Evaluation of enzyme-linked immunosorbent assay using crude *Leishmania* and recombinant antigens as a diagnostic marker for canine visceral leishmaniasis

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The performances of ELISA assays with different antigen preparations, such as Leishmania amazonensis *or* L. chagasi *lysates and the recombinant antigens rK-39 and rK-26, were compared using sera or eluates from dried blood collected on filter paper to detect anti-*Leishmania *antibodies in dogs from a visceral leishmaniasis-endemic area in Brazil. Of 115 IFAT-reactive dogs at 1:40 titre, 106 (92.2%) were positive in parasitological exams (skin and/or spleen). These animals were compared to healthy animals (n = 25), negative for IFAT at a titre of 1:40 and parasitological exams. The sensitivities of crude and recombinant antigens were similar and remarkably high for both sera and eluates (97-100%). Specificity was higher than 96% for sera and eluates for different antigens, except for* L. chagasi *antigen using eluates (88%). Concordance values among the tests were higher either for sera or eluates (J = 0.95-1.00). High concordances were observed between sera and eluates tested with different antigens (kappa = 0.93-0.97). Crude and recombinant antigens identified different clinical phases of canine leishmaniasis. These results show that eluates could be used in canine surveys to identify* L. chagasi *infection. Recombinant antigens added little when compared to crude antigen in identifying positive dogs. Cross-reactivity with other diseases whose distribution often overlaps VL-endemic areas is a limitation of crude antigen use however.*

Key words: canine visceral leishmaniasis - *Leishmania (Leishmania) chagasi* - diagnosis - recombinant antigens

Visceral leishmaniasis (VL) is a zoonosis caused by *Leishmania (Leishmania) chagasi* and transmitted by the phlebotomine sand fly *Lutzomyia* (*Lutzomyia*) *longipalpis* in Brazil. VL occurs in both rural and urban areas and has become a serious public health problem in several large Brazilian cities in recent decades (Arias et al. 1996). Domestic dogs play an important role in VL maintenance in man-made environments by serving as reservoirs of the parasite (Deane & Deane 1962). In Brazil, canine visceral leishmanisis (CVL) prevalence ranges from 1.9 to 35% in endemic areas (Evans et al. 1990, Nunes et al. 1991, França-Silva et al. 2002). The parasite can be isolated by means of tissue biopsies in 40-50% of dogs with positive immunofluorescence titres (Lanotte et al. 1979, Pozio et al. 1981) as well as asymptomatic animals (Marzochi et al. 1985). Consequently, asymptomatic and symptomatic infected dogs are both infective to the sand fly vectors (Molina et al. 1994).

Recommended VL control strategies in Brazil involve systematic treatment of human cases, elimination of seropositive dogs and residual spraying of houses and animal shelters with insecticides (Vieira & Coelho 1998). Due to the difficulty of direct detection of the parasite and the high proportion of asymptomatic dogs, serological methods are essential for CVL diagnosis. Although the immunofluorescent antibody test (IFAT) is the most widespread diagnostic method, cross-reactivity with other diseases and low sensitivity in detecting asymptomatic dogs are the main limitations of this technique. In addition, IFAT is not readily adaptable to large-scale seroepidemiological studies (Mancianti et al. 1995). The enzyme-linked immunosorbent assay (ELISA) has proved to be at least as sensitive and specific as IFAT and is suitable for such surveys (Evans et al. 1990, Paranhos-Silva et al. 1996, Braga et al. 1998). Cross-reactivity with other canine parasites and bacterial pathogens has also been reported for ELISA (Roffi et al. 1980, Mancianti et al. 1996). Crude antigen preparations made from whole promastigotes or their soluble extracts limit standardization of the tests and reproducibility of the results (Hommel et al. 1978, Mohammed et al.1986, Burns et al. 1993). Recombinant antigens have been developed and tested using ELISA to identify active subclinical and asymptomatic leishmaniasis (Badaró et al. 1996, Braz et al. 2002, Carvalho et al. 2003). These include the recombinant antigens rK-39 and rK-26 from *L. chagasi,* which have shown high sensitivity and specificity in CVL serodiagnosis (Burns et al. 1993, Bhatia et al. 1999, Scalone et al. 2002).

In Brazil, seroepidemiological canine surveys have been conducted using filter paper to collect blood for serological tests. The Brazilian Ministry of Health recently recommended the substitution of the serology using eluates from dried blood collected on filter paper with conventional serology using serum samples (Costa & Vieira 2001).

This is mainly because of the lower sensitivity of IFAT using eluates compared with sera in CVL diagnosis (Evans et al. 1990, Braga et al. 1998). However, some studies have shown similar results for IFAT using eluates and sera for CVL (Coutinho et al. 1985) and with ELISA for human VL (Gomes et al. 2001).

The aim of the present study was to compare ELISA using lysates of *L. amazonensis* and *L. chagasi* promastigotes and the recombinant antigens rK-39 and rK-26, performed with either sera and eluates to detect anti-*Leishmania* antibodies in dogs from a VL-endemic area of Minas Gerais, Brazil.

METHODS

Dogs and blood sample collection - In the present study ELISA performed on sera was compared with that using eluates from blood dried on filter paper from 140 dogs. These were made up of 115 dogs reactive for IFAT at a titer of 1:40, identified in a field survey conducted in Janaúba, MG and 25 healthy animals obtained from the kennels of the UFMG Parasitology Department, IFAT-negative at 1:40. In addition a further 101 serum samples were also tested, including 27 from negative healthy dogs kept in the Leishmaniasis Laboratory of the UFMG Parasitology Department and 74 from dogs infected with other pathogens (40 with *Trypanosoma cruzi*, 24 with *Babesia canis* and 10 with *Dirofilaria immitis*).

Blood samples were collected by venipuncture from the jugular radial vein. A drop of blood was spread immediately onto Klabin no. 125 absorbent filter paper (15×6 cm). The filter papers were dried at room temperature, placed in plastic bags with silica gel to control humidity, labeled and stored at –20°C. The remaining blood was centrifuged and stored in aliquots at –20°C.

Clinical and parasitological examinations - The dogs were clinically classified based on presence/absence of signs of infection as being asymptomatic, oligoasymptomatic and symptomatic, as described by Mancianti et al. (1988). Euthanasia of the 115 dogs from Janaúba reactive for *L. chagasi* was performed according to the standard operating practices of the National Health Foundation, Ministry of Health in the Diseases Control Centre of Montes Claros. Necropsies were performed and tissue fragments from the skin at the border of the ears and from the spleen were collected for parasitological examinations. Skin fragments from the ears were also collected from the 25 healthy dogs. The fragments were smeared onto slides, stained with Giemsa and examined under optical microscopy to identify *Leishmania* amastigotes.

Serological assessment - ELISA test was performed for the IgG assessment against whole parasite extracts and for r-K39 and r-K26. Promastigotes of *L. amazonensis* (MHOM/BR/1960/BH6) and *L. chagasi* (MHOM/BR/1070/BH46) were harvested from LIT (Liver Infusion Tryptose) cultures in stationary phase. The parasites were washed three times by centrifugation at 2000 rpm in phosphate buffer solution (PBS) at pH 7.2, for 10 min. This was followed by three cycles of 1 min at 40W in an ice bath in ultra-sound (Sonifer Cell Disruptor® – Branson Sonic Power Co, US). Sonicated culture material was then cen-trifuged at 18,500 rpm for 90 min at 4°C. The supernatant was transferred to dialysis tubes and dialyzed through PBS for 36 h, involving four PBS changes every 6 h. Finally, the remaining material was filtered in disposable sterile 22 µm filters under aseptic conditions and a single aliquot removed for calculation of protein dosage, after being adjusted to a concentration of 1 mg/ml and stored at –70°C until use.

The antigens rK-39 and rK-26 were prepared according to the procedures described by Burns et al. (1993) and Bhatia et al. (1999) and provided by Heska Corporation, US.

A canine anti-IgG peroxidase conjugate (Sigma Co., US) was used for the ELISA tests. Reactions were read at 492 nm. The cut-off value was established as the mean absorbancy value +2 SD from 52 known negative sera as well as 25 eluates from blood dried on filter paper of dogs whose sera was negative at a titre of 1:80. In order to perform the reactions, sera and filter paper were thawed and 5 µm diameter spots eluted in casein-PBS for testing by ELISA.

Test reproducibility - Twenty to 30% of the samples of sera and filter paper were randomly selected in order to evaluate test reproducibility. Each duplicate received a different number from the original sample and the assays were performed blind.

Statistical Analysis - Sensitivity and specificity for ELISA performed with different antigens were compared with parasitological examinations (gold standard) using a 2×2 table. The agreements among these comparisons were expressed by Youlden index (Szklo & Nieto 2000). Comparisons among the reactions performed with sera and eluates for each antigen and the reliability for the duplicates were evaluated by Kappa statistics (Szklo & Nieto 2000). Absorbance values between sera and eluates for each antigen were compared by Pearson correlation. A one-way ANOVA test was used to compare the mean absorbancy values of sera and eluates for different clinical forms.

RESULTS

Sensitivity and specificity - Of 115 IFAT-reactive dogs from Janaúba, 106 (92.2%) were positive in parasitological exams (skin and/or spleen) and considered to be truly positive. The 25 healthy dogs that gave negative results in parasitological exams were considered to be truly negative. A total of 131 dogs was included in sensitivity and specificity analyses.

Sensitivity and specificity of the tests are presented in Table I, together with Youlden index values. The results showed high sensitivity and specificity for ELISA performed with sera and eluates for the different antigens assayed. ELISA performed with eluates and rK39 showed the lowest sensitivity (97.2%) while that performed with eluate and *L. chagasi* antigen presented the lowest specificity (88%). The results showed good agreement, kappa values ranging from 0.88-1.0. The lowest concordance was observed for *L. chagasi* antigen in ELISA performed with eluate.

TABLE I

Sensitivity, specificity and Youlden coefficient (J) of ELISA performed with crude antigens and the recombinant antigens rK-39 and rK-26 in sera and eluates in diagnosis of canine visceral leishmaniasis

Antigens	N = 131	Sensitivity % (95% CI)	Specificity % (IC 95%)	Youlden (95% CI)
L. amazonensis	Sera	100 (95.6-100.0)	100 (83.4-100.0)	1.00 (1.00-1.00)
L. amazonensis	Eluate	100 (95.5-100.0)	96 (77.7-99.8)	0.96 (0.88-1.00)
L. chagasi	Sera	98 (92.7-99.7)	100 (83.4-100.0)	0.98 (0.95-1.00)
L. chagasi	Eluate	100 (95.6-100.0)	88 (67.7-96.8)	0.88 (0.75-1.00)
rK-39	Sera	98.1 (92.7-99.7)	100 (83.4-100.0)	0,98 (0.95-1.00)
rK-39	Eluate	97.2 (91.3-99.3)	100 (83.4-100.0)	0,97 (0.94-1.00)
rK-26	Sera	99.1 (94.1-100.0)	96 (77.7-99.8)	0.95 (0.87-1.00)
rK-26	Eluate	100 (95.6-100.0)	96 (77.7-99.8)	0.96 (0.88-1.00)

The results of sensitivity, specificity and Youlden coefficients were estimated with a 95% confidence interval; *L*: *Leishmania*

Cross-reactivity analyses - Cross-reactivity was analyzed for 74 sera of different parasitic diseases. The ELISA performed with *L. amazonensis* antigen showed cross-reactivity with *T. cruzi* (25/40) and *D. immitis* (6/10). ELISA performed with *L. chagasi* were reactive with sera of *T. cruzi* (34/40), *D. immitis* (4/10) and *B. canis* (1/24). The recombinant antigens rK-39 and rK-26 did not show any cross-reactivity with other parasitic diseases.

Comparison among serological results and clinical form - Clinical features allowed the 115 animals to be separated into three groups. The first one was composed of 48 (41.7%) asymptomatic dogs. The second consisted of 46 (40%) oligosymptomatic dogs, each with a maximum of three clinical signs (opaque bristles, localized alopecia, and moderate weight loss). The third group was composed of 21 (18.3%) symptomatic dogs with characteristic clinical signs of VL, such as opaque bristles, severe weight loss, onychogriphosis, cutaneous lesions, apathy, and keratoconjunctivitis.

Comparisons among the mean absorbancy values of sera and eluates for different clinical forms were performed by ANOVA. The differences between the mean absorbancy values of the three clinical phases for the four antigens assayed were not statistically significant (p > 0.05).

The ELISA values for the different antigen preparations for sera and eluate applied to detect the clinical phases are shown in Table II. Crude antigens of *L. amazonensis* and *L. chagasi* detected the majority of the infected dogs, irrespective of the clinical phase, for both sera and eluate samples. The recombinant antigens were more sensitive in detecting symptomatic dogs, independent whether sera or eluate samples were used. However, no statistically significant difference was observed between the two types of antigen in this respect (p > 0.05).

Comparisons between sera and eluates - These comparisons were performed using 140 dogs, of which 115 were IFAT-positive and 25 healthy. Among the 115 IFAT-positive dogs the ELISA test performed with different antigens in sera identified 114 dogs (91.2%) with *L. amazonensis*, 112 (89.6%) with *L. chagasi*, 110 (88.0%) for rK-39 and 113 (90.4%) for rK-26. Concordances between sera and eluates for different antigens are shown in Table III: *L. amazonensis* (Kappa = 0.95), *L. chagasi* (Kappa = 0.85), rK-39 (Kappa = 0.97), and rK-26 (Kappa = 0.93).

Correlations for absorbancy values between sera and eluate using each antigen were estimated by Pearson correlation. The absorbance results were transformed to log absorbance + 1. The Pearson correlations for the different

TABLE II

Comparison between clinical phases and ELISA performed with crude antigens of *Leishmania amazonensis* and *L. chagasi* and the recombinant antigens rK-39 and rK-26 in sera and eluates

	ELISA test							
	L. amazonensis		*L. chagasi*		rK-39		rK-26	
Clinical phases	Sero (%)	Eluate (%)	Sero (%)	Eluate (%)	Sero (%)	Eluate (%)	Sero (%)	Eluate (%)
Asymptomatic (n = 48)	100	100	98	100	93.7	93.7	98	100
Oligoasymtomatic (n = 46)	97.8	100	97.8	100	95.6	95.6	97.8	95.6
Symptomatic (n = 21)	100	100	95.2	100	100	95.2	100	100

Comparisons between sera and eluates from different antigens were made using c^2; no statistical difference was observed between them.

antigens were: *L. amazonensis* (r = 0.76) and *L. chagasi* (r = 0.25). The recombinant antigens produced strong correlations between sera and eluate for rK-39 (r = 0.88) and rK-26 (r = 0.63).

Test reproducibility - To evaluate the reliability of the tests, 55 (33%) duplicate samples in sera and 28 (20%) duplicate samples in eluates for each antigen were analyzed (Table IV). The agreement varied from 0.74-1.0 for the different antigens. The lowest agreement was observed with ELISA performed with *L. chagasi* in sera (Kappa = 0.74) and eluates (Kappa = 0.63). Highest agreement was detected for r-K39 antigens in both sera (Kappa = 0.81) and eluates (Kappa = 1.0).

DISCUSSION

Among the 115 dogs IFAT-positive at titers of 1:40, 106 (92.2%) were positive in parasitological exams and were considered to be truly positive, while 25 IFAT-negative, clinically healthy animals were considered to be truly negative. Sensitivities were similar between crude and recombinant antigens and remarkably high for both sera and eluates (97-100%). Specificity of sera and eluates exceeded 96% for all antigens except *L. chagasi* in eluate, for which the value was 88%. Concordances between the tests were higher either for sera or eluates (J = 0.95- 1.00) except for *L. chagasi* antigen in eluates (J = 0.88).

Similar sensitivity and specificity values in ELISA tests for CVL diagnosis were found by Mancianti et al. (1995), who reported higher sensitivity (99.5%) and specificity (97.1%) with *L. infantum* antigen. However, Ashford et al.

(1993) found sensitivity to be lower than 88% when they used *L. chagasi* antigen in FAST-ELISA performed with sera.

Sensitivity and specificity of ELISA is greatly influenced by the antigen used to perform the tests (Singh & Sivakumar 2003). Although the *L. chagasi* antigen is species-specific, in the present study its performance was slightly inferior to *L. amazonensis* in ELISA carried out with eluates. Crude antigens are traditionally derived from promastigotes cultivated in vitro and consist of a repertory of somatic antigens and several surface components that vary according to parasite species. The different species and strains that have been used in crude antigens may therefore limit standardization of tests and reproducibility of results (Hommel et al. 1978, Mohammed et al. 1986, Burns et al. 1993).

TABLE IV

Reproducibility of ELISA performed with crude and recombinants antigens rK-39 and rK-26 in sera and eluates to diagnose canine visceral leishmaniasis

Antigens	Serum duplicates Kappa (95% CI.)	Eluate duplicates Kappa (95% CI)
L. amazonensis	0.76 (0,6-0.95)	0.84 (0.53-1.00)
L. chagasi	0.74 (0.54-0.95)	0.63 (0.15-1.00)
rK-39	0.81 (0.63-0,99)	1.00 (1.00-1.00)
rK-26	0.74 (0.54-0.95)	0.84 (0.53-1.00)

CI: confidence intervals; *L.*: *Leishmania*

TABLE III

Comparison between sera and eluates used in ELISA with crude and the recombinant antigens rK-39 and rK-26 to diagnose canine visceral leishmaniasis

Sera - *L. amazonensis*					Kappa (95% CI)
		Positive (%)	Negative (%)	Total	
Eluate	Positive	114 (100)	2 (7.7)	116	0.95 (0.88-1.00)
L. amazonensis	Negative	0 (0)	24 (92.3)	24	
Total		114	26	140	
Sera - *L. chagasi*					Kappa (95% CI)
		Positive	Negative (%)	Total	
Eluate	Positive	112 (100)	6 (21.4)	118	0.85 (0.74-0.97)
L. chagasi	Negative	0 (0)	22 (78.6)	22	
Total		112	28	140	
Sera - rK-39					Kappa (95% CI)
		Positive (%)	Negative (%)	Total	
Eluate	Positive	109 (99.1)	0 (0)	109	0.97 (0.94-1.00)
rK-39	Negative	1 (0.9)	30 (100)	31	
Total		110	30	140	
Sera - rK-26					Kappa (95% CI)
		Positive (%)	Negative (%)	Total	
Eluate	Positive	112 (99.1)	2 (7.4)	114	0.93 (0.85-1.00)
rK-26	Negative	1 (0.9)	25 (92.6)	26	
Total		113	27	140	

CI: confidence intervals; *L.*: *Leishmania*

Several purified and recombinants have recently been developed and tested, of which rK-39 antigen (Badaró et al. 1996) is highly sensitive and predictive of the onset of disease manifestation in human VL patients. Badaró et al. (1996) and Scalone et al. (2002) found high sensitivity (98%) and specificity (99%) for CVL. However, there is some dispute about the predictive onset of the disease in CVL. Scalone et al. (2002), showed that rK-39 antigen did not predict the onset of disease manifestation while Rhalem et al. (1999) found that asymptomatic dogs were negative for rk-39 antigen. Scalone et al. (2002), considered the efficacy of rK-39 in detecting asymptomatic cases to be markedly different between CVL and human VL.

In our study, crude and recombinant antigens detected the anti-leishmanial antibodies of most asymptomatic and oligoasymptomatic dogs when ELISA was performed with sera. For asymptomatic dogs the highest success rate was observed for *L. amazonensis* antigen (100%), followed by *L. chagasi* and rK-26 antigens (both 98%). The lowest proportion was observed for rK-39 in eluate (93.7%). With respect to oligoasymptomatic dogs the proportions were 97.8% for *L. amazonensis* and *L. chagasi* and 95.6% for rK-26 and rK-39. The results obtained in ELISA with eluates for different antigens were similar to those observed in tests performed in sera. The proportion detected by rK-39 was similar to that found by Scalone et al. (2002) when they assayed asymptomatic dogs (92.4%).

Although both crude antigens (*L. amazonensis* and *L. chagasi*) showed high sensitivity, they presented cross-reactivity with other microorganisms as *T. cruzi, D. reconditum,* and *B. canis.* There are two primary drawbacks to tests performed with crude antigens, i.e., cross-reactivity and the inability to discriminate between past and current infections. The recombinant antigens rK-39 and rK-26 performed similarly whether in sera or eluate. They showed high sensitivity but no cross-reactivity with other microorganisms. High specificities of recombinant antigens have also been reported by other authors (Badaró et al. 1996, Scalone et al. 2002).

High concordances were observed between sera and eluates performed with different antigens (Kappa = 0.93-0.97). The lowest concordance was observed in *L. chagasi* antigen (Kappa = 0.88). According to Guimarães (1983), errors in conducting tests with filter papers could occur for two possible reasonss : difficulties in precisely estimating eluate dilutions and denaturing of antibodies during filter paper storage at high environmental temperature and relative humidity. The good concordances observed in our study with filter paper are most likely: (1) homogeneity of the blood spread on absorbent filter paper; (2) storage at –20°C and use of silica gel to control humidity; (3) elution of filter papers in casein-PBS on the same day that the tests were carried out.

The evaluation of test reproducibility revealed a good and regular agreement for ELISA in the duplication of eluates (Kappa = 0.63-1.00). Eluates of blood dried on filter paper are commonly used in laboratory tests and the validity of this technique has been confirmed for the diagnosis of leishmaniasis (Coutinho et al. 1985, Gomes et al. 2001) and for other infections such as toxoplasmosis and Chagas disease (Chiari et al. 1987, Machado-Coelho et al. 1995).

It is also important to note improvements in the leishmaniasis control program during recent decades in Brazil. The number of dog samples collected increased from 526 to 1,353,812 between 1980 and 1996 (Vieira & Coelho 1998). Collecting blood on filter paper makes collection, transport and storage of samples easier for local health services (Guimarães 1983).

The use of only one antigen to perform both condition screening and confirmatory tests could increase the proportion of false-positive results, particularly in cases of low prevalence (Gart & Buck 1966, Costa & Vieira 2001). One important point that merits consideration is the need to standardize antigens with respect to source and purity in control programs, in order to improve the reproducibility of results in different regions of Brazil.

In summary, the results of this investigation showed that ELISA using crude and recombinant antigens gave results to similar degrees of sensitivity and specificity in identifying different clinical phases of CVL. High concordance among sera and eluates confirm that filter paper could be used to collect blood in canine surveys to identify *L. chagasi* infection. Use of recombinant antigens did not markedly improve results compared with use of crude antigen in dogs identified as being positive. However, the main limitation of crude antigens is their cross-reactivity with other diseases that often occur in VL-endemic areas. The association of crude and recombinant antigens is a strategy that could be used in control programs. This could involve an initial screening using ELISA with crude antigen in eluate from blood collected on filter paper, followed by a confirmatory test using a recombinant antigen also in eluate. However recombinant antigens would need to be widely available. Recombinant antigens could also improve differential diagnosis in clinical veterinary medicine.

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