Evaluation of Different In-vitro Culture Media for Cultivation of Leishmania Parasites

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ABSTRACT
This study was held in the Institute of Endemic Diseases, University of Khartoum during the period from January 2007 to December 2007. The study compared between horse blood and rabbit blood as main constituent of Novy-MacNeal-Nicole (NNN) culture media in addition to the use of Lowestein Jensen (LJ) media for cultivation of Leishmania parasite. The parasite was inoculated in tubes contained different concentrations of horse blood (5%, 10% and 20%) and other tubes contained rabbit blood with a concentration of 10%, also the parasite was inoculated in LJ media. All the tubes were incubated for 48 hours at 23 ºC. Parasites were then counted every 48 hours. This study indicated that Leishmania parasite showed the growth rate in the NNN media which contains horse blood (especially 10%) and rabbit blood. The rabbit blood showed better growth than horse blood. There was no growth in the LJ medium.

KEYWORDS: Novy-MacNeal-Nicole medium, LJ media, horse blood, rabbit blood.

INTRODUCTION
Leishmaniasis is a major vector-borne disease caused by obligate intramacrophage protozoa of the genus Leishmania, belonging to the subphylum Mastigophora and family Trypanosomatidae. Parasites infecting numerous mammals, including humans(1). Leishmaniasis is transmitted by bite of female sand fly (Genera Phlebotomus or Lutzomyia) either from infected...
animals to humans or from person to person. Injecting drugs can also transmit visceral leishmaniasis\(^1\). Leishmaniasis is endemic in areas of the tropics, subtropics and southern Europe. Specifically, is endemic in 88 countries and is the only tropical vector borne disease that has been endemic to southern Europe for decades\(^2\). Human leishmaniasis can manifest itself into different clinical forms which include: visceral, cutaneous, mucocutaneous and post kala-azar dermal leishmaniasis\(^2\). The increase in leishmaniasis worldwide incidence is mainly attributed to the increase of several risk factors that are clearly manmade which include massive migration, deforestation, urbanization, immunosuppression, malnutrition and treatment failure \(^3\). The gold standard for diagnosis of leishmaniasis is considered as Wright or Giemsa staining of preparations of the needle aspiration material and cultivating in NNN media. While studies of the antigenic structure, biochemical properties, and infective capabilities of *Leishmania* species that can cause leishmaniasis in the Mediterranean, the Middle East, and tropical regions are being carried out. A great number of promastigotes are needed and in vitro cultivation of both amastigote and promastigote forms is carried out by using many different cultural media \(^4-6\). A variety of media have been used for the culture of leishmanias. These can be divided into three main categories: semisolid, biphasic, and liquid. While biphasic and semisolid culture media need blood, an important factor for the reproduction of parasites, most liquid media require fetal calf serum (FCS) or erythrocyte lysate \(^6\). Biphasic Novy-MacNeal-Nicolle (NNN) culture medium, which has been used for a long time, is preferred because it is far cheaper than commercial media. Although it is quite suitable for the isolation of parasites, in this medium samples taken from leishmaniasis patients fail to produce a great number of promastigotes in a short time. Long-term cultivation and excessive production of promastigotes depend largely on the serum and serum components present in the culture medium \(^7\). In order to support the development of promastigotes for long, the culture requires a balanced chemical arrangement as well as the serum. In this study, a formula which can be obtained easily and cheaply as far as commercial procedures are concerned has been tested for in vitro cultivation of *Leishmania* species. Sudan is the original focus of visceral leishmaniasis (VL); *L. donovani* \(^8\) and it has been proposed that the *Leishmania* parasite has evolved before or at the same time as *Homo sapiens* in East Africa \(^9\). The reported occurrence of VL in Sudan is wide, erratic and variable \(^10\). Following the first reported case of VL in 1904, only sporadic cases were reported until the 1930s. Then, endemic areas started to expand considerably and erratically, following an epidemic pattern \(^11,12\). Gedaref State, in northern Sudan, is the only known stable hyperendemic region in Sudan, with a consistently high annual incidence of thousands of reported cases. In 1990-1992, VL also spread northwards to the south of Kordofan State, with nomadic tribes moving between this area and Western Upper Nile, causing an outbreak in the non-immune population \(^10,13\). Since 2006, after VL had been absent for 25 years, a surge of new cases has been observed in White Nile State, central Sudan, close to Khartoum \(^14\). Transmission during epidemics is predominantly anthroponotic. However, VL occurred in game wardens in uninhabited Dinder National Park, suggesting transmission
was zoonotic in this case (15). However, no important animal reservoir has been identified since the discovery of the first case of VL in Sudan, although thousands of small and large mammals and reptiles have been investigated (10). Climatic events, such as increased annual rainfall, have preceded past epidemics on several occasions (10,16). In Gedaref state, annual rainfall and altitude were the best predictors, among many factors that were studied, for the occurrence of VL (17). A resurgence of the sand fly population, as a consequence of regrowth of Acacia and Balanites forests destroyed by floods, or the interrupted insecticides spraying have also been associated with epidemics (10,18). Post kala-azar dermal leishmaniasis (PKDL) occurs in about 55% of sudanese patients (19). Mucosal involvement in VL is uncommon; since the disease was first described in 1914, only 64 cases, mostly in adult males from several closely related tribes in western Sudan, were reported up until 1992 (20). The first autochthonous case of cutaneous leishmaniasis (CL) was found in 1911 (21). The reporting of sporadic cases was followed by three outbreaks: in the Shendi Atbara area in 1976-1977, in ElGarrasa in the White Nile area early 1985, and in Tuti island, at the junction of the Blue and White Nile, in 1985. In 1986, the number of cases increased dramatically; all agegroups were affected with a total of 100,000 cases. Currently, CL is endemic in Darfur and Kordofan, in addition to the area from Khartoum state up to Atbra, in the northern part of the country. The main objectives of this study were to compare between different types of Leishmania culture media, to determine the best culture media for Leishmania isolation and growth and to test a bacterial culture media for Leishmania inoculation.

MATERIALS and METHODS
This study was a comparative study between different types of culture media used in inoculation and diagnosis of Leishmania parasites and was done in the Institute of Endemic Diseases, University of Khartoum during the period from January to December 2007.

Samples
Leishmania parasites strain MHOM/SD/ MW 20/03 were the parasites used in this study obtained from the Institute of Endemic Diseases, University of Khartoum, the sample required was 100μL of parasites. The parasite was isolated in complete media (Ross Well Park Memorial Institute (RPMI) + 10% fetal bovine serum + 2% streptomycin and penicillin) at 23°C.

Parasite culture
Parasite was examined microscopically and subcultured in NNN media (either by adding rabbit blood or horse blood) and Lowenstein Jensen medium.

Preparation of culture media
Preparation of NNN medium
The NNN medium was prepared as described by Sundar et al. (22) in a flask by heating 1.4g of agar and 0.6g of NaCl in 90mL distilled water. The content of the flask was heated until the agar melted and kept well mixed. The molten agar was transferred into the culture vessels and sterilized by autoclaving at 121°C for 15 min. The agar was allowed cooling to about 50°C in water bath. In a safety cabinet, collected horse blood was added to the agar at different concentrations as follow: 5%, 10% and 20%. Defibrinated rabbit blood was added to agar (plain, non-nutrient agar) at a concentration 10% and the blood agar was mixed and placed into sterile vacutainer tubes (2mL each) in a sloped position until the agar settled, then stood in an upright position and
transferred to a refrigerator ready for inoculation.

**Preparation of L.J medium**

This medium is used mainly for isolation and cultivation of *Mycobacterium* species. It was prepared (as described by Hi Media Laboratories pvt. Ltd, India) by suspension of 37.24 gram of powder in 600mL distilled water containing 12mL glycerol. The mixture was heated to dissolve the medium completely and sterilized by autoclaving at 121 °C for 15mins. One mL of whole egg emulsion collected aseptically was added to agar and mixed well as described by Cheesbrough (2000) (23). The mixture was distributed in sterile capped tubes in a sloped position. The medium was coagulated in water bath for 45 minutes.

**Parasite subculture**

Two dilutions were used to subculture promastigotes into NNN medium and LJ medium. The volume required for the first dilution was calculated from the formula \( \frac{RV}{O} \) as described by Bishop (2000) (24), where R (required volume) was 100 μL, V (volume) was 14x10^3 and O (original count from complete media) was 14X10^6. So the volume required was 100μL of parasites added to 13900μL of complete media. Then 200μL of mixture was sub cultured in NNN medium which contains different concentrations of horse blood (5%, 10%, and 20%) and with 10% rabbit blood. The parasites were also subcultured in LJ medium. The vacutainer tubes were incubated for 48 °C in a cooled incubator at 23 °C and then the parasites were counted. The second dilution was used to increase the parasite growth. The R was 1000, V was 2000 and O was 500. So the volume required was 400 μL of the parasites added to 1600μL of complete media and then subcultured as the previous dilution.

**Parasite count**

In one Eppendorf tube, 20μL of incubated promastigote was added to 80μL of 1 x phosphate buffer saline (PBS) (1:5) and mixed. 20 μL were taken from mixture to new Eppendorf tube. Another 30μL of PBS were added to the mixture (1:25) as described by Evan (1989) (25). For staining, 50μL Trypan blue was added. Before counting, 10μL of 2% formaldehyde was used for fixation. The mixture was incubated for 5 minutes. Parasites were counted using chamber in 4 squares and the mean calculated. To calculate the number of parasite that must be inoculated in NNN medium, horse blood, and rabbit blood and LJ media, the following formula described by Dacie (1995) (26) was used.

\[
\text{The absolute number} = \frac{N \times D \times F}{\text{area} \times \text{depth}}
\]

\[
\frac{N \times 4 \times 25}{1 \times 0.1} / \text{cumm}
\]

\[
= \frac{N \times 10^2}{1 \times 0.1} = N \times 10^3 / \text{cumm}
\]

\[
= N \times 10^6 / \text{mL}
\]

**RESULTS**

Different culture media were used for isolation and cultivation of *Leishmania* parasites in this study. These include: NNN media in which different
concentrations of horse blood (5%, 10% and 20%) and 10% rabbit blood were used in addition to LJ media. Two dilutions were used to subculture promastigotes into NNN media and LJ media.

The results of parasite count in the NNN media (with horse blood), LJ media and NNN media (with rabbit blood) were 0x10^6 /ml, 0x10^6 /ml and 2x10^6 /ml respectively (table 1).

The results obtained by counting promastigotes in NNN that contains 5% horse blood within 5 days were 42 x10^6 /ml, 79 x10^6 /ml, 155 x10^6 /ml, 107 x10^6 /ml and 110 x10^6 /ml in the first, second, third, fourth and fifth day respectively (table 2). The results obtained by counting promastigotes in NNN that contains 10% rabbit blood within 5 days were 121 x10^6 /ml, 466 x10^6 /ml, 494x10^6 /ml, 618 x10^6 /ml and 627 x10^6 /ml in the first (1st), second (2nd), third (3rd), fourth (4th) and fifth day (5th) respectively (table 2). The results obtained by counting promastigotes in LJ media within 5 days were 3 x10^6 /ml, 1 x10^6 /ml, 0x10^6 /ml, 0 x10^6 /ml and 0 x10^6 /ml in the first, second, third, fourth and fifth day respectively (table 2). The mean of parasite count in the NNN media using 5%, 10% and 20% of horse blood was 99x10^6 /ml, 96 x10^6 /ml and 81 x10^6 /ml respectively (figure 1). The mean of parasite count in the NNN media with 5% horse blood, NNN media with 10% rabbit blood and LJ media was 99 x10^6 /ml, 465 x10^6 /ml and 0 x10^6 /ml respectively (figure 2).

### Table 1: Inoculation of 14 X 10^10 parasite/mL (first dilution)

<table>
<thead>
<tr>
<th>Media</th>
<th>Parasite count</th>
</tr>
</thead>
<tbody>
<tr>
<td>NNN media contains horse blood (5%, 10%, and 20%)</td>
<td>0.00 (No growth)</td>
</tr>
<tr>
<td>NNN media contain rabbit blood</td>
<td>2 X 10^6/mL</td>
</tr>
<tr>
<td>L.J medium</td>
<td>0.00 (No growth)</td>
</tr>
</tbody>
</table>

### Table 2: Inoculation of 400 X 10^10 parasite/mL (second dilution)

<table>
<thead>
<tr>
<th>Media</th>
<th>Parasite count (Number of parasite cells/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1^st day</td>
</tr>
<tr>
<td>NNN media contains 5% horse blood</td>
<td>42x10^6/mL</td>
</tr>
<tr>
<td>NNN media contains 10% horse blood</td>
<td>48x10^6/mL</td>
</tr>
<tr>
<td>NNN media contains 20% horse blood</td>
<td>51x10^6/mL</td>
</tr>
<tr>
<td>NNN media contains 10% rabbit blood</td>
<td>121x10^6/mL</td>
</tr>
<tr>
<td>L.J. media</td>
<td>3x10^6/mL</td>
</tr>
</tbody>
</table>
DISCUSSION
In cultivations carried out to produce promastigote or amastigote forms of *Leishmania* species outside natural media in the best possible way, care should be taken to make the medium compatible with nutritional and environmental conditions of the natural media. Therefore, evolutionary phases in *Phlebotomus* flies and vertebrate macrophages are being investigated and needs of the organism are being determined \(^{(27)}\). Due to its low price and easy preparation, NNN culture medium is especially utilized in the production of parasites obtained through bone marrow aspiration, spleen puncture biopsy, and skin biopsy. However, for many studies to be made with *Leishmania* isolates; liquid culture media producing a large number of promastigotes in a short time are needed. Ross Well Park Memorial-1640 (RPMI 1640) medium, medium 199, and Schneider’s *Drosophila* medium, the most widely used media among these, are commercially sold \(^{(28)}\). Commercially produced culture media have certain disadvantages, such as high price, unavailability when needed, and impossibility of usage due to expiration dates. The current study compared a relatively simple formulation using common, available, and inexpensive ingredients that can be used in different culture media for the *in vitro* maintenance and mass cultivation of *Leishmania* promastigote forms. This study was to compare between 10% defibrinated rabbit blood and different concentrations of horse blood (5%, 10% and 20%) as main constituents in NNN media for the cultivation and diagnosis of *Leishmania* parasite. Also Lowenstein Johnson (LJ) media, the best culture media for *Mycobacterium* cultivation (LJ media) was used. The best results were obtained with medium containing 10% rabbit blood that, as judged by a faster rate of proliferation, higher final cell density and parasite count, and the ability to culture most *Leishmania* species. By using parasite count technique, the best results were obtained by NNN medium that contains 10% rabbit blood and showed a faster rate of proliferation, higher final cell density and parasite count, and the ability to culture most *Leishmania* species. These findings agree with Yereli et al. \(^{(1997)}\) \(^{(29)}\). The results also showed that the growth rates of promastigotes increased in horse blood but it is lower than rates of rabbit blood. Different concentrations of horse blood (10%, 15% and 20%) were used and the 10% concentration was better than the other concentrations. The growth rate in LJ medium was very poor for cultivation of *Leishmania* parasites which may
need other conditions to be used. The results also showed that the growth rates of promastigotes reached the peak number in the fourth day (except in LJ media). These findings disagree with Yereli et al. (1997) (20) and started to decline in the fifth day. This might be due to consuming of needed materials in the culture and contamination. The present study concludes that Leishmania parasite showed the growth rate in the NNN media which contains horse blood (especially 10%) and rabbit blood. The rabbit blood showed better growth than horse blood. There is no growth in the LJ medium.

The present study recommends the use of horse and rabbit blood at 10% in NNN media and further studies are needed to investigate the use of LJ media by adding the new special conditions to improve the growth.

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REFERENCES