significant changes in cytokine levels were not detected in the spleen, distinct patterns of cytokine expression were observed in the liver tissue among the groups. In mice, VL is characterized by organ-specific immune responses, with different response profiles between the liver and spleen tissues. In the liver tissue, cytokines such as IL-12, IFN- γ , and TNF result in the activation and fusion of Kupffer cells to form granulomas. In addition to these cytokines, IL-4 can directly impact the formation and function of granulomas by respectively upregulating the expression of IL-12 and iNOS[30]. Once formed, these granulomas promote the development of a pro-inflammatory microenvironment that activates non-infected Kupffer cells and recruits other classes of leukocytes, leading to a significant reduction in hepatic parasite load within a period of up to 8 weeks[31].

The presented results contribute to a better understanding of the immune response profile triggered by the K39 antigen of *L. infantum*, whether purified or inactivated bacterial vector. Nevertheless, the main limitations of this study must be mentioned:

(1) The mice used in this study were of the BALB/c strain, which has an immune response profile that favors Th2-type responses[32]. Nevertheless, a significant production of IgG2a antibodies was observed in both groups, indicating some activation of the Th1-type response. However, further studies in other animal models may be required to better characterize the response induced.

(2) Detection of cytokines produced *in vivo* may be hampered by assessing cytokine production 21 days post-challenge. However, significant levels of cytokine transcripts were detected in hepatic tissue. The response profile elicited by stimulation of splenocyte cultures from animals immunized with *L. infantum* K39 antigen could be evaluated in future studies.

(3) The use of complete Freund's adjuvant in one of the formulations biases the type of immune response elicited, favoring the Th1 profile[33]. Therefore, the response elicited against the antigen of interest in combination with other adjuvants should be evaluated in future studies.

In conclusion, this study showed that an inactivated bacterial vector carrying the K39 antigen, as well as the purified antigen, could induce a long-lasting immune response in immunized animals, predominantly favoring a Th2 profile response. Based on these results, future studies using other animal models and different immunization schedules will allow us to better understand the nature of the immune response induced by this antigen.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

Authors' contributions

LSA designed the work, conducted the experimental studies, performed data acquisition and analysis, and prepared, edited, and reviewed the manuscript. BBS conducted the experimental studies, performed data acquisition and analysis, and prepared, edited, and reviewed the manuscript. ENFNS, ASB, and SSF conducted the experimental studies and reviewed the manuscript. ARM performed statistical analysis and reviewed the manuscript. EOPTF reviewed the manuscript. Both MFvT and MIFG designed the work, performed data analysis, and prepared, edited, and reviewed the manuscript.

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