Phlebotomus papatasi (Diptera: Psychodidae) as the Vector of Leishmania Major in Kharameh District, Southern Iran

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Introduction

Leishmaniases are endemic diseases in different parts of Iran. Cutaneous Leishmaniosis (CL) is an increasing public health problem in 15 provinces of the country. CL was first described in southern Iran by Nadim and colleagues. In this area, the yearly incidence of CL has increased over the last decade. Sand flies are vectors of at least three kinds of diseases to human, the most important of which is leishmaniasis. Infection is transmitted to humans via biting of the infected female sand flies.

Leishmania major is the main causative agent of wet lesions of CL. It is mainly found in the rural areas. In Iran, CL has also been found in the urban areas where the results of Leishmania tropica infection have been observed. Both Anthroponotic (ACL) and Zoonotic (ZCL) forms caused by L. tropica and L. major occur in Iran. It seems that ACL is restricted to some large and medium-sized cities whereas ZCL is endemic in rural areas of different parts of the country. Correct detection of the parasite(s) causing each case of CL is needed if the local measures for the control of the disease are to be effective.

The characterizing techniques such as microscopic examination of direct smears and/or the culture of biopsies are not sensitive enough and diagnostic methods based on deoxyribonucleic acid (DNA) have now resulted in sensitive and quick detection of the micro organism possible.

Phlebotomus species are the sole vector responsible for transmitting the disease in the old world. Characterizing of the vector in the endemic areas is vital for both chemotherapy and prognosis.
Moreover, determination of Leishmania vectors and their biology is required for planning Leishmania control policies. Sand flies frequencies and activities are influenced by temperature, relative humidity, soil moisture, and wind speed. Temperature and moisture also affect the activities of sand flies.

Fars province is one of the most important foci of leishmaniasis in Iran. In 2014, 510 cases of ZCL have been reported in Kharameh district (unpubl. obs.). In this article, the fauna, monthly activity, and geographic distribution of sand flies have been studied in this district.

Materials and Methods

Study Area

Fars province is located in southern Iran and has an area of 122,400 km². The study was carried out in Kharameh district, located 80 kilometres northeast of Shiraz, the capital city of Fars province, southern Iran. This region is situated at 29° 30' 4" N, 53° 19' 15" E and 1500 m above the sea level. Due to suitable biological conditions for Leishmania reservoir hosts, vectors and reservoirs diversity, Kharameh has always been considered as one of the CL endemic regions (Figure 1).

Collection and Examination of Sand Flies

During April 2014 and March 2015, sand flies were collected monthly by sticky paper traps (castor oil-coated white papers, 20×32 cm) from indoor and outdoor areas (rodent burrows, agricultural plantations surrounding the houses, etc.) in different areas of Kharameh district including villages of Ahmad-Abad and Korbal regions.

In each sampling, 30 sticky paper traps were set in the evening and collected in the next morning. Sand flies were mounted in Puri’s medium. Identification of the specimens was based on specific taxonomic criteria. In determining the monthly activity of sand flies, they were caught from indoor and outdoor areas of the lowland and the highland locations during this period.

DNA Extraction

Total genomic DNA of each female sand fly was extracted using the method described by Motazedian et al. (2002) with some modifications. Each sample was homogenized in a 1.5 ml microtube containing 200 µl lysis buffer [50 mM Tris-HCl (pH=7.6), 1 mM EDTA and 1% Tween 20] and 15 µl of proteinase K solution (20 µg/ml). The homogenate was incubated at 37°C overnight before 100 µl from a phenol:chloroform:isoamylalcohol mixture (25:24:1, by vol.) were added. It was centrifuged at 12,000 rpm for 5 min and the DNA in the supernatant was precipitated with 400 µl cold pure ethanol, re-suspended in 50 µl ddH2O and stored at -20°C before use in the PCR.

Figure 1: Map of Kharameh, Fars province, southern Iran
assay to detect *Leishmania*-specific kDNA. Only 5 µl portions of DNA extracts were processed for PCR.\textsuperscript{15}

**PCR Assay**

*Leishmania* typing was carried out using the standard PCR procedure as described by Azizi et al. (2008). A set of two primers (LINR4 and LIN17) were also designed within the conserved area of the minicircle DNA of *Leishmania* parasite. This set of primers including LINR4 (5'-GGG GTT GGT GTA AAA TAG GG-3'), and LIN17 (5'-TTT GAA CGG GAT TTCTG-3') was used in a PCR technique with a slight modification.\textsuperscript{10} Reference strains of *L. major* (MHOM/IR/54/LV39), and *L. tropica* (MHOM/IR/89/ ARD2) were used as standards. PCR protocol was used to amplify the variable region of the minicircle DNA of any *Leishmania* in the sand fly midgut and involved a one-round PCR. The amplification reaction mixture contained 250 µM of each deoxynucleoside triphosphate (dNTP), 1.5 mM MgCl₂, 1 unit Taq polymerase (Cinagene, Tehran), 1 µM primer LINR4, 1 µM primer LIN17 and 5 µl of DNA extract in 1X PCR buffer (Cinagene, Tehran, Iran), in a final volume of 25 µl. This reaction mixture was incubated in a CGI-96 thermocycler (Palm-cycler, CG1-96, Genetics Biotech Asia, India) set to run for 5 min at 94°C, followed by 30 cycles, each one consisting of 30 s at 94°C, 30 s at 55°C, 60 s at 72°C, and a final extension at 72°C for 10 min and kept at 4°C. Negative controls (DNA extracts from male sand flies and aliquots of ddH₂O) were run in each PCR protocol to detect possible contamination which could cause false positive results.\textsuperscript{10}

**Agarose-Gel Electrophoresis**

A 5 µl sample of each PCR end product was subjected to electrophoresis in 1.5% agarose gel. The DNA bands were stained with 1% ethidium bromide, visualized on an UV transilluminator and compared with molecular-weight markers and the relevant second-round products for the *L. major* and *L. tropica* standards.

**Results**

In this study, among 1549 sand flies collected, four species of *Phlebotomus* and six species of *Sergentomyia* were identified to comprise *P. papatasi*, *P. bergeroti*, *P. sergenti*, *P. alexandri*, *Sergentomyia antennata*, *S. clydei*, *S. Sintoni*, *S. theodori*, *S. baghdadis*, and *S. Squamipleuris* (Table 1).

Monthly activity of the dominant species (*P. papatasi*) and the total flies are shown in Table 2. To determine the sand flies naturally infected by *Leishmania* spp., 188 female sand flies consisting of 145 *P. papatasi*, 29 *P. sergenti*, and 14 *P. alexandri* were dissected and evaluated microscopically using Giemsa-stained slides. Natural infections of two out of 145 (1.38%) *P. papatasi* were positive for *Leishmania major* (Figure 2).

**Discussion**

Cutaneous leishmaniasis is the main common vector-borne disease problem in Iran, where most cases known as zoonotic CL (ZCL) are caused by *L. major*.\textsuperscript{6,17} Fars province is enriched in sand flies fauna because of its high bio-ecological diversity. *P. major*, *P. keshishiani*, *P. alexandri*, and *P. papatasi* have been described as the vector(s) of visceral leishmaniasis and ZCL in this province, respectively.\textsuperscript{3,19} *P. papatasi* has been described as the main and proven vector of *L. major* in all endemic foci of this province.\textsuperscript{3,19} In this study, *P. papatasi* was the main species (53.45%). Moreover, the high activity levels of sand flies were observed during June and September.

Due to high bio-ecological diversity of sand flies, many species of *Phlebotomus* such as *P. papatasi*, *P. major*, *P. keshishiani*, and *P. alexandri* are reported as the vector(s) of CL and VL in this focus.\textsuperscript{7,18}

Detection of *Leishmania* vectors and their biology is necessary for planning *Leishmania*control strategies (Killick-Kendrick, 1999). Molecular-based methods using PCR are well-organized and reliable.
tools for the detection of Leishmania spp. They are more sensitive and specific than microscopic methods (Aransay et al., 2000). Although molecular methods such as PCR methods and sequencing have several advantages and drawbacks, when they are associated with morphological characterization of the sand flies, they will be a powerful epidemiological tool in determination of the number of sand flies infected with Leishmania spp. in nature (Aransay et al., 2000).

On the basis of our findings, this is the first molecular detection of L. major within naturally infected P. papatasi as the main vector of ZCL in this region of Fars province, southern Iran.

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Conflict of Interest: None declared.

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