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Original Research Article

Molecular and biochemical studies on the effect of the infection by cutaneous *leishmaniasis* in Iraqi people

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Abstract

The present study is designed for the detection of cutaneous *Leishmania* by microscopic and culture methods, also for the characterization of species that are endemic in Iraq using molecular techniques, including conventional and nested polymerase chain reaction. Some biochemical tests including liver and kidney functions tests were also carried out to estimate the levels of Aspartate aminotransferase, Alanine aminotransferase, uric acid and blood urea in serum blood samples of patients before and after administered treatment. The results showed that a high percentage of patients had multiple lesions. One hundred and fifty patients enrolled in this study divided as follows; ninety patients not received any treatment, thirty patients were taken treatments and the other thirty people chose as control group. Using nested polymerase chain reaction techniques showed that the causative agents of cutaneous *leishmaniasis* in Iraq were two species: mainly *L. major* and *L.tropica*. No elevation in the level of ALT (GPT) in serum blood samples were noticed in patients before and after administering treatment. The present study also revealed no elevation in blood urea and uric acid in both groups of patients before and after administering treatment. Slight increases were noted in the values of Aspartate aminotransferase, Alanine aminotransferase in serum blood samples of patients before administered treatment compared with healthy control group, but there were no elevations in the values of Alanine aminotransferase in serum blood samples in both groups.

Keywords: Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Leishmania, Polymerase chain reaction

INTRODUCTION

Leishmania protozoan parasite belonging to the family Trypanosomatidae, is classified as one of the top three parasitic pathogens by the World Tropical Diseases Research Center (Haddad *et al.,* 2016). Cutaneous *leishmaniasis* of the old world is caused by *L. tropica, L. major* and *L. aetiopica*,

Lieshmania major, the cause of rural oriental sore, is maintained in the reservoir population by sand flies, and human disease is maintained through the vector, *Phlebotomus papatasi*. The ulcers formed are usually single, although multiple ones have been known to occur (Aoun *et al*., 2014).

In *Leishmania tropica,* the cause of urban CL, the human is considered to be the primary reservoir and source of infection. Various phlebotomine sand flies (*P. sergenti* and *P. papatasi*) transmit the infection, depending on the specific geographic area (Andrade *et al*., 2005). The lesions of urban disease caused by *L. tropica* occur singly or two at a time, develop more slowly than those caused by *L. major* and can take a year or more to heal. For the last few years, the disease has become epidemic and can be seen in many cities from

north to south of Iraq (Sharquie *et al*., 2002).

Several molecular targets for diagnostic PCR testing have been evaluated in *Leishmania*, including minicircle kinetoplast DNA (kDNA) (Gramiccia and Gradoni, 2005). The main aim of this work is the determination of *Leishmania* species which are endemic in Iraq.

MATERIALS AND METHODS

This prospective study was carried out in the period from December 2016 to December 2017 on one hundred fifty persons enrolled in this study. The patients were inhabitants of the urban and rural areas attending the dermatology department in many hospitals at Baghdad governorate; either single or multiple, located in different parts of the body**,** and clinically diagnosed as cutaneous *leishmaniasis*. Ninety of the patients studied were not receiving any treatment while thirty patients were taken treatments, and thirty healthy people are the control group.

Parasitological studies

Smear, scraping, aspiration and culture were attempted for patients. Scrapings or biopsy specimens were taken from the edge of the lesions and then were smeared on two slides and then portions were placed in a culture bottle. Smears were allowed to dry, fixed with methanol, stained with 10% Giemsa stain, and then examined for the presence of amastigotes (William *et al.,* 2004).

Preparation of *Leishmania* DNA for PCR amplification

Frozen samples were thawed and incubated at room temperature. DNA extraction from samples performed according to manufacturer's instruction of G- spin DNA extraction kit (intron biotechnology, South Korea).

The variable segments on mini**-**circles of kinetoplast DNA from any *Leishmania* present in the smear scraping were amplified with two rounds of nested PCR. (Rasti *et al*., 2016) and (Mohammad *et al*., 2016).

First reaction

The primers were CSB1XR (ATT TTT CGC GAT TTT CGC AGA ACG) and CSB2XF (CGA GTA GCA GAA ACT CCC GTT CA) for the first round.

Second Reaction

The primers were LiR (TCG CAG AAC GCC CCT) and 13Z (ACT GGG GGT TGG TGT AAA ATAG) for the second round. The product of PCR was diluted as 1:9 with ultrapure water and then 1 µl of this dilution was used as the template for the second round of PCR, using the same conditions and reaction mixture as the first round except that LiR and 13Z were used as the primers.

5-µl sample of the second-round product was subjected to electrophoresis in 1·5% agarose gel and stained with safe red and visualized by ultraviolet trans-illumination. (Moemenbellah-Fard *et al.,* 2003). The size of each amplicon detected was estimated by comparison with a 100-bp–1500-bp molecular-weight 'ladder' (Roche) run on the same gel.

Some biochemical tests including liver and kidney functions tests Aspartate aminotransferase (AST or GOT), Alanine aminotransferase (ALT or GPT, uric acid and blood urea were performed; a colored complex was measured by spectrophotometry and the sample absorbance read at 340 and 520 nm 520nm (Burtis *et al.,* 2008)

Statistical analysis of the data

The data were analyzed by using the SPSS version 17, and the approach to the data consisted of two steps (descriptive and analytic statistic), and then tested for significance by using Student t-test. P value ≤ 0.05 was considered statistically significant. Contingency tables were conducted for studying the correlations among the different responses in three groups (Kathryn and Noel, 2009).

RESULTS

Among hundred fifty patients lesions examined by microscopic examination and stained with Giemsa, the result revealed 91 (75.8%) were positive with cutaneous leishmaniasis and 29 (24.2%) were negative (Table 1). Positive results of culturing *Leishmania* media were only 74 samples (61.7%).

Figures 1 illustrated intracellular amastigote staining by Giemsa stain and (2) manifested extracellular amastigote staining by Giemsa stain.

Forty two (35%) patients had a single lesion, while seventy eight (65%) patients had multiple lesions as shown in Table 2. Plate (1) demonstrated single and multiple lesions of cutaneous *leishmaniasis* for some patients.

DNA extracted successfully in 82(91.1%)out of 90 CL patients who didn't receive any treatment; see Table 3.

According to DNA extraction, all patients in the group after treatment and healthy control groups gave negative result for DNA extraction. Among 82(91.1%) out of 90 patients gave positive result while only 8 (8.9%) were negative, Table 5, Figure 3 and 4.

Nested PCR technique obtained for *Leishmania*

Table 1. Results of Giemsa stainand culture samples for the patient group

Results	Giemsa stain	Culture (NNN)
	No. (%)	No. (%)
Positive	91 (75.8)	74 (61.7)
Negative	29 (24.2)	46 (38.3)
Total	120 (100)	120 (100)

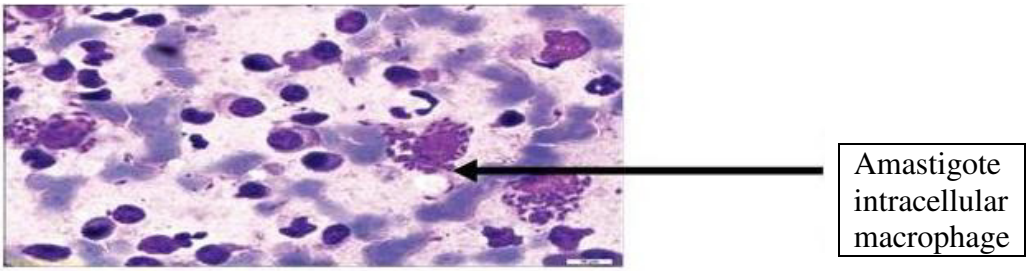

Figure 1. Intracellular amastigote staining by Giemsa stain

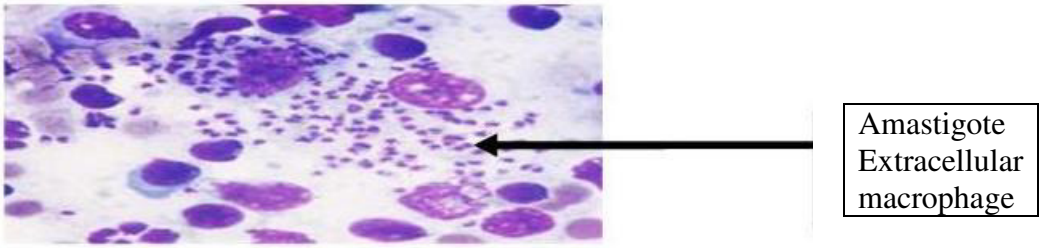

Figure 2. Extracellular amastigote staining by Giemsa stain

Table 2. Number of lesions among patients studied

Number of lesion	No.	%
Single	42	35
Multiple	78	65
Total	120	100

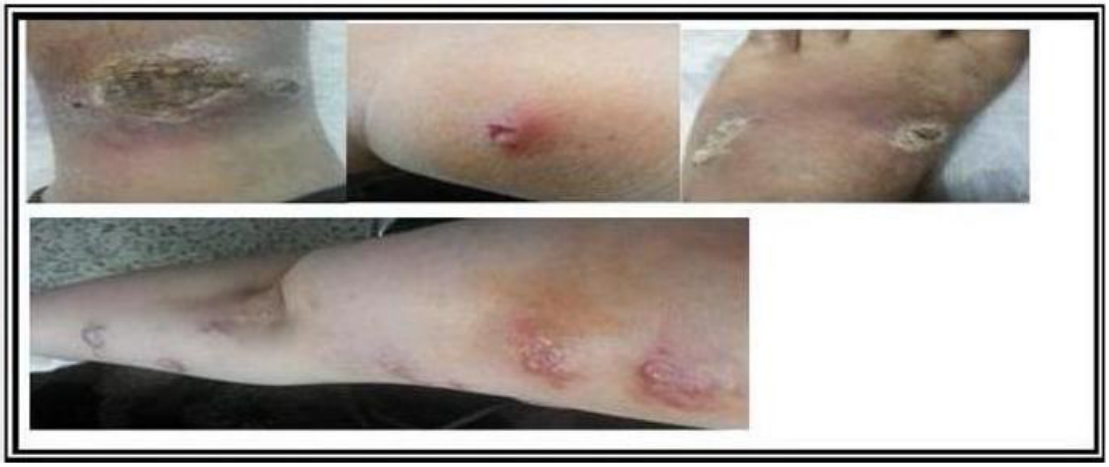

Plate 1. Single and multiple lesions among patients studied

Table 3. Result of DNA extraction among patient before treatment

DNA extraction	No.	%
Positive	82	91.1
Negative	8	8.9
Total	90	100.0

Table 4. Distribution the result of PCR technique among patients before treatment

Result of PCR	No.	%
Positive	73	89.1
Negative	9	10.9
Total	82	100.0

Table 5. Distribution of patients according to Nested PCR technique

Result of Nested PCR	No.	%
L.major	19	23.2
L.tropica	54	65.9
No result	9	10.9
Total	82	100.0

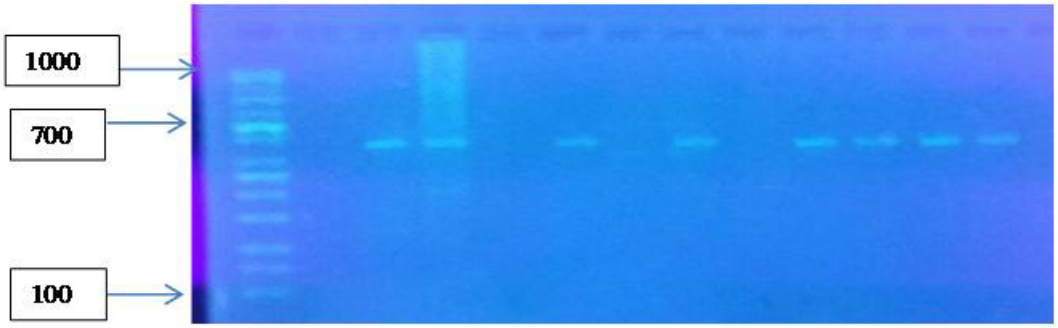

Figure 3. PCR product the band size 750bp of *Lieshmania tropica*

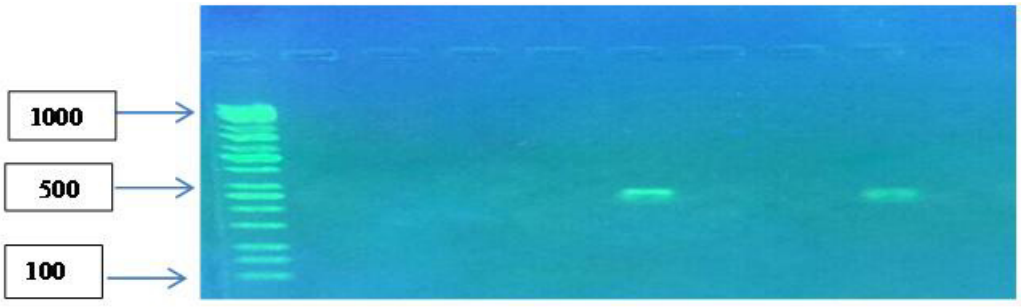

Figure 4. PCR product the band size 560 bp of *Lieshmenia major*

tropica were more frequent (54;65.9%) than *Lieshmenia major* (19;23.2%); see table 5 and Figure 5 and 6. There were 9 (10.9%) patients not positive neither for *Leishmania tropica*nor *Lieshmenia major.*

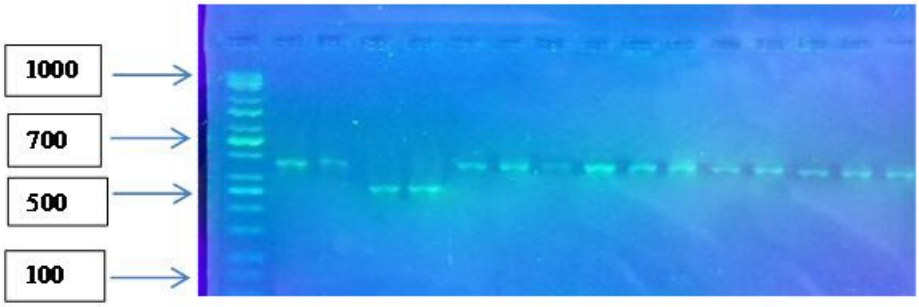

Figure 5. Nested PCR product the band size 750bp for *Lieshmaniatropica* and 560bp for *Lieshmenia* major

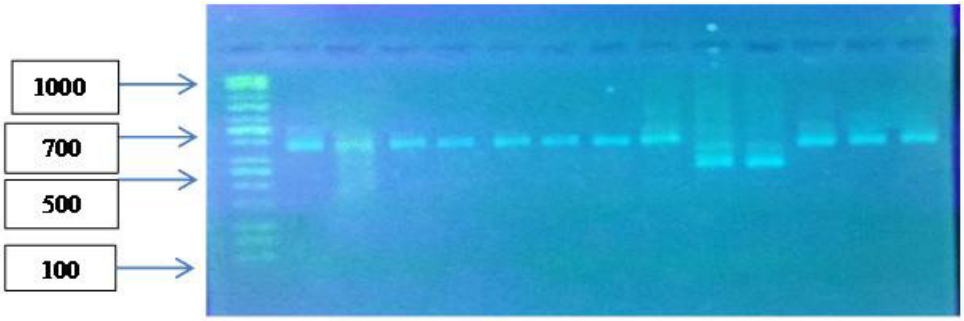

Figure 6. Nested PCR product the band size 750bp for *Lieshmania tropica* and 560bp for *Lieshmenia major*

Laboratory values of biochemical test for individuals before and after treatment compared to healthy control

Values of liver enzyme (GOT and GPT) and kidney functions (blood urea and uric acid) were shown in Table 6. Changes in liver enzymes were concerned, GOT showed slightly raised values of (47.8±34) and (43.9±211)IU/L in both groups of patients before and patients after treatment respectively. There were no significant differences at (P>0.05) as shown in Table 6.

No elevation in the level of GPT (34.3±32.5,30.0±25.0)IU/L, in both groups of patients before treatment and patients after treatment, respectively. Also there were no significant differences in serum levels (P>0.05).

Blood urea also showed no elevated values (25.9±4.9,41.7±20.8) in both groups of before treatment and patients after treatment. There were highly significant differences (at P<0.01).

The mean values of uric acid were (4.96±1.8) and (6.02±1.7) mg/dl in both groups before treatment and patients after treatment respectively, and significant differences were noticed at P<0.05 as shown in table 6.

Changes in liver enzymes were detected, as GOT showed slightly increased values of (47.8±34) IU/L mean in patients before treatment, while within normal value (21.97±7.5) IU/L mean in the healthy control group. There were highly significant differences in serum levels of GOT (P<0.01); see Table 7. No elevations in the values of GPT recorded (34.3±32.5,20.0±6.0)IU/L, in both groups of patients before treatment and in the healthy control group respectively. There were significant differences in serum levels (P<0.05);revise Table 7.

Blood urea showed no elevated values (25.9±4.9) and (17.8±2.4) in both groups of before treatment and healthy control group respectively. There were highly significant differences at P<0.01.

Uric acid recorded mean values of (4.96±1.8) and (4.19±1.1) mg/dl in both groups of before treatment and healthy control group respectively. There were no significant differences (P>0.05); see Table 7.

Values for liver enzyme (GOT and GPT) and renal (urea and uric acid) in the after treatment and the control groups are shown in Table 8.

Changes in liver enzymes were measured, GOT showed slightly increased mean of (43.9±21.1) IU/L among patients after treatment compared with healthy control group (21.97±7.5) IU/L, there were highly significant differences in serum levels of GOT at P<0.01

GPT showed no elevated values (30.0±25.0;20.0±6.0)IU/L, in both groups of patients after treatment and in the healthy control group, respectively. There were significant differences in serum levels of GPT

Table 6. Laboratory values of AST,ALT, Blood urea and Uric acid for the individuals before treatment and after treatment group (Mean ± SD)

Laboratory tests	Normal value	Before treatment	After treatment	*P* value
ST(GOT) IU/L,	5-40	47.8±34	43.9±21.1	0.563 (NS)
ALT(GPT) IU/L,	5-40	34.3±32.5	30.0±25.0	0.58 (NS)
Blood urea	14-50	25.9±4.9	41.7±20.8	0.001 (HS)
Uric acid mg/dl	Males (3.5 - 7.2) Females (2.6 - 6.0)	4.96±1.8	6.02±1.7	0.027 (S)

NS = not significant, HS = highly significant and S = significant

Table 7. Laboratory values of GOT,GPT, Blood urea and Uric acid before treatment compared with healthy control group (Mean ± SD)

Laboratory tests	Normal value	Before treatment	healthy control	P value
GOT	5-40	47.8±34	21.97±7.5	0.000(HS)
GPT	5-40	34.3±32.5	20.0±6.0	0.021 (S)
Blood urea	14-50	25.9±4.9	17.8±2.4	0.000 (HS)
Uric acid mg/dl	M (3.5-7.2) F (2.6 - 6.0)	4.96±1.8	4.19±1.1	0.059 (NS)

NS = not significant, HS = highly significant and S = significant

Table 8. Laboratory values of GOT,GPT, Blood urea and Uric acid after treatment and control groups (Mean ± SD)

Laboratory tests	Normal value	After treatment	Control	P value
GOT	5-40	43.9±21.1	21.97±7.5	.000 (HS)
GPT	5-40	30.0±25.0	20.0±6.0	.021 (S)
Blood urea	14-50	41.7±20.8	17.8±2.4	.000 (HS)
Uric acid mg/dl	M (3.5 - 7.2) F (2.6 - 6.0)	6.02±1.7	4.19±1.1	.059 (NS)

NS = not significant, HS = highly significant and S = significant

(P<0.05). Blood urea also showed no abnormal elevation in values (means of 41.7±20.8; 17.8±2.4) in both after treatment and healthy control group, respectively. There were highly significant differences in blood urea levels at P<0.01. Concerning uric acid, the mean values were (6.02±1.7) and (4.19±1.1) mg/dl in both after treatment and healthy control group respectively. There were no significant differences in the serum levels at P>0.05 (Table 8).

DISCUSSION

Parasite identification**,** performed by microscopic stained smears of scrapings and by isolation in culture and CL patients. Lesions examined by microscopic examination using Giemsa stain revealed the amastigote form with high positivity among the total sample studied. (Hassan, H.F. and Ahmed, A.S. 2015).

AL-Obaidi *et al*., (2016) believed that the sensitivity of Giemsa stain may be dependent on sampling and slide preparation of Giemsa stain storage and must be confirmed by clinical diagnoses.

This study also indicated that the incidence rate of multiple lesions in cutaneous Lieshmanasis was more predominant than single lesion**.**

Al-Qadhi *et al.* (2013) and Rahi *et al.* (2014) found that multiple sores isolates have higher percentages than single sore.In Burkina Faso, observed single lesions were also less than multiple lesions in patients with cutaneous leishmaniasis. Bamba *et al.* (2017).

Most recent epidemiological studies of old world CL had shown a similar trend with single lesions being the most common form of presentation and this could be explained by the fact that people are more exposed to phlebotomine sand flies.

According to DNA extraction, treated patients and healthy control groups gave a negative result for DNA

extraction and all the positive results were for the patients before treatment, and this is in agreement with previous studies. Abdulwahab (2013) and Karamian *et al.* (2008).

Identification of the *Leishmania* type is important, because different species may require distinct treatment regimens. Furthermore, such data are also valuable in epidemiologic studies where the distribution of *Leishmania* species in human and animal hosts is a prerequisite for designing appropriate control measures Maraghi, S. *et.al* (2013).

The results also showed that the main causative agents of CL in Iraq are two species**:** *L. major* and *L. tropica* and the highest infection rate was caused by *L. tropica*. Characterization of *leishmania* species is important, because different species may require a distinct treatment regimen. Furthermore, such information is also valuable in epidemiologic studies, since the distribution of *leishmania* species in human and animal host, as well as in insect vectors, is prerequisite for designing appropriate control measures Hossein, M.L. and Khajeh, F. (2015).

Tashakori*et al.*,(2003)mentioned that in cities *leishmaniasis* had been identified as anthroponotic cutaneous *leishmaniasis* endemic foci, while rural districts of provinces it had been known as the foci of Zoonotic cutaneous *Leishmaniasis*.

Biochemical tests included in this study are represented by assays of (GPT), aspartate aminotransferase (GOT), blood urea and uric acid. Changes in liver enzymes were concerned, GOT slightly rose in values in both groups of patients before treatment and patients after treatment. The fall of GOT and GPT levels as key liver enzymes might be correlated to treatment and indicating an anti-parasitic immune response. Since the liver is a major site of drug metabolism, increased level of GOT and GPT in blood might be due to the prolonged treatment of skin lesions and hence might have damaged the liver**,** and leakage of enzyme had occurred which quickly recovered after the termination of treatment Khan, and Zakai (2014).

Kidney functions studied in this study represented by blood urea and uric acid also showed no elevations in both groups of before treatment and healthy control.
The same results also reported by Mahajan *et al.* (2009) and Asmaa *et al.* (2017) and mentioned that the increase in uric acid level may refer to the physiological activity and the influence of destruction or catabolism, and the elevation level of uric acid may contribute much more to scavenging of free radicals in the body Baillie (2007).

CONCLUSIONS

- Detection of *leishmania* by microscopic method showed highly sensitivity with low specificity, whereas the culturing method gave high specificity but low sensitivity.
- Using nested-PCR in this study established that the causative agents of CL in Iraq are two species mainly *L. major* and *L. tropica,* and the highest infection rate was caused by *L. tropica*.
- Changes in liver enzymes showed rising in values of GOT in both patients before and after administered treatment groups and no elevation in the level of GPT in both patients before and patients after administered treatment
- Kidney function including blood urea and uric acid also showed no elevated values in both groups of patients, before and after administered treatment

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