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Molecular Detection of Suspected *Leishmania* Isolates Using Polymerase Chain Reaction

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Abstract

Leishmaniasis is a widespread parasitic disease that occurs as a result of infection with a unicellular parasite belonging to the genus *Leishmania*. Diagnosis by conventional methods is inaccurate and is not sensitive to confirm the genus infection. Here, we have investigated a methods for *Leishmania* genus diagnosis, which includes the technique of polymerase chain reaction to detect the presence of the parasite at *in vitro* for promastigote cultures using three genus-specific primer pairs to amplify HSP70, ITS, and ITS2. The results showed single band of ~1422, ~1020, and ~550 respectively. This study has proved the ability of these primer pairs to detect *Leishmania* infection and recommend them to be used for detection of leishmaniasis in hospitals and research centers.

Keywords: PCR, identification, leishmaniasis, ITS, ITS2, HSP70.

التحري الجزيئي لعزلتين مشكوك بهما لطفيلي اللشمانيا بأستخدام تفاعل البلمرة المتسلسل

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الخلاصة

يعد داء اللشمانيا من الامراض الطفيلية واسعة الانتشار والتي تحدث نتيجة الاصابة بطفيلي احادي الخلية يعود لجنس اللشمانيا. ان التشخيص بواسطة الطرق التقليدية تعد غير دقيقة وغير حساسة لتأكيد الاصابة بالمرض لذلك تم بهذه الدراسة اللجوء الى طرق احدث في تشخيص الطفيلي والتي تتضمن أستخدام تقنية تفاعل البلمرة المتسلسل للكشف عن وجود الطفيلي باستخدام الاطوار المسوطة المزروعة خارج الجسم الحي وذلك باستخدام ثلاثة بوادئ متخصصة لتضخيم كل من HSP70 و ITS و ITS2 حيث أظهرت النتائج وجود حزمة واحدة بحجم 1422~ و 1020 ~ و 550~ على التوالي .هذه النتائج تثبت امكانية استخدام هذه البوادئ للكشف عن جنس الشمانيا ونوصي باستخدامها في المستشفيات والمراكز البحثية.

Introduction

Leishmaniasis is a parasitic disease caused by infection with an obligate intracellular protozoan belongs to the genus *Leishmania* [1].The disease is endemic in 88 countries, including Iraq, where an estimated 12 million people are infected, while 350 million being at risk [2]. There are 30 species documented belong to genus *Leishmania*, but only 10 species are critical due to medical and veterinary important [3]. leishmaniasis are existing in three different forms which are cutaneous leishmaniasis (CL), visceral leishmaniasis (VL), and mucocutaneous leishmaniasis (MCL) [4].

Leishmania distribution is dichotomized into the Old (southern Europe, the Middle East, parts of southwest Asia, Central Asia and Africa) and the New (Mexico and Latin America) World groups [5]. Five species of *Leishmania* are agents of Old World leishmaniasis: *L. major*, *L. tropica*, *L. aethiopica*,

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L. donovani, and *L. infantum*. The first 3 of these are predominantly agents of CL, an infection that is limited to the skin. The last 2 are predominantly agents of VL, an infection of the liver and spleen [6]. *Leishmania* parasite is transmitted by species of sand fly belonging to the genus *Phlebotomus* [7].

The diagnosis of leishmaniasis has conventionally relied on direct microscopic examination and other serological methods. The demonstration of parasites in relevant tissues such as examination lesion biopsy smears for CL and aspirates of the spleen, bone marrow and lymph nodes for VL are examined under the microscope using giemsa staining [8]. Due to similarity of leishmaniasis symptoms with another disease, misdiagnosis originates from non-leishmaniasis infections like some of leprosy, myiasis and sarcoidosis have the same clinical signs with CL symptoms. Furthermore, some clinical symptoms perceived in chronic malaria, typhoid fever, syphilis, tuberculosis, relapsing fever, are similar to VL symptoms [9]. The invention of DNA amplification via PCR has enabled the development of fast and highly sensitive detection [10]. Conventional PCR enables the amplification of DNA or RNA through repetitive cycles, *in vitro*. This method viable for leishmaniasis diagnosis since the early 1990s because of determination several specific DNA fragments [11]. The PCR technique is the best in compare to other parasitological methods such as microscopy or cultivation, especially for samples with low parasite loads [12]. Also, PCR has applications in parasites quantification and monitoring disease progression, predicting and controlling the outcome of anti-leishmanial therapy [13]. The objective of this study was using a universal PCR technique for molecular detection of the genus of *Leishmania* parasite.

Materials and Methods

Isolate used in this study: Two *Leishmania* isolates that used in this study were kindly provided by Biology Department/ College of Science/University of Baghdad.

Cultivation of isolate: Procyclic promastigotes were cultivated in M199 medium supplemented with 10% HIFBS and 1% streptomycin/penicillin (Pen/Strep) then incubated at 26°C for 3 days for obtaining massive culture [14].

DNA extraction: DNA extraction from healthy cultures of the parasite was done by using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. Then DNA was stored at −20°C until use.

Oligonucleotide sequence: The primer pairs used in this study (listed in table1) were purchased from Alpha DNA/Canada. The first primer pair HSP70sen/HSP70ant targeting HSP70 gene. While LITSR/ LITSV targeting region located between small subunit rRNA gene and large subunit rRNA gene. The later L5.8SR/ LITSV targeting ribosomal internal transcribed spacer 2 separating the genes coding for 5.8S rRNA and large subunit rRNA gene.

Table1- Primers used to detect *Leishmania* genus:

Oligonucleotide	Sequence	Reference
HSP70 (HSP70sen/HSP70ant)	5´GACGGTGCCTGCCTACTTCAA 3´	[15]
	5´ CCGCCCATGCTCTGGTACATC 3´	
ITS (LITSR/ LITSV)	5´ CTGGATCATTTTCCGATG 3´	[16]
	5´ ACACTCAGGTCTGTAAAC 3´	
ITS2 (L5.8SR/ LITSV)	5´ AAGTGCGATAAGTGGTA 3´	
	5´ ACACTCAGGTCTGTAAAC 3´	

PCR amplification: The PCR amplification mixture which used for detection of each gene was carried out in a 25 µl volume includes GoTaq® Green Master Mix, 2X (12.5 µl), 3 µl of 25 ng DNA template, 1 µl (1 Mm) of each forwarded and reversed primers and 7.5 µl of nuclease free water to complete the amplification mixture to 25µl. Amplification was performed in a thermal cycler (Eppendorf®) programmed for 35 cycles of denaturation at 94°C for 1 min, annealing at 61°C, 57°C and 53°C for HSP70, ITS, ITS2 genus-specific primers respectively for 1 min, and extension at 72°C for 2 min, unprecedented by an initial denaturation of 2 min at 94°C. Final extension was for 3 min at 72°C.

Gel electrophoresis: PCR products were analyzed by electrophoresis on 1% agarose gel containing Ethidium bromide (0.5 mg/ml) in Tris-Acetate-EDTA buffer (TAE buffer) and photographed under UV illumination.

Results and Discussion

PCR technique conceder rapid and sensitive tool for the diagnosis of leishmaniasis. Many different PCR have targeted conserved and multiple regions of kDNA minicircles [17, 18], genomic DNA, splice leader mini-exon [19], telomeric repeats [20], rRNA gene [21] and gp63 [22].

In the present study, *Leishmania* promastigotes kDNA were successfully amplified by using three different primers that target three region which is HSP70, ITS, ITS2.

PCR amplification of HSP70

The primer pair hsp70sen/hsp70ant specifically amplified HSP70 gene of ~1422bp. The result is shown in Figure-1.

The heat shock protein performs an important role in many processes including folding, assembly, secretion, regulation, stabilization, degradation of other proteins and intracellular localization [23]. One class, the70 kDa heat-shock proteins (HSP70), are highly conserved across prokaryotes and eukaryotes both in sequence and function. Also, it is very important as molecular chaperones, transport, and protein folding [24]. Between numerous targets described for *Leishmania* detection, the heat-shock protein 70 coding gene has proven to be useful in identifying many species of different geographical origins [25]. Previous study reported that this primer set specific to detect *Leishmania* genus and also recorded the ability of same primer for successful amplification in low DNA concentration of 230 pg/µl [26].

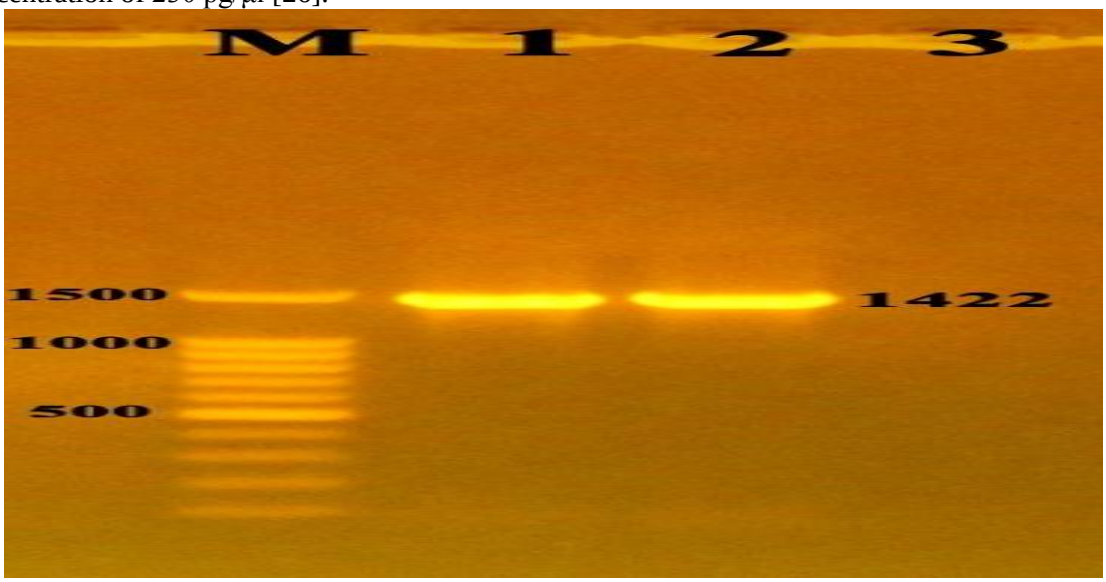

Figure 1- Agarose gel electrophoresis of PCR product of ~ 1422 bp of HSP70, M-100bp Ladder, Lane (1) first isolate, Lane (2) second isolate, and Lane (3) negative control sample (No DNA template).

The *hsp70* gene has been widely used for phylogenetic and taxonomic studies of *Leishmania*. Regions with *hsp70* homology have been found on chromosomes 26, 28, 30, and 35. These have been identified as suitable for PCR-RFLP diagnostics that does not require parasite culturing before amplification [15, 27, 28], and so may become widely-used targets.

Another previous study applied on noninvasive sample (swab smear) and by using HSP70 primer, the result proved efficient, accurate and reliable PCR amplification. The use of noninvasive sampling, along with sensitive and simple molecular analysis, can be a valuable tool for overcoming the challenges of diagnosing leishmaniasis [29].

PCR amplification of internal transcribed spacer (ITS)

Internal transcript spacer region possesses great levels of inter and intra species variation have been observed in Old and New World *Leishmania* species in the DNA internal transcribed spacers. Two PCR assays were used that amplified different parts of *Leishmania* ribosomal repeats [16]:

(I) ITS region was successfully amplified and electrophoresis, the yields were a single band of ~1020 base pair Figure-2.

(II) Ribosomal internal transcribed spacer 2 (ITS2) also was successfully amplified and electrophoresed, the yields were a single band of ~550 base pair Figure-3.

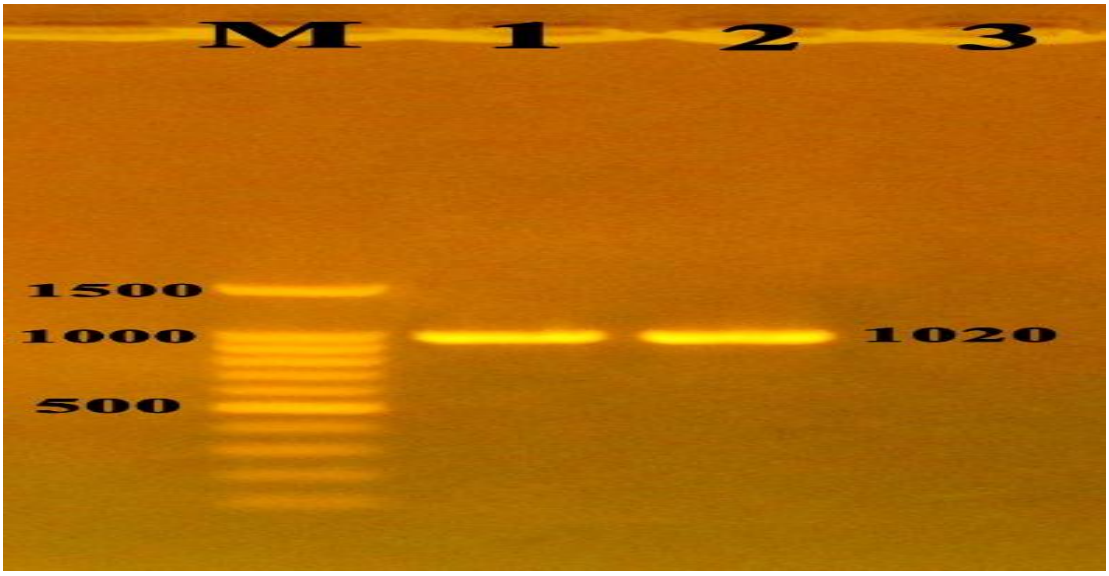

Figure 2- Agarose gel electrophoresis of PCR product of ~ 1020 bp of ITS, M-100bp Ladder, Lane (1) first isolate, Lane (2) second isolate, and Lane (3) negative control sample (No DNA template).

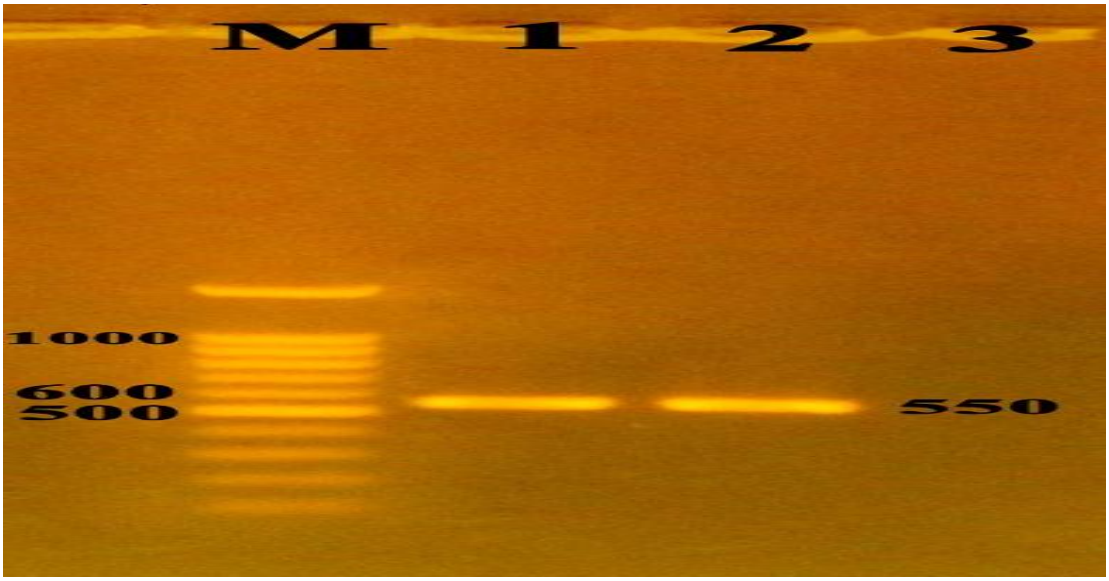

Figure 3-Agarose gel electrophoresis of PCR product of ~ 550 bp of ITS2, M-100bp Ladder, Lane (1) first isolate, Lane (2) second isolate, and Lane (3) negative control sample (No DNA template).

A previous study by [30] used ITS PCR assay proved the ability to detect *Leishmania* in different biological materials which were tested with various clinical isolates (skin biopsies, bone marrow aspirates, blood, skin scrapings on filter paper or on glass slides).

Also, there was a study by [31] on 51 Turkish *Leishmania* isolate depended on real-time PCR method based on specific region occur in the internal transcribed spacer used to diagnose many clinical forms of leishmaniasis which are visceral leishmaniasis, cutaneous leishmaniasis and canine leishmaniasis and identified the species directly.

In another study by [32] analyzed the polymorphism of the internal transcribed spacer (ITS) of 3 reference strains and 24 Mexican isolates of *L. mexicana*, by using of PCR following by digestion with restriction enzymes.

De Almeida *et al.* [33] identified a region of the ITS2 adequate for diagnostic goal and designed generic PCR primers to amplify this fragment from *Leishmania* spp. associated with human infection and found substantial differences in the ITS2 sequence region spanned by these primers that generally allowed species identification.

So there is a widely complementary application for this universal primer can be used to detect *Leishmania* species.

Conclusion

The identification of the parasite is important for diagnosis and epidemiological studies, for taxonomic and population genetic investigations for drug choice for the treatment of the disease. Our study demonstrated the usefulness of the PCR for the detection of *Leishmania* DNA. This study provides accurate molecular detection by using universal primer pairs, which can be used individually or in combination to detect *Leishmania* presence in suspected patients and recommended for central laboratories to be used rather than classical low-sensitive assays.

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