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An enzyme-linked immunosorbent assay (ELISA) for the detection of IgM antibodies against *Leishmania chagasi* in dogs[1]

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ABSTRACT.- Carvalho D., Oliveira T.M.F.S., Baldani C.D. & Machado R.Z. 2009. **An enzyme-linked immunosorbent assay (ELISA) for the detection of IgM antibodies against *Leishmania chagasi* in dogs.** *Pesquisa Veterinária Brasileira 29(2):120-124.* Departamento de Patologia Veterinária, Universidade Estadual Paulista, Faculdade de Ciências Agrárias e Veterinárias, Via de Acesso Prof. Paulo Donato Castellane s/n, Jaboticabal, SP 14870-000, Brazil. E-mail: zacarias@fcav.unesp.br

Visceral leishmaniasis is an emergent zoonosis with an increasing number of new cases in Brazil where the domestic dog is an important parasite reservoir in the infectious cycle of *Leishmania chagasi*. An enzyme-linked immunosorbent assay (ELISA), based upon the use of a total soluble antigenic preparation of *L. chagasi,* was adapted for the detection of IgM antibodies in the serum of infected dogs. Optimal dilutions of the antigen, using positive and negative reference sera, were determined by checkboard titrations. The specificity and sensitivity of the ELISA were 100 %. A total of 110 serum samples were taken from dogs in Belo Horizonte, Minas Gerais, Brazil, and examined for anti-*L. chagasi* IgM antibody by ELISA and indirect fluorescent antibody test (IFAT). About 25% (n=27) of all the dogs tested were found serologically positive for *L. chagasi* by IFAT, while 89.09% (n=98) were seropositive by ELISA. The results obtained by ELISA and IFAT were significantly different (P<0.01). The combined use of ELISA and IFAT is recommended in order to enable veterinary services to more efficiently detect canine visceral leishmaniasis.

INDEX TERMS: *Leishmania chagasi*, diagnosis, dogs, IgM, enzyme-linked immunosorbent assay.

RESUMO.- [**Ensaio imunoenziático (ELISA) para detecção de anticorpos IgM contra *Leishmania chagasi* em cães.**] A leishmaniose visceral é uma zoonose emergente, com elevado número de novos casos no Brasil, onde o cão doméstico é um importante reservatório do parasito no ciclo infeccioso da *Leishmania chagasi*. Um ensaio de imunoadsorção enzimática (ELISA) baseado em antígeno bruto de *L. chagasi* foi adaptado para a detecção de anticorpos IgM em soros de cães infectados. As diluições ótimas do antígeno e dos soros controles positivo e negativo foram determinadas através de titulação em bloco. A sensibilidade e especificidade do ELISA teste foram de 100%. Um total de 110 amostras de soros foram obtidas de cães oriundos de Belo Horizonte, Minas Gerais, Brasil, e avaliadas pelo ELISA e pela reação de imunofluorescência indireta (RIFI) para anticorpos IgM anti-*L. chagasi*. Aproximadamente 25% (n=27) dos cães testados foram sorologicamente positivos para *L. chagasi* pela RIFI, enquanto 89.09% foram soropositivos pelo ELISA. Os resultados obtidos pelo ELISA e pela RIFI foram significativamente diferentes (P<0.01). A associação do ELISA e da RIFI deve ser recomendada a fim de permitir a detecção mais eficiente da leishmaniose visceral canina pelos serviços veterinários.

TERMOS DE INDEXAÇÃO: *Leishmania chagasi,* diagnóstico, cães, IgM, ensaio imunoenzimático.

INTRODUCTION

American visceral leishmaniasis (AVL) is a chronic wasting disease characterized by the infection of mononuclear phagocytes by *Leishmania* (*Leishmania*) *chagasi*. The

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parasite life cycle includes the sand fly *Lutzomyia longipalpis* and the domestic dog as an important peridomestic reservoir. Leishmaniasis is widespread in tropical and subtropical areas of Latin America, Europe, Africa and Asia and is increasingly becoming a major public health problem (WHO 1999). An increased prevalence of human and canine AVL has been reported in Brazil, not only in terms of the number of cases and but also in terms of the geographical dispersion of the disease (Feitosa et al. 2000, Nunes et al. 2001, Silva et al. 2001, Cabrera et al. 2003, França-Silva et al. 2003). The disease is more prevalent among dogs than human beings and it has been shown that canine cases usually precede human cases. In fact, canine visceral leishmaniasis (CVL) has been considered a risk factor for human visceral leishmaniasis (HVL) (Di Lorenzo et al. 2000).

Clinical manifestations of CVL are many and include weight loss, elongated and deformed nails, mouth ulcers, skin lesions, hair loss, keratoconjuctivitis, dermatitis and lymphadenopathy. However, dogs infected with *L. chagasi* may present the disease in a severe form, remain asymptomatic, resist the clinical disease for long periods, or develop a few symptoms that may disappear spontaneously (Cordeiro-da-Silva et al. 2003). The severity of the infection depends on the parasite strain, host genetic background and host health and nutritional status. In this sense, a rapid and reliable diagnostic method would be a vital tool for the clinician, especially because clinical signs are only observed in a low proportion of infected dogs (Gradoni 1995).

Diagnosis of CVL can be made by means of parasitological, serological or molecular methods, associated with clinical and epidemiological evidence. Direct microscopic examination and in vitro culture of bone marrow aspirates, spleen and lymph node biopsy specimens are elected as methods for diagnosis and have been considered "gold standards". However, these methods are invasive, painful and even hazardous. Additionally, parasite detection in biopsy samples is influenced by several factors, including organ distribution and culturing which may be unsuccessful due to contamination (Ozbel et al. 2000).

Several serological tests have been developed and evaluated for the diagnosis of VL including indirect fluorescent antibody test (IFAT); enzyme-linked immunosorbent assay (ELISA); Dot-ELISA; immunoblot analysis; and direct agglutination test (DAT) (Islam et al. 2002). The presence of antiparasite antibodies is routinely used as a marker of infection with the production of antibody as the definition of infection or challenge. Generally serological methods present high sensitivity and specificity, although cross reactions with other infectious agents have been reported and the choice of a suitable cutoff value may not be obvious (Dye et al. 1993). Additionally, these tests may be restricted by antibody detection limits and false-positive and false-negative results (Slappendel & Greene 1990, Ferrer et al. 1995, Santa Rosa & Oliveira 1997), resulting in the unnecessary euthanasia of dogs or maintenance of a reservoir in endemic areas. Overall, the reported performance of these tests indicates that in general they are suitable tools for the serodiagnosis of CVL in both symptomatic and asymptomatic dogs.

Molecular biological techniques such as polymerase chain reaction (PCR) alone or in combination with hybridization have been used for detecting VL in humans and dogs (Berrahal et al. 1996, Martin-Sanchez et al. 2001). Despite the high sensitivity and specificity, ability to detect and identify the protozoa involved and the ability to be applied directly on clinical samples, these techniques remain quite complex and laborious, therefore most applicable for research use (Ikonomopoulos et al. 2003).

The program established in Brazil to control VL is based on the guidelines recommended by the World Health Organization (WHO 1999). Among the recommended strategies, detection and treatment of human cases; control of the vector population; and control of domestic reservoirs are most often employed. This control method is based on the interruption of the transmission cycle, using direct measures involving the main vector *Lutzomyia longipalpis* and the domestic dog (Madeira et al. 2004). Hence, efforts should be made to develop an assay capable of detecting infection as early as possible in asymptomatic carriers.

The aim of our investigation was to develop an enzyme-linked immunosorbent assay (ELISA) using total soluble antigen of *L. chagasi* for the detection of IgM antibodies anti-*L. chagasi* in dogs and compare its performance to the indirect fluorescent antibody test (IFAT). We also investigated the occurrence of IgM antibody titres in dog sera from the endemic area of Belo Horizonte, the state of Minas Gerais, Brazil.

MATERIALS AND METHODS

Source of *Leishmania chagasi* promastigotes

A *Leishmania* sp. strain isolated in Araçatuba, São Paulo, Brazil, characterized as complex Donovani, probably *L. chagasi,* using molecular techniques described by Cortes et al. (2004), was used in this study. The parasites were maintained in an RPMI-1640 medium at 25ºC and were used to prepare antigens for IFAT and ELISA.

Serum samples

The positive *Leishmania* sp. reference group consisting of 110 serum samples was kindly supplied by the Centro de Pesquisas René Rachou/ Fiocruz, Belo Horizonte, the state of Minas Gerais, Brazil, which is an endemic area for the occurrence of CVL (Barbosa-de-Deus et al. 2002). A sera panel of known origin, including 15 serum samples obtained from puppies in the city of Jaboticabal, the state of São Paulo, Brazil, was considered a negative reference group, since the area is not endemic for leishmaniasis.

Indirect fluorescent antibody test (IFAT)

The IFAT was carried out according to Oliveira et al. (2008) for IgG antibodies in order to screen positive IgM control serum samples for the ELISA test. Promastigotes of *L. chagasi,* for

antigen preparation, were washed three times in PBS with 1% BSA and resuspended in PBS-buffered formalin for 30 minutes. The parasites were washed three times again in PBS 1% BSA, and the pellet was resuspended in PBS so that 10ml yielded 20 to 25 parasites per microscope field (400x). Slides with twelve previously marked circles were supplied with 10ml for each circle and then frozen at -20ºC until use. Slides with promastigote forms of the parasite were stabilized at room temperature for the IFAT procedure. Doubling serum dilutions were used, starting at 1:40. The samples were placed over the antigen in the slides and incubated in a moist chamber at 37ºC for 30 minutes. The slides were washed three times in PBS, and incubated with anti-dog IgM serum conjugated for fluorescein isothiocyanate (KPL, USA) diluted at 1:30 in PBS containing 1mg% Evan´s Blue. The slides were washed again in PBS, covered with buffered glycerin and a cover slip, and then examined on a fluorescent microscope. In all the experiments, reference sera were included as negative and positive controls. For positive serum samples, parasites displayed a bright-green peripheral stain with a dull fluorescence of the cytoplasm and were considered positive serum samples at 1:40 or more.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA was originally described by Oliveira et al. (2008). To summarize, 100mL of *L. chagasi* total soluble antigen diluted in a sodium bicarbonate-carbonated 0.05M buffer (pH 9.6) was added per well to an ELISA plate (Nunclon ™ surface; Nunc, Denmark) and protein concentration was adjusted to 5µg/mL, 10µg/mL, 20µg/mL and 40µg/mL. After overnight incubation at 4ºC, plates were washed three times with PBS Tween-20 at 0.05%. The plates were blocked with 200mL containing 6% skim milk for 2 hours at 37ºC, to reduce non-specific binding. The blocking agent was removed, and individual dog serum diluted (1:100) in PBS Tween-20 with 5% skim milk added to each well and then incubated for 90 minutes at 37ºC, and washed as described above. One hundred mL of alkaline phosphatase conjugated anti-dog IgM (Sigma Chemical Co) diluted at 1:50 in PBS Tween-20 with 5% normal rabbit serum was added to each well and then incubated for 90 minutes at 37ºC. The plates were washed and the substrate (p-nitrophenyl phosphate) diluted in dietanolamine buffer, pH 9.8, was added. Absorbance at 405nm was read after 45 minutes incubation at room temperature using an ELISA reader (Dynex Technologies, USA). Optimal dilutions of antigen and positive and negative sera were determined by checkboard titrations (Machado et al. 1997). The immunological reactivity of each serum was calculated using the following equation: (mean sample absorbance - mean absorbance of negative serum reference)/(mean absorbance of positive reference serum - mean absorbance of negative serum reference). S/P values were grouped into ELISA levels (EL), which ranged from 0 (lowest level) to 9 (highest level), as described by Machado et al. (1997). The discriminating absorbance value (cut-off) was determined as being two and a half times the mean absorbance value of the negative group, where readings above the cut-off value were considered positive. Specificity was defined as the proportion of known negative serum samples detected as negative and the sensitivity was defined as the proportion of known positive serum samples detected as positive.

Statistical analysis

The McNemar test for nonindependent samples was used to compare the ELISA results to those of the IFAT (Baldock 1988). A *P* value of <0.01 was considered significant.

RESULTS

Checkboard titrations showed an antigen concentration of 10mg/mL in carbonate buffer, pH 9.6, as optimum. Serum samples, including positive and negative reference sera, were diluted at 1:100. The average absorbance of negative sera was 0.115±0.02, resulting in a calculated cut-off value of 0.288 (EL 3, Table 1). The mean absorbance value of the anti-*Leishmania* sp serum group (1.418+0.243) was about 12 times greater than that obtained with non-infected sera, clearly discriminating between the mean absorbances of positive and negative reference sera. This data gave a sensitivity and specificity of 100%. The EL was determined as shown in Table 1.

IFAT and ELISA results for sera samples obtained from Belo Horizonte, the state of Minas Gerais State are summarized in Table 2. Statistical analysis showed significant differences between the results (P<0.01). IgM

Table 1. S/P values for ELISA levels (EL) 0-9

EL	Absorbance value
0	0-0.155
1	0.156-0.209
2	0.210-0.282
3	0.283-0.381
4	0.382-0.514
5	0.515-0.694
6	0.695-0.937
7	0.938-1.265
8	1.266-1.708
9	≥1.709

Table 2. Summary[a] of ELISA and IFAT results for the sera samples obtained from dogs (n=110) raised in endemic area of Belo Horizonte, the state of Minas Gerais, Brazil

ELISA	IFAT	
	Leishmania chagasi positive	*L. chagasi* negative
L. chagasi positive	27	71
L. chagasi negative	0	12

[a] Results are significantly different (P<0.01).

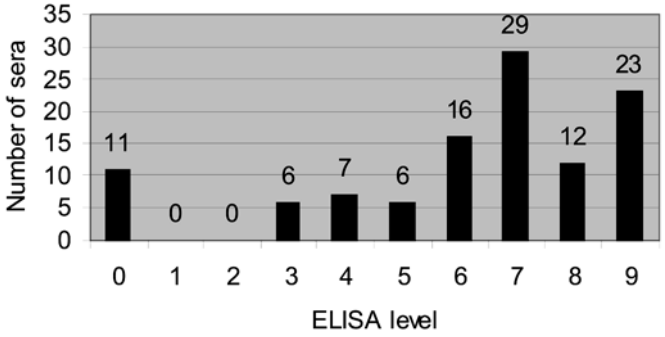

Fig.1. Number of sera from dogs (n=110) raised in endemic area of Belo Horizonte, the state of Minas Gerais, Brazil, screened by IgM ELISA against *Leishmania chagasi* soluble antigen and plotted by ELISA level (EL).

antibody against *Leishmania* sp. soluble antigen were detected in only 24.55% (n=27) of the dogs by IFAT, with titres ranging from 1:80 to 1:320. On the other hand, the estimated ELISA values demonstrated that 89.09% (n=98) of the dogs had EL 3-9, with only 10.91% (n=12) of the serum samples being negative for IgM. The results of sera number distributed through EL are shown in Figure 1.

DISCUSSION

American visceral leishmaniasis in Brazil is considered to be a zoonotic disease which was formerly restricted to rural and sub-urban areas. Today, due to the emergence of new foci of the disease in urban areas, it has become increasingly important to public health.

The diagnosis of AVL in both human beings and dogs remains a difficult task. Current methods rely on clinical signs, parasite identification in aspirated material and serology. The direct observation of amastigote forms in smears is a safe method, but the sensitivity is reduced. Serological methods, on the other hand, are widely used in endemic areas as a determining factor for the elimination of dogs but usually lack sensitivity, and frequently underestimate the real incidence of the infection. Therefore there is an increasing need for more specific and sensitive diagnostic methods to be developed.

The ELISA described in this article is based on the use of reference sera to clearly define the difference between IgM negative and positive sera and to calibrate ELISA absorbance as a score over a wide range of antibody levels. Positive controls used on the standardization of the ELISA were obtained from Belo Horizonte, the state of Minas Gerais, an endemic area for VL in Brazil. Negative controls were obtained from dogs native to Jaboticabal, the state of São Paulo, a non-endemic area for the disease. These negative reference sera were collected during a rabies vaccination program and were immunologically tested for IgG antibodies anti-*Leishmania* sp. by IFAT and ELISA (Oliveira et al., 2008). The results have demonstrated that a soluble antigen of *L. chagasi* prepared from *in vitro* culture of the parasite could be used in a sensitive and specific ELISA to detect IgM antibodies for *Leishmania* sp.

In our study, IFAT was considered the gold standard, and the results obtained from this test were considered "true". Statistical analysis showed significant differences between the ELISA and the IFAT results ($P<0.01$), indicating a poor correlation between both tests (Table 2), although the sensitivity and specificity of the IgM ELISA were 100%. An ELISA can detect antibodies binding to multiple antigenic determinants of the parasite as these are ruptured during antigen preparation and a wide range of antigen is exposed. On the other hand, IFAT detects anti-*Leishmania* sp. antibodies that are directed against surface proteins.

This study suggests that the ELISA described herein is more sensitive than IFAT for the detection of IgM antibodies anti-*Leishmania* sp. Similar results were also reported by Bouer (2001), who recorded a higher sensitivity in the ELISA compared to IFAT in the detection of anti-*Toxoplasma gondii* IgM antibodies in naturally infected monkeys (IFAT-96%, ELISA-53%). It should be mentioned, however, that when IgG anti-*T. gondii* antibodies were analyzed, the sensitivity of ELISA and IFAT were similar (IFAT- 65%, ELISA-68%). Additionally, Bouer (2005) observed that IgM anti-*T. gondii* antibodies in experimentally infected monkeys are detected up to the 15^{TH} day of infection by IFAT and up to the 120^{TH} day of infection by ELISA, while IgG antibodies were detected up to the end of the experiment. Also, Carvalho (1999) observed that ELISA-IgM is more sensitive than IFAT-IgM in experimentally infected cats.

IgM is an antibody associated with acute forms of several parasite diseases. However, IgM anti-*L. chagasi* are detected, respectively, after the first and fifth month of acute and chronic infection, maintaining high levels during the course of infection (Genaro 1993). High IgM titres are present in dogs, which are naturally infected with *L. chagasi*, and there is no difference between titres of asymptomatic, oligosymptomatic and symptomatic dogs (Reis 2001). Lucena & Ginel (1998) analyzing sera from dogs which are naturally infected with *L. chagasi* by IFAT, found that 91.7% (n=11) of the dogs presented IgM, 41.7% (n=5) IgG and 33.3% (n=4) IgA, the three imunoglobulin isotypes were not detected simultaneously.

Although IFAT is the serological method used by the Fundação Nacional de Saúde in the Brazilian program for the control of visceral leishmaniasis, several studies have demonstrated that IFAT has low sensitivity and may lead to false-negative results (Braga et al. 1998, Silva et al. 2001). Additionally, standardization is difficult considering the subjective judgement of the reader and it is a time-consuming method, especially for seroepidemiological studies. Hence, ELISA should be considered an alternative to IFAT or even a complementary method, especially because it permits the simultaneous analysis of a large sample number in a short time, and where data readings are automatically performed and antigen concentration is both small and determined.

In conclusion, we have provided convincing data demonstrating the usefulness of ELISA using total soluble antigenic preparation in the detection of anti-*L. chagasi* IgM antibodies. The combined use of ELISA and IFAT is recommended in order to enable veterinary services to more efficiently detect CVL.

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