Genotyping of Histological Cutaneous Leishmaniasis Isolated by Dental Broach Smear in Iraqi Patients

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Abstract

Cutaneous leishmaniasis is a major public health problem as a disease endemic in Iraqi population. Many diagnostic tools are used to establish the diagnosis like smear, histopathology and culture. To find more rapid, sensitive and specific diagnostic method for the diagnosis of cutaneous leishmaniasis, and to detect the genotypes of *leishmania tropica* and *leishmania major* strains in Iraq. Sixty six patients (43 males and 23 females) with clinical diagnosis of cutaneous leishmaniasis were included in present work during the period between December / 2019 to May / 2020 in Al-Yarmook Teaching Hospital and Al-Karama Teaching Hospital. The age of patients ranged from 6 months to 55 years, with median age 24 years. The following diagnostic techniques were carried out for diagnosis of cutaneous leishmaniasis including dental broach smear, histopathology examination and culture on Roswell park medium institute (RPM I 1640) and on NNN media. In addition to polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP), genotype techniques were performed for all patients. Sixty patients with other skin lesions were processed for PCR as a control group.

Key words: *Cutaneous leishmaniasis, genotypes, Al-Karama Hospital, PCR, histopathology.*

Introduction

Cutaneous leishmaniasis is a clinical manifestation in which the parasite causes one or more slow-healing ulcer on the skin. The diagnostic methods available at present are mostly based on clinical and epidemiological evidence and parasite detection [(1)] . So far, no single laboratory method has been accepted as a gold standard for diagnosis CL. Parasitological tests of a skin biopsy specimen are not always conclusive in patients with a clinical diagnosis of CL [(2)]. The different species of Leishmania present the same morphology when classical diagnostic methods, such as microscopic examination of Giemsa-stained smears or parasite culture, are used [(3)] . This led to the development of molecular approaches combining high sensitivity for direct detection and identification in clinical specimen with species specificity by amplifying either species-specific DNA sequences or genus-specific sequences that allow for subsequent differentiation of Leishmania species [(4)]. Polymerase chain reaction (PCR) is a technique that permits the exponential amplification of a known DNA sequence from minimal sample quantities, increasing the sensitivity of detection [(5)] . Due to its high sensitivity and specificity afforded, the PCR has been widely used in the diagnosis and epidemiological studies [(6).] There are in numerous DNA sequences used as targets for the diagnosis and detection of Leishmania in PCR protocols. The principal sequences used are kinetoplast DNA (kDNA), mini-exon, ribosomal DNA, and glucose-6-phosphate dehydrogenase, among others [(7)] . Only a few of the assays described in the literature permit identification of the etiological agent of American Cutaneous Leishmaniasis (ACL) at species level, the majority identifies genus and subgenus [(8).] The mini-exon gene of kinetoplastid protozoa, which is involved in the transsplicing process of nuclear mRNA, is present as 100 to 200 tandemly repeated copies per nuclear genome. Each repeat consists of three major parts: a transcribed

region comprising a highly conserved 39-nucleotide exon; a moderately conserved intron, approximately 55 to 101 bp; and a nontranscribed, intergenic spacer of variable length (51 to 1,350 bp) depending on the genus and species [9].

Material and Methods

Cutaneous leishmaniasis is a major public health problem as a disease endemic in Iraqi population. Many diagnostic tools are used to establish the diagnosis like smear, histopathology and culture. To find more rapid, sensitive and specific diagnostic method for the diagnosis of cutaneous leishmaniasis, and to detect the genotypes of *leishmania tropica* and *leishmania major* strains in Iraq. Sixty six patients (43 males and 23 females) with clinical diagnosis of cutaneous leishmaniasis were included in present work during the period between December / 2019 to May / 2020 in Al-Yarmook Teaching Hospital and Al-Karama Teaching Hospital. The age of patients ranged from 6 months to 55 years, with median age 24 years. The following diagnostic techniques were carried out for diagnosis of cutaneous leishmaniasis including dental broach smear, histopathology examination and culture on Roswell park medium institute (RPM I 1640) and on NNN media. In addition to polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP), genotype techniques were performed for all patients. Sixty six patients with other skin lesions were processed for PCR as a control group. Gene sequences the results were compared with basic local alignment reference sequences.

Statistical Analysis

The data were analyzed by using Microsoft Excel 2007 and SPSS version 18. The Chi-square test was used to compare of differences between study groups.

Results

In all age groups, males 43(65%) were more frequently infected with CL than females 21 (35%) as shown in table (1). The differences between genders were statistically highly significant (p. value < 0.05). The highest age group infected with CL was (30-44) years. The differences between age groups were statistically significant (p. value < 0.05).

Table (1): The frequency of cutaneous leishmaniasis according to age and gender in a patients group

Gender Age(year)	male	female	Total	%
0-14	11	7	18	27.3
15-29	12	7	19	28.7
30-44	13	7	20	30
45-59	7	2	9	13.6
Total	43 (65%)	23 (35%)	66	100

Chi-square 10.8, P. value = 0.001 for sex. For age Chi-square 8, P. value= 0.0460

Twenty two (33.33%) patients had a single lesion, while forty four (66.66%) patients had multiple lesions. Table (2) showed that the main part of the body infected with CL was at the upper limbs and hand (38.5%), followed by the face (25%), then the lower limbs and feet (21.5%), while the lowest frequency of infection (15%) was in other parts of the body. The statistical difference between the locations of skin lesion was not significant (P. > 0.05).

Table (2): The location of skin lesion in different parts of the body in cutaneous leishmaniasis patients

	Number	%	Location of skin lesions			
			Upper Limbs & hands	Lower Limbs & feet	face	other
Single	22	33.33%	12	4	10	5
Multiple	44	66.67%	65	39	40	25
∑	66	100%	77	43	50	30
%			38.5%	21.5%	25%	15%

*Chi-square 2.066, P.value < 0.9561.

When the number of skin lesions was from 1 to 9, it has been found that males were more affected than females, while when the number of lesions was 10 or more females were much more affected as shown in table (3). The statistical difference between numbers of lesions according to gender was highly significant (P. < 0.05)

Table (3) Number of lesions according to gender in patients group

No. of lesion	male	female	Total	%
1-3	26	7	33	50
4-6	8	4	12	24.2
7-9	7	1	8	12.1
10 or more	2	11	13	19.7

*single skin lesion in 20 cases

*multiple skin lesions in 40 case. *Chi-square 21.324, P. value < 0.0033.

All the positive cases in culture methods was considered as golden standard and compared with other methods. It had been found that PCR was more specific than other methods. All the positive cases in culture method gave positive results in PCR, so the specificity of PCR was 100%, while the specificity of dental broach smear was 75.55% and histopathology was 50% .

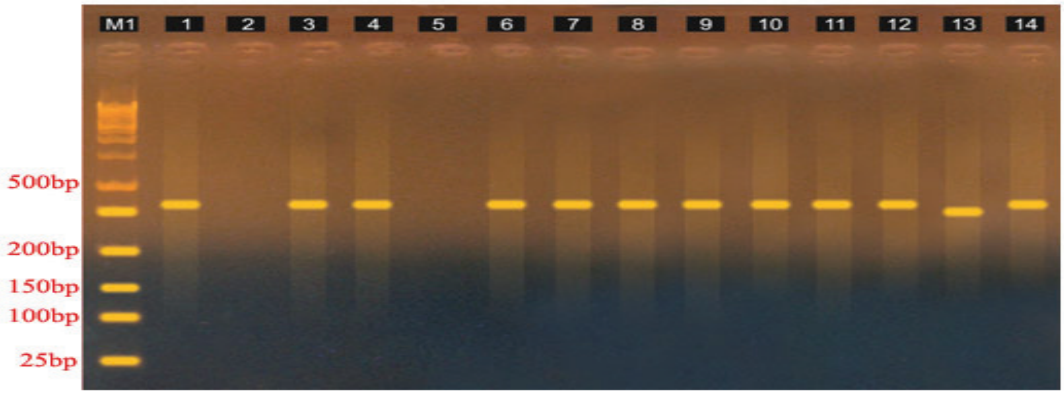

Figure (1) PCR products of mini-exon gene of Leishmania species from patients group, on 1.5% agarose gel, M1 & M2 molecular marker 1Kbp (1000-25 bp), Lane 1 positive control; Lane 2: negative control (distil water instead of DNA template); Lanes (3-14): samples, with voltage 100 for 30 min. Lanes (3,4,6,7,8,9,11,12,14): positive samples L major at M.W. 430 bp; Lane 13: positive sample L. tropica at M.W. 400bp.The mini-exon PCR assay of 60 samples by using specific primers for specific *Leishmania* gene (mini-exon gene) amplification products varied in size which was indicative of different *Leishmania spp.,* from 55 positive samples, 33 samples were identified as *L. major* (60%) with amplicon molecular Wight 430bp, and in 22 samples were identified as *L.tropica* with amplicon molecular Wight 400bp. (Fig 2).

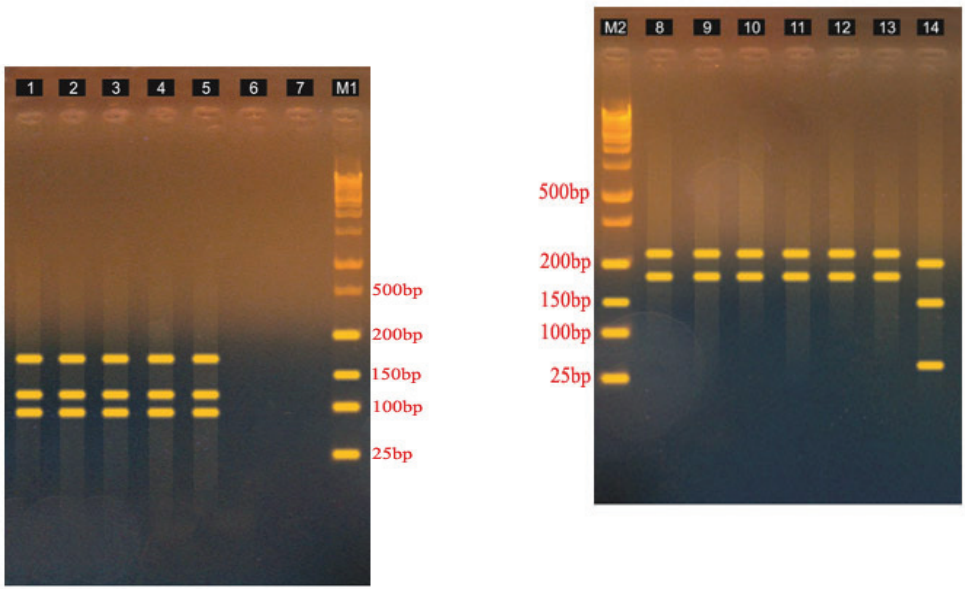

Figure (2): Genotyping of *Leishmania spp*. by Restriction Fragment Length Polymorphism (RFLP) analysis after digestion with (*Eae* I and *Hae* III) on 2.5% agarose gel. M1 & M2: 1Kbp (1000-25 bp) molecular marker; Lanes 1, 2, 3, 4, 5 L. major; Lanes 8, 9, 10, 11, 12, 13, 14 L. tropica. With voltage 100 for 30 min.

Twelve samples was makes gene sequences the results were compared with basic local alignment reference sequences

***Leishmania* tropica isolate 31 isolated internal transcribed spacer 1 and 5.8s ribosomal RNA gene partial sequence.**

Sequence ID: KP773410.1Lengh: 309 number of Matches: 1

Alignment statistic for match # 1

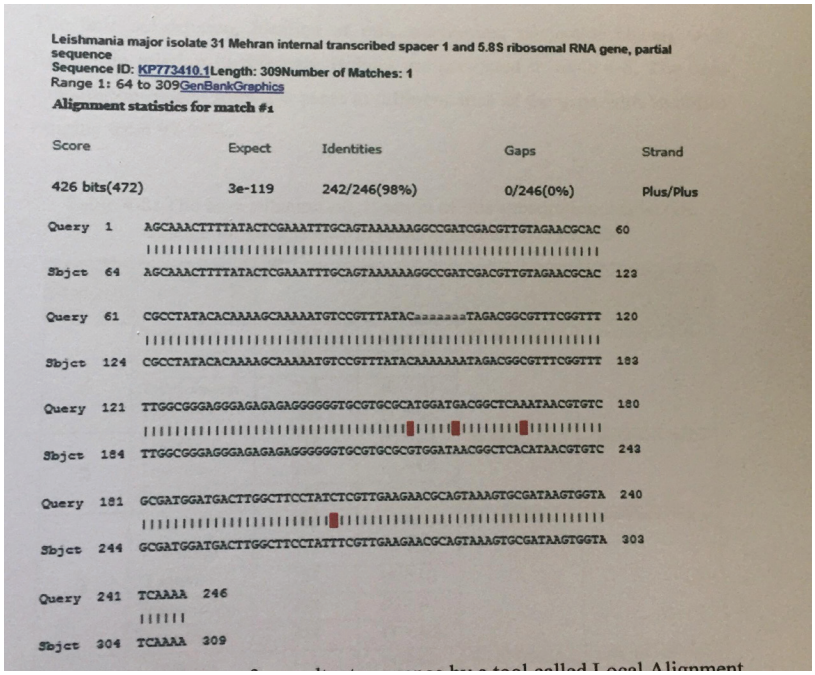

Figure (3): gene sequences results of 12 samples detected by tool of local alignment

Table (4) showed the mutation that occurrence in the numbers of positions in substantiation nucleotides in comparison of reference isolates KY612611.1.

No. of sample	Positions	Mutation	parasite
1	218	G>A	Leishmania Major
	224	A>G	
	213	C>A	
	261	T>C	
2	112	T>C	Leishmania Major
	180	G>C	
	218	G>A	
	224	A>G	
	233	C>A	
3	178	T>C	Leishmania Tropica
	179	G>A	
	185	A>G	
	194	C>A	

Discussion

Cutaneous leishmaniasis also known in Iraq as aghdad boil, is the most common form of leishmaniasis. It is a skin infection caused by a unicellular parasite that is transmitted by sand fly bites. There are about 20 species of Leishmania that may cause cutaneous leishmaniasis. The total incidence of cutaneous leishmaniasis in Iraq varies from 2.3 to 45.5 / 100000 [10] . According to age and gender in a patients group**,** The present study showed significant differences between males and females infected with CL in all age groups. In the present study males were the predominant (65%) than females (35%) were in agreement with previous Iraqi study [11] where males were (57%) and females were (31.7%). But our results were disagree with the other study done by (Saki *et al*. 2010) in Iran which has found that females were more predominant (54.68%) than males (45.31%). These results are somewhat difficult to explain and may be due to the fact that males are working out-door and also due to men are less covering than women and expose to the insect biting more than females. The more frequency of CL in this study was in age group 0-14 years showing agreement with the other studies done in Iraq [11] . This difference in age group could be due to this age group playing outdoors for long time and more exposure to the infected sand flies. The most of Cutaneous leishmaniasis lesions were present on the exposed parts of the body; the highest frequency was (38.5%) at upper limbs and hands followed at face (25%) and then lower limbs and feet (21.5%) while the lowest frequency was in other parts of the body (15%). These results were in agreement[12], in which was (57%) in upper limbs followed by face (25%) then lower limbs (15%) and also agreement with other studies done in Sri Lanka [13] , which had found that CL lesions occurred mainly on the upper limbs and the lower limbs, and less frequently on the face. When the number of lesions from 1-9, males were suffering more than females, but when the number of lesions 10 or more, females were much more suffering than males and showed the significant difference between male and female with number of lesions.

The present study showed two type of Leishmania Spp. In Iraq, by used mini-exon PCR assay, showed L. major (60%) while *L. tropica* (40%) and this agreement with other Iraqi studies [14], and other study in nearby countries such as Iran [15], hence the high frequency of *L. major* may be due to the presence of reservoir animals in large numbers, especially rodents and dogs (reservoir of *L. major)*. Obviously, dense populations of natural hosts of *L. major*, together with abundant vector sand flies, are the key elements responsible for the high rate of human infection, [16]. This study used a new genotype assay for molecular diagnosis of the different *Leishmania* species and strains in Iraq, it is based on amplification of the mini-exon gene and combined it with restriction digests of PCR product (RFLP technique), a method which is routinely used for genotyping tasks [17] . The resulting patterns of restriction fragments were characteristic for each species and achieved a high resolution and high discrimination power. The exon is highly conserved, whereas the intron and non-transcribed spacer region vary in size and sequence among different species. Species identification was performed by digesting mini-exon PCR products with one or two different restriction enzymes [15] Restriction fragment length polymorphism (RFLP) generated species-specific patterns of bands visualized in agarose gels, which allowed differentiating each species and strain unequivocally . This study was in agreement with Saki et al 2010 these all *Leishmania* species of subgenus *Leishmania* can be distinguished by mini-exon PCR-RFLP, with *Eae* I being the most informative restriction enzyme. However, in two *L. tropica* samples used in our study, *Eae* I failed to cut the mini-exon PCR products of these samples and we used another restriction enzyme *Hae* III to digest the products of PCR. The mutation that occurrence in the numbers of positions in substantiation nucleotides in comparison of reference isolates KY612611.1. *Leishmania Major*: G>A, A>G, C>A, T>C, T>C, G>C, G>A, A>G, and C>A, while *Leishmania Tropica*: T>C, G>A, A>G and C>A.

Conclusions

Sampling using dental appeared to be more active

than other sampling techniques used in this study. Histopathol ogical sections have identified the focal presence of the parasites. polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) was more specific than other methods.

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Conflict of Interest : The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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