Parasitology

cambridge.org/par

Screening diagnostic candidates from *Leishmania infantum* proteins for human visceral leishmaniasis using an immunoproteomics approach

Daniela P. Lage[1], Fernanda Ludolf[1], Patrícia C. Silveira[1], Amanda S. Machado[1], Fernanda F. Ramos[1], Daniel S. Dias[1], Patrícia A. F. Ribeiro[1], Lourena E. Costa[1], Danniele L. Vale[1], Grasiele S. V. Tavares[1], Vívian T. Martins[1], Miguel A. Chávez-Fumagalli[1], Rachel B. Caligiorne[2], Ana T. Chaves[1], Denise U. Gonçalves[1], Manoel O. C. Rocha[1], Mariana C. Duarte[1] and Eduardo A. F. Coelho[1,3]

[1]Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical, Faculdade de Medicina, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil; [2]Instituto de Ensino e Pesquisa, Santa Casa de Belo Horizonte, Belo Horizonte, Minas Gerais, Brazil and [3]Departamento de Patologia Clínica, COLTEC, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

Research Article

Cite this article: Lage DP *et al* (2019). Screening diagnostic candidates from *Leishmania infantum* proteins for human visceral leishmaniasis using an immunoproteomics approach. *Parasitology* **146**, 1467–1476. https://doi.org/10.1017/S0031182019000714

Received: 24 February 2019
Revised: 11 April 2019
Accepted: 12 May 2019
First published online: 13 June 2019

Key words:
Humans; immunoproteomics; *Leishmania infantum*; recombinant proteins; serodiagnosis; visceral leishmaniasis

Author for correspondence:
Eduardo A. F. Coelho, E-mail: eduardoferrazcoelho@yahoo.com.br

Abstract

There is no suitable vaccine against human visceral leishmaniasis (VL) and available drugs are toxic and/or present high cost. In this context, diagnostic tools should be improved for clinical management and epidemiological evaluation of disease. However, the variable sensitivity and/or specificity of the used antigens are limitations, showing the necessity to identify new molecules to be tested in a more sensitive and specific serology. In the present study, an immunoproteomics approach was performed in *Leishmania infantum* promastigotes and amastigotes employing sera samples from VL patients. Aiming to avoid undesired cross-reactivity in the serological assays, sera from Chagas disease patients and healthy subjects living in the endemic region of disease were also used in immunoblottings. The most reactive spots for VL samples were selected, and 29 and 21 proteins were identified in the promastigote and amastigote extracts, respectively. Two of them, endonuclease III and GTP-binding protein, were cloned, expressed, purified and tested in ELISA experiments against a large serological panel, and results showed high sensitivity and specificity values for the diagnosis of disease. In conclusion, the identified proteins could be considered in future studies as candidate antigens for the serodiagnosis of human VL.

Introduction

Leishmaniasis is a disease complex considered as a public health problem in tropical and subtropical regions in the world, such as Asia, Africa and the Americas. It is caused by obligate protozoan parasites of the genus *Leishmania*, occurring in 98 countries with 12 million people clinically affected, and 350 million at risk of infection (WHO, 2016). Visceral leishmaniasis (VL) is caused by parasites of the *Leishmania donovani* and *Leishmania infantum* species, and it can be fatal if acute and left untreated (Torres-Guerrero *et al*., 2017). Regarding the treatment of disease, pentavalent antimonials, liposomal and free amphotericin B, paromomycin, pentamidine and oral miltefosine are used. However, these drugs are toxic and/or present high cost (Alves *et al*., 2018; Kapil *et al*., 2018). As a consequence, the prevention of disease should be considered, such as by use of vaccination (Kumar and Samant, 2016). Although a number of antigens have been tested, and a variable degree of success has been obtained in murine and/or canine models, there is no effective vaccine to protects against human disease (Nico *et al*., 2010; Martins *et al*., 2013; Grimaldi *et al*., 2014; Mortazavidehkordi *et al*., 2016; Dias *et al*., 2018).

The control of VL requires appropriate diagnosis and adequate treatment, since the precise diagnosis is essential for effective drug regimen for patients (Vijayakumar and Das, 2018). Laboratory strategies are being employed, such as parasitological and immunological evaluations. Parasitological methods include microscopy with the identification of amastigote forms in organ aspirates, such as bone marrow, spleen, liver and/or lymph nodes. However, limitations due to the variable sensitivity, the requirement of technical expertise, the sample's collect be considered an invasive procedure, limit their efficacy (Sakkas *et al*., 2016). Immunological methods have been used; however, problems related with the sensitivity and/or specificity of the selected antigens are described; thus hampering their use as more appropriate diagnostic tools (Georgiadou *et al*., 2015; Lima *et al*., 2017).

A commercial kit, Kalazar Detect™ Test (InBios International, Inc., Seattle, Wash, USA), is an immunochromatographic assay applied for the diagnosis of human VL. However, this test

cannot discriminate between current, subclinical or past infections, and it is useless for diagnosis of relapses and as a prognostic test (Sundar and Singh, 2018). As a consequence, the possibility to identify new antigens to be employed in more sensitivity and specific diagnosis should be pointed (Didwania *et al.*, 2017). Proteomics is a technology employed for the study and characterization of the information obtained in a cell or organism about the form of protein pathways and networks (Vlahou and Fountoulakis, 2005; Garg *et al.*, 2018). Using proteomics associated with biological fluids, such as sera samples, can allow for the identification of antigens involved in the development of diseases; thus leading to the identification of biological targets that could be used as diagnostic markers and/or vaccine candidates (Fernandes *et al.*, 2012). Indeed, and due to the high amino acid conservation in *Leishmania* proteins; the identification of antigenic molecules that stimulate the humoral response in infected hosts could help for the development of more appropriated tests employing these more refined antigens (Jamal *et al.*, 2017). In this context, in the present study, an immunoproteomics approach performed in *L. infantum* promastigote and amastigote protein extracts with sera samples of VL patients was developed; with the purpose of identifying antigenic proteins in the parasites to be applied as candidates for the diagnosis of disease. To refine the selection of the candidates, sera samples of Chagas disease patients and healthy subjects living in the endemic region were used to exclude the most cross-reactive spots, aiming to avoid undesired cross-reactivity in the serological assays.

Materials and methods

Human samples

Sera samples were obtained from VL ($n = 30$, including 16 males and 14 females with ages ranging from 29 to 63 years) patients, which were collected from the endemic region of disease (Belo Horizonte). Patients were diagnosed by clinical evaluation and demonstration of *L. infantum* kDNA in spleen or bone marrow aspirates by PCR technique. Sera were also collected from healthy individuals living in an endemic ($n = 30$, including 18 males and 12 females with ages ranging from 21 to 56 years; Belo Horizonte) or non-endemic ($n = 30$, including 15 males and 15 females with ages ranging from 19 to 49 years; Poços de Caldas, Minas Gerais, Brazil) region of leishmaniasis. Subjects did not present clinical signal of disease and showed negative serological results. Samples were also obtained from Chagas disease ($n = 25$, including 15 males and 10 females with ages ranging from 29 to 60 years) patients, with the infection confirmed by hemoculture, Chagatest® recombinant ELISA v.4.0 kit or Chagatest® haemagglutination inhibition kit (Wiener Lab., Rosario, Argentina). Sera from paracoccidioidomycosis ($n = 10$, six males and four females with ages ranging from 26 to 55 years) patients were also used. The diagnosis was performed by clinical examination and positive *Paracoccidioides* culture. Samples collected from leprosy ($n = 20$, with 12 males and eight females, with ages ranging from 24 to 57 years) patients, with the diagnosis confirmed by clinical evaluation, ML Flow rapid test and lesion histopathology, as well as sera from aspergillosis ($n = 10$, including five males and five females, with ages ranging from 22 to 47 years), tuberculosis ($n = 10$, six males and four females with ages ranging from 38 to 67 years) or malaria ($n = 10$, seven males and three females with ages ranging from 20 to 46 years) patients were used in the assays.

Parasites and preparation of Leishmania protein extract

Leishmania infantum (MHOM/BR/1970/BH46) was used. Parasites were cultured at 24 °C in complete Schneider's medium (Sigma), which was supplemented with 20% inactivated foetal bovine serum (FBS; Sigma-Aldrich), 20 mM L-glutamine, 200 U mL^{-1} penicillin and 100 $\mu g\ mL^{-1}$ streptomycin, at pH 7.4 (Coelho *et al.*, 2003). To obtain the axenic amastigotes, 10^9 stationary promastigotes were washed three times in sterile phosphate buffer saline (PBS 1×), and incubated in 5 mL FBS for 48 h at 37 °C. Parasites were washed in cold PBS 1×, and their morphology was evaluated after staining by the Giemsa method in an optical microscope (Valadares *et al.*, 2011). The protein extraction and two-dimensional electrophoresis (2DE) were performed following a modified protocol (Lewis *et al.*, 2000). Briefly, cells from each stage (10^{10} cells) were washed three times in 40 mM Tris-HCl, pH 7.2, by centrifugation at 5000 × ***g*** for 10 min at 4 °C. Pellets were resuspended in lysis buffer solution [7 M urea, 2 M thiourea, 4% cholamidopropyl dimethylammonio-1-propanesulfonate (CHAPS), 40 mM dithiothreitol (DTT), 2% IPG buffer (pH 4–7), 40 mM Tris], and a protease inhibitor cocktail (GE Healthcare, Uppsala, Sweden) was added. Samples were incubated for 1 h at room temperature, with occasional vortexing. Purification was carried out by protein precipitation using a 2D Clean UpKit (GE Healthcare), according to manufacturer instructions. Whole cell extracts were measured by a bidimensional Quant-Kit (GE Healthcare), and aliquots were immediately frozen at −80 °C, until use.

Isoelectric focusing (IEF) and SDS-PAGE

For the first-dimension electrophoresis, 150 μg of protein extract was added to a volume of 250 μL with a rehydration solution [7 M urea, 2 M thiourea, 2% CHAPS, 40 mM DTT, 2% immobilized pH gradient (IPG-buffer, pH 4–7, trace bromophenol blue)]. Next, samples were applied to IPG strips (13 cm, pH 4–7; GE Healthcare) for passive rehydration overnight at room temperature. After in-gel rehydration for 12 h, isoelectric focusing was performed at 500 V for 1 h, 1.000 V for 1 h, and 8.000 V for 8 h, using a Multiphor II electrophoresis unit and EPS 3500 XL power supply (Amersham, Piscataway, NJ, USA). After IEF, each strip was incubated for 15 min in a solution made up of 10 mL of a 50 mM Tris-HCl buffer pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.002% bromophenol-blue and 125 mM DTT, followed by a second incubation step in the same buffer solution, excluding DTT, which was replaced by 125 mM iodacetamide. IPG strips were transferred to a 12% polyacrylamide and sealed with agarose solution (agarose and bromophenol blue in a Tris-glycine cathode buffer). The protein standard was purchased from Invitrogen (BenchMark™ Protein Ladder). Electrophoresis was performed in a Mini-Protean II system (BioRad) connected to a MultiTemp II cooling bath (Amersham Biosciences), in a Tris/glycine/SDS buffer. Proteins were separated at 200 V, until the dye front had reached the bottom of the gel.

Immunoblottings and protein identification

To select the best spots, immunoblottings were performed using *L. infantum* promastigote and amastigote protein extracts. For this, they were separated electrophoretically and transferred onto cellulose membranes (Schleicher and Schull, Dassel, Germany) by semi-dry blotting for 2 h at 400 mA. Then, membranes were blocked in 5% (w/v) low-fat dried milk in PBS 1× plus 0.05% Tween 20 for 2 h at room temperature. Next, they were washed seven times (10 min, each) with blocking solution and incubated with sera pools from VL ($n = 15$) or Chagas disease ($n = 10$) patients, as well as with sera pool from healthy individuals ($n = 15$) living in the endemic region of disease. All pools were performed at 1:400 in PBS 1× plus 0.05% Tween 20, and the incubation occurred for 2 h at room temperature. After,

https://doi.org/10.1017/S0031182019000714 Published online by Cambridge University Press

membranes were incubated with a peroxidase-conjugated goat anti-human IgG secondary antibody (1:10 000 diluted), for 1 h at room temperature. After having been washed seven times with PBS 1× plus 0.5% Tween 20, blots were revealed by the addition of chloronaphtol, diaminobenzidine and H_2O_2 30 vol. Bidimensional gels were stained with colloidal Coomassie Brilliant Blue G-250, according described (Neuhoff *et al.*, 1988). The stained gels were scanned using an ImageScanner III (GE Healthcare), and spots mainly recognized by antibodies in sera from VL patients were excised from the gels for protein identification. Three independent preparations were performed using independent parasite cultures, and one representative preparation is shown.

Protein digestion, peptide extraction and spot handling

Spots were excised, and fragments were washed in 25 mM ammonium bicarbonate/50% acetonitrile until completely destained. After drying, gel fragments were placed on ice in a 50 μL protease solution (20 ng mL^{-1} of a sequence grade-modified trypsin in a 25 mM ammonium bicarbonate) (Promega Biosciences, CA, USA) for 30 min. Excess protease solution was removed and replaced by 25 mM ammonium bicarbonate, when the digestion was performed for 18 h at 37 °C. Peptide extraction was performed twice for 15 min, using 30 μL of 50% acetonitrile/5% formic acid. Trypsin (Promega) digests were concentrated in a Speed-Vac (Savant, USA) to approximately 10 μL and desalted using Zip-Tip (C18 resin; P10, Millipore Corporation, Bedford, MA). Samples were mixed with a matrix (5 mg mL^{-1} recrystallized α-cyano-4-hydroxycinnamic acid) in a volume of 1 mL (1:1 ratio), and spotted for MALDI-TOF/TOF Ultraflex III (Bruker, Daltonics, Germany).

Protein identification and database search

To determine the MS spectrum of the selected spots, the digests were spotted onto 600 μm Anchorchips (Bruker Daltonics). Spotting was achieved by pipetting, in duplicate, 1 μL of analyte onto the MALDI target plate, then adding 5 mg mL^{-1} α-cyano-4-hydroxycinnamic acid diluted in 3% TFA/50% acetonitrile, which contained 2 mM ammonium phosphate. The Bruker peptide calibration mixture was spotted down for external calibration. All samples were allowed to air dry at room temperature, and 0.1% TFA was used for on-target washing. All samples were analysed in the positive-ion, reflection mode, through a MALDI-TOF/TOF Ultraflex III mass spectrometer (Bruker, Daltonics, Germany). Each spectrum was produced by accumulating data from 200 consecutive laser shots, with a frequency of 100 Hz, and an m/z range of 1.000–4.000. Instrument calibration was achieved by using peptide calibration standard II (Bruker Daltonics), a mixture of angiotensin I and II, substance P, bombesin, ACTH clip 1–17, ACTH clip 18–39 and somatostatin 28, as the internal standard. Peptide masses were measured as monoisotopic masses. The MS peaks with the highest intensities were selected for MS/MS fragmentation analyses. The resulting spectra were processed using Flex analysis software, version 2.4 (Bruker Daltonics), with the following settings: peak detection algorithm set at SNAP (Sort Neaten Assign and Place), S/N threshold at 3, precursor and product ion tolerances were set at 0.5 Da, and quality factor threshold at 50. The trypsin autodigestion ion peaks (842.51, 1045.56, 2211.10 and 2225.12 Da) were used as internal standards to validate the external calibration procedure. Matrix, and/or autoproteolytic trypsin fragments, and known contaminants (i.e. keratins) were manually removed. The resulting peptides list was used to search in the NCBI database (http://blast.ncbi.nlm.nih.gov) for the organism option of *Leishmania* (taxid: 5658). According to the obtained results, and using the peptide sequences identified for each protein, the following parameters were used as selection criteria: total score, query coverage and E value.

Cloning and purification of the parasite proteins

To evaluate the antigenic potential of selected proteins in the immunoproteomics, two antigens, endonuclease III (LINF_090005600) and GTP-binding protein (LINF_250020400) were cloned, expressed, purified and evaluated in ELISA experiments for the serodiagnosis of VL. For this, genes were cloned from *L. infantum* DNA by using the 5′-TGCTGCTAGCATGAGTAAACACTCCTTT-3′ (forward) and 5′-TCAT**GGATCC**TCACTTTATGCGTCTCT T-3′ (reverse) for endonuclease III, and 5′-TGCTGCTAGCATGCA ACAGGCACCCT-3′ (forward) and 5′-TCAT**GGATCC**TCACTC GTCATCGCCCAT-3′ (reverse) for GTP-binding protein. The restriction enzymes used were *Bam*HI and *NheI*. The DNA fragments were excised from gels, purified and linked into pGEM®-T Vector Systems (Promega, USA), and recombinant plasmids were used to transform *Escherichia coli* XL1-Blue competent cells. Positive clones were propagated and used for construction of the expression vector. DNA fragments obtained from digestion of pGEM-rENDO and pGEM-rGTP plasmids were ligated into pET28a-TEV, and *E. coli* BL21 cells (DE3; Agilent Technologies, USA) were transformed with the recombinant plasmids. Gene insertion was confirmed by PCR and sequencing by using the MegaBace 1000 automatic sequencer apparatus (Amersham Biosciences, USA). For the expression and purification of the proteins, cells were induced with IPTG (0.5 μM) and cultures were shaking at 200 × ***g*** per min for 24 h at 12 °C. Cells were ruptured by using six cycles of ultrasonication with cycles of 30 s each (38 MHz), followed by six cycles of freezing and thawing. After, cellular debris was removed by centrifugation, and recombinant proteins were purified onto a HisTrap HP affinity column connected to an AKTA system (GE Healthcare, USA). The eluted fractions containing the rENDO (29.0 kDa) and rGTP (24.0 kDa) proteins were concentrated in Amicon® ultra15 centrifugal filters 10 000 NMWL (Millipore, Germany), and further purified on a Superdex™ 200 gel-filtration column (GE Healthcare Life Sciences, USA). After, recombinant proteins were passed through a polymyxin-agarose column (Sigma) to remove any residual endotoxin content.

ELISA experiments

Previous titration curves were performed to determine the most appropriate antigen concentration and antibody dilution to be used. Falcon flexible microtitre immunoassay plates (Becton Dickinson) were coated with rENDO, rGTP or *L. infantum* SLA (0.5, 0.25 and 1.0 μg per well, respectively), which were diluted in 100 μL of coating buffer (50 mM carbonate buffer) pH 9.6, for 16 h at 4 °C. Next, free binding sites were blocked using 250 μL of PBS-T (PBS 1× plus Tween 20 0.05%) plus 5% non-fat dry milk (catalogue M7409-1BTL, Sigma-Aldrich, St. Louis, MO, USA), for 1 h at 37 °C. After washing plates five times with PBS-T, they were incubated with 100 μL of human sera (1:400 diluted in PBS-T), for 1 h at 37 °C. Plates were washed seven times in PBS-T, and incubated with anti-human IgG horseradish–peroxidase conjugated antibody (1:20 000 diluted in PBS-T; catalogue SAB3701282; Sigma-Aldrich, USA) for 1 h at 37 °C. After washing plates seven 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 in the dark. Reactions were stopped by adding 25 μL 2 N H_2SO_4, and optical density (OD) values were read in an ELISA microplate spectrophotometer (Molecular Devices, Spectra Max Plus, Canada), at 492 nm.

https://doi.org/10.1017/S0031182019000714 Published online by Cambridge University Press

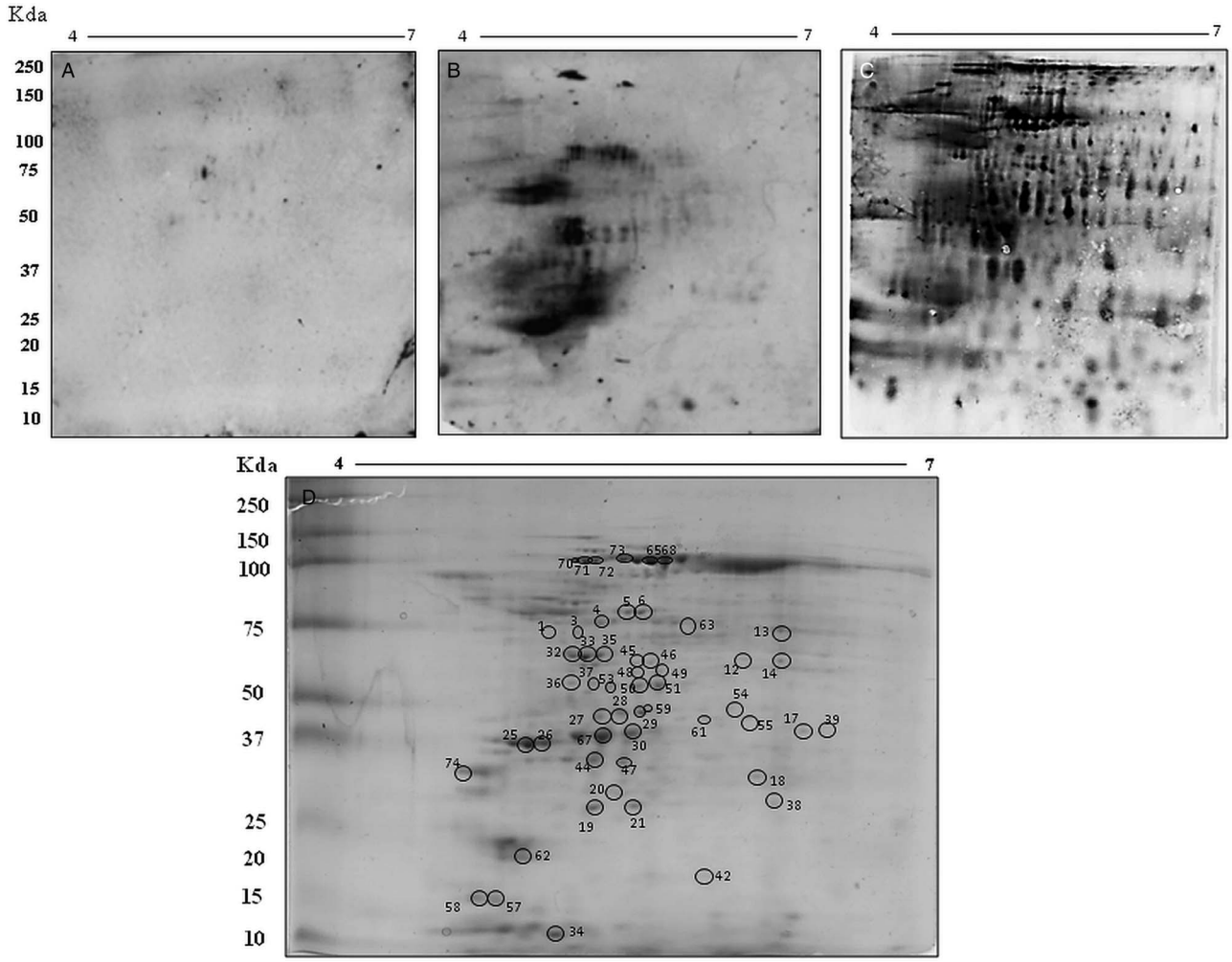

Fig. 1. Two-dimensional profile and immunoproteomic analysis in *L. infantum* promastigote protein extracts. Bidimensional gels were obtained after separation of promastigote protein extracts of the parasites (150 *μ*g) by using in the first dimension: IEF pH range 4–7, and second dimension: 12% SDS-PAGE. After, they were stained with colloidal Coomassie Brilliant Blue G-250. Immunoblots were developed after incubation of membranes with sera pools from healthy subjects (A), Chagas disease (B) or visceral leishmaniasis (C) patients, all 1:400 diluted in PBS 1× plus 0.05% Tween 20. Bound antibodies were detected with a peroxidase-conjugated goat anti-human IgG secondary antibody (1:10 000 diluted). The *x*-axis represents the isoelectric point (pI), and the *y*-axis represents the molecular weight (kDa) indicated by a commercial marker (BenchMark™ protein ladder). The identified spots after their recognition by antibodies in sera of VL patients are marked in the 2DE stained gel (D), and their identities are given in Table 1. Immunoblots are a reliable representation of three independent experiments.

Serological follow-up after VL treatment

To evaluate the serological reactivity of rGTP and rENDO proteins using sera samples from treated and untreated VL patients, samples from patients ($n = 10$, including six males and four females, with ages ranging from 32 to 57 years) were collected before and six months after treatment using pentavalent antimonials (Sanofi Aventis Farmacêutica Ltda., Suzano, São Paulo, Brazil). All patients were submitted to the same therapeutic regimen using the pharmaceutics, at a dose of 20 mg Sb^{5+} per kg during 30 days, and none of them suffered from any other infections or had any pre-existing disease. When all of them had the treatment completed, they were free of any symptom of disease.

Statistical analysis

The results were entered into Microsoft Excel (version 10.0) spreadsheets and analysed using GraphPad Prism™ (version 6.0 for Windows). Receiver operating characteristic (ROC) curves were constructed to obtain the sensitivity (Se), specificity (Sp), area under the curve (AUC) and likelihood ratio (LR); as well as the lower limit of positivity (cut-off). Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by the Bonferroni's post-test for multiple comparisons between the groups. Differences were considered significant at $P < 0.05$.

Results

Identifying the immunoreactive spots in the L. infantum *promastigotes and amastigotes*

In our study, *L. infantum* promastigote protein extracts were reacted with antibodies in sera from VL patients in an immunoproteomics approach. Samples from Chagas disease patients and healthy controls were used to exclude the most cross-reactive spots. Results showed a low reactivity when sera from healthy individuals were used (Fig. 1A). On the other hand, higher reactivity was found when sera from Chagas disease patients were employed in the analysis (Fig. 1B). The majority of the identified spots showed pH between 4.0 and 5.5, and molecular weight varying from 20 to 70 kDa. When sera from VL patients were used, about 200 protein spots were visualized (Fig. 1C). From the selected spots in *L. infantum* promastigotes (Fig. 1D), a sequencing reaction was performed and 29 valid sequences were identified in this parasite stage. Table 1 shows the identity of

https://doi.org/10.1017/S0031182019000714 Published online by Cambridge University Press

Table 1. Proteins of *L. infantum* promastigotes recognized by antibodies in visceral leishmaniasis patients in an immunoproteomic approach

Spot number[a]	Accession number[b]	Protein name[c]	pI[d]	M_r[e]
1/4/12/37	LINF_300039600	CAS/CSE/importin domain protein	5.43	108.017
3	LINF_090005600	Endonuclease III	9.86	29.288
5	LINF_290024300	Paraflagellar rod protein 1C	5.06	69.058
6	LINF_290024200	Paraflagellar rod protein 1D	5.10	68.575
13/14	LINF_320030800	Hypothetical protein	6.29	232.483
17	LINF_350027200	RNA-binding protein	8.41	30.257
18	LINF_350012100	Proteasome activator protein	5.15	23.958
19/21	LINF_230005400	Peroxidoxin	6.92	25.369
20	LINF_260024900	Hypothetical protein	4.73	89.019
25/26/72	LINF_080017700	Beta-tubulin	4.45	49.799
28/27	LINF_300018100	Pyridoxal kinase	6.37	33.128
29	LINF_340014000	Elongation factor 1-beta	4.90	25.935
30/44/47/62/67	LINF_130008400	Alpha-tubulin	4.65	49.758
32/33/35/36	LINF_330009500	Heat shock protein 83	4.79	80.549
34	LINF_130009400	ALBA-domain protein 1	4.80	13.405
38	LINF_250020400	GTP-binding protein	6.51	24.223
39/48/54/55/63	LINF_300018100	Pyridoxal kinase	6.37	33.128
42	LINF_290013500	Hypothetical protein	5.27	51.677
45	LINF_330033600	Hypothetical protein	8.23	56.498
46	LINF_120011900	Cytochrome oxidase subunit IV	5.51	39.596
49	LINF_340025300	IQ calmodulin-binding motif containing protein	10.3	128.596
50/51	LINF_360006800	Elongation factor 2	5.95	94.146
53	LINF_140018200	Poly(A) polymerase	7.47	77.146
57	LINF_120016600	Hypothetical protein	6.92	95.508
58	LINF_140014400	Calpain-like cysteine peptidase	4.40	12.931
59	LINF_340014000	Elongation factor 1-beta	4.90	25.935
61	LINF_120006200	Hypothetical protein	5.72	91.580
65//68/74	LINF_280035000	Heat-shock protein 70	5.15	71.267
70/74	LINF_290009600	Hypothetical protein	6.68	78.448

[a]Spot number in the bidimensional gel.
[b]Accession numbers according to NCBI.
[c]Name of the identified protein.
[d]Predicted isoelectric point (pI).
[e]Predicted molecular weight (M_r, in kDa).

the identified proteins, also describing their accession number, molecular weight and isoelectric point.

In this same manner, using the *L. infantum* amastigotes, sera of healthy subjects showed a low reactivity (Fig. 2A), while higher number of spots were recognized by using Chagas disease patients sera were used in the blots (Fig. 2B), with the majority of spots concentrated between 50 and 120 kDa and with pH varying from 4.5 to 5.5. Similarly to found in the promastigote extract, higher number of spots was visualized when VL patients sera were employed in the immunoblottings (Fig. 2C), when over 150 spots were visualized. From the selected spots in *L. infantum* amastigotes (Fig. 2D), a sequencing reaction was performed and 21 valid sequences were identified in this parasite stage. Table 2 shows the identity of the identified proteins in the amastigote extract, describing their accession number, molecular weight and isoelectric point.

Evaluating the biological functions of identified proteins

Among the proteins recognized by antibodies in VL patients sera in the promastigote extract, seven hypothetical and 22 known antigens were identified. Proteins related to the parasite virulence, such as alpha- and beta-tubulins (Coulson *et al.*, 1996; Coelho *et al.*, 2012) and heat-shock protein 70 (Drini *et al.*, 2016); therapeutic targets, such as RNA-binding protein (Nandan *et al.*, 2017), endonuclease III (Moreira *et al.*, 2017), GTP-binding protein (Ishemgulova *et al.*, 2017) and pyridoxal kinase (Kumar *et al.*, 2018); and vaccine candidates, such as paraflagellar rod protein (Carrillo *et al.*, 2008), peroxidoxin (Bayih *et al.*, 2014) and elongation factor (Sabur *et al.*, 2018) were found. In the amastigote extract, four hypothetical and 17 known proteins were identified. From these, virulence factors, such as leucine-rich repeat protein (Mukherjee *et al.*, 2016), diagnosis markers, such as enolase (Duarte *et al.*, 2017), vaccine candidates, such as eukaryotic initiation factor 4a (Maspi *et al.*, 2015), and

https://doi.org/10.1017/S0031182019000714 Published online by Cambridge University Press

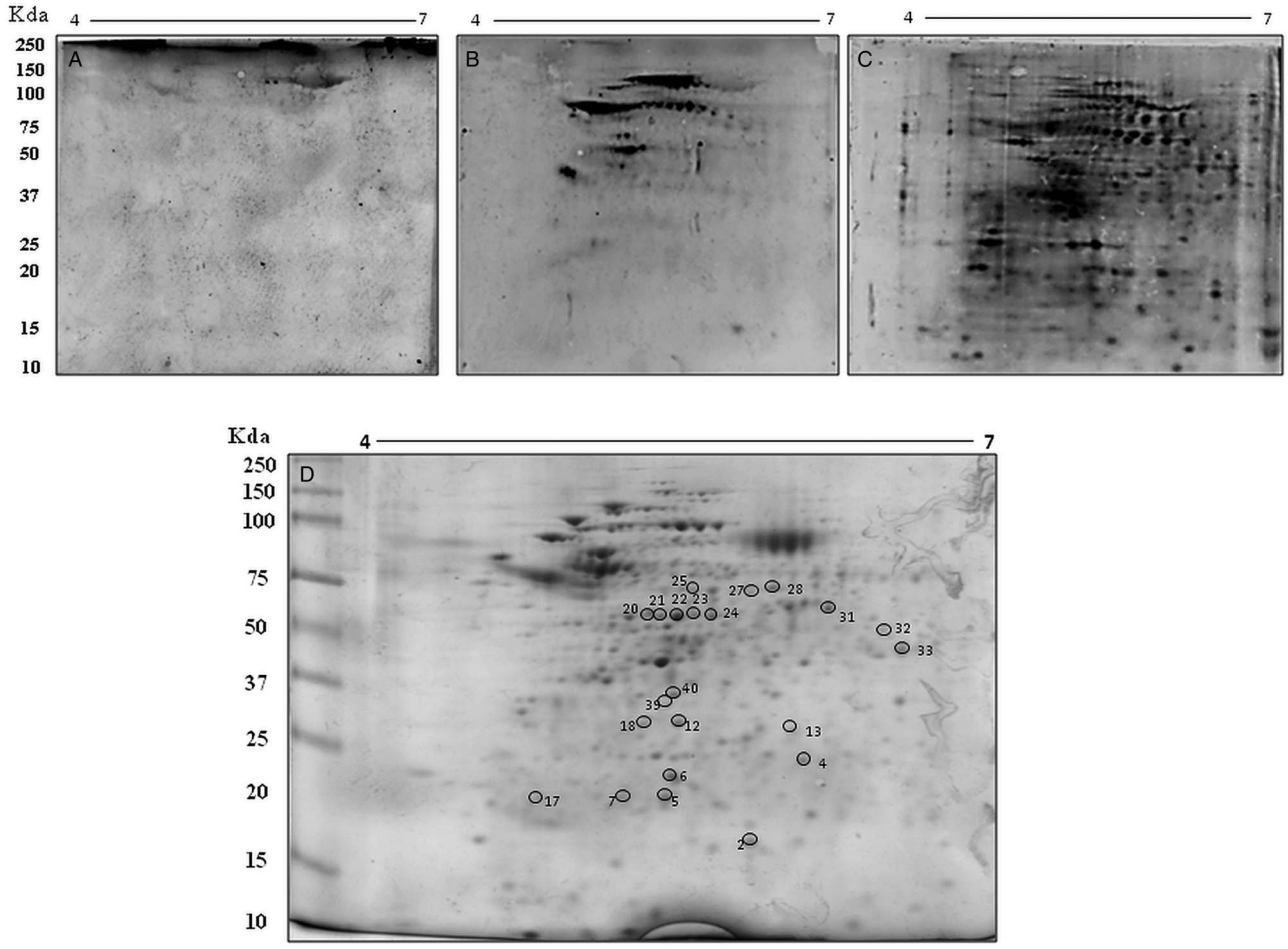

Fig. 2. Two-dimensional profile and immunoproteomic analysis in *L. infantum* amastigote protein extracts. Bidimensional gels were obtained after separation of axenic amastigote protein extracts of the parasites (150 μg) by using in the first dimension: IEF pH range 4–7, and second dimension: 12% SDS-PAGE. After, they were stained with colloidal Coomassie Brilliant Blue G-250. Immunoblots were developed after incubation of membranes with sera pools from healthy subjects (A), Chagas disease (B) or visceral leishmaniasis (C) patients, all 1:400 diluted in PBS 1× plus 0.05% Tween 20. Bound antibodies were detected with a peroxidase-conjugated goat anti-human IgG secondary antibody (1:10 000 diluted). The *x*-axis represents the isoelectric point (pI), and the *y*-axis represents the molecular weight (kDa) indicated by a commercial marker (BenchMark™ protein ladder). The identified spots after their recognition by antibodies in sera of VL patients are marked in the 2DE stained gel (D), and their identities are given in Table 2. Immunoblots are a reliable representation of three independent experiments.

drug targets, such as calpain-like cysteine peptidase (Chávez-Fumagalli *et al.*, 2017) were recognized.

Testing two recombinant proteins as diagnostic markers for VL

Two proteins that were identified in the promastigote extract, endonuclease III and GTP-binding, were cloned, expressed, purified and their recombinant versions (rENDO and rGTP, respectively) were evaluated in ELISA experiments. The individual OD values obtained against the different antigens are shown (Fig. 3), and ROC curves were constructed to obtain the cut-off values. Results showed that both proteins presented sensitivity and specificity values of 100 and 99.31%, respectively, with AUC of 1.0 and a likelihood ratio of 145; while using *L. infantum* SLA as a control antigen, sensitivity and specificity values were of 63.31 and 26.67%, respectively, with AUC of 0.67 and likelihood ratio of 38.67 (Table 3).

IgG antibody levels before and after the VL treatment

With the purpose to evaluate the serological reactivity of the patients before and after treatment, sera were collected and ELISA assays were performed. Results showed a significant decrease in the protein-specific IgG antibody levels, 6 months after the treatment of the patients (Fig. 4). Otherwise, using *L. infantum* SLA as an antigen, similar antibody levels were found before and after treatment.

Discussion

Visceral leishmaniasis is a neglected disease in which the outcome of infection can be fatal if acute and left untreated (Burza *et al.*, 2018). Diagnostic tests are still related to invasive aspiration or by means of immunochromatographic evaluations. Advancements in immunoproteomics employing bidimensional electrophoresis and mass spectrometry have enabled the identification of new markers for disease diagnosis and prognosis, as well as to they use in therapeutic monitoring and the understanding of the infected hosts' immune response (Kumari *et al.*, 2008; Ejazi *et al.*, 2018). In this study, an immunoproteomics approach was performed using VL patients sera, as well as from Chagas disease patients and healthy endemic control; those employed to exclude the most cross-reactive spots. From this approach, 29 and 21 valid sequences were identified by MALDI-TOF/TOF and mass spectrometry in the promastigote and amastigote stages of the parasites. On the basis of results obtained, these antigens can be expected to be antigenic in the human VL. Some of them, such as tubulins and heat shock proteins are housekeeping in *Leishmania* (Coelho *et al.*, 2012); while others, such as paraflagellar rod protein, elongation factor and enolase present immunological role in the disease in mammalian hosts

https://doi.org/10.1017/S0031182019000714 Published online by Cambridge University Press

Table 2. Proteins of *L. infantum* amastigotes recognized by antibodies in visceral leishmaniasis patients in an immunoproteomic approach

Spot number[a]	Access number[b]	Protein name[c]	pI[d]	M_r[e]
2	LINF_270014400	Right-handed beta helix region/periplasmic copper-binding protein	6.81	347.161
4	LINF_350012100	Proteasome activator protein pa26	5.15	23.959
5	LINF_230005400	Peroxidoxin	6.43	25.370
6	LINF_250012900	Eukaryotic translation initiation 5A	4.62	17.826
7	LINF_320037600	Leucine-rich repeat protein	4.54	96.15
12	LINF_300039600	Putative CAS/CSE/importin domain protein	5.43	108.017
13	LINF_320008900	Hypothetical protein	9.22	57.423
17/18	LINF_280039500	Glucosamine 6-phosphate n-acetyltransferase	7.41	16.32
20	LINF_280028700	Mannosyl oligosaccharide glucosidase	8.14	107.533
21	LINF_310018300	Cytochrome b5-like haem/steroid binding domain containing protein	4.37	17.225
22	LINF_010008300	Hypothetical protein	9.85	23.543
23	LINF_300018100	Pyridoxal kinase	6.37	33.128
24	LINF_350044500	PFPI/DJ-1-like protein	10.01	16.768
25	LINF_140018000	Enolase	5.12	46.36
27	LINF_270011100	Calpain-like cysteine peptidase	5.09	507.736
28	LINF_210019600	Hypothetical protein	8.20	116.161
31	LINF_010012800	Eukaryotic initiation factor 4a	6.15	45.326
32	LINF_320041000	B-box zinc finger containing protein	9.27	142.361
33	LINF_300018100	Pyridoxal kinase	6.37	33.128
39	LINF_120006200	Hypothetical protein	5.72	91.58
40	LINF_340014000	Elongation factor 1-beta	4.90	25.935

[a]Spot number in the bidimensional gel.
[b]Accession numbers according to NCBI.
[c]Name of the identified protein.
[d]Predicted isoelectric point (pI).
[e]Predicted molecular weight (M_r, in kDa).

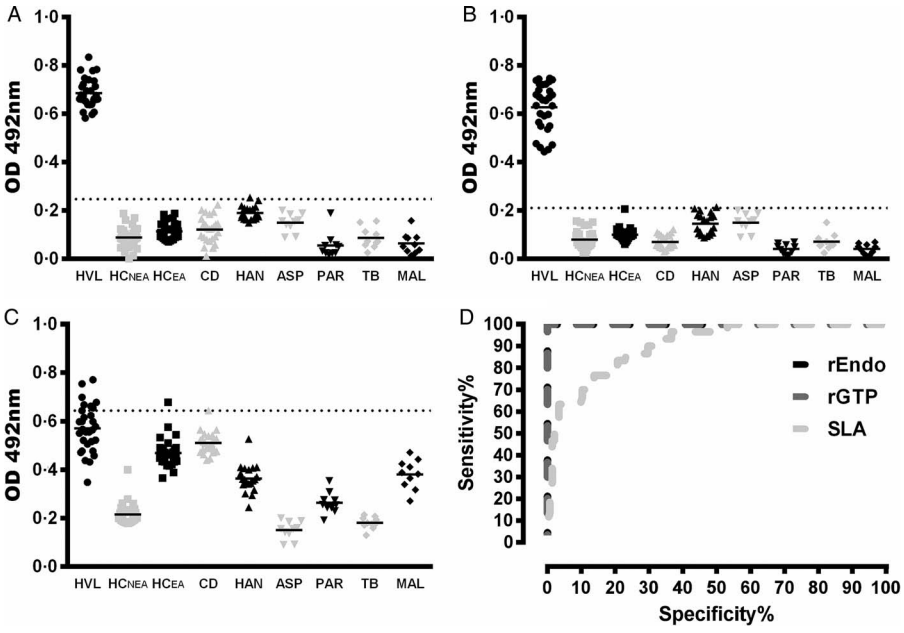

Fig. 3. Evaluation of recombinant antigens for the serodiagnosis of visceral leishmaniasis. ELISA experiments were performed using the rENDO and rGTP proteins and *L. infantum* SLA, as a control. Sera samples from visceral leishmaniasis (HVL, $n = 30$) patients and healthy individuals living in non-endemic (HC_{NEA}, $n = 30$) or endemic (HC_{EA}, $n = 30$) areas of leishmaniasis were used. To evaluate the cross-reactivity of the antigens, samples from Chagas disease (CD, $n = 25$), leprosy (HAN, $n = 20$), aspergillosis (ASP, $n = 10$), paracoccidioidomycosis (PAR, $n = 10$), tuberculosis (TB, $n = 10$) and malaria (MAL, $n = 10$) patients were used in the assays. The cut-off values were calculated by Receiver Operator Curves (ROC). Results showing the optical density (OD) values of each sample, as well as the mean of sera groups for the rENDO (A), rGTP (B) and SLA (C) antigens, are shown. ROC curves were used to determine ELISA sensitivity (95% CI), specificity (95% CI) and AUC for the diagnostic antigens (D).

(Carrillo *et al.*, 2008; Santos *et al.*, 2017; Sabur *et al.*, 2018). Not less important, a number of hypothetical proteins were identified by antibodies in VL patients sera, when both proteins extracts were tested; thus demonstrating the possibility to also evaluate these antigens as diagnostic markers and/or vaccine candidates against disease (Lage *et al.*, 2016; Ribeiro *et al.*, 2018; Chávez-Fumagalli *et al.*, 2019).

In our study, we have chosen two parasite proteins that were recognized in the promastigote extract, aiming to validate our data generated in the immunoproteomics approach, by using

https://doi.org/10.1017/S0031182019000714 Published online by Cambridge University Press

Table 3. Diagnostic performance of the antigens for the serodiagnosis of visceral leishmaniasis

Antigen	AUC	*P* value	Cut-off	Se	95% CI	Sp	95% CI	LR+
rENDO	1	<0.0001	>0.2473	100	88.43–100	99.31	96.22–99.98	145
rGTP	1	<0.0001	>0.2105	100	88.43–100	99.31	96.22–99.98	145
L. infantum SLA	0.67	<0.05	>0.6438	63.31	46.22–79.98	26.67	12.28–45.89	38.67

A human serological panel was used in ELISA experiments against rENDO, rGTP and *L. infantum* SLA. Receiver operating characteristic (ROC) curves were constructed to obtain the sensitivity (Se), specificity (Sp), area under the curve (AUC) and likelihood ratio (LR) values; as well as the lower limit of positivity (cut-off).

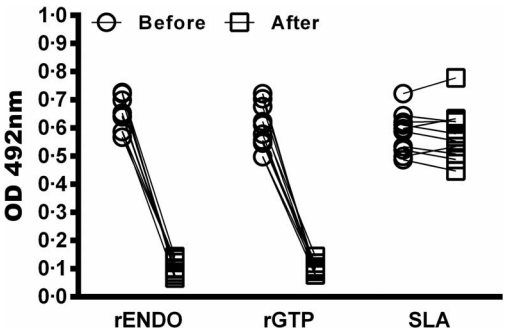

Fig. 4. Serological reactivity of the recombinant proteins before and after treatment of visceral leishmaniasis patients. Sera samples were collected before and six months after treatment of VL ($n = 10$), and the anti-protein and anti-parasite IgG levels were investigated. White circles and square shown the optical density (OD) values of the individual samples collected before and after treatment, respectively.

ELISA experiments and a large serological panel. Results showed that the recombinant proteins, rENDO and rGTP, showed high sensitivity and specificity values to identify VL samples. Additionally, they were not recognized by antibodies in sera from patients with paracoccidioidomycosis, leprosy, aspergillosis, tuberculosis or malaria; as well as by sera from non-endemic or endemic healthy controls. This fact opens the possibility to test these candidates in future studies for the serodiagnosis of human disease.

GTP-binding proteins are considered as virulence factors in *Leishmania*, being involved in the drug resistance against *L. amazonensis* species (Lang *et al.*, 1994; Ishemgulova *et al.*, 2017). These molecules participate in secretion pathways and/or environmental response of parasites, as well as in the communication with the host cell during the infection (Ishemgulova *et al.*, 2017). Recently, Magalhães *et al.* (2014) applied a proteomic approach in *L. amazonensis* to analyse the variation of the protein expression profile, when parasites were *in vitro* cultured for a 150-day period. Results showed that 37 proteins presented a significant decrease in their expression content, whereas 19 proteins showed a significant increase in their content during the cultivation. The authors associated some of these proteins as diagnosis markers, vaccine candidates and/or drug targets on leishmaniasis. One of them, the GTP-binding protein, was showed to decrease its expression content in the order of 3.13-fold during the cultivation. It and others that also decrease their expression were considered as parasites' infectivity factors, in which a significant reduction in their *in vitro* and *in vivo* infectivity was found after 150 days of cultivation (Magalhães *et al.*, 2014).

Recent evidence has shown that the characteristics of the *Leishmania* replication and repair machinery can be used as targets for the development of new therapeutic strategies. In this context, the evaluation of DNA repair enzymes as biological targets can open the way to the identification of potential targets for the development of novel diagnostic and therapeutic strategies (Rajão *et al.*, 2014). One of the proteins identified here, endonuclease III, is a DNA repair glycosylase, previously known for its repair activity on oxidative pyrimidine damage (Mishra *et al.*, 2018). In this study, the diagnostic potential of this enzyme was successfully evaluated, then speculate about the possible employment of this recombinant antigen for the serodiagnosis of disease.

Different strategies have been tested to provide understanding about the hosts' immune response against infection by different pathogens, such as *Leishmania*, such as by proteomic studies (Chambers *et al.*, 2000; Sundar and Singh, 2018). Previous work published used the immunoproteomics tool to identify antigenic proteins in *L. braziliensis* protein extracts by antibodies in sera from tegumentary leishmaniasis (TL) patients. With the purpose to reduce the cross-reactivity of the identified proteins, spots highly recognized by antibodies in sera from Chagas disease patients and healthy individuals living in an endemic region were excluded. Results showed that 20 proteins were identified in the protein extracts, and five of them were cloned, expressed, purified and tested in ELISA experiments for the diagnosis of TL, with serological results showing high sensitivity and specificity values (Duarte *et al.*, 2015).

Here, the experimental strategy was also based on the exclusion of the most reactive spots, since sera samples from Chagas disease patients and healthy controls were used. This fact is based on the high cross-reactivity found when serodiagnosis tests are applied in such patients, and could justify the low number of identified proteins. In addition, several antigens recognized by antibodies in parasite extracts were recognized as multiple spots or proteolytic fragments. Although their degradation cannot be discarded; extracts were prepared in the presence of a cocktail of protease inhibitors. As a consequence, these findings can be associated with the presence of isoforms or post-translational modifications, known to occur in *Leishmania* parasites (Brotherton *et al.*, 2010; Coelho *et al.*, 2012; Moreira *et al.*, 2015).

The serological evaluation in cured patients should also be considered, since the purpose of diagnostic tools is to develop a test that can be used in endemic regions to detect active disease. Results obtained in this study can be considered promising, since lower serological reactivity specific to rGTP and rENDO proteins was found in the cured and treated VL patients. Conversely, rA2 and SLA did not show such discriminative results, and new experiments must be performed to confirm the lack of their diagnostic role in human disease. To the best of our knowledge, the present study is the first in which these proteins were effective to discriminate patients after treatment had been completed; thus suggesting the possibility of using them as serological markers for VL, as well as to correlate the presence of low levels of specific antibodies with the clinic cure of patients. As limiting factor of the work, the small number of patients evaluated before and after treatment should be considered and certainly new studies are necessary to be performed.

Taking into account, results indicated patterns of protein recognition by antibodies in sera from *L. infantum*-infected patients by an immunoproteomics approach, and suggested that rENDO and rGTP could be evaluated as diagnostic markers for human VL, as well as in the monitoring of human disease treatment. Additional

https://doi.org/10.1017/S0031182019000714 Published online by Cambridge University Press

studies using these antigens, as well as others identified in the amastigote forms, should be performed to validate our findings about the use of these proteins as diagnostic markers for human VL.

Author ORCIDs. Eduardo A. F. Coelho, 0000-0002-6681-9014.

Financial support. The authors would like thank CAPES, CNPq and FAPEMIG for scholarships. This work was supported by grants from CNPq (APQ-408408/2016-2 and APQ-408675/2018-7).

Conflict of interest. None.

Ethical standards. The study was approved by the Human Research Ethics Committee from Federal University of Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil (protocol number CAAE-32343114.9.0000.5149).

References

Alves F, Bilbe G, Blesson S, Goyal V, Monnerat S, Mowbray C, Muthoni GO, Pécoul B, Rijal S, Rode J, Solomos A, Strub-Wourgaft N, Wasunna M, Wells S, Zijlstra EE, Arana B and Alvar J (2018) Recent development of visceral leishmaniasis treatments: successes, pitfalls, and perspectives. *Clinical Microbiology Reviews* **31**, e00048.

Bayih AG, Daifalla NS and Gedamu L (2014) DNA-protein immunization using *Leishmania* peroxidoxin-1 induces a strong CD4+ T cell response and partially protects mice from cutaneous leishmaniasis: role of fusion murine granulocyte-macrophage colony-stimulating factor DNA adjuvant. *PLoS Neglected Tropical Diseases* **8**, e3391.

Brotherton MC, Racine G, Foucher AL, Drummelsmith J, Papadopoulou B and Ouellette M (2010) Analysis of stage-specific expression of basic proteins in *Leishmania infantum*. *Journal of Proteome Research* **9**, 3842–3853.

Burza S, Croft SL and Boelaert M (2018) Leishmaniasis. *Lancet* **392**, 951–970.

Carrillo E, Crusat M, Nieto J, Chicharro C, Thomas MC, Martínez E, Valladares B, Cañavate C, Requena JM, López MC, Alvar J and Moreno J (2008) Immunogenicity of HSP-70, KMP-11 and PFR-2 leishmanial antigens in the experimental model of canine visceral leishmaniasis. *Vaccine* **26**, 1902–1911.

Chambers G, Lawrie L, Cash P and Murray GI (2000) Proteomics: a new approach to the study of disease. *The Journal of Pathology* **192**, 280–288.

Chávez-Fumagalli MA, Schneider MS, Lage DP, Machado-de-Ávila RA and Coelho EA (2017) An *in silico* functional annotation and screening of potential drug targets derived from *Leishmania* spp. hypothetical proteins identified by immunoproteomics. *Experimental Parasitology* **176**, 66–74.

Chávez-Fumagalli MA, Lage DP, Tavares GSV, Mendonça DVC, Dias DS, Ribeiro PAF, Ludolf F, Costa LE, Coelho VTS and Coelho EAF (2019) *In silico Leishmania* proteome mining applied to identify drug target potential to be used to treat against visceral and tegumentary leishmaniasis. *Journal of Molecular Graphics and Modelling* **87**, 89–97.

Coelho EA, Tavares CA, Carvalho FA, Chaves KF, Teixeira KN, Rodrigues RC, Charest H, Matlashewski G, Gazzinelli RT and Fernandes AP (2003) Immune responses induced by the *Leishmania* (*leishmania*) *donovani* a2 antigen, but not by the LACK antigen, are protective against experimental *Leishmania* (*Leishmania*) *amazonensis* infection. *Infection and Immunity* **71**, 3988–3994.

Coelho VT, Oliveira JS, Valadares DG, Chávez-Fumagalli MA, Duarte MC, Lage PS, Soto M, Santoro MM, Tavares CA, Fernandes AP and Coelho EA (2012) Identification of proteins in promastigote and amastigote-like *Leishmania* using an immunoproteomic approach. *PLoS Neglected Tropical Diseases* **6**, e1430.

Coulson RM, Connor V, Chen JC and Ajioka JW (1996) Differential expression of *Leishmania major* beta-tubulin genes during the acquisition of promastigote infectivity. *Molecular and Biochemical Parasitology* **82**, 227–236.

Dias DS, Martins VT, Ribeiro PAF, Ramos FF, Lage DP, Tavares GSV, Mendonça DVC, Chávez-Fumagalli MA, Oliveira JS, Silva ES, Gomes DA, Rodrigues MA, Duarte MC, Galdino AS, Menezes-Souza D and Coelho EAF (2018) Antigenicity, immunogenicity and protective efficacy of a conserved *Leishmania* hypothetical protein against visceral leishmaniasis. *Parasitology* **145**, 740–751.

Didwania N, Shadab M, Sabur A and Ali N (2017) Alternative to chemotherapy-the unmet demand against leishmaniasis. *Frontiers in Immunology* **8**, 1779.

Drini S, Criscuolo A, Lechat P, Imamura H, Skalický T, Rachidi N, Lukeš J, Dujardin JC and Späth GF (2016) Species- and strain-specific adaptation of the HSP70 super family in pathogenic Trypanosomatids. *Genome Biology and Evolution* **8**, 1980–1995.

Duarte MC, Pimenta DC, Menezes-Souza D, Magalhães RD, Diniz JL, Costa LE, Chávez-Fumagalli MA, Lage PS, Bartholomeu DC, Alves MJ, Fernandes AP, Soto M, Tavares CA, Gonçalves DU, Rocha MO and Coelho EA (2015) Proteins selected in *Leishmania* (*viannia*) *braziliensis* by an immunoproteomic approach with potential serodiagnosis applications for tegumentary leishmaniasis. *Clinical and Vaccine Immunology* **22**, 1187–1196.

Duarte MC, Lage DP, Martins VT, Costa LE, Salles BCS, Carvalho AMRS, Oliveira TTS, Dias DS, Ribeiro PAF, Chávez-Fumagalli MA, Machado-de-Ávila RA, Roatt BM, Menezes-Souza D, Magalhães-Soares DF and Coelho EAF (2017) Performance of *Leishmania braziliensis* enolase protein for the serodiagnosis of canine and human visceral leishmaniasis. *Veterinary Parasitology* **238**, 77–81.

Ejazi SA, Bhattacharyya A, Choudhury ST, Ghosh S, Sabur A, Pandey K, Das VNR, Das P, Rahaman M, Goswami RP and Ali N (2018) Immunoproteomic identification and characterization of *Leishmania* membrane proteins as non-invasive diagnostic candidates for clinical visceral leishmaniasis. *Scientific Reports* **8**, 12110.

Fernandes AP, Coelho EAF, Machado-Coelho GLL, Grimaldi J and Gazzinelli RT (2012) Making an anti-amastigote vaccine for visceral leishmaniasis: rational, update and perspectives. *Current Opinion in Microbiology* **15**, 1–10.

Garg G, Singh K and Ali V (2018) Proteomic approaches unravel the intricacy of secreted proteins of *Leishmania*: an updated review. *Biochimica et Biophysica Acta – Proteins and Proteomics* **1866**, 913–923.

Georgiadou SP, Makaritsis KP and Dalekos GN (2015) Leishmaniasis revisited: current aspects on epidemiology, diagnosis and treatment. *Journal of Translational Internal Medicine* **3**, 43–50.

Grimaldi Jr G, Teva A, Porrozzi R, Pinto MA, Marchevsky RS, Rocha MG, Dutra MS, Bruña-Romero O, Fernandes AP and Gazzinelli RT (2014) Clinical and parasitological protection in a *Leishmania infantum*-macaque model vaccinated with adenovirus and the recombinant A2 antigen. *PLoS Neglected Tropical Diseases* **8**, e2853.

Ishemgulova A, Kraeva N, Hlaváčová J, Zimmer SL, Butenko A, Podešvová L, Leštinová T, Lukeš J, Kostygov A, Votýpka J, Volf P and Yurchenko V (2017) A putative ATP/GTP binding protein affects *Leishmania mexicana* growth in insect vectors and vertebrate hosts. *PLoS Neglected Tropical Diseases* **11**, e0005782.

Jamal F, Shivam P, Kumari S, Singh MK, Sardar AH, Pushpanjali MS, Narayan S, Gupta AK, Pandey K, Das VNR, Ali V, Bimal S, Das P and Singh SK (2017) Identification of *Leishmania donovani* antigen in circulating immune complexes of visceral leishmaniasis subjects for diagnosis. *PLoS One* **12**, e0182474.

Kapil S, Singh PK and Silakari O (2018) An update on small molecule strategies targeting leishmaniasis. *European Journal of Medicinal Chemistry* **157**, 339–367.

Kumar A and Samant M (2016) DNA vaccine against visceral leishmaniasis: a promising approach for prevention and control. *Parasite Immunology* **38**, 273–281.

Kumar V, Sharma M, Rakesh BR, Malik CK, Neelagiri S, Neerupudi KB, Garg P and Singh S (2018) Pyridoxal kinase: a vitamin B6 salvage pathway enzyme from *Leishmania donovani*. *International Journal of Biological Macromolecules* **119**, 320–334.

Kumari S, Kumar A, Samant M, Sundar S, Singh N and Dube A (2008) Proteomic approaches for discovery of new targets for vaccine and therapeutics against visceral leishmaniasis. *Proteomics – Clinical Applications* **2**, 372–386.

Lage DP, Martins VT, Duarte MC, Costa LE, Garde E, Dimer LM, Kursancew AC, Chávez-Fumagalli MA, Magalhães-Soares DF, Menezes-Souza D, Roatt BM, Machado-de-Ávila RA, Soto M, Tavares CA and Coelho EA (2016) A new *Leishmania*-specific hypothetical protein and its non-described specific B cell conformational epitope applied in the serodiagnosis of canine visceral leishmaniasis. *Parasitology Research* **115**, 1649–1658.

Lang T, Chastellier C, Frehel C, Hellio R, Metezeau P, Leão SS and Antoine JC (1994) Distribution of MHC class I and of MHC class II molecules in macrophages infected with *Leishmania amazonensis*. *Journal of Cell Science* **107**, 69–82.

https://doi.org/10.1017/S0031182019000714 Published online by Cambridge University Press

Lewis TS, Hunt JB, Aveline LD, Jonscher KR, Louie DF, Yeh JM, Nahreini TS, Resing KA and Ahn NG (2000) Identification of novel MAP kinase pathway signalling targets by functional proteomics and mass spectrometry. *Molecular Cell* **6**, 1343–1354.

Lima MP, Costa LE, Duarte MC, Menezes-Souza D, Salles BCS, Santos TTO, Ramos FF, Chávez-Fumagalli MA, Kursancew ACS, Ambrósio RP, Roatt BM, Machado-de-Ávila RA, Gonçalves DU and Coelho EAF (2017) Evaluation of a hypothetical protein for serodiagnosis and as a potential marker for post-treatment serological evaluation of tegumentary leishmaniasis patients. *Parasitology Research* **116**, 1197–1206.

Magalhães RD, Duarte MC, Mattos EC, Martins VT, Lage PS, Chávez-Fumagalli MA, Lage DP, Menezes-Souza D, Régis WC, Alves MJ, Soto M, Tavares CA, Nagen RA and Coelho EA (2014) Identification of differentially expressed proteins from *Leishmania amazonensis* associated with the loss of virulence of the parasites. *PLoS Neglected Tropical Diseases* **8**, e2764.

Martins VT, Chávez-Fumagalli MA, Costa LE, Canavacci AM, Martins AM, Lage PS, Lage DP, Duarte MC, Valadares DG, Magalhães RD, Ribeiro TG, Nagem RA, DaRocha WD, Régis WC, Soto M, Coelho EA, Fernandes AP and Tavares CA (2013) Antigenicity and protective efficacy of a *Leishmania* amastigote-specific protein, member of the super-oxygenase family, against visceral leishmaniasis. *PLoS Neglected Tropical Diseases* **7**, e2148.

Maspi N, Ghaffarifar F, Sharifi Z and Dalimi A (2015) Cloning and constructing a plasmid encoding *Leishmania* eukaryotic initiation factor gene of *Leishmania major* fused with green fluorescent protein gene as a vaccine candidate. *West Indian Medical Journal* **65**, 256–259.

Mishra A, Khan MI, Jha PK, Kumar A, Das S, Das P and Sinha KK (2018) Oxidative stress-mediated overexpression of uracil DNA glycosylase in *Leishmania donovani* confers tolerance against antileishmanial drugs. *Oxidative Medicine and Cellular Longevity* **2018**, 4074357.

Moreira DS, Pescher P, Laurent C, Lenormand P, Späth GF and Murta SM (2015) Phosphoproteomic analysis of wild-type and antimony-resistant *Leishmania braziliensis* lines by 2D-DIGE technology. *Proteomics* **15**, 2999–3019.

Moreira VR, Jesus LCL, Soares RP, Silva LDM, Pinto BAS, Melo MN, Paes AMA and Pereira SRF (2017) Meglumine antimoniate (glucantime) causes oxidative stress-derived DNA damage in BALB/c mice infected by *Leishmania* (*Leishmania*) *infantum*. *Antimicrobial Agents and Chemotherapy* **61**, e02360.

Mortazavidehkordi N, Farjadfar A, Khanahmad H, Najafabadi ZG, Hashemi N, Fallah A, Najafi A, Kia V and Hejazi SH (2016) Evaluation of a novel lentiviral vaccine expressing KMP11-HASPB fusion protein against *Leishmania infantum* in BALB/c mice. *Parasite Immunology* **38**, 670–677.

Mukherjee I, Chakraborty A and Chakrabarti S (2016) Identification of internalin-A-like virulent proteins in *Leishmania donovani*. *Parasite & Vectors* **9**, 557.

Nandan D, Thomas SA, Nguyen A, Moon KM, Foster LJ and Reiner NE (2017) Comprehensive identification of mRNA-binding proteins of *Leishmania donovani* by interactome capture. *PLoS One* **12**, e0170068.

Neuhoff V, Arold N, Taube D and Ehrhardt W (1988) Improved staining of proteins in polyacrilamide gels including isoeletric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* **9**, 255–262.

Nico D, Claser C, Borja-Cabrera GP, Travassos LR, Palatnik M, Soares IS, Rodrigues MM and Palatnik-de-Sousa CB (2010) Adaptive immunity against *Leishmania* nucleoside hydrolase maps its c-terminal domain as the target of the $CD4^+$ T cell-driven protective response. *PLoS Neglected Tropical Diseases* **4**, e866.

Rajão MA, Furtado C, Alves CL, Passos-Silva DG, Moura MB, Schamber-Reis BL, Kunrath-Lima M, Zuma AA, Vieira-da-Rocha JP, Garcia JB, Mendes IC, Pena SD, Macedo AM, Franco GR, Souza-Pinto NC, Medeiros MH, Cruz AK, Motta MC, Teixeira SM and Machado CR (2014) Unveiling Benznidazole's mechanism of action through overexpression of DNA repair proteins in *Trypanosoma cruzi*. *Environmental and Molecular Mutagenesis* **55**, 309–321.

Ribeiro PAF, Dias DS, Novais MVM, Lage DP, Tavares GSV, Mendonça DVC, Oliveira JS, Chávez-Fumagalli MA, Roatt BM, Duarte MC, Menezes-Souza D, Ludolf F, Tavares CAP, Oliveira MC and Coelho EAF (2018) A *Leishmania* hypothetical protein-containing liposome-based formulation is highly immunogenic and induces protection against visceral leishmaniasis. *Cytokine* **111**, 131–139.

Sabur A, Bhowmick S, Chhajer R, Ejazi SA, Didwania N, Asad M, Bhattacharyya A, Sinha U and Ali N (2018) Liposomal elongation factor-1α triggers effector CD4 and CD8 T cells for induction of long-lasting protective immunity against visceral leishmaniasis. *Frontiers in Immunology* **9**, 18.

Sakkas H, Gartzonika C and Levidiotou S (2016) Laboratory diagnosis of human visceral leishmaniasis. *Journal of Vectors Borne Diseases* **53**, 8–16.

Santos TTO, Martins VT, Lage DP, Costa LE, Salles BCS, Carvalho AMRS, Dias DS, Ribeiro PAF, Chávez-Fumagalli MA, Machado-de-Ávila RA, Roatt BM, Magalhães-Soares DF, Menezes-Souza D, Coelho EAF and Duarte MC (2017) Probing the efficacy of a heterologous *Leishmania*/*l. Viannia braziliensis* recombinant enolase as a candidate vaccine to restrict the development of *L. infantum* in BALB/c mice. *Acta Tropica* **171**, 8–16.

Sundar S and Singh OP (2018) Molecular diagnosis of visceral leishmaniasis. *Molecular Diagnosis & Therapy* **22**, 443–457.

Torres-Guerrero E, Quintanilla-Cedillo MR, Ruiz-Esmenjaud J and Arenas R (2017) Leishmaniasis: a review. *F1000Research* **6**, 750.

Valadares DG, Duarte MC, Oliveira JS, Chávez-Fumagalli MA, Martins VT, Costa LE, Leite JP, Santoro MM, Régis WC, Tavares CA and Coelho EA (2011) Leishmanicidal activity of the *Agaricus blazei murill* in different *Leishmania* species. *Parasitology International* **60**, 357–363.

Vijayakumar S and Das P (2018) Recent progress in drug targets and inhibitors towards combating leishmaniasis. *Acta Tropica* **181**, 95–104.

Vlahou A and Fountoulakis M (2005) Proteomic approaches in the search for disease biomarkers. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences* **814**, 11–19.

World Health Organization (2016) Leishmaniasis. Available at http://www.who.int/topics/leishmaniasis/en/.

https://doi.org/10.1017/S0031182019000714 Published online by Cambridge University Press