

Clinical Isolation and Molecular Diagnosis of Cutaneous Leishmaniasis by using KDNA Gene PCR at some Baghdad Hospitals

Nada Noori*, Amina N.AL-Thwani*, Basaad Akrab AL.Aboody**

*Institute of genetic engineering and biotechnology- University of Baghdad

**The College of Science\ University of Dhi Qar

Abstract.

This study was aimed to find out the most effective clinical samples and diagnosis methods in chronic cutaneous leishmaniasis (CCL). Smear, aspiration fluid, and filter paper, samples were taken from 44 skin lesions of suspected cases with CCL in some Baghdad hospitals, and they were compared using microscopic examination, culture, and molecular methods. Samples from the clinical patients were examined which showed through PCR 95.45% (42/44), Microscopic examination 72.7% (32/44), culture 27% (12/44). During the study, products in KDNA –PCR, *L. tropica* and *L. major* the observation of expected bands 780 and 650bp, and the filter paper most effective clinical samples from lesion. In conclusion the PCR –based assays tested on our increased the speed and sensitivity of the diagnosis DNA –PCR very important to the species identification (*L. tropica* and *L. major*).

Keywords: *Leishmania tropica*, *Leishmania major*, Direct examination, Culture, PCR, KDNA.

العزل السريري والتشخيص الجزيئي للشمانيا الجلدية باستخدام (PCR) لجين kDNA في

بعض مستشفيات بغداد

ندى نوري يونس*، امانة نعمة الثويني*، بسعاد عقرب العبودي**

*معهد الهندسة الوراثية والتقنيات الاحيائية للدراسات العليا / جامعة بغداد

**كلية العلوم / جامعة ذي قار

المستخلص

تهدف الدراسة ايجاد الطرق التشخيصية الفعالة لمعظم العينات السريرية للشمانيا الجلدية المزمنة باخذ مسحة، سحب من سائل القرحة، ورق الترشيح. العينات التي اخذت من القرحة الجلدية (44) لحالات مشكوك بها لشمانيا جلدية من مستشفيات بغداد وبأستعمال مقارنة بالفحص المجهرى، الزرع والطرق الجزيئية. نتيجة العينات من المرضى السريريين بعد الفحص لوحظ في PCR 95% (42/44)، بالفحص المجهرى 72% (32/44)، الزرع 27% (12/44). خلال الدراسة لجين KDNA ناتج ال PCR، *L. tropica* (780) bp و *L. major* (650) bp، وافضل طريقة للحصول على عينات الشمانيا من القرحة هي ورق الترشيح. استنتجنا ان طريقة ال PCR لكل العينات كانت سريعة وحساسة لتشخيص اللشمانيا الجلدية مقارنة بالطرق التقليدية و جين kDNA مهم جدا لتشخيص جنسي اللشمانيا الجلدية.

الكلمات المفتاحية: *L. tropica*, *L. major*, الفحص المباشر، الزرع، تفاعل البوليميراز المتسلسل، جين kDNA.

Introduction

Leishmaniasis is a disease caused by Leishmania parasite and transmitted to mammals and human beings by Phlebotomine sand flies and it causes skin infections [6, 10]. Twenty-one species of Leishmania have been reported to cause human infection [6]. Each year, 1.5–2 million new cases are reported and 70,000 deaths occurred. The number of disease and death cases showed about 2.4 million people affected throughout the world [25]. Leishmaniasis can produce various symptoms in mammalian host depending on the host genetic makeup and species of the Leishmania parasite [15].

Approximately, 90% of the cases of the cutaneous leishmaniasis were observed in Iran, Afghanistan, Pakistan, Saudi Arabia, Brazil, Peru, Iraq and Syria [5]. The lesions are mostly found on the exposed areas of the skin [3, 16, 22]. The lesion or ulcer leaves a scar on infected area [24]. Secondary bacterial or fungal infection of the ulcers causes increased tissue destruction and disfiguring of the skin [18].

Several techniques have been described for the identification of Leishmania at the molecular level. These techniques include sequence analysis of multicopy genes, restriction fragment length polymorphism, inter genic spacer regions, DNA fingerprinting, polymerase chain reaction (PCR), and randomly amplified polymorphic DNA [8-1]. The accurate identification and diagnosis which are concerned with epidemiology, clinical finding, and management and treatment of the patient must be based on molecular diagnosis [4].

Materials and methods

Samples were collected from the lesions of a patient with clinical suspected cutaneous leishmaniasis from some Baghdad Hosptails (Al-Yarmook, AL-Karama, Al-Kadhimiya, Al-Kindi) The participants suspected of having CL were informed about the study and given a questionnaire. According to the results obtained by the questionnaire, a classification of existing lesions was done for the following categories: acute CL (ACL) less than 1 year in duration, CCL more than 12 months.

Samples collected by Aspiration fluid, Smear, Filter paper.

Microscopic examination

All of the smears were fixed by dipping in absolute methanol and stained with Giemsa 10% stain, and then, they were examined under a light microscope with magnification at 1,000×. Some of aspiration fluid was also smeared onto a glass slide, fixed with methanol, stained with Giemsa, and examined under a microscope. All of the preparations where amastigote was observed were accepted to be positive, and those preparations where amastigote was not observed were negative.

Culture.

Approximately 0.2 mL aspiration fluids were inoculated into a 2-mL sterile tube containing 0.1 mL Novy-MacNeal-Nicolle (NNN) medium supplemented with 10% fetal calf serum (FCS; Sigma Aldrich Chemical, France), antibiotics (penicillin and streptomycin at 50 U/mL), and an antifungal agent (fucytosine). The cultures were incubated at 26°C and observed every week for 1 month. Promastigote-observed cul-

tures were accepted to be positive, and cultures where promastigote was not observed were negative.

DNA Extraction

The samples were subjected to DNA extraction by using Isolation Kit (Qiagen, Hilden, Germany) manufacturer protocol and extracted DNA were stored at -20°C for further process

DNA Amplification

KDNA –PCR

Primers, *L.tropica* F(5-AGGTGTTTTTGGGCTTGAC-3), *L.major* F(5-TCGCGTGTCTGACTTTTGC-3), Reverse(5ACTCAAGTCCCGTCCA TCAAC-3) were carried out as described by [11,13] for amplifying the variable region of the Leishmania species[17] .PCR was carried out in 20ml reaction mixture containing Master mix (Bioneer ,Korea), 10 p mol primers and DNA 2mL .The reaction mixtures were incubated in thermocycler (Appendorf ,USA) as follows: initial denaturation at 95°C for 5min by 30 cycles of 94°C for 30 sec , 60°C for 30 sec and 72°C for 40 sec and final extension at 72°C for 5min. The PCR products were electrophoresed in 1.2% agarose gel ,in the KDNA –PCR determined by the observation of expected bands 780 and 650 bp for *L. tropica* and *L. major* respectively.

Statistical Analysis

The Statistical Analysis System- SAS (2012) program was used to effect of difference factors in study parameters . Chi-square test was used to significant compare between percentage in this study[21].

Results and Discussion

The productivity and the potential isolation of Leishmania amastigotes in NNN media were assessed during the present study for evaluating the efficiency in the diagnosis and identification of the promastigotes and other life stages of the parasite. The results of cultivation, microscopic examination, and PCR of the materials were obtained from the patients having suspected CL lesions (Table1).

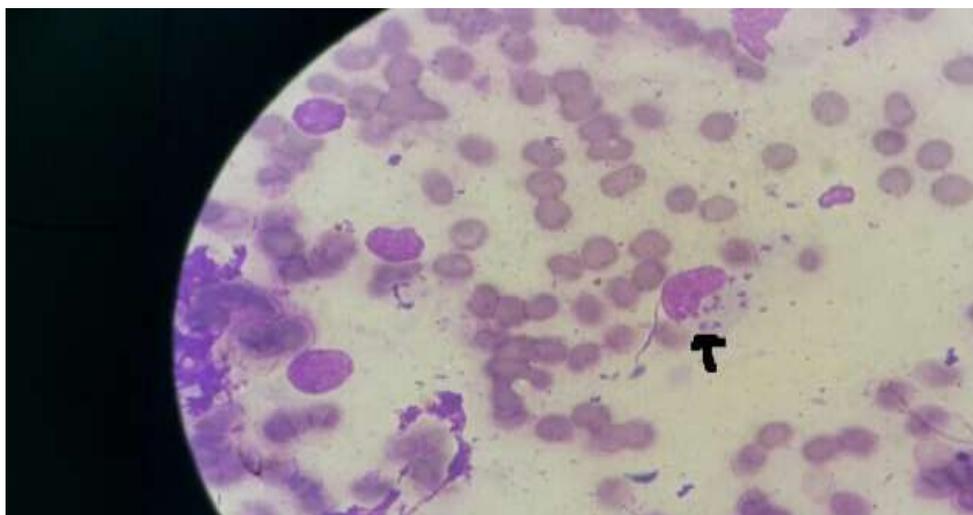
Table 1: Comparative detection of *Leishmania* in culture, direct microscopy, and PCR (KDNA)

Assay	Positive No.	%	Negative No.	%	Total	χ^2 -P- value	<i>Leishmania</i> sp.	
							<i>L.tropica</i>	<i>L.major</i>
Microscopic examination	32	72	8	18	44	12.07** (0.0001)	—	—
Culture	12	27	32	72	44	10.96** (0.0001)	—	—
KDNA-PCR	42	95	2	5	44	17.79** (0.0001)	30	12

**($P<0.01$)-Highly significant

Out of 44 suspected Cutaneous Leishmaniasis patients ,cases were positive PCR 95% (42\44), ,cases were positive by microscopic method 72 %(32)[fig 1] culture 27%(12) [fig 2].

Figer (3) present the KDNA-PCR product band of 780 bp for *L. tropica* and 650 bp for *L. major* , Figer (4) see NNN media .The result revealed that filter paper was the most effective method for samples collection from the lesions than aspiration fluid ,swab and smear [tabl2].

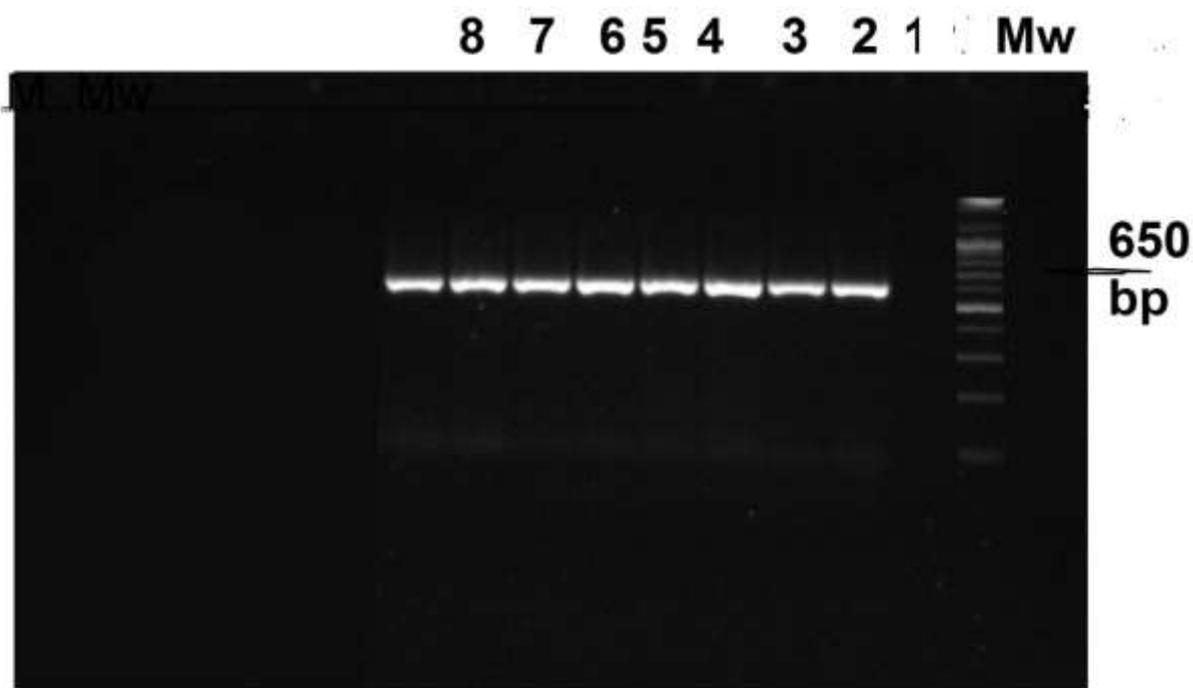


[fig1]smear from skin lesion stained with Giemsa show amastigotes in WBC.

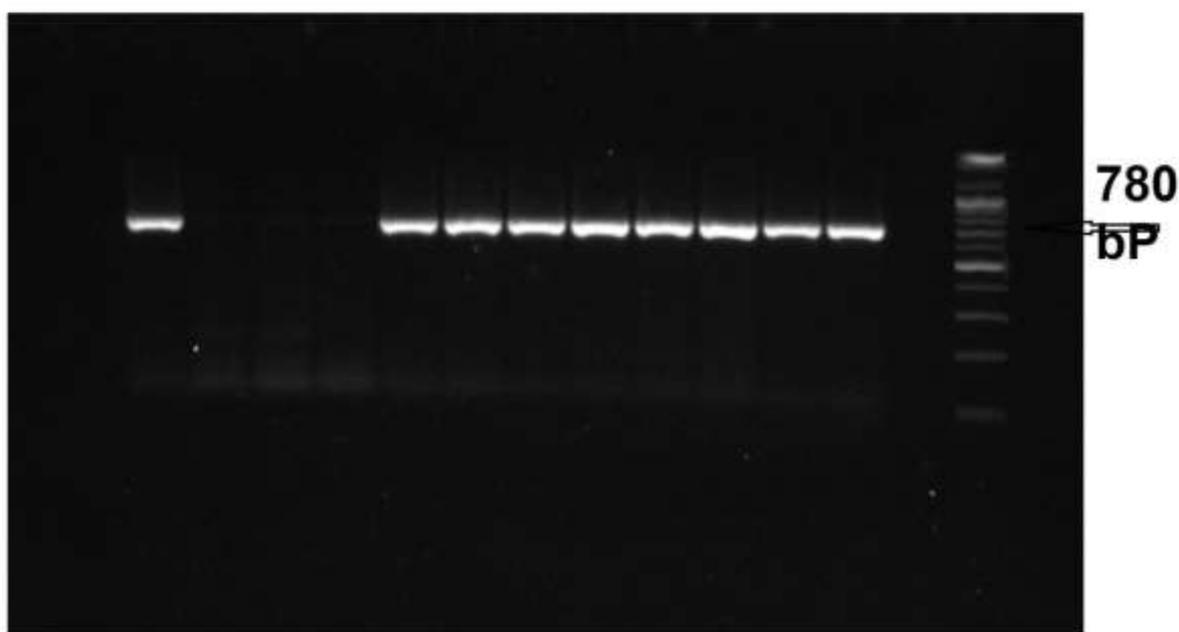


[fig 2] smear from NNN medium show promastigotes.

A



12 11 10 9 8 7 6 5 4 3 2 1 MW



B

[Fig3]:Gel electrophoresis of kDNA-PCR products, patients samples, MW 100 bp ladder(**A**)*L. major*, (**B**)*L. tropica* .

Table 2: Comparison between type of clinical samples and methods of diagnosis in Cutaneous Leishmaniasis suspected cases.

Clinical samples=44	Microscopic examination	Culture	PCR
Aspiration fluid	20	12	30
Smear (scraping)	32	0	20
Filter paper	0	0	42



[Fig 4]: NNN culture media

This study emphasized diagnosis and species identification in a large number of patients with CL in some Baghdad hospitals, The disease may originate in central Asia and was transported to Iraq either by Mongol invaders or by national extension [9].

There are many laboratory tests that help in diagnosis of CL that depended mainly on a classical characteristic morphological picture. PCR although not available in all 01tests[2].. In the present study, application of mini-exon PCR in order to characterize the *Leishmania* species causing cutaneous leishmaniasis in Iraq.

In the present study, 30 wet skin lesion(*L.tropica*) and dry skin lesion(*L.major*) was 12 with significant differences between different diagnostic methods of CL. with the type of lesions. These results were in agreement with many studies in Iraq [2] Afghanistan [7], Iran [23], Colombia [20], but disagreement with other study done in India [23] . The high frequency of wet lesions may be due to the presence of reservoir animals in large number in some areas in Iraq especially rodents and dogs [2]. The present study showed the PCR was more specific technique for diagno-

sis of cutaneous leishmaniasis with (95%) specificity; this result was in agreement with Marfurt et al [14]. 72 % of cases were positive by microscopic, whereas 27% of cases by culture.

Results in figure (3) showed the two types of *Leishmania* spp., in Iraq, by using mini-exon PCR assay, *L. major* (12) and *L. tropica* (30) and this in agreement with other Iraqi study [19] and other study in nearby countries such as Iran [5], hence the high incidence of *L. major* may be due to the presence of reservoir animals in large numbers, especially rodents and dogs. Obviously, dense populations of natural hosts of *L. major*, together with abundant vector sand flies, are the key elements responsible for the high rate of human infection.

The results of the study were probably based on sampling location ,

Primer and DNA extraction. KDNA is more sensitive than microscopy and cultuer for detection of CL [5].

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