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Molecular characterization of *Leishmania* parasites in naturally infected sand flies from the endemic focus of Kerman City, Southeastern Iran

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ABSTRACT

Objective: To identify the etiological agent, host and vector of anthroponotic cutaneous leishmaniasis in Kerman City, Southeastern Iran, using nested PCR and restriction fragment length polymorphism-PCR techniques.

Methods: Conducting this cross-sectional study in Kerman City from March to November 2014, we collected and morphologically identified 1 075 sandflies. The phlebotomine sand flies were then molecularly examined for harboring *Leishmania* parasites and blood meal preference using nested PCR and restriction fragment length polymorphism-PCR techniques respectively.

Results: *Phlebotomus sergenti* (*P. sergenti*) and *Phlebotomus papatasi* were found to comprise 94.3% and 5.7% of catches respectively. Nested PCR assay, applied for kDNA minicircles amplification, detected *Leishmania tropica* in *P. sergenti* at the rate of 3.6%. Also, restriction fragment length polymorphism-PCR assay on mtDNA fragments demonstrated that 41.8% of *P. sergenti* population preferred to feed on human blood rather than other animals.

Conclusions: This is the first study to provide molecular bases for incriminating *P. sergenti* as the main vector of *Leishmania tropica*, the causative agent of anthroponotic cutaneous leishmaniasis, in Kerman City. This study emphasized the high predominance, strong anthropophilic behavior and peridomicile adaptation of *P. sergenti* population in the focus.

1. Introduction

Despite considerable progress in the global fight against infectious diseases, some, including leishmaniasis, remain significant public health concern in many parts of the world including Iran[1]. The World Health Organization recognizes leishmaniasis to be an important parasitic disease among six prevailing in tropical and subtropical regions. Leishmaniasis is endemic in 88 countries with an estimated of 12 million cases worldwide in addition to about 2 million new cases each year, 90% of which occur in Afghanistan, Algeria, Brazil, Iraq, Peru, Saudi Arabia, Syria and Iran[2,3].

Both psychodid genera, *Phlebotomus* in the old world and *Lutzomyia* in the new world, include 81 species and subspecies of sand flies which are incriminated in transmitting 30 species of *Leishmania* to mammals[4,5]. In Iran, out of 54 species of sand fly, 45 species are involved in leishmaniasis transmission, 27 of which belong to the genus *Phlebotomus* and 18 others to the genus *Sergentomyia*[6-9].

Cutaneous leishmaniasis is still a major health problem in many urban, suburban and rural areas in 17 out of 31 provinces of Iran. Despite years of efforts by public health authorities to prevent and control the disease, the country has witnessed a recent cutaneous leishmaniasis resurgence and expansion[10]. About 20 000 new cases of leishmaniasis are reported each year, including anthroponotic cutaneous leishmaniasis (ACL), zoonotic cutaneous leishmaniasis and visceral leishmaniasis, although the true figure is believed to be 4–5 times more[3]. In some parts of the country where malaria

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prevention is a priority, the ACL has retreated due mainly to malaria control measures. However, in many other active foci, such as Tehran, Mashhad, Isfahan Shiraz and Kerman, the disease remains problematic[11]. Kerman has always been ranked the first amongst provinces of high-prevalence rate of ACL where many of its endemic foci including Kerman City has witnessed ACL resurgence as per recent national reports[12-14].

Detection of *Leishmania* parasites in unfed parous *Phlebotomus* species can be taken as a key epidemiological evidence on an established disease-vector relationship in a given foci in a bid to facilitate the disease control measures. However, this is not always an easy task using the conventional microscopic or serological methods. This study aimed at application of sensitive and specific molecular methods to identify *Leishmania* species, vectors and the feeding behavior of sandflies in Kerman City where leishmaniasis exhibits changing epidemiological patterns[7]. This is the first study to be undertaken in Kerman City to elucidate the main causative agent of cutaneous leishmaniasis and its main vector using nested PCR and restricted fragment length polymorphisms (RFLP)-PCR techniques.

2. Materials and methods

2.1. Study area and sand fly collection

This descriptive cross-sectional study was carried out from March to November 2014 during which 1 075 sandflies were collected using sticky traps (white papers 20 cm × 30 cm coated with castor oil) as well as Centers for Disease Control light traps. Samples were collected from different areas in Kerman city, including indoor and outdoor places. For PCR-based techniques the samples were kept in 70% ethanol and transferred immediately to laboratory and stored at –20 °C[15,16].

2.2. Sand fly identification

All sand flies were washed in 1% detergent (washing-up liquid) solution for 5 min and dissected in drops of sterile phosphate buffered saline (pH 7.2). The head and genitalia were mounted on a slide in Puri's medium for morphological identification using Iranian morphologic keys[17-19]. The remainder of the body of engorged and unengorged females was placed and dried overnight in 1.5 mL Eppendorf tubes before being subjected to DNA extraction[20,21].

2.3. DNA extraction

DNA was extracted from dissected thorax and attached abdomen of sand flies using AccuPrep® Genomic DNA Extraction Kit; Cat No.: K-3032 according to the manufacturer's instructions (Bioneer, South Korea). Briefly, the tissues were transferred into 1.5 mL tubes and grinded in liquid nitrogen before being homogenized in lysis buffer (200 μL tissue lysis buffer) and proteinase K solution (20 μL). The homogenates were then subjected to 1 h incubation at 60 ˚C interrupted by vortexing at 30 min intervals. Binding buffer (200 μL GC-rich PCR buffer) was then applied to the lysates followed by incubation at 60 ˚C for 10 min, addition of isopropanol (100 μL) and gentle mixing (pipetting). The lysates were finally subjected to steps of filtration through binding columns, washing and elution before being stored at 4 ˚C for later analysis.

2.4. Nested PCR for parasite identification

Nested PCR approach was applied for the detection and identification of the *Leishmania* parasites according to the Noyes *et al*. method[22]. To carry out this assay 65 unengorged parous females of sand flies were dissected and alimentary tracts were used for DNA extraction. For the first-round PCR, external primers CSB1XR (ATTTTTCGCGATTTTCGCAGAACG) and CSB2XF (CGAGTAGCAGAAACTCCCGTTCA) and for the second-round PCR, internal primers 13Z (ACTGGGGGTTGGTGTAAAATAG) and LiR (TCGCAGAACGCCCCT) were used to amplify variable minicircles of *Leishmania* kDNA. Characterization of the parasite resulted in generation of amplified fragments of about 750 bp for *Leishmania tropica* (*L. tropica*) and 560 bp for *Leishmania major*. The profile was visualized by 1.5% agarose gel electrophoresis (Uvitech, Cambridge, UK), using a 100 bp DNA ladder at 260 nm wavelength. A negative control as well as a positive control of *L. tropica* (MHO/Sudan/58/OD strain) was also run in each round of electrophoresis[23-26].

2.5. RFLP-PCR for blood meal preference determination

Host preference of engorged *Phlebotomus* females was examined by applying PCR to amplify two regions of *Cyt b* gene of hosts' mitochondrial DNA producing fragments of 358 bp and 623 bp for human and animal bloods respectively as described in Maleki-Ravasan *et al*.[27]. To amplify a 358 bp segment corresponding to human mtDNA *Cyt b* gene, a pair specific primers L14841 (5'-CCATCCAACATCTCAGCATGATGAAA-3') and H15149 (5'-CCCCTCAGAATGATATTTGTCCTCA-3') were used. However, amplification of animal segment of 623 bp was carried out using the following forward and reverse primers respectively, 5'-TGAGGACAAATATCATTCTGAGG-3' (UNFOR403) and 5'-GGTTGTCCTCCAATTCATGTTA-3' (UNREV1025). PCR solution contained 2.5 μL PCR buffer, 3 mmol/L $MgCl_2$, 200 μmol/L dNTPs, 0.4 μmol/L of each primer, 1–2 IU of *Taq* DNA polymerase, and 15 μL of extracted DNA and distilled water in a final volume of 25 μL. The PCR products were then digested with either *Xho*I or *Hae*III and analyzed against a DNA size marker by electrophoresis on 2% agarose gels stained with 2 mg/mL ethidium bromide and visualized under ultraviolet light. To prevent evaporation, the solution was over laid with 10 μL mineral oil. Samples were finally incubated at 95 ˚C

for 10 min, followed by 40 cycles each at 95 ˚C for 45 s, 58 ˚C for 45 s, and 72 ˚C for 45 s, and a final extension at 72 ˚C for 5 min. A negative control was also run each time[28-30].

3. Results

Table 1 shows that about 70% of the most abundant sand fly species, *Phlebotomus sergenti* (*P. sergenti*), were caught from outdoor places exhibiting primarily exophilic behavior. However, the less abundant species, *Phlebotomus papatasi* (*P. papatasi*), appeared to be also exophilic as 66% of them were trapped outdoors. Whereas 82% of collected *P. sergenti* were males, the relative abundances of both sexes of *P. papatasi* were almost similar with slight inclination towards male richness.

Table 1
Sand fly species collected from indoor and outdoor places and their relative abundances and sex ratios. *n* (%).

Sand flies	Female	Male	Indoor	Outdoor	Number of samples
P. sergenti	183 (18.0)	831 (82.0)	299 (29.4)	715 (70.6)	1 014 (94.3)
P. papatasi	28 (46.0)	33 (54.0)	21 (34.0)	40 (66.0)	61 (5.7)
Total	211 (20.0)	864 (80.0)	319 (30.0)	756 (70.0)	1 075

In this study, a sample of 280 female sand flies pooled from all season catches were dissected and their midguts were microscopically examined for leptomonad infection. None of these sandflies was found infected. However, application of nested PCR assays to DNA samples extracted from alimentary tracts of unengorged parous females of both *P. sergenti* and *P. papatasi* revealed the presence of *L. tropica* genome in *P. sergenti* at the rate of 3.6 % (Figure 1).

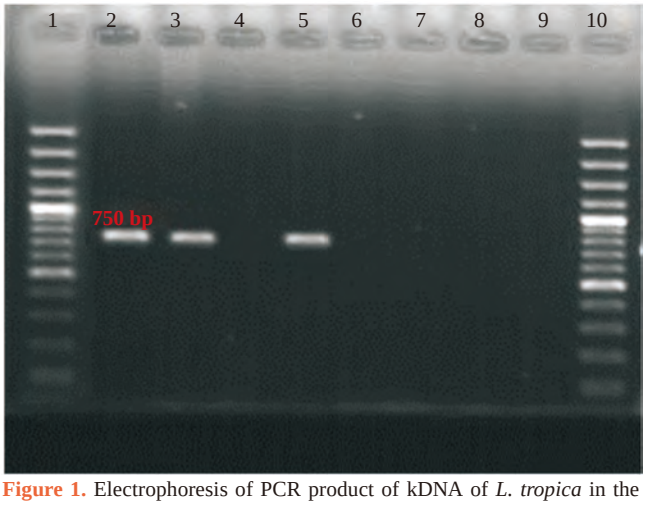

Figure 1. Electrophoresis of PCR product of kDNA of *L. tropica* in the alimentary tract of *P. sergenti* sand flies.
Lane 1: 100 bp lader; Lanes 2 and 3: Positive samples of *L. tropica* (750 bp); Lane 4: Negative control; Lane 5: Positive control (MHO/Sudan/58/OD strain); Lanes 6 to 9: Negative samples of *L. tropica.*

On the other hand, RFLP-PCR assays on DNA extracted from engorged *Phlebotomus* females demonstrated that *P. sergenti* has fed on both human and animals. Gel electrophoresis as in Figure 2 showed the presence of mtDNA fragments of 623 bp and 358 bp corresponding to animal and human *Cyt b* genes digested by *Hae*III and *Xho*I enzymes respectively. The result indicated that 41.8% of *P. sergenti* sand flies feed on human blood.

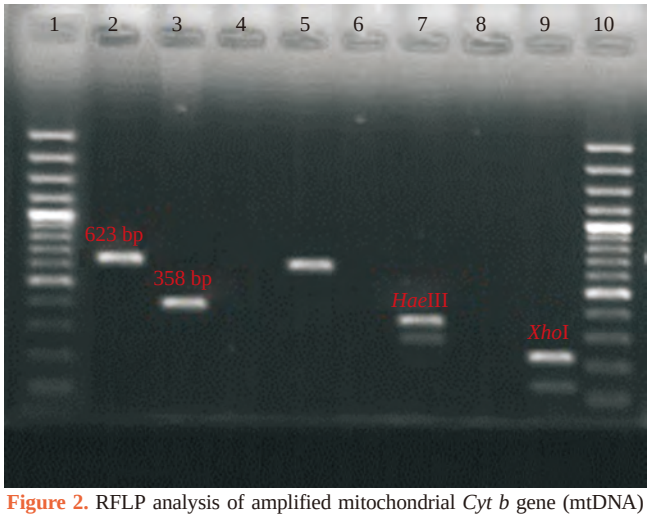

Figure 2. RFLP analysis of amplified mitochondrial *Cyt b* gene (mtDNA) digested with restriction enzymes *Hae*III and *Xho*I.
Lanes 1 and 10: 100 bp laders; Lane 2: Animal DNA fragment of blood meal (623 bp); Lane 3: Human DNA fragment of blood meal (358 bp); Lane 4: Negative control; Lane 5: Positive control (animal blood); Lanes 6 and 8: Negative control; Lane 7: Animal DNA digested by *Hae*III (300 bp and 323 bp); Lane 9: Human DNA digested by *Xho*I (143 bp and 215 bp).

4. Discussion

The province of Kerman witnesses recurrent earthquakes, as in recent years, due to its geological position on tectonically active faults in south east of Iran. This has strongly complicated epidemiological patterns of leishmaniasis in the region as an endemic focus and resulted in numerous ACL epidemics and resurgences[14]. Various risk factors and man-made determinants such as poor health and low socioeconomic status in suburbs in addition to the transit of infected immigrants from neighboring countries have significantly contributed to the changing epidemiological pattern and occasional incidence fluctuation of leishmaniasis in Kerman City[11]. This has urged the local authorities to undertake surveillance and validation researches alongside necessary preventive and control measures to keep pace with the changing scene of the disease epidemiology.

This cross-sectional study was conducted to help elucidating the current bionomics and epidemiological indices of sand fly vectors of leishmaniasis in Kerman City. Throughout this study, the only trapped sand fly species were *P. sergenti* and *P. papatasi* which exhibited low (≈ 6%) and high abundances (≈ 94%) respectively. The sand fly fauna reported by Svobodová *et al*. in cutaneous leishmaniasis focus of Sanliurfa (Turkey), also, comprised predominantly (> 99%) only these two phlebotomine species[31]. Similar relative abundance (95%) was reported for *P. sergenti* in indoor catches in Kerman by Seyedi-Rashti *et al*.[32]. The very low species diversity of sand flies in this study may be attributed to Kerman's geographical location in a semi-arid plain. However,

both sand fly species showed higher abundances in peridomiciliary than intradomiciliary areas, revealing their exophilic behaviors as demonstrated by other authors[33,34]. Given the high endemicity of *L. tropica* (95.6%) and high abundance of *P. sergenti* as a main vector in ACL prone foci in Kerman Province, the biological interaction of both agents remains to be molecularly established in Kerman City[10,11]. In this study, in order to provide evidence on the development of promastigotes stages in *P. sergenti*, engourged parous female sand flies were subjected to nested PCR assays. Our results showed that 3.6% of *P. sergenti* females harbored *L. tropica* genomes which proved its involvement as the main biological vector of ACL in Kerman City. This finding was emphasized by the fact that none of *P. papatasi* females were found to be infected with the same parasite. Aghaei *et al.* has recently reported 2.9% infection of *P. sergenti* females with *L. tropica* parasite in another focus of ACL in Kerman Province[21].

On the other hand, RFLP-PCR analysis of mitochondrial *Cyt b* gene (mtDNA) of bloods imbibed by sand flies demonstrated that 41.8% of *P. sergenti* sand flies fed on human blood. In a recent study by Ajaoud *et al*., the phlebotomine was also found to be the main vector of leishmaniasis in ACL foci of Morocco, feeding primarily on human blood at a higher percentage of 64%[35]. Although, the birds comprised the second source of blood meals for *P. sergenti* in the aforementioned study, the phlebotomine may not be considered to be ornithophilic as reported by Svobodová *et al.*[31]. The feeding behavior of *P. sergenti* in exploring various sources of blood meals around breeding sites including human and animals as in this study in one hand, and the reported predominance of this species from numerous ACL foci in Asia, middle east and north and east Africa in other hand, may rather provide evidence on its ecological compatibility and/or intraspecific variability which possibly affect its vector competence for *L. tropica*[36-39]. In conclusion, taking into consideration the high relative abundance of *P. sergenti* in all seasonal activity periods and its strong preference of human blood meal as well as its infection with *Leishmania* promastigotes, we may molecularly incriminate *P. sergenti* as being the main vector of ACL in Kerman City. This is the first study to molecularly identify parasite-vector-host interactions involved in ACL transmission in Kerman City.

Conflict of interest statement

We declare that we have no conflict of interest.

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