Successful substitution of fetal calf serum by human plasma for bulk cultivation of *Leishmania donovani* promastigotes

Abdelhafeiz Mahamoud,¹ Hussam Ali Osman,¹ Durria Mansour¹ and Abdallah el Harith²

¹Biomedical Research Laboratory, Ahfad University for Women, PO Box 167, Omdurman, Sudan ²Retired microbiologist

The potential of human plasma (HP) or serum (HS) as a replacement for fetal calf serum (FCS) was evaluated in a liver infusion tryptose (LIT) medium for bulk cultivation of *Leishmania donovani* promastigotes. The promastigote yield with the LIT-FCS standard medium was $0.4 - 1.8 \times 10^7$ ml⁻¹, and yields of $0.5 - 3.4 \times 10^7$ ($P = 0.527$) and $0.4 - 2.4 \times 10^7$ ($P = 0.062$) were recorded for two LIT medium variants containing HP or HS as supplement instead of FCS. Significantly, higher promastigote yields of $1.3 - 4.9 \times 10^7$ ml⁻¹ were demonstrated when LIT medium was supplemented with HP of blood group O but not A, B, AB or equally pooled ABO ($P = 0.007 - 0.020$). Matching ($P = 0.56$) strong positive ($1 : 10 \ 2400$ to $\geq 1 : 262 \ 144$ $00$) and weak negative ($1 : 5 - 1 : 160$) direct agglutination test (DAT) titres, respectively, were demonstrated in 24 visceral leishmaniasis (VL) and 45 non-VL sera for both standard LIT-FCS and alternative LIT-HP derived antigens. Our findings indicate strong potential for sustainable production of promastigotes for important diagnostic procedures such as DAT in the VL affected areas.

INTRODUCTION

Being ethically and technically less demanding than *in vivo* cultivation, *in vitro* cultivation of *Leishmania* species is commonly practised to obtain sufficient parasite material to conduct immunological, biochemical or molecular studies. Despite the different composition of defined or undefined liquid media used, fetal calf serum (FCS) or blood lysate supplementation is essential to obtain parasite replication (Hendricks & Wright, 1979; Berens et al., 1976). While blood lysate is generally acknowledged as vital medium supplement for primary isolation and maintenance, FCS has been proved to be crucial for mass culturing of *Leishmania* species and strains (Lemesre et al., 1988).

Since its successful introduction by Novy and McNeal as the major nutrient in NNN-medium, no blood source other than rabbit has been recommended for cultivation of *Leishmania* species (Novy & McNeal, 1904). To further augment promastigote yields, improvements, including addition of glucose-saline (Locke’s) solution and use of blood agar to serve as a solid phase, were introduced (Tobie et al., 1950). Despite significant increase in production, disadvantages were also experienced in using this biphasic media version as source for promastigotes in certain biochemical and molecular biological studies due to contamination with blood or agar artefacts (Handman, 1983; Schuster & Sullivan, 2002). The increasing need for more refined methods for promastigote mass culture has resulted in establishment of several mono-phasic liquid media. The essential supplementation, however, of these media with expensive nutrients, such as FCS, constitutes a significant obstacle to their routine use in developing countries where leishmaniasis is most prevalent.

Based on highly encouraging results initially obtained with defibrinated human blood as a substitute for its rabbit equivalent in NNN-medium (unpublished results), we decided to extend our efforts to evaluate the performance of HP and HS as alternative supplements compared to FCS in our routinely used liver infusion tryptose (LIT) medium for mass culture of *Leishmania donovani* promastigotes.

METHODS

Culture media. NNN-medium as originally described by Novy and McNeal is used in our laboratory for primary cultivation and maintenance of *Leishmania* strains (Novy & McNeal, 1904). For bulk cultivation of promastigotes, a basic version of LIT medium, as described by Sadigursky & Brodsky (1986), was prepared by mixing the following ingredients in 1000 ml of distilled water: liver infusion broth (Difco) 2.5 g, tryptose (Difco) 5 g, NaCl 4 g, glucose 2 g, KCl 0.4 g and NaHPO₄ g. After thorough mixing and adjusting pH to 7.4, the medium was filtered and sterilized by autoclaving for 20 min.

Abbreviations: β-ME, β-mercaptoethanol; DAT, direct agglutination test; FCS, fetal calf serum; HP, human plasma; HS, human serum; LIT, liver infusion tryptose medium; VL, visceral leishmaniasis.
under pressure (1 atm) at 120 °C. The sterilized basic LIT medium was then left to cool at room temperature. If not immediately needed, the ready-for-use basic medium was stored at 4 °C; shortly prior to use, penicillin and streptomycin were added.

**Serum and plasma supplements.** Depending on the experimental objective, the basic LIT medium was supplemented at up to 15% (v/v) with either FCS, pooled human sera (HS) or individual human plasma (HP) from donors with either blood group A, B, AB or O, regardless of the Rhesus factor.

FCS obtained from Sigma (Lot 019K3398) was used throughout this study. Following detailed explanation of the study objectives and consent for contribution, blood samples (5 ml), without anticoagulant were collected by intravenous puncture from 25 volunteer female medical students. All 25 serum samples (approximately 2 ml) obtained by centrifugation were pooled and stored frozen at −20 °C until required. The HP quantities needed for the performance of this study were kindly provided by the Central Blood Bank of the Federal Ministry of Health in Khartoum where all procedures suggested by the World Health Organization for designation of donors and collection of blood/plasma were strictly followed. Only plasma samples that were scheduled for destruction due to accidental thawing (electric failure), redundancy, extremely long periods (>3 years) of storage or positive diagnosis of donors with blood disorders such as polycythaemia, were approved for use in this study. Plastic bags containing 150–200 ml plasma from donors with the different blood groups were transported on ice to our laboratory in Omdurman where they were kept at −20 °C until needed.

**Parasite strain.** The *L. donovani* strain employed in this study was isolated in December 2011 by inguinal lymph-node aspiration from a 4-year-old child presenting with typical visceral leishmaniasis (VL) symptoms at the rural hospital of Elsuffi in the White-Nile State, Central Sudan. The strain was initially maintained in NNN-medium and thereafter adapted by continuous subpassage every 8–10 days in Central Sudan. The strain was initially maintained in NNN-medium and thereafter adapted by continuous subpassage every 8–10 days in Sudan, potentially cross-reactive with *L. donovani* strain adapted in Omdurman. These sera were collected from patients presenting with diseases widely known as difficult to distinguish from VL on clinical grounds and that are, according to our experience in Sudan, potentially cross-reactive with *L. donovani* antigen in DAT, including leukaemia (10), malaria (5) and typhoid (10).

**Experimental design.** Firstly, to establish grounds for comparison, it was decided to evaluate the performance of HS versus that of bovine equivalent (FCS) currently used as a standard supplement in all known mono-phasic *Leishmania* culture media including LIT. Secondly, comparison between HS and HP was necessary to justify using the latter as alternative supplement in LIT medium based on its comparable performance and the advantage over the former as being continuously available at central blood banks. To determine effect of the blood group on *L. donovani* promastigote growth, plasma samples from donors with different ABO blood types were used as supplements in LIT medium. The suitability of promastigotes cultivated in the alternative LIT-HP medium to serve as antigen in DAT was assessed by comparison with results obtained against equivalent parasite population grown in the standard LIT-FCS medium.

In experiment 1, three bottles containing LIT-FCS medium were each inoculated with 1 × 10^6 LIT-FCS adapted promastigotes of *L. donovani* (suspected in 0.5 ml of the same medium). Equal numbers of promastigotes (1 × 10^6) of the same *L. donovani* strain adapted in LIT-HP were inoculated into the second (LIT+HS) and third (LIT+HP) medium combination bottles.

In experiment 2, each of the 18 bottles was inoculated with 1.9 × 10^6 LIT-HP adapted promastigotes of *L. donovani* (suspected in 0.5 ml of LIT-HP).

All 27 bottles containing the various *Leishmania* cultures were maintained under continuous orbital shaking (120 r.p.m.) at between 24 °C and 26 °C for a period of 12–14 days.

Specimens of approximately 200 μl were aseptically taken at 2-day intervals from each bottle to check for bacterial or fungal contamination, evaluate promastigote morphology and determine parasite count ml⁻¹ by using an improved Neubauer haemocytometer method. The average parasite count determined for the three bottles representing each of the nine LIT medium combinations or subcombinations was considered as the final promastigote count on that day. Termination of the experiments was based on the predominant presence of round (degenerative) promastigote forms and an appreciable drop in parasite count.

Because of an insignificant difference (*P*=0.46) determined between promastigote yields of the LIT-HP and LIT-FCS alternative medium combinations and for obvious ethical and practical reasons, it was decided that further comparison on antigenic reactivity should be carried out between those cultured in LIT-HP and LIT-FCS only.

**Antigen processing.** Except for replacing FCS by HP from equally pooled blood of groups A, B, AB and O, the procedure for promastigote bulk cultivation and processing of the antigen for DAT was as described previously (el Harith et al., 1995). For validating diagnostic performance of LIT-HP derived antigen, promastigote culture of the same *L. donovani* strain was established in the standard LIT-FCS medium. Further steps in antigen processing, including β-mercaptoethanol (β-ME) treatment, formaldehyde fixation and Coomassie brilliant blue staining of promastigotes, remained exactly the same.

**DAT procedure.** The test was performed according to standard procedures using β-ME as a reducing agent in the serum diluent (el Harith et al., 1995). Sera were tested in twofold serial dilutions starting at 1 : 100 through full-out titration to determine the highest end point of agglutination reaction in VL sera and at 1 : 5 to critically evaluate for minimal cross-reactivity levels with non-VL conditions. As reported previously by us and others in the Sudan, titres ≥1 : 1600 were considered indicative for VL (el Harith et al., 1986).

**Serum samples.** Serum samples were obtained from three sources. Twenty-four serum samples were collected from patients with confirmed VL (positive inguinal lymph-node aspirates) diagnosed at Doka peripheral hospital in the Eastern State of Sudan during an epidemiological study carried out between September 2004 and October 2005 (el Mutasim et al., 2006). Twenty-five samples were kindly offered by the Institute of Tropical Medicine in Omdurman. These sera were collected from patients presenting with diseases widely known as difficult to distinguish from VL on clinical grounds and that are, according to our experience in Sudan, potentially cross-reactive with *L. donovani* antigen in DAT, including leukaemia (10), malaria (5) and typhoid (10).

Twenty sera from non-endemic apparently healthy female medical students of Afld University for Women, Omdurman.
All of these serum samples were stored at −20 °C.

**Data analysis.** The statistical analysis was conducted using SPSS software version 11.5. The results obtained were transformed into simple categorical data: promastigote yields into log10 and agglutination titres into the number of the twofold serial dilution showing the highest agglutination titre, starting at 1:100 (1) up to 1:262,144 (19) for VL and at 1:5 (1) up to 1:10,240 (12) for non-VL. Mean values of the parasitological and serological results obtained for each medium combination were then compared using Student’s t-test at critical x-level of 0.05; all tests were two tailed and any P-values ≤0.05 were considered to be significant.

**RESULTS**

Similar to the standard LIT-FCS, the alternative LIT-HP and LIT-HS medium combinations yielded maximum promastigote growth 10 days post-inoculation (Table 1). Also, similar to growth in the standard LIT-FCS medium was the predominance (80–90%) of typical promastigote spindle forms during the exponential phase of growth (4–10 days post infection) in both LIT-HP and LIT-HS media. A third feature of similarity between the three LIT medium variants was the noticeable drop in promastigote counts at 12–14 days post infection (Tables 1 and 2).

Despite using a promastigote inoculum \((1.9 \times 10^6 \text{ ml}^{-1})\), approximately double that used in experiment 1, a similar pattern of promastigote multiplication was observed in experiment 2, which used plasma of blood groups A, B, AB, O or equally pooled ABO as a supplement (Table 2). In experiment 2, maximum promastigote production commenced 4 days earlier, and the trend in promastigote multiplication was similar in all five LIT-HP medium subcombinations as well as in the LIT-FCS standard (Table 2).

No significant difference in promastigote yields was found between the LIT-HP \((P=0.527)\) and LIT-HS (0.062) on the one hand, and LIT-FCS on the other. However, the yield of promastigotes recorded for LIT-HP supplemented with HP of blood group O was significantly higher compared to the other four subcombinations supplemented with HP of blood groups A \((P=0.007)\), B \((P=0.015)\), AB \((P=0.020)\) or equally pooled ABO (0.015) (Table 2). The promastigote yields recorded for LIT-FCS standard medium was similar to that for medium supplemented with blood groups A, B, AB and pooled ABO (respectively, \(P=0.827, P=0.091, P=0.232\) and \(P=0.290\)).

Treatment with β-ME of promastigotes from LIT-HP or LIT-FCS did not cause any noticeable reduction in parasite motility or change in morphology indicating deformation or onset of lysis. Almost all parasite rosette formations or aggregations seen earlier in both medium combinations had disintegrated after β-ME treatment, resulting in uniformly dispersed promastigote suspensions.

Following formaldehyde fixation, both promastigote suspensions revealed similar affinity to staining with Coomassie brilliant blue. Approximately 80% of the promastigotes generated in either medium successfully retained the blue stain. Like the LIT-FCS standard, the antigen derived from the LIT-HP alternative medium settled uniformly within 3–4 h of incubation at the bottom of the V-shaped microtitre-plate wells forming sharp-edged blue spots indicating absence of auto-agglutination. Highly comparable \((P=0.56)\) DAT titre levels were also recorded for both antigens against 24 VL and 45 non-VL sera tested (Table 3).

**DISCUSSION**

Because of the high cost involved, ethical issues related to killing of animals and variability in performance of batches produced by different companies on different occasions, attempts were made to replace FCS as supplement in the liquid medium used for cultivation of *Leishmania* species. Although sera of adult mammals had shown some potential for replacing FCS, processing of these sera to completely remove or significantly minimize inherent antileishmanial activities demanded rigorous handling procedures, such as repeated heating up to 86 °C (Evans, 1986).

Our current study is the first of its type in which ready-for-transfusion HP samples of different ABO blood groups, without further processing, were used as an alternative to

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**Table 1.** Comparative replication of *L. donovani* promastigotes in LIT medium variants supplemented with HP, HS or FCS

<table>
<thead>
<tr>
<th>Days post-inoculation</th>
<th>Promastigote counts ((x10^6 \text{ ml}^{-1})) in culture medium combinations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LIT-HP</td>
</tr>
<tr>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>5.1</td>
</tr>
<tr>
<td>4</td>
<td>8.2</td>
</tr>
<tr>
<td>6</td>
<td>12.9</td>
</tr>
<tr>
<td>8</td>
<td>26.2</td>
</tr>
<tr>
<td>10</td>
<td>34.1</td>
</tr>
<tr>
<td>12</td>
<td>22.1</td>
</tr>
<tr>
<td>14</td>
<td>9.3</td>
</tr>
</tbody>
</table>
FCS with evident success. Under similar experimental conditions, including use of basic LIT medium, promastigote inoculum size and temperature of incubation, a comparable pattern of promastigote replication was observed in all three LIT medium combinations supplemented with HP, HS or FCS. Despite doubling of promastigote inoculum size and using HP belonging to donors with different ABO blood groups, appreciable promastigote yields, with typical spindle (healthy) forms, were seen during the exponential phase of growth. This clearly implies that, as for the LIT-FCS standard medium, very low or no anti-promastigote activity was present in the LIT-HP alternative medium. We think that this finding is of a particular importance as it contradicts the previously held assumption that sera from adult mammals possess lethal effects on *Leishmania* promastigotes (Pearson & Steigbigel, 1980; Schmunis & Herman, 1970). This same assumption may previously have led to the exclusion of mammalian sera as eligible substitutes for FCS in *Leishmania* culture media. However, one can argue that because of their obligatory intracellular location during infection, amastigotes but not promastigotes are most exposed to the adverse effect of the mammalian immune response. We can further assume that although amastigotes and promastigotes have several epitopes in common, each of them possesses distinctive antigenic identity. Future evaluation of plasma donated by individuals residing in known VL endemic areas is expected to reveal important information in this regard.

Except for higher promastigote yield recorded for the LIT supplemented with plasma of blood group O, no difference was observed in the promastigote cultures in any of the medium subcombinations using blood plasma. These results imply that use of HP as an alternative to FCS is not blood group/type dependent, a characteristic that will further encourage its use in laboratories with limited research budgets.

Depending on promastigote quantities needed, FCS supplementation usually varies between 10 and 30% of the total volume of culture medium used. Based on the current price range ($350–$550 per litre), the cost of procuring FCS sufficient to obtain 1 ml packed promastigote volume might well exceed budgets allocated for such studies in endemic countries. Aside from cost of purchase, the logistics involved for delivering FCS based on conditions recommended for overseas transportation, constitute additional expenses and may on some occasions lead to delays in obtaining the required volume. Having alternative sources of supplementation would help in avoiding such problems.

### Table 2. Efficiency of HP of different ABO blood groups as replacement for FCS in LIT medium for cultivation of *L. donovani* promastigotes

<table>
<thead>
<tr>
<th>Days post-inoculation</th>
<th>Promastigote count (×10^6 ml⁻¹) in LIT medium subcombination supplemented with HP of blood group</th>
<th>Promastigote count (×10^6 ml⁻¹) in LIT-FCS medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>0</td>
<td>1.9</td>
<td>1.9</td>
</tr>
<tr>
<td>2</td>
<td>10.2</td>
<td>12.8</td>
</tr>
<tr>
<td>4</td>
<td>20.5</td>
<td>26.2</td>
</tr>
<tr>
<td>6</td>
<td>30.9</td>
<td>38.2</td>
</tr>
<tr>
<td>8</td>
<td>29.6</td>
<td>38.0</td>
</tr>
<tr>
<td>10</td>
<td>28.0</td>
<td>32.0</td>
</tr>
<tr>
<td>12</td>
<td>9.0</td>
<td>15.0</td>
</tr>
</tbody>
</table>

### Table 3. Reactivity of *L. donovani* promastigote antigens derived from LIT medium variants supplemented either with HP (LIT-HP) or FCS (LIT-FCS)

<table>
<thead>
<tr>
<th>Antigen source</th>
<th>Clinical condition</th>
<th>Number of sera tested</th>
<th>Direct agglutination test titre range*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>≤7</td>
</tr>
<tr>
<td>LIT-HP</td>
<td>VL</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>LIT-FCS</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>LIT-HP</td>
<td>Clinical conditions</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td>LIT-FCS</td>
<td>other than VL†</td>
<td></td>
<td>45</td>
</tr>
</tbody>
</table>

*Titres are expressed as number of the twofold serial dilution showing the highest titre starting at 1 : 100 (dilution 1) up to 1 : 262 144 00 (dilution 19).
†Non-VL conditions included healthy Sudanese female students (20), patients with leukaemia (10), typhoid (10) or malaria (5). Sera were tested at a starting dilution of 1 : 5 (dilution 1) up to 1 : 10 240 (dilution 12).
be ineffective should duration of shipment and clearance from customs last longer than 72 h.

Though incorporation of gamma-globulin-depleted sera is satisfactory, the costs involved in their production surpass that of FCS. In addition, despite promising results achieved by addition of growth stimulatory factors such as human urine, incorporation of these factors has not yet been routinely practised as sole replacement for FCS (Howard et al., 1991). It is worth mentioning in the light of this study that our current results provided an important complementary piece of information as to why, in addition to canines, humans are the mammals most susceptible to Leishmania infection. We also believe that, beside reduction in the costs involved in VL diagnosis, our results provide an answer to the important question as to whether promastigotes raised in culture media supplemented with serum other than FCS are suitable for use as antigens in serological tests for diagnosing leishmaniasis. In the current study, the LIT-HP derived antigen has demonstrated, similarly to the LIT-FCS standard, typical high DAT titres against VL sera as opposed to extremely low ones for sera from non-VL conditions. More trials are required to further validate use of promastigotes grown in alternative medium to serve as intact or homogenate antigen in VL diagnostic procedures other than DAT.

Although it is difficult to draw firm conclusions based on the limited number of sera in this study as to whether the antigen derived from LIT-HP alternative can definitively replace that of LIT-FCS standard medium in DAT, we can nevertheless assume that our current results are highly encouraging and deserve further confirmation. We also think that these results may very well stimulate further efforts to develop affordable techniques for in vitro cultivation of micro-organisms other than Leishmania in the financially less privileged countries.

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