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# First report on natural *Leishmania* infection of *Phlebotomus sergenti* due *Leishmania tropica* by high resolution melting curve method in South-eastern Iran

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ABSTRACT

**Objective:** To identify the *Leishmania* species in infected sand flies by Real-time PCR coupled with HRM analysis. **Methods:** Real-time PCR coupled with HRM analysis targeting the first internal transcribed spacer (ITS1) of nuclear ribosomal DNA as the genetic marker was used to identify and distinguish *Leishmania* species in sand flies specimens. **Results:** Three out of 115 females of *Phlebotomus sergenti* (*P. sergenti*) (2.6%) were positive to *Leishmania tropica* (*L. tropica*). **Conclusions:** This is the first report on *P. sergenti* as the main and proven vector of anthroponitic cutaneous leishmaniasis in Dehbakri County using Real-time PCR coupled with HRM analysis. This method is rapid, sensitive and specific for diagnosing of parasites in infected Sand flies and ideal for large scale genotyping projects.

## 1. Introduction

Cutaneous leishmaniasis(CL) is endemic in many parts of the world and considered as a major public health problem[1]. The disease is one of the main health problem in Iran. Zoonotic cutaneous leishmaniasis (ZCL) due to *Leishmania* major (*L. major*) is endemic in 50% of the 31 provinces of Iran, whereas anthroponotic cutaneous leishmaniasis (ACL) associated with *Leishmania tropica* (*L. tropica*) is an old endemic disease in 8 provinces, especially in South East Iran[2-6].

Two epidemiological forms of CL occur in Bam district, ACL is mainly limited to the city, with its main reservoir of host of human. Anthroponotic Cutaneous Leishmaniasis is a serious problem due to its difficulty of treatment and longer duration, hence potentially sever long-term complications[7-9]. However ZCL is the more prevalent form. After the massive earthquake of Bam in December 2003 the prevalence of CL cases increased among school children. It increased from 2% in 2005 to 5% in 2008 (unpublished data). The disease is outspread and a new emerging focus of cutaneous leishmaniasis was confirmed in Dehbakri County, adjacent to Bam. According to published data, this paper is the first report on identifying of responsible sand flies to transmitting of disease agent to human, employing Real-time PCR coupled with HRM analysis.

In many areas, however, despite considerable research on these diseases, the main 'reservoir' hosts and the species of sand fly responsible for most transmission have still to be

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identified. In many foci of CL there is at least one species of sand fly that is sufficiently common and anthropophilic to be considered a probable vector, although good evidence to support this belief, such as the detection, in wild caught females of this species, of the parasites causing the CL, is lacking. The prevalence of infection in even the primary vector may be quite low, making the detection of sand fly infection, and particularly the detection of any temporal trends in the prevalence of sandfly infection, difficult, especially if dissection and microscopy constitute the detection method. Furthermore this traditional methods do not discriminate the organism in species level and often with variable and low sensitivity[10,11].

Molecular methods are currently being employed to detect Leishmania infection up to genus, complex or species level[12,13]. High-resolution melting (HRM) analysis is a relatively new technique that allows direct characterization of PCR amplicons in a closed system. It measures changes in the fluorescence intensity of a DNA intercalating dye during dissociation from double-stranded DNA to single-stranded DNA and can differentiate between single nucleotide polymorphisms (SNP)[14].

Real-time PCR offers several advantages over traditional PCR, including faster processing time, higher sensitivity and decreased contamination risk. A previous study used HRM PCR assay for Old World *Leishmania* based on gene 7SL-HRM that can be used to differentiate between *L. tropica*, *L. major*, and the *Leishmania donovani* complex[15].

PCR processing. Nowadays, the HRM has mostly been used in human clinical studies[16-20]. However, the application of the HRM technique to the diagnosis of parasitic organisms has been rather limited and the method has mostly been applied in molecular studies of parasitic protozoa such as the old world *Leishmania* spp[14]. The aim of the present study was to evaluate the potential use of ITS1 gene for identifying Old World *Leishmania* species by HRM method based real-time PCR. The method was successfully applied to identify *Leishmania* species in sand flies collected from Dehbakri county, Bam, South eastern Iran.

## 2. Material and methods

### 2.1. Study area

This study was carried out in Dehbakri county, Bam district, Kerman province during summer of 2011. Dehbakri is located 60 km west of Bam city. This area has a semi-desert climate temperate in summer and cold at winter. The mean of monthly maximum and minimum temperatures were 40 ℃ and -5 ℃ in July and Dec, respectively. The total annual rainfall was 220 mm. The minimum and maximum of monthly relative humidity were 45% and 92%, respectively in July and January.

### 2.2. Sample collection and identification

Sand flies were caught using aspirator from 8.00 pm till 2.00 am during summer 2011 where cases of ACL had been reported. The caught sand flies were transfered to the entomological cage and then were kept by wet towel and transported to laboratory research center for identification and Detection of Leishmania species. All the collected sand flies were mounted separately using Puri's media for species identification. Male and female were considered separately[21-23].

### 2.3. DNA extraction

Sand flies were washed in 1% detergent (washing-up liquid) solution for 5 min, Heads and last abdominal segments were kept for morphological identification based on the keys described by Leger *et al*., 1986[24]. Individual code names, consisting of a letter(s) taken from the collection area name followed by a number were assigned. DNA was extracted by maceration in a micro tube using a plastic pestle, followed by addition of 35 μL of lysis buffer (100 mM TRIS-HCl, 100 mM NaCl, 25 mM EDTA, 0.5% SDS, pH 8.0) and another maceration step. The test samples were digested overnight at 37 ℃ by proteinase K (1.25 μL of a 10 mg/mL solution) and the DNA extracted by phenol-chloroform. The DNA pellet was resuspended in 20 μL of TE (10 mM TRIS-HCl pH 8.0, 1 mM EDTA). Ten μL were used to estimate the DNA concentration and purity at 280 and 260 nm in a spectrophotometer, then discarded. The remaining 10 μL were stored at -20 ℃ until use. Double distilled water and DNA from *L. major* and *L. tropica* provided to the Iran Institute of Pasteur by the World Health Organization were used as negative and positive controls.

### 2.4. HRM-real-time PCR assay

We designed two primers AGCTGGATCATTTTCCGATG and ATCGCGACACGTTATGTGAG using the software Beacon as forward and reverse respectively. Real-time PCR was performed in a total reaction mixture of 20 mL containing 10 mL of HRM Master Mix, 10 pmole of each primer, approximately 10 ng/mL of genomic DNA and sterile deionized water using a 7500 fast real-time PCR system. Infection was detected by internal transcribed spacer 1 (ITS1) real-time PCR and high-resolution melt analysis (ITS1-HRM) PCR. All samples were tested in duplicates and results were compared with those from HRM analysis of positive controls for each assay. Positive samples also were verified by kDNA PCR.

## 3. Results

### 3.1. Sand fly identification

A total of 1 261 sand flies were captured and identified to the species level. Fourteen species including, 5 species of Phlebotomus genus and 9 species of *Sergentomyia* genus were identified. They were *Phlebotomus sergenti (P. sergenti)*, *Phlebotomus papatasi(P. papatasi)*, *Phlebotomus monglensis*, *Phlebotomus major*, *Phlebotomus longiductus*, *Sergentomyia sintoni*, *Sergentomyia baghdadis*, *Sergentomyia sumbarica*, *Sergentomyia africana*,*Sergentomyia dentata*, *Sergentomyia squemipleuris*, *Sergentomyia grekovi*, *Sergentomyia mervynae* and *Sergentomyia antennata*. Two species of *P. sergenti* (67.8%) and *P. papatasi* (19.4) were the dominant specimens respectively.

### 3.2. Leishmania species identification

We tested 164 specimens of sand flies including 115 Ph. sergenti and 49 *P. papatasi* by ITS1-HRM PCR. The overall *Leishmania* infection rate for *P. sergenti* was 2.6% (3/115) and all *P. papatasi* samples were negative. Positive specimens by ITS1-HRM PCR were also positive by kDNA PCR and produced a 750 bp kDNA product. Infected sand flies had been collected from indoors places with empty position of abdomen. This is the first report on natural infection of *P. sergenti* to *L. tropica* using high resolution melting curve (HRM) method in Iran. According our results this species was the only infected sand fly and it seems was responsible to transmitting of *L. tropica* among human.

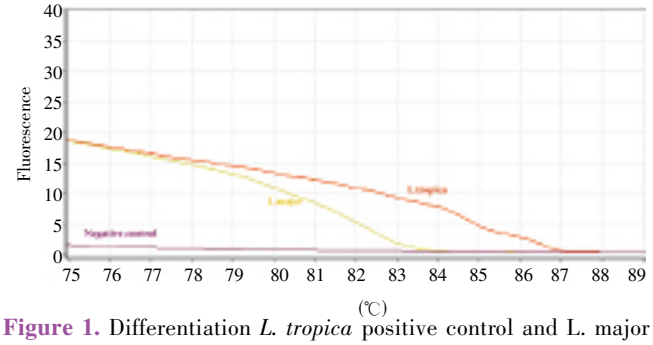


**Figure 1.** Differentiation *L. tropica* positive control and L. major positive control using HRM method.

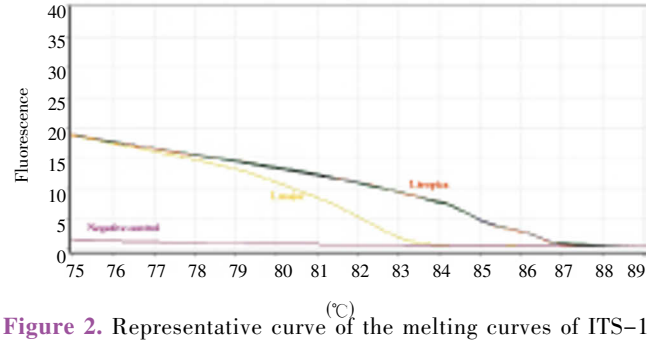


**Figure 2.** Representative curve of the melting curves of ITS-1 amplicons for infected *P. sergenti* species to *L. tropica*, Dehbakri County, Southeastern Iran, 2011.

## 4. Discussion

Control of leishmaniasis need to understand on ecology and epidemiology of disease in endemic areas. There is a major problem for epidemiologists both in identification of reservoir hosts and in detection of vectors. Therefore, finding naturally infected sand flies is essential in identifying a species as a vector of *Leishmania* and in studying infection rates in areas of endemicity[25, 26].

The applicability of kDNA for the detection and identification of *Leishmania* within sand flies by DNA hybridization have been shown previously[27]. In recent years, several molecular techniques, mainly those based on PCR have been developed for the specific identification and characterization of *Leishmania* species[28-30]. Although other techniques are sensitive and specific for identification of *Leishmania* species, they are laborious and time consuming, especially the post-PCR processing steps. In addition, there is also a higher risk of contamination, more expensive (*e.g.*, DNA sequencing) and the techniques only provide qualitative information. A highly sensitive method is needed to determine sand fly infection of *Leishmania* parasite. The choice of the gene used for HRM analysis is important in developing a successful assay, since it can take advantage of small differences in melting curves to distinguish between organisms with highly homologous sequences[14].

The ITS1 gene can be used for diagnosis of human leishmaniasis, as well as epidemiological studies on potential reservoir hosts, sand fly vectors, and parasite genotypes[12]. In the present study, we have successfully utilized HRM analysis along with real-time PCR for the rapid detection and species identification of Leishmania species in sand flies samples. To the best of our knowledge, this is the first report on the utilization of the HRM approach for rapid detection and discrimination of sand flies species by employing the ITS1 of nuclear ribosomal DNA as a genetic marker in Iran. We report *L. tropica* infections in *P. sergenti*(2.6%) specimens, the only proven vector of *L. tropica* in Iran.

In conclusion, using HRM assay for identifying of Old World *Leishmania* species is rapid, sensitive, specific, and simple and can be used to directly diagnose parasites in the sand flies vector with a minimum of operator post-PCR manipulation and interpretation.

## Conflict of interest statement

We declare that we have no conflict of interest.

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