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# CANINE VISCERAL LEISHMANIASIS: STUDY OF METHODS FOR THE DETECTION OF IgG IN SERUM AND ELUATE SAMPLES

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## SUMMARY

The Brazilian Ministry of Health recommends the culling and euthanasia of dogs with a positive serological test for canine visceral leishmaniasis (CVL). In the Municipality of Rio de Janeiro, the technique used for the diagnosis of CVL is the indirect fluorescent antibody test (IFAT), using blood samples eluted on filter paper (eluate). A dog survey was conducted over a period of one year in the region of Carapiá, in order to evaluate the diagnosis of CVL in this region. All animals underwent clinical examination, and blood samples (serum and eluate) were collected for analysis by enzyme immunoassay (ELISA) and IFAT. A skin biopsy was obtained for parasitological examination (culture). A total of 305 animals were studied and *Leishmania chagasi* was isolated from nine animals. Sensitivity and specificity were 100% and 96.6% for ELISA, respectively, 100% and 65.5% for IFAT (cut-off at a 1:40 dilution), 100% and 83.4% for IFAT (cut-off at a 1:80 dilution), and 22.2% and 97.0% for eluate IFAT. In conclusion, ELISA was the best tool for the diagnosis of CVL among the serological techniques tested. The present results suggest the need for a better evaluation of filter paper IFAT as the only diagnostic method for CVL in the Municipality of Rio de Janeiro.

**KEYWORDS:** *Leishmania*; Dog; Diagnosis; Serological methods; Rio de Janeiro.

## INTRODUCTION

Leishmaniasis is an important zoonosis and is one of the six leading world epidemics[29]. Leishmaniasis is caused by different protozoans of the genus *Leishmania* and is transmitted to the vertebrate host by insect vectors of the subfamily Phlebotominae[19,29].

In Brazil, American visceral leishmaniasis (AVL) represents a serious public health problem due to its wide geographic distribution, large number of cases, and severity of its clinical forms[20]. The epidemiology of AVL in Brazil is eminently rural, but the disease is currently spreading to mid-size and large urban areas such as Palmas (TO), Campo Grande (MS), Belo Horizonte (MG), and Rio de Janeiro (RJ). *Leishmania (Leishmania) chagasi* (syn. *L. infantum*) is the etiological agent responsible for the disease, and *Lutzomyia longipalpis* has been identified as the main transmitting vector[12,20].

In the wild, reservoirs of *Leishmania* are foxes (genera *Dusicyon* and *Cerdocyon*) and marsupials (genus *Didelphis*), whereas in urban areas the domestic dog (*Canis familiaris*) represents the main source of infection for the vector[8,19,23,24]. Therefore, one of the main measures for the control of AVL in Brazil is the identification and culling of seroreactive dogs in endemic areas in order to interrupt the cycle of transmission[20].

In the municipality of Rio de Janeiro, the technique used for the diagnosis of canine visceral leishmaniasis (CVL) is the indirect fluorescent antibody test (IFAT) using blood samples eluted on filter paper (eluate). Although eluates are used for the serological diagnosis of numerous diseases[11,13], few studies have compared the use of serum and eluate for the routine diagnosis of leishmaniasis in dogs. Using mathematical models, PALATNIK-DE-SOUSA *et al.*[22] demonstrated the inefficacy of eluate IFAT in the control of CVL and the superiority of IFAT and ELISA using serum samples. The Brazilian Ministry of Health currently recommends the use of ELISA for the screening of seroreactive animals and subsequent confirmation by IFAT[20].

The gold standard for the diagnosis of leishmaniasis is a positive parasitological test[5]. Clinical specimens for the diagnosis of CVL by parasitological examination can be obtained by aspiration of bone marrow, spleen, liver and lymph nodes and, in some cases, by a biopsy of intact skin[15,16], lesion, or viscera[2,3].

In view of the importance of the domestic dog for the maintenance of the transmission cycle of AVL in endemic areas and of discussions regarding the use of eluate samples for its diagnosis, we evaluated the eluate IFAT method used by the Municipal Health Secretary Office of Rio de Janeiro (SMS/RJ) by comparing it with parasitological

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examination (isolation in culture) and serological techniques using serum samples.

## MATERIAL AND METHODS

In 2005, the Municipal Health Secretary Office performed a dog survey in the region of Carapiá, Guaratiba, western region of the Municipality of Rio de Janeiro, which identified 351 dwellings comprising a population of 698 inhabitants and 341 dogs. The survey of CVL in the region was conducted using isolation of the parasite in culture (skin biopsy) and the findings were compared to IFAT and ELISA using serum samples and to IFAT using eluate samples. The last method was performed by the Epidemiology Service of the Municipality of Rio de Janeiro.

The responsible persons of each household visited received detailed information about the study and signed a free informed consent form before agreeing to anamnesis, and before the animals underwent clinical examination and sample collection. The project was approved by the Ethics Committee on the Use of Animals (CEUA-FIOCRUZ-L. 0298-06).

For the collection of skin fragments, the dogs were sedated by intramuscular injection of 10 mg/kg ketamine hydrochloride plus 0.2 mg/kg acepromazine. After asepsis and local anesthesia with 2% lidocaine hydrochloride, a biopsy was obtained from apparently healthy skin on the inner side of the auricle or scapular region of all animals. The tissue fragments were kept in saline containing 1000 IU penicillin, 200 µg streptomycin and 50 µg 5'-fluorocytocine per milliliter and stored at 4 °C for 24 h for isolation in culture and subsequent isoenzyme characterization according to protocols described in the literature[16].

For the serological test, a 5 mL blood sample was collected from each animal by venipuncture after trichotomy with a disposable scalpel, and local antisepsis was performed with 70% alcohol. The brachial vein was punctured in large dogs and the jugular vein in medium-size and small dogs. The presence of anti-*Leishmania* IgG antibodies in dog serum was investigated using the Canine Visceral Leishmaniasis IFAT and ELISA kits (Bio-Manguinhos/FIOCRUZ/MS). The following two criteria were adopted for interpretation of the IFAT results: samples presenting fluorescence at dilutions of 1:40, 1:80 or higher were considered to be positive. For ELISA, samples showing readings above the cut-off were considered to be positive according to manufacturer recommendations. For both tests, the same sample was analyzed in duplicate at different times.

Analysis of eluate samples was carried out by the Epidemiology Service of the Municipality of Rio de Janeiro according to the procedures indicated in the manual of the Canine Visceral Leishmaniasis IFAT kit (Bio-Manguinhos/FIOCRUZ/MS).

Sensitivity, specificity, and both positive and negative predictive values were evaluated. The Chi-square test was used for the comparison of proportions. Analyses were performed with the Statistical Package for the Social Science (SPSS), version 11, and the Epi Info 6.04 program, considering a 95% confidence interval (CI).

## RESULTS

On the basis of the dog census performed over a period of one year, 305 (89.4%) animals of a population of 341 dogs were studied. Thirty-six (10.6%) dogs were excluded, mainly because of the lack of adherence of their owners to the study, the absence of a responsible person at the residence, and losses due to the death of some animals between the census in 2005 and the present study.

*Leishmania* sp. were isolated by culture of intact skin samples from nine animals, corresponding to a prevalence of 3% (95% CI: 1.4; 5.7). These species were identified as *Leishmania (Leishmania) chagasi* by isoenzyme electrophoresis.

Of the 305 samples tested, 19 (6.2%) were positive by ELISA, 111 (36.4%) by IFAT (cut-off at a dilution of 1:40), and 58 (19%) by IFAT (cut-off at a dilution of 1:80).

The accuracy parameters of the tests for the detection of anti-*Leishmania* IgG, using parasitological examination as a reference, demonstrated a better performance of ELISA for the evaluation of dogs infected with *Leishmania chagasi* (Table 1). Although the sensitivity of IFAT using serum samples was 100%, irrespective of the positivity criterion adopted, specificity was higher at a cut-off dilution of 1:80 (83.4%), when compared to the 1:40 dilution (65.5%) (Table 1). Similarly, the difference in specificity between ELISA (96.6%) and IFAT (83.4%) was also significant ($p = 0$; 95% CI). The likelihood ratios for positive results were 29.4 for ELISA, and 2.98 and 6.02 for IFAT at cut-off dilutions of 1:40 and 1:80, respectively. IFAT using eluate samples showed a lower sensitivity (22.2%) compared to the other tests and a likelihood ratio of 7.4. The other accuracy parameters are shown in Table 1.

## DISCUSSION

In the present study, a dog census was performed using isolation of the parasite in culture as the gold standard for the diagnosis of CVL

**Table 1**
Performance of ELISA and IFAT for canine visceral leishmaniasis with serum and eluate samples

| | Sensitivity (IC 95%) | Specificity (IC 95%) | PPV (IC 95%) | NPV (IC 95%) |
|---|---|---|---|---|
| IFAT (Eluate) | 22.2 (3.9; 59.8) | 97.0 (94.1; 98.5) | 18.2 (3.2; 97.6) | 97.6 (94.9; 98.9) |
| IFAT (1:40) | 100 (62.8; 100) | 65.5 (59.8; 70.9) | 8.1 (4.0; 15.2) | 100 (97.6; 100) |
| IFAT (1:80) | 100 (62.8; 100) | 83.4 (78.6; 87.4) | 15.5 (7.8; 27.9) | 100 (98.1; 100) |
| ELISA | 100 (62.8; 100) | 96.6 (93.7; 98.3) | 47.4 (25.2; 70.5) | 100 (98.3; 100) |

IC (confidence interval); IFAT (indirect fluorescence antibody test); ELISA (enzyme immunoassay); PPV (positive predictive value); NPV (negative predictive value).

and comparing it to serological methods (IFAT and ELISA using serum samples and IFAT using eluate samples).

Parasitological confirmation by culture of intact skin fragments from seroreactive dogs has been shown to be successful in clinical practice for the diagnosis of CVL. In two studies conducted in the Municipality of Rio de Janeiro, MADEIRA *et al*.[16,17] isolated *Leishmania* from 18/20 and from 31/39 animals, respectively, using skin fragments obtained from dogs euthanized because of a suspicion of CVL for parasitological confirmation. In another study, the same authors reported a rate of isolation of 78.7% when skin samples from 394 dogs were evaluated[15]. A similar percentage (83%) has been reported by BARROUIN-MELO *et al*.[2] who analyzed spleen samples from seroreactive dogs. These results demonstrate that intact skin fragments can be used as the target sites for the parasitological confirmation of CVL based on mild intervention, with sensitivity close to that observed for the culture of organs of the phagocytic-mononuclear system. One advantage of this technique is that it is applied easily. However, operational difficulties are encountered by public health authorities in implementing this procedure in routine practice.

Culling of seroreactive dogs from AVL-endemic areas for the control of the disease is a polemic subject because of the disagreement among investigators regarding the accuracy of the tests currently employed for this purpose[1,7,27,28]. There is no scientific evidence demonstrating the impact of dog culling on human infection. Another widely discussed aspect is the cut-off value used to define positive or negative IFAT reactions[6]. In AVL endemic areas, the BRAZILIAN MINISTRY OF HEALTH[20] recommends the culling and euthanasia of dogs that are reactive in the IFAT assay at dilutions of 1:40 or higher. In the present study, the sensitivity of the IFAT assay was the same for the two cut-off dilutions used (1:40 and 1:80). On the other hand, specificity was significantly higher when a cut-off dilution of 1:80 was defined. This discordance might be due to the overlapping geographic endemicity of AVL with American tegumentary leishmaniasis and sporotrichosis, which occur at some localities in the Municipality of Rio de Janeiro, favoring low-affinity cross-reactivity that is detected at low dilutions. RIBEIRO *et al.*[25] observed reactivity in 14.3% of serum samples from dogs with sporotrichosis at dilutions of 1:40. These results suggest that, at least in areas of low prevalence, a more appropriate cut-off would be at a dilution of 1:80. With respect to the performance of ELISA and IFAT using serum samples, the results regarding the accuracy of the tests are conflicting. FERREIRA *et al*.[10] observed 52% and 64% specificity and 96% and 72% sensitivity for ELISA and IFAT, respectively. OLIVEIRA *et al.*[21] demonstrated 90% sensitivity and 100% specificity for ELISA and 40% sensitivity and 98.6% specificity for IFAT. However, LIRA *et al*.[14] observed no significant difference between the two tests. Although no differences in the sensitivity of the techniques were observed in the present study, specificity was significantly higher for ELISA and the positive predictive values for the IFAT were very low. In addition, there was a 29.4 times higher chance that a positive ELISA result is a true positive result, a value higher than that observed for IFAT. The use of intact parasites and whole parasite extracts in serological tests may result in cross-reactions with other *Leishmania* species and with *Trypanosoma cruzi*, *Ehrlichia canis* and *Sporothrix schenckii*[18,25,26]. Therefore, the specificity of 96.6% and likelihood ratio of 29.4 obtained for ELISA seem to be relevant, especially when considering that the samples analyzed were obtained from dogs living in areas that are also endemic for diseases of other etiologies and that the species isolated from seropositive dogs was characterized as *L. chagasi*.

The sensitivity of IFAT using blood dried on filter paper and then processed and analyzed at the Epidemiology Service of Rio de Janeiro was much lower than that obtained for IFAT and ELISA using serum samples. This observation was confirmed in a subsequent study carried out in the same region, in which 34.9% of positive dogs were identified by serum IFAT among 146 dogs that tested seronegative by IFAT using blood samples collected on filter paper[9]. Although the serum and eluate samples were processed and analyzed at different laboratories, the results obtained suggest that IFAT using samples collected on filter paper may not be adequate for epidemiological surveys. Aspects related to sample collection, transport and storage may explain, in part, the low performance of eluate samples when compared to serum samples. On the other hand, the accuracy of ELISA suggests that this diagnostic method would be best suited for campaigns of visceral leishmaniasis control, reducing two major problems. The first is related to the number of infected dogs that remain after culling of seropositive animals and that are not detected by other tests, and the second refers to the unnecessary sacrifice of dogs due to the large number of false-positive IFAT reactions in serum. BRAGA *et al*.[4] demonstrated that up to 2.85 times more seropositive dogs are detected by ELISA using serum samples than by IFAT testing of eluate samples.

The present results suggest that the method used in the Municipality of Rio de Janeiro for the diagnosis of CVL should be better evaluated and that the filter paper IFAT might be replaced with ELISA. In addition, the results demonstrate the superiority of ELISA over IFAT and that ELISA might be indicated for epidemiological surveys. Comparison of these results with those obtained by the Epidemiology Service of Rio de Janeiro (IFAT testing of eluates) also demonstrates the superiority of ELISA, showing 100% sensitivity and 96.6% specificity.

**RESUMO**

**Leishmaniose visceral canina: avaliação de métodos para detecção de IgG em amostras de soro e eluato**

O Ministério da Saúde recomenda a eutanásia de cães sororreatores como controle da leishmaniose visceral canina (LVC). No Município do Rio de Janeiro, a técnica utilizada para o diagnóstico da LVC é o teste de imunofluorescência indireta (IFI), utilizando amostras de sangue eluídas em papel de filtro (eluato). Um levantamento, durante um ano, foi conduzido na região de Carapiá, a fim de avaliar o diagnóstico da LVC nesta região. Todos os animais foram submetidos a exame clínico e coleta de sangue (soro e eluato) para realização do ensaio imunoenzimático (ELISA) e imunofluorescência indireta (IFI). Biópsia de pele foi obtida para o exame parasitológico (cultura). Foram avaliados 305 (89,4%) animais de uma população de 341 cães e *Leishmania chagasi* foi isolada de nove animais. A sensibilidade e especificidade do ELISA foram de 100% e 96,6%, na IFI (ponto de corte 1:40) de 100% e 65,5%, na IFI (ponto de corte 1:80) de 100% e 83,4% e na IFI (eluato) de 22,2% e 97,0%, respectivamente. A partir dos resultados obtidos podemos concluir que entre as técnicas sorológicas empregadas, o teste de ELISA apresentou-se como a melhor ferramenta para o diagnóstico da LVC. Os resultados sugerem a necessidade de uma melhor avaliação do teste de IFI realizada com eluato, como único método de diagnóstico para LVC no município do Rio de Janeiro.

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