**Original Article**

# Molecular Detection of *Leishmania major* in the Vectors and Reservoir Hosts of Cutaneous Leishmaniasis in Kalaleh District, Golestan Province, Iran

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## Abstract

**Background:** An epidemiological study was carried out on the vector(s) and reservoir(s) of cutaneous leishmaniasis in rural areas of Kalaleh District, Golestan Province during 2006 - 2007.
**Methods:** Totally 4900 sand flies were collected using sticky papers and were subjected to molecular methods for detection of leishmanial parasite.
**Results:** *Phlebotomus papatasi* was the common species in outdoor and indoor resting places. Employing PCR technique showed only 1 out of 372 *P. papatasi* (0.3%) was positive to parasite due *Leishmania major*. Sixteen rodent reservoir hosts were captured by Sherman traps and identified as *Rhombomys opimus*. Microscopic investigation on blood smear of the animals for amastigote parasites revealed 6(37.5%) infected rodents. Infection of these animals to *L. major* was then confirmed by PCR against rDNA loci of the parasite.
**Conclusion:** This is the first molecular report of parasite infection of both vector (*P. papatas*) and reservoir (*R. opimus*) to *L. major*. The results indicated that *P. papatas* was the primary vector of the disease and circulating the parasite between human and reservoirs, and *R. opimus* was the most important host reservoir for maintenance of the parasite source in the area.

**Keywords:** Cutaneous leishmaniasis, Vector, Reservoir, Iran

## Introduction

There are several reports indicating occurrence of Cutaneaous Leishmaniasis due to *Leishmania major* (CLM) in Iran (Yaghoobi-Ershadi et al.1996, Rassi et al. 2006). Based on animal reservoir host, there are four foci of disease in our country (Rassi et al. 2006) The first one has been located in central and northeast of Iran, where *Rhombomys opimus* and *Phlebotomus papatasi* play important roles as reservoir and vector of the disease (Seyedi-Rashti et al. 1967, Javadian et al. 1976, Yaghoobi-Ershadi et al. 2001).

The second focus of Zoonotic cutaneous leishmaniasis is located in the west and southwest of Iran, where *Tatera indica* replaced with *R. opymus* as a reservoir and *P. papatasi* as a vector. (Javadian et al. 1988). Baluchistan Pprovince, 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 (Seyedi-Rashti and Nadim 1984). From the reported evidences,

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it is apparent that the most rural areas of Fars Province in southern Iran can be considered as the ZCL focus where *M. libycus* is the primary and main reservoir host of the disease, while *R. opimus* and *T. indica* were absent and *P. papatasi* is considered as the proven vector of ZCL (Rassi et al. 2006, Rassi et al. 2007).

Kalaleh district from Golestan Province in northern 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 in the study area.

## Material and Methods

### Study area

The study was carried out in 3 villages (Ghare ghol-e-gharbi, Sozesh and Allah nour) of Maraveh tapeh district (55° 57 E, 37° 54 N) at an altitude 228 m above sea level and 90-100 km far from Kalaleh City. The weather is hot in the summer and cold in the winter. It receives an average of 311.3 mm of rain per year. The temperature ranges between 2.5-36.6° C and the ratio humidity ranges between 25- 85%. The total population of the district was about 156939 people in 2007 (Unpublished data). The major activities of the population are agriculture and animal farming.

### 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). 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 (Nadim and Javadian 1976, Rassi et al. 2006 a).

### Sand flies DNA Extraction

DNA was extracted through ISH-Horowicz (Ready et al., 1991). Individual female sand flies were homogenized with a sealed pasture pipette in 1.5 ml tubes. Then 100 µL lysis buffer [0.1M Tris-HCl Ph 7.5; 0.6M NaCl; 0.1 EDTA] and 10 µL [0.8M Tris-HCl Ph 9.0: 0.27M EDTA] were added and incubated at 65 °C for half an hour before 30 µL acetate potassium (8 mol) were added. After short centrifuge it was cooled for 45 min and transferred to new tube and added 350 µL cold pure ethanol and stored at -20°C for 24 h. The tube was centrifuged at 13000 rpm for 30 min then emptied the solution until dried and washed by 500 µL ethanol (75 °C) and the TE buffer was added.

### Semi-Nested PCR for detection of *Leshmania* infection in sand fly

Semi-Nested PCR was employed for detection of kinetoplast DNA (kDNA) of *L. major* in sand fly specimens. The primers were: LINR4 (Forward):5´-GGGGTTGGTGTAA-AATAGGG-3´ (20bp); LIN19 (Reverse): 5´-CAGAACGCCCCTACCCG-3´ (17bp), and LIN17 (reverse): 5´-TTTGAACGGGATTT-CTG-3´ (17bp). Positive samples were tested by PCR method against ITS gene using the primers of ITS1 (Forward): 5´- TCCGTAG-GTGAACCTGCGG-3´ and ITS2 (Reverse): 5´- GCTGCGTTCTTCATCGATGC -3´. PCR amplification was followed by RFLP technique using *Hae*III enzymes for final species identification of the parasite.

### Collection of rodents

Rodents were captured by setting the 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 in December, February, May, and July. 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. Procedure of species identification by PCR method is described previously.

### DNA Extraction from positive smears of rodents

Total DNA was extracted from positive smears by digestion in 100 µL PBS buffer and the tube was centrifuged at 10000 rpm for 10 min, then 300 µL lysis buffer and 30 µL proteinase K added. The tube was incubated for 24 h at 37 °C before adding 300 µL sacharin phenol. After adding this solution the tube was centrifuged at 9300 rpm for 5 min. After transferring upper phase to new tube, 300 µL phenol- chloroform should be added and was centrifuged at 10000 rpm for 5 min. Again transferred the upper phase to new tube and washed by pure chloroform. Thirty µL MgCl2 and 1000 µL ethanol were added to upper phase and stored at -20° C for 2 h before was centrifuged at 10000 rpm for 10 min and washed down phase by 70% ethanol with TE and was centrifuged at 10000 rpm for 10 min and the TE buffer was added.

### PCR-RFLP for detection of *Leishmania* infection in Rodents

PCR-RFLP was employed for detection and identification of *L. major* in rodent specimens by the method explained by Dweik et al. (2007). The primers were: IR1 5´-GCT GTA GGT GAA CCT GCA GCA GCT GGA TCA TT-3´ and IR2 5´-GCG GGT AGT CCT GCC AAA CAC TCA GGT CTG -3´ (Cupolillo et al. 1995). Reference strains of *L. infantum* (MCAN/IR/96/Lon49), *L. tropica* (MHOM/IR/89/ARD2) and *L. major* (MHOM/IR/54/LV39) were used as positive controls. All were obtained from the Medical Parasitology Laboratory, the School of Public Health, Tehran University of Medical Sciences, Iran. PCR production was followed by RFLP technique using *Hae*III enzymes for final identification of the parasite.

## Results

### Sand flies

Totally 4900 sand flies were collected, including *P. papatasi* (41.2%), *P. mongolensis* (5.8%), *P. caucasicus* (3.6%), *P. caucasicus* group (7.3%), *P. sergenti* (2.2%), *P. alexandri* (2%), *P.* (*adlerius*) sp. (0.02%), *P. brevis* (0.02%), *P. kazeruni* (1.5%), *S. sintoni* (36%), *S. clydei* (3%) and *S. sogdiana* (0.02%).

Three hundred and seventy two *P. papatasi* specimens were examined by Semi-Nested PCR for *Leishmania* infection. Species-specific amplification of *L. major* DNA was found in one (0.3%) of the *P. papatasi* Giemsa stained prepared for detection of promastigote (Fig.1). Furthermore ITS amplification by Nested PCR primers followed by RFLP technique confirmed the DNA of parasite in the infected *P. papatasi* sample (Fig. 2 & 4). The infected *P. papatasi* was collected from bed room with parous and empty abdomen position.

### Rodents

During this study 16 rodents were captured and identified. All of them were *R. opimus*. Although all collected animals were examined for parasite infection under light microscope, amastigotes were only found in smears of 6(37.5%) of them. Each sample from infected rodents was inoculated subcutaneously at the base of tail of one BALB/c. Results from inoculation of parasite from infected rodents, revealed the presence of amastigotes in the nodules and ulcers of the experimentally mice after 35 d of inoculation period. Parasites infection was observed in both males and females animals. Isolated parasites from infected rodents were identified as *L. major* using PCR followed by RFLP technique (Figs. 3, 4).

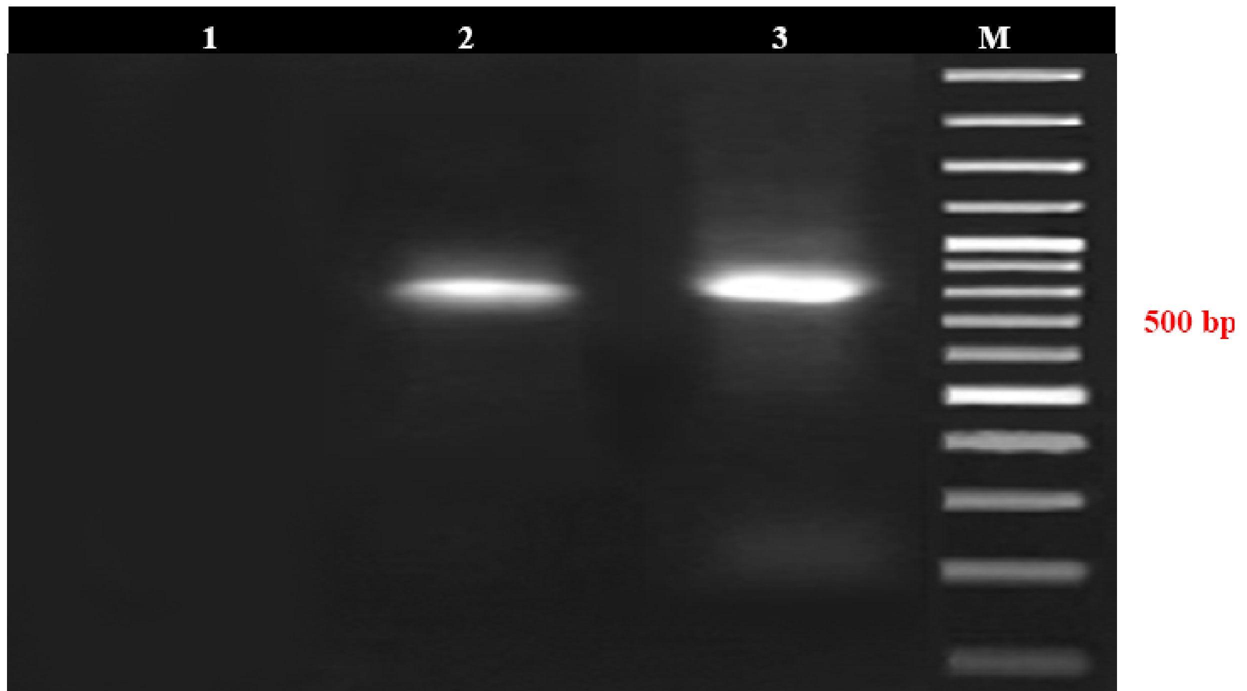


**Fig. 1.** kDNA PCR amplification of *L.major* in *P.papatasi using* Semi-nested PCR
M: Molecular size Marker, 1: Negative control, 2: Sample of *P.papatasi*, 3: positive control *L.major*

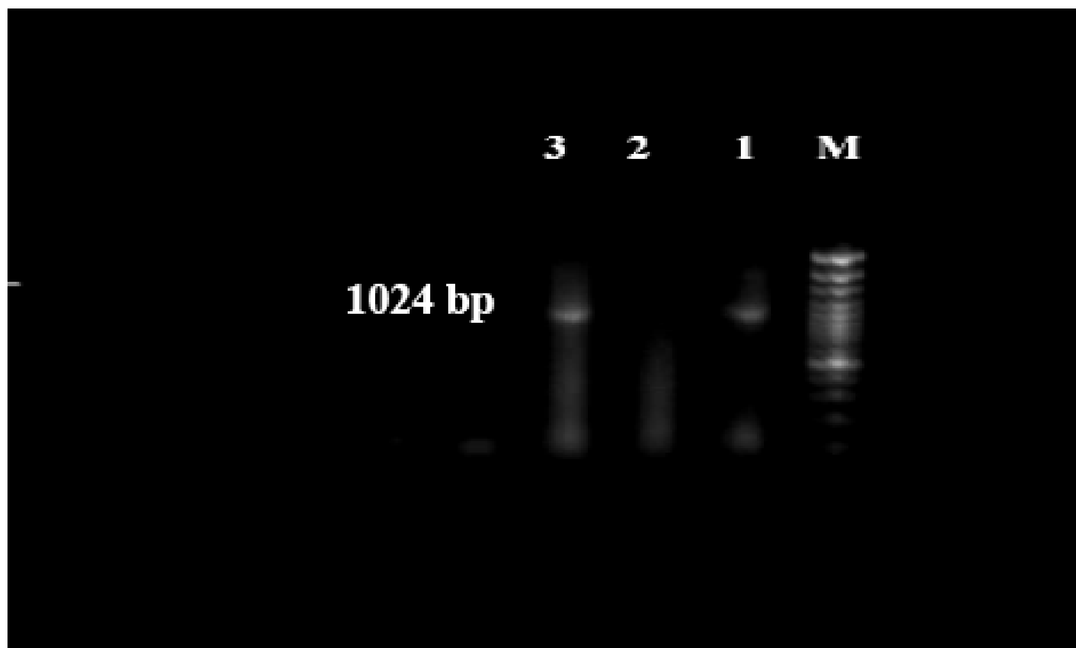


**Fig. 2.** ITS amplification of *L.major* in *P.papatasi using N*ested PCR primers
M(Marker) , 1(*L.major* standard), 2(blank), 3 (sample of *P.papatasi*)

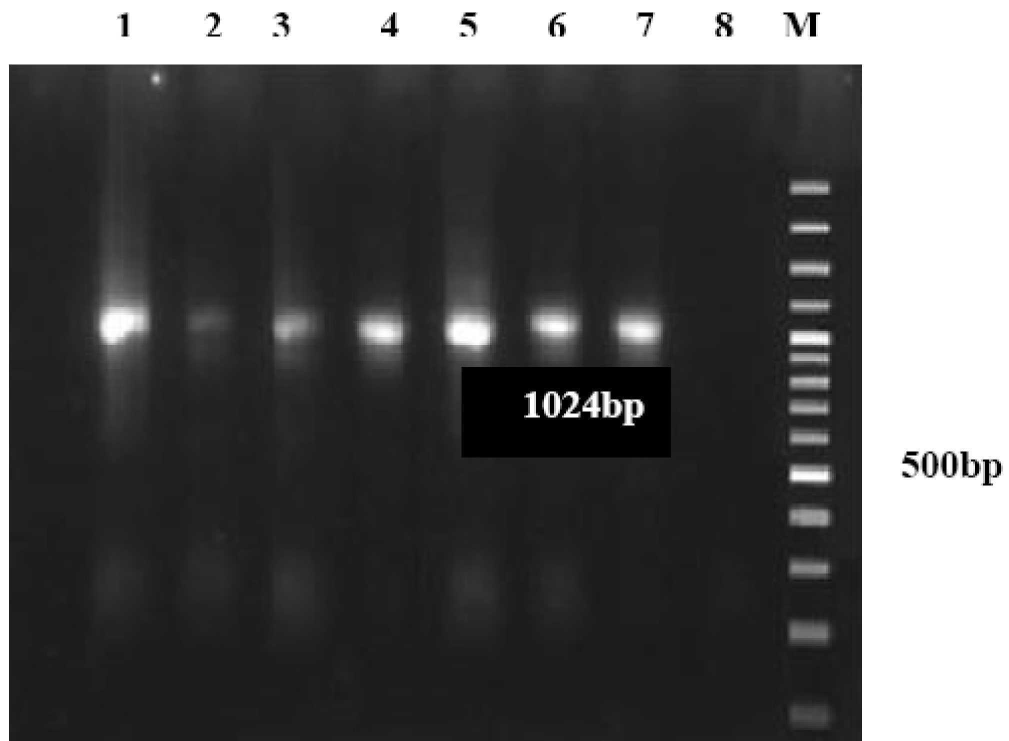


**Fig. 3.** ITS-rDNA amplification of *Leishmania* parasite in *R.opimus*
1-6(infected *R.opimus*), 7(*L.major* standard), 8(Negative Control), M (Marker)

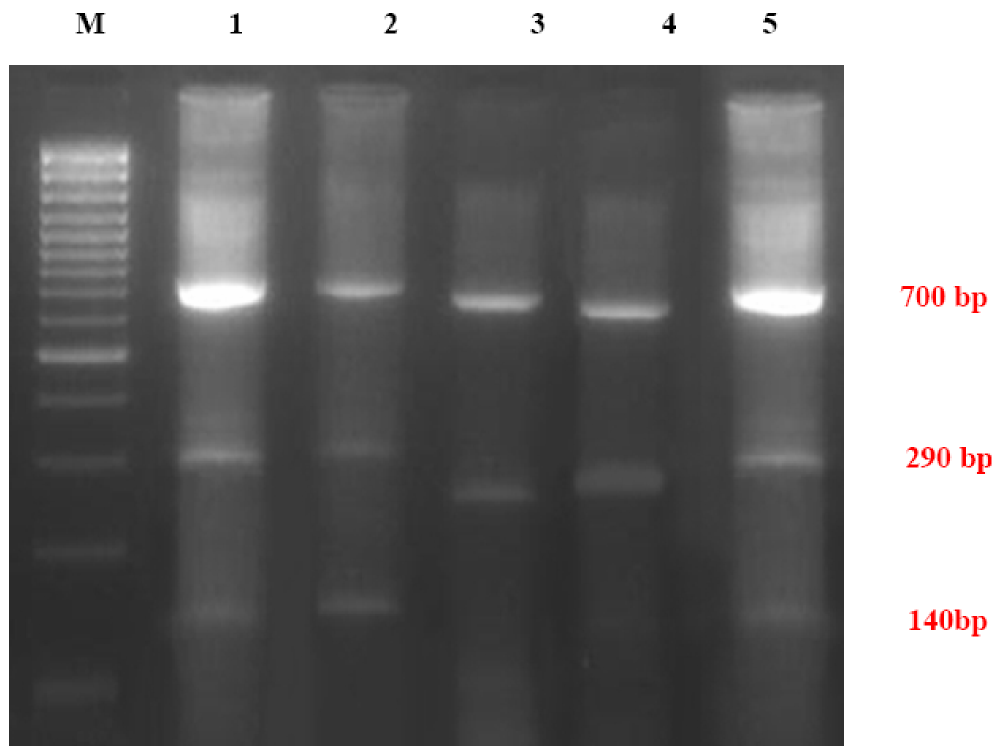


**Fig. 4.** PCR-RFLP analysis of ITS region for identification of *Leishmania* species using HaeIII
*L.major* standard (1), *P.papatasi* (2), *L.tropica* standard (3), *L.infantum* standard (4), and *Rhombomys opimus* (5)

## Discussion

Ecology and epidemiology of leishmaniasis are important measures for management and planning of the disease control. The entomological survey accompanied by epidemiological data is a major component for combating against disease. Several epidemiological and entomological finding including anthropophily, common infection of the sand-flies with the same *Leishmania* parasite that found in man in the same places, suggested the capacity of sand-fly as a vector (Killick-kendrick et al. 1990).

For further confirmation, molecular techniques (PCR) have been employed too. The highly sensitive technique of PCR has been used for detecting Leishmaniain sandflies in the world (Mukherjee et al. 1997), Iran (Azizi et al. 2006, Rassi et al. 2006, Rassi et al. 2007) and India (De Bruijn et al. 1992). Results of our study revealed that the high density of *P. patasti* in indoor resting places and infectivity with *L. major* is attributed that this species can play a major role as a principle vector in the region.

Another important finding of this survey was confirmation of *R. opimus* as the principal reservoir of ZCL in rural regions of Kalaleh 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 (Yaghoobi-Ershadi et al. 1996, Javadian et al. 1976, Seyyedi-Rashti et al. 1967). This great gerbil, a colonial, burrowing rodent, is a common species in the arid desert and steppe regions of central Asia. This species also exist in the southern territories of the former U.S.S.R. (i.e. Turkmenistan, Uzbekistan, Kazakhstan, Tajikistan) and neighboring countries where ZCL caused by *L. major* is endemic and considered as an important public health problem, therefore *R. opimus* is considered the principal mammalian host of the parasite (Strekova et al. 2001).

Human activities close to *R. opimus* burrows, the presence of high density of *P. papatas* in the rodent burrows and indoors as well as proximity of human habitat to *R. opimus* colonies lead to a rise in human contact with the disease agents and possible appearance of a new focus of leishmaniasis in the region.

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## References

Azizi K, Rassi Y, Javadian E, Motazedian MH, Rafizadeh S, Yahoobi Ershadi MR, Mohebali M (2006) *Phlebotomus (Paraphlebotomus) alexandri*: a probable vector of *Leishmania infantum* in Iran. Ann Trop Med Parasitol. 100(1): 63-68.

Cupolillo E, Grimaldi JR, Momen H, Beverley SM Intergenic region typing (IRT) (1995) a rapid molecular approach to the characterization and evolution of leishmaniasis. Mol Biochem Parasitol. 73: 145-155.

De Bruijn MH, Barker DC (1992) Diagnosis of new world leishmaniasis: specific detection of species of the *Leishmania brasiliensis* complex by amplification of kinetoplast DNA Acta Tropica. 52:5-58.

Dweik A, Schönian G, Mosleh IM, Karanis P (2007). Evaluation of PCR-RFLP (based on ITS-1 and Hae III) for the detection of leishmania species, using Greek canine isolates and Jordanian clinical material. Ann Trop Med Parasitol. 101 (5): 399-407.

Javadian E, Nadim A, Tahvidare-Bidruni GH, Assefi V (1976) Epidemiology of cutaneaous leishmaniasis in Iran. B: Khorassan area, part V; report on a focus of zoonotic cutaneaous leishmaniasis in Esferayen. Bull Soc Pathol Exot. 69:140-143.

Javadian E (1988) Reservoir host of cutaneous leishmaniasis in Iran. The XIIth International congress for tropical Medicine and Malaria, Amsterdam, the Netherlands. P.52.

Killick-kendrick R (1990).*Phlebotomus* vectors of visceral leishmaniasis, A review. Med Vet Entomol. 4:1-24.

Mukherjee S, Hassan MQ, Ghosh A, Bhattacharya A, Adhya S (1997) Short report: *Leishmania* DNA in *Phlebotomus* and *Sergentomyia* species during a kala-azar epidemic. American J Trop Med Hyg. 57:423-425.

Nadim A, Javadian E (1976) Key for the species identification of sand flies (Diptera phlebotominae) of Iran. Iranian J Publ Hlth. 5 (1): 25-28.

Rassi Y, Javadian E, Amin M, Rafizadeh S,Vatandoost H, Motazedian H (2006) *Meriones libycus* is the main reservoir of zoonotic cutaneous leishmaniasis in south Islamic Republic of Iran, East Med Hlth .12 (3-4): 475-7.

Rassi Y, Gassemi MM, Javadian E, Rafizadeh S, Motazedian H, Vatandoost H (2007) Vectors and reservoirs of cutaneous leishmaniasis in Marvdasht district, southern Islamic

Rassi Y, Hanafi Bojd A (2006a) Phlebotomine Sandflies, Vectors of Leishmaniasis.pp.251

Ready PD, Lainson R, Shaw JJ, Souza AA (1991) DNA probes for distinguishing Psychodopygus wellcomei from Psychodopygus complexus (Diptera: Psychodidae). Mem Inst Oswaldo Cruz. 86: 41-49.

Seyedi-Rashti MA, Nadim A (1967) Epidemiology of cutaneous leishmaniasis in Iran. B. Khorassan area, part I; the reservoir. Bull Soc Pathol Exot. 60: 510-518.

Seyedi-Rashti MA, Nadim A (1984) Cutaneous leishmaniasis in Baluchistan, Iran. Abstracts of and Poster Volum.XI, International Congress for Tropical Medicine and Malaria. Calgary, Canada. Sep. 16(22): 124.

Yaghoobi-Ershadi MR, Akhavan AA, Mohebali M (1996) *Meriones libycus* and *Rhombomys opimus* (Rodentia: Gerbellidae) are the main reservoir hosts in a new focus of zoonotic cutaneous leishmaniasis in Iran. Trans R Soc Trop Med Hyg. 90: 503-4.

Strekova MV, Elisev LN, Ponirovsky EN, Dergacheva TI, Annacharyava DK, PErokhin I, Evans DA (2001) Mixed leishmanial infections in *Rhombomys opimus*: a key to the persistence of *Leishmania major* from one transmission season to the next. Ann Trop Med Parasitol. 95(8): 811-19.

Yaghoobi-Ershadi MR, Hanafi-Bojd, AA, Akhavan AR, Zahrai-ramazani AR Mohebali M (2001) Epidemiological study in a new focus of cutaneous leishmaniasis due to *Leishmania major* in Ardestan town, central Iran. Acta Tropica. 79: 115-21.