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Research paper

Evaluation of two recombinant *Leishmania* proteins identified by an immunoproteomic approach as tools for the serodiagnosis of canine visceral and human tegumentary leishmaniasis

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ABSTRACT

Serological diagnostic tests for canine and human leishmaniasis present problems related with their sensitivity and/or specificity. Recently, an immunoproteomic approach performed with *Leishmania infantum* proteins identified new parasite antigens. In the present study, the diagnostic properties of two of these proteins, cytochrome *c* oxidase and IgE-dependent histamine-releasing factor, were evaluated for the serodiagnosis of canine visceral (CVL) and human tegumentary (HTL) leishmaniasis. For the CVL diagnosis, sera samples from non-infected dogs living in an endemic or non-endemic area of leishmaniasis, sera from asymptomatic or symptomatic visceral leishmaniasis (VL) dogs, from Leish-Tec®-vaccinated dogs, and sera from animals experimentally infected by *Trypanosoma cruzi* or *Ehrlichia canis* were used. For the HTL diagnosis, sera from non-infected subjects living in an endemic area of leishmaniasis, sera from active cutaneous or mucosal leishmaniasis patients, as well as those from *T. cruzi*-infected patients were employed. ELISA assays using the recombinant proteins showed both sensitivity and specificity values of 100% for the serodiagnosis of both forms of disease, with high positive and negative predictive values, showing better diagnostic properties than the parasite recombinant A2 protein or a soluble *Leishmania* antigen extract. In this context, the two new recombinant proteins could be considered to be used in the serodiagnosis of CVL and HTL.

1. Introduction

Leishmaniasis presents a broad spectrum of clinical manifestations and is caused by different species of protozoa belonging to the genus *Leishmania*. The disease exhibits a high morbidity and mortality in the world, with approximately 380 million people in 98 countries at risk of contracting the infection (Alvar et al., 2012). The clinical manifestations of the disease are dependent upon both the parasite species and the immune response from the hosts (Kaye and Scott, 2011). Some *Leishmania* species cause tegumentary leishmaniasis (TL), while others disseminate to internal organs, such as the liver, spleen, and bone marrow causing visceral leishmaniasis (VL) (Ashford, 2000). Parasitological and molecular evidences have shown that, in Brazil, that *Leishmania braziliensis* is the main specie causing TL (Marzochi and Marzochi, 1994; Silveira et al., 2004), while *Leishmania infantum* is responsible for cases of VL in humans (Lainson et al., 2002).

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Current control methods for leishmaniasis are focused on the diagnosis and treatment of human disease. While dogs are recognized as the main domestic reservoirs of VL (Gramiccia and Gradoni, 2005), there is no scientific evidence supporting the involvement of these animals in the zoonotic cycle of TL (Brandão-Filho et al., 2003; Figueredo et al., 2012). Upon infection, dogs can develop asymptomatic or symptomatic clinical forms of disease (Solano-Gallego et al., 2011). Symptomatic canine visceral leishmaniasis (CVL) usually results in death and its clinical manifestations include cutaneous alterations such as alopecia, onychogryposis, and exfoliative dermatitis, associated with organic alterations such as splenomegaly, lymphadenomegaly, renal azotemia, and neurological disorders (Ciaramella et al., 1997; Viuelas et al., 2001). Also, infected dogs can remain asymptomatic, and be classified as false-negative in both clinical and serological evaluations performed (Moreno et al., 2009). This is an important problem, since these animals can contribute to maintaining the transmission of parasites between sand flies and humans (Courtenay et al., 2002; Moshfe et al., 2009).

The Brazilian public health authorities have determined that, for a precise immunological diagnosis of CVL, the Dual Path Platform (DPP; Bio-Manguinhos, Fiocruz, Rio de Janeiro, Brazil) is used as a screening test, followed by Canine Leishmaniasis ELISA (EIE-LVC kit; BioManguinhos, Fiocruz, Rio de Janeiro, Brazil), which is used as a confirmatory test (Grimaldi et al., 2012). However, the efficacy of these tests is hampered by factors affecting their sensitivity and/or specificity (Oliveira et al., 2009; Troncarelli et al., 2009; Silva et al., 2011; Viol et al., 2012).

Human tegumentary leishmaniasis (HTL) presents distinct clinical manifestations, such as cutaneous and mucocutaneous leishmaniasis (CL and MCL, respectively) (Reithinger et al., 2007). At present, there is no gold standard test to diagnose HTL, and a combination of clinical and parasitological methods is needed to obtain a more precise diagnosis (David and Craft, 2009). Serological tests employed in the serodiagnosis of HTL present problems related to their specificity, mainly, as regards to the occurrence of cross-reactivity (Malchiodi et al., 1994; Brito et al., 2000; Goto and Lindoso, 2010). Moreover, non-infected subjects living in endemic areas of the disease can present low to moderate antileishmanial serology, and they can be classified as false-positives in serological assays (Brito et al., 2001; Colmenares et al., 2002). On the other hand, patients developing CL can present low antileishmanial serology, and they can be classified as false-negatives in serological trials. This problem, although in a minor extent, also occurs in MCL (Kubar and Fragaki, 2005; Souza et al., 2013).

In a recent study, an immunoproteomic approach was performed with *L. infantum* proteins using sera of asymptomatic and symptomatic VL dogs. In this study, antigenic proteins of the parasites were identified (Coelho et al., 2012). Among these proteins, the parasite cytochrome *c* oxidase VII (CcOx) (XP_001565615.1) and *Leishmania* orthologue of the IgE-dependent histamine-releasing factor (HRF) (CAJ05086.1) were identified as antigens recognized by sera of active VL dogs. Therefore, the purpose of the present study was to evaluate the antigenic and diagnostic characteristics of both proteins, in a recombinant form, using a large canine and human serological panel. Based on the results obtained, the improvement of the diagnostic conditions for CVL and HTL is also discussed.

2. Materials and methods

2.1. Ethics statement

The study was conducted according to the Declaration of Helsinki principles, and all patients received an individual copy of the study policy, which was reviewed by an independent person. Written informed consents were obtained from all patients, and the study was approved by Ethics Committee from Federal University of Minas Gerais (COEP/UFMG, CAAE–323431 14.9.0000.5149 protocol), Belo Horizonte, Brazil. With regards to research using dogs sera, this study was approved by Committee on the Ethical Handling of Research Animals from UFMG (CETEA/UFMG), under the protocol number 043/2011. In addition, we received consents from the domestic dog's owners to obtain samples for analysis.

2.2. Canine sera

Dog blood samples (10 mL) were collected by venipuncture of jugular vein in tubes without anticoagulant, and were kept at 37 °C for 15 min; when they were centrifuged at 3000 × *g* for 15 min, and sera were separated and kept at −80 °C, until the use. The sample size was composed of 99 domestic animals (*Canis familiaris*), which consisted of males ($n = 49$) and females ($n = 50$) of different breeds and ages. CVL-positive animals presented positive parasitological results, which were based on identification of *L. infantum* kDNA in blood and/or bone marrow samples of these animals by a PCR technique, as previous described (Reis et al., 2013). Also, these animals presented positive serological results, which were evaluated with two laboratorial tests: IFAT-LVC® and EIE-LVC® (both kits derived from BioManguinhos). In this context, symptomatic dogs (CVLs; $n = 20$) were those with positive parasitological and serological results, as well as presented three or more of the following symptoms: weight loss, alopecia, adenopathy, onychogryposis, hepatomegaly, conjunctivitis, exfoliative dermatitis on the nose, tail, and ear tips; splenomegaly, lymphadenomegaly, and renal azotemia. Asymptomatic dogs (CVL_A; $n = 9$) presented positive parasitological and serological results, but they did not present any clinical signal of leishmaniasis. Non-infected dogs were selected from an endemic (H_{EA}, $n = 17$; Belo Horizonte, Minas Gerais, Brazil) or non-endemic (H_{NEA}, $n = 17$; Poços de Caldas, Minas Gerais, Brazil) area of VL, but they presented negative parasitological and serological results; and were free of any clinical signs of VL at the moment of the samples collection. Sera of non-infected animals and immunized with Leish-Tec® vaccine (HV; $n = 16$), or those of animals experimentally infected with *Ehrlichia canis* (Ec; $n = 10$) or *Trypanosoma cruzi* (Tc; $n = 10$), all of them (vaccinated and infected) maintained in kennels to prevent their contact with transmitting vectors of leishmaniasis or other pathogens; were also used.

2.3. Human sera

The following human sera samples were used: 24 sera samples were obtained from infected individuals presenting a state of symptomatic TL ($n = 12$ for CL, and $n = 12$ for MCL). Sera from non-infected subjects living in an endemic area of TL (HC_{EA}, $n = 20$; Belo Horizonte) were used, as well as sera samples of *T. cruzi*-infected patients (CD; $n = 8$). Diagnosis of TL was confirmed when, besides epidemiological and clinical features, parasites were visualized in the Giemsa-stained smears of skin biopsies (CL) or mucosal (MCL) fragments. These patients samples, when subjected to PCR, presented positive results for *L. braziliensis* kDNA. In addition, none of these patients were treated with antileishmanial drugs, before samples collection. The non-infected subjects did not present any clinical sign or symptom of leishmaniasis. Infection with *T. cruzi* was confirmed by hemoculture, and using the Chagatest® recombinant ELISA v.4.0 kit, as well as the Chagatest® hemmaglutination inhibition (Wiener lab., Rosario, Argentina).

2.4. Parasites

L. infantum (MOM/BR/1970/BH46) and *L. braziliensis* (MHOM/BR/1975/M2903) strains were used. The stationary-