

ENTOMOLOGIE MÉDICALE

Molecular data on vectors and reservoir hosts of zoonotic cutaneous leishmaniasis in central Iran.

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Résumé : Données moléculaires sur les vecteurs et les réservoirs de leishmaniose cutanée zoonotique dans un foyer du centre de l'Iran.

Suite à l'augmentation du nombre des cas de leishmaniose cutanée et à l'émergence de nouveaux foyers, une étude des vecteurs et des réservoirs incriminés a été menée dans le district de Shahrood (centre de l'Iran) au cours des années 2005-2006. La capture des phlébotomes a été réalisée par la méthode des papiers huilés et celle des rongeurs à l'aide de pièges Sherman.

Plus de 1700 phlébotomes ont été capturés et identifiés, dont une majorité de *Phlebotomus papatasi*. La recherche de *Leishmania major* par RAPD-PCR a montré que 10 % des *P. papatasi* et 4,2 % des *P. caucasicus* étaient naturellement infestés. Deux espèces de rongeurs, réservoirs potentiels, ont été piégées : *Rhombomys opimus* (92,5 %) et *Nesokia indica* (7,5 %). Chez 91,9 % des *Rhombomys opimus* des formes amastigotes de *Leishmania major* ont été observées, isolées et identifiées par RAPD-PCR. Cette enquête épidémiologique a mis en lumière l'importance de la maladie dans la région et pourrait aider à la mise en place d'un programme de contrôle.

Summary:

Due to the increasing number of positive cases of cutaneous leishmaniasis with occurrence of new foci, a study was carried out to investigate on vectors and reservoirs of the disease in the Shahrood district, central Iran during 2005-2006. Sandflies and rodents were collected using sticky papers and Sherman live traps respectively. More than 1700 sandflies were collected and identified, mainly *Phlebotomus papatasi* species. RAPD-PCR analysis of sandflies showed that 10% of *P. papatasi* and 4.2% of *P. caucasicus* were naturally infected with *Leishmania major*. Two species of rodents, potential reservoirs, *Rhombomys opimus* (92.5%) and *Nesokia indica* (7.5%) were trapped in the district. Microscopy identification from rodents confirmed that 91.9% of the *Rhombomys opimus* were positive to amastigotes. Species identification of isolated parasites revealed *Leishmania major* DNA in the infected *Rhombomys* using RAPD-PCR technique. This epidemiological data highlight the importance of the disease in the region and could help people involved in control programs.

Introduction

There are several reports indicating occurrence of zoonotic cutaneous leishmaniasis (ZCL) in Iran (19). The first important focus of the disease has been located in the center and northeast of Iran, where *Rh. opimus* and *P. papatasi* play an important role as a reservoir and vector (20, 14, 5).

The second focus of ZCL has been reported in the west and southwest of Iran, where *Tatera indica* replaced *Rh. opimus* as a reservoir and *Pb. papatasi* as a vector (4). The Baluchistan province, in the southeast of Iran is considered as a third focus of ZCL. In this region *Meriones hurrianae* have been proved as a natural reservoir host (15). From reported evidences, it

appears that the most rural areas of the Fars province, south of Iran, can be considered as a ZCL focus and in this area *M. libycus* is the primary and main reservoir host of the disease, where *R. opimus* and *T. indica* were absent and *P. papatasi* is considered as the proven vector of ZCL (11-13).

Following the emergence of 400 new cases of cutaneous leishmaniasis in Bekran County, Shahrood district, centre of Iran with a population of 6500 people in 2005 (unpublished data) a comprehensive study was carried out in the region. The main objective was to determine the sandflies species responsible for transmission of *L. major* to human, as well as, the reservoirs of the disease in Shahrood district, Semnan province, centre of Iran.

Phlebotomus papatasi
Phlebotomus caucasicus
Leishmania major
Rhombomys opimus
Nesokia indica
leishmaniose cutanée zoonotique
épidémiologie
vecteur
hôte réservoir
PCR
Shahrood
Iran
Moyen-Orient

Phlebotomus papatasi
Phlebotomus caucasicus
Leishmania major
Rhombomys opimus
Nesokia indica
cutaneous leishmaniasis
epidemiology
vector
reservoir host
PCR
Shahrood
Iran
Middle East

Material and Methods

Study areas

The study was carried out in the two villages of Bekran and Honestan (eastern Kalate district) in Shahrood county (36° 25' N, 55° 01' E, altitude 1,345m). The total population of Shahrood was 134,920 in 2006 and their main community activities are agriculture and farming.

Collection of sandflies

Sandflies were collected from indoors (bedroom, guest room, toilet, stable) as well as outdoors (rodent burrow, wall cracks and crevices) bi-weekly, using sticky traps and aspirator during their activity period. After collections, samples were rinsed from the sticky traps and mounted in a drop of Puri's medium. Subsequently species identification was conducted using a national systematic key (16, 18).

Dissection of sandflies

Different stages of abdominal conditions including blood fed, semi gravid and gravid were dissected in a drop of normal saline. Infected sandflies with promastigotes were fixed and stained by methanol and Giemsa.

Isolation of parasite from infected sandflies

Samples of infected sandflies were inoculated subcutaneously at the base of the tail of 12 Balb/C mice. After inoculation and incubation period, parasites from the infected laboratory mice were cultured into the liquid phase of Novy-MacNeal and Nicole (NNN) medium (3).

Collection and examination of rodents

Rodents were captured by setting up Sherman live traps. 50 traps were baited with roasted walnut, cucumber, tomato and placed in active burrows. The traps were set up in the evening, monthly. To assess the infectivity of rodents by the parasites, their ears, noses and feet were examined. Impression smear was taken and then stained by Giemsa staining method. Presence of parasite was checked under microscope. Samples from infected rodents were cultured as it was done for sandfly (3).

DNA Extraction

Promastigotes from a 15 ml stationary phase of bulk culture were harvested by centrifugation (3,000 g at 4°C for 10 minutes) and washed 3 times in cold sterile PBS (pH 7.2). The pellet was re-suspended in 500 µl of cell lysis buffer (50 mM NaCl, 50 mM EDTA, 1%SDS, and 50 mM Tris- HCl, pH 8.0) with 100 microgram/ml Proteinase K, and incubated at 55°C overnight. The lysate was extracted by phenol/chloroform followed by ethanol precipitation (6). The DNA was re-suspended in double distilled water (DDW) and stored at 4°C. Working solutions were adjusted to 10 ng/µl in DDW (10).

RAPD-PCR Procedure

Amplification reactions were performed following the protocol described earlier (8). Each 20 µl of RAPD reaction contained 20 ng genomic DNA, 2.0 mM MgCl₂, 0.2 mM dNTP (Roche Biotech), 20 pmol of each primer, 1u of *Taq* polymerase (Roche Biotech) in the PCR buffer. Reactions were overlaid with 30/µl of mineral oil and amplified in a thermocycler (Techne USA) programmed for one cycle at 94°C for 5 minutes followed by 35 cycles of 94°C, 36°C, 72°C for 1 minute each, and 1 cycle of 72°C for 10 min. 20 µl of PCR products were run along with a 100 bp ladder on a 1.2%

agarose gel containing ethidium bromide for 4 hours at 50 V. The gel was observed on a UV transilluminator and then, digital photographs were prepared. The primer AB1-07 [(5-3) GGTGACGCAG, %G/C 70] (Roche Biotech) was evaluated with two *Leishmania* standard species including *L. major* (MHOM/IR/75/ER), and *L. tropica* (MHOM/IR/99).

Results

Sandflies

Overall, 1,777 sandflies (911 females and 866 males) were caught from indoors (rooms, toilet and stables) and then identified during their activities in 2005. Adult sandflies were collected only between middle of May and end of December, with the highest numbers per collection-night caught in the middle of August. The species identification revealed only 2 species: *P. papatasi* and *S. sintoni* (table 1). *P. papatasi* was the most common species in the collection, representing over 54.3% of sandflies caught.

In a parallel study, altogether 119 female and parous sandflies were caught from rodents burrows which were located less than 100 meters from human dwellings and dissected. They were *P. papatasi* (16.8 %) *P. caucasicus* (79.8%) and *S. sintoni* (3.4%). Only 10% (2/20) of *P. papatasi* and 4.2% (4/95) of *P. caucasicus* were found naturally infected with promastigotes under light microscope 40x magnification. 20 slides of promastigote from infected sandflies were prepared and stained with Giemsa (photo 1).

Results from inoculation of parasite from infected sandflies, revealed the presence of amastigotes into the nodules and ulcers of the experimentally mice after 10 days from inoculation period. All six experimental BALB/c which were infected artificially were found positive. Isolated parasites from infected mice were identified using RAPD- PCR. The results of the PCR revealed the presence of *L. major* (photo 2).

Table 1.

The species of collected sandflies from indoors and rodent burrows in Shahrood district Centre of Iran, 2005.

Espèces de phlébotomes capturées à l'intérieur des habitations et dans les terriers de rongeur dans le comté de Shahrood, Centre de l'Iran

species places	<i>P. papatasi</i>		<i>P. caucasicus</i>		<i>S. sintoni</i>	
	M	F	M	F	M	F
indoors	533	470	0	0	333	441
rodent burrows	20	0	95	0	0	4
total	533	490	0	95	333	445

V: male / F: female

Photo 1.

Giemsa stained of promastigotes of *L. major* from naturally infected of *P. papatasi*. Shahrood district, Iran, 2005-2006

Promastigotes colorés au Giemsa de *L. major* de *P. papatasi* naturellement infectées. Shahrood district, Iran, 2005-2006

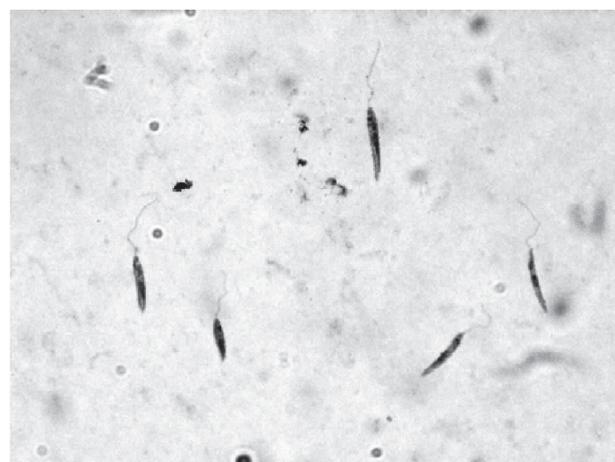
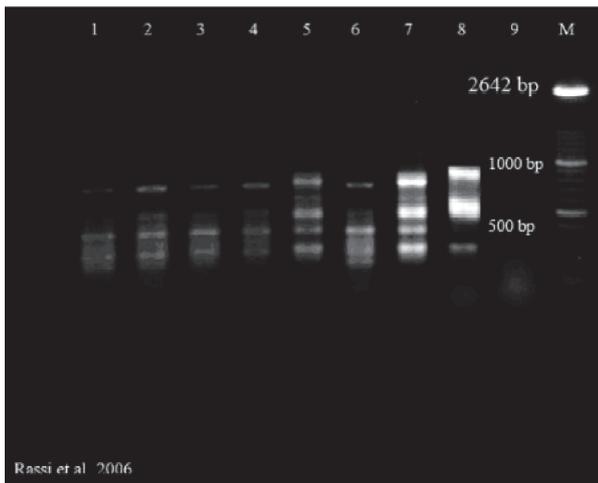


Photo 2.

Results of RAPD-PCR based on DNA extracted from sand-flies and rodents.
 Résultats de RAPD-PCR à partir d'extraction d'ADN de phlébotomes
 et de rongeurs.



The bands shown on 1.2 agarose gel stained with ethium bromide correspond to molecular weight markers (M), reference strain of *L. tropica* and *L. major* (lanes 8, 7), sample from one specimen of *P. papatasi* (lane 6), sample from one specimen of *P. caucasicus* (lane 1), sample from four specimens of *R. opimus* (lane 2, 3, 4, 5), blank (lane 9).

Photo 3.

L. major amastigotes from smear of ear tissue in *Rh. opimus* stained by Giemsa, Shahrood district, Iran, 2005-2006s.

Formes amastigotes de *L. major* provenant du frottis de tissu d'oreille de *R. opimus* coloré au Giemsa, Shahrood district, Iran

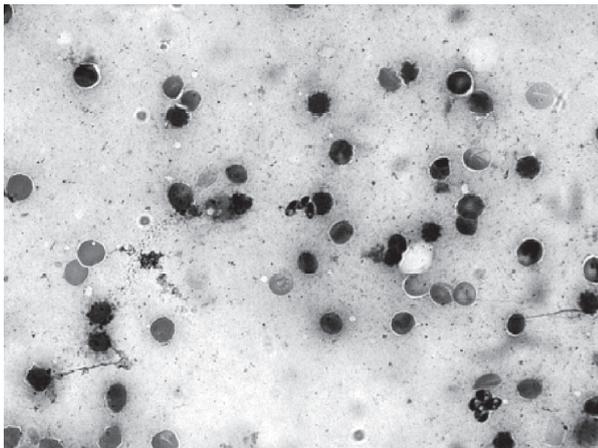


Photo 4.

The malformation ear of *Rh. opimus* infected with *L. major* in Shahrood district, Iran, 2005-2006.

Malformation d'oreille de *Rhombomys opimus* infecté par *L. major* dans le district de Shahrood, Iran, 2005-2006



After 20 days of inoculation it was observed that the tails of some infected mice showed symptoms of necrosis indicating a severe pathogenicity of the parasites. Severe ulcer with necrosis of the tail is shown.

Reservoir hosts

Among 40 collected rodents, 92.5% were identified as *Rh. opimus* and *Nesokia indica* for the remaining ones. All collected rodents were examined for the presence of parasites. To do that, direct sampling from ears, noses and feet of rodents revealed that 91.9% (34/37) of *Rh. opimus* were positive for amastigote (photo 3). Lesions were observed on ears (91.9%), noses (8.8%) and legs (0.3%) of great gerbils. In some cases ears and noses of rodents have been malformed by the parasites (photo 4). Parasites infection was observed in both male and female animals. Isolated parasites from infected rodents were identified *L. major* using RAPD-PCR (photo 2). All examined rodents of *N. indica* were free of infection.

Discussion

Ecology and epidemiology of leishmaniasis are important measures for management and planning of disease control. The entomological survey together with epidemiologists data are major components to fight against the disease. Several epidemiological and entomological findings including anthropophily, common infection of sandflies with the same *Leishmania* parasite found in man in the same places, suggested the capacity of sandfly as a vector (7).

For further confirmation, molecular techniques (PCR) have been used too. The highly sensitive technique of PCR has been used for detecting *Leishmania* in sandflies in the world (9), Iran (1, 13) and India (2). In this study, use of RAPD-PCR technique was able to detect *L. major* in both species of *P. papatasi* and *P. caucasicus*. Results of our study revealed that the high density of *P. papatasi* in indoor resting places and high infectivity with *L. major* agree with the hypothesis that this species can play a major role as a main vector in the region. Our entomological data on *P. caucasicus* suggested that this species plays a secondary role for maintenance and stability of the disease among rodents.

Another finding of this survey was the confirmation of *Rh. opimus* as the principal reservoir of ZCL in rural regions of Shahrood district. This rodent has been also reported as a main reservoir in the other foci of disease (such as Isfahan and Khorassan provinces) in Iran (5, 14, 19). This great gerbil, a colonial, burrowing rodent, is a common species in arid desert and steppe regions of central Asia. This species also exists in the southern territories of the former USSR (i.e. Turkmenistan, Uzbekistan, Kazakhstan, Tajikistan) and neighbouring countries where zoonotic cutaneous leishmaniasis (ZCL) caused by *L. major* is endemic and considered as an important public health problem, therefore *Rh. opimus* is considered as the principal mammalian host of the parasite (17). Human activities are closed to *Rh. opimus* burrows. The presence of high density of *P. papatasi* in rodent burrows and indoors and proximity of human habitat to *Rh. opimus* colonies increases the chances of human contact with the disease agents and possible appearance of a new focus of leishmaniasis in the region.

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