J. Protozool. Res., 5, 1-9 (1995)

Evaluation of Direct Agglutination Test in the Diagnosis of Sub-clinical Kala-Azar and Lymphatic Leishmaniasis

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Received 1 February 1993 / Accepted January 30 1995

Key words: DAT, lymphatic leishmaniasis, sub-clinical kala-azar

ABSTRACT

Efficacy of direct agglutination test (DAT) in diagnosing sub-clinical visceral and lymphatic leishmaniasis was assessed in the field, with an aim to incorporate the test as an early diagnostic tool in the kala-azar control programme of the State, using trypsinized whole promastigotes of *Leishmania donovani* as antigen and filter paper collected finger prick blood, as clinical sample. Bone marrow and lymph node biopsies were examined to demonstrate parasites. Frank kala-azar and persons with initial high antibody level (>3,200) were excluded from this report. Following initial DAT screening of 2,150 individuals (which included persons with fever of any duration and those with superficial lymph node enlargement with or without fever), 150 persons with low antibody level (DAT titre 400 and 800) were retested after 6 weeks. Out of these 150 individuals, 100 were diagnosed as lymphatic leishmaniasis either by demonstrating parasites in lymph nodes or by a rise in antibody level; thirty cases as kala-azar progressing from a sub-clinical stage, because of a 2-4 fold rise in antibody level and/or demonstrating parasites in bone marrow. In the remaining 20, the antibody level declined, no parasites could be demonstrated in bone marrow and thus were considered of non-leishmanial origin. It was therefore concluded that the DAT was highly effective in identifying lymphatic leishmaniasis and could be very useful in selecting out infected individuals at their subclinical state for future confirmation of the transformation to clinical state using the same tool without the need for bone marrow biopsies.

INTRODUCTION

Kala-azar, the severest clinical expression of leishmanial infection in India, is considered to constitute a minor fraction of the total problem of *Leishmania donovani* infection. Asymptomatic infection with *L. donovani* and sub-clinical

forms of the disease in some parts of the world might be very frequent, and in countries like Brazil, Italy and Kenya are thought to outnumber clinical cases by about 5:1 (WHO 1990). These forms may progress to develop classical kala-azar or may resolve spontaneously without specific treatment. Because of scanty parasites in tissue, the conventional parasitological methods usually fail to provide any result. The importance of such forms can never be over-emphasized in view of unidentified maintenance of parasites. No kala-azar control programme seems to succeed without identifying and eradicating such sources of parasite. Under this situation, the use of antibody detection based serological methods, as a tool for population screening and surveillance is highly recommended, because of their ability to identify the infection early in the disease process. Badaro et al. (1986) using ELISA in a prospective study of 86 children from an endemic area in Brazil, demonstrated a precise infection rate, and depending upon the ELISA positivity, the children were classified into four groups including a sub-clinical kala-azar with self healing and another progressing to clinical kala-azar. While parasites were always demonstrated in the clinical kala-azar in bone marrow in their study, they were positive only in one fourth cases in the sub-clinical variety. In our own earlier laboratory based study in a limited number of cases, it was observed that DAT could effectively identify visceral leishmaniasis where parasites could not be demonstrated in the bone marrow aspirate (Addy et al., 1989). It was then planned to undertake a field study in a kala-azar affected population to assess DAT's efficacy in identifying sub-clinical and lymphatic leishmaniasis (without comparing it with any other serological methods) with a final aim of replacing the invasive methods like bone marrow and/or lymph node biopsies. We present here the results of a population screening using DAT to identify lymphatic leishmaniasis and also the results of subsequent follow-up of sub-clinical kala-azar to demonstrate the transformation, if any, to clinical kala-azar.

MATERIALS AND METHODS

The present study was undertaken in the kala-azar affected districts of 24-Parganas South and 24-Parganas North, of West Bengal province of India. A detailed house to house survey in the affected areas included both clinical examination as well as questionnaire utilization.

The study subjects included individuals with fever of any duration and those with superficial lymphadenopathy with or without fever. Individuals having hepatosplenomegaly with or without fever and those diagnosed as frank kala-azar although studied were excluded from this report.

Finger prick blood samples of 2,150 individuals were collected, in triplicate, over printed circles of 12.7 mm diameter on filter paper strips (Whatman filter paper No. 3). Blood soaked filter paper strips were air dried, sealed, transported, and stored at -30° C until use, in the laboratory.

Blood samples collected from another 1,500 individuals from non-endemic

villages of the district of Midnapore and Bankura of the same State served as non-endemic controls.

All the 3,650 blood samples were subjected to DAT, and individuals with initial antibody titre of 1:400 and 1:800 were re-examined after 6 weeks, clinically, parasitologically as well as by DAT. Patients with superficial lymph node enlargement and initial anti-leishmanial antibody level of >1:1,600 were subjected to lymph node biopsy to demonstrate parasite and were excluded from this report.

Parasitological confirmation was done by demonstrating amastigotes in bone marrow and/or lymph node biopsy smears and promastigotes in NNN culture medium.

Preparation of antigen for DAT: The antigen was prepared according to the earlier methods of Harith et al. (1988) and Addy et al. (1989) with minor modifications. Promastigotes of *L. donovani* were harvested after 48-72 hrs of growth on modified Ray's medium (Nandy et al. 1987). Harvested parasites were washed 3-4 times in Locke solution (pH 7.7) at 4° C and trypsinised at 37° C for 45 min with 0.4% trypsin (Sigma Cat. No. T 8128) in Locke solution. The parasites were then washed at 4° C, 4-5 times with cold Locke solution and fixed for 20 hrs at 4° C in 2% (wt/vol) formaldehyde in Locke solution. The parasites were then washed in 0.15 M NaCl and 0.056 M sodium citrate solution (pH 7.4) thrice and were stained with 0.1% Coommassie Brilliant Blue (Sigma Cat. No. B 0149) in citrate-saline solution and resuspended in 0.43% (wt/vol) formaldehyde-citrate-saline solution. The suspension was then filtered through a nylon mesh and the parasite count was adjusted at $6.5x10^7$ to $7.5x10^7$ per ml. This parasite suspension was preserved at 4° C, in formalinised citrate-saline solution till use.

Elution of blood from filter paper strips: Filter paper blood samples of each patient was extracted from a single corresponding blood spot, overnight at 4° C in 1 ml, 0.15 M saline to provide a dilution of 1:30.

Direct agglutination test: Each eluted sample was diluted further to 1:200 in tubes and then over microtitre "V" plates (Greiner, West Germany) doubly till 12,800. The diluent used was 0.2% gelatin (Difco) in citrate-saline solution with 0.78% 2-Mercaptoethanol. The first well of each transverse row was used as antigen control without blood sample. Equal volume (50 µl) of antigen suspension was added to each well including the controls, mixed by gentle shaking over a table top and incubated at 22° C overnight. The last well showing definite agglutination of the parasite was considered as the end point.

RESULTS

As shown in Table 1, the clinical and questionnaire screening of the population resulted in the identification of 2,150 study subjects, which could be divided into two groups depending upon their clinical presentation. Group-I consisted of 550 individuals (25.58%) with superficial lymph node enlargement including the epitrochlear one, with or without fever. The other 1,600

Table1. Results of initial DAT screening of 3650 individuals.

Group (Total No.)	Number of individuals positive (end point titre) at each dilutions						
	200	400	800	1,600	3,200	6,400	12,800
Group-I Persons with LNE, with or without fever (550)	135	25	90 (115)	0	24*	9* (35)	2*
Group-II Persons with fever and no LNE (1,600)	196	17	18 (35)	0	0	0	0
Non-endemic controls (1,500)	15	2	0	0	0	0	0
Total (3,650)	346	44	108 (152)		24 *	9* (35)	2*

LNE: Lymph node enlargement; * Excluded from the follow-up study

persons (74.41%) had fever of varying duration without appreciable lymph-adenopathy, particularly of the epitrochlear region (Group-II).

Of the 2,150 endemic samples tested (Table 1), 185 individuals were serologically reactive in initial DAT screening (8.60%), and none of them had hepatosplenomegaly. In the lymphadenopathic group (Group-I), 35 had initial high antibody level (24 positive up to the dilution of 1:3,200, 9 up to 6,400 and 2 up to 12,800) and therefore were subjected to lymph node biopsy smear and culture, where 29 showed L. D. bodies in smear and in remaining 6 cases culture in NNN-medium demonstrated growth of promastigotes after 3 weeks incubation. All these 35 patients were subjected to chemotherapy without waiting for the culture report.

The rest 150 individuals, because of low antibody level (115 in Group-I and 35 in the Group-II) were advised symptomatic treatment and were clinically, parasitologically and serologically re-examined after 6 weeks from the time of initial blood collection. It may be observed that out of 1,500 non-endemic control samples only 2 showed agglutination at a dilution of 1:400 and 15 at 1:200. Also it may be noted that 135 and 196 persons from Group-I and Group-II respectively had a DAT reactivity at 1:200.

During the repeat DAT (Table 2), 15 individuals of the total 115 lymph-adenopathic patients, showed either a steady or declining antibody level, of which 10 came down from 400, 4 remained at 400 and 1 came down from 800. In the remaining 100 patients there was a 2-4 fold increase in antibody level of which 11 persons had earlier antibody level at 400 and 89 individual's

Table 2. Results of repeat DAT in 150 persons 6 weeks after the first blood collection.

Group (Total No.)	Number of individuals positive(end point titre) at each dilution						
	200	400	800	1,600	3,200	6,400	12,000
Group-I Lymphadenopathic (115)	10 * +22	5	0	70	30	0	0
Group-II Non-lymphadenopathic, with fever. (35)	4 * +53	1	0	17**	11**	2**	0
Control	3	0	0	0	0	0	0
Total	92	6	0	87	41	2	0

* Number of individuals whose antibody titre declined from 400.

** Individuals who developed hepatosplenomegaly after 6 weeks.

antibody increased from an initial level of 800, indicating that persons with higher initial antibody level are prone to develop a progressive disease as observed by more frequent rise in antibody level than those at the level of 400. All these individuals were subjected to lymph node biopsy for parasitological confirmation. Parasites could be demonstrated either in imprint smear and/or culture in 82 of them. On the basis of parasite positivity and rise in antibody level, however, all these 100 individuals were subjected to chemotherapy as lymphatic leishmaniasis.

Similarly in the non-lymphadenopathic subclinical group (Group-II), out of 35 individuals 30 persons progressed to overt kala-azar at the end of the study period. Of these 30 persons, 17 individuals had initial antibody level at 800 and 13 were DAT positive at 400 during the initial screening. All these 30 individuals developed hepatosplenomegaly along with 2-4 fold rise in the antibody level. Subsequently they were confirmed as clinical kala-azar by demonstrating L. D bodies in bone marrow smears and/or promastigotes in culture. However, 5 persons from this group did not show any progress neither clinically nor in the antibody level.

It was observed that while 57.14% of the persons from endemic area having an initial DAT positivity at a dilution of 1:400 progressed to overt disease, the chances of such progress increased with the higher initial titre (98.14% at 1:800).

Further, it was observed that majority of the persons with DAT reactivity at a dilution of 1:200 during the initial screening became subsequently negative. Thus, out of 135 persons from Group-I and 196 from Group-II initially positive at 1:200, 113 and 143 respectively became negative during

the repeat DAT after 6 weeks. Although, the remaining 22 and 53 persons in Group-I and Group-II respectively were still showing DAT reactivity at the earlier level of 1:200, none of them had any clinical sign of progressing visceral leishmaniasis and therefore were not subjected to invasive parasitological investigations. Similarly, out of 17 non-endemic control individuals showing DAT positivity at 200 and 400 during the initial screening, all but 3 (still positive at 200) became negative. None of these 3 persons were subjected to parasitological investigations.

DISCUSSION

Until recently, kala-azar and post kala-azar dermal leishmaniasis (PKDL) were considered to be the only clinical outcome of *L. donovani* infection in man in India. Identification of a new clinical entity, the lymphatic leishmaniasis in India (Nandy and Chowdhury 1984 1988) and also a sub-clinical form of kala-azar (Nandy, unpublished) provided newer dimension to the under-standing of pathogenic mechanism(s) of the infection in man. This also added a new direction to the formulation of control strategies considering the potential reservoir role these forms might play.

With the idea of implementing field based easy and dependable method for identifying early the infected individuals and with the aim to replace such invasive techniques like bone marrow/lymph node biopsy and culture for the purpose, we evaluated the efficacy of DAT in this regard and tried to justify its possible future incorporation in to the kala-azar control programme.

While Badaro et al. (1986) used ELISA for similar purpose, in the present study DAT was used because of its low cost, easiness to perform and its field applicability. Further, information available from earlier studies including our own, indicated usefulness of DAT in effectively diagnosing parasite negative cases of leishmaniasis. Thus in our earlier study (Addy et al. 1989) including 132 DAT positive kala-azar cases, parasite could be demonstrated in 88 of them in bone marrow smear. Of the remaining 44 cases of hepatosplenomegaly with fever who were DAT positive, 20 were formol gel test negative where the bone marrow culture demonstrated promastigotes after 6 weeks. In 24 cases while both formol gel and DAT became positive bone marrow did not reveal parasite in smear. All these cases, however, responded to antimony therapy successfully. A similar observation was also made by Chowdhury et al. (1991) in Bangladesh where using DAT they could identify 1,273 seropositive symptomatic cases of visceral leishmaniasis, out of which 715 were parasitologically confirmed. The rest 558 patients were subjected to antimony treatment successfully on the basis of DAT positivity. Depending upon the therapeutic response of these seropositive and parasite negative cases sensitivity and specificity of DAT positivity. Depending upon the therapeutic response of these seropositive and parasite negative cases sensitivity and specificity of DAT were calculated to be 99.6% and 97.7% respectively. The result supported the reliability of DAT in the diagnosis of kala-azar at levels

below that of parasite positivity.

In the present study in case of lymphatic leishmaniasis, high degree of parasitological correlation was observed with the antibody titre beyond a particular level which supported the view of replacing lymph node biopsy with DAT at a predetermined diagnostic titre. However, it may be noted from the observations that the diagnosis in the clinically suspected individuals even with low antibody level at the screening test, could be confirmed on re-examination after 6 weeks by demonstrating rising antibody titre which also correlated well with the parasitological examination. This was particularly true with individuals having an initial DAT positivity at 1:400 and 1:800. Similar correlation between DAT with parasite positivity was also observed in the cases who migrated from sub-clinical state to overt kala-azar. Therefore it may be concluded that the DAT titres of 400 and 800 were very valuable in selecting out infected individuals at their sub-clinical state for future confirmation of the transformation to clinical state.

It may thus be considered that DAT was quite satisfactory in identifying persons at an early stage of the disease process depending upon which such persons were advised re-examination after 6 weeks for final confirmation by demonstrating rising antibody level using the same tool and thus avoiding the use of technically demanding bone marrow aspiration or lymph node biopsy for the purpose. This was of particular importance in view of the field applicability of DAT which has been for better acceptable to the patients than the biopsy.

Badaro et al. (1986), using ELISA to study the migration of sub-clinical to clinical kala-azar. Of these, a group of 20 children remained asymptomatic and 15 developed classical kala-azar after a few weeks. Out of the remaining 51 at a sub-clinical stage, 13 (25%) progressed to overt kala-azar after a mean period of 5 months and 38 (75%) of them could resolve their illness spontaneously without any specific treatment after a mean duration of 35 months. All the transformations were confirmed either by demonstration of parasite in the bone marrow aspirates or rise in the anti-leishmanial antibody level. The result of the present study also indicated existence of a similar situation in West Bengal like that identified in Bangladesh and Brazil.

Information available so far indicates higher or similar degree of sensitivity and specificity of DAT over ELISA. Thus in a study of Sudanese patients of visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL) it was observed the while DAT and ELISA were equally sensitive in the diagnosis of VL, the former technique was found to be more sensitive than ELISA (67% over 60%) in cases of CL (EL Safi and Evans 1989). These authors preferentially suggested use of DAT in the diagnosis of kala-azar, because of its low cost and simplicity. In our own further study of 112 patients (Ghose et al. 1994), positivity of DAT and ELISA was observed to be 97% over 90% respectively.

It was further observed that DAT could efficiently discriminate lymphadenopathy of leishmanial origin. In the present study in contrast to the

parasitological confirmation of individuals with initial high antibody level, omission of such confirmation in individuals with low antibody level during initial DAT, was deliberate to simulate the future situation when DAT might remain the only method for mass surveillance under the disease control programme. In the present study, for persons with persistent DAT positivity at 1:200 after six weeks follow up (Table 2) without any clinical manifestations it would be difficult to explain whether the low level reactivity in DAT with subsequent decline or persistence at the initial level of the antibodies, in some individuals, as an anamnestic reaction, or evidence of spontaneous cure of *L. donovani* infection, as documented in other areas of the world (WHO 1990; Badaro et al. 1986).

As indicated earlier, in countries like Brazil, Kenya, and Italy, the ratio of sub-clinical to clinical cases of leishmaniasis was observed to be 5:1 (WHO 1990). However, attempt to calculate such figures from the result of the present study is not only beyond the scope of this report but also would be artificial, considering the introduction of an element of bias in to the selection criteria of the study subjects.

It was interesting to note that in a non-endemic population the DAT reactivity was extremely lower and was of no clinical or diagnostic significance. In contrast, in endemic areas degree of DAT reactivity was directly related to the severity and progress of the disease.

With these background information it might be well justified to conclude that DAT was highly useful in the field diagnosis of lymphatic leishmaniasis without the need for lymph node biopsy and also could be used as a tool for screening individuals having early infection to suggest re-examination at an appropriate later date for confirmation of the diagnosis avoiding the unacceptably invasive techniques.

ACKNOWLEDGEMENTS

The authors wish to put on record, their deep sense of gratitude to the Department of Science and Technology, Government of West Bengal, India, for financial assistance, to the Department of Health and Family Welfare, Government of West Bengal, India, for infrastructural support in the field and to the Director, Calcutta School of Tropical Medicine, Calcutta, India, for permission to publish this report.

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