J Parasit Dis (Jan-Mar 2017) 41(1):274–281
DOI 10.1007/s12639-016-0792-3

ORIGINAL ARTICLE

Molecular characterization of *Leishmania* parasites isolated from sandflies species of a zoonotic cutaneous leishmaniasis in Musiyan south west Iran

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Received: 1 August 2015 / Accepted: 25 May 2016 / Published online: 14 June 2016

Abstract Cutaneous leishmaniasis (CL) is vector borne parasitic disease, considered as public health problem especially in border of Iran and Iraq, Dehloran County (Musian district). The aim of this study was molecular identification of *Leishmania* parasites in sandfly as vectors of Leishmaniasis. Totally 280 female sandflies were trapped by sticky traps from 7 rural areas of Musiyan in September–November 2012. All sandflies were identified using morphological characters of the head and abdominal terminalia. DNA was extracted from female sandflies and *Leishmania* was identified using PCR and sequencing. All 280 trapped sandflies were identified as *Phelobotumus Papatasi* and *Leishmania* infections were detected in 3.2 % out of 280 female sandflies. All leishmania were identified as *L. major* and submitted in Gene bank as: LC014642.1, LC014641.1, LC014640.1 and LC014639.1. Frequency of *Phlebotomus Papatasi* and infection with *L. major* in studied regions showed that this vector is dominant in these areas.

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Keywords Sandfly · *L. major* · Characterization · Iran

Introduction

Leishmaniasis is a major public health problem caused by *Leishmania* protozoa from class of Kinetoplastida. Based on clinical manifestation, Leishmaniasis are seen in different forms: Cutaneous (CL), Mucocutaneous (MCL), Diffius (DCL) and Visceral Leishmaniasis (VL). Epidemiological studies show that 12 million people around the world are involved with the disease; 350 million people are at risk and annually 2 million new cases are added. More than 90 % of cases of cutaneous Leishmaniasis occur in 10 countries: Afghanistan, Algeria, Saudi Arabia, Iran, Syria, Bolivia, Brazil, Colombia, Nicaragua and Peru (WHO 2010).

Cutaneous Leishmaniasis has long existed in Iran and region is one of the endemic areas of cutaneous Leishmaniasis and known since ancient times. Currently, around 20,000 cases of the disease reported annually that the real number is probably several times more than this (Islamic Republic of Iran Ministry of Heath and Medical Education 2007).

The disease occurs as endemic areas in Central, Northeastern, West and Southern parts of Iran (Hajjaran et al. 2013) and exists in rural (Zoonotic) and urban (Anthroponotic) types. Causative agent of urban leishmaniasis or Anthroponotic Cutaneous leishmaniasis (ACL) is *Leishmania tropica* and its vector is *Phlebotomus sergenti* sandfly, the main reservoir of the disease is human. But *Leishmania major* causes Zoonotic type or Zoonotic Cutaneous Leishmaniasis (ZCL); *Phlebotomus papatasi* is the main vector and sandflies as *P. anderjevi*, *P. mongolensis*, *P. ansarii* and *P. alexandri* have

been identified as vectors in rodents. *Tatera indica* rodents are the main reservoirs of ZCL in Iran (4.5). The only confirmed sandfly as the main vector of ZCL in Iran is the haematophagous females of *P. papatasi* (Diptera: Psychodidae) (6.7). A large number of sandflies from endemic areas of Iran have been described and *L. major* Promastigote infection have been detected from *P. papatasi*, *Para phlebotomus* subgenus including *P. caucasicus*, *P. mongoliensis*, *P. ansari*, *Sergentomyia sintoni* etc. (Nadim and Seyedi-Rashti 1971; Yaghoobi-Ershadi et al. 1996; Yaghoobi-Ershadi et al. 1995; Nadim et al. 1968a, b; Javadian and Mesghali 1974; Yaghoobi-Ershadi and Javadian 1996; Tashakori et al. 2006).

Previously several traditional laboratory methods including culturing or inoculating to sensitive lab animals carried out for detection of *Leishmani* sp. from infected *Phlebotomus*.

Also, given the clinical and geographical data as well as laboratory methods, *Leishmania* species in human, animal reservoir and vectors have been identified and declared as *Leishmania major*, *L. tropica* and *L. infantum*.

However, the above method definitely lacks the efficiency of new methods as determining the nucleotide sequence of the gene under study and phylogenetic analysis (Davami et al. 2010; Muller and Schlein 2011; Strelkova et al. 2001; Mirzaei et al. 2011; Hajjaran et al. 2009).

One of Iran endemic region is located in Southwest (Ilam province).

Most of the cases reported in this area are from Musiyan district. The course of the disease over recent years shows that especially Musian district is one of the endemic foci of cutaneous Leishmaniasis. This is very important in terms of public health in this border region (Yaghoobi-Ershadi 2012; Kavarizadeh et al. 2013).

Finding *Leishmania* naturally, infected female sandflies is essential to determine a sandfly as vector of Leishmaniosis, however finding the parasite in the sandflies is not a definite reason because most of the *Leishmania* infections in vectors are temporary and life cycle of parasites in the infected sandflies may be not completed and removed from the vectors (Nadim et al. 1968a, b).

In this study, hunting of sandflies done at the end of the active seasons of adult sandflies, when the rate of vectors infection are increasing. However, limited studies have been done in the past only to determine species of sandflies in Musiyan district, no studies have been done on the amount and type of infection by *Leishmania* parasite. (RanjbarKermani 1989; Javadian et al. 1997).

Therefore, identification of *Leishmania* sp. and its molecular type in the infected vector of ZCL with Nested-PCR molecular technique was considered for the first time in the region.

Materials and methods

Study area

Ilam province is located in the west of Iran with an area of about 20,133 km^2 and located at 32°31′20″N, 47°22′3″E Dehloran county, Musiyan city, located in bordering Iraq (Fig. 1) and it neighbors Khuzestan province in the south, and has a warm climate with hot summers and mild, short winters (Mansoori et al. 2009).

Sample collection

Two hundred eighty Sandflies were sampled from 7 different villages of Dehloran county, Musiyan city; Patak-e Arab, Cham Hendi, Nahr Anbar, EynKhvosh, Shahrak-e Nasr, Shahrak-e Fath and Borom.

Samples were collected from, Rodent burrows, and indoor outdoor the places using sticky traps in September to November 2012.

Briefly, in every trapping 90 traps were established in sunset and gathered before sunrise, all trapped sandflies washed by ethanol 96 and were identified by using morphological characters of the head and Spermatheca (Nadim and Javadian 1976; Seydei-Rashti and Nadim 1992; Lewis 1982).

DNA extraction and Nested-PCR-based diagnosis

DNA was extracted from the thorax and was attached to the anterior abdomen of each sandflies using a standard

Fig. 1 Locations of *Musiyan*, districts in *Dehloran*, *Ilam province of Iran*, where sandflies screened for *Leishmania* infections

extraction procedure with the QIA DNeasy blood and tissue kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions (Parvizi et al. 2005).

DNA concentration was measured at 260 nm using a spectrophotometer. For molecular diagnosis, primers were chosen from variable segments on minicircles of kinetoplast DNA(ref) as CSB1XR (ATT TTT CGC GAT TTT CGC AGA ACG) and CSB2XF (CGAGTA GCA GAA ACT CCC GTT CA) for the first round, and LiR (TCG CAG AACGCC CCT) and 13Z (ACT GGG GGTTGG TGT AAA ATAG) for the second (Noyes et al. 1998).

Amplifications were carried out using an AccuPower PCR PreMixkit (Bioneer. Co, Daejeon, Korea).The thermo cycler used (MyCycler; Bio-Rad, Hercules, CA) was set to give 5 min at 94 °C, followed by 30 cycles, each of 30 s at 94 °C, 90 s at 55 °C and 90 s at 72 °C, and then a final extension for 5 min at 72 °C. The PCR product of first step was diluted 1:9 with ultrapure water and then 1 ml of this dilution used for the second round of PCR using the same conditions and reaction mixture as the first round.

Electrophoresis performed after adding a 5 µl sample of the PCR products to a 1.5 % agarose and the gel was stained with ethidium bromide. Bands were observed by ultraviolet transillumination. (BioSystematica, Devon, UK).

Nucleotide sequences

DNA derived from PCR amplification was subjected to sequencing by MWG (Ebersberg, Germany). The data were analyzed using Chromas software (http://www.technelysium.com.au/Chromas.htm) and compared with the sequence in the NCBI nucleotide gene bank (www.ncbi.nlm.nih.gov/BLAST/).

Statistical analysis

Statistical analysis was performed by using SPSS 18.0 software. The data was expressed as frequency and percentage.

Results

In this cross-sectional study, a total of 280 female sandflies were caught in Musian district. All hunted sandflies were *P. papatasi* that 101 caught in rodent burrows, 109 in indoors and 70 in the outdoors. Among the hunted sandflies, *P. papatasi* naturally infected with *Leishmania major* were observed (Table 1). In terms of the hunting place, 4 % of the infected sandflies were caught from rodent burrows, 2.7 % from indoors, and 2.9 % from outdoors respectively (Table 2, 3).

The species with high frequency were caught in all three places in all regions.

In the Nested-PCR molecular method, on 1.5 % agarose gel bands with size of 560, 750, 680 bp showed as *L. major*, *L. tropica* and *L. infantum* respectively.

PCR results confirm that 11 female sandflies were infected with *Leishmania* parasites and by using Nested-PCR method all cases were detected as *L. major* (Fig. 2).

To confirm and complete the identification of samples, 4 randomly selected PCR products were sequenced and submitted in the GenBank database (http://www.ncbi.nlm.nih.gov/nuccore/) with accession numbers: LC014642.1, LC014641.1, LC014640.1 and LC014639.1. Multiple sequence alignment revealed conserved nucleotides among the obtained sequences (Fig. 3).

Discussion

Controlling leishmaniasis in endemic areas requires knowledge of the ecology and epidemiology of the parasite, the reservoir host and vector of the disease. In this regard, identifying reservoirs and seeking infected carriers is one of the fundamental problems of those responsible for disease control. Finding sandflies infected with parasites is an essential step to identify the vector species and the potential disease transmission in endemic areas. In terms of low parasite infection in most centers because of Leishmaniasis, to find the Leptomonad in sandfly body, tend to eat the human blood, it is enough to introduce it as a potential vector of the disease. In this study, all caught female sandflies were *P. papatasi*, that is the dominant species in this region because in faunistic survey (by Kaveri-Zadeh et al.) in the area this species has almost included 74 % of the total caught sandflies that *P. papatasi* includes 92 % of female sandflies caught in the study.

Phlebotomus papatasi had the highest frequency in indoor, outdoor and nests of rodents, so this study is in agreement with other studies in all three locations at a rate of 36 % in rodent burrows, 25 % in outdoors and 39 % in indoors (Killick-Kendrick 1999; Nadim et al. 1968a, b).

Two conventional methods microscopic and injection into sensitive lab animals and isolating parasites through medium are done for determining and estimating the rate of infection of the vector insects and disease reservoirs. Both methods are difficult, long-term and imprecise, and on the other hand, in terms of morphological similarity of species and subspecies, the diagnostic value of these methods is low and additional studies such as isoenzyme analysis or (Monoclonal antibody) mAbs are required to identify species and subspecies of isolated parasites.

Meanwhile, problems related to medium infection and removing isolated parasites and expensive and time

Table 1 *Leishmania* species identified in *P. papatasi* in different villages based on habitats and abdomens

Village		Mounth Day										
		August 26	September 14	September 24	October 5	October 16	October 17	October 29	November 1	November 2	November 7	November 8
Patak aerab	I	8		5								
	O	7		1								
	R.B			6*								
Chamhendi	I				7							
	O		10*		5							
	R.B		9		12*							
Aynkhosh	I		2							15*		
	O		7*									
	R.B		19							2		
Borom	I					20*	18					
	O					15	1					
	R.B					22	25*					
Nahre Anbar	I							9	23			
	O							9*	2			
	R.B							4*	2			
Nasr	I											
	O										4	
	B											
Fath	I											2
	O											9
	R.B											
Total		15	47	12	24	57	44	22	27	17	4	11

I indoor, *O* outdoor, *R.B* rodent burrow

* Cases positive

Table 2 Frequency of natural *Leishmania* infected *P. papatasi* based on abdominal position and collected areas

Village	*P. papatasi*			
	Abdomen position			
	FF	G	SG	EM
Patak aerab	7	4	1	15 (1)+
Chamhendi	7 (1)+	5 (1)+	1	30
Aynkhosh	13 (1)+	2	2	28 (1)+
Borom	55 (1)+	10	2	33 (1)+
Nahre Anbar	32 (1)+	6	2 (1)+	9
Nasr	2	1	1	
Fath	6	1	1	3
Total	122	30	10	118

FF full fed, *G* gravid, *SG* semi gravid, *Em* empty

+ Cases positive

Table 3 Frequency of natural *Leishmania* infected *P. papatasi* based on abdominal position and collected areas

P. papatasi				
Location			Cases positive	
	Number	%	Number	%
Indoor	109	39	2	2.7
Outdoor	70	25	3	2.9
R. Burrow	101	36	4	4
Total	280	100	9	3.2

R.B rodent burrow

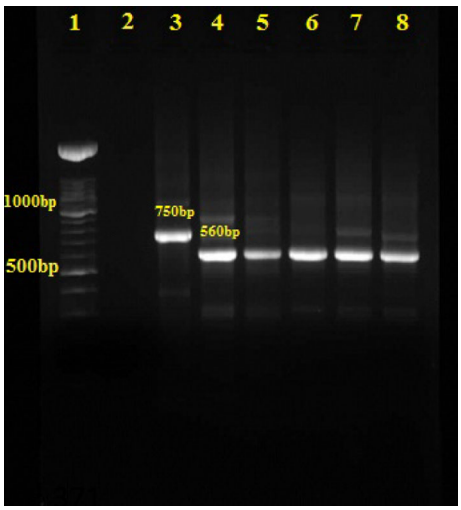

Fig. 2 Agarose gel electrophoresis of *Leishmania* isolates from sandflies in nested PCR using the primers 13Z and LiR in the second step: *Lane 1* DNA size marker 100 bp; *Lane 2* negative control; *Lane 3 L. tropica* (positive control 750 bp); *Lane 4 L. major* (positive control 560 bp); *Lanes 5–8 L. major* isolates obtained from sandflies

consuming isoenzyme analysis are the major problems faced by the classical laboratory studies (Javadian et al. 1977).

New PCR-based molecular methods have none of these problems. They are generally enhanced the DNA-based molecular techniques to determine the species of Leishmania parasites to detect infection in the past decades. Using molecular methods in endemic and hyper endemic areas is particularly useful in identifying infection of vectors.

In this study, all the sandflies identified with molecular methods and 9 isolates (3.2 %) were diagnosed as positive *Leishmania* parasites. Molecular results showed that 100 % of cases were only infected with *L. major*. Comparison of the sequence of PCR results with other sequences submitted in World Gene Bank has also confirmed the *L. major*. These findings show the difference in other studies obtained in the rest of the country.

In similar studies conducted in other areas of Iran, the dominant speciesism *P. papatasi*. In a study by Rassi et al. (2005), the main vector of cutaneous *Leishmania* in South of Iran is called *P. papatasi*. The researchers have analyzed the sandflies caught with Nested-PCR method. The results showed that about 2.7 % of *P. Papatasi* were naturally infected by *L. Major*.

The study by Roshan-Ghalb and Parvizi (2012) in the north east of Iran with molecular methods *has also confirmed the L. Major as* agent and *P. papatasi* as the main vector of ZCL (Roshan-Ghalb and Parvizi 2012).

Sharbatkhori et al. 2014 in a similar study in the North East of Iran have obtained different species, *P. caucasicus*, *P. mongolensis* in addition to *P. papatasi*. The researchers also have mentioned the most vectors as *P. papatasi*.

One of the differences between our results with similar studies in other parts of Iran is that only *L. major* was detected using molecular methods. Contrary to our results, Sharbatkhori et al. 2014 in addition to finding *L. major* have found *L. turanica* in vectors and disease reservoirs in the North East of Iran. The researchers believed that non-pathogenic *Leishmania* sp. such as *Leishmania turanica* and *Leishmania Jerbili* play a major role in the survival of *L. major* in vectors and reservoirs. (Sharbatkhori et al. 2014).

Sequences from this study were consistent with *L. major* recorded in the World Gene Bank in West of Iran (Mehran Ilam) with the access numbers KM555286.1, to KM555295.1.

Given the high frequency of *P. papatasi* in indoor, outside and nests of rodents and the introduction of the species as the certain vector of this diseases in Khuzestan Province (Province neighboring the area) and separating the *L. major* from adjacent urban areas patients such as Dehloran, it seems that the species is the main and certain

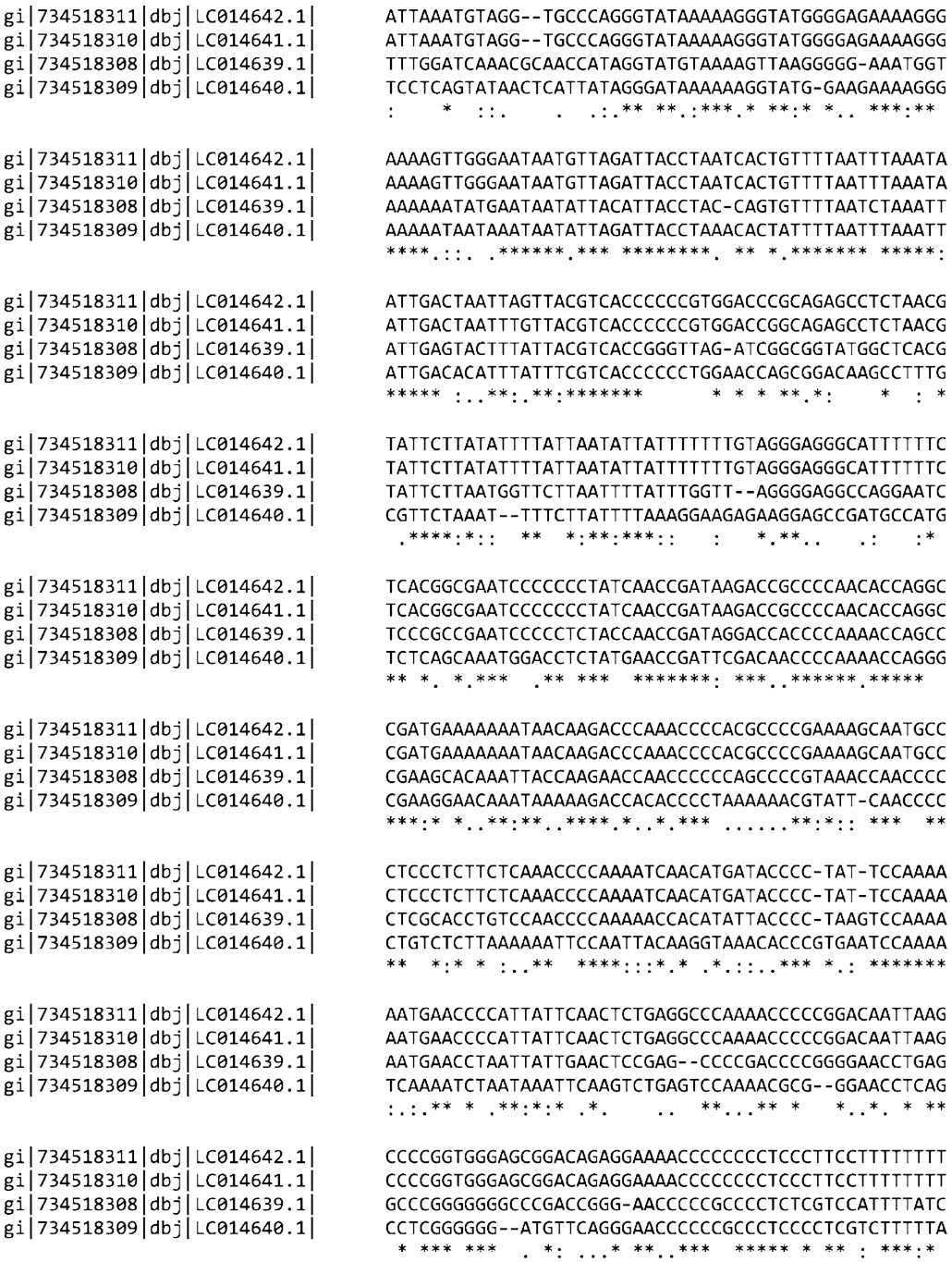

Fig. 3 Multiple alignments of nucleotide sequences of 4 isolates submitted in gene bank as: LC014642.1, LC014641.1, LC014640.1 and LC014639.1. *Dashes indicate* computer-generated gaps. *Asterisks* indicate identical nucleotides

vector of the disease in the region. The type of disease in the area is zoonotic cutaneous Leishmaniasis (ZCL).

Statistical analysis of CL over the years in Dehloran suggests that Musian District in the city is one of the areas that are mostly infected. The trend of the disease during the mentioned years shows that Dehloran and especially Musian is one of the endemic foci of cutaneous Leishmaniasis. In terms of public health in the border region, it is quite significant (Yaghoobi-Ershadi 2012; Yaghoobi-Ershadi and Javadian 1996; Killick-Kendrick 1990).

Phelobotumus papatasi highest infection rate was in October, which is in accordance with the second activity peak of the mosquito and it seems that most transmission occurs this month. So, it is recommended to protect oneself to prevent sandfly bites more than other months. Also, it is

recommended that people in affected areas not to leave their homes during the evening and night when the sandflies are active unless it is necessary. Farmers, workers and soldiers who must necessarily be present outdoors when the sandflies are active should have enough cover and use insect repellent to prevent bites. And it is recommended to use screens and mosquito nets impregnated with pesticide to prevent disease (World Health Organization 1993; Yaghoobi-Ershadi et al. 2000; Desjeux 1991).

Examining the natural contamination and determining the identity of *Leishmania* parasite in sandflies of subgenus *Phlebotomus*, the only the dominant species in this area is *P. papatasi* as the certain and main vector of disease, in fact, the first step is to determine the vectors of the disease in this area.

Considering that detecting vectors to run control programs is important, it is hoped that using the results of this study and examining the rodents of this area that are considered as the disease reservoir to help the regional planning to control the disease in the area.

Acknowledgments This study was financially supported by grants No: 91114 from Ahvaz Jundishapur Medical Sciences. The authors would like to thank Mr. Akhavan A. A. of Tehran University and health centers of Musian and Dasht Abbas for their help in sample collection.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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