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Potential application of small myristoylated protein-3 evaluated as recombinant antigen and a synthetic peptide containing its linear B-cell epitope for the serodiagnosis of canine visceral and human tegumentary leishmaniasis

Beatriz C.S. Salles[a,1], Daniel S. Dias[a,1], Bethina T. Steiner[b,1], Daniela P. Lage[a], Fernanda F. Ramos[a], Patrícia A.F. Ribeiro[a], Thaís T.O. Santos[a], Mariana P. Lima[a], Lourena E. Costa[a], Ana T. Chaves[a], Miguel A. Chávez-Fumagalli[a], Ricardo T. Fujiwaraa[c], Lílian L. Buenoa[c], Rachel B. Caligiorne[d], Danielle F. de Magalhães-Soares[e], Julia A.G. Silveira[c], Ricardo A. Machado-de-Ávila[b], Denise U. Gonçalves[a], Eduardo A.F. Coelho[a,*,1]

[a] Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical, Faculdade de Medicina, Universidade Federal de Minas Gerais, Belo Horizonte, 30130-100, Minas Gerais, Brazil
[b] Programa de Pós-Graduação em Ciências da Saúde, Universidade do Extremo Sul Catarinense, Criciúma, 88806-000, Santa Catarina, Brazil
[c] Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, 31270-901, Minas Gerais, Brazil
[d] Instituto de Ensino e Pesquisa, Santa Casa de Belo Horizonte, Rua Domingos Vieira, 590, Santa Efigênia, 30150-240, Belo Horizonte, Minas Gerais, Brazil
[e] Departamento de Medicina Veterinária Preventiva, Escola de Veterinária, Universidade Federal de Minas Gerais, Belo Horizonte, 31270-901, Minas Gerais, Brazil

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ABSTRACT

Serological tests are important tools for the diagnosis of *Leishmania* infection. However, they are not effective markers to diagnose asymptomatic cases of visceral leishmaniasis (VL) and patients developing tegumentary leishmaniasis (TL), since antileishmanial antibodies can be encountered in low levels resulting in false-negative results in the serological trials. In this context, antigens able to be recognized by antibodies in sera from both VL and TL patients will be desirable to be employed in a more sensitivity and specific diagnosis of disease. In the present study, a conserved *Leishmania* protein, small myristoylated protein-3 (SMP-3), which was showed to be conserved in different *Leishmania* species and an effective vaccine candidate against *Leishmania infantum* infection in a murine model, was cloned and the recombinant protein was evaluated as a serological marker for the diagnosis of human TL and canine VL. In addition, a linear B cell-specific epitope (MQKDEESGEFKCEL) was identified, synthetized and also investigated as a serological marker. As antigen controls, rA2 protein and antigenic *Leishmania* extracts (SLA) were used. Results showed that ELISA-rSMP-3 and ELISA-Peptide presented sensitivity and specificity values higher than 90% in both diseases in humans and canids, having identified all asymptomatic cases and did not present cross-reaction with cross-reactivity diseases in both mammalian hosts. On the other hand, sensitivity and specificity values were worst when rA2 or SLA were used as antigens in humans and dogs. In conclusion, results showed the efficacy and *Leishmania* SMP-3 protein, employed as a recombinant antigen or a B cell epitope, for the improvement of the serodiagnosis of human TL and canine VL. This candidate can be tested in other diagnostic platforms, such as rapid immunochromatographic dipstick tests, aiming its use in epidemiological studies in remote areas where laboratories are not readily accessible for conventional assays.

* Corresponding author at: Laboratório de Pesquisa do Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical, Faculdade de Medicina, Universidade Federal de Minas Gerais, Avenida Prof. Alfredo Balena, 190, 30130-100, Belo Horizonte, Minas Gerais, Brazil.
E-mail address: eduardoferrazcoelho@yahoo.com (E.A.F. Coelho).
[1] These authors contributed equally to this work.

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1. Introduction

Leishmaniases are diseases caused by protozoan parasites of the genus *Leishmania*, which are endemic in 98 countries in the world, with 350 million people living in risk areas of infection, and an annual incidence estimated at approximately 1.0–1.5 million new cases (Alvar et al., 2012; World Health Organization, 2016). The genus comprises parasite species that are transmitted to mammalian hosts through the bite of infected sand fly vectors. The protozoan presents two distinct morphological forms in its life cycle, promastigote and amastigote, which is a flagellated form and multiplies in the midgut of the sand fly or an intracellular form residing within phagocytes of the vertebrate hosts, respectively (Grimaldi and Tesh, 1993). The amastigotes can cause a broad spectrum of disease in mammalian hosts, such as cutaneous (CL), mucocutaneous (MCL), and visceral leishmaniasis (VL), depending of immune and nutritional status of the infected host, parasite virulence and species, among other factors (Vijayakumar and Das, 2018).

Visceral leishmaniasis (VL) is the most severe clinic form of disease and presents a fatality rate of almost 100%, if acute and untreated. On the other hand, CL and MCL cause nodule that undergoes progressive ulceration and may vary in severity and clinical appearance. These manifestations have a tendency to self-cure (CL) or are characterized by multiple non-ulcerative nodules that may spread to patient's body (MCL) (Copeland and Aronson, 2015; Torres-Guerrero et al., 2017). In the Americas, VL is mainly caused by infection with *Leishmania infantum* species, while CL and MCL are mainly caused by *L. braziliensis* species (Belo et al., 2013; Campos et al., 2018).

Epidemiological studies have shown that dogs are an important domestic source of infection by L. *infantum* parasites. Upon infection, these animals can develop the asymptomatic or symptomatic form of disease; although between 20–60% of the infected animals develop asymptomatic infection exhibiting higher prevalence than the human population. As a consequence, the detection of these cases is crucial in controlling epidemics and avoiding the spread of the disease in humans, since asymptomatic dogs are also reservoirs of the parasites, allowing their interaction between sand flies and humans (Foglia-Manzillo et al., 2013; Lara-Silva Fde et al., 2015).

The diagnosis of leishmaniasis comprises the association between laboratory results with clinical and epidemiological data. The classic diagnostic methods are limited by variable sensitivity of the microscopic examination, requiring repeated biopsy or aspirate sampling and a trained laboratory staff (Goto and Lindoso, 2010; Al-Jawabreh et al., 2018). In addition, tests require invasive procedures of samples collection, which limit their use (Boggild et al., 2011). Therefore, there is a need to develop more rapid, effective and simple assays to diagnose disease (Paiva-Cavalcanti et al., 2015). Laboratory methods can detect the whole parasite or their components in biological samples collected from the infected hosts. Among these tests, serology has been the most widely method used in epidemiological studies, providing additional support for control measures of disease (Fonseca et al., 2014; Oliveira et al., 2015).

Techniques such as the enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence assay (IFAT) employing parasite crude antigens for the detection of antibodies in serum samples from humans and dogs have been used, each one exhibiting variable sensitivity and specificity values (Wolf et al., 2014; Mniouil et al., 2018). However, problems such as the standardization of the production of the antigenic extracts, the delay in the processing of samples due to its laborious conditions and inadequate accuracy leading to the underestimation of infection rates, have limited the use of these antigens (Salles et al., 2017; Seyyedtabaei et al., 2017). Otherwise, recombinant molecules have been evaluated for the serodiagnosis of human and canine leishmaniasis by means of conventional methodologies. The diagnostic accuracy of these molecules has proven to be superior to serological tests using crude antigens (Faria et al., 2015; Chauhan et al., 2016).

Although *Leishmania* proteins have been evaluated, their sensitivity and/or specificity have also variable due to presence of asymptomatic infection and cross-reactive diseases, respectively (Das et al., 2014; Vallur et al., 2016). Rapid tests using parasite proteins, such as rK26, rK28, and rK39, when employed in immunochromatographic dipstick tests, appear to be suitable for the point-of-care diagnoses from symptomatic cases of disease, but the lack of sensitivity in the asymptomatic canine and human disease remains a primary problem. As a consequence, contradictory results have been reported, which will be influenced by the type of *Leishmania* antigen used, the manufacturer's production process, geographical location and clinical form of disease.

Serological tests have been well-employed for the serodiagnosis of VL; however, they present lower efficacy for the diagnosis of MCL and, mainly, to the CL cases, since the most of patients present low titers of antileishmanial antibodies and, consequently, they are classified as false-negative in the trials (Elmahallawy et al., 2014; Duarte et al., 2015; Pedral-Sampaio et al., 2016 Sep). In this context, antigens able to be recognized by antibodies in sera from both CL and MCL patients will be desirable to be evaluated for a more sensitivity and specific diagnosis of disease.

In the present study, a *Leishmania* protein namely small myristoylated protein-3 (SMP-3) (XP_003873457.1), which was recently identified by means of a proteomic approach performed in L. *amazonensis* species (Magalhães et al., 2014), and successfully evaluated as a vaccine candidate against L. *infantum* infection in a murine model (Oliveira et al., 2018), was studied regarding its diagnostic efficacy for human TL and canine VL. The protein and a relevant linear B cell-specific epitope were characterized, and the recombinant molecule and the synthetic epitope (MQKDEESGEFKCEL) were serologically evaluated by means of ELISA experiments. Results showed that both antigens presented high diagnostic performance for all clinical forms of disease, and could well be evaluated in new studies using other diagnostic platforms to detect leishmaniasis in different mammalian hosts.

2. Materials and methods

2.1. Sera samples

2.1.1. Canine patients

The present study was approved by Committee on the Ethical Handling of Research Animals from Federal University of Minas Gerais (UFMG; Belo Horizonte, Minas Gerais, Brazil), with the protocol number 043/2011. The sample size was composed of 200 domestic animals (*Canis familiaris*), and consisted of males (n = 125) and females (n = 75) of different breeds and ages. VL dogs presented positive parasitological results for L. *infantum* kDNA by PCR technique, as well as positive serological results, when evaluated by two commercial tests: IFAT (IFAT-CVL BioManguinhos® kit) and ELISA (EIE-LVC BioManguinhos® kit). Symptomatic VL animals (n = 30) presented three or more of the following clinical symptoms: weight loss, alopecia, adenopathy, onychogryposis, hepatomegaly, conjunctivitis, exfoliative dermatitis on the nose, tail, and ear tips; splenomegaly, lymphadenomegaly, and renal azotemia. Asymptomatic VL dogs (n = 25) did not present clinical signal of disease. Non-infected dogs were selected from endemic (n = 30, Belo Horizonte) or non-endemic (n = 30; Poços de Caldas, Minas Gerais, Brazil) areas of leishmaniasis. They presented negative serological results, and were free of any clinical signal of disease. As cross-reactive sera, samples from non-infected animals and immunized with Leish-Tec® vaccine (n = 30), or those that were experimentally infected with *Ehrlichia canis* (n = 20), *Babesia canis* (n = 15) or *Trypanosoma cruzi* (n = 20), all of them maintained in kennels to prevent their contact with transmitting vectors of leishmaniasis, were used.

2.1.2. Human patients

The study was also approved by the Ethics Committee of UFMG (protocol number CAAE–32,343,114.9.0000.5149). Blood samples (10 mL) were collected by venipuncture in tubes without anticoagulant, and were kept at 37 °C by 15 min, when they were centrifuged at 3000 × g for 15 min, and samples were separated and kept at −80 °C until use. Serum samples were obtained from cutaneous (CL; n = 15, including 9 males and 6 females, with ages ranging from 26 to 53 years), mucocutaneous (MCL; n = 25, including 10 males and 15 females, with ages ranging from 19 to 59 years) and visceral leishmaniasis (VL; n = 10, including 6 males and 4 females, with ages ranging from 25 to 62 years) patients. Diagnosis was confirmed when, besides clinical features, parasites were found in the Giemsa-stained smears of biopsies of skin (CL) or mucosa (MCL) fragments or bone marrow aspirates (VL). They also presented positive results for L. *braziliensis* (CL and MCL) or L. *infantum* (VL) kDNA. None of the patients had been treated with antileishmanial drugs before blood collect. Sera from non-infected subjects living in an endemic area of disease (n = 35, Belo Horizonte) were also used. They did not present clinical signal of leishmaniasis, and showed negative serological by using commercial tests (Kalazar Detect™ Test, InBios International, Inc., Seattle, Wash, USA). Samples of *T. cruzi*-infected patients (n = 30) were also used. Infection was confirmed by hemoculture, Chagatest® recombinant ELISA v.4.0 kit, and Chagatest® hemmaglutination inhibition (Wiener lab., Rosario, Argentina). Also tested as cross-reactive sera, samples collected from paracoccidioidomycosis (n = 10, 5 males and 5 females with ages ranging from 26 to 48 years), leprosy (n = 15, with 8 males and 7 females, with ages ranging from 20 to 49 years), and aspergillosis (n = 10, including 6 males and 4 females, with ages ranging from 17 to 47 years) patients were used. The diagnosis was confirmed by means of positive Paracoccidioides cultures, ML Flow rapid test, and by detection of fungal hyphae in histopathological examination, respectively.

2.2. Bioinformatics assay

The amino acid sequence of SMP-3 (XP_003873457.1) protein was evaluated by BLAST-p algorithm to comparison of similarity between Trypanosomatides protein sequences (Gish and States, 1993). The similar sequences were aligned by the Clustal Omega program (Sievers et al., 2011). The predicted epitopes were investigated by ABCpred program (Saha and Raghava, 2006) by using the following parameters: threshold of 0.84, window length of 14, and overlapping filter: ON. The molecular modeling was developed by the SwissModel program (Biasini et al., 2014), being models visualized at SwissPDBviewer program (Guex and Peitsch, 1997), and physic-chemical characteristics of the epitopes evaluated by PepCalc server (available in: https://pepcalc.com).

2.3. Parasites

L. braziliensis (MHOM/BR/1975/M2903) and *L. infantum* (MOM/BR/1970/BH46) strains were used. The stationary promastigotes were grown at 24 °C in Schneider's medium (Sigma-Aldrich, St. Louis, MO, USA), which was supplemented with 20% inactivated fetal bovine serum (FBS, Sigma), 20 mM L-glutamine, 200 U/mL penicillin, and 100 µg/mL streptomycin, at pH 7.4. The soluble *Leishmania* antigenic extracts (SLA) were prepared according described (Coelho et al., 2003). Briefly, 2×10^8 parasites were washed three times in cold sterile phosphate-buffered saline (PBS 1x). After six cycles of freezing (−196 °C) and thawing (+ 37 °C), followed by ultrasonication (Ultrasonic processor, GEX600) with six cycles of 30 s at 38 MHz, the suspension was centrifuged at 8000 × *g* for 30 min at 4 °C, and the supernatant containing SLA was collected. The protein concentration was estimated by the Bradford method (Bradford, 1976), and aliquots were stored at −80 °C until use.

2.4. Cloning and purification of recombinant SMP-3 (rSMP-3) protein

The primers used to amplify SMP-3 gene from genomic DNA were 5´-GGATCCATGCCGCGTCCGTTCGGTGTTTG-3´ (*forward*) and 5´-AAG CTTAAGCGGGGATGTAGAACTTG-3´ (*reverse*), and the *Bam*HI and *Hin*dIII restriction enzymes were used. The DNA fragment was excised from gel, purified and linked into pGEM®-T vector system (Promega, USA). The recombinant plasmid was used to transform *Escherichia coli* XL1-Blue (Phoneutria, Brazil) competent cells, and positive clones were tested by the restriction enzymes, propagated and used for construction of the expression vector. DNA fragments obtained from digestion of pGEM-SMP-3 plasmid were ligated into pET28a-TEV, and the *E. coli* BL21 cells (DE3; Agilent Technologies, USA) were transformed with the recombinant plasmid. Gene´ insertion was confirmed by PCR, and the sequencing was performed in a MegaBace 1000 automatic sequencer apparatus (Amersham Biosciences, USA). To purification of rSMP-3, bacteria were induced with IPTG (0.5 mM) for 3 h at 37 °C, and shaking at 200 × *g* per min. Cells were ruptured by seven cycles of ultrasonication, with cycles of 30 s each (38 MHz), followed by seven cycles of freezing and thawing. After, cellular debris were removed by centrifugation, and the recombinant protein was purified onto HisTrap HP affinity column connected to an AKTA system (GE Healthcare, USA), and further purified on a Superdex™ 200 gel-filtration column (GE Healthcare Life Sciences, USA). The A2 recombinant (rA2) protein was produced according described (Zhang et al., 1996). After purification, both recombinant proteins were passed through a polymyxin-agarose column (Sigma) to remove any residual endotoxins content. The purity of proteins was checked by sodium dodecyl sulfate-12% polyacrylamide gel (data not shown). A linear B cell epitope predicted in the SMP-3 sequence (MQKDEESGEFKCEL) was commercially synthesized (Genscript®, USA).

2.5. ELISA assays

2.5.1. Serodiagnosis of canine leishmaniasis

Previous titration curves were performed to determine the most appropriate antigen concentration and antibody dilution to be used. Falcon flexible microtiter immunoassay plates (Becton Dickinson) were coated with rSMP-3, Peptide, rA2 or L. *infantum* SLA (0.5, 2.0, 1.0, and 1.0 µg per well, respectively), diluted in 100 µL coating buffer (50 mM carbonate buffer), pH 9.6, for 18 h at 4 °C. Next, free binding sites were blocked using 200 µL of PBS-T (PBS 1x plus Tween 20 0.05%), containing 5% casein, for 1 h at 37 °C. After washing the plates seven times with PBS-T, they were incubated with 100 µL of canine sera (1:200 diluted in PBS-T), for 1 h at 37 °C. Plates were subsequently washed seven times in PBS-T, and incubated with anti-dog IgG horseradish-peroxidase conjugated antibody (1:10,000 diluted in PBS-T, catalog A6792, Sigma-Aldrich, USA), for 1 h at 37 °C. After washing the plates seven times with PBS-T, the reaction was developed by incubation with 100 µL per well of a solution consisting of 2 µL H_2O_2, 2 mg ortho-phenylenediamine and 10 mL citrate-phosphate buffer at pH 5.0 for 30 min in the dark. The reaction was stopped by adding 25 µL 2 N H_2SO_4, and the optical density was read in an ELISA microplate spectrophotometer (Molecular Devices, Spectra Max Plus, Canada) at 492 nm.

2.5.2. Serodiagnosis of human tegumentary leishmaniasis

Similarly for the analyses using canine sera, previous titration curves were performed to determine the most appropriate antigen concentration and antibody dilution to be used. Then, Falcon 96-well plates were coated with rSMP-3, Peptide or L. *braziliensis* SLA (0.25, 1.5 and 2.0 µg per well, respectively), diluted in 100 µL coating buffer, pH 9.6, for 18 h at 4 °C. Free binding sites were blocked using 200 µL of PBS-T plus 5% casein for 1 h at 37 °C. After washing the plates seven times with PBS-T, they were incubated with 100 µL of human sera (1:400 diluted in PBS-T), for 1 h at 37 °C. Plates were subsequently washed seven times in PBS-T, and incubated with anti-human IgG

horseradish-peroxidase conjugated antibody (1:20,000 diluted in PBS-T, catalog SAB3701282, Sigma-Aldrich), for 1 h at 37 °C. After washing the plates seven times with PBS-T, the reaction was developed according described by canine sera. The optical density was read in an ELISA microplate spectrophotometer, at 492 nm.

2.6. Statistical analysis

The results were entered into Microsoft Excel (version 10.0) spreadsheets and analyzed using GraphPad Prism™ (version 6.0 for Windows). The lower limits of positivity (cut-off) for each diagnostic antigen were established by construction of ROC curves. The curves for canine sera were plotted with the values from VL dogs *versus* those from the other groups. The curves for human sera were plotted with the values from tegumentary and visceral leishmaniasis patients *versus* those from the other groups. The diagnostic performance of each antigen was evaluated by analyzing sensitivity [95% confidence interval (95%CI)], specificity (95%CI), area under the curve (AUC), and likelihood ratio (LR).

3. Results

3.1. Characterization of SMP-3 protein

A BLAST-p assay was performed, and results showed that SMP-3 presents 99%, 98%, and 91% of structural homology among *L. infantum*, *L. major,* and *L. braziliensis* species, respectively. Then, the *L. infantum* protein sequence was cloned; the recombinant protein was purified and tested as a serological marker for the serodiagnosis of canine and human disease. The Blast-p algorithm also identified a similar Trypanosomatidae protein, CCC49075.1, and an epitope comparison between these antigens was performed, having been found a linear epitope with high similarity among *Leishmania* species, but not with other Trypanosomatides proteins, such as the molecule identified here (CCC49075.1). A molecular modeling was performed to evaluate the three-dimensional structure of SMP-3, and the B cell epitope (MQKDEESGEFKCEL) was identified by ABCpred program is showed (Fig. 1). It presents GLU-76, GLU-77 and SER-78 amino acids located in the loop. On the other hand, the three-dimensional structure of CCC49075.1 protein showed an epitope marked in green (Fig. 2), where the corresponding amino acids located in the loop were ALA-76, VAL-77 and TYR-78. It is notable that the change in these three amino acid residues modified the characteristics of the proteins, since SMP-3 was found hydrophilic and with amino acids exposed on surface, while CCC49075.1 was found hydrophobic with these amino acid residues less exposed in the molecule´ surface.

To confirm these findings, the PepCalc program was used and physic-chemical analyses of the epitopes were performed, aiming to reveal the characteristics of each amino acid residue (Fig. 3). The three amino acids located in the loops of the proteins confirmed different characteristics, and this fact could explain the low occurrence of cross-reactivity of SMP-3 with sera samples of *Trypanosoma*-infected dogs and humans. With these results, the B cell-specific epitope was synthesized and evaluated in the ELISA assays for the diagnosis of leishmaniasis.

3.2. Serological evaluation of rSMP-3 for the diagnosis of canine leishmaniasis

To evaluate the diagnostic performance of rSMP-3 for the serodiagnosis of canine leishmaniasis, ELISA assays were performed and L. *infantum* SLA and rA2 were used as comparative antigens. After calculating the ROC curves, results showed that all symptomatic and asymptomatic VL sera identified rSMP-3, presenting individual OD results above of cut-off value (Fig. 4). However, when sera samples of the other animals´ groups were used, DO results were below of cut-off value, although two Leish-Tec®-vaccinated animals have showed values in the cut-off limit. Using rA2 as an antigen, all groups presented results above of cut-off value, then demonstrating low specificity of this molecule. In addition, some sera samples from symptomatic and asymptomatic VL dogs presented results below of cut-off value, being classified as false-negative in the serological analysis. Similar results were obtained when SLA was used as an antigen (Fig. 4). The individual performance of the each antigen was determined, and results are shown (Table 1). The sensitivity and specificity values for rSMP-3 were 100% and 99.31%, respectively, with AUC of 1.0. The rA2 antigen showed sensitivity and specificity values of 58.18% and 53.10%, respectively, and AUC of 0.52; while L. *infantum* SLA showed sensitivity and specificity values of 5.45% and 9.31%, respectively, and AUC of 0.25.

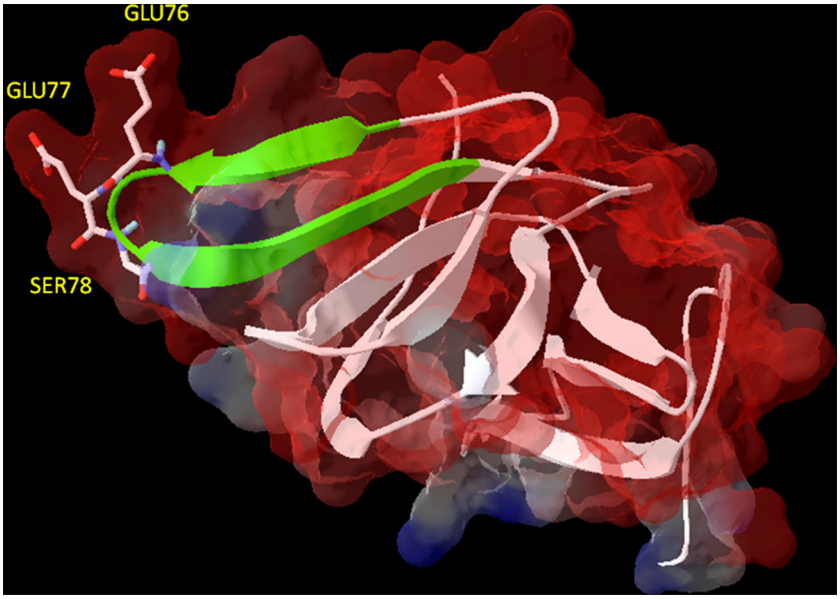

Fig. 1. Three-dimensional structure of *Leishmania* SMP-3 protein. The SwissPDBviewer program was used to construct the three-dimensional structure of SMP-3 protein. The linear B cell epitope region is showed in green, with distinct amino acids indicated in the loop (GLU-76, GLU-77 and SER-78).

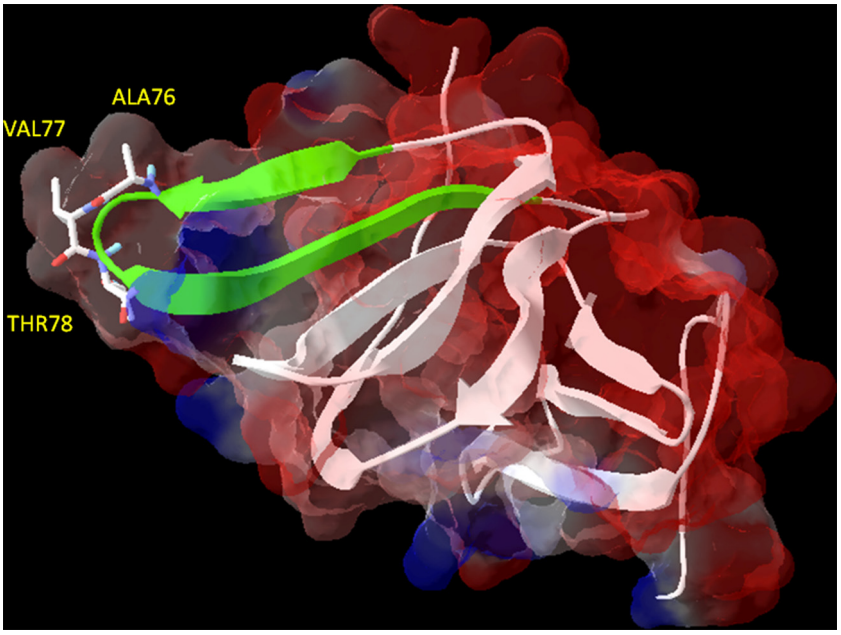

Fig. 2. Three-dimensional structure of *Trypanosoma* CCC49075.1 protein. The SwissPDBviewer program was used to construct the three-dimensional structure of CCC49075.1 protein. The epitope region similar to SMP-3 is showed in green, with distinct amino acids indicated in the loop (ALA-76, VAL-77 and TYR-78).

Fig. 3. Analysis of physical-chemical characteristics of the selected epitopes. The PepCalc program was used to analyses the physic-chemical characteristics of the selected epitopes in the SMP-3 and CCC49075.1 proteins sequence. The three amino acids located in the loop of two proteins presented different characteristics, like showed in the figure, indicating differences in the hydrophilicity and hydrophobicity of these molecules.

3.3. Evaluation of rSMP-3 for the serodiagnosis of human tegumentary leishmaniasis

Next, the diagnostic performance of rSMP-3 was evaluated for the diagnosis of human TL. Although the protein codifying gene has been cloned from L. *infantum* species, the high similarity and conservation between the main B-cell epitopes, like described above, allow deduce the probable efficacy of the antigen for the serodiagnosis of both diseases. As control, L. *braziliensis* SLA was used. Results showed that antibodies from both CL and MCL patients were highly reactive with rSMP-3, but not with *T. cruzi*-infected patients´ sera or from those developing cross-reactive diseases (Fig. 5). In this context, the individual performance of the antigens was determined, and sensitivity and specificity values for the rSMP-3 were 100% and 99%, respectively, with AUC of 1.0, while the sensitivity and specificity values for SLA were 45.0% and 73.0%, respectively, with AUC of 0.52 (Table 2).

3.4. Comparing the diagnostic efficacy between the synthetic peptide and recombinant protein

The antigenicity of B cell epitope was investigated, and compared with results obtained using rSMP-3 protein. While the recombinant antigen showed sensitivity and specificity values of 100% and 99.31%, respectively, for the serodiagnosis of canine leishmaniasis, values using the synthetic peptide were 95% and 93.5%, respectively (Table 3). For the serodiagnosis of human leishmaniasis, the sensitivity and specificity values of rSMP-3 were of 100% and 99%, respectively, while for the synthetic peptide they were 94.5% and 92.5%, respectively.

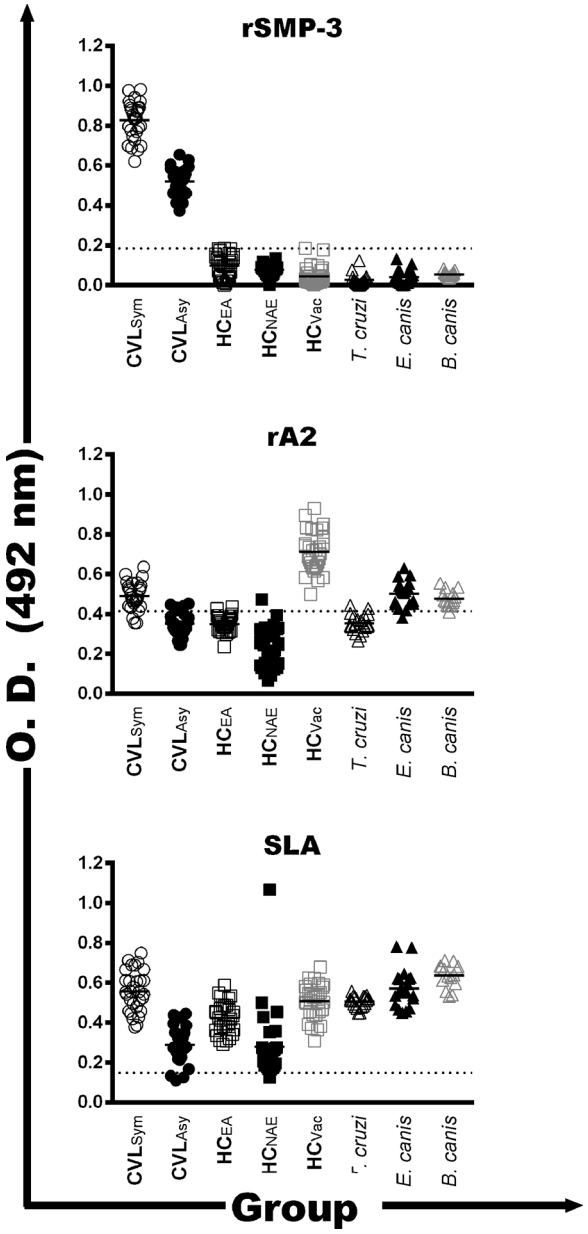

Fig. 4. Evaluation of ELISA reactivity of the antigens for the serodiagnosis of canine visceral leishmaniasis. ELISA assays were performed with sera samples from asymptomatic (CVL_{Asy}) and symptomatic (CVL_{Sym}) *Leishmania infantum*-infected dogs, from non-infected dogs living in endemic (HC_{EA}) or non-endemic (HC_{NEA}) areas of disease, as well as sera from *Trypanosoma cruzi, Ehrlichia canis or Babesia canis*-infected dogs, besides sera from Leish-Tec®-vaccinated and healthy animals (HC_{Vac}). Reactions against rSMP-3, rA2 and L. *infantum* SLA are shown. The respective ROC curves were constructed and used to calculate the cut-off values (dotted lines).

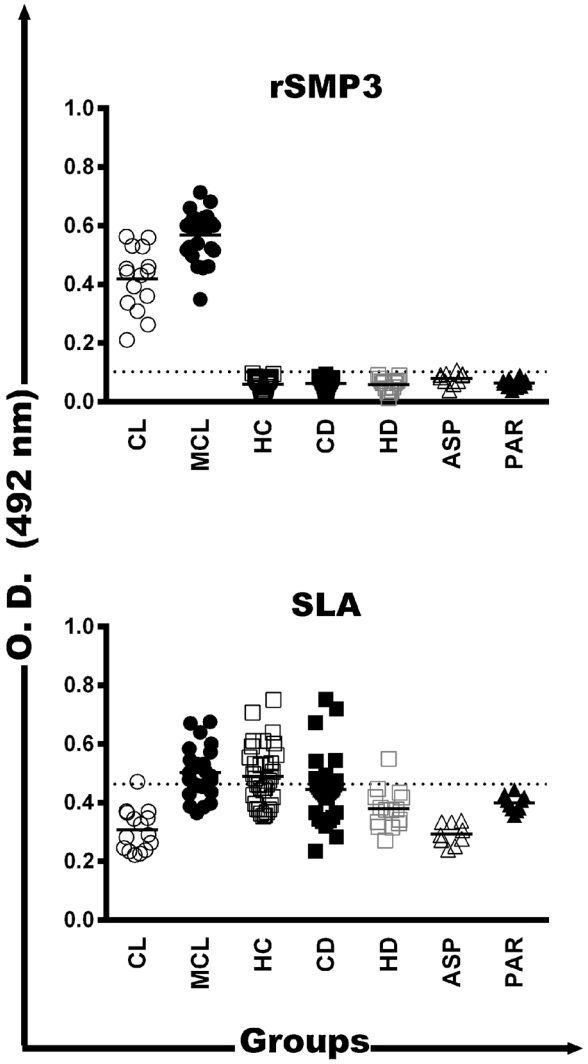

Fig. 5. Diagnostic evaluation of the antigens for the serodiagnosis of human tegumentary leishmaniasis. ELISA assays were performed using sera samples from cutaneous (CL) and mucocutaneous (MCL) leishmaniasis patients, from non-infected subjects living in an endemic area of disease (HC), as well as sera from Chagas disease (CD) patients, and those developing leprosy (HD), aspergillosis (ASP) or paracoccidioidomycosis (PAR). Reactions against rSMP-3 and L. *braziliensis* SLA are shown. The respective ROC curves were constructed and used to calculate the cut-off values (dotted lines).

4. Discussion

In the present study, the diagnostic efficacy of SMP-3 was evaluated by ELISA technique, a non-invasive diagnostic tool for leishmaniasis, by using sera from a large variety of patients´ groups composed by canine and human samples. The diagnostic performance of this recombinant

Table 1
Diagnostic performance of the antigens for the serodiagnosis of canine visceral leishmaniasis. Sera samples from asymptomatic (n = 25) and symptomatic (n = 30) VL dogs, from non-infected animals living in endemic (n = 30) or non-endemic (n = 30) areas of disease, as well as sera from *Trypanosoma cruzi* (n = 20), *Ehrlichia canis* (n = 20) or *Babesia canis* (n = 15)-infected dogs, and samples from Leish-Tec®-vaccinated animals (n = 30) were used in ELISA tests to determine the sensitivity [95% confidence interval (95%CI)], specificity (95%CI), area under the curve (AUC), and likelihood ratio (LR).

Antigen	Diagnostic performance for canine leishmaniasis								
	AUC	CI 95%	*p*-value	Cut-off	Sensitivity	CI 95%	Specificity	CI 95%	LR
rSMP-3	1	1-1	< 0.0001	> 0.18	100	93.51-100	99.31	96.22-99.98	145
rA2	0.52	0.44-0.60	0.60	> 0.41	58.18	44.11-71.35	53.10	44.65- 61.43	1.24
L. infantum SLA	0.25	0.45-0.64	0.28	> 0.15	5.45	1.14-15.12	9.31	6.22-9.98	0.91

Table 2
Evaluation of the performance of the antigens for the diagnosis of human tegumentary leishmaniasis. Sera samples from cutaneous (n = 15) and mucocutaneous (n = 25) leishmaniasis patients, from non-infected subjects living in an endemic area of leishmaniasis (n = 35), as well as sera from Chagas disease (CD) patients, and those developing leprosy (n = 15), aspergillosis (n = 10) or paracoccidioidomycosis (n = 10) were used in ELISA assays to determine the sensitivity [95% confidence interval (CI 95%)], specificity (CI 95%), area under the curve (AUC), and likelihood ratio (LR).

Antigen	Diagnostic performance for human leishmaniasis								
	AUC	CI 95%	*p*-value	Cut-off	Sensitivity	CI 95%	Specificity	CI 95%	LR
rSMP-3	1	1-1	< 0.0001	> 0.10	100	91.19-100	99.00	94.55-99.97	100
L. braziliensis SLA	0.52	0.40-0.63	0.77	> 0.46	45.00	29.26-61.51	73.00	63.20-81.39	1.67

molecule was compared with results obtained using other protein, rA2, also showed to be antigenic in the leishmaniasis (Akhoundi et al., 2013; Jusi et al., 2015; Mendes et al., 2017), as well as by using antigenic *Leishmania* extracts derived from two parasite species. Results showed that rSMP-3 presented excellent diagnostic performance for CL, MCL and VL; with sensitivity and specificity values higher than 95% in all cases, while rA2 protein and L. *infantum* or L. *braziliensis* SLA presented worst results in the serological analyses.

Biotechnological advances employed for the serodiagnosis of leishmaniasis have resulted in higher sensitivity and specificity values for tests (Ibrahim et al., 2015). Recombinant proteins have been shown to be useful for the diagnosis of *Leishmania* infection in dogs and humans (Farahmand and Nahrevanian, 2016). It should be noted that dogs are considered as a major reservoir of L. *infantum* parasites and, therefore, promote the spread of human disease (Travi et al., 2018). Some proteins, such as MAPK3 (Menezes-Souza et al., 2015), LiHyp1 (Martins et al., 2015), rLiHyD (Lage et al., 2016), rKLO8 (Abass et al., 2013), among others; showed to be effective for the serodiagnosis of canine and human leishmaniasis. In addition, *Leishmania*-kinesin family proteins, such as rK26, rK28, and rK39, have showed also to be useful for the diagnosis of disease, exhibiting satisfactory performance in the serological trials (Takagi et al., 2007; Mohapatra et al., 2010 Mar; Fraga et al., 2016; Ghosh et al., 2016). However, in the most of these cases, symptomatic sera have been tested, but problems related with the sensitivity of the antigens when asymptomatic sera or those from canids and humans presenting low levels of antileishmanial antibodies are used have been related. The cross-reactivity with samples of dogs or humans infected by *Leishmania*-related parasites has also hampered the specificity of the tests (Salles et al., 2017; Belo et al., 2017 Oct; Costa et al., 2017).

SMP-3 protein was recently identified by means of a proteomic approach based on two-dimensional electrophoresis and mass spectrometry, which was carried out to analyze the variation of protein expression content in *L. amazonensis* stationary promastigotes, which were pre-isolated from lesions of infected BALB/c mice and maintained in axenic cultures over a long period of time (Magalhães et al., 2014). In this study, SMP-3 presented a 2.48-time reduction in their expression content, when parasites were *in vitro* cultured for 150 days. *Leishmania* parasites express a conserved family of small myristoylated proteins (SMPs), which share an identical central domain but contain differential acylation signals and distinct C-terminal subdomains, being target to distinct regions of the plasma membrane including the cell body, flagellum and flagellar pocket (Tull et al., 2012; Heng et al., 2013). The most of SMPs appear to have unknown function in *Leishmania* (Tull et al., 2004); however, some family proteins, such as SMP-1, showed to be required for flagellar function, since the inhibition of the expression of this protein resulted in flagellum retraction and uncoordinated movements by parasites (Tull et al., 2010). Here, we show that SMP-3 was efficiently evaluated as a diagnostic marker for all tested cases of leishmaniasis: CL, MCL, and VL, based on the structural homology between different parasite species, then demonstrating the possibility to use this antigen for the serodiagnosis of disease. It is notable that other proteins also identified in the cited proteomic study also showed application as diagnostic markers, vaccine candidates and/or immunotherapeutic targets against leishmaniasis (Soto et al., 1996; Achour et al., 2002; Kushawaha et al., 2011).

Although recombinant *Leishmania* proteins have been used as a strategy for a more sensitive and specific diagnosis of leishmaniasis, contradictory results have been obtained and are related with the manufacturer's production process, parasite strain used, among other factors (Mohammadiha et al., 2013; Jamal et al., 2017; Lima et al., 2017). One alternative will be the search for more defined antigens to diagnose the disease, such as the use of synthetic peptides (Menezes-Souza et al., 2015; Lage et al., 2016; Chávez-Fumagalli et al., 2013). These molecules, when compared to recombinant proteins, are relatively simpler and cheaper to produce (Florez et al., 2017). Some studies have showed that the use of synthetic peptides, individually or in a mix format, when compared to the use of recombinant proteins, can increase the sensitivity and/or specificity of immunoassays for serodiagnosis of parasitic diseases, such as leishmaniasis (Costa et al., 2012; Link et al., 2017). In our study, a refined bioinformatics strategy was performed, and a linear B cell-specific epitope was identified in the SMP-3 sequence, which did not present similarity in other members of the Trypanosomatidae family. This epitope was then synthetized and evaluated comparatively with the recombinant protein, and results showed also a promising role of this antigen for the diagnosis of canine VL and human TL.

For the serodiagnosis of canine disease, results showed that both rSMP-3 and synthetic peptide presented sensitivity and specificity values higher than 90%. On the other hand, although rA2 presents satisfactory results in previous studies for VL (Carvalho et al., 2002; Porrozzi et al., 2007), in the present work, the performance observed for this recombinant antigen was worst, in comparison to results obtained using rSMP-3 and the B cell peptide. Our molecules were also

Table 3
Comparative analysis of the linear peptide and rSMP-3 for the diagnosis of human tegumentary leishmaniasis. Sera samples from cutaneous (n = 15) and mucocutaneous (n = 25) leishmaniasis patients, from non-infected subjects living in an endemic area of leishmaniasis (n = 35), as well as sera from Chagas disease (CD) patients, and those developing leprosy (n = 15), aspergillosis (n = 10) or paracoccidioidomycosis (n = 10) were used to determine the sensitivity [95% confidence interval (CI 95%)], specificity (CI 95%), area under the curve (AUC), and likelihood ratio (LR).

Antigen	Canine leishmaniasis				Human leishmaniasis			
	Sensitivity	CI 95%	Specificity	CI 95%	Sensitivity	CI 95%	Specificity	
rSMP-3	100	93.51-100	99.31	96.22-99.98	100	91.19-100	99.00	94.55-99.97
Peptide	95	88.75-98.90	93.50	84.50-97.65	94.50	87.55-98.40	92.50	83.50-97.65

effective in discriminate between VL dogs from the other groups, as well as to identify all asymptomatic and symptomatic animals. Taken together, our data suggest that the rSMP-3 and its linear peptide may be a valuable tool for the diagnosis of canine VL, as well as useful in the development and/or improvement of other simpler and faster serological tests to detect the disease.

The diagnosis of CL and MCL is based on clinical criteria, associated with laboratory results. However, problems related with the sensitivity of the tests, mainly due to the time when the lesions occurred, the low number of parasites present in late lesions, and the invasive procedures of samples collection have been observed (Souza et al., 2013; Lima et al., 2017b). Regarding antileishmanial serology, CL patients usually present low levels of anti-*Leishmania* antibodies, and are classified as false-negative in the serological assays (Sato et al., 2017). In this context, the search for more refined parasite antigens can identify molecules able to improve the sensitivity and specificity for the diagnosis of this disease, since evidence have shown that the humoral response determined by specific-IgG reactivity to *Leishmania* spp. antigens could well be used as an indicator of the development of CL, even in patients with low titers of specific antibodies (Seyyedtabaei et al., 2017). In the present study, SMP-3 was recognized by antibodies from both CL and MCL patients. In fact, results showed that this recombinant protein presented sensitivity and specificity values of 100% and 99% to detect human disease, as well as a perfect accuracy when it was used to discriminate between positive and negative samples, then suggesting its employ as a diagnostic marker for the human TL.

The choice of an accurate and reproducible diagnostic test, capable of supporting control and surveillance strategies in mammalian hosts infected by *Leishmania* parasites is relevant. In the present study, data showed the efficacy and possible use of SMP-3 protein, employed as a recombinant antigen or linear B cell-specific epitope in the improvement of the serodiagnosis of canine and human disease. This candidate could be tested in other diagnostic platforms for leishmaniasis, such as rapid dipstick tests or other immunochromatographic assays, aiming its use in remote areas where laboratories are not readily accessible for conventional assays.

Conflict of interest

The authors declare that there are no conflicts of interest.

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References

Abass, E., Bollig, N., Reinhard, K., Camara, B., Mansour, D., Visekruna, A., Lohoff, M., Steinhoff, U., 2013. rKLO8, a novel Leishmania donovani - derived recombinant immunodominant protein for sensitive detection of visceral leishmaniasis in Sudan. PLoS Negl. Trop. Dis. 7 (July (7)), e2322.

Achour, Y.B., Chenik, M., Louzir, H., Dellagi, K., 2002. Identification of a disulfide isomerase protein of *Leishmania major* as a putative virulence factor. Infect. Immun. 70, 3576–3585.

Akhoundi, B., Mohebali, M., Shojaee, S., Jalali, M., Kazemi, B., Bandehpour, M., Keshavarz, H., Edrissian, G.H., Eslami, M.B., Malekafzali, H., Kouchaki, A., 2013. Rapid detection of human and canine visceral leishmaniasis: assessment of a latex agglutination test based on the A2 antigen from amastigote forms of Leishmania infantum. Exp. Parasitol. 133 (March (3)), 307–313.

Al-Jawabreh, A., Dumaidi, K., Ereqat, S., Nasereddin, A., Azmi, K., Al-Jawabreh, H., Al-Laham, N., Abdeen, Z., 2018. A comparison of the efficiency of three sampling methods for use in the molecular and conventional diagnosis of cutaneous leishmaniasis. Acta Trop. 182 (June), 173–177.

Alvar, J., Vélez, I.D., Bern, C., Herrero, M., Desjeux, P., Cano, J., Jannin, J., Den Boer, M., WHO Leishmaniasis Control Team, 2012. Leishmaniasis worldwide and global estimates of its incidence. PLoS One 7, e35671.

Belo, V.S., Werneck, G.L., Barbosa, D.S., Simões, T.C., Nascimento, B.W., da Silva, E.S., Struchiner, C.J., 2013. Factors associated with visceral leishmaniasis in the americas: a systematic review and meta-analysis. PLoS Negl. Trop. Dis. 25–7 (April (4)), e2182.

Belo, V.S., Gregório, E.A., Teixeira-Neto, R.G., 2017 Oct Oct. Da Rocha Lima ACVM, Pereira AAS, Marcelino AP, Paz GF, da Silva ES. Reliability of techniques used in the diagnosis of canine visceral leishmaniasis by the national control program in Brazil: a survey in an area of recent transmission. Prev. Vet. Med. 1 (146), 10–15.

Biasini, M., Bienert, S., Waterhouse, A., Arnold, K., Studer, G., Schmidt, T., Kiefer, F., Cassarino, T.G., Bertoni, M., Bordoli, L., Schwede, T., 2014. SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. Nucleic Acids Res. 42, W252–W258.

Boggild, A.K., Valencia, B.M., Veland, N., Pilar-Ramos, A., Calderon, F., Arevalo, J., Low, D.E., Llanos-Cuentas, A., 2011. Non-invasive cytology brush PCR diagnostic testing in mucosal leishmaniasis: superior performance to conventional biopsy with histopathology. PLoS One 6, e26395.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Campos, M.B., Lima, L.V.D.R., de Lima, A.C.S., Vasconcelos Dos Santos, T., Ramos, P.K.S., Gomes, C.M.C., Silveira, F.T., 2018. zToll-like receptors 2, 4, and 9 expressions over the entire clinical and immunopathological spectrum of American cutaneous leishmaniasis due to Leishmania(V.) braziliensis and Leishmania (L.) amazonensis. PLoS One 13 (March (3)), e0194383.

Carvalho, F.A., Charest, H., Tavares, C.A., Matlashewski, G., Valente, E.P., Rabello, A., Gazzinelli, R.T., Fernandes, A.P., 2002. Diagnosis of American visceral leishmaniasis in humans and dogs using the recombinant Leishmania donovani A2 antigen. Diagn. Microbiol. Infect. Dis. 43 (August (4)), 289–295.

Chauhan, I.S., Shukla, R., Krishna, S., Sekhri, S., Kaushik, U., Baby, S., Pal, C., Siddiqi, M.I., Sundar, S., Singh, N., 2016. Recombinant Leishmania Rab6 (rLdRab6) is recognized by sera from visceral leishmaniasispatients. Exp. Parasitol. 170 (November), 135–147.

Chávez-Fumagalli, M.A., Martins, V.T., Testasicca, M.C., Lage, D.P., Costa, L.E., Lage, P.S., Duarte, M.C., Ker, H.G., Ribeiro, T.G., Carvalho, F.A., Régis, W.C., Reis, A.B., Tavares, C.A., Soto, M., Fernandes, A.P., Coelho, E.A., 2013. Sensitive and specific serodiagnosis of *Leishmania infantum* infection in dogs by using peptides selected from hypothetical proteins identified by an immunoproteomic approach. Clin. Vaccine Immunol. 20, 835–841.

Coelho, E.A.F., Tavares, C.A.P., Carvalho, F.A.A., et al., 2003. Immune responses induced by the *Leishmania (Leishmania) donovani* A2 antigen, but not by the LACK antigen, are protective against experimental *Leishmania (Leishmania) amazonensis* infection. Infect. Immun. 71, 3988–3994.

Copeland, N.K., Aronson, N.E., 2015. Leishmaniasis: treatment updates and clinical practice guidelines review. Curr. Opin. Infect. Dis. 28 (October5), 426–437.

Costa, M.M., Penido, M., dos Santos, M.S., Doro, D., de Freitas, E., Michalick, M.S., Grimaldi, G., Gazzinelli, R.T., Fernandes, A.P., 2012. Improved canine and human visceral leishmaniasis immunodiagnosis using combinations of synthetic peptides in enzyme-linked immunosorbent assay. PLoS Negl. Trop. Dis. 6 (5), e1622.

Costa, L.E., Salles, B.C.S., Santos, T.T.O., Ramos, F.F., Lima, M.P., Lima, M.I.S., ÁSB, Portela, Chávez-Fumagalli, M.A., Duarte, M.C., Menezes-Souza, D., Machado-de-Ávila, R.A., Silveira, J.A.G., Magalhães-Soares, D.F., Goulart, L.R., Coelho, E.A.F., 2017. Antigenicity of phage clones and their synthetic peptides for the serodiagnosis of canine and human visceral leishmaniasis. Microb. Pathog. 110 (September), 14–22.

Das, S., Matlashewski, G., Bhunia, G.S., Kesari, S., Das, P., 2014. Asymptomatic Leishmania infections in northern India: a threat for the elimination programme? Trans. R. Soc. Trop. Med. Hyg. 108 (November (11)), 679–684.

Duarte, M.C., Pimenta, D.C., Menezes-Souza, D., Magalhães, R.D., Diniz, J.L., Costa, L.E., Chávez-Fumagalli, M.A., Lage, P.S., Bartholomeu, D.C., Alves, M.J., Fernandes, A.P., Soto, M., Tavares, C.A., Gonçalves, D.U., Rocha, M.O., Coelho, E.A., 2015. Proteins selected in Leishmania (Viannia) braziliensis by an immunoproteomic approach with potential serodiagnosis applications for tegumentary leishmaniasis. Clin. Vaccine Immunol. 22 (November (11)), 1187–1196.

Elmahallawy, E.K., Sampedro Martinez, A., Rodriguez-Granger, J., Hoyos-Mallecot, Y., Agil, A., Navarro Mari, J.M., Gutierrez Fernandez, J., 2014. Diagnosis of leishmaniasis. J. Infect. Dev. 8 (August (8)), 961–972.

Farahmand, M., Nahrevanian, H., 2016. Application of recombinant proteins for serodiagnosis of visceral leishmaniasis in humans and dogs. Iran. Biomed. J. 20 (July (3)), 128–134.

Faria, A.R., de Castro Veloso, L., Coura-Vital, W., Reis, A.B., Damasceno, L.M., Gazzinelli, R.T., Andrade, H.M., 2015. Novel recombinant multiepitope proteins for the diagnosis of asymptomatic leishmania infantum-infected dogs. PLoS Negl. Trop. Dis. 9 (January (1)), e3429.

Florez, M.M., de Oliveira, C.I., Puerta, C., Guzman, F., Ayala, M., Montoya, G., Delgado, G., 2017. Synthetic peptides derived from ribosomal proteins of Leishmania spp. in Mucocutaneous Leishmaniasis: diagnostic Usefulness. Protein Pept Lett. 24 (10), 982–988.

Foglia-Manzillo, V., Di Muccio, T., Cappiello, S., Scalone, A., Paparcone, R., Fiorentino, E., Gizzarelli, M., Gramiccia, M., Gradoni, L., Oliva, G., 2013. Prospective study on the incidence and progression of clinical signs in naïve dogs naturally infected by Leishmania infantum. PLoS Negl. Trop. Dis. 7 (May (5)), e2225.

Fonseca, A.M., Faria, A.R., Rodrigues, F.T., Nagem, R.A., Magalhães, R.D., Cunha, J.L., Bartholomeu, D.C., de Andrade, H.M., 2014. Evaluation of three recombinant Leishmania infantum antigens in human and canine visceral leishmaniasis diagnosis. Acta Trop. 137 (September), 25–30.

Fraga, D.B., Pacheco, L.V., Borja, L.S., Tuy, P.G., Bastos, L.A., Solcà Mda, S., Amorim,

L.D., Veras, P.S., 2016. The rapid test based on leishmania infantum chimeric rK28 protein improves the diagnosis of canine visceral leishmaniasis by reducing the detection of false-positive dogs. PLoS Negl. Trop. Dis. 10 (January(1)), e0004333.
Ghosh, P., Bhaskar, K.R., Hossain, F., Khan, M.A., Vallur, A.C., Duthie, M.S., Hamano, S., Salam, M.A., Huda, M.M., Khan, M.G., Coler, R.N., Reed, S.G., Mondal, D., 2016. Evaluation of diagnostic performance of rK28 ELISA using urine for diagnosis of visceral leishmaniasis. Parasit. Vectors 9 (July(1)), 383.
Gish, W., States, D.J., 1993. Identification of protein coding regions by database similarity search. Nature Genet. 3, 266–272.
Goto, H., Lindoso, J.A.L., 2010. Current diagnosis and treatment of cutaneous and mucocutaneous leishmaniasis. Expert Rev. Anti. Ther. 8, 419–433.
Grimaldi Jr, G., Tesh, R.B., 1993. Leishmaniases of the New World: current concepts and implications for future research. Clin. Microbiol. Rev. 6 (July (3)), 230–250.
Guex, N., Peitsch, M.C., 1997. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis 18, 2714–2723.
Heng, J., Saunders, E.C., Gooley, P.R., McConville, M.J., Naderer, T., Tull, D., 2013. Membrane targeting of the small myristoylated protein 2 (SMP-2) in Leishmania major. Mol. Biochem. Parasitol. 190 (July(1)), 1–5.
Ibrahim, F., Thio, T.H., Faisal, T., Neuman, M., 2015. The application of biomedical engineering techniques to the diagnosis and management of tropical diseases: a review. Sensors (Basel). 15 (March (3)), 6947–6995.
Jamal, F., Shivam, P., Kumari, S., Singh, M.K., Sardar, A.H., Pushpanjali, Murugesan S., Narayan, S., Gupta, A.K., Pandey, K., Das, V.N.R., Ali, V., Bimal, S., Das, P., Singh, S.K., 2017. Identification of Leishmania donovani antigen in circulating immune complexes of visceral leishmaniasis subjects for diagnosis. PLoS One 12 (August(8)), e0182474.
Jusi, M.M., Oliveira, T.M., Nakaghi, A.C., André, M.R., Machado, R.Z., 2015. Expression of a recombinant protein, A2 family, from Leishmania infantum (Jaboticabal strain) and its evaluation in Canine Visceral Leishmaniasis serological test. Rev. Bras. Parasitol. Vet. 24 (July-September (3)), 309–316.
Kushawaha, P.K., Gupta, R., Sundar, S., Sahasrabuddhe, A.A., Dube, A., 2011. Elongation factor-2, a Th1 stimulatory protein of *Leishmania donovani*, generates strong IFN-γ and IL-12 response in cured *Leishmania*-infected patients/hamsters and protects hamsters against *Leishmania* challenge. J. Immunol. 187, 6417–6427.
Lage, D.P., Martins, V.T., Duarte, M.C., Costa, L.E., Garde, E., Dimer, L.M., Kursancew, A.C., Chávez-Fumagalli, M.A., de Magalhães-Soares, D.F., Menezes-Souza, D., Roatt, B.M., Machado-de-Ávila, R.A., Soto, M., Tavares, C.A., Coelho, E.A., 2016. A new Leishmania-specific hypothetical protein and its non-described specific B cell conformational epitope applied in the serodiagnosis of canine visceral leishmaniasis. Parasitol. Res. 115 (April (4)), 1649–1658.
Lara-Silva Fde, O., Michalsky, ÉM., Fortes-Dias, C.L., Fiuza Vde, O., Pessanha, J.E., Regina-Silva, S., de Avelar, D.M., Silva, M.A., Lima, A.C., da Costa, A.J., Machado-Coelho, G.L., Dias, E.S., 2015. Epidemiological aspects of vector, parasite, and domestic reservoir in areas of recent transmission and no reported human cases of visceral leishmaniasis in Brazil. Acta Trop. 148 (August), 128–136.
Lima, M.P., Costa, L.E., Duarte, M.C., Menezes-Souza, D., Salles, B.C.S., de Oliveira Santos, T.T., Ramos, F.F., Chávez-Fumagalli, M.A., Kursancew, A.C.S., Ambrósio, R.P., Roatt, B.M., Machado-de-Ávila, R.A., Gonçalves, D.U., Coelho, E.A.F., 2017. Evaluation of a hypothetical protein for serodiagnosis and as a potential marker for post-treatment serological evaluation of tegumentary leishmaniasis patients. Parasitol. Res. 116 (April (4)), 1197–1206.
Lima, B.S., Pires, S.F., Fialho Jr, L.C., Oliveira, E.J., Machado-de-Avila, R.A., Chávez-Olórtegui, C., Chapeaurouge, A.D., Perales, J., Andrade, H.M., 2017b. A proteomic road to acquire an accurate serological diagnosis for human tegumentary leishmaniasis. J. Proteomics 16 (January151), 174–181.
Link, J.S., Alban, S.M., Soccol, C.R., Pereira, G.V., Thomaz Soccol, V., 2017. Synthetic peptides as potential antigens for cutaneous leishmaniosis diagnosis. J. Immunol. Res. 2017, 5871043.
Magalhães, R.D., Duarte, M.C., Mattos, E.C., Martins, V.T., Lage, P.S., Chávez-Fumagalli, M.A., Lage, D.P., Menezes-Souza, D., Régis, W.C., Manso Alves, M.J., Soto, M., Tavares, C.A., Nagen, R.A., Coelho, E.A., 2014. Identification of differentially expressed proteins from Leishmania amazonensis associated with the loss of virulence of the parasites. PLoS Negl. Trop. Dis. 8 (April (4)), e2764.
Martins, V.T., Chávez-Fumagalli, M.A., Lage, D.P., Duarte, M.C., Garde, E., Costa, L.E., da Silva, V.G., Oliveira, J.S., Magalhães-Soares, D.F., Teixeira, S.M., Fernandes, A.P., Soto, M., Tavares, C.A., Coelho, E.A., 2015. antigenicity, immunogenicity and protective efficacy of three proteins expressed in the promastigote and amastigote stages of leishmania infantum against visceral leishmaniasis. PLoS One 10 (September (9)), e0137683.
Mendes, T.M., Roma, E.H., Costal-Oliveira, F., Dhom-Lemos, L.C., Toledo-Machado, C.M., Bruna-Romero, O., Bartholomeu, D.C., Fujiwara, R.T., Chávez-Olórtegui, C., 2017. Epitope mapping of recombinant Leishmania donovani virulence factor A2 (recLdVFA2) and canine leishmaniasis diagnosis using a derived synthetic bi-epitope. PLoS Negl. Trop. Dis. 11 (May (5)), e0005562.
Menezes-Souza, D., Mendes, T.A., Gomes Mde, S., Bartholomeu, D.C., Fujiwara, R.T., 2015. Improving serodiagnosis of human and canine leishmaniasis with recombinant Leishmania braziliensis cathepsin l-like protein and a synthetic peptide containing its linear B-cell epitope. PLoS Negl. Trop. Dis. 9 (January (1)), e3426.
Mniouil, M., Fellah, H., Amarir, F., Sadak, A., Et-Touys, A., Bakri, Y., Moustachi, A., Tassou, F.Z., Hida, M., Lyagoubi, M., Adlaoui, E.B., Rhajaoui, M., Sebti, F., 2018. Comparative evaluation of immunochromatographic dipstick test (ICT) rk39, soluble antigen ELISAand IFAT for the sero-diagnosis of visceral leishmaniasis in Morocco. Acta Trop. 182 (June), 185–189.
Mohammadiha, A., Mohebali, M., Haghighi, A., et al., 2013. Comparison of real-time PCR and conventional PCR with two DNA targets for detection of *Leishmania (Leishmania) infantum*infection in human and dog blood samples. Exp. Parasitol. 133, 89–94.
Mohapatra, T.M., Singh, D.P., Sen, M.R., Bharti, K., Sundar, S., 2010 Mar. Compararative evaluation of rK9, rK26 and rK39 antigens in the serodiagnosis of Indian visceral leishmaniasis. J. Infect. Dev. 4 (March (2)), 114–117.
Oliveira, I.Q., Silva, R.A., Sucupira, M.V., da Silva, E.D., Reis, A.B., Grimaldi Jr, G., Fraga, D.B., Veras, P.S., 2015. Multi-antigen print immunoassay (MAPIA)-based evaluation of novel recombinant Leishmania infantum antigens for the serodiagnosis of canine visceral leishmaniasis. Parasit. Vectors 24 (January(8)), 45.
Oliveira, M.P., Martins, V.T., Santos, T.T.O., Lage, D.P., Ramos, F.F., Salles, B.C.S., Costa, L.E., Dias, D.S., Ribeiro, P.A.F., Schneider, M.S., Machado-de-Ávila, R.A., Teixeira, A.L., Coelho, E.A.F., Chávez-Fumagalli, M.A., 2018. Small myristoylated Protein-3, identified as a potential virulence factor in Leishmania amazonensis, proves to be a protective antigen against visceral leishmaniasis. Int. J. Mol. Sci. 19 (January (1)).
Paiva-Cavalcanti, M., de Morais, R.C., Pessoa-E-Silva, R., Trajano-Silva, L.A., Gonçalves-de-Albuquerque, SdaC., Tavares Dde, H., Brelaz-de-Castro, M.C., Silva Rde, F., Pereira, V.R., 2015. Leishmaniases diagnosis: an update on the use of immunological and molecular tools. Cell Biosci. 17 (June (5)), 31.
Pedral-Sampaio, G., Alves, J.S., Schriefer, A., Magalhães, A., Meyer, R., Glesby, M.J., Carvalho, E.M., Carvalho, L.P., 2016 Sep Sep. Detection of IgG anti-leishmania antigen by flow cytometry as a diagnostic test for cutaneous leishmaniasis. PLoS One 11 (9), e0162793.
Porrozzi, R., Santos da Costa, M.V., Teva, A., Falqueto, A., Ferreira, A.L., dos Santos, C.D., Fernandes, A.P., Gazzinelli, R.T., Campos-Neto, A., Grimaldi Jr, G., 2007. Comparative evaluation of enzyme-linked immunosorbent assays based on crude and recombinant leishmanial antigens for serodiagnosis of symptomatic and asymptomatic Leishmania infantum visceral infections in dogs. Clin. Vaccine Immunol. 14 (May (5)), 544–548.
Saha, S., Raghava, G.P.S., 2006. Prediction of continuous B-cell epitopes in an antigen using recurrent neural network. Proteins 65 (1), 40–48 PMID: 16894596.
Salles, B.C., Costa, L.E., Alves, P.T., Dias, A.C., Vaz, E.R., Menezes-Souza, D., Ramos, F.F., Duarte, M.C., Roatt, B.M., Chávez-Fumagalli, M.A., Tavares, C.A., Gonçalves, D.U., Rocha, R.L., Goulart, L.R., Coelho, E.A., 2017. Leishmania infantum mimotopes and a phage-ELISA assay as tools for a sensitive and specific serodiagnosis of human visceral leishmaniasis. Diagn. Microbiol. Infect. Dis. 87 (March (3)), 219–225.
Sato, C.M., Sanchez, M.C., Celeste, B.J., Duthie, M.S., Guderian, J., Reed, S.G., de Brito, M.E., Campos, M.B., de Souza Encarnação, H.V., Guerra, J., de Mesquita, T.G., Pinheiro, S.K., Ramasawmy, R., Silveira, F.T., de Assis Souza, M., Goto, H., 2017. Use of recombinant antigens for sensitive serodiagnosis of American Tegumentary Leishmaniasis caused by different Leishmania Species. J. Clin. Microbiol. 55 (Febraury(2)), 495–503.
Seyyedtabaei, S.J., Rostami, A., Haghighi, A., Mohebali, M., Kazemi, B., Fallahi, S., Spotin, A., 2017. Detection of potentially diagnostic *Leishmania* Antigens with western blot analysis of sera from patients with cutaneous and visceral leishmaniases. Iran. J. Parasitol. 12 (April-June(2)), 206–214.
Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J., Thompson, J.D., Higgins, D.G., 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol. Syst. Biol. 7, 539.
Soto, M., Requena, J.M., Quijada, L., Alonso, C., 1996. Specific serodiagnosis of human leishmaniasis with recombinant *Leishmania* P2 acidic ribosomal proteins. Clin. Diagn. Lab. Immunol. 3, 387–391.
Souza, A.P., Soto, M., Costa, J.M., Boaventura, V.S., de Oliveira, C.I., Cristal, J.R., Barral-Netto, M., Barral, A., 2013. Towards a more precise serological diagnosis of human tegumentary leishmaniasis using Leishmania recombinant proteins. PLoS One 8 (June (6)), e66110.
Takagi, H., Islam, M.Z., Itoh, M., Islam, A.U., Saifuddin Ekram, A.R., Hussain, S.M., Hashiguchi, Y., Kimura, E., 2007. Short report: production of recombinant kinesin-related protein of Leishmania donovani and its application in the serodiagnosis of visceral leishmaniasis. Am. J. Trop. Med. Hyg. 76 (May (5)), 902–905.
Torres-Guerrero, E., Quintanilla-Cedillo, M.R., Ruiz-Esmenjaud, J., Arenas, R., 2017. Leishmaniasis: a Review. F1000Res 26. pp. 750 May6.
Travi, B.L., Cordeiro-da-Silva, A., Dantas-Torres, F., Miró, G., 2018. Canine visceral leishmaniasis: diagnosis and management of the reservoir living among us. PLoS Negl. Trop. Dis. 12 (January (1)), e0006082.
Tull, D., Vince, J.E., Callaghan, J.M., Naderer, T., Spurck, T., McFadden, G.I., Currie, G., Ferguson, K., Bacic, A., McConville, M.J., 2004. SMP-1, a member of a new family of small myristoylated proteins in kinetoplastid parasites, is targeted to the flagellum membrane in Leishmania. Mol. Biol. Cell 15 (November11)), 4775–4786.
Tull, D., Naderer, T., Spurck, T., Mertens, H.D., Heng, J., McFadden, G.I., Gooley, P.R., McConville, M.J., 2010. Membrane protein SMP-1 is required for normal flagellum function in Leishmania. J. Cell. Sci. 123 (February (Pt4)), 544–554.
Tull, D., Heng, J., Gooley, P.R., Naderer, T., McConville, M.J., 2012. Acylation-dependent and-independent membrane targeting and distinct functions of small myristoylated proteins (SMPs) in Leishmania major. Int. J. Parasitol. 42 (3), 239–247.
Vallur, A.C., Reinhart, C., Mohamath, R., Goto, Y., Ghosh, P., Mondal, D., Duthie, M.S., Reed, S.G., 2016. Accurate serodetection of asymptomatic leishmania donovani infection by use of defined antigens. J. Clin. Microbiol. 54 (April (4)), 1025–1030.
Vijayakumar, S., Das, P., 2018. Recent progress in drug targets and inhibitors towards combating leishmaniasis. Acta Trop. 181 (May), 95–104.
Wolf, D., Failing, K., Taubert, A., Pantchev, N., 2014. Serological diagnosis of canine leishmaniosis: comparison of three commercially available tests. Parasitol. Res. 113 (May(5)), 1997–2002.
World Health Organization, 2016. Leishmaniasis. http://www.who.int/topics/leishmaniasis/en/.
Zhang, W.W., Charest, H., Ghedin, E., Matlashewski, G., 1996. Identification and overexpression of the A2 amastigote-specific protein in Leishmania donovani. Mol. Biochem. Parasitol. 78, 79–90.