



Short communication

Canine visceral leishmaniasis: Performance of a rapid diagnostic test (Kalazar Detect™) in dogs with and without signs of the disease

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ABSTRACT

Current visceral leishmaniasis (VL) control programs in Brazil include the infected dog elimination but, despite this strategy, the incidence of human VL is still increasing. One of the reasons is the long delay between sample collection, analysis, control implementation and the low sensitivity of diagnostic tests. Due to the high prevalence of asymptomatic dogs, the diagnosis of these animals is important considering their vector infection capacity. Hence, a rapid and accurate diagnosis of canine visceral leishmaniasis is essential for an efficient surveillance program. In this study we evaluated the performance of rK39 antigen in an immunochromatographic format to detect symptomatic and asymptomatic *Leishmania chagasi* infection in dogs and compared the results with those using a crude antigen ELISA. The sensitivity of rK39 dipstick and ELISA were 83% vs. 95%, respectively, while the specificity was both 100%. Our results also demonstrated that the dipstick test was able to detect infected dogs presenting different clinical forms.

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1. Introduction

Leishmania (Leishmania) chagasi is the etiologic agent of visceral leishmaniasis (VL) in Brazil and dogs are the major domestic reservoirs of this protozoan disease (Deane and Deane, 1962). Due to its anthroponotic characteristic, the Brazilian VL control program emphasizes serologic surveys with elimination of seropositive dogs as an attempt to reduce human disease (Vieira and Coelho, 1998; Palatinik-de-Souza et al., 2001). In the past 5 years, more than two million dogs were screened and more than 160,000 seropositive dogs were eliminated. Despite this effort, the Ministry of Health has not been able to reduce the incidence of human disease to an acceptable level. One of the alleged reasons for that is the long delay between sample collection, sample analysis, and culling of infected dogs (as long as 80 days). In addition, the current diagnosis method adopted by the Ministry of Health as the gold standard for VL screening surveillance (immunofluorescence antibody test) lacks sensitivity and specificity, requires well trained lab technician, a flu-

orescent microscopy, and is labor-intensive and time-consuming. Hence, a rapid, sensitive and accurate diagnostic test would represent an essential tool in mass-screening survey for interventional programs.

A rapid immunochromatographic test for qualitative detection of anti-*Leishmania* antibodies has recently become available. This test is based on a recombinant *Leishmania* K39 antigen (rK39), a repetitive immunodominant epitope in a kinesin-related protein that is highly conserved among viscerotropic *Leishmania* species: *L. (L.) donovani*, *L. (L.) infantum* and *L. (L.) chagasi* (Burns et al., 1993). This dipstick test has been shown to be very sensitive and specific for human VL diagnosis (Sundar et al., 1998; Jelinik et al., 1999; Bern et al., 2000; Zijlstra et al., 2001; Carvalho et al., 2003) and there are also some publications using it for the diagnosis of VL in dogs (Mohebbi et al., 2004; Otranto et al., 2004; Toz et al., 2004; Mettler et al., 2005). In Brazil there are only two publications about this issue with conflicting results and none of them included dogs in different stages of the disease: asymptomatic, oligosymptomatic and with full blown disease (Reithinger et al., 2002; Da Costa et al., 2003).

Symptomatic dogs usually produce high levels of specific antibodies which can be easily detected (Deplazes et al., 1995). However, the sensitivity of antibody detection is generally lower in early or in asymptomatic canine infections (Leontides et al., 2002).

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Due to the high prevalence of asymptomatic dogs in endemic areas of VL, the diagnosis of these animals is very important considering their vector infectivity capacity (Molina et al., 1994; Guarga et al., 2000). Here in, we evaluated the performance of a commercially available rK39 dipstick test (Kalazar Detect™) for the detection of specific anti-*L. (L.) chagasi* antibodies in dogs with and without signs of VL. We also compared the results with those obtained by ELISA using crude antigen.

2. Materials and methods

2.1. Samples

Blood samples were taken from 76 infected dogs from two different VL endemic areas in Brazil, Araçatuba, São Paulo and Belo Horizonte, Minas Gerais. The diagnosis of VL was based on the presence of *Leishmania* amastigotes in bone marrow aspirates. Animals were classified clinically according to the presence/absence of signs of VL as follows: asymptomatic—without signs of disease; oligosymptomatic—with at most three clinical signs including opaque bristles, localized alopecia and moderate weight loss; symptomatic—with the following signs of disease: opaque bristles, severe weight loss, onychogriphosis, cutaneous lesions, apathy and keratoconjunctivitis (Mancianti et al., 1998). As controls, we tested serum from 33 clinically healthy animals from a non-endemic area. All control animals were also negative by both parasitological and serological methods (ELISA).

All procedures described in the present work were carried out in compliance with current Brazilian regulations relating to Experimental Biology and Medicine as described in the guidelines issued by the Colégio Brasileiro de Experimentação Animal (COBEA).

2.2. Kalazar Detect test

The Kalazar Detect™ (InBios International, Seattle, WA, USA) is an immunochromatographic qualitative antibody assay against *L. (L.) chagasi* rK39 antigen. Twenty microliters of serum mixed with two drops of buffer was placed on the pad of the dipstick. Following the manufacturer's instructions, a test was positive when two bands, a control and a positive test bands, appeared within 10 min. The test was negative if only the control band appeared. The test is qualitative and the manufacturer indicates that a faint band should be considered positive. The result of a dipstick was considered not valid if the internal control was not stained. An investigator blinded to the dogs underlying disease evaluated all tests.

2.3. ELISA

L. (L.) chagasi (MHOM/BR/1070/BH46) parasites were cultivated in LIT medium supplemented with 10% fetal calf serum at 26 °C. The promastigotes pellet was collected by centrifugation, washed three times with phosphate-buffered saline (PBS) and lysed by freeze thawing. The parasite suspension was homogenized using a tissue grinder in an ice bath. The homogenized material was centrifuged at 10,000 × g for 20 min at 4 °C. The supernatant was collected and used as crude soluble antigen.

Briefly, MaxiSorp plates (Nalge Nunc Intl., USA) were coated with 10 mg/ml of *L. (L.) chagasi* crude antigen and held overnight at 4 °C. Plates were washed three times with PBS and blocked with 100 µl of PBS containing 10% fetal bovine serum (FBS) for 2 h at 37 °C. After washed three times, the serum samples diluted 1/80 in PBS-T containing 10% of FBS were added to wells and incubated for 3 h at room temperature. The well was washed three times and bound antibodies were detected using canine anti-IgG peroxidase conjugated (Sigma Co., USA) diluted 1/5000 after incubation, for 1 h at

Table 1

Performance of rK39 dipstick and ELISA using crude antigen in the diagnosis of canine visceral leishmaniasis

	CVL (n = 76) ^a	Controls (n = 33) ^b	Sensitivity (95% CI)	Specificity (95% CI)
rK39 dipstick				
Positive	63	00	83% (72.2–90.2)	100% (87.0–100)
Negative	13	33		
ELISA test				
Positive	72	00	95% (86.4–98.3)	100% (87.0–100)
Negative	04	33		

^a Dogs with parasitological proven infection.

^b Healthy dogs with both negative parasitological test and ELISA serology.

room temperature. Plates were then washed three times and incubated with *o*-phenylenediamine (OPD)–H₂O₂ in citrate buffer for 5 min. The reaction was stopped with 50 µl of 1 M H₂SO₄, and the optical density (OD) was read at 492 nm. Positive and negative controls sera were run in each plate to standardize the readings and plate variations. The cut-off point between negative and positive results was calculated as the mean of the negative controls plus 3 standard deviations.

2.4. Statistical analysis

The sensitivity and specificity for each test were calculated by using the formulas: sensitivity = True positive / (True positive + False negative) × 100% and specificity = True negative / (True negative + False positive) × 100%. In addition, the degree of agreement between the evaluated tests was determined by calculating κ value. κ Value express the agreement beyond chance; a κ value of 0.60–0.80 represents substantial agreement, whereas κ value of >0.80 represents almost perfect agreement beyond chance (Altman, 1991).

3. Results

We compared the sensitivity of the rK39 dipstick and ELISA using crude antigen in two different scenarios: in the first one the sera of all positives dogs were tested regardless their clinical forms of the disease. The sensitivity was 83% and 95% for rK39 and ELISA, respectively (Table 1). The specificity for both tests was 100% and the agreement between the tests was high, demonstrated by a κ value of 0.81.

In the second scenario the sera was stratified according to the clinical forms of the disease (asymptomatic, oligosymptomatic and symptomatic). In this condition the ELISA presented higher sensitivity in all groups of animals compared to the dipstick. In the asymptomatic group the dipstick was able to detect 12 out of 16 (75%), whereas ELISA detected 15 out of 16 (94%). In the oligosymptomatic group, 15 and 16 out of 17 animals were positive in the dipstick (88%) and ELISA (94%) test, respectively. For symptomatic dogs, the dipstick detected 36 out of 43 animals (84%) and the ELISA detected 41 of out 43 dogs (95%) (Table 2).

In order to evaluate the rK39 dipstick cross-reactivity, it was also analyzed 25 sera from dogs presenting different parasitic diseases

Table 2

Performance of rK39 dipstick and ELISA in the diagnosis of canine visceral leishmaniasis according to the clinical forms of the disease

	Number of positive dogs/total (%)		
	Asymptomatic	Oligosymptomatic	Symptomatic
rK39 dipstick	12/16 (75%)	15/17 (88%)	36/43 (84%)
ELISA test	15/16 (94%)	16/17 (94%)	41/43 (95%)

such as cutaneous leishmaniasis (5), ehrlichiosis (3), toxoplasmosis (5) and Chagas disease (12). The rapid test showed cross-reactivity with ehrlichiosis (1 out of 3 sera) and Chagas disease (3 out of 12 sera) altering the specificity from 100% to 93%.

4. Discussion

In the present study we evaluated the performance of dipstick rK39 for diagnosis of dogs presenting different clinical forms of VL. Our results showed that the rK39 was able to detect 83% of VL dogs regardless the clinical form of the disease. These findings were similar to those obtained by Reithinger et al. (2002) which demonstrated that the sensitivity of rK39 dipstick varied from 72% to 77% in the VL diagnosis in dogs. However, Da Costa et al. (2003) described a better performance of rK39 test showing a sensitivity of 96%.

Concerning the different clinical forms, the ELISA presented a higher sensitivity to detect VL dogs despite the presence of symptoms. On the other hand, the dipstick showed lower sensitivity for diagnosis of asymptomatic compared to oligo- and symptomatic dogs. Our results corroborate those reported by Mettler et al. (2005) that demonstrated low sensitivity of rK39 test in detecting asymptomatic dogs naturally infected by *L. (L.) infantum*.

Although the ELISA demonstrated a better performance compared to rK39 in the diagnosis of infected dogs, the technological expertise (i.e. training personnel) necessary to perform the dipstick tests and the requirement for specialized laboratory equipment are minimal compared to ELISA. Another advantage of dipstick tests is that the dog owners can see the results by themselves, which will contribute to a better working relationship between local communities and people carrying out the surveys. In addition, from the epidemiological point of view, a rapid test allows interventions to be implemented in real time. Furthermore, it can be used to confirm VL in dogs at veterinary clinics.

Finally, we believe that, even being less sensitive than the ELISA, the rK39 dipstick could be used as a screening tool by the Brazilian VL control program due to its simplicity and the possibility of eliminating infected dogs without any further delay.

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