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ORIGINAL ARTICLE

High resolution melting assay in discrimination of the main etiologic agents of leishmaniasis in Iran

Shima Fayaz[1,2], Pezhman Fard-Esfahani[1], Fariborz Bahrami[2], Parviz Parvizi[3], Soheila Ajdary[2*]

[1]Department of Biochemistry, Pasteur Institute of Iran, Tehran, Iran
[2]Department of Immunology, Pasteur Institute of Iran, Tehran, Iran
[3]Department of Parasitology, Pasteur Institute of Iran, Tehran, Iran

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ABSTRACT

Background and Objectives: The three old world *Leishmania* species i.e., *L. major, L. tropica*, and *L. infantum* are considered as potential etiological agents of the various clinical forms of leishmaniasis in Iran. Different species co-exist in some areas. Accurate differentiation between the species is essential for choosing an appropriate therapy. Conventional and gold standard methods for the detection and characterization of parasites are time-consuming, laborious, and have low sensitivity. A polymerase chain reaction followed by high resolution melting (PCR-HRM) analysis has been employed for detection and species identification. Most of the studies suffer from the use of multiple targets and/ or requiring more than one reaction to identify a single sample. The present study aimed to design a PCR method based on the amplification of kinetoplast DNA minicircles (kDNA) and HRM analysis of the amplicons for rapid discrimination of the three mentioned species.

Materials and Methods: DNA from reference strains including *L. major, L. tropica*, and *L. infantum* and fifty-eight strains subjected to PCR-HRM analysis targeting kDNA. All the samples were also analyzed by conventional kDNA- PCR.

Results: The PCR-HRM analysis allowed discrimination between the three Old World species. The normalized HRM curves for the amplicons of kDNA indicated a unique and repeatable melting plot for each species, even in combination with human and mouse genomic DNA. Conventional kDNA- PCR could not properly discriminate *L. tropica* from *L. infantum.*

Conclusion: PCR- HRM analysis of kDNA proved to be fast and accurate for discrimination of *L. major, L. tropica*, and *L. infantum.*

Keywords: *Leishmania major; Leishmania tropica; Leishmania infantum;* High resolution melting; Kinetoplast DNA; Minicircles

INTRODUCTION

Leishmaniases are vector-borne infections caused by protozoa of the genus *Leishmania*, affecting various mammals, mainly carnivores and humans. *Leishmania* is transmitted by the bite of a female sandfly, mainly *Phlebotomus* and *Lutzomyia*. The disease is endemic in Asia, Africa, America, and the Mediterranean region. In the world 350 million people are at risk of leishmaniases, 1.5 to 2 million new cases occur each year, and it causes 70,000 death per year (1). Leishmaniasis is endemic in different parts of Iran and the three old world *Leishmania* species i.e., *L. major, L. tropica*, and *L. infantum* are considered as etiologic agents of the various clinical forms of the disease in the country (2). The ability to distinguish between *Leishmania* species is essential to establish

*Corresponding author: Soheila Ajdary, Ph.D, Department of Immunology, Pasteur Institute of Iran, Tehran, Iran.
Telefax: +98-21-66968857
Email: sohary@yahoo.com; ajdsoh@pasteur.ac.ir

the correct diagnosis and prognosis of the disease. The identification of *Leishmania* species based on isoenzyme analysis is of great value but remains a difficult and time-consuming method. For the diagnosis and identification of parasites, molecular techniques have become increasingly popular due to their higher sensitivity and specificity. Among them, PCR based methods are considered to be the most sensitive and specific technique (3). Different approaches based on coding and/or non-coding regions in the *Leishmania* genome have been employed. Kinetoplast and ribosomal DNA genes are among the most commonly used PCR targets for diagnosis and/or identification of *Leishmania* species in the Old World (4-7). Conventional Kinetoplast DNA (kDNA) minicircles PCR has limited ability to characterize *Leishmania* at the species level and usually the downstream analysis of PCR product is required (8).

Quantitative PCR (qPCR) has greatly improved molecular detection and diagnosis. The method is fast, has a broad dynamic range, and cross-contamination is drastically reduced because there is no need to open reaction tubes for post-PCR analyses. Several SYBR-green and Taqman chemistry-based single and multiplex qPCR methods have been reported with favorable sensitivity and specificity (9-12). Targeting high copy number genes to the amplification by qPCR increases the sensitivity of the method; however most of these high-copy number sequences, particularly kDNA minicircles, usually allow the identification of *Leishmania* only at the genus or subgenus level (9, 13). Approaches based on analysis of melting and high resolution melting (HRM) curves have improved the ability of PCR in discrimination of different species of *Leishmania*. Melting analysis differentiates amplicons based on dissociation characteristics of double-stranded DNA during heating. The introduction of HRM analysis, as the next-generation application of amplicon melting analysis, allowed improved technical approaches. It is cost-effective, fast, powerful, and simple. HRM characterizes amplicon samples based on their disassociation (melting) behavior and permits rapid analysis of the resulting data sets and discrimination of DNA sequences according to their composition, length, GC content, or strand complementarity (14, 15). Recently, targeting various genes including *hsp70, ITS1, 7SL* RNA, and *lack* in differentiation *Leishmania* species by HRM analysis is developed (15-18). HRM analysis of amplicons derived from kDNA conserved sequences discriminated *Leishmania* from *Viannia* subgenuses; however, differentiation of *L. infantum* from *L. amazonensis* was not possible (9, 19).

The aim of the present study was discriminating three existing Iranian *Leishmania* species: *L. infantum*, *L. tropica*, and *L. major* by HRM analysis targeting kDNA, as a simple, accurate, and rapid method. kDNA minicircles are suitable targets for sensitive *Leishmania* detection because they are present in high copy numbers and contain conserved sequence blocks in which PCR primers can be designed. This target has been employed to discriminate *L. major, L. tropica*, and *L. infantum*; however, due to the very close length of amplicons of *L. tropica* and *L. infantum* discrimination of these species has not precisely been possible.

MATERIALS AND METHODS

***Leishmania* strains.** Reference strains *L. major* (MRHO/IR/75/ER), *L. tropica* MHOM/SU/74/K27, and *L. infantum* MCAN/ES/98/LIM-877 were included. Fifty-eight *Leishmania* strains including *L. major, L. tropica*, and *L. infantum* from different parts of Iran, were cryopreserved and stored in liquid nitrogen in the Immunology Department of Pasteur Institute of Iran. All parasites have been characterized by isoenzyme analysis.

Parasite culture and DNA extraction. To revive the cryopreserved- *Leishmania* promastigotes, the parasites were cultured on (Novy- MacNeal- Nicolle) NNN biphasic culture medium and then were grown in RPMI medium (Gibco) with 10% fetal calf serum, 1U/ml penicillin, and 1 ug/ml streptomycin. Promastigotes were harvested and washed three times with PBS buffer by centrifugation at 4000 rpm for 10 min at 24°C. Lymph nodes from experimentally infected BALB/c mice were obtained 4 weeks after infection and passed through a mesh sieve; then, DNA was extracted from the cells. DNA was also extracted from uninfected human and mouse tissues. A proteinase K-phenol/chloroform method was used to extract genomic DNA from all samples (20). The obtained DNA was suspended in TE buffer (10 mM Tris-HCl, 1mM EDTA) and stored at -20°C until use. The quantity and quality of the extracted DNA were determined

using NanoDrop 1000 spectrophotometer (Thermo Scientific, USA).

Restriction fragment length polymorphism (RFLP) assays. Conventional PCR by targeting the ITS gene with direct (CTGGATCATTTTCCGATG) and reverse (ACACTCAGGTCTGTAAAC) primers was performed for all samples as previously described (21). The optimum amplification conditions were 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1min, annealing at 58°C for 1min, and extension at 72°C for 1min, with a final extension step at 72°C for 5 min. PCR products were digested by Taq1 (Thermo Scientific) endonuclease for 2 hours at 65°C. After electrophoresis on 2.5% agarose gel, the bands were stained with DNA Green Viewer (Parstous Biotechnology, IR-Iran) and visualized by UV transilluminator.

PCR-HRM method. For all samples, PCR-HRM was carried out in a Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Sciences). The direct (AGGGGTTGGTGTAAAATAGG) and reverse (TCGCAGAACGCCCCTACC) primers were used to target the conserved region of kDNA minicircle of *Leishmania* which amplifies the DNA from a wide range of *Leishmania* spp., including *L. major, L. tropica,* and *L. infantum* (8, 22). The reactions were prepared in 15 µl volumes in 0.2 ml strip tubes of a 36-well rotor. For symmetric PCR-HRM, the amount of DNA in each reaction was adjusted to 50 ng. Each reaction contained 7.5 µl of 2× PCR master mix (RealQ Plus 2× Master Mix Green, AMPLIQON), 1 µl (10 µM) of kDNA primer mix, 1 µl of DNA and nuclease-free water to a final volume of 15 µl. The thermal cycling conditions consisted of 1 min at 95°C, followed by 35 cycles at 95°C for 1 min, 60°C for 1 min and 72°C for 1 min, 72°C for 5 min as a final extension. HRM assay was performed from 70°C to 95 °C with each step raised by 0.5°C, followed by a waiting time of 2 s. Each DNA sample was analyzed in duplicate. Genomic DNA from three reference strains of *Leishmania* (mentioned above) in proportions of 1:10 and 1:100 to genomic DNA from uninfected human and BALB/c mice were used in the PCR-HRM assay. Furthermore, DNA extracted from *L. major*-infected mouse lymph nodes, uninfected human and mouse DNA were subjected to HRM analysis.

Tenfold serial dilutions containing 50 ng to 500 fg DNA from three reference species of *L. major, L. tropica,* and *L. infantum* were used to evaluate the sensitivity of the test.

Gel electrophoresis of kDNA PCR products. To compare the size of PCR products all the amplified samples were subjected to electrophoresis on 1.5% agarose gel stained with DNA Green Viewer.

RESULTS

Verification of strains by RFLP-PCR. All PCR products resulted from amplification of ITS of three reference species and samples collected from different parts of Iran were subjected to *Taq1* digestion. The sizes of bands for each *Leishmania* species were as follows: three bands for *L. major* 110, 295, and 680 bp, four bands for *L. tropica* 123, 207, 226, and 278 bp; and four bands for *L. infantum* 132, 277, 301 and 328 bp (Fig. 1).

Identification of different strains by PCR-HRM analysis. PCR was coupled with HRM analysis using kDNA as a target. The profiles of the normalized and derived fusion curves were obtained by Rotor-Gene 6000 Series Software 1.7. As shown in Fig. 2 by the normalized melting curves (Fig. 2a) and difference plot (Fig. 2b) analysis *L. major, L. tropica,* and *L. infantum* reference strains can be distinctly differentiated.

The average and standard deviation of the Tm of each amplicon was determined in duplicate from three independent experiments using DNA as a tem-

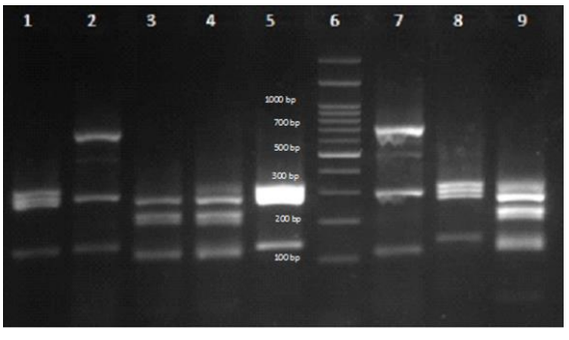

Fig. 1. ITS-PCR products digested with *Taq1*. Lanes 1 and 5 represent *L. infantum*; lane 2 denote to *L. major*, and lanes 3 and 4 signify *L. tropica*; Lane 6 is 100 bp DNA ladder; lanes 7, 8, and 9 are reference strains of *L. major* (MRHO/IR/75/ER), *L. tropica* MHOM/SU/74/K27 and *L. infantum* MCAN/ES/98/LIM-877.

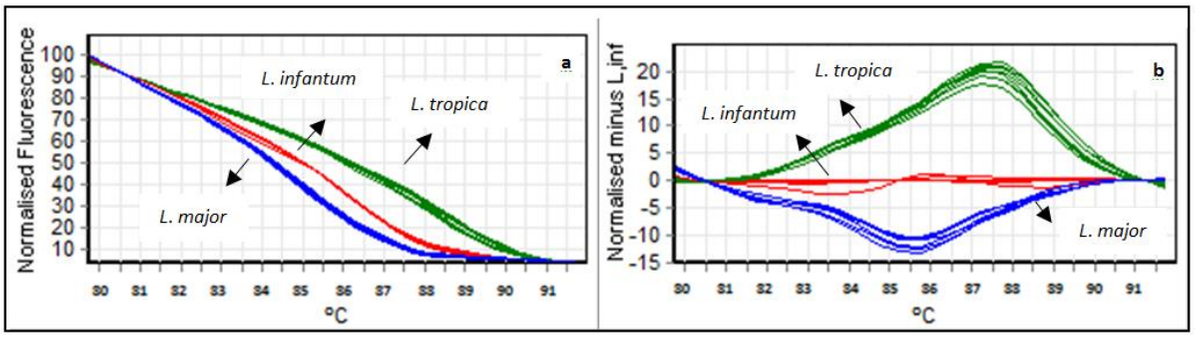

Fig. 2. HRM plots of kDNA amplicon. Normalized (a) and difference plot (minus *L. infantum*) (b) melting curves for differentiation of *L. major, L. tropica,* and *L. infantum* species.

plate from each reference species. The different average Tm values of and 85.1 ± 0.1°C, and 88.6 ± 0.3°C, and 86.2 ± 0.1°C were assigned to *L. major, L. tropica*, and *L. infantum*, respectively (Fig. 3).

To consider if the presence of high amounts of host DNA would influence the Tm value, the parasite DNA was mixed with uninfected human or mouse genomic DNA in proportions of 1:10 or 1:100. The results showed that the presence of host DNA in different proportions has no effect on Tm for none of the amplicons of different species. All of the species were differentiated correctly Fig. 4. To evaluate the specificity, uninfected human and mouse DNA (as host DNA) were subjected to PCR-HRM analysis. No amplification occurred for uninfected human or mouse DNA. Non-template controls showed no signal and primer-dimer formation was not noted.

The sensitivity of the test was assessed on a series of 10-fold dilutions of DNA. Evaluation of the sensi-

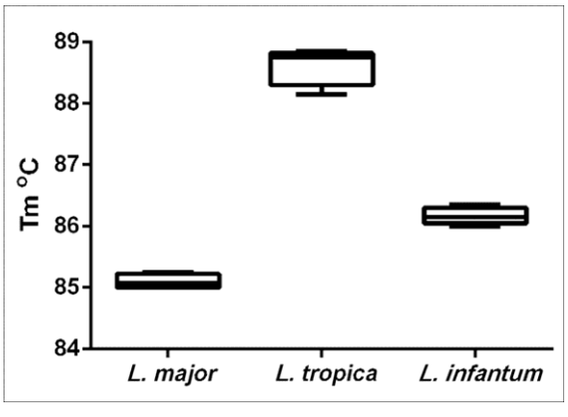

Fig. 3. Box plot of Tm values for each reference *Leishmania* strain. The chart shows the distribution of data into the mean, the smallest and the largest observation, and the standard deviation of the Tm values. Each species was tested in duplicate in three independent experiments.

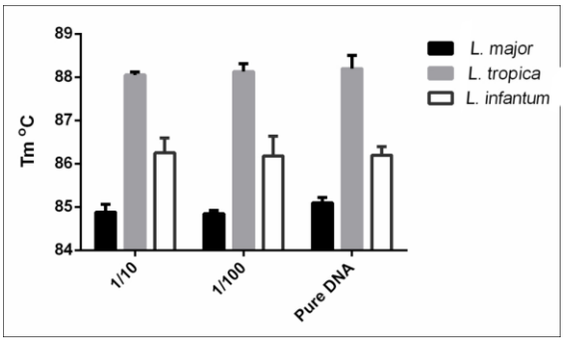

Fig. 4. Host DNA does not affect the Tm value of *Leishmania* strains. Melting profiles of kDNA amplicon obtained with *L. major, L. tropica,* and *L. infantum* pure DNA and the parasites DNA plus 10 and 100 times more genomic DNA from uninfected humans and mice.

tivity PCR-HRM by targeting kDNA indicated that the three species were easily differentiated even with 5 pg of parasite DNA. Notably, no Tm variation was observed with different concentrations of the initial target DNA of each species.

The HRM analysis was applied blind to amplicons generated from kDNA minicircles of *Leishmania* strains isolated from different parts of Iran. The results were in a 100% correlation with the Tm values of the reference strains. Tm values of 85 ± 0.4, 88.3 ± 0.4, and 86.4 ± 0.2 were allocated to *L. major, L. tropica*, and *L. infantum*, respectively. The HRM assay could successfully distinguish three Old World *Leishmania* species: *L. major, L. tropica*, and *L. infantum* (Fig. 5).

The Tm of the PCR product obtained from DNA extracted from the lymph node of *L. major* infected BALB/c mice was similar to Tm when parasite DNA was used as the template (84.8 + 0.2, n=3).

Electrophoresis of PCR products. The PCR prod-

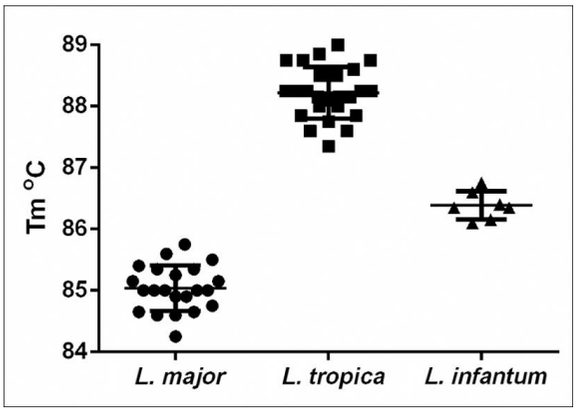

Fig. 5. Tm values distribution of PCR- HRM analysis of different strains isolated from different parts of Iran.

uct of *L. major* kDNA was about 615 bp and those of *L. tropica* and *L. infantum* were about 830, and 744 bp, respectively (Fig. 6); which is in line with other reports (8, 22). As it was shown *L. major* could be distinguished from *L. tropica* and *L. infantum*; however, amplicons sizes of *L. infantum* and *L. tropica* were very close; therefore it was not easy to discriminate them by conventional PCR targeting kDNA minicircles.

DISCUSSION

Three species of *Leishmania* are prevalent in Iran; *L. major* and *L. tropica* which cause cutaneous leishmaniasis and *L. infantum* which mainly causes visceral leishmaniasis. In some geographic areas, two or three of them coexist. It should be mentioned that *L. infantum* has been isolated from cutaneous lesions and *L. tropica* has been identified as the causative agent of visceral disease (19). Identification of parasites is essential for diagnosis, treatment, and epidemiological purposes. Conventional and gold standard methods for detection and characterization of parasites are time-consuming, laborious, and have low sensitivity. Molecular techniques in detecting and discriminating *Leishmania* species have become increasingly popular due to their notable sensitivity and specificity. Among them, real-time PCR-based methods are very effective in the diagnosis of leishmaniasis; moreover melting curve (Tm) and HRM analysis has extended this technique for species identification (23).

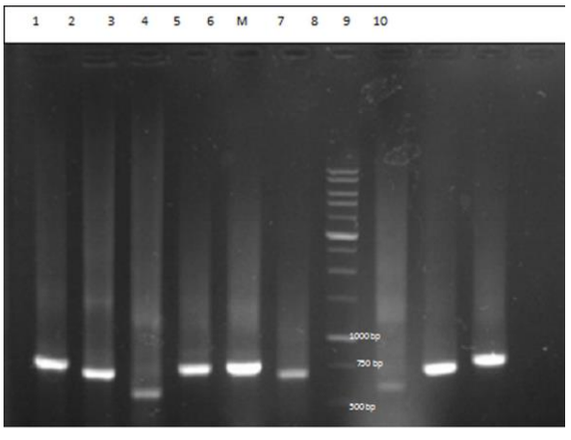

Fig. 6. Product of kDNA amplification by conventional PCR Lanes 1, 4, 5 represent *L. tropica*, lanes 2, 6 *L. infantum*, and lane 3 *L. major* species. Lanes 7, 8, 9 are reference strains *L. major* (MRHO/IR/75/ER), *L. infantum* (MCAN/ES/98/LIM-877), and *L. tropica* (MHOM/SU/74/K27), respectively, lane 10 is the negative control. M is 1 kb DNA ladder.

Different parts of the *Leishmania* genome or mitochondrial DNA have been targeted for detection and identification by molecular methods. Among them, ITS, HSP70, and kDNA have been the widest employed markers (15, 23). Toz et al. developed a PCR assay on ITS1 followed by Tm analysis for diagnosis and identification of *L. tropica* and *L. infantum* (24). It has been shown that TM analysis of kDNA minicircles fragments that were amplified by PCR was able to differentiate *Leishmania* from *Viannia* subgenera; however, species identification was not possible (9).

HRM which at first was used for the detection of single nucleotide polymorphism in genetic diseases currently is widely used for the detection of differences in double-stranded DNA. High-resolution DNA melting has several advantages over other genotyping and scanning methods, including an inexpensive closed-tube format that is homogenous, accurate, and rapid. There is no need for sequencing or gel electrophoresis to analyze the product, thus avoiding laboratory contamination with PCR products (15).

HRM analysis of PCR products derived from the ITS-1 region could differentiate Old World *Leishmania* species except for *L. infantum* from *L. donovani* (25). In another attempt, HRM analysis of hsp70 allowed for the discrimination of *Leishmania* sp. Responsible for leishmaniasis in Brazil and Eurasia and Africa; however, overlapping Tm values were obtained. Therefore, sequential discrimination was required by another set of primers (15). Likewise,

Hernandez et al. applied sequential PCR-HRM analysis of the HSP70 gene and then the ITS1 region to differentiate six New World *Leishmania* species (16). The above-mentioned studies suffer from the use of multiple targets and/or requiring more than one reaction to identify a single sample.

kDNA is organized in thousands of minicircles (0.8-1.0 kb each) and varies dozens of maxicircles (about 23 kb each) (26). The minicircles considered nearly 95% of kDNA. Due to the abundance of these minicircles, they have been used as a sensitive target for the diagnosis of leishmaniasis. However, conventional kDNA PCR has limited ability to characterize *Leishmania* at the species level. kDNA universal primers, which can amplify various minicircle classes from different *Leishmania* species, have been successfully used to discriminate *L. major* from *L. tropica* based on their amplicons size (27-29); however, the length of amplicon obtained with these primers in *L. infantum* is very close to that of *L. tropica* (8). To take advantage of the sensitivity of kDNA-based PCRs along with the ability to discriminate between *L. major, L. tropica,* and *L. infantum*, we used kDNA- based PCR- HRM. For this purpose PCR was performed using kDNA as a target, and then HRM analysis was used for the characterization of the species. Different reference species and well-characterized strains collected from various parts of Iran were used. The results indicated that each *Leishmania* species (*L. major, L. tropica,* and *L. infantum*) produced a unique repeatable melting plot that was easily distinguishable from the other species. Nicolas et al. have used a melting curve analysis of amplicons from kDNA that was amplified by primers very similar to ours, for differentiation of *L. major, L. tropica, L. donovani,* and *L. infantum.* Analysis of fragments indicated that *L. major* could be differentiated from both *L. tropica* and *L. infantum*, while the two latter species could not be discriminated against. These findings indicate that HRM analysis compared to the melting curve analysis is more powerful in the differentiation of these three common *Leishmania* species.

kDNA- based PCR- HRM analysis with primers different from ours has been used for discrimination between subgenera *Leishmania* and *Viannia*; however species identification was not possible (19). Ceccarelli et al. by applying a combination of two kDNA-based PCR-HRM assays could distinguish *L. infantum* and *L. amazonensis* (30).

High-resolution DNA melting has several advantages over other genotyping and scanning methods, including an inexpensive closed-tube format that is homogenous, accurate, and rapid. There is no need for sequencing or gel electrophoresis to analyze the product, thus avoiding laboratory contamination with PCR products (15). The results of our study indicated that the PCR-HRM test was specific since no amplification and plot were detected for uninfected human and mouse DNA. Furthermore, mixing un-infected human and mouse genomic DNA with *Leishmania* DNA as a template did not influence the results of three species discrimination, which is valuable in the clinical differential diagnosis. The results also indicated that PCR-HRM analysis was sensitive and can differentiate the species with as little as 5pg of parasite genomic DNA. Besides, the stability of Tm over a template concentration range from 50 ng to 5 pg indicated that the initial amount of template DNA did not influence the Tm Values. Furthermore, Tm patterns were consistent for *L. major* whether the DNA sample was from cultured promastigotes or amastigotes in lymph nodes of infected mice.

CONCLUSION

Altogether, we showed that the three common *Leishmania* species in Iran, *L. major, L. tropica,* and *L. infantum*, could be discriminated easily and distinctively by HRM analysis of the amplicons derived from kDNA. Performing PCR in the diagnosis of *Leishmania* and HRM analysis of the amplicons in the characterization of species would be a valuable approach in laboratories in endemic countries.

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