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Serological and infection statuses of dogs from a visceral leishmaniasis-endemic area

Status sorológico e de infecção canina em área endêmica de leishmaniose visceral

ABSTRACT

OBJECTIVE: This study investigated the serological status of dogs living in a visceral leishmaniasis-endemic area and its correlation with the parasitological condition of the animals.

METHODS: Canine humoral response was evaluated using the sera of 134 dogs by enzyme-linked immunosorbent assay and immunohistochemistry to detect parasites in the skin, lymph node, and spleen of the animals. The specific antibodies investigated were IgG, IgG1, IgG2, and IgE.

RESULTS: According to the parasitological, laboratory, and clinical findings, the dogs were placed into one of four groups: asymptomatic with (AP+, n = 21) or without (AP-, n = 36) *Leishmania* tissue parasitism and symptomatic with (SP+, n = 52) or without (SP-, n = 25) parasitism. Higher IgG and IgE levels were positively correlated with the infection condition and parasite load, but not with the clinical status. In all groups, total IgG was the predominant antibody, which occurred at the expense of IgG2 instead of IgG1. Most of the infected dogs tested positive for IgG (SP+, 98.1%; AP+, 95.2%), whereas this was not observed with IgE (SP+, 80.8%; AP+, 71.2%). The most relevant finding was the high positivity of the uninfected dogs for *Leishmania*-specific IgG (SP-, 60.0%; AP-, 44.4%), IgE (SP-, 44.0%; AP-, 27.8%), IgG1 (SP-, 28.0%; AP-, 22.2%), and IgG2 antibodies (SP-, 56.0%; AP-, 41.7%).

CONCLUSIONS: The serological status of dogs, as determined by any class or subclass of antibodies, did not accurately distinguish dogs infected with *L. (L.) infantum chagasi* from uninfected animals. The inaccuracy of the serological result may impair not only the diagnosis, but also epidemiological investigations and strategies for visceral leishmaniasis control. This complex serological scenario occurring in a visceral leishmaniasis-endemic area highlights the challenges associated with canine diagnosis and points out the difficulties experienced by veterinary clinicians and coordinators of control programs.

DESCRIPTORS: Leishmaniasis, Visceral, diagnosis. Leishmaniasis, Visceral, epidemiology. Leishmania, immunology. Dogs. Seroepidemiologic Studies. Endemic Diseases, veterinary.

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RESUMO

OBJETIVO: Foi investigado o *status* sorológico de cães, em área endêmica de leishmaniose visceral, e sua correlação com a infecção parasitológica dos animais.

MÉTODOS: A resposta humoral canina foi avaliada no soro de 134 cães pelo método ELISA e pela imuno-histoquímica, para detectar parasitos na pele, linfonodo e baço desses animais. Os anticorpos específicos investigados foram IgG, IgG1, IgG2 e IgE.

RESULTADOS: De acordo com os achados parasitológicos, laboratoriais e clínicos, os cães foram alocados em um dos quatro grupos: assintomáticos com (AP+, n = 21) e sem (AP-, n = 36) parasitismo tecidual por *Leishmania* e sintomáticos com (SP+, n = 52) ou sem (SP-, n = 25) parasitismo. Níveis mais elevados de IgG e IgE se correlacionaram positivamente com o *status* de infecção e a carga parasitária, mas não com a condição clínica. Em todos os grupos, IgG total foi o anticorpo predominante, com maior concentração de IgG2 que IgG1. O anticorpo IgG foi positivo em proporção elevada nos animais infectados (SP+ 98,1%; AP+ 95,2%), mas não o IgE (SP+ 80,8%; AP+ 71,2%). O achado mais relevante refere-se aos cães não infectados que apresentaram elevada positividade para anticorpos IgG anti-*Leishmania* (SP- 60,0%; AP- 44,4%), IgE (SP- 44,0%; AP- 27,8%), IgG1 (SP- 28,0%; AP- 22,2%) e IgG2 (SP- 56,0%; AP- 41,7%).

CONCLUSÕES: O *status* sorológico dos cães, determinado por qualquer classe ou subclasse de anticorpos, não distinguiu com acurácia cães infectados por *L. (L.) infantum chagasi* daqueles não infectados. A imprecisão do resultado sorológico pode prejudicar não só o diagnóstico, mas também as investigações epidemiológicas e as estratégias para o controle da leishmaniose visceral. Esse complexo cenário sorológico observado na área endêmica mostra quão desafiador é o diagnóstico canino, e aponta a dificuldade enfrentada pelos médicos veterinários e coordenadores dos programas de controle.

DESCRITORES: Leishmaniose Visceral, diagnóstico. Leishmaniose Visceral, epidemiologia. Leishmania, imunologia. Cães. Estudos Soroepidemiológicos. Doenças Endêmicas, veterinária.

INTRODUCTION

Visceral leishmaniasis (VL) is regarded worldwide as a public health problem of increasing importance.[15] The domestic dog is considered the main reservoir of the Leishmania pathogen, and from an epidemiological point of view, canine visceral leishmaniasis (canVL) is of considerable importance because canine disease precedes the occurrence of human cases, and a group of infected animals can serve as source of infection for the vector.[5,7] In Brazil, the major prophylactic practices for disease control include the systematic and early treatment of human cases, in addition to vector control and elimination of seropositive dogs.[15]

Serology is, by far, the most commonly used diagnostic tool in large surveys and in clinics. However, this method has been seriously criticized, as it frequently fails to recognize asymptomatic dogs,[17] is not capable of differentiating infected animals from vaccinated ones,[9] and frequently shows cross-reactivity with sera from dogs infected with other pathogens.[3] Thus, the resulting inaccuracies in diagnosing canVL has led to unnecessary culling of dogs or even the maintenance of infected dogs in areas of transmission, both of which decrease the effectiveness of the Brazilian control program.[1]

Total IgG is the only anti-*Leishmania* antibody that is routinely inspected. Other classes and IgG subclasses of antibodies have been studied; however, these effects were geared towards detecting markers related to clinical prognosis or vaccination status[2,6,14] and evaluating the efficacy of treatments.[10,24,25] Nevertheless, the practical use and the reliability of these markers for diagnostic purposes remain unclear.

The objective of this study was to investigate the serological status of dogs living in an endemic area for VL and its correlation with the parasitological condition of the animals.

METHODS

The investigation involved dogs from the municipality of Araçatuba, located in northwest Sao Paulo, Southeastern Brazil, which is a region with high endemicity for canVL (12.0% - 42.0%) and reported its first canine case in 1998.[13]

A total of 134 stray dogs were collected by the *Centro de Controle de Zoonoses (*Zoonosis Control Center) from the streets of Araçatuba, SP, without previous serological inspection for leishmaniasis and ultimately destined to euthanasia for sanitary practices. These were male and female dogs between two and six years old and of various breeds and weights. The animals were anesthetized with 25 mg/kg sodium thiopental (Cristália, Brazil), and blood samples were drawn by cardiac puncture. The sera were stored at -20°C. Necropsies were performed following euthanasia using potassium chloride, and popliteal lymph nodes and fragments of the spleen and skin were collected and fixed in 10.0% buffered formalin.

This study was conducted in accordance with the ethical principles of animal experimentation adopted by the *Colégio Brasileiro de Experimentação Animal* and was approved by the Ethics Committee of the *Faculdade de Medicina* of the *Universidade de São Paulo,* Brazil (number 706/04).

Immunohistochemistry was performed on paraffin sections of the popliteal lymph nodes, spleens, and skin from each dog.[12] Briefly, paraffin-embedded sections were dewaxed and rehydrated. Antigen retrieval was conducted by steaming the sections in a 10 mM citric acid solution (pH 6.0) for 30 min in a water bath at 95°C. Endogenous peroxidase activity was quenched with 3.0% hydrogen peroxide, and unspecific interactions were blocked with a solution of 60 g/L powdered skimmed milk diluted in distilled water. Immunolabeling was performed with a mouse anti-*Leishmania* polyclonal antibody diluted 1/800 in 0.01 M phosphate-buffered saline (PBS) containing 1.0% bovine serum albumin at 4°C overnight. After washing, the sections were incubated with a biotinylated secondary antibody and then with a streptavidin-peroxidase complex from the LSAB kit (DakoCytomation, USA). Both incubations were performed at 37°C for 30 min. Color development was conducted for five min at room temperature, using 3-3-diamenobenzidine (Sigma, USA) at 60 mg/100 mL 0.01 M PBS containing 1.0% hydrogen peroxide. The sections were counterstained with hematoxylin, dehydrated, and mounted in resin**.** After a comparative analysis of all slides, tissue parasitism was considered negative (-) when the sample did not contain any parasites in 20 fields, low (+) when a sample contained 1-10 amastigotes/field, moderate (++) for 11-25 amastigotes/field, and high (+++) for more than 25 amastigotes/field, using the 40× objective.

To optimize the enzyme-linked immunosorbent assay (ELISA), we tested different concentrations of crude and soluble antigens of *L. (L.) infantum chagasi* (MHOM/BR/72/LD46), dilutions of control positive sera with low, moderate, and high titers, protein A, alkaline phosphatase conjugate, and levels of cut-offs. After checkerboard titrations, the best discriminative condition between positive (n = 10) and negative controls (n = 30) for each isotype was defined, and we performed the ELISA as follows: a suspension of stationary-phase promastigotes was disrupted by freeze-thawing, sonicated once for 60 sec in ice bath, and then centrifuged at 10,000 g for 20 min. The supernatant was collected and microplates were coated with 10 µg/mL of this soluble antigen in 0.1 M carbonate-bicarbonate buffer (pH 9.5) at 4°C overnight for IgG and with 20 µg/mL for IgG1, IgG2, and IgE. After blocking the wells with 10.0% powdered skim milk in 0.01 M PBS with 0.05% Tween (PBS-T), 100 µL of the diluted serum samples at ratios of 1:400 for IgG, 1:200 for IgG1 and IgG2, and 1:20 for IgE were added to each well and incubated at 37°C for 2 h for IgG detection or at 4°C overnight for IgG1, IgG2, and IgE. After washing with PBS-T, 100 µL of alkaline phosphatase-labeled secondary antibodies at dilutions of 1:2,000 for IgG, 1:500 for IgG1 and IgG2, and 1:50 for IgE (Bethyl Laboratories, USA) were added to each well, and the plates were incubated at 37°C for 1h. After further washing, 100 µL/well of 1.0 mg/mL pNPP (Sigma, USA) in 0.1 M carbonate-bicarbonate buffer pH 9.5 was added, and the samples were incubated for 30 min at room temperature. The reaction was stopped with 50 µL/well of 1 M NaOH, and absorbance was measured at a wavelength of 405 nm using an ELISA reader. The minimum level of detection (cut-off) was set at the mean optical density obtained from the negative controls plus three standard deviations.

To measure the amount of cross-reactivity with *L. (L.) infantum chagasi* ELISA, sera of dogs with ehrlichiosis (n = 17), babesiosis (n = 9), toxoplasmosis (n = 9), neosporosis (n = 6), Chagas disease (n = 6), toxocariasis (n = 9), and dirofilariasis (n = 6) were tested.

The clinical signs assessed were as follows: lymphadenopathy, splenomegaly, hepatomegaly, weight loss, skin lesions (desquamation, alopecia, ulcers, and nodules), and onychogryphosis. Serum biochemistry was conducted for all dogs. The animals were initially divided into two groups: symptomatic, including dogs displaying any clinical sign compatible with canVL and/or biochemical abnormalities, and asymptomatic, composed with apparently healthy animals with serum protein levels of < 8.5 mg/dL and serum creatinine levels within normal limits, according to the International Renal Interest Society (IRIS, 2006).[a]

Considering the parasitological, laboratory, and clinical findings, the animals were finally classified as follows: asymptomatic with (AP+) and without (AP-) detectable parasitism by immunohistochemistry and symptomatic with (SP+) or without (SP-) *Leishmania* visualization in tissues.

Statistical analysis was performed with SPSS for Windows version 12.0. Kruskal-Wallis (Mann-Whitney Rank Sum test) was used to compare the anti-*Leishmania* antibody levels (optical density values) and the parasite load between groups. Spearman's rank test (r_s) was used to correlate antibody levels with the degree of tissue parasitism and clinical condition. The level of significance for all cases was set at $p \leq 0.05$. Kappa test (κ) was used to quantify the agreement between IgG results and other isotypes.

RESULTS

Of the 134 dogs studied, 57 (42.5%) were asymptomatic and 77 (57.5%) were symptomatic (Table 1). The most frequent clinical signs in the symptomatic population included cutaneous lesions (84.4%), lymphadenomegaly (76.6%), splenomegaly (66.0%), weight loss (45.5%), onychogryphosis (28.6%), and hepatomegaly (27.3%).

Tissue parasitism was detected in 21 (36.8%) of the asymptomatic cases (AP+ group) and in 52 (67.5%) of the symptomatic dogs (SP+ group). *Leishmania* was not detected in 36 (63.2%) of the asymptomatic dogs (AP- group) and in 25 (32.5%) of those with clinical signs suggestive of canVL (SP- group) (Table 1). The lymph nodes were the most common positive site in the infected animals (n = 73; 100%), followed by the spleen, (n = 60; 82.2%), and skin (n = 47; 64.4%) (Table 2). The degree of parasitism in all tissues did not differ between AP+ and SP+ dogs (p > 0.05).

A high degree of variability in the levels of anti-*Leishmania* antibody types and subtypes was observed among dogs (Figure). Total IgG was the most abundant *Leishmania*-specific antibody (optical density median = 0.61), followed by IgG2 (optical density median = 0.50), IgE (optical density median = 0.13),

and IgG1 (optical density median = 0.11) (Figure). In the infected groups, 20 (95.2%) AP+ and 51 (98.1%) SP+ dogs tested positive for *Leishmania*-specific IgG antibodies (Table 3). In the uninfected dogs, positivity for *Leishmania*-specific IgG antibodies was observed in both AP- (44.4%) and SP- (60.0%) animals (Table 3). The results examining the IgG2 showed high agreement ($\kappa > 0.90$), IgE a moderate to substantial agreement ($\kappa < 0.79$), and IgG1 a fair to moderate agreement ($\kappa < 0.50$) with those obtained for total IgG.

With respect to IgE antibodies, 71.4% (AP+) and 80.8% (SP+) of the infected dogs were positive. Uninfected dogs also had *Leishmania*-specific IgE in their sera, 27.8% in the AP- group, and 44.0% in the SP- group (Table 3).

The levels of IgG and IgE did not significantly differ between the clinically symptomatic and asymptomatic groups (SP- *versus* AP-, and SP+ *versus* AP+; p > 0.05), but the infection status showed higher levels of both types of antibodies ($p \leq 0.05$) (Figure). A correlation between total IgG and IgE levels ($p \leq 0.05$) and the amount of parasitism was observed in the lymph node ($r_s = 0.62$ and $r_s = 0.55$, respectively), spleen ($r_s = 0.56$ and $r_s = 0.47$, respectively), and skin ($r_s = 0.51$ and $r_s = 0.52$, respectively) and also IgG1, although in a weak fashion: lymph node ($r_s = 0.26$), spleen ($r_s = 0.30$), and skin ($r_s = 0.31$). IgG2 showed no correlation.

Table 1. Absolute number and percentage of infected and uninfected dogs enrolled in the study, in relation to clinical status. Araçatuba, SP, Southeastern Brazil, 2006. (N = 134)

Clinical status	Infected		Uninfected		Total	
	n	%	n	%	n	%
Asymptomatic dogs	21	36.8	36	63.2	57	42.5
Symptomatic dogs	52	67.5	25	32.5	77	57.5
Total	73	54.5	61	45.5	134	100

Table 2. Frequencies of the parasite loads in the spleen, lymph node, and skin of symptomatic (SP+) and asymptomatic (AP+) infected dogs in a visceral leishmaniasis-endemic area. Araçatuba, SP, Southeastern Brazil, 2006.

Dogs	Spleen[a]						Lymph node[a]						Skin[a]					
	(+)		(++)		(+++)		(+)		(++)		(+++)		(+)		(++)		(+++)	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
SP+ (n = 52)	16	30.8	11	21.2	14	26.9	24	46.2	10	19.2	18	34.6	9	17.3	12	23.1	15	28.9
AP+ (n = 21)	5	23.8	5	23.8	9	42.9	7	33.3	5	23.8	9	42.9	5	23.8	3	14.3	3	14.3
Total (n = 73)	21	28.8	16	21.9	23	31.5	31	42.5	15	20.5	27	37.0	14	19.2	15	20.6	18	24.7

SP+: symptomatic with parasitism dogs; AP+: asymptomatic with parasitism dogs
[a] Parasite load was graduated as: + low, ++ moderate and +++ high.

Table 3. Percentage of positive results for each type of anti-*Leishmania* antibody detected in dogs from an endemic area, according to their clinical and parasitological conditions.

Dogs	Asymptomatic								Symptomatic							
	IgG		IgG1		IgG2		IgE		IgG		IgG1		IgG2		IgE	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Infected	20	95.2	13	61.9	20	95.2	15	71.4	51	98.1	37	71.2	50	96.2	42	80.8
Uninfected	16	44.4	8	22.2	15	41.7	10	27.8	15	60.0	7	28.0	14	56.0	11	44.0
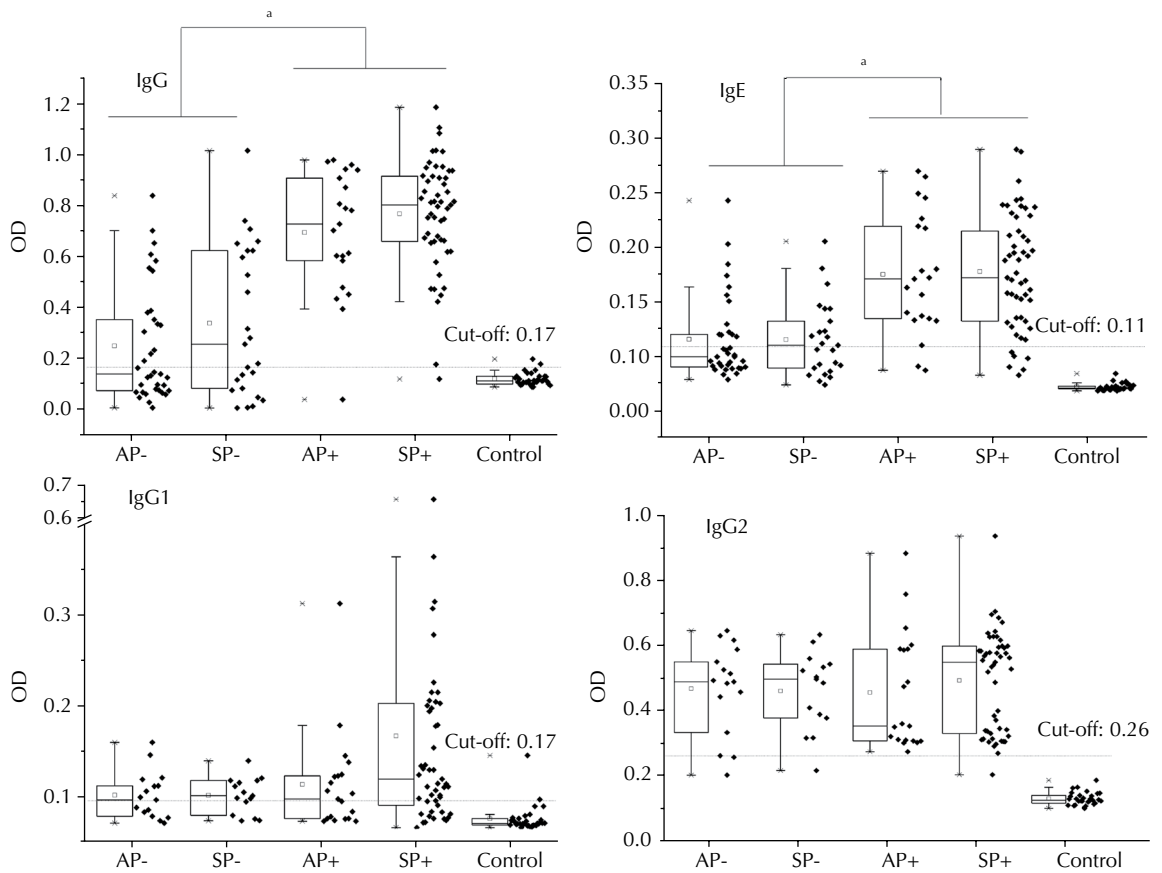
Figure. Dot-plot of the optical densities of the canine sera and the respective cut-offs for IgG, IgG1, IgG2, and IgE.

[a] p ≤ 0.05

Cross-reactivity with the *L. (L.) infantum chagasi* ELISA was detected with IgG antibodies present in the serum from two of nine animals diseased with babesiosis (22.2%), and with IgE from 1 out of 17 animals (5.9%) with ehrlichiosis, and 1 out of 9 (11.0%) with toxoplasmosis.

DISCUSSION

The non-specific and varied clinical manifestations of canVL, which can overlap with the symptoms of other canine infections, and the lack of external signs in the infected asymptomatic animals make the diagnosis of leishmaniasis one of the most significant problems concerning the disease, which in most cases may render ineffective epidemiological surveillance and VL control measures.[24]

It is clear that veterinarians and practitioners of control programs aim to diagnose a case of VL with certainty. Thus, we investigated the potential of employing serology for examining the levels and the positivity of anti-*Leishmania* IgG (total and subclasses) and IgE antibodies in a canine population living in a region with high canVL endemicity, taking into account their parasitological condition.

This study revealed four groups of dogs living in such region. There were asymptomatic animals infected with *L. (L.) infantum chagasi* (AP+) or not (AP-), symptomatic dogs with confirmed tissue parasitism (SP+), and dogs presenting signs related to canVL without detectable *Leishmania* infection (SP-).

The sera of both asymptomatic and symptomatic dogs contained detectable levels of all antibodies inspected, even those without *Leishmania* tissue parasitism. IgG was by far the most predominant *Leishmania*-specific antibody and IgG2 was the major subclass present, a result observed in previous reports,[23,25] but not by Quinnell et al,[18] who observed high levels of *Leishmania*-specific IgG1 in sick and asymptomatic parasite-positive dogs compared to the uninfected ones. In our study, all four groups were low responders for IgG1, including the SP+ dogs. Although IgG1 was higher in the SP+ dogs than in the other groups, its level in asymptomatic infected animals (AP+) did not differ from those of the non-parasitized dogs, excluding its possible use for diagnostic purposes.

Few studies have assessed IgE in canVL, but some have shown that high IgE production in symptomatic dogs strongly correlates with active disease and high

parasitism.[6,19,20] In the present study, all groups produced small amounts of *Leishmania*-specific IgE antibodies and were not significantly different from those of symptomatic dogs and their asymptomatic counterparts (AP- *versus* SP-; AP+ *versus* SP+), which was similar to the findings of Amorim et al.[2] No correlation between the levels of both IgG and IgE and the clinical status was observed in the present study. However, correlation between these antibodies and infection condition was observed, specifically with the detection of amastigotes in tissues (skin, lymph nodes, or spleen), and with the levels of the parasite burden, which did not differ between the asymptomatic (AP+) and sick dogs (SP+).

In this investigation, 98.1% and 80.8% of the symptomatic infected animals (SP+) tested positive for *Leishmania*-specific IgG and IgE antibodies, respectively, which demonstrate that serology adequately reflected the *Leishmania* infection status of these dogs, especially with respect to IgG. Regarding the infected but asymptomatic dogs (AP+), 95.2% were positive for *Leishmania*-specific IgG, but IgE failed to detect 28.6% of these animals. Our major concern was that a high percentage of the asymptomatic animals without *Leishmania* tissue parasitism (AP-) also tested positive for *Leishmania*-specific IgG (44.4%), IgG subclasses and IgE (27.8%). This finding may be attributable to recent infection without the establishment of tissue parasitism. Another is that these dogs have suffered a transient infection, characterized by initial parasitological positivity that might have become and remained negative thereafter,[16] and had already controlled the *Leishmania* infection at the time we performed sera collection. Therefore, the *Leishmania*-specific antibodies detected in the AP- group could be the result of specific antibodies that were maintained in the bloodstream after recovery. However, cross-reactivity was not ruled out, which could also explain the anti-*Leishmania* antibodies found in the SP- group, with clinical signs related to canVL, although proven to be *Leishmania*-negative.

Leishmania antigens used in serological tests are recognized by antibodies present in the sera of dogs infected with a plethora of agents, including protozoans, bacteria, fungi, intestinal and non-intestinal worms, and ectoparasites.[3,8,11,17,21,22] Among the sera we tested, cross-reactivity with IgG was observed in dogs with babesiosis, and with IgE in those with ehrlichiosis and toxoplasmosis. Thus, we may not exclude that SP- dogs were infected with other agents that can cause some clinical signs compatible with canVL, such as alopecia or desquamation, sole signs presented in a portion of our symptomatic population, which highlights the importance of conducting a differential diagnosis, as evidenced by others.[4,24]

Among the four isotypes investigated, IgG would be the only antibody that should be used for screening VL due to its high positivity in infected dogs (AP+ and SP+), but the overall results pointed that the use of serology as a sole diagnostic method is ineffective in determining if dogs are actually infected with *Leishmania*, thus requiring additional confirmatory tests.

With regard to the parasitological method used in this study to define infection status, immunohistochemistry is a technique that could provide results that were comparable to those obtained with conventional polymerase chain reaction (PCR) and is sensitive enough to detect low parasite load such as in samples from asymptomatic dogs.[13] Moreover, to ensure precise immunohistochemical parasitological diagnosis, the presence of parasites was examined in three different tissues, namely, skin, spleen, and lymph nodes, which are the most likely sites of high parasitism during a *L. (L.) infantum chagasi* infection.[13,19] For those reasons, there is a strong probability that both groups in which no amastigotes were visualized in the tissues inspected, AP- and SP-, truly included only unparasitized animals.

In conclusion, we characterized the serological and the infection statuses of dogs living in a VL-endemic region and demonstrated that serology, even using an optimized technique with specific *L. (L.) infantum chagasi* antigens, was not reliable method for discriminating between *Leishmania*-infected and uninfected dogs through any of the four isotypes tested. This complex serological scenario that we observed in the endemic area reflects the difficulties experienced by veterinary clinicians and coordinators of control programs. Thus, to enhance the effectiveness of control measures, a re-evaluation of the canine serodiagnosis is necessary, especially in countries where VL is a major public health problem.

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