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GENETICS, EVOLUTION, AND PHYLOGENY - ORIGINAL PAPER

Detection, genotyping, and phylogenetic analysis of *Leishmania* isolates collected from infected Jordanian residents and Syrian refugees who suffered from cutaneous leishmaniasis

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Abstract

Leishmania is a parasitic protozoan which is transmitted to humans through the bite of an infected female *Phlebotomus* and *Lutzomyia* sand flies. Cutaneous leishmaniasis (CL), caused by *Leishmania major* and *L. tropica*, is an endemic disease in many areas of Jordan and considered as a major public health problem. The political instability in the Syrian Arab Republic has resulted in the immigration of large number of refugees into Jordan where most of them resided in camps near the Syrian borders. Therefore, the main objective of the present study was to inspect *Leishmania* species/genotypes which are responsible for CL infections among Syrian refugees and compare them with the recovered species/genotypes isolated from Jordanian patients. Three molecular-based assays (ITS1-PCR-RFLP, Nested ITS1-5.8S rDNA PCR, and Kinetoplast DNA PCR) followed by sequencing and phylogenetic analysis were undertaken and compared for their efficiency to confirm CL diagnosis and genotype the infecting *Leishmania* species. Thereafter, the evolutionary relationships among various *Leishmania* isolates from Syrian and Jordanian CL patients were elucidated. Results from the present study indicated that 20 and 9 out of the inspected 66 patients (39 Jordanian and 27 Syrian) were infected with *L. major* and *L. tropica* respectively. ITS1-PCR RFLP typing proved to be more sensitive in the detection of *Leishmania* species (positive in 44% of the isolates) compared to both ITS1-5.8S rDNA gene and Kinetoplast DNA PCR which were successful in identifying *Leishmania* species only in 23% and 33% of the isolates respectively. Sequencing and phylogenetic analysis of ITS1 and ITS1-5.8S rDNA genes revealed high levels of heterogeneity among the sequenced isolates. One sample typed as *L. tropica* from Jordanian patient showed high similarity with *L. tropica* sample isolated from a Syrian patient in a Lebanon refugee camp; therefore, the need for comprehensive studies to confirm if any new *L. tropica* strains might be introduced to Jordan by Syrian refugees is urgently indicated. These observations highlighted the need for further studies to clarify the risk status of species and strains which might be introduced from Syria to Jordan.

Keywords *Leishmania* · Cutaneous leishmaniasis (CL) · ITS1 · 5.8S rDNA gene · PCR · RFLP · Sequence analysis

Introduction

Leishmaniasis is a vector-borne disease which is caused by the obligate intracellular protozoan parasites of the genus *Leishmania* (Maslov et al. 2010). *Leishmania* parasites are dimorphic, existing as intracellular amastigotes within the macrophage of the mammalian host, and as flagellated promastigotes in the midgut of *Phlebotomus* and *Lutzomyia* sand flies (Bates 2007; Akhoundi et al. 2016; Maroli et al. 2013). At least 30 *Leishmania* species were confirmed to cause leishmaniasis in humans (Reithinger et al. 2007; Akhoundi et al. 2016), which varied in their clinical forms from localized cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), to a wide spread disseminating visceral leishmaniasis (VL) known as kala-azar, which is a fatal disease if left untreated (Desjeux 2004). The transmission of CL can occur either by anthroponotic (from human to human) or zoonotic (from animals to human) transmission routes (Sharifi et al. 1998; Ashford 2000; Postigo 2010).

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Leishmaniasis is global in its distribution, where it has been reported in 98 countries worldwide with approximately 0.4 to 0.2 million cases and 0.7 to 1.2 million VL and CL cases, respectively, occurring every year (Alvar et al. 2012). CL is more widely distributed than the other two forms of leishmaniasis (MCL and VL), with about one third of the cases occurring in three epidemiological regions, the Americas, the Mediterranean basin, and Western Asia from the Middle East to Central Asia (Desjeux 2001). In Jordan, the main causative organism of CL was identified as *L. major* which is zoonotically transmitted (ZCL), as reported in several areas in the country and is regarded as an endemic disease especially in certain areas such as Sweimeh near the Dead Sea. However, CL caused by *L. tropica* also occurs within small endemic areas in Jordan such as Eira in the Salt governorate from where the first CL caused by *L. tropica* case was identified (Kamhawi et al. 1995a, b; Saliba et al. 1993; Mosleh et al. 2008). The epidemiology of CL and its distribution is not fully understood among different areas of Jordan, and severe underestimation of the disease burden among Jordanians has been reported (Mosleh et al. 2008). Although CL is endemic in Jordan, VL is rare with only 15 cases being reported since 1960 which is caused by *L. infantum* (Postigo, 2010; Salam et al. 2014).

Sweimeh is regarded as a hyper-endemic area for CL, where about 100% of individuals in this particular region over 5 years old were found to be reactive to the leishmanin skin test in 1992 (Arbaji et al. 1993; Salam et al. 2014). Early studies in Jordan reported outbreaks of CL caused by *Leishmania major* and therefore this species is still regarded as the most prevalent one in the country from the North to the South (Saliba et al. 1988). In 1 year (2004 to 2005(, 100–200 cases were reported from Aqaba, North Agwar, and South Shuneh; however, a spike in 2007 reported higher number of 354 cases (Mosleh et al. 2008). The most recent Jordanian annual report released by the Ministry of Health confirmed the occurrence of a total of 2560 CL cases throughout Jordan between 1994 and 2014 (Al-Abdallat et al. 2015; Hijjawi et al. 2016).

The incidence rate of CL has increased in Syria during the last 20 years from 12,027 cases in 1997 to 58,156 cases in 2011 and 2012 (Haddad et al. 2015). Moreover, the incidence rate jumped from 41,000 cases to 53,000 cases in the first two quarters of 2013 (Hayani et al. 2015). It has been reported that *L. tropica* is the main recovered species from CL Syrian patients which is anthroponotically transmitted (ACL) (Reithinger et al. 2003). ACL is still endemic in its traditional home of Aleppo, but was observed also in Edlib, Lattakia, Tortous, Hama, and the city of Damascus (Jalouk et al. 2007). CL caused by *L. tropica* represents about 90% of all CL cases and is regarded as one of the most important public health problems in Syria, especially in Aleppo (Mosleh et al. 2008). CL caused by *L. major* is less common in Syria and occurs in rural areas close to Hama, Idleb, and Homs governorates (Al-Nahhas and Kaldas 2013). Recently, due to the massive population migrations caused by the ongoing Syrian crisis, new CL outbreaks have been reported in countries bordering Syria including Turkey, Iraq, and Lebanon (Hayani et al. 2015).

Diagnosis of leishmaniasis is based mainly on clinical presentation, microscopic examination, and parasite culture (Reithinger et al. 2007). In Jordan, microscopic examination of Giemsa-stained parasites from CL scrapings from skin lesions is the most commonly used and applied diagnostic technique in the country. However, recently PCR-based methods were tested in two studies for their reliability to identify *Leishmania* species in Jordan (Mosleh et al. 2015; Hijjawi et al. 2016). Molecular identification of *Leishmania* species has become a reliable and sensitive technology (Swick 2012) where several PCR amplification methods are thought to be highly efficient techniques for the identification of *Leishmania* species. Among these PCR-based assays, ITS1-PCR-RFLP, Nested ITS1–5.8S rDNA PCR, and Kinetoplast DNA PCR followed by gene sequencing are frequently used. Several studies indicated that ITS1-PCR (which is the sequence between the 18S rRNA and 5.8S rRNA genes) is considered to be one of the most sensitive technique which is used for the detection and discrimination of the infecting *Leishmania* species (*L. major* and *L. tropica*) (Schönian et al. 2003; Schnur et al. 2004; Bensoussan et al. 2006; Parvizi et al. 2008; El-Beshbishy et al. 2013; Es-Sette et al. 2014; Mouttaki et al. 2014; Mosleh et al. 2015).

In the present study, three molecular typing techniques (ITS1-PCR-RFLP, the nested ITS1-5.8S rDNA gene PCR, and Kinetoplast DNA-PCR-RFLP) were validated for their ability to specifically type *Leishmania* isolates which were collected from Jordanian residents and Syrian refugees in Jordan. Thereafter, sequencing and phylogenetic tree analysis were applied to analyze the heterogeneity between the isolates for both Syrian and Jordanian CL patients.

Materials and methods

Sample collection

The study participants from Jordan were suspected CL patients; specimens were collected from patients who were admitted to the Ministry of Health in several clinics among Jordan cities. For Syrian refugees, specimens were collected in Al-Azraq Camp from patients who presented with suspected CL lesions acquired in several Syrian cities before they arrived to the camp. All patients signed their informed consent to participate in the study, which was reviewed by the Institutional Review Board and approved by the Research Ethics Committee at Hashemite University and the Ministry

of Health. A special approval from the Ministry of Interior was necessary to obtain a limited pass to access AL-Azraq camp. A total of 66 patients thought to be infected with CL based on clinical examination only (i.e., size, number, location, and type of lesion) were recruited in the present study. Demographic data from the recruited patients were collected after filling a designed questionnaire and thereafter, skin scrapings were individually saved on NucleoCard® (Macherey-Nagel, Germany) from CL suspected lesions.

DNA extraction from NucleoCard®

DNA extraction from the NucleoCard® was performed as described in the QIAamp® DNA Mini kit (QIAGEN, USA) protocol and the resultant DNA was quantified with NanoDrop ND-100 spectrophotometer.

Leishmania genotyping

The first step of genotyping was carried out to confirm that the lesions were caused by *Leishmania* parasites. For this purpose, ITS1 PCR-RFLP was performed. To enhance and confirm the sensitivity of the results, second PCR was applied which is the nested PCR of ITS1-5.8S rDNA gene region. Finally, RFLP analysis of the Kinetoplast minicircle DNA was performed for the characterization of isolates infected with *L. tropica*.

ITS1 PCR of *Leishmania* isolates

The extracted DNA was identified by PCR amplification of the *Leishmania*-specific ribosomal ITS1 region using L5.8S (5′-TGATACCACTTATCGCACTT-3′) and LITSR (5′-CTGGATCATTTTCCGATG-3′) primers followed by RFLP analysis. Two microliters of 15 ng/μl DNA was used for ITS1 gene amplification in a 20 μl total reaction volume. Four microliters of master mix (0.4 M Tris-HCl, 0.1 M $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 1 mM dNTPs, blue dye and yellow dye) were added to the reaction with 0.5 μl of 10 μM (L5.8S and LITSR) primers and 13 μl of nuclease free water were added to get 20 μl final volume, the cycling conditions were 95 °C for 12 min followed by 44 amplification cycles, each consisting of three steps: 94 °C for 20 s, 53 °C for 30 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 6 min. All PCR assays were performed on thermal cycler (Bio-Rad C1000 Touch™, USA). PCR amplicons were analyzed by 2.0% agarose gel electrophoresis. Five microliters of PCR products were separated by electrophoresis at 100 V in 1X TBE buffer and compared to a standard 50 bp DNA ladder.

ITS1-PCR-RFLP analysis

Ten microliters of the PCR products were digested with 2 μl of the 10 u/μl restriction endonuclease (*HaeIII*) in 2 μl Restriction Enzyme 10× Buffer (Promega, USA) (1× of buffer consist of 10 mM Tris-HCl (pH 7.4), 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mg/ml BSA, 50% glycerol) and 18 μl nuclease free water. Digestion was performed in a thermal cycler, in a total reaction volume of 32 μl, with the following conditions: 6 h at 37 °C followed by 20 min at 80 °C. Ten microliters of the PCR product were run on 3.0% agarose gel in 1× TBE for 1 h. The obtained bands were compared to a 50 bp DNA ladder.

Nested PCR of ITS1-5.8S rDNA genes of *Leishmania* isolates

The extracted DNA was further screened by targeting the ITS1-5.8S rDNA gene region. This step consisted of two stages of amplification: The first was performed using the forward IR1 (5′-GCTGTAGGTGAACCTGCAGC AGCTGGATCATT-3′) and the reverse IR2 (5′-GCGG GTAGTCCTGCCAAACACTCAGGTCTG-3′) primers, while the second was performed with the nested-forward ITS1F (5′-GCAGCTGGATCATTTTCC-3′) and the nested-reverse ITS2R4 (5′-ATATGCAGAAGAGAGGAGGC-3′) primers. In the first stage of amplification, a total reaction volume of 20 μl was prepared of 4 μl of master mix (0.4 M Tris-HCl, 0.1 M $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 1 mM dNTPs, blue dye and yellow dye), 1 μl of 10 μM IR1 and 1 μl of 10 μM IR2 primers, and 2 μl of the 15 ng/μl DNA. PCR was performed under the following conditions: initial denaturation at 95 °C for 12 min followed by 39 cycles consisting of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 90 s. The extension step was further continued for 10 min after the last cycle. In the second stage, the products obtained from the first PCR were further amplified by the nested-PCR technique. A total reaction volume of 20 μl was prepared consisting of 4 μl of master mix (0.4 M Tris-HCl, 0.1 M $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 1 mM dNTPs, blue dye and yellow dye), 1 μl of 10 μM ITS1F and 1 μl of 10 μM ITS2R4 primers, 2 μl of the previously obtained PCR product, and 13 μl of nuclease free water. PCR was performed under the same conditions as the first stage of amplification. The obtained bands were subjected to electrophoresis on a 1.5% agarose gel in 1× TBE buffer and compared to a 100 bp ladder.

RFLP analysis of the kinetoplast minicircle DNA (kDNA)

The extracted DNA was screened by kDNA minicircle for the *L. tropica* using the primer pair Uni 21 (5-GGGGTTGG TGTAAAATAGGCC-3′) and Lmj4 (5-CTAGTTTC CCGCCTCCGAG-3′). A total reaction volume of 20 μl was

prepared of 4 μl of master mix (0.4 M Tris-HCl, 0.1 M $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 1 mM dNTPs, blue dye and yellow dye), 0.5 μl of 10 μM (Uni 21 and Lmj4) primers, 2 μl of 15 ng/μl DNA, and 13 μl of nuclease free water. The cycling conditions were 94 °C for 4 min followed by 35 amplification cycles, each consisting of three steps: 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The PCR products were digested with endonuclease *Rsa1*. Six microliters of the PCR products were digested with 1 μl of the restriction endonuclease (*Rsa1*) in 2.5 μl of 10× of Restriction Enzyme 10× Buffer (Promega, USA) (1× of buffer consist of 50 mM Potassium Acetate, 20 mM Tris-acetate, 10 mM Magnesium Acetate, and 100 μg/ml BSA, pH 7.9 at 25 °C). Digestion was performed in a thermal cycler, in a total reaction volume of 9.5 μl, with the following conditions: 3 h at 37 °C. 9.5 μl of the PCR product were run on 3.0% agarose gel in 1× TBE for 1 h. The obtained bands were compared to 100 bp DNA ladder.

DNA sequencing reaction

The obtained PCR products of both PCR reactions, targeting the entire ITS1 and the ITS1-5.8S rDNA gene regions, were purified using ExoSAP-IT (Thermo Fisher Scientific, USA). PCR products were sequenced using the ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem, USA), and PCR products were sequenced by 3730xl DNA Analyzer (Life Technologies, USA).

Sequence analysis and phylogenetic tree

Sequences obtained were analyzed on CLC Main Workbench 7 (CLC BIO, Denmark) software. Sequences were aligned, and a phylogenetic tree was constructed using the Neighbor-Joining algorithm in CLC Main Workbench 7 and bootstrap values for 1000 replicates were indicated.

DNA quality assessment

For DNA quality, actin amplification was performed under the following conditions: A total reaction volume of 20 μL was prepared consisting of 4 μl of master mix (0.4 M Tris-HCl, 0.1 M $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 1 mM dNTPs, blue dye and yellow dye), 1 μl of 10 μM forward (5′-CGC TGC GCT GGT CGT CGA CA-3′) and 1 μl of 10 μM reverse (5′-GTC ACG CAC GAT TTC CCG CT-3′) primers, 2 μl of DNA, and 13 μl of nuclease free water; the reaction amplified under the following cycling conditions: initial denaturation at 95 °C for 12 min followed by 35 cycles consisting of denaturation at 94 °C for 20 s, annealing at 56 °C for 30 s, and extension at 72 °C for 1 min. Followed by a final extension step of 72 °C for 10 min, 5 μL of the obtained PCR products were loaded on a 2.0% agarose gel and compared to a 50 bp DNA ladder.

Results

Sampling sites and demographic data

Sixty-six samples were collected from suspected CL patients; 27 Syrian refugees and 39 Jordanian (Tables 1 and 2) (Fig. 1). The number of skin lesions which were observed in the 66 recruited patients ranged from 1 to 15 lesions in Syrian patients and 1 to 6 lesions in Jordanian patients; most of the inspected lesions appeared as papules or ulcers (Tables 3 and 4). Among the Jordanian patients, 66.7% of them were males and 33.3% were females; however, among the Syrian patients, 59% were males and 41% were females. In this regard, sex ratios showed no significant difference between Jordanian and Syrian patients at the chi-square test ($p = 0.7$).

DNA extraction

The extracted DNA concentrations ranged from 7.3 to 30.7 ng/μl. DNA extraction was successful, and PCR was positive only for 29 (44%) of the spotted skin scrapings samples; therefore, 37 (56%) of the spotted skin scrapings samples were negative.

ITS1 and kDNA PCR of *Leishmania* isolates

LITSR and L5.8S primers were used to detect the ITS1 gene, after performing PCR on all isolates; 29 out of 66 isolates (44%) produced a band at around 350 bp which confirmed the presence of *Leishmania* species. Also, Lmj4 and Uni 21 primers were used for the identification of *Leishmania* species; 22 out of 66 isolates (33.3%) produced clear bands; 14 with a 650 bp band for *L. major* and 8 with an 800 bp band for *L. tropica* (Tables 1 and 2).

ITS1-PCR-RFLP analysis

RFLP analysis of the 29 PCR positive clinical specimens with the endonuclease *HaeIII* restriction enzyme identified 20 (69%) samples infected with *L. major* consisting of two bands 203 bp and 140 bp, and 9 (31%) were infected with *L. tropica* consisting of two bands 185 bp and 60 bp (Tables 1 and 2) (Fig. 5a).

Characterization of *L. tropica* PCR-RFLP analysis of kDNA

Seven of the eight *L. tropica* isolates were further analyzed by RFLP. Digestion with *Rsa1* produced different kDNA RFLP profiles which consisted of two basic kinds: *Ltro*-kD1 and *Ltro*-kD2, which can be differentiated mainly by the presence of a 417 bp component only in the *L. tro*-kD2 profiles (Tables 1 and 2) (Fig. 5b, c).

Table 1 List of *L. major* isolates, their origin, and their results using different PCR methods

Seq. no.	Actin PCR	ITS1 PCR	Nested PCR	RFLP HaeIII	Lmj4/uni21 PCR	Geographical origin of patient
1	+	+*	NA	+	+	Jordan-Sweimeh
2	+	+*	+*	+	–	Jordan-Dead Sea
3	+	+*	NA	+	+	Jordan-Alkraimah
4	+	+*	+*	+	–	Jordan-Ghor Alsaffe
5	+	+*	+*	+	+	Jordan-Al-mashare'a
6	+	+*	+*	+	+	Syria-Homs
7	+	+*	+*	+	–	Syria-Homs
8	+	+*	+*	+	+	Syria-Homs
9	+	+*	NA	+	–	Jordan-Jarash
10	+	+*	+*	+	+	Jordan-Alkraimah
11	+	+*	NA	+	+	Jordan-South Shouna
12	+	+*	+*	+	+	Jordan-Sahab
13	+	+	NA	+	+	Jordan-South Shouna
14	+	+	NA	+	–	Syria-Homs
15	+	+	NA	+	+	Syria-Homs
16	+	+	NA	+	–	Syria-Aleppo
17	+	+	NA	+	+	Jordan-Al-mashare'a
18	+	+	NA	+	+	Jordan-Al-mashare'a
19	+	+	NA	+	+	Jordan- South Shouna
20	+	+	NA	+	+	Jordan-Al-mashare'a

*Sequencing was done for some selected ITS1 PCR positive isolates

*Sequencing was done for all nested-PCR positive isolates

NA not applicable

Nested PCR for amplifying the ITS1-5.8S rDNA gene of *Leishmania* isolates

Nested PCR amplification was used for the 7 *L. tropica* and 8 *L. major* isolates that were sequenced. The first PCR reaction using the IR1 and IRs primers followed by the second primers ITS1F and ITS2R4. The nested PCR reaction produced bands from 400 to 477 bp (Tables 1 and 2) (Fig. 5d).

Sequencing and phylogenetic tree analysis of ITS1-5.8S rDNA gene

The results showed several polymorphisms and variations among the 7 *L. tropica* sequences with the presence of single point mutations. Analysis of the ITS1-5.8S rDNA gene sequences further confirmed that they all belonged to *L. tropica*. The phylogenetic tree showed two different

Table 2 List of *L.tropica* isolates, their origin, and their results using different PCR methods

Seq. no.	Actin PCR	ITS1 PCR	Nested PCR	RFLP *HaeIII*	Lmj4/uni21 PCR	(Rsa1) RFLP	Geographical origin of patient
26	+	+	+*	+	+	Ltro-kD2	Jordan-Dair alla
27	+	+	+*	+	+	Ltro-kD1	Jordan-Ajloun
28	+	+	+*	+	+	Ltro-kD1	Jordan-Zarqa
29	+	+	–	+	+	Ltro-kD1	Jordan-Karak
30	–	+	+*	+	+	Ltro-kD2	Jordan-Amman
31	+	+	+*	+	+	Ltro-kD2	Syria-Homs
32	–	+	+*	+	–	–	Jordan-Alkraimah
33	+	+	+*	+	+	Ltro-kD2	Syria-Aleppo
34	+	+	–	+	+	–	Syria-Homs

*Sequencing was done for all nested-PCR positive isolates

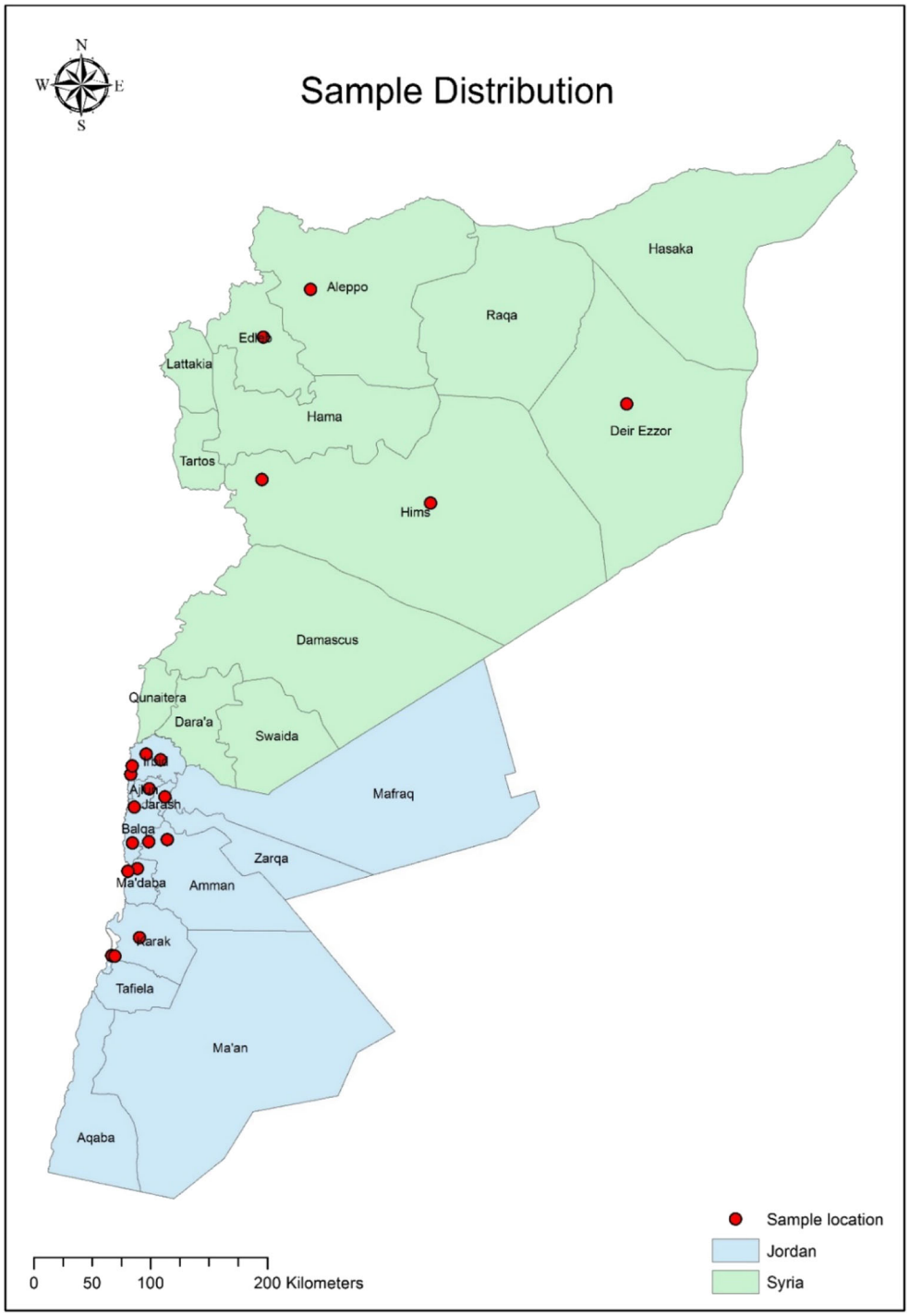

Fig. 1 Map of Jordan and Syria, showing the foci of the 66 suspected CL cases and their distribution; the red dots represent the sample location

clades. These clades included the following: (clade I) *L. tropica* (no. 27) sequence, which was isolated from a Jordanian patient from the North of Jordan which showed similarity with the Syrian isolates references extracted from NCBI BLAST (KT363779, KT363778, KT363785, and KT363786) (Salloum et al. 2016) and (clade II) *L. tropica* (no. 26, no. 30, no. 28, no. 32, no. 33, and no. 31) sequences; all these isolates showed significant similarities with few point mutations. Four isolates were collected from the middle part of Jordan and the other two isolates collected from Syria (Fig. 2). The phylogenetic tree showed two different clades for *L. major* which were also identified using of ITS1-5.8S rDNA nested PCR: (clade I) sequences, *L. major* (no. 8 and no. 6) which are Syrian isolates and (clade II) sequences, *L. major* (no. 7, Syrian isolate), and (no. 12, no. 2, no. 4, no. 5,

Table 3 Major characteristics of the study participants (Syrian) ($N = 27$)

Characteristic (*N*)					
Gender	M (16) 59%	F (11) 41%			
Age (years)	3–62 years	Average 20.11	Median 13.0	Mode 9	
Nationality	Syrian (27)				
Infected body parts	Facial (20)	Upper Limbs (15)	Lower Limbs (15)	Trunk (2)	
Stage of infection	Papule (20)	Ulcerated (6)	Cured (1)		
Number of lesions	Average 2.0	Minimum 1	Maximum 15		
Type of diagnosis	Clinical (27)	Laboratory (0)			
PCR results (*Leishmania* species)	*L. major* (6)	*L. tropica* (3)	No result (18)		
Jordan Border Entry Date	From (1/1/2016)	To (1/5/2016)			
Origin of infection	Aleppo (4)	Homs (18)	Palmyra (3)	Deirez-Zor (1)	Idlib (1)
Treatment Pentostam injection	Yes (27)	NO (0)			

N, number; *M*, male; *F*, female

and no. 10, Jordanian isolates) which every clade showed significant similarities but with several point mutations and described for *L. tropica* above (Fig. 3).

Sequencing and phylogenetic tree analysis of ITS1 gene for *L. major*

Leishmania major sequences grouped into similar clusters as for the ITS1-5.8S rDNA-nested PCR. The phylogenetic tree showed two different clades. These two clades included (clade I) sequences, *L. major* (no. 7, no. 2, no. 4, no. 5, no. 12, no. 10, no. 3, and no. 11) and (clade II) sequences, *L. major* (no. 1, no. 9, no. 6, and no. 8) which every clade showed significant similarity but with several point mutations (Fig. 4).

Discussion

Due to the political instability in Syria, millions of Syrian refugees who might be infected with CL entered the neighboring countries of Lebanon, Turkey, and Jordan. In Jordan, there are about 1.4 million Syrian refugees; only 20% of them are living in refugee camps while the other 80% reside within the Jordanian population in major cities (Ozaras et al. 2016). The establishment of Syrian refugee camps possesses a high risk for the introduction of leishmaniasis into areas which were free of the infection and mainly the ACL caused by *L. tropica* since it is the predominant species in Syria. In Jordan, CL is believed to be zoonotic in its transmission (ZCL) and is mostly caused by *L. major*; however, *L. tropica* is also present in some areas in the North of

Table 4 Major characteristics of the study participants (Jordanian resident) ($N = 39$)

Characteristic (*N*)				
Gender	M (26) 66.7%	F (13) 33.3%		
Age (years)	1–63	Average 29.3	Median 28.0	Mode 26.0
Nationality	Jordanian (32)	Other (7)		
Infected body parts	Facial (15)	Upper Limbs (27)	Lower Limbs (18)	Trunk (1)
Stage of infection	Cured (2)	Papule (17)	Ulcerated (20)	
Number of lesions	Average 1.56	Minimum 1	Maximum 6	
Type of diagnosis	Clinical (37)	Laboratory (2)		
PCR results (*Leishmania* species)	*L. major* (14)	*L. tropica* (6)	No result (19)	
Treatment	Yes (10)	No (29)*		
Origin of infection	South Shouna (6)	Al-mashare'a (4)	Amman (2)	Other (6)

N, number; *M*, male; *F*, female

*The samples collected from the patients before receiving any treatment

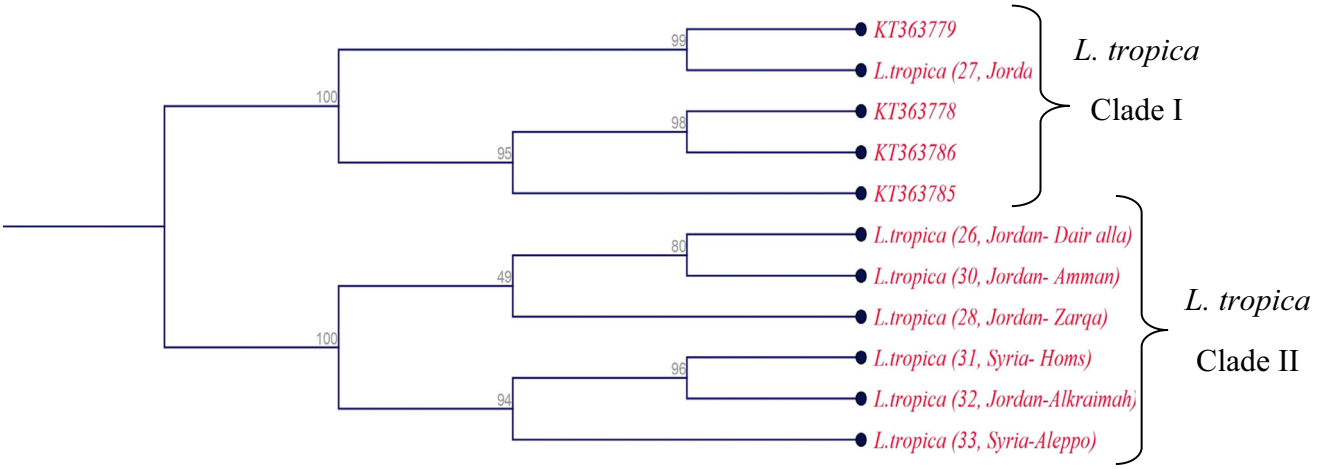

Fig. 2 Neighbor-joining tree showing the relationships of the L. tropica isolates based on the ITS1-5.8S rDNA gene sequences, using the CLC Main Workbench 7. Bootstrap values are based on 1000 replicates

Jordan but at a lower frequency than *L. major* (Saliba et al. 1988; Kamhawi et al. 1995a, b; Jumaian et al. 1998; Kamhawi et al. 2000; Postigo 2010; Mosleh et al. 2015; Hijjawi et al. 2016). As ZCL is currently believed to be the main form of CL in Jordan as reported earlier in previous many studies, the possibility for the introduction of *L. tropica* causing (ACL) into areas surrounding the Syrian refugee camps would provide a route for ACL disease transmission among Syrian and Jordanian populations.

Worldwide, there are several methods which are currently used for the diagnosis of CL; these include direct parasite detection by microscopic examination of Giemsa-stained smears prepared from the suspected lesions. Other indirect testing methods with serology and molecular-based assays (PCR) for detecting the parasite DNA is also available (Motazedian et al. 2002; de Vries et al. 2015). The molecular-based assays are more sensitive to diagnose CL than microscopic examination and parasite culture, but their use remains restricted to referral hospitals and research centers (Elmahallawy et al. 2014). Recently, two studies evaluated the use of PCR-RFLP analysis for the confirmation of CL diagnosis and for the discrimination of the infecting *Leishmania* species in Jordan (Mosleh et al. 2015; Hijjawi et al. 2016). The use of PCR-based assays are valuable in epidemiological studies where the identification and the understanding of the distribution of different *Leishmania* species is regarded as a prerequisite for designing appropriate control measures for combating the spread of the disease (Mosleh et al. 2015; Hijjawi et al. 2016). The present study was conducted in order to optimize and validate a sensitive diagnostic PCR method for the diagnosis of CL and species discrimination as well as to investigate whether the Syrian crises and the flow of refugees could influence the epidemiology and the transmission of CL in Jordan. Therefore, three molecular techniques included: ITS1 PCR-RFLP, kDNA minicircle PCR-RFLP, and nested ITS1-5.8S rDNA gene PCR were used followed by phylogenetic tree analysis to study the evolutionary relationships among various *Leishmania* species.

DNA extraction from *Leishmania*-suspected skin scraping spotted on NucleoCard® was performed based on an optimized protocol and few ng of *Leishmania* parasites DNA was recovered (Boggild et al. 2010; Hijjawi et al. 2016).

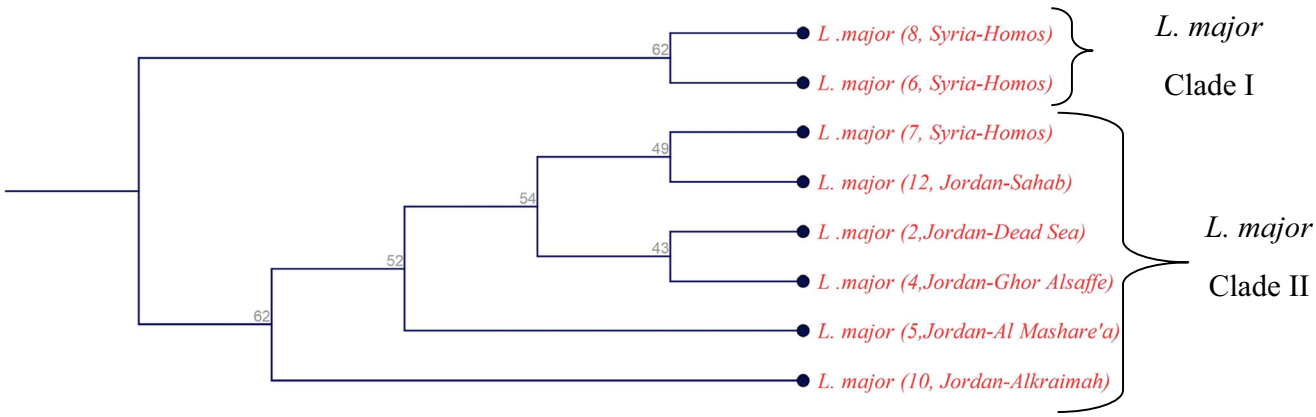

Fig. 3 Neighbor-joining tree showing the relationships of the L. major isolates based on the ITS1-5.8S rDNA gene sequences, using the CLC Main Workbench 7. Bootstrap values are based on 1000 replicates

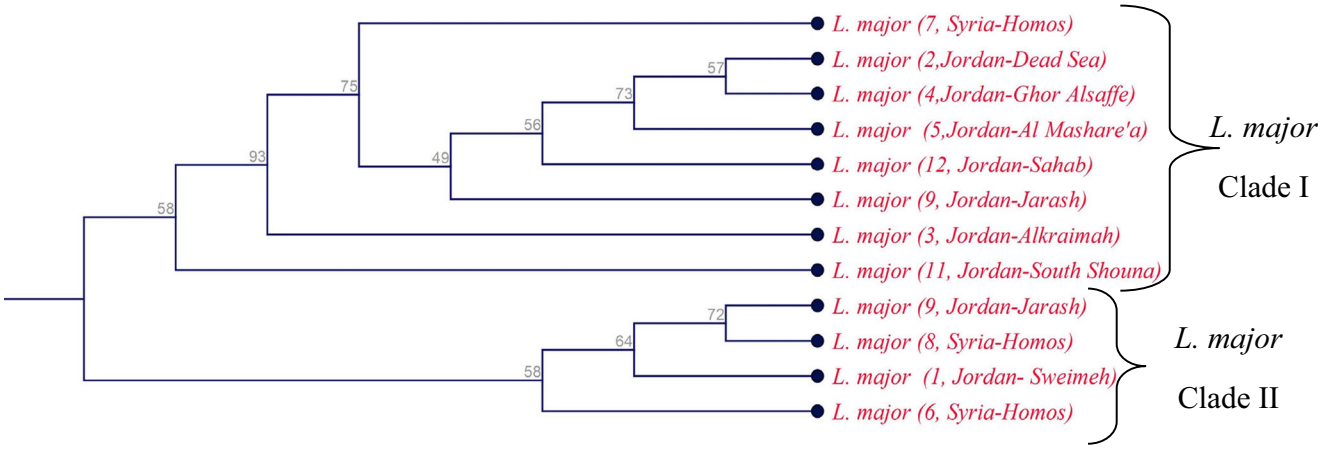

Fig. 4 Neighbor-joining tree showing the relationships of the L. major isolates based on the ITS1 gene sequences, using the CLC Main Workbench 7. Bootstrap values are based on 1000 replicates

DNA was successfully extracted from 29 out of 66 samples and these were positive by PCR. The extraction failed for the rest of the samples (37) and this might be attributed to two main factors: first is the low load of the *Leishmania* parasite spotted on the NucleoCard® which in turn resulted in the recovery of low concentration of extracted DNA (< 5 ng/㎕) and second is the early treatment of the suspected patients with (Pentostam). In clinics all over Jordan and in refugee camps, CL is directly treated when the papule is clinically suspected to be CL. Furthermore, the low DNA concentration and its quality inversely affect the sequence step which might in part explain the limited number of isolates which were sequenced for both *L. major* and *L. tropica*.

The ITS1 sequence (300–350 bp depending on the species) was chosen in the present study as the target for PCR assay of *Leishmania* species and found to be highly sensitive and specific in detecting samples that have *Leishmania* (Talmi-Frank et al. 2010; Alvar et al. 2012). Previously, several studies performed the ITS1 region amplification technique for the discrimination of *Leishmania* parasites (Monroy-ostria et al. 2014; Amro et al. 2012), as recent studies have shown that ITS1-PCR followed by restriction fragment length polymorphism analysis is a suitable tool for diagnosing and identifying *Leishmania* species (Schönian et al. 2003; Bensoussan et al. 2006). The major advantage of ITS1-PCR is that species identification can be achieved by digesting the PCR product by (*Hae III*) restriction enzyme and this is sufficient to distinguish almost all *Leishmania* species (Teixeira et al. 2013; Tsokana et al. 2014) (Fig. 5).

In the present study, it was found that most CL is caused by *L. major* (20 cases) in a mixture of nationalities of Jordanians (14) and Syrians (6). There were (9) cases of CL that were caused by *L. tropica*; (3) of them were Syrians and the other (6) were Jordanians. Although the ITS1 region serves as a marker for the differentiation of *Leishmania* at both the species and the strain levels, only few studies employed ITS1 sequence analysis to compare *L. tropica* and *L. major* isolates (Jafari et al. 2013; Ajaoud et al. 2013; Guerbouj et al. 2014; Doroodgar et al. 2015). *Leishmania tropica* is known to be a very heterogeneous species (Schwenkenbecher et al. 2006; Azmi et al. 2012). Nested ITS1-5.8S rDNA gene PCR enhanced the sensitivity of the differentiation tool by targeting two fragments in the ITS1-rDNA region one consisting of ITS1 and the other fragment in the 5.8S rDNA gene (Parvizi et al. 2008). Some studies suggest that the nested PCR of ITS1-5.8S rDNA gene is sensitive for *L. tropica* identification (Noyes et al. 1998); however, the identification for *Leishmania* species using ITS1 gene showed higher sensitivity than the ITS1-5.8S rDNA gene. One sample (no. 27) of *L. tropica* in (clade I) which was collected from the North of Jordan showed high similarity with *L. tropica* sample isolated from a Syrian patient who resided in Lebanon refugee camp (KT363779) on GenBank with 98% identity; therefore, indicating the probability of the introduction of new strains of *L. tropica* from Syria to Jordan. (clade II) sequences showed high similarity for all isolates, (no. 26, no. 28, no. 30, and no. 32) of *L. tropica* which were collected from the middle part of Jordan and similar to two isolates which were collected from Syrian refugees, and the similarities found in this study could be a base to have additional studies to proof if there is an emergence of leishmaniasis introduced by Syrian refugees. Furthermore, the establishment of a regional databases for cases of CL should be created for a better epidemiological assessment of these infectious agents and for tracing their patterns of migration between Syria and Jordan and surrounding countries. Sequencing based on the ITS1 gene was used for the analysis and genotypic variations of *L. major* since this method showed high sensitivity for the identification of *L. major* and to study the molecular variation between *L. major* isolates (Hajjaran et al. 2013; Bordbar and Parvizi 2014). In this study, *L. major* ITS1 sequencing showed differences between clade I and clade II. Seven Jordanian sequences

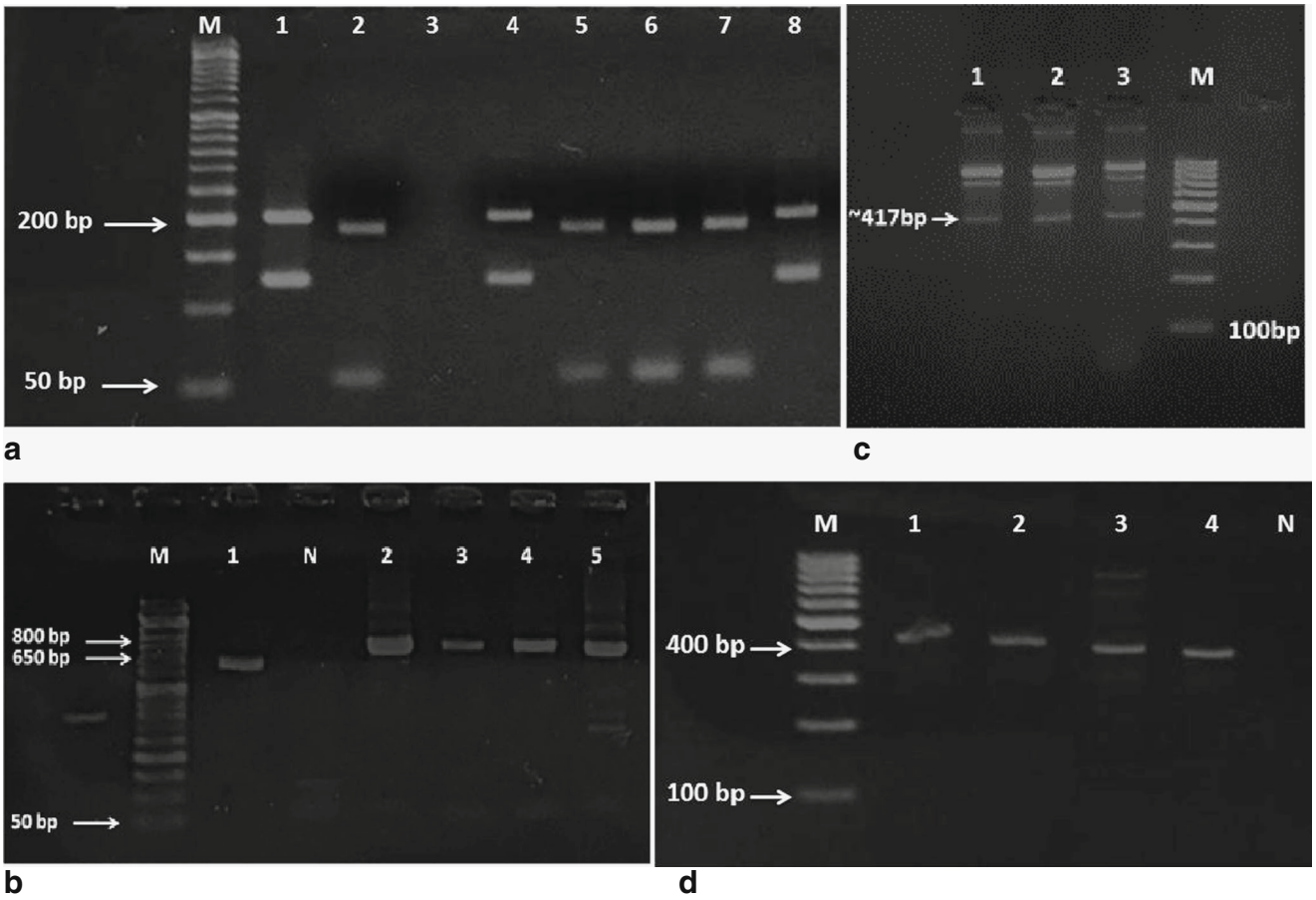

Fig. 5 **a** Representative picture showing agarose gel electrophoresis (3%) of random RFLP results which were extracted from the positive Leishmania samples. Lane M: 50 bp DNA ladder. Lanes 1–8 showing the digestion of amplified ITS1 regions for different Leishmania species with the restriction endonuclease HaeIII. Lane 3: negative control. Lane 1: L. major positive control showing two bands (203 bp and 140 bp). Lane 2: L. tropica positive control showing three bands (185 bp, 60 bp). Lanes 4 and 8: random samples for L. major detected in clinical samples. Lanes 5, 6, and 7: L. tropica detected in clinical samples. **b** The kDNA PCR results: Lane 2 L. tropica positive control, lane 3, 4, and 5, L. tropica isolates (800 bp), Lane 1 L. major positive control (650 bp). M: molecular weight marker 50 bp C) The RFLP of kDNA PCR products digested with RsaI. M: molecular weight marker 1000 bp; Lanes 1, 2, and 3 L. tro-kD2. **d**) Nested-PCR of ITS1-5.8S rDNA gene of Leishmania DNA. Agarose gel electrophoresis of representative isolates positive for Leishmania produced a DNA band around 400 bp characteristic of L. tropica. M 100 bp DNA ladder was used as a molecular marker. N: Negative Control

showed high similarity with one Syrian sequence in clade I; (clade II) sequences included two Jordanian and two Syrian sequences with high similarity. ITS1 sequencing proved to be successful to confirm the identification of CL cases and to study the phylogenetic variations between *Lesihamnia* isolates. These results indicated that new isolates/clades of CL might be introduced and imported into areas surrounding the refugee camps such as in Al-Azraq, and possible outbreaks might occur in areas which were previously free from CL in Jordan.

Furthermore, to confirm the ITS1 sequencing, ITS1-5.8S rDNA sequencing was applied on *L. major* isolates, and the results showed high similarity to ITS1 sequencing. Amplification of the kDNA minicircle sequence by using Lmj4 and Uni 21 primers successfully identified the two *Lesihamnia* species (*L. tropica* and *L. major*) except for five samples who showed negative results which make ITS1 more sensitive than the kDNA gene. The RFLP analysis of the PCR products of the 7 *L. tropica* isolates after their digestion with *RsaI* enabled their separation into the kDNA groups L.tro-kD1 and L.tro-kD2, respectively. The analysis of the kDNA RFLP profiles also exposed a further level of micro-heterogeneity, and the need for more studies to identify the *L. tropica* kDNA groups is recommended in Jordan (De Almeida et al. 2011; Rouhani et al. 2014).

Since only small number of samples (66 skin scrapings) were collected from CL lesions (39 samples from Jordanian CL patient and 27 from Syrian CL patients), therefore, the results obtained from the present study can be regarded as a preliminary investigation since larger number of CL cases are required to be analyzed to come to a clear conclusion about the possible influence of Syrian refugees on CL epidemiology and transmission dynamics. The low number of cases collected during the present study was due to limitations in time and

accessibility to the dermatology clinic inside the refugee camp to obtain samples from the CL-infected Syrian refugees. Moreover, the early treatment of CL cases makes it difficult to recover the parasite DNA in the collected samples. Therefore, an urgent need for more comprehensive studies in order to understand the molecular epidemiology of CL in Jordan and to see if new *L. tropica* strains (causing ACL) from Syria are being introduced into Jordan upon the influx of thousands of Syrian refugees due to the current political disturbance in their country.

In the present study, males were observed to be more infected with CL than females, a fact which can be explained by their higher engagement with outdoor activities such as agriculture and shepherds which usually expose them to a higher risk to be bitten by sand flies than females who mostly stay at home. Furthermore, the number of lesions recovered from the Syrian patients, who acquire the infection in Syria before their migration to Jordan, were higher (1 to 15 lesions per person) compared to those observed among Jordanian patients (1–6 lesions per person). This indicates that the exposure to sand fly bites is more among the Syrians due to the poor control of the sand flies vector which could be due to the collapsed health care infrastructure and the population displacement from leishmaniasis endemic and non-endemic regions (Hayani et al. 2015; Du et al. 2016).

The prediction of future CL outbreaks in Jordan around the established Syrian refugee camps depend on many factors among them is the investigation of the density and distribution of *Phlebotomine* sand fly species (proven vector of *L. tropica* and *L. major*) around the refugee camps, which has never been investigated in the last few years. The last study to identify the different sand flies in Jordan was done 23 years ago by Kamhawi et al. 1995a, b. Therefore, future studies to clarify the current species of sand flies as well as the possible reservoir host/s for the parasite all over Jordan and mainly around the refugee camps is urgently required.

Conclusion

In conclusion, the use of ITS1-PCR RFLP and ITS1-5.8S rDNA gene PCR enabled the accurate detection and identification of *L. tropica* in clinical samples spotted on NucleoCard®. ITS1-5.8S rDNA gene PCR proved to be a more sensitive method for *Leishmania tropica* discrimination. ITS1-PCR RFLP showed high sensitivity to identify *Leishmania* species and highly sensitive for *L. major* characterization. kDNA minicircle gene showed less sensitivity compared to the methods which were mentioned before. The obtained results highlighted the need to find a universally accepted diagnostic tool for *Leishmania* typing, that is specific, sensitive, rapid, and capable of identifying all clinically significant *Leishmania* species. Also, it was demonstrated that *L. tropica* and *L. major* are the causative agents of CL observed among Syrian and Jordanian populations. The living conditions in refugee's camps and collective shelters are characterized by poor sanitary conditions and inadequate waste disposal, thus increasing the risk of *Leishmania* transmission through the sand fly vector. The risk of CL transmission mainly ACL into the Jordanian community is high. Therefore, the present study provides a pertinent contribution and brings attention to a clear risk to Jordan from *Leishmania* infections from Syrian refugees. Thus, effective prevention methods and appropriate therapy is critical. Prevention can be as simple as using nets treated with insecticide or spraying insecticides to kill sand fly vectors (Al-Dakhil et al. 2017).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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