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In Vitro new experimentally culture media for Leishmania species cultivation

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Abstract :

A fifty four different biphasic media were used to cultivate promastigote parasite of *Leishmania tropica* and compared with ordinary NNN medium, to find a cow milk agar medium can provide a good cultivation to the parasite as well as the ordinary medium, where milk is a cheap and simple, and a good source of lipids, protein, carbohydrate, and calcium. Also, using a 20% of human urine, can enhance the growth rate of parasite, this reduced the time that needed to cultivate the parasite in the ordinary NNN medium. So, using of a modified NNN medium (medium that used the solid phase of ordinary NNN and 20% of human urine as liquid phase) in the field of laboratory can provide a short duration to cultivate *Leishmania* parasite. The count of *Leishmania tropica*, promastigotes taken from NNN medium reached, 3×10^6 /ml at the end of the 6th day in our new medium, while in NNN medium the number of organisms reached only 1×10^6 / ml. also 9.7×10^7 promastigotes found after the fourteen days of cultivation in media supplemented with urine and 22×10^6 promastigotes in the tenth days in media with milk. After several passages, the cultured medium, prepared was evaluated as being quite simple, inexpensive, and successful compared with other commercially available culture media.

المستخلص

تم استخدام خمس واربعون وسط زرع مختلف لاستنبات طفيلي اللشمانيا الاستوائية وقورنت النتائج بالوسط المتعارف عليه. ووجد ان استخدام حليب الابقار كإضافة للوسط الزرع الاعتيادي أدى الى الحصول على نتائج جيدة لاستنبات الطفيلي حيث ان الحليب ارخص ثمنا واسهل في الحصول عليه كذلك ان الحليب مصدر غني بالدهون والبروتين والكربوهيدرات والكالسيوم. كما ان استخدام 20% من ادرار الانسان يمكن ان يكون عامل مشجع لنمو الطفيلي، كما انه يختصر الزمن اللازم المطلوب للنمو في الاوساط الزرع الاعتيادية. إذن ان استخدام الوسط الزرع المحور (الوسط المكون من الجزء الصلب مع إضافة 20% من ادرار الانسان من خلال الجزء السائل) في حقل التجارب المختبرية للحصول على وقت قصير للاستنبات. ان عدد طفيلي اللشمانيا الاستوائية المستحصل من خلال استخدام هذه الاوساط كان 3×10^6 لكل مل في نهاية اليوم السادس من الاستنبات بينما كانت الاعداد المستحصلة من الاوساط المضاف لها الادرار والحليب هو 9.7×10^6 /مل في اليوم الرابع عشر من الاستنبات للوسط المضاف له الادرار بينما كان العدد 22×10^6 بعد اليوم العاشر للزرع مع إضافة الحليب للوسط. بعد عملية إعادة الزرع المتكرر وجد ان الاوساط الجديدة كانت بسيطة جدا وغير مكلفة وناجحة للاستنبات ومتوفرة واقتصادية

Introduction

A variety of media used for culturing of *leishmania*. These can be divided into three, main types; liquid; semi-solid; and biphasic, . While semi solid and biphasic, culture media need blood, an important factors for the reproduction of parasites, most of liquid media required erythrocyte lysate or fetal calf serum (FCS)⁽¹⁾. handling biphasic media is more technical demanding than handling liquid media, which were more suitable for the mass culture of *Leishmania*. biphasic media are strongly recommended for initial isolation of *Leishmania* parasites . Additionally; there were evidence that biphasic media is more favorable for the infectivity of *Leishmania* parasites ⁽²⁾. Other studies has been showed the stimulatory effect of human urine on *Leishmania* promastigotes, when supplemented with human urine, Schniders Drusophila ,culture media was found to increase the proliferation of different *Leishmania*, parasite sp. and it was fund that culturing amastigotes which isolated from *Leishmania* infected mice in a culture environment containing urine increase promastigote proliferation, differentiation compared with controls⁽³⁾. Tyndialized milk of cow, goat, and, buffalo was found to be a substitute for fetal bovine serum (FBS) ,in the medium for the cultivation of *L. donovani* promastigotes⁽⁴⁾. Excessive production of promastigotes and long, term cultivation depend largely on the serum and serum components present in the culture medium ,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 by using milk and urine, as far as, commercial procedures are concerned has. been tested for *in vitro*; cultivation of *Leishmania*. species.

Material and method

Preparation of Media:

Lesion aspirate obtained previously from patients attended to Salah Al-deen hospital and diagnosed clinically as cutaneous Leishmaniasis by Dermatology consultant were cultured into serial of fifty four tubes of three types of media:

- 1- The first group tubes containing NNN media: It's consist of solid and liquid phase, this media used for cultivation and continuation of promastigotes .stage of *lieishmania* and used fore the first time by Kagan and Norman⁽⁵⁾.
- 2- The second group tubes of media containing NNN media with Fresh healthy human Urine and made sterile by passing through 0.22 μ M filter paper .then the urine added in the medium tubes(instead of FCS. or De fibrenated rabbit blood) in percentage 20% of media⁽⁶⁾.
- 3- The third group of media tubes of media containing NNN media with 0.5g of milk which dissolved in distal water then filtered ,pH value fixed at 7.2 and autoclaved at 121C° for min⁽⁴⁾.

Then added for all medias antibiotics
0.2 ml of mixture of penicillin plus
streptomycin solution ,nystatin 250
I.U/ml⁽⁷⁾

Promastigotes were counted with a
hemocytometer slide from the 6th day to
the 14th day of culture time Furthermore,
promastigotes produced in the culture
media were cultivated in new culture
media consequently, and thus, continuity
of the passages was also kept under
control⁽¹⁾.

Results

As shown in table 1, the mean number
of *leishmania* promastigotes in NNN
media was 1×10^6 at the sixth day of
culture and reach to 6×10^6 after fourteen
days of culturing, while reach 9.7×10^7 at
the fourteenth days of culturing in NNN
supplemented with urine ,in the other
hand the highest number of
promastigotes found to be 22×10^6 at
tenth days of culturing in NNN plus
milk.

Table (1):- Reproduction of *Leshmania* promastigotes in NNN medium, NNN plus urine and NNN plus milk.

Types of Media	Mean No. of promastigotes /days								
	6	7	8	9	10	11	12	13	14
NNN	1×10^6	1.35×10^6	2.4×10^6	2.9×10^6	3.2×10^6	3.8×10^6	4.4×10^6	5.7×10^6	6×10^6
NNN+Urine	3×10^6	1×10^7	1.7×10^7	2.3×10^7	5.5×10^7	7.8×10^7	8×10^7	8.4×10^7	9.7×10^7
NNN+Milk	8.1×10^6	5.2×10^6	11×10^6	12×10^6	22×10^6	19×10^6	16×10^6	13×10^6	9×10^6

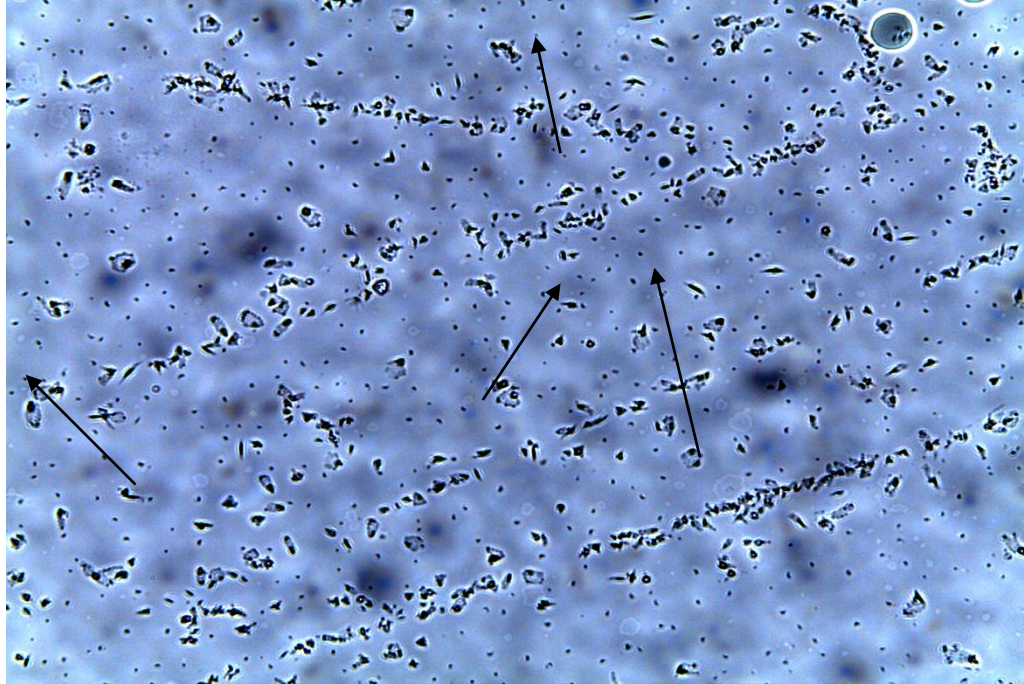


Fig.(1): Direct smear from NNN culture shows promastigote of *leishmania* (40X)

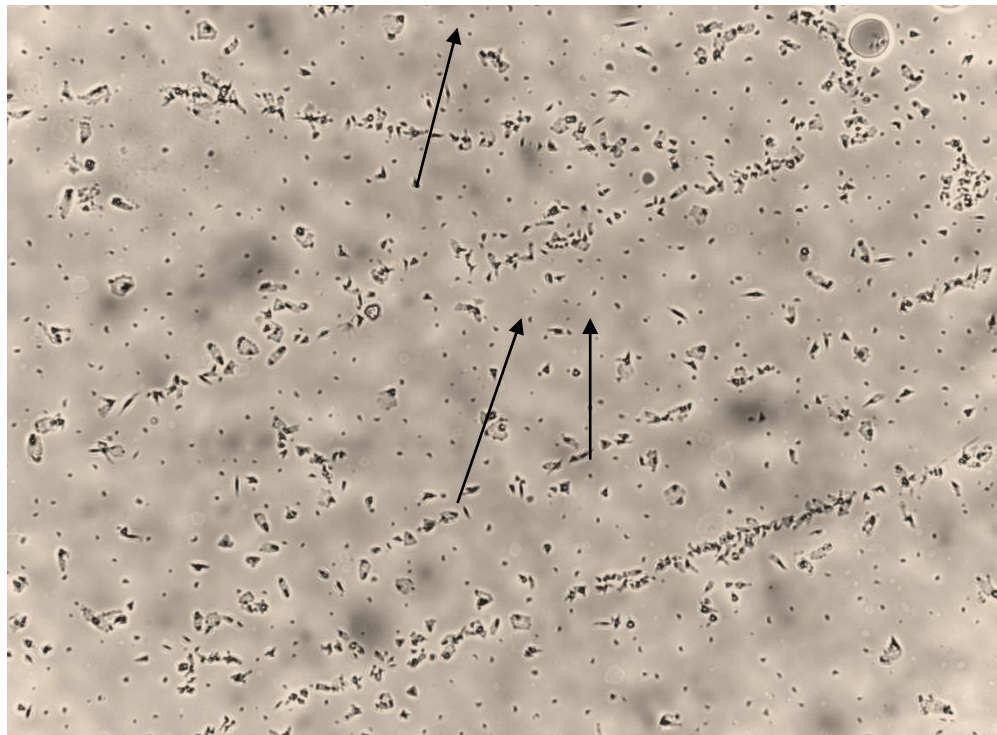


Fig.(2):- direct smear from NNN plus milk culture (40X)

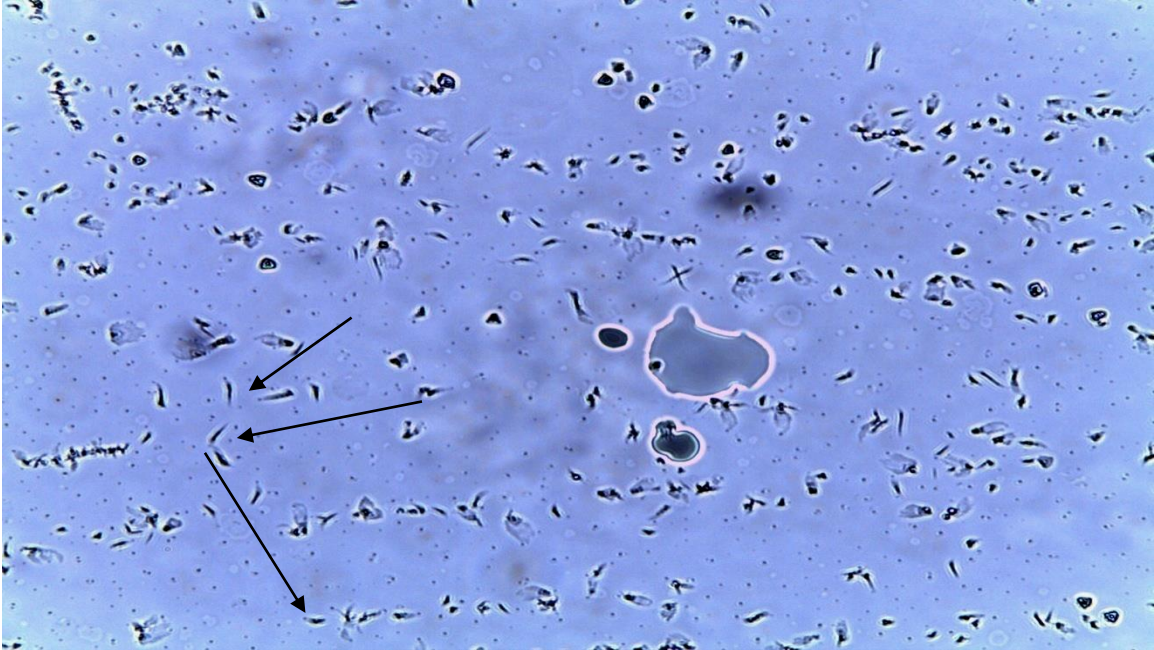


Fig.(3):- direct smear from NNN plus urine culture(40X)

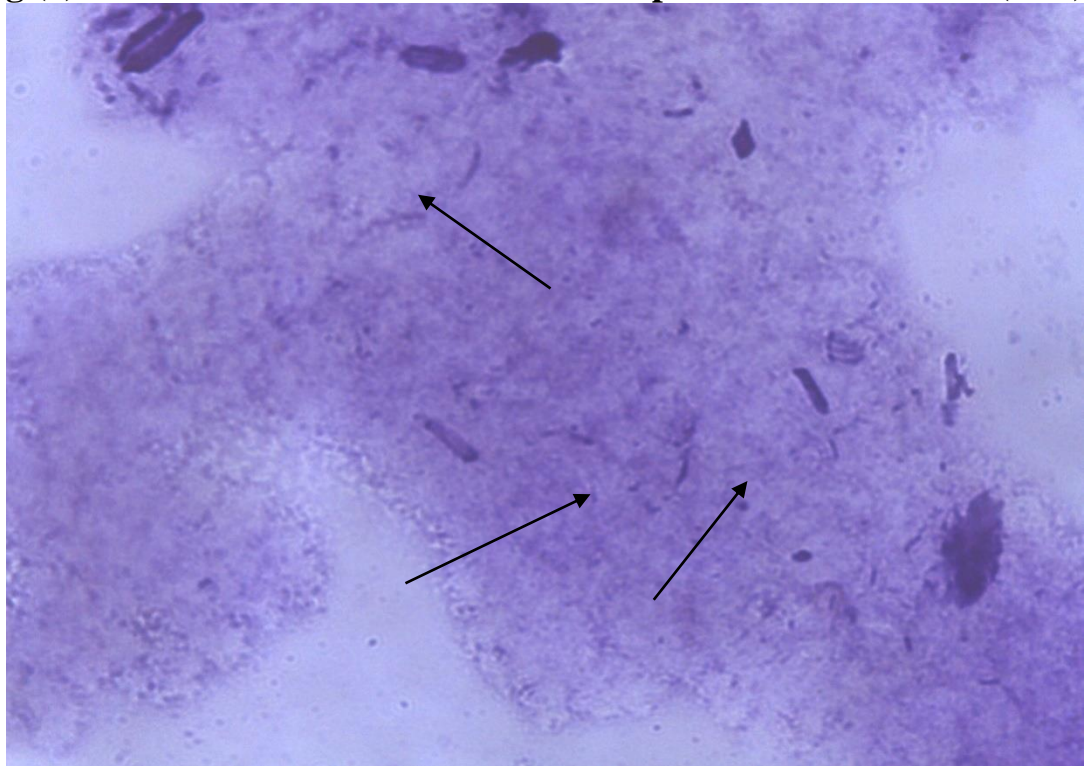


fig.(4):Direct Giemsa stained smear from NNN plus urine culture(1000X)

Discussion

The current study describes a relatively simple formulation using common, inexpensive, available ingredients that can be used in place of serum, supplemented media for *in vitro*, maintenance and mass cultivation of *Leishmania*, promastigote Forms⁽⁸⁾. Different formulations were tested, but the best results were obtained with NNN with healthy human urine medium, as judged by a faster ratio of proliferation, higher final cell density, and ability to culture most *Leishmania* species⁽⁹⁾. This completely Defined medium without serum and-or micro molecules as a serum substitute supports the continuous growth of *Leishmania*, species at rates comparable with those obtained with serum-supplemented medium and due to its easy and low price preparation, NNN cultured medium is especially utilized in the production of parasites obtained through skin biopsy. However, for many studies to be made with *Leishmania*, isolates, biphasic cultured media with supplements producing a large number of promastigotes, in a short time, are need⁽¹⁰⁾. we successfully Adapted and optimized NNN with the urine and NNN with milk biphasic medium originally developed for the cultivation of *Leishmania*, and mammalian host interactions, for the analysis of promastigotes for obtaining *Leishmania* related biological material, and for primary isolation of *Leishmania* strains. The yield of parasites is one of the most important parameters which determine the applicability of a medium for mass cultivation. The NNN with urine allowed reaching the parasite to 9.7×10^7 promastigotes after two weeks of cultivation compared with 6×10^6 and 9×10^6 for

the same time in NNN and NNN with milk respectively. In another study Allahverdiyev, *et al.* found that human urine in parasite culture affected the proliferation and infectivity of all four types of *Leishmania* parasites that were investigated *in vitro*⁽³⁾, also Howard, estimated that the addition of 1-5% urine to Schneider's Drosophila medium containing 10% fetal calf serum enhanced the growth of 11 *Leishmania* strains representing 8 different taxonomic groups⁽¹¹⁾. Using milk as a sample, cheap and a good source of protein, carbohydrate, lipids and calcium, in *leishmania* cultivation gave a good alternative of serum supplement to the culture and this result agrees with Muniaraj, study⁽⁴⁾ and Lei, study⁽¹²⁾. The present study demonstrates clearly that a completely defined culture medium for the mass culture and maintenance of these important pathogens is a reality. All the advantages, described here will be particularly important for researchers, where FCS is expensive, and difficult to purchase, transport, and where the facilities for cryopreservation are not present. In addition, serum free technology will be increasingly important in providing stability and reproducibility as research using promastigote forms moves closer to therapeutic applications.

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