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Growth Study of a Nonpathogenic Strain of *Leishmania donovani* with Different Nutrients

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ABSTRACT

The studies on growth pattern of a nonpathogenic *Leishmania donovani*, strain UR6, in different media showed that it can be regularly cultivated amd maintained in modified Ray's Medium (Agar) and three other liquid media, namely DME Medium, Medium 199 and RPMI-1640 which are manufactured in India. The well known N.N.N. Medium provided quantitatively poorer growth in comparison to these medium. Measurement of *L. donovani* cell concentration by optical density in a spectrophotometer has been worked out for expressing immunochemical observations on antigens in terms of cells per millilitre.

INTRODUCTION

Leishmania donovani, the etiological agent of kala-azar, has a digenic life cycle assuming an extracellular flagellated promastigote form in the alimentary tract of its sandfly vector and obligate intracellular form within the phagolysosomal system of spleen, liver and bone marrow macrophages of its definite mammalian hosts[1-3].

The promastigote form of most human leishmania species have been cultivated *in vitro* in various serum supplemented tissue culture media or in several chemically defined leishmania growth media[6-7]. In this paper, observations made on some readily available culture media or media prepared with indigenously available ingredients for the regular maintenance and growth of a nonpathogenic strain of *L. donovani*, are presented.

MATERIAL AND METHODS

Organisms : *L. donovani* (MHOM/IN/1978/UR6) used extensively for immunochemical experiments in India was kindly supplied by Dr. A. A. Bhaduri of Indian Institute of Chemical Biology (Calcutta). The strain was originally obtained from a patient of Calcutta School of Tropical Medicine[8].

Cultivation of cell : The strain (UR6) was grown in Rays medium[9]; modified to contain 1 per cent glucose, 1 per cent peptone, 0.6 per cent NaCl, 1.5 per cent agar powder; 50 per cent beef-heart extract with 3.3 per cent rabbit blood (pH 7.2). After 72-96 hrs. of incubation at 26°C in B.O.D. incubator, the cells were subcultured or harvested in cold phosphate-buffered saline (pH 7.2) containing 0.9 per cent NaCl, 0.25 per cent Na_2HPO_4 and 0.04 per cent NaH_2PO_4 (PBS).

The cells were also grown on liquid Ray's Media having the same composition but without agar-agar poweder.

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The other monophasic liquid media used for growth studies were medium 199, RPMI-1640, Dulbecco's Modified Eagle Medium (DME) with 20 per cent heat inactivated foetal calf serum. All these media were obtained from M/s. Hi-Media Laboratories, Bombay.

Another monophasic solid media, Brian-Heart-Infusion media (Hi-Media Laboratories) was also used for growth studies, in 3.7 per cent concentration. The biphasic media used for growth study was Novy-Mac Neal-Nicolle Medium (N.N.N.) adding 10 per cent defibrinated rabbit blood. This medium was also purchased from M/s. Hi-Media Laboratories, Bombay.

Growth study: The cells from each culture slants were suspended in 5 ml of cold PBS, and were washed three times with same buffer. The cells from the biphasic medium were harvested in the fluid media and were diluted with PBS. The culture growth in monophasic liquid medium was also diluted with PBS before cell counting or optical density measurements.

Total number of microbial cells per millilitre was calculated by haemocytometric method[10]. The growth obtained in different media was also measured by optical density (O.D.) of the respective suspensions in "Systronics" spectrophotometer Model No. 106, at 650 mm wave length with appropriate control and dilution factor.

RESULTS

Standard 2 mm loopful of 96 hrs growth was subcultured on to the respective media slants and 0.2 ml of 96 hrs growth was inoculated to 5 ml of respective liquid media. In this manner, serial sub-cultures/passages were made and the growth was quantified at each passage level by total microbial counts and optical density.

The total count of cells of *L. donovani* expressed per millilitre of original suspension/growth in liquid media is presented in Table 1. It is seen that the growth in modified solid Ray's Medium was good (10^6 or more cells/ml) at initial stge itself which further increased at third passage level and the growth was sustained even at 10th serial passage level on this medium. Brain-Heart-Infusion media (Hi-Media Laboratories) though produced initial good growth with increase at third serial passage level, the growth could not be sustained beyond fourth passage level. In liquid Ray's Medium too initial moderate growth (4×10^6 cells/ml) could not be maintained and decreasing growth through passages resulted to "no growth" in fifth passage. The growth in N.N.N. medium remained at 10^4 cells/ml even at ten serial passages. All the three liquid media-DME, Medium 199, RPMI-1640, however, produced good growth of 10^6 cells/ml or more and maintained the same upto ten serial passages.

The average optical density of the suspension having known total count of *L. donovani* have been shown in Table 2.

The optical density of 1 : 10 dilution of 7×10^7 cells/ml was 1.204 while the same for 1 : 20 dilution was 0.681. The growth in luqid medium 199, DME medium or RDMI-1640 having cell count of 6.8×10^6, 5.7×10^6 and 6.0×10^6 cells/ml,

Table 1—Growth of *Leishmania donovani* Promastigote Cells in Different Media after 96 Hours at 26°C Temperature

Media		Total count per millilitre of different passages									
Nature	Name	1	2	3	4	5	6	7	8	9	10
Solid blood Agar media	Solid Ray's medium (Modified	6×10^6	7×10^6	7×10^7	8×10^7	7.5×10^7	7×10^7	7.2×10^7	8×10^7	6.9×10^7	6.9×10^7
	Brain Heart Infusion Media	6.3×10^6	6.05×10^7	5×10^7	4×10^7	No Growth					
Liquid Monophasic Media	DME Medium	6×10^6	5×10^6	6.2×10^6	5.7×10^6	5.5×10^6	5.7×10^6	5.5×10^6	5.8×10^6	6.3×10^6	6×10^6
	Medium 199	7×10^6	7.7×10^6	7.2×10^6	6.7×10^6	6.5×10^6	5.7×10^6	6.7×10^6	6.5×10^6	6.5×10^6	5.7×10^6
	RPMI-1640	5×10^6	5.7×10^6	5.2×10^6	5.5×10^6	5.7×10^6	5.3×10^6	6×10^6	7×10^6	6×10^6	6.2×10^6
	Ray's Liquid Media	4×10^6	4.2×10^5	3×10^5	3.3×10^4	No Growth					
Biphasic Medium	Novy-MacNeal and Nicolle Medium	3×10^4	4×10^4	4.2×10^4	5×10^4	5.7×10^4	5.2×10^4	5.6×10^4	6×10^4	6.2×10^4	6×10^4

Table 2—Optical Density of *L. donovani* Promasigote Cells Suspension in Different Media

Media used	Cell Growth per Millilitre	Optical Density at 650 × mm Dilutions 1 : 10	1 : 20
Solid Ray's Medium (Modified)	7×10^7	1.204	0.681
Medium 199	6.8×10^6	0.073	0.030
DME-Medium	5.7×10^6	0.130	0.057
RPMI-1640	6.0×10^6	0.081	0.047

showed optical density of 0.073, 0.130 and 0.081 respectively (averge O.D. of 0.095) after 1 : 10 dilution. Thus, it is apparent that on an average 6×10^6 cells/ml diluted to 1 : 10, represented O.D. of 0.095 and when diluted to 1 : 20 the O.D. was 0.045.

Thus, it can be derived that:—
O.D. 1.205 = 7×10^6 cells/ml
O.D. 0.0681 = 3.5×10^6 cells/ml
O.D. 0.095 = 6×10^5 cells/ml
O.D. 0.045 = 3×10^5 cells/ml

The optical density measurement can be an easy method of assessing the cell concentration of *L. donovani* suspension used for the preparation or extraction of antigens.

DISCUSSION

Leishmania donovani (strain UR6) is used frequently for immunochemical studies, antigenic analysis and cell biology. The growth of the organism and to that extent, the chemical analysis of the antigenic components are influenced by the media in which the development and multiplication of the organism take place. Different media have been tried time to time, which have been reviewed by earlier workers[6-9]. Many of these media and the ingredient are not indigenously available. Yet, simplified media and the media promoting sustainable good growth are incontinous search. In this attempt, the present study indicate the usefulness of Ray's Media modified to contain easily available minimum chemical ingredients, shows the prospects of its utility in studies on antigenic analysis. Sustainable good growth on several passages would indicate the development of all chemical/antigenic constituents optimally in modified media. Large number of salts and aminoacids initially proposed in Ray's Media are under study, whether in the solid or in the liquid form. The commercially available three media attempted in this study also provided continued good growth through several passages, and interestingly the products manufactured in India could produce the desired growth, thus, providing a scope for dispensing with the imported products.

Measurement of growth or microbial concentration by counting of the organisms or determining the opacity is well known in microbiological techniques. Brown's opacity tube, Burrows wellcom opacity set, McFarlane opacity standards available commercially, are not useful for assessing the *L. donovani* concentration in a suspension. The present study is unique in its attempt to propose a standard of

optical density calibrated to denote known number of *L. donovani* cells per millilitre of suspension. This is particularly useful to express the concentrations of different fractions and extracts obtained from a known suspension in terms of number of cells per millilitre. it is expected that this would facilitate the expression of results of chemical analysis in terms of number of cells of *L. donovani* per millilitre instead of expressing per mg nitrogen. However, further studies in this direction are warranted.

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Microscopic Agglutination Test in Detection of *Leishmania donovani* Antibodies

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Kala-azar (visceral leishmaniasis) is one of the important health problems in the eastern states of India. Several serological methods have been developed from time to time for specific and sensitive diagnosis of the disease. However, researches are continuing for developing a simple, specific, sensitive, low cost test for serodiagnosis of kala-azar. This paper presents the observations on the microscopic agglutination test (MAT) of promastigotes of *Leishmania donovani*.

L. donovani (MHOM/IN/1978/UR6) grown on modified Ray's medium[1], (Majumder and Sen, 1990) for 72 h at 25C and suspended in phosphate-buffer-saline to have cell concentration of 7×10^7 cells/ml was used as antigen. One loop full of this suspension was mixed with equal volume of heat inactivated *anti-L. donovani* rabbit serum and spread over microscopic slide and covered with coverslip. After 3 minutes, the slide was examined under high power (40X) microscope, when it was observed that *L. donovani* cells had become immobilized and clumped together forming a rosette with spindle shaped elongated cells converging mostly at one point (Fig. 1). Similar test when performed with heat

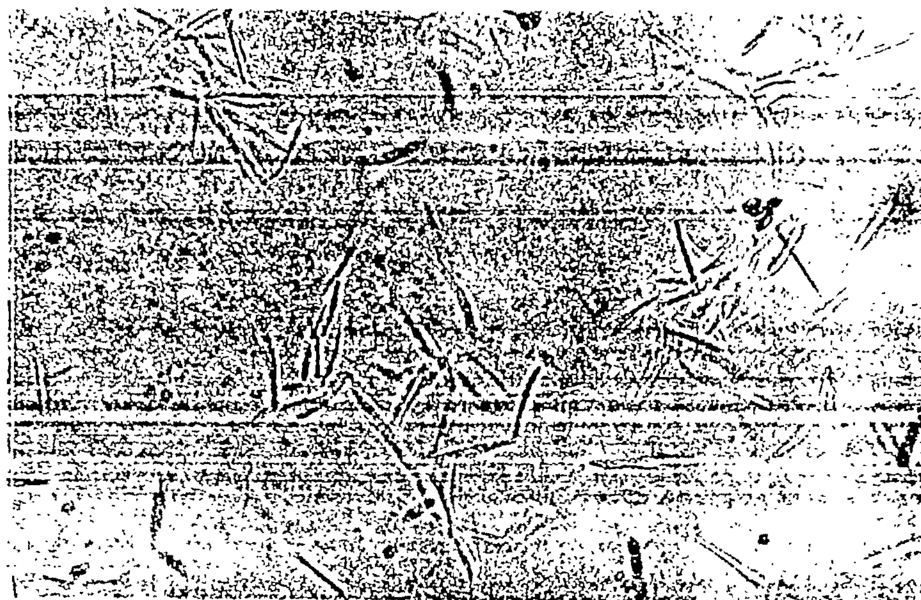

Fig. 1. Microphotograph showing agglutination of promastigotes in rosette forms with elongated parasites converging at one point (X40).

inactivated normal rabbit serum revealed mobile *L. donovani* promastigotes. The heat inactivation might have destroyed the factors in normal rabbit serum which otherwise would have immobilized *L. donovani* promastigotes[2]. The degree of agglutination was recroded by '++++' = 4 or more clumps per microscopic field, '+++' = 3 clumps; '++' = 2 clumps; '+' = 1 clump and '-' no clumps of *L. donovani* cells. The highest dilution of the hyperimmune serum showing '++++' reaction was noted at 1:40 and at 1:160 dillution of the hyperimmune serum, there was no agglutination of *L. donovani* cells.

The test described is simple and can be performed by any laboratory maintaining avirulent strain (promastigote) of *L. donovani*.

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17. Biochemical Characterization of Antigens from *Leishmania donovani*.

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Key words : *Leishmania donovani SDS-Polyacrylamide-gelelectrophoresis.*

The surface and cellular moeities isolated by different methods from a nonpathogenic strain of *Leishmania donovani* have been characterized by qualitative and quantitative Chemical assessments. Qualitatively, all the isolates were protein in nature, mostly of heat coagulable type. the molecular pattern by SDS - Polyacrylamide gel-electrophoretic Study indicated variable heterogenity and homogenity between different proteins isoluted from the organisms. The immunological studies of the fractions of known molecular pattern might throw light on existence of possible non - human sources of the parasite during inter - epidemic period.

(71.) Inhibitition of Motility as a Tool for detection of *Leishmania donovani* Antibodies

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Key words : *Leishmania donovani; Antibody detection; Motility inhibiition; MAT*

The motility of a microbe can be inhibited by specific flagellar antibodies. This principle has been applied to develop a test for detection of *Leishmania donovani* antibodies. When one drop of the serum containing *L donovani* antibodies was mixed with one drop of 5-days-old culture-growth suspension of the microbe containing $2x10^5$ cells ml on a slide, appearence of 4-5 clumps of the microbes with inhibition of motility was noticed under lowpower of microscope. Autoagglutination by normal rabbit serum was inhibited by healting at 56°c for 30 minutes. The field serum samples from clinical Kala-azar patients specifically inhibited the motility. The test is therefore termed as Microscopic Agglutination Test, (MAT).

24. Immunological Characterization of antigen isolated from a non-pathogenic strain of *Leishmania donovani*

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Key words : *Leishmania donovani ; Plate Agglutination Inhibition Test (PAIT) Microsocopic Agglutination Inhibition Test (MAIT).*

Wholecell particulate antigen and soluble antigens prepared by Autoclaving, Sonication and Autolysis; followed by chemical fractionation were studied by *in vitro* inhibition of microscopic agglutination and plate agglutination tests, and by allergic tests in presensitised rabbits. Besides formolised 'R' antigen reacting in MAT-inhibition and in the PAT-inhibition tests, autoclaved supernatant reacted well in microscopic agglutination Inhibition Test (MAIT), indicating that these antigens contained the properties of 'H' or flagellar antigen. The autoclaved supernatant, sonicated supernatant, and 25% sodium chloride extract of sonicated residual pellet inhibited the plate agglutination test, indicating there "spatial" distribution on the plate agglutination test, indicating there "spatial" distribution on the surface of the cells of *L donovani* and associated with the production of humoral antibody. Sonicated supernatant, when used as an allergen in sensitized rabbits, produced best hyperaemic delayed hyper sensitive reacting indicating thereby its association in the production of cell mediate immunity (CMI) and IgE.

67. Leucocyte Migration Inhibition Assay of different soluble antigen isolated from *L. Donovani*

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Key words : *Leishmania Donovani, Leucocyte Migration Inhibition test (LMIT)*

Cellular hypersensitivity of the Soluble Autoclaved Supernatant (SAS) antigen of *Leishmania donovani* promastigotes was studied by performing Leucocyte Migration Inhibition Test (LMIT). The sensitized lymphocytes on SAS antigenic stimulation produced more inhibitory effect than SSS (Soluble Sonicated Supernatant) antigen.