

COMPARING THE EFFECT OF MODIFICATION IN- NNNCULTURE MEDIUM FOR *LEISHMANIA* SPECIES USING DIFFERENT MAMMALS

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ABSTRACT

The efficacy of the in vitro cultivation of promastigotes of three *Leishmania* spp. were tested in modified the biphasic Novy-MacNeal

Nicolle (NNN) medium prepared using blood from different mammals (human blood group O , horse, donkey, goat and sheep).

This study was carried out to determine which modification of NNN media will be gave the best yield in the shortest time for different parasite species, in order to obtain a large crop of promastigotes for experimental work and for antigen preparation. Promastigotes of the three main parasite species *Leishmania donovani*, *Leishmania tropica* and *Leishmania major*, isolated from patient in Baghdad and cultured, at equal numbers, in the 5 different NNN preparations. At the end of the 7th day, the NNN medium using horse blood produced the greatest number of promastigotes for all *Leishmania* spp. tested, 27,15,33x10⁶ whilst goat blood provided the poorest medium, providing culture results only for *L. donovani*, 7x10⁶. Human blood group O give good results, 9.2, 5.4x10⁶. Donkey blood 6.5, 2, 1 X10⁶. Then Sheep blood gave 4.5, 2.5 X10⁶, This finding may be explained by the fact that *Leishmania* is a nicotinamide adenine dinucleotide (NAD) auxotroph and horse erythrocytes support NAD-dependent microorganisms



INTRODUCTION

More than 30 *Leishmania* species (Protozoa, Trypanosomatidae) are known worldwide, 21 of which may be transmitted to humans by the bite of infected female phlebotomine sandflies (Diptera, Psychodidae) (1) causing leishmaniasis. Even though Leishmaniasis is found in 98 countries worldwide (2), they are classified as Neglected Tropical Diseases (NTD) (3). Since the spread of the parasite and its vectors to new areas, due to factors like climatic changes and human activities, pathogenicity and drug resistance as well as for the development of new drugs. Most of these studies require a huge number of parasites, which are produced by cultivation *in vitro* using different culture media (4).

The most common culture medium used for parasite isolation from biological samples and sandflies is the biphasic Novy-MacNeal-Nicolle (NNN). Yet, it is not always easy to produce a good number of promastigotes in a short time, for all species, something vital in research regarding many aspects of the parasite and the disease.

NNN medium, usually prepared using rabbit or sheep blood is considered effective for the isolation of parasites from biological

Samples although often fails to produce a great number of promastigotes in a short time (5). In order to develop a medium which would be the most suitable for the

isolation of the largest possible number of strains in order to be practical and not to miss out strains that are new in the lab/country/area.

MATERIAL AND METHODS

Parasites

Amastigote of, *Leishmania major*, and *Leishmaniatropica* were used in this study, isolated from patient with a cutaneous lesion newly introduced during an epidemiological survey conducted in Baghdad, while

L. donovani was isolated from a patient with visceral disease from bon marrw, cultured in NNN media at $26C^{\circ} \pm 1 C^{\circ}$. The development of promastigotes was checked every 2 days(6). Promastigotes were used for inoculation on day three after the second passage(15).

Blood

Blood samples from four different mammals - donkey, horse, sheep and goat e were used to prepare the NNN biphasic culture medium. Blood originating from four to five animals of each species were used for the preparation of each kind of NNN in order to avoid factors related to animal's health condition which may give misleading results. All animals were healthy and originated from non-leishmaniasis endemic regions(7).

The blood samples were mechanically defibrinated under sterile conditions, using a cone-shaped flask containing glass beads which was shaken at low speed, for 20 min, at room temperature (8)

Preparation of NNN medium

For the preparation of the NNN culture medium: 16 g/L blood agar, D-glucos 8 g/L, antibiotic gentamycin 1.5ml, 900 mL dH₂O were added. The mixture in a glass flask and autoclaving at $121C^{\circ}$ for 30 sec, after which the defibrinated animal blood (150 ml/L) was added. The NNN tubes were prepared and kept at 4 _C for at least 2 days before they were used for parasite inoculation. Prepar liquid phase (loks solution)(8).

Inoculation and incubation of promastigotes

Each of the four *Leishmania* strains were inoculated into three tubes of each different NNN culture medium. The inoculum consisted of 0.5 ml, containing 200,000 stationary-phase promastigotes. They were incubated at 26 ± 1 C° for a total period of 2 months. The proliferation potential of the parasites in the NNN culture media containing blood of five different mammals was assessed on day 7, day 14 and then checked for live parasites at the end of month 1 and month 2. All cultures were performed in triplicate. The experiment was repeated twice and the results were confirmed.

Light microscopy

To estimate the parasite population on day 7 and day 14, 0.1 ml of the liquid medium in the NNN tube (nearly all liquid phase of the culture). The number of parasites per ml was estimated by counting the parasites using a Thomas Slide after immobilizing the promastigotes with 10% formaldehyde, to check the viability of the parasites so only live parasites were taken into account (9).

- **Statistical Analysis**

The Statistical Analysis System- SAS (17) program was used to effect of difference group in study parameters. Least significant difference –LSD test was used to significant compare between means in this study.

RESULTS

After 7 days of culture, all three *Leishmania* spp. developed only in the NNN medium containing horse blood; which provided, by far, the best crop compared to the others tried (Table 1). Human blood group O produced cultures lower numbers than horse blood. Donkey and sheep blood produced cultures, in lower numbers and for some species they required longer than 7 days to appear. Goat blood NNN exhibited the poorest results providing parasites only of *L. donovani*; the other species remained negative after two months of culture. *L. donovani* developed in all four NNN mediums tried, but provided best yield in horse NNN whilst *L. Tropica* culture became positive after 14 days in NNN using donkey and sheep blood. The proliferation ratio of *L. major* in horse blood NNN was 2.1 times higher to that of *L.*

tropica, 1.9 to that of *L. donovani* (Table 1). The positive cultures kept producing Leishmania parasites after two months. Horse blood NNN was successfully used for parasite culture three months after preparation. Horse blood (defibrinated). Significance was defined as $P \leq 0.05$.

Table 1: The proliferation potential of three *Leishmania* spp. in five different NNN media using blood of different mammals, during a seven day period. Initial inoculum in all cases .200,000 stationary -phase promastigotes.

NNN media	<i>L. donovani</i> X10 ⁶ / ml	<i>L. tropica</i> X10 ⁶ / ml	<i>L. major</i> X10 ⁶ / ml
Horse	27±2.04	15± 0.77	33± 2.09
Human blood group O	9.2± 0.83	5± 0.65	4± 0.06
Donkey	6.5± 0.52	2± 0.05	1± 0.008
Sheep	4.5± 0.30	2.5± 0.07	- ^a
Goat	7± 0.44	-	-
LSD value	4.0528*	3.603*	4.794
*CP<0.05			

^aPromastigotes appeared after 14 days of culture. The cultures which were negative (-) remained negative after two months of culture..

DISCUSSION

The NNN culture medium is widely used for isolating and culturing *Leishmania* parasites for many clinical and laboratory purposes. It is easy to prepare, can be kept in the freeze for over a month before use, if necessary, and it costs very little to make. The most common NNN culture medium used is prepared using sheep blood, which can be obtained commercially. The only drawback of this culture medium is its weakness to provide good, healthy parasites of many different species, in a short time (10). This study was carried out to prepare an NNN culture medium which would be suitable for the three main *Leishmania* spp. found in the Old World, that is: *L. donovani*, *L. tropica* and *L. major*; the first two causing visceral and the other two cutaneous disease. The horse blood NNN proved the most suitable for all three *Leishmania* spp. and yielded, by far,

The highest number of parasites after only one week incubation. Human blood group O NNN was the second most efficient allowing the production of promastigotes of three *Leishmania* species. in 7 days and the fourth after 14 days of culture; but it

provided considerably lower numbers compared to the horse blood NNN (Table 1). Donkey and Sheep blood NNN preparation allowed the propagation of all 3 *Leishmania* spp. but after 7 days for *L. tropica*, and 14 days for *L. donovani* and *L. major*; and in considerable lower numbers compared to horse blood. Goat blood allowed only the growth of *L. donovani* promastigotes, which appeared 7 days after inoculation, but the other parasite species did not grow in this medium, even after 2 months. From our findings it appears that horse blood is the most suitable of the ones tested in providing a high number of parasites in a short time for all three *Leishmania* spp.

Horse blood is relatively easy to obtain in large quantities in most countries, compared to other smaller animals as rabbits whose blood is also used for the preparation of NNN culture medium. Rabbit blood is collected either from a rabbit's ear artery or from the animal's heart.

The blood from the ear of one rabbit is enough to make only two to four NNN tubes (depending on the size of the animal), whereas it is usually a terminal procedure taking the blood using cardiac puncture (16). Different *Leishmania* spp. are found in different geographical regions; and some areas are endemic for more than one species. Therefore it is useful to have a culture medium such as horse blood NNN in which most, if not all, species can grow. Such a medium can help in the isolation of parasites for a rapid diagnosis of visceral and cutaneous disease; but also in isolating strains of newly introduced species of *Leishmania* in an area or even species circulating unnoticed since the culture medium used cannot sustain them.

For a culture medium to be favorable to an organism, it must meet its requirements for development and proliferation. The ability of horse blood to provide this, to all species tested, may be explained by the fact that *Leishmania* is a nicotinamide adenine dinucleotide (NAD) auxotroph organism (11) and since horse erythrocytes support NAD-dependent microorganisms and lack measurable NADase (12), the enzymes that participate in NAD metabolism, they may provide what is essential for parasite growth.

Nicotinamidase plays a key role in *Leishmania*'s cellular development as it requires assimilating NAD precursors (nicotinamide or vitamin B₃, nicotinic acid, nicotinamide riboside) from the host environment to synthesize NAD by a salvage pathway (11). Nicotinamidase is a key enzyme of this salvage pathway that catalyses

conversion of nicotinamide (NAM) to nicotinic acid (Na), and that is absent in higher eukaryotes. The enzyme has been shown to be important for growth and proliferation of *L. donovani* and essential for establishing an infection (13).

Goat blood, which contains a thermo labile inhibitor, an NAD inactivating enzyme, may prevent the growth of NAD-dependent microorganisms by depleting available pyridine nucleotides. Earlier work has shown that goat, bovine and sheep erythrocytes (14), all of which inhibit the growth of NAD - requiring organisms, like the bacteria *Haemophilus* spp., possess high NADase activity (12). Conversely, the erythrocytes of horse, guinea pig, and rat support *Haemophilus* species growth and lack measurable NADase. A level of about 40 times the concentration of NAD present in 5% chocolate sheep blood agar is needed to propagate *Haemophilus* spp. on unheated sheep blood agar (12). Similarly, fragility of rabbit erythrocytes in agar plates results in gradual release of their NAD and NADP contents into the medium. Due to high NADase and negligible NADPase activity of rabbit red blood cell stroma at neutral pH, the NAD released into the medium is hydrolyzed and NADP remains intact (14).

مقارنة التأثير لتحوير الوسط الزراعي NNN الخاص لاجناس اللشمانيا بأستعمال دم لمختلف اللبائن

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الخلاصة

فعالية المزروع المسوط في المختبر بوسط محور NNN لوسط ثنائي الطور اعدت باستخدام الدم من الثدييات المختلفة (فصيلة دم الانسان نوع (O)، حصان، حمار ، ماعز والغنم) وكان الهدف هو اختبار اي وسط NNN اعطى افضل عائد في اقصر وقت لانواع الطفيليات المختلفة من اجل الحصول على محصول كبير من الطور المسوط لاجل التجارب وتحضير المستضدات. لثلاث انواع من اللشمانيا نوعين جلدية عزلت من المرضى المصابين بالقرحة الجلدية ونوع احشائي عزل من نخاع العظم لمرضى من بغداد ثم زرعت باعداد متساوية في خمسة من الاوساط المحورة المحضرة، في نهاية اليوم السابع وجد باستخدام وسط دم احصان ينتج اكبر عدد من الطور المسوط لجميع انواع اللشمانيا المستخدمة بالاختبار، 27,15,33 بينما دم الماعز ثبت انه

افقر وسط فقط اعطي نتائج لطفيلى اللشمانيا الحشوية فقط، 7، ودم الانسان نوع () اعطى 9.2 و5 و4 ثم دم الحمار اعطى 6.5 و2 و1. دم الاغنام اعطى 4.5 و2.5. هناك تفسير حقيقي ان اللشمانيا وكريات دم الحصان تكون غنية بهذه المادة (NAD). تحتاج نيكوتينمايد ادنين دانيوكلبيوتايد

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