J. Protozool. Res. 11, 74-81 (2001)

Application of direct agglutination test and fast agglutination screening test for serodiagnosis of visceral leishmaniasis in endemic area of Minas Gerais, Brazil

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Keywords: Visceral leishmaniasis; diagnosis; DAT; FAST.

ABSTRACT

We have tested an indirect fluorescence technique (IFAT), fast agglutination screening test (FAST) and direct agglutination test (DAT) for the detection of anti-*Leishmania* antibodies in serum and blood samples from patients with visceral and cutaneous leishmaniasis in Brazil. For visceral leishmaniasis the results obtained with DAT showed a sensitivity and specificity of 100% with a cut-off value of 1:800. The blood and serum samples could also be clearly read in FAST using a 1:100 dilution with the same high sensitivity. Both DAT and FAST were not able to detect significant amounts of antibodies in samples from cutaneous leishmaniasis patients.

INTRODUCTION

American visceral leishmaniasis (AVL) is a protozoan disease caused by *Leishmania chagasi* parasites, constitutes a major health problem in Brazil. In the last few years the number of human cases of AVL in the Metropolitan Region of Belo Horizonte, Minas Gerais, Brazil has increased, indicating an elevation in the transmission rate of the disease (Silva et al. 2001a). Dogs and fox are considered to be the main reservoirs for this parasite (Deane and Deane 1954; 1962;

Silva et al. 2000). The diagnosis of VL is based on clinical-epidemiological characteristics, by demonstrating the parasite microscopically, indirectly by serological tests and culturing or molecular methods like the polymerase chain reaction (PCR) (Mathis and Deplazes, 1995; Silva et al. 2001b). Several techniques can be used for the sero-diagnosis of VL. The indirect immunofluorescence antibody test (IFAT) was the first choice until 1974 (Camargo 1969; Zuckerman 1975). Since then counter-current immuno-electrophoresis and enzyme-linked immunosorbent assay (ELISA) have been found to be powerful tools for the serodiagnosis of leishmaniasis (Mukerji et al. 1984). In addition, several other serological tests have been developed. The direct agglutination test (DAT) has proved to be a very important serodiagnostic tool combining high levels of intrinsic validity and ease of performance (Harith et al. 1988; Zijlstra et al. 1997; Boelaert et al 1999). The test uses whole, stained promastigotes either as a suspension or in a freeze-dried form (Harith et al. 1988; Meredith et al. 1995; Zijlstra et al. 1997; Oskam et al. 1999). By using the freeze-dried antigen, logistic problems, i.e., a cold chain is required for storage of antigen, are avoided, making the DAT very suitable for use under field conditions.

Although the DAT for the sero-diagnosis of visceral leishmaniasis has a high sensitivity and specificity, it still has some drawbacks like the relative long incubation time (18 hours) and the need for serial dilutions of blood or serum. In order to circumvent these problems Schoone et al. (2001) developed a fast agglutination screening test (FAST) for the rapid detection of anti-*Leishmania* antibodies in serum samples and in blood collected on filter paper. The FAST utilizes only one serum dilution (qualitative result) and requires 3 hours of incubation. This makes the test very suitable for the screening of large populations.

The increasing importance of the disease in man, and its high rates of lethality in the Belo Horizonte Metropolitan Region (BHMR) mean that a rapid and relatively simple method is needed for the routine diagnosis of VL. The objective of this study was to standardize DAT and FAST as potential VL diagnostic methods using clinical samples from this region in Brazil.

MATERIALS AND METHODS

Serum samples

This study was carried out utilizing over 200 clinical samples (serum and blood) of different patients groups. The serum samples examined were part of the serum collection at the Leishmaniasis Laboratory of the Centro de Pesquisas Rene Rachou - Fiocruz. All sera were sent to the laboratory under the existing health care system of Metropolitan Region of Belo Horizonte (MRBH), Minas Gerais Brazil and stored at -20 ºC. The following three groups of samples were included in the present study: i) serum and blood samples from patients with active VL microscopically proved (n=16); ii) serum samples from patients clinically suspected of VL, all patients are living in endemic area (n=99); iii) serum samples from patients clinically suspected of cutaneous leishmaniasis (n=85).

Detection of anti-*Leishmania* antibodies

The presence of anti-*Leishmania* antibodies in the samples was determined by three different tests: IFAT, FAST and DAT. The IFAT using a commercial kit for the diagnosis of human leishmaniasis (FIOCRUZ/BIO-MANGUINHOS) was performed according to the instructions of the manufacturer for detection of antibodies in serum diluted from 1:40 up to 1:640. The antigens for FAST and DAT were prepared as described earlier by Schoone et al. (2001) and Meredith et al. (1995), respectively.

For the performance of the FAST we used the protocol described previously (Schoone et al. 2001). Serum and blood samples were diluted 1:100 in physiological saline (NaCl 0.85%) to which 0.78% β-mercaptoethanol was added. Twenty µl of this 1:100 dilution was transferred to another well and 20 µl FAST antigen (2 x 10^8 promastigotes/ml) was added. The plate was carefully shaken, covered with a rid and allowed to incubate for 3 hours at room temperature after which the results were read. Appropriate positive and negative controls were always included in each plate.

The DAT was performed basically as described by Harith et al. (1988) and Meredith et al. (1995). In brief, the samples were diluted in physiological saline (0.9% NaCl) containing 0.78% β-mercaptoethanol. Two-fold dilution series of the sera were made, starting at a dilution

of 1:100 (step 1) and going up to a maximum serum dilution of 1:102,400 (step 11). Well 12 was used as a negative control. Fifty µl of DAT antigen (concentration of 5 x 10^7 parasites/ml) was added to each well containing 50 µl of diluted serum and the results were read after 18 hours of incubation.

RESULTS

Serum and blood samples from patients with active microscopically proven VL.

The blood and serum samples could be clearly read in FAST using a 1:100 dilution. All 16 blood and serum samples (100%) of active proven VL patients were positive by this test. Also in DAT using a cut-off titer of 1:800 we found 16/16 (100%) of these blood and serum samples positive (Table 1). IFAT detectd 15 out of 16 samples positive (see table 2). The blood as well as the serum sample of the patient that was missed by IFAT had DAT titer of 1: 12,800.

Serum samples from patients clinically suspected of VL.

The results obtained from IFAT and FAST are presented in Table 3. It was observed that 77/99 patients were FAST positive and 84/99 patients were IFAT positive with titers varying from 1:40 to 1:640. In addition, we found that 77/99 (77.8%) of the patients from this group were also DAT positive using a cut-off titer of 1:800 (Table 1).

Table 1. Comparison of direct agglutination test (DAT) for anti-*Leishmania* antibodies in various patient groups

Patient group	Serum dilution										
	200	400	800	1,600	3,200	6,400	12,800	25,600	51,200	10,2400	Total (n)
Group 1 (VL) serum DAT	-	-	-	-	1	-	4	1	3	7	16
Group 1 (VL) blood DAT	-	-	-	-	-	3	3	3	2	5	16
Group 2 (VL) serum DAT	20	1	1	-	3	9	8	15	5	37	99
Group2 (VL) Blood DAT	79	3	2	-	1	-	-	-	-	-	85

Table 2. Comparison of indirect immunofluorescence test (IFAT) for anti-*Leishmania* antibodies in various patient groups

	Serum dilution						
Patient group	NR	1:40	1:80	1:160	1:320	1:640	Total (n)
Group 1 (VL) Serum IFAT	1	1	6	0	6	2	16
Group 2 (VL) Serum IFAT	15	7	20	20	23	14	99
Group 3 (VL) Serum IFAT	37	3	23	12	9	1	85

Serum samples of patients clinically suspected of cutaneous leishmaniasis.

Only 3/85 patients of this group were found positive in FAST. Furthermore, 6/85 patients showed some agglutination in DAT, but titers were usually low (Table 1). Using a cut-off titer of 1:800, only 1 CL patient could be considered to have a positive DAT titer. In contrast, 38/85 suspected cutaneous leishmaniasis patients were found positive by IFAT, but 26/38 samples had low titers (<1:160) (Table 2).

DISCUSSION

In view of the public health importance of visceral leishmaniasis and the inherent difficulties of conventional diagnosis techniques, we evaluated the DAT and FAST developed by KIT Biomedical Research in the present study (Harrith et al. 1988; Meredith et al. 1995; Schoone et al. 2001). These techniques are easy to interpret and rapid as well as being specific and sensitive (Oskam et al. 1999; Schoone et al, 2001). DAT is more practical under field or rural conditions because no specialized equipment is necessary nor a cold chain is necessary for the storage of antigen and the test is easy to perform (Zijlstra et al.1997). In addition, the FAST requires only one serum dilution and the results can be read within 3 hours. The FAST can be used to screen large populations, for example in situations such as epidemics where large number of suspects are seen at the clinic or cases where immediate treatment is necessary (Schoone et al.

2001).

The antigen on which DAT and FAST are based is a strain of *L. donovani*, whereas human visceral leishmaniasis in Brazil is caused by *Leishmania chagasi*, both species belonging to the *L. donovani* complex. Apparently, the use of a heterologous antigen did not affect the performance of both tests for the detection of anti-*Leishmania* antibodies in Brazilian VL patients. The DAT and FAST tests found all proven VL patients positive, whereas the IFAT missed one patient. In addition, both DAT and FAST found the same suspected VL patients positive (77/99). The IFAT found slightly more patients positive (84/99). It remains to be established whether these are the false positive reactions as there has been now further follow up of the suspected patients.

In contrast, the FAST and DAT only detected a very limited number of positive cases in serum samples of patients clinically suspected of cutaneous leishmaniasis. This is not surprising because these patients probably only mount limited antibody responses. However, IFAT found approximately 50% of these putative patients positive, but their titers were in general very low. To our opinion, the DAT and FAST are suitable tools in the diagnosis of visceral leishmaniasis only and should not be used for the diagnosis of the cutaneous form of the disease.

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