

## Life cycle of cutaneous leishmaniasis in Larestan, southern Iran

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### ABSTRACT

**Background:** Cutaneous leishmaniasis (CL) with diverse clinical manifestations is prevalent and remains a major public health problem in Iran, while its incidence has been doubled over the last decade. This study was performed for the first time in Larestan, Fars province, located in southern part of Iran to determine infectious patterns in the reservoir hosts and sand fly vectors using parasitological and molecular methods.

**Materials and methods:** Thirty two rodents and 156 sand flies were evaluated for the patterns of *Leishmania* infection. PCR was performed to characterize the parasites using the culture of parasite in Evans medium.

**Results:** *Tatera indica*, *Gerbillus* spp. and *Meriones crassus* were the trapped animals and *Phlebotomus papatasi* was the trapped fly. *L. major* was isolated and identified as the causative agent of CL from *T. indica*, *Gerbillus* spp and two female *Ph. papatasi* (Alamarvdasht). Among the trapped rodents, four *T. indica* were positive for *L. major* in Larestan region including Alamarvdasht town (2 males), and Lamerd town (2 females). *L. major* was also isolated from two female *Gerbillus* spp in Lamerd and Lar towns in Larestan region. No infection was detected in *M. crassus* in the area.

**Conclusion:** This is the first report detecting *L. major* in naturally infected *T. indica*, *Gerbillus* spp and *Phlebotomus papatasi* in Larestan region.

**Keywords:** *Cutaneous leishmaniasis, Life cycle, Southern Iran.*

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### INTRODUCTION

The leishmaniasis are considered as a group of globally widespread parasitic diseases caused by different species of *Leishmania*. Twenty-two species of *Leishmania* were reported to be pathogenic for human (1,2). In the new world, more than 40 species of mammals are known to

harbor *Leishmania* spp., however, few species are considered to be important in the natural transmission cycle (3). Identification of the natural hosts of *Leishmania* is important to determine the natural cycle of the parasite. The important role of rodents as reservoirs in the epidemiology of the disease has already been demonstrated by Hertig et al (4).

Cutaneous leishmaniasis (CL) due to *L. major*, is prevalent in the Middle East (5), and the *Psammomys obesus* was reported as the main

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reservoir host (1). *Meriones libycus* was dominant reservoir host in Jordan (6). In Southern Iran, the annual incidence of CL has gradually risen over the last decade (7). This trend may be due to spread of human populations into the habitats of the local vectors and the rodents as reservoir hosts (8). It is not always easy to detect leishmanial infections in wild rodents since the parasites may cause no or only minor skin lesions in the reservoirs (9). CL was previously reported in 11 of the 29 provinces of the country (10). It was more frequently reported in central parts of Iran showing the role of *R. opimus* and *M. libycus erythrosus* (11,12), in western Iran (13), in Khorasan province (14) and in Tehran (15), showing the role of *Tatera indica*, *M. hurrianae*, *Nesokia indica* and *M. libycus erythrosus* as reservoir hosts of CL in south east of Iran (16). CL was also described in Southern Iran by Nadim et al in 1977 (17). In this area, L. major infection was also reported in *M. libycus* (7,18). The present study was conducted to determine the reservoirs and vectors of CL in Larestan region in Southern Iran.

## PATIENTS and METHODS

### Study area

The Fars province is located in southern Iran and has an area of about 53000 miles (8% of Iran's total area) and is at 27°3'42" longitude east. It is mountainous with an average altitude of 5000 feet above sea level. The climate is quite dusty and dry, with warm summers, generally mild winters, and a great deal of sunshine throughout the year (19). Larestan is located in southeastern part of the province with an area of 26046.57 km<sup>2</sup> including six towns with a total population of 100,000.

### Rodent trapping

The rodents were captured according to standard techniques for capturing small mammals (20). They were caught in live wire traps (35×12×12cm), baited with bread and toasted peanuts.

Totally, in a one-year period (April 2004 to April 2005) 100 traps were set in agricultural plantations surrounding the houses. The traps were then checked each subsequent morning. The identification of the specimens was based on specific taxonomic criteria (21). The captures were conducted under permission of Fars Environment Protection Organization and Ethics Code of Iran Veterinary Organization.

### Collection of sand flies

Sand flies were collected biweekly from outdoor (rodent burrows, cracks in the walls, bird holes, etc.) fixed locations in all 6 towns of Larestan region using 50 sticky traps (caster oil coated white papers 20×32cm). To determine the species, sand flies were mounted in Puris medium (22) and identified after 24 hours using the keys of Theodor and Mesghali (23) and Lewis (24).

### Preparation of smears and culture

Impression smears were provided from the ear, tail and foot of the rodents and from any patent skin lesions (25), stained with Giemsa, and examined under a compound light microscope for the amastigote form. Trapped animals were euthanized under chloroform anesthesia according to Iran Veterinary Organization Ethics and examined for any signs of cutaneous lesions and then necropsied. Sampling and touch smear from skin of ear, tail and foot-pad of rodents were provided and inoculated into the Evans medium and incubated in 25°C for 1-4 weeks with weekly subcultures (26). Resulting isolates were coded and cryopreserved in liquid nitrogen until required for identification. Samples were examined regularly to monitor the growth and presence of contaminations. The contaminated cultures were discarded. Mass cultivation of the organism was carried out on RPMI medium containing 15% fetal calf serum (Gibco). The cultures were harvested at the end of the logarithmic phase of the growth and the number of the organisms adjusted to 1-1.5×10<sup>7</sup>/ml. The cultures were centrifuged at

2000g for 20 minutes at 4°C. The supernatant was discarded and the pellet of promastigotes washed three times by resuspension and recentrifugation in cold-proline balanced salt solution. Two impression smears were provided from filings of Department of Parasitology of Shiraz Medical School in the area of our study including two skin lesion smears from two women lived in Alamarvdasht town.

#### DNA extraction

The precipitate of the cultivated sample was diluted by double distilled water (1:10) and DNA of each cultivated sample was extracted by adding proteinase K to 5 ml of each sample. Then lysis buffer was added (50 ml of Tris-HCl, pH=7.6; 1mM of EDTA, pH=8.0; 1% Tween 20, 8.5 ml of proteinase K solution, 19 mg/ml) and incubated for 24 hours at 37°C. The lysate was then extracted twice with phenol/chloroform/Isoamyl before the DNA was precipitated with absolute ethanol and resuspended in 100µL of double distilled water and stored at 4°C.

#### Polymerase chain reaction (PCR)

The set of primers LINR4 (forward: 5'-GGGGTTGGTGTAAAATAGGG-3'), LIN17 (reverse: 5'-TTTGAACGGGATTTCTG-3') was used for PCR (27). These primers were designed within the conserved area of the minicircle and contained conserved sequences blocks of CSB-3 and CSB-2, respectively. A reaction mixture containing 1.5mM of MgCl<sub>2</sub>, 200µM (each) of deoxynucleoside triphosphate, 2.5µL of 10X taq polymerase buffer, 1.5 unit of Taq DNA polymerase and 40pmol of each primer were used in a total reaction volume of 25µL including 5µL of DNA sample. The mixture was amplified in a programmable thermocycler (Thecne Cambridge, UK) for 5 min at 94°C (1 cycle) followed by 30 cycles at 94°C for 30 seconds, 52°C for 30 seconds and 72°C for 1 min followed by a final elongation at 72°C for 5 minutes (1 cycle) and kept at 4°C.

#### Agarose-gel electrophoresis

A 10-µL sample of the final PCR product was subjected to electrophoresis in 1.5% agarose gel, then 5µL of loading buffer was added to the product before electrophoresis and visualized under UV light with ethidium bromide. The WHO reference strains of *L. tropica* (MHOM/SU/71/K27), *L. major* (MHOM/SU/73/5ASKH) and *L. infantum* (MHOM/TN/80/IPT1) were obtained from Pasteur Institute, Tehran.

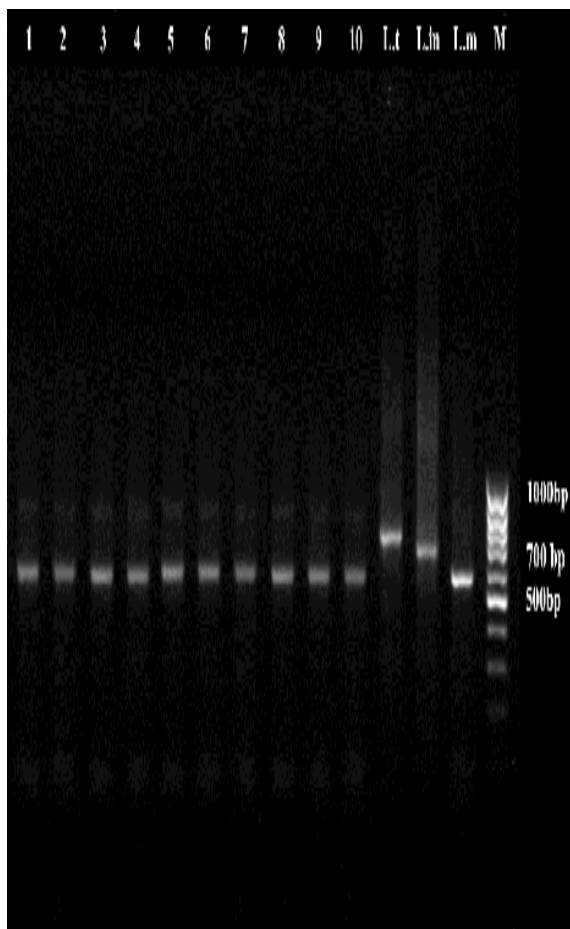
## RESULTS

#### Live trapping

The total number of rodents captured was 32, including three different species. The most abundant were *T. indica* (Indian gerbil) accounting for 20 specimens (Alamarvdasht=12, Lar=4, Lamerd=4) followed by *M. crassus* with 8 specimens (Lar=4, Lamerd=4). Only four specimen of *Gerbillus* spp. were captured in this study (Lar=2, Lamerd=2). Rodents were captured in agricultural plantations. Totally, 156 sand flies were collected from outdoors and identified as *Phlebotomus papatasi* (121 males and 35 females). The sand flies were captured in May.

#### Identification of parasites

*L. major* was isolated and identified as the causative agent of CL from 4 *T. indica*, 2 *Gerbillus* spp, 2 female *Ph. papatasi* (Alamarvdasht) and 2 women skin lesions (Alamarvdasht). Among the trapped rodents, 4 *T. indica* were positive for *L. major* in Larestan region including Alamarvdasht town (2 males) and Lamerd town (2 females). *L. major* was also isolated from 2 female *Gerbillus* spp in Lamerd and Lar towns in Larestan region. No infection was detected in *M. crassus* in the area. Figure 1 demonstrates agarose gel electrophoresis of PCR products obtained with primers specific for the *L. major* complex from rodents, sand flies and two human isolates, respectively.



**Figure 1.** Gel electrophoresis of PCR products of *Leishmania* parasites isolated from human, trapped rodents and *phlebotomus papatasi* using the primers LINR4 and LIN17 (M: Marker; L.t: *L. tropica*; L.m: *L. major*; L.in: *L. infantum*; 1 and 2: human isolates; 3 and 4: Sand fly isolates; 5: Lar, female, *Gerbillus* spp.; 6: Lamerd, female, *T. indica*; 7: Lamerd, female, *Gerbillus* spp.; 8: Alamarvdasht, male, *T. indica*; 9: Alamarvdasht, male, *T. indica*; 10: Lamerd, female, *T. indica*).

## DISCUSSION

By application of PCR method, *L. major* was isolated as the causative agent of CL in Larestan region, southern Iran in *T. indica*, *Gerbillus* spp, *Ph. papatasi* and two human isolates. In our area in southern Iran, Rassi et al (18) showed *L. major* infection in 6.8% of *Meriones libycus* in Arsanjan town as the principal reservoir of CL and Momenbellah-Fard et al (7) noticed *L. major*

infection in *Meriones libycus* in Marvdasht city. In Isfahan province (central parts of Iran), infection with *L. major* was noticed in three species of *Meriones libycus*, *Nesokia indica* and *Rhombomys opimus* (10,11,18,28-32).

In this province, *Phlebotomus papatasi* and *phlebotomus caucasicus* were reported as vectors of *L. major* (10,29-32). In our study in Larestan, the reservoir host of *L. major* were different from those reported in southern Iran in Arsanjan (18) and Marvdasht towns (7) which may be due to differences in plantation, weather, sunshine duration, humidity and type of soil in the area of our study, as Larestan region is a dry region with poor plantation and long and severe sunshine while lacking enough water supplies. *Tatera indica* and *Gerbillus* spp. were the reservoir hosts infected with *L. major* showing them as reservoir hosts for CL in the area. This is the first report of leishmaniasis in this region showing *L. major* as the source of infection in rodents, sand flies and human. Wasserberg et al. (33) showed infection with *L. major* in *Gerbillus dasyurus*, *M. crassus* and *P. obesus* demonstrating their role as reservoir hosts and their public health importance.

In Brazil, *R. rattus* and *T. apereoides* were the most abundant species presenting high prevalence of natural infection of leishmaniasis (34). Saliba et al (35) showed that none of *Gerbillus* spp. was infected with *L. major* while *P. obesus* was the main reservoir host for the parasite in Jordan. In Senegal, *L. major* was isolated from of *A. niloticus*, *M. erythroleucus* and *T. gambiana* reservoir hosts (36). Schlein and colleagues (37) showed infection with *L. major* in *M. crassus* and *P. obesus* as reservoir hosts and *Ph. papatasi* as the vector of CL along the Dead Sea region. Le Blancq and colleagues (38) identified *L. major* as the causative agent of CL in India from rodents (*R. opimus*, *P. obesus*, *T. gambiana*, *M. erythroleucus*, *Arvicanthis* spp), sand flies (*Ph. papatasi*, *Ph. Duboscqi*, *Ph. saheli*) and human isolates. Harrat et al (39) reported infection with *L. major* in reservoir hosts

(*Ps. Obesus*), vectors (*Ph. papatasi*) and human isolates in Algeria. *L. major* was isolated from *T. robusta*, *A. niloticus* and *M. natalensis* in Kenya as reservoir hosts of CL (40). *Arvicanthus niloticus* and *Ph. duboscqi* were also reported as reservoir and vector hosts of CL due to *L. major* in Kenya. Human isolates also showed a greater role in spread of the disease (41). Characterization of *L. major* in *M. natalensis*, *T. emini* and *A. kaiseri* as reservoir hosts of CL were demonstrated in Kenya by Githure et al (42).

Our results revealed that the isolates of *L. major* from reservoir hosts, vectors and the two human cases were identical using PCR method. This is the first report of *L. major* in Larestan region in Fars province located in southern Iran in wild rodents and sand flies. Rodent control is a challenge to the health authorities involved in the control of CL in southern parts of Iran. The agricultural and rural development in the area may lead to more human contact with the rodent-sand fly-rodent cycle of leishmania transmission. Rapid urbanization, construction of buildings in farms near the colonies of rodents, storage of waste materials around the town which are suitable for building nests by rodents, new agricultural projects in this area, existence of some animal shelters among old mud houses and socioeconomic changes in the area may increase the number of wild rodents and sand flies to provide a very efficient cycle for the transmission of the disease. Migration of refugees from Afghanistan has also provided suitable conditions for further spread of the disease (43).

This study showed that *T. indica* and *Gerbillus* spp. served as the reservoir host and *Ph. papatasi* as vectors for *L. major*. Taking these facts into consideration may help to clarify the key epidemiological parameters to be brought into consideration when planning preventive measures.

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