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RESEARCH ARTICLE

Genetic Diversity of Human Leishmania spp. Isolated from an Endemic Area in the Middle of Iraq

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Abstract

This study aimed to the possible characterization of cutaneous leishmaniasis species isolated from cutaneous tissue imprints from patients in an endemic area in the middle of Iraq. Samples were taken by skin scraping and dermal tissue aspiration. Direct examination and polymerase chain reaction, two types of primers used (CL for L. major, Lin R for L. tropica) for detection. DNA Sequencing and phylogenetic analysis were also used for detection. In this study, 48 patients with Cutaneous leishmaniasis were enrolled, 32 (66.7 %) of them were males and 16 (33.3%) were females. Giemsa staining results showed presence of 25 amastigote forms inside and outside the macrophages, while 25 (53 %) and 23 (47 %) were positive to direct test, 26 (54.2 %) patients had multiple lesions and 22(45.8%) had single lesions in a different locations of the body. More than 68.8% of patients had dry lesions, while 27.1% had moist and 4.2 % had mixed lesions. The results showed that 8 isolates (16.7 %) were L. tropica and 4 cases were L. major (8.3 %). Most L. tropica lesions were located on the face and other parts of the body, while lesions of L. major were located on foot. In addition, new species of Leishmania were identified as L. infantum 3 (6.3 %). Four PCR products of L. major isolates were prepared and sequenced by Macrogen Company, USA. The sequence of the isolates was recorded in the gene bank and the accession numbers of L. major were LC369673, LC369674, LC369675 and LC369676. The phylogenetic tree constructed from these sequences and the polygenetic tree of the L. major. The Iraqi isolates were close to French and Russian isolates.

Keywords: *Cutaneous leishmaniasis, PCR, Sequencing, Phylogenetic.*

Introduction

Cutaneous leishmaniasis (CL) is caused by parasites belonging to the genus Leishmania [1]. The disease is one of the most common zoonotic diseases and a major public health problem worldwide. Leishmaniasis occurs in three forms: cutaneous (oriental sore), visceral (kala-azar), and mucocutaneous [2]. Cutaneous leishmaniasis is a complex disease of a wide spectrum of clinical features, and it is one of the most common endemic diseases and the second most frequently transmitted arthropod-borne parasitic disease after malaria in the Middle East [3].

Leishmaniasis was recorded to be endemic in more than 88 countries. It mostly occurs in tropical and subtropical areas [4]. About 95% of CL cases occur in America, the Mediterranean Basin, the Middle East and Central Asia. Cutaneous leishmaniasis (CL) is a parasitic dermal disease involving the epidermis and may infect mucosal tissues which spontaneously heals and leaves a scar on the infected areas [5]. The causative agents of (CL) in the old world are Leishmania tropica, Leishmania major, Leishmania aethiopica and Leishmania infantum. CL infection is commonly found in Iraq and its neighbors such as Syria, Iran & Turkey [6]. Nearly two thirds of CL cases were reported to happen in six countries including Iran [7]. Following the civil war in Iraq and later in Syria, there was a dramatic increase in CL incidence [8].

Transmission of Leishmania major, the causative agent of (ZCL), occurs by a sand fly vector bite which belongs to the phlebotomus family and spreads at the central and southern parts of the country [9]. Leishmania infantum was recognized as a probable cause

of cutaneous leishmaniasis, and this species was isolated from dermal lesions of Mediterranean basin patients [10]. Diagnosis of (CL) depends upon clinical features and lab. Examinations, involving direct microscopic examination and / or indirect serologic and molecular diagnostic tests [11].

Molecular biological methods or isoenzyme analysis are used to identify different Leishmanial species [12]. A Main focus of this study was the possible characterization of this species from cutaneous tissue imprints in patients with cutaneous leishmaniosis in Iraq.

Materials and Methods

This study was conducted in Baghdad, Iraq during a period of time ranging between an average temperature (of 48-50ºC) in July & August to below the freezing point in January, with the majority of the rainfalls taking place between December and April which may reach 25 centimeters in some places [11].

Summer months are characterized by a dusty and dry wind with gusts that may reach to eighty kilometers / hour, and happen during April to early June, followed by late September to November.

The steady northern and northwest wind of the city dominates from mid-June to mid-September, and is accompanied by very dry air, which allows the intensive sun heat of the land surface, but also provides some cooling effects.

The patient's surrounding intact skin was first cleaned with 70% alcohol for sample collection, followed by injection of (0.2–0.5) ml saline solution in the margin between the lesion and the intact skin [11]. Smears were then dried, fixed with methanol, and stained with (10%) Giemsa stain, and examined for the existence of amastigote form [12].

Molecular techniques were applied to identify Lishmania spp., and for amplification of the kinetoplast DNA of Leishmania sp, nested PCR reaction was performed as described by [13].

Direct Examination

This study was carried out for the period from January 2017 to March 2017. Samples from 48 patients were collected from the skin with suspected cutaneous Leishmaniasis. The ages ranged from 5 to 70 years, with mean age of (32.17 ± 13.32) years, and 29 of them were men and 19 were women with mean gender of (32.77 ± 12.98). The studied individuals were inhabitants of the urban and rural areas attending dermatology department in many hospitals in Baghdad city suffering from single or multiple skin lesions located in different parts of the body.

One directional scalpel was used for skin scraping with the till oozing out of blood from lesions, and an incision was mostly made in the inflamed margin of the lesion. The samples were then placed on a slide, dried, fixed with methanol and stained with (10%) Giemsa stain and the whole slide were examined with a 100× oil immersion objective lense. All the slides were examined for the existence of amastigote forms at least twice before reporting the final results [14].

Molecular Examination

DNA Extraction

Kinetoplast DNA of Leishmania was performed according to the manufacturer's instruction of G- spin DNA extraction kit (Intron biotechnology, South Korea).

PCR Amplification and Gel Electrophoresis

For the detection of Leishmania spp by using polymerase chain reaction, two types of Primers were used (CL for L. major, Lin R for L. tropica, FLC2 for L. infantum) as listed in Table (1). The following primers were prepared by dissolving in the free (ddH2O) to give a final concentration of (100) pmol/µl as a stock solution, and a stock was kept at (-20) ºC.

To prepare (10) pmol/µl concentration as a working solution, (10) µl of the stock solution was added to (90) µl of the free (ddH2O) water to give a final volume of (100) µl (Integrated DNA Technologies company, Canada). PCR was carried out in a (25) mixture reaction containing DNA, specific primer, Taq PCR PriMix, and (ddH2O2). All mixtures were put in Multi Gene OptiMax Gradient thermo cycler (Lab net, USA). All PCR products were electrophoresed in (2%) agarose gel at (5) volt/cm² according to [15].

Table 1: Primer sequences and main PCR conditions

Sequences Primer	PCR conditions				Amplification program
	Primer p/µl	DNA µl	Taq PCR PriMix µl µl	ddH2O2 µl	
CL1 5'-CGAGTAGCAGAAACTCCCGTTCA-3'	1	1. 5	5	16.5	95°C for 3 min, 95°C for 45 sec., 57°C for 1 min, 72°C for 45 sec., 72°C for 10 min (35 cycles)
CL2 5'-ATTTTTCGCGATTTTCGCAGAACG- 3'	1				
LinR 5'-TCGCAGAACGCCCT-3'	1	1. 5			
LinR-R 5'-ACTGGGGGTTGGTGTAAAATA-3'	1				
FLC2 5'-GTCAGTGTCGGAAACTAATCCGC -3'	1	1. 5			95°C for 5 min, 95°C for 1 min., 56°C for 1 min, 72°C for 1 min., 72°C for 10 min (30 cycles)
RLC2 5'-GGGAAATTGGCCTCCCTGAG -3'	1				

DNA Sequencing and Phylogenetic Analysis

PCR products were visualized on the gel, purified and sequenced with Macrogen Company, (USA), and the results were matched with the data obtained from Gene Bank published ExPASY program which was present at the NCBI online. MEGA 4 was used for drawing a phylogenetic tree of L. spp.

Statistical Analysis

The data were analyzed by using the SPSS version 17 program, and the approach to data consisted of two steps (descriptive and analytic statistics), then analyzed by using the Student t-test. The P-value ≤ 0.05 was considered as statistically significant.

Results

Direct Examination

Giemsa staining results showed presence of 25 amastigotes forms in samples inside and outside macrophages, 25 (53 %) and 23 (47 %) were positive to direct test (Table 2).

Table 2: Demographical picture of cutenous leishmaniasis patients

CL patient's data		No(%)
Giemsa	Positive	25(53)
	Negative	23(47)
	Total	48(100)
No. of lesions	Single	22(45.8)
	Multiple	26(54.2)
	Total	48(100)
Nature of lesions	Dry	33(68.8)
	Moist	13(27.1)
	Mixed (dry and moist)	2(4.2)
	Total	48(100)
Site of lesion	Hand	20(41.7)
	Foot	16(33.3)
	Legs	1(2.1)
	Face	2(4.2)
	Total	47(100)

The results revealed that 26 (54.2 %) out of 48 patients had multiple lesions and 22 (45.8 %) patients showed single lesions in different locations of the body. More than 33 (68.8%) had dry lesions, 13 (27.1%) showed moist lesions and the remaining 2 (4.2 %) had mixed lesions.

Molecular Diagnosis

Polymerase chain reaction technique was performed for 48 individuals included in this study. The results showed that 8(16.7 %) isolates were L. tropica, 4 (8.3%) cases were L. major, 3 (6.3 %) L.infantum and 33 (68.8 %) were not detected.

The size of the amplified products of L. tropica was 750bp, L. major was 600bp and L. infantum was 230bp (Figure 1, 2).

Most L. tropical lesions were present on the face and other body parts, while the L. major lesions were present on the foot.

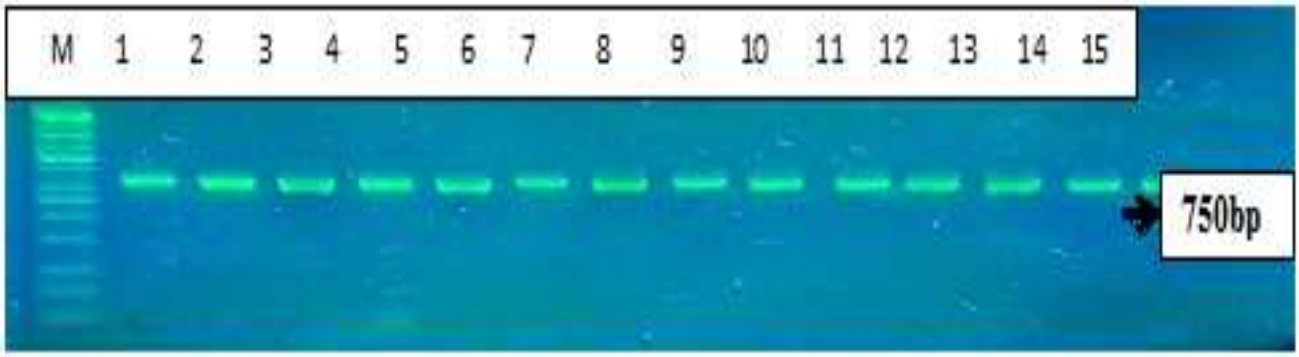

Figure 1: Gel electrophoresis of PCR products of Leshmania spp. M: DNA ladder marker, La ne1-15: L. tropica

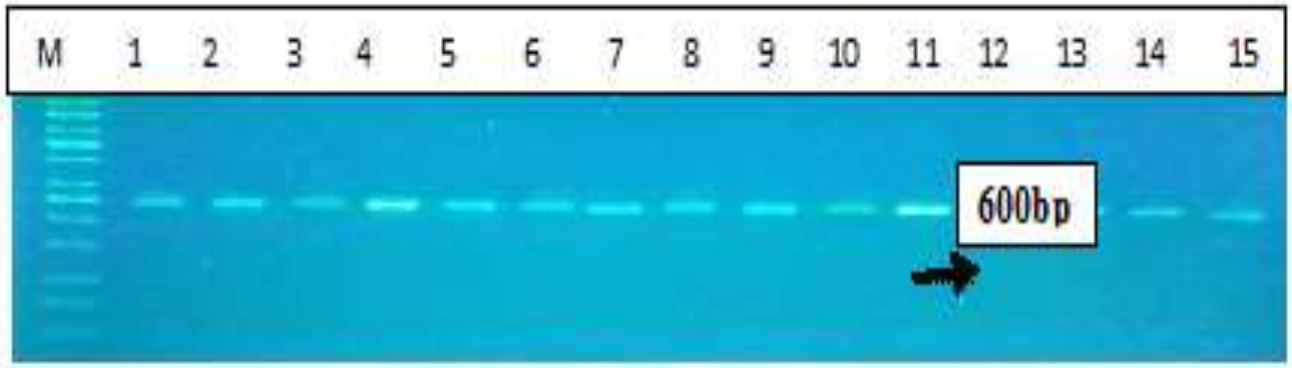

Figure 2: Gel electrophoresis of PCR products of Leshmania spp. Lane M: DNA ladder marker, Lane1-15: L. major

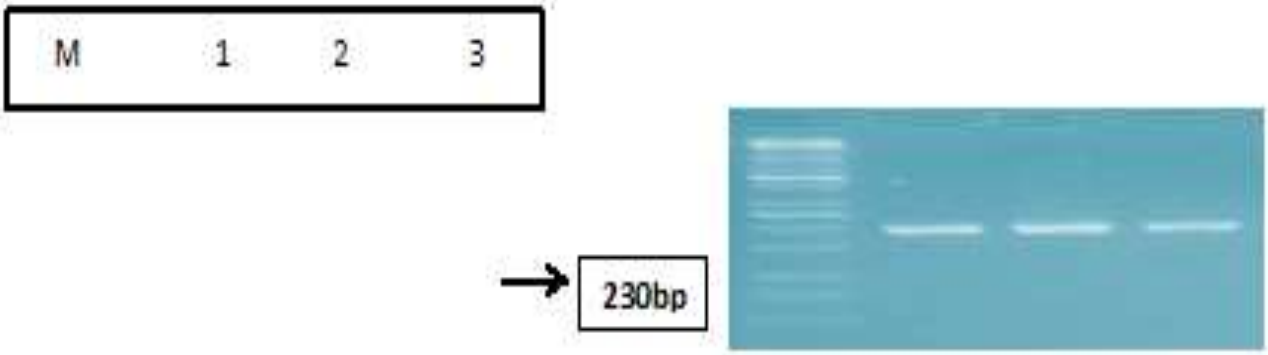

Figure 3: Gel electrophoresis of PCR products of Leshmania spp. Lane M: DNA ladder marker, Lane1-3: L. infantum

Four PCR products of isolated L. major were prepared and sequenced by Macrogen Company, USA. The sequence for isolates was recorded in the gene bank. The accession numbers of L. major were LC369673, LC369674, LC369675, and LC369676. Phylogenetic tree of Leishmania major was constructed based on KDNA gene sequencing using the Neighbor-joining. Figure 4 illustrates the genetic dimension between the isolates of Iraq and the isolates of the world in details.

The adoption of the height of the tree drawn as two clusters: The large cluster branch with five branches: The first branch represents France, Netherlands, Iraq and Russia, and the genetic dimension was 0.1, and the distance between them was 0.2. While the second branch represents Tunisia, Iran and the USA. Small clusters represent Australia and Pakistan. The genetic dimension was 0.2, and the distance between them was 0.7. The Iraqi isolates were close to the French and Russian isolates.

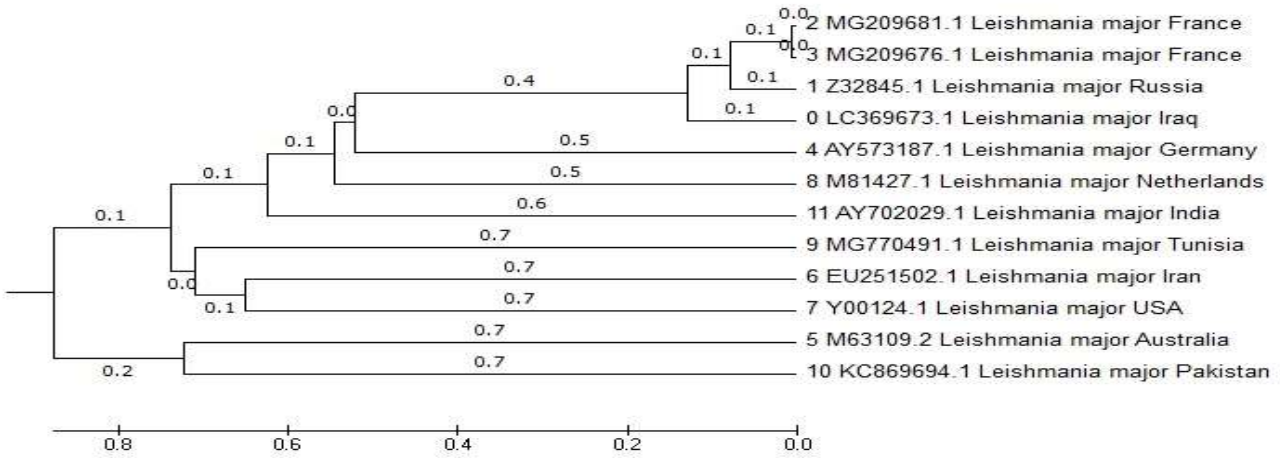

Figure 4: Neighbor-joining Leishmania major of KDNA

Discussion

The zoometric cutaneous leishmaniasis (ZCL) is still an increasing public medical problem in the Middle East area [16]. In this disease, one or more cutaneous lesions appear on areas where sand flies (vectors) have fed. Depending upon the Leishmania species and strains, nutritional status, immunologic status, the morphology of the cutaneous lesions differs and may change in shape and size over time [17].

Leishmaniasis has been reported as one of the six significant infectious diseases worldwide by the world health organization (WHO). Thus, researches have concentrated, emphasized and recommended on the different aspects of the disease [8]. Suitable environment for the disease maintenance was provided by the growth of population, immigration of non-immune individuals to endemic places, activity of sand flies, presence of rodents as well as patients suffering from lesions or sores [9].

The infection was more common in male as compared to female, and this may be due to environmental and ecological agents of the area. Direct microscopy of CL lesions smears is reported to have low sensitivity (13–60%). Similar observations were made in slit smears of CL patients. The findings in the present study were similar to Rehman et al [18].

While disagreed with the report of Abd El-Salam et al (2014) and this may be due to ecological, cultural, environmental and socioeconomic conditions of the areas [19]. Molecular characterization of Leishmania species was reported in the present study.

The identification of the species of cutaneous leishmaniasis has a great diagnostic and clinical importance. Leishmanial parasites possess similar morphology and may cause similar clinical features, thus, distinguishing between species needs molecular diagnostic techniques such as the polymerase chain reaction (PCR), electrophoresis as well as DNA probe hybridization methods [4].

In our study, we used the polymerase chain reaction with specific primers and Kinetoplast DNA of Leishmania Spp. This approach is very important for diagnosing cutaneous leishmaniasis (CL). Results of PCR in our study confirmed that both species (L. major and L. tropica) are found in different cities of Iraq. In addition, new species of Leshmania such as (L. infantam) was detected. L.tropica caused 16.7% of infections, L. major caused 8.3% and L. infantum caused 6.3% of infections.

However, in our country, L. infantum in cases of cutaneous leishmaniasis without visceral leishmaniasis history was recorded. These results are so necessary for selecting the suitable treatment since L.infantum strains may enter the visceral part of the body. More studies are also required to affirm these results. Cutaneous leishmaniasis has been mainly caused by L.major, L.tropica, L. aethiopica and sometimes by L. infantum in the old world [14].

Several studies have reported that cutaneous leishmaniasis can be caused by L. infantum strains in the Middle-East, Southern Europe, Africa, Tunisia & Iran [3, 21]. On using Zymodeme analysis method, Chargui et al [8] in Tunisia have isolated (1%) of L.infantum

from skin lesions and found that (99 %) of them were caused by L. major. Our study revealed that cutaneous leishmaniasis has been caused by L. tropica and L. major with the highest infection caused by L. tropica.

These results were in agreement with the studies conducted in Morocco 2014) [20], in Iraq (2016) [21], in Syria (2014) [22], in Iran (2013) [23] and in Turkey (2009) [24]. The characterization of leishmania spp. is necessary as various species may need distinct therapeutically regimen. Moreover, this information are also of great value in epidemiologic studies, because leishmanial species distribution in human and animal host bodies, as well as in insect vectors, is prerequisite to design the suitable controlling measures. However, the current study disagreed with a previous Iranian study by Azizi et al, 2012 [25].

Regarding this point, the PCR method is an important reliable technique for leishmanial species identification. However, PCR provides certain advantages that conventional methods can't offer to diagnose and characterize the cutaneous leishmaniasis. The technique of PCR in the diagnosis of CL is a valuable tool to detect the DNA of leishmanial parasite. The PCR technique can detect the species of parasite involved, and is more sensitive than the microscopical methods.

In the current study, the DNA sequencing of Iraqi L. major isolates was confirmed on molecular diagnosis basis by PCR and registered in Gene bank, with the accession numbers of L. major LC369673, LC369674, LC369675 and LC369676. These sequences have constructed the phylogenetic tree and the genetic heterogeneity of L. major. From these results, it can be concluded that genetic methods are efficient, perfect and rapid in the detection of leishmania isolates, thus shortening the time and cost and giving the correct treatment and accurate treatment of the disease without the error of time.

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