Major Article

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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

Shara Regina-Silva[1], Consuelo Latorre Fortes-Dias[2], Érika Monteiro Michalsky[1], João Carlos França-Silva[3], Patrícia Flávia Quaresma[1], Ana Cristina Vianna Mariano da Rocha Lima[1], Rafael Gonçalves Teixeira-Neto[1] and Edelberto Santos Dias[1]

[1]. Laboratório de Leishmanioses, Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz, Belo Horizonte, MG. [2]. Diretoria de Pesquisa e Desenvolvimento, Fundação Ezequiel Dias, Belo Horizonte, MG. [3]. Departamento de Parasitologia, Universidade Federal de Minas Gerais, Belo Horizonte, MG.

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[1].

***Address to*:** Dr. Edelberto Santos Dias. Laboratório de Leishmanioses/CPqRR/FIOCRUZ. Av. Augusto de Lima 1715, Barro Preto, 30190-002 Belo Horizonte, MG, Brasil.
Phone: 55 31 3349-7758
e-mail: edel@cpqrr.fiocruz.br
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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[2,3] and play a primary role in the maintenance of vector infection[4]. 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[5].

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[6]. 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.

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 (15º47'50"S, 43º18'31"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[7] 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'GGGGAGGGGCGTTCTGCGAA3') primers previously designed to amplify a 120bp segment from the conserved region of the kinetoplast DNA minicircle (kDNA) from the *Leishmania* genus[8]. 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*[9].

Immunochromatographic test

Canine sera were tested using the Kalazar Detect™ test (InBios International Inc., Seatle, 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*[10].

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[11]. When necessary, Fisher's exact test[11] was employed. The odds ratios and the 95% confidence intervals were calculated and adjusted for small samples[12] when applicable.

The Mann-Whitney test[13] 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[14].

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 κ value, there was little agreement between the results from the four diagnostic tests for CVL (κ = 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[15]. 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[4,16,17].

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.

		ELISA-IFAT			
		positive		negative	
Diagnostic test	Result	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.

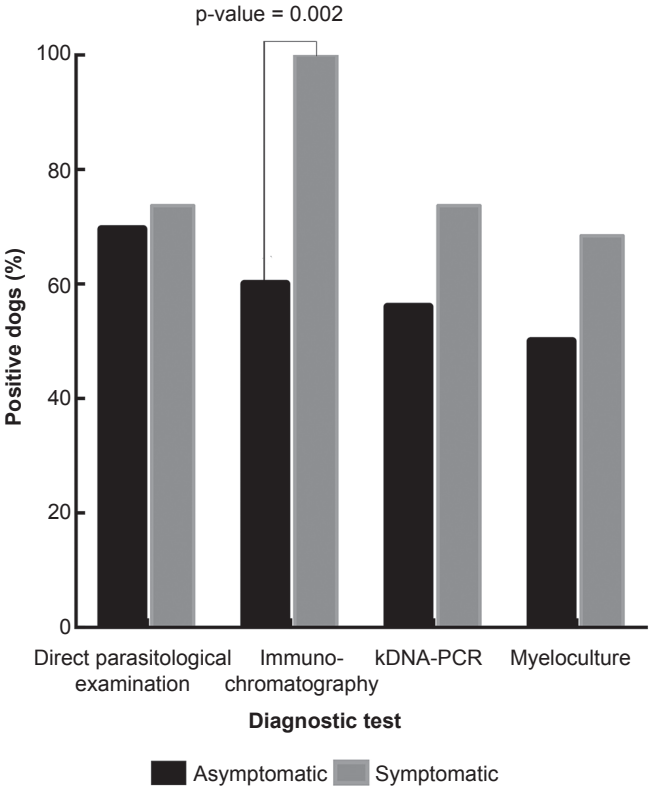

FIGURE 1 - Co-positivity of diagnostic tests for canine visceral leishmaniasis for each clinical group of dogs. The reference test was ELISA-IFAT, which was adopted by the Brazilian Ministry of Health in the screening-culling procedure for the Surveillance and Control Program of Visceral Leishmaniasis 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[18-21]. 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[22-27]. 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[28-33]. As previously noted[4], 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[34], 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[2,3,34-39]. Depending on the assay, this may be related to higher antibody titers or to higher parasite loads in clinically ill dogs[2]. 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[a]	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[b]	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[a]	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[a]	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; [a]Chi-square test; [b]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.

	Mann-Whitney test					
			Quartiles			
Clinical group	Number of dogs	mean ± standard deviation	1st	2nd	3rd	p-value
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	

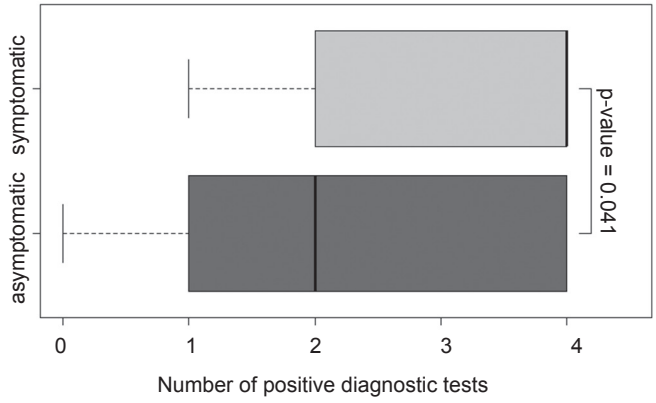

FIGURE 2 - Distribution of positive results for the entire set of diagnostic tests for canine visceral leishmaniasis for each clinical group. All of the dogs were sampled from endemic areas of visceral leishmaniasis in the State of Minas Gerais, Brazil, and were seropositive based on ELISA-IFAT. **ELISA-IFAT:** 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 κ 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[40], cross-reactivity with other diseases[41] or the presence of a pre-patent infection[42,43]. In the case of the puppy, the positive serology might also be attributable to the presence of maternal antibodies[44].

Published estimates for antibody-based immunofluorescence assays range from 72 to 100% sensitivity and 52 to 100% specificity[4]. One reason for the limited specificity of antibody-based tests is that they are generally manufactured with antigens from *Leishmania major*[45-47] instead of *Leishmania infantum* because the latter is difficult to culture and mass produce in artificial media[47,48]. 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[43]. 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[16] 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.

REFERENCES

1. Chappuis F, Sundar S, Hailu A, Ghalib H, Rijal S, Peeling RW, et al. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? Nat Rev Microbiol 2007; 5:873-882.
2. Reis AB, Martins-Filho OA, Teixeira-Carvalho A, Carvalho MG, Mayrink W, França-Silva JC, et al. Parasite density and impaired biochemical/hematological status are associated with severe clinical aspects of canine visceral leishmaniasis. Res Vet Sci 2006; 81:68-75.
3. Quaresma PF, Murta SM, Ferreira EC, Rocha-Lima AC, Xavier AA, Gontijo CM. Molecular diagnosis of canine visceral leishmaniasis: identification of *Leishmania* species by PCR-RFLP and quantification of parasite DNA by real-time PCR. Acta Trop 2009; 111:289-294.
4. Romero GA, Boelaert M. Control of visceral leishmaniasis in Latin America: a systematic review. PLoS Negl Trop Dis 2010; 4:e584.
5. Ministério da Saúde. Brasil. Manual de Vigilância e Controle da Leishmaniose Visceral. Brasília: Ministério da Saúde; 2006.

6. Faria AR, Andrade HM. Diagnosis of canine visceral leishmaniasis: major technological advances and few practical applications. Rev Pan-Amaz Saude 2012; 3:47-57.
7. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory Press; 1989.
8. Degrave W, Fernandez O, Campbell D, Bozza M, Lopez UG. Use of molecular probes and PCR for detection and typing of *Leishmania*: a mini review. Mem Inst Oswaldo Cruz 1994; 89:463-469.
9. Lins RMMA, Oliveira SG, Souza NA, Queiroz RG, Justiniano SCB, Ward RD, et al. Molecular evolution of the cacophony IVS6 region in sandflies. Insect Mol Biol 2002; 11:117-122.
10. Burns Jr JM, Shreffler WG, Benson DR, Ghalib HW, Badaro R, Reed SG. Molecular characterization of a kinesin-related antigen of *Leishmania chagasi* that detects specific antibody in African and American visceral leishmaniasis. Proc Natl Acad Sci USA 1993; 90:775-779.
11. Agresti A. Categorical data analysis. 2nd edition. Wiley Series in Probability and Statistics. New York: Wiley Interscience; 2002.
12. Jewell NP. Statistics for Epidemiology. Texts in Statistical Science 1st edition. v. 58. London: Chapman & Hall; 2003.
13. Hollander M, Douglas AW. Nonparametric Statistical Methods, Solutions Manual. 2nd edition. v. 348. New York: Wiley Series in Probability and Statistics John Wiley & Sons; 1999.
14. Fleiss JL. Statistical methods for rates and proportions. 2nd edition. New York: Wiley, John and Sons Inc; 1981.
15. Werneck GL. Geographic spread of visceral leishmaniasis in Brazil. Cad Saude Publica 2010; 26:644-645.
16. Courtenay O, Quinnell RJ, Garcez LM, Shaw JJ, Dye C. Infectiousness in a cohort of Brazilian dogs: why culling fails to control visceral leishmaniasis in areas of high transmission. J Infect Dis 2002; 186: 1314-1320.
17. Reithinger R, Quinnell RJ, Alexander B, Davies CR. Rapid detection of *Leishmania infantum* infection in dogs: comparative study using an immunochromatographic dipstick test, enzyme-linked immunosorbent assay and PCR. J Clin Microbiol 2002; 40:2352-2356.
18. Sundar S, Reed SG, Singh VP, Kumar PC, Murray HW. Rapid accurate field diagnosis of Indian visceral leishmaniasis. Lancet 1998; 351: 563-565.
19. Zijlstra E, Nur Y, Desjeux P, Khalil E, El-Hassan A, Groen J. Diagnosing visceral leishmaniasis with the recombinant K39 strip test: experience from Sudan. Trop Med Int Health 2001; 6:108-113.
20. Carvalho SF, Lemos EM, Corey R, Dietze R. Performance of recombinant K39 antigen in the diagnosis of Brazilian visceral leishmaniasis. Am J Trop Med Hyg 2003; 68:321-324.
21. Assis TS, Braga AS, Pedras MJ, Oliveira E, Barral A, Siqueira IC, et al. Multi-centric prospective evaluation of rk39 rapid test and direct agglutination test for the diagnosis of visceral leishmaniasis in Brazil. Trans R Soc Trop Med Hyg 2011; 105:81-85.
22. Reithinger R, Davies CR. Canine leishmaniasis: novel strategies for control. Trends Parasitol 2002; 18:289-290.
23. Mohebali M, Taran M, Zarei Z. Rapid detection of *Leishmania infantum* infection in dogs: comparative study using an immunochromatographic dipstick rK39 test and direct agglutination. Vet Parasitol 2004; 121:239-245.
24. Otranto D, Paradies P, Sasanelli M, Spinelli R, Brandonisio O. Rapid immuno-chromatographic test for serodiagnosis of canine leishmaniasis. J Clin Microbiol 2004; 42:2769-2770.
25. Toz SO, Chang KP, Ozbel Y, Alkan MZ. Diagnostic value of rK39 dipstick in zoonotic visceral leishmaniasis in Turkey. J Parasitol 2004; 90:1484-1486.
26. Mettler M, Grimm F, Capelli G, Camp H, Deplazes P. Evaluation of enzyme linked immunosorbent assays, an immunofluorescent-antibody test, and two rapid tests (immunochromatographic-dipstick and gel tests) for serological diagnosis of symptomatic and asymptomatic *Leishmania* infections in dogs. J Clin Microbiol 2005; 43:5515-5519.
27. Quinnell RJ, Carson C, Reithinger R, Garcez LM, Courtenay O. Evaluation of rK39 rapid diagnostic tests for canine visceral leishmaniasis: longitudinal study and meta-analysis. PLoS Negl Trop Dis 2013; 7:e1992.
28. Barrouin-Melo SM, Laranjeira DF, Andrade Filho FA, Trigo J, Julião FS, Franke CR, et al. Can spleen aspirations be safely used for the parasitological diagnosis of canine visceral leishmaniosis? A study on asymptomatic and polysymptomatic animals. Vet J 2005; 171:331-339.
29. Diniz SA, Melo MS, Borges AM, Bueno R, Reis BP, Tafuri WL, et al. Genital lesions associated with visceral leishmaniosis and shedding of *Leishmania* sp. in semen of naturally infected dogs. Vet Pathol 2005; 42:650-658.
30. Franceschi A, Merildi V, Guidi G, Mancianti F. Occurrence of *Leishmania* DNA in urines of dogs naturally infected with leishmaniasis. Vet Res Commun 2006; 31:335-341.
31. Ferreira SA, Ituassu T, Melo MN, Andrade AS. Evaluation of the conjunctival swab for canine visceral leishmaniasis diagnosis by PCR-hibridization in Minas Gerais state, Brazil. Vet Parasitol 2008; 152: 257-263.
32. Coura-Vital W, Marques MJ, Velos VM, Roatt BM, Aguiar-Soares RD, Reis LE, et al. Prevalence and factors associated with *Leishmania infantum* infection of dogs from an urban area of Brazil as identified by molecular methods. PLoS Negl Trop Dis 2011; 5:e1291.
33. Ferreira SA, Almeida GG, Silva SO, Vogas GP, Fujiwara RT, Andrade AS, et al. Nasal, oral and ear swabs for canine visceral leishmaniasis diagnosis: new practical approaches for detection of *Leishmania infantum* DNA. PLoS Negl Trop Dis 2013; 7:e2150.
34. Quinnell RJ, Courtenay O, Davidson S, Garcez L, Lambson B, Ramos P, et al. Detection of *Leishmania infantum* by PCR, serology and cellular immune response in a cohort study of Brazilian dogs. Parasitology 2001; 122:253-261.
35. Killick-Kendrick R, Killick-Kendrick M, Pinelli E, Del Real G, Molina R, Vitutia MM, et al. A laboratory model of canine leishmaniasis: the inoculation of dogs with *Leishmania infantum* promastigotes from midguts of experimentally infected phlebotomine sandflies. Parasite 1994; 1:311-318.
36. Saridomichelakis MN, Mylonakis ME, Leontides LS, Koutinas AF, Billinis C, Kontos VI. Evaluation of lymph node and bone marrow cytology in the diagnosis of canine leishmaniasis (*Leishmania infantum*) in symptomatic and asymptomatic dogs. Am J Trop Med Hyg 2005; 73:82-86.
37. Costa-Val AP, Cavalcanti RR, Gontijo NF, Michalick MS, Alexander B, Williams P, et al. Canine visceral leishmaniasis: Relationships between clinical status, humoral immune response, haematology and *Lutzomyia (Lutzomyia) longipalpis* infectivity. Vet J 2007; 174:636-643.
38. Teixeira Neto RG, Giunchetti RC, Carneiro CM, Vitor RW, Coura-Vital W, Quaresma PF, et al. Relationship of *Leishmania*-specific IgG levels and IgG avidity with parasite density and clinical signs in canine leishmaniasis. Vet Parasitol 2010; 169:248-257.
39. Almeida AB, Sousa VR, Gasparetto ND, Silva GF, Figueiredo FB, Dutra V, et al. Canine visceral leishmaniasis: diagnostic approaches based on polymerase chain reaction employing different biological samples. Diagn Microbiol Infect Dis 2013; 76:321-324.
40. Alves WA, Bevilacqua PD. Reflexões sobre a qualidade do diagnóstico da leishmaniose visceral canina em inquéritos epidemiológicos: o caso da epidemia de Belo Horizonte, Minas Gerais, Brasil, 1993-1997. Cad Saude Publica 2004; 20:259-265.
41. Vale AM, Fujiwara RT, Silva Neto AF, Miret JA, Alvarez DC, Silva JC, et al. Identification of highly specific and cross-reactive antigens of *Leishmania* species by antibodies from *Leishmania (Leishmania) chagasi* naturally infected dogs. Zoonoses Public Health 2009; 56:41-58.
42. Quinnell RJ, Courtenay O, Garcez L, Dye C. The epidemiology of canine leishmaniasis: transmission rates estimated from a cohort study in Amazonian Brazil. Parasitology 1997; 115:143-156.
43. Maia C, Campino L. Methods for diagnosis of canine leishmaniasis and immune response to infection. Vet Parasitol 2008; 158:274-287.
44. Laurenti MD. Correlação entre o diagnóstico parasitológico e sorológico na leishmaniose visceral americana canina. Bepa 2009; 6:13-23.

45. Costa CA, Genaro O, Lana M, Magalhães PA, Dias M, Michalick MS, et al. Canine visceral leishmaniasis: evaluation of the serologic method used in epidemiologic studies. Rev Soc Bras Med Trop 1991; 24:21-25.

46. Rondon FC, Bevilaqua CM, Franke CR, Barros RS, Oliveira FR, Alcântara AC, et al. Cross-sectional serological study of canine *Leishmania* infection in Fortaleza, Ceará state, Brazil. Vet Parasitol 2008; 155:24-31.

47. Silva DA, Madeira MF, Abrantes TR, Filho CJ, Figueiredo FB. Assessment of serological tests for the diagnosis of canine visceral leishmaniasis. Vet J 2013; 195:252-253.

48. Santiago MA, Ribeiro FC, Mouta-Confort E, Nascimento LD, Schubach AO, Madeira MF, et al. Differentiation between canine cutaneous and visceral leishmaniasis by the detection of immunoglobulin G specific for *Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) chagasi* antigens using flow cytometry. Vet Parasitol 2008; 154: 341-346.