Sensitive and specific serodiagnosis of tegumentary leishmaniasis using a new chimeric protein based on specific B-cell epitopes of *Leishmania* antigenic proteins

Authors

Nathalia C. Galvani[1], Amanda S. Machado[1], Daniela P. Lage[1], Vívian T. Martins[1], Daysiane de Oliveira[2], Camila S. Freitas[1], Danniele L. Vale[1], Bruna B. Fernandes[2], João A. Oliveira-da-Silva[1], Thiago A.R. Reis[1], Thaís T.O. Santos[1], Fernanda F. Ramos[1], Raquel S. Bandeira[1], Fernanda Ludolf[1], Grasiele S.V. Tavares[1], Nathalia S. Guimarães[1], Unaí Tupinambás[1], Miguel A. Chávez-Fumagalli[3], Maria V. Humbert[4], Denise U. Gonçalves[1], Myron Christodoulides[4,#], Ricardo A. Machado-de-Ávila[2,#], Eduardo A.F. Coelho[1,5,#,*]

Affiliations

[1] Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical, Faculdade de Medicina, Universidade Federal de Minas Gerais, Belo Horizonte, 30130-100, Minas Gerais, Brazil.

[2] Programa de Pós-Graduação em Ciências da Saúde, Universidade do Extremo Sul Catarinense, Criciúma, 88806-000, Santa Catarina, Brazil.

[3] Universidad Católica de Santa María, Urb. San José S/N, Umacollo, Arequipa, Perú.

[4] Neisseria Research Group, Molecular Microbiology, School of Clinical and Experimental Sciences, University of Southampton Faculty of Medicine, Southampton General Hospital, Southampton, England, SO16 6YD.

[5] Departamento de Patologia Clínica, COLTEC, Universidade Federal de Minas Gerais, Belo Horizonte, 31270-901, Minas Gerais, Brazil.

[#]Co-senior authors of the work.

[*]Corresponding author

Address: Prof. Dr. Eduardo Antonio Ferraz Coelho. Laboratório de Pesquisa do Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical, Faculdade de Medicina, Universidade Federal de Minas Gerais, Avenida Prof. Alfredo Balena, 190, 30130-100, Belo Horizonte, Minas Gerais, Brazil. Phone and fax number: +55 31 3409 4983

E-mail address: eduardoferrazcoelho@yahoo.com.br

Abstract

Serological tests used for the diagnosis of tegumentary leishmaniasis (TL) presents problems, mainly related to their variable sensitivity and/or specificity, which can be caused by low levels of antileishmanial antibodies or by presence of cross-reactive diseases, respectively. In this context, the search for new antigenic candidates presenting higher sensitivity and specificity is urgently required. In the present study, the amino acid sequences of the LiHyT, LiHyD, LiHyV, and LiHyP proteins, which were previously showed to be antigenic in the visceral leishmaniasis (VL), were evaluated and eight B-cell epitopes were predicted and used for construction of gene codifying a chimeric protein called ChimLeish. The protein was expressed, purified and evaluated as a recombinant antigen in ELISA (Enzyme-Linked Immunosorbent Assay) for the diagnosis of TL. The own B cell epitopes used to construct the chimera were synthetized and also evaluated as antigens, as well as a soluble *Leishmania braziliensis* antigenic extract (SLA). Results showed that ChimLeish presented 100% sensitivity and specificity to diagnose TL, while synthetic peptides showed sensitivity varying from 9.1% to 90.9%, while specificity reached from 98.3% to 99.1%. SLA showed sensitivity and specificity of 18.2% and 98.3%, respectively. A preliminary prognostic evaluation showed that anti-ChimLeish IgG antibodies declined in significant levels, when serological reactivity was compared before and six months after treatment, suggesting also a possible prognostic role of this antigen for TL.

Keywords: Tegumentary leishmaniasis; B-cell epitopes; chimera; diagnosis; ELISA; prognosis.

1. Introduction

Leishmaniases are neglected tropical diseases caused by protozoa parasites of the genus *Leishmania*, which are endemic in 98 countries in the world with 380 million people exposed to the infection risks, and 12 million people clinically affected [1]. There is an annual estimated incidence of 0.7 to 1.2 million cases of tegumentary leishmaniasis (TL) and 0.5 million cases of visceral leishmaniasis (VL) registered [2,3]. TL causes distinct clinical manifestations in patients, such as cutaneous leishmaniasis (CL), diffuse cutaneous leishmaniasis (DCL), and mucosal leishmaniasis (ML). This disease complex is caused by several parasite species, such as *Leishmania braziliensis, L. major, L. tropica, L. guyanensis, L. amazonensis*, among others [4].

The diagnosis of TL relies on clinical manifestations associated with epidemiological and laboratory data. As there is no gold-standard test to diagnose the disease, a combination of methods is usually required to obtain the precise diagnosis [5]. Parasitological tests, which are based on the direct examination of amastigotes in Giemsa-stained lesion smears of scrapping, biopsies or impression smears, have been used with such purpose. However, due to the scarcity of parasites in the collected samples, mainly in patients with old lesions, the sensitivity is variable and between 20% and 40% of the patients present false-negative results [6-9]. Molecular tests have also been used as more sensitive tools to detect *Leishmania* content in collected samples. PCR-based assays have obtained higher sensitivity to detect TL patients as compared to conventional parasitological methods. However, these tests are expensive, require sophisticated equipment and trained professionals to perform, as well as the sample collection is also invasive; limiting then their use [10,11].

Serological tests have been applied for the detection of antileishmanial antibodies in sera of infected hosts, and high efficacy has been reached for VL [12]. These tests are simpler and cheaper, when compared to the parasitological methods, and the sample collection is considered less invasive [12,13]. However, serological tests to diagnose TL have showed lower sensitivity, mainly due to the presence of low levels of antileishmanial antibodies [14,15]. In addition, the specificity has been also variable by the presence of cross-reactive antigens in patients developing leprosy, malaria, tuberculosis, histoplasmosis, aspergillosis, systemic lupus erythematosus, among others [14-19]. There are also cases where treated patients maintain positive serology for months and/or years after treatment and clinic cure, being difficult to differentiate them from those with active disease [20,21]. In this context, the search continues to identify more sensitive and specific diagnostic targets for TL.

Our group performed an immunoproteomic study to identify *Leishmania* proteins with application potential for the diagnosis of leishmaniasis [22]. Some of these antigens, evaluated as individual recombinant proteins, satisfactory diagnostic action against canine and/or human VL, such as LiHyT [23], LiHyD [24] and LiHyV [25]. LiHyP, a hypothetical protein also identified in the cited immunoproteomic study, showed specific B-cell epitopes in its amino acid sequence; suggesting then the potential use as diagnostic marker to detect the disease. Considering the similarities between the amino acid sequences of LiHyT, LiHyD, LiHyV, and LiHyP proteins in distinct *Leishmania* spp.; in the present study, linear B-cell epitopes were predicted in the sequences of these four proteins, and they were used to construct the gene codifying a chimeric protein. This new antigen, called ChimLeish, was cloned and the recombinant chimera was purified and evaluated in ELISA (Enzyme-Linked Immunosorbent Assay) for the diagnosis of TL. The B cell epitopes used to construct the chimeric protein were also synthetized and tested in the experiments, as well as a soluble *L. braziliensis* antigenic extract (SLA). Results suggested that ChimLeish presented 100% sensitivity and specificity to detect TL patients, including those presenting lower anti-parasite serology; while synthetic peptides and SLA showed lower sensitivity and specificity values, mainly to diagnose CL cases. Evaluating a possible prognostic role of the antigens, low levels of anti-ChimLeish IgG antibodies were found in sera of treated patients, which were similar to those obtained in healthy subjects; suggesting also a prognostic role of this antigen for TL.

2. Materials and Methods

2.1. Parasites

L. braziliensis (MHOM/BR/1975/M2904) stationary promastigotes were grown at 24ºC in Schneider's medium (Sigma, St. Louis, MO, USA) added with 20% (v/v) inactivated fetal bovine serum (FBS, Sigma), 20 mM L-glutamine, 200 U/mL penicillin, and 100 µg/mL streptomycin at pH 7.4. The antigenic extract was prepared from 10^9 parasites according to described in [26]. Briefly, stationary promastigotes were washed three times in sterile phosphate-buffered saline (PBS 1x pH 7.4) and submitted to six cycles of freezing and thawing, at which time they were submitted to ultrasonication (Ultrasonic processor, GEX600) with five cycles of 30 seconds at 38 MHz. The supernatant containing SLA was collected after centrifugation (9,000 x g for 30 min at 4^{o}C) and stored at -70^{o}C until use.

2.2. Prediction of B-cell epitopes and construction of chimeric protein

The amino acid sequences of LiHyT (XP_001465138.1), LiHyD (XP_001468360.1), LiHyV (XP_001462854.1), and LiHyP (XP_001468385.2) proteins were obtained from the SwissProt server (web.expasy.org/docs/swiss-prot) and saved in TXT format [27]. The IEDB server (www.iedb.org) was used to identify the most accessible amino acids in the primary structures using the parameters: window size of 12 and threshold value of 1.0. Next, epitopes were predicted using the ABCpred server (www.imtech.res.in/raghava/abcpred/) with the parameters: window size of 16 and threshold value of 0.85. Eight epitopes were selected and their amino acid sequences were used to construct the chimera-codifying gene. Aiming to provide flexibility and avoid spatial overlap between the epitopes in the protein sequence, two glycine residues were included between each peptide sequence. Both the ChimLeish-codifying gene, which was cloned into pET28a-TEV vector, and B cell epitopes (Pept1, Pept2, Pept3, Pept4, Pept5, Pept6, Pept7 and Pept8) were commercially produced (Genscript®, USA). The lyophilized peptides were diluted in milli-Q water before use in the experiments. The construct was inserted into *E. coli* Arctic Express cells, and the protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (1.0 mM; Promega®, Canada) for 2 h at 37ºC. Next, bacteria were centrifuged at 3,000 x *g* for 10 min at 4ºC, and then disrupted with six cycles of freezing and thawing, followed by six cycles of ultrasonication with cycles of 30 seconds each (36 MHz). Cellular debris was removed after centrifugation (5,000 x *g* for 15 min at 4ºC) and the supernatant was collected and concentrated in Amicon® ultra15 filters with a nominal molecular weight limit of 10,000 (NMWL, Millipore, Germany). The recombinant protein was purified on a HisTrap HP affinity column (GE Healthcare Life Sciences, USA) connected to an AKTA system. After purification, ChimLeish (19.2 kDa) was passed through a polymyxin-agarose column (Sigma-Aldrich, USA) in order to remove any residual endotoxin content (<10 ng of LPS per 1 mg of recombinant protein, measured by the Quantitative Chromogenic Limulus Amebocyte Assay QCL-1000 kit, BioWhittaker, USA). SDS-12% PAGE gels were used to evaluate the purification profile.

2.3. Study population and sera samples

The study was approved by the Ethics Committee of the Federal University of Minas Gerais (UFMG, Belo Horizonte, Minas Gerais, Brazil), with protocol number CAAE-32343114.9.0000.5149. Sera samples of CL (n=25; including 15 males and 10 females, with ages ranging from 27 to 56 years old) and ML (n=30; including 18 males and 12 females, with ages ranging from 29 to 63 years) patients, all living in endemic area of TL (Belo Horizonte, Minas Gerais, Brazil), were used. The diagnosis was confirmed by clinical exams and parasitological tests

to detect parasites by Giemsa-stained smears from lesion and/or mucosal fragments, as well as by identification of parasite content by PCR technique according described [28]. Briefly, DNA was extracted from collected samples using the QIAamp® DNA Blood Mini Kit (Qiagen®, MD, USA), purified and assayed by spectrophotometry. It was then diluted in sterile ultrapure water and the following primers were used to amplify a *Leishmania* kDNA region (150 base pairs) in TL patient samples: 5´-GGGKAGGGGCGTTCTSCGAA-3´ (Forward) and 5´-SSSWCTATWTTACACCAACCCC-3´ (Reverse). The parasite DNA extracted of a reference strain for TL (MHOM/BR/2002/LPC-RPV) was used as positive control, while sterile ultrapure water was used as negative control. For reactions, the following reagents were used: 2 mM $MgCl_2$, 200 µM dNTPs, 0.6 µM of each primer (Sigma-Aldrich, USA), 1 UI Taq DNA polymerase with specific buffer (Invitrogen, USA), and 20 ng DNA templates. The program applied was: (step 1) 94ºC for 10 min; (step 2) 60ºC for 30 seconds; (step 3) 72ºC for 30 seconds; (step 4) 94ºC for 30 seconds and go to step 2 for 42 times; (step 5) 72ºC for 10 min [28]. The amplified product was analysed by polyacrylamide gel. CL patients presented an assumed disease evolution time varying from 4 months to 8 years, while ML patients presented an assumed disease evolution time ranging from 6 months to 12 years. None of the TL patients had been treated before sample collection. Sera were also collected from healthy individuals living in endemic region of disease (n=25, including 14 males and 11 females, with ages ranging from 26 to 42 years; Belo Horizonte). These subjects presented no clinical signal of leishmaniasis. Samples of Chagas Disease patients (n=25, including 16 males and 9 females, with ages ranging from 33 to 59 years), which were diagnosed by hemoculture, Chagatest® recombinant ELISA v.4.0 kit and/or Chagatest® hemmaglutination inhibition (Wiener lab., Rosario, Argentina), as well as from patients with leprosy (n=20, with 12 males and 8 females, with ages ranging from 35 to 55 years), aspergillosis (n=15, including 8 males and 7 females, with ages ranging from 34 to 60 years) and histoplasmosis (n=15; including 6 males and 9 females, with ages ranging from 30 to 56 years) were used. The diagnosis of these diseases was confirmed by means of ML Flow rapid test, detection of fungal hyphae in histopathological examination, and isolation of the fungus in culture of bronchoalveolar lavage fluids and/or whole blood, respectively. Additionally, sera of HIV-infected patients (n=15, including 9 males and 6 females, with ages ranging from 28 to 50 years), which were diagnosed by means of clinical exam and laboratory tests for $CD4^+$ T cell count and determination of the viral load, were also used.

2.4. Serological follow-up after treatment

Sera samples from ML patients (n=15) were collected before and six months after treatment, when the humoral response specific to ChimLeish, synthetic peptides and SLA was evaluated. The

patients were submitted to the same therapeutic regimen using pentavalent antimonials (Sanofi Aventis Farmacêutica Ltda., Suzano, São Paulo, Brazil), at a dose of 20 mg Sb^{+5} per kg for 30 days. In addition, none of them suffered from other infection or had any pre-existing disease. After completion of the treatment, all patients were free of any sign of leishmaniasis and were classified as clinically cured.

2.5. Immunoblotting assays

Immunoblottings were performed using ChimLeish, which was submitted (10 µg) to SDS-12% PAGE gels and blotted onto a nitrocellulose membrane (0.2 µm pore size, Sigma-Aldrich, USA). Next, membranes were blocked with PBS-T plus 5% bovine serum albumin (BSA) (w/v) and incubated for 1 h at 37°C. A new incubation with sera pools of healthy subjects (n=4) or patients with CL or ML (n=4 each) was performed for 1 h at 37°C, with sera samples 1:100 diluted in PBS-T. Next, membranes were washed with PBS-T and an anti-human IgG horseradish-peroxidase conjugated antibody was added in the plates (diluted 1:10,000 in PBS-T; Sigma-Aldrich, USA), when a new incubation was performed for 1 h at 37°C. After, reactions were developed using a solution composed by chloronaphtol, diaminobenzidine and H_2O_2 for 30 min, and stopped by adding distilled water. A low range protein ladder standard (Invitrogen™, Life Technologies, USA) was used. Three independent experiments were performed and results were similar.

2.6. Serological assays

Previous titration curves were performed to determine the most appropriate concentration of antigens and sera dilutions to be used in the experiments. Microtiter immunoassay plates (Jetbiofil®, Belo Horizonte) were coated with ChimLeish (0.5 µg/well), individual peptides (5.0 µg/well, all antigens) or *L. braziliensis* SLA (2.0 µg/well), which were diluted in coating buffer (50 mM carbonate buffer at pH 9.6), for 18 h at 4°C. Next, free binding sites were blocked using 250 µL of PBS-T plus 5% BSA (w/v) per well for 1 h at 37°C. After washing the plates five times with PBS-T, they were incubated with the individual serum samples (1:100 diluted for synthetic peptides and SLA or 1:200 diluted for ChimLeish, both in PBS-T), and incubated for 1 h at 37°C. Plates were washed five times with PBS-T and incubated with an anti-human IgG peroxidase conjugated antibody (1:10,000 for peptides and SLA, and 1:20,000 for ChimLeish, both in PBS-T) for 1 h at 37°C. After washing the plates six times with PBS-T, reactions were developed by incubation with 100 µL per well of a solution composed by 2 µL H_2O_2, 2 mg ortho-phenylenediamine and 10 mL citrate-phosphate buffer at pH 5.0, for 30 min and in the dark. Reactions were stopped by adding 25 µL 2 N H_2SO_4. The optical density (OD) values were measured in a microplate reader (Molecular

Devices, Spectra Max Plus, Canada) at 492 nm. All samples were tested in parallel on the same day to reduce experimental variation.

2.7. Statistical analysis

Results were entered into Microsoft Excel (version 10.0) spreadsheets and analysed with GraphPad Prism™ software (version 6.0 for Windows). The cut-off values for each antigen were determined by constructing Receiver Operating Characteristic (ROC) curves, which were plotted with the individual OD values of TL patients group versus those from the other (healthy individuals and patients with Chagas Disease, leprosy, histoplasmosis, aspergillosis or HIV-infected) groups. ROC curves were used to calculate: sensitivity (Se), specificity (Sp), area under the curve (AUC), confidence interval using a 95% level (95% CI), likelihood ratio (LR), and Youden index (Y). The unpaired two-tailed t-test was used and differences were considered statistically significant with $P < 0.05$.

3. Results

3.1. Construction and characterization of ChimLeish protein

Eight linear B-cell epitopes were predicted in the amino acid sequences of LiHyT, LiHyD, LiHyV and LiHyP proteins, with two epitopes identified in each protein sequence. In addition, the amino acid sequences of the selected epitopes were found to be conserved in distinct *Leishmania* species (Fig. 1). With the purpose to provide flexibility and to avoid the spatial overlap between epitopes in the protein sequence, two glycine residues were included between them (Fig. 2). After purification, ChimLeish was showed to be a soluble protein and with high purification yield (higher than 95.0%), which can be visualized in a representative SDS-12% PAGE gel (Fig. 3A, lane 2). Immunoblotting assays showed that, while ChimLeish was not recognized by antibodies in sera of healthy individuals (Fig. 3B, lane 2), it was specifically recognized by samples of CL and ML patients (Fig. 3B, lanes 3 and 4, respectively). In both cases, a low range protein ladder standard was used (Figs. 3A and 3B, lane 1 in both).

3.2. Evaluation of antigens for the diagnosis of tegumentary leishmaniasis

The diagnostic efficacy for the detection of TL cases was evaluated using ChimLeish, synthetic peptides and SLA as antigen by means of ELISA against a human serological panel. Results showed that the recombinant protein was recognized by sera in CL and ML patients, but not

by those from healthy subjects or of patients presenting TL-related diseases; with statistically significant differences being found between the groups ($P < 0.0001$). Results are presented as box plots, with indication of the minimum and maximum values obtained for each antigen (Fig. 4). The higher serological variation found in the TL group, when synthetic peptides and SLA were used as antigens, can be explained by the fact that CL patients usually present lower antileishmanial serology, making difficult to be reactive against a diverse antigenic preparation, as well as by the fact that peptides are short antigens and that usually present higher difficulty to be identified by antibodies in ELISA plates. Otherwise, lower serological variation was found when ChimLeish was used as an antigen (Fig. 4); suggesting then that this antigen presented a more homogeneous recognition by antibodies present in sera of CL and ML patients. ROC curves were constructed for each antigen and results are shown (Fig. 5), as well as the sensitivity and specificity values were determined. Results showed that ChimLeish presented both 100% sensitivity and specificity, while synthetic peptides showed sensitivity ranging from 9.1% to 90.9%, and specificity varying from 98.3% to 99.1% (Table 1). SLA showed sensitivity and specificity values of 18.2% and 98.3%, respectively; thus concluding that ChimLeish was the best antigen applied for the diagnosis of TL when our serological panel was evaluated in ELISA.

3.3. Serological follow-up after treatment

The antibody response against the antigens was evaluated comparatively before and six months after patient treatment (Fig. 6). Results showed that anti-ChimLeish IgG antibodies significantly declined after therapy as compared to OD values obtained before treatment. Using using SLA as antigen, the serological reactivity was similar before and after treatment (Fig. 6A). Testing the synthetic peptides, lower humoral response was found after patient treatment, although data using the chimeric protein have been more expressive. With the OD values obtained before and after treatment, ratios between IgG levels using each antigen were calculated and results are also shown (Fig. 6B). Data indicate that anti-chimera antibodies declined in more significant levels after therapy, when compared to values obtained using the synthetic peptides or SLA.

4. Discussion

Distinct clinical manifestations are caused by leishmaniasis in humans. VL is the most serious clinical form of the disease and is responsible by death of approximately 59,000 people annually [29]. TL is not considered a fatal disease; however, it can cause since a localized

cutaneous lesion that appears ulcerated, with granular base and raised borders, until lesions causing gradual tissue destruction, affecting the upper respiratory and digestive tracts mucosa of the patients [23]. In this context, a precise diagnosis of this disease complex is essential to perform a rapid treatment aiming to improve the quality of life of the patients, and serological tests have been considered important tools for the diagnosis of leishmaniasis, due to their simplicity, high performance, reproducibility, and low cost [30]. In the VL, symptomatic patients usually develop high antileishmanial serology, and they are precisely diagnosed in the available tests [31-33]. However, in the TL, the anti-parasite antibody production is usually lower against *Leishmania* antigens, mainly in CL cases, and diagnostic tests are not effective to detect the disease [34].

In a previous work developed by our group, *L. infantum* proteins were recognized by antibodies in sera of VL dogs, and they were suggested as potential diagnostic markers for disease [22]. In fact, some of these antigens, such as LiHyT [23], LiHyD [24] and LiHyV [25], were cloned and the recombinant proteins were individually tested as diagnostic markers for VL, and promising results being obtained. However, to the best of our knowledge, none of these antigens were tested for the diagnosis of TL. Otherwise, the association of diagnostic antigens could represents better diagnostic accuracy for leishmaniasis, when compared to the use of individual proteins, since higher antigenic variety represented by distinct B cell epitopes derived from different parasite proteins could be included in the protein sequence, making it easier to be recognized by antibodies in sera of infected hosts [35]. In this context, the development of polypeptide-based chimeric proteins could result in high sensitivity and specificity values for the diagnosis of leishmaniasis, as well as present low production cost due to the fact that it is an unique protein [36].

In this context, in the present study, the amino acid sequences of LiHyT, LiHyD, LiHyP, and LiHyV proteins were mapped and the main B-cell epitopes were identified and used to construct a recombinant chimeric protein, which was tested for the diagnosis of TL. The chimera was showed to be a soluble protein and with high purification yield and it was tested in ELISA for the diagnosis of TL, here represented by sera of CL and ML patients. The B cell epitopes used to construct the chimera sequence were produced as synthetic peptides, and they were also used as antigens in the experiments, as well as a soluble *L. braziliensis* antigenic extract. Results showed that ChimLeish was identified by all TL patient sera, including CL patients samples, and presented low cross-reactivity against antibodies in sera from healthy subjects or patients developing Chagas Disease, leprosy, malaria, tuberculosis, histoplasmosis, and aspergillosis or those HIV-infected. Although some synthetic peptides showed satisfactory diagnostic performance, their sensitivity and specificity values were lower in comparison to data obtained using ChimLeish as antigen, mainly to detection of CL cases, since these patients usually present lower antileishmanial serology [37,38].

In addition, SLA as antigen showed also lower sensitivity in ELISA for the detection of CL patients.

The diagnosis of CL and ML is based on clinical exams associated with parasitological and/or immunological analyses. However, problems related to the sensitivity of tests have been registered, mainly due to the longer lesion time, which can reflect in a lower number of parasites, hampering then the sensitivity of the tests [19,39]. Regarding serological diagnosis, CL patients usually tend to present low levels of anti-*Leishmania* antibodies, and they can be mis-diagnosed as false-negative in the laboratorial assays [40]. The present study's results showed that SLA, a mixture of *Leishmania* antigenic and non-antigenic proteins and that present distinct expression levels, presented also low sensitivity to detect CL. Serological results were similar to those described in other works evaluating antigens for the diagnosis of TL [41-43], suggesting then the importance of selection of more sensitive antigenic candidates to diagnosis the disease. In addition, individual peptides showed also lower sensitivity for the detection of CL and ML patients, when compared to the use of ChimLeish. Usually, short peptides present some problems to be used as diagnostic antigens for diseases, such as the limited adsorption on polystyrene plates, the low recognition by few specific antibodies, and the variable reproducibility according the clinical and immunological state of the disease [44,45]. In this context, the grouping of the selected antigenic fragments in chimeric proteins could presents higher diagnostic efficacy, such as visualized in this study.

Distinct immune response profiles are found according to the clinical manifestation of TL, and higher levels of antibodies are often described in the ML cases, when compared to CL patients [46]. In this context, a diagnostic kit presenting high sensitivity and specificity to diagnose CL and ML cases will be desirable, since this disease complex can cause a range of clinical manifestations in the patients, reaching from self-limiting lesions to mutilating scars. In addition, problems related to the available therapeutics, which are toxic and/or present high cost have been described [47,48]. *Leishmania* antigenic preparations have showed variable sensitivity and specificity values to diagnose TL, when sera of patients with Chagas Disease, leprosy, malaria, tuberculosis, histoplasmosis, aspergillosis, among others, or those HIV-infected; have been evaluated in the serological assays [49-52]. In fact, sera of patients developing such diseases, which present direct or indirect relation with the clinical and/or laboratorial diagnosis of TL, have been also used in other studies as cross-reactive disease groups, when distinct antigenic candidates are tested to detect TL [53-55]. In this context and according to the results found in this study, ChimLeish showed high sensitivity to be recognized by antibodies in sera of CL and ML patients, as well as high specificity due to the low reactivity against sera of cross-reactive diseases; suggesting then a diagnostic

potential for this antigen in the TL. However, additional studies are certainly necessary to be performed, aiming to prove the high performance of ChimLeish for the TL diagnosis, as well as evaluating this antigen in other diagnostic platforms.

The antileishmanial serological follow-up of the patients after their treatment and clinical cure should be considered as an immunological control measure. However, antigens usually present similar or little variation in the post-therapy antibody follow-up, since OD values are similar to those found before treatment; suggesting then the absence of a relevant prognostic role [13, 56-58]. Here, significantly lower levels of anti-ChimLeish IgG antibodies were found in sera of treated ML patients, when samples were collected six months post-therapy; suggesting then that the chimeric protein could be considered as an immunological candidate for the prognosis of disease. On the other hand, anti-SLA antibody levels were found to be similar before and after treatment, suggesting that this parasite antigenic preparation was not suitable to indicate the clinical cure of the patients. Similar results were also found by others, when SLA was evaluated as an antigen in serological follow-up after treatment against leishmaniasis [13,21,59]. Overall, our results can be considered interesting, mainly due to the fact that current diagnostic tests for leishmaniasis show variable sensitivity to monitor the treatment and clinic cure of patients, as well as to differentiate between past and active infections [20,28,60].

The sample size used in this study can be considered a limiting factor, as well as the absence of sera of patients with other clinical and/or laboratorial TL-related diseases. In addition, the serological follow-up of treated patients should also be performed for a longer period of time, aiming to prove the long-term prognostic efficacy of ChimLeish. Thus, present study's data can be taken as proof-of-concept of the capacity of this novel recombinant antigen to be applied for the diagnosis and prognosis of TL, and would serve as reference for future diagnostic studies, applied through the ELISA or in other diagnostic methods, such as immunochromatographic strips.

Conflicts of interest

The authors hereby declare that they have no conflicts of interest.

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FIGURE LEGENDS

Fig. 1. Bioinformatic assays and identification of linear B-cell epitopes. The amino acid sequences of LiHyT (XP_001465138.1), LiHyD (XP_001468360.1), LiHyV (XP_001462854.1) and LiHyP (XP_001468385.2) proteins were evaluated by bioinformatic tools, and two linear B-cell epitopes were identified in each sequence. The selected epitopes are shown in grey squares. "*" indicates identical amino acid, ":" indicates similar amino acid, and "." indicates low similarity amino acid.

Fig. 2. Construction of the chimeric protein. The selected B-cell epitopes were grouped in a linear sequence with the inclusion of two glycine residues between each epitope, and the chimeric protein sequence is shown.

Fig. 3. SDS-12% PAGE gels and immunoblottings. The ChimLeish protein was evaluated by means of SDS-12% PAGE gels and one representative preparation is shown (in A). A low range protein ladder standard was used (lane 1), and the purified protein presented purity degree higher than 95.0% (lane 2). Immunoblottings were performed using TL patients and healthy subject sera, and results are shown (in B). A low range protein ladder standard was used (lane 1), and the reaction of protein against sera pools (n=4 in each) of healthy subjects (lane 2) and CL (lane 3) and ML (lane 4) patients is also shown.

Fig. 4. Serological assays using ChimLeish, synthetic peptides and SLA as antigens. ELISA was performed with ChimLeish, synthetic peptides (Pept 1, Pept 2, Pept 3, Pept 4, Pept 5, Pept 6, Pept 7, and Pept 8) and SLA as antigens, which were reacted against sera of CL (n=25) and ML (n=30) patients characterized as "TL group", as well as against samples of healthy individuals living in endemic region of disease (n=25) or patients with Chagas Disease (n=25), leprosy (n=20), histoplasmosis (n=15) and aspergillosis (n=15) or those HIV-infected (n=15); which were characterized as "control group". The individual optical density (OD) values and box plots representing the minimum and maximum values are shown. The dotted lines indicate the cut off values for each antigen evaluated. Statistically significant difference ($P < 0.0001$) between (TL and Controls) groups is also indicated. Abbreviation: SLA- Soluble *L. braziliensis* Antigenic Extract.

Fig. 5. Serological follow-up after treatment. Levels of IgG antibodies specific to ChimLeish, synthetic peptides (Pept 1, Pept 2, Pept 3, Pept 4, Pept 5, Pept 6, Pept 7, and Pept 8) and SLA were evaluated by ELISA using sera of ML patients collected before and six months after treatment (n=15). Black and grey circles represent the optical density (OD) values of the samples obtained before and after treatment, respectively (in A). Ratios between antibody levels before and after therapy were also calculated, and results are shown (in B). Abbreviation: SLA- Soluble *L. braziliensis* Antigenic Extract. (***) indicate statistically significant difference in relation to the other groups ($P < 0.05$).

Table 1. Diagnostic evaluation of antigens for tegumentary leishmaniasis. Sera of healthy individuals living in endemic region of TL (n=25), as well as from patients with CL (n=25), ML (n=30), Chagas Disease (n=25), leprosy (n=20), histoplasmosis (n=15) and aspergillosis (n=15) or those HIV-infected (n=15) were used in ELISA against ChimLeish, synthetic peptides (Pept 1, Pept 2, Pept 3, Pept 4, Pept 5, Pept 6, Pept 7, and Pept 8) and SLA. The individual optical density (OD) values obtained for each antigen were used to construct ROC curves, and sensitivity (Se), specificity (Sp), area under the curve (AUC), confidence interval using a 95% level (95% CI), likelihood ratio (LR), and Youden index (Y) were calculated. Abbreviation: SLA- Soluble *L. braziliensis* Antigenic Extract.