

Real-time PCR using high-resolution melting analysis technology for diagnosis of *Leishmania* and determination of types of clinical samples

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Received: 19.07.2018 • Accepted/Published Online: 03.11.2018 • Final Version: 12.12.2018

Background/aim: Leishmaniasis is a disease group carried by *Phlebotomus* and *Lutzomyia* sand flies infected with *Leishmania* and is mostly observed in rural areas. In this study, using high-resolution melting analysis (HRMA), we aim to identify the active types of leishmaniasis, which are inadequately identified by classical methods.

Materials and methods: Samples of 85 patients were examined in the study. Six of the patients were suspected of having visceral leishmaniasis (VL) and 79 cutaneous leishmaniasis (CL). The slides prepared from the samples were stained with Giemsa stain and examined under a light microscope. The results were compared with those determined in real-time PCR. When the real-time PCR result was positive for *Leishmania*, we determined the type by HRMA.

Results: Among 85 *Leishmania* amastigote samples, 28 (32.9%) of them were detected and accepted as positive by microscopic examination. On the other hand, 25 (29.4%) of the 85 samples were found as positive using real-time PCR. In addition, when 25 *Leishmania*-positive samples were examined by HRMA, the results showed that 21 (84%) were *L. tropica*, 3 (12%) were *L. major*, and 1 (4%) was *L. infantum*.

Conclusion: Based on our findings, we conclude that using real-time PCR and HRMA is useful in determining the cause of the disease in leishmaniasis.

Key words: Genotyping, high-resolution melting analysis, *Leishmania*, leishmaniasis, real-time PCR

1. Introduction

Leishmaniasis appears when female sand flies (*Phlebotomus* species in the Old World, *Lutzomyia* species in the New World), which have *Leishmania* parasites as compulsory agents in the cell, suck blood from humans. According to the World Health Organization (WHO), *Leishmania* is one of the seven most important tropical diseases, and it is endemic in 98 countries (1,2). In clinical terms, *Leishmania* can be separated into two subclasses: cutaneous leishmaniasis (CL), which heals by leaving a trace after causing deformations, and visceral leishmaniasis (VL), which may be fatal when not treated. Neither form is not dependent on certain *Leishmania* types (2,3).

The conventional opinion is that the causes of VL are *L. infantum* and *L. donovani* and that the causes of CL are *L. tropica* and *L. major* (4,5). However, in recent years, we have also seen cases in which *L. tropica* causes VL and *L. infantum* causes CL. Also, new *Leishmania* types have been detected among the nearly 4 million immigrants who fled from Syria during the recent internal conflicts

and found refuge in Turkey (6). In Turkey, leishmaniasis is seen clinically in two forms: CL and VL. In Turkey, anthroponotic CL, in which *L. tropica* is the active agent, has been reported since 1833, sometimes as epidemics. Half of the cases were reported in the province of Şanlıurfa. Although VL (in which *L. infantum* is the active agent) is mostly seen in the Aegean and Mediterranean regions, there are cases reported from nearly all regions of Turkey (7,8).

In addition, with increased travel opportunities and human relations, developing technologies, and changes in climatic, socioeconomic, and sociopolitical conditions, new drug-resistant *Leishmania* types have developed (6,9,10).

The prognoses and severity of the disease vary and depend on the immune response of the patient and on the types of the *Leishmania*, and even on its parasite strains. To avoid infection and plan proper treatment, it is important that the disease-causing parasite be defined in a fast and accurate manner. In this respect, various protocols have

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been optimized and developed to discriminate between the different *Leishmania* types, and the efficiency of the most proper medication is targeted (11).

Various molecular methods have been developed to detect the *Leishmania* parasites in clinical samples. One such method is high-resolution melting analysis (HRMA) (12,13). HRMA is a simple and extremely sensitive method realized in the existing real-time PCR systems. It is cost-effective and simple to use when compared with the other sequencing methods for genotyping (14,15).

Here, using a molecular approach, we determine the types of *Leishmania* that are difficult to detect by classical methods. Also, we use the HRMA method to determine the various *Leishmania* types in Turkey.

2. Materials and methods

2.1. Study area

The study was conducted in İstanbul, located in the western part of Turkey, and in Şanlıurfa, which has a border with Syria and is located in an endemic area, the Southeast Anatolia region of Turkey.

2.2. Sample collection

In this present study, which was conducted between November 2015 and March 2017, bone marrow samples were taken from six patients who were prediagnosed with VL. The samples were sent to the İstanbul University Cerrahpaşa Faculty of Medicine's medical microbiology department laboratory for diagnosis. The serous fluid samples of the skin lesions of 14 patients who were suspected of CL were also used in the study. In addition, skin exudate materials taken from 65 patients who were prediagnosed with CL were also used in the present study. These patients had applied to the Republic of Turkey Ministry of Health, Şanlıurfa Oriental Sore Diagnosis and Treatment Center. The protocol of the study was approved by the İstanbul University Cerrahpaşa Faculty of Medicine's Clinical Research Ethics Board (No: 83045809/604.01/02-313761).

2.3. Direct examination (light microscopy)

The samples were spread onto glass slides and were left to dry in open air. Then the slides that were fixed with methanol were stained with Giemsa stain. All the slides were subjected to main examination under a 100× oil-immersion objective. For the purpose of increasing the sensitivity, both slides were examined by two different researchers experienced in the field of parasitology. The slides on which the amastigote form was detected were defined as positive, and those on which the amastigote form was not detected were defined as negative.

2.4. DNA extraction, PCR assays, HRMA, and sequencing

To perform DNA extraction from direct samples taken from patients, the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) was

used according to the instructions of the manufacturing company. When the process was completed, 100 µL of DNA was obtained and kept at -20 °C until the PCR process was completed.

The DNA samples were reproduced using two specific primers (CJ7SLF and QRT7SLR) that targeted the 7SL-RNA gene areas (13). The primer series and protocols used in the amplification are given in the Table.

Real-time PCR amplification and HRMA were performed in the LightCycler Nano System (Roche Diagnostics) with LightCycler 480 High Resolution Melting Master (Roche Applied Science) by optimization under the cycle conditions given in the Table. After 40 cycles, the temperature was increased at a rate of 0.02 °C per second from 65 °C to 95 °C, and the melting curves were obtained according to the measured fluorescent values. The melting curves were analyzed using the PCR software and the HRMA profiles were defined.

All of the isolates that were determined as positive in real-time PCR with HRMA and that were included in groups were subjected to series analysis. A T100 Thermal Cycler (Bio-Rad, France) was used to perform the PCRs.

In the present study, the primers that encoded the ITS1 (internal transcribed spacer 1) gene area of the *Leishmania* types were used. The PCR process in which we used the LITSR and L5.8S primers were performed under the thermal cycle and conditions given in the Table.

The base sequencing analysis was performed with the MegaBACE 1000 automatic base sequencing instrument. The values were read in light of the instructions of the manufacturer company and the chromatogram data were received. The chromatogram files were converted into FASTA files and were compared manually by using the Sequencher 4.10.1, MEGA 4.1, and FinchTV sequence programs. The gene series that were obtained were analyzed by comparing them with the other *Leishmania* types recorded by using the BLAST program from the National Center for Biotechnology Information (NCBI) website (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

2.5. Statistical analysis

The frequency, percentage, average, and mean values were computed for descriptive statistics. The statistical analysis of the results was performed using the IBM SPSS 21.0 (IBM Corp., Armonk, NY, USA).

3. Results

3.1. Microscopic examination

By microscopic examination of Giemsa-stained slides, in 27 (34.2%) of 79 skin lesion samples *Leishmania* amastigotes were detected; hence, these were considered as positive samples. Among Giemsa-stained slides of the bone marrow samples, only one (16.7%) sample was defined as positive.

Table. The primer series and PCR protocols of the PCR methods.

| PCR-based method | Target | Primer sequence | Amplification conditions | PCR conditions | | | | |
|------------------|---------|----------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------|-----------|-------------|-------------------------|----------|
| | | | | MgCl ₂ (mM) | dNTP (mM) | Primer (μM) | Taq DNA polymerase (μL) | DNA (μL) |
| PCR-HRMA | 7SL RNA | 5'-ACG TGG ACC AGC GAG GGT-3' | 95 °C for 10 min, 95 °C for 10 s, 59 °C for 25 s, 72 °C for 15 s, 95 °C for 1 min, 40 °C for 1 min, 65 °C for 1 s, 95 °C for 1 s (40 cycles) | 2 | 0.2 | 0.2 | 0.25 | 5 |
| | | 5'-CGG TTC CCT CGC TTC AAC-3' | | | | | | |
| PCR sequencing | ITS1 | 5'-CTG GAT CAT TTT CCG ATG-3' | 95 °C for 10 min, 95 °C for 20 s, 56 °C for 40 s, 68 °C for 1 min, 68 °C for 5 min (30 cycles) | 1.5 | 1 | 5 | 0.25 | 3 |
| | | 5'-TGA TAC CAC TTA TCG CAC TT-3' | | | | | | |

3.2. *Leishmania* species identification by PCR-HRMA

According to the real-time PCR, positive results were found in 25 (31.6%) of 79 CL cases. There was no positive result by real-time PCR in samples from VL-suspected cases.

Our HRMA findings are shown in the Figure. The HRMA curves were divided into three groups, and of the 25 *Leishmania*-positive samples, 21 were *L. tropica*, three were *L. major*, and one was *L. infantum*. Those isolates positive for *Leishmania* were divided into groups (*L. major*, *L. infantum*, and *L. tropica*) and a series of analyses were performed.

When we compared the sequences obtained from our samples with *Leishmania* types recorded in the NCBI GenBank, the *L. tropica* samples revealed 93%–97% similarity, *L. major* 96%–97% similarity, and *L. infantum* 96% similarity.

4. Discussion

When we consider the data on leishmaniasis since 2000 in Turkey, the mean annual number of VL cases is 27, and the total number of the CL cases is 2020 (14). According to the data released by the WHO in 2014, more than 1 billion people (556 million VL, and 399 million CL) are at risk of leishmaniasis. In addition, 44% of Turkey's population faces the risk of being infected with CL and 9% of the population faces the risk of being infected with VL (16,17).

Many laboratory methods are used to diagnose leishmaniasis. It has been reported that molecular methods are more sensitive compared to traditional microscopy and culture methods (18–20). In our study, we detected 28 positive cases by light microscopy and 25 positive cases by real-time PCR. The samples detected in at least any of the two diagnostic methods were considered as positive. In this case, 27 (34.2%) of 79 patients were diagnosed with

CL and 1 (16.7%) of the 6 patients was diagnosed with VL.

Abd El-Salam et al. (21) conducted a study on CL patients with microscopy, culture, and PCR techniques and reported positivity rates of 54%, 47%, and 88%, respectively. These authors claimed that PCR was more sensitive than the other methods. Ertabaklar et al. (22) compared the microscopy, culture, and PCR methods in CL diagnosis. In this study the authors detected 29 (53%) amastigotes, 34 (62%) promastigotes, and 30 (55%) parasite-specific amplicons by using direct microscopic examination, culture, and PCR methods, respectively. These authors reported that the culture method was the most sensitive method in diagnosing CL. Rasti et al. (20) conducted a comparative study and used microscopy, culture, and molecular methods in 130 cases, reporting positivity rates of 67%, 56%, and 75%, respectively. They reported that the most sensitive method was PCR in their study and added that the sensitivity in diagnosis increased when PCR and microscopy were used together.

In previous studies, the PCR method has been ranked first among the methods used in diagnosing leishmaniasis, whereas here we found that the microscopic examination method was more sensitive. This might be because of a lack of proper sampling and the existence of a PCR inhibitor in the samples. Therefore, we recommend that PCR be used together with other methods, rather than using it alone, which will enable the diagnosis of more cases and therefore will be more useful in diagnosis and follow-up stages of the disease.

Considering the recent studies carried out in Turkey, *L. infantum* and *L. major* were detected in CL cases and *L. tropica* was detected in VL (7,8,23). This might be a clue that the *Leishmania* that causes CL and VL seen in Turkey has started to modify, so it is important to know the *Leishmania* parasite types in Turkey, which is located in a

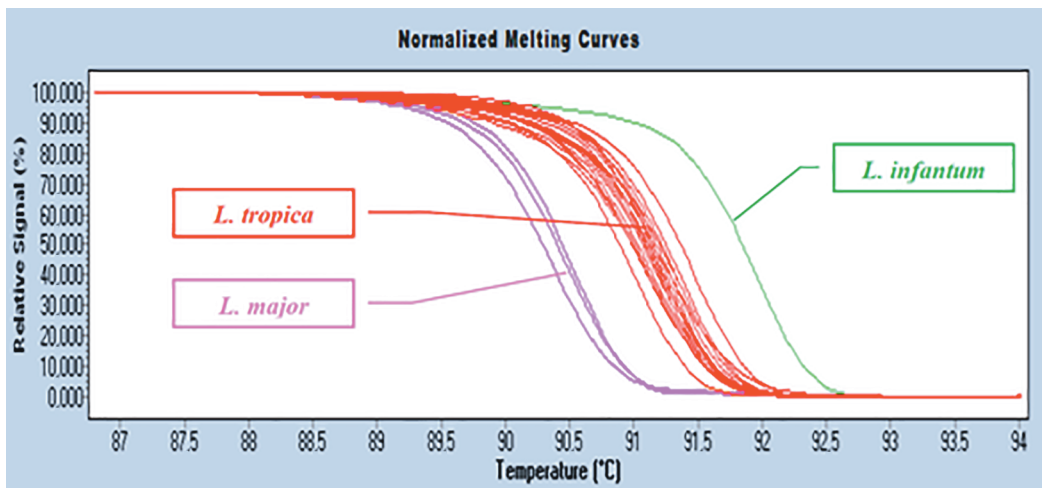


Figure. HRMA results.

wide geographical area with different climatic conditions.

Due to Turkey's geographical location, increasing travel opportunities, and nearly 4 million immigrants who fled to Turkey from Syria due to civil war in December 2011, there has been an increase in foreign-originated leishmaniasis cases in Turkey. Globally, Iran is ranked first as having the most *Leishmania* cases, and Turkey is second (24). Hence, it is important to characterize *Leishmania* strains at the molecular level to exhibit the drug resistance or clinical form. In our study, we detected *L. infantum* in a CL patient who did not have a history of VL. In addition, 2 of the 3 *L. major* cases with CL occurred in Syrian citizens who came from Syria to İstanbul, and one belonged to a Turkish citizen who had a history of travel to Afghanistan. Thus, we considered the *L. major* types as being foreign-originated. Similar cases were also detected in other countries. As an example, although *L. donovani* is more commonly associated with VL, it was reported that *L. donovani* was detected with CL in some countries, such as France (25). Thus, we considered the *L. major* types to be foreign-originated.

The prognosis and clinical presentation of the disease vary according to the types of *Leishmania*, and this results in difficulties in diagnosis. For this reason, the discrimination of the *Leishmania* parasite is important. In genotyping the *Leishmania* types, methods such as restriction fragment length polymorphism (RFLP), multilocus enzyme electrophoresis (MLEE), and multilocus sequence typing (MLST) have been used for many years (26,27). The costs of these methods and long procedures (such as the interpretation of complex data) are disadvantageous. A

method that enables cost-effective, fast, sensitive, easily repeatable detection must be employed to overcome these disadvantages (28).

HRMA is used for genotyping of various bacteria, fungi, and parasites, and it can be used in defining and genotyping *Leishmania* (29–31). It has been reported that HRMA is 3-fold faster and 5-fold cheaper than RFLP and MLST analyses. It has also been reported that HRMA has considerable potential for the determination of *Leishmania* types and genetic characterization (32).

In the present study, real-time PCR and HRMA were used to define the *Leishmania* types. Three groups of curves were obtained by HRMA examination of the *Leishmania*-positive (real-time PCR) samples. In the HRMA examination of the 25 *Leishmania*-positive samples by real-time PCR, 21 (84%) were *L. tropica*, three (12%) were *L. major*, and one (4%) was *L. infantum*.

In conclusion, leishmaniasis is an important public health problem in Turkey, as well as many other countries of the world. Since the prognosis and clinical presentation of the disease vary according to the type of *Leishmania*, we believe that clinicians and researchers must consider the changing active agent profile for possible precautions in public healthcare systems. For this reason, we believe that HRMA and genotyping will be useful in the diagnosis of *Leishmania* and to monitor its changing epidemiology.

Acknowledgment

This work was supported by the research funds of İstanbul University with Project No. 54948.

References

- Alvar J, Velez ID, Bern C, Herrero M, Desjeux P, Cano J, Jannin J, den Boer M; WHO Leishmaniasis Control Team. Leishmaniasis worldwide and global estimates of its incidence. PLoS One 2012; 7: e35671.
- Özbel Y, Toz S. Leishmaniosis. In: Özcel MA, editor. Tıbbi Parazit Hastalıkları. İzmir, Turkey: Meta Basım Matbaacılık Hizmetleri; 2007. pp. 197-244 (in Turkish).
- Desjeux P. Leishmaniasis: current situation and new perspectives. Comp Immunol Microbiol Infect Dis 2004; 27: 305-318.
- Aygün G. Kan ve Doku Protozoonları. In: Kiraz N, Samastı M, Aygün G, editors. Mikrobiyoloji ve Klinik Mikrobiyoloji Ders Kitabı. İstanbul, Turkey: Cerrahpaşa Tıp Fakültesi Yayınları; 2011. pp. 1353-1364 (in Turkish).
- Unat EK, Yücel A, Altaş K, Samastı M. Unat'ın Tıp Parazitolojisi. 5th ed. İstanbul, Turkey: Cerrahpaşa Tıp Fakültesi Vakfı Yayınları; 1995 (in Turkish).
- Koltas IS, Eroglu F, Alabaz D, Uzun S. The emergence of *Leishmania major* and *Leishmania donovani* in southern Turkey. Trans R Soc Trop Med Hyg 2014; 108: 154-158.
- World Health Organization. Leishmaniasis in the European Region, A WHO Consultative Intercountry Meeting; 17–19 November 2009; İstanbul, Turkey. Geneva, Switzerland: WHO; 2009.
- Gürel MS, Yeşilova Y, Ölgen MK, Özbel Y. Cutaneous leishmaniasis in Turkey. Turkiye Parazit Derg 2012; 36: 121-129 (in Turkish with abstract in English).
- Alborzi A, Rasouli M, Shamsizadeh A. *Leishmania tropica*-isolated patient with visceral leishmaniasis in southern Iran. Am J Trop Med Hyg 2006; 74: 306-307.
- Toz SO, Nasereddin A, Ozbel Y, Ertabaklar H, Culha G, Sevil N, Ziya Alkan M, Jaffe CL. Leishmaniasis in Turkey: molecular characterization of *Leishmania* from human and canine clinical samples. Trop Med Int Health 2009; 14: 1401-1406.
- Zampieri RA, Laranjeira-Silva MF, Muxel SM, Stocco de Lima AC, Shaw JJ, Floeter-Winter LM. High resolution melting analysis targeting hsp70 as a fast and efficient method for the discrimination of *Leishmania* species. PLoS Negl Trop Dis 2016; 10: e0004485.

12. Reithinger R, Dujardin JC. Molecular diagnosis of leishmaniasis: current status and future applications. *J Clin Microbiol* 2007; 45: 21-25.
13. Nasereddin A, Jaffe CL. Rapid diagnosis of Old World Leishmaniasis by high-resolution melting analysis of the 7SL RNA gene. *J Clin Microbiol* 2010; 48: 2240-2242.
14. Wittwer CT. High-resolution DNA melting analysis: advancements and limitations. *Hum Mutat* 2009; 30: 857-859.
15. Vossen RH, Aten E, Roos A, den Dunnen JT. High-resolution melting analysis (HRMA): more than just sequence variant screening. *Hum Mutat* 2009; 30: 860-866.
16. World Health Organization. Leishmaniasis in high-burden countries: an epidemiological update based on data reported in 2014. *Releve Epidemiologique Hebdomadaire* 2016; 91: 287-296.
17. World Health Organization. Leishmaniasis, Turkey, 2014. Geneva, Switzerland: WHO; 2016.
18. Khademvatan S, Neisi N, Maraghi S, Saki J. Diagnosis and identification of *Leishmania* spp. from Giemsa-stained slides, by real-time PCR and melting curve analysis in south-west of Iran. *Ann Trop Med Parasitol* 2011; 105: 559-565.
19. Ozerdem D, Eroglu F, Genc A, Demirkazik M, Koltas IS. Comparison of microscopic examination, rK39, and PCR for visceral leishmaniasis diagnosis in Turkey. *Parasitol Res* 2009; 106: 197-200.
20. Rasti S, Ghorbanzadeh B, Kheirandish F, Mousavi SG, Pirozmand A, Hooshyar H, Abani B. Comparison of molecular, microscopic, and culture methods for diagnosis of cutaneous leishmaniasis. *J Clin Lab Anal* 2016; 30: 610-615.
21. Abd El-Salam NM, Ayaz S, Ullah R. PCR and microscopic identification of isolated *Leishmania tropica* from clinical samples of cutaneous leishmaniasis in human population of Kohat region in Khyber Pakhtunkhwa. *Biomed Res Int* 2014; 2014: 861831.
22. Ertabaklar H, Özlem Çalışkan S, Boduç E, Ertuğ S. Comparison of direct microscopy, culture and polymerase chain reaction methods for the diagnosis of cutaneous leishmaniasis. *Mikrobiyol Bul* 2015; 49: 77-84 (in Turkish with abstract in English).
23. Zeyrek FY, Gürses G, Uluca N, Yentür Doni N, Toprak Ş, Yeşilova Y, Çulha G. Is the agent of cutaneous leishmaniasis in Sanliurfa changing? First cases of *Leishmania major*. *Turkiye Parazitoloj Derg* 2014; 38: 270-274 (in Turkish with abstract in English).
24. Pratloug F, Bastien P, Perello R, Lami P, Dedet JP. Human cutaneous leishmaniasis caused by *Leishmania donovani* sensu stricto in Yemen. *Trans R Soc Trop Med Hyg*. 1995; 89: 398-399.
25. World Health Organization. Leishmaniasis. Geneva, Switzerland: WHO; 2016.
26. Arana M, Evans DA, Zolessi A, Cuentas AL, Arevalo J. Biochemical characterization of *Leishmania (Viannia) braziliensis* and *Leishmania (Viannia) peruviana* by isoenzyme electrophoresis. *Trans R Soc Trop Med Hyg* 1990; 84: 526-529.
27. Evans DA, Lanham SM, Baldwin CI, Peters W. The isolation and isoenzyme characterization of *Leishmania braziliensis* subsp. from patients with cutaneous leishmaniasis acquired in Belize. *Trans R Soc Trop Med Hyg* 1984; 78: 35-42.
28. Hernandez C, Alvarez C, Gonzalez C, Ayala MS, Leon CM, Ramirez JD. Identification of six New World *Leishmania* species through the implementation of a High-Resolution Melting (HRM) genotyping assay. *Parasit Vectors* 2014; 7: 501.
29. Arancia S, Sandini S, De Bernardis F, Fortini D. Rapid, simple, and low-cost identification of *Candida* species using high-resolution melting analysis. *Diagn Microbiol Infect Dis* 2011; 69: 283-285.
30. Santos GB, Espinola SM, Ferreira HB, Margis R, Zaha A. Rapid detection of *Echinococcus* species by a high-resolution melting (HRM) approach. *Parasit Vectors* 2013; 6: 327.
31. Tong SY, Xie S, Richardson LJ, Ballard SA, Dakh F, Grabsch EA, Grayson ML, Howden BP, Johnson PDR, Giffard PM. High-resolution melting genotyping of *Enterococcus faecium* based on multilocus sequence typing derived single nucleotide polymorphisms. *PLoS One* 2011; 6: e29189.
32. Tsukayama P, Nunez JH, De Los Santos M, Soberon V, Lucas CM, Matlashewski G, Llanos-Cuentas A, Ore M, Baldeviano GC, Edgel KA et al. A FRET-based real-time PCR assay to identify the main causal agents of New World tegumentary leishmaniasis. *PLoS Negl Trop Dis* 2013; 7: e1956.