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EPIDEMIOLOGICAL AND GENOTYPIC STUDY OF CUTANEOUS LEISHMANIASIS IN AL-DIWANIYAH PROVINCE, IRAQ

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ABSTRACT

The current study included examining 73 samples, the number of positive samples reached 72 samples and one negative sample. Samples were collected from expatriates patients in Diwaniyah Province General hospital during the study period from October 2019 to March 2020.

The study included identifying the cutaneous *Leishmania* parasite species using the Nested PCR technique. The results showed the successful of the DNA amplifications extracted Scraped from the edges of the skin lesion of the infected 56 (77.77%) cases were recorded for *L. major*, corresponding to *L. tropica* 12 (16.66%) and the mixed infection was 4 (5.55%) as for the epidemiology of the parasite, it was found that the percentage of males was 54.16 compared to females with a rate of 45.83%. It was also found that AL-Hamza AL-Sharqi district recorded the highest rate of infection (40.27%), while the district recorded (4.16%). Multiple ulcers recorded 81.94%, compared to single ulcers at 18.05%. October recorded the lowest percentage (1.38%), while January recorded (51.38%).

Keywords: Genotypic study; Leishmaniasis, ulcers, *L. tropica*

Introduction

The term Leishmaniasis refers to an important human disease that is caused by the parasite called *Leishmania*. This parasite grows in the invertebrate host and then moves to the vertebral host like a human through the bite of the infected sand flies (Akhoundi *et al.*, 2016; Alemayehu & Alemayehu, 2017).

The diagnosis of leishmaniasis is based on clinical signs, epidemiological and laboratory data. As for laboratory methods, there is no accurate diagnostic base for infected humans or animals (Rodríguez-Cortés *et al.*, 2010), This led to the weakness in the collection of accurate epidemiological data and thus limited disease control. Moreover, false negative results can delay treatment. Several immunological and molecular diagnostic tools have been developed to diagnose leishmaniasis recently (De Paiva-Cavalcanti *et al.*, 2015). In particular, the use of molecular techniques is increasing due to their high sensitivity and specificities over diverse sets of clinical samples.

L. spp. contains 34-36 chromosomes and unique genomic organization, in which the protein coding genes are organized into polycistronic units and do not contain introns. Moreover, gene expression is regulated after transcription (mRNA stability and translation) (Llanes, Restrepo, Del Vecchio, Anguizola, & Leonart, 2015). Leishmaniasis also has a mitochondrial genome called kinetoplast DNA, which is organized into thousands of Minicircles (0.8-1.0 kbp each) and several dozen Maxicircles (about 23 kbp each) linked into a network The Concatenated network (Galluzzi, Ceccarelli, Diotallevi, Menotta, & Magnani, 2018).

Material and Methods

Samples Collection

Scraped from the edge of the skin lesion were collected from the ulcer of 73 patients before the treatment of the patients with cutaneous leishmaniasis, and the expatriates in Diwaniyah General hospital, as samples were taken for the process of the Nested PCR.

Diagnosis of Samples

Clinical diagnosis: Clinical diagnosis is made by a dermatologist.

Laboratory diagnosis: A direct smear method prepared from the edge of a stained ulcer using Giemsa was used and examined by a high-powered microscope using oil immersion (David & William, 2006).

Genomic DNA Extraction

Genomic DNA was extracted from skin lesion samples by using gSYAN DNA kit extraction kit Geneaid. Taiwan, and done according to company instructions as following steps: A 200mg of skin lesion sample was transferred to sterile 1.5ml microcentrifuge tube, and then added 20µl of proteinase K and GST buffer then, mixed by vortex. And incubated at 60 for 30 minutes. 200µl of GSB cell lysis buffer was added to each tube and mixed by vortex vigorously, and then all tubes were incubated at 60 for 15 minutes, and inverted every 3 minutes through incubation periods. 200µl absolute ethanol were added to lysate and immediately mixed by shaking vigorously. DNA filter column was placed in a 2 ml collection tube and transferred all of the mixture (including any precipitate) to column. Then centrifuged at 10000rpm for 5 minutes. And the 2 ml collection tube containing the flow through were discarded and placed the

column in a new 2 ml collection tube.400µl W1 buffer were added to the DNA filter column, then centrifuge at 10000rpm for 30 seconds. The flow through was discarded and placed the column back in the 2 ml collection tube.600µl Wash Buffer (ethanol) was added to each column. Then centrifuged at 10000rpm for 30 seconds. The flow through was discarded and placed the column back in the 2 ml collection tube. All the tubes were centrifuged again for 3 minutes at 10000 rpm to dry the column matrix. The dried DNA filter column was transferred to tubes 1.5 ml and 50 µl of elution buffer were added to them. The tubes were let stand for at least 5 minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 10000 rpm for 30 seconds to elute the purified DNA.

Nested PCR

The Nested PCR technique was performed for detection cutaneous *L. major* based on The kinetoplast DNA (kDNA). This method was carried out according to method described by(Noyes, Reyburn, Bailey, & Smith, 1998).

Primers

The Nested PCR primers based kinetoplast DNA (kDNA) for *L. major* were design (Noyes *et al.*, 1998) and provided by (Macrogen company, Korea) as in the following table1:

Table 1: Primer genes of molecular diagnosis Nested PCR

Primer	Sequence (5'-3')
External primers	CSB2XF CGAGTAGCAGAAACTCCCGTTCA
	CSB1X R ATTTTTCGCGATTTTCGCAGAACG
Internal primers	13Z ACTGGGGGTTGGTGTAATAATAG
	LiR TCGCAGAACGCCCT

Nested PCR master mix preparation

Nested PCR master mix was by using Maxime PCR PreMix and done according to company instructions as following table2:

Table 2: Nested PCR external master

PCR master mix	Volume
Genomic DNA 5-50ng	5µL
Primary primers forward (10pmol)	1µL
Primary primers reverse (10pmol)	1µL
PCR water	13µL
Total	20µL

After that, these PCR master mix reaction components that mentioned above, placed in standard PCR tubes containing the PCR PreMix as lyophilized materials containing all other components needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, MgCl₂, stabilizer, and tracking dye). Then the tube placed in Exispin vortex centrifuge for 3 minutes. Then transferred in PCR thermocycler.

External thermocycler reaction conditions

PCR Thermocycler conditions was designed for external primer was done according to (Noyes *et al.*, 1998) as following table 3:

Table 3: The design of thermocycler reaction for the external primers.

PCR cycle	Temp.	Time	Repeat
Initial Denaturation	95°C	5min	1
Denaturation	95 °C	30sec.	30cycle
Annealing	55 °C	30sec.	
Extension	72 °C	1min.	
Final extension	72 °C	5min.	1
Hold	4 °C	Forever	-

Gel electrophoresis

PCR products were analyzed by loading in 1% Agarose as following steps:1% Agarose gel was prepared in using 1X TBE and dissolving in water bath at 100 °C for 15 minutes, after that, left to cool 50°C. 3µL of ethidium bromide stain were added into agarose gel solution. Agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray and 10µl of PCR product were added in to each comb well and 5ul of (100bp Ladder) in one well.The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer. Then electric current was performed at 100 volt and 80 AM for 1hour.PCR products were visualized by using ultraviolet transilluminator.

Results

Molecular Diagnosis of *L. major* Using Nested PCR

The *L. major* parasite was diagnosed in the current study using Nested PCR, as the electrophoresis analysis on acarose gel showed the results of the Nested PCR technique.

56 cases were recorded, with a rate of 77.77% for the type *L. major*, compared to the type *L. tropica*, with a number of infected 12, with a rate of 16.66%. As for the people who were found to have mixed infection with both types of leishmaniasis, the number was 4 infected by 5.55%. Table 4 and Figure 1 illustrate these types, with the numbers of infected people and the percentages for each.

Table 4: Molecular Diagnosis of *L. major* Using Nested PCR

Parasite	No.	%
<i>L. major</i>	56	77.77
<i>L. tropica</i>	12	16.66
mixed infection	3	5.55
P value		0*

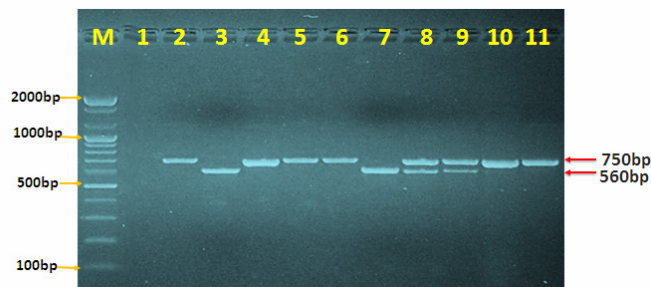


Fig. 1: Agarose gel electrophoresis image that show the Nested PCR product analysis of kDNA in Cutaneous *L. major* positive isolates from skin lesion samples. Where M: marker (2000-100bp), lane (1) Negative control, lane (3, 7, 8, and 9) positive *L. major* at (560bp) PCR product size and lane (2,4,5,6,8,9,10, and 11) positive *L. tropica* at (750bp) PCR product.

Results of the epidemiological study

The current study showed that the highest percentage of leishmaniasis was recorded in males, reaching 54.16% (39 infected), while the lowest percentage of infections was in females, which recorded 45.83 (33 infected) out of the total number of infections of 72 infected.

The study also recorded that the highest rate of infection depending on the residential area was in AL-Hamza AL-Sharqi district, with 29 infecteds out of the total number of infections, and by 40.27%. On the other hand, Sunnia district recorded the lowest percentage of the infections, with only 3 infecteds, and by 4.16%.

When studying the distribution of Leishmania parasite infections according to the number of ulcers, it was found that 59 infected people had multiple ulcers, with a rate of 81.94%. While it appeared that 13 patients had one ulcer, by 18.05%.

The highest rate of infections was in January, when 37 infections were recorded, or 51.38%. This corresponds to the lowest percentage recorded in October, which was for one person only, at 1.38%.

Discussion

Both types of *L. tropica* and *L. major* were diagnosed using the primers of the mitochondrial kinetoplast DNA.

The results of this study are in agreement with (Mohammad & Hmood, 2018) conducted in Al-Diwaniyah province/ Iraq, which stated that the percentage of *L. major* in the positive samples was 70%, while the percentage of 14% was from the type *L. tropica* and the negative samples for the type *L. major* were 16%.

It also agreed with a study (Ghasemian, Maraghi, Samarbafzadeh, Jelowdar, & Kalantari, 2011) in the city of Ahwaz (the capital of Khuzestan province, in southwestern Iran), which found that 97 of the 100 cases examined were of *L. major*, while the remaining three cases were of type *L. tropica*, and thus the predominant species causing cutaneous leishmaniasis in Ahwaz is *L. major*.

The similarity of our study with the results of studies conducted in the regions of Al-Diwaniyah province may be an indication that the rural type of *L.* is more endemic than the tropical type in the province, and the nearby provinces.

The current study showed that there were no significant differences between Leishmaniasis infection between males and females. The incidence of leishmaniasis among males was 54.16%, and its rate among females was 45.83%.

Our study agreed with some studies in Iraq and the world. At the country level, it agreed with the study (Al-Difaie & Ghyda, 2014) that was conducted in Diwaniyah province, as the infection rate in this study was 50.90% for males, and for females 49.09%. Thus, it did not record significant differences between the genders.

The results of this study differed with (Khademvatan *et al.*, 2017) in southwestern Iran, which recorded an infection rate of 60.1% in males compared to 39.9% in females.

Perhaps the reason for this slight increase in the number of infections to males is that males are more susceptible to sand fly stings than females because they move

more than females, in addition to their constant presence in farms and orchards.

Our current study showed significant differences in infection rates in the cities of Al-Diwaniyah province, as AL-Hamzah AL-Sharqi district acquired the largest percentage (40.27%), while Sunnia had the lowest percentage (4.16%).

Our study agreed with a study (Al-Waaly & Shubber, 2020), which found that the infection rate in Hamza district is 63.79%.

The reason may be due to the spread of the sand fly (Hares) in this province in general. In most Leishmania foci, the vast majority of sand flies are uninfected, yet infected flies are highly effective vectors. Infected flies are likely to remain so for a lifetime, which takes 4-18 days. Under Optimum Conditions (Garcia & American Society for, 2016).

The results of this study indicated that 81.94% of the patients had multiple ulcers, compared to 18.05% of the patients who had one ulcer.

Our study was in agreement with the study (Al-Waaly & Shubber, 2020) that recorded people with multiple ulcers by 60.44%, while those with one ulcer accounted for 39.65%.

It disagreed with (Al-Samarai & Al-Obaidi, 2009), which found that 58% of patients had multiple ulcers, and 42% had one ulcer.

The reason for the number of ulcers may be due to the availability of optimum opportunities for sand fly bites, such as the nature and size of the exposed area of the body, as well as the number of flies that sting, or if the same fly bites more than one bite at the same time.

The highest incidence rate in our study was in January, when it was recorded at 51.38%. This corresponds to the lowest percentage recorded in October, which is 1.38%.

(Al-Waaly & Shubber, 2020) recorded the highest rate of infection in December, January and February, at 57.62%, 51.16% and 52.25%, respectively, while the lowest percentage was in July and August by 2.70% and 2.06%, respectively.

The cause of the spread of *L.* parasite in January may be attributed to the proliferation of insects and the increase in their numbers in March and April, and the transmission of infection to humans after two months, and after the incubation period of the parasite within 4-6 months, symptoms of the disease appear in winter.

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