Original Article

Molecular Survey on Detection of Leishmania Infection in Rodent Reservoirs in Jahrom District, Southern Iran

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Abstract

Background: Zoonotic Cutaneous Leishmaniasis (ZCL) is endemic in many parts of Iran. Recently its incidence is considerable in different parts of Jahrom district, in Fars Province, southern Iran. The aims of our study were to investigate the prevalence of leishmania infection, and identify and characterize the *Leishmania* species present, among the rodents by molecular methods in a new endemic focus of ZCL, in an urban and rural area of the Jahrom district, Fars Province, southern Iran.

Methods: From May to November 2010), 55 rodents in four regions of Jahrom focus were caught and checked for leishmania infection by the microscopical examination of liver, spleen, ears, and footpads' smears.

Results: Overall 18 *Meriones persicus*, 15 *Tatera indica*, 14 *Mus musculus*, and 8 *Rattus rattus* were caught. Totally, four (16.5%) and two (13.3%) of the *Me. persicus* and *Ta. indica*, but only one of *Mu. musculus* and *Ra. rattus* were found smear-positive for leishmania amastigotes, respectively. In the nested-PCR assay 8 (14.6%) smears were found positive for *Leishmania major*, none was found positive for any other *Leishmania* species. Sequencing based detection of *Leishmania* confirmed the microscopic and PCR findings. All positive specimens were shown 95–96% similarity with *L. major* Friedlin.

Conclusion: *Tatera indica* and *Me. persicus* are incriminated as the main 'reservoir' hosts of *L. major* in the rural area of Jahrom, moreover, *Mu. musculus* and *Ra. rattus* have the minor but remarkable role in the maintenance of the disease in the urban regions of Jahrom focus.

Keywords: Rodent, *Leishmania*, PCR, Sequencing, Iran

Introduction

The leishmaniases are parasitic diseases with an extensive variety of clinical symptoms that have an effect on over 12 million people in 88 countries, mainly in the tropical and subtropical situated regions, as well as Iran (Javadian et al. 1976, Mohebali et al. 2004, Guedes et al. 2008). Zoonotic Cutaneous Leishmaniasis (ZCL) takes place in many rural endemic foci in different parts of Iran (Yaghoobi-Ershadi et al. 2005). Sandflies (Diptera: Phlebotominae) have been naturally found infected with *Leishmania major* and considered as the sole vectors of leishmaniasis. Moreover, gerbil rodents (Muridae: Gerbillinae) are the reservoir hosts of ZCL in Iran (Azizi et al. 2011 a). Rodents, as the 'reservoir' hosts, are the main bases in the ep-

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idemiology and control strategy of the ZCL (Pourmohammadi et al. 2008). Due to high similarity of different species of the parasite, various sources of *Leishmania* DNA such as kinetoplast DNA (kDNA), ssu rRNA, ITS1 and repetitive sequences have been used for molecular characterizations (Van Eys et al. 1992, Noyes et al. 1998, Parvizi and Ready 2008, Davami et al. 2011, Ghasemian et al. 2011).

Recently, PCR-based assays are characteristically used to detect *Leishmania* species in patients, vectors and reservoir hosts (Rodgers et al. 1990, Bulat et al. 1992, Parhizkari et al. 2011).

Materials and Methods

Study Area

Fars Province placed in southern Iran and covers an area of about 122,400 km². Jahrom district is located in the south-east of province and situated at 30° 4' 45" North, 51° 43' 29" East, and 1050m above the sea level (Fars Budget and Planning Organization 2000). Due to a favorable ecological conditions for vectors and reservoirs of leishmaniasis, Jahrom has always been considered as one of the foci of cutaneous and visceral leishmaniasis in south of Iran (Fig. 1) (Davami et al. 2010, Davami et al. 2011).

Collection and Examination of Rodents

Between May to November 2010, rodents were caught alive in wire traps on agricultural plantations, surrounding and near the houses in four regions including Jahrom City and three villages in the area including Mousavieh, Ghotb-Abad and Fath-Abad. Each trap was set in the evening and checked in the next early morning. There were set at least 50 'trap-nights' per months. Each rodent was identified to species (Eisenberg and Redford 1999), killed by over-anesthesia and dissected, so that four impressions smears (two of the liver, spleen, ear and footpad) could be prepared. Each smear was fixed in methanol, Giemsa-stained and checked for amastigotes under a light microscope (Mohebali et al. 2004).

DNA Extraction

All specimens were checked in a nested-PCR assay for *Leishmania* kDNA. Therefore, each dry smear was scraped from the slides and mixed with 200µl of lysis buffer [1 mM EDTA, 50 mM Tris-HCl (pH 7.6, 1% (v/v) Tween 20] containing 5µl of a proteinase K solution which already had 23mg/ ml of enzyme. The specimen was incubated for 2 h at 56 ºC or 12h at 37 ºC before 75µl of a phenol: chloroform: isoamyl-alcohol solution (25:24: 1, by vol.) was added (Motazedian et al. 2002). After being shaken vigorously, specimen was centrifuged at 6000× g for 10min. DNA in the supernatant solution was precipitated with 300µl of cold ethanol, resuspended in 100µl of double distilled water, and stored at 4 ºC before using in the PCR- assay (Noyes et al. 1998).

Nested-PCR assay

The specific and sensitive nested-PCR was used to amplify the variable area of the minicircle kDNA of *Leishmania* spp. in the rodent liver, spleen, ear and footpad as previously described by Noyes et al. (1998) with slight modification (Moemenbellah-Fard et al. 2003, Davami et al. 2011, Ghasemian et al. 2011). The first-round (external) primers were CSB1XR (ATTTTTCGCGATTTTCGCAGA ACG) and CSB2XF (CGAGTAGCAGAAA CTCCCGTTCA). A reaction mixture containing of 1.5 mM of MgCl2, 2 mM of dntp, 2.5µL of 10 X PCR buffer (Boehringer Mannheim, Mannheim, Germany), 1 unit of Taq DNA polymerase (Cinagene, Tehran), and 10pmol of each primer were used in a total reaction volume of 25µL including 5µL of DNA sample. The second-round (internal) primers were 13Z (ACTGGGGGTTGGTGTAAAA TAG) and LIR (TCGCAGAACGCCCCT). The reaction mixture was used in a total volume of 30µL including 2µL of DNA product of the

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first round. These mixtures were amplified in a programmable thermocycler (Eppendorf AG (Mastercycler gradient), Germany) for 5min at 94 ºC (1 cycle) followed by 30 cycles at 94 ºC for 30 seconds, 55 ºC for 60 seconds and 72 ºC for 1.5min followed by a final elongation step at 72 ºC for 5 minutes. The WHO reference strains of *L. major* (MHOM/IL/67/ LV561) was used as standard DNA. A band of 560bp indicated that *L. major* kDNA is present in the sample (Davami et al. 2011).

Electrophoresis

A 6µl sample of each second-round product of PCR was subjected to electrophoresis in 1.5% agarose gel, stained with ethidium bromide and visualized by ultraviolet transillumination (Davami et al. 2011).

Sequencing

The PCR products of all positive samples were purified by Gel Purification Kit (Accu Prep®, Cat. No. k-3035-1, Bioneer, USA). The strands of amplified DNA were sequenced (Both forward and reverse sequencing) with the PCR primers on an automated sequencer (Applied Biosystems 377XL). The nucleotide homologies of the sequenced products were investigated with the TritrypDB blast programme. The characterization of consensus sequences was performed by using FASTA formatted sequences aligned with the Chromas programme.

Results

The 55 rodents trapped over the study belonged to four species; totally, 18 (32.7%) *Me. persicus*, 15 (27.3%) *Ta. indica*, 14 (25.5%) *Mu. musculus*, and 8 (14.5%) *Ra. rattus* were caught from Jahrom district (Table 1).

Amastigotes were seen in the liver, spleen, ear and/or footpad smears of seven (12.7%) of the rodents- three (16.7%) of the *Me. persicus*, two (13.3%) of the *T. indica*, one (7.1%) of the *Mu. Musculus*, and one (12.5%) of the *Ra. Rattus*- caught in Jahrom district (Table 2).

The nested-PCR results showed that 8 (14.6%) of rodents- four (22.2%) of the *Me. persicus*, two (13.3%) of the *Ta. indica*, one (7.1%) of the *Mu. Musculus*, and one (12.5%) of the *Ra. Rattus*- were positive for leishmania kDNA, Also 10 out of the 220 smears belonged to different parts of rodents organs (liver, spleen, ear and/or footpad) were found positive, separately (Table 2,3).

All the kDNA detected appeared to come from *L. major* (Fig. 2). Using TritrypDB sequence analysis against Trypanosomatidae species, the target sequence of PCR products showed 95–96% similarity with *L. major* strain IranJWmaj (GenBank accession no. AB67 8349.1) (Fig. 3).

Fig. 1. Map of Iran, showing the locations of Fars Province and the city of Jahrom

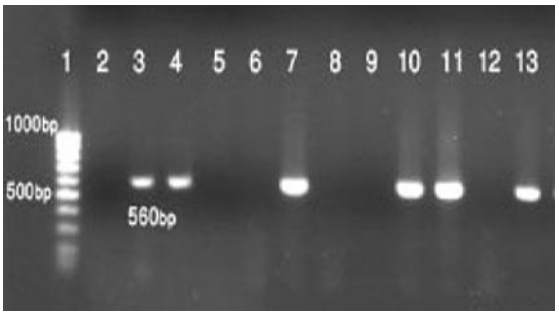

Fig. 2. The results of the nested PCR-based amplification of kDNA recovered either from a negative control (lane 2), and the reference samples of *Leishmania major* (lane 3), or positive and negative smears of liver, spleen, ear, or footpad of *Mus musculus* (lane 4, 5 and 6), *Rattus rattus* (lanes 7, 8), *Meriones persicus* (lanes 9, 10 and 11), and *Tatera indica* (lanes 12 and 13). Molecular-weight markers were run in lanes 1

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Table 1. The species, numbers and geographical distributions of the rodents caught in Jahrom district

Study areas	No. and (%) of rodents caught				
	Meriones persicus	***Tatera indica***	***Mus musculus***	***Rattus rattus***	**All species**
***Jahrom**	14 (77.8)	13 (86.7)	6 (42.9)	8 (100)	41 (74.5)
Fath-Abad	3 (16.7)	0 (0)	3 (21.4)	0 (0)	6 (10.9)
Mousavieh	0 (0)	2 (13.3)	4 (28.6)	0 (0)	6 (10.9)
Ghotb-Abad	1 (5.5)	0 (0)	1 (7.1)	0 (0)	2 (3.6)
All	18 (100)	15 (100)	14 (100)	8 (100)	55 (100)

* Urban and suburban areas of Jahrom

Table 2. The prevalences of leishmania infection in the rodents, as revealed by microscopy and, nested-PCR based detection of *Leishmania major* kDNA in Jahrom district

	No. and (%) of rodents found positive														
	Meriones persicus			***Tatera indica***			***Mus musculus***			***Rattus rattus***			**All species**		
Study areas	*Ch.	** Mic.	PCR	Ch.	Mic.	PCR	Ch.	Mic.	PCR	Ch.	Mic.	PCR	Ch.	Mic.	PCR
*****Jahrom**	14	1(7.1)	2(14.3)	13	0(0)	0(0)	6	0 (0)	0 (0)	8	1(12.5)	1(12.5)	41	2(4.9)	3 (7.3)
Fath-Abad	3	2(2.67)	2(66.7)	0	0 (0)	0(0)	3	0 (0)	0 (0)	0	0 (0)	0 (0)	6	2(33.3)	2 (33.3)
Mousavieh	0	0(0)	0(0)	2	2(100)	2(100)	4	1 (25)	1(25)	0	0 (0)	0 (0)	6	3 (50)	3 (50)
Ghotb-Abad	1	0(0)	0(0)	0	0 (0)	0 (0)	1	0 (0)	0 (0)	0	0 (0)	0 (0)	2	0 (0)	0 (0)
All	18	3(16.7)	4 (22.2)	15	2(13.3)	2(13.3)	14	1(7.1)	1(7.1)	8	1(12.5)	1(12.5)	55	7 (12.7)	8 (14.6)

*Checked, **Microscopy, ***Urban and suburban areas of Jahrom district

Table 3. The prevalence of leishmania infection in the studied organs of rodents, as revealed by the nested-PCR in Jahrom district

	Studied organs of rodents (No. and %)											
	Footpad		**Ear**			**Liver**			**Spleen**			**All**		
Species	*Ch.	** Pos. (%)	Ch.	Pos.	(%)	Ch.	Pos.	(%)	Ch.	Pos.	(%)	Ch.	Pos.	(%)
Meriones persicus	18	2 (11.1)	18	1	(5.6)	18	2	(11.1)	18	0	(0)	72	5	(6.9)
Tatera indica	15	2 (13.3)	15	1	(6.7)	15	0	(0)	15	0	(0)	60	3	(5)
Mus musculus	14	1 (7.1)	14	0	(0)	14	0	(0)	14	0	(0)	56	1	(1.8)
Rattus rattus	8	0 (0)	8	0	(0)	8	1	(12.5)	8	0	(0)	32	1	(3.1)
All	55	5 (9.1)	55	2	(3.6)	55	3	(5.5)	55	0	(0)	220	10	(4.6)

*Checked, **Positive

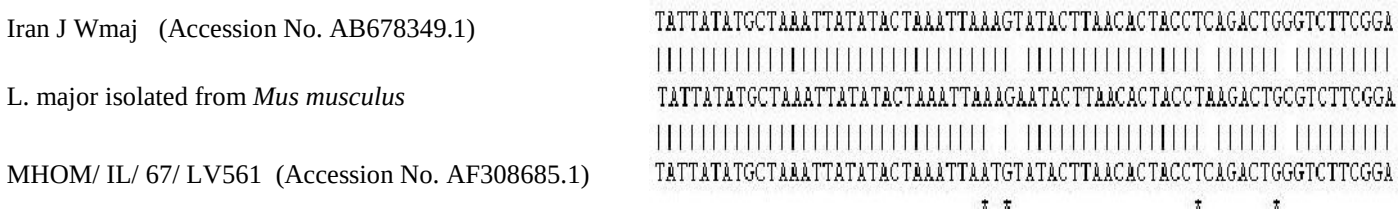

Fig. 3. Alignment analysis of the kDNA of *Leishmania major* isolated from *Mus musculus* foot-pad, *L. major* strain Iran J Wmaj (GenBank accession no. AB678349.1) and *L. major* isolate MHOM/IL/67/LV561 (accession no. AF308685.1). Target sequence of PCR product of *Mu. musculus* showed 95% similarity with *L. major* strains, of IranJWmaj. Stars indicate the different regions between the isolates

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Discussion

Detection of the mammalian 'reservoir' hosts is one of the main problems in front of researchers who make an effort to evaluate the epidemiology of ZCL to control the disease (Pourmohammadi et al. 2008). In the present study using a specific and sensitive nested-PCR on scrapings from Giemsa-stained smears of rodent tissues, the overall prevalence of rodents with leishmania infection was outcome as 14.6% that showed the higher infection than microscopical observations (12.7%). Different biochemical, immunological, and molecular assays such as iso-enzyme electrophoresis, monoclonal antibodies, and PCR have been used to characterize the causative agents of leishmaniasis (Fakhar et al. 2008, Parvizi et al. 2008, Fakhar et al. 2010, Pourmohammadi et al. 2010, Azizi et al. 2011b). Usuallym, in the leshmanial detections molecular assays are more sensitive than the other diagnostic methods such as micriscopical examinations or culture (Fakhar et al. 2011). As it is doable to use the molecular techniques to detect and identify the leishmania parasites in fixed and stained smears utilized for microscopy, recently, those methods chiefly based on nested-PCR and sequencing have been remarkably raised to detect the leishmania infections (Noyes et al. 1998).

In Iran, *Rhombomys opimus*, *Ta. indica*, *Me. lybicus*, and *Me. hurrianae* have been reported as the major 'reservoir' hosts of ZCL in endemic foci of the central and north-east, west and south-west, south, and south-eastern parts of Iran, respectively (Yaghoobi-Ershadi et al. 1996, Moemenbellah-Fard et al. 2003, Parvizi et al. 2005, Asgari et al. 2007).

Meriones lybicus, *Me. persicus*, *Me. Hurrianae* and *Ta. indica* have been found positive for *Leishmania* kDNA in Fars Province (Rassi et al. 2001, Moemenbellah-Fard et al. 2003, Rassi et al. 2006, Mehrabani et al. 2007, Mehrabani et al. 2011, Parhizkari et al. 2011, Azizi et al. 2012). In our study, the results of rodents trapped in Jahrom district of Fars Province, indicated that *Ta. indica* and *Me. persicus* were the most common rodents in that part of the province, and the results of the nested-PCR showed that 22.2% and 13.3% of mentioned rodents were found to be positive for *L. major*. *Me. persicus* and *Ta. indica* are the most important mammalian hosts of *L. major* in the rural areas of Jahrom district. No *R. opimus* and *Me. hurrianae* were caught in the present study in Jahrom.

Nesokia indica, *Gerbillus nanus* have been described as an accidental, main or a probable 'reservoir' hosts in different parts of Iran (Pourmohammadi et al. 2008, Azizi et al. 2011a, Azizi et al. 2011c, Azizi et al. 2012). In our investigation any *Nesokia* or *Gerbilus* species were not caught.

Mu. Musculus has been found naturally infected with *L. major* in Fars Province (Parhizkari et al. 2011). In the present study, the footpad of one out of 14 (7.1%) *Mu. Musculus* were found to be both microscpical and molecular positive.

Recently Motazedian et al. has isolated the causative agents of ZCL (*L. major*) from *Ra. norvegicus* in Fars Province (Motazedian et al. 2010). In this investigation one liver of *Ra. rattus* caught in urban area of Jahrom district was found to be infected with *Leishmania* kDNA, the present report appears to be the first to describe natural infection of *Ra. rattus* with *L. major* in Iran. *Mu. Musculus* and *Ra. rattus* infection with *L. major* may explain why, in recent years, ZCL causative has been remarkably increased in some urban areas of Iran (Razmjou et al. 2009, Davami et al. 2010).

The importance of the 'reservoir' hosts in the epidemiology of ZCL appreciated with the local sandflies feeding on that species and the infectivity of the leishmania infections

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in each host species (Motazedian et al. 2006). Even though *Mu. musculus* and *Ra. rattus* were found infected with *L. major*, but some reports show that the prevalence of leishmania infection have been observed from those which caught from other regions of Iran (Parhizkari et al. 2011).

There was no evidence of *L. infantum or L. tropica* in the tested smears. This study includes the first isolation and characterisation of *L. major* from Iranian *Me. persicus* and *Ta. indica*, caught in an area where zoonotic CL has recently occurred.

In Jahrom, whatever the time of the year, more than half of all rodents attracted to walnut-baited traps are infected with *L. major*. If local sandflies take their bloodmeals from both rodents and humans, there is clearly much scope for transmission of these parasites to humans.

Conclusion

Based on observing of amastigotes in livers, spleens, ears, and footpads smears, using a high sensitive and specific nested-PCR designed for kDNA of *Leishmania*, and comparing the kDNA of sequenced products with GenBank that confirmed the highest homology of 95–96% with *L. major*, concluded that the species isolated form rodents is *L. major*.

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