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Isotype patterns of immunoglobulins: Hallmarks for clinical status and tissue parasite density in brazilian dogs naturally infected by *Leishmania* (*Leishmania*) *chagasi*

Alexandre B. Reis [a,b,*], Andréa Teixeira-Carvalho [a,b], André M. Vale [c], Marcos J. Marques [d], Rodolfo C. Giunchetti [b], Wilson Mayrink [c], Luanda Liboreiro Guerra [a], Renata A. Andrade [e], Rodrigo Corrêa-Oliveira [a], Olindo A. Martins-Filho [e]

[a] *Laboratório de Imunologia Celular e Molecular, Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz, Belo Horizonte, Minas Gerais, Brazil*
[b] *Laboratório de Parasitologia e Histopatologia, Núcleo de Pesquisas em Ciências Biológicas/NUPEB; Departamento de Análises Clínicas, Escola de Farmácia, ICEB II – Morro do Cruzeiro, Universidade Federal de Ouro Preto, Ouro Preto, CEP 35400-000, Minas Gerais, Brazil*
[c] *Laboratório de Leishmanioses, Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil*
[d] *Laboratório de Biologia Molecular de Microorganismos, Universidade Federal de Alfenas, Alfenas, Minas Gerais, Brazil*
[e] *Laboratório de Doença de Chagas, Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz, Belo Horizonte, Minas Gerais, Brazil*

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Abstract

The role of anti-leishmanial immune response underlying the susceptibility/resistance during canine visceral leishmaniasis (CVL) has been recognized throughout ex vivo and in vitro investigations. Recently, we demonstrated that immunoglobulin levels (Igs), as well as the parasite load are relevant hallmarks of distinct clinical status of CVL. To further characterize and upgrade the background on this issue, herein, we have evaluated, in *Leishmania* (*Leishmania*) *chagasi* naturally infected dogs, the relationship between tissue parasitism (skin, bone marrow, spleen, liver and lymph node), the CVL clinical status (asymptomatic (AD), with no suggestive signs of the disease; oligosymptomatic (OD), with maximum three clinical signs—opaque bristles; localized alopecia and moderate loss of weight; symptomatic (SD), serologically positive with severe clinical signs of visceral leishmaniasis), and the humoral immunological profile of anti-*Leishmania* immunoglobulins (IgG, IgG1, IgG2, IgM, IgA and IgE). Our major statistically significant findings revealed distinct patterns of tissue parasite density

* Corresponding author at: Laboratório de Parasitologia e Histopatologia, Núcleo de Pesquisas em Ciências Biológicas/NUPEB, Escola de Farmácia, ICEB II – Morro do Cruzeiro, Universidade Federal de Ouro Preto, Ouro Preto, CEP 35400-000, Minas Gerais, Brazil. Tel.: +55 21 31 3559 1694; fax: +55 21 31 3559 1680.
E-mail address: alexreis@nupeb.ufop.br (A.B. Reis).

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within *L. chagasi*-infected dogs despite their clinical status, pointing out the spleen and skin as the most relevant sites of high parasitism during ongoing CVL. Parasite density of bone marrow and spleen were the most reliable parasitological markers to decode the clinical status of CVL. Moreover, the parasite density of bone marrow better correlates with most anti-*Leishmania* Igs reactivity. Additionally, a prognostic hallmark for canine visceral leishmaniasis was found, highlighting strong correlation between IgG1 and asymptomatic disease, but with IgA, IgE and IgG2 displaying better association with symptomatic disease. The new aspects of this study highlighted pioneer findings that correlated the degree of tissue parasite density (low (LP), medium (MP) and high (HP) parasitism) with distinct patterns of anti-*Leishmania* Igs reactivity. In this scope, our data re-enforce the anti-*Leishmania* IgG but with IgA reactivity as the better marker for overall tissue parasitism. The association between clinical status, Ig profile and the tissue parasitism support a novel investigation on the impact of humoral immune response and susceptibility/resistance mechanism during ongoing CVL.
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Keywords: Canine visceral leishmaniasis; Clinical forms; Tissue parasitism; Ig profile; *Leishmania chagasi*

1. Introduction

Visceral leishmaniasis caused by *Leishmania (Leishmania) infantum* syn. *Leishmania* (*Leishmania*) *chagasi* affects wild and domestic animals as well as humans in several parts of the Old and New World. Peridomestic sand flies acquire the etiological agent by feeding on infected wild/domestic reservoirs leading to transmission to humans causing severe disease fatal if not treated immediately after the onset of early symptoms (WHO, 2000). The major prophylactic practice to control this human disease, as recommended by the World Health Organization, involves a systematic treatment of human cases besides vector control by insecticide and elimination of the domestic reservoir, mainly seropositive infected dogs (Tesh, 1995).

Canine visceral leishmaniasis (CVL) is one of the most important emerging diseases with high prevalence in Latin American countries (Tesh, 1995). The major signs of CVL include hepatosplenomegaly, lymphadenopathy, cutaneous lesions, keratoconjunctivitis, opaque bristles, alopecia, apathy, onychogriphosis, anorexia and severe weight loss (Bettini and Gradoni, 1986). Hypergammaglobulinemia is also one of the classic signs of CVL as the disease progresses and it is accompanied by a suppression of cellular immune response, both mitogen-triggered and antigen-specific as well as a strong up-regulation humoral response (Pinelli et al., 1994; Cabral et al., 1998). According to Mancianti et al. (1988), CVL can be categorized into three distinct clinical forms, based on major features observed for infected dogs, which can be classified as asymptomatic (AD), with no suggestive signs of the disease; oligosymptomatic (OD), with maximum three clinical signs including opaque bristles and/or localized alopecia, and/or moderate loss of weight and symptomatic (SD), with characteristic clinical signs of visceral leishmaniasis, such as opaque bristles, severe loss of weight, onychogriphosis, cutaneous lesions, apathy and keratoconjunctivitis, showing the most severe signs of CVL.

Several reports have focused attention on the relationship between distinct clinical forms of CVL, disease progression and the IgG isotype levels, in both, experimental and natural *L.* (*L.*) *infantum* and *L.* (*L.*) *chagasi* infections (Solano-Gallego et al., 2001; Leandro et al., 2001; Quinnell et al., 2003; Cordeiro-da-Silva et al., 2003; Vercammen et al., 2002). Although the majority of these investigations have being performed based on well-established ELISA and Western-blotting protocols, controversial data on immunoglobulin isotype profiles are frequently documented.

Increased levels of IgG and IgG2 have been indiscriminately reported for AD and SD as described by Bourdoiseau et al. (1997) and Vercammen et al. (2002). However, according to Deplazes et al. (1995), Nieto et al. (1999) and Solano-Gallego et al. (2001), SD showed considerably higher anti-*Leishmania* IgG1 antibodies in comparison to asymptomatic carriers. Additionally, Courtenay et al. (2002) and Quinnell et al. (2003) reported that higher levels of anti-*Leishmania* IgG/IgG1 and lower levels of IgG2 were also observed in SD. However, Leandro et al. (2001) and Cordeiro-da-Silva et al. (2003) have documented

increased levels of IgG2 in sera samples from infected animals, particularly in the case of SD.

Despite these controversial findings regarding the immunoglobulin isotype profile associated with CVL, it is clear that during canine *Leishmania* infection a dichotomous humoral immune response is triggered, similarly to that found in human infection (Anam et al., 1999). However, it is important to mention that the small number of infected animals evaluated on these studies may count for these contradictory results, especially considering the broad spectrum of humoral immune response observed during CVL (Alvar et al., 2004).

In order to further investigate this controversial issue on the context of CVL, herein we have performed a detailed analysis of anti-*Leishmania* immunoglobulin isotypes on a larger number of infected dogs, focusing attention on the possible association between clinical status and immunoglobulin isotype profile. As a pioneer investigation, we have also assessed the levels of *Leishmania*-specific antibody isotypes in dogs with visceral leishmaniasis classified by their degree of parasite load. The major goal of this new strategy is to evaluate the possible association between immunoglobulin isotype profiles and tissue parasitism that may offer additional scientific data to support further investigations on the immunopathological progress of CVL.

2. Materials and methods

2.1. Dogs and study design

In the current study, 60 mixed breed adult dogs of both genders aging from 2- to 6-year-old were captured by Control Zoonosis Center in Belo Horizonte City Hall (Minas Gerais state), where the clinical pre-selection was carried out and maintained in quarantine, confined in kennels at the Institute of Biological Sciences, Universidade Federal de Minas Gerais (UFMG), Brazil. Prior to the inclusion in this study, all animals were treated for intestinal helminthic (Endal plus®) infections and immunized against parvovirosis, leptospirosis, distemper, parainfluenza and hepatitis (HTLP 5CV-L vaccine Pfizer®). All animals received drinking water and a balanced feed (Kinus®- BRASWEY-AS) ad libitum.

The dogs inserted in this study were strayed or domiciled mongrel dogs, selected based on their serological results on IFAT, used as a ''gold standard'' immunological test for diagnosis of CVL. Forty dogs presenting IFAT titers $\geq$1:40 were considered positive and included into one of the groups constituted of infected animals. Twenty non-infected dogs with IFAT negative at 1:40 sera dilution and negative parasitological exams for *Leishmania* were considered non-infected and included as a control group (CD).

After quarantine, blood samples were collected in 10cc disposable sterile syringes, preferentially from jugular and/or cephalic veins. Five milliliter samples were transferred to a tube with no anticoagulant. The serum samples were stored in aliquots at 20 °C, until use for the serological examinations.

Leishmania infected dogs did not receive any treatment for CVL and were euthanized prior to collection of tissue samples which include skin, spleen, liver and lymph nodes. As chemotherapeutical practices for CVL is not officially allowed in Brazil, all infected dogs must be submitted to euthanasia.

This study was approved by the Ethical Committee for the use of Experimental Animals of the Universidade Federal de Minas Gerais, Brazil (CETEA).

2.2. Clinical evaluations

The mongrel dogs serologically positive by IFAT, were clinically classified according to presence/absence of clinical signs into three distinct categories, including: ''asymptomatic'' (AD, $n = 12$), with no suggestive signs of the disease; ''oligosymptomatic'' (OD, $n = 12$), with maximum three clinical signs, including opaque bristles and/or localized alopecia, and/or moderate loss of weight; ''symptomatic'' (SD, $n = 16$), with characteristic clinical signs of visceral leishmaniasis, such as opaque bristles, severe loss of weight, onychogriphosis, cutaneous lesions, apathy and keratoconjunctivitis.

2.3. Serological diagnosis of CVL—indirect immunofluorescence antibody test

Diagnosis of CVL was made by IFAT through the specific anti-*Leishmania* IgG reactivity using promastigote forms of *Leishmania amazonensis* (MHOM/

BR/1960/BH6). The parasites were maintained in logarithmic growth, in liver infusion tryptose (LIT) medium as described by Camargo and Rebonato (1969). FITC-conjugated-anti-dog IgG antibody was used to access the IgG reactivity (Biomanguinhos, FIOCRUZ, RJ, Brazil). Animals with antibody titration higher than 1:40 were considered to have a positive diagnosis of CVL.

Infection with *L. chagasi* was confirmed in all IFAT positive dogs by at least one additional serological approach, including ELISA-extract and ELISA r-K39, as previously described (Reis et al., 2006) and/or parasitological examination as described below.

2.4. Parasitological analysis—parasite load index

All seropositive dogs were euthanized under sedation (Thiopental®) and submitted to parasitological examinations for *Leishmania* and classified according to the parasite load.

Bone marrow aspiration was undertaken from the inferior region of the sternum or from the iliac crest, under sedation with intravenous doses of sodium thiopental—Thionembutal® (8.4 mg/kg of body weight).

Parasitological diagnosis in tissue smears (ear skin, spleen, liver and lymph node) was performed after necropsy of the animals. Tissue samples were randomly collected regardless the presence or absence of lesions. Imprints were performed on two microscopic slides and after air-drying, samples were fixed in methanol, stained with Giemsa, and examined under optical microscopy, for the identification of *Leishmania* amastigote forms. The parasite density evaluation was performed in ear skin, bone marrow, spleen, liver and popliteal lymph node imprints, and the results expressed as ''Leishman Donovan Units'' (LDU index), according to Stauber (1955), which correspond to the number of *Leishmania* amastigotes per 1000 nucleated cells.

2.5. Immunological study—immunoglobulin isotype profile by enzyme linked immunosorbent assay (ELISA)

The ''in-house'' ELISA tests were performed to determine the anti-*Leishmania* immunoglobulin pattern and were carried out using soluble *L. chagasi* (MHOM/BR/1972/BH46) promastigotes antigen (SLA) from axenic culture in LIT medium. The parasites were cultured for 7 days (Mancianti et al., 1995), washed three times by centrifugation at 2000 rpm in phosphate buffer solution (PBS) pH 7.2, for 10 min, followed by three ultrasound cycles per 1 min at 40 W on ice bath (Sonifier Cell Disruptor®–Branson Sonic Power Co., USA). The sonicated material was centrifuged at 18,500 rpm for 1 h and 30 min at 4 °C. The supernatant was transferred to dialysis tubes and dialyzed against PBS for 36 h, and submitted to four PBS changes every 6 h. Finally, the remaining material was filtered through disposable sterile filters of 22 μm under aseptic conditions; one aliquot was taken for protein concentration by the method of Lowry et al. (1951), and adjusted to the concentration of 1000 μg/mL and stored in small aliquots at 70 °C prior to use.

Ninty-six-well microplates (MaxiSorp™, Nalge Nunc Intl., USA) were coated with SLA at a concentration of 10 μg/well, overnight at 4 °C. After coating and washing procedures, sera samples were added at the dilution 1:80 followed by washes and addition of peroxidase conjugated goat anti-dog IgG1 (anti-heavy chain specific), IgM (anti-μ chain specific), IgA (anti-α chain specific) and IgE (anti-ε chain specific) or sheep anti-dog IgG and IgG2 (both anti-heavy chain specific). All reagents were purchased from Bethyl Laboratories Inc., Montgomery, TX, USA. Wells were then washed, substrate and chromogen (*O*-Phenylenediamine, Sigma–Aldrich Co., USA) were added and absorbance was read on an automatic ELISA microplate reader (Multiskan® MCC 340, Labsystems, Helsinki, Finland) at 492 nm. The conjugate concentrations were determined by a block titration method with positive and negative standard sera. The conjugate anti-IgG1, IgM, IgA, IgE was used at dilution 1:1000 and the anti-IgG and IgG2 were used at dilutions 1:8000 and 1:16,000, respectively.

2.6. Statistical analysis

Statistical analysis was performed using the Minitab 9.2 Statistical Software (Minitab Inc., Pennsylvania, USA). One-way analysis of variance (ANOVA) was used for the comparative studies of Immunoglobulins absorbance values between groups

of clinical form and parasitic status. Student's *t*-test was used to identify significant differences between the group absorbance averages. In the non-parametric data, Kruskal–Wallis test was used for the comparative study between groups, followed by Dunns test. Comparative analysis between clinical form and parasitic status was carried out by the chi-square test. Spearman's rank correlations (r_S) was computed to investigate associations among immunoglobulin levels (Igs), parasite density and clinical status parameters. In all cases, the differences were considered significant when the probabilities of equality, *p*-values, were ≤ 0.05.

3. Results

3.1. Distinct patterns of tissue parasite density are observed in dogs infected with L. chagasi

We have evaluated the tissue parasitism in different organs from *L. chagasi* naturally infected dogs (skin, bone marrow, spleen, liver and lymph node). Results were expressed as parasite density (LDU) (Fig. 1). Tissue parasitism for each compartment was initially classified as low (LP), medium (MP) or high (HP) parasitism based on tissue-specific LDU values statistically categorized into tertiles, as follow: skin (LP: 0–9; MP: 10–130; HP: 133–7246), bone marrow (LP: 0-0; MP: 2–33; HP: 46–1104), spleen (LP: 0–11; MP: 12–170; HP: 184–2564), liver (LP: 0–1; MP: 6–41; HP: 44–2196), lymph node (LP: 0–1; MP: 3–24; HP: 37–616). This approach strengthens further statistical analyses on similar number of dogs into each subgroup. The number of animals included on each subgroup was approximately 12–14 animals despite the tissue evaluated. Data analysis pointed out the spleen and the skin as the major targets of higher LDU values (Fig. 1), wherein the parasite density was much higher than other evaluated tissues (skin compared to bone marrow, $p = 0.0027$; liver, $p = 0.0035$; lymph node, $p = 0.0006$ and spleen compared to bone marrow, $p = 0.0006$; liver, $p = 0.0011$; lymph node, $p = 0.0025$).

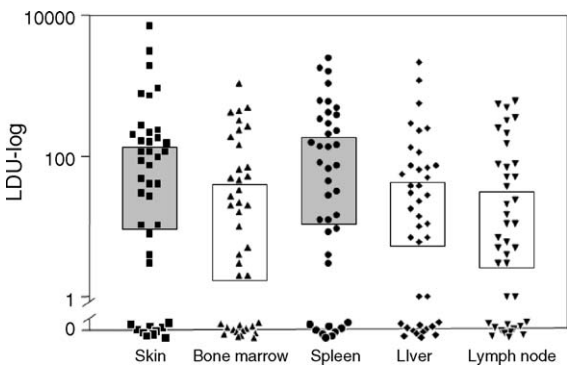


Fig. 1. Tissue parasitism in different organs from *L. chagasi* naturally infected dogs. Results were expressed as parasite density (LDU) for ear skin (■), bone marrow (▲), spleen (●), liver (◆) and Lymph node (▼). Tissue parasitism for each compartment was classified as low (LP), medium (MP) or high (HP) parasitism based on LDU values statistically categorized into tertiles. Significant differences at $p < 0.05$ were highlighted in gray boxes showing the skin and the spleen as the most relevant sites of high parasite density as compared to the other tissues.

3.2. Despite the clinical status, spleen and skin are the major sites of high parasite density during ongoing canine visceral leishmaniasis

Analysis of LDU values has been further addressed by calculating the 75% percentile for each tissue evaluated (Fig. 2). Our results demonstrated distinct profile of parasite density depending on both the tissue and the clinical form evaluated. In this context, the skin (as compared to bone marrow, $p = 0.0027$; liver, $p = 0.0035$; lymph node, $p = 0.0006$) and the spleen (as compared to bone marrow, $p = 0.0006$; liver, $p = 0.0011$; lymph node, $p = 0.0025$) have been pointed out as the most relevant sites of high parasite density in comparison to all other sites, when taking together all infected dogs.

Analysis of LDU values regarding the clinical status confirmed the skin and the spleen as the major parasitic site as compared to bone marrow in AD ($p = 0.0313$ and $p = 0.0313$, respectively), bone marrow and lymph node in OD ($p = 0.0195$, $p = 0.0078$ and $p = 0.0371$, $p = 0.0039$, respectively) and liver and lymph node in SD ($p = 0.0102$, $p = 0.0065$ and $p = 0.0088$, $p = 0.015$, respectively).

The other evaluated organs, liver and lymph node, showed similar values of parasite density. It is important to notice that bone marrow from AD showed lower parasite density in comparison to OD and SD (Fig. 2).

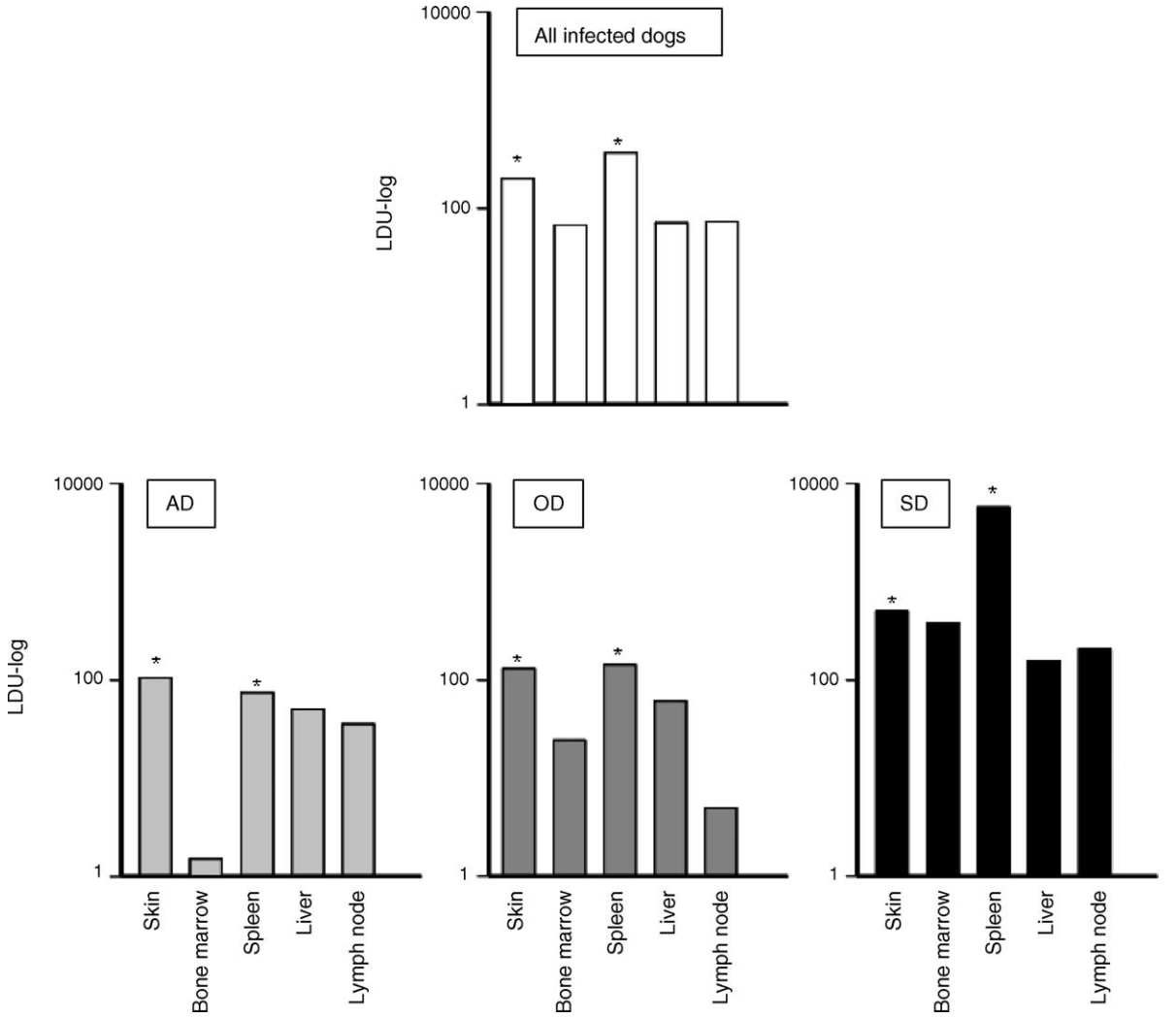


Fig. 2. Parasite density (LDU) in different organs from *L. chagasi* naturally infected dogs (□), classified based on their clinical status as asymptomatic (AD: ▒), oligosymptomatic (OD: ▓) and symptomatic (SD: ■) dogs. Results were expressed as 75% percentile for each organ. Significant differences at $p < 0.05$ were identified by "*" highlighting the skin and the spleen as the most relevant sites of high parasite density as compared to bone marrow, liver and lymph node in all infected dogs, bone marrow in AD, bone marrow and lymph node in OD and liver and lymph node in SD.

3.3. Bone marrow and spleen parasite density are the most reliable parasitological markers to decode the clinical status of canine visceral leishmaniasis

The frequency of low, medium and high parasite density in different tissues of *L. chagasi* naturally infected dogs categorized according to their clinical status are shown in Table 1. Data analysis demonstrated that clinical forms of CVL have a strong association with parasite density status, with connection between LP and AD, whereas HP showed to be more associated with SD. Interestingly, bone marrow parasitism pointed out that 75% of dogs with LP display connection with AD group, whereas 25% and 12.5% of animals displaying LP were confined into OD and SD groups, respectively. In contrast, 68.8% of dogs presenting high parasitism belong to the SD

Table 1
Frequency of low, medium and high parasite density (LDU) in different tissues of *L. chagasi* naturally infected dogs categorized according to their clinical status

Tissues	Parasite density (LDU)	Clinical status					
		AD		OD		SD	
		n (%)	% of total	*n* (%)	% of total	*n* (%)	% of total
Skin ($r = 0.4416$, $p = 0.0043$)	LP	6 (50.0%)	15.0	5 (41.7%)	12.5	2 (12.5%)	5.0
	MP	3 (25.0%)	7.5	4 (33.3%)	10.0	6 (37.5%)	15.0
	HP	3 (25.0%)	7.5	3 (25.0%)	7.5	8 (50.0%)	20.0
Bone marrow ($r = 0.6508$, $p < 0.0001$)	LP	9 (75.0%)	22.6	3 (25.0%)*	7.5	2 (12.5%)*	5.0
	MP	2 (16.7%)	5.0	7 (58.3%)	17.5	3 (18.7%)	7.5
	HP	1 (8.3%)	2.5	2 (16.7%)	5.0	11 (68.8%)*,†	27.5
Spleen ($r = 0.5048$, $p = 0.0010$)	LP	7 (58.3%)	17.9	4 (36.3%)	10.4	2 (12.5%)*	5.1
	MP	3 (25.0%)	7.7	5 (45.5%)	12.8	5 (31.3%)	12.8
	HP	2 (16.7%)	5.1	2 (18.2%)	5.1	9 (56.2%)	23.1
Liver ($r = 0.4066$, $p = 0.0092$)	LP	7 (58.3%)	18.5	6 (50.0%)	15.0	1 (6.3%)*,†	2.5
	MP	2 (16.7%)	5.0	2 (16.7%)	5.0	8 (50.0%)	20.0
	HP	3 (25.0%)	7.5	4 (33.3%)	10.0	7 (43.7%)	17.5
Lymph node ($r = 0.4795$, $p = 0.0017$)	LP	7 (58.3%)	17.5	5 (41.7%)	12.5	2 (12.5%)*	5.0
	MP	2 (16.7%)	5.0	6 (50.0%)	15.0	4 (25.0%)	10.0
	HP	3 (25.0%)	7.5	1 (8.3%)	2.5	10 (62.5%)†	25.0

LDU, Leishman Donovan Units. The results are expressed as frequency of animals displaying a given parasite density score. Parasite density degree (low (LP), medium (MP) and high (HP) parasitism); clinical status (asymptomatic (AD), oligosymptomatic (OD) and symptomatic (SD) dogs).
*Differences statistically significant in comparison to AD at $p < 0.05$.
†Differences statistically significant in comparison to OD at $p < 0.05$.
Spearman correlation indexes (r) at $p < 0.05$ are shown in table.

group, whereas only 8.3% and 16.7% of dogs with HP fit into AD and OD groups, respectively. Analysis of spleen parasitism demonstrated that 58.3% of dogs showing LP belong to AD group in contrast with 12.5% of dogs from SD group. Similar results were documented in the liver compartment, with 58.3% of dogs with LP fitting into the AD group in contrast with 6.3% of dogs from SD group. Analysis of parasite density of lymph node demonstrated that 58.3% of dogs with LD belong to AD group and 62.5% of dogs with high parasitism were from SD group, whereas only 8.3% of dogs with HP fit into OD group (Table 1).

In addition, in order to validate the association between tissue parasite load in the organs more affected during CVL, we further performed a correlation analysis between parasite density in specific organs and clinical status of CVL (Table 1). Our results demonstrated that, although parasite density in all organs was positively correlated with the clinical forms of CVL, parasite density of bone marrow and spleen display the stronger correlation with clinical status ($r = 0.6508$, $p < 0.0001$ and $r = 0.5048$, $p = 0.0010$, respectively).

3.4. Analysis of immunoglobulin isotypes point out a prognostic hallmark for canine visceral leishmaniasis highlighting strong correlation between IgG1 and AD but IgA, IgE and IgG2 displaying better association with SD

The reactivity of seric anti-*Leishmania* immunoglobulin isotypes (IgG, IgG1, IgG2, IgM, IgA and IgE) from dogs naturally infected, categorized according to the clinical status of CVL are shown in Fig. 3. Our results demonstrated that all non-infected dogs displayed negative IgG, IgG1 and IgG2 reactivity considering the cut-off edges for the anti-*Leishmania* immunoglobulin reactivity detected by ELISA (0.140 for total IgG; 0.170 for IgG1 and 0.180 for IgG2). Moreover, all infected dogs displayed significant higher anti-*Leishmania* total IgG, IgG2, IgM and IgA in comparison to control group

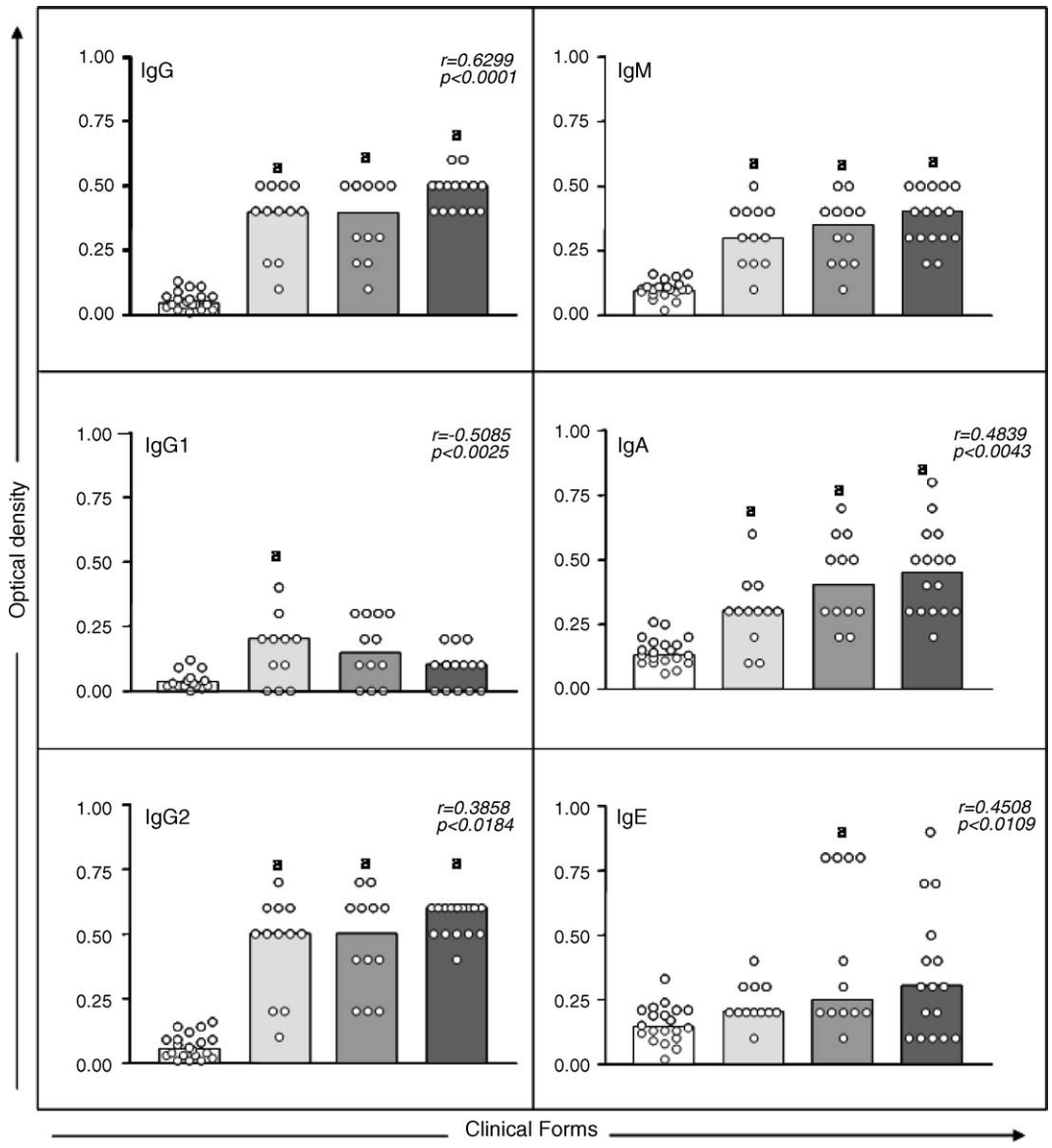


Fig. 3. Anti-*Leishmania* immunoglobulin isotypes reactivity in *L. chagasi* naturally infected dogs categorized according to their clinical status as asymptomatic (AD: ▒), oligosymptomatic (OD: ■) and symptomatic (SD: ■) dogs. Uninfected dogs were used as a control group (CD: □). Results were expressed as scattering of individual values and mean optical density for each immunoglobulin isotype. Significant differences at $p < 0.05$ were identified by the letter "*a*" in comparison to CD. Confirmatory analysis was also performed and the spearman correlation indexes (r) at $p < 0.05$ are shown in figure.

($p < 0.05$). Further, only AD and OD groups presented higher levels of anti-*Leishmania* IgG1 and IgE, respectively, in comparison to control group ($p < 0.05$).

The correlation between immunoglobulins levels and the clinical status of CVL was also evaluated and demonstrated a positive association between most anti-*Leishmania* immunoglobulin isotypes investigated and the clinical status of CVL, except for IgG1 and IgM: (IgG, $r = 0.6299$; $p < 0.0001$), (IgA, $r = 0.4839$; $p < 0.0043$), (IgE, $r = 0.4508$; $p < 0.0109$) and (IgG2, $r = 0.3858$; $p < 0.0184$). Interestingly, a

negative correlation was observed between IgG1 and disease morbidity ($r = -0.5085$; $p < 0.0025$) (Fig. 3).

3.5. Anti-Leishmania IgG and IgA reactivity better reflect the overall tissue parasitism

The analysis of the anti-*Leishmania* total IgG reactivity in *L. chagasi* naturally infected dogs categorized according to parasite density from different tissues are shown in Fig. 4. Tissue parasitism was classified as LP, MP and HP as previously described. Data analysis revealed that animals displaying MP or HP presented increased levels of IgG in most tissues. Interestingly, only animals displaying HP at bone marrow also presented increased levels of IgG. In addition, the analysis of correlation demonstrated a significant positive association between anti-*Leishmania* IgG reactivity and parasite density in all evaluated organs: (skin, $r = 0.5146$; $p = 0.0016$), (bone marrow, $r = 0.4816$; $p = 0.0034$), (spleen, $r = 0.5200$; $p = 0.0016$), (liver, $r = 0.4420$; $p = 0.0078$) and (lymph node, $r = 0.4536$; $p = 0.0062$) (Fig. 4).

The evaluation of the other anti-*Leishmania* immunoglobulin isotypes (IgG1, IgG2, IgM, IgA and IgE) in *L. chagasi* naturally infected dogs categorized according to parasite density in different tissues is shown in Table 2. Our data demonstrated that IgG1 and IgE are not associated with tissue parasitism. Higher IgG2 levels were observed in dogs with MP and/or HP in the liver and lymph node sites, in comparison with LP. On the other hand, although higher IgM levels were also observed in dogs with MP and/or HP in the lymph node, an increase in the IgM levels was detected only in dogs with HP in the liver. Analysis of IgA reactivity also exhibited higher association with tissue parasitism, since higher IgA levels were observed in dogs displaying MP and/or HP in most tissues, except skin (Table 2).

3.6. Bone marrow parasite density better correlates with immunoglobulin reactivity during canine visceral leishmaniasis

In order to identify which organ confirms better association between parasite load and anti-*Leishmania* immunoglobulin isotype pattern, we have performed additional correlation analysis between tissue parasite density of different organs and anti-*Leishmania*

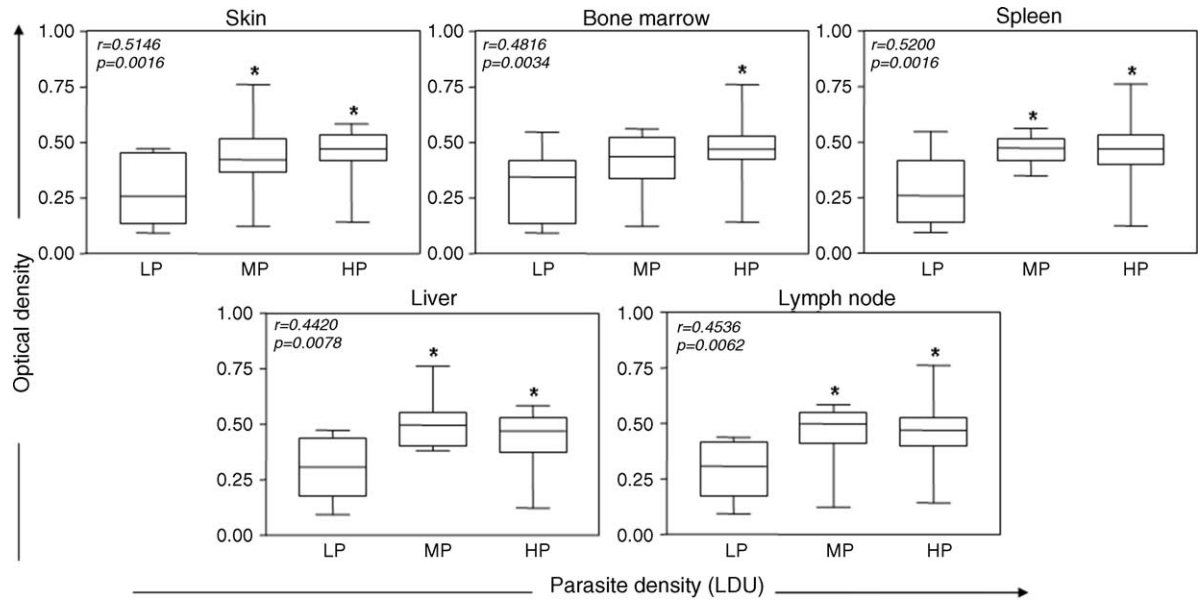


Fig. 4. Anti-*Leishmania* IgG reactivity in *L. chagasi* naturally infected dogs categorized according to parasite density (LDU) in different tissues as low (LP), medium (MP) and high (HP) parasitism. The results are expressed in box-plot format highlighting the gap of 50% of data set measurement. *Significant differences in comparison to LP at $p < 0.05$. Confirmatory correlation analysis was also performed and the spearman correlation indexes (r) at $p < 0.05$ are shown in figure.

Table 2
Anti-*Leishmania* immunoglobulins isotypes reactivity in *L. chagasi* naturally infected dogs categorized according to parasite density (LDU) in different tissues

Tissues	Parasite density (LDU)	Immunoglobulin isotypes				
		IgG1	IgG2	IgM	IgA	IgE
Skin	LP	0.15 (0.0–1.0)	0.45 (0.1–0.6)	0.28 (0.1–0.4)	0.30 (0.1–0.6)	0.26 (0.2–0.8)
	MP	0.12 (0.0–0.2)	0.53 (0.2–0.6)	0.28 (0.2–0.5)	0.34 (0.2–0.7)	0.22 (0.1–0.6)
	HP	0.12 (0.0–0.3)	0.57 (0.4–0.7)	0.42 (0.2–0.5)	0.50 (0.3–0.8)	0.40 (0.2–1.0)
Bone marrow	LP	0.19 (0.0–1.0)	0.49 (0.1–0.6)	0.27 (0.1–0.4)	0.29 (0.1–0.5)	0.26 (0.2–0.8)
	MP	0.11 (0.3–0.3)	0.49 (0.2–0.7)	0.39 (0.2–0.5)	0.51 (0.3–0.8)*	0.22 (0.2–0.6)
	HP	0.09 (0.0–0.3)	0.57 (0.4–0.7)	0.41 (0.2–0.5)	0.46 (0.3–0.7)*	0.54 (0.1–1.0)
Spleen	LP	0.19 (0.0–1.0)	0.45 (0.1–0.6)	0.26 (0.1–0.4)	0.28 (0.1–0.5)	0.26 (0.2–0.8)
	MP	0.14 (0.0–0.3)	0.54 (0.2–0.6)	0.37 (0.2–0.5)	0.42 (0.3–0.8)*	0.23 (0.2–0.8)
	HP	0.09 (0.0–0.2)	0.57 (0.4–0.7)	0.41 (0.2–0.5)	0.43 (0.3–0.7)*	0.43 (0.1–1.0)
Liver	LP	0.16 (0.0–1.0)	0.43 (0.1–0.6)	0.25 (0.1–0.5)	0.30 (0.1–0.6)	0.25 (0.2–0.4)
	MP	0.14 (0.0–0.3)	0.57 (0.4–0.6)*	0.36 (0.2–0.5)	0.51 (0.2–0.8)*	0.30 (0.2–0.8)
	HP	0.11 (0.0–0.3)	0.57 (0.4–0.7)*	0.41 (0.2–0.5)*	0.40 (0.3–0.7)	0.25 (0.1–1.0)
Lymph node	LP	0.12 (0.0–1.0)	0.43 (0.1–0.6)	0.23 (0.1–0.5)	0.29 (0.1–0.3)	0.24 (0.2–0.4)
	MP	0.11 (0.0–0.3)	0.60 (0.4–0.7)*	0.41 (0.2–0.5)*	0.55 (0.2–0.8)*	0.24 (0.2–0.8)
	HP	0.14 (0.0–0.3)	0.57 (0.4–0.7)*	0.38 (0.2–0.5)*	0.38 0.3–0.7)*	0.37 (0.1–1.0)

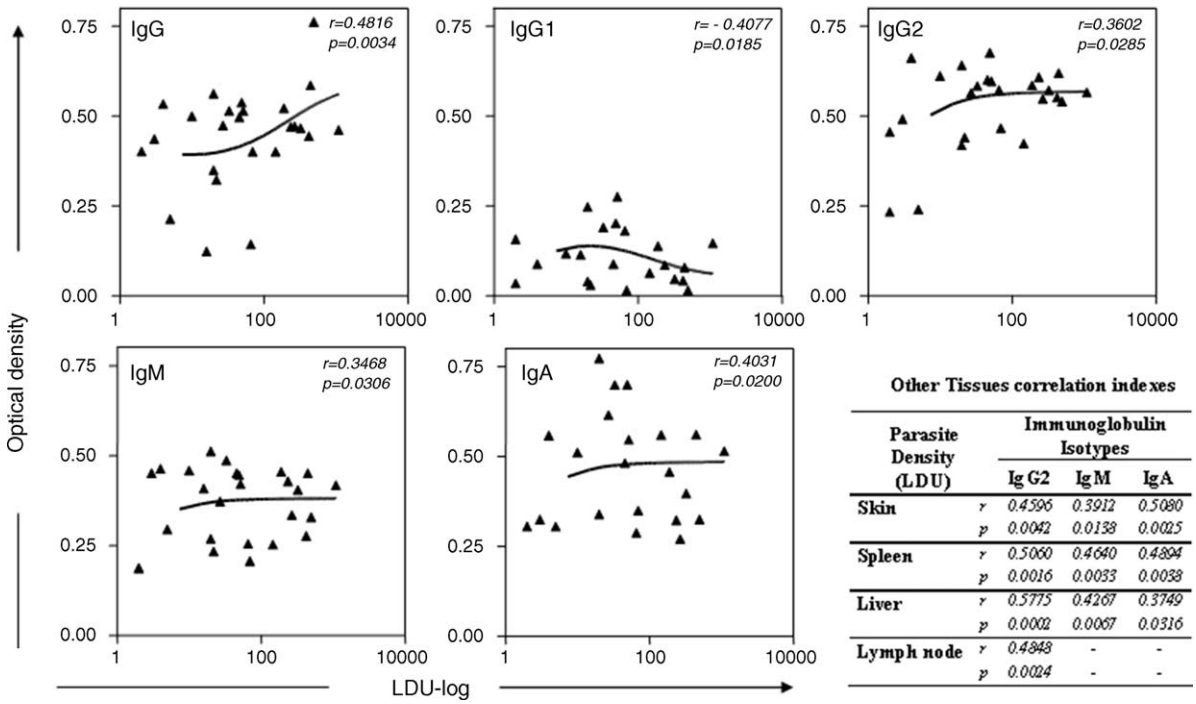
LDU, Leishman Donovan Units. LP, low parasitism; MP, medium parasitism; HP, high parasitism. The results are expressed as median of optical density (minimum–maximum values). The anti-*Leishmania* OD values were measured at 492 nm in the sera diluted at 1:80.
* Differences statistically significant in comparison to LP at $p < 0.05$.

immunoglobulin isotype reactivity (Fig. 5). Interestingly, our results pointed out the bone marrow as the top organ showing positive correlation with most immunoglobulin isotypes (IgG, $r = 0.4816$; $p = 0.0034$), (IgG1, $r = -0.4077$; $p = 0.0185$), (IgG2, $r = 0.3602$; $p = 0.0285$), (IgM, $r = 0.3468$; $p = 0.0306$) and (IgA, $r = 0.4031$; $p = 0.0200$) (Fig. 5). Positive correlation was also observed between skin, spleen and liver parasite density and levels of anti-*Leishmania* IgG2, IgM and IgA. Similar data was detected between lymph node and IgG2 levels (table inserted in Fig. 5).

4. Discussion

Besides its relevance as the major reservoir for human visceral leishmaniasis, dogs have been considered to be a relevant model for *L.* (*L.*) *infantum and L.* (*L.*) *chagasi* infection, since many aspects resemble those observed for the human disease (Keenan et al., 1984; Hommel et al., 1995; Alvar et al., 2004). The analysis of clinical and pathological features in CVL disease disclosed three major groups of infected animals, as they respond to chronic infection, generally named asymptomatic, oligosymptomatic and symptomatic, both in natural and experimental infection (Pinelli et al., 1994; Keenan et al., 1984; Abranches et al., 1991; Cordeiro-da-Silva et al., 2003). Despite the importance of canines as reservoirs for the human disease, little information is available on the immunological basis of CVL (Alvar et al., 2004). The goal of this present investigation was to evaluate the humoral immune response in naturally *L.* (*L.*) *chagasi*-infected dogs focusing on a possible prognostic marker such as a correlation among immunoglobulin isotypes profile, clinical status as well as tissue parasite density.

Firstly, we have assessed the tissue parasite density of relevant organs in order to identify the major sites of parasite spreading. According to Tafuri et al. (2004), the clinical manifestations of CVL do not necessarily correlate with the parasite load. However, other investigations have reported a good correlation between the parasite load of the spleen and lymph node and the clinical manifestations of CVL (Barrouin-Melo et al., 2004; Sanchez et al., 2004). Our findings pointed out the spleen and the skin as the



Other Tissues correlation indexes

Parasite Density (LDU)		Immunoglobulin Isotypes IgG2	IgM	IgA
Skin	r	0.4596	0.3912	0.5080
	p	0.0042	0.0138	0.0025
Spleen	r	0.5060	0.4640	0.4894
	p	0.0016	0.0033	0.0038
Liver	r	0.5775	0.4267	0.3749
	p	0.0002	0.0067	0.0316
Lymph node	r	0.4848	-	-
	p	0.0024	-	-

Fig. 5. Correlation between parasite density (LDU) in bone marrow (graphs) and other tissues (table) and anti-*Leishmania* immunoglobulin isotypes reactivity in *L. chagasi* naturally infected dogs. The results were expressed on graphs as scattering of individual values. Spearman correlation indexes (r) at $p < 0.05$ are shown on graphs and table. Connecting lines illustrated positive and negative correlation indexes.

major targets of higher LDU values (Fig. 1), where high parasite density showed to be distinct from other evaluated tissues. In addition, we have evaluated in *L.* (*L.*) *chagasi* naturally infected dogs, the relationship between tissue parasitism (skin, bone marrow, spleen, liver and lymph node) and CVL clinical status (asymptomatic (AD), oligosymptomatic (OD) and symptomatic (SD)). We have documented that despite the clinical status, the spleen and the skin remain as the top sites of high LDU values (Fig. 2). It was important to notice that bone marrow from AD showed lower parasite density in comparison to OD and SD. Together, these findings re-emphasize our previous observations demonstrating the lower performance of bone marrow impression smears in diagnosing CVL in AD (Reis et al., 2006). Moreover, the higher parasite density of the spleen and the skin also reflects the higher sensitivity of spleen and skin impression smears to CVL diagnosis (Reis et al., 2006). It was interesting to notice that despite parasite density of bone marrow represents the lower LDU values (Fig. 1), additional analysis pointed out this organ as the major site that correlates with the clinical status of CVL (Table 1). Indeed, the present work demonstrated that better correlations between parasite density and clinical forms are documented this way: bone marrow > spleen > lymph node > skin > liver (Table 1). Our data showed also that whereas AD display skin, liver and lymph node parasitism similarly to that observed for symptomatic dogs, SD exhibited intense parasite load in most evaluated tissue a higher density was found in the bone marrow and in the spleen when compared to AD (Fig. 2). Together, these data may explain discordant reports regarding the association between clinical status and tissue parasite load during CVL (Tafuri et al., 2004; Sanchez et al., 2004; Barrouin-Melo et al., 2004). It is possible that a correlation analysis between clinical features and tissue parasitism performed in distinct sites is the major responsible for this controversy. Therefore, it is important to highlight the major contribution of our present investigation to clarify the influence of compartmentalized parasite density when evaluating its association with the clinical status of CVL.

In addition, when searching for laboratory prognosis markers, we have aimed to correlate clinical status and tissue parasite density with humoral immune response of *L* (*L.*) *chagasi*-infected dogs. While some reports have suggested that the sole presence of anti-*Leishmania* antibodies is not a conclusive marker for the disease progression during canine *L.* (*L.*) *infantum* infection (Gicheru et al., 1995; Nieto et al., 1999), herein, we have observed that the reactivity of serum anti-*Leishmania* immunoglobulin isotypes from dogs naturally infected with *L.* (*L.*) *chagasi*, showed a marked association between clinical status and tissue parasite density and therefore represents a good indicator of disease morbidity. Our data demonstrated that anti-*Leishmania* IgG and IgA reactivities were the best markers for overall tissue parasitism (Fig. 4 and Table 2, respectively). We indeed observed a positive correlation between clinical forms of CVL with the levels of IgG, IgG2, IgA and IgE (Fig. 3) and tissue parasite density with the levels of IgG, IgG2, IgM and IgA (Fig. 5). Although no correlation was observed among the levels of IgM as well as IgA and lymph node parasitism (table inserted in Fig. 5), we have detected an increase in the levels of these immunoglobulins in animals displaying both medium and high lymph node parasite density (Table 2). On the other hand, we verified a negative correlation between the IgG1 reactivity and both clinical status and bone marrow parasitism with (Figs. 3 and 5). Moreover, the bone marrow parasite density strikingly correlated with most immunoglobulin reactivity, except for IgE (Fig. 5).

Abranches et al. (1991) verified that specific antibodies production starts at 1.5–3 months after the *L.* (*L.*) *infantum* experimental infection. Although Abranches et al. (1991) did not find any correlation between IgG titers and disease progression, the major findings from Lanotte et al. (1979) and Oliveira et al. (1993) suggested that higher levels of IgG observed in asymptomatic dogs from endemic areas (Oliveira et al., 1993) seem to be consistent with the fact that 90% of these dogs would develop the advanced and symptomatic form of the disease (Lanotte et al., 1979). According to this proposal, it has been reported that dogs infected with *L.* (*L.*) *infantum* showed increased levels of IgG anti-*Leishmania* prior to the appearance of the first clinical signals (Hommel et al., 1995). Our data support these observations, since the antibody levels (mainly IgG, IgM, IgA and IgG2) are elevated in all infected dogs, not depending on their clinical status (Fig. 3). Similar findings were also reported by Bourdoiseau et al. (1997), Leandro et al. (2001), Vercammen et al. (2002) and Cordeiro-da-Silva et al. (2003). These authors observed an increase of total IgG level in asymptomatic and symptomatic dogs. Furthermore, the IgG2 levels appeared to be the predominant subclass present in the sera of all infected animals (Bourdoiseau et al., 1997; Vercammen et al., 2002), particularly in the case of symptomatic dogs (Leandro et al., 2001; Cordeiro-da-Silva et al., 2003).

Additional correlation analysis pointed out that antibody response was closely related to CVL morbidity, since as the clinical signals become evident, higher levels of IgG, IgA, IgG2 and IgE are observed (Fig. 3). In the present work, the levels of IgG1 suggest an association with immunoprotector mechanisms of the canine infection, since they were frequently elevated in AD (Fig. 3) as well as in animals bearing low bone marrow parasite density (Fig. 5). In contrast, circulating IgG2 seems to be more associated with CVL morbidity, been positively correlated with more severe clinical status and higher parasite density (Figs. 3 and 5). Previous investigations have postulated an association between high levels of IgG2 and asymptomatic form of CVL, whereas IgG1 levels better correlate with symptomatic disease (Deplazes et al., 1995; Nieto et al., 1999; Solano-Gallego et al., 2001; Courtenay et al., 2002; Quinnell et al., 2003). On the other hand, additional investigations failed to demonstrate this trend suggesting that in fact lower levels of IgG1 better associated with symptomatic dogs (Bourdoiseau et al., 1997; Leandro et al., 2001; Vercammen et al., 2002; Cordeiro-da-Silva et al., 2003). Our results resembled in part those obtained by Bourdoiseau et al. (1997) and Vercammen et al. (2002) that described an association between high levels of IgG1 and the establishment/maintenance on asymptomatic chronic disease. We believed that the small number of infected animals enrolled on these studies might account for these controversial results (Solano-Gallego et al., 2001).

Although IgM is classically associated with the acute forms of parasitic diseases, it has been demonstrated that in canine *L.* (*L.*) *chagasi* infection, the levels of IgM, detected early after the first month

during acute infection persist throughout chronic infections (Genaro et al., 1992). Our data re-emphasize these findings, since high IgM levels were found in all *L.* (*L.*) *chagasi*-infected dogs, despite their clinical status (Fig. 3). However, despite no correlation between IgM levels and CVL clinical status was detected (Fig. 3), our findings revealed that IgM levels were correlated with skin, bone marrow, spleen, and liver parasite density (table inserted in Fig. 5). The spleen is an important immunologically active compartment, where cells from the immune system interact with parasite-derived antigens during *L.* (*L.*) *chagasi* infection. Considering the spleen as the main site of lymphoid cells interposed into the blood stream, the large amount of circulating *Leishmania* antigens, besides high local parasite density frequently in contact with splenocytes, would lead to a strong humoral response. Moreover, as the spleen contain the larger amount of B-1 lymphocytes (Tischendorf, 1985), the major source of poly-reactive IgM anti-carbohydrate (Garzelli et al., 1994; Karpatkin et al., 1995), it is plausible to infer that the increment of IgM reflects the fundamental response to parasite antigens.

Our results also emphasize that disease progression was additionally characterized by increment of other specific immunoglobulins isotypes (mainly IgA and IgE) that may contribute to aggravation of clinical status during CVL, as previous suggested by Iniesta et al. (2005). Our data demonstrated that levels of anti-*Leishmania* IgA were positively correlated with clinical status (Fig. 3) as well as skin, bone marrow, spleen and liver parasite density (Fig. 5). Interestingly, as IgA deposits are identified in the kidneys during CVL, it has been proposed that IgA may contribute for the genesis of CVL-associated glomerulonefritis (Nieto et al., 1992).

Likewise IgA, but independently of tissue parasite density, we have found that IgE levels were correlated with CVL clinical status (Fig. 3). Similar results were reported by Iniesta et al. (2005), pointing out higher IgE levels in symptomatic animals.

The association between clinical status, Ig profile and the tissue parasitism support a novel investigation on the impact of humoral immune response and susceptibility/resistance mechanism during ongoing CVL. In conclusion, the new aspects of this study highlighted pioneer findings that revealed a correlation between distinct patterns of tissue parasite density and the profile of anti-*Leishmania* immunoglobulin isotypes during CVL, pointing out the spleen and skin as the most relevant sites of high parasite density and the parasitism in the bone marrow and spleen as the most reliable parameters to decode CVL clinical status. Moreover, the parasite density of bone marrow better correlates with most anti-*Leishmania* Igs reactivity. An outstanding prognostic marker for canine visceral leishmaniasis was identified, highlighting IgG1 for asymptomatic infection and in relevance IgA, IgE and IgG2 for symptomatic disease. Moreover, our data re-enforce the anti-*Leishmania* IgG but with IgA reactivity as the better marker for overall tissue parasitism.

Taken together, our findings supported that the association between clinical status, Igs profile and tissue parasitism should be taken into account in further investigations focusing the impact of humoral immune response on susceptibility/resistance mechanism during ongoing CVL.

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