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The molecular detection of leishmaniasis agent in Dhi-Qar Province patients

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Abstract

Objective: One of the most common parasitic diseases is cutaneous leishmaniasis, which is seen especially in the Middle East and North Africa and in the Old World. Female sand flies carry the disease. Visitors, immigrants, and military members are infected with these flies every year .

Aims of study: the aim of this thesis study is to determine the prevalence of cutaneous leishmaniasis in Dhi-Qar Province, as well as to distinguish between the three species of this genus using the PCR-RFLP.

Materials and methods: In this study, 100 patients who applied to dermatology department of the Dhi-Qar Hospital located in Dhi-Qar, Iraq and had clinically confirmed cutaneous leishmaniasis were included.

Results: The PCR results showed that 84 samples were positive in total. The positivity of the samples was distributed in terms of their demographic characteristics. According to the gender distribution, 54 samples of men were positive, and 30 samples were female. According to the age distribution, the highest number of positive samples was observed in the age group of 61-70 years (36 samples, 42%), followed by the older age group of >80 (24 samples, 28%). Both age groups of 51-60 and 71-80 years showed similar sample sizes (12 samples,14%). The distribution of blood groups showed that the higher percentage of samples (71%) belonged to the blood group O+ , followed by both AB+ and A- (14%). The source of the infections showed a higher sample number (42%) by arms, followed by hands, (28%), then face and foot had a similar sample (12 samples, 14%).

Keywords: Leishmaniasis, 18sRNA Gene, Hea enzyme, RFLP, Sequencing

Introduction

Leishmaniasis, a parasitic illness, has various clinical manifestations, including cutaneous (CL) and visceral leishmaniasis (VL). Some skin lesions are able to heal by themselves. CL poses serious public health risk [1] in cases where lesions fail to heal. Moreover, they can cause chronic ulcers and bacterial infections, thus leading to high treatment costs and issues over medication.

Traveling to places where vector-borne illnesses are endemic (if one is coming from a non-endemic region) is a risk worth taking seriously. CL is a parasitic illness caused by bites of sand flies. This disease has been recorded in people who have visited endemic locations [2].

The diagnosis of CL is commonly established under the laboratory conditions by finding out amastigote *Leishmania* forms in Giemsa-stained smears using light microscopy and culture techniques. However, these methods are not used to differentiate *Leishmania* species. DNA-based methods such as various PCR techniques, RFLP analysis, and sequencing have been widely utilized to identify *Leishmania* species in people, animal reservoir hosts, and infected vectors. High-resolution polymerase chain reaction (PCR) tests are used to detect and identify *Leishmania* parasites in people, animal reservoirs, and phlebotomine sand flies [3]. The aim of this study is to investigate the molecular detection of the leishmaniasis agent (*L. tropica* or *L. major*) in Iraqi patients.

Materials and methods

sample

swabs were collected from the lesion of patients and preserved in 300 µL of RNA/DNA shield (CAT; R1100-50). All the patients and controls were examined for their age, gender, blood group, weight, height, and period of infection.

Study design

The study was conducted in Nasiriyah Teaching Hospital, southern Iraq. The samples came from different regions of Dhi Qar Governorate. Infections were diagnosed in the Advisory Division of the Department of Dermatology. The infections of the cutaneous leishmaniasis parasite were in both sexes, males and females, and at different ages, ranging from 40 years to more than 80 years, and in different locations in the body, for example in the hand, face, arms and feet. In this study, skin lesions were taken from these sites and preserved in test tubes. Then we used a special kit to extract the DNA of the cutaneous Leishmania parasite, measured the DNA concentration using a nanodrop spectrophotometer, and transferred onto agarose gel electrophoresis. Then we used conventional PCR technique and fragment length polymorphism technique, using specific gene-targeting primers manufactured by Pioneer (Korea). Those samples were selected from skin lesions with the highest infectious period. Sequences were matched to any sequences in the NCBI genBank database and screened using NCBI software.

Ethical approval

The study was approved by the ethics committee of the Dhi-Qar Hospital and was conducted in accordance with the Declaration of Helsinki (1975).

Statistical analysis

Chi-square test was used to show the difference between the groups in terms of their demographic characteristics.

Results

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Table 4.1 shows the distribution of the samples in terms of their demographic characteristics. The samples showed a higher frequency in males than females (68 VS. 32, respectively). The distribution of samples according to age group showed higher frequency (32) within the age group of 40-50 years, which was followed by the age group of 51-60 years (22) and the age group of 71-80 years (20). Furthermore, the lower frequency was detected in age group of 61-70 years (16). The blood grouping of the samples showed higher frequency in the blood group O+ (36), followed by AB+ (18). Both the blood groups A+ and B- showed a similar frequency, then followed by the blood groups A- (8), and O-(2). The distribution of the samples according to the source of isolation showed higher frequency within the foot (30), followed by arms (28), hands (26), and the face (16).

Table1: Distribution of the CL samples according to gender, age, blood group, and source of infection

	Parameter	Frequency	Percent (%)
Gender	Male	68	68%
	Female	32	32%
Age	40-50	32	32%
	51-60	22	22%
	61-70	16	16%
	71-80	20	20%
	>80	10	10%
Blood Group	AB+	18	18%
	AB-	4	4%
	A+	16	16%
	A-	8	8%
	O-	2	2%
	O+	36	36%
	B-	16	16%
localization	Face	16	16%
	Hands	26	26%
	Arms	28	28%
	Feet	30	30%

DNA was extracted from the 100 samples, the eluted DNA was subjected to electrophoresis through 1% agarose gel to detect the accuracy of the extraction process. Figure 4.1 shows the results of electrophoresis

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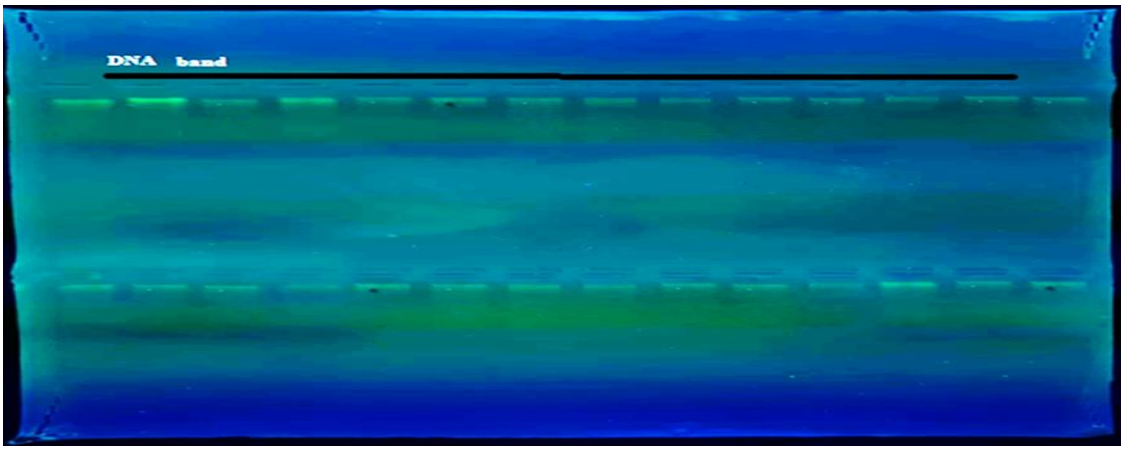

Figure 4.1 Gel electrophoresis of genomic DNA extraction from samples in 1% agarose gel for 30 min

The first primer set utilized in this PCR procedure was designed by NCBI for the Uni21 gene and had a product length of 650-800 bp, as indicated in Figure 4.2. The positive samples showed two sharp band possibilities: (1) 800bp (L. tropica) and (2) 650 bp (L. major / L. infantum.)

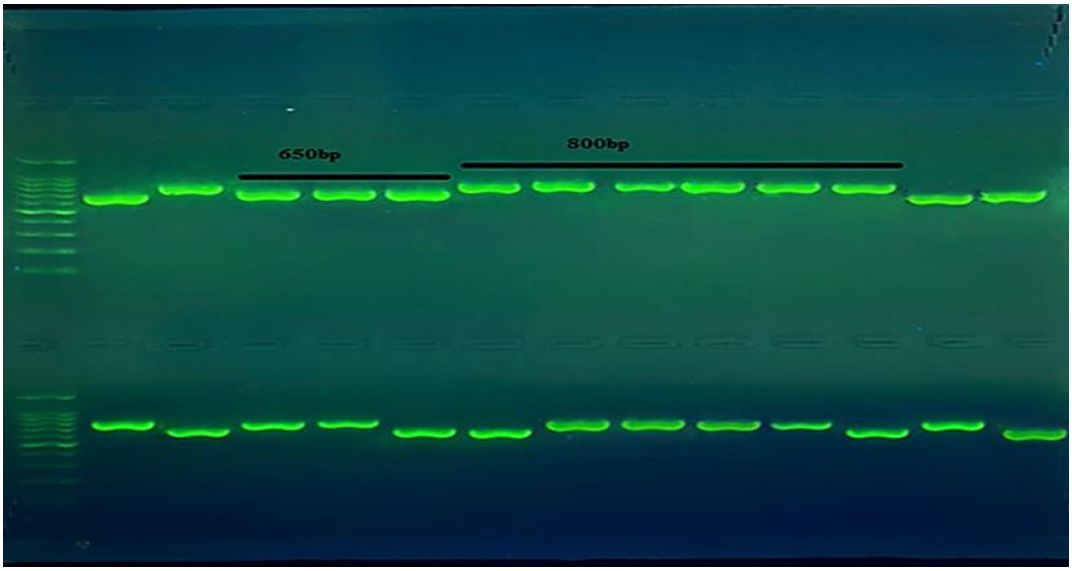

Figure 4.2 *Uni21/Lmj4* PCR result with a band size of 800 bp. Electrophoresis on 2% agarose at 5 volt/cm2 produced the final product.

The second primer set used in this PCR procedure was specific for the *LITSR/L5.8S* gene and was designed by NCBI (350bp). Furthermore, the products of PCR were subjected to gel electrophoresis, as shown in Figure 4.3.

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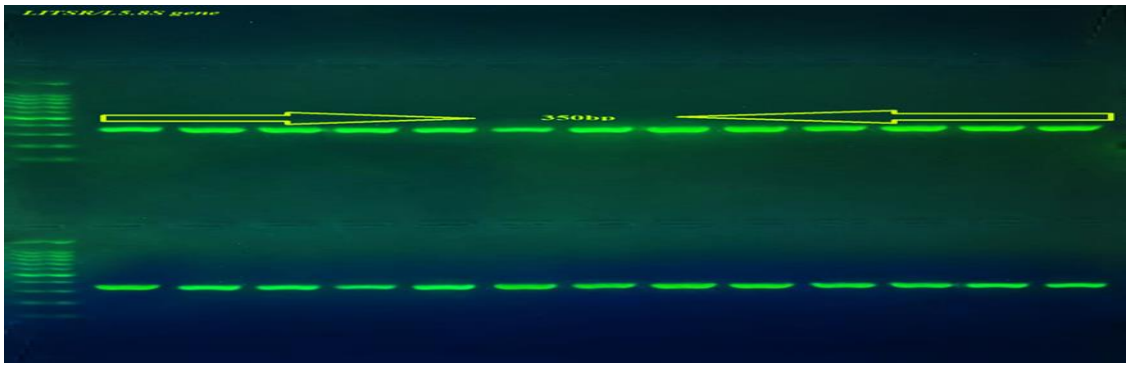

Figure 4.3 LITSR/L5.8S PCR result with a band size of 350 bp. Electrophoresis on 2% agarose at 5 volt/cm2

With the restriction enzyme HaeIII, an endonuclease, RFLP examination of the 84 clinical specimens that were positive for PCR revealed that 41 samples were L. infantum with three bands (200/80/60 bp), 24 samples were L. major with two bands (220/140 bp), and 19 samples were infected with L. tropica with two bands (220/50 bp). Figure 4.4 shows the gel electrophoresis findings. DNA molecular marker with a size of >50 bp on lane 1. Lane 2 represents *L. major*, Lane 3 represents *L. tropica*, and Lane 4 represents *L. infantum*.

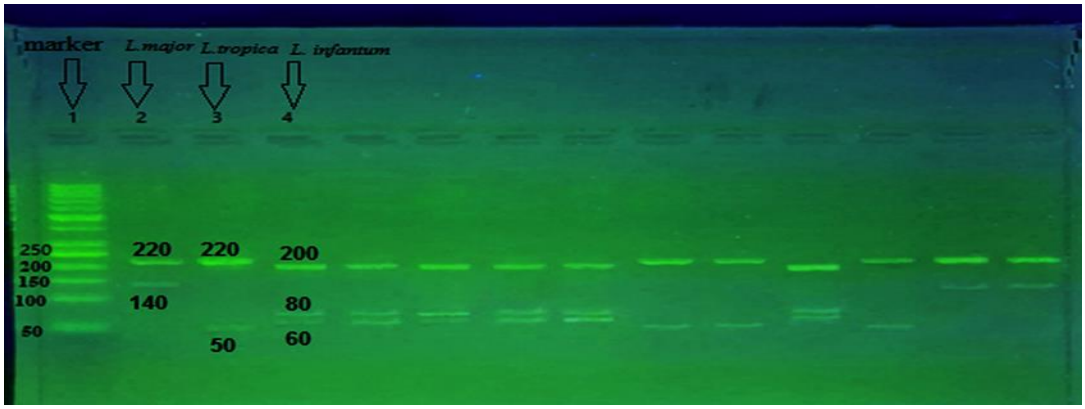

Figure 4.4 Electrophoresis pattern of the PCR product digested with the HaeIII restriction enzyme (2.5% agarose gel). DNA molecular marker with a size of >50 bp. The safe red stain was used to stain the bands in the gel

Leishmania species is shown in Figure 4.5, the higher number of the samples was detected in *L. infantum* (41), followed by 24 samples from *L. major*, and 19 samples from *L. tropica*.

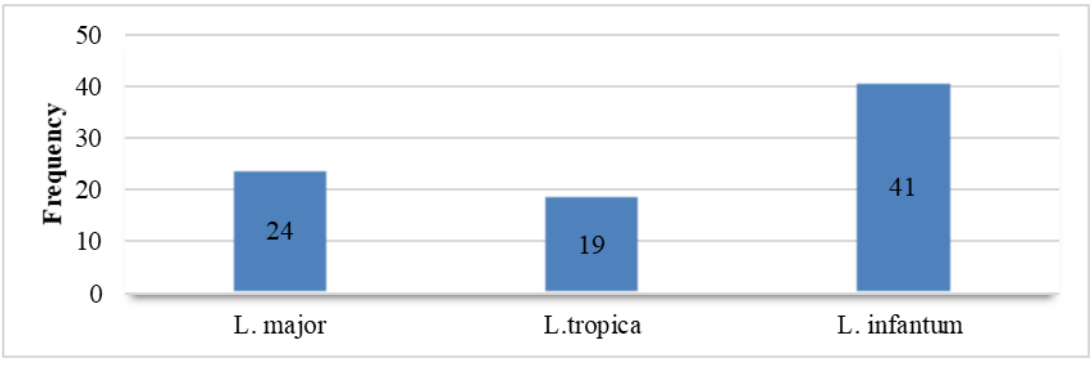

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Figure 4.5 Distribution of positive samples based on the species

Table 4.3 summarizes the sequencing information of nucleotides. The results showed that the five samples of *L. infantum* were 100% complementary to the database sequence under the ID; EF653268.1 (strain MCAN/IR/97/LON 49) 18S ribosomal RNA gene, without any variations between the query and subjects

Source: *Leishmania infantum*			
No.	**Sequence ID**	**Source**	**Identities**
1	ID: EF653268.1	*18S ribosomal RNA gene of Leishmania infantum strain MCAN/IR/97/LON 49*	100%
2	ID: EF653268.1	*MCAN/IR/97/LON 49 18S ribosomal RNA gene from Leishmania infantum strain MCAN/IR/97/LON*	100%
3	ID: EF653268.1	*18S ribosomal RNA gene of Leishmania infantum strain MCAN/IR/97/LON 49*	100%
4	ID: EF653268.1	*MCAN/IR/97/LON 49 18S ribosomal RNA gene from Leishmania infantum strain MCAN/IR/97/LON*	100%
5	ID: EF653268.1	*The 18S ribosomal RNA gene of Leishmania infantum strain MCAN/IR/97/LON 49*	100%

Table 4.3 Sequence alignment information summary for L. infantum samples

The sequencing results were analyzed by BLAST and showed 100% complementary to nucleotides sequence of L. major strain AA6 (internal transcribed spacer 1), which can be reached under the ID MT071546.1 within the NCBI database. The sequence alignment for L. major is summarized in Table 4.4

No	SEQUENCE ID	Source	IDENTITIES
1	ID: MT071546.1	*Leishmania major* strain AA6 internal transcribed spacer 1	100%
2	ID: MT071546.1	*Leishmania major* strain AA6 internal transcribed spacer 1	100%

Table 4.4 Information of sequence alignment for L. major samples

The sequencing results aligned to the sequence from the NCBI database and showed 100% complementary to the L. tropica under the ID: MT071547. Table 4.5 shows the alignment summary.

Source: *Leishmania tropica*			
No.	Sequence ID	Source	Identities
1	ID: MT071547.1	*Leishmania tropical* strain AA1 internal transcribed spacer 1	100%

Table 4.5 Information of sequence alignment for L. tropica samples

Discussion

This study showed a high prevalence of leishmaniasis (84 out of the 100 samples), which is compatible with a previous study [4]. It was used to test molecular techniques to diagnose

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cutaneous *Leishmania* species. The findings revealed that molecular approaches were more specific than traditional serological tests. This study found that males had a greater incidence rate compared to females, which is supported by a previous study. Leishmaniasis affects adult males more frequently than their female counterparts [5].

This study indicated that older patients had a higher incidence rate of CL than younger patients, which is supported by a previous study [6]. Older people make more interleukin-10 and less interferon-gamma, which may help parasites stay around and spread infections.

When it comes to the distribution of positive samples by blood groups, a previous study reported that cutaneous leishmaniasis was more common in certain blood groups [7]. In this study, three major species (*L. tropica, L. major, and L. infantum*) which are present in the Iraqi population, were examined in CL. CL is found all over the country, except for three provinces in the northeastern part. It is transmitted by the sand flies *P. sergenti* and *P. papatasi*, which carry the causative agent *L. major* and *L. tropica* [8].

In 2008, a CL epidemic broke out in Al Diwaniyah, with 300 cases reported. Additional 400 cases were reported in Rahmania province in 2009 [9]. The results are also compatible with another study done in the neighboring country, Saudi Arabia. In that study, it was determined that 49.5% of 206 CL biopsies tested positive for Leishmania major (*L. major)*, 28.6% tested positive for *Leishmania tropica (L. tropica)*, and 3.9% tested positive for *Leishmania infantum/donovani* (*L. infantum/donovani*) [10].

A previous study examined the kDNA 13A/13B gene for PCR and reported that while this gene can provide a profoundly delicate symptomatic device for CL, it cannot distinguish between *Leishmania* species [11]. In another study, PCR and specific primers *Lmj4/Uni21* were used for patients with CL to differentiate between *L. major* and *L. tropica* [12].

The primer that was utilized in this consider and the ponder done by Andres was outlined based on a distributed grouping from the *L. major* DNA, and the items of PCR items appeared the consistent species-specific contrasts in estimate between *L. major* (650 bp), and the other species yielded 800 bp [13].

In their study,[14] examined ninety-eight patients with suspected CL using (*Uni21/Lmj4*) primers and revealed that they were appropriate for amplifying and detecting *Leishmania tropica*. As positive control PCR (650 bp), the primer used in this study could benefit a large number of CL patients.

Conclusions

Frequency incidence was higher in men than women. The blood group affects the incidence rate of CL. A higher rate is seen in the blood group O+. Three major species are present in the Iraqi population; L. infantum, L. major, and L. tropica.

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