Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading doi:

Phlebotomus papatasi and *Meriones libycus* as the vector and reservoir host of cutaneous leishmaniasis in Qomrood District, Qom Province, central Iran

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ARTICLE INFO

Article history:
Received 25 October 2010
Received in revised form 27 November 2010
Accepted 15 December 2010
Available online 20 February 2011

Keywords:
Cutaneous leishmaniasis
Vector
Reservoir
Iran

ABSTRACT

Objective: To determine the sand flies species responsible for most transmission of *Leishmania major (L. major)* to human, as well as to determine the main reservoir hosts of the disease. **Methods:** Sand flies were collected using sticky papers and mounted in Puri´s medium for species identification. Rodents were trapped by live Sherman traps. Both sand flies and rodents were subjected to molecular methods for detection of leishmanial parasite. **Results:** *Phlebotomus papatasi (P. papatasi)* was the common species in outdoor and indoor resting places. Employing PCR technique only three specimens of 150 *P. papatasi* (2%) were found naturally infected by parasites with a band of 350 bp which is equal to the *L. major* parasite. Forty six rodents were captured by Sherman traps and identified. Microscopic investigation on blood smear of the animals for amastigote parasites revealed 1 (3.22%) infected *Meriones libycus (M. libycus)*. Infection of this animal to *L. major* was confirmed by PCR against rDNA loci of the parasite. **Conclusions:** This is the first molecular report of parasite infection of both vector *(P. papatasi)* and reservoir *(M. libycus)* to *L. major* in the region. The results indicated that *P. papatasi* was the primary vector of the disease and circulating the parasite between human and reservoirs and *M. libycus* was the most important host reservoir for maintenance of the parasite source in the area.

1. Introduction

Cutaneous leishmaniasis (CL) is a worldwide public health and a social problem in many developing countries. It can affect the skin and mucous membranes, and is caused by different *Leishmania* species widespread in 88 countries in the New and Old World.

Old World cutaneous leishmaniasis (OWCL) is present in many endemic areas in North Africa, the Mediterranean, the Middle East, the Indian subcontinent and Central Asia. The species responsible for OWCL are mainly *Leishmania major (L. major)* and *Leishmania tropica(L. tropica)*. *Leishmania infantum* and *Leishmania donovani* can also cause localised CL but are observed less frequently in the Mediterranean areas. Diffuse CL is uncommon and is caused by *Leishmania aethiopica* in Africa[1,2].

The annual incidence of cutaneous leishmaniasis has gradually increased in Iran and about 30000 cases has been reported in 2009 (Unpublished data). This increasing outbreak is related to human-sandfly-rodent contacts, itself probably the product of the development of irrigation schemes and the spread of human populations into the habitats of the vector, *Phlebotomus papatasi* Scopoli *(P. papatasi)*, and the rodents that act as reservoir hosts.

Based on animal reservoir host, there are four foci of disease in the country[3]. The first one has been documented in the center and northeast of Iran, where *Rhombomys opimus* (*R. opimus)* and *P. papatasi* play an important role as the reservoir and the vector of the disease[4-7].

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Foundation project: Supported by the School of Public Health, Tehran University of Medical Science (No.8952).

The second focus of zoonotic cutaneous leishmaniasis is located in the west and southwest of Iran, where *Tatera indica* substituted with *R. opymus* as a reservoir and *P. papatasi* as a vector[8].

Baluchistan Province, in the southeast of Iran is considered as the third focus of ZCL. In this region *Meriones hurrianae* has been approved as a natural reservoir host[9].

From the reported documents, it is apparent that most of the rural areas of Fars Province in southern Iran can be considered as the ZCL focus where *Meriones libycus (M. libycus)* is the primary and the main reservoir host of the disease, while *R. opimus* and *Tatera indica* were not found and *P. papatasi* is considered as the proven vector of ZCL [3,10,11].

Qomrood district from Qom Province in Center of Iran is a ZCL focus and this study was performed to put light through the epidemiology of the disease in the region. The main objectives were to determine the sand flies species responsible for most transmission of *L. major* to human, as well as to determine the main reservoir hosts of the disease.

2. Material and methods

2.1. Study area

The study was carried out in 3 villages (Kooh sefid, Faraj Abad and Kaj) of Qoomrood area (located in east of Qom province) (50° 52 E, 34° 35 N) at an altitude of 900 m above sea level and 25-35 km far from Qom City. The weather is hot in summer and cold in winter.

It receives an average of 165 mm of rain per year. The temperature ranges between 0.9-41.1 ℃ and the ratio humidity range between 10%- 92%. The total population of the district was about 5007 people in 2007 (Unpublished data). The major activities of the population are agriculture and animal farming.

2.2. Collection of sand flies

Sand flies were collected from indoors (bedroom, guestroom, toilet) as well as outdoors (rodent burrows, wall cracks) biweekly using sticky traps. Three above mentioned villages were selected and 180 sticky traps were set up during the sand flies activity period (May-November) of 2009. Sand flies were rinsed from the sticky traps and mounted in a drop of Puri's medium and identified after 24 h using a valid key[12].

2.3. Collection of rodents

Rodents were captured by setting the 40 Sherman live traps. Traps were baited with roasted walnut, cucumber, tomato and placed in the active burrows. The traps were set up early morning and evening (December & February of 2009; May & July of 2010). In order to approve the infectivity of rodents by the parasites, their ears were examined and an impression smear was taken and stained by Geimsa staining method. The presence of the parasite was checked under microscope. Samples from infected rodents were inoculated subcutaneously at the base of tail of BALB/c.

2.4. DNA extraction

DNA of sand flies was extracted individually or as a group of 10 samples through ISH-Horowicz[13]. The smear on the Giemsa stained slides of rodent specimens was scrapped off the slides and treated as follow. Total DNA was extracted from positive smears by digestion in 100 μL PBS buffer and the tube was centrifuged at 10 000 rpm for 10 minutes, then 300 μL Lysis buffer and 30 μL proteinase K was added. The tube was incubated for 24 hours at 37 ℃ before adding 300 μL sacharin phenols. After adding this solution the tube was centrifuged at 9 000 rpm for five minutes. After transferring upper phase to new tube, 300 μL phenol-chloroform was added and centrifuged at 10 000 rpm for five minutes. This step was repeated again using pure chloroform. 20 μL acetate potassium (5 M) and 1 000 μL ethanol were added to upper phase and stored at -20 ℃ for two hours before centrifugation at 10 000 rpm for 10 minutes. The pellet was washed twice by 70% ethanol and finally diluted with TE buffer.

2.5. ITS1-PCR

All extracted samples were tested by PCR method against ITS1 gene[14] using primers LITSR 5′-CTG GAT CAT TTT CCG ATG-3′ and L5.8S 5′-TGA TAC CAC TTA TCG CAC TT-3′. In all of the PCR experiments ddH_2O, and DNA of male sand flies were used as negative controls. Reference strain of *L. major* (MHOM/IR/54/LV39) was used as positive controls. This reference strain was obtained from the Medical Parasitology Laboratory, Faculty of Health, Tehran University of Medical Sciences.

2.6. PCR-RFLP

ITS1-rDNA PCR amplification was followed by RFLP technique using HaeIII enzymes in condition recommended by manufacture (Fermentase) for final species identification of the parasite as explained by Dweik[15]. Restriction analysis was performed in a total volume of 25 μL solution including 15-20 μL ITS1-rDNA PCR products, 5 U (1 μL) restriction enzyme (HaeIII), 2.5 μL Enzyme reaction buffer, and 1.5-6.5 μL ddH_2O and overlaid by a drop of mineral oil. Restriction fragments were analyzed in 2% agarose gel using 1× TBE buffer, and etidium bromide staining.

3. Results

3.1. Sand flies

Totally 10 246 sand flies were collected, including *P.*

papatasi (68.75%), *Phlebotomus salehi* (0.01%), *Phlebotomus sergenti*(0.98%), *Phlebotomus caucasicus* (0.56%), *Phlebotomus alexandri* (13.00%), *Sergentomyia sintoni* (28.95%), *Sergentomyia dentata* (0.12%), *Sergentomyia clydei* (0.11%), *Sergentomyia theodori* (0.37%) and *Sergentomyia pawlowski* (0.03%).

One hundred and eighty specimens of female sand flies from indoor and outdoors including 150 *P. papatasi*, 20 *Phlebotomus sergenti* and 10 *Phlebotomus caucasicus* were examined for *Leishmania* infection using PCR-RFLP.

Only three specimens (2.0%) of *P. papatasi* were found naturally infected by parasites with a band of 350 bp which is equal to the *L. major* parasite (Figure 1). Furthermore ITS amplification by Nested PCR, primers followed by RFLP technique confirmed the DNA of *L. major* in the infected *P. papatasi* samples (Figure 2). All infected sand flies were parous. Based on abdominal condition, they were unfed (2 specimen) and semi-gravid. Sand flies with *Leishmania* infection were collected from rodents burrow and toilet.

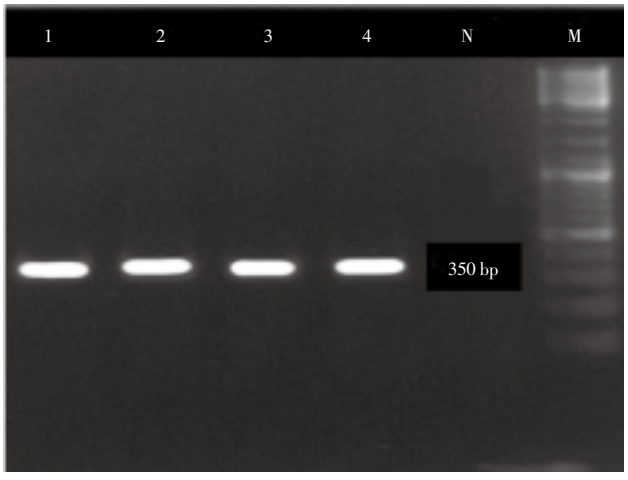

Figure 1. ITS1-PCR amplification (350 bp) of *L. major* in *P. papatasi* (1-2), *M. libycus* (3), negative control(N), positive control of *L. major* (4) and M, 100 bp molecular weight marker (Fermentase).

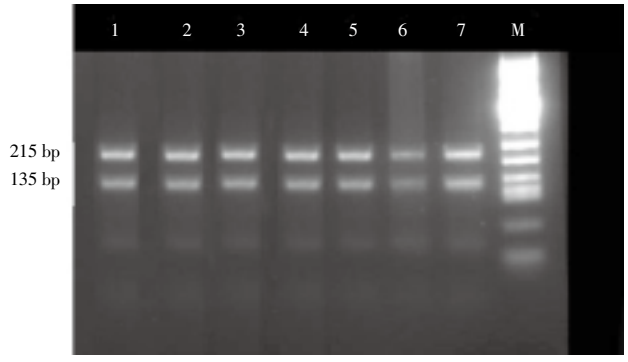

Figure 2. PCR-RFLP analysis of 350 bp of ITS1-rDNA of *Leishmania* species using HaeIII restriction enzyme.
L. major in *P. papatasi* (1-4), *L. major* in *M. libycus*(5), *L. major* in positive human sample (6), standard *L. major* (7), 100 bp molecular weight marker (M) (Fermentase).

3.2. Rodents

For determination of reservoir hosts of the disease, altogether 46 small mammals were captured by live Sherman traps and identified.

They were *M. libycus* (67.4%), *Nesokia indica* (4.35%), *Dipodidae* (17.4%), *Mus musculus* (8.7%) and *Hemiechinus auritis* (2.15%).

Although all collected animals were examined for parasite infection under light microscope, only one out of 31 *M. libycus* (3.7%) were found to be infected by *L. major* amastigotes ,identified ITS1 gene and followed by PCR-RFLP (Figure 1, 2). Three samples from infected rodent was inoculated subcutaneously at the base of tail of 3 BALB/c mice. Our result revealed the presence of amastigotes in the nodules and ulcers of the experimentally mice after 35 d of inoculation period. There was no parasites infection observed in other animals.

High density of *M. libycus* in rural area, closing of their burrows to human dwellings with infection of them to *L. major* (3.7%) are the most important reasons to introduced of *M. libycus* as the main reservoir host of disease in this region. This is the first molecular report on vector(s) and reservoir host(s) of cutaneous leishmaniasis due *L. major* in the rural parts of Qomrood District, Qom Province, Central of Iran.

4. Discussion

Our results showed only *P. papatasi* is incriminated as the principal vectors of *L. major* among the examined female sand flies with an infection rate of (2%) in the studied area.

Collection of wild specimens is an essential need in incriminating a phlebotomine sand fly species as a vector. Parasite isolation and its molecular identification could then confirm its vector status.

Recently, molecular techniques (PCR) have been employed for vector incrimination of sand flies[16-23]. The highly sensitive technique of PCR has been used for *Leishmania* in sand flies in some endemic areas including Iran and India [16-24]. Results of our study revealed that the high density of *P. patasti* in indoor resting places as well as rodent burrows and their infectivity with *L. major* is attributed to the fact that this species plays a major role as a principle vector in the region. According of our data, all infected *P. papatasi* were collected in the second half of August of 2009, that indicate the probable transmission time of parasite to human

In this survey we isolated some *L. major* parasites from *M. libycus* where its PCR-RFLP results was identical to that of isolates from human and *P. papatasi* sand flies.

Our previous study showed the rodent of *M. libycus* is primary reservoir host of cutaneous leishmaniasis in south of Iran[3]. This fact is confirming that *M. libycus* is the principal reservoir of CLM in the rural regions of Qomrood District and circulate the parasites between *P. papatasi* and dwelling

peoples.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The author would like to appreciate the kind collaboration of Health Center staff of Qomrood District. This study was financially support by the School of Public Health, Tehran University of Medical Sciences: Project No. 8952.

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