

Serological cross-reactivity of *Trypanosoma cruzi*, *Ehrlichia canis*, *Toxoplasma gondii*, *Neospora caninum* and *Babesia canis* to *Leishmania infantum chagasi* tests in dogs

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ABSTRACT

Introduction: The aim of this study was to evaluate the serological cross-reactivity between *Leishmania* sp. and other canine pathogens. **Methods:** Positive serum samples for *Ehrlichia canis*, *Babesia canis*, *Toxoplasma gondii*, *Neospora caninum* and *Trypanosoma cruzi* were tested using three serological methods enzyme linked immunosorbent assay (ELISA), indirect immunofluorescent antibody test (IFAT) and Kalazar Detect™, for canine visceral leishmaniasis. **Results:** Of the 57 dog samples tested, 24 (42.1%) tested positive using one of the three serological methods: 10/57 (17.5%) for ELISA, 11/57 (19.3%) for IFAT and 3/57 (5.3%) for Kalazar Detect™. **Conclusions:** Our results demonstrated that the presence of other infectious agents may lead to cross-reactivity on leishmaniasis serological tests.

Keywords: Diagnosis. Serology. Visceral leishmaniasis.

Visceral leishmaniasis (VL) is a widespread protozoan zoonosis caused by the genus *Leishmania*. *Leishmania chagasi* (*Leishmania infantum*) is the reported species present in the New World¹. Dogs are considered the main epidemiological reservoirs of VL in the domestic environment, and mandatory euthanasia of positive dogs has been the basis of disease control in several developing countries, including Brazil². Because the clinical diagnosis of canine visceral leishmaniasis (CVL) is not reliable due to the wide variety of clinical signs of CVL and the high percentage of asymptomatic dogs, serological methods have been used for the definitive diagnosis of CVL¹.

Although the detection of amastigote forms of *Leishmania* sp. may be considered the gold standard for the diagnosis of VL, this procedure is laborious and difficult to routinely apply under field conditions. Serological methods, such as the enzyme linked immunosorbent assay (ELISA) and the indirect immunofluorescent antibody test (IFAT), have been indicated as better, rapid and reliable diagnostic methods that are easily standardized and suitable for mass testing³. Despite the high sensitivity and specificity of serological assays for

canine leishmaniasis, the presence of cross-reactivity remains controversial. The aim of the present study was to evaluate the cross-reactivity of serum samples from dogs serologically reactive for *Trypanosoma cruzi*, *Ehrlichia canis*, *Babesia canis*, *Toxoplasma gondii* and *Neospora caninum* by ELISA, IFAT and an immunochromatographic test (Kalazar Detect™, InBios Inc., Seattle, WA, USA) for canine leishmaniasis. All dogs were from areas that were non-endemic for leishmaniasis, Chagas disease and *Trypanosoma evansi*, free of vectors and with no single reported case to date.

A total of fifty-seven serum samples were used: 14 dogs experimentally infected with *T. cruzi* (Colombian strain) with chronic cardiomyopathy and anti-*T. cruzi* IgG antibodies, as determined by IFAT, with titers ranging from 160 to 640⁴; 12 dogs naturally infected with *B. canis* and presenting pale mucous membranes, anemia, intraerythrocytic inclusions of *Babesia* sp. and anti-*B. canis* antibodies, as determined by IFAT, with titers ranging from 400 to 800⁵; 13 dogs naturally infected with *E. canis* and presenting thrombocytopenia, *Ehrlichia* morulae within macrophages on capillary blood smears and antibodies, as detected by a commercially available kit (SNAP® 3Dx®, IDEXX Laboratories Inc., Westbrook, ME, USA); 10 dogs naturally infected with *T. gondii* and presenting clinical signs of the disease and anti-*T. gondii* antibodies, as detected by IFAT, with titers ranging from 1:128 to 1:1024⁶; and eight dogs presenting clinical signs of *N. caninum* infection, with anti-*N. caninum* antibodies, no detected anti-*T. gondii* antibodies and IFAT antibody titers ranging from 400 to 800⁷.

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The serological detection of anti-*Leishmania chagasi* IgG antibodies was performed by ELISA as previously described⁸ using an *L. chagasi* total promastigote lysate (strain MHOM/BR/74/PP75) and alkaline phosphatase-conjugated anti-dog IgG (Sigma-Aldrich, St. Louis, MO, USA). A cut-off value of 0.270 was determined using the analysis of serum samples from 50 healthy dogs from an area that was non-endemic for leishmaniasis (mean + 3 SD). The detection of *L. chagasi* antibodies by IFAT was determined as previously described⁹ using a suspension of parasites (MHOM/BR/72) in buffered saline solution (PBS) at a concentration of 8.1×10^6 promastigotes/mL and a fluorescein isothiocyanate-conjugated anti-dog IgG (Sigma-Aldrich, St. Louis, MO, USA). The samples were considered positive when titers were $\geq 1:40$. Positive sera were serially diluted and tested to establish the maximum reaction titer. The samples were also tested for *Leishmania* sp. using an immunochromatographic qualitative antibody assay against *L. chagasi* recombinant K39 (rK39) antigen (Kalazar Detect™, InBios Inc., Seattle, WA, USA) according to the manufacturer's protocol.

A total of 24/57 (42.1%) dog samples tested positive using any of the three serological methods for *Leishmania* sp. (Table 1). Of the positive ELISA samples, the optical density (OD) values of the *T. cruzi* samples ranged from 0.299 to 0.941, with an average of 0.531 ± 0.226 , and the OD value of the *E. canis* sample was 0.385 (Figure 1). Of the positive IFAT samples, the titer values ranged from 40 to 160 and from 40 to 80 for the *T. cruzi* and *T. gondii* samples, respectively. Of the positive dipstick samples, the number of samples that cross-reacted using this method was lower than those observed using the ELISA and IFAT methods (Table 1).

Although the ELISA and IFAT tests were conducted using *Leishmania* crude antigens and although the observed cross-reactivity may be explained by a decrease in test specificity², a previous study also used crude antigens and observed no cross-reactivity with *E. canis*, *T. gondii* and *B. canis*⁸. Antibodies against *T. cruzi* have been recognized using conventional serological methods as the main cause of cross-reactivity with *Leishmania* due to the phylogenetic similarity between *Leishmania* sp. and *Trypanosoma cruzi*, which poses

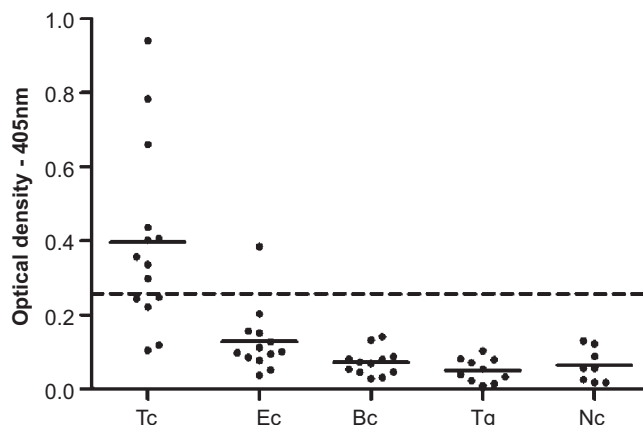


FIGURE 1 - Distribution of individual enzyme linked immunosorbent assay (ELISA) results for the diagnosis of *Leishmania chagasi* in 57 seropositive dogs with other infectious and parasitic agents: 14 with *Trypanosoma cruzi* (Tc), 13 with *Ehrlichia canis* (Ec), 12 with *Babesia canis* (Bc), ten with *Toxoplasma gondii* (Tg), and eight with *Neospora caninum* (Nc). The horizontal line within each data group is the arithmetic mean. The horizontal line at 0.270 represents the cut-off optical density value.

a problem for overlapping endemic areas^{10,11}. In the present study, *T. cruzi* displayed a high percentage of cross-reactivity with *Leishmania* when using ELISA and IFAT, both of which are tests that are used as standard diagnostic methods for CVL in Brazil. Samples from five dogs cross-reacted with *T. cruzi* in both the ELISA and the IFAT. The dipstick test was the only test displaying no cross-reactivity between *T. cruzi* and *Leishmania* sp., confirming that rK39 does not cross-react with *T. cruzi*¹².

The identification of ELISA cross-reactivity with *E. canis* (1/13; 7.7%) corroborated previous studies that reported 2/3 (66.6%) cross-reactivity on the IFAT and 1/3 (33.3%) cross-reactivity on the ELISA¹³. Although positive, titers were within 2-fold of the cut-off value (OD value 0.385), and these samples tested negative by IFAT. None of the 12 *Babesia canis* samples

TABLE 1 - Results obtained by ELISA, IFAT and Kalazar Detect™ for the detection of cross-reactivity between *Leishmania chagasi* and other infectious and parasitic agents in dog serum samples.

Infectious/parasitic agents	Positive dogs (n/%)						
	n	ELISA		IFAT		Kalazar Detect™	
		n	%	n	%	n	%
<i>Trypanosoma cruzi</i>	14	9	64.3	6	42.9	0	0.0
<i>Ehrlichia canis</i>	13	1	7.7	0	0.0	1	7.7
<i>Babesia canis</i>	12	0	0.0	0	0.0	0	0.0
<i>Toxoplasma gondii</i>	10	0	0.0	5	50.0	1	10.0
<i>Neospora caninum</i>	8	0	0.0	0	0.0	1	12.5
Total	57	10		11		3	

ELISA: enzyme linked immunosorbent assay; IFAT: indirect immunofluorescent antibody test.

displayed cross-reactivity with *Leishmania* by either ELISA or IFAT, as previously demonstrated⁸.

In a previous study, cross-reactivity between *Leishmania* sp. and *T. gondii* was not identified when dog serum samples were tested by ELISA and IFAT¹³. Although IFAT is considered highly specific³, the present study has demonstrated that half of the dogs (5/10; 50%) with anti-*T. gondii* antibodies were mistakenly considered serologically positive for visceral leishmaniasis.

Despite the fact that the dipstick test did not display cross-reactivity with Chagas disease, dogs seroreactive for *E. canis* (1/13; 7.7%), *T. gondii* (1/10; 10%), and *N. caninum* (1/8; 12.5%) induced false positive results on this test. Our results differed from those of a previous study that reported 3/12 (25%) samples cross-reacting with Chagas disease, and our results differed from those of a previous study, which found 3/12 (25%) cross-reacted with Chagas disease and no false positives with *T. gondii*, although 1/3 (33.3%) dogs with ehrlichiosis presented false positive results on the dipstick¹⁴. Although the dipstick test has been developed for the diagnosis of visceral leishmaniasis in humans, previous studies have demonstrated that the sensitivity and specificity of this test ranged from 83% to 91.5% and from 94.7% to 100%, respectively, for disease diagnosis in dogs^{8,14}. False positive results were also observed in a rapid commercial ELISA test for dog leishmaniasis, with cross-reactivity found in 3/6 samples (50%) of *T. gondii*, 1/2 (50%) samples of *T. gondii* and *N. caninum*, 3/4 (75%) samples of *B. canis* and *E. canis* and 4/7 samples (57.1%) of *T. cruzi*¹⁵. Thus, rapid in-clinic diagnostic tests should be used in association with other diagnostic tests and should be used carefully when screening for canine visceral leishmaniasis in areas that are endemic for ehrlichiosis, babesiosis, toxoplasmosis and neosporosis. In the present study, with the sole use of the ELISA, IFAT or commercial immunochromatographic tests, approximately 19%, 14% and 11% of the dogs tested would have been considered positive for leishmaniasis, respectively. A recent guideline consensus has concluded that dogs should be considered infected by serology only when antibody titers are 2- to 4-fold higher than the positive threshold value. Otherwise, cytology and polymerase chain reaction (PCR) using bone marrow and/or lymph node samples should be conducted to accurately confirm the diagnosis¹¹.

Despite the territorial expansion of VL in Brazil, where the disease has reached even the southern states of the country, when the present study was conducted, the number of municipalities in which vectors and reported cases were present was lower than the current number of such municipalities, and many areas were still considered to be disease-free. Thus, the possibility of dogs having been infected prior to the time of this study by *Leishmania chagasi* (*L. infantum*) is remote.

In summary, our results indicate that confounding infectious agents such as *Trypanosoma cruzi*, *Ehrlichia canis*, *Babesia canis*, *Toxoplasma gondii* and *Neospora caninum* may cross-react with leishmaniasis serological tests and lead clinicians to falsely diagnose visceral leishmaniasis. Thus, the use of serological methods with low antibody titers for the diagnosis of *Leishmania*-infected dogs as a criterion for euthanasia should be reassessed.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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