STABLE FREEZE DRIED ANTIGEN FROM LEISHMANIA DONOVANI PROMASTIGOTES FOR SERODIAGNOSIS OF KALA-AZAR BY DIRECT AGGLUTINATION TEST (DAT)

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Amongst several serological tests reported for the diagnosis of VL, Direct Agglutination Test (DAT) is promising in meeting these requirements. DAT makes use of aqueous (AQ) antigen prepared from L. donovani Promastigotes whose major drawbacks are limited stability, requirement of uninterrupted cold chain during its storage and transportation and shock sensitiveness. In view of the shortcomings of AQ antigen and to further explore its field utility, particularly in Indian VL, we have independently and indigenously developed stable Freeze Dried (FD) antigen from L. donovani (MHOM/IN/80/Dd8) Promastigotes. Several stabilizers (Sucrose, Trehalose, TritonX, Sodium Dodecyl Sulphate) were tried alone and in different combinations and ratios with AQ antigen before freeze drying and then diagnostic potentials were judged. Of the various stabilizers used, SDS was found to be most promising in stabilizing the aqueous antigen. The FD antigen was found stable at 56°C for 24 months period. Both FD and AQ antigens on evaluation on positive and negative serum samples showed that the end point titres of FD antigen were on the average 0.86-step dilution lower than that of AQ antigen (P>0.01). At a cut off dilution of 1:1600, no difference in quality of agglutination was observed in any case with both the antigens. Thus, the improved stability of FD antigen will facilitate production of large batches of the antigen with reproducible results over a longer period of time.

Visceral Leishmaniasis (VL) is a potentially fatal disease that affects an estimated 500,000 persons per year. Ninety percent of the cases occur in Bangladesh, India, Nepal and Sudan. In India, the disease primarily prevails in Bihar and neighboring eastern states. Treating patients on clinical presumption is inadequate because of the potential severe side effects of currently available drugs. Since communities affected by Kala-azar often lived in remote areas and have poor access to health services, research has been focused on the development of a simple, cheap and reliable diagnostic test for VL. Demonstration of parasites in the spleen or bone marrow biopsy is extremely difficult (in patients living in underdeveloped endemic area of India) especially in the early stage of the disease. The Direct Agglutination test (DAT) remains the first line diagnostic tool for Visceral Leishmaniasis (VL) in many developing countries. The DAT is a relatively simple test with high sensitivity and specificity.

DAT makes use of aqueous (AQ) antigen prepared from L. donovani Promastigotes. It is associated with major drawbacks like limited stability and requirement of uninterrupted cold chain during its storage and transportation. The shaking during transportation also significantly affects the reproducibility of results with AQ antigen. To overcome these drawbacks Meredith and co-workers 4 first reported use of stable freeze dried (FD) antigen for serodiagnosis of VL in field conditions. Subsequently, few more workers5-8 also demonstrated its use in diagnosis of canine and human VL. We have also independently and indigenously developed FD antigen from L. donovani/Promastigotes for diagnosis of Indian VL. None of the above workers disclosed exact procedure for making freeze dried antigen. In this manuscript we wish to describe first time the procedure of making the FD antigen. This assay offers promise as a rapid, non-invasive assay that is applicable to most clinical settings.

MATERIALS AND METHODS
Preparation of the AQ antigen: The AQ antigen was prepared from promastigotes of L. donovani (MHOM/IN/80/Dd8) by the method of Harith et al., 3,11 except that the organism was grown in L-15 medium supplemented with 10% Fetal Calf Serum (GIBCO BRL, Grand Island, N.Y.) instead of RPMI-1640 and in place of counting the promastigotes at each step of antigen preparation the percent promastigote was determined by microcentrifuge technique. In brief, the promastigotes were harvested in late log to stationary phase and washed with Locke’s solution. 10% suspension of the promastigotes was treated with equal volume of 0.8% trypsin in Locke’s solution at 37°C for 45 minutes. After washing with Locke’s solution 10% suspension was fixed by treatment with equal volume of 4% (w/v) formaldehyde in Locke’s solution for 20 hrs at 4°C followed by washing in cold...
saline citrate. 1% suspension of fixed promastigotes was stained overnight with equal volume of 0.05% comassie brilliant blue in saline citrate. Subsequently, the stained promastigotes were washed with the saline citrate. One part of the promastigotes was suspended in Antigen Stabilizing Solution (ASS) and the remainder in saline citrate in 0.35% to 0.4% concentrations and stored after filtration through nylon gauge. Remaining AQ antigen was stored at 4°C for further use.

Preparation of freeze-dried antigen: Several stabilizers alone and in combinations Viz. (Sucrose (SE), Trehalose (TE), Triton X (TX), Sodium Lauryl Sulphate (SL), TE+SE, TX+SE & SL+SE) were tried to select best antigen stabilizer 10 mg of TE, TX & SL and 100 mg of SE were dissolved in 10 ml of citrate saline. All were filtered through 0.22µm membranes before mixing to aqueous antigen. 65µl of each stabilizer was mixed homogenously with 1000µl antigen and freeze dried. After freeze-drying, the antigen samples were reconstituted in 600µl of citrate saline divided into two parts. One part was used as such and in other part formaline (4.3%) was added. All samples were tested by DAT using pooled positive & negative sera. End point titre was taken into consideration for comparing diagnostic potential. After selecting best one different concentrations (0.10%, 0.15%, 0.25%, 0.30% & 0.50%) of that stabilizers were assessed with the antigen & diagnostic potentials were checked as above. Comparison of antibody titre was made with AQ antigen of same batch.

Particle loss on freeze-drying: Particle loss on freeze drying was calculated by subtracting mean packed cell volume of FD antigen from mean packed cell volume of AQ antigen which were determined by centrifuging 10% promastigote suspension of AQ and FD antigen in graduated capillary tubes at 3000 rpm for 10 minutes. As the original column of promastigote suspension in the capillary tube is 100 mm long, the volume of packed cells can be expressed directly as a percentage.

Reconstitution of FD Antigen prior to use: Each aliquot of FD antigen was reconstituted in 0.6 ml of normal saline (0.9% NaCl) and 4.3 µl formaline was added & kept at room temperature for 30 minutes before performing DAT.

Performance of the test: DAT was carried out as per method of Harith et al.11 in V-shaped 96 well microtitre plates. Serum samples were serially diluted (1:100 to 1:6553600) in 0.2% gelatin (SIGMA Chemical Company, ST. Louis, USA) containing 0.9% NaCl (w/v) and 0.78% (v/v) 2-mercaptoethanol. End point titre was taken into consideration for comparing diagnostic potential of FD antigens with different stabilizers. The end point was determined as the highest dilution at which agglutination was still visible, as a blue mat, enlarged dot with frayed edges, or enlarged blue dot, compared with negative control wells, which has clear blue dots.

Serum Samples: Final FD antigen was evaluated with ten proven Kala-azar (KA) sera and ten normal sera and comparison was made with aqueous antigen.

Stability of AQ and FD antigens: AQ antigen was kept at 4°C, room temperature and at 37°C. FD Antigen, FD pool positive and FD pool negative sera samples were stored at 4°C, 37°C and 56°C as recommended by WHO.12 The reactivity of AQ and FD antigen was tested at intervals of 7 and 15 days respectively in order to establish their stability.

Statistical Analysis: The Wilcoxon signed rank sum test13 was used for comparing two groups of paired observations. Comparison of two proportions was done by "Z" test14

RESULTS AND DISCUSSION

Particle loss on freeze drying and subsequent reconstitution: A particle loss of 41.48% (±8.45%) was observed upon freeze-drying the promastigote suspension stored in ASS. Accordingly each aliquot of FD antigen was reconstituted in 0.6 ml of normal saline to achieve an initial 0.35% to 0.4% promastigote concentration (25-30 million promastigotes per ml of the antigen, a concentration equivalent to concentration of promastigotes in AQ antigen) and to attain comparable agglutination. If the FD antigen is reconstituted in an initial volume of 1 ml, the promastigote concentration is lower than the promastigote concentration in AQ antigen which results in difference in agglutination patterns with AQ and FD antigen and therefore in reading of final results. Actual comparison of results is thus possible only when particle loss during freeze-drying is taken into consideration and further reconstitution of FD antigen is done accordingly. Earlier workers4,5,7 have not reported such an observation. Our observation, therefore, assumes significance in view of the fact that a certain percentage or number of promastigotes are necessary for performing DAT.
Endpoint titres of various stabilizers: Endpoint titres observed with SE, TE, TX, TE+SE, TX+SE, SL and SL+SE were: 3200, 1; 6400, 1; 3200, 1; 12800, 1; 25600, 1; 1:819200 and 1:25600 respectively. "SL" gave best reactivity (endpoint titre) with pooled positive KA sera. Amongst the different concentrations of SL (0.10%, 0.15%, 0.20%, 0.30%, 0.50%), SL-30 (0.30%) was found best. The endpoint titre with SL-30 was found to be 1:1638400.

Comparative endpoint titres of Kala-azar samples in DAT with FD and AQ antigen: Results have been presented in Table-1. All ten serum samples were tested with both the FD and AQ antigens. Statistical analysis showed that the endpoint titres of FD antigen were on the average 0.86-step dilution lower than the AQ antigen (P > 0.01). At a cut off dilution of 1:1600, no difference in quality of agglutination was observed in any case with both the antigens.

Stability of AQ and FD antigens: The AQ antigen remained fully reactive for 9 months when stored at 4°C but deteriorated at 37°C after 3 weeks of storage, which is in agreement with the reported findings. Since AQ antigen did not deteriorate at 4°C, it was used as standard antigen in all comparative studies including stability and endpoint titres calculations. The FD antigen was found stable at 4°C, 37°C and 56°C for 24 months period studied so far. The freeze-dried pool positive and pool negative sera were also found to be stable at 56°C. This indicates the feasibility of its use in DAT even under adverse field conditions.

The FD antigen is at par with AQ antigen. The shortcomings associated with AQ DAT antigen have been overcome by development of FD Antigen. In addition to it, FD antigen may be a better diagnostic tool in terms of commercial viability and cost effectiveness. The major advantage of FD Antigen is that because of its improved stability, the production of its large batches will facilitate reproducible results over a longer period of time. The DAT, particularly with FD antigen, also appears to be simple, easy to perform, highly sensitive and specific. It is, therefore, more suitable and economically feasible for diagnosis of VL under adverse field conditions prevailing in endemic zones in many countries, like Sudan, Bangladesh, Nepal and India and in African countries where most of the affected population is below poverty line.

Table 1: Comparison of End Point Antibody Titres of Sera Sample using the Freeze-Dried and Aqueous antigens.

<table>
<thead>
<tr>
<th>No. of serum samples</th>
<th>Reciprocal Antibody titre</th>
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<tbody>
<tr>
<td></td>
<td>AQ antigen</td>
</tr>
<tr>
<td>1.</td>
<td>409600</td>
</tr>
<tr>
<td>2.</td>
<td>6553600</td>
</tr>
<tr>
<td>3.</td>
<td>6553600</td>
</tr>
<tr>
<td>4.</td>
<td>3200</td>
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<tr>
<td>5.</td>
<td>6400</td>
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<tr>
<td>6.</td>
<td>12800</td>
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<tr>
<td>7.</td>
<td>25600</td>
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<tr>
<td>8.</td>
<td>51200</td>
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<td>9.</td>
<td>102400</td>
</tr>
<tr>
<td>10.</td>
<td>204800</td>
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</table>

Note: The end point was expressed as the highest dilution at which agglutination was still visible, as a blue mat, enlarged dot with frayed edges, or enlarged blue dot, compared with negative control wells, which shows clear blue dots.

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