

Leishmania, *Babesia* and *Ehrlichia* in urban pet dogs: co-infection or cross-reaction in serological methods?

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ABSTRACT

Introduction: The present study was designed to assess the occurrence of co-infection or cross-reaction in the serological techniques used for detecting the anti-*Leishmania* spp., -*Babesia canis vogeli* and -*Ehrlichia canis* antibodies in urban dogs from an area endemic to these parasites. **Methods:** The serum samples from dogs were tested for the *Babesia canis vogeli* strain Belo Horizonte antigen and *Ehrlichia canis* strain São Paulo by immunofluorescence antibody test (IFAT) and by anti-*Leishmania* immunoglobulin G (IgG) antibody detection to assess *Leishmania* infection. We used the following four commercial kits for canine visceral leishmaniasis: ELISA, IFAT, Dual Path Platform (DPP) (Bio Manguinhos®/FIOCRUZ/MS) and a rK39 RDT (Kalazar Detect Canine Rapid Test; Inbios). **Results:** Of 96 serum samples submitted to serological assays, 4 (4.2%) were positive for *Leishmania* as determined by ELISA; 12 (12.5%), by IFAT; 14 (14.6%) by rK39 RDT; and 20 (20.8%), by DPP. Antibodies against *Ehrlichia* and *Babesia* were detected in 23/96 (23.9%) and 30/96 (31.2%) samples, respectively. No significant association was identified between the results of tests for detecting *Babesia* or *Ehrlichia* and those for detecting *Leishmania* (p -value>0.05). **Conclusions:** In the present study, we demonstrated co-infection with *Ehrlichia* or *Babesia* and *Leishmania* in dogs from Minas Gerais (Brazil); we also found that the serological tests that were used did not cross-react.

Keywords: Immunochromatographic test. Enzyme-linked immunosorbent assay. Immunofluorescence antibody test. Canine visceral leishmaniasis.

INTRODUCTION

Vector-borne dog infections such as ehrlichiosis, babesiosis and leishmaniasis are among the most common canine diseases in the tropical, sub-tropical and temperate climate regions of the world. Co-infection with more than one pathogen is common due to the abundance of hematophagous vectors such as ticks and sand flies and due to the ability of arthropod vectors to host and transmit several pathogens simultaneously⁽¹⁾. *Ehrlichia canis* (Rickettsiales: Anaplasmataceae), an intracellular Gram-negative bacterium that infects monocytes, is the causative agent of canine monocytic ehrlichiosis and is transmitted by the tick *Rhipicephalus sanguineus*⁽²⁾. Piroplasmids are tick-borne apicomplexan parasites that infect the erythrocytes of domestic and wild animals.

Babesia (Piroplasmida: Babesiidae) infections in dogs are caused by at least six genetically distinct species; babesiosis in Brazil is caused predominantly by *Babesia canis vogeli*^{(3) (4)}.

Canine leishmaniasis is a vector-borne disease caused by protozoa of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae), and the most important etiological agent of canine leishmaniasis is *Leishmania infantum* (syn. *Leishmania chagasi*). The control of canine visceral leishmaniasis (CVL) in Brazil is based primarily on eliminating seropositive dogs. Until 2012, CVL had been diagnosed using the immunofluorescent antibody test (IFAT), a method recommended for confirming positive cases detected by enzyme-linked immunosorbent assay (ELISA). Recently, to improve the accuracy of diagnosing CVL, the Brazilian Ministry of Health has recommended the use of an immunochromatographic rapid test consisting of rK26 and rK39 recombinant antigens, the Dual-Path Platform (DPP; Bio-Manguinhos/FIOCRUZ, Rio de Janeiro, Brazil) to screen infected dogs, and ELISA Bio-Manguinhos/FIOCRUZ to confirm the positive results⁽⁵⁾.

Some researchers have reported that antibodies against *E. canis* and *B. canis* are cross-reactive with *Leishmania* spp. in the common serological tests that have been applied to detect

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dogs infected by *Leishmania* spp.⁽⁶⁾⁽⁷⁾⁽⁸⁾. The aim of this study was to evaluate the possible relationship between the naturally occurring anti-*Leishmania* spp., -*Babesia* spp. and -*Ehrlichia* spp. antibodies in Brazilian dogs.

METHODS

Study area

This study was conducted in a region to the Northeast of Belo Horizonte (latitude: 19°55'15" S; longitude: 43°56'16" W) that is endemic for canine vector-borne parasitic diseases caused by organisms of the genera *Leishmania*, *Babesia*, and *Ehrlichia*⁽⁹⁾⁽¹⁰⁾⁽¹¹⁾. The study area was selected based on environmental and socio-economical characteristics and consisted of a relatively flat, densely populated urban district containing predominantly one- or two-story buildings with woody vegetation between the plots.

Animals

The canine population within the study area consisted of 120 domestic dogs that were domiciliated, corresponding to almost every resident dog in the area during the period of our study (according to the local health department); however, only 96 (80%) of these dogs could be surveyed. The remaining dogs could not be evaluated because either the owners refused to participate in the study or the residence was closed at the start of the study.

Blood samples were collected by venipuncture during July-August 2013 and transferred to tubes without anticoagulant. Serum samples were obtained by centrifugation 2,500rpm for 15 minutes and stored at -70°C until use.

To detect the presence of clinical signs of disease, each dog was subjected to one clinical examination that was conducted during the experimental period. Dog owners were interviewed using a semi-structured questionnaire with questions covering the gender, age and breed of each dog.

Serological methods

Serum samples were tested for the presence of anti-*Leishmania* immunoglobulin G (IgG) antibodies using the following four commercial canine visceral leishmaniasis kits: enzyme-linked immunosorbent assay (ELISA)

(Bio-Manguinhos®/FIOCRUZ/MS), immunofluorescence antibody test (IFAT) (Bio-manguinhos®/FIOCRUZ/MS), Dual Path Platform (DPP) (Bio-manguinhos®/FIOCRUZ/MS), and rK39 RDT (Kalazar Detect Canine Rapid Test; Inbios). Dogs were considered positive for *Leishmania* infection if found to be reactive by IFAT, DPP, ELISA or rK39 RDT. To detect anti-*Ehrlichia canis* antibodies, sera were tested by IFAT *in house* using *E. canis* strain São Paulo antigen⁽¹²⁾ with a cut-off titer of 1:80⁽¹³⁾⁽¹⁴⁾. To detect anti-*B. canis vogeli* antibodies, sera were tested by IFAT *in house* using *B. canis vogeli* strain Belo Horizonte antigen⁽¹⁵⁾ with a cut-off titer of 1:40⁽¹⁶⁾.

Statistical analysis

Fisher's exact test was used for statistical analysis (p-value<0.05) to evaluate the possible association between the results of tests for detecting *Babesia* or *Ehrlichia* infection with those for detecting *Leishmania* infection.

This study was submitted to and approved by the Ethics Committee for Research with Animals of the Oswaldo Cruz Foundation [(Comissão de Ética no Uso de Animais/Fundação Oswaldo Cruz (CEUA/FIOCRUZ))] under protocol number LW-76/12. All procedures involving experimental animals were conducted according to the guidelines of the Brazilian College for Experiments with Animals [Colégio Brasileiro de Experimentação Animal (COBEA)].

RESULTS

The studied dog population consisted of 42 (43.7%) males and 54 (56.3%) females, and the average age was 5.8±1.1 years (range: 3 months to 17 years). The breeds included in the population were mongrels (53.1%), poodles (24%), Doberman pinschers (8.3%), Yorkshire terriers (3.1%), boxers, cocker spaniels, Labradors and German shepherds (8.4%) and others (3.1%).

Clinical examination conducted at the time of blood sample collection revealed that all dogs were apparently healthy and did not show any visible signs of disease.

Tables 1, 2 and 3 summarize the results of the different diagnostic tests used in this comparative study. Of the 96 serum samples submitted to serological assays, 4 (4.2%) were considered positive for *Leishmania* by ELISA; 12 (12.5%), by IFAT; 14 (14.6%), by rK39 RDT; and 20 (20.8%), by DPP.

TABLE 1 - Frequency of infection and co-infection by *Ehrlichia canis* and *Leishmania* spp. according to IFAT, ELISA, DPP and rK39 RDT.

	<i>Leishmania</i> spp. (positive results)							
	IFAT		ELISA		DPP		rK39 RDT	
	n	%	n	%	n	%	n	%
<i>Ehrlichia canis</i>								
Negative (n=73)	11	15.1	3	4.1	17	23.3	7	9.6
Positive (n=23)	1	4.3	1	4.3	3	13.0	1	4.3
Total (n=96)	12	12.5	4	4.2	20	20.8	8	8.3

IFAT: immunofluorescence antibody test; ELISA: enzyme-linked immunosorbent assay; DPP: Dual Path Platform; rK39 RDT: Kalazar Detect Canine Rapid Test; Inbios. Fisher's exact test for association: IFI (p-value=0.2826), ELISA (p-value=1.000); DPP (p-value=0.3851), and Kalazar (p-value=0.6752).

TABLE 2 - Frequency of infection and co-infection by *Babesia canis vogeli* and *Leishmania* spp. according to IFAT, ELISA, DPP and rK39 RDT.

<i>Babesia canis vogeli</i>	<i>Leishmania</i> spp. (positive results)							
	IFAT		ELISA		DPP		rK39 RDT	
	n	%	n	%	n	%	n	%
Negative (n=66)	7	10.6	2	3.3	12	18.2	4	6.1
Positive (n=30)	5	16.7	2	6.7	8	26.7	4	13.3
Total (n=96)	12	12.5	4	4.2	20	20.8	8	8.3

IFAT: immunofluorescence antibody test; **ELISA:** enzyme-linked immunosorbent assay; **DPP:** Dual Path Platform; **rK39 RDT:** Kalazar Detect Canine Rapid Test; Inbios. Fisher's exact test for association: IFAT (p-value=0.5075), ELISA (p-value=0.5867), DPP (p-value=0.4179), and Kalazar (p-value=0.2521).

TABLE 3 - Frequency of infection and co-infection by *Ehrlichia canis* and *Babesia canis vogeli* according to IFAT.

<i>Ehrlichia canis</i>	<i>Babesia canis</i>				Total	
	negative		positive			
Negative	52	71.2	21	28.8	73	76.0
Positive	14	60.9	9	39.1	23	24.0
Total	66	68.8	30	31.2	96	100.0

IFAT: immunofluorescence antibody test. Fisher's exact test for association: p-value=0.4399.

For the ELISA+IFAT, ELISA+DPP, and IFAT+DPP combinations, the percentages of positive sera were 4.2%, 4.2% and 9.5%, respectively.

For the anti-*Ehrlichia canis* and anti-*Babesia canis vogeli* antibodies, 23/96 (23.9%) and 30/96 (31.2%) samples had detectable antibodies against these agents, respectively.

Simultaneous reactivity to *Ehrlichia canis* and to *Babesia canis vogeli* was observed in nine dogs (by IFAT). Simultaneous reactivity to *E. canis* and *Leishmania*, considering each of the four *Leishmania* serological tests, was observed in 4.3% (by IFAT), 4.3% (by ELISA), 13% (by DPP), and 4.3% (by rK39 RDT) of the dogs. Additionally, simultaneous reactivity to *B. canis vogeli* and *Leishmania* was found in 16.7% (IFAT), 6.7% (ELISA), 26.7% (DPP), and 13.3% (rK39 RDT) of the dogs. No significant association was identified between the results of the tests for detecting *Babesia* and *Ehrlichia* and those for detecting *Leishmania* (p-value>0.05).

DISCUSSION

In endemic areas, serological tests, which are helpful tools in epidemiological studies, are useful for identifying asymptomatic carrier dogs and for diagnosing chronic infections. Canine monocytotropic ehrlichiosis and canine babesiosis are multisystemic diseases manifesting in acute, subclinical or chronic forms^{(3) (17)}. Canine leishmaniasis is a disease in which infection does not always result in clinical illness due to the high prevalence of subclinical infection⁽¹⁸⁾. In the present

study, all 96 dogs were diagnosed as asymptomatic according to one clinical examination of each dog, which was conducted during the experimental period. However, this classification has limited value because it does not consider clinicopathological abnormalities and disregards dogs that have widespread organ dysfunction without apparent visual manifestations⁽¹⁹⁾.

The requirements of a serological test for canine leishmaniasis may vary. To confirm clinically suspected cases, the test specificity is the most important feature, whereas a high sensitivity is the most essential feature for surveillance programs or for tests of dogs imported from endemic areas, facilitating the detection of asymptomatic carriers. In our study, all four commercial canine visceral leishmaniasis kits had both high sensitivities and specificities. However, these kits detected considerably fewer asymptomatic cases and varied significantly with respect to their sensitivity and specificity for the different antigens^{(20) (21)}.

Immunofluorescent antibody test, enzyme-linked immunosorbent assay and immunochromatographic devices are the most commonly used techniques for detecting antileishmanial antibodies. False positive results due to serological cross-reactivity with other pathogens have been described in all of the above-mentioned serological techniques, particularly for *Trypanosoma cruzi* infection in North, Central and South America or for other species of *Leishmania* and tests using whole-parasite crude antigens. Cross-reactions are less likely to occur when using recombinant peptides such as rA2, rK9, rK26 and rK39⁽²²⁾.

Veterinarians are the key professionals at the companion animal-human interface. Not only do veterinarians provide traditional medical and surgical services for pets, their training in the disciplines of parasitology, microbiology, epidemiology, and public health place them in a unique position to provide advice about matters pertaining to vector-borne diseases.

Researchers and veterinarians have often inferred the existence of cross-reaction in serological assays for detecting canine leishmaniasis in dogs co-infected with *E. canis* or *B. canis vogeli*. In fact, Mancianti et al.⁽⁶⁾ demonstrated cross-reactivity of *B. canis vogeli* with anti-*Leishmania* antibodies using dot-ELISA. Furthermore, Gomes and Cordeiro⁽²³⁾ also observed cross-reaction with *Leishmania* during serological diagnostic assessments of the disease in dogs with acute phases of *E. canis* or *B. canis vogeli* infections. These authors suggested that primary treatment of infection with these two pathogens in dogs will be crucial to reverse cross-reaction activity with leishmaniasis. However, notably, dogs may remain seropositive for long periods even after chemosterilization^{(24) (25)}.

Co-infection of canine piroplasms with other tick-borne pathogens, such as *Ehrlichia canis*, *Hepatozoon canis*, and sand fly-borne *Leishmania infantum*, is frequent in areas where exposure to ectoparasites is extensive⁽²⁶⁾. A recent study tested the sera samples from endemic and non-endemic areas of Brazilian leishmaniasis using IFAT and ELISA, as well as IFAT for *Ehrlichia* and *Babesia* detection; the authors reported that the serological reactivity to *Leishmania* and *Ehrlichia* or *Babesia* was from co-infection rather than from cross-reaction between the agents⁽²⁷⁾. In our study, these findings are statistically supported because we found evidence of a lack of association between the serological results for detecting anti-*Ehrlichia*, -*Babesia* and -*Leishmania* antibodies, suggesting that co-infection is more plausible than cross-reaction.

This situation could be explained if we consider the taxonomic classification of these pathogens. The microorganisms of the *Ehrlichia* genus are intracellular bacteria⁽²⁸⁾, and *Babesia* and *Leishmania* are two protozoan agents that are phylogenetically different, belonging to the Piroplasmida and Kinetoplastida orders, respectively, indicating that differences in their biology exist^{(29) (30)}. This phylogenetic distance between these three pathogenic agents could explain the lack of cross-reactions. Nonetheless, cross-reaction activity between nearly related protozoa species has been observed. For example, Zanette et al.⁽³¹⁾ used several serological techniques (IFAT, ELISA and rK39 RDT) for detecting *Trypanosoma cruzi* and *Leishmania infantum chagasi*, two protozoa species belonging to the Trypanosomatidae family, and observed cross-reaction between these species. In the same study, supporting our results, no cross-reaction was observed between *Babesia* and *Leishmania* when using IFAT as a diagnostic test. In contrast to our results, these authors observed cross-reaction between *Leishmania* and *E. canis* with ELISAs. Moreover, these authors suggest that *Neospora caninum* and *Toxoplasma gondii* were also responsible for the cross-reaction with CVL. However, these findings remain controversial because other studies have demonstrated the absence of cross-reaction between *Leishmania* and *T. gondii*⁽⁷⁾, *E. canis* and *B. canis*⁽²⁷⁾.

In conclusion, our study suggests the occurrence of a haphazard co-infection pattern in the endemic studied area, and our results do not support serological cross-reaction between *Leishmania* spp., *E. canis* and *B. canis vogeli*.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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