Immunological and Molecular Characterization of Leishmania Parasite in Kirkuk, Iraq

Husain F. Hassan[1], Hiyam R. Shahab[2]*
[1,2] Department of Biology, College of Science, University of Kirkuk, Iraq.
ahmedheyam45@gmail.com

Abstract
The strains characterization of leishmanial parasite were investigated using immunological and molecular methods. The mean concentration of cytokines interleukin 10 (438.5pg/ml) and interleukin 7 (18.06 pg/ml) were significantly increased in sera of patients with cutaneous leishmaniasis as compared to control healthy groups (85.05 pg/ml and 10.8 pg/ml) respectively. Hematological investigation revealed decrease in level of hemoglobin and platelet and an increase in the level of leuckocytes, neutrophils, and lymphocytes in patients with cutaneous Leishmaniasis. The use of PCR system and DNA sequences provided evidence that *Leishmania tropica* and L. *major* are the only etiological agents of cutaneous leishmaniasis in Kirkuk governorate.
Key words: Leishmania parasite; Immunological; molecular method; Interleukin 7 and 10; PCR; Kirkuk, Iraq

1. Introduction

Leishmania cause a wide spectrum of human disease that ranges from localized cutaneous lesions to disseminated systemic infections [1]. The leishmanial parasites infect macrophages and may thereby be shielded from many immune defense mechanisms [2]. In certain instances, notably infections with Leishmania cutaneous strains, intracellular parasites are destroyed by host responsed and the disease resolves usually without treatment [3]. Interleukins are known to play a role in inflammation and have multiple effects, acting both in the induction of proinflammatory cytokines and in the recruitment and activation of leukocytes [4]. Here we evaluated the role of IL-7 and IL-10 in the pathogenesis of cutaneous leishmaniasis. In addition, we investigated the validation of kDNA amplification for the diagnosis of cutaneous leishmaniasis as part of an epidemiological surveys confirmed in Kirkuk province, Iraq

2. Materials and Methods

Patients: The sera from patients (41) and healthy donors (30) were collected for IL-7 and IL-10 dosage. Assessment of IL-7 and IL-10 serum levels: The assessment was carried out by using two ELISA kits, IL-7 (Boster,USA) and IL-10 (Bioassay Technology, China) with reference standard curves using known amounts of the respective murine recombinant cytokines [5].

Extraction of DNA: DNA was extracted from promastigotes of L.tropica by using the Genomic Prep cells and Tissue DNA isolation kit (Bioneer, Korea) according to the protocol of the manufacturer instructions. The purity and concentration of extracted DNA samples were analysed by Nanodrop.

Polymerase chain reaction amplification: The entire 760bp minicircle kinetoplastid DNA (kDNA) of Leishmania species was amplified using primers forward (5´ATTTTTCGCGATTTTCGCAGAAACG-3´) and reverse (5´CGAGTAGCAGAAACTCCCGTTCA-3´). The PCR amplification was conducted using PTC-100 thermo cycler (MJ Research Inc., Waltham,MA) following the thermal profile previously used [5] as indicated in Tables (1) and (2)

Table (1): Pre-Mix components

Size	Components
12.5 ml	Taq PCR Pre Mix
1 ml of 10 pmol/ml	Primer Forward
1 ml of 10 pmol/m	Primer Revers
2 ml	DNA template
8.5 ml	Nuclease free water
25	Total

Table (2) PCR program

Number of cycles	Time	Temp. Cº	Steps
1 cycle	5 min.	94ºC	Initial Denaturation
30 cycle	30 sec	94ºC	Denaturation
	30 sec	55ºC	Annealing
	30 sec	72ºC	Extension-1
1 cycle	5 min.	72ºC	Extension-2
	10 min.	4ºC	Hold

The amplified PCR products were subjected to electrophoresis on 1% agaroce gel using X TBE running buffer (0.045 M Tris borated, 1 mM EDTA) and PCR product bands visualized by staining with Red safe (0.4 mg/ml).

Standard sequencing: The purified PCR product was sequenced by Macrogen Corporation, Korea using Sanger sequencing (ABI 3730XL automated DNA sequencer). The results were received by email then analysed using Geneious software.

Phylogenetic analysis: The Phylogenetic analysis were performed based on Bio Edit programs and NCBI BLAST (Basic Local Alignment Search Tool) that available at NCBI (National Center for Biotechnology Information) website at (http://www.ncbi.nim.nih.gov).

C-Reactive protein: was assessed using Dutch Diagnostic kit according to the protocol of the manufacturer instructions.

The total count of leukocytes, erythrocytes, platelets and hemoglobin were estimated according to procedures indicated by Powers [6].

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3. Results and Discussion

The result as shown in table (3) revealed significant increase in the IL-10 level in patients suffering with cutaneous leishmaniasis (438.5 pg/ml) as compared to the healthy control (85.04 pg/ml). The level of IL-10 in females (411.3 pg/ml) was more than in males (281.8 pg/ml) suffering with cutaneous leishmaniasis. Interleukin 10 is one kind of multi cell derived and multifunctional cytokine with potent anti flammatory properties that plays a central role in limitating host immune response to pathogens thereby preventing tissue damage and immune pathology [7]. The elevated IL-10 in sera of patients with cutaneous leishmaniasis presented in this investigation, is in agreement with [8] who observe that the lesion progression infection with L.major preferentially induce the production of high amount of IL-10. Thus this study point to an important role for IL-10 in regulating immune response to this intracellular pathogens.

Table (3) Comparison of IL-10 levels between sera of patients with cutaneous leishmaniasis and healthy control groups.

Serum level mean (Pg/ ml) ± Standard Error	Groups
438.5 ± 12	Infected patients
85.04± 4	Control
281.8 ± 9	Infected males
411.3 ± 14	Infected female
There are significant differences at the level of probability ≤0.05	

Table (4) Comparison of IL-7 levels between sera of patients with cutaneous leishmaniasis and healthy control group.

Serum level mean (Pg/ ml) ± Standard Error	Groups
18.06± 3	Infected patients
10.8±2	Control
30.5± 4	Infected males
27.58± 3	Infected female
There are significant differences at the level of probability ≤0.05	

On the other hand, an increase in the level of IL-7 was observed in patients with cutaneous leishmaniasis (18.06 pg/ml) as compared to the healthy control groups (10.8 pg/ml). The level of IL-7 in females (27.58 pg/ml) was less than in males (30.5 pg/ml) suffering with cutaneous leishmaniasis.

IL7 is a bone marrow-derived cytokine that affects B cells. It enhances the proliferation of Thymocytes, and mature T cells, and proliferation of killer cell [9] Interleukin 7 has been shown to induce secretion of cytokines by human monocytes critically involved in defense against Leishmania [10].The potential role of IL7 as an immune-regulating cytokine during anti-parasite immune response and activation of phagocytes to destroy Leishmania parasites was investigated [11]. Consistent with other investigations, our finding of increased level of IL-7 in the sera of patient with cutaneous leishmaniasis represents a promising an stimulatory capacity for different cell types of immune system critically involved in the defense against leishmanial parasite such as macrophages which are activated for the elimination of the parasite by IL-7.

As shown in Table (5) the results confirmed the presence of positive cases of C-reactive protein (19.5%) in patients with cutaneous Leishmaniasis, being (15.7%) in female groups of patient as compared to male groups patient (22.7%), where as no positive cases appeared in uninfected people groups. This protein is one of the main acute phase proteins that are made in the liver and regulated by (IL6, IL1, TNF-α). The increasing in its concentration is an early response to any kind of parasitic and bacterial infections, and tissue damage. The results of current study agreed with the observation of Nemati et al. [12] who indicated that C-reactive protein is an important component during most infections due to its association with nitric oxide (NO) and suggested that NO and C-reactive protein participate in the protective or causative responses to Leishmaniasis.

Table (5) The rate of C-reactive protein in patients with cutaneous Leishmaniasis in Kirkuk governorate.

Positive Casesnmg/L		The Number	Groups
The Number	%		
8	19.5	41	Infected Patients
5	22.7	22	Male
3	15.7	19	Female
0.0	0.0	30	Control

Table (6) shows the average blood components of patients with cutaneous Leishmaniasis

The Control	Infected patients	Test
4.84 x 10^6 ± 0.16	4.43 x 10^6 ± 0.1	RBC (cell/mm^3)±Standard Error
13.1 ± 0.4	12.1 ± 0.31	Hb (g/dL)
311.48 x 10^3 ± 23	204 x 10^3 ± 18	PLT (cell/mm^3. Blood)
5521 ± 48	6530 ± 66	WBC (cell/mm^3)
1826.03 ± 31	5500 ± 39	Lymphocytes (cell/mm^3. Blood)
2812.9 ± 49	3700 ± 66	Nutrophilis (cell/mm^3. Blood)

Hematological studies revealed decrease in the rate of measuring red blood cells, hemoglobin and platelets in patients with cutaneous leishmaniasis. It has been shown that the sequence of CD4 + T cells leads to a defect in the bone marrow environment, which leads to a defect in hematopoietic stem cells and precursor cells [13]. In contrast, there will be high average in the rate of WBC, lymphocytes and neutrophils in patients with cutaneous leishmaniasis.

These increasment are explained by the fact that the neutrophils and other cell types are among the first non-specific defensive elements that head to the site of infection to carry out their defensive function in devouring and killing the parasite [14].

DNA extraction

DNA was successfully extracted and assessed by electrophoresis on the Agarose gel. The extracted DNA was used as templates in PCR reaction. Molecular characterization of the isolates revealed amplification of the characteristic 760 bp minicircle band as shown in figure (1).

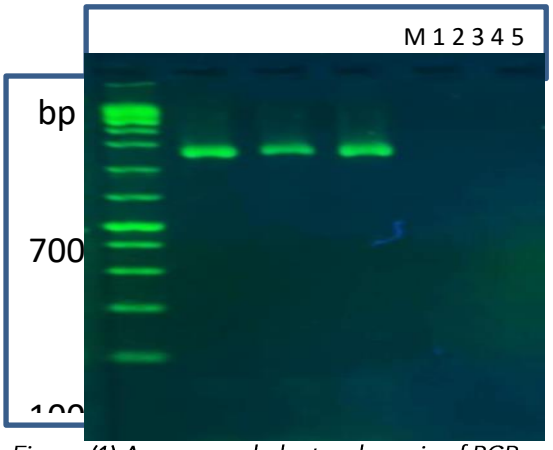

Figure (1) Agarose gel electrophoresis of PCR products obtained trom biopsy samples with primers specific for L.tropica. M represent 123 bp DNA lader ; Lane 1-3 represent positive control ; Lane 4-5 negative control.

The amplified PCR products of 760 bp were sequenced and analyzed by alignments with reported references sequences of Leishmania genotypes using Gene bank. The result of phylogenetic analysis showed that the Leishmania genotype was due to L.*tropica* and L.*major* strains. Analysis of alignment via online blast demonstrates correspondence of 97%,98% and 100% of an examined sequence fragment when compared with the data base of genbank of Leishmania accessible data. Also, it was evident and interesting that there are two codons transvertion and transition mutations between nucleotides (Table 7, Table 8 and Table 9) (Figure 2 and 3).

Table (7) Sequencing results represent the presence of two types of Leishmania according to registration in NCBI.

Gene: 18S ribosomal RNA gene						
Identities	**Source**	**Sequence ID with compare**	**Nucleotide**	**Location**	**Type of substitution**	**No.**
100%	Leishmania tropica	ID: MT071547.1	-----	-------	------	1
100%	Leishmania tropica	ID: MT071547.1	-------	-------	------	2
99%	Leishmania major	ID: MT071546.1	A\T	82	Transvertion	3
			T\G	84	Transvertion	

Table (8) Shows the matching percentage of L. *tropica* isolated samples with the database in the Information Center for Biotechnology

Compatibility	*Source*	Country	Gene	ACCESSION ID	
100%	*Leishmania tropica*	Iraq	ribosomal RNA gene	MZ635502	1
97%	*Leishmania infantum*	Iran	ribosomal RNA gene	EF653268	2
100%	*Leishmania braziliensis*	Brazil	ribosomal RNA gene	MT606274	3
100%	*Leishmania sp.*	Panama	ribosomal RNA gene	MH195209	4
100%	*Leishmania lainsoni*	USA	ribosomal RNA gene	MG776303	5
100%	*Leishmania guyanensis*	Austria	ribosomal RNA gene	JN671917	6
100%	*Leishmania naiffi*	Brazil	ribosomal RNA gene	MT606263	7
98%	*Leishmania braziliensis*	Argentina	ribosomal RNA gene	MN497062	8
98%	*Leishmania peruviana*	GERMANY	ribosomal RNA gene	FN398340	9

Table (9) Shows the matching percentage of L. *major* isolated samples with the database in the Information Center for Biotechnology

Compatibility	*Source*	Country	Gene	ACCESSION ID	
99%	*Leishmania donovani*	France	ribosomal RNA gene	CP022642	1
99%	*Leishmania major*	Iran	ribosomal RNA gene	KU680847.1	2
99%	*Leishmania infantum*	Morocco	ribosomal RNA gene	KX664452	3
99%	*Leishmania donovani*	India	ribosomal RNA gene	MN031876	4
99%	*Leishmania donovani complex sp*	Georgia	ribosomal RNA gene	KT438663	5
99%	*Leishmania sp.*	UNITED KINGDOM	ribosomal RNA gene	LT577674	6
99%	*Leishmania sp.*	USA	ribosomal RNA gene	MT791386	7
99%	*Leishmania infantum*	Spain	ribosomal RNA gene	LR812960	8
99%	*Leishmania major*	Iraq	ribosomal RNA gene	MZ618780	9

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                                10 20 30 40 50
                                ....|....|....|....|....|....|....|....|....|....|
MZ635502 GCGTGCATGG ATGACGGCTC AAATAACGTG TCGCGATGGA TGACTTGGCT
EF653268 GCGTGCATGG ATGACGGCTC AAATAACGTG TCGCGATGGA TGACTTGGCT
MT606274 GCGTGCGTGG ATAACGGCTC ACATAACGTG TCGCGATGGA TGACTTGGCT
MH195209 GCGTGCGTGG ATAACGGCTC ACATAACGTG TCGCGATGGA TGACTTGGCT
MG776303 GCGTGCGTGG ATAACGGCTC ACATAACGTG TCGCGATGGA TGACTTGGCT
JN671917 GCGTGCGTGG ATAACGGCTC ACATAACGTG TCGCGATGGA TGACTTGGCT
MT606263 -CGTGCGTGG ATAACGGCTC ACATAACGTG TCGCGATGGA TGACTTGGCT
MN497062 GCGTGCGTGG ATAACGGCTC ACATAACGTG TCGCGATGGA TGACTTGGCT
FN398340 GCGTGCGTGG ATAACGGCTC ACATAACGTG TCGCGATGGA TGACTTGGCT
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60 70 80 90
....|....|....|....|....|....|....|....|....|.
MZ635502 TCCTATCTCG TTGAAGAACG CAGTAAAGTG CGATAAGTGG TATCAA
EF653268 TCCTATCTCG TTGAAGAACG CAGTAAAGTG CGATAAGTGG TAT---
MT606274 TCCTATTTCG TTGAAGAACG CAGTAAAGTG CGATAAGTGG TATCAA
MH195209 TCCTATTTCG TTGAAGAACG CAGTAAAGTG CGATAAGTGG TATCAA
MG776303 TCCTATTTCG TTGAAGAACG CAGTAAAGTG CGATAAGTGG TATCAA
JN671917 TCCTATTTCG TTGAAGAACG CAGTAAAGTG CGATAAGTGG TATCAA
MT606263 TCCTATTTCG TTGAAGAACG CAGTAAAGTG CGATAAGTGG TATCAA
MN497062 TCCTATTTCG TTGAAGAACG CAGTAAAGTG CGATAAGTGG TATCA-
FN398340 TCCTATNTCG TTGAARAACG CAGTAAAGTG CGATAAGTGG TATCA-
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Figure (2) Analysis of alignment to L. tropica

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10 20 30 40 50
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MZ618780 GCATGGATGA CGGCTCAAAT AACGTGTCGC GATGGATGAC TTGGCTTCCT
MN031876 ---TGGATAA CGGCTCACAT AACGTGTCGC GATGGATGAC TTGGCTTCCT
CP022642 ---TGGATAA CGGCTCACAT AACGTGTCGC GATGGATGAC TTGGCTTCCT
KX664452 ---TGGATAA CGGCTCACAT AACGTGTCGC GATGGATGAC TTGGCTTCCT
KU680847 GCGTGGATAA CGGCTCACAT AACGTGTCGC GATGGATGAC TTGGCTTCCT
LT577674 ---TGGATAA CGGCTCACAT AACGTGTCGC GATGGATGAC TTGGCTTCCT
KT438663 ---TGGATAA CGGCTCACAT AACGTGTCGC GATGGATGAC TTGGCTTCCT
MT791386 ---TGGATAA CGGCTCACAT AACGTGTCGC GATGGATGAC TTGGCTTCCT
LR812960 ---TGGATAA CGGCTCACAT AACGTGTCGC GATGGATGAC TTGGCTTCCT
60 70 80 90 100
....|....|....|....|....|....|....|....|....|....|
MZ618780 ATCTCGTTGA AGAACGCAGT AAAGTGCGAT ATGGGGTATC AATTGCAGAA
MN031876 ATTTCGTTGA AGAACGCAGT AAAGTGCGAT AAGTGGTATC AATTGCAGAA
CP022642 ATTTCGTTGA AGAACGCAGT AAAGTGCGAT AAGTGGTATC AATTGCAGAA
KX664452 ATTTCGTTGA AGAACGCAGT AAAGTGCGAT AAGTGGTATC AATTGCAGAA
KU680847 ATTTCGTTGA AGAACGCAGT AAAGTGCGAT AAGTGGTATC AATTGCAGAA
LT577674 ATTTCGTTGA AGAACGCAGT AAAGTGCGAT AAGTGGTATC AATTGCAGAA
KT438663 ATTTCGTTGA AGAACGCAGT AAAGTGCGAT AAGTGGTATC AATTGCAGAA
MT791386 ATTTCGTTGA AGAACGCAGT AAAGTGCGAT AAGTGGTATC AATTGCAGAA
LR812960 ATTTCGTTGA AGAACGCAGT AAAGTGCGAT AAGTGGTATC AATTGCAGAA
110 120 130 140 150
....|....|....|....|....|....|....|....|....|....|
MZ618780 TCATTCAATT ACCGAATCTT TGAACGCAAA CGGCGCATGG GAGAAGCTCT
MN031876 TCATTCAATT ACCGAATCTT TGAACGCAAA CGGCGCATGG GAGAAGCTCT
CP022642 TCATTCAATT ACCGAATCTT TGAACGCAAA CGGCGCATGG GAGAAGCTCT
KX664452 TCATTCAATT ACCGAATCTT TGAACGCAAA CGGCGCATGG GAGAAGCTCT
KU680847 TCATTCAATT ACCGAATCTT TGAACGCAAA CGGCGCATGG GAGAAGCTCT
LT577674 TCATTCAATT ACCGAATCTT TGAACGCAAA CGGCGCATGG GAGAAGCTCT
KT438663 TCATTCAATT ACCGAATCTT TGAACGCAAA CGGCGCATGG GAGAAGCTCT
MT791386 TCATTCAATT ACCGAATCTT TGAACGCAAA CGGCGCATGG GAGAAGCTCT
LR812960 TCATTCAATT ACCGAATCTT TGAACGCAAA CGGCGCATGG GAGAAGCTCT
160 170 180 190 200
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MZ618780 ATTGTGTCAT CCCCGTGCAT GCCATATTCT CAGTGTCGAA CAAAAAACAA
MN031876 ATTGTGTCAT CCCCGTGCAT GCCATATTCT CAGTGTCGAA CAAAAAACAA
CP022642 ATTGTGTCAT CCCCGTGCAT GCCATATTCT CAGTGTCGAA CAAAAAACAA
KX664452 ATTGTGTCAT CCCCGTGCAT GCCATATTCT CAGTGTCGAA CAAAAAACAA
KU680847 ATTGTGTCAT CCCCGTGCAT GCCATATTCT CAGTGTCGAA CAAAAAACAA
LT577674 ATTGTGTCAT CCCCGTGCAT GCCATATTCT CAGTGTCGAA CAAAAAACAA
KT438663 ATTGTGTCAT CCCCGTGCAT GCCATATTCT CAGTGTCGAA CAAAAAACAA
MT791386 ATTGTGTCAT CCCCGTGCAT GCCATATTCT CAGTGTCGAA CAAAAAACAA
LR812960 ATTGTGTCAT CCCCGTGCAT GCCATATTCT CAGTGTCGAA CAAAAAACAA
MZ618780 CA
MN031876 CA
CP022642 CA
KX664452 CA
KU680847 CA
LT577674 CA
KT438663 CA
MT791386 CA
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Figure (3) Analysis of alignment to L.major

A phylogenetic tree for the Iraqi isolate of L. *tropica* was drawn using Mega 6 and NCBI program and the results slowed a match between samples 8 and 9 was 98 % and samples 1,3,4,5,6 and 7 was 100%, whereas match between samples 1 and 2 was 97 % (Figure 4). On the other hand, the phylogenetic tree for Iraqi isolate of L.major show a match between all samples was 99% (Figure 5).

Figure (4) Phylogenetic of the Iraqi isolated of L.*tropica* compared to other isolates registered in Gene bank.

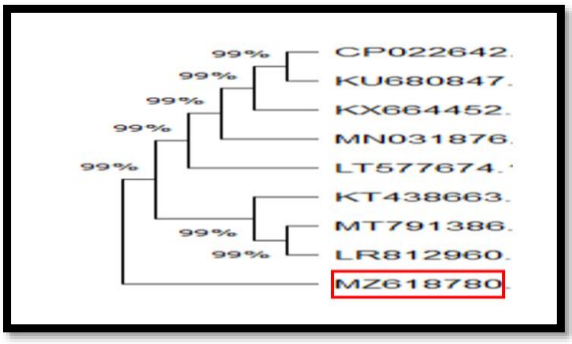

Figure (5) Phylogenetic of the Iraqi isolated of L.major compared to other isolates registered in Gene bank.

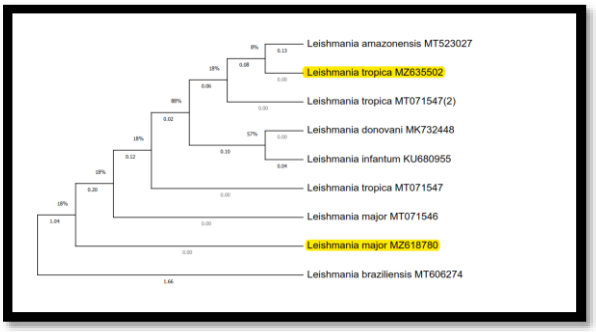

Figure (6) Neighbor joining tree

The Neighbor joining tree shows that the evolutionary history of the similar sequences of the two species recorded in the current study with related species in the NCBI data set. This analysis includes 9 nucleotide sequences, and there were a total of 1146 similar sites in the final data set. L. barazilinensis was also considered as a comparison group (Figure 6).

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