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Clinical Isolation and Molecular Diagnosis of Cutaneous Leishmaniasis using RFLIP for *ITS1* Gene in some Baghdad Hospitals

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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 88 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 examid which showed throug PCR 93%(82/88) ,Microscopic examination 70%(62/88), culture 25%(22/88).During the study ,products in RFLIP for *ITS1*, by restriction the amplified *ITS1* gene product of *Leishmania* by the endonuclease Hae III, gave two fragments 60 and 200 bp were identified(57 patient) as *L.tropica* and two fragments of 140 and 220 bp (25 patient) as *L. major* ,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*).

Introdaction

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 [18]. 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] The lesion or ulcer leaves a scar on infected area [7]. Secondary bacterial or fungalinfection of the ulcers causes increased tissue destruction and disfiguring of the skin [6] .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].

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Materials and methods

Samples were collected from the lesions of patients 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, aclassification 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 cultures were accepted to be positive, and cultures where promastigote was not observed were negative.

Filter paper

The skin lesion area was cleaned with 70% alcohol andsterile Whatman 5-mmfilter papers (Whatman House, Maidstone, United Kingdom) was gentlypattedonto the lesion around the edges of the cuts and allowed to air drythoroughly and then cut and but in the appendrof conten 400ml BPS to use for DNA extraction.

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

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Agarose Gel Electrophoresis

After genomic DNA extraction for skin samples, agarose gelelectrophoresis was adopted to confirm the presence and integrity of the extracted DNA [17].

DNA Amplification

-ITS1 PCR- RFLP

The **ITS1** PCR was performed on all 88 extracted DNA samples. Amplification reaction was performed in volum 20 *m*l containing PCR Master mix (Bioneer,Koera) with primers Forward LITSR(5\- CTGGATCATTTTCCGATG-3\) and ReverseITS1R-TR1 (5\-GAAGCCAAGTCATCCATCGC-3\) 10p mol as mentioned by Davila and Momen(2000) and DNA 5*ml*nucleus free water as negative control .

Rection were amplified in thermocycler (Appendorf USA) as follows ;initial denaturation at 95^{o}C for 5min by 35 cycles of 94^{o}Cfor 30 sec ,48^{o}C for 30 sec ,72^{o}C for 1min and final extension 72^{o}Cfor 10min.At the end PCR products were analyzed using 1.5% gel electrophoresis .The diagnostic criteria of cutaneous Leishmaniasis in ITS1 PCR was based on the observation of expected band 350 bpinPCR product and after enzymatic digestion by the observation of its pattern, species of *Leishmania*were identified.

.RFLIP analysis of amplified ITS1

All positive samples of ITS1 PCR product were digested with restriction enzyme HaeIII and the *Leishmania*species were identified based on pattern of enzymatic digestion the PCR products [2], (10*m*l) of PCR product weredigested with 1*m*l of HaeIII (fast digest fermentas)at 37^{o}C for 30 min using conditions recommended by the supplier, the restriction fragments were subjected to electrophoresis in 4% agarose gel.

Statistical Analysis

Chi-square test was used to significant compare betweenpercentage in this study(SAS, 2012).

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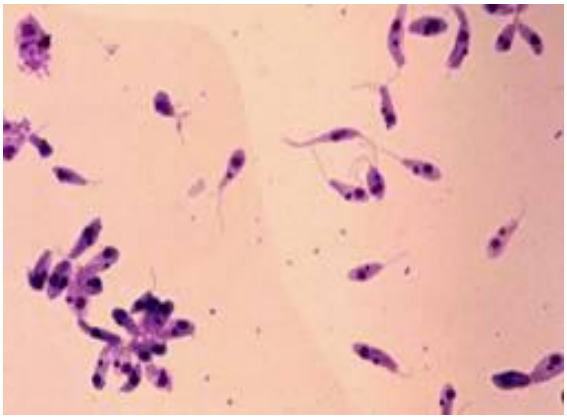
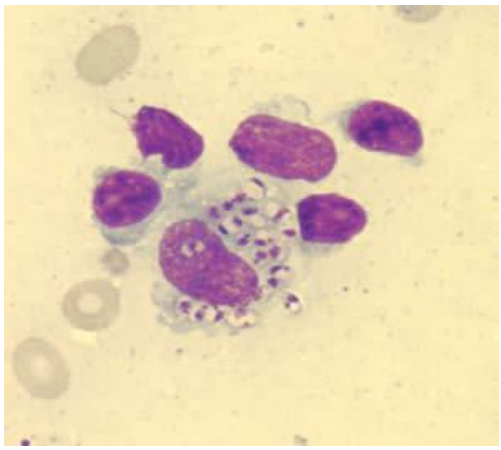
Results and Discussion

Leishmania spp the productivity and the potential isolation of (amastigotes) in NNN media .were assessed during the present study for evaluating the efficiency in the diagnosis of different life stages of the parasite. The results of microscopic examination, cultivation , extracted DNA for ITS1 gen**RFLIP** PCR of the specimens from the patients having suspected CL lesions.

The results revealed that 70%(62/88) of cases were positive by the Microscopic examination)(fig- 1) , where as only 25%(22/88) by culture(fig- 2). In molecular method by **RFLIP** PCR it was found 93%(82/88) for ITS1 gene(fig-3) ; 69%(57/82) were typed as *L.tropica* and 31%(25/82) were typed as *L.major* (fig-4) while PCR positive for ITS1 gene .Highly Significant ($P<0.01$) correlation was observed between diagnosis methods (Table 1). .



(Fig -2) Promastigote in culture NNN



(fig- 1) Amastigote in WBC(monocyte)

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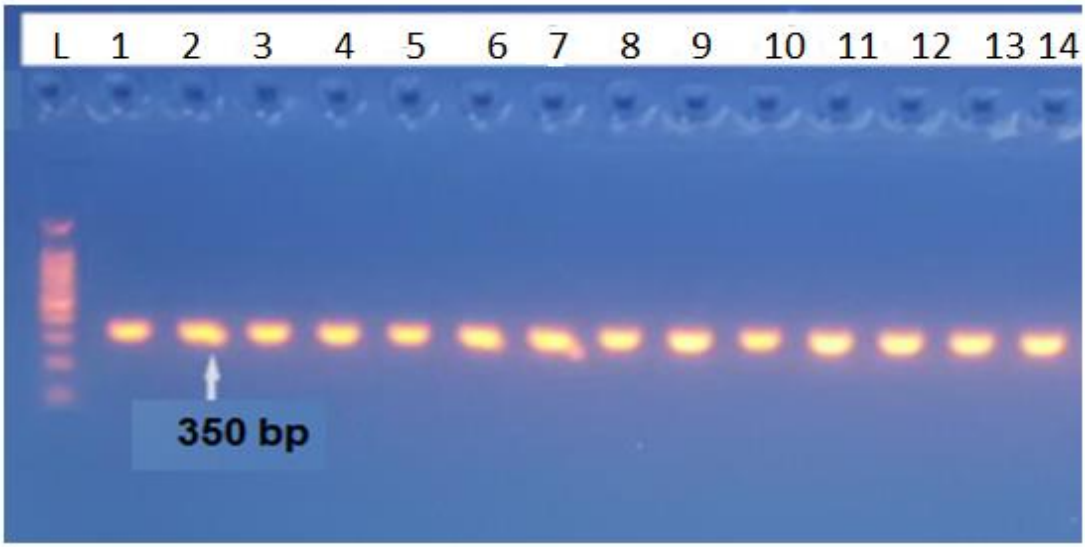

Fig- 3] Gel electrophoresis of *ITS1*PCR products of *Leishmania* on 4% Agarose gel ,Wells 1-14 ,L 100bp DNA ladder marker

Table 1: Comparative detection of *Leishmania* in direct microscopy, culture and **RFLIP** for (ITS1) genes

Assay	Positive No.	%	Negative No.	Total	χ^2: P-value
Microscopic examination	62	70	26	88	5.032 * (0.039)
Culture	22	25	66	88	13.406 ** (0.001)
RFLIP(ITS1)for L.tropica	57	69	25	88	14.215 ** (0.001)
RFLIP(ITS1)for L.major	25	31	57	88	13.215 ** (0.001)

** (P<0.01)-Highly significant

This result confirmed by previous study conducted by [9] obtained same result when used conventional PCR for amplification *ITS1* gene.

The results of restriction the amplified *ITS1* gene product of *Leishmania* by the endonucleaseHae III,gave two fragments 60 and 200 bp were identified as *L.tropica* and two fragments of 140 and 220 bp as *L. major*(Figure, 2).

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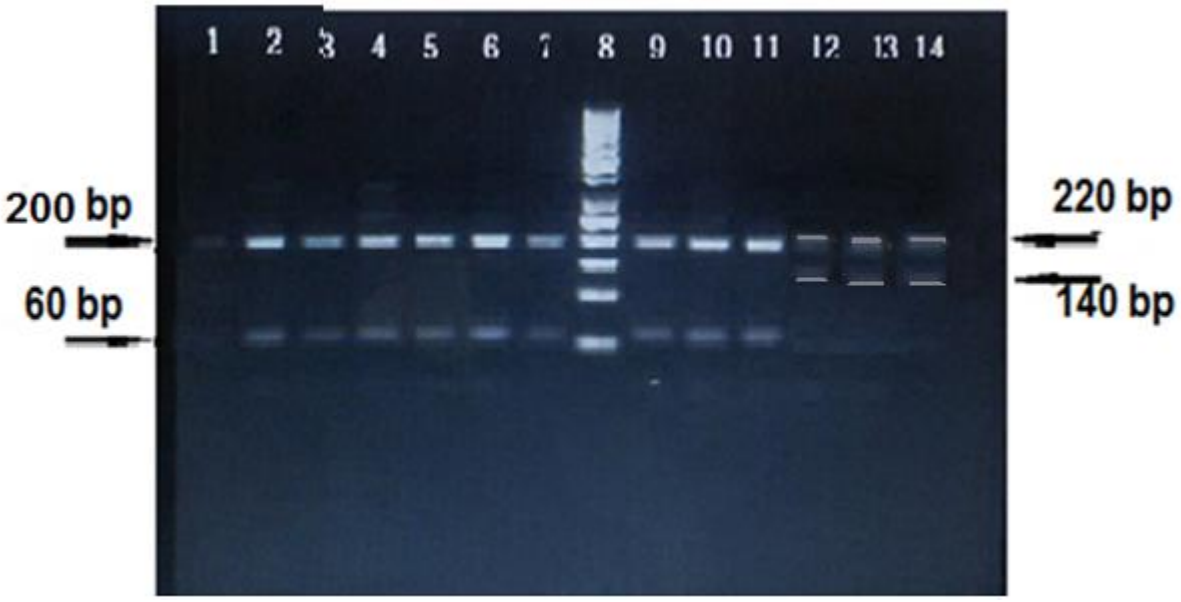

Fig (4)Gel electrophoresis of PCR- RFLP products of *Leishmania* isolates using HaeIII enzyme on 4% gel agarose 1-7 and 9-11:*Leishmania tropica* isolates,8:50 bp DNA ladder marker 12 ,13,14: *L. major*

In the present study, 57 *L.tropica* (dry skin lesion)and 25 *L.major* (wet skin lesion)were diagnosed with different diagnostic methods of CL. These results were in agreement with many studies, in Iraq [16, 3] and Afghnistan [10 ,12, 13] in Colombia [11] who found the dry more than wet lesions, but in contrast with other study done in Indiaby [15] and in America [3] reported that dry skin lesion less than wet skin lesion.

The high frequency of *L.tropica* in this and other studies may be due to the presence of reservoir animals in large number in some areas in Iraq especially rodents and dogs as reported by CDC [6] .

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