A COMPARISON OF INDIRECT FLUORESCENT ANTIBODY TEST, INDIRECT ENZIMATIC IMMUNOASSAY AND SERUM GELIFICATION TECHNIQUE IN 40% FORMOL IN THE DIAGNOSIS OF CANINE VISCERAL LEISHMANIASIS

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SUMMARY

One hundred fifty-five samples of canine sera, divided in five groups, were compared by indirect fluorescent antibody test (IFAT), serum gelification in 40% formol (GF) and the indirect enzyme-linked immunosorbent assay (indirect ELISA) for serological diagnosis of canine visceral leishmaniasis. Group A had 36 dogs from endemic area of visceral leishmaniasis, serologically positive to IFAT, and showing clinical signs; group B had 27 dogs from endemic area, serologically positive, and no clinical signs; group C had 34 healthy dogs from endemic area, serologically negative; group D had 25 healthy dogs from non-endemic area, serologically negative; group E had 33 dogs from non-endemic area, serologically negative, but with other diseases. There was not statistical difference between the sera samples of the animals from groups A and B (p = 0.065). GF presented a sensitivity of 44.44%, specificity of 82.61%, positive predictive value of 63.64% and negative predictive value of 68.47%, observed agreement of 67.10%. The indirect ELISA presented a sensitivity of 84.13%, specificity of 93.48%, positive predictive value of 89.83%, negative predictive value of 89.58%, observed agreement of 89.68%. Serum samples of dogs from group E were not positive in indirect ELISA, and presented lower sensitivity and specificity than IFAT. Both indirect ELISA and GF presented a positive correlation in comparison with the results of IFAT. In conclusion, IFAT should be recommended in serological surveys for canine visceral leishmaniasis, in animals with either clinical or subclinical disease, since there is no significant statistical difference.

KEY-WORDS: Leishmaniasis. Dog. IFAT. Indirect ELISA. Serum Gelification.

RESUMO

Amostras de 155 soros de cães foram separadas em cinco grupos e comparadas pelas técnicas de reação de

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Improperly infected characteristics of acute reticuloendotheliosis and adolescents are the most infected, with the subclinical disease, in endemic areas, in which children behaving once as a simple infection, other as clinical or mononuclear fagocitic cells, with variable intensity, compromises the hematologic system and, mainly, 1993). It attends to a systemic protozoosis that responsible for the disease in Brazil (GRIMALDI & TESH, disease caused by a protozoan, 

The visceral leishmaniasis (VL) is a parasitic disease caused by a protozoan, Leishmania (L.) chagasi, responsible for the disease in Brazil (GRIMALDI & TESH, 1993). It attends to a systemic protozoosis that compromises the haematologic system and, mainly, mononuclear fagocitic cells, with variable intensity, behaving once as a simple infection, other as clinical or subclinical disease, in endemic areas, in which children and adolescents are the most infected, with the characteristics of an acute reticuloendotheliosis (BARBOSA & BARBOSA, 1994).

INTRODUCTION

The dogs (Canis canis) and the crab-eating foxes (Cerdocyon thous) are the most important vertebrate reservoirs of this parasite, characterizing this disease as a zoonoses (COUTERNAY et al., 1996). Lutzomyia longipalpis, realizes the function of vector of the protozoan responsible for American visceral leishmaniasis. This sandfly has 2 to 3 mm of size, with peridomestics and domestics habits, and transmits to humans by the bite, with recognized avidity at night, principally during the dusk, when the host is resting (ARIAS et al., 1996).

Since 1907, there were signs of the existence of the visceral leishmaniasis in Brazil. Between 1911 and 1912,
Carlos Chagas suspected that it would happen in some Amazonian regions (MONTEIRO et al., 1994). It is believed that the first case of the autochthonous visceral leishmaniasis was diagnosed in 1913 (SHAW & LAINSON, 1980). The disease was identified only in 1934, where the parasites were found in 41 samples of liver from patients from Ceará and Pará states, which died of yellow fever (MONTEIRO et al., 1994). It is known by many names: calazar, dundun fever and dog’s disease (BRASIL, 2000).

Due to the increased dissemination of AIDS and other immunodepressive diseases, the opportunistic infection by *Leishmania* in human patients is becoming common and the prevention and control of this parasitic disease of zoonotic character (BADARÔ, 1997; SASAKI et al., 1997), turned out to be very important.

The dog is the most important reservoir (DEDET, 1994) of this parasite, contemplated in 1908, in Mediterranean, in spite of the existence of wild reservoir responsible to maintain the wild cycle of the disease, like the hoary fox (*Lycalopex vetulus*), crab-eating fox (*Cerdocyon thous*) and marsupials (*Didelphis marsupialis* and *D. albiventris*) (LAINSON et al., 1969). Synanthropic animals, as rats (*Rattus rattus*) (GRADONI et al., 1983), realize an important epidemiologic role, being capable of the maintenance of the cycle of this disease.

There are several diagnostic methods, such as: parasitologic, serodiagnosis (Indirect Fluorescent Antibody Test – IFAT, Enzimatic Immunoassay and a fast test to detection antibodies of *Leishmania donovani* (TRALd), methods of molecular biology as DNA Hybridization and the Polymerase Chain Reaction – PCR, and others. EL SAFI & EVANS (1989) comparing ELISA and the direct agglutination test (DAT) using human positive and negative sera for visceral leishmaniasis, showed 100% of sensibility and specificity between both tests, although sera of patients with others parasitary infections were not tested. HARITH et al. (1989) working with 220 canine sera, 26 parasitologically confirmed, 12 suspected but not confirmed, and 182 in others conditions, by the direct agglutination test for diagnosis of the visceral leishmaniasis, determined sensibility of 100% and specificity of 98.9%, using as positive cut off point dilution of 1:320, in spite that 25 of 26 confirmed cases obtained titers higher than 2560, and only a 640 titer.

GENARO et al. (1990) found 2.76% of positivity in 276 dogs examined by IFAT, in Belo Horizonte-MG. NUNES et al. (1991) realized a canine serological survey to visceral and cutaneous leishmaniasis, in Rio de Janeiro state, in an area of high risk of transmission, after adoption of prophylactic measures. For six successive years, a positivity of 0.91% (206 / 22828) sera samples using IFAT was observed.

Considering the importance of canine visceral leishmaniasis and its zoonotic aspects, the present work had the objective of verifying the relation among IFAT, the indirect ELISA and the Serum Gelification Test in 40% Formol - GF for serological diagnosis of canine visceral leishmaniasis; to evaluate the levels of sera antibodies of IgG class using IFAT in dogs with visceral leishmaniasis, with and without clinical signs, and to compare the sensibility, specificity, predictive values and agreement rate of GF and the indirect ELISA and IFAT, in sera samples of the dogs with visceral leishmaniasis, with and without clinical signs.

**MATERIAL AND METHODS**

One hundred fifty-five samples of canine sera were used, as follows: Group A with 36 dogs from endemic area of visceral leishmaniasis, positive serologically with titer ≥ 40 by IFAT, with clinical signs (inappetence, hepatoesplenomegaly, onychogryphosis, breakable fur, lymphadenopathy); Group B with 27 dogs from endemic area of visceral leishmaniasis, positive serologically (titer ≥ 40 to IFAT), without clinical signs; Group C with 34 dogs from endemic area of visceral leishmaniasis, healthy and negative serologically; Group D with 25 dogs from non-endemic area of visceral leishmaniasis, healthy and negative serologically, and Group E with 33 dogs from non-endemic area of visceral leishmaniasis, negative serologically, but with other diseases like toxoplasmosis, leptospirosis, erlichiosis, babesiosis or transmissible venereal tumor.

The animals of groups A, B and C were from Araçatuba-SP, group D from Botucatu, with no description of autochthonous cases of leishmaniasis (LANGONI et al., 2001), and group E, dogs with others diseases, diagnosed previously at the Veterinary Hospital from FMVZ / UNESP / Botucatu-SP.

The serum samples were harvested by jugular vein puncture and maintained at −20°C until the analysis, at the Laboratory of the Zoonosis Research Nucleus – NUPEZO, at the Veterinary Hygiene and Public Health Department of the Veterinary Medicine and Zootechnology Faculty of UNESP – Campus Botucatu – SP.

For the indirect fluorescent antibody test, the samples were diluted initially to 1:40 as positive cut off point (EVANS et al., 1990), following successive dilutions in 2 ratio in polystyrene micro plates with 96 wells, and added to impregnated slides with promastigotes of *Leishmania (L.) major*, for the detection of antibodies of
IgG class, according with CAMARGO (1966) and MANCIANTI et al. (1988), using an anti-dog IgG secondary antibody-labelled with FITC diluted to 1:200, produced and gracefully supplied by Zoonosis Control Center (CCZ) of São Paulo. As positive controls, sera of animals from endemic area were used and, as negative controls sera of animals from non-endemic areas. The sera that presented fluorescence of equal or superior intensity to the positive control were considered positive. This technique was taken as golden standard for comparison to the other methods applied.

The ELISA technique was realized, according with GUIMARÃES et al. (1981), with modifications for the extraction and quantification of the soluble protein used as antigen, that were produced from promastigote forms of Leishmania (L.) major, obtained in LIT culture media (Liver Infusion Tryptose) in its growth’s exponential level. The promastigote forms were washed three times in buffered saline solution (PBS), adjusted to 2 x 10^5/mL in PBS, and stocked at –20ºC. After, in this alkaline solution was added an inhibitor of protease, phenylmethylsulfonylfluoride (PMSF), in a concentration of 250 mM. This suspension was submitted to freezing at –86ºC and thaw at 37ºC for four successive times and, next, centrifuged at 3000 x g to 4ºC for five minutes, with separation of the suspension and the supernatant to use as alkaline antigen. Protein concentration of this antigenic suspension was dosed by bicinchoninic acid (BCA) method (BOLLAG et al., 1996) and was maintained at –86ºC, until the moment of use.

The concentrations of the conjugates and antigens were determined by block titration, diluting optimally positive and negative standard sera. The conjugate consisted of anti-dog IgG labelled to peroxidase (SIGMA). We used 5 μg/mL of the conjugate to dog, according with the known titration. Aliquots of 100 μL of the antigenic suspension in adequate concentration were prepared as above described, and deposited in wells of new plates (wells of bottom in “U” – MaxiSorp NUNC®), that were maintained at 4ºC between 15 and 20 hours, for sensibilization.

To increase the confiability of the technique, it was established that the optic density of the average of the positive control sera would be at least ten times more than the average of the negative control sera (positives: 0.847 + 0.790 + 0.802 = 2.439 / 3 = 0.813 and negatives: 0.089 + 0.057 + 0.108 = 0.254 / 3 = 0.084) in the concentration of 5 μg/mL, and that demonstrated the real difference of optic density at posterior serum dilution (between the dilution 1:40 and 1:80), establishing, therefore, the dilution 1:80 as standard.

The sensibilizated plates were washed with PBS–Tween 0.05% and the antigen, adsorbed to the wells, was blocked with 100 μL/well of PBS–T caseined (PBS–T–Skim milk 5%) for 30 minutes to 37ºC. The plates were washed three times with 200 μL/well with PBS–T–0.05%. The sera diluted to 1:80 in duplicates, adding it on respective wells in volumes of 100 μL, incubating at 37ºC/1 hour. Then, the plates were washed again for three times with 200 L/well, adding the chromogen orthophenylene diamine (OPD) in a concentration of 10 mg to 12.5 mL of phosphate-citrate buffer 0.15M, pH 4.5 and 10 μL of oxygen water (H₂O₂) at 30%. The plates were incubated for approximately 30 minutes in room temperature, under luminous protection, and the reaction interrupted with 50 μL/well of sulfuric acid 4N. The lecture was realized in a plate spectrophotometer (Multiskan EX – Labsystems®) under wave length of 492 nm (VOLLER et al., 1976; VOLLER et al., 1978).

The sera were considered positive according to the following criteria (BIONDI et al., 1996): The values were expressed as: S / P = mS – mN / mP – mN, where mS = average of absorbance between the duplicates of test sample, mN = average of absorbance between the duplicate of the negative reference; mP = average of absorbance between the duplicate of the positive reference. This model makes possible the use of positive and negative references to determine corrections.

For the serum gelification in 40% formol (GF) 1 mL of the total serum was added to one drop of 40% formol, waiting the reaction for 5 minutes, observing the formation of gelification, according with the following degrees: + means a light gelification with difficulty of loosening the material of the tube; ++ bright formation of the gel, but with a translucent secretion; and +++ revealing gelification and opacity of the tube contents (PESSÔA, 1972).

The serological tests were compared (sensibility, specificity, predictive values and agreement rate) using the statistical program Instat and repeated by Epi-Info, 6.04d version. Each serological test was compared among the groups for linear correlation test, by Winstat statistical program. The confidence level used was α = 0.05 (CURI, 1997). IFAT was compared between the groups A and B, for the tendency test of orderly data with help of Epi-Info program, 6.04d version, taking the group A as control.

RESULTS AND DISCUSSION

All serological results of the groups to all tests applied are shown in Table 1.
On the IFAT results in animal samples of the groups A and B, with or without clinical signs, the comparison of the tendency was realized in orderly data, in according the serological titters (40, 80, 160, 320, 640, 1280 and 2560), increasingly orderly and compared the number of the result revealing that there was not a statistical difference among them, with the value $p = 0.065$, with average (mean ridit) = 0.366 and $z = -1.847$ (Tab. 2).

The study of sensibility (S), specificity (Sp), predictive values (PV) and agreement rate (AR) among the serological tests, between IFAT and GF, showed for the last one a sensibility of 44.44% (AR$_{95\%}$ = 31.92 – 57.51%), specificity of 82.61% (AR$_{95\%}$ = 73.3 – 89.72%), positive predictive value (PPV) of 63.64% (AR$_{95\%}$ = 47.77 – 77.59%), negative predictive value (NPV) of 68.47% (AR$_{95\%}$ = 59.10 – 74.42%), linear correlation coefficient (r) of 0.2947 (AR$_{95\%}$ = 0.1437 – 0.4323%) and $p$ (2-tailed) < 0.0002. GF presented three false-positive results in group C, three in group D and ten in group E, that compromises its use in surveys of visceral leishmaniasis.

Between IFAT and the indirect ELISA, taking the total soluble protein as antigen, this presented the estimated sensibility of 84.13% (AR$_{95\%}$ = 72.74 – 92.12%), specificity of 93.8% (AR$_{95\%}$ = 86.4 – 97.7%), positive predictive value of 89.83% (AR$_{95\%}$ = 79.17 – 96.18%) and negative predictive value of 89.58% (AR$_{95\%}$ = 81.68 – 94.89%), with Kappa rate ($\kappa$) of 0.7839 and observed agreement of 89.68% (AR$_{95\%}$ = 83.78 – 93.98%) and linear correlation coefficient (r) of 0.7850 (AR$_{95\%}$ = 0.7160 – 0.8389%) and $p$ (2-tailed) < 0.0001 (Tab. 3). The optic density of the negative control was 0.128, while the optic density of the positive control serum was 1.264. The indirect ELISA didn’t react with serum samples of dogs from group E, and presented six false-positive sera in group C.

In this study, IFAT was adopted as “golden test”, or standard, taking in account its large diffusion through Brazil, with best technique qualification personal, and by indication of the same by the Ministry of Health (BRASIL, 1994).

As controls, animal sera were used from endemic area recognized positives for aspirative punction and, as negative controls, negative animals from non-endemic area (LUVIZOTTO et al., 1999; LANDONI et al., 2001). Using the method described by BIONDI et al. (1996), was possible the correction to ELISA and, therefore, a better prediction of the results by this technique, fundamental data to affirm the validity of the same.

In regard to the technique of antigen production to ELISA, this result disagree with REED et al. (1986), that used the successive freezing and thawing, for six times, to obtain total soluble proteic antigen, in spite of, in the present, had altered the initial protocol by four times.

DELGADO et al. (1998) evaluating the situation of american visceral leishmaniasis in an old focus, at Guayabita village, in Aragua state, in the north of Venezuela, realized an epidemiologic survey in dogs, using as serological method of screening the GF and counterimmunoelectrophoresis – CIEP reactions. The positive samples were submitted to IFAT and Western Blot – WB, detecting positivity in 6 (8.5%) in a total of 71 analyzed dogs by GF, in 11 by CIEP, and in 4 (5.6%) by IFAT and WB, whereas in only one of these samples was detected leishmania in smears, after the punction. In present study, the comparison between IFAT and GF presented a specificity of 82.61%, in spite of a low coefficient of linear correlation and sensibility.

With regard to the results obtained for groups A and B, that didn’t reveal a statistical difference with reference to the animals with clinical or subclinical disease, VEXENAT et al. (1994) revealed amastigote forms of *Leishmania* spp in skin biopsy material in 60% of positive dogs with clinical signs and, in 30% of the animals without clinical signs by IFAT, demonstrating the importance of this in the detection in apparently healthy animals.

PARANHOS-SILVA et al. (1996) comparing IFAT and ELISA in a study involving a canine serological survey, using a total of 148 samples – 46 positives and 102 negatives – obtained, respectively, the sensibility of 78% and 98% and specificity of 100% and 99%, presenting, therefore, better results. In the present study, the IFAT as standard test demonstrated a low sensibility (84.13%) and good specificity (93.48%).

GARCEZ et al. (1996) comparing the Direct Agglutination Technique – TAD with IFAT and the Indirect ELISA, achieved 100% of sensibility in the three techniques and 98.4%, 97.5% and 84.8% of specificity, respectively, using *L. (L.) donovani* as antigen. Studying the same canine sera, obtained for TAD, IFAT and ELISA with *L. (L.) chagasi* antigen, 71.43%, 100% and 71.4% of sensibility, respectively. TAD, however, has shown to be less specific than ELISA and IFAT. With regards to the sensibility, this comes near of the obtained in the present study, that was 84.13%.

Concerning the formation of the groups, there wasn’t statistical difference regarding groups A and B, but the distribution revealed to be important because CABRERA et al. (1999), standardizing the FML-ELISA, used canine sera with other diseases to challenge the test proposed by them. This fact reafirms the importance of the choice of the five distinct groups. It still confirms that ELISA, proposed in this research, didn’t show crossed reaction in animal groups with others diseases also.

The present study permits to conclude that IFAT
Table 1 – Frequency of positivity and negativity of each group to the serological tests realized. Botucatu city - São Paulo. 2004.

<table>
<thead>
<tr>
<th>GRUPOS</th>
<th>IFAT</th>
<th>Indirect ELISA</th>
<th>GF-40%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>A</td>
<td>36</td>
<td>30</td>
<td>6</td>
</tr>
<tr>
<td>B</td>
<td>27</td>
<td>23</td>
<td>4</td>
</tr>
<tr>
<td>C</td>
<td>0</td>
<td>6</td>
<td>28</td>
</tr>
<tr>
<td>D</td>
<td>0</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>E</td>
<td>0</td>
<td>33</td>
<td>0</td>
</tr>
<tr>
<td>TOTAL</td>
<td>63</td>
<td>59</td>
<td>96</td>
</tr>
</tbody>
</table>

(IFAT: indirect fluorescent antibody test, Indirect ELISA: indirect enzyme-linked immunosorbent assay, GF-40%: serum gelification technique in 40% formol)

A: 36 IFAT (+), with clinical signs
B: 27 IFAT (+), without clinical signs
C: 34 IFAT (+), healthy from endemic area
D: 25 IFAT (+), healthy from non endemic area
E: 33 IFAT (-), other diseases

Table 2 - Ordained number of analyzed serum samples of the groups A and B, in central tendency test. Botucatu city - São Paulo. 2004.

<table>
<thead>
<tr>
<th>TITERS</th>
<th>IFAT</th>
<th>A</th>
<th>GROUPS</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>5</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>6</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>160</td>
<td>11</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>320</td>
<td>6</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>640</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1280</td>
<td>5</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2560</td>
<td>1</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(A: Positive animals to leishmaniasis with symptoms, B: Positive animals to leishmaniasis without symptoms)

Table 3 – Percent number of analyzed serum samples for group, to the diagnostic of visceral leishmaniasis. Botucatu city - São Paulo. 2004.

<table>
<thead>
<tr>
<th>TEST</th>
<th>S</th>
<th>Sp</th>
<th>PPV</th>
<th>NPV</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFAT</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>GF</td>
<td>44.44</td>
<td>82.61</td>
<td>63.64</td>
<td>68.47</td>
<td>&lt; 0.0002</td>
</tr>
<tr>
<td>ELISA</td>
<td>84.13</td>
<td>93.48</td>
<td>89.83</td>
<td>89.58</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

(S: Sensibility, Sp: Specificity, PPV: Positive Predictive Value, NPV: Negative Predictive Value, p: Confidence Level)
should be recommended in serological surveys to canine visceral leishmaniasis, to animals with or without clinical signs, considering the smallest sensibility presented for the others studied tests. The different titers compared, with and without clinical signs of the disease, didn’t present statistical difference, therefore, doesn’t suggest that the animals with clinical signs present higher or lower titers, comparing to animals without clinical signs. The indirect ELISA with antigen constituted by soluble total protein presented sensibility and specificity lower than IFAT. At the same time, such antigen didn’t detect positivity in samples of animals with others diseases.

It permits to conclude that both indirect ELISA and GF presented positive correlation with the results obtained in IFAT, and that GF revealed low sensibility and specificity than the others. Thus, although cheaper, GF should not be used as a screening test in surveys to canine visceral leishmaniasis.


