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Diagnostic accuracy of rKLO8 versus rK26 ELISAs for screening of canine visceral leishmaniasis

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ABSTRACT

Canine visceral leishmaniasis (CVL) represents an important public health issue. Despite numerous diagnostic tests available, CVL diagnosis still needs to be improved to achieve a more accurate detection rate. Recently, rKLO8, a new antigenic protein of Sudanese *Leishmania donovani,* was studied for the first time in diagnosis of human visceral leishmaniasis (HVL) and showed good performance. The present study aimed to evaluate serum reactivity to rKL08 and the reference antigen rK26, and to compare both diagnostic proteins with the combined DPP® CVL rapid test and ELISA (EIE-Bio-Manguinhos) confirmatory test, which are both recommended for the diagnosis of CVL in Brazil. Serum samples of dogs were grouped into: (I) DPP®/EIE negative (n = 100) and (II) DPP®/EIE positive sera (n = 100). Enhanced levels of IgG, mainly IgG2, to both rKLO8 and rK26 were found in group II. Sensitivity was 68% and 77% and specificity was 92% and 91%, for rKLO8 and rK26 antigens, respectively. Moreover, the combination of rKLO8 and rK26 antigens (rKLO8 + rK26) exhibited higher sensitivity (85%) and specificity (93%). Thus, our results show that apart from the improved diagnostic power of rKLO8 in HVL, this new antigen is also suitable for the diagnosis of CVL. Further, the combination of rKLO8 and rK26 antigens increases the diagnostic accuracy of CVL.

1. Introduction

Visceral leishmaniasis (VL) is an emerging parasitic zoonosis caused by intracellular protozoan parasites of the *Leishmania genus* (WHO, 2015). Infected dogs are a potential source of infection for the phlebotomine vector, thus posing risks for indirect transmission of the parasite to humans (Quinnell and Courtenay, 2009). Canine visceral leishmaniasis (CVL) ranges from subclinical infections with apparently healthy animals, to widespread chronic infections that inevitably lead to the death of the animal if not adequately treated (Solano-Gallego et al., 2011). In the Americas, it is estimated that most cases of CVL are caused by *Leishmania infantum (syn. Leishmania chagasi)* and also by other species of *Leishmania*, such as *Leishmania (V.) braziliensis* (Dantas-Torres, 2009). In Brazil, the seroprevalence rates in dogs vary widely reaching up to 75% in highly endemic foci (Paranhos-Silva et al., 1996; Cortada et al., 2004; Dantas-Torres, 2009). Governador Valadares is an endemic area for leishmaniasis in Minas Gerais, Brazil. The first cases of HVL in Governador Valadares were recorded in the 60s. In a study carried out between 2008 and 2011 in 35 districts of Governador Valadares, 86 indigenous cases of HVL were reported and 4992 (30.2%) dogs out of 16,529 were seropositive for CVL (Barata et al., 2013).

For the diagnosis of CVL, several strategies have been established, based on parasitological methods, immunological and serological tests, and molecular techniques in association with clinical and epidemiological parameters (Miro et al., 2008). The clinical diagnosis of CVL is difficult to determine because of the

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large percentage of existing asymptomatic dogs, and similarity to other infectious diseases affecting dogs (Alves et al., 2012). Serological tests are commonly used for the diagnosis of CVL (Gomes et al., 2008), including the direct agglutination test (DAT), the enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence (IFI). Currently, ELISA is the method of choice in population-based surveys (Morales-Yuste et al., 2012), because of the suitability to test large number of samples in a short period of time, and the versatility to use several types of antigens, such as purified antigens, synthetic peptides and recombinant proteins (Maia and Campino, 2008). However, it presents important limitations for the diagnosis of CVL, including lack of sensitivity and specificity, which are strongly influenced by the type of antigen used (Sundar and Rai, 2002).

The recombinant antigens rK26, a hydrophilic protein of 247 amino acids specifically expressed in *L. donovani* and *L. infantum*, and rK39 of *L. infantum* have been shown to be suitable for CVL diagnosis (Bhatia et al., 1999; Da Costa et al., 2003; Mettler et al., 2005). The rK39 antigen is more sensitive for the diagnosis of symptomatic cases of CVL (100%) compared to asymptomatic CVL (66%) (Porrozzi et al., 2007). Recently, rK28, a multi-epitope recombinant chimeric protein obtained by fusing *L. infantum* k9 gene with single repeat units of k39 and k26 genes (Boarino et al., 2005), was used to develop an immunochromatographic rapid test, the Dual-Path Platform (DPP; Bio-Manguinhos/Fiocruz, Rio de Janeiro, Brazil), which was recommended for the screening of infected dogs (Grimaldi et al., 2012; Coura-Vital et al., 2014).

Although DPP demonstrates excellent sensitivity for identifying symptomatic dogs (98%), it showed lower efficacy for the diagnosis of asymptomatic animals (47%) (Grimaldi et al., 2012). Since 2011, the Brazilian Department of Communicable Disease Control, Ministry of Health, recommended the use of DPP in combination with ELISA-BioManguinhos (EIE) as screening method in CVL surveys. EIE employs total antigens of *L. major* and thus confirms positive results (Grimaldi et al., 2012; Coura-Vital et al., 2014).

Despite numerous diagnostic tests available for CVL, the issue continues to represent a challenge. There is no single method to obtain maximum sensitivity and specificity in order to allow an accurate diagnosis of the disease (Coura-Vital et al., 2014), and since asymptomatic dogs can participate in the natural transmission cycle of VL, the importance of vector and host surveillance with an early, reliable and rapid diagnostic test requires the refinement of current methods to improve CVL diagnosis (Quinnell et al., 2013). Recently, a new kinesin-homolog, rKLO8, from a Sudanese *strain of L. donovani* has been cloned and tested for its diagnostic value in VL patients. By ELISA it was demonstrated that rKLO8 had a higher sensitivity (98.1% vs 96.2%) and specificity (96.1% vs 94.8%) in East African VL patients as compared to the currently used rK39 from *L. infantum*, respectively (Abass et al., 2013). In the present study, we evaluated for the first time canine serum antibody reactivity towards rKL08 in comparison to rK26. Receiver Operator Characteristic Curve (ROC) analysis was applied to study differences between the rKLO8 and rK26 in the diagnosis of CVL using serum samples from dogs tested negative ($DPP^{-}EIE^{-}$) and positive ($DPP^{+}EIE^{+}$) for CVL.

2. Materials and methods

2.1. Serum samples

A total of 200 sera collected from dogs were obtained from the Zoonosis Control Center (ZCC) serum repository, Municipal Health Secretariat, Governador Valadares (GV), Minas Gerais, an area endemic for visceral and tegumentar leishmaniasis in Brazil. The serum samples were first tested, in a pre-evaluation trial conducted by technicians from the ZCC-GV, with the qualitative rapid test "Dual Path Platform" (TR DPP® CVL – Bio-Manguinhos) (DPP), and ELISA Bio-Manguinhos (EIE-CVL, FIOCRUZ, Rio de Janeiro, Brazil). DPP, based on the use of rK28 protein, and EIE Bio-Manguinhos, which employs total *L. major* antigens, are tests currently recommended by the Brazilian Ministry of Health for screening CVL (Grimaldi et al., 2012). Based on data from the manufacturer's, the DPP test presents a sensitivity of 100% and specificity of 87.5 – 91.7%, (62 dogs tested), and the EIE presents 94.54% sensitivity and 91.76% specificity. Serum samples were grouped as follows: (1) DPP-/EIE- group (n = 100), with sera from dogs tested negative for DPP and negative for EIE (considered the seronegative control), and (2) DPP+/EIE+ group (n = 100), with sera from dogs tested positive for DPP and positive for EIE (considered the seropositive control). All samples were stored at −20 °C until testing in rKLO8-ELISA. The Ethics Committee on Animal Experimentation of the Federal University of Juiz de Fora approved the study protocol (no. 016/2015).

2.2. Antigens

The recombinant protein rKLO8 were produced and quality controlled by Philipps University of Marburg using *Escherichia coli* M15 transformed with the pQE41/KLO8 plasmid. Briefly, genes were amplified by PCR and cloned into the bacterial expression vector pQ41 (Qiagen GmbH, Germany), containing an N-terminal histidine tag. The proteins were over-expressed in *E. coli* M15 (Qiagen GmbH, Germany) and purified, as described previously (Abass et al., 2013). Purity and size were checked by gel electrophoresis and western blotting with anti-His antibodies and sera of VL patients. Recombinant antigens were freeze-dried, shipped to Juiz de Fora/Brazil, reconstituted in PBS and kept at −80 °C until testing in ELISA. The recombinant protein of *L. infantum* rK26 was kindly provided by the Infectious Disease Research Institute (IDRI), Seattle, Washingnton, USA. Both rKLO8 and rK26 proteins are specific to *L. donovani complex.*

2.3. ELISA

Ninety-six well-plates (Plast-Bio, Curitiba, Brazil) were coated overnight with 100 μl/well rKLO8 and rK26 individually (0.5 μg/mL) or in combination (0.25 μg/mL of each) diluted in 0.1 M $NaCO_3$ buffer (pH 9.6) solution. Plates were then washed with phosphate buffered saline (PBS) containing 0.05% (v/v) Tween-20 (PBS-T) and blocked with 2% (w/v) BSA in PBS-T for 1 h at room temperature. After washing with PBS-T, 50 μl of diluted serum samples (1:2000 for IgG; 1:1000 for both IgG1 and IgG2) were added to each well and the plates incubated for 1 h at room temperature. After washing with PBS-T, 50 μl of either peroxidase-conjugated rabbit anti-dog IgG (1:10,000 dilution) (Sigma, St. Louis, MO), peroxidase-conjugated goat polyclonal anti-dog IgG1 (1:10,000 dilution) (AbD Serotec, Bio-Rad Company, USA), or peroxidase-conjugated polyclonal sheep anti-dog IgG2 (1:5000 dilution) (AbD Serotec) were added. Plates were incubated at room temperature for 1 h, washed, and a substrate solution containing tetramethylbenzidine (TMB) and H_2O_2 (BD, Sao Paulo-Brazil) was used. The reaction was stopped with 2N H_2SO_4 and the optical density (OD) measured at 450 nm (Spectramax-190, Molecular Devices, Sunnyvale, CA, USA). Each sample was tested in duplicates and the mean OD was determined.

2.4. Statistical analysis

Two strategies were used to study differences between the rKLO8 and rK26, using group I (DPP^{-}/EIE^{-}) and group II (DPP^{+}/EIE^{+}) as seropositive and seronegative controls: (i) Median differences between optical densities and (ii) comparison of ROC curves.