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ORIGINAL PAPER

A cross-sectional study on canine *Leishmania* (*L.*) *infantum chagasi* infection in Amazonian Brazil ratifies a higher prevalence of specific IgG-antibody response than delayed-type hypersensitivity in symptomatic and asymptomatic dogs

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Abstract This was a cross-sectional study which analyzed the prevalence and the clinical and immunological spectrum of canine *Leishmania* (*L.*) *infantum chagasi* infection in a cohort of 320 mongrel dogs living in an endemic area of American visceral leishmaniasis in the Amazonian Brazil by using, mainly, the indirect fluorescence antibody test (IFAT-IgG) and the delayed-type hypersensitivity (DTH), and the parasite research by the popliteal lymph node aspiration. The IFAT and DTH reactivity recognized three different immune response profiles: (1) $IFAT^{(+)}/DTH^{(-)}$ (107 dogs), (2) $IFAT^{(-)}/DTH^{(+)}$ (18 dogs), and (3) $IFAT^{(+)}/DTH^{(+)}$ (13 dogs), providing an overall prevalence of infection of 43 % (138/320). Thus, the specific prevalence of $IFAT^{(+)}/DTH^{(-)}$ 33.4 % (107/320) was higher than those of $IFAT^{(-)}/DTH^{(+)}$ 5.6 % (18/320) and $IFAT^{(+)}/DTH^{(+)}$ 4.0 % (13/320). Moreover, the frequency of these profiles among 138 infected dogs showed that the $IFAT^{(+)}/DTH^{(-)}$ rate of 77.5 % (107/138) was also higher than those of 13.0 % (18/138) of $IFAT^{(-)}/DTH^{(+)}$ and 9.5 % (13/138) of $IFAT^{(+)}/DTH^{(+)}$ rates. The frequency of asymptomatic dogs (76 %—105) was higher than those of symptomatic (16.6 %—23) and oligosymptomatic ones (7.4 %—10). A total of 16 (11.6 %) *L.* (*L.*) *i. chagasi* isolates were obtained from infected dogs, all from the $IFAT^{(+)}/DTH^{(-)}$ profile: 41 % (9/22) from symptomatic,

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33.3 % (3/9) from oligosymptomatic, and 5.2 % (4/76) from asymptomatic dogs. These findings strongly suggested that despite the higher frequency of asymptomatic dogs (76 %—105), the majority (72.4 %—76) was characterized by the IFAT$^{(+)}$/DTH$^{(-)}$ profile with a doubtful immunogenetic resistance against infection.

Introduction

The New World canine visceral leishmaniasis (CVL) is a parasite protozoal zoonosis widely spread in most countries of Latin America and caused specifically by *Leishmania* (*L.*) *infantum chagasi* Lainson and Shaw 2005 (Silveira and Corbett 2010). In contrast to this, however, the New World canine tegumentary leishmaniasis (CTL) may be due to a variety of *Leishmania Viannia* spp., such as *L.* (*V.*) *braziliensis* (Mayrink et al. 1979), *L.* (*V.*) *panamensis* (Herrer and Christensen 1976), *L.* (*V.*) *peruviana* (Herrer 1951), and *L.* (*V.*) *colombiensis* (Delgado et al. 1993). Recently, two single CVL cases were associated to another commonly dermotropic agent of human disease, *Leishmania* (*L.*) *amazonensis*, in Araçatuba municipality, São Paulo State, Brazil, but the serological diagnosis of both cases through the indirect fluorescence antibody test (IFAT) had unexpected negative results and the diagnosis of the disease was only confirmed by PCR assay (Tolezano et al. 2007). In general, it is assumed that CVL always precedes the emergence of a new focus of the human disease, American visceral leishmaniasis (AVL). Moreover, although *L.* (*L.*) *i. chagasi* infection might be the cause of severe canine pathology, the majority of infected dogs are asymptomatic for extended periods (Madeira et al. 2004; Queiroz et al. 2008). Thus, it has been noted that in the endemic area of Mediterranean region in Europe, such as in Spain and Italy, where the causative agent of infection is *Leishmania* (*L.*) *infantum*, a parasite closely related to *L.* (*L.*) *i. chagasi*, this asymptomatic status of infected dogs has ranged from 3 months to nearly 7 years (Solano-Gallego et al. 2001; Oliva et al. 2006).

In Brazil, despite some studies designed to characterize the clinical and immunological status of canine *L.* (*L.*) *i. chagasi* infection, this has mainly been carried out by using parasitological and serological (IgG-antibody response) methods for the diagnosis of CVL, which has raised some difficulties concerning a complete view of the canine immune response against infection (Paranhos-Silva et al. 1996; Ikeda-Garcia et al. 2007; Ferreira et al. 2007). In other words, these studies have generally used only serological methods, such as IFAT or enzyme-linked immunosorbent assay (ELISA), to determine the immunodiagnosis of canine *L.* (*L.*) *i. chagasi* infection, leading to an underestimation of the possibility that certain clinical and/or immunological features of this infection would also be associated with the T-cell immune response of infected dogs living in an endemic area. This is the situation, for example, of canine infection of Mediterranean region in Europe where the T-cell immune response of *L.* (*L.*) *infantum*-infected dogs has already been demonstrated principally in subclinical or asymptomatic dogs, either by the lymphocyte proliferative assay or delayed-type hypersensitivity (DTH) skin reaction, confirming its likely role on the apparent immune resistance against canine infection (Cabral et al. 1992, 1993, 1998; Pinelli et al. 1994; Cardoso et al. 2007).

More recently, however, some studies in the northeast region of Brazil used both IgG-antibody response performed by ELISA and delayed-type hypersensitivity (DTH), elicited by the leishmanin skin test (LST) to evaluate the humoral and T-cell immune responses of naturally infected dogs from the endemic area (Baleeiro et al. 2006; Dos-Santos et al. 2008; Pinheiro et al. 2009). Nevertheless, despite these studies that have dealt with both T-cell and humoral immune responses of naturally infected dogs, there was not any impact withdrawn concerning their role on the clinical development of canine infection in an endemic area.

In Pará State, Amazonian Brazil, we have just introduced a diagnostic approach based on the IFAT and DTH immunodiagnostic methods for simultaneously evaluating the humoral and T-cell immune responses of a cohort of mongrel dogs from an AVL endemic area, aiming to improve our current understanding concerning the repertory of immune responses responsible for the development of the clinical spectrum of the canine *L.* (*L.*) *i. chagasi* infection. In the first step, we present here the results of a cross-sectional analysis regarding the prevalence as well as the clinical and immunological spectrum of canine infection, based mainly on the IFAT and DTH assays, but also on conventional parasitology by culturing samples of popliteal lymph node aspiration.

Materials and methods

Study area

This study was carried out in Santa Maria village, situated 7 km from the administrative center of Barcarena municipality (01° 30′ S; 48° 37′ W), which is considered to be within the metropolitan region of Belém, the capital of Pará State, in north of Brazil. The climate is typically equatorial, with an average temperature of 28 °C and high humidity. The annual rainfall in the region is of 2,500 mm or more, with the period from January to June forming the principal rainy season. The field activities of this study conducted from March to May (2010), within the period (January–May) of the highest density of the sandfly vector, *Lutzomyia longipalpis*, of *L.* (*L.*) *i. chagasi* in this region (Souza et al.

2005). In the same period in 2009, we had previously estimated the prevalence (23.3 %) of human *L.* (*L.*) *i. chagasi* infection in this area (Silveira et al. unpublished data). Following extensive destruction of the primary forest, the area now consists mainly of plantations, with occasional patches of developing secondary forest.

Canine population examined

The canine population enrolled in this study consisted in a cohort of 320 mongrel dogs, of both genders (174/54.4 % males and 146/45.6 % females) and different ages (the mean age was 2 years and 3 months old), living in the endemic area of AVL in Pará State, north of Brazil.

Study design

This study was addressed to identify the asymptomatic and symptomatic infected dogs with *L.* (*L.*) *i. chagasi*, to study the infection both clinically and immunologically, and to evaluate the infection prevalence, attempting to better understand transmission dynamics, as well as the clinical and immunological features for each pattern of the spectrum of canine *L.* (*L.*) *i. chagasi* infection. In order to obtain a clearer idea regarding the transmission dynamics of infection, the total dog population examined (320) was divided into three age groups: (1) <1 year old, (2) >1 and <7 years old and, (3) >7 years old, each group consisting of 120 (37.5 %), 186 (58.1 %), and 14 (4.4 %) dogs, respectively. For the infection diagnosis, the simultaneous use of IFAT and DTH skin reaction was performed in all dogs previously selected for the prevalence survey. However, before these immunodiagnostic methods were performed, all dogs were clinically examined in order to identify any signs that could be associated with classical CVL. Furthermore, parasitological research of *L.* (*L.*) *i. chagasi* was performed by examining popliteal lymph node aspiration samples of all dogs.

Clinical evaluation of dogs

This was based on the classical clinical signs described for CVL, such as lymphadenopathy, splenomegaly, hepatomegaly, weight loss, alopecia, and onychogryphosis (Ferrer 1999; Feitosa et al. 2000). Dogs with one to three clinical signs were regarded as oligosymptomatic, those with more than three clinical signs were considered as symptomatic, and those with no clinical sign as asymptomatic. Thus, despite this clinical approach that may be regarded with limited value, it has recently been used on clinical and immunological surveys to access the clinical spectrum of canine infection due to *L.* (*L.*) *infantum* or *L.* (*L.*) *i. chagasi* (Cardoso et al. 2007; Alves et al. 2009; Reis et al. 2009; Moreira et al. 2010; Santarém et al. 2010).

Collection of tissue samples

The dogs were anesthetized with sodium thiopental (25 mg/kg), and blood samples (2.0 mL) were collected by jugular puncture and stored at −20 °C for evaluating the specific IgG-antibody response (IFAT). Also, the needle popliteal lymph node aspiration from each dog for the parasitological aspects was used in the Difco B45 blood agar culture medium (Walton et al. 1977).

Criteria for identification of canine infection

The definition of canine *L.* (*L.*) *i. chagasi* infection was mainly based on the presence of reactivity to either IFAT or DTH skin reaction or both immune assays. Nevertheless, as IFAT evidences the humoral response (susceptibility) and DTH the T-cell response (resistance) (Pinelli et al. 1994; Cardoso et al. 1998, 2007), the definition of canine *L.* (*L.*) *i. chagasi* infection was assumed to be the presence of reactivity to either one or both immune assays, in association or not with positive parasitological research. Thus, it was assumed that IFAT with 1:80 (IgG) titer and DTH skin reactions forming papules or indurations of ≥5 mm in diameter were regarded as a positive cut-off for IFAT and DTH, respectively (Pinelli et al. 1994; De Jesus et al. 2003; Silveira et al. 2002).

Immunological test procedures

The methods for DTH skin reactions in dogs were based on those described in other studies for human American cutaneous leishmaniasis (Silveira et al. 1991, 1998). The *Leishmania* antigen was performed with the cultured promastigote forms from the stationary phase (RPMI 1640 media) of a regional strain of *L.* (*L.*) *i. chagasi* (MCAO/BR/2003/M22697/Barcarena, Pará State). They were inactivated in a merthiolate solution (1:10,000) with a final concentration of approximately 4×10^8 parasites/mL (~6.5 mg of protein/mL); 0.1 mL of this suspension (4×10^7 parasites; ~0.65 mg of protein) was the dosage used for intradermal injection in the inner surface of the right thigh of each dog. This dosage was approximately 40 times more concentrated than that (10^6 parasites/0.1 mL) recently used with *L.* (*L.*) *i. chagasi* antigen for DTH skin reactions in humans (Silveira et al. 2009, 2010) and not more concentrated than that (3×10^8 parasites/mL) used by Pinelli et al. (1994) for eliciting DTH in naturally and experimentally infected beagle dogs in Spain. As a control for DTH with *L.* (*L.*) *i. chagasi* antigen, 0.1 mL of the merthiolate solution (1:10,000) was intradermally used in the opposite thigh of each dog. The skin reactions were measured after 48–72 h and those indurations with diameter ≥5 mm were considered positive. Despite this antigen not previously been tested in experimentally

L. (*L.*) *i. chagasi*-infected dogs, its potential immunogenicity was confirmed by promoting positive DTH skin reactions in two specimens of *Cebus apella* monkey experimentally inoculated (IV) with 3×10^7 amastigotes of *L.* (*L.*) *i. chagasi*, even though one had previously failed to develop DTH skin reaction for a *L.* (*L.*) *i. chagasi* promastigotes antigen with a smaller concentration (10^6 parasites/dose) (Carneiro et al. 2011).

The IFAT was performed according to De Jesus et al. (2003), who demonstrated that IFAT using amastigote antigen of *L.* (*L.*) *i. chagasi* had a higher specificity and sensitivity than the available kits of IFAT, using promastigote antigen of *Leishmania* (*L.*) *major*-like, as well as of ELISA, using a soluble *L.* (*L.*) *i. chagasi* antigen, both from Bio-Manguinhos, Brazil. Briefly, the amastigote antigen was impregnated in the IFAT slides by printing small fragments of spleen and liver from "Golden hamster" (*Mesocricetus auratus*) experimentally infected with the same strain of *L.* (*L.*) *i. chagasi* (MCAO/BR/2003/M22697/Barcarena, Pará State) used for preparation of the cultured stationary phase promastigote forms antigen for DTH skin reactions in dogs. Thus, the serological reactions (IFAT) with 1:80 (IgG) titer were regarded as positive in according with the pioneer sero-epidemiological survey on canine visceral leishmaniasis in the municipality of Belém, the capital of Pará State, Brazil (Silveira et al. 2002). The positive (1:1,280 IgG) and negative control serum samples used in the IFAT assay were from a dog which supplied the *L.* (*L.*) *i. chagasi* strain (MCAO/BR/2003/M22697/Barcarena, Pará State) used to perform the antigens for both DTH and IFAT assays and another animal born and raised in the municipality of Belém, a non-endemic area for AVL, respectively. In addition, the possibility of any canine serological cross-reaction with other mammal trypanosomatids from Amazonian Brazil, such as *Trypanosoma cruzi* or *Trypanosoma rangeli*, was strongly minimized due to the absence of well-known domiciliary triatomine vectors (Reduviidae: Triatominae) of these protozoan parasites in the study area.

Parasitological research proceeding

The parasitological diagnosis of canine *L.* (*L.*) *i. chagasi* infection was based on the Difco B45 blood agar culture of the needle popliteal lymph node aspiration into 0.6 mL of sterile PBS for each dog examined; this volume was distributed into three blood agar culture tubes, which were examined over a 3-week period.

Distribution of IgG-antibody response (IFAT)

This analysis was based in the following semi-quantitative scale of specific IgG-antibody response (IFAT): 80–160=weak, 320–640=moderate, and ≥1,280=strong, which was only performed with infected dogs (107) within the $IFAT^{(+)}/DTH^{(-)}$ profile.

Distribution of delayed-type hypersensitivity (DTH) according to the age groups of dogs

This analysis was based in the following semi-quantitative scale of DTH skin reactions: 5–8 mm=weak, 9–13 mm=moderate, and ≥14 mm=strong, which was performed only with infected dogs (18) within the $IFAT^{(-)}/DTH^{(+)}$ profile.

Distribution of immune response profiles according to age groups

This analysis was based on the frequency of each immune response profile within the age groups of canine *L.* (*L.*) *i. chagasi* infection.

Data analysis

Data were analyzed by the Bio-Estat 4.0 software (Ayres et al. 2004), and the χ^2 and binomial tests were used to determine the significant differences among the prevalence and the clinical and immunological profiles of infection with a confidence interval of 95 %.

Ethical approval

This study was approved by the Ethics Committee in Animal Research of the "Instituto Evandro Chagas (IEC)", Surveillance Secretary of Health, Ministry of Health, Brazil, with the protocol number 018/2005/CEPAN/IEC/SVS/MS/Brazil.

Results

Canine *L.* (*L.*) *i. chagasi* infection prevalence

The IFAT and DTH reactivity of dogs revealed the following three immune response profiles: (1) $IFAT^{(+)}/DTH^{(-)}$=107 dogs, (2) $IFAT^{(-)}/DTH^{(+)}$=18 dogs, and (3) $IFAT^{(+)}/DTH^{(+)}$=13 dogs. Thus, these three combined results (107 dogs reacting only by IFAT, 18 only by DTH, and 13 by both tests) provided an overall prevalence of 43 % (138/320) in this dog cohort. In other words, the specific prevalence of infection according to each immune response profile demonstrated that the rate of 33.4 % (107/320) of $IFAT^{(+)}/DTH^{(-)}$ profile was higher ($p<0.05$) than those of 5.6 % (18/320) of $IFAT^{(-)}/DTH^{(+)}$ and 4.0 % (13/320) of $IFAT^{(+)}/DTH^{(+)}$ profile, despite there being no difference ($p>0.05$) between the last two (Table 1). Moreover, comparing the frequency of these three immune response

Table 1 Specific prevalence of infection according to the immune response profiles of canine *L.* (*L.*) *i. chagasi* infection in Santa Maria village, Barcarena municipality, Pará State, Brazil

Survey	Immune response profiles (%) *n*		
	$IFAT^{(+)}/DTH^{(-)}$	$IFAT^{(-)}/DTH^{(+)}$	$IFAT^{(+)}/DTH^{(+)}$
Specific prevalence (*n*=320 dogs)	33.4 % (107)	5.6 % (18)	4.0 % (13)

profiles among the 138 infected dogs, it was observed that the $IFAT^{(+)}/DTH^{(-)}$ rate of 77.5 % (107/138) was also higher ($p<0.05$) than those of $IFAT^{(-)}/DTH^{(+)}$ 13.0 % (18/138) and $IFAT^{(+)}/DTH^{(+)}$ 9.5 % (13/138), although there was no difference ($p>0.05$) between the last two (Table 2). However, it is important to note that the rate of 22.5 % of both DTH reactivity profiles [$IFAT^{(-)}/DTH^{(+)}$ and $IFAT^{(+)}/DTH^{(+)}$] was lower ($p<0.05$) than the 77.5 % of the $IFAT^{(+)}/DTH^{(-)}$ profile.

The prevalence of canine infection according to the gender showed no difference ($p>0.05$) between males (22.2 %—71/138) and females (21 %—67/138). However, regarding the age groups of infected dogs, a higher ($p<0.05$) prevalence was noted of the age group >1 and <7 years old (26 %—83) than those of <1 year old (14.6 %—47) and >7 years old (2.5 %—8) in this cohort (320 dogs).

The clinical status of 138 infected dogs showed that asymptomatic hosts (76 %—105) had a higher frequency ($p<0.05$) than symptomatic (16.6 %—23) and oligosymptomatic ones (7.4 %—10), as well as than symptomatic and oligosymptomatic together (24 %—33). Moreover, the clinical status of infection has also showed that the prevalence of asymptomatic dogs (32.8 %) was also higher ($p<0.05$) than that of symptomatic ones (10.3 %). In addition, when the clinical status of infection was examined within each immune response profile, it was observed that among 107 dogs of the $IFAT^{(+)}/DTH^{(-)}$ profile there was a higher ($p<0.05$) rate of asymptomatic (71 %) than those of symptomatic (20.6 %) and oligosymptomatic ones (8.4 %). Furthermore, among 18 dogs of $IFAT^{(-)}/DTH^{(+)}$ profile, 100 % were asymptomatic and, among 13 dogs of the $IFAT^{(+)}/DTH^{(+)}$ profile, there was also a higher ($p<0.05$) rate of asymptomatic (84.6 %) than those of symptomatic (7.7 %) and oligosymptomatic ones (7.7 %) (Table 3).

L. (*L.*) *i. chagasi* isolation from popliteal lymph node aspiration

A total of 16 (11.6 %) *L.* (*L.*) *i. chagasi* isolates were obtained in the Difco B45 blood agar culture medium from the popliteal lymph node aspiration of the 138 infected dogs; however, it is important to record that all isolates were obtained from infected dogs within the $IFAT^{(+)}/DTH^{(-)}$ profile (107 dogs—15 % isolation). In other words, no isolate was obtained from infected dogs belonging to the profiles with DTH reactivity, neither from the $IFAT^{(-)}/DTH^{(+)}$ nor the $IFAT^{(+)}/DTH^{(+)}$. Moreover, it is also important to record that among the 107 infected dogs within the $IFAT^{(+)}/DTH^{(-)}$ profile, the distribution of isolates has a higher ($p<0.05$) frequency in symptomatic (41 %—9/22) and oligosymptomatic (33.3 %—3/9) dogs than in asymptomatic ones (5.2 %—4/76).

Distribution of IgG-antibody response (IFAT) according to the clinical status of canine *L.* (*L.*) *i. chagasi* infection

This analysis was performed only with infected dogs (107) within the $IFAT^{(+)}/DTH^{(-)}$ profile. Thus, among 22 (20.6 %) symptomatic dogs, 36.4 % (8) showed strong IgG response (≥1,280), while others 36.4 % (8) presented moderate response (320–640) and 27.2 % (6) weak response (80–160). Although there were no differences ($p>0.05$) among these three categories of IgG responses, there was a tendency of a stronger response (from moderate toward to strong=72.8 %) among symptomatic dogs. Moreover, among nine (8.4 %) oligosymptomatic dogs, there was a higher ($p<0.05$) rate (44.5 %—4) of strong IgG response only than that of moderate (22.2 %—2) but not than that of weak response (33.3 %—3); thus, in a similar way to that observed with symptomatic dogs, there was also only a tendency of a stronger IgG response (from moderate toward to strong=66.7 %) among oligosymptomatic dogs. In contrast to these, however, among 76 (71 %) asymptomatic dogs although there was no higher ($p>0.05$) rate (44.7 %—34) of weak IgG response over that of moderate response (30.3 %—23), these two categories together (75 %) were higher ($p<0.05$) than that of the strong response (25 %—19) (Table 4).

Distribution of delayed-type hypersensitivity (DTH) according to the age groups of canine *L.* (*L.*) *i. chagasi* infection

This analysis was performed only with infected dogs (18) within the $IFAT^{(-)}/DTH^{(+)}$ profile. Thus, among all asymptomatic dogs (18) with DTH reactivity, 66.7 % (12) were in

Table 2 Frequency of each immune response profile among 138 infected dogs by *L.* (*L.*) *i. chagasi* in Santa Maria village, Barcarena municipality, Pará State, Brazil

Frequency	Immune response profiles (%) *n*		
	$IFAT^{(+)}/DTH^{(-)}$	$IFAT^{(-)}/DTH^{(+)}$	$IFAT^{(+)}/DTH^{(+)}$
Infected dogs (*n*=138)	77.5 % (107)	13 % (18)	9.5 % (13)

Table 3 Frequency of the clinical status of canine *L.* (*L.*) *i. chagasi* infection according with each immune response profile in Santa Maria village, Barcarena municipality, Pará State, Brazil

Immune response profiles	Clinical status (%) *n*		
	Asymptomatic	Symptomatic	Oligosymptomatic
$IFAT^{(+)}/DTH^{(-)}$ (n=107)	71 % (76)	20.6 % (22)	8.4 % (9)
$IFAT^{(-)}/DTH^{(+)}$ (n=18)	100 % (18)	–	–
$IFAT^{(+)}/DTH^{(+)}$ (n=13)	84.6 % (11)	7.7 % (1)	7.7 % (1)

the >1- and <7-year age group, 33.4 % being moderate, 27.8 % weak, and 5.5 % strong responders. Beside these, 22.2 % (4) were in the <1-year-old age group, with 16.7 % being moderate and 5.5 % strong responders. Finally, there were only two (11.1 %) at the >7-year age group, all (100 %) being moderate responders. Thus, dogs with DTH reactivity at the >1 and <7 years age group had a higher frequency (66.7 %) ($p<0.05$) than those at <1 year (22.2 %) and at >7 years (11.1 %) (Table 5).

Distribution of immune response profiles according to the age groups of canine *L.* (*L.*) *i. chagasi* infection

The >1- and <7-year-old age group recorded 74.6 % (103) of 138 infected dogs concerning the three immune response profiles, with 57.2 % $IFAT^{(+)}/DTH^{(-)}$, 8.7 % $IFAT^{(-)}/DTH^{(+)}$, and other 8.7 % $IFAT^{(+)}/DTH^{(+)}$. Besides these, 18.8 % (26) were at the <1-year-old age group, with 15.2 % $IFAT^{(+)}/DTH^{(-)}$, 2.9 % $IFAT^{(-)}/DTH^{(+)}$, and only 0.7 % $IFAT^{(+)}/DTH^{(+)}$. At last, there were 6.6 % (9) at the >7-year-old age group, being 5.1 % $IFAT^{(+)}/DTH^{(-)}$ and 1.5 % $IFAT^{(-)}/DTH^{(+)}$. In other words, there was a rate of 74.6 % immune response profiles at the >1- and <7-year-old age group, higher ($p<0.05$) than those 18.8 % (26) at the <1-year-old and 6.6 % at the >7-year-old age group (Table 6).

Discussion

The present paper represents the first cross-sectional study carried out in an endemic area of AVL in Amazonian Brazil that examined a cohort of 320 mongrel dogs in order to determine the prevalence and the clinical and immunological spectrum of canine *L.* (*L.*) *i. chagasi* infection, combining two immunodiagnostic assays: IFAT (IgG) and DTH for the humoral and cellular responses. The combined results of the IFAT and DTH assays indicated an overall prevalence of 43 % in the canine cohort, which was higher than the 23.5 % and 24 % found by ELISA in the States of Bahia and Ceará, respectively, both in northeastern Brazil (Paranhos-Silva et al. 1996; Rondon et al. 2008). The overall prevalence (43 %) found in our study was also higher than the 16 % recorded by IFAT (1:40) in the State of Pernambuco, also in northeastern Brazil (Santos et al. 2010). An estimated infection prevalence of 40.3 % by IFAT (1:80) observed in the same State of Pernambuco (Dantas-Torres et al. 2006) was similar to that found in the present study, indicating that, in exceptional situations, the use of low-specificity antigens (such as *L. major*-like) may give rise to unexpectedly high infection prevalence rates due (most likely) to high percentages of false-positive reactions (Silveira et al. 2002; De Jesus et al. 2003).

Of interest is the specific infection prevalence based on three immune response profiles: (1) $IFAT^{(+)}/DTH^{(-)}$, (2) $IFAT^{(-)}/DTH^{(+)}$, and (3) $IFAT^{(+)}/DTH^{(+)}$. The 33.4 % specific prevalence of the $IFAT^{(+)}/DTH^{(-)}$ profile was higher ($p<0.05$) than the 5.6 % $IFAT^{(-)}/DTH^{(+)}$ and 4.0 % $IFAT^{(+)}/DTH^{(+)}$ profiles, indicating that the $IFAT^{(+)}/DTH^{(-)}$ profile was the most prevalent expression of canine immune response in the endemic area. Also, among the 138 infected dogs, the $IFAT^{(+)}/DTH^{(-)}$ profile rate (77.5 %) was higher ($p<0.05$) than the $IFAT^{(-)}/DTH^{(+)}$ (13 %) or $IFAT^{(+)}/DTH^{(+)}$ (9.5 %) profiles, confirming the importance of the $IFAT^{(+)}/DTH^{(-)}$ profile as the most frequent immune expression of

Table 4 Distribution of IgG-antibody response (IFAT) according to the clinical status of canine *L.* (*L.*) *i. chagasi* infection in Santa Maria village, Barcarena municipality, Pará State, Brazil

Clinical status of dogs	IgG-antibody response (IFAT) *n* (%)		
	80–160	320–640	≥1,280
Symptomatic n=22	6 (27.2 %)	8 (36.4 %)	8 (36.4 %)
Asymptomatic n=76	34 (44.7 %)	23 (30.3 %)	19 (25 %)
Oligosymptomatic n=9	3 (33.3 %)	2 (22.2 %)	4 (44.5 %)

80–160 IgG=weak, 320–640 IgG=moderate, ≥1,280 IgG=strong

Table 5 Age distribution of DTH skin reactions of canine *L.* (*L.*) *i. chagasi* infection in Santa Maria village, Barcarena municipality, Pará State, Brazil

Age groups—years old	DTH skin reaction (mm) *n* (%)		
	5–8	9–13	≥14
<1 (n=4)	–	3 (16.7 %)	1 (5.5 %)
>1 and <7 (n=12)	5 (27.8 %)	6 (33.4 %)	1 (5.5 %)
>7 (n=2)	–	2 (11.1 %)	–

5–8 mm=weak, 9–13 mm=moderate, ≥14 mm=strong

Table 6 Age distribution of immune response profiles of canine *L.* (*L.*) *i. chagasi* infection in Santa Maria village, Barcarena municipality, Pará State, Brazil

Age groups—years old	Immune response profiles *n* (%)		
	$IFAT^{(+)}/DTH^{(-)}$	$IFAT^{(-)}/DTH^{(+)}$	$IFAT^{(+)}/DTH^{(+)}$
<1 (*n*=26)	21 (15.2 %)	4 (2.9 %)	1 (0.7 %)
>1 and <7 (*n*=103)	79 (57.2 %)	12 (8.7 %)	12 (8.7 %)
>7 (*n*=9)	7 (5.1 %)	2 (1.5 %)	–

canine *L.* (*L.*) *i. chagasi* infection, despite its doubtful capacity of immunoprotecting (Reis et al. 2006; Baneth et al. 2008; Solano-Gallego et al. 2009). It should also be stressed that the 77.5 % $IFAT^{(+)}/DTH^{(-)}$ profile was much higher than both assays showing DTH reactivity [$IFAT^{(-)}/DTH^{(+)}$ and $IFAT^{(+)}/DTH^{(+)}$], which together corresponded to only 22.5 % of the infected dogs. This result appears important in understanding the dynamics of T-cell and humoral immune responses against canine *L.* (*L.*) *i. chagasi* infection. As there is yet no clear evidence of a dichotomy between T-cell and humoral responses to infection (Barbieri 2006), it seems obvious that the humoral IgG-antibody response was prominent (77.5 %) among symptomatic and asymptomatic infected dogs, and suggests that most naturally infected dogs in the endemic area may be unable to develop an effective T-cell response.

As humoral response has doubtful capacity for immunoprotection against infection (Pinelli et al. 1994; Cabral et al. 1998; Baneth et al. 2008), we would expect that a significant number of infected dogs within the $IFAT^{(+)}/DTH^{(-)}$ profile would be unable to control their infections irrespective of their clinical status (i.e., either asymptomatic or symptomatic). Thus, it is important to note that 100 % of the *L.* (*L.*) *i. chagasi* isolates obtained from needle popliteal lymph node aspiration were of the $IFAT^{(+)}/DTH^{(-)}$ profile, being 41 % from symptomatic, 33.3 % from oligosymptomatic, and 5.2 % from asymptomatic dogs, confirming the high susceptibility of dogs with $IFAT^{(+)}/DTH^{(-)}$ profile to *L.* (*L.*) *i. chagasi* infection.

No differences were observed in canine *L.* (*L.*) *i. chagasi* infection rates between males (22.2 %) and females (21 %), suggesting that both were equally attractive blood meals for the sandfly vector, *Lutzomyia longipalpis*, and may therefore play identical roles in the epidemiology of AVL—not only as susceptible hosts but as infection sources for the vector. In the Pernambuco State, in northeastern Brazil, Santos et al. (2010) found similar gender infection rates in mongrel dogs, but in an urban area of the same state, Dantas-Torres et al. (2006) noted that male pet dogs had higher infection rates. We found no differences in the prevalence of symptomatic male (6.2 %—20/320) and female (4.0 %—13/320) dogs, indicating that gender has little influence on infection outcomes. This agrees with evidence presented by Abranches et al. (1991) and Miró et al. (2007) for CVL due to *L.* (*L.*) *infantum* in an endemic area in the Mediterranean region, but contrasted with the findings of Zaffaroni et al. (1999) and Zivicnjak et al. (2005) for the same region.

With regards to the age classes of infected dogs, a higher prevalence of infection in the >1- and <7-year-old class was noted (26 %—83) than in the <1-year-old (14.6 %—47) or >7-year-old classes (2.5 %—8) in the cohort examined, suggesting that most infections appeared following the first year of life when the mother's protective antibodies had declined. Nevertheless, we cannot dismiss the importance of the 14.6 % infection prevalence in <1-year-old dogs, which demonstrated that even before completing their first year, a significant percentage of juvenile dogs became infected. On the other hand, the low infection prevalence (2.5 %) found in dogs >7 years old might indicate that most infected dogs in the >1- and <7-year-old age group succumb to CVL before reaching their seventh year. However, there is evidence from the Mediterranean region that asymptomatic infected dogs convert to symptomatic states following periods ranging from 3 months to 7 years (Solano-Gallego et al. 2001; Oliva et al. 2006). We found higher percentages of symptomatic individuals in the <1-year-old (42.4 %) and >1- and <7-year-old (48.5 %) classes than in the >7-year-old class (9.1 %), with the following mean ages: 9.3 months old, 2.7 years old and, 9 years old, respectively. Based on these mean ages, we can predict that 42.4 % will become symptomatic before reaching their first year and 48.5 % before their third year—suggesting that more than 90 % of the animals would become symptomatic while younger than 3 years old, reinforcing the suspicion that most infected dogs in the >1- and <7-year-old class will succumb to CVL before reaching their seventh year.

The clinical status of infected dogs indicated that asymptomatic animals (76 %—105) were more frequent than symptomatic (16.6 %—23) or oligosymptomatic animals (7.4 %—10), or even symptomatic and oligosymptomatic animals together (24 %—33). Although this has been a usual finding in many New and Old World endemic areas (Madeira et al. 2004; Dantas-Torres et al. 2006; Queiroz et al. 2008; Cabral et al. 1998; Baneth et al. 2008; Solano-Gallego et al. 2009), it does not guarantee that asymptomatic infections will refrain from converting to symptomatic conditions in significant numbers of infected dogs. In this

regard, it is worth noting that only 22.5 % of infected dogs were found to show DTH reactivity [IFAT$^{(-)}$/DTH$^{(+)}$=13 % and IFAT$^{(+)}$/DTH$^{(+)}$=9.5 %] associated with resistance to infection by either *L.* (*L.*) *i. chagasi* (Barbieri 2006; Dos-Santos et al. 2008; Pinheiro et al. 2009) or *L.* (*L.*) *infantum* (Pinelli et al. 1994; Cabral et al. 1998; Cardoso et al. 2007). Thus, although there is some evidence for a 45 % resistance rate of *L.* (*L.*) *infantum*-infected dogs in endemic areas of the Mediterranean region (27 % with both T-cell and humoral responses, and 18 % with only T-cell response) (Cabral et al. 1998; Solano-Gallego et al. 2000), this is a polemic issue that still needs to be clarified. In the present study, only 9.5 % of the dogs were found to have both T-cell and humoral responses [IFAT$^{(+)}$/DTH$^{(+)}$ profile] and 13 % had only T-cell response [IFAT$^{(-)}$/DTH$^{(+)}$ profile].

An examination of the distribution of specific IgG-antibody responses in terms of the clinical status of 22 symptomatic dogs revealed a tendency towards strong responses in 72.8 % of animals, ranging from moderate (36.4 %) to strong (36.4 %) responders—which means that we could expect a well-developed specific IgG-antibody response as high as 1:320 in these animals. On the other hand, although there was no predominance of weak IgG-antibody response (44.7 %) over a moderate response (30.3 %) among asymptomatic dogs, these two categories together (75 %) were more frequent than strong responders (25 %). This suggests not only a lower parasite burden but also a weaker IgG-antibody response, which is supported by observations from an earlier survey carried out in Santana do Cafezal (bordering the current study area in Santa Maria, in the municipality of Barcarena) (Lima et al. 2010).

Regarding the distribution of DTH skin reactions among the different classes of infected dogs, it is important to note that most positive responses were seen in asymptomatic dogs in the >1 and <7-year-old class, being significantly higher than in the <1-year-old age class or the >7-year-old age class—suggesting that canine T-cell response against *L.* (*L.*) *i. chagasi* infection appears to be better developed in adults than in juveniles or older animals, an observation that might be of interest in vaccination programs against CVL. It should also be noted that 61.2 % of these responses were of moderate intensity (9–13 mm), as opposed to weak (5–8 mm; 27.8 %) or strong responses (≥14 mm; 11 %), indicating that most dogs with DTH reactivity developed proportional T-cell response.

Finally, it should be stressed that the high (74.6 %) immune response profile in the >1 and <7-year-old class [57.2 % IFAT$^{(+)}$/DTH$^{(-)}$, 8.7 % IFAT$^{(-)}$/DTH$^{(+)}$, and 8.7 % IFAT$^{(+)}$/DTH$^{(+)}$ profiles] reflected the fact that most infections appear to become established after the first year of life and that the IFAT$^{(+)}$/DTH$^{(-)}$ profile is the most prominent immune response to infection.

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References

Abranches P, Silva-Pereira MC, Conceição-Silva FM, Santos-Gomes GM, Janz JG (1991) Canine leishmaniasis: pathological and ecological factors influencing transmission of infection. J Parasitol 77:557–561

Alves CF, Amorim IFG, Moura EP, Ribeiro RR, Alves CF, Michalick MS, Kalapothakis E, Bruna-Romero O, Tafuri W, Teixeira MM, Melo MN (2009) Expression of IFN-g, TNF-a, IL-10 and TGF-b in lymph nodes associates with parasite load and clinical form of disease in dogs naturally infected with *Leishmania (Leishmania) chagasi*. Vet Immunol Immunopathol 128:349–358

Ayres M, Ayres M Jr, Ayres D, Santos AS (2004) Bioestat 4.0: Aplicações estatísticas nas áreas das Ciências Biológicas e Médicas. Sociedade Civil Mamirauá-Brasília CNPq, Belém

Baleeiro CO, Paranhos-Silva M, dos Santos JC, Oliveira GGS, Nascimento EG, de Carvalho LP, dos Santos WLC (2006) Montenegro's skin reactions and antibodies against different *Leishmania* species in dogs from a visceral leishmaniosis endemic area. Vet Parasitol 139:21–28

Baneth G, Koutinas AF, Solano-Gallego L, Bourdeau P, Ferrer L (2008) Canine leishmaniosis—new concepts and insights on a expanding zoonosis: part one. Trends Parasitol 24:324–330

Barbieri CL (2006) Immunology of canine leishmaniasis. Parasite Immunol 28:329–337

Cabral M, O'Grady J, Alexander J (1992) Demonstration of *Leishmania*-specific cell mediated and humoral immunity in asymptomatic dogs. Parasite Immunol 14:531–539

Cabral M, McNerney R, Gomes S, O'Grady J, Frame I, Sousa JC, Miles MA, Alexander J (1993) Demonstration of natural *Leishmania* infection in asymptomatic dogs in the absence of specific humoral immunity. Arc Inst Pasteur Tunis 70:473–479

Cabral M, O'Grady J, Gomes S, Sousa JC, Thompson H, Alexander J (1998) The immunology of canine leishmaniosis: strong evidence for a developing disease spectrum from asymptomatic dogs. Vet Parasitol 76:173–180

Cardoso L, Neto F, Sousa JC, Rodrigues M, Cabral M (1998) Use of a leishmanin skin test in the detection of canine *Leishmania*-specific cellular immunity. Vet Parasitol 79:213–220

Cardoso L, Schallig HDFH, Cordeiro-da-Silva A, Cabral M, Alunda JM, Rodrigues M (2007) Anti-*Leishmania* humoral and cellular immune responses in naturally infected symptomatic and asymptomatic dogs. Vet Immunol Immunopathol 117:35–41

Carneiro LA, Silveira FT, Campos MB, Brígido COM, Gomes CMC, Corbett CEP, Laurenti MD (2011) Susceptibility of *Cebus apella* monkey (Primates: Cebidae) to experimental *Leishmania (L.) infantum chagasi*-infection. Rev Inst Med Trop São Paulo 53:45–50

Dantas-Torres F, de Brito MEF, Brandão-Filho SP (2006) Seroepidemiological survey on canine leishmaniasis among dogs from an urban area of Brazil. Vet Parasitol 140:54–60

De Jesus RCS, Corrêa ZC, Everdosa DR, Martins AP, Eliseu LS, Campos MC, Jenning YAA, Ishikawa EAI, De Souza AAA, Silveira FT (2003) A comparison between the indirect fluorescent

antibody test (IFAT), Evandro Chagas Institute-antigen *versus* Bio-Manguinhos-antigen, and the enzyme-linked immunosorbent assay (ELISA) in the serological diagnosis of canine visceral leishmaniasis in Pará State, Brazil. Rev Soc Bras Med Trop 36:323

Delgado O, Castes M, White AC Jr, Kreutzer RD (1993) *Leishmania colombiensis* in Venezuela. AmJTrop Med Hyg 48:145–147

Dos-Santos WL, Jesus EE, Paranhos-Silva M, Pereira AM, Santos JC, Baleeiro CO, Nascimento EG, Moreira ED, Oliveira GG, Pontes-de-Carvalo LC (2008) Associations among immunological, parasitological and clinical parameters in canine visceral leishmaniasis: emaciation, spleen parasitism, specific antibodies and leishmanin skin test reaction. Vet Immunol Immunopathol 123:251–259

Feitosa MM, Ikeda FA, Luvizotto MCR, Perri SHV (2000) Aspectos clínicos de cães com leishmaniose visceral no município de Araçatuba, São Paulo (Brasil). Clin Vet 28:36–44

Ferreira EC, Lana M, Carneiro M, Reis AB, Paes DV, da Silva ES, Schallig H, Gontijo CMF (2007) Comparison of serological assays for the diagnosis of canine visceral leishmaniasis in animals presenting different clinical manifestations. Vet Parasitol 146:235–241

Ferrer L (1999). Clinical aspects of canine leishmaniasis. In: Killick-Kendricks, R. (ed.) Canine leishmaniasis: an update. Proc First Inter Canine Leish Forum, Hoeschst Roussel Vet, Barcelona, Spain, 6–10

Herrer A (1951) Estudios sobre leishmaniasis tegumentaria en el Peru. Leishmaniasis natural en perros procedentes de localidades utügenas. Rev Med Exp Lima 8:87–117

Herrer A, Christensen HA (1976) Natural cutaneous leishmaniasis among dogs in Panama. AmJTrop Med Hyg 25:59–63

Ikeda-Garcia FA, Lopes RS, Marques FJ, de Lima VM, Morinishi CK, Bonello FL, Zanette MF, Perri SH, Feitosa MM (2007) Clinical and parasitological evaluation of dogs naturally infected by *Leishmania* (*Leishmania*) *chagasi* submitted to treatment with meglumine antimoniate. Vet Parasitol 143:254–259

Lima LVR, Carneiro LA, Campos MB, Chagas EJ, Laurenti MD, Corbett CEP, Lainson R, Silveira FT (2010) Canine visceral leishmaniasis due to *Leishmania* (*L.*) *infantum chagasi* in Amazonian Brazil: comparison of the parasite density from the skin, lymph node and visceral tissues between symptomatic and asymptomatic, seropositive dogs. Rev Inst Med Trop São Paulo 52:259–265

Madeira MF, Shubach AO, Shubach TMP, Leal CA, Marzochi MCA (2004) Identification of *Leishmania* (*Leishmania*) *chagasi* isolated from healthy skin of symptomatic and asymptomatic dogs seropositive for leishmaniasis in the municipality of Rio de Janeiro, Brazil. Braz J Infec Dis 8:440–444

Mayrink W, Williams P, Coelho MV, Dias M, Martins AV, Magalhães PA, da Costa CA, Falcão AR, Melo MN, Falcão AL (1979) Epidemiology of dermal leishmaniasis in the Rio Doce Valley, State of Minas Gerais, Brazil. Ann Trop Med Parasitol 73:123–137

Miró G, Montoya A, Mateo M, Alonso A, Garcia S, Garcia A, Caballero MJ, Molina R (2007) A leishmaniosis surveillance system among stray dogs in the region of Madrid: ten years of serodiagnosis (1996–2006). Parasitol Res 101:253–257

Moreira PRR, Vieira LM, de Andrade MMC, Bandarra MB, Machado GF, Munari DP, Vasconcelos RO (2010) Immune response pattern of the popliteal lymph nodes of dogs with visceral leishmaniasis. Parasitol Res 107:605–613

Oliva G, Scalone A, Foglia Manzillo V, Gramiccia M, Pagano A, Di Muccio T, Gradoni L (2006) Incidence and time course of *Leishmania infantum* infections examined by parasitological, serologic and nested-PCR techniques in a cohort of naive dogs exposed to three consecutive transmission seasons. J Clin Microbiol 44:1318–1322

Paranhos-Silva M, Freitas LA, Santos WC, Grimaldi G Jr, Pontes-deCarvalho LC, Oliveira-dos-Santos AJ (1996) A cross-sectional serodiagnostic survey of canine leishmaniasis due to *Leishmania chagasi*. Am J Trop Med Hyg 55:39–44

Pinelli E, Killick-Kendrick R, Wagenaar J, Bernardina W, del Real G, Ruitenberg J (1994) Cellular and humoral immune responses in dogs experimentally and naturally infected with *Leishmania infantum*. Infect Immun 62:229–261

Pinheiro PH, Pinheiro AN, Ferreira JH, Costa FA, Katz S, Barbieri CL (2009) A recombinant cysteine proteinase from *Leishmania* (*Leishmania*) *chagasi* as an antigen for delayed-type hypersensibility assays and serodiagnosis of canine visceral leishmaniasis. Vet Parasitol 162:32–39

Queiroz PV, Monteiro GR, Macedo VP, Rovha MA, Batista LM, Queiroz JW, Jerônimo SM, Ximenes MF (2008) Canine visceral leishmaniasis in urban and rural areas of northeast Brazil. Res Vet Sci 86:267–273

Reis AB, Teixeira-Carvalho A, Vale AM, Marques MJ, Giunchetti RC, Mayrink W, Guerra LL, Andrade RA, Correa-Oliveira R, Martins-Filho OA (2006) Isotype patterns of immunoglobulins: hallmarks for clinical status and tissue parasite density in Brazilian dogs naturally infected by *Leishmania* (*Leishmania*) *chagasi*. Vet Immunol Immunopathol 112:102–116

Reis AB, Martins-Filho AO, Teixeira-Carvalho A, Giunchetti RC, Carneiro CM, Mayrink W, Tafuri WL, Correa-Oliveira R (2009) Systemic and compartmentalized immune response in canine visceral leishmaniasis. Vet Immunol Immunopath 128:87–95

Rondon FC, Bevilaqua CM, Franke CR, Barros RS, Oliveira FR, Alcântara AC, Diniz AT (2008) Cross-sectional serological survey of canine *Leishmania* infection in Fortaleza, Ceará State, Brazil. Vet Parasitol 155:24–31

Santarém N, Silvestre R, Cardoso L, Shallig H, Reed SG, Codeiro-da-Silva A (2010) Application of an improved enzyme-linked immunosorbent assay method for serological diagnosis of canine leishmaniasis. J Clin Microbiol 48:1866–1874

Santos JM, Dantas-Torres F, Mattos MR, Lino FR, Andrade LS, Souza RC, Brito FL, Brito ME, Brandão-Filho SP, Simões-Mattos L (2010) Prevalence of anti-*Leishmania* spp. antibodies in dogs from Garanhuns, in the middle scrub zone (Agreste) of Pernambuco. Rev Soc Bras Med Trop 43:41–45

Silveira FT, Corbett CEP (2010) *Leishmania chagasi* Cunha & Chagas, 1937: indigenous or introduced? A brief review. Rev Pan-Amaz Saúde 1:143–147. doi:10.5123/S2176-62232010000200018

Silveira FT, Lainson R, Shaw JJ, de Souza AA, Ishikawa EAI, Braga RR (1991) Cutaneous leishmaniasis due to *Leishmania* (*Leishmania*) *amazonensis* in Amazonian Brazil, and the significance of a negative Montenegro skin-test in human infections. Trans R Soc Trop Med Hyg 85:735–738

Silveira FT, Blackwell JM, Ishikawa EA, Braga RR, Shaw JJ, Quinnell RJ, Soong L, Kima P, McMahon-Pratt D, Black GF, Shaw M-A (1998) T cell responses to crude and defined leishmanial antigens in patients from the lower Amazon region of Brazil infected with different species of *Leishmania* of the subgenera *Leishmania* and *Viannia*. Parasite Immunol 20:19–26

Silveira FT, Pinto ZB, Carneiro LA, Tabosa K, Dias L, Brígido MC, De Jesus R, Corrêa Z, De Sousa AAA, Ishikawa EA (2002) Preliminary results on the first seroepidemiologic survey of canine visceral leishmaniasis in the municipality of Belém, Pará State, Brazil. Rev Soc Bras Med Trop 35:341

Silveira FT, Lainson R, Pereira EA, de Souza AAA, Campos MB, Chagas EJ, Gomes CMC, Laurenti MD, Corbett CEP (2009) A longitudinal study on the transmission dynamics of human *Leishmania* (*L.*) *infantum chagasi*-infection in Amazonian Brazil, with special reference to its prevalence and incidence. Parasitol Res 20:19–26

Silveira FT, Lainson R, de Souza AAA, Crescente JAB, Campos MB, Gomes CMC, Laurenti MD, Corbett CEP (2010) A prospective

study on the dynamics of clinical and immunological evolution of human *Leishmania* (*L.*) *infantum chagasi*-infection in the Brazilian Amazon region. Trans Roy Soc Trop Med Hyg 104:529–535

Solano-Gallego L, Llull J, Ramos G, Riera C, Arboix M, Alberola J, Ferrer L (2000) The Ibizian hound presents a predominantly cellular immune response against natural *Leishmania* infection. Vet Parasitol 90:37–45

Solano-Gallego L, Riera C, Roura X, Iniesta L, Gallego M, Valladares JE, Fisa R, Castilejo S, Alberola J, Ferrer L, Arboix M, Portus M (2001) *Leishmania infantum*-specific IgG, IgG1 and IgG2 antibody responses in health and ill dogs from endemic areas. Evolution in the course of infection and after the treatment. Vet Parasitol 96:265–276

Solano-Gallego L, Koutinas A, Miró G, Cardoso L, Pennisi MG, Ferrer L, Bourdeau P, Oliva G, Baneth G (2009) Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniosis. Vet Parasitol 165:1–18

Souza AAA, Barata I, Lima JAN, Martins AFP, Barbosa RNP, Ishikawa EAI, Chagas EJ, Pereira E, Silveira FT (2005) Evaluation of the seasonality of *Lutzomyia longipalpis* (Diptera:Psychodidae) in an endemic area of visceral leishmaniasis in Para State, Brazil. Third World Congress on Leishmaniasis, Palermo-Terrasini, Sicily, Italy, Epidemiology-Poster Section: 134

Tolezano JE, Uliana SRB, Taniguchi HH, Araújo MFL, Barbosa JAR, Barbosa JER, Floeter-Winter LM, Shaw JJ (2007) The first records of *Leishmania* (*Leishmania*) *amazonensis* in dogs (*Canis familiaris*) diagnosed clinically as having canine visceral leishmaniasis from Araçatuba county, São Paulo State, Brazil. Vet Parasitol 149:280–284

Walton BC, Shaw JJ, Lainson R (1977) Observations on the *in vitro* cultivation of *Leishmania braziliensis*. J Parasitol 63:1118–1119

Zaffaroni E, Rubaudo L, Lanfranchi P, Mignone W (1999) Epidemiological patterns of canine leishmaniasis in Western Liguria (Italy). Vet Parasitol 81:11–19

Zivicnjak T, Martinkovic F, Marinculic A, Mrljak V, Kucer N, Matijtko V, Mihaljevic Z, Baric-Rafaj R (2005) A seroepidemiologic survey of canine visceral leishmaniosis among apparently health dogs in Croatia. Vet Parasitol 131:35–43