

Molecular And Diagnostic Study Of Cutaneous Leishmaniasis Species By Nested- PCR Technique In Babylon Province/ Iraq

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Abstract: *The study included identification of Leishmania parasite from cases of cutaneous leishmaniasis, by examination of smears from patients and Nested-PCR technique, Out of 215 cases suspected for cutaneous leishmaniasis included (61) pus and (178) tissue these pus sample were diagnosed by direct smear method to see the Amastigote phase. The cases included in this study belonged to some hospitals in the Babylon as Morjan Medical City Hospital (24) , Musayyib General Hospital(17), Thu Al-Kifl General Hospital (29) and Hashimyah General Hospital(145), during the period from (October 2019 to March 2020). According to stain results,(60.6%) from the (61) total pus sample cases were positive to giemsa stain and after that used the DNA extraction from total number (215) sample in Nested-PCR and the results appear in percent (82.7%) ,these percent divided in to (20.7%) to Leishmania. Tropica and (79.2%) to Leishmania. Major. The study aimed to detection of Leishmania species in Babylon province.*

Keywords: *Cutaneous leishmaniasis, Leishmania, Nested-PCR, Leishmania major, Leishmania Tropic, Babylon province, Iraq.*

1. INTRODUCTION

Leishmaniasis is a disease spread through the bites of a female sandfly and triggered through different varieties of leishmaniasis, which is expressed in three main clinical shapes: cutaneous, mucous and visceral leishmaniasis (1–4). Prevalent in tropical and subtropical regions, about (12) million people are affected in 98 countries, with (350) million people more at infection risk worldwide (5,6) mostly reported from Afghanistan, Algeria, Pakistan, Saudi Arabia, Iran, Iraq, Peru and Brazil (7–9). Cutaneous leishmaniasis exists in at least two shapes (10): 1. Amastigote from Leishmania was elliptical and non-flagellated, 3-5 µm long. 2. Promastigote was a cutaneous type contained in the host sand fly. Worldwide, vector-borne transmission is the most common mode of transmission. Other theories of transmission could occur, such as person-to-person transmission, occupational, sexual, congenital, and injection (11–13). Cutaneous Leishmaniasis cases were more abundant in winter, with a peak in February. The rate of infection then started to decline from April and reaches its lowest in July and August (14).

Cutaneous Leishmaniasis, commonly known as called Baghdad boil, is a very old disease in Iraq. It is a less severe disease which manifests self-healing ulcers. *Leishmania major* and *Leishmania tropica* are causative agents of Cutaneous Leishmaniasis in Iraq (15). The first demonstration of the parasite of CL in Baghdad was by (Wenyon, 1911). (16) noted the relation of the disease to *Phlebotomus sergenti*. In 1930 they were able to infect a human volunteer with Baghdad canine strain and produced a lesion identical to Baghdad boil.

A total reported cases of Leishmaniasis infections in the period from 2008 to 2015 in Iraq, were 17001 cases were reported ranging from the (2.9 - 10.5) / 100,000 individuals. The greatest number of cases were noted in the year 2015 (4000 cases) (17). There are about 30 known species of Leishmaniasis, and more than 10 species that are medically and veterinarily important (18,19). Leishmaniasis is one of the six important infections on the WHO list of tropical diseases research (20).

According to species of parasite and immune response of the patients, the symptoms differ in regions, that beginning as erythematous papule, increase in size producing a nodule, ulcerate and crusts (21,22). The zoonotic type is caused by *Leishmania major* and anthroponotic type is caused by *Leishmania tropica* (23,24).

Leishmaniasis is transmitted by sand flies which leads to parasite inoculation into skin after a bite. The lesions occur as inflammatory erythematous papules which further develop to nodules and crust in the center of the lesion. The lesions are painless and heal spontaneously after several months, and sometimes develop into disfiguring permanent scars (25). CL caused by *L.tropica* and *L.major* are indistinguishable on clinical bases as both erupt in the same way. The size of the lesion ranges from a few millimeters to 4 centimeter or more. The site and number of lesions are an indication of the type of CL. *L. major* usually presents as multiple lesions more than 3 millimeters in diameter and *L. tropica* is more often on the nose (26).

The type of lesion developing in susceptible patients after a bite from a sand fly is dependent on the number of promastigotes inoculated, numbers of bites received, pre-existing state of immunity strain of *Leishmania*, age of patient and type of sand fly (29). The flagellated promastigote stage is transmitted to the mammalian host by the bite of an infected sand fly during a blood meal. Neutrophils are the first cells recruited to the site of the bite and take up promastigotes by phagocytosis [39]. Parasites are then taken up by dendritic cells and macrophages, either via phagocytosis of free parasites or of infected neutrophils (30).

The parasite Leishmaniasis is also capable of infecting fibroblasts, which may serve as a reservoir of infection (31), note that the parasites undergo incubation for a period of weeks or months before they show and the skin develops at the site of the biting and become red in color. After that, it ulcerate and possibly gets injured. Secondary bacteria in many species, for example *L. major* often heal the lesion spontaneously with scarring atrophic and formed leaching in disease Leishmaniasis in the skin site of the bite contains the macrophages in the first place as well as the cells of lymphocyte cells and plasma (32).

Molecular diagnosis may depend on the parasite examination so that only parasites fragments are identified. This technique has nearly 100 percent access to privacy and response values, but these values can vary depending on the types of samples (33). *Leishmania* species recognition is important for the assessment of clinical prognosis and a species therapeutic approach (34). Nested PCR is one of the better parasite genome parts for sequencing to classify multiple *Leishmania* species (35).

2. MATERIALS AND METHODOLOGIES

2.1. Specimen collection and diagnosis

Two hundred fifteen samples (pus and tissue) from patients with skin lesion suspected of cutaneous leishmaniasis (CL) patients through the period from October/ 2019 to March / 2020 in the out patients clinic of the dermatology department in Morjan Medical City Hospital, Musayyib General Hospital, Thu Al-Kifl General Hospital and Hashimyah General Hospital, Babylon Province in Iraq. Cases were diagnosed clinically by a special dermatologist as cutaneous Leishmaniasis and confirmed as CL patients based on clinical symptoms and parasitological parameters. The samples were aspirated from the edges of the skin lesions examined in the advanced parasitology laboratory and DNA laboratory in the Biology department / College of Science-Babylon University at first by direct smear method to see the Amastigote phase and also samples preserved in deepfreeze (-20 °C) for molecular analysis by Nested- PCR technique for all sample because this method more sensitive and to identify different Leishmania species (40) thru working of this study occurred.

2.2. DNA Extraction

Genomic DNA was extracted from specimen of skin lesion by using g SYAN DNA Extraction Kit, the manufacturer's instructions (Geneaid USA). The purified DNA kept at -20 °C for later analysis.

2.3. Molecular Assays.

A. Designing Primer for ITS2 amplification

Designing Primers earlier and utilized to amplify a 230 bp product in *L. major*, a 215 bp product in *L. tropica*. The external primers, Leish out F (5'-AAA CTC CTC TCT GGT GCT TGC-3') and Leish out R

(5'-AAA CAA AGG TTG TCG GGG G-3'), and internal primers, Leish in F (5'-AAT TCA ACT TCG CGT TGG CC-3') and Leish in R (5'-CCT CTC TTT TTT CTC TGT GC3')

The primers were design by (40,41) and provided by (macrogen company, Korea)

B. Nested-PCR

It has been utilized nested-PCR to classify the organisms of Leishmania. The PCR cases and parameters have been described as previously with the minor modification (40). All samples (215) have been checked (40). Nested PCR was performed, the total volume of component of primary reaction was 25 µL, involving 1µL, Genomic DNA, 1 µL of each primers and 12 µL. master combination (Polymerase Taq DNA, 2X Red Master Mixture , Amplicon, Germany) and 10µL H₂O . The first-round PCR was conducted under the listing cases: first denaturation at 95 ° C for 5 minutes; accompanied by 35 cycles of denaturation at 95 ° C for 30 s; annealing at 56 ° C for 30 s; and extension at 72 ° C for 45 s; and a final extension at 72 ° C for 5 minutes. AdditionallyIn the second round (nested) PCR the PCR materials of the positive controls of the first round PCR were utilized as the same first round exception for annealing at 58 ° C for 30 s.

C. Gel electrophoresis

PCR materials were examined by loading in 1 per cent Agarose gel comprising 3µL of ethidium bromide and electrical current was visualized at 75 v, 20 mA for 1 hour (10µl in each well).

3. RESULTS AND DISCUSSIONS

Table 1. Results of parasitological examination by Giemsa stain.

Result	No.	%	X2	P value
Positive	37	60.6 %	2.770	0.096
Negative	24	39.3 %		
Total	61	100 %		

X2: represent chi-square value

S: P > 0.05 Non- Significant

This table illustrate positive sample in percent (60.6 %) from the total number (61), this result was in agreement with what was reported by (42,43). These techniques have limited sensitivities because they require direct visualization of the parasites and the paucity of parasites within the lesion is a hallmark of lesions with old age. Leishmania species recognition is important for evaluating the clinical prognosis and a particular therapeutic approach (18,20,34,44).



Figure 1. Amastigote phase of cutaneous leishmaniasis by direct smear (Giemsa stain, X100)

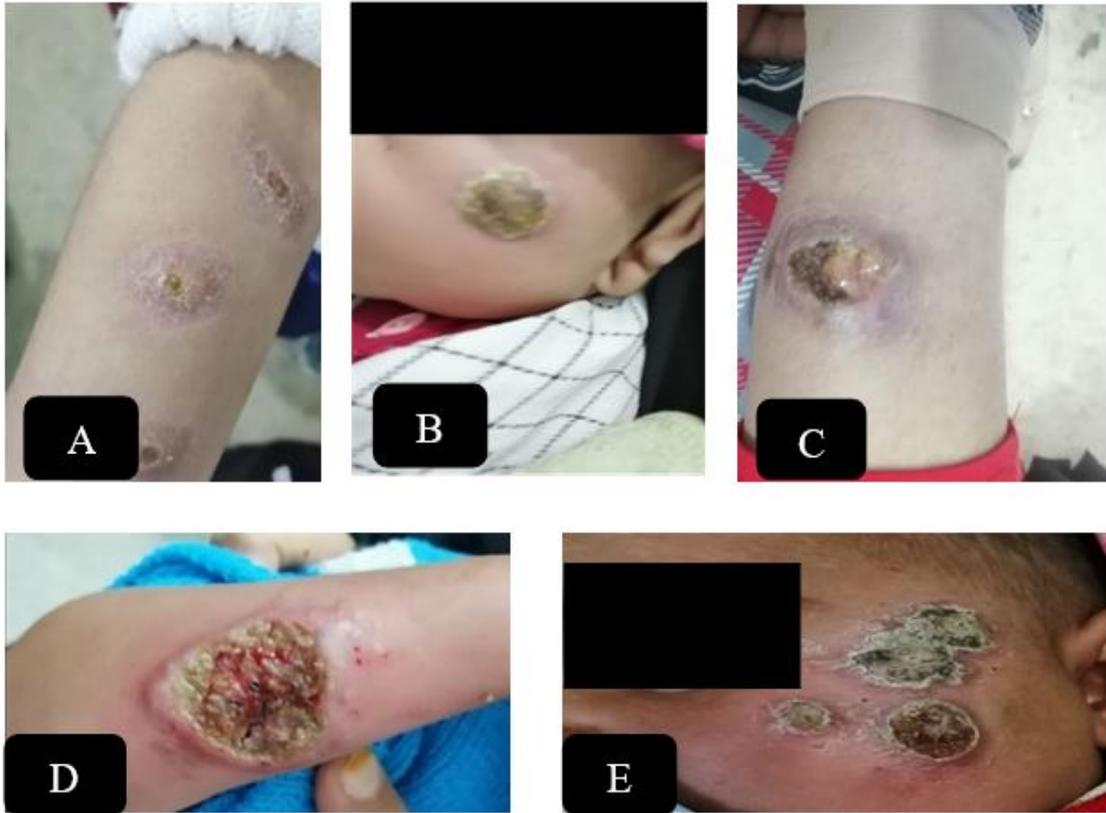


Figure 2. (A, B, C, D & E): Some picture of cutaneous leishmaniasis patients.

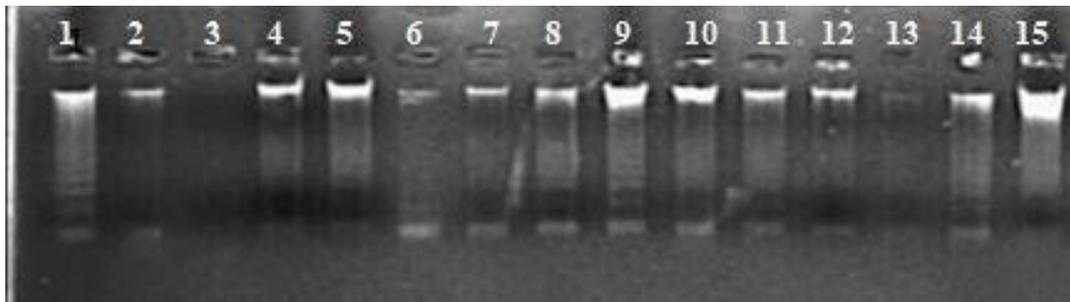


Figure 3. Electrophoreses pattern of DNA extraction of cutaneous leishmaniasis sample, 1% Agarose, 75 v, 20 mA for 1 hour (10µl in each well). lane (1-10) DNA from tissue sample, (11-15) DNA from pus sample.

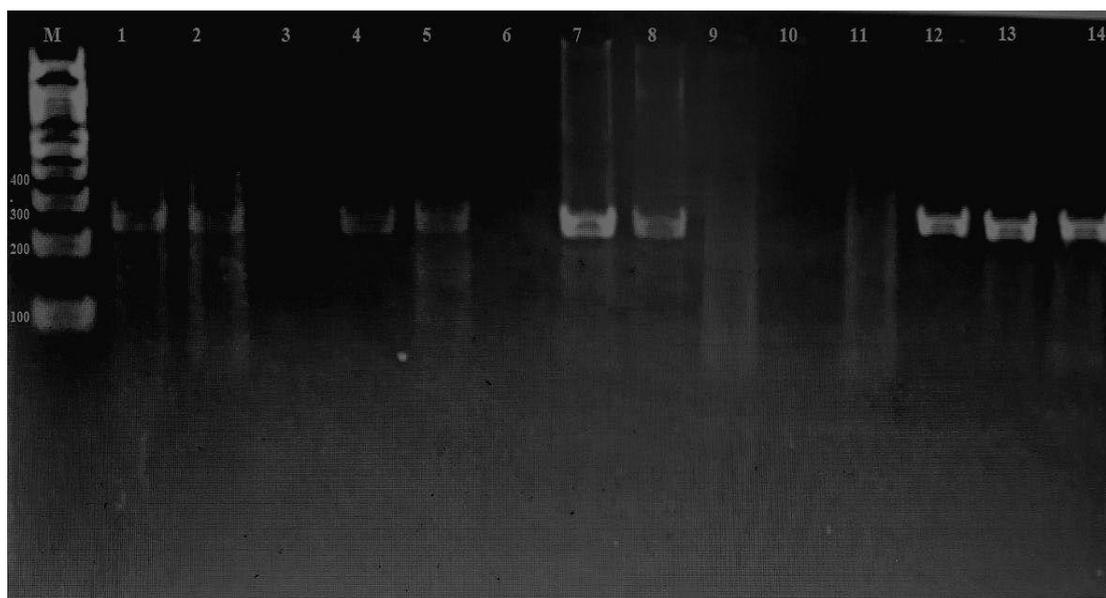


Figure 4. Electrophoresis pattern of Nested-PCR analysis for ITS2 gene: ladder (100-1500.bp), Lane (1-10) leishmanai major and lane (11-14) leishmanai tropica ,PCR product (230) and (215bp) respectively, 1 % Agarose, 75 v, 20 mA for 1 hour (10µl in each well).

Table(2):Detection of Cutaneous Leishmaniasis of all sample by Nested-PCR

Detection method	Total number of sample	Positive		Negative		χ^2	P value
		NO.	%	NO.	%		
Nested-PCR	215	178	82.7 %	37	17.2 %	92.470	0.000

X² :represent chi-square value

S: P ≤ 0.05 Significant

Table 3. Detection cutaneous leishmaniasis species by Nested-PCR.

Species of cutaneous leishmaniasis	No.	%	X ²	P value
<i>L.major</i>	37	20.7 %	60.764	0.000
<i>L.tropica</i>	141	79.2 %		
Total	178	100 %		

X²: Represent chi-square value

S: P ≤ 0.05 Significant

The bands of DNA that appear in figure (3) and the results of current study showed there are (178) samples of *leishmania*, 37 (20.7%) refer to *L.tropica* with band size 215 bp whereas 141 isolates (79.2 %) was *L.major* with band size 230 bp figure (4), that's mean CL foci of *L. major* and *L. tropica* can coexist in the same area, at least in some conditions that remain to be investigated. These results agree with (15,23,40,45), Also the study of (3) confirm that *L.major* was endemic in Jask country more than the *leishmania* species and consider the

zoonotic or rural cutaneous leishmaniasis. And the study for (46) in Qom province found the PCR results confirmed the parasite causes cutaneous leishmaniasis was *L.major*.

4. CONCLUSIONS:

The positive sample of the genetic study was (82.7 %) compared to the microscopically examined samples (60.6 %). After that, the results from the positive samples were used in the Nested-PCR technique by adding another primer to the polymerase chain reaction product to identify the different types of *Leishmania*. The results of the genetic examinations showed that the species *Leishmania major* is the most dominant, at (79.1 %) compared to the type of *Leishmania tropica* at (20.7%).

We conclude from this study that the diagnosis using the polymerase chain reaction (PCR) is more accurate and specialized than the microscopic method in detecting the different types of *Leishmania* parasite and this dangerous parasite is still spreading widely if we compare our results with those of neighboring countries.

Conflict of Interest

The authors declare that there was no conflict of interest in the present research project.

Ethical approval

Ethical approval is taken from the Department of Life Sciences at the Faculty of Science, University of Babylon, as well as the Babylon Health Department.

Source of funding

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