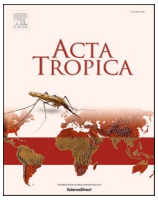
Serodiagnosis of leishmaniasis in asymptomatic and symptomatic dogs by use of the recombinant dynamin-1-like protein from Leishmania infantum: A preliminary study

Acta Tropica 239 (2023) 106827

Contents lists available at ScienceDirect

Acta Tropica

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



Serodiagnosis of leishmaniasis in asymptomatic and symptomatic dogs by use of the recombinant dynamin-1-like protein from *Leishmania infantum*: A preliminary study

Williane Fernanda Siqueira [a,d,1], Mariana Santos Cardoso [b,1], Marianna de Carvalho Clímaco [b,1], Ana Luiza Teixeira Silva [c], Benjamin Heidt [d], Kasper Eersels [d], Bart van Grinsven [d], Daniella Castanheira Bartholomeu [b], Lilian Lacerda Bueno [a,b], Thomas Cleij [d], Ricardo Toshio Fujiwara [a,b,*]

[a] *Programa de Pós-graduação em Ciências da Saúde: Infectologia e Medicina Tropical, Faculdade de Medicina, Universidade Federal de Minas Gerais, Belo Horizonte MG, Brazil*
[b] *Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte MG, Brazil*
[c] *UNIFESP - Universidade Federal de São Paulo, São Paulo SP, Brasil*
[d] *Sensor Engineering Department, Faculty of Science and Engineering, Maastricht University, Maastricht, Limburg, Netherlands*

ARTICLE INFO

Keywords:
Canine leishmaniasis
Immunodiagnostic
Recombinant antigens
Asymptomatic dogs
Symptomatic dog
L. infantum

ABSTRACT

Visceral leishmaniasis (VL) is a fatal manifestation of an infection caused by intracellular protozoa of the *Leishmania* genus. In New World countries, VL is classified as a zoonotic disease with domestic dogs acting as its main reservoir. Asymptomatic dogs are as competent to transmit *Leishmania* to the vectors as symptomatic dogs, however current diagnostic tests are limited and present low sensitivity for this important group. The development of accurate tests is fundamental to the early diagnosis, treatment, and control of canine leishmaniasis. In this study, we investigated the use of a recombinant protein (dynamin-1-like protein, Dyn-1) from *L. infantum,* as a potential target antigen for leishmaniasis serodiagnosis in both symptomatic and asymptomatic dogs. The antigenic performance of the protein was evaluated by means of ELISA assays using sera from symptomatic (n = 25), asymptomatic (n = 34) and non-infected dogs (n = 36) using ELISA. In addition, sera from dogs experimentally infected with *Trypanosoma cruzi* (n = 49) and naturally infected with *Babesia* sp. (n = 8) were tested to evaluate possible cross-reactivity. A crude soluble antigen (CSA) of *Leishmania* was used as an antigen control and K39 and K26 were used as reference antigens because they are already widely used in commercial tests. rDyn-1-based assay showed the highest sensitivity (97%) compared to the antigens K39 (88%), K26 (86%) and crude extract (95%). The highest specificity among the tests was also obtained with the protein rDyn-1 (94%), compared with the other antigens K39 (81%), K26 (87%), and crude extract (77%). This study showed that the rDyn-1 ELISA assay was able to identify 100% of asymptomatic dogs, establishing its potential as a target for the diagnosis of canine leishmaniasis.

1. Introduction

Visceral leishmaniasis (VL) still holds an endemic status in approximately 80 countries worldwide, remaining one of the highest mortality rate parasitic diseases (IHME, 2020). Transmission occurs almost exclusively by sandfly bite, particularly in areas where the precariousness of housing and basic sanitation favors vector reproduction and the maintenance of the infection cycle (Luigi, 2018). This complex cycle can vary according to the geographic region, species of *Leishmania*, and vertebrate and invertebrate hosts involved (Anversa et al., 2018). In Old World countries where VL is caused by *Leishmania donovani*, the disease is classified as an anthroponosis since it occurs mostly in the absence of reservoirs. On the other hand, in the New World, leishmaniasis is considered a zoonosis caused by *L. infantum* species, which inherently

* Corresponding author.
E-mail address: fujiwara@icb.ufmg.br (R.T. Fujiwara).
[1] Equal contribution.

https://doi.org/10.1016/j.actatropica.2023.106827
Received 17 October 2022; Received in revised form 23 December 2022; Accepted 3 January 2023
Available online 5 January 2023

establishes the importance of reservoirs in the maintenance and transmission of the disease (Ready, 2014).

Among wild and urban animals, dogs (*Canis familiaris*) are considered the main reservoir of leishmaniasis due to their close relationship with humans (Soares et al., 2022) combined with the fact that most infections remain asymptomatic and therefore are not timely treated (Roque and Jansen, 2014). Similar to it occurs with humans, clinical diagnosis of canine leishmaniasis is very difficult as there is often a lack of pathognomonic clinical signs (Ciaramella et al., 1997; Molina et al., 2020; Pinelli et al., 1994). In addition, not every infected animal develops clinical manifestations (Maia and Campino, 2008). In this context, stand out mainly dogs residing or staying for a long time in areas considered endemic for leishmaniasis, without clinical signs compatible with the disease and that present positive diagnostic in a combination of tests (serological, molecular, and/or parasitological) characterized as asymptomatics (Ibarra-Meneses et al., 2022). Dogs with asymptomatic infections are just as important as dogs with overt clinical manifestations for many reasons. Despite low levels of circulating parasites often observed in asymptomatic dogs they are as capable of transmitting the parasite to the vector with a high rate of parasitic infection as well as symptomatic dogs (Laurenti et al., 2013). Thus, these animals play an important role in maintaining the cycle and dissemination of the disease, mainly in endemic areas, presenting a risk to public health (Moshfe et al., 2009). Concerning the diagnosis, even though asymptomatic dogs are positive for *Leishmania*, current serological tests present limitations to identify these animals (Medeiros et al., 2017; Moreno et al., 2009). Therefore, a sensitive and specific test that successfully detects both symptomatic and asymptomatic infections is highly desirable.

Several serological tests are commonly used for the diagnosis of leishmaniasis and also are valuable tools in epidemiological studies, disease control programs, field studies, and laboratories (Zijlstra, 2021). The enzyme-linked immunosorbent assay (ELISA) (Badaro et al., 1993), the latex agglutination test (LAT), the direct agglutination test (DAT) (Harith et al., 1986), and Immunochromatographic tests (ICT) (da Costa et al., 2003) are some tools currently used for this purpose. DAT has been used extensively over the last decade for the serological diagnosis of leishmaniasis as well as in seroepidemiological studies particularly in Iran and some African countries (Mohebali, 2013; Mohebali et al., 2006). Attributes such as simplicity and safety, good specificity and sensitivity, economy, and field applicability have led to extensive application of DAT in leishmaniasis endemic regions of Iran (Mohebali et al., 2020, 2018). DAT is a semi-quantitative assay based on the agglutination of biological material when in contact with the dead *Leishmania* sp parasite stained (Kühne and Büscher, 2019). However, as with other serological tests, the DAT has limitations that must be considered (Molaie et al., 2017). Unlike DAT, ELISA has as its principle the coating of plates with antigens of interest that, when in contact with positive samples, are quantified using immunoglobulins labeled with enzymes (Kühne and Büscher, 2019). ELISA and ICT that use crude soluble antigens (CSA) (Reithinger et al., 2002; Rosário et al., 2005) or recombinant proteins from *Leishmania* (Farahmand and Nahrevanian, 2016; Siripattanapipong et al., 2017) are commonly employed for leishmaniasis diagnosis (Ibiapina et al., 2022; Lévêque et al., 2020). Among the antigens explored, kinesin-derived proteins such as rK39 (Burns et al., 1993), rKDDR (Dhom-Lemos et al., 2019) and rKDDR-plus (Siqueira et al., 2021), are excellent targets with the ability to discriminate both human and canine VL from healthy individuals (Burns et al., 1993; Dhom-Lemos et al., 2019; Siqueira et al., 2021). However, the diagnostic performance of assays targeting kinesin-derived proteins may vary according to the geographical region surveyed and, for canine leishmaniasis, according to the clinical sings (Zijlstra et al., 1992, 2001).

In the search for new recombinant molecules capable of improving the accuracy of serological tests, previous studies performed in our laboratory have demonstrated that housekeeping genes of *Leishmania* have a high potential for the serodiagnosis of leishmaniasis (Menezes-Souza et al., 2015, 2014). In this study, we evaluate the performance of a new antigen - rDyn-1 and compare it with crude soluble antigens (CSA) from the culture of *L. infantum* promastigotes, rK39 and rK26 proteins, which are widely used in diagnostic kits. The results obtained in this study demonstrate that ELISA test based on rDyn-1 identified all serum samples from asymptomatic dogs identifying animals in early stages with high sensitivity rates. Additionally, when compared to the other antigens evaluated, rDyn-1 showed less cross-reactivity in ELISA using sera from animals infected with other relevant parasites, such as *T. cruzi* and *Babesia* sp. Taken together, our results demonstrate that rDyn-1 is a highly interesting antigen for CanL immunodiagnostic, especially in asymptomatic dogs.

2. Material and methods

2.1. Ethics statement and study design and population

The use of the samples in this study was approved by the Institutional Animal Care and Ethics Committee on Animal Use (CEUA) from the Federal University of Minas Gerais (protocol number 44/2012).

The sera panel consisted of 59 samples from dogs naturally infected with *L. infantum* (25 symptomatic and 34 asymptomatic) obtained from the endemic area for CanL in Montes Claros, Minas Gerais State, Brazil. To confirm the positivity of the animals, samples of bone marrow (BM) and whole blood from all animals were collected. Part of the bone marrow material collected was were cultivated in Novy-Mac Neal-Nicolle (NNN) medium supplemented with 20% FBS (Fetal Bovine Serum, Gibco BRL, New York, USA) and 100 μg/mL of gentamicin. The cultures were maintained at 24 °C for four weeks and examined weekly for the presence of parasites (Barrouin-Melo et al., 2004). The remainder of the BM together with the whole blood samples were subjected to DNA extraction techniques followed by specific qPCR assays for Leishmania kDNA. The qPCR method for evaluating the parasitic load (DNA copies/μL) in the samples was performed using the qPCR method (Real-Time PCR with TaqMan Probe) based on a probe with a standard curve and direction of the genome kDNA minicircle sequence from *L. infantum* (TECSA Laboratories®, Belo Horizonte, Minas Gerais, Brazil). The absence of clinical signs suggestive of *Leishmania* infection was used to define dogs with asymptomatic clinical status. On the other hand, dogs that presented some of the most common signs of the disease such as skin changes (alopecia, furfuraceous eczema, ulcers, hyperkeratosis), onychogryphosis, weight loss, keratoconjunctivitis and hindlimb paresis were characterized as symptomatic. It used 36 sera from non-infected dogs (negative samples) from non-endemic areas for the disease confirmed by qPCR assays, specific for Leishmania kDNA, from peripheral blood and serological tests (ELISA and ICT) from serum. To evaluate possible cross-reactivity and coinfection cases 49 samples from dogs experimentally infected with *T. cruzi* and 8 from dogs naturally infected with *Babesia* sp. were tested. Serum samples infected with T. cruzi were kindly provided from the Department of Clinical Analysis of the School of Pharmacy/UFOP. The dogs were inoculated with 2.0×10^3 bloodstream trypomastigotes per kg of body weight belonging to two strains. The Y strain (DTU TcII), isolated from an acute human case of Chagas' disease and the Berenice-78 (Be-78) strain (DTU TcII) isolated by xenodiagnosis of a patient with an indeterminate form of the disease. The positivity to *T. cruzi* was confirmed by hemoculture or by combined positivity indicated by Chagatest-ELISA Recombinante version 3.0 kit (Wiener Laboratorios, Santa Fé, Argentina) and Chagatest Indirect Hemagglutination Assay (IHA; Wiener Laboratorios).

Samples from dogs naturally infected with Babesia sp. were kindly provided from a private veterinary laboratory (Contagem/Minas Gerais State, Brazil). The infection with *Babesia* sp. was confirmed by real-time PCR performed after DNA extraction from EDTA-anti-coagulated blood samples in the commercial laboratory. Approximately 6 ml of blood per animal were collected from the saphenous vein of the hind limb or the cephalic vein of the forelimb. The collected blood was distributed into

two collection tubes (3.0 ml in each tube), one containing EDTA anticoagulant and the other without anticoagulant for serum separation. Blood stored in EDTA tubes was pipetted and aliquoted into microtubes. The blood contained in the tubes without anticoagulant was centrifuged at 2500 rpm for 10 min and the serum resulting from the centrifugation was pipetted and aliquoted into new tubes. Subsequently, all samples were stored at −80 °C.

2.2. Selection of the protein and analysis and linear B-cell epitope prediction

Dynamins are guanosine triphosphatases (GTPase) that are related to several processes linked to membrane dynamics and functioning, having an especially important role in endocytosis (Daumke and Praefcke, 2016). The dynamin superfamily in eukaryotic cells includes several molecules involved in numerous intracellular membrane trafficking events, including the Dyn-1 (dynamin-like proteins) (Kar et al., 2017). The sequence of the dynamin-1-like protein, from *L. infantum* (ID: LINF_290,029,700), starin JPCM5, used in this work were obtained from the predicted proteomes available in the TriTrypDB database (http://tritrypdb.org), from which a sub-bank was generated after the exclusion of pseudogenes and partial genes. For the selection of possible targets for the serodiagnosis of leishmaniasis, *L. infantum* proteins were chosen based on their similarity with protein sequences related to the host's immune system and/or pathogen defense, deposited on the ImmunoneBase database (Dantas-Torres et al., 2019). Similarity analysis was performed using the BLASTp algorithm (Altschul et al., 1990, 1997), with an e-value cutoff of less than $1e^{-50}$ was employed for the NCBI Reference Sequence (RefSeq) database. Linear B-cell epitopes were predicted using the BepiPred 1.0 program (http://www.cbs.dtu.dk/services/BepiPred-1.0/) (Larsen et al., 2006), with a cutoff of 0.8. Intrinsically unstructured/disordered regions were predicted by IUPred program (http://iupred.elte.hu/) (Dosztányi et al., 2005) with a cut-off of 0.5.

2.3. Total extract of L. infantum

A total extract of the parasite was prepared from *L. infantum* (MHOM/BR/1974/PP75) promastigotes maintained at 24 °C in Schneider's Insect Medium (Sigma-Aldrich, USA), supplemented with 10% inactivated fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco/Thermo Fisher Scientific, USA). Approximately 5×10^8 parasites were washed and resuspended in 1 mL PBS, followed by lysis through 15 cycles of freezing in liquid nitrogen and thawing at 37 °C. The concentration of the total proteins of the parasite was quantified using the Pierce BCA Protein Assay (Thermo Fisher Scientific, USA).

2.4. Cloning, protein expression and purification

The primers used to amplify the entire coding region of the dynamin-1-like protein from *L. infantum* (rDyn-1) were Forward, 5′ ACT**CATATG**GACCAGTTGATCAGCGTGATC 3′ and Reverse, 5′ GTT**GTCGAC**TTAGGCGCCGGCTTGCATGGAC 3′. Restriction enzyme (*Nde*I and *Sal*I, respectively) sites that were added to the sequence to facilitate cloning are shown in bold. The resulting 2.1 kb DNA fragment was excised from an agarose gel, purified, and linked into the pGEM-T Easy vector (Promega, USA). Recombinant plasmid pGEM-rDyn-1 was used to transform *Escherichia coli* XL1-Blue competent cells (Phoneutria, Brazil). Positive clones were confirmed by colony PCR using M13 universal primers, and later for constructing the expression vector. The DNA fragment obtained from the digestion of pGEM-rDyn-1 with *Nde*I and *Sal*I was ligated into pET28a-TEV, previously digested with the same enzymes. The pET28a-TEV expression vector contains a sequence encoding a histidine tag, in their N-terminal region, to facilitate the protein purification process. Electrocompetent *E. coli* ArcticExpress (DE3) cells (Agilent Technologies, USA) were transformed by electroporation using a MicroPulser Electroporation Apparatus (Bio-Rad Laboratories, USA) with the recombinant plasmid pET28a-TEV-rDyn-1. Gene insertion was confirmed by colony PCR and sequencing, using T7 universal primers (Macrogen, South Korea).

Expression of the recombinant rDyn-1 protein was induced in the positive clones by the addition of 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Promega, USA), for 24 h at 200 rpm and 12 °C. Cells were ruptured by sonication and centrifuged at 6000 × g for 30 min at 4°C. The recombinant protein was purified using a HisTrap HP affinity column connected to the ÄKTAprime chromatography system (GE Healthcare, USA).

2.5. ELISA serological assay

Through tests, ELISA evaluated the performance of Dyn-1 protein and the other antigens. The rK39 and rK26 antigens were kindly provided by Steven G. Reed (Infectious Disease Research Institute-IDRI, Seattle, Washington). The rDyn-1 recombinant protein, as well as total *L. infantum* extract and the kinesin-derived proteins rK26 and rK39, widely used in commercial diagnostic kits, were coated onto 96-well microplates (Nalge Nunc Intl., USA) overnight at 2–8 °C, with an amount of 50 ng/well for rDyn-1, 1000 ng/well for total extract, and 100 ng/well for rK39 and rK26. The plates were blocked with 200 μL of 2% PBS-BSA for 1 h at 37 °C and treated successively with 1:100 dilutions of the canine serum samples for 1 h at 37 °C. Peroxidase-labeled antibodies specific to dog IgG (Sigma-Aldrich, USA) were diluted at 1:5000 and added for 1 h at 37 °C. The wells were washed, and *o*-phenylenediamine dihydrochloride (OPD) substrate (Sigma-Aldrich, USA) in citrate buffer containing hydrogen peroxide (Sigma-Aldrich, USA) was added. The plates were incubated for 20 min in the dark and reactions were stopped by the addition of 4 N H_2SO_4. The absorbance at 492 nm was determined on an automatic microplate reader (Versamax, Molecular Devices, USA). Each sera sample was assayed in duplicate. The results of the ELISA using rDyn-1 as antigens were compared with total *L. infantum* extract, rK26 and rK39.

2.6. Statistical analysis

Results were entered into Microsoft Excel (version 10.0) spreadsheets and analyzed with GraphPad Prism™ software (version 6.0 for Windows). The cut-off values were calculated by constructing Receiver Operating Characteristic (ROC) curves, which were plotted with the individual OD values of CanL (symptomatic and asymptomatic) dogs group versus those from the other (healthy dogs, with *T. cruzi* and *Babesia* sp.) groups. ROC curves were used to calculate: sensitivity, specificity, area under the curve (AUC), 95% confidence interval (95% CI), positive predictive value (PPV), and negative predictive value (NPV). Through the ROC curve was possible to evaluate all the combinations of sensibility and specificity and determine the best cut-off value based on the most appropriate sensitivity and specificity ratio for each antigen tested. Agreement beyond chance was assessed using the kappa coefficient (95% CI) and interpreted according to the following scale: 0.00 to 0.20 (negligible), 0.21 to 0.40 (weak), 0.41 to 0.60 (moderate), 0.61 to 0.80 (good), and 0.81 to 1.00 (Very good).

3. Results

3.1. Selection of the L. infantum dynamin-1-like protein

For the selection of a potential target of *L. infantum* for use in the serological diagnosis of CanL, proteins of the parasite with protein sequences related to the host's immune system, deposited in the ImmunoneBase database, were analyzed. The dynamin-1-like protein from *L. infantum* (rDyn-1) was selected for presenting similarities with proteins associated with the host defense processes.

3.2. Prediction of linear B-cell epitopes and structural disorder of the rDyn-1

To assess *in silico* the antigenic potential of the rDyn-1 protein, the complete amino acid sequence was inserted in the BepiPred and IUPred programs, to predict linear B cell epitopes and structural disorder respectively. The protein showed nine linear B cell epitopes, as well as disordered regions (Fig. 1). Since the prediction of B cell epitopes is based on the linear sequence of the protein (primary sequence), the overlap of regions of structural disorder with predicted B cell epitopes increases the possibility that this region of the protein will be recognized by lymphocytes from the immune system of the host.

3.3. Expression and purification of the rDyn-1 protein

The full-length coding region of the *L. infantum* rDyn-1 protein was amplified by PCR, cloned into the vector pGEM, and confirmed by sequencing. The gene was then transferred to the pET28a-TEV expression vector, and the rDyn-1 was expressed in *E. coli* BL21 and purified by affinity chromatography. A 78 kDa band referring to the rDyn-1 protein can be observed by polyacrylamide gel electrophoresis (SDS-PAGE) of the expression in bacteria and the purified fraction (Supplementary Fig. 1).

3.4. Serological recognition of rDyn-1 by ELISA for the diagnosis of canine leishmaniasis

After purification by affinity chromatography, the recombinant rDyn-1 protein (LINF_290,029,700) was used as an antigen in an ELISA experiment to assess its reactivity against canine sera. The rDyn-1 protein showed an improved performance against canine sera in the ELISA experiment, when compared to the antigens K26 and K39 and the crude extract of *L. infantum* (Fig. 2). The assay using rDyn-1 as an antigen proved to be the only one capable of detecting 100% of asymptomatic infected dogs for CanL and also showed the lowest percentage of cross-reactivity with dogs infected with *T. cruzi* and *Babesia* sp. The K39, K26 and crude extract antigens identified only 79%, 76% and 94% of asymptomatic dogs, respectively, and showed greater cross-reactivity mainly with dogs infected with *T. cruzi*. The antigens K39 and K26 were also recognized 6% of healthy dogs (negative control). The ROC curve was used to determine the cut-off for each antigen and the area under the curve (AUC) was obtained (Fig. 3). The rDyn-1 protein assay showed the highest AUC value (0.9858), followed by K26, rK39 and the crude extract of *L. infantum*, which presented the values 0.9405, 0.9196, and 0.8917, respectively.

When examining the diagnostic performance of the assays, the rDyn-1-based assay showed the highest sensitivity and specificity (97% [IC 95%; 88.29%–99.59%] and 94% [IC 95%; 86.48–97.60%], respectively) compared to the essays using antigens K39 (88% [IC 95%; 77.07–95.09%] and 81% [IC 95%; 71.15–88.11%], respectively), K26 (86% [IC 95%; 75.02–93.96%] and 87% [IC 95%;78.55–93,15%], respectively) and crude extract (95% [IC 95%; 85.85–98.94%] and 77% [IC 95%; 67.58–85.45%], respectively) (Table 1). In addition, accuracy, PPV and NPV values the rDyn-1 antigen were also bigger 95%, 90% and 98%, respectively compared to the other antigens. The highest kappa agreement index was also obtained by the rDyn-1 protein (0.891) (Table 2), showing an almost very good degree of agreement, suggesting its potential use for canine serodiagnosis.

4. Discussion

Dogs with asymptomatic *Leishmania* infections are more present than symptomatic dogs in the population of endemic areas. However, research has shown there is no statistically significant difference in the rate of vector infectivity between symptomatic and asymptomatic dogs (Alvar et al., 2004; Molina et al., 1994). In those regions, infected but asymptomatic dogs may not be adequately diagnosed by current serological tests, due to the low number of antibodies produced by the host's immune system or because it is the initial stage of the disease (García-Castro et al., 2022). In Brazil, the governmental policy to control human infection based on the identification of seropositive dogs (Dantas-Torres et al., 2019; Nunes et al., 2010). The Brazilian Ministry of Health officially established a rapid chromatographic immunoassay for canine survey based on dual path platform (DPP®) for screening of the disease and ELISA as a confirmatory test (Fraga et al., 2016). Serologic tests, currently, are methods of choice in population studies because it is a relatively simple and fast tool that can be performed manually or automated (Lévêque et al., 2020; Singh, 2006), and it allows a large number of samples to be analyzed simultaneously in a short period. The key to controlling *Leishmania* infection and associated diseases in the human population, therefore, it's in the development of new, improved serological tests that are able to detect *Leishmania* infection in one of their main reservoirs, dogs that do not display any clear symptoms of infection (Travi et al., 2018).

The prevalence of CanL is often underestimated, mainly due to the fact that the detection of antibodies in dogs considered asymptomatic or with recent infections is generally lower in serological tests (Dye et al., 1993). For years, crude extracts have been used in the serological diagnosis of leishmaniasis mainly in ELISA-based assays. Despite the relatively low cost of production, our results indicate that although ELISA assays using crude soluble antigens demonstrated a relatively high degree of sensitivity when used to identify both asymptomatic and symptomatic dogs (95% and 94%, respectively) their major limitation was the high rate of cross-reaction of dog sera with *T. cruzi*. Resulting in sub-optimally low specificity levels (77%). The use of recombinant antigens, such as kinesin-derived proteins in the serological diagnosis of CanL allowed a better standardization of the assays and therefore more robust results. Among them, the rK39 (Burns et al., 1993) is the most widely used protein from CanL testing. However, literature data indicate that the protein rK39 is more sensitive for the diagnosis of symptomatic cases (100%) compared to asymptomatic (66%) (Porrozzi et al., 2007). The results obtained in the work confirm that rK39 antigen-based assays have a good capacity to identify dogs with active disease (100%), but their performance drops considerably when detecting asymptomatic

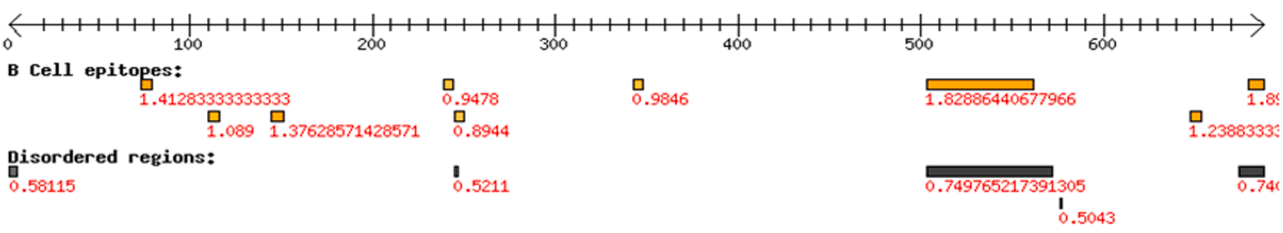


Fig. 1. Predictions of linear B-cell epitopes and structural disorder regions of the dynamin-1-like protein (rDyn-1) from *L. infantum*. The dashed arrow represents the complete amino acid sequence of the rDyn-1 protein (LINF_290,029,700). The linear epitopes of B cells, predicted by the BepiPred program, are represented by orange boxes. The regions of structural protein disorder, predicted by the IUPred program, are represented by gray boxes. The value below each box corresponds to the score of each prediction.

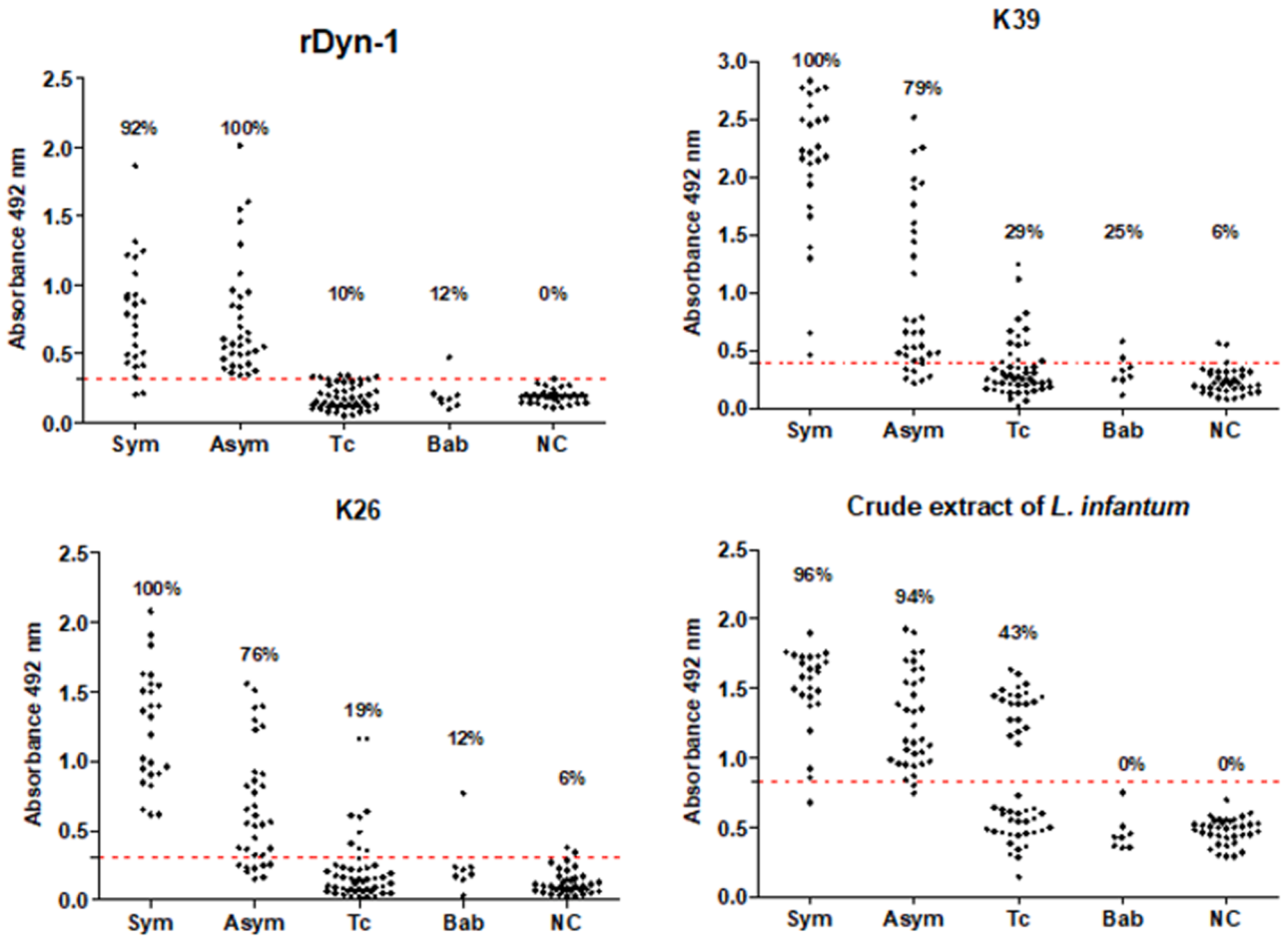


Fig. 2. Evaluation of the recombinant rDyn-1, K39 and K26 antigens, and *L. infantum* crude extract for the serodiagnosis of CanL. Comparison of the performance of ELISA was performed using canine sera from the following groups: dogs infected with *Leishmania*, 25 symptomatic (Sym) and 34 asymptomatic (Asym); 36 non-infected healthy dogs (NC); 49 experimentally infected dogs with *T. cruzi* (Tc); and 8 naturally infected dogs with *Babesia* sp. (Bab, $n = 27$), to evaluate possible cross-reactivity. The dashed red line represents the cut-off determined by the ROC curve of each test. The index above each column in the plot indicates the percentage of samples that are above the cut-off.

infections (79%). In addition, a high rate of cross-reactions with sera from dogs with *T. cruzi* (29%) and *Babesia* sp. (25%) is observed yet. The rDyn-1 antigen-based assay developed in this study has been demonstrated to overcome most of these problems, as its sensitivity and specificity are superior to those obtained with assays based on the rK39 antigen. Furthermore, the recombinant K26/HASPb1 protein, also included in this work as a reference technique, is considered an antigen capable of increasing the diagnostic accuracy of leishmaniasis (Martínez Abad et al., 2017). A study conducted in Iran indicated a sensitivity of 96.8% and a specificity of 100% for this antigen (Farajnia et al., 2008). Another study conducted in an endemic area in northwestern Brazil showed that the rK26 antigen has high sensitivity in dogs with symptoms, but low sensitivity in asymptomatic dogs, (94% and 64%, respectively) (Porrozzi et al., 2007). Following the literature data, the rK26 protein in this study showed high sensitivity (100%) in symptomatic dogs and low sensitivity (76%) in dogs without symptoms. Following the low values of sensitivity and specificity recorded in the literature, in this study, we found an overall sensitivity and specificity of 87% and 87.1%, respectively.

In this study, we compared the efficacy of assays based on the different recombinant antigens already tested in the literature, K26 and K39 (Farahmand and Nahrevanian, 2016; Farajnia et al., 2008; Kumar et al., 2001; Porrozzi et al., 2007), the total extract of the parasite of *L. infantum* promastigotes, and the antigen produced by our rDyn-1 group. Although a bank of sera with a limited number of samples previously characterized from a single area considered endemic for CanL in Brazil was used, not being representative of the universal population the rDyn-1 protein, a new antigenic protein derived from L. infantum, was studied here for first time in terms of application in ELISA-based CanL diagnosis. The assay based on the new antigen showed excellent performance, given its high ability to identify asymptomatic dogs (100%), without losing the ability to detect symptomatic dogs (92%). Overall, the ability of this protein to identify dogs with CanL was 97% [IC95%; 88.29–99.59%] (sensitivity).

In addition to the problematic diagnosis of asymptomatic dogs (leading to a limited sensitivity), current diagnostic tests also suffer from cross-reactivity. Antibodies against *T. cruzi* have been recognized using conventional serological methods as the main cause of cross-reactivity with *Leishmania* due to the phylogenetic similarity between *Leishmania* sp. and *T. cruzi*, which poses a problem for overlapping endemic areas (Paltrinieri et al., 2010; Umezawa et al., 2009). Researchers have often inferred the existence of cross-reaction in serological assays for detecting canine leishmaniasis in dogs co-infected with *E. canis or B. canis vogel* (Silva et al., 2015). In fact, Mancianti et al. (Mancianti and Meciani, 1988) demonstrated cross-reactivity of *B. canis vogeli* with anti--*Leishmania* antibodies using dot-ELISA. Although rDyn-1 showed cross-reactivity with other diseases, the specificity (94% [IC95%; 86.48–97.60%]) of this protein remained good and higher than the other antigens compared (CSA, rK39 and rK26).

The results in this study show that rDyn-1-based ELISA assays could

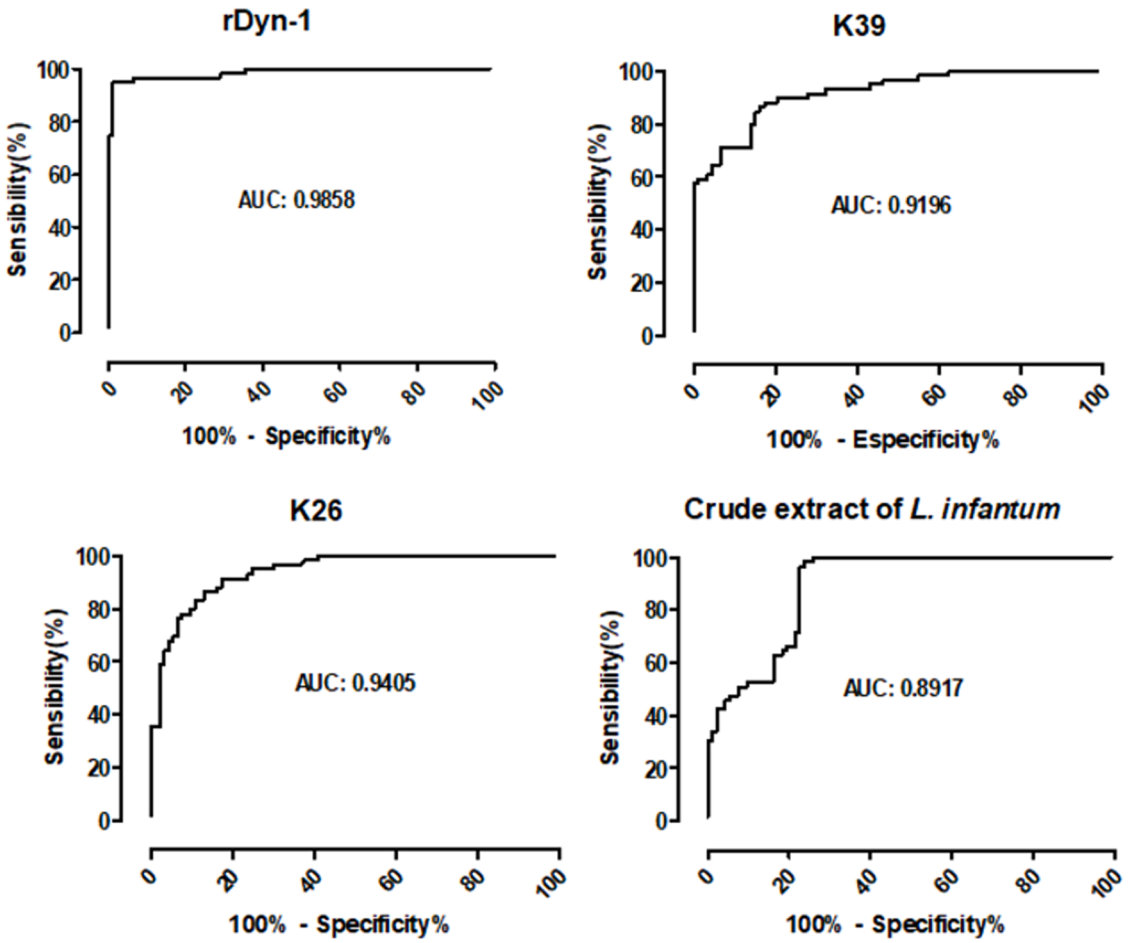


Fig. 3. Comparison of ROC curve of each canine serological test obtained for ELISA using recombinant rDyn-1, K39 and K26 antigens, and *L. infantum* crude. The sensitivity, specificity and area under the curve (AUC) were determined by ROC curve.

Table 1
Diagnostic performance of ELISA tests, using recombinant rDyn-1, K39 and K26 antigens, and *L. infantum* crude, to detect antibodies against CanL.

ELISA Test	Cut-off	Sensitivity (%) [CI 95%; $n = 59$]	Specificity (%) [CI 95%; $n = 93$]	PPV (%)	NPV (%)	AC (%)
rDyn-1	**0.3228**	**97** [88.29% - 99.59%]	**94** [86.48% - 97.60%]	**90**	**98**	**95**
K39	0.3964	88 77.07% - 95.09%	81 71.15% - 88.11%	74	91	84
K26	0.3142	86 75.02% - 93.96%	87 78.55% - 93.15%	81	91	87
Crude extract	0.8208	95 85.85% - 98.94%	77 67.58% - 85.45%	73	96	84

Abbreviations: (CI) confidence interval; (PPV) positive predictive value; (NPV) negative predictive value; (AC) accuracy.

be advantageous over the classic diagnostic test that lacks the sensitivity to properly diagnose asymptomatic carriers, which is a major limitation to epidemiology studies and hence for control programs (da Costa et al., 1991; Singh et al., 1995). Thus, a rapid, sensitive, and specific tool for the detection of *L. infantum* infection in dogs would be highly desirable because it would allow for effective control interventions in areas where zoonotic VL is endemic. The data presented here suggest that further studying the antigenic properties of each protein by carefully analyzing the portions/fragments responsible for its strength and subsequently combining the supremum of each protein in a multiple fragment model may be the way to a 100% accurate diagnosis capable of recognizing all *Leishmania* infections (symptomatic and asymptomatic), without cross-reactions with other diseases. Furthermore, the use of rDyn-1 might be considered in combination with other recombinant antigens (e.g. rK26, rK39, rKDDR, among others) that are remarkable to detect the parasitism in symptomatic animals, which might provide an improvement of overall sensitivity that is often impaired when detecting infection in asymptomatic dogs.

Funding

This study was supported by the following grants: RTF received financial support from Fundação de Amparo a Pesquisa do Estado de Minas Gerais/FAPEMIG, Brazil (http://www.fapemig.br) (Grant# APQ-04035-17 and APQ-02592-17); Conselho Nacional de Desenvolvimento Científico e Tecnológico/CNPq, Brazil (http://www.cnpq.br) (Grant# 303345/2018-7 and 421424/ 2018-4).

Table 2
Agreement analysis of ELISA tests using rDyn-1, rK39, rk26 and crude extract with the parasitological diagnosis of CanL.

ELISA Test	AUC	tP	FN	tN	FP	K [CI 95%]	Agreement
rDyn-1	0.9858	57	2	87	6	0.891 0.817 to 0.964	Very good
K39	0.9196	52	7	75	18	0.665 0.547 to 0.784	Good
K26	0.9405	51	8	81	12	0.726 0.615 to 0.838	Good
Crude extract	0.8917	56	3	72	21	0.685 0.573 to 0.797	Good

Abbreviations: (AUC) area under the curve; (CI) confidence interval; (tP) test positive; (tN) test negative; (FP) false positive; (FN) false negative; (k) Kappa index.

CRediT authorship contribution statement

Williane Fernanda Siqueira: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Mariana Santos Cardoso:** Conceptualization, Methodology, Validation, Formal analysis, Visualization, Investigation, Data curation, Writing – original draft. **Marianna de Carvalho Clímaco:** Writing – review & editing. **Ana Luiza Teixeira Silva:** Validation, Writing – original draft. **Benjamin Heidt:** Writing – review & editing. **Kasper Eersels:** Writing – review & editing. **Bart van Grinsven:** Writing – review & editing. **Daniella Castanheira Bartholomeu:** Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Lilian Lacerda Bueno:** Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Thomas Cleij:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Ricardo Toshio Fujiwara:** Conceptualization, Methodology, Resources, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

We are grateful to Michele Silva de Matos for performing serological tests and technical support.

Supplementary materials

Supplementary Fig. 1. **Analysis in polyacrylamide gel electrophoresis (SDS-PAGE) of the expression in bacteria and purification of rDyn-1 protein**. (A) Extracts of *E. coli* BL21 Star containing the plasmid pET28a-TEV/rDyn-1, before (0 h) and after (24 h) induction of the recombinant protein with IPTG. (B) After purification by affinity chromatography of the insoluble fraction of the bacterial lysate, the purified fraction presented a band of approximately 78 kDa, corresponding to rDyn-1. The blue box indicates the rDyn-1 protein band on the gels. Ladder: molecular mass marker; kDa: kilodalton.

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2023.106827.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215 (3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic. Acids. Res. 25 (17), 3389–3402. https://doi.org/10.1093/nar/25.17.3389.

Alvar, J., Cañavate, C., Molina, R., Moreno, J., Nieto, J., 2004. Canine leishmaniasis. Adv. Parasitol. 57, 1–88. https://doi.org/10.1016/S0065-308X(04)57001-X.

Anversa, L., Tiburcio, M.G.S., Richini-Pereira, V.B., Ramirez, L.E., 2018. Human leishmaniasis in Brazil: a general review. Rev. Assoc. Med. Bras. (1992) 64 (3), 281–289. https://doi.org/10.1590/1806-9282.64.03.281.

Badaro, R., Eulalio, M.C., Benson, D., Freire, M., Miranda, J.C., Pedral-Sampaio, D., Reed, S.G., 1993. Sensitivity and specificity of a recombinant Leishmania chagasi antigen in the serodiagnosis of visceral leishmaniasis. Arch. Inst. Pasteur Tunis. 70 (3–4), 331–332.

Barrouin-Melo, S.M., Larangeira, D.F., Trigo, J., Aguiar, P.H., dos-Santos, W.L., Pontes-de-Carvalho, L., 2004. Comparison between splenic and lymph node aspirations as sampling methods for the parasitological detection of Leishmania chagasi infection in dogs. Mem. Inst. Oswaldo Cruz. 99 (2), 195–197. https://doi.org/10.1590/s0074-02762004000200014.

Burns, J.M., Shreffler, W.G., Benson, D.R., Ghalib, H.W., Badaro, R., Reed, S.G., 1993. Molecular characterization of a kinesin-related antigen of Leishmania chagasi that detects specific antibody in African and American visceral leishmaniasis. Proc. Natl. Acad. Sci. USA 90 (2), 775–779.

Ciaramella, P., Oliva, G., Luna, R.D., Gradoni, L., Ambrosio, R., Cortese, L., Persechino, A., 1997. A retrospective clinical study of canine leishmaniasis in 150 dogs naturally infected by Leishmania infantum. Vet. Rec. 141 (21), 539–543. https://doi.org/10.1136/vr.141.21.539.

da Costa, C.A., Genaro, O., de Lana, M., Magalhães, P.A., Dias, M., Michalick, M.S., Mayrink, W., 1991. Canine visceral leishmaniasis: evaluation of the serologic method used in epidemiologic studies. Rev. Soc. Bras. Med. Trop. 24 (1), 21–25. https://doi.org/10.1590/s0037-86821991000100004.

da Costa, R.T., França, J.C., Mayrink, W., Nascimento, E., Genaro, O., Campos-Neto, A., 2003. Standardization of a rapid immunochromatographic test with the recombinant antigens K39 and K26 for the diagnosis of canine visceral leishmaniasis. Trans. R. Soc. Trop. Med. Hyg. 97 (6), 678–682. https://doi.org/10.1016/s0035-9203(03)80102-5.

Dantas-Torres, F., Miró, G., Baneth, G., Bourdeau, P., Breitschwerdt, E., Capelli, G., Otranto, D., 2019. Canine leishmaniasis control in the context of one health. Emerg. Infect. Dis. 25 (12), 1–4. https://doi.org/10.3201/eid2512.190164.

Daumke, O., Praefcke, G.J., 2016. Invited review: mechanisms of GTP hydrolysis and conformational transitions in the dynamin superfamily. Biopolymers 105 (8), 580–593. https://doi.org/10.1002/bip.22855.

Dhom-Lemos, L., Viana, A.G., Cunha, J.L.R., Cardoso, M.S., Mendes, T.A.O., Pinheiro, G. R.G., Fujiwara, R.T., 2019. Leishmania infantum recombinant kinesin degenerated derived repeat (rKDDR): a novel potential antigen for serodiagnosis of visceral leishmaniasis. PLoS One 14 (1), e0211719. https://doi.org/10.1371/journal.pone.0211719.

Dosztányi, Z., Csizmok, V., Tompa, P., Simon, I., 2005. IUPred: web server for the prediction of intrinsically unstructured regions of proteins based on estimated energy content. Bioinformatics 21 (16), 3433–3434. https://doi.org/10.1093/bioinformatics/bti541.

Dye, C., Vidor, E., Dereure, J., 1993. Serological diagnosis of leishmaniasis: on detecting infection as well as disease. Epidemiol. Infect. 110 (3), 647–656. https://doi.org/10.1017/s0950268800051074.

Farahmand, M., Nahrevanian, H., 2016. Application of recombinant proteins for serodiagnosis of visceral leishmaniasis in humans and dogs. Iran Biomed. J. 20 (3), 128–134. https://doi.org/10.7508/ibj.2016.03.001.

Farajnia, S., Darbani, B., Babaei, H., Alimohammadian, M.H., Mahboudi, F., Gavgani, A. M., 2008. Development and evaluation of Leishmania infantum rK26 ELISA for serodiagnosis of visceral leishmaniasis in Iran. Parasitology 135 (9), 1035–1041. https://doi.org/10.1017/S003118200800454X.

Fraga, D.B., Pacheco, L.V., Borja, L.S., Tuy, P.G., Bastos, L.A., Solcà, M.a.S., 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 (1), e0004333 https://doi.org/10.1371/journal.pntd.0004333.

García-Castro, A., Egui, A., Thomas, M.C., López, M.C., 2022. Humoral and cellular immune response in asymptomatic dogs with visceral Leishmaniasis: a review. Vaccines 10 (6). https://doi.org/10.3390/vaccines10060947.

Harith, A.E., Kolk, A.H., Kager, P.A., Leeuwenburg, J., Muigai, R., Kiugu, S., Laarman, J. J., 1986. A simple and economical direct agglutination test for serodiagnosis and sero-epidemiological studies of visceral leishmaniasis. Trans. R. Soc. Trop. Med. Hyg. 80 (4), 536–583. https://doi.org/10.1016/0035-9203(86)90149-5.

Ibarra-Meneses, A.V., Corbeil, A., Wagner, V., Onwuchekwa, C., Fernandez-Prada, C., 2022. Identification of asymptomatic Leishmania infections: a scoping review. Parasit. Vectors 15 (1), 5. https://doi.org/10.1186/s13071-021-05129-y.

Ibiapina, A.B., Batista, F.M.A., Aguiar, B.G.A., Mendonça, V.J., Costa, D.L., Costa, C.H.N., Abdala, C.V.M., 2022. Evidence map of diagnosis, treatment, prognosis, prevention, and control in visceral leishmaniasis. Rev. Panam. Salud Publica 46, e89. https://doi.org/10.26633/RPSP.2022.89.

IHME (2020). *Protocol for the global burden of diseases, injuries, and risk factors study (GBD)*.

Kar, U.P., Dey, H., Rahaman, A., 2017. Regulation of dynamin family proteins by post-translational modifications. J. Biosci. 42 (2), 333–344. https://doi.org/10.1007/s12038-017-9680-y.

Kumar, R., Pai, K., Pathak, K., Sundar, S., 2001. Enzyme-linked immunosorbent assay for recombinant K39 antigen in diagnosis and prognosis of Indian visceral leishmaniasis. Clin. Diagn. Lab. Immunol. 8 (6), 1220–1224. https://doi.org/10.1128/CDLI.8.6.1220-1224.2001.

Kühne, V., Büscher, P., 2019. The Unknown nature of the antigen in the direct agglutination test for visceral Leishmaniasis hampers development of serodiagnostic tests. Am. J. Trop. Med. Hyg. 100 (2), 246–255. https://doi.org/10.4269/ajtmh.18-0740.

Larsen, J.E., Lund, O., Nielsen, M., 2006. Improved method for predicting linear B-cell epitopes. Immunome Res. 2, 2. https://doi.org/10.1186/1745-7580-2-2.

Laurenti, M.D., Rossi, C.N., da Matta, V.L., Tomokane, T.Y., Corbett, C.E., Secundino, N. F., Marcondes, M., 2013. Asymptomatic dogs are highly competent to transmit Leishmania (Leishmania) infantum chagasi to the natural vector. Vet. Parasitol. 196 (3–4), 296–300. https://doi.org/10.1016/j.vetpar.2013.03.017.

Luigi, G. (2018). A brief introduction to leishmaniasis epidemiology. In. Biomedical and Life Sciences: 13 January 2018.

Lévêque, M.F., Lachaud, L., Simon, L., Battery, E., Marty, P., Pomares, C., 2020. Place of serology in the diagnosis of zoonotic leishmaniases with a focus on visceral Leishmaniasis due to. Front. Cell Infect. Microbiol. 10, 67. https://doi.org/10.3389/fcimb.2020.00067.

Maia, C., Campino, L., 2008. Methods for diagnosis of canine leishmaniasis and immune response to infection. Vet. Parasitol. 158 (4), 274–287. https://doi.org/10.1016/j.vetpar.2008.07.028.

Mancianti, F., Meciani, N., 1988. Specific serodiagnosis of canine leishmaniasis by indirect immunofluorescence, indirect hemagglutination, and counterimmunoelectrophoresis. Am. J. Vet. Res. 49 (8), 1409–1411.

Martínez Abad, L.P., Almeida, C.S., Mattos, A.M.M., Mendonça, A.C.P., Alves, M.J.M., Pinheiro, A.C., Teixeira, H.C., 2017. Diagnostic accuracy of rKLO8 versus rK26 ELISAs for screening of canine visceral leishmaniasis. Acta Trop. 166, 133–138. https://doi.org/10.1016/j.actatropica.2016.11.021.

Medeiros, F.A., Gomes, L.I., Oliveira, E., de Souza, C.S., Mourão, M.V., Cota, G.F., Rabello, A., 2017. Development and validation of a PCR-ELISA for the diagnosis of symptomatic and asymptomatic infection by. J. Trop. Med. 2017, 7364854 https://doi.org/10.1155/2017/7364854.

Menezes-Souza, D., de Oliveira Mendes, T.A., de Araújo Leão, A.C., de Souza Gomes, M., Fujiwara, R.T., Bartholomeu, D.C., 2015. Linear B-cell epitope mapping of MAPK3 and MAPK4 from Leishmania braziliensis: implications for the serodiagnosis of human and canine leishmaniasis. Appl. Microbiol. Biotechnol. 99 (3), 1323–1336. https://doi.org/10.1007/s00253-014-6168-7.

Menezes-Souza, D., Mendes, T.A., Nagem, R.A., Santos, T.T., Silva, A.L., Santoro, M.M., Fujiwara, R.T., 2014. Mapping B-cell epitopes for the peroxidoxin of Leishmania (Viannia) braziliensis and its potential for the clinical diagnosis of tegumentary and visceral leishmaniasis. PLoS One 9 (6), e99216. https://doi.org/10.1371/journal.pone.0099216.

Mohebali, M., 2013. Visceral leishmaniasis in Iran: review of the epidemiological and clinical features. Iran J. Parasitol. 8 (3), 348–358.

Mohebali, M., Edrisian, G.H., Nadim, A., Hajaran, H., AkhoundI, B., Houshmand, B., Fakhar, M., 2006. Application of direct agglutination test (DAT) for the diagnosis and seroepidemiological studies of visceral leishmaniasis in Iran. Iranian J. Parasitol. 1, 15–25.

Mohebali, M., Keshavarz, H., Shirmohammad, S., Akhoundi, B., Borjian, A., Hassanpour, G., Mahmoudi, S., 2020. The diagnostic accuracy of direct agglutination test for serodiagnosis of human visceral leishmaniasis: a systematic review with meta-analysis. BMC Infect. Dis. 20 (1), 946. https://doi.org/10.1186/s12879-020-05558-7.

Mohebali, M., Moradi-Asl, E., Rassi, Y., 2018. Geographic distribution and spatial analysis of *Leishmania infantum* infection in domestic and wild animal reservoir hosts of zoonotic visceral leishmaniasis in Iran: a systematic review. J. Vector Borne Dis. 55 (3), 173–183. https://doi.org/10.4103/0972-9062.249125.

Molaie, S., Mohebali, M., Abai, M.R., Molaie, A., Akhoundi, B., Asl, E.M., 2017. Alteration of Direct Agglutination Test (DAT) results in Iranian Kala-Azar patients: a case series. J. Parasit. Dis. 41 (2), 446–449. https://doi.org/10.1007/s12639-016-0825-y.

Molina, R., Amela, C., Nieto, J., San-Andrés, M., González, F., Castillo, J.A., Alvar, J., 1994. Infectivity of dogs naturally infected with Leishmania infantum to colonized Phlebotomus perniciosus. Trans. R. Soc. Trop. Med. Hyg. 88 (4), 491–493. https://doi.org/10.1016/0035-9203(94)90446-4.

Molina, R., Jiménez, M., García-Martínez, J., San Martín, J.V., Carrillo, E., Sánchez, C., Alvar, J., 2020. Role of asymptomatic and symptomatic humans as reservoirs of visceral leishmaniasis in a Mediterranean context. PLoS Negl. Trop. Dis. 14 (4), e0008253 https://doi.org/10.1371/journal.pntd.0008253.

Moreno, E.C., Gonçalves, A.V., Chaves, A.V., Melo, M.N., Lambertucci, J.R., Andrade, A. S., Carneiro, M., 2009. Inaccuracy of enzyme-linked immunosorbent assay using soluble and recombinant antigens to detect asymptomatic infection by Leishmania infantum. PLoS Negl. Trop. Dis. 3 (10), e536. https://doi.org/10.1371/journal.pntd.0000536.

Moshfe, A., Mohebali, M., Edrissian, G., Zarei, Z., Akhoundi, B., Kazemi, B., Mahmoodi, M., 2009. Canine visceral leishmaniasis: asymptomatic infected dogs as a source of L. infantum infection. Acta Trop. 112 (2), 101–105. https://doi.org/10.1016/j.actatropica.2009.07.004.

Nunes, C.M., Pires, M.M., da Silva, K.M., Assis, F.D., Gonçalves Filho, J., Perri, S.H., 2010. Relationship between dog culling and incidence of human visceral leishmaniasis in an endemic area. Vet. Parasitol. 170 (1–2), 131–133. https://doi.org/10.1016/j.vetpar.2010.01.044.

Paltrinieri, S., Solano-Gallego, L., Fondati, A., Lubas, G., Gradoni, L., Castagnaro, M., Canine Leishmaniasis Working Group, I. a. S. o. V. o. C. A., 2010. Guidelines for diagnosis and clinical classification of leishmaniasis in dogs. J. Am. Vet. Med. Assoc. 236 (11), 1184–1191. https://doi.org/10.2460/javma.236.11.1184.

Pinelli, E., Killick-Kendrick, R., Wagenaar, J., Bernadina, W., del Real, G., Ruitenberg, J., 1994. Cellular and humoral immune responses in dogs experimentally and naturally infected with Leishmania infantum. Infect. Immun. 62 (1), 229–235. https://doi.org/10.1128/iai.62.1.229-235.1994.

Porrozzi, R., Santos da Costa, M.V., Teva, A., Falqueto, A., Ferreira, A.L., dos Santos, C. D., Grimaldi, 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 (5), 544–548. https://doi.org/10.1128/CVI.00420-06.

Ready, P.D., 2014. Epidemiology of visceral leishmaniasis. Clin. Epidemiol. 6, 147–154. https://doi.org/10.2147/CLEP.S44267.

Reithinger, R., Quinnell, R.J., Alexander, B., Davies, C.R., 2002. Rapid detection of Leishmania infantum infection in dogs: comparative study using an immunochromatographic dipstick test, enzyme-linked immunosorbent assay, and PCR. J. Clin. Microbiol. 40 (7), 2352–2356. https://doi.org/10.1128/JCM.40.7.2352-2356.2002.

Roque, A.L., Jansen, A.M., 2014. Wild and synanthropic reservoirs of Leishmania species in the Americas. Int. J. Parasitol. Parasites Wildl. 3 (3), 251–262. https://doi.org/10.1016/j.ijppaw.2014.08.004.

Rosário, E.Y., Genaro, O., Franca-Silva, J.C., da Costa, R.T., Mayrink, W., Reis, A.B., Carneiro, M., 2005. Evaluation of enzyme-linked immunosorbent assay using crude Leishmania and recombinant antigens as a diagnostic marker for canine visceral leishmaniasis. Mem. Inst. Oswaldo Cruz. 100 (2), 197–203. /S0074-02762005000200015.

Silva, K.F.d., Afonso, R.I., Angélica, d.S.J., Moreira, A.D., Pain, M.A., Loureiro, W.G., Fontes, P.G., 2015. *Leishmania, Babesia* and *Ehrlichia* in urban pet dogs: co-infection or cross-reaction in serological methods?. In: Rev. Soc. Bras. Med. Trop: 10 Feb 2015, 48.

Singh, S., 2006. New developments in diagnosis of leishmaniasis. Indian J. Med. Res. 123 (3), 311–330.

Singh, S., Gilman-Sachs, A., Chang, K.P., Reed, S.G., 1995. Diagnostic and prognostic value of K39 recombinant antigen in Indian leishmaniasis. J. Parasitol. 81 (6), 1000–1003.

Siqueira, W.F., Viana, A.G., Reis Cunha, J.L., Rosa, L.M., Bueno, L.L., Bartholomeu, D.C., Fujiwara, R.T., 2021. The increased presence of repetitive motifs in the KDDR-plus recombinant protein, a kinesin-derived antigen from Leishmania infantum, improves the diagnostic performance of serological tests for human and canine visceral leishmaniasis. PLoS Negl. Trop. Dis. 15 (9), e0009759 https://doi.org/10.1371/journal.pntd.0009759.

Siripattanapipong, S., Kato, H., Tan-Ariya, P., Mungthin, M., Leelayoova, S., 2017. Comparison of recombinant proteins of kinesin 39, heat shock protein 70, heat shock protein 83, and glycoprotein 63 for antibody detection of *Leishmania martiniquensis* infection. J. Eukaryot. Microbiol. 64 (6), 820–828. https://doi.org/10.1111/jeu.12415.

Soares, P.H.A., da Silva, E.S., Penaforte, K.M., Ribeiro, R.A.N., de Melo, M.O.G., Cardoso, D.T., Belo, V.S., 2022. Responsible companion animal guardianship is associated with canine visceral leishmaniasis: an analytical cross-sectional survey in an urban area of southeastern Brazil. BMC Vet. Res. 18 (1), 135. https://doi.org/10.1186/s12917-022-03238-z.

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 (1), e0006082. https://doi.org/10.1371/journal.pntd.0006082.

Umezawa, E.S., Souza, A.I., Pinedo-Cancino, V., Marcondes, M., Marcili, A., Camargo, L. M., Teixeira, M.M., 2009. TESA-blot for the diagnosis of Chagas disease in dogs from co-endemic regions for Trypanosoma cruzi, Trypanosoma evansi and Leishmania chagasi. Acta Trop. 111 (1), 15–20. https://doi.org/10.1016/j.actatropica.2009.01.006.

Zijlstra, E.E., 2021. Precision medicine in control of visceral leishmaniasis caused by *L. donovani*. Front. Cell Infect. Microbiol. 11, 707619 https://doi.org/10.3389/fcimb.2021.707619.

Zijlstra, E.E., Ali, M.S., el-Hassan, A.M., el-Toum, I.A., Satti, M., Ghalib, H.W., Kager, P. A., 1992. Kala-azar: a comparative study of parasitological methods and the direct agglutination test in diagnosis. Trans. R. Soc. Trop. Med. Hyg. 86 (5), 505–507.

Zijlstra, E.E., Nur, Y., Desjeux, P., Khalil, E.A., El-Hassan, A.M., Groen, J., 2001. Diagnosing visceral leishmaniasis with the recombinant K39 strip test: experience from the Sudan. Trop. Med. Int. Health 6 (2), 108–113. https://doi.org/10.1046/j.1365-3156.2001.00680.x.