

Associations among immunological, parasitological and clinical parameters in canine visceral leishmaniasis: Emaciation, spleen parasitism, specific antibodies and leishmanin skin test reaction

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Received 13 August 2007; received in revised form 27 December 2007; accepted 8 February 2008

Abstract

Associations among parameters commonly used as markers of infection by *Leishmania* sp., or of susceptibility to visceral leishmaniasis, were investigated in 325 stray dogs from an area where this disease is endemic. Evidence of infection (presence of *Leishmania* in splenic cultures, positive leishmanin skin test (LST) or detection of anti-*Leishmania* antibody activity in the serum) was found in 57% of the animals. Both evidence of weight loss (χ^2 -test, $P = 0.0005$) and presence of specific antibody activity in the serum (χ^2 -test, $P < 0.0001$) were directly associated with positive splenic culture. The frequencies of animals with positive splenic culture were directly correlated with the intensities of antibody activity in the serum as measured by ELISA (relative risk of 3.4 for animals with moderate antibody levels and relative risk of 8.43 for animals with high-antibody levels). A negative association was observed between positive leishmanin skin test results and emaciation (χ^2 , $P = 0.0089$). Furthermore, animals with positive splenic cultures and negative leishmanin skin test results had higher levels of total serum IgG (Kruskal–Wallis test, $P = 0.001$) and IgG2 (Kruskal–Wallis test, $P = 0.05$) than animals with negative splenic cultures, and were more emaciated than animals with negative LST results and positive splenic cultures. The data presented herein suggest that associating these common parameters may improve their performance in predicting susceptibility to canine visceral leishmaniasis.

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Keywords: Leishmanin skin test; Canine visceral leishmaniasis; *Leishmania chagasi*; *Leishmania infantum*; Spleen; Immunoglobulin isotypes

1. Introduction

Leishmania chagasi/Leishmania infantum is the causal agent of human and canine visceral leishma-

niases (CVL) in Europe and America (Paranhos-Silva et al., 1996; Alvar et al., 1997). Recovery or resistance to these diseases is associated with cellular, and not humoral, immune responses (Carvalho et al., 1992; Alvar et al., 1997). A large effort has been made to identify resistance and susceptibility markers to visceral leishmaniasis in dogs, based on the profile of the anti-parasite immune response in combination with clinical and parasitological data. The main parameters used to

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access the status of susceptibility or resistance in CVL are: (i) the presence of parasites in lymph nodes or internal organs (Pinelli et al., 1994; Paranhos-Silva et al., 2003; Gradoni et al., 2005; Lemesre et al., 2005); (ii) the parasite burden in different organs (Molano et al., 2003; Reis et al., 2006); (iii) the levels of specific antibodies (Pinelli et al., 1994; Paranhos-Silva et al., 2003); (iv) the specific antibody isotypes (Leandro et al., 2001; de Oliveira Mendes et al., 2003; Quinnell et al., 2003); (v) the leishmanin skin test (LST) reaction (Pinelli et al., 1994; Martinez-Moreno et al., 1995; Solano-Gallego et al., 2000; Borja-Cabrera et al., 2002; Molano et al., 2003); (vi) the intensity of *in vitro* lymphoproliferative response and cytokine production in the presence of *Leishmania* antigen (Pinelli et al., 1994; Santos-Gomes et al., 2002; Lemesre et al., 2005); and (viii) the presence of clinical signs of disease (Oliveira et al., 1993; Pinelli et al., 1994; Martinez-Moreno et al., 1995; Leandro et al., 2001; Paranhos-Silva et al., 2003; Gradoni et al., 2005; Reis et al., 2006). None of these parameters is definitive and the determination of some of them requires extensive laboratory work. This may be the reason why many studies on visceral leishmaniasis use serology, parasite identification, leishmanin skin test, and clinical data to assess the state of host immunity. The relationship between these tests with susceptibility to leishmaniasis has been defined in human beings (Badaro et al., 1986; Carvalho et al., 1992) and in murine models of visceral and cutaneous leishmaniasis (Honore et al., 1998; Melby et al., 2001; Guimaraes et al., 2006). The accurate interpretation of such parameters in CVL still requires additional study.

In this work, we studied the distribution of the following parameters in dogs naturally infected with *L. chagasi*: specific antibody activity in the serum, leishmanin skin test results, emaciation, and splenic parasitism. The study was aimed at determining the degree of concomitance of these parameters in individual animals. Our hypothesis was that parameters usually considered markers of susceptibility to CVL, such as spleen parasitism, emaciation and high levels of anti-*Leishmania* antibodies would be frequently found in the same animal and, conversely, such susceptibility markers would be absent or less intensely expressed in dogs with positive LST result (which is considered a marker of resistance to CVL by some authors). Understanding the distribution of these parameters in *L. chagasi*-infected animals may contribute to their more appropriate use in the evaluation of new therapies and candidate vaccines.

2. Material and methods

2.1. Animals

325 stray dogs of different breeds and estimated ages were collected from the streets of the town of Jequié (Bahia, Brazil), at different periods, between 1997 and 2006, in collaboration with the endemic disease surveillance program of the Bahia State Health Service. Visceral leishmaniasis is endemic to the urban area of Jequié (Dos Santos et al., 1993; Paranhos-Silva et al., 1996) and *L. chagasi* has been the only species isolated from dogs (Paranhos-Silva et al., 1996) and human beings (Paranhos-Silva et al., 2001) with the disease. The dogs were identified with sequential numbers and subjected to the tests/examinations described below. The animals were kept for at least 48 h in the municipal kennel. If *Leishmania* infection was confirmed or if the dogs were not claimed by their rightful owners, they were sacrificed as recommended by the Brazilian program for control of zoonotic diseases (http://portal.saude.gov.br/portal/saude/visualizar_texto.cfm?idtxt=22139). All experiments were conducted in accordance with the Oswaldo Cruz Foundation guidelines for experimentation with animals (http://www.presidencia.fiocruz.br/vppdt1/com_etn_uso_anim_.php).

2.2. Parasite antigens

L. chagasi (MHOM/BR2000/Merivaldo2) and *Leishmania amazonensis* (Leila strain, MHOM/BR88/BA-125) promastigotes were characterized by isoenzymes and reactivity with species-specific monoclonal antibodies (Dr. G. Grimaldi, Fundação Oswaldo Cruz, Rio de Janeiro). The parasites were grown *in vitro* using Schneider's medium (Gibco) containing 10% of fetal calf serum (Cultlab). They were washed three times with 0.15 M phosphate-buffered saline, pH 7.4 (PBS) and lysed by repeated cycles of freezing and thawing followed by sonication. Antigen batches to be used in ELISA were processed in the presence of a combination of enzymatic inhibitors: 2 µg/mL of phenylmethanesulfonyl fluoride (PMSF), 2 µg/mL of *N-p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 2 µg/mL of *N*-alfa-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), 2 µg/mL of 4-nitrophenyl *p'*-guanidinobenzoate (NPGb) and 2 µg/mL of *N*-(*trans*-epoxysuccinyl)-L-leucine 4-guanidinobutylamide; *trans*-3-carboxyoxirane-2-carbonyl-L-leucylagmatine; *trans*-epoxysuccinyl-L-leucylamido (4-guanidino) butane; L-*trans*-3-carboxyoxiran-2-carbonyl-L-leucylagmatine (E64). Antigen batches to be used in the leishmanin skin tests

were sterily prepared and no enzyme inhibitors or preserving agents were added. Antigen suspensions were stored at -70°C until use.

2.3. *Leishmanin skin test*

L. chagasi antigen was diluted in saline to a final concentration of 2.5 mg of protein per mL. Each animal was intradermally injected with 100 μL volumes of the antigen solution at different sites of the abdominal skin (Baleeiro et al., 2006). As a control, 100 μL of the diluent (saline) was injected at a different site on the abdominal skin. Forty-eight hours after the injections, the sizes of indurations were measured by observers without previous knowledge of the site of injections. In order to reduce experimental variations, both the tests and their readings were always performed by the same two authors (WLCS and MPS for the leishmanin injection; WLCS and GGSO for the readings). The readings were aided by the use of a ballpoint pen. A cellotape stamp of the measured area was always taken and preserved in the experimental book, for eventual confirmation if necessary. The results are expressed as the mean of the two largest perpendicular axes of the induration area. Skin reactions with induration size equal or larger than 5 mm were considered positive.

2.4. *Anti-Leishmania antibody activity in the serum*

This was measured by ELISA as previously described (Baleeiro et al., 2006). Briefly, 96-well plates were sensitized with crude antigen obtained from *L. amazonensis* or *L. chagasi*. The plates were washed, blocked with PBS containing 10% of skimmed milk, and the serum of each animal was applied at the dilution of 1:400, followed by an anti-dog total IgG peroxidase conjugate (Sigma). The enzymatic reaction was developed with tetramethyl benzidine (Sigma). A cut-off was established using serum samples obtained from 48 animals from Porto Alegre (Rio Grande do Sul State, Brazil) and from Salvador (Bahia State, Brazil). Both Salvador and Porto Alegre are non-endemic areas for visceral leishmaniasis. The mean \pm S.D. of the anti-*Leishmania* antibody activity in the serum of this group of animals was 0.117 ± 0.052 with Gaussian distribution (Kolmogorov–Smirnov test, 0.062). Values greater than the mean plus three S.D. values of the results obtained from 48 health dogs were considered positive. The measurements of IgG1 and IgG2 anti-*Leishmania* antibodies in the serum were performed using an ELISA similar to the one described above, with the following modification: horseradish peroxidase–anti-dog IgG1 or

anti-IgG2 conjugates, both produced in goat and supplied by Bethyl Laboratories Inc., replaced the anti-total IgG conjugate. All measurements were performed in duplicate and the results were confirmed in another independent assay.

A group of 203 animals (all animals from number 31 to 249 for which serum samples were available) were comparatively examined for their serum anti-*Leishmania* antibody activity. In order to allow for a more accurate comparison among blood samples tested at different periods, minimizing the occurrence of problems such as variability among assays and loss of reactivity of stored sera, the following procedures were adopted: (1) a pool of sera collected from three dogs naturally infected with *L. chagasi*, having high titers of anti-*Leishmania* specific antibody, was added in quadruplicate and used to normalize results between assays. This pool of sera was used at the dilution corresponding to the middle point titer of its anti-*Leishmania* antibody activity. Among these 203 animals, the intensity of anti-*Leishmania* antibody response in the serum varied between 10 and 406% of the O.D. values obtained using the reference pool of positive sera. (2) In order to control for the preservation of the serum samples, the results obtained in these tests were compared with those obtained during the initial tests performed at the moment of capture of the animals. No decrease in serum reactivity was observed. Following these measurements the animals were distributed into four categories according with the intensity of antibody response: negative serology: relative O.D. $\leq 60\%$; positive low: relative O.D. of 61–120%; positive intermediate: relative O.D. of 121–180% and positive high: relative O.D. $\geq 181\%$.

2.5. *Parasite isolation in cultures*

Immediately after sacrifice, splenic samples were collected from the animals by puncture using an 18 G \times 38 mm gauge needle connected to a 20-mL syringe. Spleen aspirates were cultured in biphasic agar–blood–Schneider’s medium, supplemented with 10% fetal bovine serum as previously described (Paranhos-Silva et al., 2003). Cultures were examined weekly for identification of promastigotes, and examinations continued for a period lasting up to 2 months if they remained negative.

2.6. *Nutritional status*

Animals were assigned to two categories of nutritional status: (i) with evidence of emaciation (if

prominence of bones of vertebral column, iliac crest, and ribs was present); (ii) normal (if bone prominence was absent).

2.7. Expression and significance of the results

Numerical data are shown in tables and graphs, representing absolute values, means or proportions when stated. The significance of the differences among the means of the groups was tested using the Kruskal–Wallis test. When the test was found significant, the difference between two groups was identified using Dunn's Multiple Comparison test (Glantz, 1997). For comparisons involving proportions, the χ^2 -test with Yates' correction or Fisher's exact probability test were used when recommended. The critical level of significance was established at $P \leq 0.05\%$.

3. Results

3.1. Main characteristics of the animals used in the study

Among the 278 animals for which gender was recorded, 167 (60.1%) were male and 111 (39.9%) were female. Nutritional status was recorded for 301 animals and signs of weight loss were observed in 122 (40.5%) (Table 1).

3.2. Evidence of natural infection by *Leishmania*

We considered as evidence of *Leishmania* infection the presence of a positive splenic culture, positive serology or positive LST result.

Splenic cultures were performed in 282 animals and were positive for *Leishmania* in 84 (29.8%).

Table 1
Characteristics of the population of 325 stray dogs from an endemic area of infection caused by *L. chagasi*

Parameter	N	%
Gender		
Male	167	60.1
Female	111	39.9
Total	278	100
Signs of infection/disease		
Emaciation (EN = 301) ^a	122	40.5
Positive spleen culture (EN = 282)	84	29.8
Positive ELISA (EN = 307)	132	43.0
Positive leishmanin skin test (EN = 282)	44	15.6

^a Total number of animals examined in each category.

The presence of anti-*Leishmania* antibody activity was investigated in the sera of 307 animals and was positive in 132 (43%). The values of optical density in the ELISA performed with the serum of the 203 comparatively examined animals varied from 10 to 406% of that observed with a reference positive serum. The distribution of the values were, however, non-Gaussian (KS distance = 0.207, $P < 0.001$), skewed to the right (Pearson's skewness index = 1.829 ± 0.171), and displayed no obvious bimodal profile. The median was 47%, the 25th percentile was 26% and the 75th percentile was 85%. The values were distributed into categories of negative, low, intermediate or high optical densities as follows: 127 (62.6%) were negative (relative O.D. ≤ 60), 41 (20.2%) were low positive (relative O.D. of 61–120); 14 (6.9%) were intermediate positive (relative O.D. of 120–180) and 21 (10.3%) were high positive (Table 2).

The leishmanin skin test was performed in 282 animals (Table 1). Induration areas of 2.5–12.5 mm were observed in 61 animals (21.6%), and in 44 (15.6%) of the animals, the induration areas were equal to or larger than 5 mm.

Evidence of *Leishmania* infection (positive splenic culture, serology or LST) was present in 171 (54.1%) of the 316 animals subjected to at least one of the tests. Among the 252 dogs subjected to all three tests (splenic culture, serology and LST), 109 (43.2%) were negative in all and 143 (56.8%) were positive in at least one of these tests (Fig. 1).

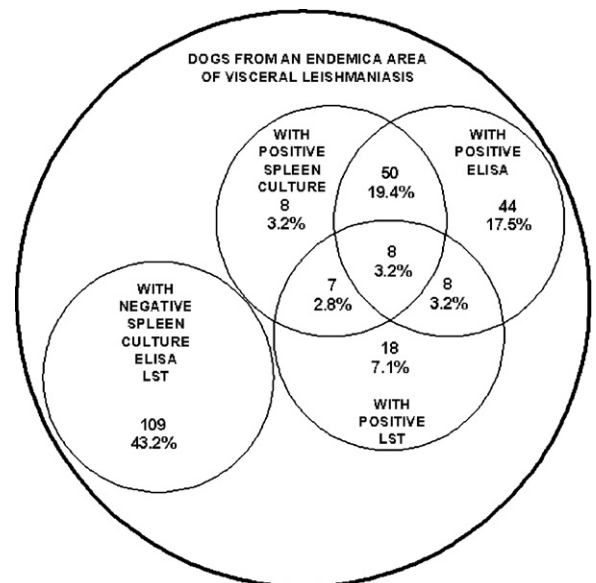


Fig. 1. Distribution of dogs from an endemic area of visceral leishmaniasis according to different markers of infection and immunity to the disease. LST = leishmanin skin test.

Table 2

Distribution of dogs from an endemic area of visceral leishmaniasis according to the intensity of the anti-*Leishmania* antibody response and the leishmanin skin test

Immunological test	N	Spleen culture		Prevalence ratio	95% C.I.	P-value
		Total	%			
Serological categories (O.D.%)						
Negative (0–60)	127	12/113	10.7			
Positive low (61–120)	41	8/38	21.1	1.98	0.88–4.48	0.1724
Positive intermediate (121–180)	14	4/12	33.3	3.14	1.20–8.22	0.0476
Positive high (181–406)	21	17/19	89.5	8.43	4.83–14.70	<10 ⁻⁶
LST categories (average axis) ^a						
Negative (0.0–4.9 mm)	238	59/216	27.3			
Positive low (5.0–9.9 mm)	33	15/33	45.5	1.33	0.97–1.84	0.0550
Positive high (10.0–12.5 mm)	11	2/11	18.2	0.89	0.66–1.19	0.7505

Relationship with splenic parasitism.

^a LST: leishmanin skin test.

3.3. Associations between findings

The associations between each of the studied parameters (sex, emaciation, spleen parasitism, positive

LST result or serology against *Leishmania* antigens) are shown in Table 3 and Fig. 1.

No significant associations involving gender were observed (Table 3).

Table 3

Distribution and association of infection/disease markers in stray dogs from an endemic area of visceral leishmaniasis caused by *L. chagasi*

	Gender		ELISA		Emaciation		LST ^a	
	Male (%)	Female (%)	+	-	+	-	+	-
Spleen culture								
+	50 (34)	29 (29)	64 (53)*	17 (11)	46 (42)***	35 (22)	17 (39)	59 (27)
-	96 (66)	70 (71)	56 (46)	137 (89)	63 (58)	126 (78)	27 (61)	157 (73)
Total	146 (100)	99 (100)	120 (100)	154 (100)	109 (100)	161 (100)	44 (100)	216 (100)
ELISA								
+	72 (45)	46 (44)			60 (51)**	64 (37)	16 (39)	102 (44)
-	89 (55)	59 (56)			58 (49)	107 (63)	25 (61)	130 (56)
Total	161 (100)	105 (100)			118 (100)	171 (100)	41 (100)	232 (100)
Emaciation								
+	60 (37)	47 (45)	60 (48)**	58 (35)			9 (21)****	99 (44)
-	101 (73)	58 (55)	64 (52)	107 (65)			34 (79)	128 (56)
Total	161 (100)	105 (100)	124 (100)	165 (100)			43 (100)	227 (100)
LST ^a								
+	27 (18)	11 (12)	16 (14)	25 (16)	9 (8)****	34 (21)		
-	123 (82)	83 (88)	102 (86)	130 (84)	99 (92)	128 (79)		
Total	150 (100)	94 (100)	118 (100)	155 (100)	108 (100)	162 (100)		
Gender								
Male			72 (61)	89 (60)	60 (56)	101 (64)	27 (71)	123 (60)
Female			46 (39)	59 (40)	47 (44)	58 (36)	11 (29)	83 (40)
Total			118 (100)	148 (100)	107 (100)	159 (100)	38 (100)	206 (100)

* $P < 0.0001$, ** $P = 0.032$, *** $P = 0.0005$ and **** $P = 0.0089$.

^a LST = leishmanin' skin test. χ^2 -test.

Table 4

Anti-*Leishmania* antibody activity in the serum and cases of emaciation in dogs with parasites in the spleen and negative or positive leishmanin' skin test from an endemic area for visceral leishmaniasis

Spleen culture	LST ^a	Anti- <i>Leishmania</i> antibody activity in the serum									Emaciation ^d	%
		Total IgG ^b			IgG1 ^c			IgG2 ^c				
		Median	[25–75%]	N	MEDIAN	[25%–75%]	N	Median	[25%–75%]	N		
–	–	38.5	[24.0–64.0]	104	0.22	[0.17–0.51]	7	0.47	[0.25–1.26]	8	55/151	36
–	+	44.0	[24.0–73.5]	23	0.19	[0.16–0.35]	11	0.91	[0.58–1.39]	11	6/26	23
+	+	52.5	[38.0–117.5]	10	0.20	[0.17–0.25]	8	0.84	[0.66–1.19]	8	3/17	18
+	–	179.0	[77.0–215.0]	25 ^e	0.21	[0.15–0.81]	19	1.51	[1.20–1.55]	19 ^f	37/56	66 ^g

^a LST = leishmanin skin test.

^b The O.D. expressed as the median and 25th and 75th percentiles of the relative values obtained using a pool of positive serum as reference.

^c The O.D. is expressed as the median and the 25th and 75th percentiles of the absolute values.

^d The number of animals with emaciation and total examined in each category.

^e Significantly higher than the than the groups with negative spleen culture (Kruskal–Wallis test, $P < 0.001$).

^f Significantly higher than the than the groups with negative spleen culture (Kruskal–Wallis test, $P < 0.05$).

^g Proportion is significantly higher than that observed the other groups (χ^2 -test, $P = 0.0001$).

A positive anti-*Leishmania* ELISA result was associated with a positive splenic culture; a positive splenic culture was observed in 64 (53%) out of 120 animals with positive serology and in 17 (11%) out of 154 animals with negative serology (χ^2 -test, $P < 0.0001$). The frequency of animals with positive spleen parasitism increased with each change in category of anti-*Leishmania* antibody activity in the serum (Table 2): positive splenic culture was observed in 12/113 (10.7%) of the animals with negative serology; in 8/38 (21.1%) of the animals in the low-positive category; in 4/12 (33.3%) of the animals in the intermediate-positive category; and in 17/19 (89.5%) in the high-positive category. The prevalence ratios were significantly higher for animals with intermediate-positive (3.14, $P = 0.0476$) or high-positive serology (8.43, $P < 10^{-6}$) than for animals in the other categories.

Positive serology was also associated with emaciation (Table 3). Evidence of weight loss was present in 60 (48%) out of 124 animals with positive ELISA and in 58 (35%) out of 165 animals with negative serology (χ^2 -test, $P = 0.032$).

Emaciation was associated with positive splenic culture. Among the 109 dogs with evidence of weight loss, 46 (42%) had a positive splenic culture and among the 161 animals without emaciation, 35 (22%) had positive splenic culture ($P < 0.0005$, χ^2 -test). Emaciation was also inversely associated with positive LST results: only nine (8.3%) out of the 108 animals with emaciation had a positive LST result, while 34 (21%) out of 162 animals without evidence of weight loss had a positive LST result (χ^2 -test, $P = 0.0089$).

No other significant association was observed between LST results and the other variables in the whole group of animals. Although the frequency of dogs with positive splenic culture appears to increase in animals with induration diameters between 5 and 9.9 mm, declining in animals with larger induration areas (Table 2), such differences were not statistically significant. In order to examine in more detail the relationship between LST results and *Leishmania* infection, we further investigated the association between LST results and other immunological parameters (total IgG, IgG1 and IgG2 anti-*Leishmania* antibody activity in the serum) and emaciation, in animals with or without spleen parasitism.

As shown in Table 4, the animals with positive splenic culture and negative LST results had higher anti-*Leishmania* antibody activity in serum, both for total IgG (Kruskal–Wallis test, $P < 0.001$) and IgG2 (Kruskal–Wallis test, $P < 0.001$), than the animals with positive LST without splenic parasitism or than the animals that were negative both in the LST and splenic cultures. Animals with a positive splenic culture and a negative LST result also displayed emaciation more frequently (χ^2 -test, $P < 0.0001$) than the animals of other groups.

4. Discussion

In this work, we examined the relationships among several parameters commonly used as markers of *Leishmania* infection and susceptibility to CVL. We found statistically significant associations of positive serology with both positive splenic culture for *Leishmania* and emaciation, indicating that positive

serology could be considered a marker for both infection and susceptibility to CVL. On the other hand, positive LST results were negatively associated with emaciation. In addition, animals with splenic parasitism only had significantly high anti-*Leishmania* antibody activity in the serum and higher frequency of emaciation when they were negative in the LST. Such findings suggest that the presence of an LST reaction may indicate that the immune system is controlling the infection even in animals with a positive splenic culture.

The association between positive serology and active parasitism in different organs has been reported in the literature (Killick-Kendrick et al., 1994; Ashford et al., 1995; Ozensoy et al., 1998; Paranhos-Silva et al., 2003; Barrouin-Melo et al., 2004, 2006; Baleeiro et al., 2006). We extended this observation by showing an exponential increase in the frequency of spleen parasitism paralleling the increased levels of anti-*Leishmania* antibodies in the serum. One possible explanation for this finding is that the parasite burden may determine the intensity of the antibody response (Reis et al., 2006), either by increasing the availability of minor parasite epitopes, such that a larger number of B lymphocytes are stimulated (Rhalem et al., 1999), or by favoring the development of an antibody-producing Th2 immune response (Bretscher et al., 1992). It is interesting to note that, in this work, 17 (11%) animals with negative serology had detectable *Leishmania* in their spleens. This finding may indicate either that those animals were at an initial stage of the infection, i.e., they had not yet developed the antibody response, or that they had a sub-clinical infection, in which splenic cultures were intermittently positive (Paranhos-Silva et al., 2003). In fact, 9 of the 17 animals with negative ELISA and positive splenic culture showed no evidence of weight loss, in contrast to the association that was observed between positive serology and emaciation in the entire group of animals with spleen parasitism.

In this series, 48% of the animals with positive serology were underweight. This prevalence is higher than that reported for dogs referred to veterinary clinics (Amusatogui et al., 2003). Although CVL leads to weight loss and caquexia (Pearson et al., 1990, 1992), a fact that has been confirmed in this study, other causes of emaciation, such as malnutrition and other infections (Andreotti et al., 2006; Barrouin-Melo et al., 2006; Trapp et al., 2006), should be considered in the present study. In fact, signs of weight loss were found in 33% of the animals with negative serology and negative splenic culture. Longitudinal studies are necessary in order to investigate the potential for other infections and

malnutrition to increase the CVL susceptibility of stray animals in endemic areas.

It is interesting to note that the associations of emaciation with spleen parasitism and positive serology were absent in animals with positive LST results, and that an inverse association was found between LST reactions and underweight status. These findings may reflect either an impairment of cellular immune response secondary to emaciation or a causal effect of disease progression on both immune energy and nutritional impairment. In order to evaluate whether the presence of the skin cellular hypersensitivity to *Leishmania* antigens would reflect changes in other parameters of immunity to the parasite, we examined the distribution of total IgG, IgG1 and IgG2 anti-*Leishmania* antibodies in these animals. Negative LST results were associated with higher levels of specific antibodies in the serum, especially for the IgG2 isotype in *Leishmania*-infected animals. Such observations seem to be in disagreement with Solano-Gallego and collaborators' results (2000), who found an association between specific IgG levels and LST results in a population of Ibizian hounds. Such disagreement may be due to genetic or phenotypic differences in the studied populations and/or differences in the interpretation of LST results. Erythematous responses without induration were considered positive by Solano-Gallego and collaborators' results (2000) and negative in our study.

Although a clear dichotomy of IgG1/IgG2a antibody response to *Leishmania* antigens is found in mice (Guimaraes et al., 2006), this is not well defined in dogs. Whereas most studies show an increase in IgG2 anti-*Leishmania* antibody activity in infected dogs (Deplazes et al., 1995; Bourdoiseau et al., 1997; Nieto et al., 1999; Leandro et al., 2001; Solano-Gallego et al., 2001; Almeida et al., 2005), the data on IgG1 antibodies is more controversial (Deplazes et al., 1995; Cavaliero et al., 1999; Solano-Gallego et al., 2001; Reis et al., 2006). In fact, Quinnell et al. (2003), using a set of monoclonal antibodies, were not able to demonstrate a clear polarity in antibody response in infected animals. In our study, we found an elevation of IgG2 anti-*Leishmania* antibodies in infected dogs, very similar to that observed with the total IgG, but not a clear association between IgG1 and infection or emaciation. Taken together, these data suggest that, if a dichotomous pattern of antibody isotypes exists in CVL, their determinants must be complex and do not lead to a clear-cut association with clinical status, presence of parasite in tissues or cellular hypersensitivity to *Leishmania* antigens. Studies on the comparability of

the reagents used for the detection of IgG isotypes in dogs may help to solve some of the discrepancies observed among different studies.

5. Conclusions

- (1) Our data confirm the associations between serology and active parasitism and the presence of clinical signs of disease in *L. chagasi*-infected dogs;
- (2) Our results show that the frequency of spleen parasitism increases with a relative risk of 8.43 in animals with high levels of anti-*Leishmania* antibody in the serum;
- (3) Our study also shows that *Leishmania*-infected dogs with negative LST results have positive serology and emaciation more frequently than *Leishmania*-infected dogs with positive LST.

Acknowledgments

This work was supported by the Brazilian Ministry of Science and Technology (Brazilian National Research Council – CNPq, grant no. 521260/98-1, and PRONEX) and FAPESB.

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