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Title
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Author(s)
Gupta, Suman; Tiwari, Suman; Jain, Girish K.

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Direct Agglutination Test Based on Freeze Dried Antigen from *Leishmania donovani*: Development and Comparative Evaluation with Aqueous Antigen and Recombinant rK$_{39}$ Antigen for Sero-diagnosis of Visceral Leishmaniasis

Suman GUPTA$^1$, Suman TIWARI$^1$ and Girish K. JAIN$^2$

$^1$Division of Parasitology & $^2$Division of Pharmaceutics, Central Drug Research Institute, Lucknow-226001 India

Abstract: Direct Agglutination Test (DAT) and rK$_{39}$ strip tests have been considered useful for the diagnosis of Visceral Leishmaniasis (VL). DAT with aqueous (AQ) antigen has drawbacks like limited stability and requirement of uninterrupted cold chain till its end use. The present study describes the development of modified DAT based on freeze-dried (FD) antigen from *L. donovani* promastigotes. FD antigen was found to be stable for 24 months at 56°C. FD antigen, AQ antigen and rK$_{39}$ strips were evaluated with 708 serum samples of different disease spectrum of VL. Findings revealed that both FD as well as AQ antigen showed 96.5% sensitivity and 100% specificity, where as rK$_{39}$ strip test exhibited 86% sensitivity and 100% specificity. Statistically the performance of FD and AQ antigen was better than rK$_{39}$ ($P<0.05$) in Indian VL patients. It also appeared that the production of large batches of FD antigen might be highly commercially viable and economically feasible for the diagnosis of VL in millions of poverty ridden endemic population under adverse field conditions.

Key words: Visceral Leishmaniasis, Serodiagnosis, DAT, AQ Antigen, FD Antigen, rK$_{39}$ Strip Test

INTRODUCTION

VL is caused by protozoan parasites of *L. donovani* complex. It is geographically distributed in tropical and subtropical regions of the world. The disease is often fatal if not diagnosed timely and left untreated. The occurrence of disastrous VL epidemics in several regions of world in the past has created a pressing demand for appropriate diagnostic tools. Its definitive diagnosis requires a high order of sensitivity, specificity, cost effectiveness and field utility of various methods and techniques. Pathological methods are mainly based on
demonstration of parasites in biopsies or aspirates from infected spleen, bone marrow and lymphnodes (Zijlstra et al., 1992). Their sensitivities are in the range of 90-95% (Manson Bahr 1987; 1988; Zijlstra et al., 1992). These procedures are invasive, troublesome and are not conveniently applicable under field conditions (Osman, 1998). They have also often resulted in deaths of clinical suspects (Boelaert et al., 1983; 1999; Thakur, 1997). A venous or capillary blood sample is, therefore, more acceptable. PCR amplification testing for Leishmanial DNA is also highly sensitive and specific but its field applicability has not yet been established (Meredith et al., 1995).

An ideal immuno-diagnostic test should be simple, quick, cost effective, highly sensitive and specific and be applicable in the fields even in the adverse conditions that prevail in many areas where Leishmaniasis is endemic. Several serological tests has been reported for the diagnosis of VL (Osman, 1998), of which direct agglutination test (DAT) (Harith et al., 1986) and rK₉₉ dipstick, which is based on ELISA with rK₉₉ recombinant protein having a 39 amino acid repeat, that is a part of a 230 kDa kinesin related protein predominant in L. chagasi tissue amastigotes (Sundar et al., 1998; Zijlstra et al., 1998), are promising in meeting these requirements.

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 (1995) first reported use of stable freeze dried (FD) antigen for sero-diagnosis of VL under field conditions. Subsequently, Oskam et al. (1996, 1999), Zijlstra et al., (1997) and Ozbel et al., (2000) also demonstrated its use in diagnosis of canine and human VL. To further explore its field utility, particularly in Indian VL cases, we, in our laboratory, have independently and indigenously developed FD antigen from L. donovani (MHOM/IN/80/Dd8). The present study describes comparative diagnostic usefulness of AQ and FD Antigen and rK₉₉ dipsticks.

**MATERIALS AND METHODS**

**Preparation of the AQ and FD antigen:**

The AQ antigen was prepared from promastigotes of L. donovani (MHOM/IN/80k/Dd 8) by the method of Harith et al., (1986,1988) 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% coomassie 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. 1-ml aliquots of promastigotes, suspended in ASS, were freeze-dried and sealed under vacuum. Remaining AQ antigen was stored at 4°C for further use.

**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 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., (1986) 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. 1:1600 serum dilutions were estimated to be the best cut off between sensitivity an specificity. This parameter was exclusively based on results of cases from active VL patients, endemic controls and from patients with other diseases. End point titre was taken into consideration for comparing diagnostic potential of FD and AQ antigens. For comparing FD and AQ antigen with rK39 strip test both the AQ and FD antigens were tested at a cut off dilution of 1:1600. 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.

**rK39 Strip Test:**

rK39 strips received from WHO were tested both with the neat serum samples as well as with their 1:100 dilutions as described by Sundar et al. (1998).

**Serum Samples:**

A total of 708 serum samples comprising 86 parasitologically proven cases, 8 PKDL (Post Kala-azar Dermal Leishmaniasis) cases, 43 successfully treated cases, 150 asymptomatic endemic controls, 150 non-endemic controls and 271 cases (from non-endemic area) suffering from other diseases namely AIDS (17), amoebiasis (20), echinococcosis (3), filariasis (40), giardiasis (15), hepatitis-B (11), leprosy (63), malaria (41), neurocysticercosis (5), typhoid (3),
trypanosomiasis (1), and tuberculosis (50) were included in the study.

**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 (Jerne and Perry, 1956). 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 test (Altman, 1991) was used for comparing two groups of paired observations. Comparison of two proportions was done by “Z” test (Zar, 1974).

**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 workers (Meredith et al., 1995, Oskam et al., 1996 and Zijlstra et al., 1997) 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 (Meredith et al., 1995, Oskam et al., 1996 and Zijlstra et al., 1997).

**Cut off titre:**

271 serum samples from different groups of subjects suffering from diseases other than VL were tested to establish cut off dilution. Our results reveal that not a single case showed positivity if cut off value is set at 1 : 1600, thus this dilution can be treated as a cut off dilution for testing both the FD as well as AQ antigen

**Stability of the AQ and FD antigen:**

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 (Harith et al., 1988, Meredith et al., 1995). Since AQ antigen did not deteriorate at 4°C, it was used as standard antigen in all comparative studies including stability, sensitivity and specif-
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 indicate the feasibility of its use in DAT even under adverse field conditions.

**Comparative End point titres of Kala-azar sera samples in DAT with FD and AQ antigens:**

Results have been presented in Table 1. All serum samples were tested with both the FD & AQ antigens. Out of 86 serum samples from Kala-azar active cases 83 serum samples were tested positive with the FD and AQ antigens in DAT. Statistical analysis showed that the end point titres of FD antigen were on the average 0.86 step dilution lower than 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.

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

<table>
<thead>
<tr>
<th>Category</th>
<th>No. of serum samples</th>
<th>Reciprocal Antibody Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AQ Antigen</td>
</tr>
<tr>
<td>Category I</td>
<td>20</td>
<td>409600</td>
</tr>
<tr>
<td>Category II</td>
<td>59</td>
<td>3200</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>6400</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>12800</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>25600</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>51200</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>102400</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>204800</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>409600</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>819200</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>1638400</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>3276800</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>6553600</td>
</tr>
<tr>
<td>Category III</td>
<td>2</td>
<td>6553600</td>
</tr>
<tr>
<td>Category IV</td>
<td>2</td>
<td>6553600</td>
</tr>
</tbody>
</table>

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.
Comparative sensitivity and specificity of FD and AQ antigen and rK39 strips:

The results of the comparative evaluation from different groups of subjects are presented in Table 2. Of 86 proven cases, 83 showed positive response by DAT, both with FD & AQ antigens, whereas only 74 showed positivity with rK39 strips, representing a sensitivity of 96.5% and 86% respectively (P<0.05). In all the 74 cases, rK39 strips showed positivity, both with neat sera as well as with their 1:100 dilution. These cases were also positive with DAT. Similarly all 3 cases that were negative with DAT were also negative with rK39 strips. In PKDL cases, there was no significant difference in response with rK39 strips, AQ and FD antigen (P>0.05). Both DAT and rK39 strip tests were unable to distinguish between active VL and past treated cases because positive responses of 81% and 86% respectively were observed in past treated cases (P<0.05). This indicated that these tests were not capable of distinguishing between active, sub-clinical and past treated cases. It corroborates earlier finding that high levels of anti-Leishmanial antibodies remain circulating in blood for a long time even after completion of treatment (Oskam et al., 1996; Zijlstra et al., 1998).

9 of the 150 healthy control subjects from the VL endemic area showed positive response both with AQ and FD antigen whereas 24 cases were positive with rK39 strips thus representing positivity of 6% and 16% respectively (P>0.05). None of the 150 subjects from non-endemic area showed a positive response either by DAT or rK39 strip test. All 271 serum samples from non endemic areas from patients suffering from diseases other than VL did not show any cross reactivity, FD & AQ antigen and rK39 strips test thus representing 100% specificity with each of test systems.

The FD antigen is, therefore, at par with AQ antigen and rK39 strip test in all respects. The merits of strip test are that it is quick, suitable for use under field conditions, requires a tiny amount of peripheral blood and no laboratory facilities, and is simple to carry out and read. Singh et al. (1997) has reported 100% sensitivity with the strip test while the studies conducted on European and African VL cases using rK39 strip test have shown only 71.4% and 60% sensitivity respectively (Jelinek et al. 1999; Anon, 1999). It has also been reported

Table 2: Comparative performance of FD, AQ Antigen & rK39 Strips in the diagnosis of VL (Total 708 cases)

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Total cases</th>
<th>No and (%) of cases found positive by</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DAT (AQ Antigen)</td>
<td>DAT (FD Antigen)</td>
</tr>
<tr>
<td>Proven cases</td>
<td>86</td>
<td>83 (96.5)</td>
<td>83 (96.5)</td>
</tr>
<tr>
<td>PKDL cases</td>
<td>8</td>
<td>3 (37)</td>
<td>3 (37)</td>
</tr>
<tr>
<td>Past treated cases</td>
<td>43</td>
<td>35 (81)</td>
<td>35 (81)</td>
</tr>
<tr>
<td>Endemic controls</td>
<td>150</td>
<td>9 (6)</td>
<td>9 (6)</td>
</tr>
<tr>
<td>Non-endemic controls</td>
<td>150</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Cases with other diseases</td>
<td>271</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>
that rK39 ELISA remains positive even after 24 months of treatment (Zijlstra et al., 1998). 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 around in many countries, like Sudan, Bangladesh, Nepal and India and in African countries where most of the affected population is below poverty line.

REFERENCES