

# Evaluation of parasitological examination, kDNA polymerase chain reaction and rK39-based immunochromatography for the diagnosis of visceral leishmaniasis in seropositive dogs from the screening-culling program in Brazil

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## ABSTRACT

**Introduction:** Dogs play a primary role in the zoonotic cycle of visceral leishmaniasis (VL). Therefore, the accurate diagnosis of infected dogs, primarily asymptomatic dogs, is crucial to the efficiency of VL control programs. **Methods:** We investigated the agreement of four diagnostic tests for canine visceral leishmaniasis (CVL): parasite detection, either after myeloculture or by direct microscopic examination of tissue imprints; kinetoplast-deoxyribonucleic acid-polymerase chain reaction (kDNA-PCR); and an immunochromatographic test (ICT). An enzyme-linked immunosorbent assay (ELISA) and an indirect immunofluorescence test (IFAT), both of which were adopted as part of the screening-culling program in Brazil, were used as reference tests. Our sample set consisted of 44 seropositive dogs, 25 of which were clinically asymptomatic and 19 were symptomatic for CVL according to ELISA-IFAT. **Results:** The highest and lowest test co-positivities were observed for ICT (77.3%) and myeloculture (58.1%), respectively. When analyzed together, the overall percentage of co-positive tests was significantly higher for the symptomatic group compared to the asymptomatic group. However, only ICT was significantly different based on the results of a separate analysis per test for each group of dogs. The majority (93.8%) of animals exhibited at least one positive test result, with an average of 2.66 positive tests per dog. Half of the symptomatic dogs tested positive for all four tests administered. **Conclusions:** The variability between test results reinforces the need for more efficient and reliable methods to accurately diagnose canine VL, particularly in asymptomatic animals.

**Keywords:** Visceral leishmaniasis. Canine visceral leishmaniasis. Diagnosis. *Leishmania*.

## INTRODUCTION

Visceral leishmaniasis (VL) is a chronic parasitic disease caused by the *Leishmania donovani* complex in East Africa and the Indian subcontinent and by *Leishmania infantum* (syn. *Leishmania chagasi*) in Europe, North Africa and Latin America. VL is fatal if left untreated and is transmitted in one of two ways. Zoonotic VL is transmitted from animal to vector to human, and anthroponotic VL is transmitted from human to vector to human. In the former, humans are occasional hosts and dogs are the main reservoir for *Leishmania*, particularly in rural and urban domestic environments<sup>1</sup>.

Canine visceral leishmaniasis (CVL) is an important public health problem and is a disease of great interest to private veterinary medicine due to its high prevalence in urban areas of Brazil. Infected dogs may present intense cutaneous parasitism<sup>2,3</sup> and play a primary role in the maintenance of vector infection<sup>4</sup>. Along with measures to control the vector population and early treatment of human cases, euthanasia of infected dogs is part of the VL control policy adopted by the Brazilian Ministry of Health<sup>5</sup>.

Early and accurate diagnosis of CVL is of major importance to the effectiveness of control programs. Although several techniques have been proposed to serologically diagnose CVL, a fast and safe diagnostic method with optimal sensitivity and specificity is not currently available<sup>6</sup>. Until very recently, standard serology in canine surveys of the government VL control program used enzyme-linked immunosorbent assay (ELISA) as a screening method followed by an indirect immunofluorescence test (IFAT) to confirm ELISA-positive samples. Currently, this methodology is being replaced by immunochromatographic screening using the Dual Path Platform (DPP® CVL, *Biomanguinhos*, Rio de Janeiro, Brazil) followed by ELISA confirmation.

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The main limitation of most diagnostic methods is the detection of asymptomatic but infected dogs. In this context, the present study evaluated the results of four different diagnostic tests for CVL in symptomatic and asymptomatic seropositive dogs from control program surveys in endemic areas of Brazil. Two tests are based on *Leishmania* visualization, either after culturing or by direct microscopic examination; one test, kinetoplast-deoxyribonucleic acid-polymerase chain reaction, kDNA-PCR, is a molecular biology-based method, and one test is antibody-based but uses a recombinant protein rather than natural *Leishmania* antigens.

## METHODS

### Clinical samples

Forty-four dogs that tested seropositive for CVL according to the standard methodology adopted by the Brazilian Ministry of Health (ELISA and IFAT kits, both produced by the *Biomanguinhos* Institute, Rio de Janeiro, Brazil) were used in this study. These animals were collected by Zoonosis Control Centers located in three endemic cities for VL in the Brazilian State of Minas Gerais, Montes Claros (16°44'02"S, 43°51'23"W), Janaúba (15°47'50"S, 43°18'31"W) and Paracatu (17°13'01"S, 46°52'17"W), all of which are classified as intense transmission areas for the disease (Brazil 2006). The dogs were examined by veterinary physicians and classified as asymptomatic or symptomatic according to the absence or presence of at least one clinical sign suggestive of canine VL. Serum samples were collected, and bone marrow aspirates were harvested by sterilely puncturing the tibial crest for the preparation of slide smears and parasite cultures. Ear skin, spleen and mesenteric lymph nodes were biopsied, and the tissue fragments were used to prepare slide imprints and to extract total deoxyribonucleic acid (DNA). Euthanasia followed the technical norms defined by Resolution no 714 from the Federal Council of Veterinary Medicine (dated 06/02/2002) and by the screening-culling procedure adopted by the Brazilian Ministry of Health in its Program for Visceral Leishmaniasis Control.

### Parasite culture

Novy-MacNeal-Nicolle/liver infusion tryptose (NNN/LIT) culture medium was inoculated with bone marrow aspirates and incubated at 25°C. The cultures were examined weekly for the presence of *Leishmania* promastigotes. After the culture was expanded to approximately 100 million cells, the cells were washed with phosphate-buffered saline (PBS), and positive samples were frozen at -20°C until use. Negative samples were discarded after five weeks of monitoring.

### Polymerase chain reaction

Total DNA was organically extracted<sup>7</sup> from *Leishmania* cultures and biopsied tissues. DNA amplification was performed with A [5'(C/G)(C/G)(G/C)CC(C/A)CTAT(T/A)TTACACCAACCCC3'] and B (5'GGGGAGGGCGTTCTGCGAA3') primers previously designed to amplify a 120bp segment from the conserved region of the kinetoplast DNA minicircle (kDNA) from the

*Leishmania* genus<sup>8</sup>. The amplified products were visualized by electrophoresis on a 6% polyacrylamide gel after silver staining. The presence of a 120bp band from any of the amplified tissue samples (ear skin, spleen or mesenteric lymph nodes) was considered a positive result. The quality of DNA extraction was verified by amplifying the IV S6 region from a constitutive gene (cacophony) from *Lutzomyia*<sup>9</sup>.

### Immunochromatographic test

Canine sera were tested using the Kalazar Detect™ test (InBios International Inc., Seattle, Washington, USA), which is a rapid immunochromatographic test (ICT) that qualitatively detects antibodies specific for a recombinant *Leishmania* antigen, rK39, formed by repeats of 39 amino acids. This peptide is a portion of a 230kDa antigen homologous to the kinesin superfamily of motor proteins and encodes a repetitive, highly conserved epitope in *L. infantum* and *L. donovani*<sup>10</sup>.

### Direct parasitological examination

After Giemsa or panoptic staining, tissue imprints and bone marrow smears were examined for the presence of *Leishmania* amastigotes using optical microscopy. The observation of parasites in the imprints from any biopsied tissue (ear skin, spleen or mesenteric lymph nodes) was considered a positive test result.

### Statistical analysis

The standard serological methodology for canine visceral leishmaniasis (ELISA-IFAT) was used as a reference, and the performance of the tests evaluated in this study was calculated compared to this standard method. Because all of the dogs were positive according to the standard serological method, diagnostic test performance was measured based on precision or accuracy and expressed as the total number of correct classifications. In this case, the test *precision* is equivalent to *sensitivity*. In addition, given that the reference did not identify certainty of the absence of the disease but instead a serological result, the term *sensitivity* was replaced with *co-positivity* (which is equivalent to *relative sensitivity*).

The co-positivity of the four tests as well as the respective positivity percentages for the two clinical groups of dogs - symptomatic and asymptomatic - were compared using the chi-square test<sup>11</sup>. When necessary, Fisher's exact test<sup>11</sup> was employed. The odds ratios and the 95% confidence intervals were calculated and adjusted for small samples<sup>12</sup> when applicable.

The Mann-Whitney test<sup>13</sup> was employed to compare the number of positive tests among the four tests, in the two clinical groups of dogs (symptomatic and asymptomatic). Agreement between the results of the four tests was verified by calculating the Kappa coefficient<sup>14</sup>.

All statistical analyses were performed with R software (version 3.0.1) with a 5% significance level.

### Ethical considerations

All of the procedures involving dogs followed the ethical conditions established by the Ethics Committee on Animal Use (CEUA/Fundação Oswaldo Cruz under license no. L013/09),

the Ethics Committee on Animal Experimentation (CETEA/ Universidade Federal de Minas Gerais under license no. 35/2007) and the Federal Board of Veterinary Medicine (CFMV, Resolution no. 714/2002).

## RESULTS

Forty-four dogs seropositive for CVL by ELISA-IFAT were assayed using direct parasitological examination, myeloculture, ICT and kDNA-PCR, and the degree of co-positive tests were evaluated. The highest and lowest co-positivities were obtained for ICT (77.3%) and myeloculture (58.1%), respectively (Table 1). However, differences in co-positivity were not statistically significant for any of the tests ( $p$ -value = 0.237). kDNA-PCR showed variable co-positivity percentages based on the tissue used for DNA extraction: 27.3% for the mesenteric lymph nodes, 36.4% for the spleen and 50% for the skin (data not shown). These differences in percentages were not analyzed because our positivity criterion was the presence of a positive result in any of the three tissues tested. The DNA extracted from the *Leishmania* biomass served as an internal control for the extraction process, with positive results in 100% of the samples (data not shown).

Of the 44 dogs in our sample, 25 were clinically symptomatic and 19 were asymptomatic. Table 2 depicts the test results for each clinical condition. Although we observed a tendency towards a higher percentage of co-positivity in the symptomatic group compared to the asymptomatic group, the differences for each test were not significantly different except for ICT. This particular assay exhibited 100% co-positivity for symptomatic dogs compared to 60% in asymptomatic dogs, a statistically significant difference (Figure 1). According to the odds ratio, the

chance of a positive ICT result in the asymptomatic group was 11.88 [1.43; 487.0] times greater than in the symptomatic group.

The majority (93.8%) of dogs exhibited at least one positive CVL result for the diagnostic tests used in this study, with an average of 2.66 positive test results per dog (Table 3). When the clinical group of dogs was analyzed separately, the average number of positive tests decreased to 2.28 in the asymptomatic group and increased to 3.16 in the symptomatic group (Table 3). Further analysis calculating the median value per clinical group indicated that at least 50% of the asymptomatic animals tested positive for two of the diagnostic tests for CVL. In the symptomatic group, half of the animals showed positive results for all four tests. Comparing the canine clinical groups, the total number of positive tests was significantly different ( $p$ -value = 0.041) (Figure 2). Three of the asymptomatic dogs were not co-positive for any of the diagnostic tests (data not shown).

According to the calculated  $\kappa$  value, there was little agreement between the results from the four diagnostic tests for CVL ( $\kappa$  = 0.348). However, the corresponding  $p$ -value ( $p$ -value = 0.000) indicates that the tests exhibited significant agreement.

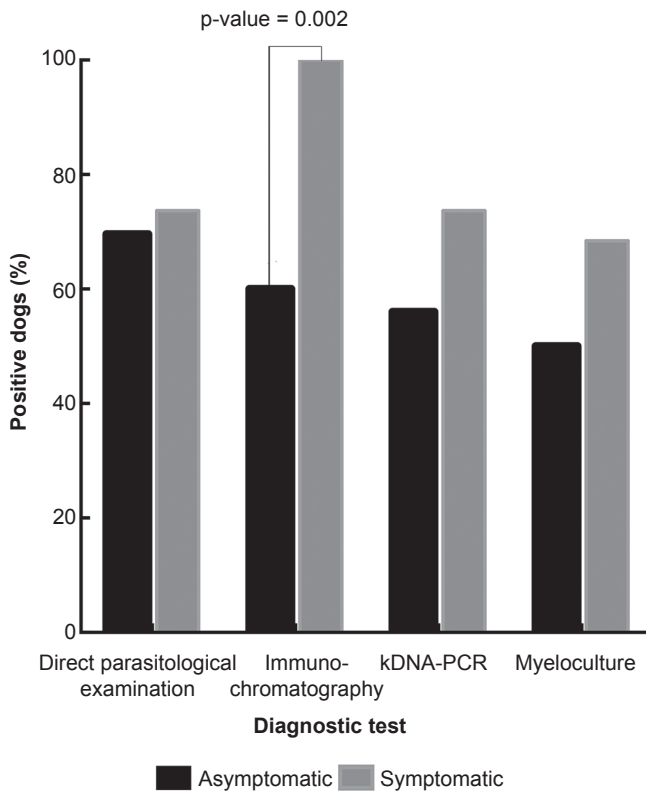
## DISCUSSION

In Brazil, despite efforts from the Ministry of Health to control CVL vectors and reservoirs, VL is undergoing rapid territorial expansion<sup>15</sup>. The recent adoption of DPP® CVL as the screening step for canine surveys is advantageous because the delay between diagnosis and culling/treatment is decreased. Currently, the long duration between sample collection and culling, as well as the complexity of performing the procedure, have been suggested as reasons for its low efficacy<sup>4,16,17</sup>.

**TABLE 1 - Contingency table of the co-positivities (relative sensitivity) of diagnostic tests for canine visceral leishmaniasis. The reference test was ELISA-IFAT, which was adopted by the Brazilian Ministry of Health in the screening-culling procedure for the Surveillance and Control Program of Visceral Leishmaniasis at the time of our study. Forty-four seropositive dogs (n=44) were sampled from endemic areas of visceral leishmaniasis in the State of Minas Gerais, Brazil.**

Diagnostic test	Result	ELISA-IFAT			
		negative		positive	
		n	%	n	%
Direct parasitological examination (n=42*)	negative	-	-	12	28.6
	positive	-	-	30	71.4
Immunochromatography (n=44)	negative	-	-	10	22.7
	positive	-	-	34	77.3
kDNA-PCR (n=44)	negative	-	-	16	36.4
	positive	-	-	28	63.6
Myeloculture (n=43*)	negative	-	-	18	41.9
	positive	-	-	25	58.1

**ELISA-IFAT:** enzyme-linked immunosorbent assay-indirect immunofluorescence test; **kDNA-PCR:** kinetoplast-deoxyribonucleic acid-polymerase chain reaction. \*n<44 due to sample loss.



**FIGURE 1 - Co-positivity of diagnostic tests for canine visceral leishmaniasis for each clinical group of dogs. The reference test was ELISA-IFAT, which was adopted by the Brazilian Ministry of Health in the screening-culling procedure for the Surveillance and Control Program of Visceral Leishmaniasis at the time of our study. The animals were sampled from endemic areas of visceral leishmaniasis in the State of Minas Gerais, Brazil. Statistically significant differences are indicated with a p-value. kDNA-PCR: kinetoplast-deoxyribonucleic acid-polymerase chain reaction; ELISA-IFAT: enzyme-linked immunosorbent assay-indirect immunofluorescence test.**

When diagnosing human patients with the goal of detecting the clinical disease, other rapid tests have proven to be sensitive and specific<sup>18-21</sup>. However, when the goal is detecting canine infection for control purposes, these tests demonstrated low sensitivity and were useful only for the confirmation of infection in suspicious clinical cases<sup>22-27</sup>. Therefore, it follows that ICT identified 100% of the symptomatic dogs in our hands but only identified 60% in the asymptomatic group.

PCR is considered highly sensitive and specific, can be automated, and is applicable to different types of biological samples<sup>28-33</sup>. As previously noted<sup>4</sup>, in contrast to antibody-based assays, antigen-based methods such as PCR might become more relevant indicators of infection in the future, as these tests can still be used in vaccinated dogs that will test as antibody-positive. In our hands, kDNA PCR identified only 64% of the samples as positive. Two reasons could justify this unexpectedly low percentage: the methodology we employed for DNA extraction and/or a limitation of the technique itself. In the first case, residue of PCR inhibitors from the organic DNA extraction could lead to false negative results. However, this is unlikely because the internal control (DNA extracted from the *Leishmania* biomass) was assayed with the same methodology and tested 100% positive. Concerning the technique itself<sup>34</sup>, a previous study reported a decrease in PCR sensitivity over the course of CVL infection, from 78-88% at the 135th day post-infection to approximately 50% after 300 days. Thus, the sensitivity of PCR for CVL diagnosis might depend on the sampling time during infection. For our samples, this time point is unknown.

The tendency towards higher co-positivity percentages in symptomatic dogs compared with asymptomatic dogs, which was consistently observed in this study, agrees with the literature for several diagnostic tests<sup>2,3,34-39</sup>. Depending on the assay, this may be related to higher antibody titers or to higher parasite loads in clinically ill dogs<sup>2</sup>. However, a statistically significant

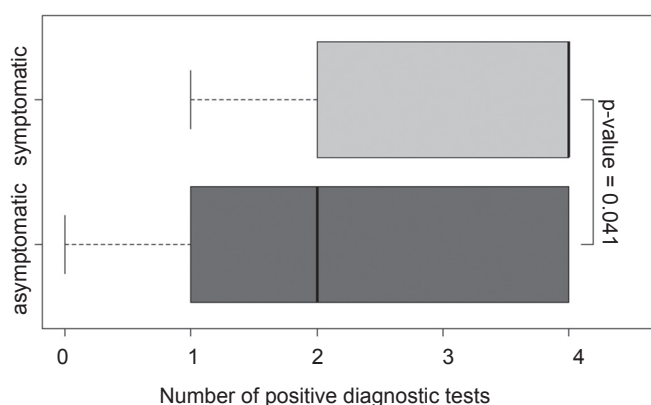
**TABLE 2 - Contingency table of the co-positivities (relative sensitivity) of diagnostic tests for canine visceral leishmaniasis for each clinical group. The reference test was ELISA-IFAT, which was adopted by the Brazilian Ministry of Health in the screening-culling procedure for the Surveillance and Control Program of Visceral Leishmaniasis at the time of our study. The dogs were sampled from endemic areas of visceral leishmaniasis in the State of Minas Gerais, Brazil.**

Diagnostic test		Asymptomatic		Symptomatic		p-value	Odds ratio	Confidence interval (95%)
		n	%	n	%			
Direct parasitological examination (n=42)	Negative	7	30.4	5	26.3	0.769 <sup>a</sup>	1	
	Positive	16	69.6	14	73.7		1.23	[0.31; 4.74]
Immunochromatography (n=44)	Negative	10	40.0	0	0.0	0.002 <sup>b</sup>	1	
	Positive	15	60.0	19	100.0		11.88	[1.43; 487.1]
kDNA-PCR (n=44)	Negative	11	44.0	5	26.3	0.227 <sup>a</sup>	1	
	Positive	14	56.0	14	73.7		2.2	[0.61; 7.99]
Myeloculture (n=43)	Negative	12	50.0	6	31.6	0.224 <sup>a</sup>	1	
	Positive	12	50.0	13	68.4		2.17	[0.62; 7.60]

**ELISA-IFAT:** enzyme-linked immunosorbent assay-indirect immunofluorescence test; **kDNA-PCR:** kinetoplast-deoxyribonucleic acid-polymerase chain reaction; <sup>a</sup>Chi-square test; <sup>b</sup>Fisher's exact test.

**TABLE 3 - Number of positive diagnostic tests for canine visceral leishmaniasis for each clinical group. The dogs were sampled from endemic areas of visceral leishmaniasis in the State of Minas Gerais, Brazil.**

Clinical group	Number of dogs	mean ± standard deviation	Mann-Whitney test			p-value
			Quartiles			
			1 <sup>st</sup>	2 <sup>nd</sup>	3 <sup>rd</sup>	
Asymptomatic	25	2.28 ± 0.29	1	2	4	0.041
Symptomatic	19	3.16 ± 0.26	2	4	4	
Total	44	2.66 ± 0.21	1.5	3	4	

**FIGURE 2 - Distribution of positive results for the entire set of diagnostic tests for canine visceral leishmaniasis for each clinical group. All of the dogs were sampled from endemic areas of visceral leishmaniasis in the State of Minas Gerais, Brazil, and were seropositive based on ELISA-IFAT. ELISA-IFAT: enzyme-linked immunosorbent assay-indirect immunofluorescence test.**

difference in test co-positivities between the two clinical groups of dogs was only observed for ICT. Unfortunately, a final conclusion determining the agreement of the results from the four tests employed in this study was not possible. The  $\kappa$  value and the p-value suggest different conclusions, possibly due to our sample size, which precluded a more thorough analysis.

Despite testing positive serologically, three dogs from the asymptomatic group, including a three-to-four-month-old puppy, tested negatively for all of the administered tests. Possible explanations include false seropositivity<sup>40</sup>, cross-reactivity with other diseases<sup>41</sup> or the presence of a pre-patent infection<sup>42,43</sup>. In the case of the puppy, the positive serology might also be attributable to the presence of maternal antibodies<sup>44</sup>.

Published estimates for antibody-based immunofluorescence assays range from 72 to 100% sensitivity and 52 to 100% specificity<sup>4</sup>. One reason for the limited specificity of antibody-based tests is that they are generally manufactured with antigens from *Leishmania major*<sup>45-47</sup> instead of *Leishmania infantum* because the latter is difficult to culture and mass produce in artificial media<sup>47,48</sup>. Differences between these *Leishmania*

species likely compromise the final test results. In our hands, even parasitological diagnosis, which is considered the gold standard for CVL diagnosis, did not reach 100% co-positivity, even in the symptomatic group.

Ideally, a test for CVL diagnosis should detect asymptomatic infection, have high sensitivity, specificity and reproducibility, and be simple, easy to perform, inexpensive, and viable to use in regional laboratories or adaptable to field conditions. This test should detect all dogs infected with *Leishmania*, and use samples that can be collected non-invasively<sup>43</sup>. Despite the technological advances made in the development of CVL diagnostic tests, the primary limitation for most tests is the diagnosis of asymptomatic dogs. A previous study<sup>16</sup> suggests that the effectiveness of control programs could be higher if euthanasia was directed towards infective dogs not infected, dogs, which would require a specific test to predict infectivity. Unfortunately, the development of such a test is a hurdle to improving the success of CVL control programs. This study reinforces the idea that more sensitive and specific tests need to be developed before the efficient diagnosis of canine VL can be realized.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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## ERRATUM

The *Revista da Sociedade Brasileira de Medicina Tropical* / Journal of the Brazilian Society of Tropical Medicine retained the original content of the manuscript related to this erratum and the final version (after paging and layout) was submitted for review by the authors.

In the article entitled “Evaluation of parasitological examination, kDNA polymerase chain reaction and rK39-based immunochromatography for the diagnosis of visceral leishmaniasis in seropositive dogs from the screening-culling program in Brazil” by Regina-Silva S, et al. (*Rev Soc Bras Med Trop* 2014; 47:462-468), there are 2 errors in Table 1. The second line of the third column should read as “negative” instead of “positive.” Further, the second line of the fourth column should read as “positive” instead of “negative.”

These corrections have been made to the online version as of October 24, 2014.