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Detection, molecular typing and phylogenetic analysis of *Leishmania* isolated from cases of leishmaniasis among Syrian refugees in Lebanon

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ABSTRACT

Leishmania is a parasitic protozoan with more than two-dozen species causing the disease leishmaniasis. It is transmitted to humans through the bite of an infected female phlebotomine sand-fly vector. In the past two years the incidence of leishmaniasis has been drastically increasing in Lebanon. This was in parallel with the deterioration of the security in Syria forcing thousands to flee and seek shelter in poorly maintained refugee camps and collective shelters. Cutaneous leishmaniasis (CL) is now considered a public health problem, but its epidemiology has not been fully elucidated. To our knowledge, this is the first study comparing two different molecular methods for the detection and identification of *Leishmania tropica* in Lebanon.
Two molecular typing methods of 39 FFPE *Leishmania* isolates were used: the ITS1-PCR RFLP and the nested ITS1-5.8S rDNA gene amplification followed by sequencing and phylogenetic analysis. The efficiency of these two techniques in *Leishmania* identification was compared and the phylogenetic relationships among these isolates were illustrated based on the neighbor-joining (NJ) method. The results were statistically correlated with the parasitic index (PI). The DNA storage in formalin-fixed paraffin embedded (FFPE) tissues was assessed as well. The parasites identified were all *L. tropica* as determined by both techniques. ITS1-5.8S rDNA gene based typing proved to be more sensitive in the detection of parasites (positive in 69.2% of the isolates) as opposed to the ITS1-PCR RFLP method that was successful in identifying *L. tropica* in only 43.6% of the isolates. Sequencing and phylogenetic analysis revealed high levels of heterogeneity. A statistically significant correlation was observed between PI and the results of the nested ITS1-5.8S rDNA gene PCR. Genotyping at the species level is essential for monitoring the relative frequency of CL in the Mediterranean area that is correlated to three different *Leishmania* species (*Leishmania infantum*, *Leishmania major* and *L. tropica*), each characterized by distinct epidemiological features. The obtained results highlight the need to find a universally accepted diagnostic tool for *Leishmania* typing.

1. Background

Leishmania is a digenetic parasitic protozoan of the *Leishmania* genus, family Trypanosomatidae and Kinetoplastida order (Ramos et al., 2013). *Leishmania* parasites are transmitted to humans by phlebotomine female sand fly vectors (Teixeira et al.,

Abbreviations: Bp, base pair; CL, cutaneous leishmaniasis; FFPE, formalin-fixed paraffin embedded; ITS, internal transcribed spacer; *L. tropica, Leishmania tropica*; MLST, multilocus sequence typing; NJ, neighbor-joining; PCR, polymerase chain reaction; PI, parasitic index; RFLP, restriction fragment length polymorphism

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2013) and are the causative agents of leishmaniasis. At least 21 species have showed to cause disease in humans (World Health Organization, 1990). The disease ranges from localized cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) to widespread visceral leishmaniasis (VL), also known as kala-azar which is fatal if left untreated (Tsukamaya et al., 2008; Zhang et al., 2013). CL is the most common with more than 95% of the cases occurring in the Americas, the Mediterranean basin, the Near East and Central Asia especially in Afghanistan, Algeria, Brazil, Colombia, Iran and Syria. In fact, leishmaniasis is sometimes referred to as "Aleppo boil" in the medical literature (Hayani et al., 2014). Although, an estimated of 0.7 to 1.3 million new cases of CL occur worldwide each year (World Health Organization, 2014), leishmaniasis is still considered one of the world's most neglected diseases (Ramos et al., 2013).

The crisis in the Syrian Arab Republic that started in March 2011 has resulted in outbreaks of several previously overlooked diseases (World Health Organization, 2013). Since then, cases of CL in Lebanon have been drastically increasing relative to the previous years (2001–2012) (Alawieh et al., 2014; Alvar et al., 2012). From 2004 to 2008 no CL cases were reported in Lebanon compared to 22,882 cases in Syria during the same period (World Health Organization, 2013). Recently, Saroufim et al. (2014) identified *Leishmania major* in 15% and *L. tropica* in 85% out of 948 Syrian refugee patients living in Lebanon, the latter species being endemic to the Aleppo region in Syria.

Since different *Leishmania* species have been shown to cause CL and due to population travel and migration, unexpected *Leishmania* species can appear in unexpected regions (Dujardin, 2006). Also, different species show different susceptibility to drugs (Blum et al., 2004) and primary and secondary resistance (Desjeux, 2004). The occurrence of natural interspecies hybrids and sexual recombination hinders species discrimination (Tojal da Silva et al., 2006; Nolder et al., 2007; Odiwuor et al., 2011a; Ravel et al., 2006).

Several PCR-based methods have been employed for typing *Leishmania* parasites. MLEE (Multilocus Enzyme Electrophoresis) is sometimes considered the gold standard for *Leishmania* identification (Bañuls et al., 2007; Schönian et al., 2001). DNA sequencing, PCR-RFLP (Restriction Fragment Length polymorphism) (Yehia et al., 2012) and MLST (Multilocus Sequence Typing) have all been employed for this purpose (da Silva et al., 2010; Montalvo et al., 2010; Maiden et al., 1998). In terms of sensitivity and validation, the ITS1 region offers the best resolution for *Leishmania* discrimination in the Old World (Odiwuor et al., 2011b). ITS1 is the sequence in between the 18S rRNA and 5.8S rRNA genes. It contains enough conservation to serve as a PCR target but sufficient polymorphisms to facilitate species typing and identification (Roelfsema et al., 2011). However, these PCR based methods are often hindered by low sensitivity results that fail to detect amastigotes in samples that proved to be positive by microscopy. This caveat in molecular typing is often due to a low parasite load in the original sample (Shahbazi et al., 2008).

In this study, the identification and typing of *Leishmania* isolates obtained from Syrian refugees in Lebanon using two molecular typing techniques: the ITS1-PCR-RFLP and the nested ITS1-5.8S rDNA gene PCR both followed by sequencing and phylogenetic tree analysis were conducted. The obtained results were used in comparing the efficiency of these two techniques in *Leishmania* identification and assessing the evolutionary and phylogenetic relationships among these isolates. The results were correlated with the parasitic index (PI). The DNA storage in formalin-fixed paraffin embedded (FFPE) tissues was assessed as well.

2. Methods

2.1. Ethical approval

This study was approved by the American University of Beirut Institutional Review Board and the patient data used in this study was anonymized.

2.2. Sample collection and description

A total of 39 FFPE blocks were obtained from the American University of Beirut Medical Center (AUBMC) in Lebanon. FFPE blocks contained skin biopsies from patients having histologically confirmed CL (PI ≥ 2) during the years of 2013–2014. Each patient had one biopsy performed, and one FFPE block per biopsy was available. Cases included in the study were restricted to cutaneous lesions of patients who did not receive treatment prior to the biopsy. Clinical information pertaining to the lesion was also collected including: number, duration, location and dermatologic appearance. In addition, the patient's age, gender and country of residency were tabulated. Organisms collected from cultures were used as positive controls to validate the analysis. IRB approval was granted prior to the initiation of this study (PALM I.K.01).

2.3. Histopathology

Sections from each of 39 FFPE tissue blocks were stained for hematoxylin and eosin, Giemsa, Acid Fast bacilli, Gomori Methylamine Silver and Periodic Acid-Schiff. All cases were reviewed by four pathologists (JS, RK, FF and IK) and classified according to the modified Ridley's parasitic index (PI) (Table 1). The correlation between the PI, the duration of the lesion, the age of the patients and the results of the ITS1-PCR-RFLP and the nested ITS1-5.8S rDNA gene PCR was performed using the Pearson's correlation test.

Table 1
Modified Ridley's parasitic index (Ridley and Ridley, 1983).

1+	1 or more amastigotes per standard section
2+	10 or more amastigotes per standard section
3+	100 or more amastigotes per standard section
4+	1000 or more amastigotes per standard section
5+	10,000 or more amastigotes per standard section
6+	100,000 or more amastigotes per standard section

2.4. DNA extraction

DNA extraction was performed as described in Yehia et al. (2012). The resultant DNA was quantified with NanoDrop ND-100 spectrophotometer and Qubit fluorometer (Thermo Fisher Scientific, USA) and stored at 4 °C.

2.5. ITS1 PCR of Leishmania isolates

The extracted DNA was examined for the *Leishmania*-specific ribosomal ITS1 region by polymerase chain reaction (PCR) amplification using L5.8S and LITSR primers (Table 2) followed by RFLP analysis. The volume of DNA to be amplified was calculated to normalize the quantity among all isolates. 5 μL of DNA was used for ITS1 gene amplification in a 50 μL total reaction volume. A master mix was prepared consisting of 0.2 mM dNTPs, 1 X Taq buffer, 1.5 mM $MgCl_2$, 500 nM of both the forward and the reverse primers and 2 U of Hot-Start Taq Gold DNA polymerase. The cycling conditions were 95 °C for 12 min followed by 32 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 a PerkinElmer GeneAmp 9700 thermal cycler (PerkinElmer, Wellesly, Massachusetts).

Nested PCR was performed on all isolates that were negative by conventional PCR. 10 μL of the ITS1 PCR amplicon were amplified in a 50 μL total reaction volume using the same primers and the same cycling conditions.

PCR amplicons were analyzed by 1.5% agarose gel electrophoresis (1.5% TAE gels stained with ethidium bromide). 25 μL of PCR products were electrophoresed at 90 V in 1X TAE buffer (400 mM Tris-acetate, 10 mM EDTA, pH: 8.2–8.4) and compared to a standard 100 bp DNA ladder. A DNA band of 300–350 bp was considered as a positive indicator for the presence of *Leishmania*.

2.6. RFLP

10 μL of the PCR products were digested with 2 μL of the restriction endonuclease BsuRI (*HaeIII*) in 2 μL of 10 X Buffer R (Thermo Fisher Scientific, USA) (1 X of buffer R consists of 10 mM Tris HCl (pH 8.5 at 37 °C), 10 mM $MgCl_2$, 100 mM KCl, 0.1 mg/mL BSA) and 18 μL H_2O. 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. 25 μL of the PCR product were run on 2.5% agarose gel in 1 X TAE (0.04 M Tris-acetate, 1 mM EDTA, pH = 8) for 1 h. The obtained bands were compared to 100 bp and 20 bp ladders.

2.7. Nested PCR of ITS1-5.8S rDNA genes of Leishmania isolates

The previously extracted DNA was further analyzed 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 and the reverse IR2 primers, while the second was performed with the nested-forward ITS1F and the nested-reverse ITS2R4 primers (Table 2). In the first stage of amplification: A total reaction volume of 20 μL was prepared of 1 X PCR reaction buffer, 1.5 mM of $MgCl_2$, 60 μM of each dNTP, 1 μM of IR1 and 1 μM of IR2 primers, 1 U of Taq polymerase and 2 μL of the template DNA. PCR was performed under the following conditions: 95 °C for 12 min followed by 39 cycles consisting of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 90 s. The extension step was further continued for 10 min after the last cycle.

Table 2
The primers used in this study, their corresponding sequences and target amplicon size.

Targeted gene	Primer sequences	Amplicon size
LITSR	5′-CTGGATCATTTTCCGATG-3′	300–350 bp
L5.8S	5′-TGATACCACTTATCGCACTT-3′	
IR1	5′-GCTGTAGGTGAACCTGCAGCAGCTGGATCATT-3′	320 bp
IR2	5′-GCGGGTAGTCCTGCCAAACACTCAGGTCTG-3′	
ITS1F	5′-GCAGCTGGATCATTTTC-C-3′	400 bp
ITS2R4	5′-ATATGCAGAAGAGAGGAGGC-3′	
Actin	5′-CGC TGC GCT GGT CGT CGA CA-3′	600 bp
	5′-GTC ACG CAC GAT TTC CCG CT-3′	
GAPDH	5′-TGGTGCTCAGTGTAGCCCAG-3′	110 bp
	5′-GGACCTGACCTGCCGTCTAG-3′	

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 1 X PCR reaction buffer, 1.5 mM of $MgCl_2$, 60 μM of each dNTP, 1 μM of ITS1F and 1 μM of ITS2R4 primers, 1 U of Taq polymerase and 2 μL of the previously obtained PCR product. Nested PCR was performed under the same conditions as the 1st stage of amplification. Bands were electrophoresed on a 1.5% agarose gel in 1 X TAE buffer and compared to a 100 bp ladder.

2.8. 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).

The amplicons were sequenced using the ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem, USA). Two sequencing reactions were performed for each isolate. The sequencing reaction consisted of the BigDye premix, 0.2 pmol of either forward or reverse primer, and the cleaned PCR product in a total volume of 10 μL. The same primers used in the PCR were used for sequencing (Table 2). All sequencing reactions were performed with 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. PCR products were sequenced by Genetic Analyzer 3500 (Life Technologies, USA) using the BigDye XTerminator purification kit (Applied Biosystems, USA).

2.9. DNA sequencing and phylogenetic analysis

Sequences obtained were analyzed on CLC Main Workbench v5.5 and deposited to GenBank under the accession numbers indicated below. Sequences were aligned using the multiple alignment program MEGA6 (available at http://www.megasoftware.net/) (Tamura et al., 2013) and Clustal Omega multiple sequence alignment program (available at http://www.clustal.org/omega/) (Sievers et al., 2011) with default parameters. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and were in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. Bootstrap replicates were performed to estimate the node reliability, and values were obtained from 1000 randomly selected samples of the aligned sequence data. Sequences obtained from both procedures were compared to seven entries retrieved from Genbank (Table 3).

2.10. DNA quality assessment

In order to assess the DNA quality of the extracted DNA, Actin and GAPDH amplification were performed under the following conditions: A total reaction volume of 50 μL was prepared consisting of 0.2 mM dNTPS, 1 X Taq buffer, 1.5 mM $MgCl_2$, 500 nM of both forward and reverse primers and 2 U of Taq DNA polymerase. For actin, 10 μL of DNA were amplified under the following cycling conditions: 95 °C for 12 min followed by 35 cycles consisting of 94 °C for 20 s, 56 °C for 30 s and 72 °C for 1 min. Followed by a final extension step of 72 °C for 10 min. For actin, 10 μL of DNA were amplified under the following cycling conditions: Initial denaturation at 95 °C for 12 min followed by 40 cycles consisting of 94 °C for 20 s, 60 °C for 30 s and 72 °C for 1 min. Followed by a final extension step of 72 °C for 10 min. 25 μL of the obtained PCR product were loaded on a 1.5% agarose gel and compared to a 100 bp and a 20 bp ladders.

3. Results

3.1. DNA extraction

Skin biopsies from 39 patients from Lebanon and Syria with clinical diagnosis of CL were assessed. The specimen was obtained by punch biopsy, and the duration of the lesions ranged from 1 to 12 months (mean: 4.51 ± 2.36; median: 4). Ages of patients varied from 1 to 83 years (mean: 25.08 ± 23.22; median: 16). 53.9% of patients were females and 46.1% were males. Most of the samples (38.5%) were from the 0–10 year's old age group followed by the above 60 years old age group (15.4%). The obtained DNA concentrations ranged from 8.5 ng/μL to 462.3 ng/μL (mean: 66.16 ± 99.62 ng/μL; median: 37.6 ng/μL).

Table 3
The sizes and G + C content of the reference sequences included in the phylogenetic trees. References were extracted from NCBI BLAST. References used cover the ITS 1, partial sequence; 5.8S rRNA gene, complete sequence and ITS 2, partial sequence.

Reference	Size (bp)	G + C content (%)	GenBank accession no.
L. tropica	390	44.10	KJ567476
L. tropica	390	44.36	KJ567477
L. tropica	390	44.10	KJ567478
L. tropica	390	45.90	KJ567479
L. infantum	391	43.73	KJ567480
L. infantum	392	43.62	KJ567481
L. infantum	392	43.88	KJ567482

3.2. ITS1 PCR of Leishmania isolates

None of the isolates were positive for the first PCR step targeting the ITS1 region. After nested PCR using the same primers, 17 out of 39 isolates (43.6%) produced a band at around 350 bp, indicating the presence of *Leishmania* parasites (Fig. 1).

3.3. RFLP

The digestion of the PCR product with endonuclease *HaeIII* revealed the profiles of *L. tropica* consisting of two bands at 200 bp and 60 bp, respectively.

3.4. Sequencing and phylogenetic tree analysis

Analysis of the ITS1 rDNA gene sequences further confirmed that they all contain *L. tropica*. Sequences showed a size range of 90 to 332 bp (mean: 236.06 ± 76.83 bp; median: 277 bp) and a G + C content ranging from 41.22% to 53.43% (mean: 45.74 ± 4.14%; median: 42.51%). GenBank accession numbers were obtained for 10 of the sequences [KT376735-KT376744] as the remaining sequences were too ambiguous even after re-sequencing. The complete alignment of the 10 sequences revealed a percent conservation of 6% to 94% (mean: 50.85 ± 19%; median: 56%) per nucleotide. The aligned sequences showed several blocks of conserved nucleotides in addition to a high occurrence of single nucleotide polymorphisms (SNPs) (Fig. 2). Variation was reflected by the low bootstrap values obtained in the NJ phylogenetic tree (Fig. 3).

3.5. Nested PCR for amplifying the ITS1-5.8S rDNA gene

The first PCR reaction using the ITS1 and ITS2 primers showed no bands on gel electrophoresis for all 39 isolates. Nested PCR reaction produced bands at around 400 bp for 27 isolates (69.2%) confirming that they belong to *L. tropica* (Fig. 4).

3.6. Sequencing and phylogenetic tree analysis

Analysis of the ITS1-5.8S rDNA gene sequences further confirmed that they all contain *L. tropica*. Alignment revealed a higher level of conservation along with the presence of numerous single point mutations (SNPs) (Fig. 5). Sizes ranged from 254 bp to 512 bp (mean: 426.79 ± 38.81 bp; median: 432 bp) and the G + C content from 38.17% to 50.58% (mean: 43.70 ± 2.84%; median: 39.9%). Percent conservation ranged from 4% to 100% (as per every nucleotide) (mean: 62.5% ± 29.75; median: 75%). Genbank accession numbers were obtained for the 27 sequences [KT363773-KT363799] (Table S2) (Fig. 6).

3.7. Assessing DNA quality through actin and GAPDH amplification

In order to rule out possible DNA quality reasons affecting the results, two housekeeping genes, Actin and GAPDH, were amplified. The first PCR amplification yielded no bands. Nested PCR resulted in bands at around 600 bp and 200 bp for Actin and GAPDH, respectively. All the isolates used in this study were positive for Actin and GAPDH after performing nested PCR.

3.8. Statistical analysis

A statistically significant correlation was observed between the PI and the nested ITS1-5.8S rDNA gene PCR ($p = 0.04 \leq 0.05$ level of significance). PIs of 2 and 3 were always correlated with positive molecular results. Increasing PI was correlated with an increase in the number of false negatives. These were microscopically determined to contain parasites but proved negative for molecular typing. False negatives appeared with ratios of 1:2, 1:1.6 and 2:1 for PI 4, 5 and 6, respectively. For PI = 6, a two fold increase in the number of false negatives might be due in part to a low frequency number (Fig. 7). No significant correlation was obtained between the PI and the results of the ITS-1-PCR RFLP, between the duration of the lesions and the results of the molecular tests and between PI and the duration of the lesion.

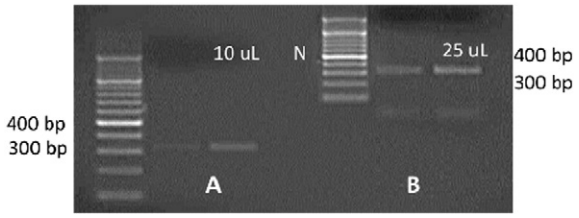

Fig 1. Agarose gel electrophoresis of the amplicons of ITS1-PCR for two representative isolates obtained after nested PCR. 10 μL (A) and 25 μL (B) of the PCR amplicon were loaded on the gel. Thicker bands at 350 bp were observed for loading 25 μL indicating the presence of *Leishmania*. A 100 bp ladder was used as a molecular marker. N: negative control.

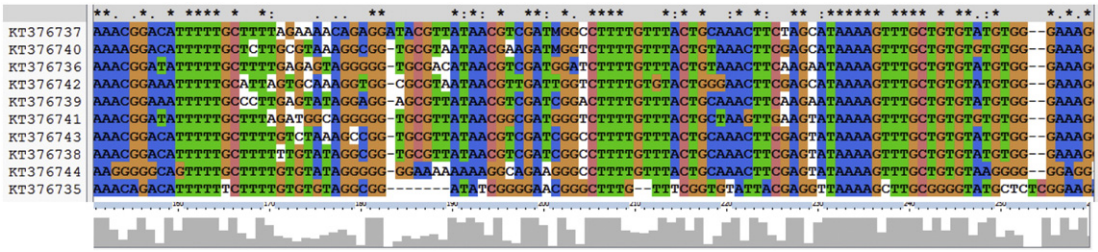

Fig 2. ITS1-PCR gene sequence alignment of *L. tropica* isolates using the Clustal Omega multiple sequence alignment program. Matching residues are highlighted with the same color. Gaps are denoted as dashes. "*" indicates positions which have a single, fully conserved residue. ":" indicates that a 'strong' group is fully conserved. "." indicates that a 'weaker' group is fully conserved. These are all the positively scoring groups that occur in the Gonnet Pam250 matrix. The strong and weak groups are defined as strong score > 0.5 and weak score = <0.5 respectively.

4. Discussion

4.1. DNA extraction from FFPE tissue

In this study, commercial reagents (Qiagen GmbH) were used to extract purified DNA from FFPE skin biopsies (Yehia et al., 2012). *Leishmania* parasites contain only 10^{-13} g of DNA, meaning that 10 million parasites would be required to generate 1 μg of workable DNA (Barker, 1989). This makes gene detection in *Leishmania* isolates extremely difficult. In order to obtain sufficient amount of DNA for further analysis, the process of DNA extraction has to be critically efficient (Momeni et al., 1996). In this study, all the conducted PCR reactions, including the isolates having a DNA concentration as high as 400 ng/μL, required a second PCR amplification to obtain a visible band, making the analysis process more costly, tedious and time consuming. Previously, it has been reported that DNA often gets degraded during the process of formalin fixation as well as during the extraction process itself (Momeni et al., 1996; Huijsmans et al., 2010). Also, negative results can be obtained despite a high yield due to the presence of crosslinks in the extracted DNA (Ludyga et al., 2012).

4.2. Leishmania typing using ITS1-PCR-RFLP and ITS1-5.8S rDNA gene

PCR-based methods have been proven to be highly sensitive and specific for the detection of *Leishmania* parasites in cultures or clinical samples (Ghatee et al., 2013). Previously, several studies exploited the ITS1 region amplification technique for the discrimination of *Leishmania* parasites (Roelfsema et al., 2011; Saitou and Nei, 1987; Schonian et al., 2003; Amro et al., 2009). Nested ITS1-5.8S rDNA gene PCR enhanced the sensitivity of this diagnostic tool by targeting two fragments in the ITS-rDNA region, one region consisting of ITS1 with the 5.8S rDNA gene (Parvizi and Ready, 2008). In this study, nested PCR of ITS1-5.8S rDNA gene proved to be more sensitive for *Leishmania* species identification. This proved to be consistent with a number of previously obtained results (Ajouad et al., 2013; El Tai et al., 2000; El Tai et al., 2001; Spanakos et al., 2008; Schallig and Oskam, 2002; Parvizi et al., 2008; Es-Sette et al., 2014). All of the cases studied were caused by *L. tropica*, a species endemic to the Aleppo region in Syria (Saroufim et al., 2014) thus supporting the import of this species into Lebanon from across the Syrian borders.

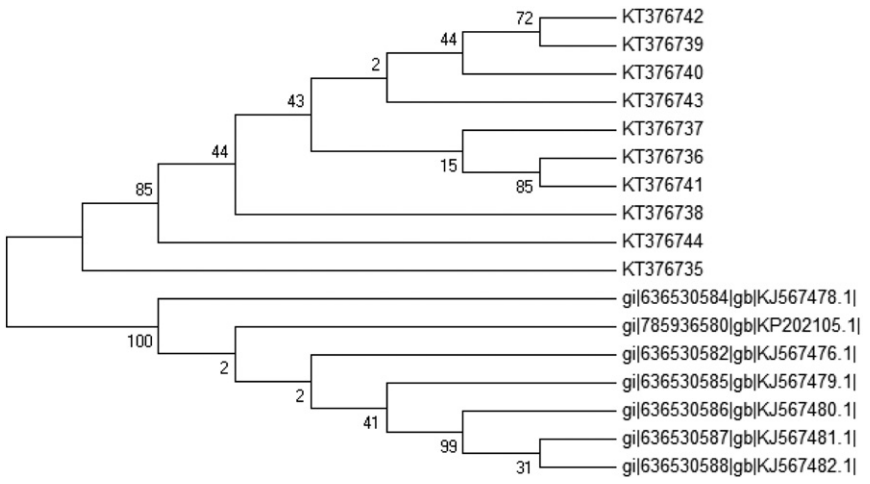

Fig 3. Neighbor-joining (NJ) tree showing the relationships of the 17 *L. tropica* isolates based on the ITS1 sequences. Bootstrap values are based on 1000 replicates.

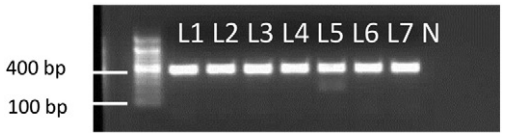

Fig 4. Nested-PCR of ITS1-5.8S rDNA gene of *Leishmania* DNA. Isolates positive for *Leishmania* gave a band around 400 bp characteristic of *L. tropica*. A 100 bp ladder was used as a molecular marker. N: negative control.

Diagnosis of CL relying solely on the microscopical examination of the lesions lacks enough sensitivity (Al-Jawabreh et al., 2004). And thus, it needs to be complemented by an efficient molecular typing method (Zhang et al., 2013) or preferably a combination of different techniques to achieve maximal sensitivity in parasite detection and eliminate false negative results. Several factors can affect the number of amastigotes in a lesion including the strain type, the stage of the disease and the immune defense of the host (Croft et al., 2006). Although histopathology-based diagnosis is the first step towards appropriate targeted therapy (Salman et al., 1999), it can be inadequate as all *Leishmania* species are morphologically similar and have variable numbers of amastigotes (Schonian et al., 2003; Singh et al., 2005). Furthermore, histopathological examination fails to detect amastigotes in up to 47% of cases (PI < 2) (Ameen, 2010). A PI of 2 or more is confirmatory of the presence of *Leishmania* parasites in the lesion. At this level the patient can start treatment (Ridley and Ridley, 1983). However, the species still needs to be determined because *L. major* and *L. tropica* infections are administered different treatment protocols (González et al., 2010). In this study, false negative results in ITS1-5.8S rDNA gene PCR were observed with Ridley's PI = 5 and 6 which could be attributed to the high parasite load (Yehia et al., 2012; Bensoussan et al., 2006). It can be inferred from the correlation analysis that samples with PI = 2 and 3 can be further subjected to molecular sub-species identification as opposed to the samples having a PI = 4 and more that are more likely to fail in ITS1-5.8S rDNA gene detection.

Moreover, ITS1 PCR-RFLP is the most widely used assay for direct detection and identification of *Leishmania* species in the Old World (Schonian et al., 2011), which identifies all clinically significant *Leishmania* species using only one enzyme (Yehia et al., 2012; Al-Nahhas and Kaldas, 2013). The comparison between three PCR-based methods involving two kinetoplast DNA (kDNA) PCRs and ITS1-PCR (LITSR/L5.8S primers) suggested that the ITS1 PCR-RFLP method was the most sensitive (Mouttaki et al., 2014). Based on the RFLP pattern of the ITS1 PCR product, isolates were identified as *L. tropica* (Schönian et al., 2001; Schonian et al., 2003; Al-Nahhas and Kaldas, 2013; Doudi et al., 2010; Rotureau et al., 2006). Previous attempts of extending the RFLP to include the 5.8S rDNA gene region did not show any advantage (Parvizi and Ready, 2008).

4.3. Sequencing and phylogenetic tree analysis

Although the ITS region serves as a marker for the differentiation of *Leishmania* at both the species and the strain levels, only few studies employed ITS sequence analysis to compare *L. tropica* isolates (Schönian et al., 2001; Khanra et al., 2011; Talmi-Frank

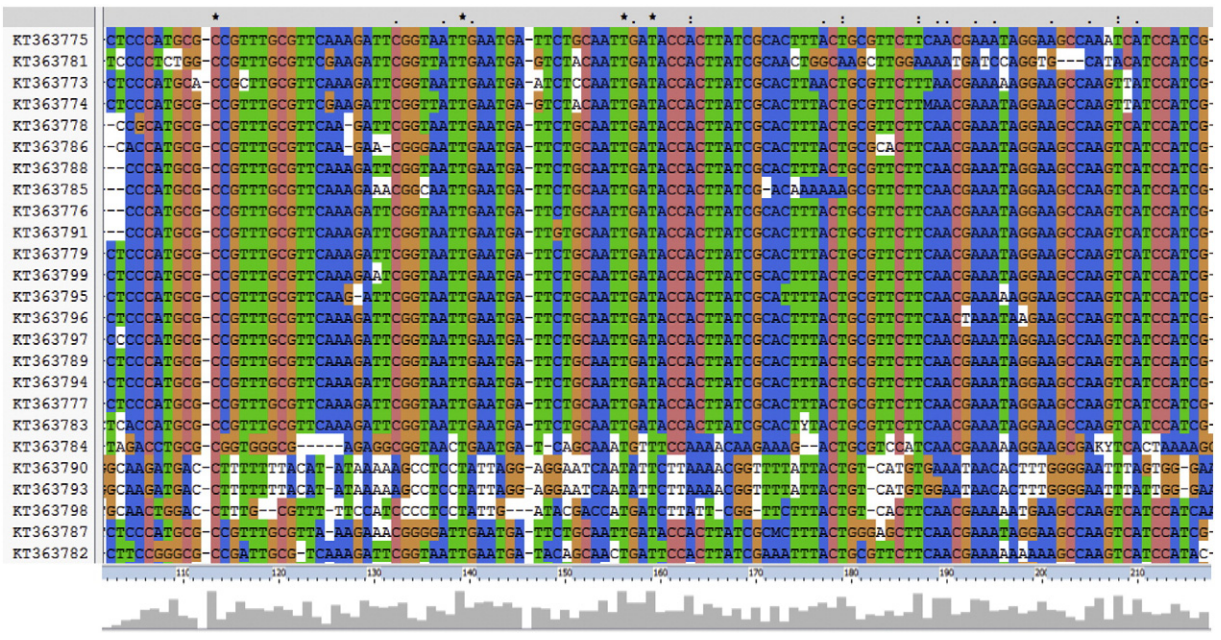

Fig 5. ITS1-5.8S rDNA gene sequence alignment of *L. tropica* isolates using the Clustal Omega multiple sequence alignment program. Matching residues are highlighted with the same color. Gaps are denoted as dashes. "*" indicates positions which have a single, fully conserved residue.

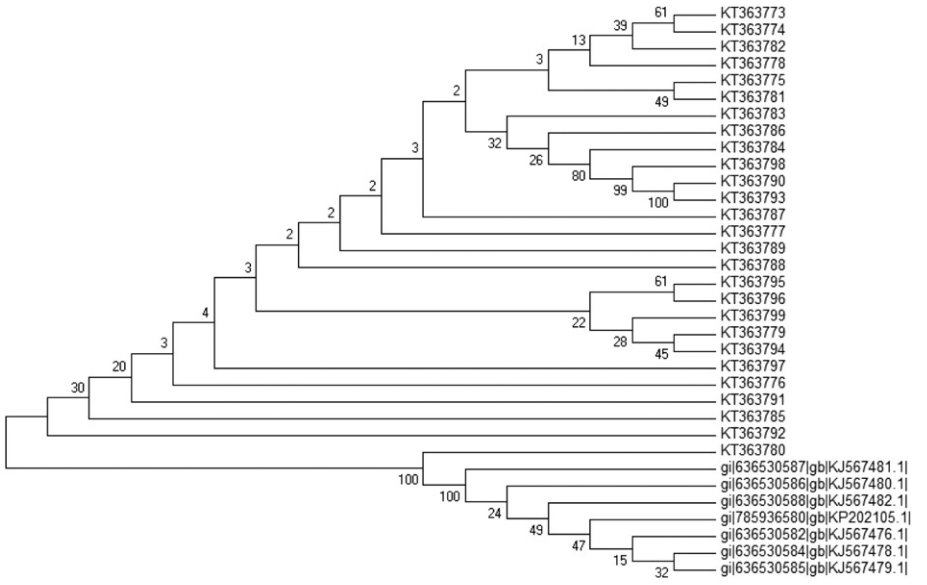

Fig 6. Neighbor-joining (NJ) tree showing the relationships of the 27 *L. tropica* isolates based on the ITS1-5.8S rDNA gene sequences. Bootstrap values are based on 1000 replicates.

et al., 2010; Mahdy et al., 2012; Ghatee et al., 2014). *L. tropica* is a diploid organism and such heterozygosity renders sequencing analysis more complicated (Yeo et al., 2011). Heterozygosity from electropherograms can be inferred by a double peak with two bases at the same variable bi-allelic site (Tavanti et al., 2005). A sequence of multiple bi-allelic sites is largely ambiguous. However, diploid sequence data can be modified, concatenated across multiple loci and applied in distance based phylogenetic methods for lineage assignment (Yeo et al., 2011; Tavanti et al., 2005). *L. tropica* is known to be a very heterogenous species (Schönian et al., 2001; Azmi et al., 2012; Le Blancq and Peters, 1986) and this was clearly observed in the alignment of the PCR amplification products and the low bootstrap values obtained on the phylogenetic tree. The reason behind this is the occurrence of at least two alleles for ITS in ribosomal DNA in *Leishmania* spp. (Ghatee et al., 2013; Mauricio et al., 2004).

Alignment based on the ITS1-5.8S rDNA gene revealed a significantly higher level of conservation than the ITS1 based alignment. ITS1 alone was very divers yielding several unreadable sequences. It was commonly reported that samples positive for leishmaniasis often show unreadable sequences upon ITS-rDNA fragment examination (Mirzaei et al., 2013). This can be explained by extremely high heterogeneity levels or sometimes may be due to mixed infections of two or more *Leishmania* species (Parvizi and Ready, 2008; Parvizi et al., 2008; Mirzaei et al., 2013; Strelkova et al., 2001). The obtained data also infers evolutionary relatedness and helps in detecting gene mosaics within or between homozygous gene loci (Odds and Jacobsen, 2005).

The total G + C content in *Leishmania* species was determined to be 57% (Alonso et al., 1992) whereas the G + C content in ITS1 and ITS1-5.8S rDNA gene was on average 45.74% and 43.70%, respectively. Although *Leishmania* species show a high preference for C- and G-ending codons (Langford et al., 1992), the low G + C content in the ITS region confirms that the selective pressure is not identical in coding and non-coding regions (Syvanen, 1994; Goncalves and Rosato, 2002).

In this study, two techniques for the molecular detection of *Leishmania* isolates were used: ITS1-PCR RFLP and ITS1-5.8S rDNA gene PCR amplification. ITS1-PCR RFLP profiles proved that the isolates were all *L. tropica*. This was further validated by sequencing the ITS1-PCR product as well as the ITS1-5.8S rDNA gene product. ITS1-5.8S rDNA PCR proved to be a more sensitive identification method for *Leishmania* isolates. Genotyping at the species level contributes to monitor the relative frequency of CL (Al-Jawabreh et al., 2004), especially that in the Mediterranean area CL can be caused by three different *Leishmania* species (*Leishmania infantum*, *L. major* and *L. tropica*), each characterized by distinct epidemiological features (Schonian et al., 2011). The obtained results highlight the need to find a universally accepted diagnostic tool for *Leishmania* typing, that is sensitive and capable of identifying all clinically significant *Leishmania* species. The risk of transmitting this “flesh-eating” parasite into

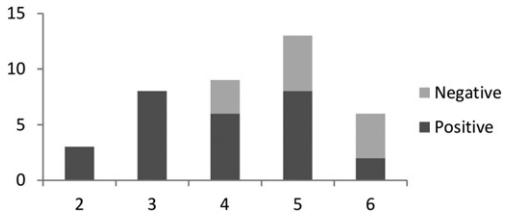

Fig 7. Correlation between the parasitic index (PI) of the lesion and the results of the ITS1-5.8 S rDNA gene PCR.

the Lebanese community is high. 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 (Barrett and Croft, 2012). For the differentiation of *Leishmania* at the sub-species level, it is recommended to perform multi-locus sequence typing (MLST) method. Moreover, whole-genome sequencing (WGS) provides a greater potential to identify genetic component of health problems and infectious diseases (Schonian et al., 2011; van El et al., 2013). Accessible international databases for cases of leishmaniasis in the MENA region (Middle East and North Africa) should be created for a better epidemiological assessment of these infectious agents and for tracing their patterns of migration between countries and continents.

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