Tropical Biomedicine 29(1): 1–8 (2012)

First molecular detection of *Leishmania major* within naturally infected *Phlebotomus salehi* from a zoonotic cutaneous leishmaniasis focus in southern Iran

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Received 12 December 2010; received in revised form 14 May 2011; accepted 20 May 2011

Abstract. Human cutaneous leishmaniasis (CL) is a major notifiable public health problem in many parts of Iran. It is often caused by the zooflagellate parasite *Leishmania major* which is mainly transmitted by the bites of female phlebotomine sandflies belonging to the genus *Phlebotomus* (Diptera: Psychodidae). The annual incidence of CL in Fars province, southern Iran, was about 108-144 in 2007. The leishmanial infections of wild sandflies that may act as vectors were thus investigated at an endemic focus in this province. In all 330 female *Phlebotomus* sandflies were screened for the detection of *Leishmania*-specific kinetoplast DNA (kDNA) by polymerase chain reaction (PCR) methods. A two stage nested PCR protocol was used to establish the identity of *Leishmania major* species in naturally infected sandflies. The *L. major* kDNA was detected in 18 (5.5%) individual sandflies which belonged to four different *Phlebotomus* species (*Phlebotomus papatasi*, *Phlebotomus salehi*, *Phlebotomus sergenti* and *P. major* group). For the first time, one naturally infected *P. salehi* specimen contained the kDNA of the protozoan parasite, *L. major*, with a main band of 560 base pairs identified using the nested PCR method. It seems most likely therefore that *P. salehi* is potentially a rare sylvatic vector of *L. major* parasites in parts of this province. This is the first combined morphological and molecular studies of *P. salehi* in Iran.

INTRODUCTION

Members of the zooflagellate genus *Leishmania* Yakimoff and Schokhor (Kinetoplastida: Trypanosomatidae) cause complex protozoan diseases in humans known as leishmaniases. These are important re-emerging parasitic zoonoses (Ashford, 2000) vectored by the bites of female phlebotomine sandflies belonging to the genus *Phlebotomus* (Diptera: Psychodidae)(Dedet *et al.*, 1982; Depaquit *et al.*, 2008). *Leishmania* reproduces as an amastigote form within the mammalian host macrophages (Ashford, 2001). About two dozen distinct *Leishmania* species are known causing human leishmaniases and clinical features are mostly indicative of the species (Murray *et al.*, 2005). Description of the *Leishmania* species in endemic areas is thus crucial for both chemotherapy and prognosis.

The geographical range of leishmaniases is within that of the sandfly vectors and reservoir hosts. Although <5% of them are probably associated with human health problems, about 1,000 sandfly species are recognized (Service, 2008; Ready, 2010), though none of them are exclusively anthropophilic (Alexander, 2000). In addition, only a limited number of sandfly species can support the growth of specific species of *Leishmania* and transmit them in due course (Lane, 1993). Thus, it is important to detect

and identify both *Leishmania* and sandfly species which are vital to incriminate putative vectors, elucidate natural transmission cycles, develop effective control strategies, and predict the risk of rapid disease expansion in endemic areas (Killick-Kendrick, 1999; Alexander & Maroli, 2003).

The sandfly *Phlebotomus (Phlebotomus) salehi* Mesghali, 1965 was first described from the southeast Sistan-Baluchestan and Hormozgan provinces of Iran. Its natural promastigote infection was recorded from the former province only a decade ago (Kasiri & Javadian, 2000). No report has yet been published which incriminates this species by molecular means from any Iranian provinces. It appears that this species is a vector of the unstable Old World zoonotic cutaneous leishmaniasis (ZCL) among rodent reservoir hosts, like *Meriones* species (Lewis, 1982; Gramiccia & Gradoni, 2005). It shares a close phylogenetic affinity with *Phlebotomus duboscqi* Neveu-Lemaire, 1906 and less closely with *Phlebotomus papatasi* Scopoli, 1786; both of which are proven to be the main vectors of *Leishmania (Leishmania) major*, the causative agent of ZCL (Killick-Kendrick, 1999; Gramiccia & Gradoni, 2005; Depaquit *et al*., 2008).

In this study, a method of detecting *Leishmania* species within naturally infected sandfly individuals by polymerase chain reaction (PCR) with minicircle kinetoplast DNA-specific primers was applied.

The main aim of this investigation was to incriminate the vector of *Leishmania* using direct microscopy as well as molecular methods. There appear to be no previous reports on the inclusion of *P. (P.) salehi* as a probable vector of *L. major* using molecular tools in Iran.

MATERIALS AND METHODS

Study area

The county town of Ghir-Karzin (53º03'E, 28º28'N at an altitude of about 780 m a.s.l.) lies in the south of Fars province and has a population of >35,000 people (Figure 1). Ghir-Karzin is characterized by a hilly landscape, south of the Zagros Mountain ranges, and a semi-arid habitat. It has a hot dry climate with mean annual temperature of about 21ºC. The relative humidity varies between 27-85% and the mean annual precipitation rate is ≈170 mm.

Sandfly collection and species identification

Sandflies were generally collected using sticky paper traps outdoors in three villages of Ghir-Karzin. This was carried out monthly during peak seasonal density for 3-5 consecutive days every two weeks from June to August 2006. The peak season was considered since it was more likely to find infected sandflies in this time period. Male sandflies were kept in 80% ethanol for mounting and morphological species identification, while females were processed for dissection and kDNA extraction.

Sandflies were dissected in a drop of normal PBS (pH=7.3) under a stereo-microscope to search for promastigotes. Heads and last abdominal segments were rinsed in a drop of Puri's medium (Smart, 1965) on a microscope slide to identify each sandfly to species level according to the taxonomic keys of Lewis (1982). The remaining body segments of unfed parous female sandflies of the common phlebotomine species (*P. papatasi*, *P. salehi*, *Phlebotomus sergenti*, *Phlebotomus alexandri* and *P. major* group) were used for DNA extraction and subsequent PCR processing.

DNA extraction

The total genomic DNA of each female sandfly and any parasite within it were extracted. Each sample was homogenized in a 1.5 ml microtube containing 200 µl lysis buffer [50 mM Tris-HCl (pH=7.6), 1 mM EDTA and 1% Tween 20] and 12 µl of proteinase K solution (19 µg/ml). The homogenate was incubated at 37ºC overnight before 300 µl from a phenol: chloroform: isoamylalcohol mixture (25:24:1, by vol.) were added. It was centrifuged at 10,000 rpm for 10 min and the DNA in the supernatant was precipitated with 400 µl cold pure ethanol, resuspended in 50 µl ddH_2O and stored at -20ºC before being

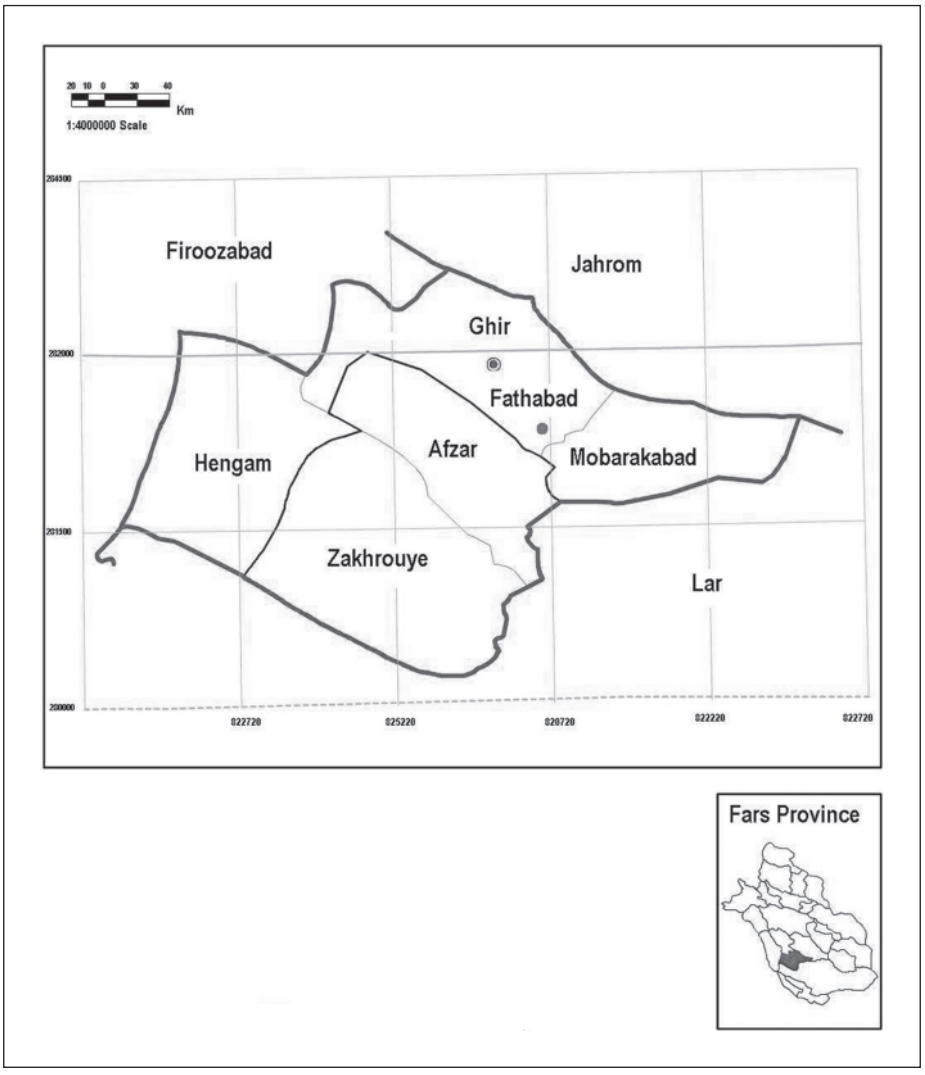

Figure 1. Local map of study area, Ghir-Karzin

used in the nested PCR assay to detect *Leishmania*-specific kDNA. Only 5 µl portions of DNA extracts were processed for PCR amplification.

Leishmania typing

This was carried out using the standard PCR procedure as described by Azizi *et al.* (2008). A set of three primers (LINR4, LIN17 and LIN19) were also designed within the conserved area of the minicircle kDNA which comprised conserved sequence blocks (CSB) CSB3, CSB2 and CSB1, respectively. This set of primers including the forward primer LINR4 (5'-GGG GTT GGT GTA AAA TAG GG-3'), LIN17 as the reverse primer for the first round (5'-TTT GAA CGG GAT TTC TG-3') and LIN19 as the reverse primer for the second round (5'-CAG AAC GCC CCT ACC CG-3'), was used in a nested PCR technique (Aransay *et al.*, 2000). Reference strains of *L. major* (MHOM/IR/54/LV39), *Leishmania tropica* (MHOM/IR/89/ARD2) and *Leishmania infantum* (MCAN/IR/96/Lon49) were used as standards.

Semi-nested PCR protocol was used to amplify the variable region of the minicircle kDNA of any *Leishmania* in the sandfly midgut. It involved a two-round nested PCR in which the two rounds were carried out in

separate tubes and 2 µl of the first round product diluted with ddH_2O (4:1) was used as template for the nested PCR.

The first-round amplification reaction mixture contained 250 µM of each deoxynucleoside triphosphate (dNTP), 1.5 mM $MgCl_2$, 1 unit *Taq* polymerase (Cinagene, Tehran), 1 µM primer LINR4, 1 µM primer LIN17 and 5 µl of DNA extract in 1X PCR buffer (Boehringer Mannheim, Mannheim, Germany), in a final volume of 25 µl. This reaction mixture was incubated in a CGI-96 thermocycler (Corbett Research, Sydney, Australia) set to run for 5 min at 94°C, followed by 30 cycles, each one consisting of 30 s at 94°C, 30 s at 52°C, 60 s at 72°C, and a final extension at 72°C for 10 min and kept at 4°C.

The second-round nested amplification was carried out in a separate tube having a 20 µl reaction mixture containing 1X *Taq* polymerase buffer, $MgCl_2$, dNTPs and *Taq* polymerase as described for the first round, added to 1 µM primer LINR4, 1 µM primer LIN19 and 2 µl of the first amplification reaction mixture (diluted 4:1 in ddH_2O). The PCR amplification profile was run for 33 cycles.

Negative controls (DNA extracts from male sandflies and aliquotes of ddH_2O) were run in each PCR protocol to detect possible contamination which could cause false positive results.

Agarose-gel electrophoresis

A 5 µl sample of each PCR end product was subjected to electrophoresis in 1.5% agarose gel. The DNA bands were stained with 1% ethidium bromide, visualized on an UV transilluminator and compared with molecular-weight markers and the relevant second-round products for the *L. major*, *L. tropica* and *L. infantum* standards.

RESULTS

Phlebotomine fauna

In all >5000 specimens were identified, more than 60% of which were species belonging to the genus *Phlebotomus*. In total, at least two distinct genera and 12 different phlebotomine sandfly species (six *Phlebotomus* and six *Sergentomyia* species) were morphologically identified. They included 52.4% female and 47.6% male sandfly specimens (Table 1). Female sandflies were more frequently collected than males in at least eight out of 12 different sandfly species (ratio 2/1); particularly among species in the subgenus *Sergentomyia* (5/6 compared with 3/6 among those in the subgenus *Phlebotomus*). The most and the least frequently identified sandfly species were *Phlebotomus papatasi* (31.29%) and *Sergentomyia tiberiadis* (1.15%), respectively. Furthermore, the least abundant *Phlebotomus* sandfly species was *P. salehi* which comprised only 2.44% of the total number of collected sandfly species. Despite this small percentage frequency, *P. salehi* revealed negative morphological and positive molecular features of infection with *Leishmania* parasites at the time of study (Figure 2).

***Leishmania*-infected sandflies**

Overall, 330 female *Phlebotomus* sandflies were dissected to search for *L. major* infection. At least nine (2.7%) out of these dissected female phlebotomine specimens appeared microscopically to have *Leishmania* promastigote parasites in their midgut. From these only five *P. papatasi* specimens and four specimens in the *P. major* group collected in the vicinity of rodent burrows were promastigote-positive by routine microscopy (Figure 2). Although *P. salehi*, *P. alexandri* and *P. sergenti* specimens contained no *Leishmania* promastigote parasites using routine microscopically-dissected methods, a few appeared to be infected with *Leishmania* promastigotes by molecular methods.

Parasite PCR

The kinetoplast DNA of the parasite *L. major* was detected in 18 (5.5%) out of the 330 female phlebotomine sandflies subjected to PCR protocols. At least one *P. salehi* specimen was positive for the kDNA of the parasite *L. major* with a main band of 560 bp (Figure 3) identified using the nested PCR method. This compared with the higher detection of kDNA in the other phlebotomine

Table 1. The types and numbers of wild male and female phlebotomine sandflies caught at Ghir-Karzin focus

Sandfly species	Females (%)	Males (%)	Total (%)
Ph. (Phlebotomus) papatasi	642 (40.8)	932 (59.2)	1574 (31.3)
Ph. (Phlebotomus) salehi	70 (56.9)	53 (43.1)	123 (2.4)
Ph. (Plebotomus) bergeroti	105 (49.8)	106 (50.2)	211 (4.2)
Ph. (Paraphlebotomus) sergenti	111 (37.2)	187 (62.8)	298 (5.9)
Ph. (Paraphlebotomus) alexandri	217 (52.4)	197 (47.6)	414 (8.2)
Ph. (Larroussius) major group*	345 (60.4)	226 (39.6)	571 (11.4)
S. (Sergentomyia) dentata	563 (56.6)	432 (43.4)	995 (19.8)
S. (Sergentomyia) sintoni	162 (69.2)	72 (30.8)	234 (4.7)
S. (Sergentomyia) theodori	101 (63.5)	58 (36.5)	159 (3.2)
S. (Parrotomyia) baghdadis	166 (77.2)	49 (22.8)	215 (4.3)
S. (Sintonius) clydei	128 (71.9)	50 (28.1)	178 (3.5)
S. (Sintonius) tiberiadis	26 (44.8)	32 (55.2)	58 (1.1)
Total	2636	2394	5030 (100)

*Species unidentified

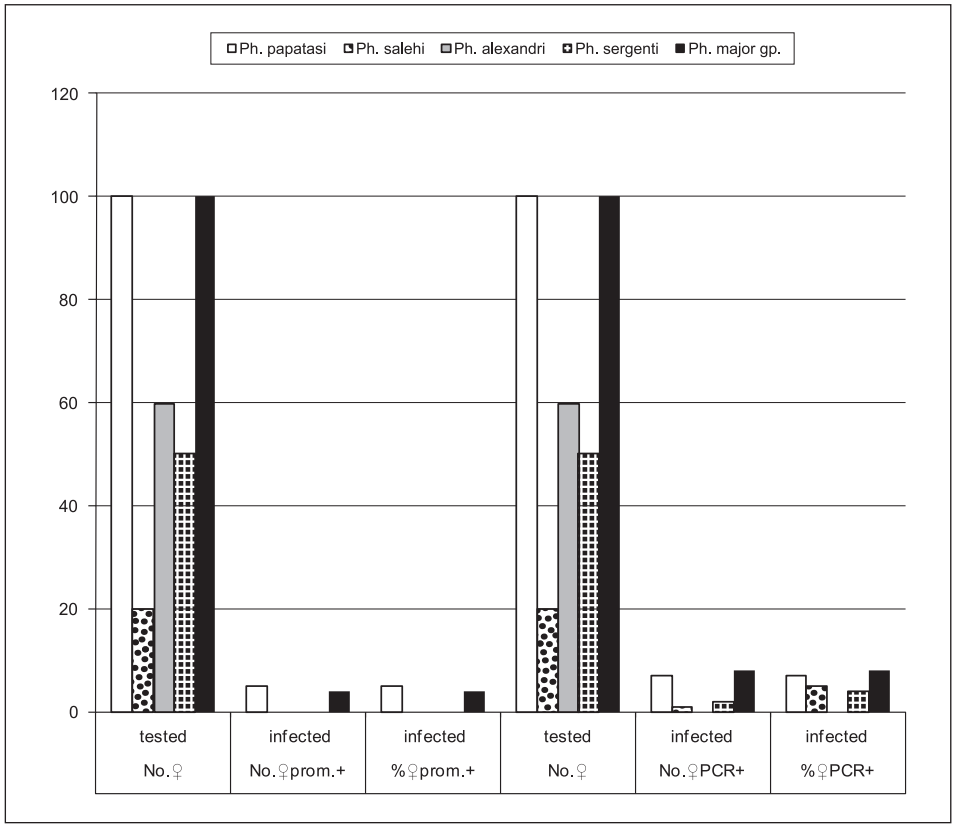

Figure 2. Histogram exhibiting the types, numbers (No.) and percentages (%) of female (♀) phlebotomine sandflies (*Ph. papatasi*, clear □; *Ph. salehi*, dotted ⊡; *Ph. alexandri*, grey ▨; *Ph. sergenti*, striated ▦; *Ph. major* group, black ■) infected with *Leishmania major* promastigotes (prom.+) by routine microscopy or by PCR method

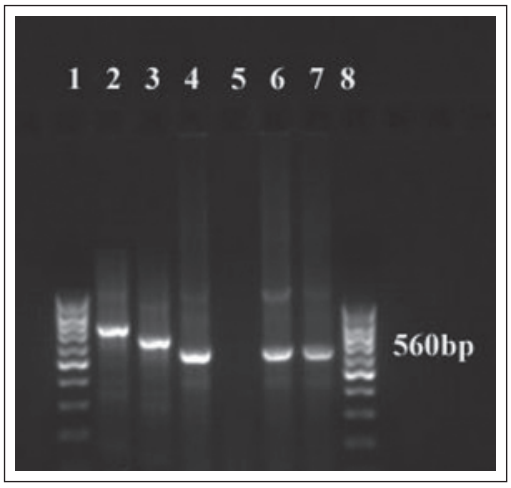

Figure 3. Gel electrophoresis profile of the nested-PCR based amplification products. The bands correspond to molecular weight marker (Lanes 1 & 8) reference strains of *L. tropica* (Lane 2), *L. infantum* (Lane 3), *L. major* (Lane 4), *Ph. papatasi* (Lane 6), *Ph. salehi* (Lane 7) and a male sandfly (Lane 5) as a control

sandflies was a major step forward. Although *P. alexandri* specimens were *L. major*-negative by PCR method, the other four *Phlebotomus* species were all positive for the kDNA of the *L. major* parasites. All sandflies were parous which meant they had produced eggs.

DISCUSSION

The challenge of elucidating the structure of highly complex ecological systems in zoonotic infections is very essential for the effective implementation of control measures (Desjeux, 2001; Reithinger *et al.*, 2007). Cutaneous leishmaniasis (CL) appears to be a major public health problem in Iran where most cases known as zoonotic CL (ZCL) are caused by *L. major* (Moemenbellah-Fard *et al.*, 2003; Fakoorziba & Nazari, 2006). It is increasingly reported from the southern, central and eastern provinces of Iran, and is a notifiable disease (Fakoorziba *et al.*, 2011). The incidence of CL in Fars province has been reported to be around 108-144 per 100,000 in 2007 (WHO, 2008). It is a debilitating disease with endemic foci in southern Iran.

Natural foci of ZCL with structures similar to that in north-western India (Rajasthan region) exist in the southern Iranian province of Fars in which only gerbillid rodents like the Libyan gird *Meriones libycus* and *Meriones hurrianae* occur outside the range of *Rhombomys opimus* and seem to be the main vertebrate reservoir hosts and *P. salehi* (Mesghali, 1965) is the vector in the region (Molyneux & Ashford, 1983; Killick-Kendrick, 1999). Moreover, it appears that *P. salehi* could probably have a wider geographical distribution than previously known partly as a result of increase in detection.

Although the percentage rate of infection with *L. major* parasites among female sandflies of *P. salehi* appeared to be very low (5%), our results corroborated the 1.7% infection rate of this species by routine microscopy in Chahbahar area about a decade ago (Kasiri & Javadian, 2000). Since *P. salehi* and *P. papatasi* appear to occur sympatrically and simultaneously in a few ZCL foci of southern Iran, it is most likely that the latter species is a proven principal general vector (Killick-Kendrick, 1990) and *P. salehi* is a secondary maintenance vector in the transmission cycle of infection between humans and rodents in Iran (Kellick-Kendrick, 1999).

Sandflies were morphologically characterized based mainly on internal structures such as the spermatheca, cibarium and pharynx in females and terminal genitalia in males. This involved modified maintenance conditions for specimens, a well-skilled technique and taxonomic expertise. Natural infection of female sandflies with *Leishmania* promastigotes were checked by dissection under a microscope. Fresh sandflies need to be dissected with a highly skilled technique to study small individuals. This is relatively time-consuming and a large sample size has to be examined to get useful data in each case, since the rate of infection of sandflies with *Leishmania* is generally very low (0.01-1%) even in endemic areas (Kato *et al.*, 2007). Thus the application of alternative methods like molecular techniques for the detection and identification of *Leishmania* parasites in sandflies is highly desirable. The

application of the kDNA PCR diagnostic assay was found to be highly sensitive (98.7%) for *Leishmania* parasites (Bensoussan *et al.*, 2006). This method is sensitive and it needs minimum effort and thus will be a powerful tool for further research on sandflies and the relationships between *Leishmania* species and their vectors.

Although PCR detection of a natural infection of *Leishmania* in a phlebotomine sandfly like *P. salehi* alone does not, however, prove that it carries the parasites to humans (Aransay *et al.*, 2000) or establish its vector identity, it helps to elucidate partially which sandflies are permissive to this protozoan parasite. In addition, the distinction between different *L. major* parasitic stages (*i.e.* infective *vs.* non-infective forms) can not be verified through detection of kDNA in the PCR assays. Nevertheless, all naturally-infected sandflies, including *P. salehi*, were at least uniparous, which means that females have produced eggs once. These females were used since they provided enough time (>24 hours) for the transformation of non-infective *Leishmania* into infective forms in the sandfly gut. The female sandflies were depleted since the blood meal was digested in them and the non-infective *Leishmania* had enough time to be transformed into infective forms and so the resulting kDNA detection belonged almost certainly to infective (promastigote or flagellated) forms. This promastigote-positive sandfly species could then be introduced as a potential vector of *Leishmania* parasites.

Acknowledgements. The authors appreciate the improvements to this article that were meticulously proposed by the anonymous peer reviewers. We are deeply indebted to the vice-chancellor for research and technology at the Shiraz University of Medical Sciences (SUMS) for permitting the use of facilities at the university. The contents of this publication are the sole responsibility of the authors and do not necessarily reflect the views of the relevant institution. No competing financial interests exist.

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