



Relationship of *Leishmania*-specific IgG levels and IgG avidity with parasite density and clinical signs in canine leishmaniasis

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ABSTRACT

The clinical status and tissue parasite burden of the skin and spleen of 40 dogs naturally infected with *Leishmania chagasi* (syn. *Leishmania infantum*), together with 5 uninfected control dogs, were assessed. On the basis of the clinical evaluation, infected dogs were classified as asymptomatic (AD) or symptomatic (SD). Infected animals were also grouped according to their parasite load as exhibiting low (LP), medium (MP) and high (HP) parasitism. The results indicated a high parasite load in the skin samples of SD animals in relation to the AD group. The serum immunoglobulin isotype profiles of the studied animals revealed increased levels of IgG₁ in the AD and LP dogs, whereas high levels of IgG₂ were correlated with SD and HP dogs. The avidity index (AI) of IgG_{total} in the SD group was high in comparison of that of the AD group. Moreover, animals with a larger parasite burden either in the spleen or skin showed higher AI values than animals with lower parasitism. Based on these findings, it is suggested that CVL commences with an asymptomatic clinical form with low parasitism, high production of IgG₁ and low affinity of IgG_{total} molecules, and evolves into a symptomatic clinical form with higher parasitism intensity, higher IgG₂ levels, and high affinity of IgG_{total}.

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1. Introduction

Visceral leishmaniasis (VL) is the most severe clinical form of *Leishmania chagasi* (syn. *L. infantum*) infection, giving rise to some 500,000 new cases and 59,000 deaths each year according to data published by the World Health

Organisation (WHO, 2005). The disease is endemic in 87 countries, although 90% of notified cases occur in India, Sudan, Bangladesh, Nepal and Brazil (WHO, 2005), and its epidemiological spectrum is broad, extending from tropical and subtropical areas through to temperate regions of the globe (Deane and Deane, 1962; Alvar et al., 2004; Desjeux, 2004).

Dogs represent important elements in the transmission of VL and constitute the main domestic reservoirs of *L. chagasi* (Deane and Deane, 1954; Molina et al., 1994; Giunchetti et al., 2006). Indeed, the major focal areas of human VL are strongly associated with locations that exhibit a high prevalence of seropositive dogs (Oliveira

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et al., 2001). In this context, even asymptomatic animals present intense cutaneous parasitism that facilitates the infection of insect vectors. From an epidemiological point of view, therefore, canine visceral leishmaniasis (CVL) is more important than the human form of the disease since efficient control of the former reduces considerably the number of reservoirs and, consequently, the rate of infection in humans (Ashford, 1996).

Much research effort has been devoted to the elucidation of the factors responsible for the resistance or susceptibility of dogs to *L. chagasi* infection. Thus, the natural history of CVL has been well described, particularly with respect to parasite load in different tissues and to the immunopathological changes relating to the progression of clinical forms of the disease (Reis et al., 2006a,b,c, 2009; Day, 2007).

In an early report, Keenan et al. (1984) predicted that the presence of anti-*Leishmania* specific antibodies would not be sufficient to confer protection against the disease, although such defence would not be possible in the absence of antibodies. Latter studies have concentrated on correlations between the various classes and subclasses of immunoglobulin (Ig) and the response produced by the host during the infection process (Bourdoiseau et al., 1997; Solano-Gallego et al., 2001; Cordeiro-da-Silva et al., 2003; Quinnell et al., 2003; Almeida et al., 2005; Reis et al., 2006a). For example, Deplazes et al. (1995) observed that symptomatic dogs presented high levels of IgG₁, whereas IgG₂ was more abundant in asymptomatic animals. More recent investigations have demonstrated that IgG_{total} concentrations were greater in dogs that presented clinical symptoms of CVL (Solano-Gallego et al., 2001; Almeida et al., 2005; Iniesta et al., 2005; Reis et al., 2006a). In addition, it has been reported that the presence of high amounts of anti-*Leishmania* specific IgG in dogs were correlated with lower specific immune cells (Fernandez-Perez et al., 2003), and that levels of IgE were directly correlated with susceptibility to *L. chagasi* infection (Almeida et al., 2005; Iniesta et al., 2005; Reis et al., 2006a). Such findings have led to the suggestion that the Ig profile could be used as a biomarker for monitoring resistance or susceptibility to the clinical manifestations of CVL. Additionally, some researchers have described the occurrence in Brazil and Spain of symptomatic dogs presenting elevated levels of IgM (Rodríguez-Cortés et al., 2007), although evidence indicating a lack of correlation between clinical status and IgM levels has also been reported (Reis et al., 2006a).

In fact, Quinnell et al. (2003) had already anticipated that IgG subclasses alone might not constitute satisfactory markers of susceptibility and resistance to CVL. In consideration of this, a new approach was proposed by Reis et al. (2006a) involving study of the possible association between parasite load and the clinical/immune response, thus establishing a new strategy for the elucidation of immunopathological aspects of CVL. Within this context, the determination of the IgG avidity index (AI) may contribute to the understanding of CVL. Moreover, according to Redhu et al. (2006), the degree of avidity of IgG towards the *Leishmania* antigen is able to predict the

time of infection in VL-affected patients, thus allowing the discrimination between recent (<6 months) and chronic (>6 months) patients.

The objective of the present work was to correlate the clinical forms of CVL (asymptomatic and symptomatic) and the parasite load with IgG subclasses (IgG₁ and IgG₂). Moreover, the IgG avidity index has been correlated for the first time with the clinical forms of CVL, and possible associations with parasite load in the skin and spleen evaluated. The resulting data open up new prospects for the clinical prognosis of CVL and evaluation of efficacy of vaccines and drugs in the future.

2. Materials and methods

2.1. Animals

The study population consisted of 45 mongrel dogs (*Canis familiaris*) being 21 male and 24 female of various ages. Forty of the dogs were naturally infected with *L. chagasi* and originated from the Centre for Zoonosis Control, Belo Horizonte, MG, Brazil. The infected animals were subdivided on the basis of the classification of Mancianti et al. (1988) into two groups, one of which ($n = 20$) comprised asymptomatic dogs (AD) (9 male and 11 female) whilst the other ($n = 20$) constituted symptomatic dogs (SD) (10 male and 10 female). The remaining five animals (control group) had been bred and raised in the kennels of the Universidade Federal de Ouro Preto, MG, Brazil, and were considered non-infected even though the area is endemic for CVL. All procedures in this study were according to the guidelines set by the Brazilian Animal Experimental College (COBEA). This study was approved by the ethical Committee for the use of Experimental Animals of the Federal University of Ouro Preto, Minas Gerais state, Brazil (CETEA).

2.2. Confirmation of *L. chagasi* seropositivity and infection

The presence of anti-*L. chagasi* antibodies in the animal population was confirmed by indirect immunofluorescence assay (IIFA) and enzyme linked immunosorbent assay (ELISA), which were performed by the laboratory of the Centre for Zoonosis Control. Infection by *Leishmania* was confirmed by PCR at least one of the tissues evaluated (skin, lymph node, spleen, bone marrow and liver) using the primers 5'(C/G)(C/G)(G/C)CC(C/A)CTAT(T/A)TTACACCAACCCC3' and 5'GGGGAGGGGCGTTCTGCGAA3' (Degraeve et al., 1994). The determination of the *Leishmania* species responsible for infection was performed using a PCR-RFLP method (Volpini et al., 2004) with *L. chagasi* strain MHOM/BR/74/PP75 as control.

2.3. Collection and analysis of samples

Ten milliliters of peripheral blood samples were collected by intravenous puncture in the radial vein of the dogs using disposable 20 mL syringes (21G 25 X 8) and placed into vacuum vials containing clot activator (Vacuette, Campinas, SP, Brazil). The resulting serum was stored in 1.8 mL sterile cryogenic vials (Sarstedt, Newton, NC, USA) at -20°C until required for assay.

After euthanasia, skin samples were collected by 5 mm punch biopsy from the right ear. Spleen biopsies, weighing approximately 30 mg, were collected using a sterile scalpel. Tissue fragments were placed onto microscope slides and stained with Giemsa for parasitological diagnosis. Parasite loads, expressed in Leishman-donovan units (LDU), were determined by counting the number of amastigote forms amongst 1000 nucleated cells according to the method of Stauber (1955) as modified by Reis et al. (2006a). Following the evaluation of tissue samples, dogs were grouped according to parasite load as follows: low (LP), medium (MP) and high (HP) parasitism based on spleen and skin specific LDU values categorised statistically into tertiles according to Reis et al. (2006a) and Guerra et al. (2008).

2.4. Determination of Ig pattern using ELISA

Anti-*Leishmania* Ig patterns were determined on the basis of in-house ELISA tests carried out using soluble promastigote antigen (SLA) obtained according to the method of Reis et al. (2006b) from *L. chagasi* (MHOM/BR/1972/BH46) grown in axenic culture on LIT medium. Ninety-six-well microplates (MaxiSorp™, Nalge Nunc Int., Rochester, NY, USA) were coated overnight at 4 °C with SLA at a concentration of 10 mg/well. The coated wells were washed and serum samples added at 1:80 dilution. Following further washes, peroxidase-conjugated goat anti-dog IgG₁ (1:1000 dilution), together with sheep anti-dog IgG (1:8000 dilution) and IgG₂ (1:16,000 dilution), all anti-heavy chain specific and purchased from Bethyl Laboratories, Montgomery, TX, USA. Wells were subsequently washed, substrate and chromogen (*O*-phenylenediamine; Sigma-Aldrich Co., St. Louis, MO, USA) added and absorbances read on an automatic ELISA microplate reader (Multiskan1 MCC 340, Labsystems, Helsinki, Finland) at 492 nm. The conjugate concentrations were determined by a block titration method with positive and negative standard sera.

2.5. IgG avidity test

A modified IgG avidity assays were performed using the ELISA protocol as described in Section 2.4. except that wells forming columns 1–6 (left-hand side of the microplate) received an extra washing with PBS/Tween following application of serum samples, whilst wells forming columns 7–12 (right-hand side of the microplate) were washed with 100 µL PBS/Tween and 6 M urea. The plate was incubated at 37 °C for 10 min and absorbances read at 490 nm. Similar techniques have been applied in the study of a number of other parasitic diseases (Suarez-Aranda et al., 2000; Montoya et al., 2004; Redhu et al., 2006; Clementino et al., 2007). AI values were determined from the ratios between the absorbances of urea-treated samples and those of non-treated samples, and the results expressed in percentage form.

Owing to the lack of information regarding IgG avidity in *Leishmania*-infected dogs, it was necessary to establish an IgG affinity profile during the development of infection in order to obtain appropriate parameters for comparison.

Thus, serum samples from dogs that had been experimentally infected with *L. chagasi* strain MHOM/BR/1972/BH46 for between 1 and 19 months were submitted to IgG avidity assay as described above.

2.6. Statistical analyses

Statistic analyses were performed with the aid of Prism 4.0 and Minitab 13 statistical software. The Student *t*-test or the non-parametric Mann-Whitney test were used to compare parasite loads between the different clinical groups and IgG AI values between groups exhibiting different clinical signs. Analysis of variance (ANOVA) followed by the multiple comparison Tukey test, or the non-parametric Kruskal-Wallis test followed by the multiple comparison Dunn test, were used to compare ELISA absorbance values with the clinical profiles of infected dogs. Similar methods were used to compare the ELISA and AI values with the parasite loads of infected dogs. Correlations were established using the Spearman rank test. All tests were carried out at the 95% confidence level.

3. Results

3.1. Clinical and molecular characteristics of the studied dogs

Uninfected control dogs and infected animals of the AD group presented no clinical signs of *Leishmania* infection. In contrast, the SD group exhibited various signs of CVL (Fig. 1) such as localized alopecia and onychogryphosis (present in 70% of dogs), accentuated wasting (50%), furfuraceous dermatitis (35%), skin ulcers (30%), slight wasting, moderate wasting, dull coat (25%), keratoconjunctivitis (20%), generalised alopecia and opaque corneas (15%). All 40 animals forming the infected groups AD and SD were seropositive for CVL as demonstrated by IIFA and ELISA tests. PCR and PCR/RFLP patterns obtained from samples derived from *Leishmania*-infected animals were similar to those produced by *L. chagasi* strain MHOM/BR/74/PP75.

3.2. Parasite loads of skin and spleen tissues

There were no differences between groups AD and SD regarding the parasite load of the spleen (Fig. 2A), although the LDU values for the skin of SD animals were greater than those of dogs in the AD group (Fig. 2B). Animals in the AD and SD groups were further stratified according to parasite loads in the studied tissues as represented in Fig. 2C and D. The majority of AD animals were classified as LP for skin ($n = 10$) and spleen ($n = 9$), whereas most of the dogs within the SD group were classified as HP for skin ($n = 11$) and spleen ($n = 10$). However the differences were significant ($\rho = 0.006$) only in skin samples.

3.3. IgG profiles

The absorbance values of IgG_{total} and IgG₂ in the serum of the whole population of *Leishmania*-infected dogs were significantly ($\rho < 0.05$) greater than those of the control dogs (Fig. 3A, panels I and III respectively). More

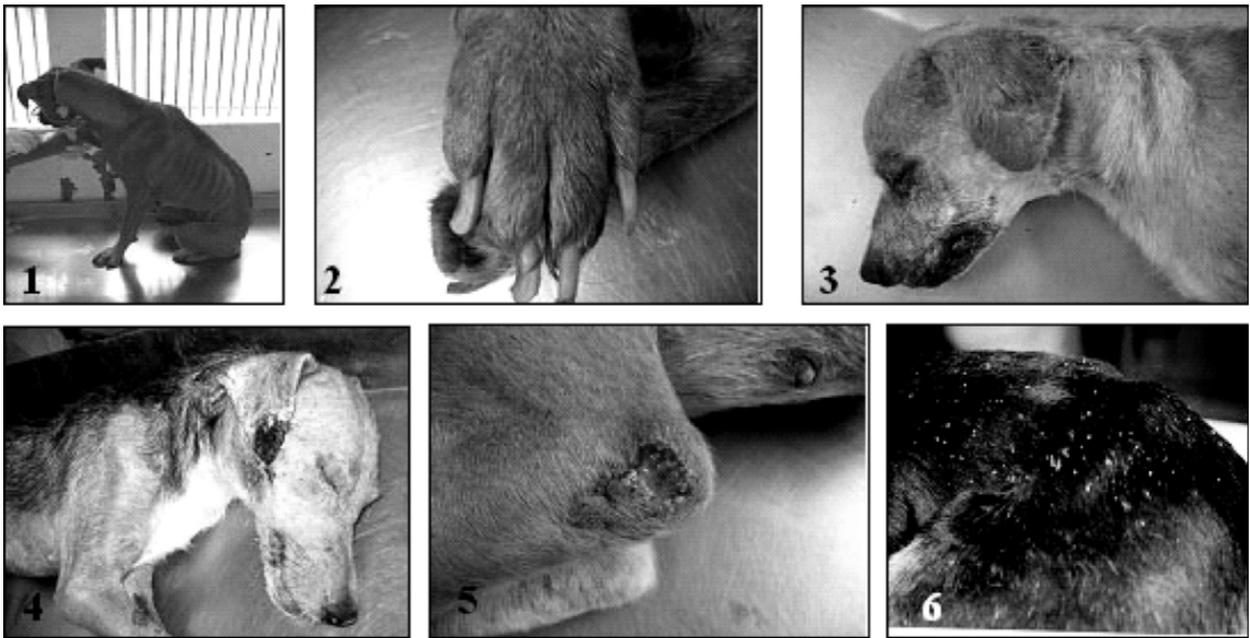


Fig. 1. Main clinical signs observed in symptomatic dogs affected by canine visceral leishmaniasis showing: 1. wasting and apathy; 2. onychogryphosis; 3. alopecia/hypotrichosis in the periorbital region and ear tip; 4. lesion in the ear tip; 5. ulcer in the left hock area; 6. furfureous dermatitis in the dorsal area.

specifically, the levels of IgG_{total} within the AD and SD groups were significantly larger ($\rho < 0.05$ and 0.001 , respectively) than that of the control group (Fig. 3B, panel I), and the level of IgG₁ within the AD group was significantly larger ($\rho < 0.05$) than those of the SD and control groups (Fig. 3B, panel II). Additionally, the levels of IgG₂ were significantly higher in AD ($\rho < 0.01$) and SD ($\rho < 0.001$) group animals compared with the control group (Fig. 3B, panel III).

The reactivities of IgG, IgG₁ and IgG₂ in serum samples were also appraised with respect to the parasite loads of skin and spleen tissue samples. Considering spleen samples (Fig. 4A), the reactivity of serum IgG_{total} was significantly lower ($\rho < 0.05$) in LP dogs compared with MP and HP animals (Fig. 4A, panel I), whilst IgG₁ reactivity was significantly smaller ($\rho = 0.0232$) in HP animals compared with LP animals (Fig. 4A, panel II). The results for IgG₂ were similar to those observed for IgG_{total}, i.e. the reactivity in LP dogs was smaller than in MP and HP animals (Fig. 4A, panel III) and the differences were statistically significant ($\rho < 0.05$ and 0.01 , respectively). In the case of skin samples (Fig. 4B), LP dogs presented significantly lower levels of IgG_{total} and IgG₂ compared with MP and HP animals ($\rho < 0.05$ and 0.01 , respectively), but there were no differences between LP, MP and HP animals regarding the values of IgG₁.

3.4. Correlations between IgG reactivities and parasite load (LDU value)

Considering the total population of *Leishmania*-infected dogs, there were weak, but significant, correlations between IgG_{total} ($\rho = 0.0164/r = 0.3684$), IgG₁ ($\rho = 0.0261/$

$r = -0.3563$) and IgG₂ ($\rho = 0.0025/r = 0.4594$), and the parasite load of the spleen (Fig. 5A–C). However, when the different clinical forms of CVL were taken into account, strong positive correlations between the reactivities of IgG_{total} ($\rho = 0.0002/r = 0.7404$) and IgG₂ ($\rho < 0.0001/r = 0.7791$) with spleen LDU values were found within the AD group (Fig. 5D and E).

No correlations were detected between the reactivities of IgG forms and the parasite load of skin samples within the population of symptomatic *Leishmania*-infected dogs.

3.5. IgG avidities

The IgG avidity profile determined for dogs experimentally infected with *L. chagasi* revealed that AI values tended to increase with the duration of infection. With respect to the studied population of *Leishmania*-infected dogs, those of the AD group presented significantly smaller ($\rho = 0.008$) AI values in comparison with SD group animals (Fig. 6A). When animals were categorised with respect to parasite load of the spleen, AI values were found to be significantly lower ($\rho < 0.01$) in LP animals than in MP and HP animals (Fig. 6B). In the case of parasite load of the skin, it was observed that the AI values in the HP group were significantly higher ($\rho < 0.01$) than those in the LP group (Fig. 6C).

In order to determine which of the IgG subclasses were associated with AI, the correlations between AI values and the absorbance values of IgG₁ and IgG₂ were determined. The results revealed that AI was strongly correlated ($\rho < 0.0001/r = 0.7315$) with IgG₂ levels (Fig. 6E) but not with IgG₁ levels ($\rho = 0.8356/r = 0.03387$; Fig. 6D).

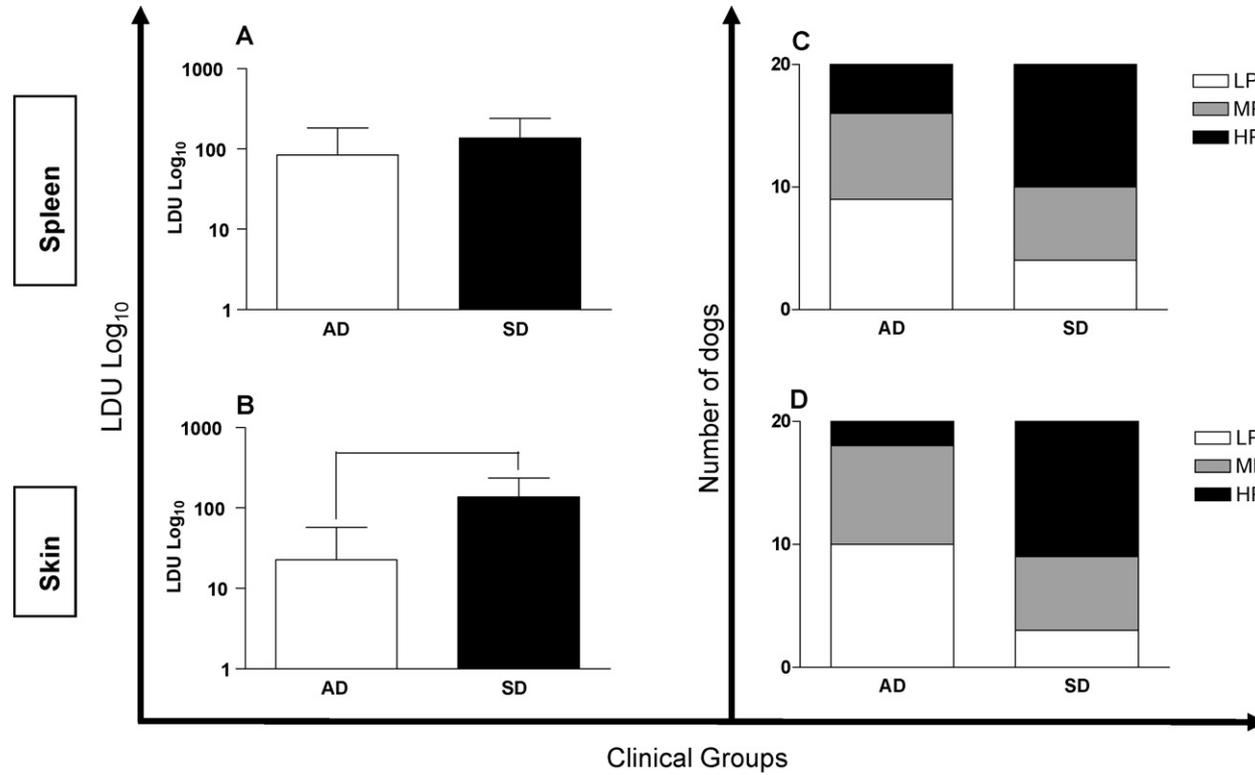


Fig. 2. Association between the parasite loads of the spleen (A) and skin (B) and the clinical forms of canine visceral leishmaniasis, and their distribution according to the parasite load in the spleen (C; $p = 0.102$) and skin (D; $p = 0.006$). AD = asymptomatic dogs, SD = symptomatic dogs, LP = low parasitism, MP = medium parasitism and HP = high parasitism. Bars representing values that are significantly different one from another are shown connected.

4. Discussion

CVL is a multisystemic disease with chronic evolution that mainly affects visceral and cutaneous tissue. The development of the various clinical forms of CVL depends upon complex interactions between the parasite and the immune system of the host. This means that some infected animals are susceptible and develop an active form of the infection, whereas others are resistant and remain asymptomatic. In addition, the nutritional status of the dog may directly influence the development of the disease. It is of interest to note that, following a study carried out in a region that was endemic for CVL, Cabral et al. (1998) reported that a large proportion (60–80%) of dogs presenting specific antibody and immune cell responses showed no signs of *Leishmania* infection.

The categorization of animals according to clinical manifestations and parasite load has been very helpful in understanding the immunopathological aspects of the disease. Such an approach has been used in the present investigation, in which the animal population was divided into AD and SD groups as suggested by Mancianti et al. (1988). The high incidence (85%) of cutaneous alterations, such as alopecia and dermatitis, observed in SD dogs agreed with those given in earlier reports (i.e. 81–89%; Koutinas et al., 1999; Ciaramella et al., 1997), whilst the frequencies of ocular alterations, such as keratoconjunctivitis (20%) and opaque corneas (15%), also corresponded with published values (i.e. 24%; Peña et al., 2000). In

contrast, however, the incidence of onychogryphosis (70%) amongst SD dogs was much higher in the present study than has been observed earlier (i.e. 20–30%; Koutinas et al., 1999; Baneth et al., 2008).

Various sophisticated techniques based on LDU values (Reis et al., 2006a), quantitative nucleic acid sequence-based amplification (QT-NASBA; Van Der Meide et al., 2005), immunohistochemistry (Tafari et al., 2004; Giunchetti et al., 2006) and real time PCR (qPCR; Rolão et al., 2004; Vitale et al., 2004) have been applied to the quantitative evaluation of tissue parasitism in animals affected by CVL. Since skin and spleen appear to be the tissues most densely parasitised by *Leishmania* independent of the clinical forms of the disease (Reis et al., 2006a), the present study attempted to correlate the humoral immune response of infected animals with clinical signs and the parasite density of these tissues. One of the most interesting findings was the lower parasite load of AD dogs compared with SD animals (Fig. 2B), suggesting that cutaneous parasitism evolves concomitantly with the clinical manifestations of CVL. Indeed, Molina (1997) has reported that, whilst AD animals presented positive xenodiagnosis, the proportions of infected phlebotomines were highest in those that had fed from dogs with severe signs of CVL, probably due to the intense parasitism in such animals. Furthermore, according to Rodríguez-Cortés et al. (2007), the clinical profiles of experimentally infected dogs were directly correlated with the degree of parasitism. In contrast, Solano-Gallego et al. (2004)

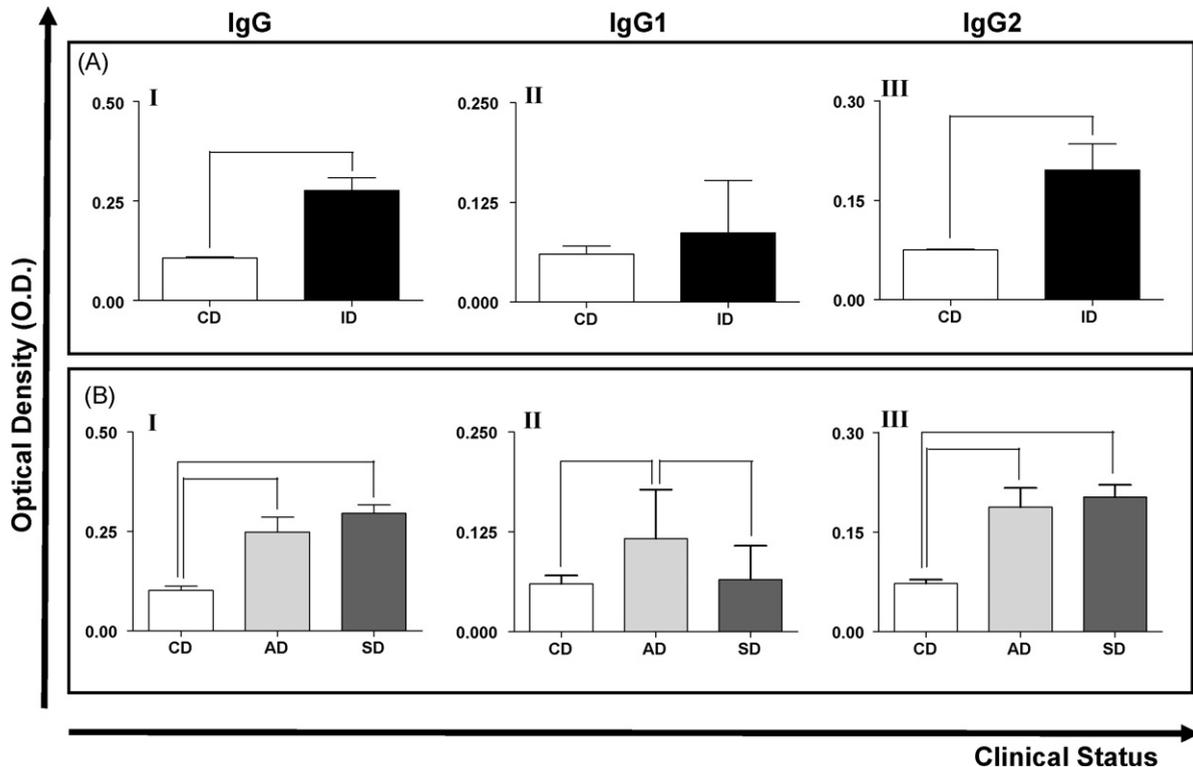


Fig. 3. IgGtotal (I), IgG1 (II) and IgG2 (III) reactivities in (A) the serum of dogs naturally infected with *L. chagasi* and (B) distributed according to clinical form. CD = control dogs, ID = infected dogs, AD = asymptomatic dogs and SD = symptomatic dogs. Bars representing values that are significantly different one from another are shown connected.

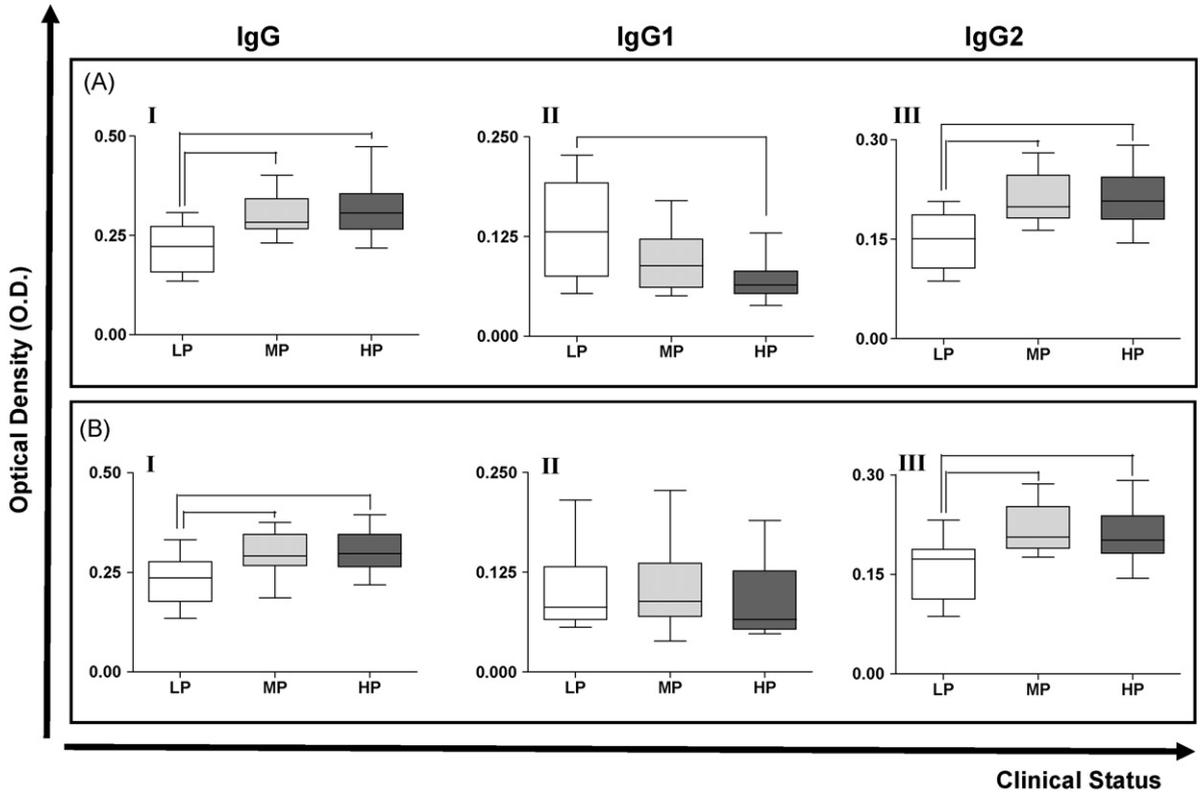


Fig. 4. IgGtotal, IgG1 and IgG2 reactivities in the serum of dogs naturally infected with *L. chagasi* categorised according to the parasite load of (A) the spleen, and (B) the skin. LP = low parasitism, MP = medium parasitism and HP = high parasitism. Bars representing values that are significantly different one from another are shown connected.

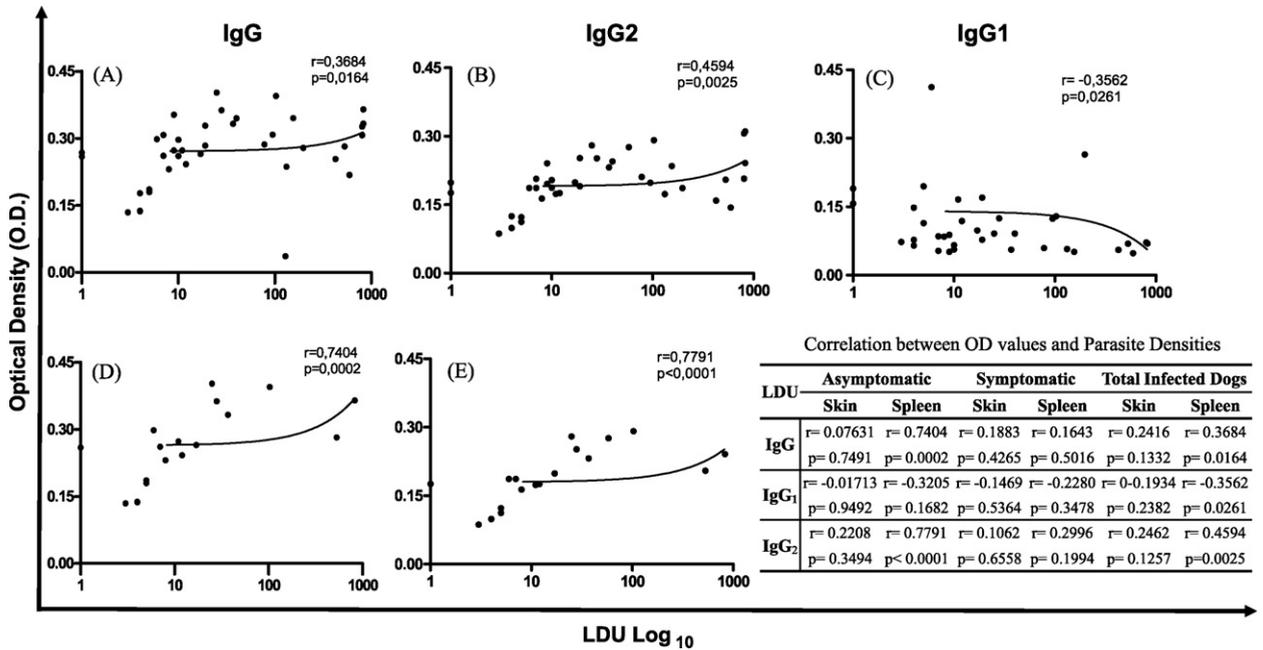


Fig. 5. Correlations between parasite load in spleen samples and anti-*Leishmania* IgG isotypes in the serum of dogs naturally infected with *L. chagasi* (panels A, B and C) and in asymptomatic dogs (panels C and D). Other potential correlations studied that were not statistically significant are detailed in the Table insert.

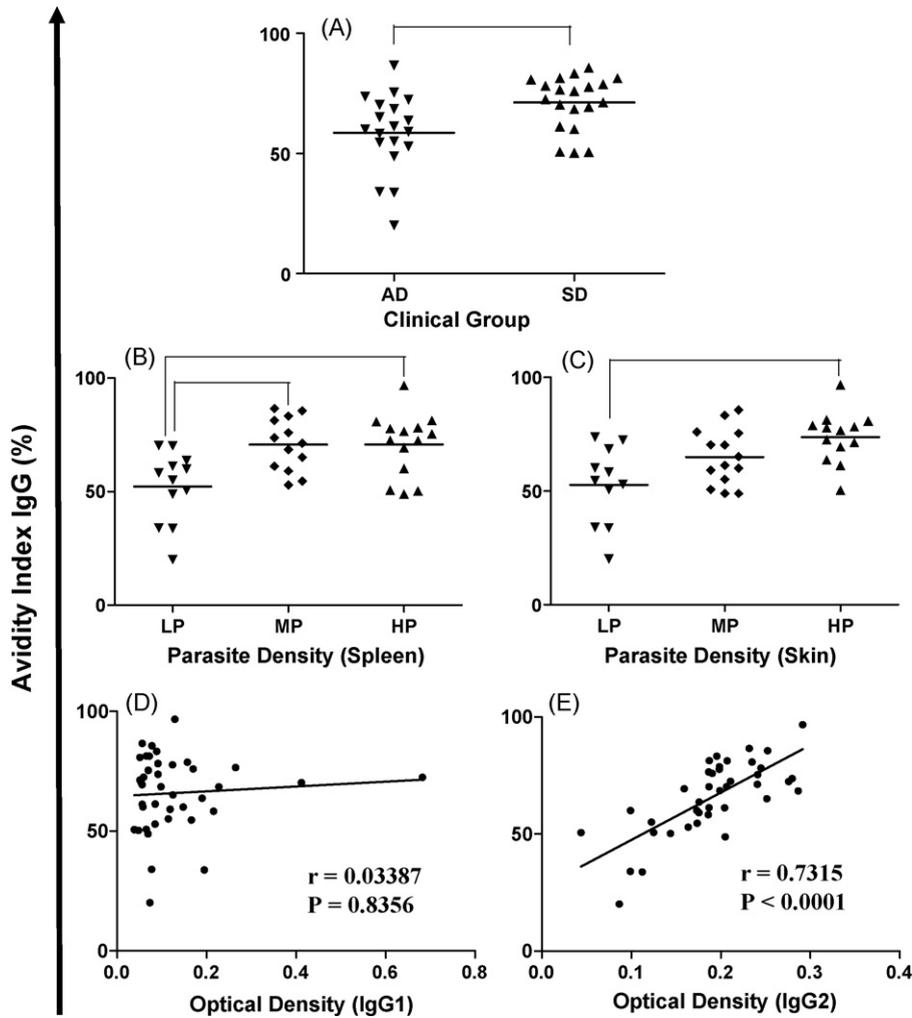


Fig. 6. IgG avidity index (AI) in the serum of dogs naturally infected with *L. chagasi* stratified according to clinical form (A), and the degrees of parasitism in the spleen (B) and skin (C). Correlations between the IgG AI and the absorbances of IgG1 and IgG2 are shown in panels D and E, respectively. AD = asymptomatic dogs, SD = symptomatic dogs, LP = low Parasitism, MP = medium parasitism and HP = high parasitism. Bars representing values that are significantly different one from another are shown connected.

observed that the numbers of parasites in the skin of symptomatic animals were no larger than those in asymptomatic animals, hence emphasising the importance of AD animals in the maintenance and transmission of CVL.

In respect of the potential prognostic value of IgG isotypes, some authors (Deplazes et al., 1995; Iniesta et al., 2005) have demonstrated that high levels of IgG₁ were correlated with symptomatic infection, whereas high levels of IgG₂ were related with asymptomatic CVL. These authors concluded that such a pattern could represent a dichotomous response of CVL. Conflicting results have, however, been reported by other researchers (Nieto et al., 1999; Boceta et al., 2000; Almeida et al., 2005; Reis et al., 2006a; Cardoso et al., 2007) in which high levels of IgG₂ were associated with active clinical infection. Such inconsistent results highlight the difficulty in establishing a typical immunological response in CVL and emphasize the importance of continued investigation.

The IgG₁ levels of the AD animals presently studied were found to be higher in comparison with those of the SD group, in agreement with the findings of Bourdoiseau et al. (1997) and Reis et al. (2006a), thus suggesting a possible association with mechanisms of immunoprotection against CVL. Conversely, serum IgG_{total} and IgG₂ appeared to be associated with CVL morbidity since higher levels were found in animals presenting severe clinical forms. On the other hand, Solano-Gallego et al. (2001) claimed, on the basis of a cross-sectional study involving 280 animals in different stages of infection, that there was no correlation between clinical status and IgG subclass. Furthermore, Quinnell et al. (2003) suggested that IgG subclasses were not satisfactory markers of resistance or susceptibility towards CVL.

In the present study, IgG profiles were evaluated in relation to LDU values revealing that increased IgG_{total} and IgG₂ reactivities were associated with the enhancement of parasite load in the skin and spleen. Although the correlations between IgG_{total} and IgG₂ and parasite load

were positive, there was a negative correlation with IgG₁, strengthening the hypothesis that this IgG is associated with immunoprotection mechanisms during *L. chagasi* infection. Indeed, animals with high concentrations of IgG₁ were asymptomatic and presented low parasitism, whereas animals with high levels of IgG₂ were associated with more severe clinical signs and high parasitism, hence with greater susceptibility to infection. These results support previous claims (Reis et al., 2006a) that some serum anti-*Leishmania* IgGs could be of value as markers of clinical status and parasite load of tissues of naturally infected dogs, thus representing a good indicator of disease morbidity. On the other hand, Nieto et al. (1999) stated that the presence of anti-*Leishmania* antibodies cannot be considered as conclusive markers of CVL progression.

IgG avidity tests were established in the present study with a view to expanding the debate over the role of IgG subtypes in CVL. Similar techniques have been applied in the study of a number of other parasitic diseases (Montoya et al., 2004; Clementino et al., 2007). In the case of infection by *Toxoplasma gondii*, Suarez-Aranda et al. (2000) demonstrated that AI values >50% indicated chronic toxoplasmosis whilst AI values <50% suggested an acute form of the disease. One of the few studies that have employed AI in the differentiation of chronic from acute VL was conducted by Redhu et al. (2006), and these authors demonstrated that the IgG avidity test could be employed to estimate the time of development of *Leishmania* infection in the host.

Results from the present study demonstrate that SD animals exhibit higher AI values compared with the AD group, and that animals showing elevated LDU values in the skin and spleen also present high AI values compared with those exhibiting low parasitism. Moreover, dogs with high IgG₂ reactivity also presented high AI values. These findings open up the possibility of applying AI values in the characterisation of CVL-affected dogs, although the limiting values of the index that differentiate recent and old forms of infection have not yet been fully established.

The results presented in this study support the idea that *L. chagasi*-infected dogs exhibit immunological characteristics that suggest a gradual evolution of CVL. Initially the disease is characterised by an absence of clinical signs (asymptomatic form), but a variety of symptoms (oligo-symptomatic form) emerge during CVL progression, and finally numerous clinical signs appear simultaneously that are characteristic of the classical symptomatic form of the disease, which probably leads to death.

In the present study, the IgG avidity test has been used for the first time in the diagnosis of CVL and shows promise as a biomarker for determining the progression of *Leishmania* infection. It has been demonstrated that the combined analysis of the various aspects connected with CVL enables the various clinical forms of the disease to be characterised. Thus, AD animals presented lower tissue parasitism (mainly in the skin), low affinity for IgG and a humoral immunoresponse with high levels of IgG₁, all of which characterise the initial phase of infection. On the other hand, SD animals exhibited greater tissue parasitism, particularly in the skin, high affinity for IgG, and a humoral immunoresponse with high levels of IgG₂, characterising the chronic phase of infection. It is thus concluded that

determination of AI values could provide an alternative tool with which to evaluate the morbidity of CVL, thus increasing the accuracy of prognosis for the animal.

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