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Original Article

Detection of *Leishmania major* In Naturally Infected Sand Flies Using Semi Nested-PCR

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Abstract

Background: The aim of this study was to assess *Leishmania* infection in sand fly species from areas where leishmaniasis is endemic. This is important for prediction of the risk and expansion of the disease.

Methods: In this cross-sectional study we used a PCR-based method for detection of *Leishmania* minicircle DNA within individual sand flies from Orzoieh, a new endemic leishmaniasis focus in southern Iran.

Results: We detected minicircle DNA in 6 of 92 (6.5%) *Phlebotomus (Phlebotomus) papatasi* collected indoor, while all of previous microscopic examination of sand flies specimens was negative for *Leishmania* promastigotes in the region. The species were identified as *Leishmania (Leishmania) major* by comparison of PCR products with a *L. major* positive control. All the *Leishmania*-positive sand flies were confirmed as *P. (P.) papatasi* by using a morphological key of Iranian sand flies.

Conclusion: Since PCR method is relatively easy and can process a large number of samples, it will be a powerful tool for the rapid identification of *Leishmania* species as well as monitoring the infection rate in sand fly populations in areas of low endemicity of leishmaniasis.

Keywords: Leishmania major, *Sand fly, Leishmaniasis, PCR, Iran*

Introduction

The leishmaniases are distributed worldwide, especially in tropical and subtropical areas, and affect at least 14 million people annually (1). In many countries of the Mediterranean region, Cutaneous Leishmaniasis (CL) is endemic and considered as a major public-health problem (1-2). *Leishmania (L.) major, L. (L.) tropica* and *L. (L.) aethiopica* cause CL in the Old World (3). Although the disease rarely causes severe morbidity, the lesions may take several months to heal and often leave ugly scars on the face or other exposed skin. CL due to *L. tropica* (CLT) is a very old endemic disease in many urban areas of Iran (4) whereas CL due to *L. major* (CLM) is prevalent in many rural areas in 15 of 30 provinces in Iran (5).

The rate of naturally infected sand flies in endemic areas and the correct identification of the infecting *Leishmania* parasites in a determined phlebotomine species are of prime importance in vectorial and epidemiological studies of leishmaniasis (6). The infection of sand flies with *Leishmania* promastigotes has usually been assessed by dissection of individual sand flies under microscopy. For this purpose, sand flies should be fresh and a procedure requiring considerable skill and expertise is needed for the dissection of these tiny insects. Furthermore, for the identification of the *Leishmania* species infecting sand flies, the isolation of parasites in culture without bacterial and/or fungal contamination is required for each dissected sand fly sample and isolated parasites are required for further zymodem (7), serodeme (8), schizodeme (9), karyotype (10), or PCR analysis (11). However, this procedure takes a relatively long time to perform and is not suitable for the examination of a large number of sand flies.

In the last decade, molecular methods have been developed for identification of certain species of *Leishmania*, either isolated from cultures or

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from patients (6) as well as in the detection of the parasite in individual or pooled phlebotomine specimens (12). The main advantages of these methods are their sensitivity and specificity, independently of the number, stage and localization of the *Leishmania* in the digestive tract of the vector (13). In the last few years, it has been successfully applied to field studies on the vectorial competence of phlebotomine sand flies (14), even in areas with low rates of infection (12).

Leishmania are members of the order Kinetoplastidae which possess a massive, compact network structure of DNA known as kinetoplast DNA (kDNA). The kDNA presents within the single mitochondrion of the organism and is comprised of two main components, the maxicircle and the minicircles, which are topologically interlocked within the kDNA network (15). The remarkable kDNA network has recently emerged as an important tool in the classification and identification of the organism (16). The discovery of minicircle sequences unique to species (17) and their use as species-specific probes has been found to be a promising solution to the problem of characterization of unknown isolates (18). Combinations of several primers within the conserved area of the kDNA minicircle have been tested for their ability to increase the sensitivity of the standard PCR.

In this study the set of LINR4, LIN17, and LIN 19 developed by Aransay et al. (14) and Parvizi et al. (19) was successfully used in a semi-nested PCR assay that was carried out in two amplification steps to assess the sensitivity of PCR in the detection of *Leishmania* parasites in individual sand flies naturally infected from three villages of Orzoieh county, Baft district, Kerman Province, south of Iran which was a new endemic focus with a few hundred leishmaniasis cases annually.

Materials and Methods

Sand fly collection

In this cross-sectional study, sand flies were caught in three villages of Shahmaran, Dowlat-Abad and Vakil-Abad from Orzoieh County, Baft, Kerman Province, Iran (Fig. 1) where CL is prevalent. Sample collections were carried out in September 2005, during the main seasonal activity of adult sand flies and leishmaniasis transmission peak in the region either using sticky papers (castor oil coated white papers, 21x30 cm) or aspirator. The traps were set overnight at indoor places such bed rooms, stables, and ware houses, as well as entrances of gerbil burrows. Specimens were stored in 96% ethanol and kept in -20º C before dissection. Dissection was carried out with sterilize microneedles and slide mounted in Pouri solution. Identification of female sand flies was based on internal morphological characters of the head and abdominal terminalia (20).

DNA extraction

DNA was extracted using the method of Ish-Horowize with minor modification (19). Extraction was carried out by grinding of individual sand flies in a microtube using glass pestle and followed by addition of 100 µl of grinding mix [500 µl 10X grinding Buffer (0.1M Tris-HCl pH:7.5, 0.6M NaCl, 0.1M EDTA), 250 µl 20X spermine/spermidine (3mM spermine, 3mM spermidine), 2.5 ml sucrose 10%, sterile water to 5 ml], and 10 µl SDS mix [1.8 ml 2X SDS Buffer (0.8 M Tris-HCl pH:9, 0.27 M EDTA), 2.5 ml sucrose, 600 µl SDS 10%, 17 µl Diethylpyrocarbonate] and incubation for 30-120 min in water bath. Then adding 30 µl 8M Potassium acetate and putting on ice for 45-120 min, centrifuged for 3 min, and supernatant was transported to a new microtube. The test samples were stored overnight at -20 ºC by addition of 350 µl ethanol 96%. The samples were centrifuged for 30 min at 13000 rpm. The DNA pellet was resuspended in 15 µl of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Three microliters were used to estimate the DNA concentration and purity at 280 and 260 nm in a spectrophotometer, then discarded. The remaining 12 µl were stored at -20 ºC until use.

DNA amplification

A portion of minicircle DNA was amplified by hot start PCR in a Thermocycler (perssonel Eppendorf). The set of primers forward LINR4: 5'-GGG GTT GGT GTA AAA TAG GG-3'), LIN17 (first-step reverse: 5'-TTT GAA CGG GAT TTC TG-3') and LIN19 (second-step reverse: 5'-CAG AAC GCC CCT ACC CG-3') was used for a semi-nested PCR that was carried out in two amplification steps, both in the same tube (14).

The first-step amplification reaction was carried out in a total of 10 µl containing 1µM 10X Buffer, 1.5 mM MgCI2, 200 µM of each dNTP, 1µM primer LINR4, 0.2 µM primer LIN17, 1 unit *Taq* polymerase and 1.5 µl of target DNA extracted from individual wild-caught sand flies. The mixture was incubated in the thermocycler (0.2 ml block) at 94° C for 5 min followed by 17 cycles, each consisting of 30 s at 94° C, 30 s at 52° C and 30 s at 72° C. After the last cycle, the extension was continued for a further 10 min then held at 4° C. The second-step amplification was then carried out following the addition of 90 µl of buffer containing MgCl2, dNTPs and *Taq* polymerase (as described for the first step), and primer LINl9 (final concentration 1 µM) for 33 cycles (94° C for 30s, 58° C for 30s and 72° C for 1 min). DNA of reference strain of *L. major* (MRHO/IR/75/ER) and DNA of male sand fly were used as positive and negative controls respectively. 15 µl of each PCR products were resolved in 5 µl loading buffer and then electrophoresed in a 1.5% agarose LE gel (6.4 cm x 10.0 cm) in TBE buffer (0.089 M Tris-HCl, 0.089 M boric acid, 0.02 M EDTA) containing 0.75% ethidium bromide) and visualized under ultraviolet transilluminator. *Leishmania* infections were identified by comparison of PCR products of specimens with the reference strains and molecular weight markers.

Results

A total of 172 female sand flies from Orzoieh region were collected. They comprised three phlebotomine species of *P. (P.) papatasi*, *Sergentomyia (Sintonius) clydei* and *S. (Parrotomyia) baghdadis*. Details of the collected samples are outlined in Fig. 1. Among the *P. (P.) papatasi* sand flies collected indoors 40% were unfed, 8.6% gravid, 15.2% semi gravid, and 33.3% blood fed. *P. (P.) papatasi* with more than 77% was the most dominant species in both indoor and rodent burrow sites.

Leishmania DNA was found in 6 out of 92 (6.5%) specimens and only in *P. (P.) papatasi* species. There was no *Leishmania* infection in other sand flies species. The visualized obtained bands in the infected specimens were similar to the standard strain of *L. major*, which was equal to 650 bp (Fig. 2). All of the infected sand flies had been collected from indoor places and some were either gravid or empty indicating there was enough time for the parasites to develop and to transform to promastigote, the infective form, which in turn by bite could be transmitted to a new host.

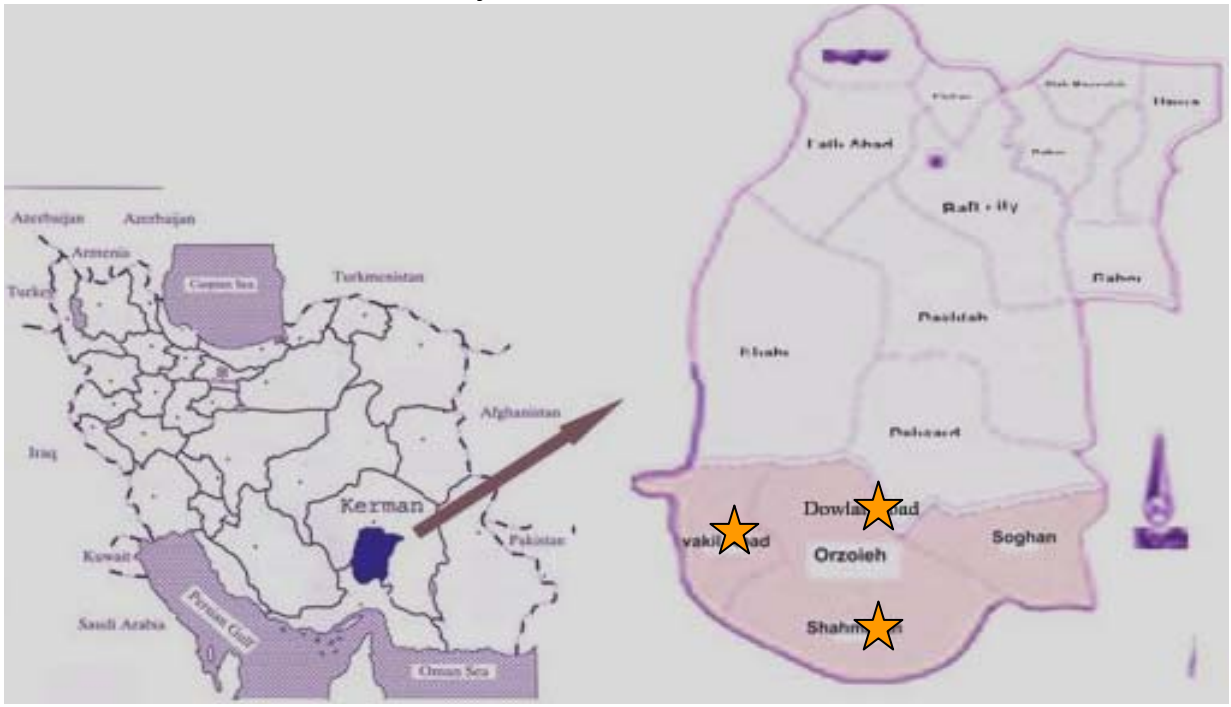

Fig. 1: Map of the area study and the position of three selected villages (stars) from Orzoieh region, Baft, Kerman Province, south of Iran

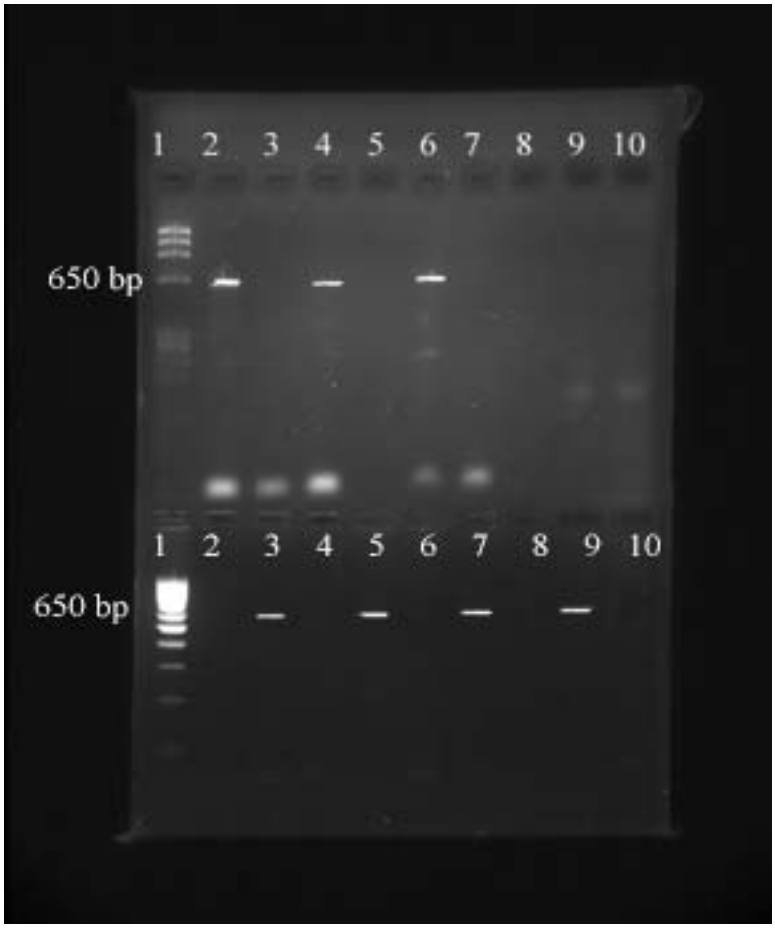

Fig. 2: Semi nested PCR amplification of *Leishmania* DNA with primers LINR4, LIN17, and LIN19 within sand flies speciesmens of Orzoieh, Baft, Kerman province, Iran. 1: DNA size marker (VI: Roche Germany), 2 (top): positive control (*L. major* strain MRHO/IR/75/ER), 3 (top) negative control (DNA of male sand fly), number 4 and 5 from top row and 3, 5, 7, and 9 from down row: infected *P. (P.) papatasi* with *Leishmania* DNA, 10: negative controls

Discussion

Information and knowledge on ecology and epidemiology of leishmaniasis is very important for control of the disease. This knowledge could be focused on identification of reservoir host(s) as well as identification and detection of the parasite and vector(s).

This is the first report on naturally infected *P. (P.) papatasi* sand flies with *L. major* in the region. Support for true parasite species identification came from previous epidemiological study using RAPD-PCR method that showed human infection with *L. major* parasite in the region (5).

In this study, *P. (P.) papatasi* was the main dominant species in both indoor and rodent burrow places. The fact that *P. papatasi* females were found indoors, whether gravid or with empty abdomen, suggest a considerable vectorial capacity of this species in transmission of *Leishmania* to humans. These findings are in agreement with the direct effects of population density and anthropophilic behavior of a given species in vector incrimination of arthropod vector borne diseases. It can be concluded that *L. major* is the cause of and *P. papatasi* is the primary vector of CL in Orzoieh. It is noticeable that *P. papatasi* is the main proven vector of CL in Iran (21).

The present study revealed the high sensitivity of the PCR technique for detection of *Leishmania* parasites infecting sand flies in an hypoendemic area. A previous study in the region, using a microscopy method, could not

detect and identify any parasites in 149 tested vector samples (5). Isoenzyme detection method could provide the gold-standard characters for identifying species and reference strain of *Leishmania*, but this method has disadvantages due to, firstly, requiring the culture of large number of parasites and, secondly, primary isolates can easily become contaminated or in a mixed infection only the strain that grows fast in laboratory conditions is yielded.

In contrast, molecular biology techniques such as PCR have resulted advantageous showing greater sensitivity, specificity, versatility and speed for the processing of large sample numbers (22-24). The applicability of molecular techniques (PCR) for detection and identification of *Leishmania* within sand flies by kDNA amplification currently is widely used by many researchers in different leishmaniasis foci (19, 25-26).

As a final conclusion, the results of this study in combination with further studies could be used in epidemiological surveys and leishmaniasis control programs in the region.

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