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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-Nicolle (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.

المستخلص

اجريت هذه الدراسة بمعهد الامراض المتوطنة، جامعة الخرطوم خلال الفترة من يناير 2007 إلى ديسمبر 2007م. قارنت الدراسة بين دم الحصان و دم الارنب كمكون للوسط الغذائى Novy-MacNeal-Nicolle (NNN)بالإضافة لإستخدام الوسط الغذائى Lowestein Jensen (LJ) لتزريع طفيل اللشمانيا. تم تزريع الطفيل فى انابيب تحتوى على تراكيز مختلفة من دم الحصان (5%, 10% and 20%) و اخرى تحتوى على دم الارنب بتركيز (10%) كما تم تزريعه فى الوسط L J. كل الانابيب حضنت لمدة 48 ساعة فى درجة حرارة 23 درجة مئوية. وتم حساب اعداد الطفيل كل 48 ساعة.اظهرت هذه الدراسة أن معدل نمو اللشمانيا يكون فى الوسط NNN الذى يحتوى على دم الحصان (خاصة التركيز 10%) و دم الارنب. دم الارنب اظهر نمو افضل من دم الحصان. و لم يحدث النمو فى الوسط LJ.

***KEYWORDS**:*Novy-MacNeal-Nicolle 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 subphylumMastigophora and family Trypansomatidae. 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 WesternUpper 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 uninhabitedDinder 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 Darfurand 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 23ºC.

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 121ºC for 15 min. The agar was allowed cooling to about 50ºC 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 ºC 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 L.J 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 ºC in a cooled incubator at 23 ºC 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.

The absolute number = $\frac{N \times D.F}{area \times depth} / cumm$

$\frac{N \times 4 \times 25}{1 \times 0.1} / cumm$

$= \frac{N \times 10^2}{1 \times 0.1} = N \times 10^3 / cumm$

= N X 10^6/mL

First dilution

The first dilution was done by adding few promastigotes (100µl) to 13900µl of complete media (14 X 6^{10} parasite/mL). From the mixture, 200µl were subcultured in NNN media and LJ media.

Second dilution

The second dilution aimed to increase the parasite growth and was done by adding 400µl of promastigotes to 1600µl of complete media (400 X 6^{10} parasite/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% horse blood within 5 days were 48 $x10^6$ /ml, 58 $x10^6$ /ml, 87 $x10^6$ /ml, 169 $x10^6$ /ml and 118 $x10^6$ /ml in the first, second, third, fourth and fifth day respectively (table 2). The results obtained by counting promastigotes in NNN that contains 20% horse blood within 5 days were 51 $x10^6$ /ml, 53 $x10^6$ /ml, 73 $x10^6$ /ml, 110 $x10^6$ /ml and 117 $x10^6$ /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, 494$x10^6$ /ml, 618 $x10^6$ /ml and 627 $x10^6$ /ml in the first (1^{st}), second (2^{nd}), third (3^{rd}), fourth (4^{th}) and fifth day (5^{th}) 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 6^{10} parasite/mL (first dilution)

Media	**Parasite count**
NNN media contains horse blood (5%, 10%, and 20%)	0.00 (No growth)
NNN media contain rabbit blood	2 X 10^6/mL
L.J medium	0.00 (No growth)

Table 2: Inoculation of 400 X 6^{10} parasite/mL (second dilution)

Media	**Parasite count (Number of parasite cells/mL)**				
	1^{st} day	**2^{nd} day**	**3^{rd} day**	**4^{th} day**	**5^{th} day**
NNN media contains 5% horse blood	$42x10^6$/mL	$79x10^6$/mL	$155x10^6$/mL	$107x10^6$/mL	$110x10^6$/mL
NNN media contains 10% horse blood	$48x10^6$/mL	$58x10^6$/mL	$87x10^6$/mL	$169x10^6$/mL	$118x10^6$/mL
NNN media contains 20% horse blood	$51x10^6$/mL	$53x10^6$/mL	$73x10^6$/mL	$110x10^6$/mL	$117x10^6$/mL
NNN media contains 10% rabbit blood	$121x10^6$/mL	$466x10^6$/mL	$494x10^6$/mL	$618x10^6$/mL	$627x10^6$/mL
L.J. media	$3x10^6$/mL	$1x10^6$/mL	$0x10^6$/mL	$0x10^6$/mL	$0x10^6$/mL

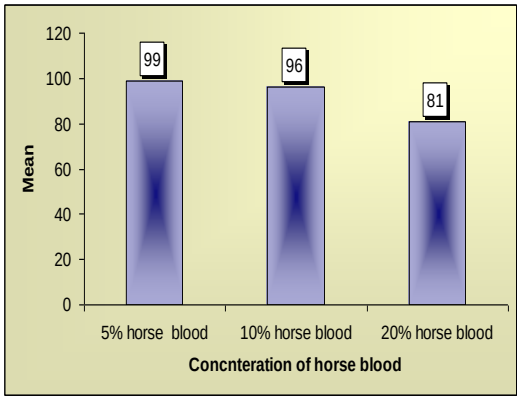

Figure 1: Mean of parasite count by different concentrations of horse blood

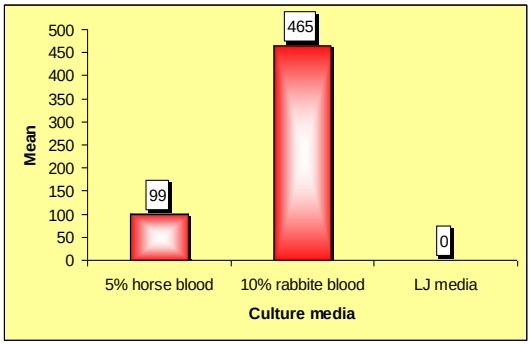

Figure 2: Mean of parasite count by different culture media

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 Lowstein Johnson (LJ) media, the best culture media for *Mycobacterium* cultivation) 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 L.J 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) [29] and started to decline in the fifth day. This might be due to consuming of needed materials in the culture and contamination.Thepresent 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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