

Research Article

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Diagnostic application of sensitive and specific phage-exposed epitopes for visceral leishmaniasis and human immunodeficiency virus coinfection

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Abstract

The diagnosis of visceral leishmaniasis (VL) has improved with the search of novel antigens; however, their performance is limited when samples from VL/human immunodeficiency virus (HIV)-coinfected patients are tested. In this context, studies conducted to identify more suitable antigens to detect both VL and VL/HIV coinfection cases should be performed. In the current study, phage display was performed using serum samples from healthy subjects and VL, HIV-infected and VL/HIV-coinfected patients; aiming to identify novel phage-exposed epitopes to be evaluated with this diagnostic purpose. Nine non-repetitive and valid sequences were identified, synthesized and tested as peptides in enzyme-linked immunosorbent assay experiments. Results showed that three (Pep2, Pep3 and Pep4) peptides showed excellent performance to diagnose VL and VL/HIV coinfection, with 100% sensitivity and specificity values. The other peptides showed sensitivity varying from 50.9 to 80.0%, as well as specificity ranging from 60.0 to 95.6%. Pep2, Pep3 and Pep4 also showed a potential prognostic effect, since specific serological reactivity was significantly decreased after patient treatment. Bioinformatics assays indicated that *Leishmania* trypanothione reductase protein was predicted to contain these three conformational epitopes. In conclusion, data suggest that Pep2, Pep3 and Pep4 could be tested for the diagnosis of VL and VL/HIV coinfection.

Introduction

Leishmaniasis is a disease complex caused by protozoa parasites of the *Leishmania* genus, which affects more than 12 million people worldwide. It presents an annual incidence of 1.5–2.0 million new cases, with 380 million people at risk of contracting the infection (WHO, 2018). The clinical manifestations of this disease complex are varied, reaching single cutaneous lesions caused by cutaneous leishmaniasis until the fatal visceral infection caused by visceral leishmaniasis (VL) (Torres-Guerrero *et al.*, 2017); and the occurrence of symptoms depends primarily on the immunological status of the infected hosts and the infecting parasite species (Tajebe *et al.*, 2017). No vaccination protocol is available to prevent human disease, and treatment presents problems due to toxicity, high cost and/or emergence of parasite resistance (Srivastava *et al.*, 2016; Ponte-Sucre *et al.*, 2017; Kaye *et al.*, 2020). In this context, a safe, rapid and precise diagnosis of leishmaniasis could allow one to identify the patients and promptly treat them in an initial clinical phase of disease, as well as reduce the side effects of the available drugs (Sakkas *et al.*, 2016). Symptomatic VL causes such symptoms as fever, splenomegaly, hepatomegaly, weight loss, anaemia and pancytopenia, among others. In addition, it is nearly always fatal

if acute and left untreated (Coutinho *et al.*, 2017); therefore, early diagnosis can prevent mortality. Similarly, there is an estimated incidence of 35 million people living with human immunodeficiency virus (HIV) in the world. It is well-known that *Leishmania* can cause an opportunistic infection among HIV-infected patients in areas where both diseases coexist (Luz *et al.*, 2018). The immunosuppression caused by the virus may reactivate latent parasite infection in asymptomatic patients (Lindoso *et al.*, 2018). Moreover, the impact of coinfection hampers the diagnosis of the diseases, as well as the therapeutic response against them (Alvar *et al.*, 2008; Cota *et al.*, 2011).

Parasitological methods are considered the gold standard for VL diagnoses. However, their sensitivity is variable, since parasites, or their genetic content, need to be present in the analysed biological samples (Ruiter *et al.*, 2014). On the contrary, immunological methods, such as enzyme-linked immunosorbent assay (ELISA) and direct agglutination tests (DATs), have also been used for VL diagnoses (Thakur *et al.*, 2020). However, in the VL/HIV coinfection cases, the sensitivity has been hampered, limiting the diagnostic value, since only about 40% of the coinfecting patients are diagnosed, as compared to the 90–95% of the cases detected in immunocompetent patients (Srividya *et al.*, 2012; Silva *et al.*, 2018). In this context, and due to the scarcity of sensitive markers to diagnose VL/HIV-coinfecting patients, new studies to find novel and more suitable diagnostic antigens should be performed (Santos-Gomes *et al.*, 2000; Kumar *et al.*, 2002; Cota *et al.*, 2012). Under such conditions, in addition to testing recombinant proteins and/or B-cell chimeras for the VL diagnosis, other types of antigens, such as synthetic peptides, could also be considered, as they are simpler, more specific, more stable, and cheaper to produce, when compared to the recombinant proteins (Carvalho *et al.*, 2018; Rodrigues *et al.*, 2019).

Phage display is a proteomic technology, considering that phage-surface-exposed epitopes, called mimotopes, recognize specific ligands (Almagro *et al.*, 2019). Large repertoires of random peptide sequences in the libraries offer the advantage that a high number of sequences can be screened in relatively short-experimental procedures (Kuhn *et al.*, 2016). The biopanning cycles consist of isolating phage clones that bind specifically to a target molecule and then identifying their insert through sequencing (Manoutcharian, 2005). This technique has been used to identify mimotopes, with applications as new diagnostic markers, vaccine candidates and/or immunotherapeutic targets against leishmaniasis (Costa *et al.*, 2014, 2015, 2017; Toledo-Machado *et al.*, 2015a; Link *et al.*, 2017). Costa *et al.* (2014) identified eight mimotopes, which are reactive against antibodies purified from VL dogs, and tested them for canine VL diagnosis, with results showing a 100% sensitivity, specificity and accuracy in the ELISA experiments. In another study, two mimotopes, called B10 and C01, as well as their synthetic peptides, were tested for the diagnosis of canine and human VL, and the results showed high sensitivity and specificity values to diagnose the diseases (Costa *et al.*, 2017). Therefore, in the current study, phage display technology was used to select antigens to be evaluated for the diagnosis of VL and VL/HIV coinfection. Biopanning cycles using negative and positive selection processes were performed, and 43 phage clones were selected by antibodies in sera from VL and VL/HIV patients, but not by those from healthy subjects and HIV-infected patients; these were then isolated and sequenced. Results showed nine valid and non-repetitive sequences, and the epitopes were produced as synthetic peptides and evaluated in ELISA experiments against a human serological panel. In this context, the current study described, for the first time, the application of phage display technology, using sera from healthy subjects, as well as of VL, HIV and VL/HIV-coinfecting patients, to select novel antigens to be used in the diagnosis of VL and VL/HIV coinfection.

Materials and methods

Parasites and antigen preparation

Leishmania infantum (MOM/BR/1970/BH46) parasites were grown at 24 °C in Schneider's medium (Sigma, St. Louis, MO, USA), together with 20% heat-inactivated fetal bovine serum (Sigma), 20 mM L-glutamine, 200 U mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin at pH 7.4. The soluble *Leishmania* antigenic extract (SLA) was prepared from stationary-phase promastigotes according to that described elsewhere (Coelho *et al.*, 2003). For this, 10⁹ stationary promastigotes were washed three times in cold sterile phosphate-buffered saline (PBS 1×) and subjected to six cycles of freezing (−196°C) and thawing (37°C), followed by ultrasonication (Ultrasonic processor, GEX600) with five cycles of 30 s at 38 MHz. The suspension was then centrifuged at 8000 g for 30 min at 4°C, the supernatant containing SLA was collected, and the protein concentration was estimated by the Bradford method (Bradford, 1976). Aliquots (500 µL, each) were prepared and stored at −80°C until use.

Ethics statement and serum samples

Serum samples of VL (*n* = 30), VL/HIV-coinfecting (*n* = 25) and HIV-infected (*n* = 20) patients were used. *Leishmania* infection was confirmed by the polymerase chain reaction technique to identify *L. infantum* kDNA in spleen or bone marrow aspirates (Cota *et al.*, 2013). HIV diagnosis was based on clinical evaluation and blood collection to determine the CD4⁺ T-cell count to determine the viral load in the patients. Samples were also collected from healthy individuals living in an endemic region of VL (*n* = 25), who presented no clinical signal of disease and showed negative serological results (Kalazar Detect™ Rapid Test Kit). Sera from Chagas disease patients (*n* = 15), diagnosed by haemoculture, Chagatest® recombinant ELISA v.4.0 kit and/or Chagatest® haemagglutination inhibition (Wiener Lab., Rosario, Argentina), as well as from leprosy (*n* = 10), tuberculosis (*n* = 10) and malaria (*n* = 10) patients were used in the serological assays.

Biopanning cycles using negative and positive selection processes

A Ph.D. library (Ph.D.[®]-C7C library, New England BioLabs, USA) was used to select conformational epitopes by phage display, according to that described elsewhere (Costa *et al.*, 2014). Serum pools of VL (*n* = 10), HIV (*n* = 10) and VL/HIV-coinfecting (*n* = 10) patients, in addition to the sera from healthy subjects (*n* = 10), were also used. For this, 10¹² viral particles of the library were diluted in 250 µL of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.1% Tween 20 buffer (TBS-T). The phages were incubated for 30 min at room temperature with microspheres coupled to the healthy subject sera, which were magnetically captured in order to perform the negative selection process. The non-ligated phages were recovered and transferred to a new tube, which was also subjected to negative selection using HIV-infected patient sera. Phages in the supernatant were recovered and positive selection processes were performed by incubating them first with VL patient sera and, subsequently, with VL/HIV-coinfecting patient sera. The supernatant was removed, and the bound phages were washed five times in 1 mL TBS-T buffer and eluted in 500 µL of 0.2 M glycine buffer, pH 2.0. Next, 75 µL of 1 M Tris-base (pH 9.0) was added to neutralize the acidic pH of the solution. After the biopanning cycles, 43 clones were individually chosen for a sterile culture microplate (96-well microtest™ plate, BD Falcon™ clear, USA) in LB medium. A sequencing reaction was performed by capillary electrophoresis on the ABI 3130 equipment with the BigDye v 3.1 and POP7 polymer (Myleus Biotechnology®, Belo

Horizonte), and sequence scanner software (Applied Biosystems) was used for analyses of ABI output files. Of the 43 selected clones, nine valid and non-repeated amino acid sequences were identified.

Bioinformatics assays

The analyses were performed with the 7-mer peptide sequence and the amino acid sequences (AC-XXXXXXXX-CGGGS) contained in the fusion with the pIII phage capsid protein, as described elsewhere (Alves *et al.*, 2014). The identified epitopes were analysed by Expasy (<http://web.expasy.org/translate/>) and Basic Local Alignment Search Tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) servers against *Leishmania* proteins of the Protein Database (PDB), and the domain regions were validated using the protein family database (<http://pfam.xfam.org/>). The conformational alignment was performed with PepSurf software (<http://pepitope.tau.ac.il/>), and results were based on the values of combination (match), incompatibility (mismatch) and the absence of the corresponding amino acids (in the match) between the aligned peptides, which were filtered according to the greatest similarity presented to identify the possible host proteins.

ELISA experiments

For the ELISA experiments, previous titration curves were performed to determine the most appropriate conditions for the serological assays. In this context, antigen concentrations of 1.25 µg per well for Pep5, Pep6, Pep7 and Pep9; of 2.50 µg per well for Pep1, Pep4 and Pep8 and of 5.0 µg per well for Pep2 and Pep3, were used. *Leishmania infantum* SLA was used at a concentration of 1.0 µg per well. Microtitre immunoassay plates (Jetbiofil®, Belo Horizonte) received the antigens diluted in a 100 µL coating buffer (50 mM carbonate buffer) at pH 9.6 for 16 h at 4°C. Free-binding sites were blocked with a blocking solution (250 µL per well), consisting of PBS 1× and 0.05% Tween 20 (PBS-T) plus 5% non-fat dry milk solution, for 1 h at 37°C. Plates were washed and incubated with 100 µL serum samples (diluted 1:100 for Pep5 and SLA or 1:200 for the other peptides) for 1 h at 37°C, at which time they were washed with PBS-T. Next, the anti-human immunoglobulin G (IgG) peroxidase-conjugated antibody (Sigma, St. Louis, USA) was added (diluted 1:5000 for Pep1, Pep3, Pep5, Pep7, Pep8, Pep9 and SLA or 1:10 000 for Pep2, Pep4 and Pep6) for 1 h at 37°C. Plates were then washed with PBS-T, and reactions were developed using 100 µL of a solution consisting of 2 µL H₂O₂, 2 mg *ortho*-phenylenediamine and 10 mL citrate-phosphate buffer (pH 5.0) for 30 min in the dark. Reactions were then stopped by adding 25 µL 2 N H₂SO₄, and optical density (OD) values were read in an ELISA microplate reader (Molecular Devices, Spectra Max Plus, Canada) at 492 nm.

Prognostic evaluation after VL treatment

The synthetic peptides that presented better sensitivity and specificity values in the previous ELISA experiments were tested against serum samples of VL patients (*n* = 8), which were collected before and 6 months after treatment, performed using pentavalent antimonials (Sanofi Aventis Farmacêutica Ltda., Suzano, São Paulo, Brazil). All patients were subjected to the same therapeutic regimen, using these pharmaceutical drugs at a dose of 20 mg Sb⁵⁺ per kg for 30 days. None of the patients suffered from any other infections or had any pre-existing disease. Additionally, when they completed the treatment, no sign or symptom of VL was observed, and patients presented no parasite content in their spleen and/or bone marrow aspirates.

Table 1. Amino acid sequences from identified and selected epitopes by phage display

Clone	Name	Sequence
G1	Peptide 1	AC-TSKFWDT-CGGGS
G2	Peptide 2	AC-HITANES-CGGGS
G7	Peptide 3	AC-LRINNQS-CGGGS
A4	Peptide 4	AC-TTHTYFG-CGGGS
C5	Peptide 5	AC-PAPSRMV-CGGGS
D1	Peptide 6	AC-DPSPWRQ-CGGGS
F4	Peptide 7	AC-HRYSPSF-CGGGS
F5	Peptide 8	AC-DPTTQYS-CGGGS
F7	Peptide 9	AC-SNYHSRW-CGGGS

The sequences of the nine identified and selected phage-exposed epitopes are shown underlined between the 'AC-' and '-CGGGS' linkers of the capsid molecule.

Statistical analysis

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 cut-off values, as well as sensitivity (Se), specificity (Sp) and area under the curve (AUC) of the antigens. The one-way analysis of variance, followed by Bonferroni's post-test, was used for comparisons between groups. Differences were significant with *P* < 0.05.

Results

Selection of the best phage-exposed epitopes by phage display

Negative and positive selection processes were performed by phage display using IgG antibodies purified from serum samples of healthy subjects or VL, HIV-infected and VL/HIV-coinfected patients, aiming to select phage-exposed epitopes that were reactive with VL and VL/HIV sera. Results showed that 43 phage clones were selected and their DNA sequences were deduced by the Expasy server, when nine valid and non-repetitive sequences were identified. These peptide sequences were commercially synthesized with the AC-XXXXXXXX-CGGGS conformation (Table 1), aiming to increase the quality of interaction in the ELISA plates.

Diagnostic evaluation of the selected phage-exposed epitopes tested as synthetic peptides

Immunological assays were performed using the nine synthetic peptides (Pep1, Pep2, Pep3, Pep4, Pep5, Pep6, Pep7, Pep8 and Pep9) against a human serological panel. *Leishmania infantum* SLA was used as the control. Results indicating the individual OD values against each peptide and SLA are shown (Fig. 1). Data suggest that Pep2, Pep3 and Pep4 were recognized by all VL and VL/HIV-coinfected patient sera, since the OD values were above the respective cut-off values. The other peptides and SLA showed a moderate performance, with OD values of VL and VL/HIV patients presenting variable reactivity against such antigens. ROC curves were constructed to determine the AUC values, which were of 1.0 for Pep2, Pep3 and Pep4, and ranged from 0.70 to 0.82 for the other peptides, as well as 0.82 for SLA (Table 2). The sensitivity and specificity were also calculated, and sensitivity and specificity values were both of 100% for Pep2, Pep3 and Pep4, while the sensitivity varied from 50.9 to 80.0% for the other peptides, and specificity values ranged from

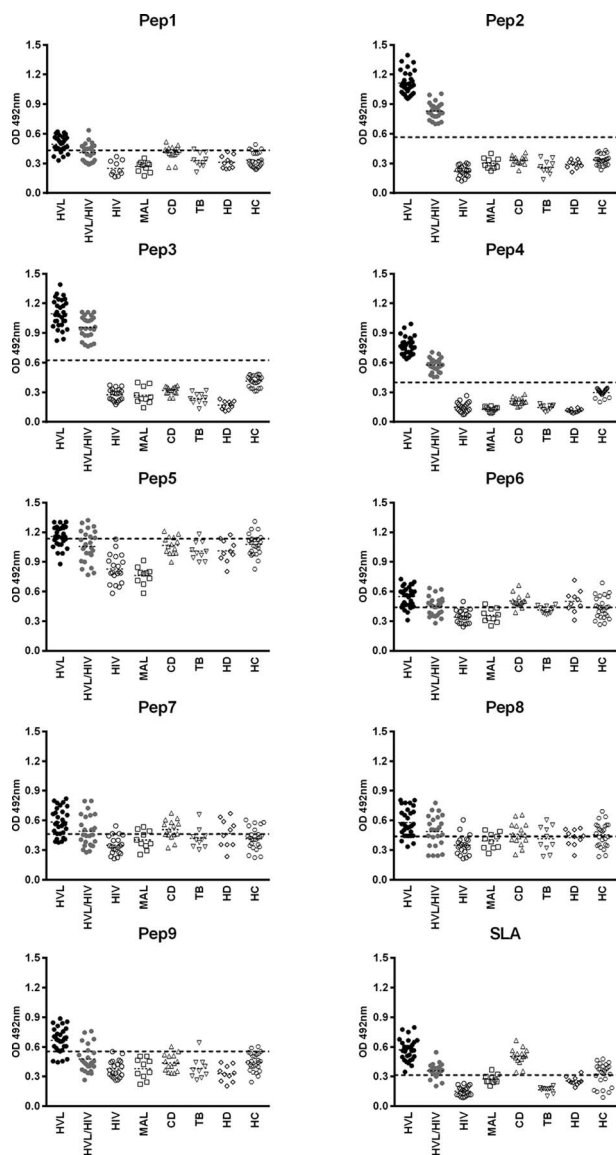


Fig. 1. Serological evaluation of the antigens for VL and VL/HIV coinfection. ELISA experiments were performed using 145 serum samples that were collected from VL (HVL; $n = 30$), VL and HIV (HVL/HIV; $n = 25$) coinfecting, HIV-infected (HIV; $n = 20$), malaria (MAL; $n = 10$), Chagas disease (CD; $n = 15$), tuberculosis (TB; $n = 10$) and leprosy (HD; $n = 10$) patients. Samples from healthy individuals living in an endemic region (HC; $n = 25$) were also used. Reactions against the individual peptides and *Leishmania infantum* SLA are shown here. The cut-off values (dotted lines) for positive (VL and VL/HIV samples) and negative (other samples) discrimination were determined by calculating ROC curves. Pep1, peptide 1; Pep2, peptide 2; Pep3, peptide 3; Pep4, peptide 4; Pep5, peptide 5; Pep6, peptide 6; Pep7, peptide 7; Pep8, peptide 8; Pep9, peptide 9; SLA, *Leishmania* antigenic extract.

60.0 to 95.6% (Table 3). Using SLA, the sensitivity and specificity values were of 92.7 and 62.2%, respectively.

Bioinformatics assays

A three-dimensional (3D) alignment of the nine identified epitopes against *L. infantum* proteins was performed using PepSurf software (<http://peptide.tau.ac.il/>), and results showed that three proteins had a high probability to contain these conformational sequences: trypanothione reductase (ID: 2JK6_A), with Pep1, Pep2, Pep3, Pep4 and Pep7; glyoxalase II (ID: 2P18_A), with Pep4 and Pep5 and tyrosine aminotransferase (ID: 4IX8_A), with Pep1, Pep2, Pep6 and Pep9 (Table 4). In addition, Pep8 did not present significant similarity with any *L. infantum* protein. In this context, a schematic representation of the location

Table 2. Performance of the diagnostic antigens

	Pep1	Pep2	Pep3	Pep4	Pep5	Pep6	Pep7	Pep8	Pep9	SLA
AUC	0.846	1.0	1.0	1.0	0.75	0.70	0.72	0.73	0.82	0.82
Std. Error	0.032	0	0	0	0.04	0.04	0.04	0.04	0.04	0.03
95% CI	0.78–0.91	1.0	1.0	1.0	0.67–0.83	0.61–0.79	0.63–0.80	0.64–0.81	0.75–0.89	0.75–0.88
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Pep1: peptide 1; Pep2: peptide 2; Pep3: peptide 3; Pep4: peptide 4; Pep5: peptide 5; Pep6: peptide 6; Pep7: peptide 7; Pep8: peptide 8; Pep9: peptide 9; SLA: *Leishmania* antigenic extract. The AUC values of each peptide and SLA were calculated after the ELISA experiments, using serum samples from VL ($n = 30$), VL/HIV-coinfecting ($n = 25$) and HIV-infected ($n = 20$) patients. In addition, samples from healthy individuals living in an endemic region of VL ($n = 25$), as well as from those diagnosed with Chagas disease ($n = 15$), leprosy ($n = 10$), tuberculosis ($n = 10$) and malaria ($n = 10$), were collected. The confidence interval (95% CI) and P values were calculated, and the results are shown here.

Table 3. Diagnostic evaluation of the synthetic peptides and SLA for VL and HIV coinfection

Antigen	Fisher's test	Se	95% CI	Sp	95% CI	PPV	95% CI	NPV	95% CI	LR	Y
Pep1	<0.0001	67.27	53.29–79.32	86.67	77.87–92.92	75.51	61.13–86.66	81.25	72.00–88.49	5.04	0.53
Pep2	<0.0001	100	93.51–100	100	95.98–100	100	93.51–100	100	95.98–100	NC	1
Pep3	<0.0001	100	93.51–100	100	95.98–100	100	93.51–100	100	95.98–100	NC	1
Pep4	<0.0001	100	93.51–100	100	95.98–100	100	93.51–100	100	95.98–100	NC	1
Pep5	<0.0001	50.91	37.07–64.65	86.67	77.87–92.92	70.00	53.47–83.44	74.29	64.83–82.32	3.82	0.38
Pep6	<0.0001	74.55	61.00–85.33	60.00	49.13–70.19	53.25	41.52–64.71	79.41	67.88–88.26	1.86	0.34
Pep7	<0.0001	63.64	49.56–76.19	68.89	58.26–78.23	55.56	42.49–68.08	75.61	64.88–84.42	2.04	0.32
Pep8	<0.0001	80.00	67.03–89.57	60.00	49.13–70.19	55.00	43.47–66.15	83.08	71.73–91.24	2.00	0.40
Pep9	<0.0001	58.18	44.11–71.35	95.56	89.01–98.78	88.89	73.94–96.89	78.9	70.04–86.13	13.09	0.54
SLA	<0.0001	92.73	82.41–97.98	62.22	51.38–72.23	60.00	48.80–70.48	93.33	83.80–98.15	2.45	0.55

NC, not calculated; Pep1, peptide 1; Pep2, peptide 2; Pep3, peptide 3; Pep4, peptide 4; Pep5, peptide 5; Pep6, peptide 6; Pep7, peptide 7; Pep8, peptide 8; Pep9, peptide 9; SLA, *Leishmania* antigenic extract. Serum samples of patients with VL (n = 30), VL/HIV-coinfected (n = 25), HIV-infected (n = 20), Chagas disease (n = 15), leprosy (n = 10), tuberculosis (n = 10) and malaria (n = 10) were used. Samples from healthy individuals living in an endemic region of VL (n = 25) were also used in the ELISA experiments to calculate the sensitivity (Se), specificity (Sp), confidence interval (95% CI), positive predictive value (PPV), negative predictive value (NPV), likelihood ratio (LR) and Youden index (Y).

Table 4. Identification of the *Leishmania infantum* proteins containing the conformational epitopes selected by phage display

Protein/species	Protein identity	Peptide sequence
Trypanothione reductase <i>L. infantum</i>	2JK6_A	Pep1: TSKFWDT
		Pep2: HITANES
		Pep3: LRINNQS
		Pep4: TTHTYFG
		Pep7: HRYSPSF
Glyoxalase II <i>L. infantum</i>	2P18_A	Pep4: TTHTYFG Pep5: PAPS RMV
Tyrosine aminotransferase <i>L. infantum</i>	4IX8_A	Pep1: TSKFWDT Pep2: HITANES Pep6: DPSPWRQ Pep9: SNYHSRW

Pep1, peptide 1; Pep2, peptide 2; Pep3, peptide 3; Pep4, peptide 4; Pep5, peptide 5; Pep6, peptide 6; Pep7, peptide 7; Pep9, peptide 9. Bioinformatics assays were performed to identify parasite proteins where the conformational epitopes could be located. Results are shown here, where the protein name and identity, as well as the selected peptide, are indicated. Pep8 showed no similarity with any *L. infantum* protein sequence.

of Pep2, Pep3 and Pep4 in the amino acid sequence of the trypanothione reductase protein is also shown (Fig. 2).

Prognostic evaluation of the selected epitopes

With serological data showing better diagnostic performance for Pep2, Pep3 and Pep4, these antigens were also tested in VL patient sera before and 6 months after treatment. Results showed that the anti-peptide serological reactivity declined by significant levels after the patient's treatment, as compared to the values found before treatment (Fig. 3). By contrast, using SLA as an antigen, a similar humoral reactivity was observed when samples collected before and after treatment were tested, thus suggesting that such an antigenic preparation is not suitable as a prognostic marker for VL.

Discussion

The VL/HIV coinfection is endemic in several countries around the world, and diagnostic tests used to identify such cases are not suitable, since low sensitivity tends to be found in coinfecting patient samples (Cota et al., 2012; Silva et al., 2018). In this context, there is a need to identify more sensitive antigens to be used in the diagnosis of VL and VL/HIV coinfection. Phage display is an in vitro selection strategy in which a peptide, protein or antibody is fused to a coat protein of phage capsid, which is displayed together with a fusion protein on the exogenous surface of the capsid (Alfaleh et al., 2020). This technique has been applied to identify novel vaccine candidates and/or diagnostic targets against a variety of diseases, such as leishmaniasis (Costa et al., 2014; Toledo-Machado et al., 2015a, 2015b; Carvalho et al., 2019). In this context, in the current study, the phage display technology applying a subtractive selection strategy was performed, aiming to identify new diagnostic targets for VL and VL/HIV coinfection.

VL is an opportunistic infection in HIV-infected patients, and this coinfection presents a relevant clinical role in endemic regions, since patients usually present higher duration time for the onset of VL, as well as a lower therapeutic response against disease (Lindoso et al., 2018). In addition, a dysfunction of T-cell and B-cell responses hampers the antibody production,

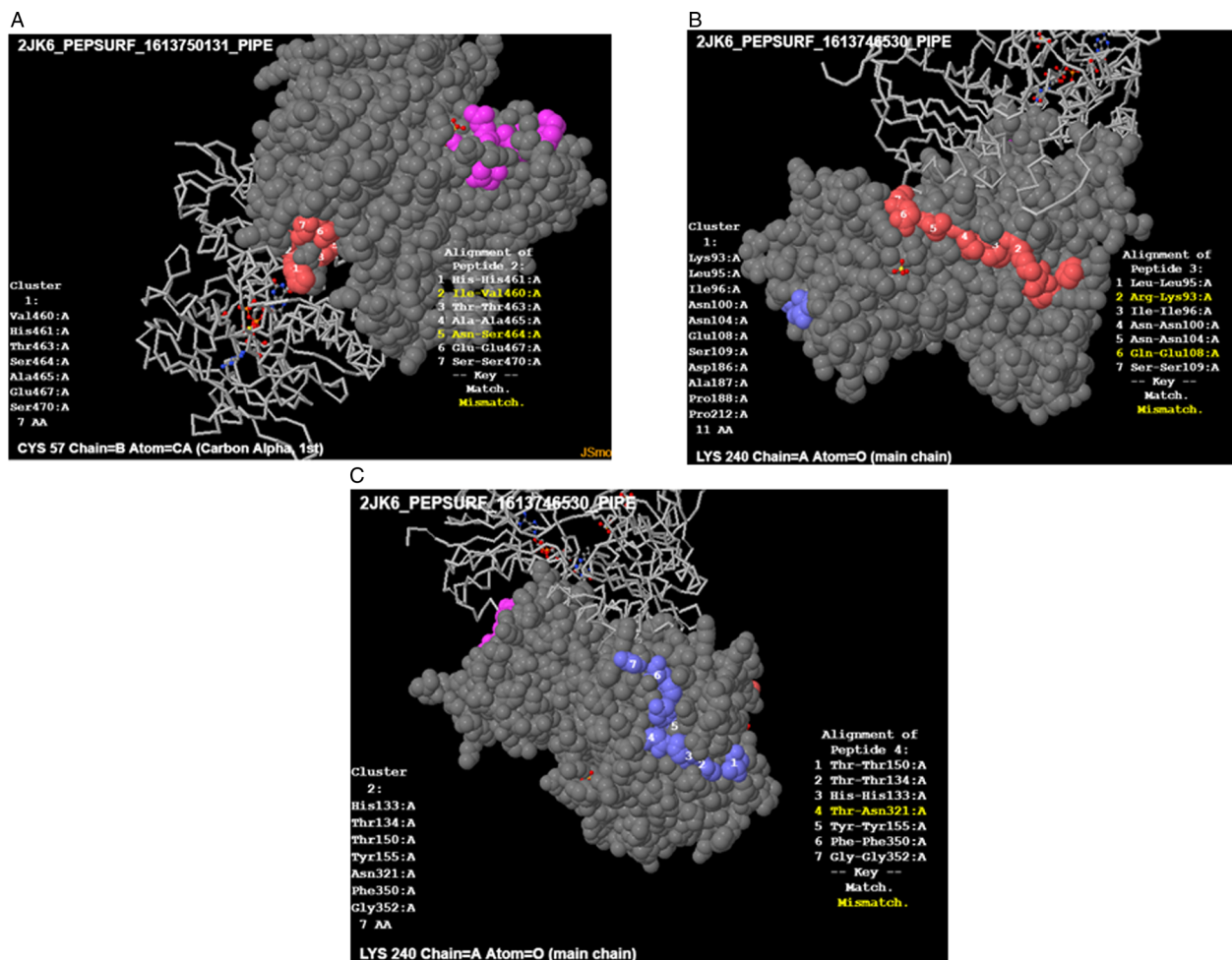


Fig. 2. 3D representation of the *L. infantum* trypanothione reductase protein and location of Pep2, Pep3 and Pep4. The Peptide server was used to predict the location of the epitopes in the protein's 3D structure. The amino acid sequences of Pep2 (A), Pep3 (B) and Pep4 (C) are numbered and shown. The equivalent amino acids are represented in white (match), the incompatibilities are shown in yellow (mismatch) and gaps where the absence of amino acids was verified are shown in pink (in match). A list containing the amino acids that make up the selected peptide is shown on the left (cluster).

causing lower sensitivity in the diagnostic tests (Silva *et al.*, 2018). Few studies have evaluated these tests for VL/HIV patients, mainly due to the lack of a gold standard. Cota *et al.* (2012) showed that the DAT and immunoblotting presented a sensitivity of 81.0 and 84.0%, respectively, to diagnose VL/HIV, which were better when compared to the use of ELISA and immunofluorescence antibody tests. Kassa *et al.* (2020) evaluated the diagnostic efficacy of distinct commercial tests in HIV-coinfected Ethiopian patients. The authors showed that the sensitivity decreased in the VL/HIV-coinfected group, with some tests being more affected than others, thus suggesting that the performance of these commercial diagnostic kits is variable and sub-optimal, and that improvement in their sensitivity and specificity will lead to a better diagnostic efficacy.

Compared to the use of recombinant proteins, synthetic peptides can increase the diagnostic performance in immunological assays, since cross-reactivity with other undesired epitopes will be lower due to their exclusion. In addition, peptides can maintain the relative spatial structure and biological activity with high specificity, low false-negative rates and an easy clinical application (Meloan *et al.*, 2003). Therefore, such antigens have represented a valuable contribution to laboratory diagnoses of distinct diseases, such as leishmaniasis (Costa *et al.*, 2012, 2017). The epitopes selected by phage display are of conformational nature (Xin *et al.*, 2014), which consists of discontinuous amino acids present in a target antigen, which in turn combine with the antigen binding site of the antibody molecule, and then simulate the

characteristics of an epitope. A whole protein, in addition to containing desired epitopes (Gazarian *et al.*, 2012), may also contain cross-reactivity epitopes for diagnosis, inhibitory epitopes and/or auto-antigen epitopes, hampering the specificity of the tests (Faria *et al.*, 2015). In this sense, the use of well-defined epitopes, such as synthetic peptides, could be beneficial to improving the sensitivity and specificity of the diagnosis of VL and VL/HIV coinfection.

Serological results obtained here showed that three of the nine selected epitopes, Pep2, Pep3 and Pep4, presented 100% sensitivity and specificity to diagnose VL and VL/HIV coinfection. Otherwise, SLA presented sensitivity and specificity values of 92.7 and 62.2%, respectively, suggesting a lower diagnostic performance as compared to these selected peptides. In one study, a promastigote crude antigenic preparation was able to identify 80% of VL patient sera by means of ELISA experiments, although high cross-reactivity was observed when patient samples presenting malaria and Chagas disease were used (Fargeas *et al.*, 1996). The authors also tested single peptides, and found better sensitivity and specificity values than the crude antigenic preparation, leading to the suggestion that the combination of peptides could result in higher diagnostic accuracy for VL. However, in this study, samples from VL/HIV-coinfected patients were not tested. Other more recent studies evaluating synthetic peptides as antigens for the VL diagnosis did not test this type of serum class in their serological panels (Costa *et al.*, 2017; Ejazi *et al.*, 2018; Machado *et al.*, 2020). In this context, data presented here using the Pep2, Pep3 and Pep4 suggest that such antigens present a high efficacy in the

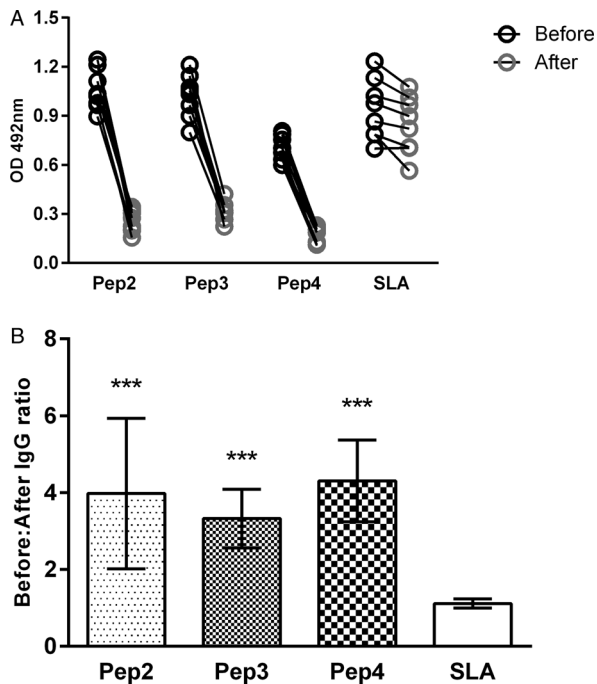


Fig. 3. Post-therapeutic serological follow-up in VL patients. ELISA experiments were conducted using Pep2, Pep3 and Pep4 against serum samples collected from VL patients ($n=8$) before and 6 months after treatment, as well as against *L. infantum* SLA. Levels of IgG antibodies were measured and the individual OD values are shown. The white and grey circles indicate the mean plus standard deviation of the samples collected before and after treatment, respectively (A). In addition, the ratio between antibody levels before and after treatment was also calculated and data are shown (B). *** indicates a statistically significant difference in relation to the SLA antigen ($P<0.0001$). Pep2: peptide 2; Pep3: peptide 3; Pep4: peptide 4; SLA: *Leishmania* antigenic extract.

diagnosis of VL, as well as in VL/HIV coinfection cases, and could be used in future studies to diagnose both clinical conditions.

A 3D alignment performed in the amino acid sequences of the selected phage exposed with *Leishmania* proteins showed the identification of the *L. infantum* trypanothione reductase protein as the target possibly containing the three epitopes that presented the best serological response for VL and VL/HIV coinfection. This protein is an essential enzyme and is of the Trypanosomatidae family, which has been validated as a drug target in *Leishmania* and *Trypanosoma*, since its downregulation causes strong impairment in the parasite's infectivity (Battista *et al.*, 2020). In addition, such drugs as antimonials interfere with trypanothione metabolism and inhibit the trypanothione reductase, suggesting that this protein could be a therapeutic option for the treatment against such diseases (Ilari *et al.*, 2018). Moreover, a significant amino acid sequence homology of trypanothione reductase proteins from different sources makes it a valuable target for developing a single, broad spectrum drug active against all Trypanosomatidae (Beig *et al.*, 2015), in addition to the fact that they are specific to the Trypanosomatidae family, showing low similarity to human protein sequences, making them suitable candidates for the diagnosis of these diseases (Holloway *et al.*, 2009).

Limitations of this study include the absence of a higher serological panel, as well as the use of other diagnostic platforms that incorporate the best selected peptides, such as those used in immunochromatographics. However, this study can be considered the first to employ the phage display technology and antibodies from VL, HIV-infected and VL/HIV-coinfected patients to select novel diagnostic targets, such as synthetic peptides, and test them for the serodiagnosis of VL and VL/HIV coinfection.

Author contributions. Conceived and designed the experiments: EAFC, FFR, LRG, PTA, VGF, ACS, FL and GSVT. Performed the experiments:

FFR, FL, ASM, TTOS, IAPG, ACS, PTA, VGF, RSB, JAOS, TARR, DPL, VTM and CSF. Analysed the data: EAFC, FFR, LRG, FL, GSVT and MACF. Contributed reagents/materials/analysis tools: ATC, NSG, UT, MOCR, GFC, RTF and LLB. Wrote the paper: EAFC, LRG and FFR.

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

Ethical standards. The Human Research Ethics Committee from the Federal University of Minas Gerais (UFMG; Belo Horizonte, Minas Gerais, Brazil) approved the study, logged under protocol number CAAE-32343114.9.0000.5149. All patients have agreed to participate in the study, and those who chose not to participate had the same attention and medical care provided as did the enrolled participants, all of whom received the same treatment, follow-up and necessary care.

References

- Alfaleh MA, Alsaab HO, Mahmoud AB, Alkayyal AA, Jones ML, Mahler SM and Hashem AM (2020) Phage display derived monoclonal antibodies: from bench to bedside. *Frontiers in Immunology* **11**, 1986.
- Almagro JC, Pedraza-Escalona M, Arrieta HI and Pérez-Tapia SM (2019) Phage display libraries for antibody therapeutic discovery and development. *Antibodies (Basel)* **8**, 44.
- Alvar J, Aparicio P, Aseffa A, Den Boer M, Cañavate C, Dedet JP, Gradoni L, Ter Horst R, López-Vélez R and Moreno J (2008) The relationship between leishmaniasis and AIDS: the second 10 years. *Clinical Microbiology Reviews* **21**, 334–359.
- Alves PT, Fujimura PT, Morais LD and Goulart LR (2014) Revisiting the CD14: epitope mapping by phage display. *Immunobiology* **219**, 822–829.
- Battista T, Colotti G, Ilari A and Fiorillo A (2020) Targeting trypanothione reductase, a key enzyme in the redox trypanosomatid metabolism, to develop new drugs against leishmaniasis and trypanosomiasis. *Molecules* **25**, 1924.
- Beig M, Oellien F, Garo L, Noack S, Krauth-Siegel RL and Selzer PM (2015) Trypanothione reductase: a target protein for a combined *in vitro* and *in silico* screening approach. *PLoS Neglected Tropical Diseases* **9**, e0003773.
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.
- Carvalho AMRS, Mendes TAO, Coelho EAF, Duarte MC and Menezes-Souza D (2018) New antigens for the serological diagnosis of human visceral leishmaniasis identified by immunogenomic screening. *PLoS One* **13**, e0209599.
- Carvalho GB, Costa LE, Lage DP, Ramos FF, Santos TTO, Ribeiro PAF, Dias DS, Salles BCS, Lima MP, Carvalho LM, Dias ACS, Alves PT, Franklin ML, Silva RAM, Duarte MC, Menezes-Souza D, Roatt BM, Chávez-Fumagalli MA, Goulart LR, Teixeira AL and Coelho EAF (2019) High-through identification of T cell-specific phage-exposed mimotopes using PBMCs from tegumentary leishmaniasis patients and their use as vaccine candidates against *Leishmania amazonensis* infection. *Parasitology* **146**, 322–332.
- Coelho EAF, 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.
- Costa MM, Penido M, Santos MS, Doro D, Freitas E, Michalick MS, Grimaldi G, Gazzinelli RT and Fernandes AP (2012) Improved canine and human visceral leishmaniasis immunodiagnosis using combinations of synthetic peptides in enzyme-linked immunosorbent assay. *PLoS Neglected Tropical Diseases* **6**, e1622.
- Costa LE, Lima MIS, Chávez-Fumagalli MA, Menezes-Souza D, Martins VT, Duarte MC, Lopes EGP, Lage DP, Ribeiro TG, Andrade PHR,

- Magalhães-Soares DF, Soto M, Tavares CAP, Goulart LR and Coelho EAF (2014) Subtractive phage display selection from canine visceral leishmaniasis identifies novel epitopes that mimic *Leishmania infantum* antigens with potential serodiagnosis applications. *Clinical and Vaccine Immunology* **21**, 96–106.
- Costa LE, Chávez-Fumagalli MA, Martins VT, Duarte MC, Lage DP, Lima MI, Pereira NC, Soto M, Tavares CA, Goulart LR and Coelho EA (2015) Phage-fused epitopes from *Leishmania infantum* used as immunogenic vaccines confer partial protection against *Leishmania amazonensis* infection. *Parasitology* **142**, 1335–1347.
- Costa LE, Salles BCS, Santos TTO, Ramos FF, Lima MP, Lima MIS, Portela ÁSB, Chávez-Fumagalli MA, Duarte MC, Menezes-Souza D, Machado-de-Ávila RA, Silveira JAG, Magalhães-Soares DF, Goulart LR and Coelho EAF (2017) Antigenicity of phage clones and their synthetic peptides for the serodiagnosis of canine and human visceral leishmaniasis. *Microbial Pathogenesis* **110**, 14–22.
- Cota GF, Sousa MR and Rabello A (2011) Predictors of visceral leishmaniasis relapse in HIV-infected patients: a systematic review. *PLoS Neglected Tropical Diseases* **5**, e1153.
- Cota GF, Sousa MR, Demarqui FN and Rabello A (2012) The diagnostic accuracy of serologic and molecular methods for detecting visceral leishmaniasis in HIV-infected patients: meta-analysis. *PLoS Neglected Tropical Diseases* **6**, e1665.
- Cota GF, Sousa MR, Nogueira BMF, Gomes LI, Oliveira E, Assis TS, Mendonça AL, Pinto BF, Saliba JW and Rabello A (2013) Comparison of parasitological, serological, and molecular tests for visceral leishmaniasis in HIV-infected patients: a cross-sectional delayed-type study. *American Journal of Tropical Medicine and Hygiene* **89**, 570–577.
- Coutinho JVSC, Santos FSD, Ribeiro RDSP, Oliveira IBB, Dantas VB, Santos ABFS and Tauhata JR (2017) Visceral leishmaniasis and leishmaniasis-HIV coinfection: comparative study. *Revista da Sociedade Brasileira de Medicina Tropical* **50**, 670–674.
- 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.
- Fargeas C, Hommel M, Maingon R, Dourado C, Monsigny M and Mayer R (1996) Synthetic peptide-based enzyme-linked immunosorbent assay for serodiagnosis of visceral leishmaniasis. *Journal of Clinical Microbiology* **34**, 241–248.
- Faria AR, Veloso LC, Coura-Vital W, Reis AB, Damasceno LM, Gazzinelli RT and Andrade HM (2015) Novel recombinant multi-epitope proteins for the diagnosis of asymptomatic *Leishmania infantum*-infected dogs. *PLoS Neglected Tropical Diseases* **9**, e3429.
- Gazarian K, Rowley M, Gazarian T, Vazquez-Buchelli JE and Hernández-González M (2012) Mimotope peptides selected from phage display combinatorial library by serum antibodies of pigs experimentally infected with *Taenia solium* as leads to developing diagnostic antigens for human neurocysticercosis. *Peptides* **38**, 381–388.
- Holloway GA, Charman WN, Fairlamb AH, Brun R, Kaiser M, Kostewicz E, Novello PM, Parisot JP, Richardson J, Street IP, Watson KG and Baell JB (2009) Trypanothione reductase high-throughput screening campaign identifies novel classes of inhibitors with antiparasitic activity. *Antimicrobial and Agents Chemotherapy* **53**, 2824–2833.
- Ilari A, Genovese I, Fiorillo F, Battista T, De Ionna I, Fiorillo A and Colotti G (2018) Toward a drug against all Kinetoplastids: from Leishbox to specific and potent trypanothione reductase inhibitors. *Molecular Pharmacology* **15**, 3069–3078.
- Kassa M, Abdellati S, Cnops L, Bremer-Hinckel BC, Yeshanew A, Hailemichael W, Vogt F, Adriaensen W, Mertens P, Diro E, Van Griensven J and Van den Bossche D (2020) Diagnostic accuracy of direct agglutination test, rK39 ELISA and six rapid diagnostic tests among visceral leishmaniasis patients with and without HIV coinfection in Ethiopia. *PLoS Neglected Tropical Diseases* **14**, e0008963.
- Kaye PM, Cruz I, Picado A, Van Bocxlaer K and Croft SL (2020) Leishmaniasis immunopathology-impact on design and use of vaccines, diagnostics and drugs. *Seminars in Immunopathology* **42**, 247–264.
- Kuhn P, Fühner V, Unkauf T, Moreira GM, Frenzel A, Miethe S and Hust M (2016) Recombinant antibodies for diagnostics and therapy against pathogens and toxins generated by phage display. *Proteomics – Clinical Applications* **10**, 922–948.
- Kumar P, Pai K, Tripathi K, Pandey HP and Sundar S (2002) Immunoblot analysis of the humoral immune response to *Leishmania donovani* polypeptides in cases of human visceral leishmaniasis: its usefulness in prognosis. *Clinical and Diagnostic Laboratory Immunology* **9**, 1119–1123.
- Lindoso JAL, Moreira CHV, Cunha MA and Queiroz IT (2018) Visceral leishmaniasis and HIV coinfection: current perspectives. *HIV AIDS (Auckland)* **10**, 193–201.
- Link JS, Alban SM, Soccol CR, Pereira GV and Soccol VT (2017) Synthetic peptides as potential antigens for cutaneous leishmaniasis diagnosis. *Journal of Immunological Research* **2017**, 5871043.
- Luz JGG, Naves DB, Carvalho AG, Meira GA, Dias JVL and Fontes CJF (2018) Visceral leishmaniasis in a Brazilian endemic area: an overview of occurrence, HIV coinfection and lethality. *Revista do Instituto de Medicina Tropical de São Paulo* **60**, e12.
- Machado AS, Ramos FF, Oliveira-da-Silva JA, Santos TTO, Ludolf F, Tavares GSV, Costa LE, Lage DP, Steiner BT, Chaves AT, Chávez-Fumagalli MA, Magalhães-Soares DF, Silveira JAG, Napoles KMN, Tupinambás U, Duarte MC, Machado-de-Ávila RA, Bueno LL, Fujiwara RT, Moreira RLF, Rocha MOC, Caligiorne RB and Coelho EAF (2020) A *Leishmania infantum* hypothetical protein evaluated as a recombinant protein and specific B-cell epitope for the serodiagnosis and prognosis of visceral leishmaniasis. *Acta Tropica* **203**, 105318.
- Manoutcharian K (2005) Bacteriophages as tools for vaccine and drug development. *Expert Review of Vaccines* **4**, 5–7.
- Meloen RH, Puijk WC, Langeveld JP, Langedijk JP and Timmerman P (2003) Design of synthetic peptides for diagnostics. *Current Protein & Peptide Science* **4**, 253–260.
- Ponte-Sucre A, Gamarro F, Dujardin JC, Barrett MP, López-Vélez R, García-Hernández R, Pountain AW, Mwenchanya R and Papadopoulos B (2017) Drug resistance and treatment failure in leishmaniasis: a 21st century challenge. *PLoS Neglected Tropical Diseases* **11**, e0006052.
- Rodrigues MR, Santos LMO, Miyazaki CK, Martins VT, Ludolf FR, Kursancew AC, Ramos FF, Dias DS, Oliveira JS, Vieira PMA, Roatt BM, Machado-de-Ávila RA, Gonçalves DU, Menezes-Souza D, Coelho EAF and Duarte MC (2019) Immunodiagnosis of human and canine visceral leishmaniasis using recombinant *Leishmania infantum* prohibitin protein and a synthetic peptide containing its conformational B-cell epitope. *Journal of Immunological Methods* **474**, 112641.
- Ruiter CM, Van Der Veer C, Leeftang MM, Deborggraeve S, Lucas C and Adams ER (2014) Molecular tools for diagnosis of visceral leishmaniasis: systematic review and meta-analysis of diagnostic test accuracy. *Journal of Clinical Microbiology* **52**, 3147–3155.
- Sakkas H, Gartzonika C and Levidiotou S (2016) Laboratory diagnosis of human visceral leishmaniasis. *Journal of Vector Borne Diseases* **53**, 8–16.
- Santos-Gomes G, Gomes-Pereira S, Campino L, Araújo MD and Abranches P (2000) Performance of immunoblotting in diagnosis of visceral *Leishmaniasis* in human immunodeficiency virus-*Leishmania* sp.-coinfecting patients. *Journal of Clinical Microbiology* **38**, 175–178.
- Silva MRB, Brandão NAA, Colovati M, Sousa MMP, Lima LC, Dorta ML, Ribeiro-Dias F, Costa DL, Costa CHN and Oliveira MAP (2018) Performance of two immunochromatographic tests for diagnosis of visceral leishmaniasis in patients coinfecting with HIV. *Parasitology Research* **117**, 419–427.
- Srivastava S, Shankar P, Mishra J and Singh S (2016) Possibilities and challenges for developing a successful vaccine for leishmaniasis. *Parasite & Vectors* **9**, 277.
- Srividya G, Kulshrestha A, Singh R and Salotra P (2012) Diagnosis of visceral leishmaniasis: developments over the last decade. *Parasitology Research* **110**, 1065–1078.
- Tajebe F, Getahun M, Adem E, Hailu A, Lemma M, Fikre H, Raynes J, Tamiru A, Mulugeta Z, Diro E, Toulza F, Shkedy Z, Ayele T, Modolell M, Munder M, Müller I, Takele Y and Kropf P (2017) Disease severity in patients with visceral leishmaniasis is not altered by co-infection with intestinal parasites. *PLoS Neglected Tropical Diseases* **11**, e0005727.
- Thakur S, Joshi J and Kaur S (2020) Leishmaniasis diagnosis: an update on the use of parasitological, immunological and molecular methods. *Journal of Parasitic Diseases* **44**, 1–20.
- Toledo-Machado CM, Avila RA, Nguyen C, Granier C, Bueno LL, Carneiro CM, Menezes-Souza D, Carneiro RA, Chávez-Olórtegui C and Fujiwara RT (2015a) Immunodiagnosis of canine visceral leishmaniasis using mimotope peptides selected from phage displayed combinatorial libraries. *BioMed Research International* **2015**, 401509.
- Toledo-Machado CM, Bueno LL, Menezes-Souza D, Machado-de-Ávila RA, Nguyen C, Granier C, Bartholomeu DC, Chávez-Olórtegui C and

- Fujiwara RT** (2015*b*) Use of phage display technology in development of canine visceral leishmaniasis vaccine using synthetic peptide trapped in sphingomyelin/cholesterol liposomes. *Parasite & Vectors* **8**, 133.
- Torres-Guerrero E, Quintanilla-Cedillo MR, Ruiz-Esmenjaud J and Arenas R** (2017) Leishmaniasis: a review. *F1000 Research* **6**, 750.
- World Health Organization** (2018) Leishmaniasis. See <http://www.who.int/topics/leishmaniasis/en/>.
- Xin L, Jinyan G, Shengfa H, Yuanyuan W and Hongbing C** (2014) Identification of conformational antigenic epitopes and dominant amino acids of buffalo β -lactoglobulin. *Journal of Food Science* **79**, T748–T756.