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Immunodiagnosis of human and canine visceral leishmaniasis using recombinant *Leishmania infantum* Prohibitin protein and a synthetic peptide containing its conformational B-cell epitope

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ABSTRACT

In the present study, *Leishmania infantum's* Prohibitin was cloned and, alongside a synthetic peptide, evaluated for the serodiagnosis of visceral and tegumentary leishmaniasis (CVL and TL, respectively) in dogs and humans. For TL diagnosis, this study analyzed serum samples from cutaneous (n = 20) or mucosal (n = 39) leishmaniasis patients, and from Chagas disease (CD) patients (n = 8) and non-infected patients (n = 45). For CVL diagnosis, serum samples from asymptomatic (n = 14), symptomatic (n = 71), non-infected (n = 116), and Leish-Tec®-vaccinated (n = 79) dogs were examined, as well as *T. cruzi* (n = 11) and *Ehrlichia canis* (n = 10) infected animals. An indirect ELISA method using rProhibitin showed diagnostic sensitivity and specificity values of 91.76% and 89.91%, respectively. *L. infantum* SLA showed 86.11% and 48.24% of specificity and sensitivity, respectively, for CVL serodiagnosis, and 98.31% and 84.91% sensitivity and specificity, respectively for TL diagnosis. *L. braziliensis* SLA showed 75.47% and 83.05% of specificity and sensitivity, respectively, for TL diagnosis. The synthetic peptide showed a better result in TL than in CVL diagnosis. In conclusion, preliminary results suggest that the detection of antibodies against the rProhibitin protein and the synthetic peptide improves the serodiagnosis of TL and CVL.

1. Introduction

Leishmaniasis is a public health problem in > 98 countries. It is caused by *Leishmania* species and is transmitted to humans by phlebotomine sandflies (Alvar et al., 2012; Davies et al., 2003; de Vries et al., 2015).

Leishmaniasis presents different clinical manifestations and can be classified into diffuse cutaneous leishmaniasis (DCL), cutaneous leishmaniasis (CL), muco-cutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL). DCL impaires cellular immune response in patients, CL can cause disabilities when multiple lesions are present, whereas MCL is associated with hematogenic dissemination of the parasite and is characterized by mucosal infiltration, which may be associated to deformities and destruction of facial structures. Finally, VL is a systemic disease and is fatal if left untreated. (Desjeux, 2004; Goto and Lindoso, 2010). Moreover, VL is a zoonotic disease and dogs (*Canis familiaris*) are considered the main domestic reservoir of *Leishmania infantum* in the Old and New Worlds. As a zoonotic disease, it shows a diverse clinical spectrum, ranging from asymptomatic to highly symptomatic cases. By presenting intense skin parasitism, dogs are potential sources of infection for phlebotomine sandflies, even when not showing symptoms (Alvar et al., 2004; Baneth et al., 2008; Moreno and Alvar, 2002).

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According to the Brazilian Ministry of Health, the control of the disease is based in the early diagnosis and treatment of human cases, control of insect vectors, identification and culling of seropositive infected dogs, and health education. The euthanasia of animals is a controversial measure and this is no longer the only option. Dog owners may now decide to treat their dogs with miltefosine that recently become available in Brazil for the treatment of VL in seroreactive dogs. (Brasil; MAPA, 2016; Coura-Vital et al., 2014; Lira et al., 2006; Nunes et al., 2018; Romero and Boelaert, 2010).

The serological methods used to identify *Leishmania*-infected dogs are based on the DPP® CVL rapid test and the enzyme linked immunosorbentassay (ELISA) produced by Bio-Manguinhos/FIOCRUZ, Brazil (Coura-Vital et al., 2014; Grimaldi et al., 2012). However, low sensitivity and high cross-reactivity of these tests make the detection of all infected dogs difficult, compromising the effectiveness of the control of the disease (Grimaldi et al., 2012; Porrozzi et al., 2007).

Regarding the diagnosis of TL, available tests do not provide enough accuracy to be considered the gold standard. For this reason, the diagnosis is often made by clinical, epidemiological, and laboratory analysis (parasitological and immunological tests). Parasitological tests are accurate but laborious to perform, and sensitivity is variable, depending on the disease duration, sampling technique, and skills of the microscopist (Goto and Lindoso, 2010). Moreover, immunologic assays, such as ELISA, are not routine tests for the diagnosis of TL due to the need of antigen preparation and low sensitivity, as it occurs in CL. (Kar, 1995; Celeste, 2014).

The early diagnosis of infected humans and dogs is important not only for an effective treatment but also for the control of the disease. In this regard, the detection of antibodies to Leishmania antigens by immunological methods has been a useful alternative for a rapid diagnosis. These serological methods are non-invasive, allow the screening of multiple samples, and are capable of early detection, before the formation of lesions in TL. It is important to note that in these cases positive results generally indicate current infection, since anti-Leishmania antibody levels do not remain high after treatment (Goto and Lindoso, 2010; Menezes-Souza et al., 2014b).

Using different techniques such as proteomics, genomics, and bioinformatics, new molecules have been identified and successfully evaluated as biomarkers for the diagnosis, vaccine development, and therapeutics against various diseases, including leishmaniasis (Coelho et al., 2012; Duarte et al., 2015; Menezes-Souza et al., 2015). In this context, new antigens have been studied aiming to provide a rapid test that does not require a laboratory or refrigeration, suitable for use in remote settings, and able to identify treatment failures. Recombinant proteins are particularly interesting, improving test performances, and represent an alternative of research (Coelho et al., 2016; Menezes-Souza et al., 2014a; Sato et al., 2017). Furthermore, these proteins could be used in immunochromatographic assays as *Leishmania* rK39 (rK39), *Leishmania* rK36 (rk26), and peroxidoxin antigens (CL-Detect™ IC-RDT) (Silva G., et al. 2017). Indeed, previous work has identified new potential candidates for TL diagnosis in an immunoproteomic study using stationary promastigote and axenic amastigotes of L. *braziliensis* and sera from TL patients (Coelho et al., 2012; Duarte et al., 2015).

Herein, the recombinant protein Prohibitin (PHB), identified by the above immunoproteomic study, and a synthetic peptide with a conformational epitope, were evaluated for the serological diagnosis of TL and CVL. This protein comprises evolutionary conserved proteins present in eukaryotes, with multiple capacities. PHB has been found to be localized in several cellular compartments, such as the nucleus, cell membrane, and the mitochondrion. Moreover, it has been implicated in an increasing number of cell processes, such as cell signaling, proliferation, and apoptosis, transcriptional control, and B-cell maturation (Joshi et al., 2003; Merkwirth and Langer, 2009; Mishra et al., 2005; Woodlock et al., 2001). Finally, *Leishmania* PHB was also able to generate a strong humoral response in VL patients (Jain et al., 2010). Together, these findings corroborate the hypothesis that PHB could be used in the serodiagnosis of TL and CVL.

2. Material and methods

2.1. Sera samples

Experiments involving human samples were approved by the Ethics Committee of the Federal University of Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil, with protocol number CAAE–323431 14.9.0000.5149.The sample size determination for accuracy of diagnostic tests was established using the receiver operating characteristic (ROC curve) generated by the PASS software (version 15, NCSS Statistical Software). For CVL [sample allocation ratio (R): $R = 6$ Groups negative for CVL/2 Groups positive for CVL = 6/2 = 3]. A minimum sample of 76 from the positive group (CVLS + CVLA) and 213 from the negative group (HDNEA + HDEA + HDV + Tc + Ec) achieve 80% power to detect a difference of 0.05 between the area under the ROC curve (AUC) under the null hypothesis of 0.80 and an AUC under the alternative hypothesis of 0.85 using a two-sided z-test at a significance level of 0.05. For TL diagnosis, the minimum sample size for detect a difference in diagnostic accuracy between the several tests evaluated in the present study involving ROC curve indices was calculated based on minimum of 80.0% of power (probability of rejecting a false null hypothesis) and above 0.1 area under curve (AUC) of difference, as described in output table obtained after statistical analyses based on methods of comparing the areas under the ROC curve (Hanley and McNeil, 1983; Obuchowski, 1997).

A total of 59 serum samples from TL patients with cutaneous [cutaneous leishmaniasis (CL), $n = 20$] or mucosal [mucosal leishmaniasis (ML), $n = 39$], were analyzed. Blood samples were also taken from a control group consisting of non-infected individuals ($n = 45$). Subjects were selected from healthy people living in an area of disease endemicity (Belo Horizonte, Minas Gerais, Brazil). These subjects were clinically evaluated through anamnesis and physical examination, and they did not present any clinical signal or suspicion of disease. To evaluate the cross-reactivity, serum samples from Chagas disease (CD) patients ($n = 8$) were also evaluated. Infection was confirmed by hemoculture or by the Chagatest recombinant enzyme-linked immunosorbent assay (ELISA) v.3.0 kit (Wiener Lab, Argentina) and the Chagatest hemagglutination inhibition (HAI) assay (Wiener Lab). The use of dog samples was approved by the Institutional Animal Care and Committee on Ethics of Animal Experimentation (CETEA) of UFMG, with protocol number 044/2012. Dog serum samples were obtained from an endemic area for CVL in Minas Gerais, Brazil. The sample size was composed of 301 dogs of different breeds and ages. CVL-positive animals presented positive parasitological results, which were based on identification of L. *infantum* kDNA in blood and/or bone marrow samples of these animals by a PCR technique, as previously described (Reis et al., 2013). In addition, these animals presented positive serological results by two laboratorial tests: IFAT-LVC® and EIE-LVC® (developed by BioManguinhos). In this context, symptomatic dogs (CVLS; $n = 71$) were those with positive parasitological and serological results. Asymptomatic dogs (CVLA; $n = 14$) presented positive parasitological and serological results, but did not present any clinical signal of leishmaniasis. Sera of dogs from a non-endemic area (HDNEA; $n = 40$) for CVL and negative to *Leishmania* from a endemic area (HDEA, $n = 76$) for VL, were included as a control group. Samples from dogs experimentally infected with *T. cruzi* (Tc; $n = 11$) or infected with *Ehrlichia canis* (Ec; $n = 10$) but negative for leishmaniasis, and from non-infected animals immunized with Leish-Tec® vaccine (HDV; $n = 78$) were used to evaluate cross-reactivity.

2.2. Cloning, expression, and purification of the recombinant protein

To evaluate the *L. infantum* protein Prohibitin (LinJ.35.0070) in the

diagnosis of CVL and TL, we expressed this protein as a His-tagged recombinant protein. The recombinant protein was cloned from *L. infantum* kDNA using specific primers. The fragment was excised from the gel, purified, digested with restriction enzymes, and ligated to a similarly digested pET28a-TEV vector. The recombinant plasmid was introduced to electrocompetent *E. coli* BL21 Arctic Express (DE3) cells (Agilent Technologies, USA) by electroporation using a MicroPulser Electroporation Apparatus (Bio-Rad Laboratories, USA). Gene insertion was confirmed by colony PCR and sequencing using T5 primers (Macrogen, South Korea). The recombinant Prohibitin protein expression was performed by adding 1.0 mM IPTG (Isopropyl-b-D-thiogalactopyranoside, Promega, Canada) for 24 h at 12 °C with shaking at 200 rev min21. The cells were then lysed by sonication and centrifuged at 10,000*g* for 30 min at 4 °C. The recombinant Prohibitin protein was purified using a HisTrap HP affinity column connected to an A¨ KTAprime chromatography system (GE Healthcare, USA). The eluted fractions containing rProhibitin were concentrated using Amicon Ultra 15 Centrifugal Filters, 10,000 NMWL (Millipore, Germany), and further purified on a SuperdexTM 200 gel filtration column (GE Healthcare Life Sciences, USA).

2.3. Mapping and synthesis of specific B cell epitope

The conformational epitope containing the sequence YNRTYGETLRDHGNGRYY was predicted using a combination of the results of three algorithms: ABCpred Prediction Server (Saha and Raghava, 2006), with parameters: window length: 16 and threshold: 0.8; Emini Surface Accessibility Prediction (Emini et al., 1985), with parameters: window size: 7 and threshold: 1.0; and Epitopia (Rubinstein, 2009). The epitope contains a combination of amino acids from two different protein regions: YNRTYGE (58–64 positions) and TLRDHGNGRYY (261–271 positions). The peptide was synthesized via the F-moc technique following a published procedure, albeit with modifications (Woolley and Merrifield, 1963; Machado de Avila et al., 2011). Briefly, the peptide was released from the amine resin upon treatment with trifluoracetic acid in the presence of the appropriate scavengers and it was further purified by high-performance liquid chromatography (HPLC) on a C18 reverse phase column (flow rate 1.0 mL/min; Vydac). The purified peptide was analyzed and confirmed by MALDI-TOF-TOF mass spectrometry.

2.4. Parasites and Soluble L. braziliensis (SLbA) and L. infantum antigen (SLiA)

Leishmania infantum (MOM/BR/1970/BH46) and *Leishmania braziliensis* (MHOM/BR/75/M2904) was grown at 24 °C in Schneider's medium (Sigma) supplemented with 20% heat-inactivated fetal bovine serum (Sigma), 20 mM L-glutamine, 200 U/ml penicillin, and 100 g/ml streptomycin, at pH 7.4. For the soluble antigen, a total of 1×10^9 *L. infantum* and *L. braziliensis* promastigotes stationary phase were washed three times with cold phosphate-buffered saline followed by three cycles of freezing in liquid nitrogen and thawing (42 °C). After ultrasonication with ten alternating cycles of 30 s at 35 MHz, the lysate was centrifuged at 6000 ×*g* at 4 °C for 15 min. The supernatant containing SLA was collected, and the protein concentration was estimated using the Pierce™ BCA™ Protein Assay (Thermo Scientific).

2.5. ELISA

For the ELISA assay, titration curves were performed to determine the most appropriate antigen concentration and antibody dilution to be used. For this, Falcon 96-well microplates (Becton Dickinson) were coated with 1.0 µg/well of rPHB in 100 µL of coating buffer (50 mM carbonate buffer), pH 9.6 for 18 h, at 4 °C. As controls, soluble antigens of *L. braziliensis* and *L. infantum* were also evaluated using the same conditions. For the peptide, flat-bottom plates (Costar, USA) were coated with 10.0 µg/well of soluble peptide and left overnight at 37 °C. After this, free binding sites were blocked using 200 µL of PBS-T (0.05% Tween-20 in a phosphate buffer saline solution), containing 5% casein, for 1 h at 37 °C. Plates were then washed five times with PBS-T, and incubated with individual canine or human sera (1:100 and 1:100, respectively, diluted in PBS-T), for 1 h at 37 °C. For the synthetic peptide, the dilution was 1:250 for canine or 1:50 for human sera. Plates were again washed five times using PBS-T, and incubated with anti-dog or anti-human IgG horseradish-peroxidase conjugated antibodies (1:2500 and 1:5000 diluted in PBS-T; catalog A6792 and I5260, respectively, Sigma-Aldrich, USA) for 1 h at 37 °C (dilution was 1:1000 and 1:5000, respectively, for the synthetic peptide). After washing five times with PBS-T, the reaction was developed through incubation with H_2O_2, ortho-phenylenediamine and citrate-phosphate buffer, pH 5.0, for 30 min in the dark. The reaction was stopped by adding 25 µL of 2 N H_2SO_4, and optical density was read in an ELISA microplate spectrophotometer (Molecular Devices, Spectra Max Plus, Canada), at 492 nm.

2.6. Statistical analysis

Results were analyzed using GraphPad PrismTM (version 6.0 for Windows) and Winpepi (version 11.0 for Windows). The mean optical density (OD) value was calculated by subtracting the mean blank OD from the mean OD for each sample by using specific values obtained in the ELISA assays. Accuracy was evaluated according to the area under the curve (AUC) relative to the receiver-operator curve (ROC), considering a 95% confidence interval (CI). Differences were considered statistically significant when $P < .05$. The cut-off values for rPHB and synthetic peptide were established using ROC. The cut-off was chosen based on the point that provides the maximum sum of sensitivity and specificity. Each test was evaluated for sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV), accuracy (AC), and AUC. Statistical analysis of the data from HDEA, HDNEA, CVLS, CVLA, HDV, TC, and Ec groups was performed by one-way analysis of variance (ANOVA), following Bonferroni's post-test for multiple comparisons between the groups. Results are shown as dotted graphs, where mean ± standard deviation (SD) of each group is shown. Differences were considered significant when $P < .05$.

3. Results

3.1. Recombinant expression of PHB and synthesis of peptide

To assess the potential of the PHB from *L. infantum* to diagnose LT and CVL, we expressed this protein as a His-tagged recombinant protein (rPHB). With a predicted molecular weight of 32.3 kDa, the recombinant protein was expressed in *E. coli* BL21 arctic express (DE3) cells as a soluble protein and obtained at a high level of purity (Fig. 1). A bioinformatic study was conducted to predict the lymphocyte B epitopes of the PHB of *L. infantum* using a combination of the results of three algorithms. The synthetic peptide is a conformational epitope containing the sequence YNRTYGETLRDHGNGRYY. This epitope contains a combination of amino acids from two different regions of PHB: YNRTYGE (58–64 positions) and TLRDHGNGRYY (261–271 positions), that are conserved in different species of *Leishmania* (Fig. 2).

3.2. Performance of rPHB and synthetic peptide for the serodiagnosis of tegumentary leishmaniasis

ELISA assays were performed to evaluate the reactivity of sera from patients with tegumentary (TL) against rPHB, peptide, and SLbA (Fig. 3). In addition, samples from Chagasic patients and non-infected individuals were used to measure the performance of each antigen. The results for each parameter (Sp, Se, FP and FN, TP and TN, PPV and NPV values, and AC) are summarized in Table 1. The sensibility of rPHB and the synthetic peptide (98.31% for both) were higher than SLbA

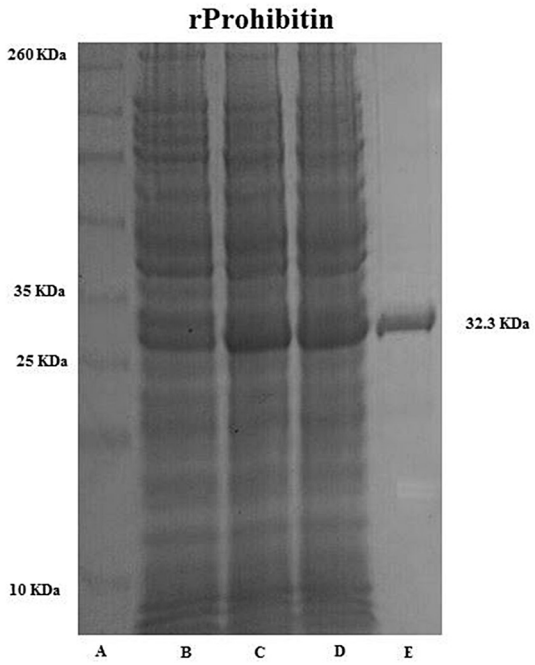

Fig. 1. Expression and purification of the recombinant Prohibitin protein. (A) Molecular weight standard (BenchMark™ Protein Ladder, Invitrogen), (B) lysate of culture before induction with IPTG, (C) lysate of culture after 3 h and (D) after 4 h of induction with IPTG (1 mM, 37 °C), and (E) recombinant Prohibitin protein (MW 32.3 KDa) purified by gel filtration.

(83.05%) and the specificity was 84.91% for rPHB, 100.00% for synthetic peptide, and 75.47% for SLb. The synthetic peptide showed increased accuracy for TL (99.12%) when compared to rPHB (91.96%) and SLbA (79.46%). The maximum PPV was achieved using the synthetic peptide (100.00%), followed by rPHB (87.88%) and SLbA (79.03%). A high NPV was also observed for the synthetic peptide (98.15%) compared with SLbA (80.00%). The synthetic peptide presented the highest AUC (0.9997; 95% CI: 0.9986–1.001) and accuracy (AC = 99.12), followed by rPBH (AUC = 0.9095; 95%CI: 0.8458–0.932) and (AC = 91.26) as contrasted with SLbA ELISA (AUC = 0.8235; 95% CI: 0.7448–0.9021) and (AC = 79.46).

3.3. Performance of rPHB and synthetic peptide for the serodiagnosis of canine visceral leishmaniasis

The recombinant proteins were also used to evaluate the potential to serodiagnosis CVL. Samples from *E. canis, T. cruzi,* and negative for leishmaniasis from endemic and non-endemic area were analyzed. Sera from non-infected, immunized with Leish-Tec® vaccine animals were used to measure the performance of each antigen. The reactivity of samples against rPHB and the peptide was compared with SLiA (Fig. 4). rPHB showed the higher sensitivity (91.76%) and discrete increase in specificity (89.81%). The peptide showed 77.65% of sensitivity and 68.52% of specificity. SLiA showed 86.11% of specificity; however, its sensitivity was only 48.24%, the lowest value. The PHB protein showed an increased accuracy for CVL (90.67%) when compared to the synthetic peptide (71.09%) or SLbA (75.41%). The maximum PPV was achieved by the synthetic peptide (88.62%), followed by the PHB protein (78.78%), and SLiA (57.75%). A higher NPV was observed for the PBH protein (96.53%) when compared to the SLiA (80.87%). Finally, the PBH protein presented the highest area under the curve (0.9574; 95% CI: 0.9272–0.9877), followed by the synthetic peptide (0.8127; 95%CI: 0.8852–0.9877), and in contrast to SLiA ELISA (0.5260; 95% CI: 0.4282–0.6237)(Table 2).

4. Discussion

In the last few years, many studies have contributed to a better understanding of the challenges of diagnosing *Leishmania* infections. Indeed, a gold standard test is necessary in order to establish effective strategic programs to control the disease. The use of serological assays has been playing an essential role in the diagnosis of leishmaniasis; however, a test with satisfactory efficiency is not yet available for the disease.

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L. infantum (XP_001468827)      1    MAAEARKKMNAYGGFGNIIGMSALVGVGCVSIYALYKSIFFVPGGFRAVKFNCITGLYNRTYGEGANFAI 70
L. donovani (XP_003864600)      1    MAAEARKKMNAYGGFGNIIGMSALVGVGCVSIYALYKSIFFVPGGFRAVKFNCITGLYNRTYGEGANFAI 70
L. major (XP_003722404)         1    MAAEARKKMNAYGGFGNIIGMSALVGVGCVSIYALYKSVFFVPGGFRAVKFNCITGLYNRTYGEGANFAI 70
L. braziliensis (XP_001568126)  1    MAAEARKKMNAYGGFGNIVGMSALVGVGCVSIYALYKSVFFVPGGFRAVKFNSITGLYNRTYGEGANFAI 70
L. guyanensis (CCM18788)        1    MAAEARKKMNAYGGFGNIVGMSALVGVGCVSIYALYKSVFFVPGGFRAVKFNSITGLYNRTYGEGANFAI 83

L. infantum (XP_001468827)      71   PFLETPVVFDIRNKPIEVPTASGSRDLQTVNMAVRVLYQPNVENLYHIYRHIGVNYAETVLPSLINEIIR 140
L. donovani (XP_003864600)      71   PFLETPVVFDIRNKPIEVPTASGSRDLQTVNMAVRVLYQPNVDNLYHIYRHIGVNYAETVLPSLINEIIR 140
L. major (XP_003722404)         71   PFLETPVVFDIRNKPIEVPTASGSRDLQTVNMAVRVLYQPNVENLYHIYRHIGVNYAETVLPSLINEIIR 140
L. braziliensis (XP_001568126)  71   PFLETPVVFDIRNKPIEVPTASGSRDLQTVNMAVRVLYQPNVENLHHIYRHIGINYAETVLPSLINEIIR 140
L. guyanensis (CCM18788)        84   PFLETPVVFDIRNKPIEVPTASGSRDLQTVNMAVRVLYQPNVENLHHIYRHIGINYAETVLPSLINEIIR 153

L. infantum (XP_001468827)      141  AVIAQFNASDLLIKRPEVSHRIGVMLAERAKRFNIDITDVSITQMSFGKEYTNAVEAKQVAQQMAERAKF 210
L. donovani (XP_003864600)      141  AVIAQFNASDLLIKRPEVSHRIGVMLAERAKRFNIDITDVSITQMSFGKEYTNAVEAKQVAQQMAERAKF 210
L. major (XP_003722404)         141  AVIAQFNASDLLIKRPEVSHRIGVMLAERAKRFNIDITDVSITQMSFGKEYTNAVEAKQVAQQMAERAKF 210
L. braziliensis (XP_001568126)  141  AVIAQFNASDLLIKRPEVSHRIGVMLAERAKRFNIDITDVSITQMSFGKEYTNAVEAKQVAQQMAERAKF 210
L. guyanensis (CCM18788)        154  AVIAQFNASDLLIKRPEVSHRIGVMLAERAKRFNIDITDVSITQMSFGKEYTNAVEAKQVAQQMAERAKF 223

L. infantum (XP_001468827)      211  RVEQAEQEKQAAILLAQGEAEAATLVGNAVKRNPAFLELRGLEAARTIAKTLRDHGNGRYYLDSDSLYVN 280
L. donovani (XP_003864600)      211  RVEQAEQEKQAAILLAQGEAEAATLVGNAVKRNPAFLELRGLEAARTIAKTLRDHGNGRYYLDSDSLYVN 280
L. major (XP_003722404)         211  RVEQAEQEKQAAILLAQGEAEAATLVGNAVKRNPAFLELRGLEAARTIAKTLRDHGNGRYYLDSDSLYVN 280
L. braziliensis (XP_001568126)  211  RVEQAEQEKQAAILLAQGEAEAATLVGNAVKRNPAFLELRGLEAARTIAKTLRDHGNGRYYLDSDSLYVN 280
L. guyanensis (CCM18788)        224  RVEQAEQEKQAAILLAQGEAEAATLVGNAVKRNPAFLELRGLEAARTIAKTLRDHGNGRYYLDSDSLYVN 293
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Fig. 2. Sequence and prediction of B-cell linear epitope expressed in different *Leishmania* spp. Alignment of *L. infantum* Prohibitin (XP_001468827) and similarity among sequences deposited in non-redundant protein databases was performed by comparison with the databases of the following *Leishmania* species using the BLAST tool: *L. major* (XP_003722404), *L. donovani* (XP_003864600), *L. braziliensis* (XP_001568126), and *L. guyanensis* (CCM18788). The amino acid sequences were aligned with the distinct residues in red color and the red boxes mark predicted B-cell epitopes. The conformational epitope contains a combination of amino acids from two different protein regions of protein: YNRTYGE (58–64 positions) and TLRDHGNGRYY (261–271 positions), and was predicted using a combination of the results of three algorithms: ABCpred Prediction Server, Emini Surface Accessibility Prediction and Epitopia. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

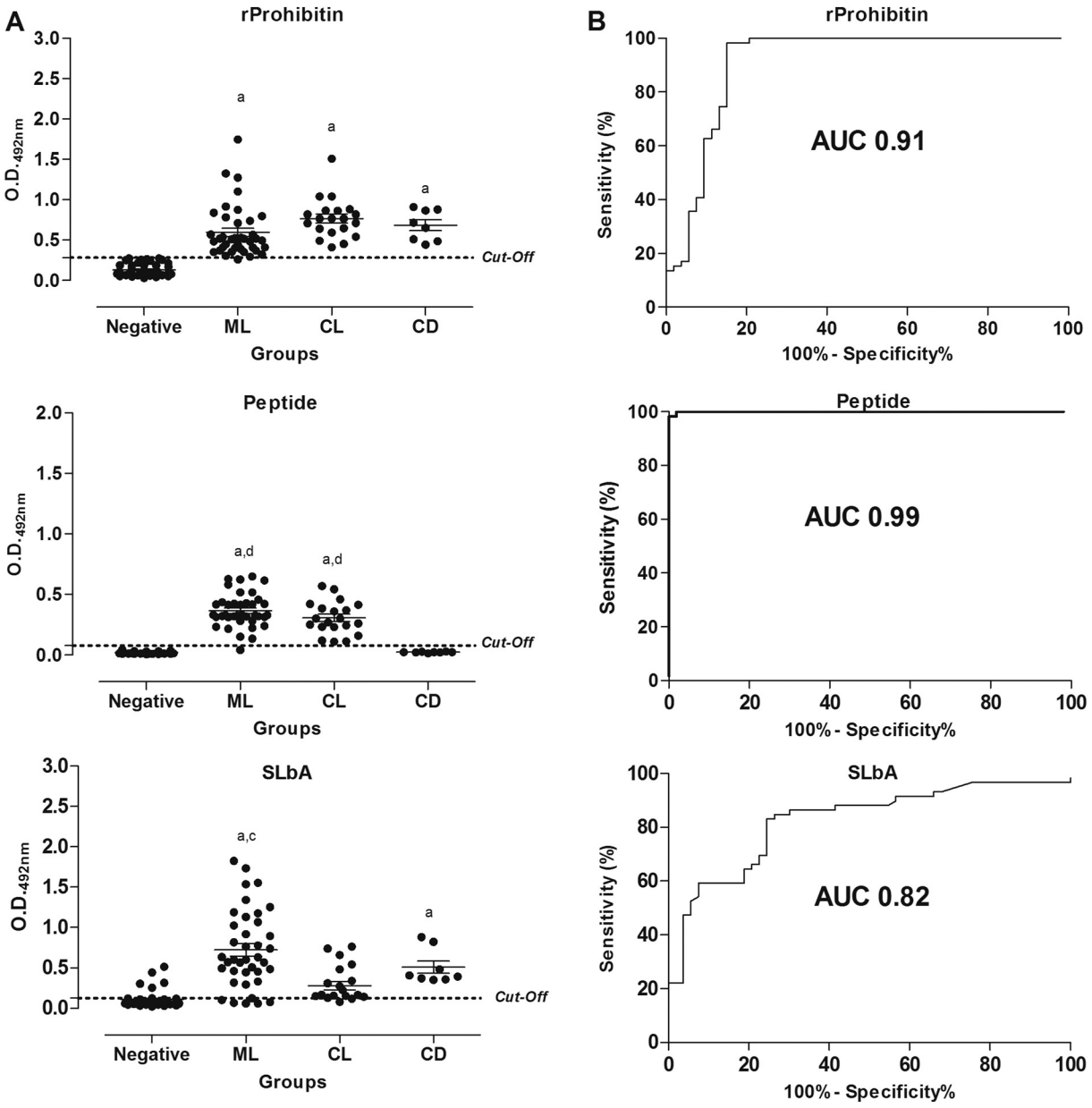

Fig. 3. Comparison of the reactivity of ELISAs in TL diagnosis.
(A)TL: ELISAs were performed on samples from different groups of individuals (CT, control group, $n = 45$; CD, Chagas disease patients, $n = 8$; CL, cutaneous leishmaniasis, $n = 20$; ML, mucosal leishmaniasis, $n = 39$). (B) ROC curves obtained from TL patients. *Cut-off obtained by ROC curve. # Cut-off obtained according to the manufacturer. Bars represent the mean ± standard deviation (SD) of the groups. (*a,b,c and d*) indicates significant difference ($P < .001$) in relation to the CT, ML, CL, and CD group, respectively.

Table 1
Measurement of diagnostic performance for rProhibitin, Peptide and SLbA.

Parameters*															
Test	Disease	Cut-off	TSe	CI95%	TSp	CI95%	AUC	CI95%	PPV (%)	NPV (%)	TP	TN	FP	FN	AC%
Prohibitin	TL	0.283	98.31	90.91 to 99.96	84.91	72.41 to 93.25	0.9095	0.8458 to 0.932	87.88	97.82	58	45	8	1	91.96
Peptide	TL	0.077	98.31	90.91 to 99.96	100.0	93.28 to 100.0	0.9997	0.9986 to 1.001	100.00	98.15	58	53	0	1	99.12
SlbA	TL	0.1270	83.05	71.03 to 91.56	75.47	61.72 to 86.24	0.8235	0.7448 to 0.9021	79.03	80.00	49	40	13	10	79.46

Parameter was calculated using all samples presented in this work for TL (CT + ML + CL + CD, n = 112).
Abbreviations: TSe: total sensitivity; TSp: total specificity; AUC: area under the curve; CI: confidence interval; PPV: positive predictive value; NPV: negative predictive value; TP: true positive; TN: true negative; FP: false positive; FN: false negative; AC: accuracy.
* Cut-off obtained by ROC Curve.

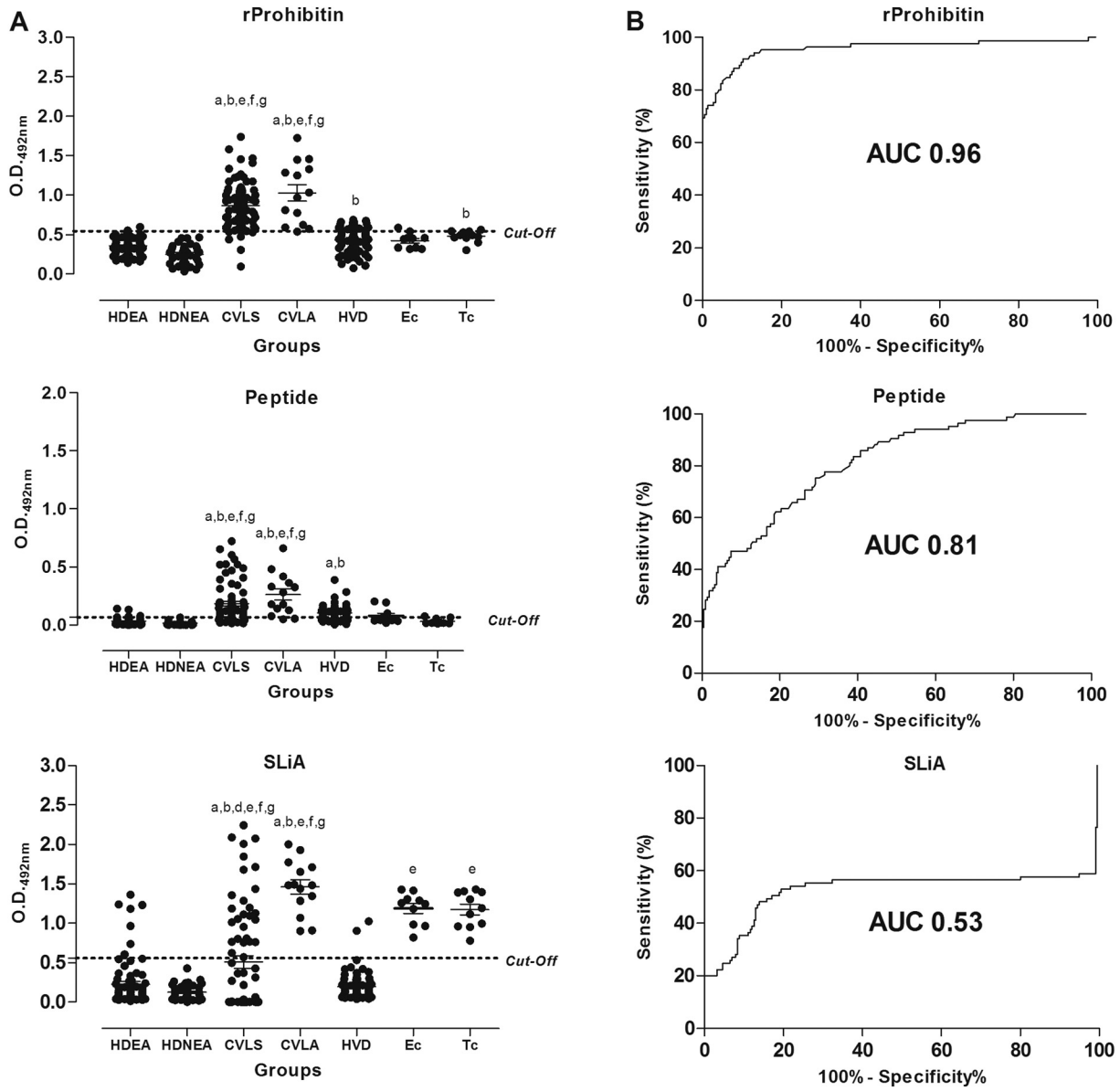

Fig. 4. Comparison of the reactivity of ELISAs in CVL diagnosis:
(A) ELISAs were performed on samples from different groups of dogs: healthy dogs from endemic area (HDEA, $n = 40$); healthy dogs from non-endemic area (HDNEA, $n = 76$); symptomatic canine visceral leishmaniasis (CVLS $n = 71$); asymptomatic canine visceral leishmaniasis (CVLA, $n = 14$);healthy, vaccinated dogs (HDV;$n = 78$); *Ehrlichia canis* (Ec; $n = 10$); *T. cruzi* (Tc; $n = 11$). (B) ROC curves obtained from *L. infantum*-infected dogs (CVL). *Cut-off obtained by ROC curve. # Cut-off obtained according to the manufacturer. Bars represent the mean ± standard deviation (SD) of the groups. (*a,b,c,d,e,f and g*) indicates significant difference ($P < .001$) in relation to the HDEA, HDNEA, CVLS, CVLA, HDV, TC, and Ec group, respectively.

Table 2
Measurement of diagnostic performance for rProhibitin, Peptide and SliA.

Parameters*															
Test	Disease	Cut-off	TSe	CI95%	TSp	CI95%	AUC	CI95%	PPV (%)	NPV (%)	TP	TN	FP	FN	AC%
Prohibitin	CVL	0.5455	91.76	83.77 to 96.62	89.81	84.99 to 93.51	0.9574	0.9272 to 0.9877	78.78	96.53	78	195	21	7	90.67
Peptide	CVL	0.06658	77.65	67.31 to 85.97	68.52	61.87 to 74.65	0.8127	0.8852 to 0.9581	88.62	49.25	66	148	68	19	71.09
SliA	CVL	0.5605	48.24	37.26 to 59.34	86.11	84.45 to 93.13	0.5260	0.4282 to 0.6237	57.75	80.87	41	186	30	44	75.41

Parameter was calculated using all samples presented in this work for CVL (HDEA + HDNEA + CVLS + CVLA + HDV + Ec + Tc $n = 301$).
Abbreviations: TSe: total sensitivity; TSp: total specificity; AUC: area under the curve; CI: confidence interval; PPV: positive predictive value; NPV: negative predictive value; TP: true positive; TN: true negative; FP: false positive; FN: false negative; AC: accuracy.
* Cut-off obtained by ROC Curve.

The diagnosis of CVL is complex and serological tests may not distinguish between symptomatic and naturally-infected, vaccinated dogs, or other canine infections. In addition, false negative results of asymptomatic dogs may contribute to the maintenance of the transmission cycle of parasites (Ferreira Ede et al., 2007; Porrozzi et al., 2007; Solano-Gallego et al., 2017, 2009).

Moreover, the diagnosis of TL based on a combination of epidemiological data, clinical, and laboratory test results, can be difficult due to its varied clinical manifestations (Gomes et al., 2014; Goto and Lindoso, 2010). Serological assays are not a routine procedure for diagnosis of TL, due to the lack of antigens with the potential to promote high sensitivity and specificity (Duarte et al., 2015; Menezes-Souza et al., 2014a).

Therefore, there is still a serious need for the development of more sensitive and specific serological assays. The use of recombinant proteins and synthetic peptides for the diagnosis of leishmaniasis is promising because it provides reproducibility of the tests due to the stability of these molecules and does not depend on processing live parasites as crude antigens (Chavez-Fumagalli et al., 2013; Coelho et al., 2016; Duarte et al., 2017).

Herein, we developed an enzyme-linked immunosorbent assay for the detection of antibodies against the rProhibitin and against a conformational B-cell epitope present in this protein for its potential use in the immunodiagnosis of TL and CVL, when compared with soluble *Leishmania* antigen used as control. This protein was characterized in an immunoproteomic study using protein extract of amastigote-*like* stage parasites against LV sera (Coelho et al., 2012). Previous studies have demonstrated that antigens characterized by proteomic studies or by epitope prediction tools have been used for the development of recombinant proteins. In addition, these antigens have been tested aiming to improve the diagnostic performance by the ELISA technique for the CVL and TL serodiagnosis (Carvalho et al., 2017; Coelho et al., 2012; Duarte et al., 2015; Lage et al., 2016).

Proteins that are expressed during specific stages in *Leishmania* parasites have been associated with virulence, high antigenicity during the active disease phase, and could be potentially used in diagnosis (Costa et al., 2011; Menezes-Souza et al., 2014b; Santarem et al., 2005). The Prohibitin protein has been described as an important mediator in host-parasite interactions, showing increased expression levels in the metacyclic promastigote form. In*L. donovani*, PHB has been shown to have an important role in pathogenesis and is able to induce a strong humoral response in patients with VL (Jain et al., 2010). In addition, the antigenicity of this protein has been confirmed in a serological assay using human and dogs sera infected by *L. infantum* (Coelho et al., 2012; Dias et al., 2018). Taken together, these results demonstrate the potential of this protein as a possible diagnostic marker in serological tests for the diagnosis of leishmaniasis.

PHB protein was found to be highly conserved among different *Leishmania* spp., with similarities higher than 98%, including all T and B cell epitopes conserved. In addition, this protein presents a low similarity with other organisms, such as human proteins and trypanosomatids (Dias et al., 2018; Jain et al., 2010). This conservation of proteins among different *Leishmania* species has already been described as an interesting strategy for the development of a serological test, since this antigen could be used to diagnose different clinical forms of leishmaniasis (Carvalho et al., 2017; Coelho et al., 2016; Menezes-Souza et al., 2015). Indeed, herein we evaluated rPHB as a marker for the serological diagnosis of CVL and TL and our results show a higher performance for the ELISA assay using this protein.

As a valuable part of this work, we have carried out a search for antigenic determinants of Prohibitin protein, using one synthetic peptide corresponding to predicted B-cell epitopes. This epitope was derived from this protein that was able to recognize specific antibodies in an ELISA for CVL and TL. The synthetic peptide presents a conformational epitope containing the sequence YNRTYGETLRDHGNGRYY. This amino acid sequence of *L. infantum* showed a high homology between distinct *Leishmania* species able to cause tegumentary and visceral leishmaniasis. Furthermore, synthetic peptides can be used as alternative antigens for serological tests, as they are relatively simple to synthesize, cheaper, and can be used in automated processes (Menezes-Souza et al., 2015; Lage et al., 2016).

The finding that PHB protein of *L. infantum* is strongly recognized by sera from TL patients is in agreement with the higher conserved sequences distribution along the entire protein of antigenic determinants in different *Leishmania* spp., which would allow for it to be used in the diagnosis of TL and CVL (Carvalho et al., 2017; Dias et al., 2018; Jain et al., 2010). In spite of our results showing that rPHB has a higher performance for the diagnosis of TL than CVL, the synthetic peptide showed an excellent result with 99.12% of accuracy, when compared with rPHB in the use of individual tegumentary leishmaniasis serum. This difference has already been described and corresponds to higher immunogenic epitopes that are absent in other organisms, such as the host, and that are associated with cross-reactions in the serological assays (Menezes-Souza et al., 2015; Oliveira et al., 2006).

There are several serological tests used for the diagnosis of dogs and the improvement of these methods for the correct identification of CVL is necessary to better comprehend the disease's status in dogs. The protocol adopted by the Brazilian PCLV for the detection of Leishmania-infected dogs is DPP/ELISA. However, this protocol is controversial due to the variability of sensitivity and specificity, especially with asymptomatic dogs and the serological cross-reactions with other canine's diseases (Coura-Vital et al., 2013; Grimaldi et al., 2012; Laurenti et al., 2014).

In the present study, it was observed that CVL sera recognized the rPHB protein. Our results demonstrated a higher sensibility and specificity (91.76% and 89.81%, respectively) than Sla of *L. infantum* (48.24% and 86.11%, respectively). Moreover, the recombinant protein PHB showed better sensitivity and specificity results than the synthetic peptide. This could be explained by the higher number of cross-reactivity with the vaccinated dogs that live in an endemic area of the disease. Further experiments with a larger panel of sera and with other correlated diseases such as *Toxoplasma gondii, Neospora caninum,* and *Babesia canis* should be tested to improve the performance of this peptide in CVL serological tests, since the results with TL showed 100% of sensitivity.

In conclusion, we showed that the use of the PHB protein and the synthetic peptide increased the sensibility and specificity of immunoassays when compared to crude antigens. More importantly, they could be valuable targets when composing an antigen panel that could significantly improve leishmaniasis diagnosis. Moreover, these results could be associated with specific immunogenic and potentially immunodominant regions in parasite proteins that are absent in the host or other organisms, and frequently associated with cross-reactions in the leishmaniasis serological tests (Souza et al., 2013). Finally, our work suggests that the rPHB and the synthetic peptide-based ELISA strategy may be useful for the development of a sensitive and highly specific serodiagnosis for TL and CVL or other parasitic diseases.

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Declaration of Competing Interest

The authors declare no commercial or financial conflict of interest.

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