



Genotyping *Leishmania* promastigotes isolated from patients with cutaneous leishmaniasis in south-eastern Turkey

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Abstract

Objective: Cutaneous leishmaniasis (CL) is a significant disease in south-eastern Anatolia because it is prevalent among Syrian refugees. We identified the causative *Leishmania* species in CL patients using molecular methods.

Methods: Novy–MacNeal–Nicolle medium was inoculated with aspirated fluid from suspected CL lesions and tested for amastigotes with Giemsa staining. PCR amplified the internal transcribed spacer 1 (ITS1) of the *Leishmania* genome in cultures containing *Leishmania* promastigotes from 100 patients, which were genotyped with a restriction fragment length polymorphism (RFLP) analysis. A phylogenetic tree was constructed from ITS1 sequences of 95 culture fluid samples from these patients.

Results: *Leishmania* amastigotes were detected in 92% of cultures with growth. *Leishmania* promastigotes were typed as *Leishmania tropica* with both PCR–RFLP and sequencing.

Conclusions: Identification of *L. tropica* as the causative agent of CL in our region allows the clinical course to be predicted, and guides treatment decisions and preventive measures.

Keywords

Cutaneous leishmaniasis, culture, PCR–RFLP, sequence analysis, phylogenetic tree

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Introduction

As in other parts of the world, cutaneous leishmaniasis (CL) is a major public health problem in Turkey. Anthroponotic CL caused by *Leishmania tropica* has been reported in Turkey since 1883, sometimes

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occurring as epidemics. Leishmaniasis is endemic to many countries in Africa, America, Asia, and Europe, but the majority of CL cases (> 90%) occur in Afghanistan, Brazil, Iran, Peru, Saudi Arabia, and Syria.^{1,2} Recently, millions of Syrians have entered Turkey in response to conflicts in the Syrian Arab Republic and are still residing in this country. Although refugee camps have been established in many cities, including Gaziantep, many Syrian refugees are also living in other parts of Turkey.³

The accurate diagnosis of CL, its treatment, disease prevention, strategies for its control, and management decisions require the identification of the causative species of the *Leishmania* parasite.⁴⁻⁶ The diagnosis of CL is based on clinical features and laboratory tests, including a direct parasitological examination and/or indirect testing with serology and molecular diagnostics.⁷ A universal PCR method targeting the internal transcribed spacer 1 (ITS1) region, which occurs between the genes encoding 18S rRNA and 5.8S rRNA, has proved useful in the direct diagnosis and identification of the *Leishmania* parasite because this region is highly conserved among species.⁸

Anthroponotic CL caused by *L. tropica* is highly endemic in south-eastern Anatolia and the eastern Mediterranean and Aegean regions of Turkey. In south Anatolia, both *L. infantum* and *L. tropica* have been reported as the causative agents of human CL. *Leishmania major* is known to be endemic in the countries bordering southern Turkey: Syria, Iraq, and Iran.⁹ In Syria, CL has two forms: zoonotic CL caused by *L. major* and anthroponotic CL caused by *L. tropica*.¹⁰

In this study, we identified the causative *Leishmania* species using PCR–restriction fragment length polymorphism (RFLP) and sequencing analyses of patients diagnosed with CL.

Materials and methods

Study population

Approval for this study was obtained from the Ethics Committee of Gaziantep University Faculty of Medicine (reference number: 221). In total, 458 patients were enrolled in the treatment program, including 433 patients of Syrian origin (94.5%) residing in refugee camps and 25 Turkish citizens (5.5%) with a diagnosis of CL, who were referred by primary and secondary health-care facilities in the city of Gaziantep to the Leishmaniasis Detection and Treatment Center established at the Department of Dermatology and Sexually Transmitted Diseases in Gaziantep University Sahinbey Research and Practice Hospital between April 2014 and April 2015. The diagnosis of CL was made based on clinical features and microscopic \pm culture methods that detected the presence of amastigotes in suspected CL lesions of the patients.^{11,12} Of these patients, 100 (96 Syrian refugees and four Turkish citizens) in whom promastigotes were demonstrated in culture were included in the study to detect and type the *Leishmania* parasites. The mean age of our patients (62% males, 38% females) was 19.4 years. Culture fluids from these 100 patients were analysed with PCR–RFLP, and a sequencing analysis was also performed in 95 of these patients to type the *Leishmania* parasite.

Sampling

To collect aspiration fluid from the lesion sites, 0.5 ml of a sterile 0.9% sodium chloride (NaCl) solution was drawn into a syringe. After the lesion site was cleaned with 70% alcohol, the lesion was penetrated with the syringe through the margin of the lesion close to the intact skin. Sterile NaCl solution was delivered into the lesion and the intralesional fluid was collected into the syringe.

The aspiration fluid was used to inoculate Now–McNeal–Nicolle culture medium (Besimik Ltd, Sti, Turkey), and a portion of the collected sample was transferred onto a clean slide to prepare a smear. The smear was then stained with Giemsa staining solution and examined under a light microscope (Olympus CX31, Tokyo, Japan) with a 100× objective lens. Preparations showing amastigotes were considered to be positive (+) for *Leishmania* spp. and preparations with no amastigotes were considered negative (–) for *Leishmania* spp. All results were recorded. The inoculated culture tubes were stored at 23–25°C and monitored every second day for 1 month for the presence of promastigotes. Promastigotes were detected with a direct microscopic examination and Giemsa staining of the culture fluid.¹¹ The samples of culture fluid containing promastigotes were collected in sterile Eppendorf tubes and stored at –80°C until typing.

Isolation of *Leishmania* DNA

Leishmania spp. DNA was isolated from the culture fluids with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and stored at –20°C.

PCR–RFLP analysis of ITS1 region

The forward primer (LITSR) 5'-CTGGATC ATTTTCCGATG-3' and reverse primer (L5.8S) 5'-TGATACCACTTATCGCAC TT-3', specific to the ribosomal ITS1 region that occurs between the genes encoding the small subunit (18S) ribosomal RNA and 5.8S rRNA, were used in this study.^{13,14} A 50 µl reaction mix was prepared in a PCR tube by adding 1 µl of 10 nmol forward primer, 1 µl of 10 nmol reverse primer, 25 µl of the Taq 2× master mix (25 mM Taq 2× master mix; New England Biolabs Inc., UK), 13 µl of nuclease-free water, and 10 µl of template DNA. The 50 µl reaction

mix was processed in a thermal cycler (Explera, Olly 96, Italy) using the programmed PCR protocol: one cycle at 95°C for 30 s; 30 cycles of 20 s at 95°C, 40 s at 56°C, and 60 s at 68°C; one cycle at 68°C for 5 min; and hold at 4°C. A 1.5% agarose gel was prepared containing 3.75 g of agarose (AB Analytica, Italy), 250 ml of 10× TBE solution (Bio Basic Canada Inc., Canada), and 25 µl of ethidium bromide (Amresco Inc., OH, USA). A 10 µl aliquot of the PCR product was loaded onto the agarose gel together with 10-bp and 100-bp markers and the DNA of *L. infantum*, *L. major*, and *L. tropica* reference strains (as the positive controls), after combination with 3 µl of loading dye. The samples were run on the agarose gel for 1–1.5 h at 80 mA and 130 V, and visualized with a UV transilluminator (UVP Upland, USA). The DNA of reference strains *L. infantum* MHOM/TN/1980/IPT1, *L. major* MHOM/IR/173, and *L. tropica* MHOM/AZ/1974/SAF-K27 was used in the analysis.

RFLP

The RFLP procedure was performed with the *Hae*III restriction enzyme (New England BioLabs, Inc.).¹³ In this step, 2.5 µl of 10× buffer, 1.5 µl of H₂O, 1 µl of *Hae*III restriction enzyme, and 15 µl of PCR product were added to a PCR tube and incubated at 37°C for 2 h. A 2% agarose gel was prepared containing 5 g of Metaphor agarose (BioShop Canada Inc., Canada), 250 ml of 10× TBE solution (Bio Basic Canada Inc.), and 25 µl of ethidium bromide (Amresco Inc.). The PCR products digested with the restriction enzyme were loaded onto the gel together with 10-bp and 100-bp markers and the DNA of the *L. infantum*, *L. major*, and *L. tropica* reference strains (as positive controls), after combination with 3 µl of loading dye. The samples were run for 3–4 h at 80 mA and 130 V, and visualized with a

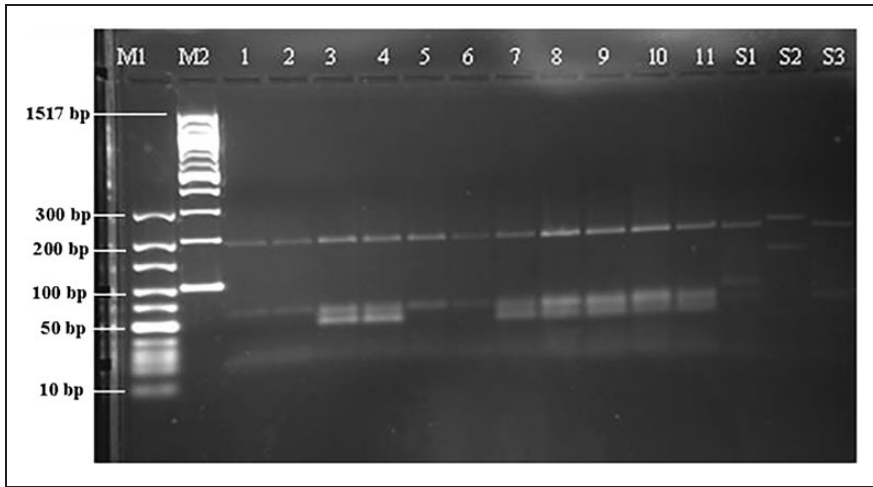


Figure 1. PCR-RFLP image. M1:10 bp marker, M2: 100 bp marker, Patient no: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, S1: *L. infantum* MHOM/TN/1980/IPT1, S2: *L. major* MHOM/IR/173, S3: *L. tropica* MHOM/AZ/1974/SAF-K27.

UV transilluminator (UVP Inc., Upland, CA, USA). The samples were evaluated by comparing them with the markers and positive controls (Figure 1).

Sequencing the *ITS1* region

The DNA extracted from the *Leishmania* samples was analysed in the laboratories of Iontek Anonim Sirketi (Iontec A.S., Istanbul, Turkey). The extracted DNA was amplified by PCR using primers specific for *Leishmania*. The primers used for sequencing (LITSR 5'-CTGGATCATTTTCCGATG-3' and L5.8S 5'-TGATACCACTTATCGACTT-3') targeted the *ITS1* region. After the PCR procedure, the PCR product was run on an agarose gel together with a marker of known size and concentration to check whether the PCR reaction had occurred. The samples visualized on the gel were purified and then sequenced with an ABI Prism 310 Genetic Analyzer using an ABI PRISM® BigDye Terminator Cycle Sequencing Kit. A BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was performed for all the sequences and the sequences were typed.

A phylogenetic tree was constructed by the Iontek Anonim Sirketi laboratory (Iontec A.S.). The phylogenetic relationships were inferred from an *ITS1* nucleotide sequence alignment produced with the MAFFT multiple alignment program using a combination of the E-INS-i alignment options.¹⁵ 'Neighbor joining method conserved sites' was chosen as the method of phylogenetic tree construction. For the bootstrap analysis, 1000 re-samplings was assigned in the program code.

Results

The 100 patient samples that showed growth of *Leishmania* promastigotes in culture were included in the study and genotyped. A light microscopic examination of the aspirated samples stained with Giemsa staining solution showed the presence of *Leishmania* amastigotes in 92 (92%) patients and an absence of amastigotes in the remaining eight patients (8%). A PCR-RFLP analysis of the DNA from the *Leishmania* promastigotes grown in culture identified all of them as *L. tropica* (Figure 1). A sequencing analysis of 95 of the *Leishmania* DNA samples

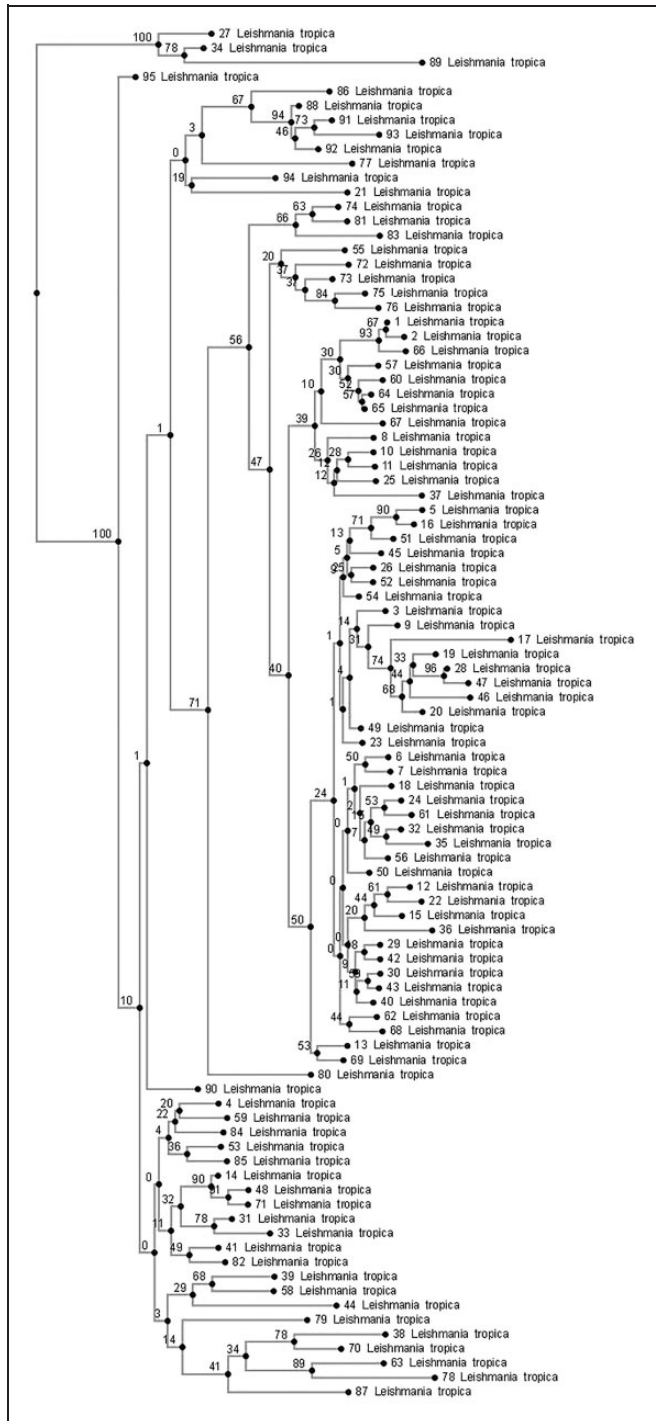


Figure 2. Numbers on the branches of the phylogenetic tree indicate bootstrap values and numbers given next to *L. tropica* indicate the number of study patients. The phylogenetic tree shows *L. tropica* genotypes isolated from Turkish patients (no. 12, 37, 58 and 68) and Syrian refugees.

confirmed the presence of *L. tropica*. A phylogenetic tree constructed from these sequences and the genetic heterogeneity of the *L. tropica* parasite are shown in Figure 2.

Discussion

Leishmaniasis is endemic in more than 80 countries. Its overall global prevalence is estimated to be around 12 million cases and this figure increases by 1.5–2 million each year.¹⁶ According to data for 2008 from the World Health Organization (WHO), CL occurs in 82 countries and 1.5 million new cases are recorded every year.¹⁷ Migration, travel, and ecological changes contribute to this increase. Host, parasite, and vector characteristics determine the occurrence and course of the disease.² CL is highly prevalent in our neighbouring countries of Iraq, Iran, and Syria and there are massive migratory flows into Turkey from these countries, both controlled and uncontrolled. According to a report issued by the United Nations High Commissioner for Refugees, 2,750,000 Syrian refugees are currently living in Turkey. Some Syrians with manifest CL have come to Turkey and live in camps or various cities.³ According to a WHO Report of 2010, Syria is one of the countries most affected by CL, with more than 25,000 cases per year.¹⁸ CL has been endemic in the Aleppo region of Syria for a very long time. However, there has been a gradual increase in the number of cases reported since the 1990s, with a maximum number of 58,156 cases reported in 2011 in the Idlib, Hamah, and Halab Provinces.¹⁹ An outbreak has occurred with the current war in Syria and measures to combat the disease are lacking, particularly in the besieged and medically underserved areas. New publications from the Turkish Ministry of Health reveal shocking statistics. An incidence rate of 53,000 cases was observed in 2012, and 41,000 cases were reported in the first 6 months of 2013.²⁰

The identification of the different species of the *Leishmania* parasite is based on an isoenzyme analysis or molecular biological methods.²¹ In the present study, *Leishmania* promastigotes isolated from patient samples were typed with PCR–RFLP and DNA sequencing using primers specific for the ITS1 region. The ITS1 fragment that were similar to those obtained with standard *Leishmania* strains was chosen as the target for the diagnostic PCR analysis,²² because ITS1 of the ribosomal DNA repeat unit has previously been used to distinguish Old World *Leishmania* species using RFLP and DNA sequencing.^{22,23} The sensitivity of the ITS1 analysis has been demonstrated and PCR–RFLP with *Hae*III is an efficient technique for the identification of *Leishmania* species.²²

In patients with CL, the lesions differ throughout the clinical course of the disease and in response to treatment, depending on the causative agent. In the present study, the *Leishmania* species isolated from samples from Turkish citizens was identified as *L. tropica*. *Leishmania tropica* is the predominant cause of CL in the south-eastern Anatolia region,² although in recent years, *L. major* and *L. donovani* have also been shown to be causative agents of CL in Turkey.²⁴ Toz et al.²³ examined the parasites in samples obtained from humans and dogs living in 31 cities in Turkey, with the majority of samples originating from Izmir, Aydın, Hatay, and Sanliurfa, and identified 80.43% of the human and canine visceral leishmaniasis samples as *L. infantum* and 6.52% as *L. tropica*, and 52.46% of the CL samples as *L. infantum* and 26.90% as *L. tropica*.

In the present study, the *Leishmania* parasite isolated from lesions of Syrian refugees was *L. tropica* and no other species was identified. Several studies have reported that the most prevalent causative agents of CL in Syria are *L. tropica*^{8,25} and *L. major*.²⁶ Consistent with our findings, Yehia et al.²⁷ established *L. tropica* as the

causative *Leishmania* species in biopsy samples isolated from lesions of Lebanese, Syrian, and Saudi Arabian patients with suspected Leishmaniasis, using PCR–RFLP. In Lebanon, Saroufim et al.²⁸ identified *L. tropica* in 85% and *L. major* in 15% of *Leishmania* isolated from the lesions of Syrian refugees with CL.

Significant heterogeneity was observed in the genetic structure of the *L. tropica* parasite that was analysed phylogenetically in the present study. *Leishmania tropica* is a very heterogeneous species and a high degree of intra-species polymorphism has been described based on an isoenzyme analysis and other molecular methods.^{29,30} This genetic variation may produce different phenotypes that are associated with a diversity of clinical manifestations and geographic distributions.³¹ In their study, Ajaoud et al.³² reported that an analysis of the ITS1–5.8S rRNA gene–ITS2 sequence in 31 specimens of the *Phlebotomus sergenti* vector showed great heterogeneity among *L. tropica*, segregating them into 16 haplotypes and demonstrating their phylogenetic relatedness to Indian strains and one Moroccan strain isolated from a CL patient. The heterozygosity of *L. tropica* isolates has also been suggested in a previous study.³³ Schwenkenbecher et al.³⁴ developed 16 polymorphic microsatellite markers for the phylogenetic analysis of *L. tropica*. They found a high degree of allelic heterozygosity among the strains, which suggested sexual recombination within the species. The variations reported in the Old World *Leishmania* species using ITS1 PCR–RFLP analyses range from highest to lowest in the order: *L. tropica* > *L. aethiopica* > *L. major* > *L. donovani*.⁹ Eroglu et al.³⁵ reported that DNA sequencing revealed genetic variation in both *L. infantum* (variants 1–3) and *L. tropica* (variants 1–5), and suggested that the increase in disease occurrence may be attributable to the geographic expansion of the disease, changing patterns of

international travel, population migrations, and the entry of non-immune people into endemic regions and of infected people into non-endemic regions.

Conclusion

There are many diseases prevalent among the Syrian and Iraqi refugees fleeing the civil wars in their countries, primarily CL. The risk of epidemics is greater for anthroponotic infections because of the transmission cycle from infected human to vector to human. Infected migrants may carry the disease to our region, particularly Nizip and its surrounds, where environmental/climatic conditions favour the vector. There is also a risk that the disease will spread to other regions. The identification of the causative *Leishmania* parasite in the cases of CL detected in our region will guide treatment decisions and the implementation of the necessary preventive measures and vector control strategies.

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Declaration of conflicting interest

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References

1. Özbel Y and Töz SÖ. Leishmaniosis. In: Özcel MA (ed.) *Tıbbi parazit hastalıkları 1*. Baskı, İzmir: Mete Basım Matbaacılık Hizmetleri, 2007, pp.198–230.
2. Gürel MS, Yeşilova Y, Olgen MK, et al. Cutaneous leishmaniasis in Turkey. *Türkiye Parazit Derg* 2012; 36: 121–129. [in Turkish, English Abstract].
3. UNHCR - BM Mülteciler Yüksek Komiserliği (www.unhcr.org.tr).
4. Wortmann G, Hochberg L, Houg HH, et al. Rapid identification of *Leishmania* complexes by a real-time PCR assay. *Am J Trop Med Hyg* 2005; 73: 999–1004.
5. Ben Abda I, de Monbrison F, Bousslimi N, et al. Advantages and limits of real-time PCR assay and PCR-restriction fragment length polymorphism for the identification of cutaneous *Leishmania* species in Tunisia. *Trans R Soc Trop Med Hyg* 2011; 105: 17–22.
6. Jirků M, Zemanová E, Al-Jawabreh A, et al. Development of a direct species-specific PCR assay for differential diagnosis of *Leishmania tropica*. *Diagn Microbiol Infect Dis* 2006; 55: 75–79.
7. de Viries HJ, Reedijk SH and Schallig HD. Cutaneous leishmaniasis: recent developments in diagnosis and management. *Am J Clin Dermatol* 2015; 16: 99–109.
8. Al-Nahhas SA and Kaldas RM. Characterization of *leishmania* species isolated from cutaneous human samples from central region of Syria by RFLP analysis. *ISRN Parasitol* 2013; 2013: 308726.
9. Toz SO, Nasereddin A, Ozbel Y, et al. Leishmaniasis in Turkey: molecular characterization of *Leishmania* from human and canine clinical samples. *Trop Med Int Health* 2009; 14: 1401–1406.
10. Knio K, Baydoun E, Tawk R, et al. Isoenzyme characterization of *Leishmania* isolates from Lebanon and Syria. *Am J Trop Med Hyg* 2000; 63: 43–47.
11. Özbel Y and Töz SO. Leishmaniasis. In: Korkmaz M and Ok ÜZ (ed) *Parazitolojide laboratuvar*. Türkiye Parazitoloji Derneği, Yayın No:23, 2011, pp.307–320.
12. Handler MZ, Patel PA, Kapila R, et al. Cutaneous and mucocutaneous leishmaniasis: differential diagnosis, diagnosis, histopathology, and management. *J Am Acad Dermatol* 2014; 73: 911–926.
13. Manual molecular procedures. Training course Molecular Epidemiology Leishmaniasis. Rio de Janeiro Brazil: Instituto Oswaldo Cruz, 2009, pp. 27–32. Available at: <http://clioc.fiocruz.br/documents/mmp.pdf>.
14. el Tai NO, Osman OF, el Fari M, et al. Genetic heterogeneity of ribosomal internal transcribed spacer in clinical samples of *Leishmania donovani* spotted on filter paper as revealed by single-strand conformation polymorphisms and sequencing. *Trans R Soc Trop Med Hyg* 2000; 94: 575–579.
15. Katoh K and Toh H. Recent developments in the MAFFT multiple sequence alignment program. *Brief Bioinform* 2008; 9: 286–298.
16. Ameen M. Cutaneous leishmaniasis: advances in disease pathogenesis, diagnostics and therapeutics. *Clin Exp Dermatol* 2010; 35: 699–705.
17. WHO. Report of the consultative meeting on cutaneous leishmaniasis Geneva, WHO Headquarters, 30 April to 2 May 2007; WHO/HTM/NTD/IDM/2008.7 http://www.who.int/leishmaniasis/resources/Cutaneous_leish_cm_2008.pdf.
18. Anonymous, WHO Working to overcome the global impact of neglected tropical diseases. First WHO report on neglected tropical diseases, 2010, pp.91–96.
19. Salam N, Al-Shaqha WM and Azzi A. Leishmaniasis in the middle East: incidence and epidemiology. *PLOS Negl Trop Dis* 2014; 8: e3208.
20. National reports for the Ministry of Health, Syrian Arab Republic. (2012, 2013) <http://www.moh.gov.sy>.
21. Klaus SN, Frankenburg S and Ingber A. Epidemiology of cutaneous leishmaniasis. *Clin Dermatol* 1999; 17: 257–260.
22. Schönián G, Nasereddin A, Dinse N, et al. PCR diagnosis and characterization of *Leishmania* in local and imported clinical samples. *Diagn Microbiol Infect Dis* 2003; 47: 349–358.
23. Toz SO, Culha G, Zeyrek FY, et al. A real-time ITS1-PCR based method in the

- diagnosis and species identification of *Leishmania* parasite from human and dog clinical samples in Turkey. *PLOS Negl Trop Dis* 2013; 7: e2205.
24. Koltas IS, Eroglu F, Alabaz D, et al. The emergence of *Leishmania major* and *Leishmania donovani* in southern Turkey. *Trans R Soc Trop Med Hyg* 2014; 108: 154–158.
 25. Haddad N, Saliba H, Altawil A, et al. Cutaneous leishmaniasis in the central provinces of Hama and Edlib in Syria: vector identification and parasite typing. *Parasit Vectors* 2015; 8: 524.
 26. World Health Organization. Leishmaniasis, http://www.who.int/leishmaniasis/resources/SYRIAN_ARAB_REPUBLIC.pdf (Accessed 15 September 2014).
 27. Yehia L, Adib-Houreh M, Raslan WF, et al. Molecular diagnosis of cutaneous leishmaniasis and species identification: analysis of 122 biopsies with varied parasite index. *J Cutan Pathol* 2012; 39: 347–355.
 28. Saroufim M, Charafeddine K, Issa G, et al. Ongoing epidemic of cutaneous leishmaniasis among Syrian refugees, Lebanon. *Emerg Infect Dis* 2014; 20: 1712–1715.
 29. Azmi K, Schnur L, Schonian G, et al. Genetic, serological and biochemical characterization of *Leishmania tropica* from foci in northern Palestine and discovery of zymodeme MON-307. *Parasit Vectors* 2012; 5: 121.
 30. Schönian G, Schnur L, el Fari M, et al. Genetic heterogeneity in the species *Leishmania tropica* revealed by different PCR-based methods. *Trans R Soc Trop Med Hyg* 2001; 95: 217–224.
 31. Mahnaz T, Al-jawabreh A, Kuhis K, et al. Multilocus microsatellite typing shows three different genetic clusters of *Leishmania major* in Iran. *Microbes Infect* 2011; 13: 937–942.
 32. Ajaoud M, Es-Sette N, Charrel RN, et al. *Phlebotomus sergenti* in a cutaneous leishmaniasis focus in Azilal province (High Atlas, Morocco): molecular detection and genotyping of *Leishmania tropica*, and feeding behavior. *PLOS Negl Trop Dis* 2015; doi:10.1371/journal.pntd.000368.
 33. Odiwuor S, Muia A, Magiri C, et al. Identification of *Leishmania tropica* from micro-foci of cutaneous leishmaniasis in the Kenyan Rift valley. *Pathog Glob Health* 2012; 106: 159–165.
 34. Schwenkenbecher JM, Fröhlich C, Gehre F, et al. Evolution and conservation of microsatellite markers for *Leishmania tropica*. *Infect Genet Evol* 2004; 4: 99–105.
 35. Eroglu F, Koltas IS, Alabaz D, et al. Clinical manifestations and genetic variation of *Leishmania infantum* and *Leishmania tropica* in Southern Turkey. *Exp Parasitol* 2015; 154: 67–74.