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Leishmania infantum mimotopes and a phage–ELISA assay as tools for a sensitive and specific serodiagnosis of human visceral leishmaniasis

Beatriz C.S. Salles [a,1], Lourena E. Costa [a,1], Patrícia T. Alves [b], Ana C.S. Dias [b], Emília R. Vaz [b], Daniel Menezes-Souza [a,c], Fernanda F. Ramos [a], Mariana C. Duarte [a,c], Bruno M. Roatt [c], Miguel A. Chávez-Fumagalli [a], Carlos A.P. Tavares [d], Denise U. Gonçalves [a], Regina L. Rocha [a], Luiz R. Goulart [b,e,*,2], Eduardo A.F. Coelho [a,c,**,2]

[a] *Programa de Pós-Graduação em Ciências Saúde: Infectologia e Medicina Tropical, Faculdade de Medicina, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, 30130-100, Belo Horizonte, Minas Gerais, Brazil*
[b] *Laboratório de Nanobiotecnologia, Instituto de Genética e Bioquímica, Universidade Federal de Uberlândia, Av. Amazonas s/n, Campus Umuarama, Bloco 2E, Sala 248, 38400-902, Uberlândia, Minas Gerais, Brazil*
[c] *Departamento de Patologia Clínica, Coltec, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, 31270-901, Belo Horizonte, Minas Gerais, Brazil*
[d] *Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, 31270-901, Belo Horizonte, Minas Gerais, Brazil.*
[e] *Department of Medical Microbiology and Immunology, University of California-Davis, 95616, Davis, CA, USA*

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ABSTRACT

Serological methods used to diagnose visceral leishmaniasis (VL) are considered minimally invasive, but they present problems related with their sensitivity and/or specificity. In this study, a subtractive selection using the phage display technology against antibodies from healthy subjects living in endemic and non-endemic areas of disease, as well as from Chagas disease patients and those developing active VL, was developed. The aim of this study was to select bacteriophage-fused epitopes to be used in the serodiagnosis of human VL. Eight phage clones were selected after the bio-panning rounds, and their reactivity was evaluated in a phage-ELISA assay against a human serological panel. A wild-type clone and the recombinant K39-based immunochromatographic test were used as controls. In the results, it was shown that all clones showed an excellent performance to serologically identify VL patients, demonstrating the feasibility of the isolated phages for developing a specific and sensitive serodiagnosis of human VL.

1. Introduction

Leishmaniasis is a parasitic disease complex occurring in tropical and subtropical countries worldwide (World Health Organization, 2010). Among the 2 million of cases of the human leishmaniasis, between 200,000 and 400,000 correspond to cases from visceral leishmaniasis (VL), culminating in approximately 20,000 to 30,000 deaths annually (Alvar et al., 2012; Desjeux, 2004). *Leishmania* parasites are distributed in the world, and in some geographic areas, more than one species can be found causing distinct clinical manifestations of the disease. In the Americas, Brazil is responsible by 90% of the cases registered of VL, and *Leishmania infantum* species is the most common parasite responsible by this disease in Brazilian dogs and humans (Alvar et al., 2012).

The human infection outcome varies from an asymptomatic and/or subclinical disease to the acute symptomatic disease. While infected subjects developing asymptomatic VL present apparently no impact on their healthy state, symptomatic patients usually develop diverse clinical manifestations, such as lymphadenopathy, fever, diarrhea, malaise, hepatomegaly, and splenomegaly (Michel et al., 2011; Sundar and Chakravarty, 2010). Chemotherapy treatment for VL has been based on parenteral administration of pentavalent antimonials, but several side effects such as anorexia, myalgia, fever, urticaria, and arthralgia, besides toxicity in the liver, spleen, and kidneys, have been registered in the patients (Chávez-Fumagalli et al., 2015; Moore and Lockwoodm, 2010).

A precise diagnosis of VL may allow to a faster and more effective treatment against the disease, which increases the possibility of cure, as well as to induce less toxic effects due to a lower time exposition for the chemotherapeutics. However, the parasitological diagnosis based on direct demonstration of *Leishmania* amastigotes presents low sensitivity and requires invasive samples collection procedures, which limit their use (Srividya et al., 2012; Tavares et al., 2003). Although the detection of *Leishmania* DNA by polymerase chain reaction (PCR) technique possesses high specificity, its sensitivity remains variable

* Corresponding author. Tel.: +55-34-3225-8440.
** Corresponding author. Tel.: +55-31-3409-4983.
E-mail addresses: lrgoulart@ufu.br (L.R. Goulart), eduardoferrazcoelho@yahoo.com.br (E.A.F. Coelho).
[1] These authors contributed equally to this work.
[2] Co-senior authors.

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due to the random dissemination of the parasites in the collected aspirates (Chatzis et al., 2014). Therefore, antileishmanial serology becomes an ideal diagnostic tool for detection of anti-parasite antibodies in sera of patients, due to its simplicity and low cost, besides being considered minimally invasive (Tavares et al., 2003).

In this context, recombinant antigens have been evaluated for the serodiagnosis of VL, showing satisfactory results when compared to crude, soluble and/or semi-purified *Leishmania* antigenic preparations. Among the most promising molecules, recombinant K39 (rK39) protein has shown good results for the serodiagnosis of disease (Singh et al., 2010). A commercial kit, namely Kalazar Detect™ Test (InBios International, Inc., Seattle, WA, USA), was developed and is considered a non-invasive immunochromatographic strip assay for the qualitative detection of rK39-specific antibodies for the *L. donovani* complex in patients sera. However, problems related to cross-reactivity with serum samples from patients with related-VL diseases, such as Chagas disease, as well as false-positive results found in healthy subjects living in endemic areas of the disease; have been registered (Chappuis et al., 2007; Singh and Sundar, 2015; Srivastava et al., 2011; Sundar et al., 2002). When comparing rK39 strip test and the direct agglutination test (DAT), it was verified that the last one presents the ability to detect patients with low levels of antileishmanial antibodies, due to the mosaic of antigens present in the antigenic preparations; although its specificity is hampered due to the fact that antibodies can also interact with cross-reactive antigens (Diro et al., 2007). Strip tests present have limitations, since patients can develop high levels of anti-*Leishmania* antibodies for months after the treatments, as well as false-negative results are possible to be detected (Maia et al., 2012; Sundar and Rai, 2002). Thus, there is an urgent need to identify new antigenic markers to be tested in a more sensitive and specific serodiagnosis of VL.

Efforts to identify novel diagnostic antigens have recently relied on the phage display, which is a subtractive proteomic approach employed to select random peptides expressed in fusion with the outer surface of phage clones (Smith, 1985). In this technology, bacteriophage-fused peptide libraries are submitted to successive bio-selection rounds, when specific phage clones are selected and their exogenous peptides are further identified by DNA sequencing (Barbas et al., 2001; Smith and Petrenko, 1997; Wang and Yu, 2004). Phage display generates short peptides that mimic epitopes (namely mimotopes), which present antigenic and immunogenic potential to be applied as diagnostic markers and/or vaccine candidates against diseases, such as malaria (Demangel et al., 1996; Greenwood et al., 1991; Monette et al., 2001), toxoplasmosis (Beghetto et al., 2003; Cunha-Junior et al., 2010), canine VL (Costa et al., 2014a, b, 2015), Chagas disease (Pitcovsky et al., 2001), strongyloidiasis (Feliciano et al., 2014) and others.

In this context, in the present study, a stringent subtractive phage display selection was employed to identify new mimotopes to be applied as antigens for the serodiagnosis of human VL. Besides the non-described subtractive selection procedure, the selected mimotopes proved to be highly relevant for the serological analysis, when evaluated by a phage–ELISA technique. This study presents novel diagnostic markers using a simple phage–ELISA test showing high accuracy, and which could well be considered as an alternative tool for the sensitive and specific serodiagnosis of VL.

2. Materials and methods

2.1. Ethics statement

This study was approved by Ethics Committee from Federal University of Minas Gerais (COEP/UFMG, protocol CAAE–32343114.9.0000.5149), Belo Horizonte, Minas Gerais, Brazil, and was conducted according to the Declaration of Helsinki principles. A written informed consent was obtained from all patients, who received an individual copy of the study policy, which was reviewed by an independent person.

2.2. Parasite and antigen preparation

L. infantum (MHOM/BR/1970/BH46) strain was used. Stationary-phase promastigotes of the parasites were grown at 24 °C in complete Schneider's medium (Sigma, St. Louis, MO, USA), which was composed by Schneider´s medium supplemented with 20% inactivated fetal bovine serum (FBS, Sigma), 20 mM L-glutamine, 200 U/mL penicillin and 100 μg/mL streptomycin, at pH 7.4. The soluble *L. infantum* antigenic extract (SLA) was prepared as described (Coelho et al., 2003), and the protein content was estimated by the Bradford method (Bradford, 1976).

2.3. Serum samples

Serum samples from patients with symptomatic VL (n=30, including 17 males and 13 females, with ages ranging from 22 to 58 years) were used. Infection was confirmed by PCR technique targeting *L. infantum* kDNA in aspirates from spleen and/or bone marrow of the patients. None of them have been previously treated with antileishmanial drugs prior to samples collection. All patients were selected from an endemic area of disease (Belo Horizonte, Minas Gerais, Brazil). The control group consisted of healthy individuals living in an endemic (Belo Horizonte; n=27, including 15 males and 12 females, with ages ranging from 17 to 42 years) or non-endemic (Poços de Caldas, Minas Gerais, Brazil; n=30, including 18 males and 12 females, with ages ranging from 19 to 48 years) areas of VL, and none of them presented clinical signs or suspicious of leishmaniasis. Serum samples from Chagas disease patients (n=15, including 9 males and 6 females, with ages ranging from 27 to 55 years) were used in the bio-panning rounds, aiming to exclude the cross-reactive phage clones. The infection was confirmed by hemoculture and by Chagatest recombinant ELISA v. 3.0 kit or Chagatest hemagglutination inhibition (HAI) assay (Wiener Lab, Argentina). Serum samples from tegumentary leishmaniasis (TL) patients, developing either mucosal (ML; n=34, including 23 males and 11 females, with ages ranging from 22 to 62 years) or cutaneous (CL; n=10, including 6 males and 4 females, with ages ranging from 18 to 47 years) leishmaniasis, were also employed in the phage-ELISA assays. Diagnosis was confirmed when parasites were visualized in Giemsa-stained smears obtained from mucosal fragments or skin biopsies, respectively. Positivity for the samples was also confirmed by the presence of *L. braziliensis* kDNA through PCR technique, and by a *L. braziliensis* SLA-ELISA reaction (data not shown).

2.4. Purification of the IgG antibodies

The purification of IgG antibodies was performed by coupling them onto magnetic microspheres (magnetic beads) conjugated to protein G (Dynabeads, Invitrogen), as described (Costa et al., 2014a). Briefly, 2×10^{10} particles of the microspheres were washed three times in 1 mL of 0.1 M MES buffer [2-(*N*-morpholino)ethanesulfonic acid] pH 5.0, and 350 μL of sera pool from healthy subjects or from those patients developing Chagas disease or VL (n=5, per group) were added to the microspheres and submitted to a 40-min incubation, at room temperature and under constant agitation. The IgG-coupled microspheres were washed four times in 1 mL of 0.1 M MES buffer, aiming to remove the non-adhered antibodies. The beads-antibody complex was washed three times in 1 mL of 0.2 M triethanolamine buffer pH 8.0, and resuspended in 1 mL of covalent coupling buffer (20 mM dimethyl pimelimidate/HCl diluted in triethanolamine buffer), for 30 min at room temperature and under constant agitation. The neutralization of unbound reactive sites was performed using 1 mL of 50 mM Tris-base pH 7.5, for 15 min and at room temperature. Microspheres were washed twice in 1 mL of TBS-T buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.1% Tween 20), blocked with a 2 mL blocking solution (5% BSA diluted in TBS-T) for 1 h at 37 °C, and resuspended in 200 μL of TBS buffer (50 mM Tris-HCl pH 7.5 and 150 mM NaCl). To verify the coupling, 5 μL of IgG-coupled beads were incubated with an anti-human IgG