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Short communication

Performance of *Leishmania braziliensis* enolase protein for the serodiagnosis of canine and human visceral leishmaniosis

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ABSTRACT

In the present study, *Leishmania braziliensis* enolase was cloned and the recombinant protein (rEnolase) was evaluated for the serodiagnosis of canine and human visceral leishmaniosis (VL). For the canine VL diagnosis, this study examined serum samples of *Leishmania infantum*-infected dogs, from non-infected animals living in endemic or non-endemic areas of leishmaniosis, as well as those from Leish-Tec®-vaccinated dogs and *Trypanosoma cruzi* or *Ehrlichia canis* experimentally infected animals. For the human VL diagnosis, this study analyzed serum samples from VL patients, from non-infected subjects living in endemic or non-endemic areas of leishmaniosis, as well as those from *T. cruzi*-infected patients. In the results, an indirect ELISA method using rEnolase showed diagnostic sensitivity and specificity values of 100% and 98.57%, respectively, for canine VL serodiagnosis, and of 100% and 97.87%, respectively, for human VL diagnosis. These results showed rEnolase with an improved diagnostic performance when compared to the recombinant A2 protein, the crude soluble *Leishmania* antigenic preparation, and the recombinant K39-based immunochromatographic test. In conclusion, preliminary results suggest that the detection of antibodies against rEnolase improves the serodiagnosis of human and canine visceral leishmaniosis.

1. Introduction

Visceral leishmaniosis (VL) is a disease caused by the parasitic protozoan *Leishmania infantum* in the Americas, of which dogs are considered domestic reservoirs of these parasites. To reduce the disease's dissemination, strategies to control canine visceral leishmaniosis (CVL) are essential, since seroprevalence varies in endemic regions, ranging from 3.4% to 40% of the animals, and reveals the potential for surveillance of canine infection as a marker of transmission between humans and sand flies (Dantas-Torres et al., 2006; Prado et al., 2011).

Early VL diagnosis makes it possible to formulate a quicker and more effective treatment against the disease, which could increase the possibility of a cure for the patients, as well as reduce the toxicity of the drugs (Coelho et al., 2009); however, conventional and molecular parasitological methods presents variable sensitivity (Tavares et al., 2003; Srividya et al., 2012). Therefore, serological tests could be considered for the detection of antileishmanial antibodies, due to its simplicity, cost effectiveness, and less invasive procedure for sample collection (Coelho et al., 2015).

A commercial kit, namely Kalazar Detect™ Test (InBios International®, Inc., Seattle, Wash, USA), was developed, which is a non-invasive immunochromatographic method to detect rK39-specific antibodies in VL patients' sera (Sundar et al., 2002; Grimaldi et al.,

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2012). However, problems, such as false-positive results in VL-related diseases patients, as well as false-negative results in subjects with low levels of antileishmanial antibodies, in immunocompromised subjects, or in subjects living in endemic areas, can hamper its diagnostic efficacy (Chappuis et al., 2007; Maia et al., 2012; Singh et al., 2012).

Recent advances in genomics, proteomics, and bioinformatics are resulting in new strategies to identify antigenic markers for the diseases (Costa et al., 2011; Duarte et al., 2015). In one immunoproteomic study, enolase protein was identified in *L. infantum* amastigotes by antibodies in VL dog sera (Coelho et al., 2012). In another work, this antigen was recognized by antibodies in cutaneous and mucosal leishmaniosis patients' sera (Duarte et al., 2015). In the present study, the diagnostic percentage of sensitivity and specificity values using recombinant enolase (rEnolase) protein were investigated in canine and human serum samples by an indirect ELISA method. Preliminary results showed that the detection of antibodies against recombinant protein can improve the serodiagnosis of human and canine visceral leishmaniosis.

2. Materials and methods

2.1. Canine sera

This study was approved by the Ethics Committee on the Handling of Research Animals (043/2011) and Ethics Committee on Human Research (CAAE-323431 14.9.0000.5149) from Federal University of Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil. Visceral leishmaniosis (n = 20) animals presented positive polymerase chain reaction (PCR) in blood and/or bone marrow aspirates, and three or more clinical signs of disease. Non-infected dogs were selected from endemic (n = 17, Belo Horizonte) or non-endemic (n = 17, Poços de Caldas, Minas Gerais, Brazil) areas of leishmaniosis, and were free of any clinical signs of the disease. Sera from Leish-Tec®-vaccinated dogs (HV, n = 16), as well as from those *Trypanosoma cruzi* (n = 10) or *Ehrlichia canis* (n = 10) infected experimentally animals, were also used.

2.2. Human sera

Serum samples from VL patients (n = 30, including 14 males and 16 females, with ages ranging from 23 to 54 years) were obtained from an endemic area (Belo Horizonte) of leishmaniosis. Infection was confirmed by PCR in spleen and/or bone marrow aspirates. Serum samples from non-infected subjects (n = 35) living in endemic (n = 18, Belo Horizonte) or non-endemic (n = 17, Poços de Caldas) areas of disease were also used. These individuals did not present the clinical signs of disease, nor negative serological results. Sera from Chagas disease patients (n = 12) were used to evaluate the cross-reactivity. None of the patients were treated before sample collection.

2.3. Antigens and parasites

The cloning, expression, and purification of rEnolase were performed according to that described in Duarte et al. (2015). The recombinant A2 (rA2) protein was purified as described in Zhang et al. (1996). *Leishmania infantum* (MOM/BR/1970/BH46) was used, and parasites were cultured according to that described in Coelho et al. (2003).

2.4. Mapping B cell-specific epitopes from enolase

The *L. infantum* and *L. braziliensis* enolase amino acid sequences were aligned using the Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/), as described in Sievers et al. (2011). The B cell epitope prediction was performed using the BepiPred 1.0 Server (http://www.cbs.dtu.dk/services/BepiPred/), according to that described in Larsen et al. (2006).

2.5. ELISA for the VL serodiagnosis

ELISA plates (JetBiofil®, Belo Horizonte) were coated with rEnolase, rA2 or *L. infantum* SLA (0.5, 1.0, and 1.0 μg/well, respectively), which were diluted in a coating buffer pH 9.6, for 18 h at 4 °C. Free binding sites were blocked with a solution comprised of PBS 1x and Tween 20 0.05% (PBS-T), plus 5% casein, for 1 h at 37 °C. After, plates were washed five times with PBS-T and incubated with 100 μL of canine or human sera (1:100 or 1:200 diluted in PBS-T, respectively), for 1 h at 37 °C. Plates were washed seven times and incubated with anti-dog or anti-human IgG horseradish-peroxidase conjugated antibodies (1:5000 or 1:10,000 diluted in PBS-T, A6792 and SAB3701282 catalogs, respectively, Sigma-Aldrich), for 1 h at 37 °C. After washing the plates seven times, reactions were developed by incubation with H_2O_2, ortophenylenediamine and citrate-phosphate buffer pH 5.0, for 30 min in the dark. The reaction was stopped by adding 2 N H_2SO_4, and the optical density (OD) was read in an ELISA microplate spectrophotometer (Molecular Devices, Spectra Max Plus, Canada), at 492 nanometers. The Kalazar Detect™ Test was used according to manufacturer's instructions (InBios International®, USA).

2.6. Statistical analysis

The results were entered into Microsoft Excel (version 10.0) spreadsheets and analyzed using GraphPad Prism™ (version 6.0 for Windows). The lower limits of positivity (cut-off) for the diagnostic antigens were established for a percent diagnostic specificity value of 100%. The curves for human sera were plotted with the values from VL patients (n = 35) versus those from the control groups (n = 47), which consisted of sera from healthy individuals living in endemic or non-endemic areas of leishmaniosis and Chagas disease patients. On the other hand, curves for canine sera were plotted with the values from CVL sera (n = 20) versus those from the control groups (n = 70), which consisted of sera from non-infected dogs living in endemic and non-endemic areas of disease, as well as from Leish-Tec®-vaccinated dog sera and from those presenting VL-related diseases. Tables of contingency and Fisher's exact test were used to compare the results obtained for each antigen, which were estimated by assessing their percent diagnostic sensitivity (DSe%), percent diagnostic specificity (DSp%), 95% confidence interval (95%CI), positive predictive value (PPV), negative predictive value (NPV), and positive likehood ratio (LR +). Differences were considered significant when $P < 0.05$.

3. Results

3.1. Antigenicity of the rEnolase against canine sera

The analyses of the *L. infantum* and *L. braziliensis* enolase amino acid sequences showed a 92% homology between them, in addition to the conservation of the main specific-B cell epitopes of this protein. The recombinant antigen was then cloned, and a protein of approximately 46.0 kDa was purified, when its potential for being used in the CVL serodiagnosis was evaluated by an indirect ELISA method. In the results, VL and healthy dog sera were found to be strongly and weakly reactive, respectively, against rEnolase (Fig. 1). A low reactivity was found when VL-related disease dog sera were employed. On the other hand, a high reactivity was observed against these sera, when *L. infantum* SLA was used as an antigen. In addition, a moderate reactivity was found using rA2 in the serological assays against CVL samples, as well as against VL-related diseases or Leish-Tec®-vaccinated dog sera. The percent diagnostic sensitivity and specificity values of the antigens were calculated and are shown in Table 1. The rEnolase protein showed the best diagnostic results, with values of 100% and 98.57%, respectively, while *L. infantum* SLA and rA2 showed worse results in their