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MOLECULAR IDENTIFICATION OF *LEISHMANIA* SPP. IN CUTANEOUS LEISHMANIASIS

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Abstract

Cutaneous leishmaniasis (CL) remain a major public health problem and an endemic disease in Iraq, therefore the aim of this study was to identify the *Leishmania* species found in the skin lesions of cutaneous leishmaniasis by molecular methods and study some demographic characteristics. Samples were collected from 50 suspected CL patients in Department of Dermatology Al-Karamaa, AL Yarmouk and Medical City Teaching Hospitals in Baghdad Province between November 2017 to March 2018. The diagnosis was made by parasitological (examination of Giemsa-stained smear and in vitro cultivation) methods and by amplifying two popular markers in kinetoplast DNA and internal transcribed spacer 1 loci by nested-PCR and conventional PCR respectively. The sequences data was analyzed and phylogenetic tree was drawn. The Results showed that the majority of patients at the age of 18-45 years (76%). The infected Males (76%) higher than females (24%), highest percentage of infection was in upper limbs (36%), single lesion was documented in (56%) of patients, whereas multiple lesions were observed in 44%, most infection occurred in border area in Al Mosul (48%) and AL Ramadi (12%) and on the outskirts of Baghdad the peak of propagation in January (44%). Microscopy revealed amastigotes in 23 samples (46 %) and in vitro cultivation gave positive results in 12 cases (24 %), whereas PCR detected *Leishmania* in 40 samples (80 %). Out of 40 well-documented cases in our study; 24(60 %) were caused by *Leishmania* major and 16 (40 %) by *Leishmania tropica*.

Key words: Cutaneous leishmaniasis, Nested PCR, Aspirate

Introduction

Leishmaniasis is a group of diseases which are caused by many intracellular protozoa species of the Leishmania genus and transmitted by sand flies (*Diptera, Phlebotominae*) (Srivastava *et al.,* 2016; WHO, 2016). Its located in thirteen tropical diseases more neglected, leishmaniasis Globally is responsible for great rates of morbidity and mortality (Thomas, 2018), affect Mediterranean and other endemic countries that putting a population of 350 million people at risk of infection (Torres-Guerrero *et al.,* 2017**)**. Twelve million cases worldwide is the estimation of overall recrudescence of leishmaniasis and about 1.5 million global annually incidence of all clinical forms of the disease (WHO, 2017). Different species of *Leishmania* causes the disease with specific tendency of each species to a particular geographical region. It is endemic in 98 countries such as Afghanistan, Algeria, Brazil, Peru, Iraq, Saudi Arabia, Syria and Iran of which occur (WHO, 2015; Azizi *et al.,* 2016). There are three main types of leishmaniasis: cutaneous leishmaniasis (CL) caused by *Leishmania tropica*, *L. mexicana* and *L. major*. visceral leishmaniasis (VL) caused by *L. donovani* and *L. infantum* and mucocutaneous leishmaniasis (MCL) caused by *L. braziliensis* (Loría and Andrade, 2014; Igbineweka *et al.,* 2012). Just the infect female Phlebotomine sand fly is responsible of the diseases transmission and spread its intracellular parasites which have a complex digenetic life cycle needing a susceptible vertebrate host and a tolerant insect vector, which permits their transmission, assured the signicance of animal reservoirs in facilitating transmission of CL (Figueira *et al.,* 2017).

There are many methods for diagnosis parasite, the traditional which include microscopic examination of direct smears using Giemsa stain, cultured on Novy–MacNeal–Nicolle (NNN) medium, histopathology and serology these based diagnostic methods are being replaced by rapid, highly sensitive and specific molecular

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techniques based on detecting the parasite DNA as polymerase chain reaction (PCR) that target coding and noncoding regions of the Leishmania genome (Fahriye *et al.,* 2017).

In Iraq leishmaniasis considered an important health problem due to its high endemicity and it required high annual expenses when it is running in an epidemic state CDC recorded 18,200 case between 2008 to 2016 (Hassan, 2017). Presence of the wars and bad conditions, poor sanitation and bad situation for people who exposed to the displacing and dived in camps, presence of swamps near their camps that was very important for reproduction sand fly, all that leads to marked increasing in percentage of infection.

Materials and Methods

Clinical Sample Collection: This study was performed in the dermatology Consultant of Al-Yarmouk, Al-Karamaa and Medical City Teaching Hospitals in Baghdad Province, Samples were collected from patients who were referred to Baghdad hospitals from November 2017 to March of 2018. A total of 50 cases of suspected cutaneous leishmaniasis were inclusive in this study.

The samples from the cutaneous lesion were taken by the chancre fluid was aspirated from swollen edge of lesions of patients collected by sterile syringe and divided into three parts, The first part of the sample use to prepared smears then air-dried, fixed in methanol, stained by Giemsa and examined under a light microscope under 1000× magnifications. A second part was inoculated on Novy-MacNeal-Nicolle (NNN) medium (Limoncu *et al.,* 1997). The cultures were incubated at 24°C and observed every week for 1 month. The third part for PCR was placed directly in lysis buffer (10 mM Tris-HCI, 10 mM EDTA).

Microscopical Examination: Apart of the sample were smeared on glass slides according to (Bensoussan *et al.,* 2006; Amro *et al.,* 2012), air dried and fixed with methanol for a few seconds. Giemsa stain was filtered and diluted 1:20 with phosphate buffer (pH 7.2). After 20 minutes of staining the slides were washed with tap water and air dried. The stained smears were examined under the microscope with a 40 x lens and with a 100 x oil immersion lens. If at least one intra- or extra-cellular amastigote with a distinctive Kinetoplast was found the smear was declared positive. When no amastigotes were seen after 15 minutes of inspection, the smear was declared negative.

Culture: The lesions and the adjacent normal-looking skin around them were cultured on Novy-MacNeal-Nicolle medium (Limoncu *et al.,* 1997).

DNA Extraction:

DNA extraction from tissue lesions was processed using the G-spin DNA extraction Kit from (Intron/ Korea), according to the manufacturer's protocol. The quantification and quality control of the DNA extraction procedures were performed using anano-spectrophotometer (NanoDrop1000, Thermo Fisher Scientific). The extracted DNA was stored at "20°C until the time of use (Spotin *et al.,* 2014).

PCR

Two PCR protocols were applied to the amplified DNAs using thermocycler (Applied Biosystems/USA). The first PCR targeted a Leishmania-specific ITS1 gene as a universal *Leishmania* species detection mark. The specific primer of ITS1-2 gene (Es-Sette *et al.,* 2014). The primer set that used in study is 5'-GCA GCT GGA TCA TTT TCC - 3' as a Forward and 5'-ATA TGC AGA AGA GAG GAG GC- 3' Reverse. The amplification was carried out by using the PCR ready premix (Intron, Korea) in 25µl total reactions comprising 5 µl premix, 10µl forward and reverse primers (10 pmol), 1.5 µl DNA template and 16.5 µl distilled water.

The PCR conditions consisted of one initial denaturing cycle at 95 ÚC for 10 min, followed by 35 cycles at 95°C for 30s, 58°C for 30 s, 72°C for 1min. This was followed by a final extension cycle at 72°C for 10 min. The PCR products were visualized after staining with red safe by 2% agarose gel electrophored by 5vol/cm in TBA buffer, the expected band will be sized 462 bp then sent to Macrogene (Korean Company) for sequencing.

Nested PCR amplified

Kinetoplast DNA (kDNA) gene was amplified according to the (Noyes *et al.,* 1998) (Selvapandiyan *et al.,* 2008). Primers for *L. major* and *L. tropica* were provided by IDT. Nested PCR first around including the extracted DNA and the first set of primers CSB, with sequence of Forward 5'- CGA GTA GCA GAA ACT CCC GTT CA - 3' and Reverse 5'-ATT TTT CGC GAT TTT CGC AGA ACG - 3'.

The PCR conditions consisted of one initial denaturing cycle at 95°C for 10 min, followed by 35 cycles of 95°C for 30s, 55°C for 30 s, 72°C for 1min. This was followed by a final extension 1 cycle at 72°C for 7 min. amplifying the Kinetoplast minicircle partial flanking region approximately band size 750bp. The second around including the CSB products from the first around and the second set of LiR primer that amplifying 604 bp, the sequence of LiR primer Forward 5'-ACT GGG GGT TGG TGT AAA ATA G- 3' Reverse 5'- TCG CAG AAC GCC CCT - 3'. The PCR conditions consisted of one

initial denaturing cycle at 95°C for 10 min, followed by 35 cycles of 95°C for 45sec, 57°C for 45sec, 72°C for 45sec. This was followed by a final extension 1 cycle at 72 ÚC for 7 min.

Sequences

The PCR products 25 µl for ITS1-2, Kinetoplast genes for each samples amplified were patching with 10 µl of each forward primers and then were sent to Macrogene (Korean Company) for sequencing. Sequenced data was analyzed using sequence analyzer software, finch software. Nucleotides were blasted with alignment search toll (BLAST) analysis software (http:/ /www.ncbi.nlm.nih.gov). The phylogenetic tree was constructed using the UPGMA method using MEGA 6.0 version software.

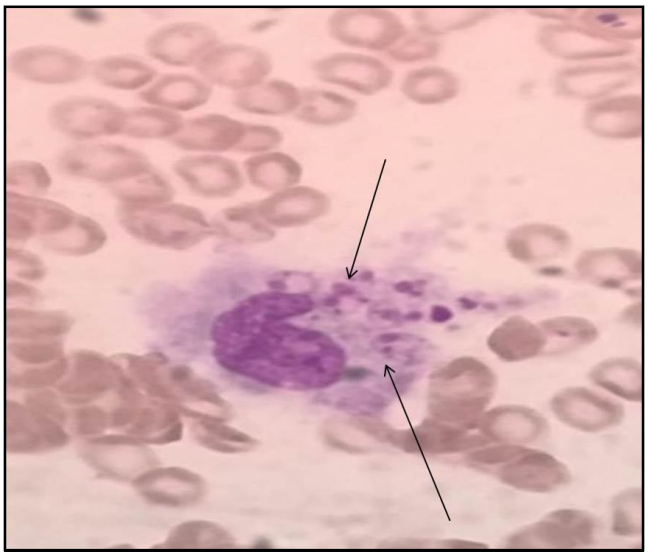

Fig. 1: Smear from skin lesion stained With Giemsa show amastigotes in WBC.

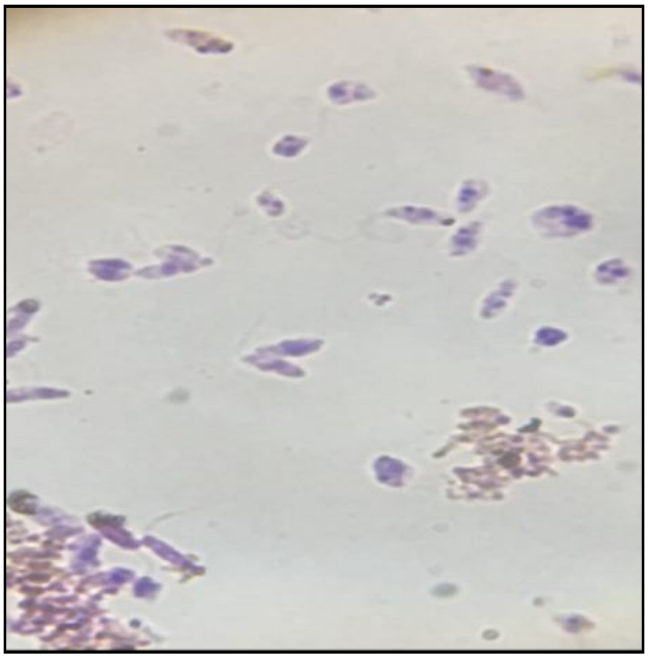

Fig. 2: Smear from skin lesion stained with Giemsa show promastigote under 10X.

Results

The demographic study results showed that the CL infection in males were 38 (76%) higher than in females 12 (24 %), cases of CL were largely reported among those aged 18–45 years old it was 38(76%), highest percentage of infection was in upper limbs (36%)

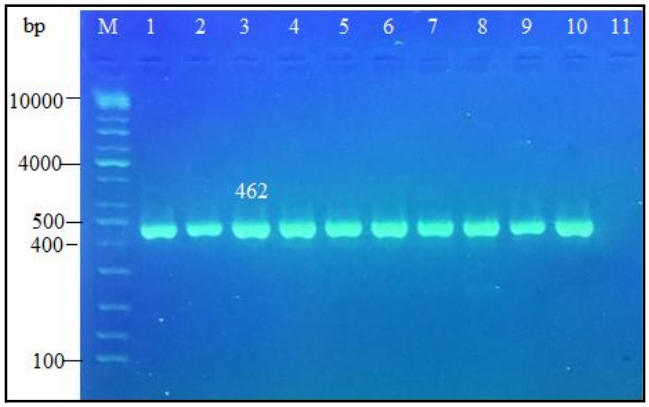

Fig. 3: Gel electrophoresis profile PCR products of ITS1- gene *Leishmania spp*. Using 2 % agarose stained with red safe DNA dye and electrophoresed by 5vol/cm in TBA buffer line 1-10 of the molecular size 462 bp.

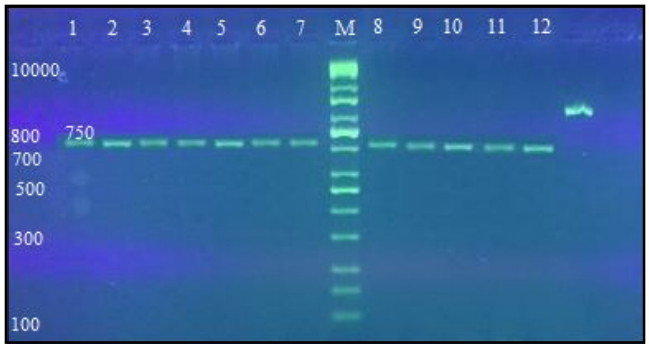

Fig. 4: PCR product the band 750 bp. The product was electrophoresis on 2% agarose at 5 volt/cm^2. 1x TBE buffer for 1:30 hours. N: DNA ladder (100).

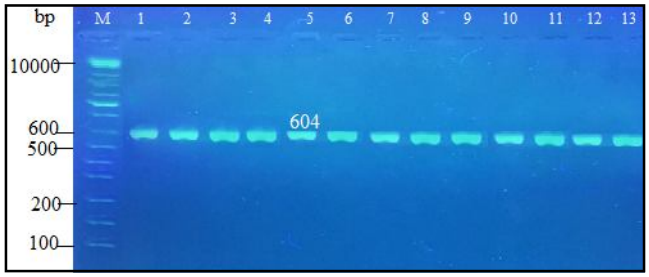

Fig. 5: Gel electrophoresis DNA profile of ITS1-5.8-2 partial gene product electrophoresed on 2% agarose at 5 volt/cm^2. 1x TBE buffer. N: Lane M represent DNA ladder (100)bp , lane 1-13 amplicons sized 604 bp indicated *L. major* species.

compared with other sites of infection, single lesion was documented in 56% of patients, whereas multiple lesions were observed in 44% and in relation to the clinical feature it seemed that *L. major* (wet) 36) 72%) infection whereas *L. Tropica* (dry) 14 (28%), that mean the wet type is higher than the dry one, most infection occurred in border area in Al Mosul (48%) and and, AL Ramadi(12%) and on the outskirts of Baghdad the peak of propagation in January (44 %).

Several techniques were used for diagnosis of CL including routine method like direct microscopic smear from lesion which showed amastigotes in the microphage in 23 positive cases Fig. 1, then aspiration fluid from lesions was cultured on Novy-MacNeal-Nicolle (NNN) media for Promastigote detection (infective stage) showed only 12 samples gave a pure growth Fig. 2, 16 samples had a

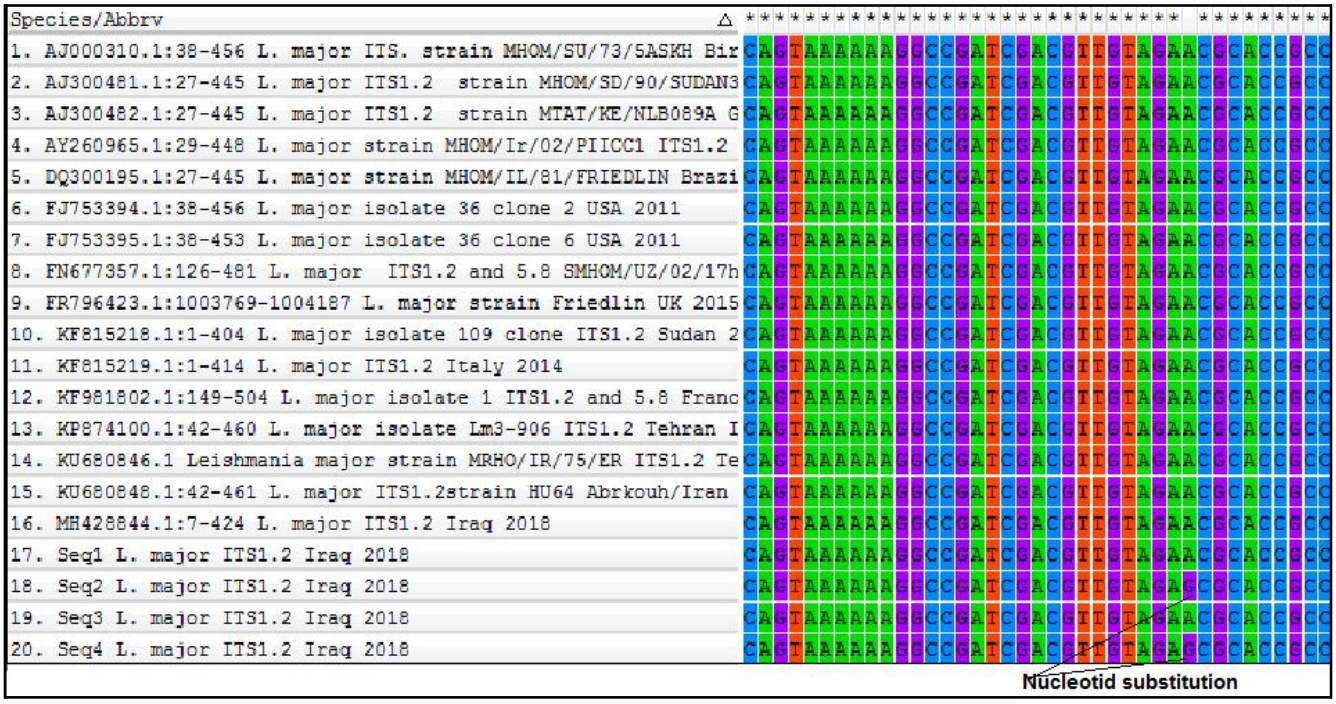

Fig. 6: Nucleotide sequence of sense flanking the partial ITS1- 5.8-2 gene compared with the related identity to gene bank strains of *L. major*MEGA6 program.

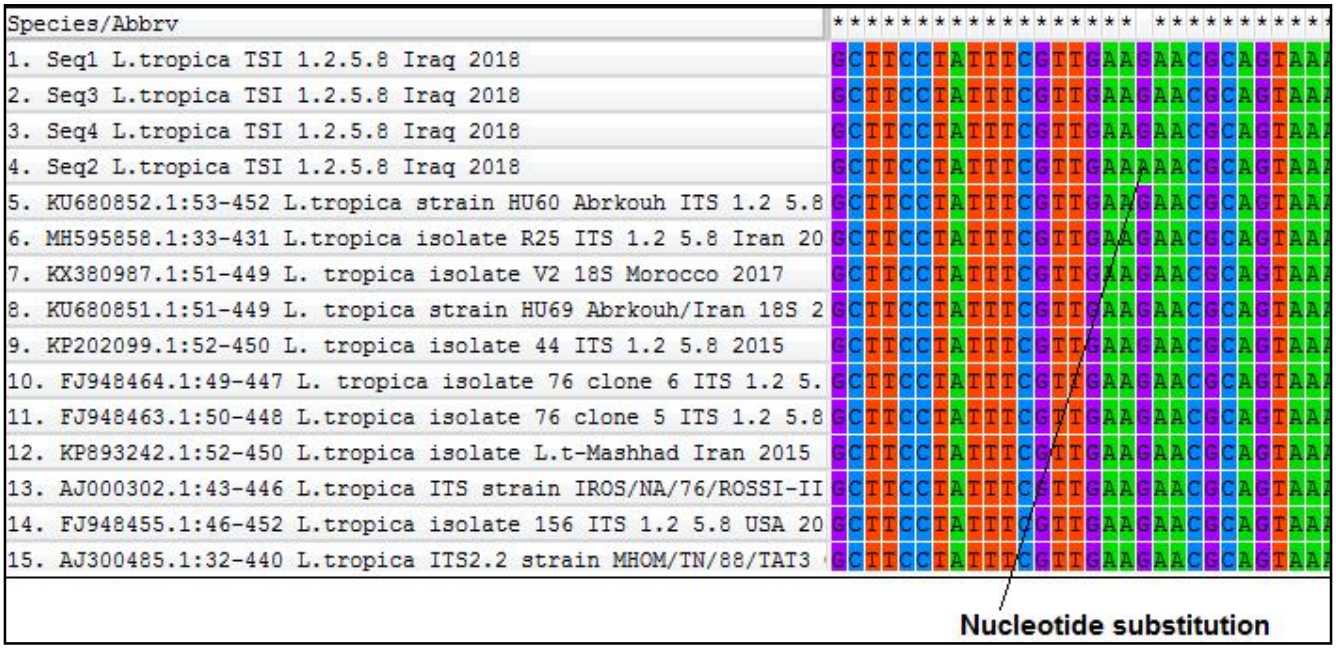

Fig. 7: Nucleotide sequence of sense flanking the partial ITS1-5.8-2 gene compared with the related identity to gene bank strains of *L. tropica* MEGA6 program.

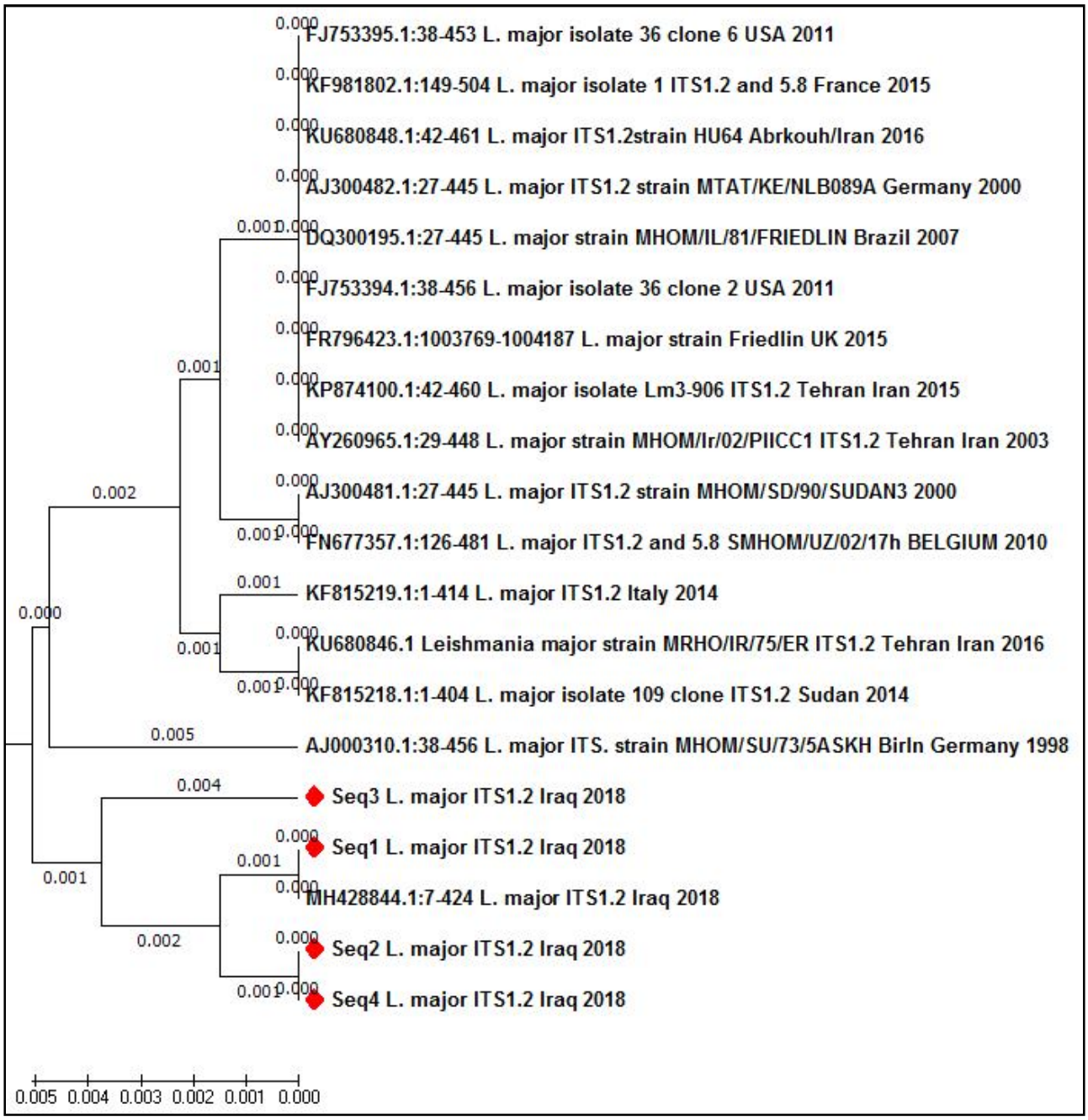

Fig. 8: The phylogenetic tree of sense flanking the partial ITS1-5.8-2 gene in *L.major* isolated in Iraq during 2018 in compared with the 16 related identity to gene bank strains. Gb| MH428844.1 | was used as the root for the tree. Scale bars indicate nucleotide substitutions per site. Phylogenetic analysis was conducted using MEGA6.

contamination (bacteria and fungus) and 22 samples showed no growth at all. The Molecular study was carried out to detect the two genes ITS1-5.8-2 (internal transcribed spacer) and KDNA (Kinetoplast DNA) by polymerase chain reaction (PCR). DNA extracted from 50 samples (aspirate fluid) showed 40 (80 %) were amplified ITS1-5.8-2 gene Fig. 3, while the amplification KDNA gene indicated 24(60%) isolates were belong to *L.major* and 16(40%) isolates were belong to *L. tropica* as in Fig. 4, 5.

Discussion

The conventional laboratory diagnosis methods of CL in general can be made on the basis of microscopic examination of the stained smear, culture,, employed for the diagnosis of CL has modest to low rates of positivity because of require the presence of a relatively high number of viable or morphologically intact parasites; this may pose a problem particularly in the chronic phase of CL where parasite levels in skin lesions are very low also the microscopic examination contributes to the

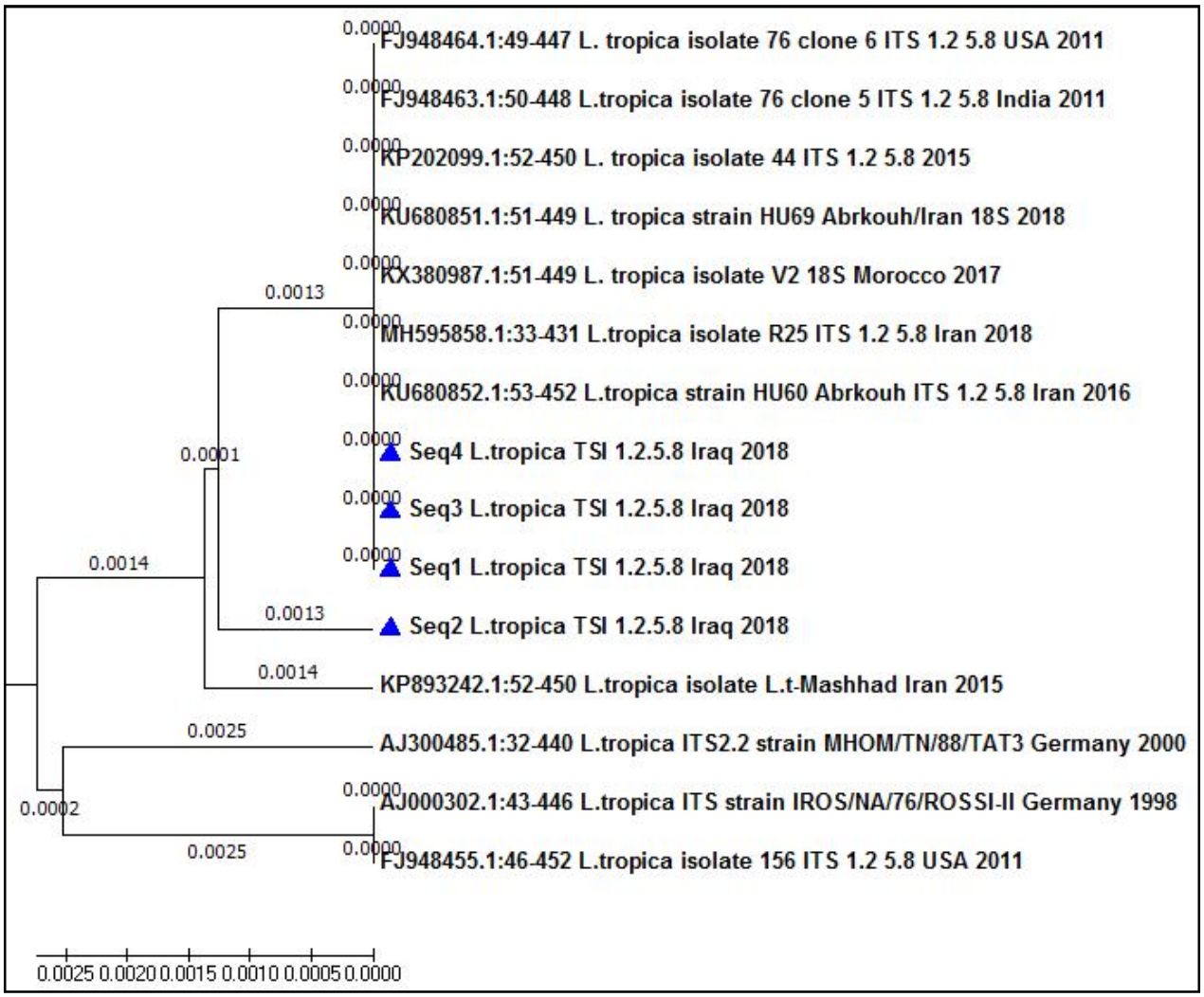

Fig. 9: The phylogenetic tree of sense flanking the partial ITS1-5.8-2 gene in *L.tropica* isolated in Iraq during 2018 in compared with the 11 related identity to gene bank strains. Gb: KU680852.1 was used as the root for the tree. Scale bars indicate nucleotide substitutions per site. Phylogenetic analysis was conducted using MEGA6.

identification of the infected parasite but is unable to identify the parasite at the species level. In the resent study we reported sensitivity of microscopic examination data integrated from Giemsa-stained smears which is consistent with (Marfurt *et al* 2003) in compared with molecular detection based on ITS1-5.8-2 marker and sequence results table 1. *L. major* Iraqi isolates were 99% identical with (high 415-416 score and 0.0 E.Value) blasted with NCBI standard species gene bank under accession number MH428844.1 with two nucleotide substitution in gene bank position 118 A>G and 72T>A, Fig. 6. On the other hand 50% the *L.tropica* Iraqi isolates were 100% and the rest were 99% identical with (high 400-397 score and 0.0 E.Value) blasted with NCBI standard species gene bank under accession number KU680852.1. Indicated nucleotide substitution in gene bank position 295 G>A, Fig. 7. The expectation value (e value) is defined to give an estimate of the number of times expected to get the same similarity coincidental and the lower the value of E (0.0) in both *L.major* and *L.tropica* sequences indicates that the degree of similarity was high between sequences which give greater confidence. The value of a very close to zero means that these sequences are identical and the higher bit Score of *L. mjore* sequences was (415-416) and *in L.tropica* was (397-400) (appendix 2) which is a statistical measure of the moral similarity and the higher value indicates that the high degree of similarity and if dropped from the class of 50 points, the sense that there is no similarity mention. That resulting sequences were edited in MEGA6.0 and aligned using ClustalW 1.8 with reference sequences from gene bank under accession number MH428844.1 The phylogenetic tree of *L.major* was inferred using the UPGMA method,and the evolutionary distances were computed using the Maximum Composite Likelihood method, the sum of branch length was = 0.02534151 is shown in Fig. 8. The analysis involved 20 nucleotide sequences 15 of them was imported from NCBI and are

in the units of the number of base substitutions per site. All positions in the sequence containing gaps and missing data were eliminated. There were a total of 336 positions in the final dataset. Phylogenetic tree of *L.major* including two major branches the upper one including all strain from Iran, Germany, Sudan, USA, Belgaum, France and the lower branch including the Iraqi isolate MH428844.1 that very closed to our isolates.

Evolutionary analyses of *L.tropica* were conducted in MEGA6 The phylogenetic tree was drawn using UPGMA method and aligned using ClustalW 1.8 with reference sequences from gene bank under accession number KU680852.1 and the evolutionary distances were computed using the Maximum Composite Likelihood method, the sum of branch length was = 0.01061598 is shown in Fig. 9. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated. There were a total of 398 positions in the final dataset. The phylogenetic tree of its including two main branches the Iraqi isolates location in the upper one closed to Iran isolates and other isolated from India,Morocco,and USA. On the other hand the lower branch including the German and USA isolates. Out of the real desire to find a fast, secure, increased sensitivity and speciation accurate diagnosis way without the need for parasite culture may be useful for to check Leishmania species and treatment in clinical settings. The Kinetoplast minicircle DNA chosen because of their heterogeneity in sequence and is present in many thousands of copies in Leishmania as mentioned by Selvapandiyan *et al.,* 2008. Nested PCR technique amplification specific region of Kinetoplast minicircle that flanked by two sets of primer (CSB, and LiR) amplified two different sized fragments. The nested PCR result showed dominant *L.major* species on *L.tropica* species in Iraq.Signify to characterize the *Leishmania* species that Causing cutaneous leishmaniasis in Iraq. *L. major* as mentioned previously by (Al-Saqur and AL-Obaidi, 2013) and *L. tropica* as mentioned previously by (Sharma *et al.,* 2015) and that promoted other Iraqi study (Rahi *et al.,* 2013) and other study in nearby countries such as Iran (Azizi *et al.,* 2012) and Saudi Arabia (Amin *et al.,* 2013). The current study appeared the PCR was more specific and speed technique for diagnosis of cutaneous leishmaniasis, DNA analysis test was consider one of important techniques to give the powerful and stable criteria for the taxonomy for *Leishmania* parasite and can detected as few as 50 – 100 parasite, so have been successfully applied in order to detected the parasite DNA (Mirahmadi *et al.,* 2017). The high incidence of *L. major* may be due to the presence of reservoir animals in large numbers, especially rodents and dogs. 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; also it must know vector sand flies responsible for human infection by *L. topica* only (Craig *et al.,* 2013). Molecular methods as PCR Remains the beast, in spite of its not available in all tests but have a decisive role in diagnosis in level of species (Hailu and Boelaert, 2016). The PCR-based checking of appears to be reasonably, sensitive and specific in revealing the presence of *Leishmania* parasites in such chronic lesions. PCR can clearly help improve the diagnosis of CL in these difficult cases.

Conclusions

Characterization of the *Leishmania* isolates which collected from several parts of Iraq appeared that *L.major* and *L.tropica* are the agents of CL. Moreover, this study revealed that PCR procedure depending on KDNA marker is very dependable technique for diagnose cutaneous Leishmaniasis and can applied in epidemiologic investigations in Iraq. Identify the parasite origin which causes cutaneous leishmaniasis and the harmonization of the genetic traits with the strains spread over the world from through Phylogenetic tree; DNA sequencing to isolate cutaneous leishmaniasis is recorded in the gene bank.

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