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Serological diagnosis and prognostic of tegumentary and visceral leishmaniasis using a conserved *Leishmania* hypothetical protein

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ABSTRACT

New candidates for serological markers against leishmaniasis are required to be identified, since the presence of high titers of anti-*Leishmania* antibodies remain detected in sera of treated and cured patients, when current antigens have being employed. In this study, the diagnostic performance of a conserved *Leishmania* hypothetical protein was evaluated against a human and canine serological panel. The serological follow-up of the patients was also evaluated, using this recombinant antigen (rLiHyS) in ELISA assays. In the results, high sensitivity and specificity values were found when rLiHyS was used in the serological tests, while when the recombinant A2 (rA2) protein or an antigenic *Leishmania* preparation were used as controls, low sensitivity and specificity were found. Regarding the serological follow-up of the patients, significant reductions in the anti-rLiHyS antibody levels were found and, one year after the treatments, the anti-protein IgG production was similar to this found in the non-infected groups, reflecting a drop of the anti-rLiHyS antibody production. In conclusion, the present study shows for the first time a new recombinant antigen used to identify tegumentary and visceral leishmaniasis, as well as being able to serologically distinguish treated and cured patients from those developing active disease.

1. Introduction

Leishmaniasis is a disease complex caused by parasites from *Leishmania* genus, presenting an incidence of 0.2 to 0.4 million visceral leishmaniasis (VL) cases, and 0.7 to 1.2 million of tegumentary leishmaniasis (TL) cases [45]. Tegumentary leishmaniasis (TL) exhibits distinct clinical manifestations ranging from cutaneous leishmaniasis (CL), diffuse cutaneous leishmaniasis (DCL), to mucosal leishmaniasis (ML). *Leishmania braziliensis* is the main species responsible by the cases of the disease in the Americas, while *L. infantum* is the main responsible for the VL cases [34].

Dogs are considered as important reservoirs for visceral disease transmission, due to their close relationship with humans [10]. Regarding the canine disease, animals can develop from asymptomatic infection, when they are apparently healthy, to widespread chronic infections, which can lead to the death [25,40]. Regarding TL, of which dogs are accidental hosts, marsupials, rodents, and wild canids species have been found as reservoirs of the parasites [28].

Serological tests used to diagnose leishmaniasis present variable efficacy, indicating that there is a need for new studies to reach a safer diagnostic result [18]. In addition, distinct *Leishmania* species exhibit a distribution that overlap in many geographical regions, making it

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difficult to isolate the parasite species causing the disease [16]. Also, anti-*Leishmania* antibodies in cured and treated patients usually remain positive for months and years after the treatments, making it difficult to distinguish between past, current and cured infections [23,43].

Despite diagnostic methods available for VL, some problems persist and maximum sensitivity and specificity values are not reached [11]. In addition, most of the asymptomatic animals are not identified by the serological assays. In this context, an early and reliable diagnosis could refine the identification of these animals, as well as allow to control the spread of this disease in the world [25]. The serodiagnosis of TL also presents problems, since most of the patients present low levels of antileishmanial antibodies and, consequently, false-negative results are usually found in the serological assays [32,33]. As a consequence, the search for identifying new candidates to be employed in the improvement of the diagnosis of TL still remains, aiming to improve sensitivity and specific values.

Modern laboratorial techniques have allowed the identification of recombinant antigens to be evaluated in the serodiagnosis of canine and human leishmaniasis. Recombinant proteins, such as cytochrome *c* oxidase and IgE-dependent histamine-releasing factor [9], rLiHyD [22], rKLO8 [25], rLbHyM [23], rLiHyV [24], rHSP83 [6], rA2 [1,5], among others, have been studied with this purpose. However, although they present satisfactory results to identify the active disease, their optimization is still required to obtain maximum sensitivity and specificity values aiming to identify asymptomatic cases, as well as leishmaniasis-related diseases developing patients. In addition, no antigen is currently used to serologically follow-up the evolution of the patients treatment, since antibody titers remain positive for a long period of time after their parasitological and/or clinical cure [15,19].

In this context, the search for new *Leishmania* proteins, are able to stimulate the humoral response in the infected hosts, and allow distinguishing between treated and untreated patients, should help the development of more sophisticated tests to detect the disease [13]. In this context, in the present study, a conserved parasite protein, namely LiHyS (XP_001467126.1), which was recognized by VL dogs sera, but not by sera from healthy dogs or from those infected with *Trypanosoma cruzi* [8]; was cloned and its recombinant version (rLiHyS) was evaluated for the serodiagnosis of human and canine leishmaniasis. In our work, rLiHyS was also employed as a diagnostic antigen in ELISA assays to compare its reactivity in sera samples of untreated and treated patients.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Committee on the Ethical Handling of Research Animals of Federal University of Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil (protocol number 0333/2015). The work was also approved by the Human Research Ethics Committee of UFMG (protocol number CAAE–32343114.9.0000.5149).

2.2. Canine sera

The sample used was composed by 113 domestic animals (*Canis familiaris*), and consisted of males (n = 67) and females (n = 46) of different breeds and ages. Healthy dogs (n = 21) were free of any clinical signs of disease and were selected from an endemic area of VL (Belo Horizonte). They also presented negative serological results when using a commercial kit (EIE-LVC Biomanguinhos kit, Biomanguinhos®, Rio de Janeiro, Rio de Janeiro, Brazil). VL dogs were diagnosed by parasitological exams to identify *L. infantum* kDNA in bone marrow aspirates by PCR technique, like described [31]. In addition, they presented positive serological results with two commercial tests: IFAT-LVC Bio-Manguinhos kit and EIE-LVC Bio-Manguinhos kit. The animals were classified as symptomatic (n = 25), when they presented three or more of the following symptoms: weight loss, alopecia, adenopathy, onychogryposis, hepatomegaly, conjunctivitis, exfoliative dermatitis, splenomegaly, lymphadenomegaly, and renal azotemia; or asymptomatic (n = 18), when they did not present any clinical signs of disease. To evaluate the cross-reactivity, sera samples of Leish-Tec®-vaccinated healthy dogs (n = 20), as well as from those experimentally infected with *Ehrlichia canis* (n = 13) or *Babesia canis* (n = 15) were used.

2.3. Human sera

Sera samples were obtained from ML (n = 23; including 15 males and 8 females with ages ranging from 22 to 55 years) or VL (n = 45, including 30 males and 15 females with ages ranging from 19 to 56 years) patients, which were collected from an endemic area of the disease (Belo Horizonte). Mucosal leishmaniasis patients were diagnosed by clinical evaluation and by the demonstration of parasites in Giemsa-stained smears of mucosal fragments, besides of a PCR assay to identify *L. braziliensis* kDNA. Visceral leishmaniasis patients were diagnosed by clinical evaluation and demonstration of *L. infantum* kDNA in bone marrow aspirates, when a PCR technique was performed. Sera samples were also obtained from healthy individuals living in an endemic area of leishmaniasis (n = 35, including 23 males and 12 females with ages ranging from 20 to 51 years). These subjects did not present any clinical signs of disease and showed negative serological results when using commercial kits. Samples were also obtained from Chagas disease patients (n = 235, including 15 males and 8 females with ages ranging from 24 to 58 years), being the infection confirmed by hemoculture using the Chagatest® recombinant ELISA v.4.0 kit or the Chagatest® hemmaglutination inhibition kit (Wiener lab., Rosario, Argentina). To evaluate the rLiHyS reactivity in patients before and after the treatment, sera samples of ML (n = 10, including 6 males and 4 females with ages ranging from 26 to 55 years) and VL (n = 10, including 7 males and 3 females with ages ranging from 22 to 58 years) patients were collected before, six and 12 months after performing the treatments. All of them were treated with pentavalent antimonials, which were administered during 20 days (Sanofi Aventis Farmacêutica Ltda., Suzano, São Paulo, Brazil). The patients were submitted to parasitological exams using the PCR technique with mucosal fragments (ML) and bone marrow aspirates (VL), 12 months after treatment, when negative results were found in all evaluations.

2.4. Parasites

Leishmania infantum (MHOM/BR/1970/BH46) and *L. braziliensis* (MHOM/BR/1975/M2903) were used. The stationary-phase promastigotes and the soluble *Leishmania* antigenic extract (SLA) were prepared according described [7].

2.5. Production of the recombinant antigens and evaluation of the LiHyS amino acid sequence

The LiHyS gene (XP_001467126.1) was cloned from *L. infantum* DNA using: 5′-TCTCGGATCCATGCGCCAGCGAAAGCAC-3′ (*forward*) and 5′-TGAAAAGCTTCCATGCGATCCAGTAGATG-3′ (*reverse*) primers, for the restriction enzymes *BamHI* and *HindIII*. The DNA fragment was excised from gel, purified and linked into a pGEM®-T vector system (Promega, USA). The recombinant plasmid was used to transform *E. coli* XL1-Blue competent cells, and clones were tested by restriction enzymes analysis. The DNA fragment obtained from digestion of pGEM-LiHyS plasmid was ligated into a pET28a-TEV vector, and *E. coli* BL21 cells were transformed with the recombinant plasmid. Gene insertion was confirmed by colony PCR, and the sequencing was performed in a MegaBace 1000 automatic sequencer apparatus (Amersham Biosciences, USA). For the expression and purification of rLiHyS, cells were induced with 1.0 μM IPTG and cultures were incubated for 2 h at 37 °C, shaking at 200 ×*g* per min. Then, they were ruptured by seven