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## Detection of *Leishmania tropica* Using Nested-PCR and Some of Their Virulence Factors in Thi-Qar Province, Iraq

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### Abstract:

Cutaneous leishmaniasis is one of endemic diseases in Iraq. It is considered as widely health problem and is an uncontrolled disease. The aim of the study is to identify of *Leishmania* species that cause skin lesions among patients in Thi-Qar Province, South of Iraq, also to detect some virulence factors of *L. tropica*. This study includes three local locations, Al-Hussein Teaching, Suq Al-Shyokh General and Al-Shatrah General Hospitals in Province for the period from the beginning of December 2018 to the end of September 2019. The samples were collected from 80 patients suffering from cutaneous leishmaniasis, both genders, different ages, various residence places and single and multiple lesions. Nested-PCR technique was used to amplify kinetoplast minicircle fragments DNA (*kDNA*). Conventional-PCR was performed for determination of some virulence factors (*LPG1*, *GP63*, *CPA* and *PPG1* genes). The electrophoresis findings of *kDNA* gene showed two species of the parasite found in the study area, 65 samples were positive for cutaneous leishmaniasis, *L. tropica* at 750bp and *L. major* at 560bp. Generally, *L. tropica* (57.5%) was the most common specie and *L. major* (23.75%) appeared in a low level. There are no significant differences between the infections of males and females, while there are significant differences at the comparison between age groups. All virulence genes (*LPG1*, *GP63*, *CPA* and *PPG1*) appeared in all *L. tropica* isolates with high percentage (100%). *L. tropica* is the major specie which that caused CL in Thi-Qar province, while *L. major* appeared in low incidence. The virulence genes, which were reviewed, are necessary and important in pathogenesis of *L. tropica*.

**Keywords:** CPA, GP63, KDNA, *Leishmania tropica*, LPG1, PPG1.

### Introduction:

Cutaneous leishmaniasis (CL) is considered as a global health problem, it is caused by the intracellular protozoa that belongs to the *Leishmania* genus, that is transmitted by the bite of the sand fly species (1). The most common leishmaniasis form is CL which infects skin epidermis to form papule usually painless and chronic, often at site of infected sand fly bite (2). *Leishmania tropica* and *L. major* cause CL and have same life cycle, but they are spread in different localities and have different intermediate and reservoir hosts (3). The disease transmits into human when human, sand fly and the reservoir share same the environment (4). *L. tropica* causes anthroponotic cutaneous leishmaniasis (ACL), while *L. major* causes the zoonotic cutaneous leishmaniasis (ZCL) (5). *Leishmania* must evade

from the innate and adaptive immune responses. It is engulfed by macrophage but resist proteolysis and degradation in the phagolysosome (6). An infection of any type of *Leishmania* depends on complex, virulence factors and host immunologic response (7). *Leishmania* produces various virulence factors to facilitate transmission of parasite and infect mammalian host (8). Lipophosphoglycan (LPG) has an important role not only in invertebrate and vertebrate host, but too during early steps for the establishment of the infection (9). However, LPG plays an important role to avoid of the parasite lysis by the host complement system, either by preventing complement molecule attachment or inactivate the assembly of complement complex on the promastigote surface (10). It binds with complement receptor (CR3) and anchored

molecules on macrophage surface to facilitate parasite engulfment (11). LPG1 is one of the key enzymes in the LPG biosynthesis, that are involved in the synthesis of the LPG glycan core. As well as, *Leishmania* that lack *lpg1* gene express incomplete LPG, not contain PG domain (12). GP63 is as known leishmanolysin or is called Major Surface Protease (MSP) or promastigote surface protease (PSP), also it is called a zinc- dependent metalloprotease (13). GP63s vary in their sequence (especially in untranslated regions), array in the *Leishmania* genome (14,15). GP63 cleaves complement protein C3b and converts to C3bi, this leads to silent entry of parasite into macrophage (16). GP63 is responsible for a migration of the parasite through the extracellular matrix, avoid lysis by the complement system, evasion from macrophagic intracellular hydrolysis (12,17). Cysteine proteases (CPs) are degrading enzymes which cleave various proteins, as well play an essential role in many pathogenic protozoa and other microorganisms (18). CPs appear to have an essential role in pathogenicity, modulation or evasion of the host immune response, cell/ tissue degradation and damage, catalyze hydrolysis of various host proteins differentiation of promastigote and autophagy (19,20). There are several types of PPGs, including filamentous proteophosphoglycan and GPI-anchored membranous proteophosphoglycan (21). PPG is proved to bind to macrophage receptors in order to facilitate parasite invasion; this may explain that PPG plays an early role in infection and the prevalence (22,23). The *PPG1* gene encodes a membrane-bound PPG (24). Lima *et al.* (25) confirmed a role of *L. infantum*-derived LPG which it plays in the pro-inflammatory response in *Leishmania* infection. Mohammad and Hmood (26) recorded gp63 and GIPLs by a ratio of 100% in all *L. major* samples in AL-Qadisiyah Province in Iraq.

Despite of the importance of cutaneous leishmaniasis, and to our knowledge, there is limited information about *Leishmania* species prevalent in the area. This study aims to identify *Leishmania species* that cause human skin lesions in Thi-Qar Province, south of Iraq by kinetoplast minicircle DNA gene, as well as determine some virulence genes of *L. tropica*.

## Materials and Methods:

### Study area and sample collection

The protocol of this study was approved by management of training and human development department/ Thi-Qar Health Office (32877 at 2/12/2018). The study includes three locations to the samples collected: Al-Hussein

Teaching, Suq Al-Shyokh General and Al-Shatrah General Hospitals in Thi-Qar Province, South of Iraq for the period from the beginning of December 2018 to the end of September 2019. The hospitals have received patients suffering from cutaneous diseases. All medical information was taken from the hospital records. Written consent is obtained from all participants by the collector and the participation in the study is voluntary and they were free to withdraw at any time from the study. The samples were collected from lesion fluid of 80 patients with CL, both genders, different ages, various residence places in the province and from single and multiple lesions. Expected skin infection lesions were injected using normal saline (0.2 ml) in lesion edge, then pulled again, after that the fluid was kept in plain tube (27,28).

### Genomic DNA extraction

DNA was extracted from lesion fluid using gSYAN DNA kit extraction kit Geneaid according to protocol of produced company (Geneaid/ Taiwan). DNA concentration was examined using a Nanodrop spectrophotometer and then stored at - 20°C until used in PCR amplification.

### Nested-PCR amplification

The *kDNA* was amplified for identification of *Leishmania* isolates, using Nested-PCR, with some modification of PCR according to (29), which included two steps. Target DNA undergone the first run with external primers: CSB2XF and CSB1XR (Table 1), then the first run product undergone second run with internal specific primers:13Z and LiR.PCR-master mix was prepared by (AccuPower® PCR PreMix kit. Bioneer, Korea). Nested PCR primers were provided by Macrogen Company, Korea. Nested PCR master mix prepared 5µL of genomic DNA, 10 pmol of each external primer and 13 µL of PCR water and placed in standard PCR tubes. Thermal condition of the PCR reaction included an initial denaturation at 95°C for 5 min followed by 30 cycles at 95°C for 30 sec., 55°C for 30 sec. and 72°C for 1min and finally final extension 72°C for 5min. A nested PCR master mix of second run included 3µL of first run product, 10 pmol of each internal primer and 15 µL of PCR water and placed in standard PCR tubes with thermal conditions for the PCR reaction. The PCR products passed electrophoresis in 1% agarose gel with 3µL of ethidium bromide. PCR product (10 µl) was added into each comb well and 5 µl of (100bp ladder) in each well. Gel tray was fixed in electrophoresis chamber and filled by 1X TBE buffer. The electric current was connected at 100 volts and 80 mA for 1 hr. PCR products were visualized using an ultraviolet transilluminator.

### Conventional-PCR amplification

The PCR technique was performed for detecting virulence factor genes in *L. tropica*. This method was carried out according to the method described by Al-Difaie (27). The primers were provided by Macrogen company, Korea. PCR master mix prepared 5µL of genomic DNA, 10 pmol of each R and F primers and 13 µL of PCR

water and placed in standard PCR tubes. Thermal conditions and agarose electrophoresis are similar to the above.

### Statistical Analysis

In the study, method of Chi-Square ( $\chi^2$ ) test was used in order to analyze the data using SPSS statistical package software V.17. Significant level was at  $P < 0.05$ .

**Table 1. Oligonucleotide primers used in Nested PCR in the study**

Primer name	Sequence 5'-3'	PCR Product size	Reference
CSB2XF	ATT TTT CGC GAT TTT CGC AGA ACG	750bp	(29)
CSB1XR	CGA GTA GCA GAA ACT CCC GTT CA		
13Z F	ACT GGG GGT TGG TGT AAA ATA G	750bp	(29)
LiR R	TCG CAG AAC GCC CCT		
LPG1 F	ACG CAT ACG GCA TCT TTT TC	311bp	(27)
LPG1 R	GCG AAA CAG CTC ATT GTT CA		
GP63 F	AGCAACACCGACTTCGTGAT	565bp	(26)
GP63 R	TGCACTTCTCGCTGAGGAAG		
CPA F	TGGGCTTTGAAAAACCACTC	437bp	(27)
CPA R	CACGAGTTCCTCACGATCCA		
PPG1 F	CAT TAT GGG TGG GAA ACC TG	568bp	(27)
PPG1 R	GAT GGG CAA GTT AGG TGG AA		

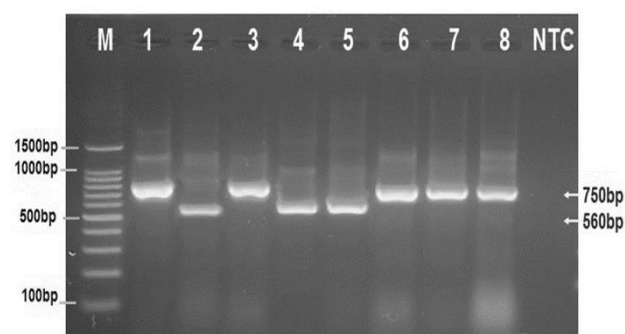
### Results:

Agarose gel electrophoresis results for 80 samples of mitochondrial kinetoplast minicircle fragments DNA (*kDNA*) gene were amplified by Nested PCR, discovered 65 (81.25%) positive for cutaneous leishmaniasis (contain *Leishmania* parasite) and 15 (18.75%) negative samples, where observed significant difference ( $P > 0.05$ ) between them (Table 2). Furthermore, the results showed that two species of the parasite which that found in the study area. Statistically, there is a significant difference between *L. tropica* and *L. major*. *L. tropica* recorded the most common species, while *L. major* was a low incidence. 46 (57.5%) positive samples were *L. tropica* at 750bp and 19 (23.75%) positive samples were *L. major* at 560bp (Fig. 1). There are no significant differences between the infection of 24 (30%) males and 22 (27.5%) females. The age groups  $\leq 20$ , 21-40 and  $>40$  years old recorded 33 (41.25%), 10 (12.5%) and 3 (6.5%) respectively, and showed significant differences ( $P$ -value = 0.000) when the compared with other age groups.

The electrophoresis results of the PCR product of *LPG1*, *GP63*, *CPA* and *PPG1* genes observed in all of cutaneous *Leishmania* which were recorded as *L. tropica* isolates in the study (Figs. 2,3,4 and 5) at 311bp, 565 bp, 437 bp and 568 bp PCR product sizes, respectively.

**Table 2. Molecular diagnosis of *Leishmania* species using Nested PCR**

<i>Leishmania</i> spp.	Infected Samples No (%)	Positive Samples No (%)	Negative Samples No (%)	P-value
<i>L. tropica</i>	46 (57.5)			
<i>L. major</i>	19 (23.75)	65 (81.25)	15 (18.75)	0.00
P-value	0.001			



**Figure 1. Agarose gel (1%) electrophoresis that showed Nested PCR product analysis of kDNA in Cutaneous Leishmaniasis positive isolates from human skin lesion. Where M: marker (100-1500 bp), lanes 1, 3, 6, 7, and 8 positive of *L. tropica* at 750 bp PCR product size and lanes 2, 4, and 5 positive of *L. major* at 560 bp PCR product.**

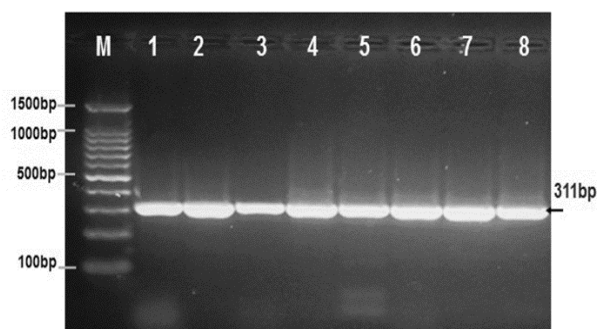


Figure 2. The image of agarose gel (1%) electrophoresis that showed the PCR product analysis of virulence factor *LPGI* gene in *L. tropica* positive samples. Where M: marker (100-1500 bp), lanes 1-8 positive *LPGI* at 311 bp PCR product size.

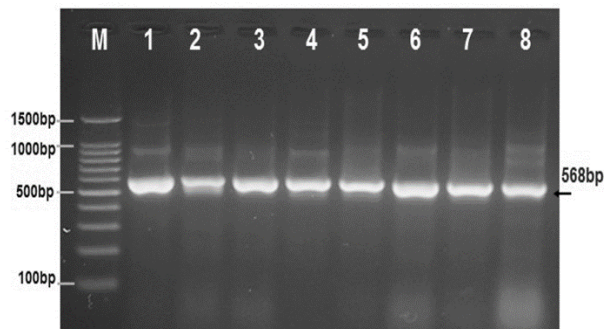


Figure 5. The image of agarose (1%) gel electrophoresis that showed the PCR product analysis of virulence factor *PPGI* gene in *L. tropica* positive samples. Where M: marker (100-1500 bp), lanes 1-8 positive *PPGI* at 568 bp PCR product size.

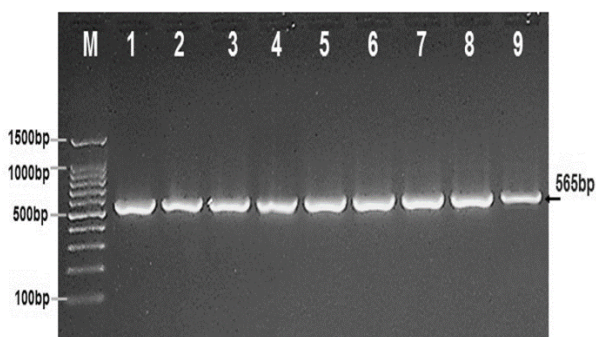


Figure 3. Agarose gel (1%) electrophoresis that showed the PCR product analysis of virulence *GP63* gene in *L. tropica* positive samples. Where M: marker (100-1500 bp), lanes 1-9 positive *GP63* at 565 bp PCR product size.

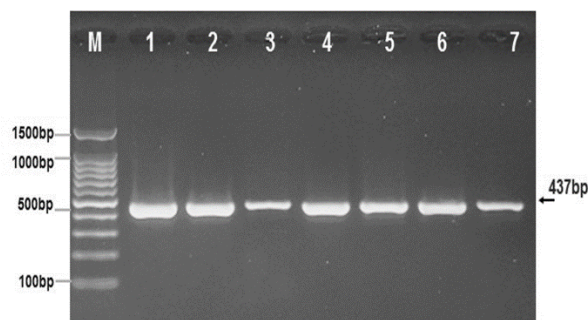


Figure 4. Agarose gel (1%) electrophoresis image that showed the PCR product analysis of virulence factor *CPA* gene in *L. tropica* positive samples. Where M: marker (100-1500 bp), lanes 1-7 positive *CPA* at 437 bp PCR product size.

### Discussion:

Cutaneous leishmaniasis is an endemic disease in several countries. Foci of *L. major* in Egypt, Jordan, Libya, Sudan and Tunisia, while Afghanistan, Pakistan, Iran, Saudi Arabia, Syria, Iraq, Morocco, and Yemen are endemic for both *L. tropica* and *L. major* (30). In this study, *Leishmania spp.* was identified by *kDNA*. Amplification *kDNA* gene is a suitable tool for differentiating between CL species and numerous diagnostic tests (31,32). *kDNA* gene is the most sensitive since kinetoplast contains about 10,000 circular and convoluted *kDNA* minicircles per parasite (31,33).

Leishmanial DNAs have been recorded in 65 (81.25%) cases and the rest were negative samples. The appearance of negative sample revealed not withdraw the parasite during the collection and may lack DNA in the sample or its crash. In addition, the results have shown that most CL patients in Thi-Qar Province were infected with *L. tropica*, while *L. major* was in a little number of patients. Geographical expansion and globally increase of leishmaniasis is still associated with vector population expansion (34). Also, increase in migration, rapid urbanization, deforestation and *Leishmania* adaptation with mammalian hosts and additional vectors (35). CL spread from endemic to non-endemic regions, if both sandfly vectors and reservoir hosts are present (36). The results of current study were no significant differences between infection of the males and the females, this may be due to the fact that *Leishmania* parasites infect both sexes at the same extent. Furthermore, there is no clear explanation for infection differences according to the gender. It might be individual and behavioral factors that play a role as mentioned in previous studies (37). This result is somewhat close to the results of Al-Difaie (27), Al-Hassani (28) in Al-Qadisiya Province, Khosravi *et*

al. (37), Ghatee *et al.* (1) in Iran and El Hamouchi *et al.*(38) in Morocco. This result is inconsistent with Alsamarai and Alobaidi (39) Al-Haweja City and Rahi (40) in Wasit Province.

The study recorded significant differences ( $p < 0.05$ ) between other age groups. The highest rate of infection by *L. tropica* was among age group of  $\leq 20$  years old (41.25%), then followed by age group 21-40 years old. This can be explained as a result to previous exposure of the infection which give permanent immune for individuals (39). This is in consistent with the findings of Al-Difaie (27), El Hamouchi *et al.*(38) and Hassan *et al.* (41) in Kirkuk City. It is also inconsistent with Rahi (40) in Wasit Province, Khosravi *et al.* (37) and Ramezany *et al.*(32) in Iran.

The current study recorded the presence of Lipophosphoglycan (*LPG1*), leishmanolysin (*GP63*), Cysteine proteinase A (*CPA*) and Proteophosphoglycan (*PPG1*) genes in all of *L. tropica* isolates (100%). Al-Difaie (27). has found *LPG*, *PPG* and *CP* in all of *L. major* isolates in Al-Qadisiya Province. In Brazil, Lázaro-Souza *et al.* (12) have mentioned a growth of *L. infantum* delayed if deletion of *LPG1* gene. *L. major* mutants which lack *LPG1* and *LPG2* genes are sensitive to host complement system, this leads to unable survival in both intermediate or definitive host cells (23). *GP63* is not only to degradation and damage of transcription factors and various kinases, but also it modulates negative regulatory mechanisms of signaling pathways (15). Cysteine protease inhibition appears as an important strategy for elimination of disease. They are necessary for metabolism, intracellular survival and reproduction of parasite (42). *CPA* plays an essential key in the interactions of parasite with host cells, but is not important to replication promastigotes (43). Other studies have shown that inhibition of *CPA* and *CPB* or deletion their genes not only alters autophagy pathway, but for prevents transformation into amastigotes, thus support the hypothesis of autophagy are required for the differentiation (44,45). Samant *et al.* (45) have suggested that possibilities of *PPG* in drug resistance mechanisms and *PPG* abundance of *L. donovani* is as an evidence for resistant clinical isolates. *PPG1* may play a direct important role in host-parasite interactions (46).

### Conclusion:

The importance of Thi-Qar Province comes from frequent infections of CL. As all know, there is a very little data about the identification of *Leishmania* species responsible of human CL. The findings have showed two species co-existing in the

study area that were *L. tropica* and *L. major*. The most cases of cutaneous lesions have been infected with *L. tropica*, that is the prevalent species. Some virulence genes have been investigated which confirmed their necessity and importance in pathogenesis of *L. tropica*. Therefore, it is essential to conduct more studies on the sandfly vectors and reservoirs of *Leishmania* species. However, nested-PCR is more suitable for direct diagnosis of *Leishmania* at species level.

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### Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- The author has signed an animal welfare statement.
- Ethical Clearance: The project was approved by the local ethical committee in University of Southern Technical.

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## تحديد طفيلي *Leishmania tropica* باستخدام تقنية Nested-PCR وبعض عوامل الضراوة في محافظة ذي قار، العراق

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### الخلاصة:

تعد اللشمانيا الجلدية هي واحدة من الامراض المتوطنة في العراق، وهي تسبب من مشاكل صحية واسعة النطاق فضلا عن كونها أحد الامراض الغير مسيطر عليها. الدراسة الحالية هدفت الى تشخيص أنواع اللشمانيا المسببة للآفات الجلدية بين المرضى في محافظة ذي قار - جنوب العراق، كذلك لتحديد بعض عوامل الضراوة لطفيلي *L. tropica*. تضمنت هذه الدراسة ثلاث مستشفيات وهي الحسين التعليمي، سوق الشيوخ العام والشرطة العام في المحافظة للفترة من بداية شهر كانون الأول 2018 ولنهاية شهر أيلول 2019. تم جمع العينات من 80 مريض يعانون من الإصابة بداء اللشمانيا الجلدية ومن كلا الجنسين وبأعمار متنوعة، مواقع سكن مختلفة في المحافظة وآفات جلدية مفردة ومتعددة. استخدمت تقنية Nested-PCR لتضخيم جين *kDNA* لتشخيص أنواع اللشمانيا فضلا عن استخدام تقنية Conventional-PCR في التحري عن تواجد بعض جينات الضراوة. بينت نتائج الدراسة تواجد نوعين من الطفيلي في منطقة الدراسة. وبينت نتائج الترحيل الكهربائي لجين *kDNA* وجود 65 عينة موجبة لداء اللشمانيا الجلدية وبحجم 750bp للـ *L. tropica* و 560bp للـ *L. major*. سجل طفيلي *L. tropica* نسبة بلغت 57.5% وكان النوع الأكثر شيوعا بينما سجل طفيلي *L. major* نسبة بلغت 23.75% حيث ظهرت بمستوى اقل. لم يلاحظ وجود فروق معنوية عند المقارنة بين إصابات الذكور والاناث، في حين بينت نتائج التحليل الاحصائي وجود فروق معنوية عند المقارنة بين المجاميع العمرية. كشفت الدراسة الحالية وجود جينات الضراوة *LPG1*, *GP63*, *CPA* and *PPG1* في كل العزلات لطفيلي *L. tropica* وبنسبة تواجد بلغت 100%. كذلك كشفت الدراسة عن ان طفيلي *L. tropica* هو النوع الرئيسي المسبب لداء اللشمانيا الجلدي في محافظة ذي قار، وجينات الضراوة هي ضرورية ومهمة لإمراضية الطفيلي.

الكلمات المفتاحية: طفيلي *Leishmania tropica*, *LPG1*, *GP63*, *CPA*, *PPG1*, *kDNA*