Vectors and reservoirs of cutaneous leishmaniasis in Marvdasht district, southern Islamic Republic of Iran

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نواقل ومستودعات داء الليشمانيات الجلدي في منطقة مارفداشت، جنوب جمهورية إيران الإسلامية

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الخلاصة: أُجريت دراسة وبائية حول نواقل ومستودعات داء الليشمانيات الجلدي في المناطق الريفية في مارفداشت، بإقليم فارس، جنوب جمهورية إيران الإسلامية، خلال الفترة الواقعة بين عامَيْ 2003 و2004. وتم، باستخدام المصائد، جمع 126 من القوارض، 75.4% منها من الجرذان الليبية، و14.3% من القداد *Cricetulus*، و10.3% من الكعبر *Meriones*. وتبيَّن أن ثمانية من الجرذان الليبية الـ 95 (8.4%) مصابة بالليشمانية الكبيرة، أمكن تحديدها عن طريق اختبار التفاعل السلسلي للبلمرة المتشابك. ولم تأت نتائج الاختبار إيجابية لأي من القوارض الأخرى. وجمعت إناث الفواصد من مواقع داخل البيوت: 75% منها من نوع الفاصدة الباباستية، ووجد أن 2.7% منها فقط مصابة طبيعياً بالليشمانية الكبيرة. ويُعَدُّ هذا أول تقرير يدلّ على نحو مُثْبَت، أن الفاصدة الباباستية هي من نواقل داء الليشمانيات الجلدي الحيواني المصدر في هذه المنطقة.

ABSTRACT An epidemiological study was made of vectors and reservoirs of cutaneous leishmaniasis in rural regions of Marvdasht, Fars province, southern Islamic Republic of Iran during 2003–04. Using live traps, 126 rodents were collected: 75.4% were *Meriones libycus*, 14.3% *Cricetulus migratorius* and 10.3% *Microtus arvalis*. Eight out of 95 *Meriones libycus* (8.4%) were found to be infected with *Leishmania major*, identified by nested-PCR; none of the other rodents were positive. Female sandflies were collected from indoor locations: 75% were *Phlebotomus papatasi* and only 2.7% were found naturally infected with *L. major*. This is the first report of *P. papatasi* as a proven vector of zoonotic cutaneous leishmaniasis in this area.

Vecteurs et réservoirs de la leishmaniose cutanée dans le district de Marvdasht au sud de la République islamique d'Iran

RÉSUMÉ Au cours des années 2003 et 2004, une enquête épidémiologique a été menée sur les vecteurs et réservoirs de la leishmaniose cutanée dans les zones rurales de Marvdasht, province de Fars, au sud de la République islamique d'Iran. À l'aide de pièges permettant de capturer l'animal vivant, 126 rongeurs ont été collectés dont 75,4 % de l'espèce *Meriones libycus* (Mérion de Libye), 14,3 % de l'espèce *Cricetulus migratorius* (Hamster migrateur) et 10,3 % de l'espèce *Microtus arvalis* (Campagnol des champs). Huit des 95 Mérions de Libye (8,4 %) se sont avérés infectés par *Leishmania major*, identifié par PCR (réaction de polymérisation en chaîne) nichée, aucun des autres rongeurs n'étant positif pour ce protozoaire. Des phlébotomes femelles ont été capturées en intérieur : 75 % appartenaient au genre *Phlebotomus papatasi* et seules 2,7 % étaient naturellement infectées par *L. major*. Il s'agit ici du premier rapport confirmant le rôle de *P. papatasi* comme vecteur de la leishmaniose cutanée zoonosique dans cette région.

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Introduction

Cutaneous leishmaniasis (CL) is still endemic and an important health problem in many countries of the Eastern Mediterranean Region [*1–4*]. Three species of *Leishmania* parasites cause CL in the old world: *L. major, L .tropica* and *L. aethiopica* [*5*].

Zoonotic cutaneous leishmaniasis (ZCL) is endemic in half of the 28 provinces of the Islamic Republic of Iran. The most important hyperendemic focus of ZCL has been located in Isfahan province, in the central part of the country, where the great gerbil, *Rhombomys opimus*, is the principle reservoir host of the disease [*6*]. In the other high risk parts of the country (the west and south-west regions), *Tatera indica* is the primary reservoir host [*7*]. In both the above-mentioned areas, *Meriones libycus* plays a secondary role as a source for transmitting of *L. major* by sandfly vectors [*6,7*]. In the central parts of the Islamic Republic of Iran (Isfahan province) as well as southeast of the country, female sandflies of *Phlebotomus papatasi* have been reported as the main vector of CL responsible for *L. major* infection [*8,9*].

In southern Islamic Republic of Iran, there is concern that the annual incidence of CL has gradually risen over the past decade, increasing from 1560 cases in 1991 to 3861 in 2001 (unpublished data). The most likely reason for this trend is an increase in human–sandfly contact. This is attributed to the development of villages and the spread of the human population into the habitats of the local vectors, *P. papatasi*, as well as rodents which act as reservoir hosts. It should be emphasized that most Iranian cases of CL are zoonotic. Our recent studies in some foci of ZCL in southern Islamic Republic of Iran showed that *Mer. libycus* was the main reservoir host, and *R. opimus* and *T. indica* were absent [*10,11*]. Although there are several studies on different aspects of disease in the region, there has been no comprehensive study on vectors of ZCL in the southern part of the Islamic Republic of Iran.

The aim of this study was to determine the species composition of reservoirs and vectors from the field in the Marvdasht focus of ZCL, southern Islamic Republic of Iran. In addition, we evaluated a new molecular technique, nested polymerase chain reaction (PCR), for species determination.

Methods

Study area

The study was carried out in 3 villages (Raja-Abad, Ghorbanlak and Soltan-Velayat) in the Mohammad Abad district (52° 56′ E, 29° 54′ N) at an altitude 1595 m above sea level and 9–11 km from Marvdasht city. The weather is very hot in the summer and cold and snowy in the winter. The major activities of the population are agriculture and animal farming.

Collection of rodents and preparation of smears

Rodents were captured monthly by Sherman live traps during October to December 2003 and April to October 2004. Traps were baited with roasted walnuts and placed in the active burrows. The traps were set in the early morning and evening once a month. Around 20–25 traps were used for each collection. Collected animals were transferred to the laboratory for preparation of smears from the ears or any suspected skin lesion of the animals. The prepared smears were stained with Giemsa and checked under light microscope for the presence of *Leishmania* parasites. Similar smears were prepared from the hand of 2 local cases of CL (a 5-year-old girl from Ghorban-Lak village

and a 15-year-old boy from Raja-Abad village). Identification was made using the standard reference for rodents in the Islamic Republic of Iran [*12*].

Extraction of DNA from positive smears of rodents

Total DNA was extracted from positive smears by digestion in lysis buffer. The dry smear was scraped with a sterile scalpel and dissolved in 200 µL buffer [50 mM Tris-HCL (pH 7.6), 1 mmol/L EDTA, 1% Tween 20] containing 8.5 µL proteinase K solution (19 µg/µL), in a 1.5 mL tube. The tube was incubated for 2 hours at 56 °C before adding 200 µL phenol–chloroform: isoamyl alcohol (25:24:1 by volume). After being shaken vigorously, the tube was centrifuged at 6000 × g for 10 minutes and then DNA was precipitated with 400 µL pure ethanol, re-suspended in 100 µL double-distilled water and stored at –20 °C before being used in PCR.

Nested PCR of minicircle kinetoplast (k) DNA for identifying *L. major*

The variable segment on minicircles of kinetoplast DNA from any *Leishmania* parasites present in the smear scraping was amplified using 2 rounds of nested PCR [*13*].

The primers were CSB1XR (CGA GTA GCAGAA ACT CCC GTT GA) and CSB2XF (ATT TTT CGC GAT TTT CGC AGA ACG) for the first round and LiR (TCG CAG AAC GCC CCT) and 13Z (ACT GGG GGT TGG TGT AAA ATA G) for the second round. Reference strains of *L. major* MHOM/IR/XX/LV114) and *L. tropica* (MHOM/IR/89/ARD22) from Tehran University of Medical Sciences were used as standards.

The first round reaction mixture contained 250 µmol/L deoxynucleoside-triphosphate (dNTP), 1.5 µmol/L $MgCl_2$, 1.0 U Taq polymerase, 50 mmol/L Tris-HCl (pH 7.6), 1% Tween 20, and 40 ng each of primers CSB1XR and CSB2XF, in a final volume of 25 µL. The DNA was amplified for 25 cycles in a Progen thermocycler (Thecne, Cambridge, UK) set to run at 94 °C for 1 min, 54 °C for 1 min, and then 72 °C for 1 min in each cycle.

The first round product (1.0 µL of a 9:1 dilution in distilled water) was used as template for the second round, in a total volume of 25 µL and under similar conditions to those for the first round except using LiR and 13Z as the primers.

Collection and dissection of sandflies

Sandflies were collected bi-weekly by an aspirator from indoor areas (guest rooms, sleeping rooms, latrines) and livestock stables, 2 hours after sunset during August and September 2004 (the main activity season of sandflies). Temperature and humidity were also recorded.

The collected sandflies were transferred to the laboratory and dissected in a drop of normal saline. For each female, the head with the 3 last segments of abdomen was cut and mounted in a slide with a drop of Puri medium for species identification. The remaining body parts of each specimen were transferred to a microtube filled with methanol 95% for DNA extraction and PCR examination.

Extraction of DNA and PCR of sandflies

DNA was extracted as described by Motazedian et al. [*14*]. Briefly, individual female, unfed and parous sandfly bodies were homogenized with a sealed pasture pipette in 1.5 mL tubes. Then 200 µL lysis buffer [50 µmol/L Tris-HCl (pH, 7.6); 1 µmol/L EDTA; 1% Tween 20] and 12 µL proteinase K (19 µg/mL) were added and incubated in 37 °C overnight before 300 µL

phenol–chloroform–isoamylalcohol (25:24:1 by volume) were added. After being shaken vigorously, the tube was centrifuged at 10 000 rpm for 10 min and then the DNA in the supernatant solution was precipitated with 400 µL cold, pure ethanol, re-suspended in 50 µL double-distilled water and stored at –20 °C before being used in PCR. Nested-PCR was employed for detection of *L. major* in sandfly specimens [*13*].

Results

Rodent and human cases

During this study 126 rodents were captured and identified as *Mer. libycus* (75.4%), *Microtus arvalis* (10.3 %) and *Cricetulus migratorius* (14.3%) (Table 1). Although all collected animals were examined for parasite infection under light microscope, amastigotes were only found in smears of 8 (8.4%) from 95 *Mer. libycus* (Table 2). Each infected *Mer. libycus* had at least 1 lesion either on an ear or at the base of its tail. Amastigotes from infected *Mer. libycus* were similar, with a mean diameter of 4 µm (Figure 1).

Parasite infection was observed among males and females animals from 2 of 3 study villages. In our study area, *Mer. libycus* had diurnal activity and each *Meriones* colony was around 2 m deep, with several burrow entrances.

All 30 smears from host and reservoir were amastigote-positive using microscopy. Out of 30 smears tested, 24 were from *Mer. libycus* and 6 from 2 local cases of ZCL, which were also shown positive for *Leishmania* DNA by PCR. For each PCR-positive sample, the second-round products of the nested PCR were identical to those of the *L. major* reference strain with a main band of 650 bp and distinct from those of the *L. tropica* standard with its main band of 750 bp (Figures 2 and 3).

Sandflies

A total of 200 parous, unfed female sandflies were checked by PCR for the presence of *Leishmania* parasite. They were *P. papatasi* (75%), *P. segenti* (15%) and *P. caucasicus* (10%). Among the examined sandflies, only 4 out of 150 *P. papatasi* (2.7%) were found naturally infected with *L. major* (Figure 3).

Discussion

Control of leishmaniasis in areas of endemicity requires a thorough knowledge of *Leishmania* ecology and epidemiology. There is a major problem for epidemiolo-

Table 1 **Density of collected rodents in Marvdasht rural district, Fars province, southern Islamic Republic of Iran, 2003–04**

Rodent species	Village			Total No.	%
	Raja-Abad No.	Ghorbanlak No.	Soltan-Velayat No.		
Meriones libycus	49	27	19	95	75.4
Cricetulus migratorius	9	5	4	18	14.3
Microtus arvalis	6	2	5	13	10.3
Total	64	34	28	126	100.0

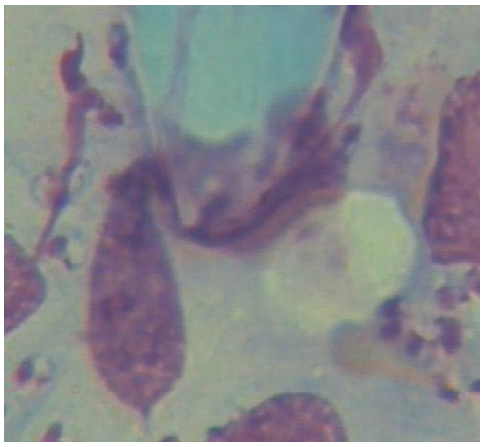

Figure 1 ***Leishmania major*** **amastigotes from smear of ear tissue in** ***Meriones libycus*** **stained by Giemsa**

gists both in the identification of reservoir hosts and in the detection of vectors.

The suggested criteria for incrimination of a vector are anthropophily and common infection with the same *Leishmania* parasite as that found in man in the same places [*15*]. Dissection of sandflies for detection of *Leishmania* parasite is difficult, and accurate estimates of infection are not possible [*16*]. Isoenzymes provide the gold standard for species identification and reference strains of *Leishmania* [*3*]. This method was used by Yaghoobi-Ershadi et al. [*8,17*] who identified single infections of the strain (zymodeme MON26) of *L. major* in *P. papatasi* and *P. caucasicus* in Isfahan province, central Islamic Republic of Iran [*8,17*]. However, this method has the disadvantage of requiring the culture of a large number of parasites, and primary isolates can easily become contaminated, or in mixed infection yield only the strain that grows fastest in laboratory conditions [*9*].

At the present time the applicability of molecular techniques (PCR) including kinetoplast DNA for detection and identification of *Leishmania* within sandflies by DNA hybridization has been demonstrated [*18–20*]. The highly sensitive technique of PCR has been used before for detecting *Leishmania* in sandflies of the new world [*21*], Islamic Republic of Iran [*9*] and India [*22*]. This is the first report of *P. papatasi* (collected from indoors) as a main and primary vector of *L. major* in the southern part of the Islamic Republic of Iran. The results of our study showed that nested-PCR is faster and more reliable for directly detecting *L. major* in wild populations of *P. papatasi* in comparison with other methods, i.e. dissection and isoenzyme techniques [*9,16*]. This species has been reported as a principle vector of ZCL transmitted to humans in other parts of the Islamic Republic of Iran [*8,9*].

Table 2 **Prevalence of leishmaniasis in captured rodents in Marvdasht rural district, Fars province, southern Islamic Republic of Iran, 2003–04**

Species of rodents	No. examined	No. positive	% positive
Meriones libycus	95	8	8.4
Cricetulus migratorius	18	0	0
Microtus arvalis	13	0	0
Total	126	8	6.4

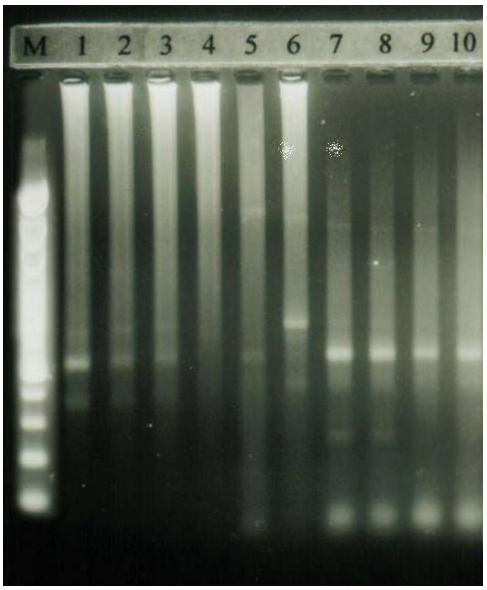

Figure 2 **Nested-PCR based amplification of DNA extracted from Giemsa-stained smears. The bands shown correspond to reference strains of** *Leishmania major* **(lane 1) and** *L. tropica* **(lane 6), molecular-weight markers (M) and smear samples of DNA from human skin lesions caused by** *L. major* **(lanes 2, 3 and 5); a human skin lesion caused by agents other than** *L. major* **(lane 4), and** *Meriones libycus* **skin lesions caused by** *L. major* **(lanes 7,8,9 and 10).**

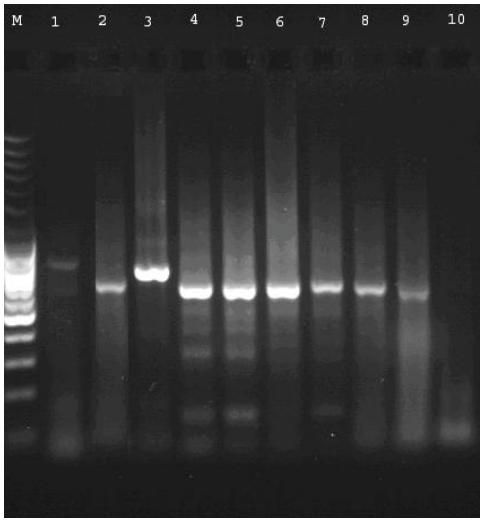

Figure 3 **Nested-PCR based amplification of DNA extracted from** *Phlebotomus papatasi* **and from Giemsa-stained smears. The bands, correspond to reference strains of** *Leishmania tropica* **(lane 1) and** *L. major* **(lane 2), molecular-weight markers (M)** *L. infantum* **(lane 3); a sample from infected** *P. papatasi* **(lane 4); a sample from uninfected** *P. papatasi* **(lane 10); a sample from infected** *Meriones libycus* **(lane 5); a sample from infected human (lane 6); two sample of infected** *Mer. libycus* **(lanes 7,8); and a sample from other infected human (lane 9).**

Another important finding of this survey was confirmation of *Mer. libycus* as a principal reservoir of ZCL in rural regions of Marvdasht focus. This rodent has been reported as a main reservoir in the foci of Arsanjan and Neiriz, adjacent to our study areas [*10,11*]. In the central and west of the Islamic Republic of Iran, *Mer. libycus* is a secondary reservoir host, where *R. opimus* and *T. indica* are present [*1,7*].

L. major has also been isolated from *Mer. libycus* in ZCL foci of Saudi Arabia [*23*] and Uzbekistan [*24*]. This animal is common and widespread in central and southern Islamic Republic of Iran. It seems that agriculture and rural development in many parts of this region will lead to more human contact with the rodent–sandfly–rodent cycle of *Leishmania* transmission.

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Dedication

This study is dedicated to our colleague and friend M.M. Gassemi who passed away in a car accident during this study.

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