Evaluation of Blood agar micro-titer plates for culturing *Leishmania* parasites to titrate the parasite burden in spleen and peripheral blood of VL patients

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Running Title- Microtiter plate Culture of *Leishmania* from splenic tissue and peripheral blood

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Abstract

Serial dilutions of blood and spleen biopsy specimen, plated on NNN-blood agar using micro-titer culture plates is a sensitive and reproducible method for detection and growth of *Leishmania* parasites. Plates could easily be monitored and growth rapidly detected. Moreover, parasite number may be estimated using this technique.

Key words- visceral leishmaniasis, peripheral blood, splenic biopsies, microtiter culture, parasite culture.
Demonstration of parasites in tissue biopsy by microscopy is the most reliable and conventional method for diagnosing visceral leishmaniasis (VL), also known as kala-azar. The sensitivity of detection of amastigotes in the splenic aspirate (SA) smear by microscopy is 96-98% and is regarded as the gold standard for diagnosis of VL (1-3). Bone-marrow (BM) and lymph-node (LN) aspirate smears have lower sensitivity, around 70% and 58%, respectively (1, 4, 5). The parasite density score is determined using a logarithmic scale ranging from 0 (no parasites per 1,000 oil-immersion fields) to +6 (>100 parasites per field) (6), and is sometimes used as an indication of severity of infection, e.g. in drug evaluation trials.

An improved, affordable, diagnostic procedure for VL is desirable as splenic aspiration is an invasive procedure associated with risk of severe hemorrhage. Bone marrow aspiration, on the other hand, requires anesthesia and is painful for the patient (7, 8). Patient’s peripheral blood contains living amastigotes (9) and may be a good specimen for diagnosis of VL, if made sensitive enough. However, the isolation of parasites from the patients’ blood may be difficult because of low parasitemia, and it is not very well documented.

Culturing of Leishmania parasites from patient biopsies are usually done using NNN-blood agar in tubes. The sensitivity of SA-culture in NNN-tube is around 97-100%, whereas the sensitivities of bone-marrow and lymph-node aspirate are estimated at 70-80% and 60-70% respectively.
In this study, we have evaluated a serial dilution micro-titer culture technique for the isolation and detection of *Leishmania* parasites from SA and white blood cells [peripheral blood mononuclear cells (PBMC), and buffy coat cells] isolated from 2-3 ml of peripheral blood. Experimentally this method has been used to estimate parasite burden in infected animals.

Sixty-eight microscopically confirmed cases of VL with an average splenic score of 2+ were included in the study. Patients were from the Kala-azar Medical Research Centre, Muzafarpur, India. The Ethical Committee at the Centre approved the study.

Splenic aspirates were collected in heparinzed tube containing RPMI 1640 supplemented with serum and antibiotics. Peripheral blood was collected in heparinzed vacutainer tubes. Samples were transported to Central laboratory at 15-20°C (blood) or 4-8°C (splenic aspirates). All samples were processed in less than 24 hours from the time of collection. Buffy coat cells (WBC) were isolated from 3 ml of blood by centrifugation at 1300 g for 15 min, Red blood cells in Buffy coat layer were reduced by lysis using hypotonic (0.2%) NaCl solution for 20 seconds, to achieve an isotonic solution an equal volume of hypertonic (1.6%) NaCl solution was added. Mononuclear cells (PBMC) were isolated from 2 ml of blood by Ficoll gradient centrifugation according to manufactures instructions (GE Healthcare, USA).

Blood agar (BA) was prepared by mixing 3 parts defibrinated rabbit blood with 7 parts warmed (43°C) sterile NNN-medium (5g Bacto beef, 2g Neopeptone, 2g Bactoagar and 0.5g NaCl, per 100 ml water, pH 7.4). 50 µl blood agar was added to each wells of a 96-well plate tilted at a 45° angle until coagulated. This generates an open window that
allows examination of parasites growth using an inverted microscope. M199-medium (100 µl), supplemented with serum, antibiotics and trace amounts of hemin, adenine, biotin and triethanolamine, as previously described (10), was added to each well except first vertical row of the plate, which was inoculated with 150 µl of splenic aspirate, WBC or PBMC. A serial 3-fold dilution from the first to the 12th well was made. The culture plate was sealed and incubated at 27°C. Cultures were examined for growth on every second day using an inverted microscope.

Culturing parasites from leishmaniasis patients is sometimes needed to confirm the diagnosis, particularly when microscopic evaluation is uncertain and when species determination is desired. Sensitive and rapid outgrowth of parasites in cultures is thus helpful for diagnostic purposes. To determine if plates were advantageous to tubes for growing out Leishmania and suitable for routine diagnosis of kala-azar; the sensitivity, parasite load and time to detection were evaluated.

This serial dilution method had a sensitivity of 100% for detection of parasites in splenic aspirates. Detection of parasites in WBC was 85%, which is close to what Hide et al. found (84%), using a similar technique. In contrast to results by Hide et al., who were unable to culture parasites from PBMC, we were able to grow out parasites from 91% of PBMC samples (table 1a). The culture success in WBC and PBMC cultures were better or at par with those described for BM and LN aspirates. The time to detection was shorter in titrated cultures as compared to using the larger NNN-culture tubes for which samples are not diluted.

Titration of parasites allows a rough estimation of the parasite load in peripheral blood, which may give an indication of the severity of infection. There was, however, no
correlation between splenic score and the parasite load as determined by titration ($R^2 = 0.00005$; Pearson test). The average numbers, determined by serial dilution of parasites in the different blood or tissue preparations, are shown in table 1a. The numbers are, however, underestimated, as no end titration (i.e. growth detected in the last well of the titration, table 1b) was determined in 43% of SA and in 16% of Buffy coat / WBC cultures. The levels of parasites found in blood were low compared to the spleen. However, it is clear that in most cases it is feasible to diagnose VL by culturing either PBMC or Buffy coat / WBC isolated from a small volume of blood. Whole blood cannot be used (not shown). In addition to concentrating the sample in the enriched leukocyte preparations, the removal of serum/plasma is probably required for transformation and growth as promastigotes are complement sensitive.

The number of parasites was lower in PBMC compared to Buffy coat/WBC, as determined by serial dilution, however, the sensitivity was slightly better and more importantly, the time to detection was faster using purified PBMC. Similar observations were made when mononuclear cells (MNC) from SA were cultured (unpublished observation). Due to limitations in sample volumes a proper comparison of larger sample size has not been made using splenic MNC.

From our results it is evident that dilution of the samples may benefit detection of parasites. *Leishmania* did not always grow in the least diluted wells, and were often first detected in the 4th or 5th well (243 x and 729 x dilution factor, respectively). The serial dilution may improve parasite growth because the high concentration of blood cells dying as a result of culture conditions optimized for promastigote growth produces an environment toxic to the parasites.
Replacing the traditional agar tubes with plates may be advantageous in several aspects: The method consumes less medium and micro-titer plates can be monitored for growth while in culture and time to detection shortened. Moreover, it can give a crude estimate of infection burden, which, may be more accurate than the traditional 1-6 scoring system.

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References


Table 1

a) NNN tube and Blood Agar Micro-titer culture technique using splenic aspirates, WBC / Buffy coat and PBMCs. b) Last well of titration in which parasites were detected.

Table 1a.

<table>
<thead>
<tr>
<th>Techniques</th>
<th>NNN tube culture</th>
<th>Blood agar Micro-plate culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subject (Confirmed VL)</td>
<td>Splenic Aspirates</td>
<td>Splenic Aspirates</td>
</tr>
<tr>
<td>Time for detection of parasite growth</td>
<td>5-10 days</td>
<td>3-7 days</td>
</tr>
<tr>
<td>n</td>
<td>68</td>
<td>68</td>
</tr>
<tr>
<td>No. of Leish positive samples</td>
<td>67</td>
<td>68</td>
</tr>
<tr>
<td>% of Sensitivity</td>
<td>98.5%</td>
<td>100%</td>
</tr>
<tr>
<td>Average end titer in BA plate</td>
<td>n/a</td>
<td>8.9</td>
</tr>
<tr>
<td>Dilution Factor (1:3)</td>
<td></td>
<td>$3^{8.86}$</td>
</tr>
<tr>
<td>Parasites/ml specimen</td>
<td>~19,68,300*</td>
<td>~243**</td>
</tr>
</tbody>
</table>

Table 1b.

<table>
<thead>
<tr>
<th>Techniques</th>
<th>No. of patient (n)</th>
<th>Growth of parasite at end titer (In blood agar micro-plate culture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>End titer well ****</td>
<td>0</td>
<td>1-3</td>
</tr>
<tr>
<td>SA culture</td>
<td>68</td>
<td>-</td>
</tr>
<tr>
<td>Buffy coat culture</td>
<td>68</td>
<td>10</td>
</tr>
<tr>
<td>PBMCs culture</td>
<td>47</td>
<td>4</td>
</tr>
</tbody>
</table>

*SA (around 100 µl) was collected in 900 µl of cRPMI. The 10X diluted SA (150 µl) was plated in first well of 96-well micro- title plate, followed by serial 1:3 dilutions out to the 12th well.

** WBC Buffy coat was separated from 3 ml of peripheral blood, resuspended in 150 µl of cRPMI and plated in serial dilution (1:3) out to the 12th well.
*** PBMCs separated from 2 ml of peripheral blood, resuspended in 150 µl of cRPMI and plated in serial
dilution (1:3) out to the 12th well.
**** “0” represents no growth.