

# Real Time-PCR for the detection of the Leishmania sp. and genomic sequencing for the detected the parasite in Babylon, Iraq

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## Abstract

The most frequent type of leishmaniasis to infect people is cutaneous leishmaniasis (CL). A single-celled parasite that is spread by the bite of a Phlebotomine sp. sand fly causes a skin lesion. There are around thirty different kind of Leishmania sp., all of them can cause cutaneous leishmaniasis. In many parts of Iraq, it is one of the most prevalent parasitic infections. This significantly hinders the socioeconomic development of this impoverished province. The samples current study was skin snip and skin swab which are diagnosed through RT-PCR for the detection of the parasite and gene sequencing done for the detected parasite, The result shows that 45 (55.6) was positive microscopical and 36 (44.4) was negative while 53 (65.4%) Positive result of Nested multiplex PCR. The objective of the current study was to molecularly identify the different species of cutaneous leishmaniasis in Babylon, Iraq, patients.

**Keyword:** cutaneous leishmaniasis, molecular method (Nested multiplex PCR), RT-PCR

## 1. Introduction

Leishmaniasis is caused by a protozoa parasite from over twenty species of Leishmania and over than ninety sand fly species are known to transmit Leishmania parasites.

There are 3 main forms of the disease 1st Cutaneous leishmaniasis (CL), 2nd Visceral leishmaniasis (VL), and 3rd Mucocutaneousleishmaniasis (CL) on the other hand the most prevalent type of leishmaniasis was in skin lesions. In 2020, Afghanistan, Algeria, Brazil, Colombia, Iraq, Libya, Pakistan, Peru, the Syrian Arab Republic, and Tunisia accounted for more than 85% of all new CL cases. An estimated 600 000 to 1 million new cases worldwide are reported each year. (1 and 2).

Cutaneous leishmaniasis (CL) is one of Iraq's erratic endemic illnesses, which results in the reappearance of an intracellular obligate protestant parasite to the genus *Leishmania* (3). There are numerous reasons for the rise in leishmaniasis infections that has been seen over the past 25 years in different parts of the world, Other risk factors for the establishment and spread of leishmaniasis include war and instability (4). Globalization and climate change are two variables that contribute to the disease's spread to non-endemic places (5 and 6). Although Leishmania species differ in pathogenicity, virulence, and clinical signs, it also depends on the type of sand fly and the genetic makeup of the host (7). In general, the infection starts as a

PCR primers for Leishmania species detection by ITS1 gene (10)

tiny erythema at the site of the sand fly bite, gradually changes into an inflammatory papule, enlarges, and eventually develops into a painless nodule. Finally, a scarred discolouration is visible (8).

According to (9) the diagnosis of CL is based on the clinical case as well as laboratory investigations such as parasitological, serological, and molecular assays.

## 2. Materials and Methods

A cross-sectional study was carried out from the beginning of October 2021 to the end of April 2022, Specialized doctors diagnosed all patients in this study, and the diagnosis was verified by the history of patients and clinical features. The record of patients consists of the following: ages, genders, family history, other diseases (Diabetes, hypertension), and treatment(drugs).

Eight one skin aspiration samples were collected (48 males and 33 females) from official hospital in Babylon province. The average age varied from 4 months to 45 years old. An aspiration histology samples from the end edge lesion to detect the parasite (amastigote stage), PCR technique also used for DNA detection, and samples are tested by RT. PCR for the detection of the parasite. Genomic sequencing done to the parasite.

There two set of oligonucleotide primers that used in this study:

Gene	Primer	Sequence (5'-3')	Product size
ITS1	F	CTGGATCATTTTCCGATG	300–350 bp
	R	TGATACCACTTATCGCACTT	

### Nested PCR primers for Leishmania species typing by kDNA genome (11)

Gene	Primer	Sequence (5'-3')	Product size
First PCR kDNA primer	F	GGGGTTGGTGAAAATAGGG	720bp
	R	TTTGAACGGGATTTCTG	
Second Nested PCR kDNA primer	F	GGGGTTGGTGAAAATAGGG	650bp
	R	CAGAACGCCCTACCCG	

Making the PCR master mix Each gene's PCR master mix was created using the Maxime PCR PreMix kit and carried out in accordance with the manufacturer's instructions as shown in the table below.:

PCR Master mix	Volume
DNA template 5-50ng	5µL
ITS1 gene Forward primer (10pmol)	1µL
ITS1 gene Reverse primer (10pmol)	1µL
PCR water	13µL
Total volume	20 µL

### 3. Results

#### Demographic characteristics of patients with cutaneous leishmaniasis

Characteristic	Results
Age (years)	
≤ 5 year, n (%)	16 (19.8%)
5-9 year, n (%)	16 (19.8%)
10-19 year, n (%)	25 (30.8%)
20-29 year, n (%)	13 (16.0%)
30-39 year, n (%)	9 (11.1%)
>40 year, n (%)	2 (2.5%)
Mean ±SD	14.74 ± 10.91
Range	4 month – 45 years
Gender	
Male, n (%)	48 (59.3%)
Female, n (%)	33 (40.7%)

SD: standard deviation; n: number of cases

The results of the present study revealed that the age groups are prone to injury were distributed among a wide range of ages, generally from 4 months up to 45 years, but the higher infection rate appeared in age group 10-19 years 25 (30.8%) followed by the age group 5 and 9 years of age accounted for 16 (19.8%). This study noted that the incidence of less than the greater age and can be explained by the development of immunity with the passage of time for the people or the result of previous exposure to infection, and that gives people permanent immunity (12). The low rate of infection in the elderly may be due to their acquisition of immunity as a result of previous infection with the parasite and recovery from it during its early stages of life (13)

#### Frequency distribution of some Associated Clinical features in patient with Cutaneous Leishmaniasis

Characteristic	Patients	
	N	%
<b>Number of lesions</b>		
Single, n (%)	31	38.3%
<b>Multiple</b>		
2-4, n (%)	39	48.1%
5-6, n (%)	8	9.9%
>6, n (%)	3	3.7%
<b>Site of lesion</b>		
Face, n (%)	22	27.2%
Leg, n (%)	4	4.9%
Foot, n (%)	11	13.6%
Hand, n (%)	29	35.8%
Head, n (%)	3	3.7%
Abdomen, n (%)	1	1.2%
Both face and Hand, n (%)	3	3.7%
Both Hand and Leg, n (%)	3	3.7%
Both foot and hand, n (%)	2	2.4%
Both face and foot, n (%)	1	1.2%
Both face, hand and leg, n (%)	1	1.2%
Both face, hand and foot, n (%)	1	1.2%

n: number of cases.

The clinical feature accompanying cutaneous leishmaniasis are shown in table The frequency distribution of patients according to number of lesion was as following, were showed high frequency of infection regarded to 2-4 lesions 39 (48.1%), in compared to 31 (38.3%) and 8 (9.9%) of patients with cutaneous leishmaniasis having from single lesion and 5-6 lesion respectively, while the lowest frequency of patients with more than 6 lesion 3 (3.7%), table (4-2) and This results disagreed with the study which that showed (37.35%) have only a single lesion and (62.65%) with multiple lesions (14). Some approximate results were noticed in other studies, like studies of (15, 16 and 17) . in Iraq, but this was not in agreement with those reported by Musa (18) who mentioned that most of the patients were with single lesions. According to (15), the host's sensitivity and the exposed body parts determine the presence and distribution of lesions. L. major typically manifests as several lesions (up to three), whereas L. minor typically manifests as a single lesion. Tropica tends to be on the nose more frequently (19).

### Comparison of frequency distribution of age and mean age of patients with cutaneous leishmaniasis according to the results of PCR (18SrRNA).

Characteristic	PCR (18SrRNA) results		P
	<i>Leishmania major</i> N= 50	<i>Leishmaniatropica</i> N= 3	
Age (years)			
≤ 5year, n (%)	12 (24.0%)	0 (0 %)	0.100 ¥ NS
5-9year, n (%)	13 (26.0 %)	1 (33.3 %)	
10-19 year, n (%)	10 (20.0%)	1 (33.3 %)	
20-29 year, n (%)	8 (16.0%)	0 (0%)	
30-39 year, n (%)	6 (12.0%)	0 (0 %)	
>40 year, n (%)	1 (2.0 %)	1 (33.3%)	
Mean ±SD	13.69 ± 11.62	24.33 ± 18.58	0.141
Range	4 month – 44 years	9 -45 years	† NS

SD: standard deviation; n: number of cases; ¥: chi-square test; †: Independent T test; NS: not significant at  $p > 0.05$

### Comparison of frequency distribution of gender of patients with cutaneous leishmaniasis according to the results of PCR (18SrRNA).

Characteristic	PCR (18SrRNA) results		p
	<i>Leishmania major</i> N= 50	<i>Leishmaniatropica</i> N= 3	
Gender			
Male, n (%)	30 (60.0%)	2 (66.7%)	0.819
Female, n (%)	20 (40.0 %)	1 (33.3%)	NS

SD: standard deviation; n: number of cases; ¥: chi-square test; NS: not significant at  $p > 0.05$  Liesaccording to the results of multiplex-PCR.

samples was shown in an agarose gel electrophoresis image. M (Marker ladder 2000-100bp). Lane (1-10) *Leishmania major* samples at 650bp product size showed positive results.

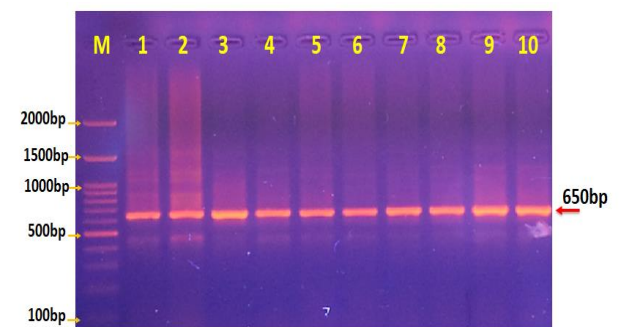
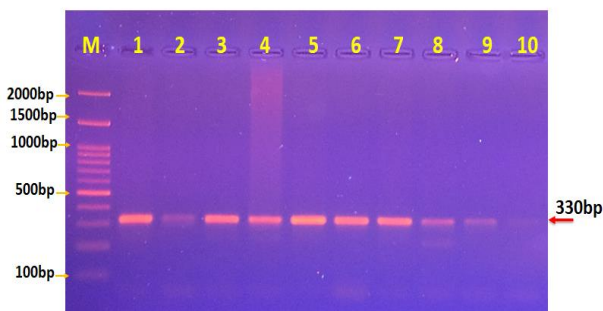
### 4. Discussion

Frequency distribution the gender of patients with cutaneous leishmaniasis according to the results of PCR (18SrRNA). There was no significant association between gender and results of PCR (18SrRNA), ( $p = 0.819$ ).

Males and boys are more likely to be exposed during outdoor activities, which may account for these discrepancies (20). Various researchers think that sex hormone levels may also impact the onset and progression of some parasitic infections, making males more susceptible to the protozoan infectious agent (21). Our findings concur with those of earlier research projects in Iraq(13) People aged 5 to 14 and 15 to 45 have a higher overall incidence of CL. These age ranges represent the school and working years in Iraq for both sexes, and they are more likely than other age ranges to engage in outdoor activities and be exposed to sandfly-related environmental factors. Additionally, infections with *L. tropica* result in the formation of lifetime immunity(15).

In some areas of Iraq, population shifts occur often, causing the disease to affect people of all ages. There are numerous factors, notably in Diyala and Salahuddin, which had the highest number of cases, that are crucial to the incidence and distribution of CL in various regions of Iraq. These include the existence of animal reservoirs for ZCL and the utilization of clay in some village homes in these regions (22). Clay preserves an adequate degree of moisture in the sand fly larval environment, enabling sand fly reproduction in these structures. Most CL cases occurred in provinces with a more rural population; these provinces have sizable agricultural areas that draw and harbor a variety of bug species. The present result showed the 18S ribosomal RNA gene for detection *Leishmania major* were reported in 50 (94.3%) , and the 18S ribosomal RNA gene for detection *Leishmaniatropica*were showed 3 (5.7%).

ITS1 ribosomal RNA gene analysis of PCR products for *Leishmania* species detection from skin lesion samples was shown on an agarose gel electrophoresis image. M (Marker ladder 2000-100bp). Lane (1-10) 330 bp product size samples that were positive for *Leishmania* species.



Nested PCR product analysis of mitochondrial kDNA for detection of *Leishmania* species from skin lesion

The findings of this study confirmed that *L. major* was more predominant in Babylon province and this was identical to other studies in Iraq. This parasite was found to be the cause of Cutaneous Leishmaniasis by many studies like Al-Tamemy and Al-Qurashi (19) who investigated that out of 80 samples, 68 (85%) were positive for *Leishmania* using Nested-PCR. Only seven positive cases were detected as *L. tropica* and sixty one were *L. major* in different area of Wasit province.

The present study agreed with (10) who mentioned that 52 cases were studied in Al-Najaf province using Nested – PCR method to detect CL species. They discovered that 750 bp DNA fragments from *L. major* and *L. tropica* were present in 7 (13.4%) patients and 45 (86.5%) cases in the synthesis of 560 bp DNA, respectively.

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