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Molecular confirmation of main vector and reservoir host of cutaneous leishmaniasis in new focus of disease in South of Iran

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Abstract
Zoonotic cutaneous leishmaniasis (ZCL) is an endemic disease in many rural areas of Iran. In order to determine of vector(s) and reservoir of disease, a study was conducted in south part of Shiraz city (new focus of disease), Fars Province in 2014. A total of 2161 sand flies were collected. They were recognized as *Phlebotomus papatasi*, *P. sergenti*, *P. tobbi*, *Sergentomyia dentata*, *S. sintoni*, *S. clydei*, *S. sogdiana* and *S. mervynae*. *Phlebotomus papatasi* was the dominant species. Employing k DNA-nested PCR technique on 200 females of sand flies only 5 out of *P. papatasi* (4.5%), were positive to *Leishmania major*.
Using Sherman live traps, 26 rodents were captured. They all were *Meriones libycus*. Among the collected animals only 2 out of them (7.7%) were infected with *L. major*.
The results indicated that *P. papatasi* and *M. libycus* were the main vector and reservoir of disease in the studied area respectively.

Keywords: *Phlebotomus papatasi*, *Meriones libycus*, *Leishmania major*, Iran

1. Introduction
Leishmaniasis is a neglected tropical disease caused by a protozoan parasite from *Leishmania* species. Approximately 350 million people are at risk of infecting with leishmaniasis. The disease is reported from about 100 countries with a prevalence of 12 million infected people and an incidence of 2 million people per year [1]. Estimate of the disease burden is 2357000 disability adjusted life years [2].
Leishmaniasis is a complex disease with clinical forms of cutaneous leishmaniasis (CL), visceral leishmaniasis (VL), post kala-azar dermal leishmaniasis (PKDL) and mucucutaneous leishmaniasis (MCL). Visceral leishmaniasis is the most serious form which is fatal if left untreated. Cutaneous leishmaniasis is a public health problem but not fatal and is caused by a number of various *Leishmania* species. About 90% of CL worldwide cases are reported from Afghanistan, Algeria, Iran, Iraq, Saudi Arabia, Syria, Brazil and Peru [3-5].
Cutaneous leishmaniasis in the Old World is categorized to anthroponotic cutaneous leishmaniasis (ACL) and zoonotic cutaneous leishmaniasis (ZCL) with respect to the transmission. In the Old World, geographic distribution of ZCL is in rural areas in the Middle East, North Africa and China. Zoonotic cutaneous leishmaniasis is caused by *L. major*, *L. aethiopica* and dermotropic *L. infantum* in Old World countries [6].
Approximately 800 species of phlebotomine sand flies have been described from which about 10 percent have been incriminated as vectors of leishmaniasis [7].
Zoonotic cutaneous leishmaniasis is mainly transmitted by the sand fly belonging subgenus *Phlebotomus*. *Phlebotomus papatasi* as the principal vector of ZCL has a very wide distribution: in North Africa, central Asia, India, Middle East and is genetically conserved with little variation throughout its geographical distribution and takes a blood meal from a wide variety of reservoir hosts from rodent species [1, 6].
Rodents are the main and proven reservoirs of *Leishmania* parasite in the old world. Three species of *Rhombomys opimus*, *Meriones spp*. and *Psammomys obesus* are the major reservoir of causative agent of disease that maintain infection in most of Central Asia, Middle East and North Africa [8].
Others that are implicated in various parts of the old world include *Mastomys spp*., *Xerus* spp., *Mus musculls*, *Tatera indica*, *Arvicanthis spp*. and *Nesokia indica* [5, 9-15].

Recent studies in south of Iran showed that , two species of G*erbillus nanus* and *Rattus norvegicus* are naturally infected with *L. major* and maybe considered as the new reservoir of zoonotic cutaneous leishmaniasi [16, 17].
South part of Shiraz city, southern Iran is a new focus of ZCL, and this study was performed to determine the main vector(s) as well as host reservoir of the disease employing PCR techniques.

2. Material and methods

2.1 Study area

The study was carried out in Shiraz city, Fars province, and south of Iran during May to October of 2014. Shiraz is the sixth most populous city of Iran and the capital of Fars Province. In 2009 the population of the city was 1,455,073. Shiraz is located in the south of Iran and the northwest of Fars Province. It is built in a green plain at the foot of the Zagros Mountains 1,500 m (above sea level. Shiraz is 919 km south of Tehran.
Shiraz's climate has distinct seasons, and is overall classed as a hot semi-arid climate, though it is only a little short of a hot-summer Mediterranean climate. Summers are hot, with a July average high of 37.8 °C. Winters are cool, with average low temperatures below freezing in December and January. Around 300 mm of rain falls each year, almost entirely in the winter months.

2.2 Collection of sand flies

Based on prevalence of disease four places in south part of Shiraz city were selected. Sand flies were collected biweekly from indoors (e.g. bedroom, guest bedroom, toilet, and stable) , outdoors (wall cracks and crevices) as well as animal burrows by using sticky paper (60 papers per place) during May to October of 2014. All traps were installed at sunset and collected near sunrise. The sand fly specimens were washed in 96% ethanol alcohol to get rid of the sticky materials and to preserve them. Dissection of preserved sand flies was done in phosphate buffered saline (PBS) solution. The terminal segments of the abdomen containing the spermatheca and the heads of females were removed and mounted in a drop of Puri's medium and identified to species level using key of Theodor and Mesghali, 1964 [18]. The remains of the bodies of the sand flies were kept individually in 96% alcohol and stored at –20 ºC for molecular analysis.

2.3 Collection of rodents

Active colonies of rodents near human houses were identified, and rodents were captured using 20 Sherman live traps. The traps were baited with roasted walnut, cucumber, and tomato, and placed in active burrows. The traps were set up early morning and evening during July to October, 2014.
The genus and species of the rodents were determined by external characteristics: color, body measurements, ears, tail, feet, teeth, and cranium [19, 20]. Parasite infection in rodents was examined microscopically by preparing an impression smear from their ears after Giemsa staining. Positive smear samples were collected from infected rodents and were inoculated subcutaneously at the base of the tail of BALB/c. The procedure followed for species identification by polymerase chain reaction (PCR) method is described later.

2.4 DNA amplification and PCR-RFLP

Examination for infection of female specimens of sand flies as well as rodents with *Leishmania* species was performed using nested- polymerase chain reaction (PCR) against the mini circle kinetoplast kDNA using the primers (Table1) and protocol described by Noyes *et al* [21]. Amplification was carried out in two steps, both in the same tube. This PCR protocol is able to identify *Leishmania* parasites by producing a 680 bp for *L. infantum*/*L. donovani*, 560 bp for *L. major*, and a 750 bp for *L. tropica*. The cycling conditions were 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 seconds, 55 °C for 60 seconds, and 72 °C for 90 seconds. One micro liter of a 9:1 dilution in water of the first-round product was used as template DNA for the second round in a total volume of 30 mL under the same conditions as those for the first round, except with primers LIR and 13Z. All PCR products were analyzed by 1–1.5% agarose gel electrophoresis, followed by ethidium bromide staining and visualized under UV light. Standard DNA fragments (100 bp ladder, Fermentas) were used to permit sizing [21].

Table 1: Details of primers used in this study

Target	PCR step	Name	Sequences(5'-3')
kDNA	First	CSB2XF	CGAGTA GCAGAAACTCCCGTTA
		CSB1XR	ATTTTTCGCGATTTTCGCAGAACG
	second	13Z	ACTGGGGGTTGGTAAAATAG
		LIR	TCGCAGAACGCCCCT

3. Results

3.1 Sand flies

Altogether, 800 sticky traps were installed and 2161 specimens comprising 8 species of sand flies were collected and identified. They were *Phlebotomus papatasi*, *P .sergenti*, *P. tobbi*, *Sergentomyia dentata*, *S. sintoni*, *S. clydei*, *S. sogdiana* and *S. mervynae.* Among the collected sand flies 960 specimens were collected from indoor places. They identified as, *P. papatasi* (61%), *P. sergenti* (10%) *S. dentata* (15%) and *S. sintoni* (14%). About 350 specimens of sand flies including: *P .papatasi* (75%), *S. dentata* (12%) and *S. sintoni* (13%) were identified from rodent burrows. Eight species of *P. papatasi* (30 %), *P. sergenti* (18%), *P. tobbi* (2%), *S. dentata* (19%), *S. sintoni* (13%), *S. clydei* (6%), *S. mervynae* (3.5%) and *S. sogdiana* (2.5%) were caught from outdoor places. The species of *P. papatasi* was the dominant specimen in outdoors, indoors and rodent burrows. The species richness of sand flies in outdoor were higher than indoors and rodent burrows.
Based on medical importance of female sand flies and their abundance in outdoors, indoors and rodent burrows, in all, 200 females of *P. papatasi* (55%), *P. sergenti* (20%), *P. tobbi* (3%), *S. dentata* (13%) and *S. sintoni* (9%) were tested against

the *Leishmania* parasite genome. Among all specimens, only 5 out of *P. papatasi* females (4.5%) were positive for *L. major* using the nested PCR against kinetoplast DNA. This was observed in the kDNA nested-PCR amplification assays where a 560 bp PCR band was produced for *Leishmania major* (Figure 1). Their abdominal stages was empty indicating that there was enough time for the parasites to develop and transform to Meta cyclic promastigote, the infective form to human.

3.2 Rodents

A total of 26 rodents were captured and identified. They all were *Meriones libycus.* Although all collected animals were examined for parasite infection under a light microscope, amastigotes were found only in smears of 2 (7.7%) rodents. Each sample from infected rodents was inoculated subcutaneously at the base of tail of one BALB/c. Inoculation of the parasite from infected rodents revealed the presence of amastigotes into the nodules and ulcer of the experimental mice after 22 days of the inoculation period. Parasite infections were observed in both male and female animals. Isolated parasites from infected rodents were identified as *L. major* using kDNA nested PCR with 560 bp band (Figure 1).

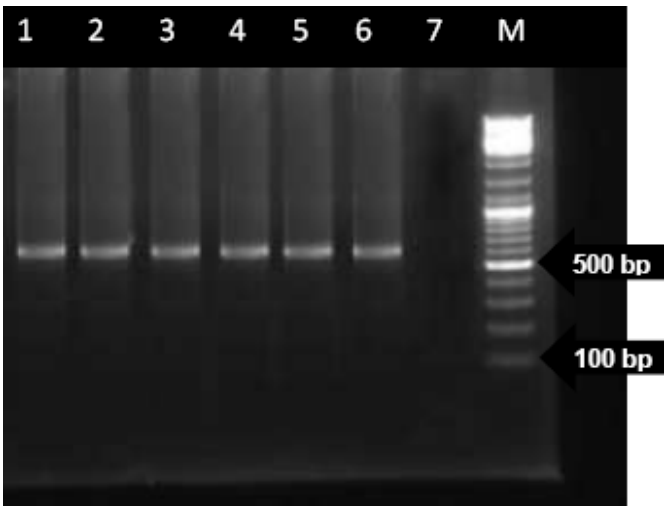

Fig 1: Minicircle kDNA PCR amplification of *L. major* in *P. papatasi* using Nested PCR. **M**: molecular size marker (100 bp ladder); **Lane 6**: *Leishmania major* standard; **Lane** 3–5: samples of infected *P. papatasi* to *L. major*; **Lane** 1-2: samples of infected *M. libycus* to *L. major*; **Lane** 7: negative control.

4. Discussion

Several evidences including the presence of natural infections, the ability to feed on the reservoir host(s), relative anthropophilic behaviour, the ability to support the growth of the parasite and the ability to transmit the parasite by bites are important criteria to implicate *P. papatasi* as the potential vector for circulation of leishmaniasis among rodents as the reservoir and human in the endemic areas of disease [2].

The Afro-Asian leishmaniasis vector, *Phlebotomus papatasi* (Scopoli), the type species of the genus, has a wide distribution throughout the Old World [23]. *Phlebotomus papatasi* has been established as the vector of *Leishmania major*, the causative agent of zoonotic cutaneous leishmaniasis (ZCL) in most parts of the Old World as well as in Iran [10, 14, 23-25].

The current study identified 8 species of sand fly. However, the results revealed no evidence for the presence of infection in most of the sand flies collected; for instance, the only species found infected with *Leishmania major* was *P. papatasi*, with an infection rate close to 4.5% using molecular methods. At the present study, high density of *P. papatasi* in outdoors, indoors rodent burrows and its natural infection with *L. major* is attributed to the fact that this species play a major role as a main vector in the region.

The animal reservoir populations and their roles in disease transmission were also considered in our study. Rodents are one of the most important small mammalian groups which may carry a wide variety of pathogens responsible for many tropical zoonotic diseases, including those caused by the trypanosomatid parasitic protozoa, *Leishmania* [26]. So far sixty five species of rodents have been cited in Iran [27]. In Iran and the rest of the ZCL endemic countries in the Old World, rodents in Gerbillinae subfamily play the main role as the reservoir hosts. Gerbils are the most frequent mammals in natural deserts of Old World [9, 10, 12, 14, 15, 25, 28, 29].

At present, nine species of *Rhombomys opimus*, *Meriones libycus*, *Tatera indica*, *Nesokia indica*, *Meriones hurrianae*, *Meriones persicus* , *Gerbillus nanus* and *Rattus norvegicus* are thus reported to be infected with *L. major* in Iran [12, 14-17, 25, 28, 29].

However, the current study revealed the presence of infections in *M. libycus* (7.7%), the only collected species, in the studied region. Similar observations were reported in central and south districts of Iran [10, 14, 15]. This species plays as the main reservoir host of ZCL in several endemic foci of disease in Iran, where two rodents of *Rhombomys opimus* and *Tatera indica* are absent. In conclusion, the result of the current study revealed the important factors present for the establishment of the disease in the region. These include human activities close to *M. libycus* burrows, the presence of high density of *P. papatasi* in the rodent burrows, outdoors and indoors, and proximity of human habitat to *M. libycus* colonies, which have led to emergence of a new focus of *L. major* in the region.

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