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Short Communication

Comparison between ELISA using total antigen and immunochromatography with antigen rK39 in the diagnosis of canine visceral leishmaniasis

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ABSTRACT

In this study, an enzyme-linked immunosorbent assay using crude total antigen (CTA-ELISA) and immunochromatography with antigen rK39 were compared in the diagnosis of canine visceral leishmaniasis (CVL). Fifty-two total blood samples from symptomatic dogs obtained from a location endemic for leishmaniasis and 52 blood samples from healthy dogs from a nonendemic region were tested. Polymerase chain reaction (PCR) was used to detect DNA from *Leishmania* spp. in both groups. Symptomatic dogs with positive PCR were considered infected by *Leishmania* spp. and the PCR technique was chosen as a gold standard test. The sensitivity determined for CTA-ELISA was 100%, with specificity of 91.2%, while the immunochromatographic assay with the antigen rK39 showed sensitivity of 91.5%, with specificity of 94.7%. A strong correlation was verified between CTA-ELISA and immunochromatography with antigen rK39, with a kappa coefficient of agreement of 0.88. Analysis of the results suggested that both assays presented good sensitivity and specificity for diagnosing CVL; however, immunochromatography with the antigen rK39 may be more advantageous when a fast field test is required.

1. Introduction

Visceral leishmaniasis (VL) is a chronic disease caused by the species *Leishmania* spp., pertaining to the *Leishmania donovani* complex. This disease affects 47 countries and its mean annual incidence is estimated to be approximately 500,000 new cases. The main historical foci of endemic VL are located, east to west, in China, India, Central Asia, East Africa, the Mediterranean Basin and Brazil. Brazil has the second highest number of human VL cases (Desjeux, 2004). Domestic canids (*Canis familiaris*) are known to establish the peridomestic cycle of VL (Courtenay et al., 1994). Dogs have also been implicated as reservoir hosts for *Leishmania* spp. (Vercosa et al., 2008). The canine prevalence of leishmaniasis can reach 20–40% of the population in endemic locations (Slappendel and Ferrer, 1990). The high parasitism observed in the skin of *Leishmania* spp.-infected

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dogs shows that they play a role in the dissemination of leishmaniasis. In dogs, the infection can be asymptomatic or symptomatic, with more common clinical/pathological manifestations. Clinical signs of the disease can vary, but more often include: hepatosplenomegaly; sweating; leukopenia, lymphadenopathy; onychogryphosis; and skin alterations, such as alopecia, rash, skin and hyperkeratinization cushions, depigmentation. Some animals can also present ulcers and intradermal nodules. Nonspecific signs of leishmaniasis, such as fever, cachexia and weight loss, may be verified in infected animals and other signs commonly observed include uveitis and nasal epistaxis. The occurrence of secondary illnesses is also common.

Diagnosis may be achieved by direct methods, such as cytology, parasite isolation and cultivation, or molecular detection of the parasite with polymerase chain reaction (PCR) to amplify the kinetoplast DNA of the parasite. The detection of the parasite is the gold standard for diagnosis of VL, but the procedure is difficult to apply in field conditions.

Due to the limitations of direct diagnostic methods, indirect and noninvasive techniques have been investigated, especially those relating to the detection of specific antibodies in the animal serum. The current diagnostic methods used for *Leishmania* spp. mass-screening surveys investigate specific anti-*Leishmania* spp. antibodies and include a large variety of techniques and antigens (Alvar et al., 2004).

In the last few years, several *Leishmania* spp. antigens have been characterized. One of the important advances in the indirect diagnosis of canine visceral leishmaniasis (CVL) was the production and identification of recombinant antigens (Burns et al., 1993). Specifically, the antigen rK39, a recombinant protein, resembles a part of a 230-kDa protein of the kinesin family, coded for the kinetoplast DNA of *Leishmania*. The protein rK39 presents identical sequences in seven species of *Leishmania*. Reactivity with other trypanosomatids is negative and the presence of the antibody, anti-rK39, indicates an active infection (Bisugo et al., 2007).

The recombinant protein K39 has been used in fast immunochromatographic tests (using dipsticks) and revealed great utility in the serological diagnosis of CVL and prognosis in the field and the laboratory in Africa and India (Sundar et al., 1998).

Considering the epidemiological importance of early serological diagnosis in CVL, the performance of CTA obtained from promastigotes used in ELISA and immunochromatographic tests of rK39 was evaluated by comparing the sensitivity and specificity of these tests in dogs from endemic and nonendemic locations for CVL.

2. Materials and methods

2.1. Sample

Fifty-two symptomatic dogs from endemic locations were used and 52 healthy dogs from a nonendemic region were used as controls. The symptomatic dogs presented at least three clinical signs of CVL. These could include fever, dermatitis, lymphoadenopathy, onychogryphosis, weight loss, cachexia, locomotor abnormalities, conjunctivitis, epistaxis, hepatosplenomegaly, edema and apathy.

From each dog, 5.0 mL of blood was collected from the cephalic veins, clotted at room temperature for 4 h and subsequently centrifuged to extract the serum. The serum samples were stored at −20 °C prior to analysis and additional blood samples were collected with anticoagulant (sodium EDTA) and stored at 4 °C for PCR.

2.2. ELISA (enzyme-linked immunosorbent assay)

The ELISA was based on the protocol described by Lima et al. (2003). Briefly, microplates (Greiner-bio One Microloan 600, Germany) were coated with 20 μg/mL total antigen from *Leishmania chagasi* (MHOM/BR00/MERO2) in 100 μL buffer (0.05 M carbonate buffer, pH 9.6) and incubated overnight at 4 °C. The plates were then washed in washing buffer (PBS + 0.05% Tween 20®) and blocked for 1 h at room temperature with 150 μL PBS containing 10% fetal bovine serum. After a further wash, 100 μL of serum sample diluted in PBS containing 10% fetal bovine serum and 0.05% Tween 20® (concentration 1:400) were added and the plates were incubated for 2.5 h at room temperature. After incubation, the plates were washed again and 100 μL of anti-IgG dog peroxidase conjugate (Sigma, USA) were added and incubated for 1 h. The washing process was repeated and the substrate added (*o*-phenylenediamine (Sigma, USA) and H_2O_2 in phosphate-citrate buffer). The reaction was stopped by the addition of 50 μL 1N HCl. Absorbance was measured at 490 nm using an automatic reader (Spectra Count, Packard®). The cutoff point was determined with serum from healthy dogs from a nonendemic location, using as reference the mean plus 3 times the standard deviation obtained for the group. The samples were analyzed in duplicate and a blank well (PBS + 0.05% Tween 20® solution) was included in all plates.

2.3. Rapid test rK39

To conduct this test, blood samples were collected and centrifuged to separate the serum. The test was performed in accordance with the manufacturer's instructions.

2.4. PCR

DNA obtained from peripheral blood cells was extracted by freezing and thawing the cells 3 times and washing them in 1 × SSC buffer solution (3 M NaCl, 0.3 M sodium citrate, pH 7.0). For cell lysis and protein digestion, 300 μL of lysing solution was added (10% SDS in 0.2 M sodium acetate) together with 20 μg/mL of proteinase K. The samples were incubated at 56 °C for 2 h and DNA was extracted using the phenol/chloroform/isoamyl alcohol method (25:24:1), as described by Sambrook et al. (1989). After extraction, DNA was resuspended in 50 μL TE (10 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0) and stored at −20 °C until used. For PCR, DNA was previously incubated for 3 min at 60 °C. Primers 13A (3′-GTG GGG GAG GGG CGT TCT-5′) and 13B (3′-ATT TTA CAC CAA CCC CCA GTT-5′) were used (Rodgers et al., 1990) to amplify a 120-bp fragment located in the con-

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Table 1
Comparison between the results of immunochromatographic strips with rK39 antigen, and indirect ELISA with total antigen of *Leishmania chagasi* the PCR reaction using DNA, from *L. chagasi*, considered the gold standard for the diagnosis of LVC.

	PCR		*S* (%)	Spec (%)	McN	κ
	Pos.	Neg.				
rK39						
Pos.	43	3	91.5	94.7	$P = 0.0313$	0.88
Neg.	4	54				
ELISA						
Pos.	47	5	100	91.2		
Neg.	0	52				

Pos = positive, Neg. = Negative, S = sensitivity, Spec = specificity; $P < 0.01$. κ (kappa index), McN (McNemar).

stant region of the kinetoplast minicircles in all *Leishmania* species. PCR was performed in a final volume of 60 μL, containing 30 pmol of each indicator (Invitrogen®), 0.2 mM DNTPs (Invitrogen®), 1.5 mM $MgCl_2$ (Invitrogen®), 5 U Taq DNA Polymerase (Invitrogen®), 50 nM buffer solution, MilliQ water and DNA. Amplification was performed in an Eppendorf® Mastercycler Thermocycler gradient with initial heating to 95 °C for 5 min, followed by 33 cycles at 95 °C for 1.5 min, 57 °C for 1.5 min and 72 °C for 2 min. Extension was performed at 72 °C for 10 min and the final product was stored at −20 °C until analysis. Reaction mixes containing either no DNA or DNA extracted from a *L. chagasi* (5×10^5) promastigote culture (MHOM/BR00/MER02) were used as negative and positive controls, respectively. The amplified 120-bp product was analyzed by electrophoresis on acrylamide gels followed by silver staining.

2.5. Statistical analysis

Statistical analysis was based on the McNemar nonparametric test, used to compare the proportion of positive results of the two groups. Statistical analyses were performed by the computer program SAS, 9.1 (Statistical Analysis System). The results were considered significant when $P < 0.01$. Sensitivity and specificity were determined using the MedCalc statistical software. The kappa coefficient was used to measure the degree of agreement between the techniques for immunochromatography with rK39 and CTA-ELISA.

3. Results

To examine the sensitivity and specificity of immunochromatographic test rK39 and CTA-ELISA, 104 dogs were included in the analysis. Of the 52 dogs from Araçatuba, SP (endemic location for CVL), 47 were positive by PCR (using peripheral blood) and were therefore considered infected by *Leishmania* spp. The 52 healthy dogs from a location nonendemic for CVL that were negative by PCR were considered noninfected.

The immunochromatographic test rK39 and CTA-ELISA presented sensitivity of 91.5% and 100% and specificity of 94.7% and 91.2%, respectively. No significant differences were observed between the two methods (Table 1).

A strong correlation was verified between the tests involving the immunochromatographic strip with rK39 antigen and indirect CTA-ELISA (κ 0.88). Among the three tests, PCR was considered the gold standard, because of its high sensitivity and specificity in detecting positive dogs (Mettler et al., 2005).

4. Discussion

Despite intense research regarding the diagnosis of VL, a zoonotic disease that affects humans, there is currently no perfect test for the diagnosis or epidemiological surveys of dogs. The use of accurate diagnostic tools is crucial to establish and implement measures to control the spread of this zoonosis. In this investigation, the effectiveness of CTA in an ELISA and immunochromatographic tests with antigen rK39 were compared in order to evaluate a panel of sera from infected dogs and sera from healthy dogs.

CTA-ELISA presented high sensitivity and specificity with values of 100% and 91.2%, respectively. These results were similar to those determined by Mettler et al. (2005), in an ELISA with promastigote antigens of *L. infantum*, who also used PCR as a gold standard to confirm symptomatic CVL. However, Reithinger et al. (2002) obtained low sensitivity and specificity in the diagnosis of CVL by ELISA. This difference can be attributed to the antigen, since they used sonicated *L. donovani* instead of crude total antigen from *L. chagasi*. In fact, the specificity and sensitivity of different methods depends on several factors that can be influenced by the nature of the antigen used for the test (Mohammed et al., 1985; Singh and Sivakumar, 2003).

The advantage of using the CTA promastigote in an ELISA is that this antigen is easily obtained in large quantities under standardized conditions and can provide a relatively stable source of a wide range of determinants, including some that are not available in soluble antigen preparations. In addition, CTA is cheaper than purified or recombinant antigens. Moreover, CTA has been used in studies on humans and presents greater sensitivity than sonicated antigen (Mohammed et al., 1985). The disadvantage is that CTA can present cross-reactivity with other diseases (Rosário et al., 2005), although this issue also depends on serum dilutions. Additionally, sera from dogs with *Ehrlichia canis*, *Toxoplasma gondii*, *Babesia canis* or *Dirifilaria immitis* were negative using CTA-ELISA (Lima et al., 2005).

The present results of the immunochromatographic test with rK39 antigen were better than those observed by Reithinger et al. (2002) who reported sensitivity of 77%, which was less than that observed in this work (91.5%). At the study location, a high incidence of infected dogs has been verified (Nunes et al., 2008) and most of the canines

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presented clinical signs compatible with CVL. Since the group of dogs studied by Reithinger et al. (2002) was not clearly described, they may have included asymptomatic dogs, which would lower the sensitivity of the test with rK39 antigen (Mettler et al., 2005) and explain the differences observed.

Analysis of the results of this study showed that both diagnostic methods, CTA-ELISA and immunochromatography with rK39 antigen, presented as sensitive and specific diagnostic tools to detect CVL and can be applied in substitution of each other as serology tests in symptomatic dogs. The choice between using either method depends on the conditions present and the purpose of the study, since a strong correlation was observed between the tests. A similar result was verified by Lemos et al. (2008) using an ELISA with crude soluble antigen and rk39. This group considered the detection of the presence of *Leishmania* spp. amastigotes in bone marrow aspirate as a gold standard test.

In conclusion, the immunochromatographic test with antigen rK39 proved to be a simple, low-cost and rapid test compared to PCR and CTA-ELISA, diagnostic methods that are time consuming and expensive when performed on a large scale. In endemic locations, the availability of diagnostic tools that are simple to use, such as the immunochromatographic test with antigen rK39, is of fundamental importance when monitoring public health to control the spread of leishmaniasis.

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