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USE OF ELISA EMPLOYING HOMOLOGOUS AND HETEROLOGOUS ANTIGENS FOR THE DETECTION OF IgG AND SUBCLASSES (IgG1 AND IgG2) IN THE DIAGNOSIS OF CANINE VISCERAL LEISHMANIASIS

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SUMMARY

Indirect immunofluorescence is the method recommended for the diagnosis of visceral leishmanisis in dogs, however, the accuracy of this technique is low and its use on a large scale is limited. Since ELISA does not present these limitations, this technique might be an option for the detection of IgG or specific IgG1 and IgG2 subclasses. Canine ehrlichiosis is an important differential diagnosis of American Visceral Leishmaniasis (AVL). The present study compared ELISA using *Leishmania chagasi* and *Leishmania braziliensis* antigen for the detection of anti-*Leishmania* IgG and subclasses in serum samples from 37 dogs naturally infected with *L. chagasi* (AVL) and in samples from four dogs co-infected with *L. braziliensis* and *L. chagasi* (CI). The occurrence of cross-reactivity was investigated in control serum samples of 17 healthy dogs (HC) and 35 infected with *Ehrlichia canis* (EC). The mean optical density obtained for the detection of IgG was significantly higher when *L. chagasi* antigen was used, and was also higher in subgroup VLs (symptomatic) compared to subgroup Vla (asymptomatic). The correlation between IgG and IgG1 was low. The present results suggest that IgG ELISA using homologous antigen yields the best results, permitting the diagnosis of asymptomatic *L. chagasi* infection and the discrimination between cases of AVL and ehrlichiosis in dogs.

KEYWORDS: American visceral leishmaniasis; Dog; IgG subclasses; *L. (V.). braziliensis*, *L. (L.) chagasi*.

INTRODUCTION

Leishmania (Leishmania) chagasi is a viscerotropic parasite of lymphoid organs and the causative agent of American visceral leishmaniasis (AVL), also known as Kala-azar[1]. AVL is found in various American countries. In Brazil, AVL presents a high incidence in urban and periurban areas[39]. In the municipality of Rio de Janeiro, AVL emerged at the end of the 1970s in periurban areas of the northern zone of the city, especially at the hillside of the Pedra Branca massif[32]. The domestic dog (*Canis familiaris*) is the main reservoir of AVL due to intense parasitism of healthy skin and viscera since the onset of infection[15,28,32,41,45]. It is believed that most infected dogs do not develop clinical signs and remain asymptomatic for variable periods of time[19,32]. In Brazil, AVL requires obligatory notification and control programs involve the treatment of human cases, control of the insect vector, and euthanasia of seropositive infected dogs. The Brazilian Ministry of Health recommends serological testing by indirect immunofluorescence (IIF), with antibody titers of 1:40 or higher being used as a criterion for the culling of dogs[39].

The control measures adopted in Brazil are a matter of discussion since new AVL foci continue to appear in the country[13]. The relative inefficiency of these measures has contributed to the emergence of AVL, especially because of the underestimated seroprevalence of canine infection provided by the IIF test[51]. Some authors suggest that a highly sensitive test, combined with rapid culling of the dogs, is necessary for a significant reduction in the transmission of the disease and effective control[11,16]. According to BRAGA *et al.*[8], only 35.4% of infected dogs identified by enzyme-linked immunosorbent assay (ELISA) would be detected by IIF. As a consequence, some infected dogs would not be identified during control actions and would remain in endemic areas, a fact facilitating propagation of the infection. In addition to its lower sensitivity and specificity when compared to ELISA[30], IIF is more time consuming and requires trained professionals[5,17]. The rapidity and possibility of automation of ELISA are additional advantages that favor its use on a large scale.

Once clinical signals are installed, AVL should be differentiated from ehrlichiosis, lymphosarcoma, myeloma, seborrhea, pemphigus and systemic fungal infections, as well as from other diseases causing proliferation of the reticuloendothelial system[23]. Ehrlichiosis is a disease caused by the rickettsia *Ehrlichia canis*, which primarily parasitizes cytoplasmic vacuoles of monocytes and granulocytes[47,55], causing a

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potentially fatal multisystemic infection that is characterized by clinical and hematological anomalies similar to those observed in AVL, including fever, lymphadenopathy, anorexia, lethargy and thrombocytopenia[31,36,42]. *Ehrlichia canis* presents a wide geographic distribution and mainly occurs in tropical and subtropical areas. In Rio de Janeiro, ehrlichiosis is one of the most common causes of visits to veterinary clinicians, with a mean prevalence of the disease of 26.8% in thrombocytopenic dogs[27]. Despite the clinical and epidemiological similarity to AVL, there is no indication for euthanasia of dogs as a public health measure. Thus, the test used for the serodiagnosis of canine AVL should be able to differentiate the two infections[48].

The IgG subclasses, whose expression depends on the nature of the antigen and antigenic stimulus, are involved in different biological activities. In dogs, four IgG subclasses have been defined[33,34,35], which can be applied to the diagnosis of infectious diseases, including leishmaniasis[24,52]. The study of IgG subclasses in canine AVL might have a practical application both to the diagnosis and to the development of control strategies[50].

In the present study, we compared the results of ELISA using *L. (L.) chagasi* (homologous) and *L. (V.) braziliensis* (heterologous) antigen for the detection of anti-*Leishmania* IgG, IgG1 and IgG2 antibodies for the diagnosis of symptomatic and asymptomatic infection of dogs with *L. (L.) chagasi* and for the possible discrimination with cases of canine ehrlichiosis.

MATERIAL AND METHODS

Population and biological samples: Serum samples obtained from 93 domestic dogs were tested for leishmaniasis by total IgG and subclass IgG1 and IgG2 ELISA. The animals were classified into three well-defined groups: group VL (n = 37) included animals infected with *L. (L.) chagasi*. This group was subdivided into two subgroups: asymptomatic animals (VLa, n = 12), and symptomatic animals (VLs, n = 25) that presented two or more clinical signs of AVL: skin ulcerations, desquamation, adenitis, weight loss, alopecia, hepatosplenomegaly, onychogryphosis, anemia, fever, apathy, and keratoconjunctivitis. Group CI (n = 4) consisted of dogs co-infected with *L. (L.) chagasi* and *L. (V.) braziliensis*. All dogs included in groups VL and CI were from AVL-endemic areas in the municipality of Rio de Janeiro, tested seropositive by IIF, and were euthanized according to the municipal zoonosis control program. In all animals of the two groups, the diagnosis of infection was established by isolation of *Leishmania* from cultured viscera or skin and species characterization by isoenzyme electrophoresis[29].

The control group consisted of 52 dogs subdivided into two subgroups: healthy controls (HC, n = 17) and *E. canis* infected controls (EC, n = 35). Group HC included healthy dogs with no clinical alterations and normal blood count and biochemical parameters (protein, globulin, glutamate-oxaloacetate transaminase/aspartate aminotransferase (GOT/AST), glutamate-pyruvate transaminase/alanine aminotransferase (GPT/ALT), albumin, blood urea and creatinine) that lived outside AVL endemic areas and had a negative parasitological diagnosis of canine ehrlichiosis. Group EC included dogs with two or more clinical signs compatible with AVL but that lived outside AVL endemic areas and in which *E. canis* was visualized by direct parasitological examination of blood smears.

Indirect immunofluorescence: IIF for the detection of anti-*Leishmania* IgG was performed according to manufacturer instructions (Biomanguinhos/FIOCRUZ kit, Rio de Janeiro, Brazil). Titers ≥ 1:40 were considered to be positive. Serum samples of dogs from the VL and CI groups (tested within the leishmaniasis control program as inclusion criterion for this study) were retested and serum samples of dogs from the HC and EC groups were tested for the first time.

Antigens used for ELISA: Partially soluble fractions of promastigote forms of *L. (V.) braziliensis* (MHOM/BR/75/M2903) and *L. (L.) chagasi* (MHOM/BR/74/PP75) obtained during the stationary phase of growth according to the protocol of RIBEIRO *et al.*[46] were used as antigens for ELISA.

ELISA: ELISA for the detection of anti-*Leishmania* IgG, IgG1 and IgG2 was carried out according to the method of VOLLER *et al.*[54] using *L. (V.) braziliensis or L. (L.) chagasi* antigen. The cut-off point was determined as described by RIBEIRO *et al.*[46]. Briefly, the antigens were diluted as follows: *L. (V.) braziliensis* at concentrations of 5 µg/mL for the detection of IgG and 7.5 µg/mL for the detection of IgG1 and IgG2, and *L. (L.) chagasi* at concentrations of 10 µg/mL for the detection of IgG and IgG1 and 2.5 µg/mL for the detection of IgG2. Next, the serum samples were diluted 1:20 for *L. (V.) braziliensis* and 1:100 for *L. (L.) chagasi* antigen in a solution of 1% skim milk (Molico-Nestlé®) in PBS/0.05% Tween 20 (v/v). The plates were then incubated with peroxidase-conjugated anti-dog IgG (Sigma Chemical Co., St. Louis, MO, USA) and anti-dog IgG1 and IgG2 immunoglobulins (Bethyl Laboratories, Inc., Montgomery, TX, USA) at dilutions of 1:40,000, 1:10,000 and 1:30,000 for reactions with *L. (V.) braziliensis* antigen and at dilutions of 1:20,000, 1:5,000 and 1:30,000 for reactions with *L. (L.) chagasi* antigen, respectively.

Different concentrations of antigen and conjugates used were set independently for each class and subclass of IgG, using criteria for choosing the lowest concentration of antigen and greater dilution of the conjugate, which maximized the parameters of sensitivity and specificity of the test.

Statistical analysis: The cut-off was determined from the ROC curves calculated with the MedCalc statistical program (version 8.2.0.2). The nonparametric Spearman test was used for the comparison of the position and variation in optical density (OD) and their eventual correlation since the data did not follow a normal distribution. Mean OD values were compared by the paired Student t-test using the SPSS version 11.0 statistical software package. Differences at $p < 0.05$ were considered to be significant.

RESULTS

Indirect immunofluorescence: All sera from dogs of group VL reacted at different dilutions: four (10.8%) at a titer of 1:80, seven (18.9%) at titers ranging from 1:160 to 1:320, and 26 (70.3%) at a titer ≥1:640. In the mixed infection group (CI), one serum sample reacted at a titer of 1:160 and three at a titer of 1:320. The sera of one dog in the HC group (5.9%) and two dogs (5.7%) in the EC group reacted at a titer of 1:40.

ELISA

Correlation between ODs in the different reactions: In all groups, high and significant Spearman correlations were observed between the

IgG and IgG2 values for all sera analyzed when *L. (V.) braziliensis* (r = 0.86, $p < 0.01$) or *L. (L.) chagasi* antigen (r = 0.95, $p < 0.01$) was used, respectively. In contrast, the correlation between IgG and IgG1 was low but significant when *L. (V.) braziliensis* (r = 0.49, $p < 0.01$) and *L. (L.) chagasi* antigen (r = 0.45, $p < 0.01$) was used.

In group VL, no significant correlation for the detection of IgG1 was observed between symptomatic and asymptomatic animals with either *L. (V.) braziliensis* (r = 0.12, p = .35) or *L. (L.) chagasi* (r = 0.09, p = 0.46) antigen.

Determination of the cut-off: The determination of the cut-off was established by ROC curve, considering all sera from infected (LVs, VLA, IC) and uninfected animals (HC and EC), choosing the point on the curve that maximizes sensitivity and specificity provided by the program MedCalc (version 8.2.0.2).

The cut-off value that permitted the best discrimination between the infected groups (VL and CI) and control animals (HC and EC) was determined from the respective ROC curves (Fig. 1 and 2) plotted for each ELISA reaction (IgG, IgG1 and IgG2) obtained with the *L. (V.) braziliensis* and *L. (L.) chagasi* antigens. The following cut-off values were obtained for the IgG, IgG1 and IgG2 reactions: 0.212, 0.081 and 0.347 for *L. (V.) braziliensis* antigen, and 0.32, 0.085 and 0.15 for *L. (L.) chagasi* antigen, respectively.

Comparison of the area under the ROC curve: No significant differences were observed when comparing the areas under the curve (AUC) for the detection of IgG and IgG2 using either *L. (V.) braziliensis* or *L. (L.) chagasi* as antigen (p = 0.85) (Table 1). Significantly different AUCs ($p < 0.05$) were observed when comparing IgG and IgG2 with IgG1 for both *L. (V.) braziliensis* and *L. (L.) chagasi* antigen, respectively.

OD values: The following mean OD values and their standard deviations were obtained for the VL group: 0.811 ± 0.142 for IgG, 0.124 ± 0.061 for IgG1 and 1.429 ± 0.426 for IgG2 when *L. (V.) braziliensis* antigen was used, respectively, and 1.728 ± 0.426, 0.239 ± 0.253 and 1.177 ± 0.337 when *L. (L.) chagasi* antigen was used. The mean OD values obtained for the detection of IgG (Fig.1 and 2) and IgG1 were significantly higher when *L. (L.) chagasi* was used as antigen ($p < 0.01$).

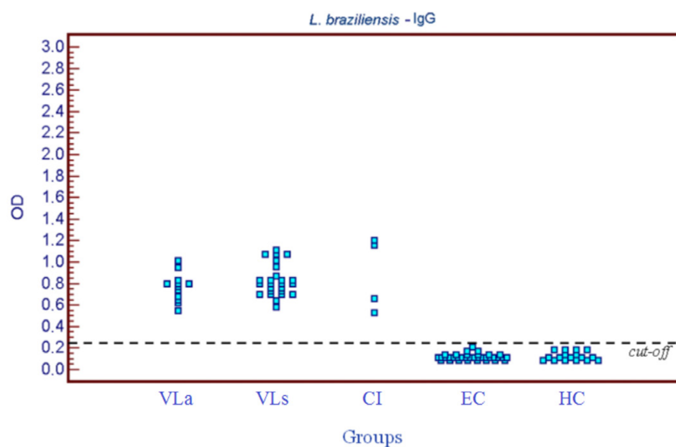

Fig. 1 - Distribution of OD values in ELISA reactions (IgG) using *L. (V.) braziliensis* antigen obtained for the visceral leishmaniasis group (VL) (symptomatic subgroup, VLs, and asymptomatic subgroup, VLa), group co-infected with *L. (L.) chagasi* and *L. (V.) braziliensis* (CI), and control group (healthy subgroup, HC, and ehrlichiosis subgroup, EC).

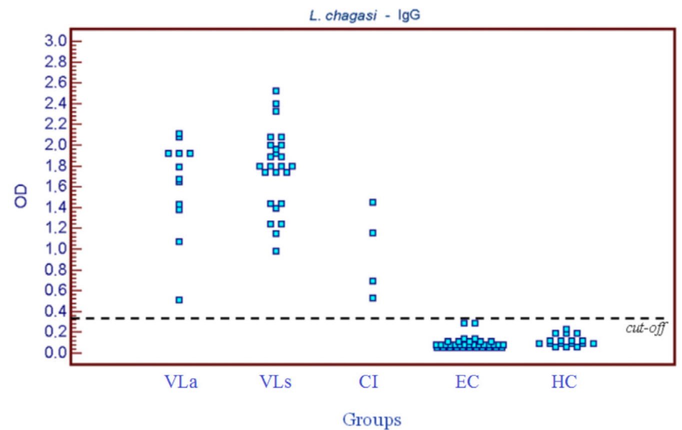

Fig. 2 - Distribution of OD values in ELISA reactions (IgG) using *L. (L.) chagasi* antigen obtained for the visceral leishmaniasis group (VL) (symptomatic subgroup, VLs, and asymptomatic subgroup, VLa), group co-infected with *L. (L.) chagasi* and *L. (V.) braziliensis* (CI), and control group (healthy subgroup, HC, and ehrlichiosis subgroup, EC).

Table 1
Area under the curve values for IgG, IgG1 and IgG2 for antigens of *L. braziliensis* and *L. chagasi*.

L. braziliensis	Area under the curve	*L. chagasi*	Area under the curve
IgG	1,000	**IgG**	1,000
IgG1	0.913	**IgG1**	0.858
IgG2	1,000	**IgG2**	1,000

The seroprevalence of IgG and IgG2 was 100% in both symptomatic and asymptomatic dogs, irrespective of the antigen used. When using *L. (L.) chagasi* as antigen to compare symptomatic and asymptomatic dogs, no significant difference was found in the mean OD values obtained for the detection of IgG (1.778 ± 0.387 and 1.622 ± 0.466, p = 0.20, respectively) (Fig. 1 and 2), IgG1 (0.252 ± 0.287 and 0.212 ± 0.166, p = 0.65) or IgG2 (1.244 ± 0.281 and 1.037 ± 0.410, p = 0.08).

The seroprevalence of IgG1 did not differ significantly between symptomatic and asymptomatic dogs when *L. (V.) braziliensis* (84% versus 75%, p = 0.84) or *L. (L.) chagasi* antigen (88% versus 83.3%, p = 0.90) was used.

In the CI group, mean OD values and their standard deviations for the detection of IgG, IgG1 and IgG2 were, respectively: 0.886 ± 0.342, 0.134 ± 0.048 and 0.897 ± 0.134 when *L. (V.) braziliensis* antigen was used, and 0.957 ± 0.423, 0.137 ± 0.076 and 0.418 ± 0.204 when *L. (L.) chagasi* antigen was used. No significant difference in mean OD values for the detection of IgG was observed between antigens (p = 0.790) (Fig. 1 and 2).

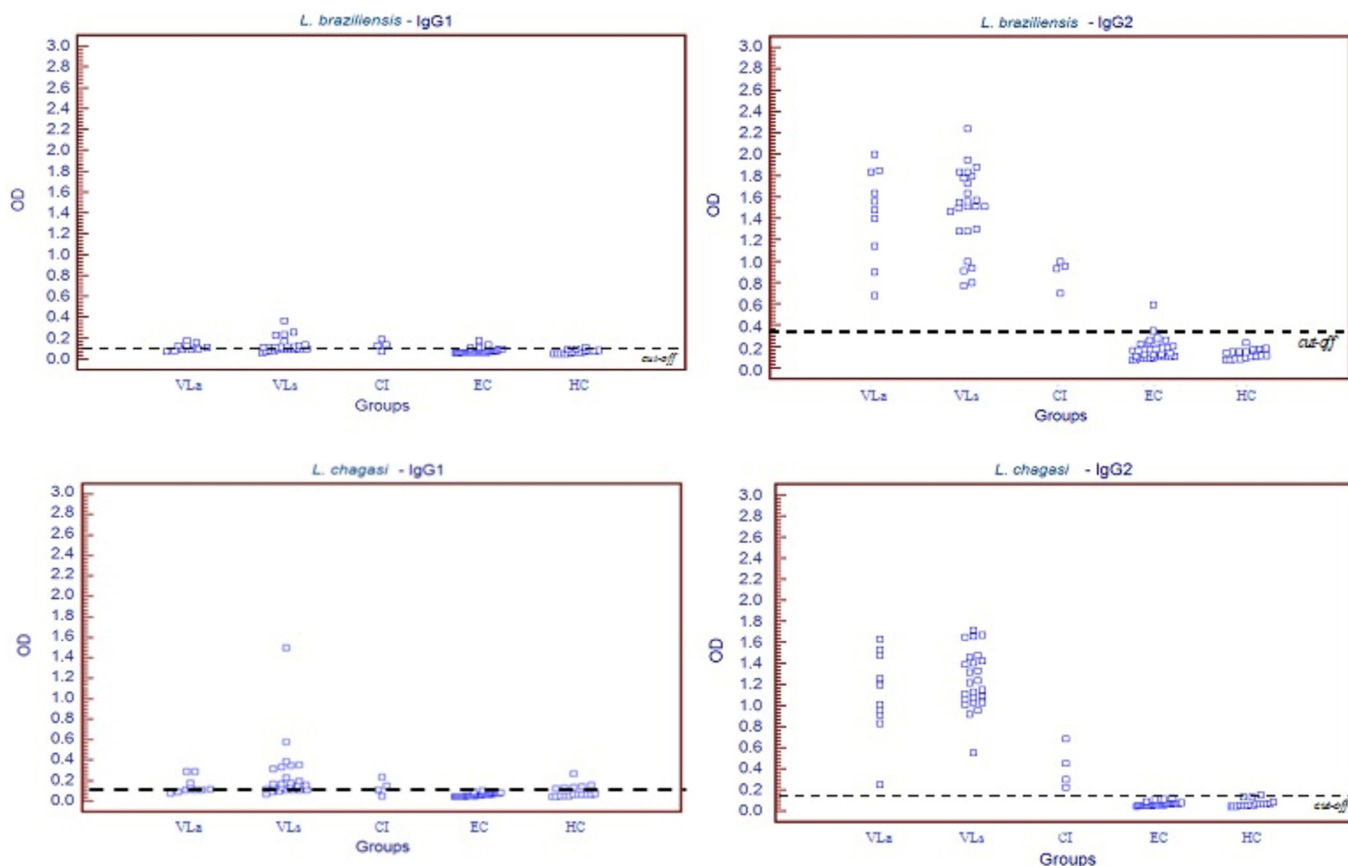

Fig. 3 - Distribution of OD values in ELISA reactions obtained for the visceral leishmaniasis group (VL) (symptomatic subgroup, VLs, and asymptomatic subgroup, VLa), group co-infected with *L. (L.) chagasi* and *L. (V.) braziliensis* (CI), and control group (healthy subgroup, HC, and ehrlichiosis subgroup, EC).: **A**- IgG1; **B**- IgG2 using *L. (V.) braziliensis* antigen and **C**- IgG1 and **D**- IgG2 using *L. (L.) chagasi* antigen.

In the HC and EC control groups, low mean OD values and standard deviations were obtained for the detection of IgG (Fig. 1 and 2), IgG1 and IgG2 when *L. (V.) braziliensis* (0.126 ± 0.031, 0.076 ± 0.023, and 0.158 ± 0.088, respectively) and *L. (L.) chagasi* antigens (0.111 ± 0.076, 0.073 ± 0.039, and 0.069 ± 0.024) were used. In the EC group, IgG1 was detected in 29.0% and 14.3% of serum samples tested with *L. (V.) braziliensis* and *L. (L.) chagasi* antigen, respectively.

The distribution of OD values in ELISA reactions obtained for IgG1 and IgG2 for all groups are shown in Fig. 3.

DISCUSSION

In the present study, we evaluated the detection of IgG and its subclasses by ELISA comparing *L. (L.) chagasi* (homologous) and *L. (V.) braziliensis* (heterologous) antigens in order to improve the performance of the assay for the diagnosis of AVL in symptomatic or asymptomatic naturally infected dogs.

Higher mean OD values were obtained for the detection of IgG, IgG1 and IgG2 in sera from dogs with AVL when *L. (L.) chagasi* antigen was employed. The use of homologous antigen for the diagnosis of leishmaniasis has been reported to provide the best results for both canine[4] and human[2,21] AVL, as well for canine[46] and human[6] American cutaneous leishmaniasis[3]. BADARÓ *et al.*[3] demonstrated that the use of antigens from *Leishmania* sp. isolated from the same area where the cases occurred increased the accuracy of the assay. DA COSTA *et al.*[12] observed no significant differences in the sensitivity of IIF reactions for the diagnosis of canine AVL when different *Leishmania* species were used as antigens.

Overlapping transmission of AVL caused by *L. (L.) chagasi* and American cutaneous leishmaniasis caused by *L. (V.) braziliensis* has been observed in various regions of the Municipality of Rio de Janeiro[39,40]. In the group of dogs co-infected with *L. (V.) braziliensis* and *L. (L.) chagasi*, similar mean OD values for the detection of IgG were obtained with the two antigens, a finding suggesting a balance in the humoral immune response. Since euthanasia is not indicated for dogs infected with *L. (V.) braziliensis* from areas with overlapping transmission of the two species, ELISA employing both antigens could be used to select dogs for parasitological investigation[46].

Some investigators have reported an association between the immune response (Th1/Th2) and IgG subclasses in dogs infected with *L. (L.) infantum*[7,15]. However, other authors do not agree that the IgG2 or IgG1 subclass is a marker of a Th1 or Th2 immune response in dogs, respectively[14,33]. This discrepancy might be explained by the different antisera used.

AVL is frequently associated with a marked specific humoral response and anti-*Leishmania* IgG subclass levels have been suggested to be markers of disease susceptibility and resistance, with IgG1 being associated with the development of the disease and IgG2 with asymptomatic infection[7,9,15,25,43,49]. However, this association was not observed by QUINELL *et al.* (2003) when using monoclonal antisera.

In the present study, mean OD values obtained for the IgG, IgG1 and IgG2 reactions were higher in symptomatic (group VLs) than in asymptomatic dogs (group VLa), a finding also reported by INIESTA *et al.*[22]. IgG2 was the most seroprevalent subclass in both subgroups, with a seroprevalence close to that of IgG, a fact that did not increase the performance of the diagnostic test. These results agree with similar studies involving dogs infected with *L. infantum*[7,10,20,38,53].

The seroprevalence of IgG1 was relatively low in infected animals but slightly higher in symptomatic (group VLs) dogs when compared to asymptomatic animals (group VLa). This low seroprevalence of IgG1 indicates its limited use for the diagnosis of AVL. The limitation of IgG1 in the diagnosis of canine AVL has been suggested previously[38]. Other investigators proposed that this subclass predominates in dogs infected with *L. (L.) chagasi* or *L. (L.) infantum*[37,44], or that IgG1 levels are only elevated in the presence of signs of visceral leishmaniasis[49].

In the present study, the correlation between OD values for the detection of IgG and IgG2 was high and significant. However, the correlation of OD values for IgG or IgG2 with IgG1 was low. LEANDRO *et al.*[25], studying symptomatic infected dogs, also observed high levels of IgG2, with a strong correlation between IgG and IgG2. In agreement with these findings, there was no significant difference between the AUCs of IgG and IgG2, whereas a significant difference was observed when comparing each marker to the AUC of IgG1.

Canine ehrlichiosis is an important differential diagnosis of AVL due to the similar clinical findings and high prevalence of dogs infected with *E. canis* in Rio de Janeiro[27]. Serology has been used to differentiate the two infections[48]. LLERA *et al.*[26] observed no cross-reaction between sera of dogs infected with *E. canis* and *L. infantum*. However, we observed cross-reactivity in IIF and in ELISA (IgG1). Similar findings have been reported by FERREIRA *et al.*[18].

In conclusion, the present study suggests that IgG ELISA using *L. (L.) chagasi* antigen yielded the best results for the diagnosis of AVL, permitting the discrimination between cases of AVL and ehrlichiosis in dogs. However, the 100% positivity of IgG observed for serum samples from dogs of the VL group might be related to the selection criterion adopted in this study, i.e., dogs testing seropositive by IIF. We suggest a comparative study of IIF and IgG ELISA using *L. (L.) chagasi* antigen for the evaluation of dogs from AVL-endemic areas under field conditions.

RESUMO

A utilização do ELISA empregando antígenos homólogos e heterólogos para a detecção de IgG e subclasses (IgG1 e IgG2) no diagnóstico de Leishmaniose Visceral Canina

A imunofluorescência indireta é o método recomendado para o diagnóstico de leishmaniose visceral em cães, entretanto, a acurácia dessa técnica é baixa e seu uso em grande escala é limitado. Uma vez que o ELISA não apresenta essas limitações, essa técnica poderia ser uma opção para a detecção de IgG ou subclasses IgG1 e IgG2 específicas. A ehrlichiose canina é um importante diagnóstico diferencial de Leishmaniose Visceral Americana (LVA). O presente estudo comparou o ELISA usando antígenos de *Leishmania chagasi* e *Leishmania braziliensis* para a detecção de IgG e subclasses anti-*Leishmania* em amostras de soro de 37 cães naturalmente infectados com *L. chagasi* (LVA) e em amostras de quatro cães co-infectados (CI). A ocorrência de reatividade cruzada foi investigada em amostras de soro controle de 17 animais saudáveis (HC) e 35 de infectados por *Ehrlichia canis* (EC). A média de densidade óptica obtida para a detecção de IgG foi significantemente maior quando o antígeno de *L. chagasi* foi usado e também mais elevada no subgrupo LVs (sintomático) quando comparado ao subgrupo LVa (assintomático). A correlação entre IgG e IgG1 foi baixa. O presente resultado sugere que ELISA IgG empregando antígeno homólogo, produz os melhores resultados, permitindo o diagnóstico de infecção assintomática por *L. chagasi* e a discriminação entre casos de LVA e ehrlichiose em cães.

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