Diagnostic Microbiology and Infectious Disease 95 (2019) 134–143

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Parasitology

Diagnostic evaluation of the amastin protein from *Leishmania infantum* in canine and human visceral leishmaniasis and immunogenicity in human cells derived from patients and healthy controls

Danniele L. Vale [a], Daniel S. Dias [a], Amanda S. Machado [b], Patrícia A.F. Ribeiro [a], Daniela P. Lage [a], Lourena E. Costa [a], Bethina T. Steiner [c], Grasiele S.V. Tavares [a], Fernanda F. Ramos [a], Abel Martínez-Rodrigo [d], Miguel A. Chávez-Fumagalli [a], Rachel B. Caligiorne [b], Danielle F. de Magalhães-Soares [e], Julia A.G. Silveira [f], Ricardo A. Machado-de-Ávila [c], Antônio L. Teixeira [a,g], Eduardo A.F. Coelho [a,h,*]

[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] *Instituto de Ensino e Pesquisa, Santa Casa de Belo Horizonte. Rua Domingos Vieira, 590, Santa Efigênia, 30150-240, Belo Horizonte, Minas Gerais, Brazil*
[c] *Programa de Pós-Graduação em Ciências da Saúde, Universidade do Extremo Sul Catarinense, Criciúma, 88806-000, Santa Catarina, Brazil*
[d] *Department of Animal Health, Faculty of Veterinary Science, Complutense University of Madrid, 28040 Madrid, Spain*
[e] *Departamento de Medicina Veterinária Preventiva, Escola de Veterinária, Universidade Federal de Minas Gerais, Belo Horizonte, 31270-901, Minas Gerais, Brazil*
[f] *Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, 31270-901, Minas Gerais, Brazil*
[g] *Neuropsychiatry Program, Department of Psychiatry and Behavioral Sciences, McGovern Medical School, The University of Texas Health Science Center at Houston, Houston, TX, USA; 1941 East Road, Houston, TX, 77041*
[h] *Departamento de Patologia Clínica, COLTEC, Universidade Federal de Minas Gerais, Belo Horizonte, 31270-901, Minas Gerais, Brazil*

ARTICLE INFO

Article history:
Received 20 December 2018
Received in revised form 9 April 2019
Accepted 29 April 2019
Available online 6 May 2019

Keywords:
Amastin
Recombinant proteins
Synthetic peptides
Diagnosis
Response immune
Visceral leishmaniasis

ABSTRACT

The diagnosis of visceral leishmaniasis (VL) presents problems due to the toxicity and/or high cost of drugs. In addition, no vaccine exists to protect against human disease. In this study, the antigenicity and immunogenicity of amastin protein were evaluated in *L. infantum*–infected dogs and humans. For the diagnosis, besides the recombinant protein, 1 linear B-cell epitope was synthetized and evaluated in serological assays. Results showed high sensitivity and specificity values to detect the disease when both antigens were employed against a canine and human serological panel. By contrast, when using rA2 and a soluble *Leishmania* antigenic preparation, sensitivity and specificity values proved to be lower. A preliminary immunogenicity study showed that the amastin protein induced high IFN-γ and low IL-10 production in stimulated PBMC derived from treated VL patients and healthy subjects, thus suggesting a potential use of this protein as an immunogen to protect against human disease.

1. Introduction

Leishmania protozoa are the causative agents of leishmaniasis in 98 countries worldwide, threatening over 380 million people, more than 12 million of whom are infected by these parasites (WHO, 2016). Visceral leishmaniasis (VL) is a severe clinical manifestation of disease, which accounts for approximately 0.5 million cases annually (Burza et al. 2018). In the Americas, the etiological agent is the *Leishmania infantum* species, which is transmitted through the bite of the phlebotomine vector, with dogs representing the main urban reservoirs of the parasites (Savoia 2015). *Leishmania* parasites are found in the form of metacyclic promastigotes that are flagellated and able to transmit the disease to mammalian hosts, whereas the amastigote forms are intracellular and nonflagellated forms found in phagolysosomes from infected mammalian cells, such as neutrophils, macrophages, and dendritic cells, among others (Subramanian and Sarkar 2018).

Clinical manifestations from canine VL (CVL) are variable, ranging from a subclinical or asymptomatic infection to the severe and symptomatic disease (Ferreira et al. 2007). Cutaneous and organic alterations, such as lymph node enlargement, skin lesions, loss of weight, lethargy, splenomegaly, fever, ocular alterations, epistaxis, and lameness, have all been found in symptomatic VL (Solano-Gallego et al. 2011). In

* 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. Tel./fax: +55 31 3409 4983.
E-mail address: eduardoferrazcoelho@yahoo.com.br (E.A.F. Coelho).

https://doi.org/10.1016/j.diagmicrobio.2019.04.015
0732-8893/

addition, neurological and cardiorespiratory disorders have also been observed (Maia et al. 2015). However, many asymptomatic cases can be found in cross-sectional surveys, which can hamper the identification of the infected animals and the success of disease control programs (Almeida-Leal et al. 2014). Moreover, human infection can present variable results of the disease, from asymptomatic to subclinical diseases in the hosts. Asymptomatic patients present practically no impact on their state of health, while symptomatic patients can develop clinical manifestations, including lymphadenopathy, fever, diarrhea, malaise, hepatomegaly, and splenomegaly (Michel et al. 2011).

The diagnosis of VL is based on parasite detection in aspirates from the spleen, lymph nodes, and/or bone marrow (Santos et al. 2018). However, this method presents variable sensitivity and involves invasive procedures for sample collection. Consequently, infected hosts can remain untreated and develop the symptomatic disease (Duthie et al., 2018). More-refined parasitological methods, such as those employing molecular biology, have shown higher performance to diagnose the disease, since they allow for the detection of a small number of circulating parasites in biological samples, as compared to the conventional parasitological methods (Monteiro et al. 2018). However, these incur high costs, require sophisticated equipment, and have no standard methodology for the large-scale molecular diagnosis based on PCR (Nunes et al. 2018).

Serological tests have been employed for the diagnosis of disease; however, these also present advantages and disadvantages (Nunes et al. 2015). Rapid immunochromatographic tests based on recombinant proteins, such as rK28 and rk36, have been used and have become the preferred diagnostic method for CVL in Brazil, followed by ELISA (EIE-LVC) as a confirmatory test (Souza-Filho et al. 2016). Regarding the human disease, the Kalazar Detect™ Test Kit (InBios International, Inc., Seattle, WA) is an immunochromatographic assay applied for the diagnosis of human VL. However, this test cannot discriminate between current, subclinical, or past infections and has proven to be useless for the diagnosis of relapses and as a prognostic test (Sundar and Singh 2018). As a consequence and in addition to the progress in alternate therapeutic strategies, the possibility of developing preventive measures against the disease has been pointed out, such as through the identification of new antigens to be employed for a more specific and sensitive diagnosis (Didwania et al. 2017).

However, in both mammalian hosts, tests commonly fail to detect asymptomatic case, because the analyzed material contains few anti-*Leishmania* antibodies, which can present cross-reaction with *Leishmania*-related diseases (Chaabouni et al. 2018). Moreover, false-positive results can occur in healthy subjects living in endemic areas of the disease (Salles et al., 2019). Distinct *Leishmania* proteins have been evaluated as antigens for the serodiagnosis of VL (Dias et al. 2017; Farahmand and Nahrevanian 2016; Magalhães et al. 2017). However, variable sensitivity and/or specificity results have been found. As an alternative, the use of synthetic peptides should also be considered. Since they present a lower cost to produce, a higher yield in the purification production is obtained, thus presenting a higher stability when compared to recombinant proteins or parasite extracts (Chávez-Fumagalli et al. 2013; Lage et al. 2015; Noya et al. 2003).

A family of closely related transmembrane glycoproteins, namely, amastins, was identified in the *Leishmania* spp. These molecules belong to a family of developmentally regulated proteins consisting of up to 45 members, codified for small proteins of about 200 amino acid residues, dispersed throughout the parasite genome (Jackson 2010). Amastin proteins seem to operate at the host–parasite interface and are likely to be involved in the development of diseases, such as Chagas disease (Teixeira et al. 1994) and human VL (Rafati et al. 2006). In the present study, the *L. infantum* amastin gene (XP_003392700.1) was cloned, and the recombinant protein was purified to be evaluated for the serodiagnosis of canine and human VL using serological panels from Brazilian hosts. In addition, bioinformatics tools were used to identify a specific B-cell epitope derived from the protein, which was also evaluated in the serological assays. In addition, preliminary studies were developed to evaluate the immune response in human PBMCs derived from treated and untreated VL patients, as well as from healthy subjects.

2. Materials and methods

2.1. Cloning, expression, and purification of Leishmania amastin protein

The amino acid sequence of the amastin (XP_003392700.1) protein was cloned from *L. infantum* DNA genome using the following primers: 5′-TCATGGATCCATGCCGCAAGGCCAGTA-3′ (forward) and 5′-TGATAAGCTTCTAGACCATCCGGTTGGC-3′ (reverse), with the *Bam*HI and *Hin*dIII restriction enzymes. The DNA fragment was excised from gel, purified, and linked into the pGEM®-T vector (Promega, USA). Thee recombinant plasmid was then transformed into *E. coli* XL1-Blue (Phoneutria, Brazil) cells, and positive clones were used for the construction of the expression vector. DNA fragments obtained from the digestion of pGEM-amastin plasmid were ligated into the pET28a-TEV vector, and Arctic Express cells (Agilent Technologies, USA) were transformed with the recombinant plasmid. Gene insertion was confirmed by colony PCR, and the sequencing was performed in a MegaBace 1000 automatic sequencer apparatus (Amersham Biosciences, USA). For the purification of the recombinant protein (~19.8 kDa), bacteria were induced with 0.8 mM IPTG for 24 h at 12 °C and were shaken at 200 ×*g* per min. Cells were ruptured by 6 cycles of ultrasound, with cycles of 30 s each (38 MHz), followed by 7 cycles of freezing and thawing. Cell debris were removed by centrifugation, and the recombinant protein was passed onto a 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 as described by Zhang et al. (1996). Briefly, pET16b-A2 plasmid, which was kindly provided by Dr. Greg Matlashewski (Microbiology and Immunology Department, McGill University, Montreal, Quebec, Canada), was used to express and purify the recombinant protein (~53 kDa). For this, transformed *E. coli* DH5α cells were grown in the presence of 1 mM IPTG for 2 h at 34 °C and later disrupted by 7 cycles of freezing and thawing followed by mild ultrasound treatment (5 cycles of 30 s each with an ultrasound processor), in which they were centrifuged at 13,000 ×*g* for 30 min at 4 °C. The protein was purified by using the gel-filtration column. After purification, both the recombinant amastin and rA2 proteins were passed through a polymyxin-agarose column (Sigma-Aldrich, St. Louis, MO) in order to remove any residual endotoxin content (<10 ng of LPS per 1 mg of recombinant protein, measured by the Quantitative Chromogenic Limulus Amebocyte Assay QCL-1000, BioWhittaker, MD).

2.2. Bioinformatics to obtain specific B-cell epitope

The process of *in silico* analysis of the amastin (XP_003392700.1) sequence consisted of a comparison with the amino acid sequence from distinct parasite species, where it showed structural similarity: *L. donovani* (XP_003862861.1), *L. major* (XP_001684736.1), *L. major* (XP_001684735.1), *L. mexicana* (XP_003877293.1), and *L. braziliensis* (XP_001566782.1). A comparison with the *L. infantum* amastin sequence was then performed with sequences found in other trypanosomatides: *T. theileri* (ORC76762.1), *T. cruzi Brener* (XP_810211.1), *T. cruzi* (PBJ80659.1), *T. cruzi* (PWU91633.1), *T. rangeli* (AGN32994.1), and *T. rangeli* (XP_009310501.1). In both cases, the BLAST-p algorithm (Gish and States 1993) was used, and alignments were performed by the Clustal Omega program (Sievers et al. 2011). The presence of linear B-cell epitopes was then investigated by the ABCpred server (Saha and Raghava 2006) using the following parameters: window size of 14, threshold of 0.85, and overlapping filter: ON. The peptide sequences presenting values higher than 0.85 were considered positive, and the best value (0.89) was selected as being an

amastin-specific B-cell epitope. The peptide (LPFSISCVFASETRRLARERYGISG) sequence was then synthetized (Genscript®, USA).

2.3. Parasites

L. infantum (MOM/BR/1970/BH46) was used. Stationary promastigotes were grown at 24 °C in Schneider's medium (Sigma-Aldrich, USA), which was comprised of the medium plus 20% inactivated fetal bovine serum (FBS, Sigma-Aldrich, USA), 20 mM L-glutamine, 200 U/mL penicillin, and 100 μg/mL streptomycin, pH 7.4. The soluble *Leishmania* antigenic extract (SLA) was produced according to that described by (Coelho et al. 2003). To obtain the axenic amastigotes, previous titration curves were performed to determine the best temperature and time of conversion of the promastigotes in amastigote forms. The technical protocol was adapted from Doyle et al. (1991) by Mendonça et al. (2018). Briefly, 10^9 *L. infantum* stationary promastigotes were washed 3 times in sterile phosphate buffer saline (PBS 1×) at pH 7.4 and incubated in 5 mL FBS for 72 h at 37 °C. The parasites were then washed in cold PBS 1× pH 7.4, and cell density was estimated by counting in the Newbauer chamber. The conversion of the parasites was evaluated after staining with Giemsa, and the results in the conversion percentage were higher than 90.0%.

2.4. Canine sera

This study was approved by the Committee on the Ethical Handling of Research Animals (protocol number 0333/2015) of Federal University of Minas Gerais (UFMG). Dogs developing VL were classified as asymptomatic ($n = 25$) or symptomatic ($n = 25$) based on a score calculated according to the intensity of the following evaluated clinical signs: emaciation, alopecia, anemia, conjunctivitis, dehydration, dermatitis, erosion, ulcerations, lymphadenopathy, and onychogryposis. In this context, asymptomatic animals showed a score between 0 and 3, and symptomatic dogs presented a score of higher than 3, according to that described by Solcà et al. (2014). All samples tested positive when applying PCR to detect *L. infantum* kDNA, and infected animals presented positive results by using the IFAT-CVL and EIE-CVL BioManguinhos® kits (Rio de Janeiro, Rio de Janeiro, Brazil). Sera obtained from healthy dogs living in endemic ($n = 25$; Belo Horizonte) or nonendemic ($n = 25$; Poços de Caldas, Minas Gerais, Brazil) areas of leishmaniasis were used. These animals showed no clinical sign of the disease and presented negative serological results. Samples collected from healthy animals that had been immunized with a commercial vaccine (Leish-Tec®; $n = 25$) and from those that had been experimentally infected with *Ehrlichia canis* ($n = 15$), *Babesia canis* ($n = 15$), or *Trypanosoma cruzi* ($n = 15$), all of which were maintained in kennels to prevent their contact with transmitting vectors of leishmaniasis, were also used in the serological assays.

2.5. Human sera

Serum samples were also collected from VL patients ($n = 30$, including 19 males and 11 females, with ages ranging from 26 to 56 years). Infection was confirmed by PCR targeting *L. infantum* kDNA in aspirates from the spleen and/or bone marrow of the patients. The patients were selected from an endemic region of disease (Belo Horizonte). The control group consisted of healthy individuals living in an endemic (Belo Horizonte; $n = 30$, including 17 males and 13 females, with ages ranging from 24 to 55 years) or nonendemic (Poços de Caldas, Minas Gerais, Brazil; $n = 30$, including 16 males and 14 females, with ages ranging from 23 to 54 years) areas of VL, none of which presented clinical signs or suspicions of leishmaniasis. Serum samples from Chagas disease patients ($n = 20$, including 13 males and 7 females, with ages ranging from 24 to 52 years) were also used, with the infection confirmed by hemoculture and Chagatest recombinant ELISA v. 3.0 kit or Chagatest hemagglutination inhibition (HAI) assay (Wiener Lab, Argentina). Samples from paracoccidioidomycosis ($n = 10$, 5 males and 5 females, with ages ranging from 29 to 51 years), leprosy ($n = 10$, with 6 males and 4 females, with ages ranging from 30 to 61 years), and aspergillosis ($n = 10$, including 4 males and 6 females, with ages ranging from 24 to 45 years) patients were used in the assays.

2.6. ELISA for the serodiagnosis of VL

Previous titration curves were performed to determine the most appropriate antigen concentration and antibody dilution to be used. The flexible microtiter immunoassay plates (Jetbiofil®, Belo Horizonte)

```
L. infantum (XP_003392700.1)     1   -MPQGQYSQKPNDGPTQPVKTRPGQNVYTG----------N-------------P-----MNAPGSRPAVYYREGPWHY   50
L. donovani(XP_003862861.1)      1   -MPQGQYSQKPNDGPTQPVKTRPGQNVYTG----------N-------------P-----MNAPGSRPAVYYREGPWHY   50
L. major(XP_001684736.1)         1   -MLQGQYDQKPNGGPQYNQPQGPGQNVYTG----------N-------------P-----SNPPGSQSAVYYREGPWHY   50
L. major(XP_001684735.1)         1   -MLQGQYDQKPNGGPQYNQPQGPGQNVYTG----------N-------------P-----MNAPGSRPAVHSRLGNWHY   50
L. mexicana(XP_003877293.1)      1   -MQPAPLYQKPKGDAPYYQPQGAAQNAYNG----------N-------------P-----MNAPASRSAVQNRDGLWHY   50
L. braziliensis (XP_001566782.1)1   MHTQPQALGAPNAASQGKGVPGNGADVYKTSAPPGAQWPSNDQPIPYNQRFNMLPHVKDSSQFHQLDGTIAVNRGPWHF   79
                                        *:           . :.*.           *             *      :      ::  . *:**:

L. infantum (XP_003392700.1)     51  SLCVCCEDMDSCCEACCCFPCQVSRQCNMFMYNRREIHWPYCLLMIVFDLYL-PFSISCVFASETRRLARERYGISGTG   128
L. donovani(XP_003862861.1)      51  SLCVCCEDMDSCCEACCCFPCQVSRQCNMFMYNRREIHWPYCLLMIVFDLYL-PFSISCIFAGETRRLARERYGISGTG   128
L. major(XP_001684736.1)         51  SLCVCCEDMDSCCEACCCFSCQVSRQCNMFMYNRREIHWPYCLLMTFLDLYL-PFTISWVFVGETRRLARERYGISGSS   128
L. major(XP_001684735.1)         51  SLCVCCNDMDSCCEACCCFPCQVSRQCNMLVSNRREIHWPYCLLMTLCDVSLLFFSVTCVFASETRRLARERYGISGSG   129
L. mexicana(XP_003877293.1)      51  SLCVCCEDMDSCCEACCCFPCQVSRQCNMLMNSRREIHWPYCLLMTLCDFTLFIFSVSCVFASETRRLARERYGISGSS   129
L. braziliensis (XP_001566782.1)77  SLCVCCQGIDSCCEAWCCMPCQLSRQCNMLTNNRKEIHWPYCLLMTFCDYTWLVFNASCIFASETRRMARERYSISGSS   160
                                    ******:.:****** **: **:******:  .*:********** . *     *. : :*..****:*****.***:.

L. infantum (XP_003392700.1)     129 CEDFCIGYWCRTCSAQQVLLEMIVMNEFPGAMCFEAAPQPAANRMV    174   Similarity
L. donovani(XP_003862861.1)      129 CEDFCIGYWCRTCSAQQVLLEMIVMNEFPGAMCFEAAPQPAANRMV    174      99%
L. major(XP_001684736.1)         129 CEDFCIGYWCRTCSAQQVLLEMIVMNEFPGATCYEAAPQPAANRMV    174      85%
L. major(XP_001684735.1)         130 CDDCCIGYWCRTCSAQQVLLEMTAMSEFPGATCYEAAPQPAANRMV    175      80%
L. mexicana(XP_003877293.1)      130 LEDCCLAYWCRTCSTQQVLLEMTTMNEFPGATCYEAAPQPMGNRMV    175      72%
L. braziliensis (XP_001566782.1)161 CEDCCIGYFCRPCSTQQVLLEMTLMNEFPGATCYEATSHPAGRRMV    204      68%
                                  :* *:.*:** **:*******  *.***** *:**: :* ..***
```

Fig. 1. Evaluation of the amino acid sequence from amastin protein in *Leishmania*. An *in silico* analysis of the *L. infantum* amastin (XP_003392700.1) protein was performed by BLAST tool, where a search for structural similarity among sequences in the database of other parasite species was performed with *L. donovani* (XP_003862861.1), *L. major* (XP_001684736.1), *L. major* (XP_001684735.1), *L. mexicana* (XP_003877293.1), and *L. braziliensis* (XP_001566782.1). The amino acid sequences were aligned by the Clustal Omega program, and the specific linear B-cell epitope (marked in bold and underlined) was predicted by the ABCpred server.

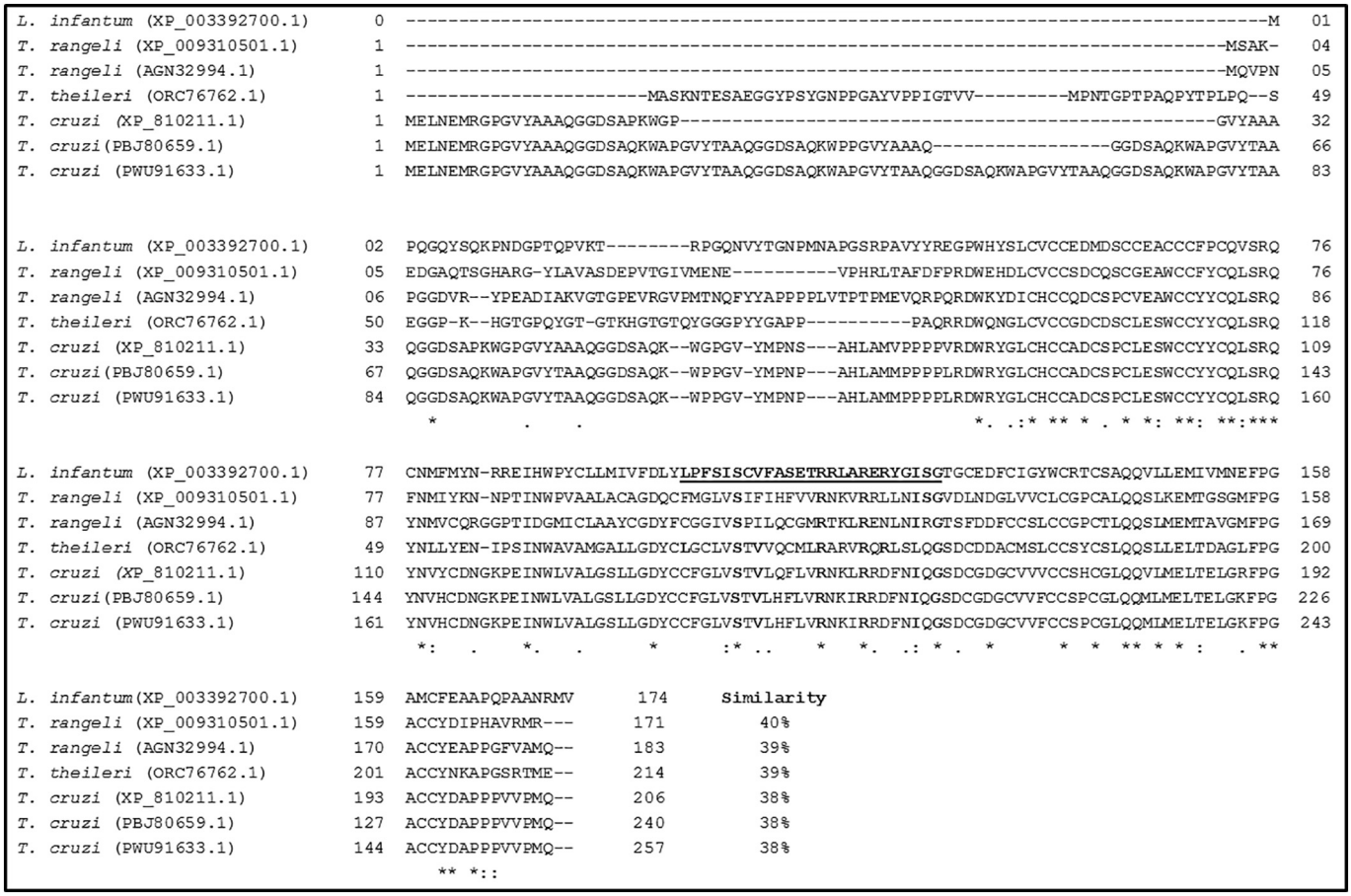

Fig. 2. Evaluation of the amino acid sequence from amastin protein in other trypanosomatides. An *in silico* analysis of the *L. infantum* amastin (XP_003392700.1) protein was performed by the BLAST tool, and the search for structural similarity among protein sequences expressed in other trypanosomatides was performed: *T. rangeli* (XP_009310501.1), *T. rangeli* (AGN32994.1), *T. theileri* (ORC76762.1), *T. cruzi* (XP_810211.1), *T. cruzi* (PBJ80659.1), and *T. cruzi* (PWU91633.1). The amino acid sequences were aligned by the Clustal Omega program, and the *Leishmania*-specific B-cell epitope is shown (marked in bold and underlined).

were then coated with amastin, synthetic peptide, rA2, or *L. infantum* SLA (0.5, 1.0, 2.0, and 2.0 μg per well, respectively), which were diluted in a 100-μL coating buffer (50 mM carbonate buffer), at pH 9.6, for 16 h at 4 °C. After, free binding sites were blocked using 250 μL of PBS 1× plus Tween 20 0.05% (PBS-T) combined with 5% casein for 1 h at 37 °C. Next, plates were washed 5 times using PBS-T and were incubated with 100 μL of canine or human sera (1:200 and 1:400 diluted in PBS-T, respectively) for 1 h at 37 °C. Plates were washed 7 times in PBS-T and incubated with anti-human or anti-dog IgG horseradish-peroxidase–conjugated antibodies (1:20,000 or 1:10,000, respectively, both diluted in PBS-T; SAB3701282 and A6792 catalogs, respectively; Sigma-Aldrich, USA) for 1 h at 37 °C. The plates were then washed 7 times with PBS-T, and the reaction was developed by incubation with 100 μL of a solution comprised of H_2O_2 30 vol., ortho-phenylenediamine, and citrate-phosphate buffer at pH 5.0 for 30 min in the dark. The reaction was then stopped by adding 2 N H_2SO_4, and the optical density (OD) was read in an ELISA microplate spectrophotometer reader (Molecular Devices, Spectra Max Plus, Canada) at 492 nm. The diagnostic testing was performed under blinded conditions, which means that test readers interpreted the results obtained from each diagnostic technique for a given sample with no knowledge of the results from other analyses.

2.7. In vitro stimulation of human PBMC

The present study was approved by the Human Ethics Committee from UFMG (protocol number CAAE-32343114.9.0000.5149). Blood samples (20 mL) were collected from VL patients ($n = 6$, including 4 males and 2 females, with ages ranging from 27 to 51 years). Infection was confirmed by PCR in aspirates from spleen and/or bone marrow. Samples were collected before and 6 months after treatment using pentavalent antimonials (Sanofi Aventis Farmacêutica Ltda., Suzano, São Paulo, Brazil). Patients were submitted to the same therapeutic regimen (20 mg Sb^{+5} per kg during 30 days), none of whom suffered from any other infections or had any preexisting disease. Upon completing the treatment, a PCR was performed, and no parasite DNA was found in the aspirates of the spleen and/or bone marrow. The control group consisted of noninfected individuals ($n = 6$, including 3 males and 3 females, with ages ranging from 24 to 48 years) living in an endemic area (Belo Horizonte) of disease. The subjects present no clinical signs and exhibited negative results when tested by the Kalazar Detect™ Test kit. For the PBMC culture, cells (10^6 cells) were purified by density centrifugation through Ficoll–Hypaque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and cultured in 48-well flat-bottomed tissue culture plates (Costar, Cambridge, MA) in RPMI 1640 medium, which was added together with 20% FBS, 2 mM L-glutamine, 200 U/mL penicillin, 100 μg/mL streptomycin, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, and 1× nonessential amino acid. PBMCs were incubated alone (medium) or stimulated with the amastin protein or SLA (10.0 and 25.0 μg/mL, respectively) for 5 days at 37 °C in 5% CO_2. The supernatants were then collected, and the IFN-γ and IL-10 production was evaluated by capture ELISA (Human IFN-γ and IL-10 ELISA Sets, BD Biosciences, USA) according to manufacturer's instructions. Results were interpolated from a standard curve using recombinant cytokines. The detection sensitivity for IFN-γ and IL-10 was 2.2 and 3.9 pg/mL, respectively.

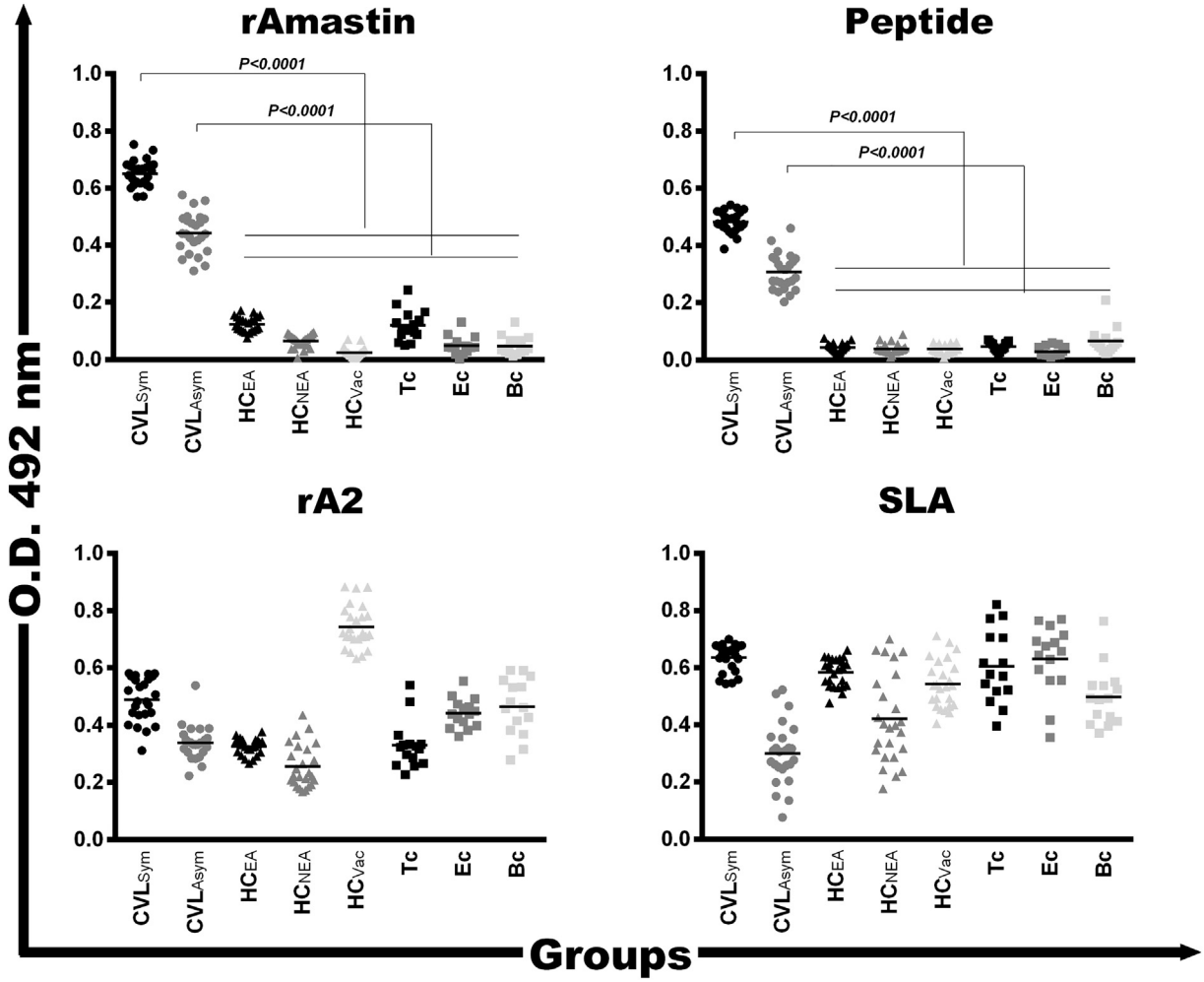

Fig. 3. ELISA reactivity using the antigens against canine sera. ELISAs were performed using sera samples from asymptomatic (CVL_{Sym}, $n = 25$) and symptomatic (CVL_{Asym}, $n = 25$) visceral leishmaniasis dogs, as well as sera from healthy dogs living in an endemic (HC_{EA}, $n = 25$) or nonendemic (HC_{NEA}, $n = 25$) area of leishmaniasis; samples from healthy animals and vaccinated with the Leish-Tec® vaccine (HC_{Vac}, $n = 25$) and from those infected with *Trypanosoma cruzi* (Tc, $n = 15$), *Ehrlichia canis* (Ec, $n = 15$), or *Babesia canis* (Bc, $n = 15$). The individual OD values for each sample against the recombinant amastin protein, synthetic peptide, rA2, and *L. infantum* SLA are shown. The mean of each group is indicated, as well as significant differences between CVL and the other groups ($P < 0.0001$).

2.8. Statistical analysis

Results were entered into Microsoft Excel (version 10.0) spreadsheets and analyzed using GraphPad Prism™ (version 6.0 for Windows). Receiver–operator characteristic (ROC) curves were constructed to determine the cutoff values. Curves were plotted using the OD values from VL (asymptomatic and symptomatic) dogs *versus* those obtained from control or cross-reactive sera. The diagnostic evaluation of the antigens was performed by calculating the sensitivity, specificity, positive predictive value, negative predictive value, area under the curve (AUC), and likelihood ratio. Confidence intervals (CIs) were calculated using a 95% confidence level (95% CI). Differences were considered significant when $P < 0.05$.

3. Results

3.1. Sequence alignment of the amastin protein

A BLAST-p assay showed that the amino acid sequence of the amastin protein presents high structural homology between distinct *Leishmania* spp.: *L. donovani* (99%), *L. major* (85%), *L. mexicana* (72%), and *L. braziliensis* (68%) (Fig. 1), but not in other trypanosomatides, such as *T. rangeli* (40%), *T theileri* (39%), and *T. cruzi* (38%) (Fig. 2). Results also showed a specific B-cell epitope (LPFSISCVFASETRRLARERYGISG), which presented high structural homology between *Leishmania* species but not in other trypanosomatides (Figs. 1 and 2).

3.2. Evaluation of the antigens for the serodiagnosis of canine VL

Serological assays were performed using the recombinant protein and the synthetic peptide, with *L. infantum* SLA and the rA2 protein used as controls. Results showed that symptomatic and asymptomatic CVL sera reacted with the amastin protein and synthetic peptide, with OD values significantly higher when compared to those found using rA2 or SLA (Fig. 3). ROC curves were constructed, and the maximum sensitivity and specificity values were found for the protein and peptide, with an AUC of 1.0, whereas when using rA2, the AUC value was 0.55 to discriminate CVL sera from healthy controls (HC) and 0.50 to discriminate between CVL sera and cross-reactive disease sera (CRD, comprised of vaccinated animals and those infected with *E. canis*, *Babesia canis*, or *T. cruzi*) (Fig. 4). Using *L. infantum* SLA, AUC values were 0.53 and 0.65

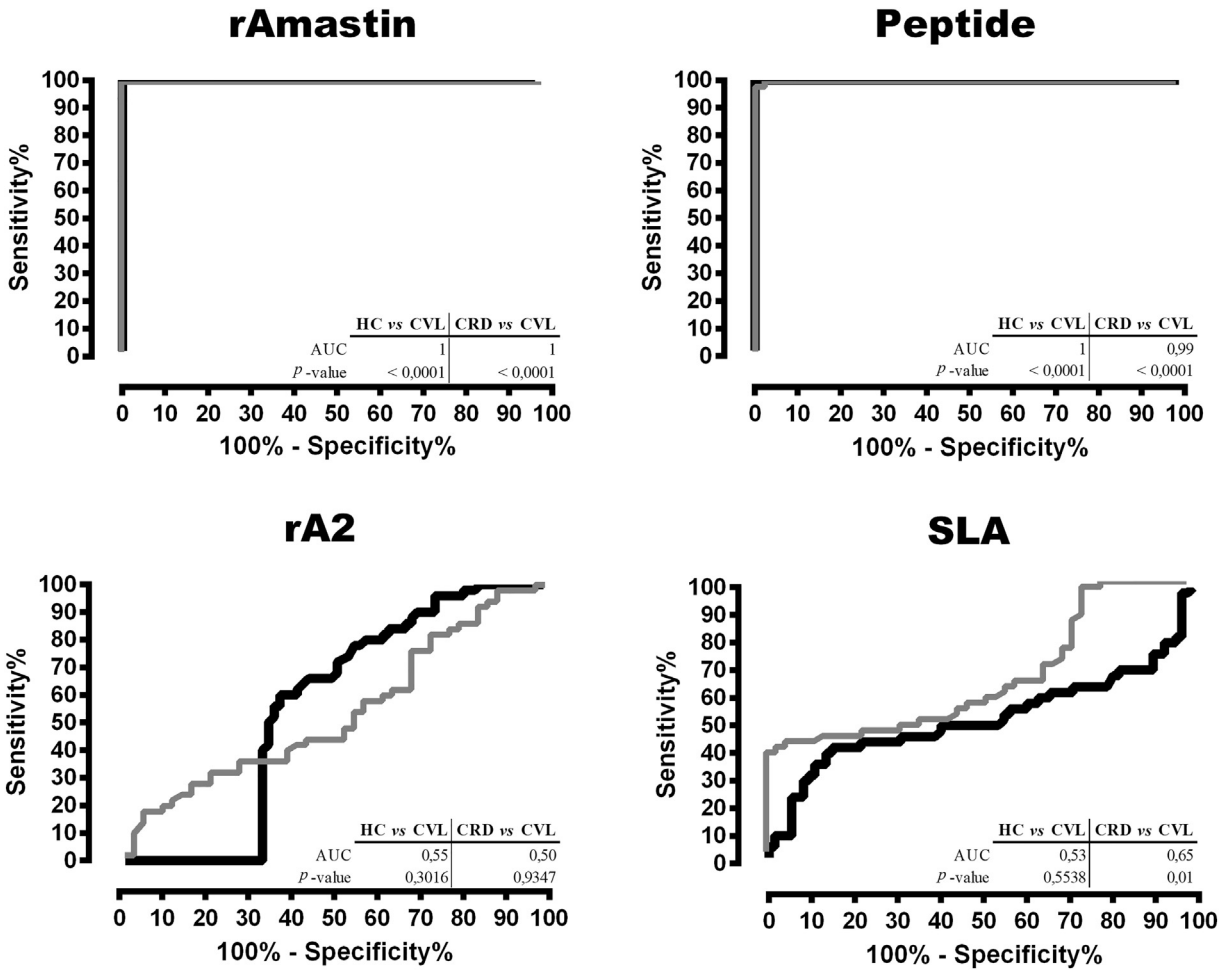

Fig. 4. ROC curves for the diagnosis of canine leishmaniasis. ROC curves were constructed with the results obtained in the ELISAs with the canine serological panel against the amastin protein, synthetic peptide, rA2, and *L. infantum* SLA. The sensitivity (95% CI), specificity (95% CI), and AUC values were determined and are shown.

to discriminate CVL sera from HC and CRD samples, respectively. Regarding the sensitivity and specificity values, the recombinant protein and synthetic peptide showed results of 100% in both cases, whereas when using rA2 or SLA, the sensitivity values were of 78.0% and 42.0% to discriminate positive and negative samples, respectively, and specificity values were of 45.3% and 75.3%, respectively (Table 1).

3.3. Testing the antigens for the serodiagnosis of human VL

The recombinant amastin protein and synthetic peptide were also evaluated in ELISA experiments for the diagnosis of human disease. For this, the rA2 protein and *L. infantum* SLA were also used as controls. Results showed that both protein and peptide presented both sensitivity and specificity values of 100%, respectively, with an AUC of 1.0 to identify *L. infantum*–infected patients, whereas when using *L. infantum* SLA and rA2, the sensitivity values were 86.67% and 100%, respectively, with an AUC of 0.84 and 0.91, respectively, and specificity values were 73.33% and 75.00%, respectively (Fig. 5). ROC curves were also constructed, and results are shown in Fig. 6. Parameters used to evaluate the diagnostic efficacy of the recombinant protein and synthetic peptide are also shown (Table 2).

3.4. Cell response generated in human PBMCs

The stimulation of human PBMC with the amastin protein was also performed. For this, cells derived from blood samples of VL patients

Table 1
Evaluation of the antigens for the serodiagnosis of canine leishmaniasis. Sera samples from asymptomatic and symptomatic visceral leishmaniasis dogs, as well as from healthy dogs living in endemic or nonendemic areas of leishmaniasis; samples from healthy animals but immunized with the Leish-Tec® vaccine; and sera from those infected with *Trypanosoma cruzi*, *Ehrlichia canis*, or *Babesia canis* were used in the assays. ROC curves were constructed to determine the cutoff values of the antigens. The sensitivity (Se; 95% CI), specificity (Sp; 95% CI), and Youden index (*J*) were calculated between the CVL (asymptomatic plus symptomatic cases) and HC (healthy animals) groups, as well as between CVL (asymptomatic plus symptomatic cases) and CRD (cross-reactive diseases) groups.

Antigen	CVL *versus* HC						CVL *versus* CRD					
	Cutoff	Se	95% CI	Sp	95% CI	*J*	Cutoff	Se	95% CI	Sp	95% CI	*J*
rAmastin	>0.2405	100	92.9–100	100	95.2–100	1.00	>0.2770	100	92.9–100	100	92,13–100	1.00
Peptide	>0.1455	100	92.9–100	100	95.2–100	1.00	>0.2170	98.0	89.4–100	100	92,13–100	0.98
rA2	>0.3328	78.0	64.0–88.5	45.3	33.8–57.3	0.23	>0.5573	16.0	7.2–29.1	73.3	81.7–92.3	0.09
SLA	<0.3870	42.0	28.2–56.8	75.3	65.3–89.4	0.27	<0.3550	38.0	24.7–52.8	68.8	55.6–89.9	0.38

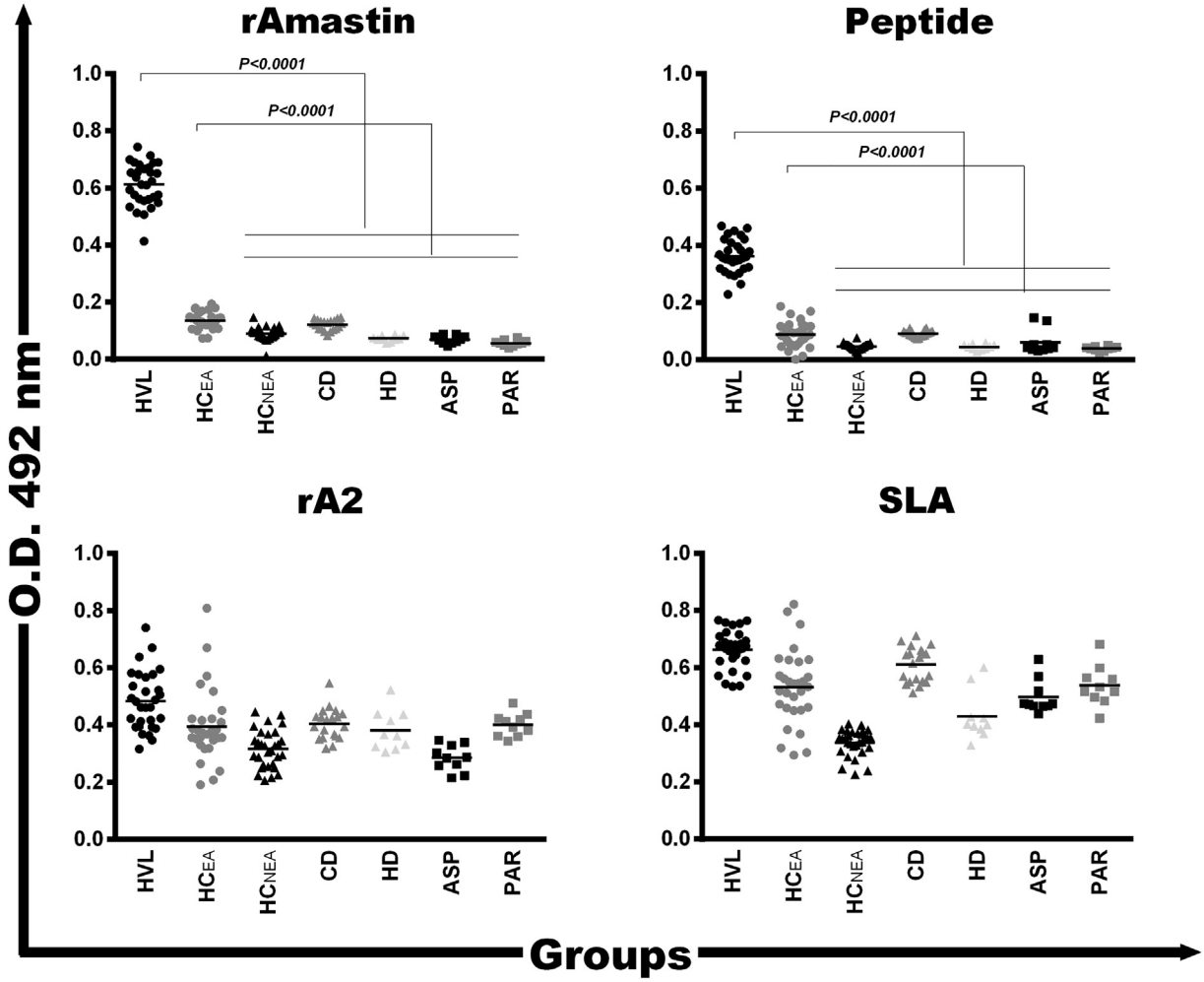

Fig. 5. Diagnostic evaluation of the antigens to detect human leishmaniasis. ELISAs were performed using sera samples from visceral leishmaniasis patients (VL, $n = 30$), from noninfected subjects living in endemic (H_{EA}, $n = 30$) or nonendemic (H_{NEA}, $n = 15$) regions of disease, as well as from patients with Chagas disease (CD, $n = 20$), leprosy (HAN, $n = 10$), aspergillosis (ASP, $n = 10$), or paracoccidioidomycosis (PAR, $n = 10$). The individual OD values for each sample against the recombinant amastin protein, synthetic peptide, rA2, and *L. infantum* SLA are shown. The mean of each group is indicated, as well as significant differences between HVL and the other groups ($P < 0.0001$).

were collected before and after treatment, and were stimulated with the recombinant protein. As a control, cells were also obtained from healthy subjects living in an endemic area of VL. After, the IFN-γ and IL-10 production was measured in the cell culture supernatants. Results showed higher amastin-specific IFN-γ levels in treated VL patients and healthy subjects, which were associated with lower IL-10 production in these groups (Fig. 7). On the other hand, when *L. infantum* SLA was used as a stimulus, higher IL-10 levels were found in the untreated patient group.

4. Discussion

Amastins comprise a glycoproteins family encoded by trypanosomatides genes. In spite of descriptions of this protein family in distinct *Leishmania* species, a biological role of these proteins has not yet been established (Azizi et al. 2009). In the present work, the *L. infantum* amastin protein was cloned and evaluated as antigenic and immunogenic in the canine and human VL. Due to the fact that this protein was recently identified in an immunoproteomics study developed in *L. infantum* amastigote extracts by antibodies in sera of asymptomatic and symptomatic VL dogs (Coelho et al. 2012), its recombinant version was evaluated for the serodiagnosis of the disease. A preliminary study was also conducted to evaluate the immunogenicity of this protein by using PBMC cultures stimulated with the protein, which were derived from treated or nontreated VL patients as well as from healthy subjects.

The success of the serological diagnosis of VL is related to the sensitivity and specificity of the tests employed in this study, and their performance depends on many factors, such as infection status and the type of diagnostic antigen (Travi et al. 2018). In this context, when the infection by the parasite is considered subclinical or asymptomatic, the sensitivity of the tests presents variable results (Carvalho et al. 2017; Nogueira et al. 2018). This fact can be due mainly to the low titers of antileishmanial antibodies found in these infected hosts (Mettler et al. 2005). On the other hand, the nature of the antigen is also important since most of them are present in the promastigote forms of the parasites (Pinheiro et al. 2009). The difficulty in cultivating axenic amastigotes, as well as the low degree of purification of these forms, can be considered limiting factors in the identification of specific antigens. On the other hand, these molecules should be considered as suitable targets to compose a diagnostic test since just a few hours after the infection, as well as during the active disease, amastigotes can already be found circulating in infected mammalian hosts (Fernandes et al. 2012).

Since amastin proteins are considered to be transmembrane glycoproteins documented as present in *Leishmania* amastigotes, they could be considered for the serodiagnosis of canine and human disease. Rafati et al. (2006) evaluated an amastin-derived peptide as a

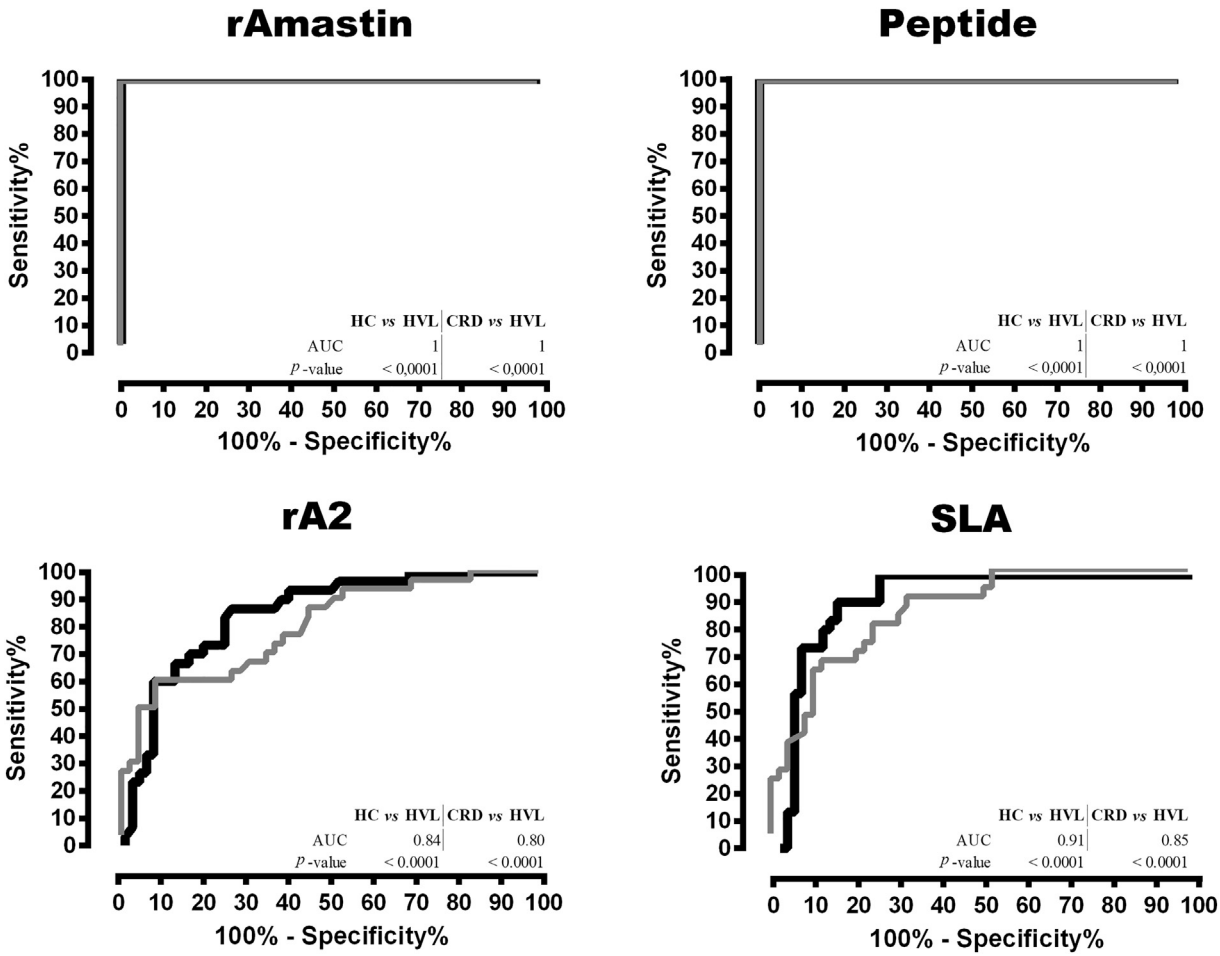

Fig. 6. ROC curves for the diagnosis of human leishmaniasis. ROC curves were constructed with the results obtained in the ELISAs with the human serological panel against the amastin protein, synthetic peptide, rA2, and *L. infantum* SLA. The sensitivity (95% CI), specificity (95% CI), and AUC values were determined and are shown.

biomarker for the diagnosis of human VL. Results showed that amastin-specific antibodies were present in VL patient sera, with a 95% reactivity. The authors suggested that this molecule could be considered a serological marker for human disease. In the present study, the recombinant amastin protein also showed high sensitivity and specificity values to detect VL patients, thus allowing one to infer about the biological role of this antigen in the identification of *L. infantum*–infected humans.

The production of recombinant proteins by cloning and heterologous expression in prokaryotic systems represents a relevant, but costly, alternative to preparing large amounts of purified antigens (Chávez-Fumagalli et al. 2013; Florez et al. 2017). By contrast, synthetic peptides, predicted as specific B-cell epitopes, could be considered since they are simpler, more stable, and less costly to produce than the recombinant proteins (Menezes-Souza et al. 2015; Noya et al. 2003). Amastins can be found conserved in distinct *Leishmania* spp. (Jackson 2010); as a consequence, a B-cell epitope was predicted to have a high structural homology between distinct parasite species but also presents a low homology when amino acid sequences from other trypanosomatides are evaluated. The synthetized epitope was tested for the serodiagnosis of VL, and results showed high sensitivity and specificity values to identify positive but not negative or cross-reactive sera, thus demonstrating the feasibility to use this molecule, when compared to the recombinant protein, for the serodiagnosis of the disease.

To the best of our knowledge, the present study is the first of its kind to evaluate the diagnostic role of both the amastin protein and a conserved and linear B-cell epitope for the serodiagnosis of CVL, as well as a possible immunological marker for human disease. The protein was also tested by using a cohort of Brazilian VL cases, and results corroborated those obtained by Rafati et al. (2006) using a cohort based on Iranian patient sera. On the other hand, although A2 proteins have also proven to be specific of *Leishmania* amastigotes (Resende et al.

Table 2
Evaluation of the antigens for the serodiagnosis of human leishmaniasis. A human serological panel comprised of sera samples from healthy individuals living in endemic or nonendemic areas of VL, as well as from VL, Chagas disease, paracoccidioidomycosis, leprosy, and aspergillosis patients, was used in ELISA experiments against the amastin protein and synthetic peptide. The rA2 protein and *L. infantum* SLA were used as controls. Results obtained were used to calculate sensitivity, specificity, 95% CI, AUC, and Youden index (*J*).

Antigen	HVL *versus* HC						HVL *versus* CRD					
	Cutoff	Se	95% CI	Sp	95% CI	*J*	Cutoff	Se	95% CI	Sp	95% CI	*J*
rAmastin	>0.3043	100	88.43–100	100	94.04–100	1.00	>0.2805	100	88.43–100	100	92,89–100	1.00
Peptide	>0.2079	100	88.43–100	100	94.04–100	1.00	>0.1880	100	88.43–100	100	92,89–100	1.00
rA2	>0.3860	86.67	69.28–96.24	73,33	60.34–83.93	0.60	>0.4558	60.00	40.60–77.34	92.00	80.77–97.78	0.52
SLA	>0.5333	100	88.43–100	75,00	62.14–85.28	0.75	>0.5703	90.00	73.47–97.89	68.00	53.30–80.48	0.58

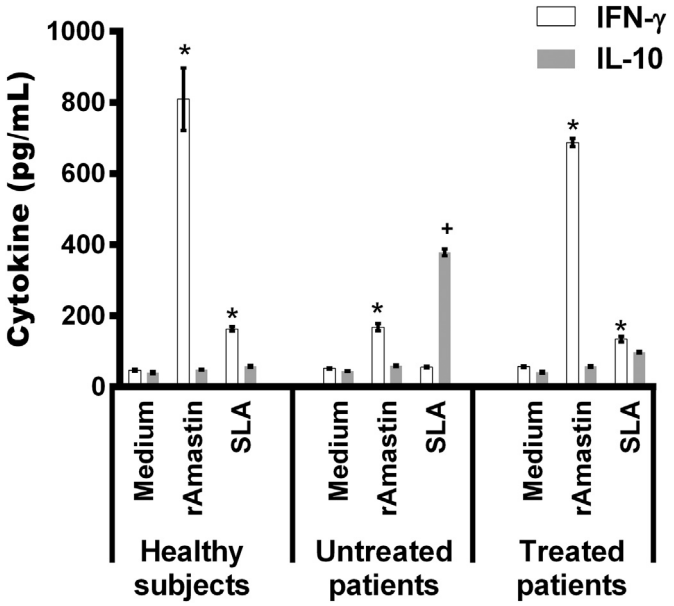

Fig. 7. Immunogenicity in PBMCs from untreated and treated VL patients. To evaluate the immunogenicity in human PBMCs from untreated and treated visceral leishmaniasis patients ($n = 6$ each), as well as from healthy subjects living in an endemic region of disease ($n = 6$), cells (10^6) were plated in 48-well flat-bottomed tissue culture plates (Costar, Cambridge, MA) and either incubated alone (medium) or stimulated with the amastin protein or SLA (10.0 and 25.0 μg/mL, respectively) for 5 days at 37 °C in 5% CO_2. Supernatants were collected, and IFN-γ and IL-10 production was evaluated by ELISA capture (Human IFN-γ and IL-10 ELISA Sets, BD Biosciences, USA) according to manufacturer's instructions. Bars indicate the mean ± standard deviation of the groups. *Statistically significant difference in relation to the SLA stimulus ($P < 0.0001$). +Statistically significant difference in relation to the rAmastin stimulus ($P < 0.0001$).

2008) and used in some studies for the serodiagnosis of canine and human disease (Akhoundi et al. 2013; Carvalho et al. 2002; Porrozzi et al. 2007), results obtained here showed worst sensitivity and specificity values when compared to the recombinant amastin protein used to identify both canine and human VL cases.

Some studies have used amastin proteins as vaccine candidates against trypanosomatides, such as *T. cruzi* (Minning et al. 2003; Teixeira et al. 1994) and *L. major* (Stober et al. 2006); however, there is a need to perform new studies to stimulate immune cells that come from sources other than those derived from mice. It is well known that, during active VL, a depressed cell-mediated immune response, characterized by the failure of patients' PBMCs to respond when stimulated by *Leishmania* antigens, is commonly found, and the production of cytokines, such as IFN-γ and IL-12, is hampered (Peruhype-Magalhães et al. 2005). On the other hand, when PBMCs from cured and/or treated patients are stimulated, they produce higher levels of proinflammatory cytokines (Singh et al. 2012). Here, PBMCs collected from untreated and treated VL patients were *in vitro* stimulated with the amastin protein, and IFN-γ and IL-10 production was measured in the cell culture supernatant. Our findings showed a significant IFN-γ response in treated patients when compared to the IL-10 levels. In addition, higher levels of IFN- were also found in healthy subjects living in an endemic region of disease. In fact, new studies are warranted, especially those using immune cells collected from a larger number of patients and controls; however, results obtained here suggest the occurrence of an immunogenic action based on the Th1 profile when cells from both patients and controls were stimulated with the amastin protein, opening the possibility of testing this protein in other more controlled studies against human VL.

In addition, the absence of a higher serological panel, as well as of other diagnostic platforms to test both the recombinant protein and synthetic peptides, could also be considered limiting factors of our study. However, the results presented here suggest that amastin can be considered a candidate for the serodiagnosis of canine and human VL, and suggest an immunogen role of this protein when it was used to stimulate immune cells derived from VL patients and healthy individuals.

Declaration of competing interest

The authors declare no commercial or financial conflict of interest.

Acknowledgments

The authors would like thank to CAPES, CNPq, and FAPEMIG for the scholarships. This work was supported by grants from CNPq (APQ-408675/2018-7).

References

Akhoundi B, Mohebali M, Shojaee S, Jalali M, Kazemi B, Bandehpour M, et al. 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 2013;133:307–13.

Almeida-Leal GG, Roatt BM, Oliveira Aguiar-Soares RD, Carneiro CM, Giunchetti RC, et al. Immunological profile of resistance and susceptibility in naturally infected dogs by *Leishmania infantum*. Vet Parasitol 2014;205:472–82.

Azizi H, Hassani K, Taslimi Y, Najafabadi HS, Papadopoulou B, Rafati S. Searching for virulence factors in the non-pathogenic parasite to humans Leishmania tarentolae. Parasitology 2009;136:723–35.

Burza S, Croft SL, Boelaert M. Leishmaniasis. Lancet 2018;392:951–70.

Carvalho AMRS, Costa LE, Salles BCS, Santos TTO, Ramos FF, Lima MP, et al. An ELISA immunoassay employing a conserved *Leishmania* hypothetical protein for the serodiagnosis of visceral and tegumentary leishmaniasis in dogs and humans. Cell Immunol 2017;318:42–8.

Carvalho FA, Charest H, Tavares CA, Matlashewski G, Valente EP, Rabello A, et al. Diagnosis of American visceral leishmaniasis in humans and dogs using the recombinant *Leishmania donovani* A2 antigen. Diagn Microbiol Infect Dis 2002;43:289–95.

Chaabouni A, Boubaker Elandoulsi R, Mhadhbi M, Gharbi M, Sassi A. Comparative analysis of the *Leishmania infantum*-specific antibody repertoires and the autoantibody repertoires between asymptomatic and symptomatic dogs. Vet Parasitol 2018;261:9–17.

Chávez-Fumagalli MA, Martins VT, Testasicca MC, Lage DP, Costa LE, Lage PS, et al. 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 2013;20:835–41.

Coelho EAF, Tavares CAP, Carvalho FAA, Chaves KF, Teixeira KN, Rodrigues RC, et al. 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 2003;71:3988–94.

Coelho VTS, Oliveira JS, Valadares DG, Chávez-Fumagalli MA, Duarte MC, Lage PS, et al. Identification of proteins in promastigote and amastigote-like *Leishmania* using an immunoproteomic approach. PLoS Negl Trop Dis 2012;6, e1430.

Dias DS, Martins VT, Ribeiro PAF, Ramos FF, Lage DP, Tavares GSV, et al. Antigenicity, immunogenicity and protective efficacy of a conserved *Leishmania* hypothetical protein against visceral leishmaniasis. Parasitology 2017;8:1–12.

Doyle PS, Engel JC, Pimenta PFP, Silva PP, Dwyer DM. *Leishmania donovani*: long-term culture of axenic amastigotes at 37°C. Exp Parasitol 1991;73:326–34.

Duthie MS, Lison A, Courtenay O. Advances toward diagnostic tools for managing zoonotic visceral leishmaniasis. Trends Parasitol 2018;34:881–90.

Farahmand M, Nahrevanian H. Application of recombinant proteins for serodiagnosis of visceral leishmaniasis in humans and dogs. Iran Biomed J 2016;20:128–34.

Fernandes AP, Coelho EAF, Machado-Coelho GLL, Grimaldi JrG, Gazzinelli RT. Making an anti-amastigote vaccine for visceral leishmaniasis: rational, update and perspectives. Curr Opin Microbiol 2012;15:1–10.

Ferreira EC, Lana M, Carneiro M, Reis AB, Paes DV, Silva ES, et al. Comparison of serological assays for the diagnosis of canine visceral leishmaniasis in animals presenting different clinical manifestations. Vet Parasitol 2007;146:235–41.

Florez MM, Oliveira CI, Puerta C, Guzman F, Ayala M, Montoya G, et al. Synthetic peptides derived from ribosomal proteins of *Leishmania* spp. in mucocutaneous leishmaniasis: diagnostic usefulness. Protein Pept Lett 2017;24:982–8.

Gish W, States DJ. Identification of protein coding regions by database similarity search. Nat Genet 1993;3:266–72.

Jackson AP. The evolution of amastin surface glycoproteins in trypanosomatid parasites. Mol Biol Evol 2010;27:33–45.

Lage DP, Martins VT, Duarte MC, Garde E, Chávez-Fumagalli MA, Menezes-Souza D, et al. Prophylactic properties of a *Leishmania*-specific hypothetical protein in a murine model of visceral leishmaniasis. Parasite Immunol 2015;37:646–56.

Magalhães FB, Castro-Neto AL, Nascimento MB, Santos WJT, Medeiros ZM, Neto ASL, et al. Evaluation of a new set of recombinant antigens for the serological diagnosis of human and canine visceral leishmaniasis. PLoS One 2017;12, e0184867.

Maia CS, Monteiro MC, Gavioli EC, Oliveira FR, Oliveira GB, Romão PR. Neurological disease in human and canine leishmaniasis: clinical features and immunopathogenesis. Parasite Immunol 2015;37:385–93.

Mendonça DVC, Lage DP, Calixto SL, Ottoni FM, Tavares GSV, Ludolf F, et al. Antileishmanial activity of a naphthoquinone derivate against promastigote and amastigote stages of *Leishmania infantum* and *Leishmania amazonensis* and its mechanism of action against *L. amazonensis* species. Parasitol Res 2018;117:391–403.

Menezes-Souza D, Mendes TAO, Leão ACA, Gomes MS, Fujiwara RT, Bartholomeu DC. Linear B-cell epitope mapping of MAPK3 and MAPK4 from *Leishmania braziliensis*: implications for the serodiagnosis of human and canine leishmaniasis. Appl Microbiol Biotechnol 2015;99:1323–36.

Mettler M, Grimm F, Capelli G, Camp H, Deplazes P. Evaluation of enzyme-linked immunosorbent assays, an immunofluorescent-antibody test, and two rapid tests (immunochromatographic-dipstick and gel tests) for serological diagnosis of symptomatic and asymptomatic *Leishmania* infections in dogs. J Clin Microbiol 2005;43:5515–9.

Michel G, Pomares C, Ferrua B, Marty P. Importance of worldwide asymptomatic carriers of *Leishmania infantum (L. chagasi)* in human. Acta Trop 2011;119:69–75.

Minning TA, Bua J, Garcia GA, McGraw RA, Tarleton RL. Microarray profiling of gene expression during trypomastigote to amastigote transition in *Trypanosoma cruzi*. Mol Biochem Parasitol 2003;131:55–64.

Monteiro FM, Machado AS, Rocha-Silva F, Assunção CB, Graciele-Melo C, Costa LE, et al. Canine visceral leishmaniasis: detection of *Leishmania* spp. genome in peripheral blood of seropositive dogs by real-time polymerase chain reaction (RT-PCR). Microb Pathog 2018;126:263–8.

Nogueira CT, Cistia MLD, Urbaczek AC, Jusi MM, Velásquez AMA, Machado RZ, et al. Potential application of rLc36 protein for diagnosis of canine visceral leishmaniasis. Mem Inst Oswaldo Cruz 2018;113:197–201.

Noya O, Patarroyo ME, Guzmán F, Alarcón de Noya B. Immunodiagnosis of parasitic diseases with synthetic peptides. Curr Protein Pept Sci 2003;4:299–308.

Nunes CM, Lima VM, Melo GD, Paula HB, Pereira ME, Tronco CM, et al. Serological, parasitological and molecular tests for canine visceral leishmaniosis diagnosis in a longitudinal study. Rev Bras Parasitol Vet 2015;24:402–9.

Nunes JB, Coura-Vital W, Colombo FA, Baêta FJM, Pinheiro AC, Roatt BM, et al. Comparative analysis of real-time PCR assays in the detection of canine visceral leishmaniasis. Parasitol Res 2018;117:3341–6.

Peruhype-Magalhães V, Martins-Filho OA, Prata A, Silva LA, Rabello A, Teixeira-Carvalho A, et al. Immune response in human visceral leishmaniasis: analysis of the correlation between innate immunity cytokine profile and disease outcome. Scand J Immunol 2005;62:487–95.

Pinheiro PH, Pinheiro AN, Ferreira JH, Costa FA, Katz S, Barbiéri CL. A recombinant cysteine proteinase from *Leishmania (Leishmania) chagasi* as an antigen for delayed-type hypersensitivity assays and serodiagnosis of canine visceral leishmaniasis. Vet Parasitol 2009;162:32–9.

Porrozzi R, Costa MVS, Teva A, Falqueto A, Ferreira AL, Santos CD, et al. 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 2007;14:544–8.

Rafati S, Hassani N, Taslimi Y, Movassagh H, Rochette A, Papadopoulou B. Amastin peptide-binding antibodies as biomarkers of active human visceral leishmaniasis. Clin Vaccine Immunol 2006;13:1104–10.

Resende DM, Caetano BC, Dutra MS, Penido ML, Abrantes CF, Verly RM, et al. Epitope mapping and protective immunity elicited by adenovirus expressing the *Leishmania* amastigote specific A2 antigen: correlation with IFN-gamma and cytolytic activity by CD8+ T cells. Vaccine 2008;26:4585–93.

Saha S, Raghava GPS. Prediction of continuous B-cell epitopes in an antigen using recurrent neural network. Proteins 2006;65:40–8.

Salles BCS, Dias DS, Steiner BT, Lage DP, Ramos FF, Ribeiro PAF, et al. 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. Immunobiology 2019;224:163–71.

Santos RCD, Pinho FA, Passos GP, Larangeira DF, Barrouin-Melo SM. Isolation of naturally infecting *Leishmania infantum* from canine samples in Novy-MacNeal-Nicolle medium prepared with defibrinated blood from different animal species. Vet Parasitol 2018;257:10–4.

Savoia D. Recent updates and perspectives on leishmaniasis. J Infect Dev Ctries 2015;9:588–96.

Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol Syst Biol 2011;7:539.

Singh OP, Stober CB, Singh AK, Blackwell JM, Sundar S. Cytokine responses to novel antigens in an Indian population living in an area endemic for visceral leishmaniasis. PLoS Negl Trop Dis 2012;6, e1874.

Solano-Gallego L, Miró G, Koutinas A, Cardoso L, Pennisi MG, Ferrer L, et al. LeishVet guidelines for the practical management of canine leishmaniosis. Parasit Vectors 2011;4:86.

Solcà MS, Bastos LA, Guedes CE, Bordoni M, Borja LS, Larangeira DF, et al. Evaluating the accuracy of molecular diagnostic testing for canine visceral leishmaniasis using latent class analysis. PLoS One 2014;9, e103635.

Souza-Filho JA, Barbosa JR, Figueiredo FB, Júnior Mendes AA, Silva SR, et al. Performance of Alere™ immunochromathographic test for the diagnosis of canine visceral leishmaniasis. Vet Parasitol 2016;225:114–6.

Stober CB, Lange UG, Roberts MT, Gilmartin B, Francis R, Almeida R, et al. From genome to vaccines for leishmaniasis: screening 100 novel vaccine candidates against murine *Leishmania major* infection. Vaccine 2006;24:2602–16.

Subramanian A, Sarkar RR. Perspectives on *Leishmania* species and stage-specific adaptive mechanisms. Trends Parasitol 2018;34:1068–81.

Sundar S, Singh OP. Molecular diagnosis of visceral leishmaniasis. Mol Diagn Ther 2018;22:443–57.

Teixeira SM, Russell DG, Kirchhoff LV, Donelson JE. A differentially expressed gene family encoding "amastin," a surface protein of Trypanosoma cruzi amastigotes. J Biol Chem 1994;269:20509–16.

Travi BL, Cordeiro-da-Silva A, Dantas-Torres F, Miró G. Canine visceral leishmaniasis: Diagnosis and management of the reservoir living among us. PLoS Negl Trop Dis 2018;12, e0006082.

World Health Organization. Leishmaniasis. http://www.who.int/topics/leishmaniasis/en/, 2016.

Zhang WW, Charest H, Ghedin E, Matlashewski G. Identification and overexpression of the A2 amastigote-specific protein in *Leishmania donovani*. Mol Biochem Parasitol 1996;78:79–90.