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*Brief Communication*

# SUPERIORITY OF DAT OVER ELISA AS A DIAGNOSTIC AND SEROEPIDEMIOLOGICAL TOOL FOR THE DIAGNOSIS OF INDIAN KALA-AZAR

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## Abstract

The aim of this study was to evaluate two methods for the diagnosis of Kala-azar. The sera of 160 individuals were evaluated by ELISA using soluble antigen and direct agglutination test (DAT) for Kala-azar. These were categorized as 100 cases of clinically and parasitologically confirmed Kala-azar and 60 controls. The controls included clinically suspected but parasitologically not confirmed Kala-azar patients (10), endemic normals (15), non-endemic normals (19), typhoid fever (10) and malaria (15). The positivity rate amongst the clinically and parasitologically confirmed Kala-azar patients by ELISA and DAT were 93% and 98% respectively. Out of 10 clinically suspected Kala-azar cases three showed positive reaction in ELISA and two in DAT. Of the endemic normals, one case was found positive by both the tests whereas ELISA was found positive in one additional case. DAT did not show any cross reactivity with malaria while ELISA was found positive in one case. Both endemic normals and typhoid fever cases showed no reaction by both tests. ELISA showed a sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of 93%, 90%, 93% and 90% respectively while for DAT these values were 98%, 95%, 98 and 95% respectively. The diagnostic accuracy for ELISA and DAT was found to be 91.9% and 96.9%, respectively. The present study shows that DAT is a simple, sensitive, specific and cost effective test with high PPV and NPV along with approximately 97% diagnostic accuracy and is comparable to ELISA. It may be applied for the routine diagnosis as well as seroepidemiological study of Kala-azar.

**Key words:** *Serodiagnosis, Kala-azar, DAT, ELISA*

Since its resurgence, the increasing incidence of Kala-azar remains unabated despite aggressive treatment and control measures. It has taken an endemic form in Bihar. For diagnostic purposes, the demonstration of the parasite in tissue fluids like splenic aspirate/bone marrow aspirates, though considered to be the gold standard, are cumbersome, invasive, painful and difficult procedures associated with complication like haemorrhage and rupture. In addition to the microscopic examination, there are some non-specific tests like Napier's aldehyde test, Sia water test and antimony test. Some of them are widely used in hospitals. A variety of serological tests have been tried and evaluated with the aim of replacing the conventional parasitological techniques.[1,2] Most of these serological methods are equivocal in their sensitivity and specificity. More recently, detection of antibody using rK39 antigen is being used in some centers in India.

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This study was designed to evaluate direct agglutination test (DAT) and compare its results with ELISA using in-house prepared soluble antigen (SA-ELISA) in terms of sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and diagnostic accuracy (DA), in order to assess its applicability and its value as diagnostic and a seroepidemiological tool.

### Materials and Methods

*Patients*

Serum was collected from a total of 160 individuals. They were categorized as 100 Kala-azar cases and 60 controls. The confirmed cases of Kala-azar had persistent fever, splenomegaly, anaemia and weight loss. In addition, microscopic examination of the stained smear of spleen/bone marrow aspirates revealed the presence of *Leishmania donovanii* (LD) bodies and all of them were positive for Napier's aldehyde test.

The controls consisted of clinically suspected Kala-azar patients (10), endemic normal (15), non- endemic normal (10), typhoid (10) and malaria (15). Out of 10 clinically suspected Kala-azar patients belonging to same endemic area, four were positive for aldehyde test but

none of them had demonstrable LD bodies in the stained smear of bone marrow/splenic aspirate.

The endemic normals were the first degree relatives of the parasitologically confirmed Kala-azar patients residing in the same endemic area. They neither showed any clinical symptom nor received any anti Kala-azar treatment. Ten apparently healthy students admitted to this University from Europe were designated as non-endemic normals. They were never exposed to the parasite and were bled at first encounter. Twenty-five febrile cases were diagnosed as typhoid (10) and malaria (15). The typhoid cases had *Salmonella typhi* isolated from the blood cultures. On subsequent serological investigations they were found to be positive for Widal test. Clinically diagnosed malaria cases had *P.vivax* in their peripheral smear. Aldehyde test was negative in all the endemic normals, non- endemic normals and febrile cases. In all the febrile cases co-infection with Kala-azar was ruled out on careful scrutiny.

*Serological tests*

ELISA and DAT were done on coded sera obtained from cases and controls. After obtaining the results these were de-coded. Personnel performing the tests were masked to the category of the serum samples.

**Enzyme Linked Immuno Sorbent Assay (ELISA)**

The ELISA test was performed using the standard procedure described earlier with modifications.[1,2] The antigen was prepared in-house from the promastigotes of *L.donovanii* (MHOM/IN/80/Dd8) grown in monophasic medium.[3] The protein content of the antigen was estimated by Lowry's method.[4] The standardization of optimum concentration of antigen, was done by chequer board titration method. For coating the wells, 0.3 µg of *L.donovani* antigen, diluted in antigen coating buffer (carbonate–bicarbonate buffer, pH 9.6) was used and the plates were incubated at 4°C overnight. On the following day the wells were washed thrice with PBS (pH 7.2) containing 0.05% (V/V) Tween 20 (PBS-Tween 20) and blocked with 100 µL of 0.5 % (w/v) BSA in PBS and incubated for 2 hours at room temperature. Sera from different categories of cases and control were initially diluted to 1:100 with 0.5% (W/V) BSA in PBS. The sera were serially diluted in two fold and 100 µL diluted sera were added to each well. Plates were incubated at 37°C for one hour. After washing three times with washing solution, 100µL of antihuman gammaglobulin conjugated with HRP (1: 1000) was added to each well and incubated at 37°C for one hour. The plates were thoroughly washed and 100 µL OPD solution (as substrate) was added to each well. The reaction was stopped after 15 minutes with 100 µL of 5N - $H_2SO_4$. The results were read on an automatic ELISA reader using a filter of 492 nm. An optical density above 0.270 (mean ± 3 SD) was taken as positive for the presence of antileishmanial antibodies.

Direct Agglutination Test (DAT)

The antigen was prepared from *Leishmania donovani* (MHOM/IN/80/Dd8) obtained from WHO reference center. The antigen was prepared as per Harith *et al.*[4] Promastigotes were taken from the monophasic medium and washed five times by cold (4°C) Locke's solution and the count was adjusted to 100 x 106 promastigotes/mL of suspension. These washed parasites were trypsinized at 37°C (0.4% trypsin in Locke's solution).

After washing 5 times with Locke's solution, the trypsinised parasites were treated with 2% formaldehyde for fixation and retained at 4°C for 2 hours. Following fixation, the promastigotes were washed twice with cold citrated saline and stained with 0.02% Coomassie brilliant blue for 90 minutes using a magnetic stirrer at a gentle speed by maintaining the promastigotes concentration at 100 x $10^6$ /mL. Stained parasites were washed three times with physiological saline and resuspended at the same parasite concentration in citrate saline with 1% formaldehyde. The morphology of stained promastigotes was checked by microscopic examination. This suspension was passed through a nylon gauze of 30 µm mesh size to remove the clumps of parasites. They were wrapped in aluminium foil and stored at 4°C until used.

Microtitre plates with V-shaped wells were used for the test performance. Serum samples were serially two fold diluted with saline-0.2% gelatin solution (w/v) from 1:100 to 1:6400. Fifty microlitre of parasite suspension was added to an equal volume of diluted serum in each well. After completion of the test, microtitre plates were shaken on a plane surface and were incubated at 22°C overnight, after wrapping it with aluminum foil. The results were recorded in titres. From our past experience, a titre of 800 and above was considered as positive, which was expressed as the highest dilution of serum that showed definite agglutination of parasite. Uniform mat formation suggested a positive result. Hence, end point was estimated by localizing a clear sharp edged spot identical to one observed in the negative control (saline–gelatin solution) wells.

**Statistical Analysis**

Statistical analysis of results of both the tests was done in terms of sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy, as described by Galen and Gambio.[6]

## Results

Of the 100 confirmed Kala-azar cases, 93 (93%) were found to be positive by ELISA, considering the OD cut off value of 0.270(± 3 SD). Two out of 15 close relatives of the patients showed positive reaction where as 3 out of 10 clinically suspected Kala-azar patients were also found positive by the test. One clinically diagnosed malarial fever case also showed positive reaction by ELISA (Table-1). The summarized results for DAT presented in table 1 show that 98 (98%) of confirmed Kala-azar were found positive on the basis of positive agglutination titre of ≥800. Two out of 10 clinically suspected persons also showed positive reactions. One out of 15 close contacts of the patients (endemic normals) was found positive by DAT.

**Table 1 : Antileishmanial antibodies as detected by ELISA and DAT in Kala-azar cases and controls**

| Clinical Status | No. | ELISA Positivity | | DAT Positivity | |
|---|---|---|---|---|---|
| | | Positive No. (%) | Negative No. (%) | Positive No. (%) | Negative No. (%) |
| Confirmed Kala-azar cases | 100 | 93 (93.0) | 7 (7.0) | 98 (98.0) | 2 (2.0) |
| Clinically suspected Kala-azar | 10 | 3 (30.0) | 7 (70.0) | 2 (20.0) | 8 (80.0) |
| Endemic normals | 15 | 2 (13.3) | 13 (86.7) | 1 (6.6) | 14 (93.4) |
| Non-endemic normals | 10 | 0 (0.0) | 10 (100) | 0 (0.0) | 10 (100) |
| Typhoid fever cases | 10 | 0 (0.0) | 10 (100) | 0 (0.0) | 10 (100) |
| Malarial fever cases | 15 | 1 (6.6) | 14 (93.4) | 0 (0.0) | 15 (100) |
| **Total** | **160** | **99** | **61** | **101** | **59** |

*Statistical Analysis of ELISA and DAT*

The comparative statistical analysis of both tests is shown in table 2.

**Table 2 : Comparative evaluation of ELISA and DAT**

| | Sensitivity | Specificity | PPV | NPV | DA |
|---|---|---|---|---|---|
| ELISA | 93% | 90% | 93% | 90% | 91.9% |
| DAT | 98% | 95% | 98% | 95% | 96.9% |

PPV – positive predictive value
NPV – negative predictive value
DA – diagnostic accuracy

## Discussion

Kala-azar or visceral leishmaniasis (VL), a serious health hazard of tropical and subtropical countries, has plagued mankind since antiquity. The main problem to control the VL is to establish a definite diagnosis. Till date the demonstration of the parasite in splenic/bone marrow aspirates is considered to be the gold standard.[7] But these invasive procedures have their own drawbacks. These tests may sometimes be found to be false negative, if the parasite density is low. Hence, there is a need for an alternative approach for diagnosis of VL.

In the present study, ELISA showed a sensitivity of 93% and specificity of 90% by using the sonicated soluble antigen at a cut off OD value of 0.270(± 3 SD). Edrissian and Darabian[8] showed the sensitivity of ELISA to be 92.3% while Sinha *et al*[9] showed the sensitivity of 91% with a specificity of 91.6%. Hommel *et al*,[1] Srivastava[10] and Alam *et al*1[1] showed the sensitivity of ELISA in the range of 97-100%.

In this study, a cut-off titre of 800 was employed for DAT. Our results showed a sensitivity and specificity of 98% and 95% respectively. These results well corroborate with the results of others[12-15] who showed a sensitivity and specificity in the range of 95-100%. Low socioeconomic status of our patients may account for low level of antibodies. Higher cut off titres (1600 or 3200) have been used by others because of the presence of trypanosomiasis in their study areas, which is not the case in our country.[4,16]

In the present study, DAT showed a higher sensitivity, specificity, PPV, NPV and DA in comparison to ELISA. The diagnostic accuracy of DAT was also higher. In addition, DAT did not show any cross reactivity with other diseases like malaria, where as one case of malaria was found positive by ELISA. Srivastava *et al*[10] and Edrissian and Darabian[8] observed similar cross reactivity between VL and malarial fever patients. DAT failed to detect antileishmanial antibodies in two parasitologically confirmed cases of VL while ELISA failed to detect in seven parasitologically confirmed cases. The negative reaction in these cases may be due to prior treatment of the patients. The other reasons could be advanced stage of the disease, which may lead to a state of anergy (Kala-azar is known to produce immune suppression). Hence these patients may be

considered as low responders so far as antibody production is concerned.

The clinically suspected persons who showed positive reactions by DAT and ELISA may indicate low parasitaemia in these patients which could not be detected by parasitological examination of their splenic /bone marrow aspirates. One of the endemic control/ contact by DAT and two by ELISA showed positivity for Kala-azar. This positivity in endemic healthy individuals is explained by constant exposure of individuals to sand fly bite resulting in latent or subclinical infection. Alternatively, the patients might be in the incubation period yet to show sign and symptoms of disease.

From our study, it appears that DAT is a sensitive and specific test with high diagnostic accuracy. It is a cost effective test, as it does not require any sophisticated equipment. It is also a simple test and can be carried out as well as interpreted even by unskilled paramedical staff. Considering the merits of DAT, in our opinion, it is an ideal test for diagnosis and seroepidemiological studies of Kala-azar.

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