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In Vitro Cultivation of Promastigotes of Iraqi Leishmanial Species in Serum-Free Liquid Medium Containing Human Urine

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A B S T R A C T

A new serum free liquid culture medium containing sterile fresh human urine was described for in vitro cultivation of Iraqi leishmanial promastigotes. The medium is simple to prepare, easy to purchase and successful to obtain bulk cultivation and maintenance of *Leishmania donovani*, *L. tropica* and *L. major* promastigotes.

Introduction

A variety of culture media have been designed for cultivating the promastigote forms of different leishmanial species (Berens *et al.*, 1976, McCarthy – Burke *et al.*, 1991, Kar, 1997, Bagrova *et al.*, 2012). There are three basic types of culture media: biphasic, semisolid and liquid. Biphasic and semisolid media need blood as one of their essential ingredients for isolation and maintenance of the parasites, whereas liquid media require foetal calf serum (FCS) or blood lysate for bulk cultivation and sustained cell multiplication (Berens and Marr, 1978, Steiger and Black, 1980, Ozbilgin *et al.*, 1995, Limoncu *et al.*, 1997, Somanna *et al.*, 2002). Cell culture in blood or FCS supplemented media has certain disadvantages. Biphasic media is susceptible to bacterial contamination and fail to support the long term in vitro growth of promastigotes to obtain larger number for various studies (Visvesvara and Garcia, 2002). Foetal calf serum is highly expensive, difficult to transport and unavailable when needed specially in developing countries (Gupta and Saran, 1991; Merlen *et al.*,1999). In this communication we describe a cheap, an autoclavable serum free liquid medium and easy to purchase when needed in countries where leishmaniasis is endemic.

Materials and Methods

Parasites and Growth Conditions

Promastigote forms of *Leishmania donovani* (MHOM/IQ/81/BRC), *L. tropica* (MHOM IQ/93/MRC1) *L. major*, originally isolated from cutaneous leishmaniasis patients, were

maintained routinely in modified Tobies biphasic (NNN) medium at 26°C as described (Chang and Hendricks, 1985). The composition of experimental media are given in Table 1. The pH of these media was adjusted to 7.4 with 1 N NaOH/HCl. Medium 199, RPMI – 1640 and individual ingredients (i.e. folic acid, and adenosine) were filtered through 0.22 µm Miller GV filters (Millipore). Medium HDI1, HDI2, HDI3 and HDI4 were generally sterilised by autoclaving at 15 psi for 15 min. After the culture medium was either filter sterilised or autoclaved, 100 U/ml of penicillin and 0.1 mg/ml streptomycin were added. Fresh urine was collected from a single adult male volunteer, cleared by centrifugation at 2000 g for 5 min and sterilised by passage through a 0.22 µm membrane filter. FCS was heat inactivated at 55°C for 1 hr. Biphasic cultured promastigotes in late log growth were sedimented by centrifugation at 4000 g for 10 min., washed twice with sterile Hanks' balanced salt solution (HBSS), and 0.5 ml of the inoculum containing 106 parasites/ml were introduced aseptically into 4.5 ml of experimental culture medium. Cellular growth was estimated by counting the parasites in a Neubauer chamber. Cellular viability was assessed, before and after incubations, by motility as well as by light microscopy observation using Trypan blue cell dye exclusion for promastigote forms. The generation time (G) of the parasite was determined as described by Kar (1997).

Results and Discussion

Typically promastigote forms of all species of *Leishmania*, when inoculated in to one of the experimental media to give initial concentrations of 105 cell/ml, were able to grow to late log phase in about 5-6 days in the presence of 5% human urine.Long lag periods, approximately 24-48 hrs were observed in early transfere from biphasic Tobies' medium to experimental media. These periods could reduced or eliminated by obtaining inocula from log phase populations. During the first transfere, it has been observed that the generation times were lower than previously reported values for *Leishmania*. The observed G values were 12.2, 11.4, and 11.8 in the early stationary growth phase, for *L. donovani*, *L. tropica* and *L. major*, respectively. By the second transfere, the generation time had increased to a relatively stable value depending on the medium and the leishmanial species used (Tables 2-4). It did not determine which components were responsible for the slightly better growth rate for *Leishmania* in experimental medium containing human urine, but it did seem to be due to supplementation with brain heart infusion(BHI) and peptone or BHI and folic acid. Significant stimulation of growth was found with 100 mg/liter (w/v) folic acid. Maximum numbers of parasite of approximately (1.9×107_ 3.8×107 cells/ml) were found in the early stationary phase after 6-7 days. Growth was observed also after 10 days incubation at 26°C. Reproduction of parasites in experimental medium and original Tobies' medium is shown in Tables 2-4. The morphology of cells grown in experimental medium were all demonstrated the typical elongate, flagellated promastigote morphology (Figure 1).

Several reports have indicated the requirement of inorganic salts, glucose and amino acids for the propagation of the leishmanial promastigotes (Steiger and Steiger, 1997, Chaudhuri *et al.*, 1986, Beltran *et al.*, 1988). In addition, purine as well as many vitamins and related growth factors including folic acid and hemin are essential for *Leishmania* (Steiger and Meshnick, 1977, Merino and Gabaldon, 1978, Melo *et al.*, 1985, Lemesre *et al.*, 1988).

Table.1 Composition of the Expermental Media

Component	HDI 1	HDI 2	HDI 3	HDI 4	M199	RPMI 1640	NNN
NaCl	0.8	0.8	0.8	0.8	0.8	0.8	0.8^{+}
KCl	0.04	0.04	0.04	0.04	0.04	0.04	0.04^{+}
$CaCl_2$	0.014	0.014	0.014	0.014	0.014	0.014	0.16^{+}
$MgCl_2.6H_2O$	0.02	0.02	0.02	0.02	0.02	0.02	
Na_2HPO_4	0.015	0.015	0.015	0.015	0.015	0.015	
KH_2PO_4	0.006	0.006	0.006	0.006	0.006	0.006	
$NaHCO_3$	0.035	0.035	0.035	0.035	0.035	0.035	0.02^{+}
Glucose	0.1	0.1	0.1	0.1	0.1	0.1	0.2^{+}
Yeast extract*	0.25						
Beef extract**	0.35	0.35	0.35	0.35			0.04^{++}
Bacto Peptone**	1.0			1.0			0.052^{++}
BHI*		3.7	3.7	3.7			
Folic acid		0.001			0.004	0.004	
Glutamine			0.1				
M199					1.0		
MEM Eagle					0.18	0.18	
RPMI 1640						1.3	
Agar							2.0^{++}
Adenosine					0.005	0.005	

* Oxoid London, United Kingdom.
** Gibco/BRL, Gaithersburg, MD.
\+ Liquid phase of NNN medium.
++ Solid phase of NNN medium, in addition defibrinated, heat inactivated and hemolysed (with equal volume of distilled water) rabbit blood was used.

Table.2 Cultivation of *L. donovani* Promastigotes in Different Media

Medium	No. of promastigotes* on day				
	2	4	6	8	G**
HDI 1	3.8×10^{6}	1.8×10^{7}	2.7×10^{7}	2.9×10^{7}	17.606
HDI 2	5×10^{6}	3.1×10^{7}	3.8×10^{7}	4.0×10^{7}	16.658
HDI 3	2.9×10^{6}	1.4×10^{7}	2.2×10^{7}	2.4×10^{7}	18.214
HDI 4	3.3×10^{6}	2.1×10^{7}	3.2×10^{7}	3.4×10^{7}	17.126
M199	4.5×10^{6}	3.6×10^{7}	9×10^{7}	9.8×10^{7}	14.67
RPMI 1640	5×10^{6}	5.5×10^{7}	6.4×10^{7}	6.8×10^{7}	15.44
NNN	9.6×10^{5}	7×10^{6}	8.1×10^{6}	9.6×10^{6}	22.7

*Initial inoculation, 105 promastigotes.
**Generation time on day 6.
M199 and RPMI 1640 medium were supplemented with 10% heat inactivated foetal calf Serum.

Table.3 Cultivation of L. *major* Promastigotes in Different Media

Medium	No. of promastigotes* on day				
	2	4	6	8	G**
HDI 1	5.6×10^5	5×10^6	1.08×10^7	1.1×10^7	19.737
HDI 2	2×10^6	1.1×10^7	1.9×10^7	2×10^7	19.027
HDI 3	1.6×10^6	8×10^6	1.15×10^7	1.2×10^7	21.043
HDI 4	1.25×10^6	1.05×10^7	1.35×10^7	1.4×10^7	20.350
M199	4×10^6	3×10^7	8×10^7	7.4×10^7	14.93
RPMI 1640	3.4×10^6	3.8×10^7	5.6×10^7	5.8×10^7	15.77
NNN	1.1×10^6	6.4×10^6	7.3×10^6	8.4×10^6	23.26

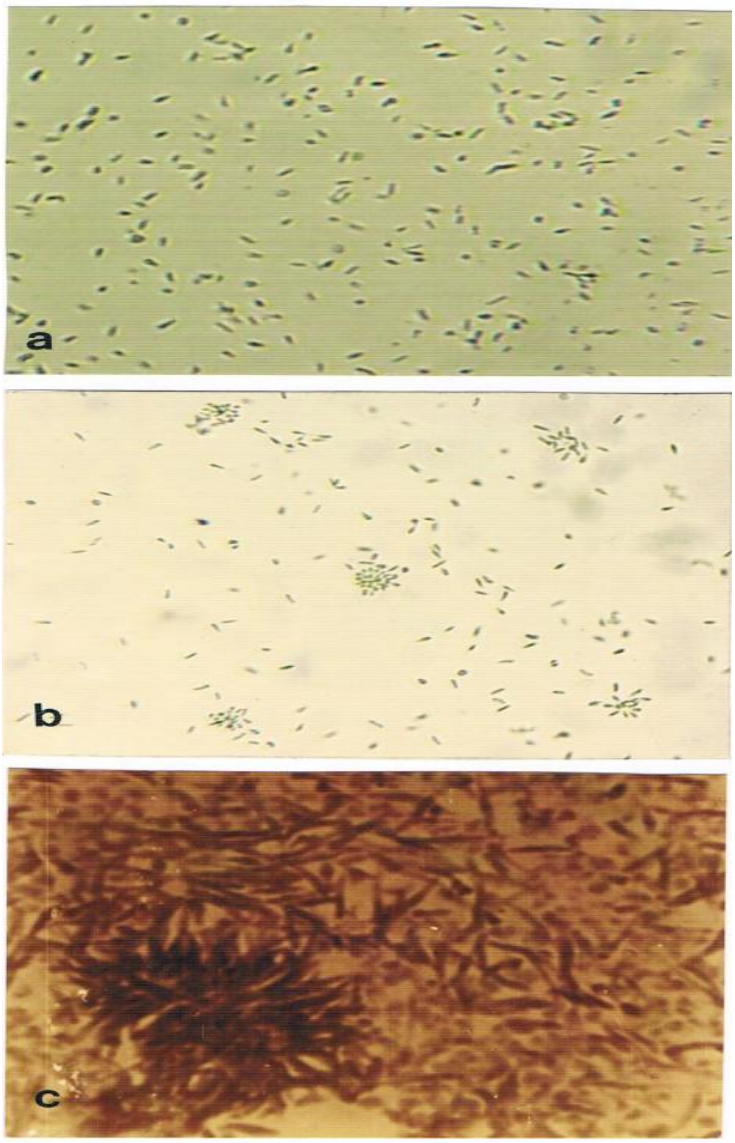
*Initial inoculation, 105 promastigotes.
** Generation time on day 6.
M199 and RPMI 1640 medium were supplemented with 10% heat inactivated foetal calf Serum.

Table.4 Cultivation of L. *tropica* Promastigotes in Different Media

Medium	No. of promastigotes* on day				
	2	4	6	8	G**
HDI 1	3.5×10^6	1.7×10^7	2.26×10^7	2.4×10^7	18.414
HDI 2	3×10^6	1.7×10^7	2.4×10^7	2.6×10^7	18.214
HDI 3	4×10^6	1.4×10^7	2.1×10^7	2.1×10^7	18.667
HDI 4	2.5×10^6	2×10^7	2.3×10^7	2.3×10^7	18.360
M199	3.4×10^6	2.7×10^7	6×10^7	6.4×10^7	15.6
RPMI 1640	4.2×10^6	4.6×10^7	5.6×10^7	6×10^7	15.77
NNN	1×10^6	6×10^6	7.8×10^6	8×10^6	22.91

*Initial inoculation, 105 promastigotes.
** Generation time on day 6.
M199 and RPMI 1640 medium were supplemented with 10% heat inactivated foetal calf Serum.

Figure.1 Growth of Promastigotes of L. *tropica* (400x) (a) L. *Major* (400x) (b) and L. *donovani* (1000x) (c) in HDI 4 Media Supplemented with 5% Human Urine



Several defined and semidefined liquid medium have been used for maintenance of leishmanias at 26°C and FCS favors the cultivation of promastigotes, as itis considered to be essential source for heme, which *Leishmania* unable to synthesize (Chang and Chang, 1985, Gupta and Saran, 1991).Fetal calf serum could be substituted by human urine to sustain the multiplication of many leishmanial promastigotes (Evans, 1986, Howard *et al.*,1991, Armstrong and Patterson, 1994) infering that the urine offers the advantage of being not only as mere source of a complex various reagents but also as free, easily available when needed anywhere. In the present study, we describe the feasibility of culturing three leishmanial promastigotes through serially propagated in HDI1, HDI2, HDI3 and HDI4 urine supplemented medium. Supplementation of beef extract and brain heart infusion(BHI), was found beneficial

for not only routine serial subpassages but also for providing large numbers of cells. However *L. donovani* shows a more rapid growth and a higher final density (3.8×107 cell/ml; G 16.65) than *L. tropica* (2.4×107 cells/ml; G 18.21) and *L. major* (1.9×107 cells/ml; G 19.02). Beef extract and BHI serves as major source for heme and amino acids repectively, which are essential for the survival of the parasite (Kar, 1997, Ali *et al.*, 1998). It is also noted that the supplementation of peptone, a source of amino acids, supported the propagation of the promastigotes. Glutamine in the course of this investigation, seems to be not essential for growth of promastigotes probably may be synthesized de novo by the parasite. The supplementation of proline was found to be prominent in the sustaining the multiplication of the parasite due to its use as a source of energy (Krassner and Flory, 1972). To compare our medium with other defined or semidefined media (Medium 199 and RPMI-1640) used for the cultivation of pathogenic hemoflagellate, we found that the culture inoculated at 105 cells/ml can last 6-7 days to reach log phase, whereas, in medium 199 and RPMI-1640 the parasite reach the log phase within 3-4 days postinoculation. Our medium are considerably simple to prepare, easy to purchase and may therefore be the mixture of choice in the future for studies dealing with the cell biology, biochemistry, nutrition, immunology and chemotherapy of pathogenic species of *Leishmania*.

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