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Evaluation of Modified Novy-MacNeal-Nicolle Medium for Isolation of *Leishmania* Parasites from Cutaneous Lesions of Patients in Iran

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Abstract This study aims to evaluate the modified Novy-MacNeal-Nicolle (NNN) medium by adding mycosel agar to inhibit the growth of fungi and comparing it to traditional culture for the isolation of *Leishmania* parasites from cutaneous lesions of patients presenting to a specialized leishmaniasis clinics in Iran. A total 128 (70 female and 58 male) consecutive patients aged 3 months to 75 years (mean age of 11.25 years), presenting to Lesion biopsies were cultured in duplicate and parallel in traditional culture tubes containing NNN medium and modified NNN medium. The contamination rate in two groups of media was assessed. After sufficient incubation we have investigated that the media tubes for the presence of fungi. Through 90 samples, in 46 NNN media and 58 mycoNNN promastigotes were growth and culture positive. The difference between NNN medium and Mycosel NNN was quite significant ($p < 0.05$). The overall sensitivity of Modified NNN medium were 75%, while traditional culture had sensitivity of 64.8%. We have demonstrated that modified NNN medium is a more sensitive and time-efficient means of isolating *Leishmania* parasites from cutaneous lesions than traditional culture. Our study showed that contamination of NNN medium as base culture medium for isolation of *Leishmania* agents could be diminished significantly by addition of mycosel agar to NNN medium.

Key words: *Leishmania*, Novy-MacNeal-Nicolle medium, fungi

INTRODUCTION

Leishmaniasis is a protozoan disease cause by *Leishmania* genus. *Leishmania* cause major health problems with a spectrum ranging from self-curing such as cutaneous to fatal visceral Leishmaniasis (Ostria *et al.*, 2000; Rioux and Lanotte, 1990; Desjeux, 2004; Guerin *et al.*, 2002). Leishmaniasis is endemic in many parts of the world and remains a serious public health problem (Jorquera *et al.*, 2005). Cutaneous and visceral leishmaniasis

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are endemic in Iran (Fazaeli *et al.*, 2009; Ashkan and Rahim, 2008; Talari *et al.*, 2006). Two species, *Leishmania tropica* and *L. major*, are involved in cutaneous leishmaniasis in Iran which causative agents of anthroponotic and zoonotic cutaneous leishmaniasis, respectively (ACL and ZCL) (Asilian *et al.*, 2004). Traditional culture methods consist of biphasic culture systems that are sampled periodically throughout the short-time incubation for the presence of motile promastigotes. Allahverdiyev *et al.* (2004) suggested that prolonged incubation (approximately 30 days) is often required, as are large numbers of amastigotes in the culture inoculum that described previously (Lightner *et al.*, 1983; Wortmann *et al.*, 2007), due to the relatively large volume of liquid overlay which must be sampled repeatedly .

In vitro cultivation of *Leishmania* is carried out by using many different culture media such as NNN (Novy-MCNeal-Nicolle), RPMI 1640, Evans and Schneider, for isolating of *Leishmania* genus (Pierres and Antuñano, 1997). The NNN medium is more common and world known medium for isolation of the agent of *Leishmania* (Chouih *et al.*, 2009). In this way, particularly in the field study, the bacterial and saprophytic fungi growth is among unwanted agents which inhibit the *Leishmania* culture process. There are some antibiotics which can use to termination the growth of bacteria in the *Leishmania* culture without negative effect on the multiplication of parasite but, there are limitations in using antifungal agents. Therefore, the aim of this study was evaluation of the modified NNN medium by adding mycosel agar to inhibit the growth of fungi and comparing it to traditional culture for the isolation of *Leishmania* parasites from cutaneous lesions of patients presenting to a specialized leishmaniasis clinics in Iran.

MATERIALS AND METHODS

Study Site and Population

This is a double blind study of total 128 consecutive patients aged 3 months to 75 years (mean age of 11.25 years; 70 female and 58 male), conducted at the Leishmaniasis Clinic of the Tehran University and Ahwaz Jondishapour University of Medical Sciences (AJUMS), between August 2007 and May 2008. Consecutive patients presenting to the Leishmaniasis Clinic for the evaluation of skin lesions were approached to participate in this study and screened for eligibility criteria. The study was approved by the University Hospital and Ahwaz Jondishapour University of Medical Sciences Ethics Committees, and all subjects granted informed consent to participate.

Inclusion Criteria

Patients, who were referred to the Leishmaniasis Clinic for suspected cutaneous Leishmaniasis had a clinical indication for skin scraping or aspirate.

Exclusion Criteria

Patients with intercurrent bacterial or fungal super-infection of the ulcer and those undergoing active treatment for cutaneous Leishmaniasis were excluded.

Preparation of the Media

To prepare MycoNNN medium; at first make a solution by adding 5 mg chloramphenicol, 50 mg cycloheximide and 1.5 g agar to 100 mL of distilled water. The second solution was made by mixing 1.4 g agar and 1.2 g NaCl in 90 mL distilled water. The obtained two solutions were mixed together, boiled and autoclaved at 121°C for 20 min, after reaching to 50°C in room temperature, 20 mL defibrinated rabbit blood was added, and 3 mL of the mixture were divided

in 14 mL tubes. Just before inoculating of a sample, 0.5-1 mL of phosphate buffer saline supplemented with 200 IU mL⁻¹ penicillin-streptomycin was added to each tube as aquatic phase. To prepare traditional NNN as described by Chouhi *et al.* (2009); 1.4 g agar and 0.6 g NaCl were suspended in 90 mL distilled water and followed by the same above mentioned steps.

Sampling

To more evaluating the results, sampling was conducted in two different times. At first, 30 samples of cutaneous leishmaniasis were inoculated in NNN and mycoNNN media. After transferring to the laboratory, the cultures were incubated at 25°C; fungi contamination was checked after 4 days and continued by 4 days intervals for monitoring the growth of *Leishmania* within 30 days. In the second phase of study, 60 patient samples were examined. All of the samples were prepared based on Evans protocol (Evans *et al.*, 1984).

Isolation of DNA

The mass cultured promastigotes were harvested by centrifugation (4000 rpm at 4°C for 15 min) and washed three times in cold sterile PBS (pH 7.2). The DNA was extracted by QIA DNeasy blood and tissue kit (QIAGEN, Hilden, Germany) according to the manufacturers instructions. Purified DNA was eluted in 100 µL of elution buffer and stored in -20°C until use. The PCR control DNA preparations were also extracted from *L. major* MHOM/IR/75/ER, *L. tropica* MHOM/IR/02/Mash 10 and *L. infantum* MCAN/IR/97/LON 49 (Iranian standard strains).

PCR

Amplification of the mini-exon gene was performed as a single PCR with forward (5'-TATTGGTATGCGAACTTCCG-3') and reverse (5'-ACAGAACTGATACTTATATAGCG-3') primers as described before (Marfurt *et al.*, 2003). Two-seven microlitter of DNA solution (75-100 ng) were amplified in 20 µL of modified Taq DNA polymerase Master Mix RED reaction (Bioneer Korea) containing 75 mM Tris-HCl (pH 8.5), 20 mM (NH₄)₂SO₄, 1.5 mM MgCl₂, 0.1% Tween 20, 0.2 mM dNTPs, 0.25 unit Amplicon Taq DNA polymerase, 12% DMSO (78.13 g mol⁻¹ Cinnagen Iran), 10 pM of each primer, inert red dye and a stabilizer. DNA was amplified using thermal cycler (Eppendorf AG 22331, Hamburg, Germany) under the following conditions: 5 min at 94°C followed by 35 cycles of 30 sec at 94°C, 30 sec at 51.5°C, 45 sec at 72°C and a final elongation at 72°C for 10 min. For each sample one positive control and one negative control were included. The PCR products were separated on a 1.5% (w/v) agarose gel and visualized by staining with ethidium bromide.

Statistical Analysis

Differences were compared using one-tailed t-test. Differences in sensitivities were compared and the level of significance was set as a p of <0.05. The True-Positive (TP), True-Negative (TN), False-Positive (FP) and False-Negative (FN) results were defined. Diagnosis sensitivity, specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV) and accuracy were all calculated. Sensitivity = (TP) / (TP + FN) and specificity = (TN) / (TN+FP), where TP = true positives, FN = false negatives, TN = true negatives and FP = false-positives. The Predictive Values (PV), whether positive (+) or negative (-), were similarly calculated, with +PV being (TP) / (TP+FP), -PV being (TN) / (TN+FN) and accuracy is being (TN + TP) / (TN+FP+TP). Statistical analysis were performed using SPSS 13.0 (SPSS Inc., Chicago, IL).

RESULTS

One hundred and twentyeight patients with skin lesions were enrolled in the study; 58 were males and 70 females. The median age was 11.25 years (range, 3 months to 75 years). Monitoring of the media showed that totally 19 of 90 NNN and 8 of 90 mycoNNN media were contaminated with fungi agents (Table 1). Through 90 samples, in 46 NNN media and 58 mycoNNN promastigotes were growth and culture positive (Table 1). The production of promastigotes in all culture media was checked and the organisms were counted with a microscope slide (hemocytometer). Propagation in NNN medium and that in mycoNNN medium appeared close (Table 2). From promastigote positive samples, 0.5-1 mL transferred to RPMI-1640 supplemented with 10% of heat inactivated Fetal Bovine Serum (FBS) for massive proliferation. Propagated parasites in RPMI-1640 medium were applied for DNA extraction by DNAeasy blood and tissue QIAGEN kit (Hilden, Germany). The PCR products of *L. tropica*, *L. major* and *L. infantum* reference strains and samples were around 410-440 bp (Fig. 1).

Table 1: Comparison of fungal contamination and *Leishmania* growth in NNN and modified NNN culture media through two stages

Sampling	Number of media No. (%)		Fungal contamination No. (%)		Transform to promastigotes No. (%)	
	M1	M2	M1	M2	M1	M2
First stage	30 (100)	30 (100)	5(16.7)	2(6.7)	14 (46.7%)	19 (63.3)
Second stage	60(100)	60(100)	14(23.3)	6 (10)	32 (53.3)	39 (65)
Total	90(100)	90(100)	19 (21.1)	8 (8.8)	46(51.1)	58(64.4)

M1: Medium 1; NNN; M2: Medium 2; modified NNN

Table 2: Proliferation of *Leishmania promastigotes* in MycoNNN medium and traditional NNN

Medium	No. of promastigotes (day)				
	4	8	12	16	20
M1	10 ⁴	1.5×10 ⁶	2.5×10 ⁶	3.9×10 ⁶	3.7×10 ⁶
M2	10 ⁴	1.4×10 ⁶	2.5×10 ⁶	3.7×10 ⁶	3.6×10 ⁶

M1: Medium 1; NNN; M2: Medium 2; modified NNN

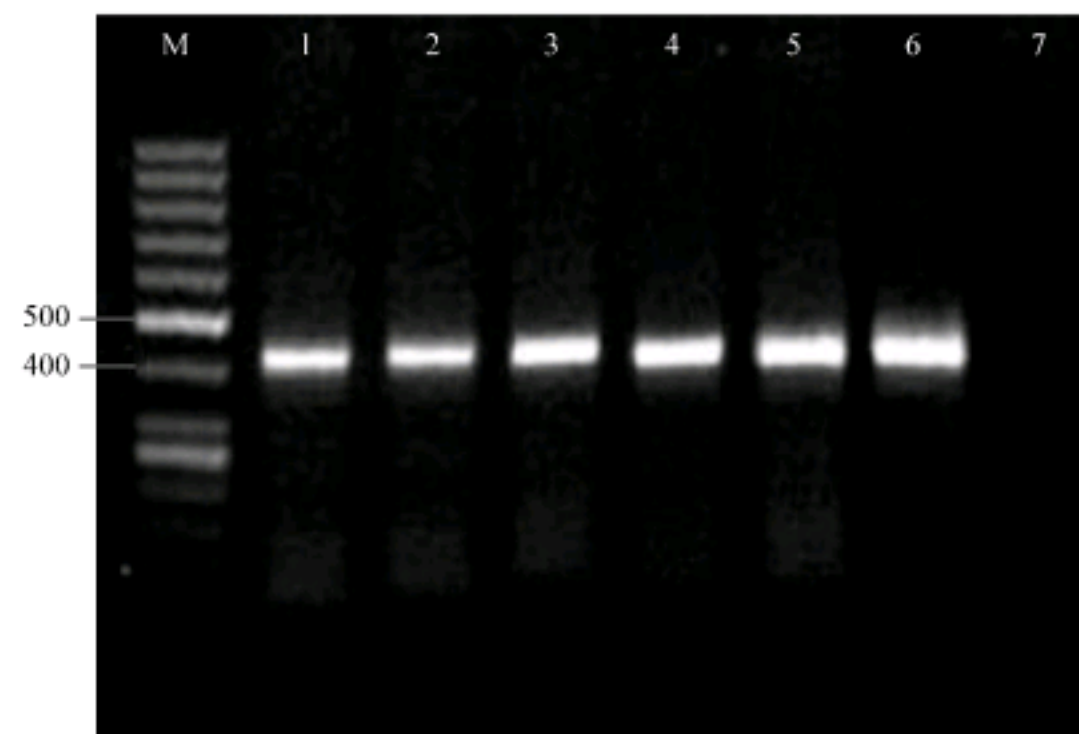


Fig. 1: Agarose gel stained with ethidium bromide following PCR amplification of DNA specific to *Leishmania* of Khuzestan isolates, Lane M size marker 50 bp, lanes 1-6: Khuzestan *Leishmania* isolates and lane 7: Negative control

Table 3: Analysis of two medium used in the evaluation of subjects suspected to be cutaneous leishmaniasis

Medium	No. of positive patients No. (%)	No. of negative patients No. (%)	Sn	Sp	+PV (%)	-PV	Accuracy
M1 (n = 90)	46 (51.1)	44 (48.9)	64.8	100	100	92.3	90
M2 (n = 90)	58 (64.4)	32 (35.6)	75	100	100	87.7	92

M1: Medium 1; NNN; M2: Medium 2; modified NNN; True-Positive (TP); True-Negative (TN); False-Positive (FP); False-Negative (FN); Positive Predictive Value (PPV); Negative Predictive Value (NPV)

The overall sensitivity, specificity, positive predicted value, negative predicted value and accuracy of Modified NNN medium were 75, 100, 100, 87.7 and 92%, respectively while traditional culture had sensitivity, specificity, positive predicted value, negative predicted value and accuracy of 64.8, 100, 100, 92.3 and 90% (Table 3).

DISCUSSION

Through all of cutaneous leishmaniasis in the world, 90% cases occur in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria (Pratlong *et al.*, 2009; Ahmed *et al.*, 2009). In order to control of the infection, epidemiological study and therapeutic aspects of the causative agents is essential. Conduction of these studies is in dire need of survive the parasites in culture medium. Also, for the isoenzymes study of the causative agents as the standard method for identification of *Leishmania*, the alive and pure parasites are necessary (Evans *et al.*, 1984). Although, there are several media are available to culture of the *Leishmania* isolates, but there is not a perfect medium for preventing the fungi contamination in the culture. Regarding to the inhibition effect of mycosel agar on the growth of saprophytic fungi, we decided to modify the NNN medium by adding mycosel agar for cultivation of cutaneous *Leishmania* agents in Khuzestan Province.

We have demonstrated that the modified NNN medium by adding mycosel agar to inhibit the growth of fungi, offers a sensitive alternative to NNN, traditional culture systems. Modified NNN medium demonstrated a 75% in sensitivity compared to traditional culture with NNN medium with a value of 64.8%. Boggild *et al.* (2007) evaluated herein the microculture method by comparing it to traditional culture for the isolation of *Leishmania* parasites from cutaneous lesions of patients presenting to a specialized leishmaniasis clinic in Lima, Peru. They showed that the sensitivity of microculture was 71.7, versus 54.7% for traditional culture with NNN. Present study evaluated 75% in sensitivity of modified NNN medium compare to 64.8% for traditional culture with NNN.

The results of current study showed that although, the present of cycloheximide in the medium, saprophytic fungi growth was not completely blocked. It maybe results of the present of resistant saprophyte fungi and diversity of saprophyte fungi species in the area. Therefore, to more evaluation and to achieve complete goals, different kind and quantities of the mycostatic agents should be examined.

Contamination by bacteria or fungi is a frequent problem with the *in vitro* isolation and propagation of protozoan parasites. Mäser *et al.* (2002) developed an antibiotic combination of broad anti-contaminant activity but minimal toxicity to protozoa. This anti-contamination cocktail well tolerated by African and South American trypanosomes, *Leishmania* sp., *Giardia duodenalis*, *Entamoeba histolytica* and *Plasmodium falciparum*, and it provides an effective means for the treatment of contaminated cultures. The anti-contamination cocktail meets numerous prophylactic applications in which sterile handling is impracticable or impossible. In particular, it facilitates the direct *in vitro* isolation of parasites from an infected host. Present study showed 8.8 % of fungal contamination in the modified NNN medium versus 21.1% in traditional NNN medium.

In conclusion, the present study showed that addition mycosel agar to the conventional NNN medium can significantly decline the saprophyte fungi contamination as well as is a more sensitive and time efficient method. Therefore, this compound by controlling the growth of fungi agents can help the researchers to protect their valuable samples, especially in field studies.

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