Some epidemiological aspects of cutaneous leishmaniasis with emphasis on vectors and reservoirs of disease in the borderline of Iran and Iraq

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Received: 28 October 2017 / Accepted: 23 March 2018 / Published online: 12 April 2018
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Abstract Cutaneous leishmaniasis (CL) is endemic and a major health problem in 17 provinces out of 31 in Iran. This study aimed to determine vectors and reservoirs of the disease using molecular techniques in the borderline of Iran and Iraq. Sand flies and rodents were sampled using sticky paper traps and metal wire live traps, respectively, in the selected villages. About 10% of archived confirmed human positive slides was randomly checked for *Leishmania* by PCR–RFLP assay. The female sand flies were dissected in alcohol 96% in a sterile condition, the head and two segments of the abdomen end permanently mounted for identification and the remaining of body used for DNA extraction. The direct parasitological tests were carried out on the stained slides of rodents for *Leishmania* as well as PCR–RFLP assay used for molecular detection of parasite. A total of 2050 sand flies were identified comprising of *Phlebotomus papatasi*, *Sergentomyia sintoni*, *Se. clydei*, *Se. mervynae*, *Se. theodori*, *Se. dentate* and *Se. iranica*. The *Ph. papatasi* was ranked as a prevailing sand fly species. Molecular tests on female sand flies revealed infection of *Ph. papatasi* to *Leishmania major*. Direct parasitology and

molecular tests confirmed of 20% infection to *L. major* among the sole rodents species “*Tatera indica*”. Due to wide dispersion of rodents colonies in the area and long favorite climate condition for sand flies, the CL foci will be provided the health risk for the religious tourists.

Keywords Cutaneous leishmaniasis · *Tatera indica* · *Phlebotomus papatasi* · *Leishmania major* · PCR–RFLP · Iran

Introduction

Leishmaniasis is endemic in more than 98 countries including Iran, and about 350 million people are at risk (World Health Organization 2010). The annual incidence of leishmaniasis is estimated to be about 2 million that 1.5 million cases are in cutaneous form. Over 90% of the cutaneous leishmaniasis (CL) is reported from Iran, Afghanistan, Syria, Saudi Arabia, Brazil and Peru (World Health Organization 2017). CL has the highest prevalence in the Old World and especially in the Eastern Mediterranean and consider as a major health problem (Salam et al. 2014). In this area, Iran is one of the leading countries in terms of zoonotic cutaneous leishmaniasis (ZCL) cases, so that the disease is endemic in 17 out of 31 provinces and the most important ZCL foci are located in Isfahan, Yazd, Khorasan, Semnan and Fars Provinces. Each year more than 20,000 new cases are reported from different parts of Iran and due to failure in referring of patients to the Health Centers, the number of cases should be four–five-folds of latter census (Shirzadi and Gouya 2010). CL is occurred in ZCL form in most parts of the country but the anthroponotic cutaneous leishmaniasis (ACL) form is also prevalent in some districts such as Bam, Kerman, Mashhad

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and Shiraz. Although the ZCL is not usually associated with mortality, but the high levels of infection and the scars of lesion, which remain in some cases for more than a year, even with standard treatment until the end of life. On the other hand, the presence of epidemiological complications in the transmission cycle of ZCL (diversity of reservoirs and vectors) has led to be the important surveillance and control of this disease. Therefore, by improvement in surveillance networking and quickly detection of ZCL cases in a timely manner as well as determining type of parasite in human, reservoirs and sand flies as a primary requirement for effective control of the ZCL (World Health Organization 2000).

There are five distinct structures for ZCL reservoirs in Iran. The first well-known structure is comprised of a geographical long belt started from northeastern parts near borderline of the Turkmenistan toward the central Iranian plateau where the great gerbils “*Rhombomys opimus*” was the main reservoir host (Abai et al. 2010; Akhavan et al. 2010; Javadian et al. 1976; Jafari et al. 2013; Mirzaei et al. 2011; Nadim and Faghih 1968; Nadim et al. 1968, 1977; Nadim and Tahvildar-Bidruni 1977; Parvizi et al. 2008, 2012; Rassi et al. 2008a, b, 2010, 2011; Rouhani et al. 2014; Seyedi-Rashti and Nadim 1967; Yaghoobi-Ershadi et al. 1996, 2004). The second reservoir structure was *Meriones libycus* (Moemenbellah-Fard et al. 2003; Parvizi et al. 2008; Pourmohammadi et al. 2008; Rassi et al. 2001, 2006, 2011; Rouhani et al. 2014; Yaghoobi-Ershadi et al. 2001a, b, 2004) which well established in southern temperate climates especially as a main reservoir in Fars Province where its territories begins just after ending of the first structure in the central plateau of Iran. The ending limit of second reservoir structure is all area where the semitropical climate is a limit for colonizing of *Meriones libycus*. The third structure of reservoirs was detected in south and southwestern parts of Iran where the main reservoir was *Tatera indica* (Azizi et al. 2012b; Javadian et al. 1976; Mehrabani et al. 2007; Motazedian et al. 2006; Rassi et al. 2013; Yaghoobi-Ershadi et al. 2013, 2015) and the *Nesokia indica* (Hajjaran et al. 2009; Mehrabani et al. 2007; Pourmohammadi et al. 2008; Yaghoobi-Ershadi et al. 2001a, 2013) have the secondary role as the ZCL reservoir. The fourth reservoir structure was limited to southeastern parts of the country in the Sistan and Baluchistan province where the main reservoir was reported *Meriones hurrianae* (Kassiri et al. 2013; Azizi et al. 2012a). The fifth ZCL reservoir structure was recently recorded from Bushehr and Fars provinces, southern Iran but with scattered dispersion and three rodent incriminated as reservoirs hosts comprising of *Rattus norvegicus* (Yaghoobi-Ershadi et al. 2013; Motazedian et al. 2010), *Gerbillus nanus* (Azizi et al. 2011, 2012b; Mehrabani et al. 2007; Motazedian et al. 2006). The sixth

structure of reservoir was recently detected in Poledokhtar, Lorestan province, southwestern parts of Iran where the main reservoir incriminated *Meriones persicus* which found by molecular assay positive to *L. major* (Shirmohammadi et al. 2017). The *M. persicus* was found infected with *L. major* in the past years in the East Azarbaijan province where is not endemic for ZCL till the present (Edrissian et al. 1975). Also *Hemiechinus auritus* which prevalent in most ZCL foci was found positive using Nested-PCR assay (Rouhani et al. 2014).

The main vector of *L. major* is the predominant species *Ph. papatasi* in Iran and natural leishmanial infection (Abai et al. 2007; Azizi et al. 2010; Akhavan et al. 2007; Javadian and Mesghali 1974; Javadian et al. 1976; Mesghali et al. 1967; Nadim and Seyedi-Rashti 1971; Rassi et al. 2008a, 2011; Rafizadeh et al. 2016; Yaghoobi-Ershadi et al. 1995, 2001a, b, 2004, 2005, 2010; Yaghoobi-Ershadi 2012a, b) or incriminating by molecular assays (Darvishi et al. 2015; Mohebbi et al. 2002; Motazedian et al. 2002; Oshaghi et al. 2008; Rassi et al. 2008a, b, 2011; Vahabi et al. 2016; Parvizi and Ready 2008) was recorded from different parts of Iran. However, other species such as *Ph. caucasicus* (Rassi et al. 2004; Yaghoobi-Ershadi et al. 1994, 2004), *Ph. bergeroti* (Azizi et al. 2016; Bakhshi et al. 2013; Parvizi et al. 2013), *Ph. mongoliensis* (Akhoundi et al. 2013b), *Ph. alexanderi* (Bakhshi et al. 2013) and *Ph. salehi* (Azizi et al. 2012c; Kassiri et al. 2012a; Rafizadeh et al. 2016) were found with *L. major* infection but at low densities, so the four latter species considered as secondary vectors. Western borderline of Iran and Iraq especially in the Mehran district is the most important territories due to yearly passing of the religious tourists and presence of the enzootic ZCL foci in this area could be a threat for spreading of disease to other localities. The molecular characteristic and identity of *Leishmania* parasite in human, sand flies and reservoir hosts were also investigated in order to understand the epidemiology of ZCL.

Materials and methods

Study area

Some villages were selected in the Mehran district where the ZCL was endemic including Farrukh-Abad, Seifi, Islamiye, Seyed-Hassan, Naft-Azar Company road as well as urban residential localities in the Mehran city. The coordinate of the study area varies between longitude N 46°07'–46°13' and latitude E 33°05'–33°07' and the elevation ranged 155–173 m above sea level. The studied area has a warm and dry to temperate climate with average annual rainfall of 185.9 mm and the absolute temperature varied from – 6 to 52 °C. The native vegetation in warm

and dry woodlands are comprising of oak, wild pistachio, wild almond, ash, fig, hawthorn, and jujube. Most of the people are farmers and wheat and barley are the dominant cultivated crops. From aspect of tourism, the Mehran city hosts many passengers who depart for visiting the Holy cities in the Iraq each year.

Collection of sand flies

Sand flies are collected during July and August 2016 using sticky paper traps at indoors such as animal shelters, rooms, toilets. For outdoor sampling, the rodent burrows was selected. The collected sand flies was put in alcohol 96% for both oil removal and preservation.

In the laboratory, the male sand flies were mounted in the Puri's medium and were identified using reliable identification key of Iranian sand flies (Rassi and Hanafi-Bojd 2006). In the case of female, the head and the end of abdomen was mounted in the mentioned media and the remaining of body was transferred to 1.5-μl microtubes contains 96% alcohol under sterile condition and used for DNA extraction and molecular assays in order to detect *Leishmania* infection.

Rodent sampling

Sherman live traps were set to catch the wild rodent with cucumbers and dates as the baits. The traps were installed before sunset and pick up them before sunrise to prevent the loss of collected rodents due to warm weather. Species identification of rodents was carried out by examining morphological characters using national Iranian rodent key (Etemad 1978).

In order to detect the *Leishmania* infection in the rodents, first they were anesthetized with injection of Ketamin at 100 μl IM for each rodent and the ears washed with detergent and rinsed with water. Then the ears were disinfected using alcohol 85% and povidone-iodine. After drying of the position, the edge of ears was scraped using vaccinostyle pen and the serosity transferred on the slides which followed by fixing with methanol and staining by Giemsa method. These imprint stained smears were searched for Leishman bodies under 100 × of compound microscope. All the positive samples were followed using restriction fragment length polymorphism (PCR–RFLP) for identification of leishmanial parasites.

Study on human infection

Human infection was checked on patients referred to the health centers of the Mehran city. After disinfecting the lesions with ethanol (70%), the serosity was sampled from edge of lesions using the vaccinostyle pen. From any each

patient, at least two microscopic slides were prepared and the patient code was written on the slide using a diamond point markers. The prepared slides were fixed by methanol and stained by Giemsa method and then searched for the parasite under magnification of 100 × of a compound microscope, respectively. The examined slides were stored in slide boxes for the molecular assays.

DNA amplification and PCR–RFLP

DNA extraction of leishmanial parasites within female sand flies as well as rodents were performed using GeneAll purification kit according to the kit's instrument. ITS1 locus was employed to detect and identify of the *Leishmania* species among examined specimens using the primers of: LITSR 5'CTGGATCATTTTCCGATG3' (Forward) and L5.8S-5'TGATACCACTTATCGCACTT3' (Reverse). The restriction enzyme of HaeIII was used for clarifying of *Leishmania* species for positive PCR products. PCR productions and restriction fragments were analyzed using 2 and 2.5% agarose gel electrophoresis, respectively. Standard parasite of *L. major* (MHOM/IR/75/ER) and distilled water were used as positive and negative control, respectively.

Results

Species composition of sand flies

A total of 2050 sand flies comprising of 8 species were caught both from indoors (31.5%) and outdoors (68.5%) of different localities in the Mehran district. The only species from genus *Phlebotomus* was *Ph. (Phl.) papatasi* which was also the prevalent species (43.5%) both at indoor (61.7%) and outdoor (38.3%) including rodent burrows. Seven species from genus *Sergentomyia* were revealed both in indoors and outdoors. The prevalent species from genus *Sergentomyia* were *Se. (Ser.) sintoni* and *Se. (Ser.) dentata* which predominantly were collected at outdoors (90.5 and 86.3%, respectively). Five other species from *Sergentomyia* were caught only at outdoors comprising *Se. (Par.) iranica*, *Se. (Ser.) theodori*, *Se. (Ser.) mervynae* and *Se. (Sin.) tiberiadis* (Table 1).

Molecular detection of *Leishmania* in sand flies

Overall, 230 female *Ph. papatasi* were collected both from indoors and outdoors. The physiological condition of sand flies was blood-fed (8.7%), semi-gravid (21.8%) and gravid (34.7%) and unfed (34.8%). The presence of *L. major* DNA was detected in 10 specimens of *Ph. papatasi* (4.34%). ITS1 PCR–RFLP analysis by HaeIII revealed the

Table 1 Species composition of sand flies both from outdoors and indoors Mehran district, Ilam province, 2016

Species	Male	Female	Indoor	Outdoor	Total	Abundance (%)
<i>Phlebotomus (Phlebotomus) papatasi</i>	330	557	547	340	887	43.5
<i>Sergentomyia (Sergentomyia) sintoni</i>	116	338	43	411	454	22.5
<i>Sergentomyia (Sergentomyia) dentata</i>	156	252	56	352	408	20.0
<i>Sergentomyia (Sintonius) clydei</i>	20	81	0	101	101	5.0
<i>Sergentomyia (Parvidens) iranica</i>	56	32	0	88	88	4.0
<i>Sergentomyia (Sergentomyia) theodori</i>	58	16	0	74	74	3.5
<i>Sergentomyia (Sergentomyia) mervynae</i>	8	16	0	24	24	1.0
<i>Sergentomyia (Sintonius) tiberiadis</i>	14	0	0	14	14	0.5
Total	758	1292	646	1404	2050	100

Fig. 1 RFLP of ITS1 positive samples of *Ph.papatasi* to *L. major* by HaeIII using 2.5%-agarose gel. Lanes: M, molecular weight marker 50 bp; lanes S1–S2, samples of *Ph. papatasi* positive to *L. major*; N, negative control; P, positive control (MHOM/IR/75/ER) of *L. major*

fragments of 210 and 140 bp which are characteristic for *L. major* (Fig. 1). All positive sand flies (*Ph. papatasi*) were unfed or gravid.

Detection of *Leishmania* in the rodents

A total of 15 rodents were sampled from different parts of the Mehran district, Ilam province which all identified as *Tatera indica*. Both direct microscopy and PCR–RFLP of ITS1 region showed the same rate of *L. major* infection among the sampled rodents (Fig. 2).

Detection of *Leishmania* in the CL cases

The trend of annual incidence had increasing trend in the studied area during past years, and the CL affected peoples

Fig. 2 RFLP of ITS1 positive samples of *Tatera indica* to *L. major* by HaeIII using 2.5% agarose gel. Lanes: M, molecular weight marker 50 bp; lanes S1–S3, samples of *Tatera indica* positive to *L. major*; N, negative control; P, positive control (MHOM/IR/75/ER) of *L. major*

have been referred to the Health Center of the Mehran district for laboratory diagnosis. About 10% of archived confirmed positive slides which were stained by Geimsa method was randomly selected for direct re-examination microscopy as well as using same slides for the molecular detection of the *Leishmania*. The parasites were identified *L. major* using PCR–RFLP on ITS1 region.

Discussion

Cutaneous leishmaniasis (CL) is one of the most important parasitic disease which is endemic and the major health problems in 17 out of 31 provinces of Iran (Yaghoobi-Ershadi et al. 2015). The main goals of this study was to identify the *Leishmania* parasites from human cases, animal reservoirs and sand fly vectors by the molecular assays. The characterizing of identity the *Leishmania*

parasites are critical for effective treatment and planning control program in the main endemic foci of ZCL in Ilam province, west of Iran. The occurrence of CL cases in southwestern of the country was first notified in 1977 (Nadim and Tahvildar-Bidruni 1977). A descriptive crosssectional survey which performed during 2000–2007 showed an expected feature of ZCL, e.g., higher distribution and more lesions at the lower part of body compared to upper one as well as the highest prevalence at winter in the Ilam province (Kassiri et al. 2012b). A spatial distribution analysis of ZCL and its relationship with climatic parameters in Ilam province showed that there was a significant association between ZCL cases and temperature. The ZCL foci are located in hot and dry districts, e.g., Mehran and Dehloran where temperature was high and rainfall low with highest incidence of ZCL in winter (Yazdanpanah and Rostamianpur 2013). About 10% of archived confirmed positive slides which were stained by Geimsa method was randomly selected for direct re-examination microscopy as well as using same slides for the molecular detection of the *Leishmania*. The parasites were identified *L. major* using PCR–RFLP on the ITS1 region. In another study, the isolates collected from different parts of the country including Isfahan, Ilam, Khuzestan and Semnan provinces were identified *L. major* by PCR–RFLP (Tashakori et al. 2003). In confirmation with finding of this study, it was shown that CL in Mehran district was rural type and 100% of isolate samples were *L. major* (Kermanjani et al. 2017; Feiz Haddad et al. 2016).

In the ZCL foci located in the central and northeastern of the country, the main reservoir host is great gerbil “*R. opimus*” (Abai et al. 2010; Akhavan et al. 2010; Javadian et al. 1976; Mirzaei et al. 2011; Nadim et al. 1968; Nadim and Faghih 1968; Nadim and Tahvildar-Bidruni 1977; Parvizi et al. 2008, 2012; Rassi et al. 2008a, 2011; Rouhani et al. 2014; Seyedi-Rashti and Nadim 1967; Yaghoobi-Ershadi et al. 1996, 2003, 2004; Yaghoobi-Ershadi and Javadian 1996). The great gerbils are absent in south and southwestern borderline of Iran with Iraq including Khuzistan and Ilam provinces where the CL is main health problem among military personnel as well as local residents. All the rodents were collected from different parts of the study area at borderline of Iran and Iraq identified as *Tatera indica* which showed 22.5% infection to *L. major* using direct microscopy examination as well as PCR–RFLP on ITS1 gene. Previous studies showed that dispersion of *Nesokia indica* in the Mehran district but without any *Leishmania* infection whereas *Tatera indica* had wider dispersion with 12.5% of infection rate and characterized the isolated *L. major* as zymodeme MON 26 (= LON 1) (Javadian et al. 1998). The infection of this rodent was also reported from west, south and southwest and southeast of Iran (Akhoundi et al. 2013a;

Kassiri et al. 2011; Mehrabani et al. 2007; Mohammadi and Parvizi 2016; Mohammadi et al. 2016; Mohebbali et al. 2004; Rab et al. 1986; Rassi et al. 2013, 2015; Vazirian-zadeh et al. 2013). The same zymodeme of *L. major* was recorded from two main reservoirs *Rhombomys opimus*, *Meriones libycus* which dispersed in the other ZCL foci in Iran (Yaghoobi-Ershadi and Javadian 1996).

Eight species of sand flies was collected both from outdoors and indoors as well as rodent burrows in the studied area comprising *Ph. papatasi*, *Se. sintoni*, *Se. theodori*, *Se. mervynae*, *Se. iranica*, *Se. clydei* and *Se. tiberiadis* which *Ph. papatasi* was prevailing species (43.3%) both at indoors, outdoor including rodent burrows which confirmed with finding of Javadian et al. (1997) except for three *Phlebotomus* species. All positive sand flies (*Ph. papatasi*) were unfed or gravid, which indicated that the *L. major* had enough time to develop to infective metacyclic form of promastigote in order to transmit the parasite to human or reservoir host. These evidences were the basic reasons for incriminating of *Ph. papatasi* as vector of ZCL in this area. In the similar study conducted in the Musiyan County at neighboring with the present studied area, *Ph. papatasi* was also recorded prevalent species (74%) and 92% of female sand flies at indoor, outdoor and rodent borrows. *Leishmania* infections were detected in 3.2% out of 280 female sandflies (Kavarizadeh et al. 2017). Also in Dehloran district, Ilam province located at south of the studied area, it was shown that 1.8% of *Ph. papatasi* was positive to *L. major* (Vahabi et al. 2016).

Several biotic and abiotic factors including high density of *Ph. papatasi* at indoors and rodent burrows, natural infection of *Ph. papatasi* and *T. indica* to *L. major*, tendency of sand flies to harbor to the human habitat, abundance of reservoir rodents and proximity of them to the human dwellings and presence of favorable humidity and temperature in the area for long-term activity of sandflies had a major roles on establishment of ZCL focus in the studied area.

Conclusion

This study conducted in several active ZCL foci where the high density of sand flies as well as *T. indica* was explored. The identity of *L. major* in CL patients, *Ph. papatasi* and *T. indica* were shown at different selected localities across the western borderline of Iran and Iraq in the Mehran district. The area is the most important territories due to passing as well as temporary accommodation of the religious tourists at open area in order to continue the routes to visit the Holy Shrine places in the Iraq. Due to turning of the lunar months, the probability of exposure of religious

people will be increased due to the presence of various CL foci and the country health risk will be the highest after coming back toward their localities which may act as a secondary source for infecting the sand flies and establishing of ZCL cycle at new areas.

Acknowledgements The authors sincerely acknowledge their gratitude to the efforts of CDC staff of the Mehran Health Center. Authors are highly thankful to Mr. Hossein Davoodi, technician of Museum of Medical Entomology (TUME), School of Public Health (SPH), Tehran University of Medical Sciences (TUMS) for assistance in archiving the sand flies slides. Thanks also to Mr. Yassin Ghasemi, technician of SPH Animal House, TUMS, for his assist in maintenance of the field caught rodents. This study was supported by Tehran University of Medical Sciences; Project No. 28137.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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