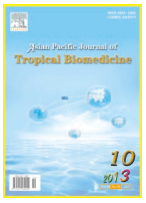
Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

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



Document heading doi:10.1016/S2221-1691(13)60163-X

First detection of *Leishmania infantum* DNA in wild caught *Phlebotomus papatasi* in endemic focus of cutaneous leishmaniasis, South of Iran

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PEER REVIEW

Peer reviewer

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Comments

This is an interesting study which used molecular markers for identification of *Leishmania* parasites at species and strain levels and identification of probable vectors and reservoir host of leishmaniasis. It provided useful information to address the key epidemiological questions for control of disease.
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ABSTRACT

Objective: To identify the vectors and reservoirs of cutaneous leishmaniasis in the endemic focus of Farashband, Fars Province, South of Iran.
Methods: Sticky papers and Sherman trap were used for collection of sand flies and rodents, respectively. Polymerase chain reaction (PCR) of kDNA, ITS1-rDNA were used for identification of *Leishmania* parasite in sand flies as well as rodents.
Results: Totally 2010 sand flies were collected and the species of *Phlebotomus papatasi* Scopoli was the common specimen in outdoors and indoors places. PCR technique was employed on 130 females of *Phlebotomus papatasi*. One of them (0.76%) was positive to parasite *Leishmania major* (*L. major*) and one specimen (0.76%) was positive to *Leishmania infantum*. Microscopic investigation on blood smear of the animal reservoirs for amastigote parasites revealed 16 (44%) infected *Tatera indica*. Infection of them to *L. major* was confirmed by PCR against kDNA loci of the parasite.
Conclusions: The results indicated that *Phlebotomus papatasi* was the dominant species circulating two species of parasites including *L. major* and *Leishmania infantum* among human and reservoirs. Furthermore, *Tatera indica* is the only main host reservoir for maintenance of the parasite source in the area.

KEYWORDS

Leishmania infantum, *Leishmania major*, Vector, Reservoir, Iran

1. Introduction

Cutaneous leishmaniasis (CL) is a worldwide public health and a social problem in many developing countries. It can affect skin and mucous membranes, and is caused by different *Leishmania* species widespread in 98 countries in the New and Old World.

Old World cutaneous leishmaniasis is present in many endemic areas in North Africa, the Mediterranean, the Middle East, the Indian subcontinent and Central Asia. The species responsible for Old World cutaneous leishmaniasis are mainly *Leishmania major* (*L. major*) and *Leishmania tropica* (*L. tropica*). *Leishmania infantum* (*L. infantum*). *Leishmania donovani* (*L. donovani*) can also cause localized CL but are observed less

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Foundation Project: Supported by the School of Public Health, Tehran University of Medical Sciences (Project No.13946).

Article history:
Received 23 Jun 2013
Received in revised form 1 Jul, 2nd revised form 12 Jul, 3rd revised form 15 Jul 2013
Accepted 28 Aug 2013
Available online 28 Oct 2013

frequently in the Mediterranean areas. Diffuse CL is uncommon and is caused by *Leishmania aethiopica* in Africa[1,2].

There are several reports indicating occurrence of CL due to *L. major* in Iran[3-6]. CL is reported from 55% of the 31 provinces in Iran[7]. The annual incidence of cutaneous leishmaniasis has gradually increased in Iran and about 30000 cases has been reported in 2011 (unpublished data). This increaseing outbreak is related to human-sand fly-rodent contacts, itself probably the product of the development of irrigation schemes and the spread of human populations into the habitats of the vector and the rodents that act as reservoir hosts.

Based on animal reservoir hosts there are four foci of disease in Iran[3]. The first one has been located in central and northeast of Iran, where *Rhombomys opimus* Lichtenstein and *Phlebotomus papatasi* Scopoli (*P. papatasi*) play important roles as reservoirs and vectors of the disease[3]. The second foci are located in the west and southwest of Iran, where *Tatera indica* Hardwicke (*T. indica*) is replaced with *Rhombomys 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 *L. major* and *Meriones hurrianae* Jordon has been approved as its natural reservoir host[9]. The most rural areas of Fars province in southern Iran can be considered as the *L. major* focus where *Meriones libycus* Lichtenstein is the primary and main reservoir host of the disease, while *Rhombomys opimus* and *T. indica* were absent and *P. papatasi* is considered as the proven vector of *L. major*[4,5].

Farashband district of Fars province in the south of Iran is an important *L. major* focus and this study was performed to study 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 host reservoir of the disease.

2. Material and methods

2.1. Study area

The study was carried out in Farashband city located in Farahband County, Fars province in southern Iran. The capital of the county is Farashband. At the 2006 census, the county's population was 38679, in 8474 families. The county is subdivided into two districts: the Central district and Dehram district with three cities: Farashband, Nujin, and Dehram. Farashband city is located at an altitude of 775 m above sea level. Farashband's climate has distinct seasons, and is overall classed as a semi-arid climate. Summers are hot, with an average of 37.8 °C. Winters are cool, with average low temperatures below freezing in December and January. Around 250-3000 mm of rain falls each year.

2.2. Sand flies collection and identification

Sand flies were collected from indoors (bedroom, guestroom, and toilet) as well as outdoors (rodent burrows, wall cracks) biweekly using sticky papers in the city with *L. major* cases during the summer of 2011. For each collection time, 300 sticky traps were set up during the sand flies high active period (July-September) in the ten selected places of city. Collected sand fly specimens were washed once in 1% detergent then twice in sterile distilled water. Each specimen was then dissected in a drop of fresh sterile normal saline by cutting off the head and abdominal terminalia with sterilized forceps and disposable needles. The rest of the body was stored in the sterile Eppendorf® micro tubes for DNA extraction. Specimens were mounted on glass slides using Puri's medium and identified using the identification keys for species within Larrousius group and for species of other groups and subgenera[10-12].

2.3. DNA extraction

DNA was extracted by using the Bioneer® Genomic DNA Extraction Kit. Extraction was carried out by grinding of individual sand fly in a micro tube using glass pestle following the kit protocol and stored at 4 °C. Double distilled water was used as a negative control and DNA from *L. major* (MHOM/IR/75/ER) and *L. tropica* (MHOM/IR/03/Mash-878) provided by the Parasitology Department, School of Public Health, Tehran University of Medical Sciences were used as positive controls[13].

2.4. Detection and identification of Leishmania species

Initial screening of sand flies was performed by nested-PCR amplification of kinetoplast DNA (kDNA) using the primers (Table 1) and protocol described by Noyes *et al*[14]. This method is highly sensitive and is recommended for initial screening. 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. Due to presence of many DNA polymorphisms in kDNA of each *Leishmania* species, sequencing of kDNA is problematic. Therefore, further identification of *Leishmania* parasites was done using the ITS1-PCR[14,15]. A set of primers (Table 1) LITSR and L5.8S was used to amplify 340 bp of rDNA including parts of 3′ end of the 18S rDNA gene, complete ITS1, and part of 5′ end of the 5.8S rDNA gene. Also, the ITS1 PCR products (340 bp) of the samples that demonstrated *Leishmania* profile were sequenced (Seqlab, Göttingen, Germany), employing the same primers used for the PCR. The sequences obtained were processed and aligned, using the multiple alignment program clustal X[16]. Homologies with the available sequence data in GenBank was checked by using BLAST analysis software (http://www.ncbi.nlm.nih.gov/BLAST). A PCR protocol of 30 cycles of denaturation was performed for 30

s at 94 °C, annealing 1 min at 62 °C and elongation for 1 min at 72 °C, followed by a final elongation of 10 min at 72 °C. All PCR products were analyzed by 1%–1.5% agarose gel electrophoresis, followed by ethidium bromide staining and visualization under UV light. Standard DNA fragments (100 bp ladder, Fermentas®) were used to permit sizing.

Table 1

List and details of primers used in this study.

Target	PCR step	Name	Sequences (5′–3′)
kDNA	First	CSB2XF	CGAGTAGCAGAAACTCCCGTTCA
		CSB1XR	ATTTTTCGCGATTTTCGCAGAACG
	Second	13Z	ACTGGGGGTTGGTGTAAAATAG
		LIR	TCGCAGAACGCCCCT
ITS1	One	LITSR	CTGGATCATTTTCCGATG
		L5.8S	TGATACCACTTATCGCACTT

3. Results

3.1. Sand flies

Altogether 1 800 sticky traps were installed and 2010 specimens comprising 7 species of sand flies were collected and identified. They were *P. papatasi*, *Phlebotomus alexandri*, *Sergentomyia sintoni*, *Sergentomyia sumbarica*, *Sergentomyia clydei*, *Sergentomyia baghdadis* (*S. baghdadis*) and *Sergentomyia theodori*. Among the collected sand flies 850 specimens were belong indoor places including: *P. papatasi* (88%), *S. baghdadis* (10%) and *Sergentomyia sintoni* (2%). About 702 specimens of sand flies were collected from rodent burrows. They were *P. papatasi* (61%), *Sergentomyia theodori* (28%), *Sergentomyia clydei* (8%), *Sergentomyia sumbarica* (1%), *S. sintoni* (1%) and *S. baghdadis* (1%). Four specie of *P. papatasi* (68%), *P. alexandri* (14%), *Sergentomyia sintoni* (16%) and *S. baghdadis* (2%) were caught from out door places. The species of *P. papatasi* was dominant in out doors, indoors and rodent burrows. The species richness of sand flies in rodent burrows were higher than indoors and out door places.

In all, 130 females of *P. papatasi* were tested against the *Leishmania* parasite genome. Only 2 out of them were positive for *L. infantum* and *L. major* using the nested PCR against kDNA. This was observed in the kDNA nested-PCR amplification assays where a 680 and 560 bp PCR band were produced for *L. infantum* and *L. major* respectively (Figure 1). Examination of the two infected specimens showed that their abdomen were empty. Further analysis showed that they were positive against ITS1 locus and produced a band of approximately 340 bp. This is the first report of *P. papatasi* naturally infected with *L. infantum* in Iran or maybe in the world. The ITS1 DNA sequences were obtained for only one of these specimens and submitted to Genbank database with accession number (KC57045) in comparison with other available data, confirming it as *L. infantum*. The specimen was identical (99%) or very similar to several *L. infantum* sequences deposited in Genbank, including isolates from Uzbekistan (Accession No. FN398341), Iran (Accession Nos. KC347301 and HQ535858), France (Accession No. AJ634340) and India (Accession No. EU326227). Also it was found to be 99% similar to *Leishmania chagasi* from Brazil (Accession No. AJ000304) and 99% similar to *L. donovani* from Bangladesh (Accession No. AB725909).

3.2. Rodents

During this study 36 rodents were captured and identified. They were *T. indica*. Although all collected animals were examined for parasite infection under a light microscope, *Leishmania* parasite were found only in smears of 16 (44%) of them. 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 30 d 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).

Furthermore we observed mix infection of some rodents to amastigote and promastigote after two month keeping in animal house (Figure 2).

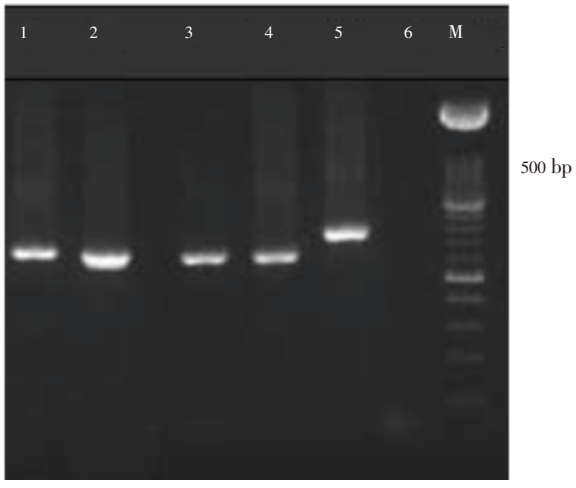

Figure 1. kDNA nested PCR amplification (680 bp).
L. infantum in *P. papatasi* (Lane 1, 560 bp); *L. major* in *P. papatasi* and *T. indica* (Lanes 2 and 3); Positive control of *L. major* (Lane 4, 720 bp); Positive control of *L. tropica* (Lane 5); Negative control (Lane 6) and (M)100 bp molecular weight marker (Fermentase).

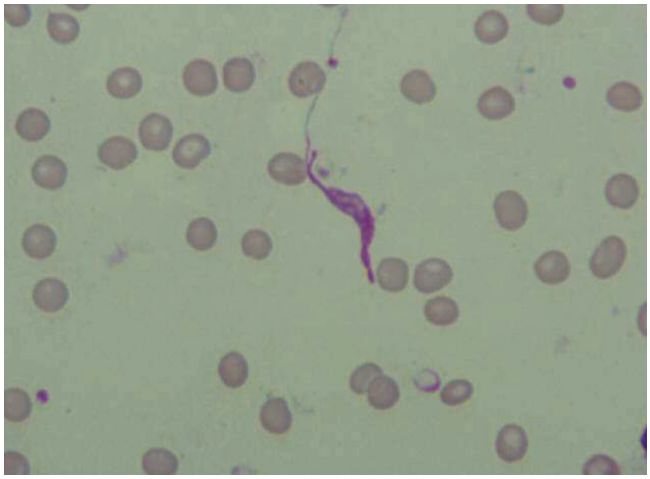

Figure 2. Promastigote and amastigote infection of wild caught *Tatera indica* after two month keeping in animal house.

4. Discussion

Ecology and epidemiology of leishmaniasis are important measures for management and planning of disease control. The entomological survey accompanied by epidemiological data is a major component for combating against disease. Several epidemiological and entomological findings, including anthropophily and common infection of the sand flies with the same *Leishmania* parasite that found in patient in the same places, suggested the capacity of sand fly as a vector[17,18]. Recently, molecular techniques (PCR) have been employed for vector incrimination of sand flies[18-20]. The highly sensitive technique of PCR has been used for *Leishmania* in sand flies in some endemic areas, including Iran and India[3,4,18,19,21-23]. Results of our study revealed that the high density of *P. papatas*i in indoor, out door and rodent burrow resting places and its infection with *L. major* is attributed to the fact that this species plays a major role as a principal vector in the region.

In this study we found natural infection of *P. papatasi* to *L. infantum*. This parasite is causative agent of infantile visceral leishmaniasis in the old world. There are several vectors of *L. infantum* in the subgenus Laroussius, including *Phlebotomus* (*Laroussius*) *perfiliewi*, *P.* (*Lar.*) *neglectus*, *P.* (*Lar.*) *syriacus*, *P.* (*Lar.*) *major*, *P.* (*Lar.*) *kandelakii* and *P.* (*Lar.*) *tobbi* have been reported from the eastern Mediterranean basin[17,18,20]. We could not find any reports on natural infection of wild caught *P. papatasi* to *L. infantum* and this is the first one in Iran and maybe in the world. Our study showed that this sand fly circulating two species of *Leishmania* parasite including *L. major* and *L. infantum* among human and reservoirs. There is no human visceral leishmaniasis cases in the study areas.

In this survey we isolated some *L. major* parasites from *T. indica*, the only collected rodents species. This fact confirms that *T. indica* is the principal reservoir of *L. major* in Farashband city. *Leishmania* infection of *T. indica* has been reported in west and southwest of Iran with 12.5% and 9.0% positive cases respectively[8]. In a study in southeastern Iran *L. major* was found to be 3.7% infection in this region. There is a report on *Leishmania* infection of *T. indica* as a reservoir of cutaneous leishmaniasis in Baluchistan, Pakistan[24].

T. indica ranges from northern Arabia throughout the Indomalayan region[25]. This species commonly known as the Indian gerbil, was first recorded from southeastern Turkey by Misonne[26].

This is the first report on natural infection of wild *T. indica* to *L. major* in Fars province, south of Iran. This rodent is the only infected animal and it seems to play as a reservoir host of the disease in the study areas.

The result of the current study revealed all of the important factors present for establishment of the disease in the region. These include human activities close to *T. indica* burrows, the presence of high density of *P. papatas*i in the rodent burrows and indoors, and proximity of human habitat to *T. indica* colonies, which have led to emergence of a new focus of *L. major* in the region. Further study is needed for confirming the presence of *L. infantum* in this region.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The authors gratefully thank Farashband Health Center, Fars province, south of Iran for its field assistance. This study was financially supported by the School of Public Health, Tehran University of Medical Sciences (Project No.13946).

Comments

Background

Cutaneous leishmaniasis 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 98 countries in the New and Old World. Old World cutaneous leishmaniasis is present in many endemic areas in North Africa, the Mediterranean, the Middle East, the Indian subcontinent and Central Asia. Phlebotomine sand flies are the only biological vectors, and rodents play as the reservoir hosts of the disease.

Research frontiers

This article is discussing on the detection and identification of *Leishmania* parasite and their corresponding vectors as well as the reservoir host of the disease using molecular methods.

Related reports

Ecology and epidemiology of leishmaniasis are important measures for management and planning of 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 patient in the same places, suggested the capacity of sand fly as a vector. Recently, molecular techniques (PCR) have been employed for vector incrimination of sand flies. The highly sensitive technique of PCR has been used for *Leishmania* in sand flies in some endemic areas including Iran, India and several countries with leishmaniasis problem. Rodents are regarded as resources of rural type of cutaneous leishmaniasis.

T. indica is main reservoir host of cutaneous leishmaniasis in west and southwest of Iran. This species of rodent has been reported from Pakistan as a reservoir host of disease.

Innovations and breakthroughs

This study for the first time found *L. infantum* in *P. papatasi* in Iran and maybe in the world. And it is also the first time to find *L. major* in *T. indica* in Fars province, South of Iran. This study also revealed mix infection of wild caught *T. indica* with amastigote and promastigot after two month

keeping in animal house.

Applications

Determination and identification of vectors and reservoir host of cutaneous leishmaniasis are two important things for planning of effectiveness program for control of the disease in rural areas.

Peer review

This is an interesting study which used molecular markers for identification of *Leishmania* parasites at species and strain levels and identification of probable vectors and reservoir host of leishmaniasis. It provided useful information to address the key epidemiological questions for control of disease.

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