EVALUATION OF DIRECT AGGLUTINATION TEST (DAT) AS AN IMMUNODIAGNOSTIC TOOL FOR DIAGNOSIS OF VISCERAL LEISHMANIASIS IN NEPAL

Joshi AB[1], Singhasivanon P[2], Khusmith S[2], Fungladda W[2] and Nandy A[3]

[1]Department of Community Medicine and Family Health, Institute of Medicine, Tribhuvan University, Nepal; [2]Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; [3]Calcutta School of Tropical Medicine, Calcutta, India

Abstract. Before field application of the direct agglutination test (DAT) for leishmaniasis, it was assessed as a diagnostic tool. Fifteen confirmed visceral leishmaniasis cases (bone marrow aspiration positive), 120 tuberculosis, 58 leprosy, 15 malaria, 26 intestinal parasitic infection cases, 24 endemic healthy controls from adjacent to the study area, and 18 controls from Kathmandu (who had never visited the VL endemic areas) were tested for anti-leishmanial antibody agglutination titers. Two of the tuberculosis cases were positive for anti-leishmanial agglutinating antibodies at 1:800. All the visceral leishmaniasis confirmed cases were reactive to anti-leishmanial antibody at ≥1:3,200. Other specimens were negative for serology. The sensitivity of the direct agglutination test was 100% and the specificity was 99.2%. The direct agglutination test had positive and negative predictive values of 100% and 99.2% respectively. The direct agglutination test has been found to be simple, rapid, reliable, economic, safe and adaptable to micro-techniques using micro-titer plates. It is specific and sensitive. The direct agglutination test is simple enough for it to be performed in a field laboratory.

INTRODUCTION

The lack of efficient surveillance tools is considered to be a major factor responsible for early detection of epidemics. Anti-leishmanial antibodies have been demonstrated in clinical cases of visceral leishmaniasis (VL) by a variety of immunodiagnostic tests (Bray and Lainson, 1965) but these tests may give false positive results with cases of typhoid, malaria and tuberculosis (Allain and Kagan, 1975; Harith, *et al*, 1986, 1987). At present, in India and Nepal, laboratory diagnosis of kala-azar is dependent upon demonstration of amastigotes of *Leishmania donovani* in bone marrow or splenic aspirate materials and positive Napier's aldehyde test (Addy *et al*, 1989). The serodiagnosis of VL for the detection of anti-leishmanial antibody has been modified by Harith *et al*, (1987). The modified DAT was described as being simple, economic and sensitive (Addy *et al*, 1989; Pal *et al*, 1991; Singla *et al*, 1993; Harith *et al*, 1987; Abdel-Hameed *et al*, 1989). All the previous studies in human visceral leishmaniasis, demonstrate that the DAT is highly suitable for wide-scale epidemiological field work (Addy *et al*, 1989). In several reports on visceral leishmaniasis, a DAT titer of 1:3,200 is used as a diagnostic titer (Zijlstra *et al*, 1991; Hailu, 1990; El-Masum *et al*, 1995; Harith *et al*, 1987). It was also useful in demonstrating the transformation of sub-clinical to clinical kala-azar in West Bengal, India (Addy and Nandy, 1995). Better diagnostic tools have been made available in the recent past, pending, however, their large scale field evaluation. Therefore, this study was designed to assess direct agglutination test (DAT) as a diagnostic tool, before its field application.

METERIALS AND METHODS

Antigen preparation

The antigen was prepared at Calcutta School of Tropical Medicine with the collaboration of Professor A Nandy and Dr M Addy, using the protocol described elsewhere (Addy *et al*, 1989). Briefly, promastigotes of *Leishmania donovani* isolated in NNN medium from cases of kala-azar were adapted to grow on the modified monophasic medium of Ray (1932) containing peptone (2%), dextrose (1%), sodium chloride (0.5%) and bacto agar (2%) in beef heart extract. The final medium was prepared by adding 8% rabbit blood together with penicillin 100 IU/ml and streptomycin 100 µg/ml, followed by preparation of blood agar slants.

Promastigotes of *Leishmania donovani* were harvested after 48-72 hours of growth on modified Ray's medium or brain heart infusion agar supple-

Table 1
Results of direct agglutination test for VL.

Clinical status	No. of examination	Reactive to DAT at dilution 1:800
VL (confirmed)	15	15
Pulmonary tuberculosis	120	2 (-ve in higher dilution)
Leprosy	58	0
Malaria	15	0
Intestinal parasites	26	0
Endemic healthy controls	24	0
Non endemic healthy controls	18	0
Total	276	17

mented with rabbit's blood. Harvested parasites were washed 3-4 times with Locke's solution at 4°C and trypsinized at 37°C for 45 minutes with 0.4% trypsin (Difco, 1:250) in Locke's solution (pH 7.7). After trypsinization, the parasites were washed at 4°C, 4-5 times with cold Locke's solution. Washed parasites were then formalinized with 2% formaldehyde in Locke's solution for 20 hours at 4°C. The parasites were then washed thrice with saline citrate in cold and subjected to staining with 0.02% Coomassie Brilliant Blue in saline citrate for 90 minutes in cold with a magnetic stirrer. The stained parasites were then washed and passed through nylon gauze to get rid of clumps and resuspended in 1% formaldehyde in saline citrate. The final antigen was prepared by bringing the parasite count to 5-7 x 10^7 per ml and preserved at 4°C until use.

Standardization of DAT

Before field application, the diagnostic efficacy and sensitivity as well as the specificity of the DAT were re-evaluated. Blood samples were collected on filter papers from parasitologically confirmed VL cases, patients suffering from tuberculosis, leprosy, malaria, intestinal parasites and healthy endemic (selected from family members of the clinically and laboratory confirmed cases) and non-endemic individuals (from permanent residents of Kathmandu who have never visited the endemic areas for kala-azar in the past) and were subjected to DAT to screen for the presence of anti-leishmanial agglutinating antibody.

The direct agglutination test was performed in commercially available v-shaped microtiter plates (Greiner, West Germany) with a twofold dilution of blood samples from 1:100 to 1:6,400. The diluent used was saline citrate containing 0.2% gelatin and 0.78% 2-mercaptoethanol. Blood samples were diluted over the microtiter plates over horizontal rows of wells, each well having 50µl of the diluted sample. However, the first well of each horizontal row was charged with 50µl diluent only, to serve as control. Following addition of 50µl of antigen to each well including the control wells, the plates were incubated at room temperature over night and examined for development of agglutination (Addy *et al*, 1989). For each sample, the last well showing definite agglutination and bigger than the bottom of the control well (first well of each row) was considered the end point.

RESULTS

The direct agglutination test (DAT) was assessed as a diagnostic tool. A total number of 276 subjects were examined for anti-leishmanial antibodies. Fifteen confirmed VL cases (bone marrow aspiration positive), 120 tuberculosis, 58 leprosy, 15 malaria, 26 intestinal parasitic infection cases, 24 endemic healthy controls from adjacent to the study area, and 18 non-endemic controls from Kathmandu (who had never visited the VL endemic areas) were tested for anti-leishmanial antibody agglutination titer. All the VL confirmed cases were reactive to anti-leishmanial antibodies at 1:3,200 or more. For other diseases, none of the sera had positive agglutination titers higher than 1:400 except two cases of tuberculosis. One of the tuberculosis cases had a past history of VL and another had anti-leishmanial agglutinating antibodies at the cut-off value of 1:800 (Table 1). The sensitivity of the direct agglutination test was 100% and the specificity was 99.2%. The direct agglutination test had positive and negative predictive value of 100% and 99.2% respectively.

DISCUSSION

The direct agglutination test (DAT) was assessed as a diagnostic tool for the diagnosis of visceral leishmaniasis in Nepal. All the VL confirmed cases were reactive for anti-leishmanial antibodies at 1:3,200 and higher. For other diseases, none of the sera had positive agglutination titer higher than 1:400 except in two cases of tuberculosis. One of the tuberculosis cases had a past history of VL and another had anti-leishmanial agglutinating antibodies at the cut-off value of 1:800. From this subject, the blood sample was redrawn and past history of the patient was sought. The same results were appeared. The sensitivity of the direct agglutination test was 100% and the specificity was 99.2%. The direct agglutination test had positive and negative predictive value of 100% and 99.2% respectively. In several reports on visceral leishmaniasis, a DAT titer of 1:3,200 is used as a diagnostic limit, since it gives highest specificity and sensitivity (Harith *et al*, 1988; Zijlstra *et al*, 1991; Hailu, 1990; El-Masum *et al*, 1995; Harith *et al*, 1987). The direct agglutination test has been observed to be very useful in the diagnosis of sub-clinical kala-azar under field conditions. It was also useful in demonstrating the transformation of sub-clinical to clinical kala-azar in West Bengal, India (Addy and Nandy, 1995). The direct agglutination test has been found to be simple, rapid, reliable, economical, safe and adaptable to micro-techniques using micro-titer plates. It is specific (with few false positive) and sensitive (with few false negatives). The field application of the direct agglutination test is simple enough for it to be performed in a field laboratory. This study suggests to use a DAT titer of 1:800 for the screening and detection of sub-clinical cases and titer of ≥1:3,200 for confirmatory diagnosis.

ACKNOWLEDGEMENTS

This study was supported by UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases.

REFERENCES

Abdel-Hameed AA, Harith AE, Abdel-Rahim IM. Potential of a direct agglutination test (DAT) for detection of visceral leishmaniasis in a known endemic area in Sudan. *Trop Med Parasitol* 1989; 40: 470-1.

Addy M, Som DK, Das RC, *et al.* Evaluation of direct agglutination test (DAT) in the diagnosis and screening of kala-azar. *Indian Med Gazette* 1989; CXXIII: 184-7.

Addy M, Nandy A. Evaluation of direct agglutination test in the diagnosis of sub-clinical kala-azar in lymphatic leishmaniasis. *J Protozool Res* 1995; 5: 1-9.

Allain DS, Kagan IG. A direct agglutination test for leishmaniasis. *Am J Trop Med Hyg* 1975; 24: 232-6.

Bray RS, Lainson R. The immunology and serology of leishmaniasis. 1. The fluorescent antibody staining technique. *Trans R Soc Trop Med Hyg* 1965; 59: 535-44.

El-Masum MA, Evans DA, Minter DM, El Harith A. Visceral leishmaniasis in Bangladesh: the value of DAT as a diagnostic tool. *Trans R Soc Trop Med Hyg* 1995; 89: 185-6.

Hailu A. Pre-and post treatment antibody levels in visceral leishmaniasis. *Trans R Soc Trop Med Hyg* 1990; 84: 673-5.

Harith AE, Kolk AHJ, Kager PA, *et al.* A simple and economical direct agglutination test for serodiagnosis and sero-epidemiological studies of visceral leishmaniasis. *Trans R Soc Trop Med Hyg* 1986; 80: 583-6.

Harith AE, Kolk AHJ, Kager PA, *et al.* Evaluation of newly developed direct agglutination test (DAT) for sero-diagnosis and sero-epidemiological studies of visceral leishmaniasis: comparison with IFAT and ELISA. *Trans R Soc Trop Med Hyg* 1987; 81: 603-6.

Pal A, Mukerji K, Basu D, *et al.* Evaluation of direct agglutination test (DAT) and ELISA for sero-diagnosis of visceral leishmaniasis in India. *J Clin Lab Anal* 1991; 5: 303-6.

Ray JC. Cultivation of various *Leishmania* parasites on solid medium. *Indian J Med Res* 1932; 20: 355-7.

Singla N, Singh GS, Sunder, Vinayak VK. Evaluation of direct agglutination test as an immunodiagnostic tool for kala-azar in India. *Trans R Soc Trop Med Hyg* 1993, 87: 276-8.

Zijlstra EE, Siddig AM, El Hassan AM, *et al.* Direct agglutination test for diagnosis and sero-epidemiological survey for kala-azar in the Sudan. *Trans R Soc Trop Med Hyg* 1991; 85: 474-6.