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Molecular detection of *Leishmania* infection in sand flies in border line of Iran–Turkmenistan: Restricted and permissive vectors

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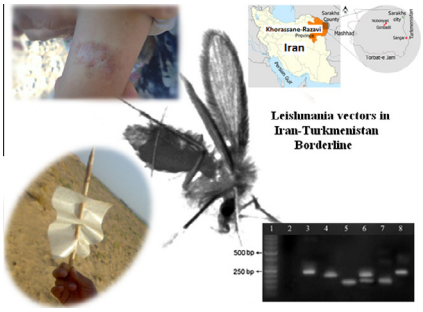
HIGHLIGHTS

- Sand fly vectors of cutaneous leishmaniasis (CL) in Sarakhs district, tested for *Leishmania* infection.
- Three *Sergentomyia* and six *Phlebotomus* species were found in the region.
- *Ph. papatasi* was the most common Phlebotomine species in outdoor and indoor resting places.
- *Leishmania* infection was found at in *Ph. papatasi*, *Ph. caucasicus*, *Ph. alexandri*, and *S. sintoni*.
- The parasites were *L. major* ($n = 5$), *L. turanica* ($n = 10$), and *L. gerbilli* ($n = 4$).

GRAPHICAL ABSTRACT



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ABSTRACT

A molecular study was carried out to incriminate sand fly vectors of cutaneous leishmaniasis (CL) in rural areas of Sarakhs district, Khorassane-Razavi Province, northeastern Iran, in 2011. Sand flies of *Sergentomyia* with three species and *Phlebotomus* with six species respectively comprised 73.3% and 26.7% of the specimens. *Phlebotomus papatasi* was the most common *Phlebotomine* species in outdoor and indoor resting places. *Leishmania* infection was found at least in 17 (22%) specimens including *Ph. papatasi* ($n = 9$ pool samples), *Phlebotomus caucasicus* ($n = 6$), *Phlebotomus alexandri* ($n = 1$), and *Sergentomyia sintoni* ($n = 1$). The parasites were found comprised *Leishmania major* ($n = 5$), *Leishmania turanica* ($n = 10$), and *Leishmania gerbilli* ($n = 4$). Infection of *Ph. papatasi* with both *L. major* and *L. turanica* supporting the new suggestion indicating that it is not restricted only with *L. major*. Circulation of *L. major* by *Ph. alexandri*, and both *L. gerbilli* and *L. turanica* by *Ph. caucasicus*, in addition to previous data indicating the ability of *Ph. alexandri* to circulate *Leishmania infantum* and *Leishmania donovani*, and *Ph. caucasicus* to circulate *L. major*, suggests that these two species can be permissive vectors. The results suggest that *Ph. papatasi* and *Ph. alexandri* are the primary and secondary vectors of CL where circulating *L. major* between human and reservoirs, whereas *Ph. caucasicus* is circulating *L. turanica* and *L. gerbilli* between the rodents in the region.

1. Introduction

Leishmaniasis is considered an emerging and re-emergent disease, where its incidence has been increased in the last decades (Reithinger et al., 2007). Its global prevalence is estimated to be of 12 million cases, with approximately 0.2–0.4 cases and

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0.7–1.2 million visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL) cases, respectively, occurs each year (Alvar et al., 2012). At the present moment the disease occurs in 98 countries throughout five continents, and 350 million people are at risk of catching the disease (Ameen, 2010; WHO, 2012; Alvar et al., 2012). Seventy to seventy-five percent of annual cases are reported from Afghanistan, Algeria, Colombia, Brazil, Iran, Peru, Saudi Arabia, Syria, Ethiopia, North Sudan, Costa Rica and Peru (Alvar et al., 2012). Some of the most important causes of this upward trend are the spread of human populations into the vector habitats and the development of irrigation schemes (Desjeux, 2004).

Cutaneous leishmaniasis (CL) is one of the infectious increasing diseases in Iran where it almost doubled (from 11,505 to 22,705 cases) over a nine-year period in 2001–2009 (IMHME, 2010). Khorassane-Razavi Province located in northeastern Iran is one of the endemic classical foci of CL in the country. The number of reported CL cases in this Province has increased in recent years: from 2005 to 2011, a total of 31,490 cases (about 22% of total cases in the country) were officially reported. Consequently the number of reported CL cases in Sarakhs has been amplified from 17 to 248 from 2006 to 2011 (IMHME, 2010). It is worth mentioning that these official reports were probably underestimates. In the neighboring country, Turkmenistan, about 1562 cases of CL in a 9 year period (2000–2009) have been reported; especially from southern and eastern parts of this country, bordering Iran and Afghanistan. Most of these cases occur in the regions bordering Sarakhs County, Iran (WHO, 2009). In spite of increasing CL cases, however, little is known about the vectors of the disease in the region.

Cutaneous leishmaniasis in Iran has been reported as Anthroponotic Cutaneous Leishmaniasis (ACL) due to *Leishmania tropica* and Zoonotic Cutaneous Leishmaniasis (ZCL) due to *Leishmania major* (Nadim and Seyedi-Rashti, 1971; Nadim and Tahvildari-Bidruni, 1977). *Phlebotomus sergenti* and *Phlebotomus papatasi* are the main vectors of these parasites respectively (Yaghoobi-Ershadi et al., 1995, 2003; Moin-Vaziri et al., 2007; Oshaghi et al., 2008, 2010; Rassi et al., 2011). All of the proven sand fly vectors of ZCL belong to subgenus *Phlebotomus*, including *Ph. papatasi* and related species *Phlebotomus salehi* and *Phlebotomus duboscqi* (Killick-Kendrick, 1990; Yaghoobi-Ershadi et al., 2003; Gramiccia and Gradoni, 2005). Other species of sand flies including *Phlebotomus andrejevi, Phlebotomus alexandri, Phlebotomus mongolensis, Phlebotomus ansarii* and *Phlebotomus caucasicus* have been reported as *L. major* vectors are either proven or probable vectors among rodents in rural areas of Iran (Nadim and Faghih, 1968; Javadian et al., 1976; Yaghoobi-Ershadi et al., 1995).

It is known that some phlebotomine species are restricted to transmit a specific *Leishmania* parasite whereas some others are permissive and able to transmit more than one *Leishmania* species (Kamhawi et al., 2000; Oshaghi et al., 2009a; Dobson et al., 2010). The interactions between parasite and sand fly midgut control this specificity that is regulated by polymorphic structures on the parasite lipophosphoglycan (LPG). LPG mediates promastigote attachment to the midgut epithelium and prevents their loss during blood-meal excretion (Kamhawi et al., 2000; Volf et al., 2008).

The abdominal status of female sand flies is a useful biological character for vector incrimination investigation. It is known that the development of *Leishmania* parasites in the sand fly gut continues for 1–2 weeks, when the blood meal has been fully digested, females are full gravid or laid eggs and abdominal status is empty. This status results in a mature transmissible infection with metacyclic promastigotes located in the anterior of the gut. Then infective metacyclic promastigotes are egested when the sand fly takes a subsequent blood meal (Bates, 2007).

Some rodents of subfamily Gerbillinae are the main reservoir hosts of ZCL in Iran and the countries where ZCL is endemic (Dubrovsky, 1979; Yaghoobi-Ershadi and Javadian, 1996; Strelkova, 1996; Abai et al., 2010; Oshaghi et al., 2011). *Rhombomys opimus* of this subfamily acts as one of the main reservoir hosts in Central Asia, Northern Afghanistan and Iran (Sosnina, 1979; Mallon, 1985; Yaghoobi-Ershadi and Javadian, 1996; Strelkova et al., 2001; Molur et al., 2005; Smith and Xie, 2008; Shar et al., 2009; Akhavan et al., 2010a–c). Some traditional techniques such as culture and direct examination have been used for *Leishmania* parasites diagnosis. These techniques are less sensitive than molecular techniques and are not able to differentiate *Leishmania* species (Ben-Ismail et al., 1992; Faber et al., 2003; Shahbazi et al., 2008). The present study has used a nested-PCR method, which provides a sensitive, rapid and specific alternative to traditional techniques (Akhavan et al., 2010a). This method is simple and reliable assay for detection and identification of *Leishmania* species involved in ZCL foci. The designed primers in this method can amplify a portion of the ITS2 region of three *Leishmania* species including *L. major, Leishmania gerbilli* and *Leishmania turanica*, the most common parasites of *R. opimus* where sand flies live in their burrows (Akhavan et al., 2010a). The main aims of this investigation were (1) to detect the natural infection of sand fly while considering their abdominal status, (2) to identify the *Leishmania* species within the sand flies, and (3) to determine *Leishmania* transmission specificity of sand flies in Sarakhs County, Khorassane-Razavi Province, northeastern Iran, located in border line of Turkmenistan.

2. Materials and methods

2.1. Study area

The study was performed in Sarakhs County which covers an area of about 5472 km^2 (36°18′N 60°49′E) with the population of about 85,000 people at the 2006 census and have a long border with Turkmenistan country (Fig. 1).

2.2. Sand fly collection and species identification

Sand flies were collected using sticky paper traps (castor oil coated white papers, 21 × 30 cm) in three villages named Sangar, Gonbadli and Nobonyad of Sarakhs County (Fig. 1) where ZCL is endemic. The traps were set overnight at indoor places including store, bathroom, stable, toilet, yard, and sleeping room as well as outdoor places of gerbil burrows entrance near the villages. Sampling was carried out during peak seasonal activity of adult sand flies and leishmaniasis transmission for 4 consecutive days per month in August and September 2011. The specimens were stored in 96% ethanol and then kept in −20 °C for further morphological and molecular investigation. The specimens were dissected using sterilize micro-needles where the head and abdominal terminalia were cut off and slide mounted in Pouri's medium and identified using the identification keys of Theodor and Mesghali (1964), Nadim and Javadian (1976) and Lewis (1982). The remainder of the body was stored in the sterile eppendorf microtube for DNA extraction.

2.3. DNA extraction and nested-PCR

DNA was extracted from the dissected thorax and attached anterior abdomen of individual sandflies using DNeasy® Blood & Tissue Kit (Qiagen), according to the manufactuer's instructions. Double distilled water and DNA from *L. major* provided MRHO/IR/75/ER, (Accession No. EF653269) was used as negative and positive controls respectively.

To detect and to identify *Leishmania* parasites we used the nested PCR assay developed by Akhavan et al. (2010a). PCR products were separated by 1.5% (w/v) agarose gel electrophoresis in