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Molecular Characterization of *Leishmania* Infection in Sand flies From Sistan Va Baluchistan Province, Southeastern Iran

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ABSTRACT

Background: Cutaneous leishmaniasis (CL) is a zoonotic disease that is caused by various species of the genus *Leishmania*. The disease is considered a major health problem in different areas of Iran and is an endemic disease in rural areas of Mirjaveh, Chabahar, and Konarak Counties, Sistan Va Baluchistan Province.
Objectives: The aim of this study was to identify *Leishmania* species that was isolated from potential sand fly vectors by molecular analysis in Chabahar County.
Materials and Methods: To collect Sand flies, sticky traps were placed at the entrance of rodents burrows in Dashtiyari division of Chabahar County, where CL is endemic. Freshly collected Sand flies were identified with regard to species, dissected in normal saline using binocular, and examined for leptomonads under a microscope. Leptomonads from the Sand flies were used to inoculate the base of Balb/c mice tails subcutaneously; after an incubation period and the development of lesions, the parasites were transferred to NNN + LIT medium culture. The harvested *Leishmania* parasites were subjected to DNA extraction and analyzed by random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR).
Results: DNA from *Leishmania* species from *Phlebotomus papatasi* and *P. salehi* Sand flies produced distinctive patterns of bands of *L. major* with all primers. However, the products at approximately 2100 bp and 800 bp that were amplified with primer 329 were stable and reproducible in all assays. This is the first report on the isolation and identification of *L. major* in *P. salehi* from Iran and *P. papatasi* from Sistan va Baluchistan.
Conclusions: The study shows that *P. papatasi* and *P. salehi* Sand flies play a major role in the maintenance and transmission of disease to humans in this area.

Implication for health policy/practice/research/medical education:
Identification of *P. papatasi* and *P. salehi* Sand flies as the insect vectors as well as the *Leishmania* species they harbor would be the key elements in understanding the epidemiology of leishmaniasis and its control in Chabahar County.

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1. Background

Cutaneous leishmaniasis (CL) is a zoonotic disease that is caused by various species of the genus *Leishmania*. The disease is considered a major health problem in different areas of Iran. At present, about 54 sand fly species are known to exist in Iran; however, only 12 of them can transmit a particular *Leishmania* species (1-4). Identification of potential sand fly vectors as well as their associ-

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ated *Leishmania* species and their comparison with those obtained from mammal hosts and humans provide the major elements to hypothesize on transmission cycle of *Leishmania spp*. in an endemic area.

The advent of molecular biology has provided various DNA-based methods for the identification of *Leishmania* species from human patients, animal reservoirs, and sandflies (5-9). Amplification of target sequences, followed by sequencing or digestion with restriction enzymes and the design of species-specific primers, has enabled *Leishmania* species to be identified through the visualization of DNA fragments of known sizes (10-14). However, these methods require prior knowledge of the target sequences, and in some cases, they do not discriminate sufficiently between closely related species (15). Random amplified polymorphic DNA (RAPD) markers are DNA fragments from PCR amplification of random segments of genomic DNA using a single random primer. Unlike conventional PCR methods, RAPD does not require any specific knowledge of the DNA sequence of the target organism. This method has been used successfully to identify *Leishmania* species in Iran (2, 7).

2. Objectives

Despite the presence of CL in Sistan Va Baluchistan, no information is available on the prevalent parasite species in sandfly vectors in this area, due in part to difficulties inherent in performing field studies here. The aim of this study was to identify *Leishmania* species from potential sandfly vectors by molecular analysis in Chabahar County.

3. Materials and Methods

3.1. Study Area

Chabahar is located on the Oman Sea littoral in far southeastern Province of Sistan va Baluchistan, southeastern Iran. Chabahar County is a low landing area, with geographical coordinates of 25° 17' North, 60° 38' East. The climate of this county is classified as very warm desert due to its low annual precipitation. The average annual temperature and humidity are 36.4°C and 75.9%, respectively. Chabahar County covers an area of 24,729 Km^2, with a population of about 230,000. The majority of the city's inhabitants are ethnic Baluch, who speak the Baluchi language. This study was carried out in Dashtiyari Division of Chabahar, where cutaneous leishmaniasis has emerged as an endemic disease.

3.2. Sample Collection

Sand flies were collected bi-weekly using sticky traps that were placed at the entrance of rodent burrows during the spring and summer of 1997.The traps were placed at sunset and collected at dawn. All, Sand flies, including gravid, semi-gravid, blood-fed, and non-fed Sand flies, were washed in 1% detergent solution for 5 min and dissected in a drop of normal saline using minute pins under binocular Heads; the last abdominal segments were used for morphological identification using entomological keys that have been described by others (16). The leptemonads from individual infected sandflies were used to inoculate the base of Balb/C mice, which was the only available method of isolating and maintaining the parasites under field conditions. After an incubation period (3 to 3.5 months) and the development of lesions, parasites from the lesions were transferred to Novy-MacNeal-Nicolle (NNN) and liver infusion broth tryptose (LIT) cultures and then to RPMI 1640 that contained 10% heat-inactivated fetal calf serum. The parasites were grown in RPMI 1640 medium, harvested at the stationary phase, and kept at -20°C until use.

3.3. DNA Extraction

The harvested parasites were washed in cold sterile PBS (pH 7.2). The recovered pellet was re-suspended in 300 μL cell lysis buffer (50 mM NaCl, 50 mM EDTA, 1%SDS, and 50 mM Tris- HCl, pH 8.0) with 20 μL of 20 mg/mL proteinase K and incubated at 55°C overnight. The lysate was extracted with phenol/chloroform followed by ethanol precipitation. The DNA was re-suspended in distilled water, and working solutions were adjusted to 5 ng/μL in distilled water.

3.4. RAPD-PCR Analysis

The RARD-PCR assays were performed as described (4, 17). Each 25-μL reaction contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 200 μM of each dNTP, 50 pmol of one of the primers (*Table 1*), 1 unit of *Taq* DNA polymerase, and 10 ng of DNA. Reactions were overlaid with 25 μL of mineral oil and amplified with the following program: one cycle at 94°C for 5 min followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 37°C to 38°C for 1 min, and extension at 72°C for 2 min and a final extension step at 72°C for 5 min. A negative control, containing all components except DNA, was included in all assays. Amplification products (8 μL) were run on a 1.2% agarose gel containing ethidium bromide and visualized on a UV transilluminator.

Table 1. The Primers Used in RAPD-PCR Analysis

	Code	Sequence	GC%
1	AB1-07	GGT GAC GCA G	70
2	327	ATA CGG CGT C	60
3	329	GCG AAC CTC C	70
4	333	GAA TGC GAD G	60
5	335	TGG ACC ACC C	70

4. Results

P. papatasi and *P. salehi* Sand flies were the only species that were obtained from the burrows of rodents (*Figures 1 and 2*). Fourteen of 667 (2.1%) *P. papatasi* and 5 of

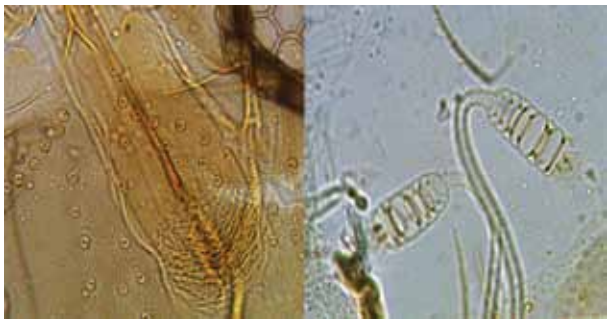


Figure 1. The Pharynx (Left) and Spermatheca (Right) of *P. papatasi*

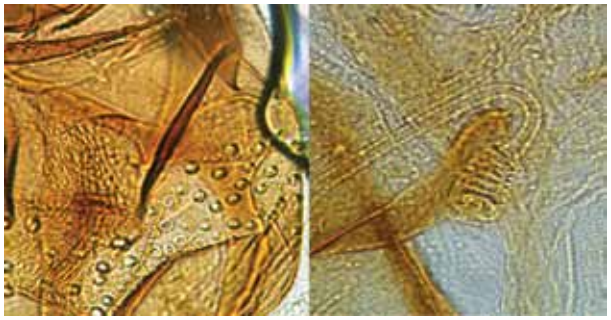
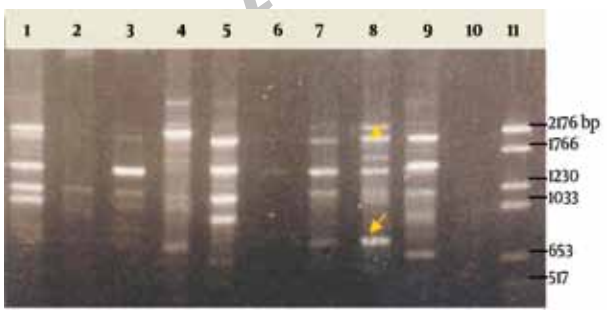


Figure 2. The Pharynx (Left) and Spermatheca (Right) of *P. salehi*

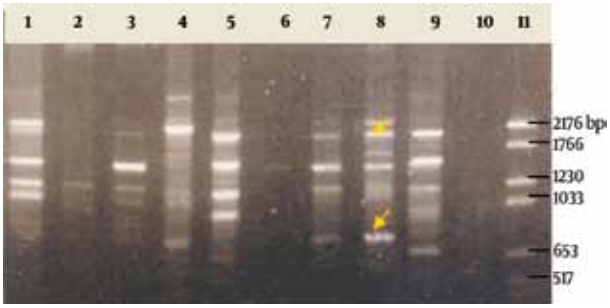
465 (1.08%) *P. salehi* Sand flies showed evidence of leptemonad infection. The leptomonads from infected *P. papatasi* and *P. salehi* Sand flies produced lesions in 7 and 3 Balb/C mice, respectively. The parasites from 10 animals were grown successfully in culture medium. DNA amplification by RAPD-PCR of DNA from 10 isolates yielded distinctive bands that were characteristic of *L. major* with all primers, but the approximately 2100-bp and 800-bp products that were amplified with primer 329 were stable and reproducible in all assays (*Figures 3 and 4*). This is the first report that implicates *P. salehi* in Iran and *P. papatasi* from Sistan va Baluchistan as 2 vectors of zoonotic cutaneous leishmaniasis.

Figure 3. Electrophoresis of Gene Fragments Amplified With RAPD-PCR Using the Primer 329.



Lane 1 and 4, Different Strains of *L. infantum*; lane 2, *L. major*; lane 3, the strain originated from *P. papatasi* (This study) ; lane 5, *L. major* standard strain; lane 6, *L. infantum* standard strain ; lane 7, *L. tropica* standard strain; lane 8, DNA size marker VI (Roche, Germany)

Figure 4. Electrophoresis of Gene Fragments Amplified With RAPD-PCR Using the Primer 329.



Lanes 1, 3, 6 and 7, different strains of *L. major*; lanes 2, 4, 5, and 9, different strains of *L. infantum*; lane 8, the strain originated from *P. salehi* (This study) ; lane10, control; lane 11, DNA size marker VI (Roche, Germany)

5. Discussion

In Iran, there are 54 reported species of Sand flies, only 12 of which play a role in the transmission of disease. *P. papatasi* and *P. salehi* are the main and secondary vectors of zoonotic cutaneous leishmaniasis (ZCL) that is caused by *Leishmania major* in Iran. *Leishmania major* has also been isolated from *P. caucasicus* and *P. ansarii* that dwell in rodent burrows. The geographical distribution of rodents that serve as reservoir hosts for ZCl have been described. *Rhombomys opimus*, the great gerbil (Cricetidae; Gerbillinae), is known as the main reservoir of ZCL in central and northeast Iran. *Meriones libycus,* the *Libyan jird* (Cricetidae; Gerbillinae), is the primary reservoir of ZCL in some areas of central and southern Iran. In the south and southwest of the country, including the Iran-Iraq border, the reservoir is *Tatera indica L*, the Indian jird (Cricetidae: Gerbillinae). In Baluchistan of Iran, *M. hurrianae,* the Indian desert jird (Cricetidae: Gerbillinae), is the suspected reservoir host for ZCL (18-21).

Control of ZCL in endemic areas requires a thorough knowledge of *Leishmania* ecology and epidemiology of the disease. The identification and comparison of *Leishmania* species from Sand flies, mammalian hosts, and humans can allow us to hypothesize on the transmission cycles of *Leishmania spp*. in endemic areas. PCR-based diagnostic techniques have shown high sensitivity in detecting and identifying *Leishmania* DNA in mammalian hosts, Sand flies, and humans. Application of RAPD-PCR to identify *Leishmania* species in Iran has been demonstrated. In separate studies, isolated *Leishmania* parasites were identified successfully at the species level using RAPD-PCR (4, 5).

Recently, 3 cutaneous leishmainasis foci Chabahar, Konarak, and Mirjaveh were identified in Sistan va Baluchistan Province. The most prevalent sandfly species in our study area (Chabahar) were *P. papatasi* and *P. salehi*. In this study, *Leishmania* parasites were isolated from both species and identified as *L. major* by RAPD-PCR. *P. papatasi* is a proven vector of *L. major* in different areas of

the old world, but there is no record of *Leishmania* infection in *P. salehi* except in India, where 4 *P. salehi* Sand flies were shown to be infected with *Leishmania;* the specimens were captured from burrows of the Indian desert jird *Meriones hurrianae* in Rajestan Province (22).

P. salehi is believed to transmit CL in rodents in India. However, isoenzyme analysis of an isolate from a Jird and a *P. salehi* sandfly showed that they were different zymodemes of *L. major (23). P. salehi* is also a suspected vector of cutaneous leishmaniasis in Pakistan, but its vectorial role has yet to be established (24). The species may be responsible for the maintenance and stability of infections in rodents in Chabahar, but further studies are needed to confirm its function as a vector.

Culturing *Leishmania* parasite from Sand flies, reservoir hosts, and humans, followed by RAPD-PCR analysis, is a useful method for the preliminary identification of *Leishmania* species.

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