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Identification of *Leishmania donovani* Isolates by Polymerase Chain Reaction

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Abstract: Leishmaniasis is endemic of Iraq in both cutaneous and visceral form. The available tools for diagnosis and detection of *Leishmania* are nonspecific and may interfere with other species. In this study, Polymerase Chain Reaction (PCR) has been used to identify Iraqi isolate of visceral leishmaniasis (MHOM/ IQ/2005/MRU15) which a previously diagnosed by classical serological tests. PCR amplification was carried out using species-specific primers of *Leishmania donovani.* Four primer pairs of mini-circle DNA and ITS-1 were used.13A/13B, which is used to identify *Leishmania* as a genus, NM12, LITSR/L5.8S and BHUL18S, were used to detect the sub species of *L. donovani.* The result of PCR amplification of 13A/13B kDNA revealed that a band of ~ 120 bp. NM12, LITSR/L5.8S and BHUL18S primer pairs demonstrated bands of 204 bp, 320 bp and 311 bp, respectively. The results of this study are recommended to be used for identification of visceral leishmaniasis identification instead of time consuming and non-specific classical methods.

Keywords: Visceral leishmaniasis, Molecular diagnosis, species-specific primer.

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Introduction:

Leishmaniasis is a vector-borne zoonotic disease caused by obligate intracellular parasitic protozoa of the genus *Leishmania*. The disease gets into human population when human, flies and the reservoir hosts share the same environment (1). Leishmaniasis is transmitted by the bite of phlebotomine female sand flies of the genera *Phlebotomus* and *Lutzomyia*, in the old and new worlds, respectively (2). According to reports, Leishmaniasis is endemic in 98 countries, and around 1.3 million new cases are reported every year, with an estimated 20,000 to 40,000 deaths every year (3).

The clinical picture of leishmaniasis is heterogeneous and can be roughly classified into three major forms of increasing severity: Cutaneous leishmaniasis (CL), Mucocutaneous leishmaniasis (MCL), and Visceral leishmaniasis (VL) (4). Visceral leishmaniasis is a symptomatic infection of the liver, spleen, and bone marrow caused by organisms of *Leishmania donovani* complex. The annual incidence and prevalence of VL cases worldwide are 0.5 million and 2.5 million respectively. Of these 90% of cases occur in India, Nepal, Bangladesh, Sudan, Bolivia, Peru, Afghanistan, Iran, Saudi Arabia, Syria and Iraq (5).

The definitive diagnosis of VL or post-kala-azar dermal leishmaniasis cases is essential for providing individual treatments and understanding the disease epidemiology. A definitive

diagnosis is usually made by detecting parasites in aspirates from the spleen or other tissues (such as bone marrow, lymph nodes, or skin), parasite DNA in the tissue and blood samples, and, with lesser specificity, parasite antigen or antibody in blood or urine.(6) Although the demonstration of parasites is most specific, the techniques are invasive and require skilled personnel and proper facilities, and the sensitivity of bone marrow aspirates has been reported to be variable (7).

Polymerase Chain Reaction (PCR) has been applied successfully in recent years to detect *Leishmania* spp. in cases with any of the clinical manifestations of leishmaniasis. Some PCR methods also allow differentiation between parasite strains, which can facilitate more-efficient treatment (8). Several PCR protocols for combined detection and differentiation of parasites exist, including multiplex PCR (9), PCR plus sequencing (10), and restriction fragment length polymorphism (RFLP) analysis (11).

Diagnostic assays for leishmaniasis have been developed based on the amplification of several DNA targets such as the minicircle of kDNA (12) , the rRNA gene (13), the miniexon-derived RNA (14), and repeated genomic sequences (15).

The objective of this study was using of species – specific oligonucleotides for molecular detection of Visceral Leishmaniasis (VL), *Leishmania donovani*, of an Iraqi isolate previously diagnosed with VL based on classical methods, such as, clinical features and serological tests.

Methodology:

Parasite isolate:

Leishmania donovani (MHOM/IQ/ 2005/MRU15) isolate from a patient clinically diagnosed with VL by serology was kindly provided from Medical Research Unit, College of Medicine, AL- Nahrain University.

Parasite culture:

Procyclic promastigotes was cultured in M199 media supplemented with 10% HIFCS and incubated at 26°C. Subculture was maintained twice a week1(6).

DNA extraction:

DNA extraction of healthy culture from *Leishmania donovani* was done by using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. The DNA was stored at −20°C.

The Primer pairs:

All primers used in this study were purchased from Alpha DNA (Canada) in lyophilized form, dissolved in TE buffer to a final concentration of 100 pmol/ µl for each and stored in a - 20 until use.

Oligonucleotide sequence:

- **13A/13B (accession no. U19811) (17):**

  This sequence is conserved in all minicircle classes of all *Leishmania* species as a genus, this primer pair amplify a fragment of approximately~120bp, (Table 1).

Table (1): The primers with their sequences, product size and optimization:

Primer	Sequence	Product size	
13A/13B	GTG GGG GAG GGG CGT TCT	120 bp	
	ATT TTC CAC CAA CCC CCA GTT		
Optimization	Temp. C	Time	Cycle No.
Initial denaturation	94	4 min	1
Denaturation	94	1 min	30
Annealing	60	1 min	
Extension	72	1 min	
Final extension	72	10 min	1
Hold	4	10 min	

- **NM12 (accession no. X98348) (18):**

This oligonucleotide was used for detection of kDNA gene (NM12) which is considered a species-specific for *L. donovani*. It amplifies a fragment of approximately 204 bp (Table 2).

Table (2): The primers with their sequences, product size and optimization.

Primer	Sequence	Product size	
NM12	TGA TAC CAC TTA TCG CAC TT	204 bp	
	GTGGTCGTGGCGCTTATGTG		
Optimization	Temp. C	Time	Cycle No.
Initial denaturation	94	3 min	1
Denaturation	94	45 sec	35
Annealing	55	30 sec	
Extension	72	90 sec	
Final extension	72	10 min	1
Hold	4	10 min	

- (**LITSR, L5.8S**) **(Accession no. AM901448.1) (19):**

LITSR, L5.8S this gene which is specific for *L. donovani.* It amplifies a fragment of approximately 320bp, (Table 3).

Table (3): The primers with their sequences, product size and optimization:

Primer	Sequence	Product size	
LITSR, L5.8S	CTG GAT CAT TTT CCG ATG	320 bp	
	TGA TAC CAC TTA TCG CAC TT		
Optimization	Temp. C	Time	Cycle No.
Initial denaturation	95	2 min	1
Denaturation	95	20 sec	40
Annealing	53	30 sec	
Extension	72	1 min	
Final extension	72	6 min	1
Hold	4	10 min	

- **BHUL18S**) **(accession no. X0773) (20):**

This primer pair was used for detection of BHUL18S gene which is specific for *L. donovani.* The primer pair amplify a fragment of approximately 311 bp. (Table 4).

PCR amplification:

Master mix for each primer pair was made for a total volume of 24 µl, including 25ng DNA and 1 µM F and R primer.

Table (4): The primers with their sequences, product size and optimization:

Primer	Sequence	Product size	
BHUL18S	CGTAACGCCTTTTCAACTCAC	311 bp	
	GCCGAATAGAAAAGATACGTAAG		
Optimization	Temp. C	Time	Cycle No.
Initial denaturation	96	1 min	1
Denaturation	96	10 sec	30
Annealing	50	10 sec	
Extension	60	30 secn	
Final extension	60	4 min	1
Hold	4	10 min	

The Gel Electrophoresis:

PCR product of each amplification was run on agarose gel 1.5%,electric current was set up at 100 Volt and 70 AM for 1hour (18).

Results and Discussion:

The PCR amplification of *Leishmania* genus-specific primer 13A/13B:

Result showed ~120 bp of the primer pair 13A/13B as in (Figure 1). Previous study showed that this primer is being able to detect an equivalent of 0.1 promastigote cells per PCR tube. This may be explained in part by the higher copy number of the kDNA target (approximately 10,000 copies) (21). Another study reported high sensitivity (98.7%) of this primer in PCR (22). Similar study proved that *Leishmania* investigation in dog's blood samples was 93.7% more specific positive results than that of IFAT serological test, which was 85.3% (23).

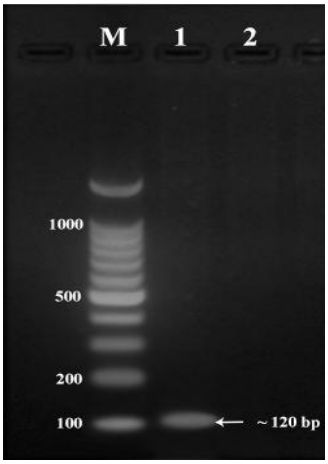

Figure (1): Agarose gel electrophoresis of PCR product of ~120 bp of 13A/13B, M-100bp Ladder, Lane1: DNA template (25 ng), Lane2: negative control sample.

NM12 amplification for identification of *L. donovani*:

The PCR product was of 204 bp on gel, (Figure 2). Similar studies showed this primer pair to be used for identification of *L. donovani* in Brazil against DNA samples from different *Leishmania* species isolates of *L. donovani, L. infantum, L. tropica* and *L. braziliensis*, in which amplification was only amplified with isolate of *L. donovani*. NM12 primer is therefore are highly specific for *L. donovani* species and can distinguish between cutaneous and visceral form (18).

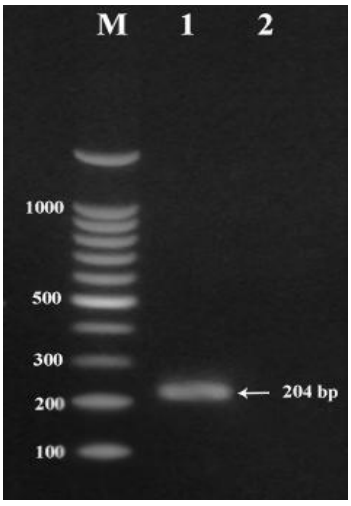

Figure (2): Agarose gel electrophoresis of PCR product of 204 bp of NM12, M-100bp Ladder, Lane (1) DNA template (25 ng), and Lane (2) negative control.

LITSR/L5.8S amplification for identification of *L. donovani*:

This primer pair specifically amplified the mini-circle fragment of 320bp from total DNA extracted from the studied isolate (Figure 3). Different parasite loads were amplified by ITS1-PCR by using LITSR/L5.8S**,** the amplified PCR products from 16 samples exhibiting moderate to strong ITS1 320bp bands with DNA of *L. donovani* (24). Similar study used ITS1 PCR-RFLP and the digestion revealed three bands for *L. infantum* (200, 100, and 50 bp), two bands for *L. tropica* (220 and 50 bp), and two for *L. major* (220 and 127 bp) (17).

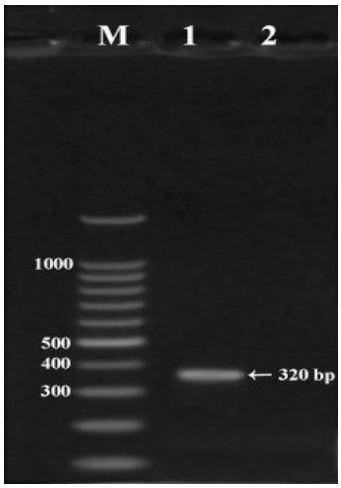

Figure (3): Agarose gel electrophoresis of PCR product of 320 bp of LITSR/L5.8S, M-100bp Ladder, Lane (1) DNA template (25 ng), and Lane (2) negative control.

BHUL18S amplification for identification of *L. donovani*:

The amplification of PCR of BHUL18Sprimerpair which was used to detect *L. donovani*, bp of 311 was observed for our isolate, and no side-product or artifacts appeared on the gel; (Figure 4).

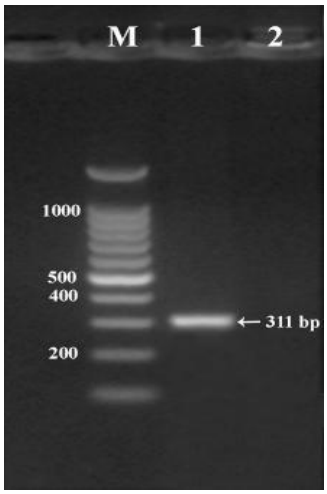

Figure (4): Agarose gel electrophoresis of PCR product of 311 bp of BHUL18S, M-100bp Ladder, Lane (1) DNA template (25 ng), and Lane (2) negative control.

According to previous study, out of 500 parasitologically confirmed clinical samples with kala azar, 439 were PCR positive with BHUL18S primer pair. The test provided successful diagnosis of VL with 87.8% sensitivity using patient's whole peripheral blood, in endemic controls, the specificity was 84%. None of the 250 non-endemic controls were positive and thus, the specificity in this group was 100%, the data from the different disease group also showed 100% specificity as none of the 250 non-leishmanial disease samples were PCR positive. The overall specificity was 94.6% (20).

This study is one of the first identification to characterize *Leishmania donovani* by PCR in Iraq and it is recommended to be adopted to the Iraqi strain of *L. donovani*.

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