Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

ISSN 2320-7078
JEZS 2014; 2 (6): 314-317

Received: 05-10-2014
Accepted: 25-11-2014

Sayena Rafizadeh
Department of Biochemistry and Genetic, Qazvin University of Medical Sciences, Qazvin, Iran.

Mehrzad Saraei
Department of Parasitology and Mycology, Cellular and Molecular Research Center, Qazvin University of Medical Sciences, Qazvin, Iran.

Mohammad Reza Abai
Department of Medical Entomology & Vector Control, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran.

Mohammad Ali Oshaghi
Department of Medical Entomology & Vector Control, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran.

Mehdi Mohebali
Department of Parasitology and Mycology, Tehran University of Medical Sciences, Tehran, Iran.

Amir Peymani
Cellular and Molecular Research Center, Qazvin University of Medical Sciences, Qazvin, Iran.

Taghi Naserpour Farivar
Cellular and Molecular Research Center, Qazvin University of Medical Sciences, Qazvin, Iran.

Hassan Bakhshi
Department of Medical Entomology & Vector Control, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran.

Alireza Akrami
Esfarayen Health Center, Esfarayen University of Medical Sciences, Esfarayen, Iran.

Yavar Rassi
Department of Medical Entomology & Vector Control, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran.

Correspondence:
Yavar Rassi
Department of Medical Entomology & Vector Control, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran.

Study on reservoirs of cutaneous leishmaniasis using molecular methods of PCR-RFLP in endemic foci of disease, north east of Iran

Sayena Rafizadeh, Mehrzad Saraei, Mohammad Reza Abai, Mohammad Ali Oshaghi, Mehdi Mohebali, Amir Peymani, Taghi Naserpour Farivar, Hassan Bakhshi, Alireza Akrami, Yavar Rassi

Abstract
Zoonotic cutaneous leishmaniasis (ZCL) due to *Leishmania major* is a great public health problem in the Old World. Rodents are important reservoir of causative agent of disease is widely distributed in arid and savannah regions. Esfaryen district is one of the most important endemic foci of zoonotic cutaneous leishmaniasis (ZCL) in Northeast of Iran. To identify the reservoir(s) of *Leishmania* in this focus, a total of 44 rodents were collected using Sherman traps during September to December 2013.
They were identified as *Rhombomys opimus* Lichtenstein (72.7%) and *Meriones libycus* Lichtenstein (27.3%). Microscopic investigation on blood smear of the animals for amastigote parasites revealed 32 (72.7%) rodents of *R. opimus* infected with amastigote. *L. major* infection in these animals was then confirmed by polymerase chain reaction against internal transcribed spacer ribosomal DNA (rDNA) loci of the parasite followed by restriction fragment length polymorphism. Further, sequence analysis of 297 bp of ITS1-rDNA loci revealed the presence of *L. major* in *R. opimus*.
This is the first molecular report of *L. major* infection within *R. opimus* in this region. Our study showed that *R. opimus* is the most important host reservoir for maintenance of the parasite source in the area.

Keywords: Cutaneous leishmaniasis, *Leishmania major*, Reservoir, Iran.

1. Introduction
Zoonotic cutaneous leishmaniasis (ZCL) is an important public health problem in many regions of low incoming countries in the Old World. The causative agent of disease, *Leishmania major* is widely distributed in various rodent populations in arid and savannah regions [1]. Rodents of the sub family Gerbillidae are the main reservoir hosts of causative agent of disease in Iran and other countries where ZCL is endemic [2-5]. In natural ecosystems of Old World deserts, the rodents of gerbils are the most abundant mammals [2].
There are several species of rodents that play as reservoir hosts of *leishmania* due to *L. major*: Great gerbil of *Rhombomys opimus* in Central Asia, Northern Afghanistan and Iran; Libyan jirid of *Meriones libycus* in the Arabian Peninsula, Central Asia and Iran; Indian jirid of *Meriones hurrianae* in India and Iran; *Psammomys obesus* (fat sand rat) and *Meriones crassus* in Northern Africa and Middle East; *Rattus rattus and Arvicanthis niloticus* in Sudan [6] and Tatera spp. in sub-Saharan Africa and Iran [1].
The rodent species of *R. opimus* (Cricetidae: Gerbillinae) has been reported as the main and proven reservoir host of *L. major* in west and south Kazakhstan , Central Asia with adjacent parts in Afghanistan and Iran, Mongolia, and in some provinces of China [2]. Based on animal reservoir hosts of ZCL, four foci of disease have been reported in Iran [4]. In central and northeast of Iran, the great gerbil of *Rhombomys opimus* Lichtenstein and *Phlebotomus papatasi* Scopoli play important roles as reservoir and vector of the disease [7-10]. The second foci is located in the west and southwest of Iran, where *Tatera indica* Hardwicke replaced *R. opimus* as a reservoir and *P. papatasi* as a vector [9]. Baluchistan province, in the southeast of Iran, is considered as the third foci of ZCL, and *Meriones hurrianae* Jordon has been approved as a natural reservoir host of disease [11].
The most rural areas of Fars province in southern Iran can be considered as the fourth foci of ZCL, where, *Meriones libycus* Lichtenstein is the primary and main reservoir host of the

disease, while *R. opimus* and *T. indica* were absent and *P. papatasi* is the proven vector of ZCL [4, 10].
Esfarayen district in north eastern Iran is an important focus of ZCL, and this study was performed to determine the main host reservoir of the disease using PCR-RFLP.

2. Material and Methods

2.1 Study area

This study was conducted in Esfarayen district, Khorasane shomali province, Northeast of Iran during September to December, 2013.
The capital of the county is Esfarayen. At the 2006 census, the county's population was 119,152, in 30, 307 families. In general, the northern part of the Esfarayen has a temperate climate due to its proximity to mountainous areas and the south and southwest areas with hot summers and cold winters. The average annual precipitation is nine mm. The main occupations of the population are farming and raising animal.

2.2 Rodents collection

Based on prevalence of disease with positive human cases, four villages of Kalatereza, Esmaeilabad, Kalatehshor and Hosseinabad were selected. Active colonies of rodents were identified, and rodents were captured using Sherman live traps in various parts of mentioned villages. Forty live traps were used each time and the traps were baited with roasted walnut, cucumber, and tomato, and placed in active burrows. The traps were set up early morning and evening during September to December, 2013.
The genus and species of the rodents were determined by external characteristics: color, body measurements, ears, tail, feet, teeth, and cranium [12, 13]. Parasite infection in rodents was examined microscopically by preparing an impression smear from their ears after Giemsa staining. Positive smear samples were collected from infected rodents and were inoculated subcutaneously at the base of the tail of BALB/c. The procedure followed for species identification by polymerase chain reaction (PCR) method is described later.

2.3 DNA extraction

DNA of the all 44 rodent specimens was extracted using the Bioneer Genomic DNA Extraction Kit Cat. No.K-3032 Lot. No. 1204D, (North Korea), according to the manufacturer's instructions. Extraction was carried out on the prepared smears slides of rodents. Double distilled water as a negative control and DNA from *L. major* and *L. tropica,* provided by Iranian Institute of Pasteur by the World Health Organization, were used as positive controls.

2.4 DNA amplification and PCR-RFLP

Primary examination for infection of rodents with *Leishmania* species was performed using nested- polymerase chain reaction (PCR) against the mini circle kinetoplast kDNA using the following primers [14] CSB2XF (forward): 5′-C/GA/GTA/GCAGAAAC/TCCCGTTCA-3′(20bp);CSB1XR(reverse):5′-ATTTTTCG/CGA/TTTT/CGCAGAACG-3′ (20 bp); 13Z (forward): 5′-ACTGGGGGTTGGTGTAAAATAG-3′ (22 bp); LIR (reverse): 5′-TCGCAGAACGCCCCT-3′ (15 bp).
Positive samples against kDNA were tested against the ribosomal internal transcribed spacer 1 (ITS1) region using the primers LITSR (5'-CTGGATCATTTTCCGATG-3') and L5.8S (5'-TGATACCACTTATCGCACTT-3') followed by digestion with *Hae* III [15]. The PCR products were run along with a 100 bp ladder on 1.2% agarose gel containing ethidium bromide for 1 h at 80 V. The gel was observed on an ultraviolet (UV) transilluminator and then digital photographs were taken. Parasites were identified by comparison with positive controls of *L. major* and *L. tropica* and molecular weight markers. Two µL *Hae* III was added to the ITS1 PCR products (20 µL) at 37 °C for 12 h with conditions recommended by the supplier (Fermentas, Germany). The restriction fragments were subjected to electrophoresis in 3% agarose gel containing ethidium bromide (0.5 µg/mL) for 3 h at 65 V and observed on a UV transilluminator [16, 17].

3. Results

Altogether, 44 rodents were captured and identified. They were *Rhombomys opimus* (72.7%) and *Meriones libycus* (27.3%). All collected animals were examined for parasite infection under a light microscope, amastigotes were found in smears of all specimens 32 (72.7%) of *R. opimus*. Some samples from infected rodents were inoculated subcutaneously at the base of tail of one BALB/c. Inoculation of the parasite from infected rodents revealed the presence of amastigotes into the nodules and ulcer of the experimental mice after 25 days of the inoculation period. Parasite infections were observed in both male and female rodents of *R. opimus*. Isolated parasites from infected rodents were identified as *L. major* using kDNA nested PCR. We observed in the kDNA nested-PCR amplification assays where a ~560 bp PCR band was produced. This length of PCR in the system is assigned to *L. major* (Figure 1). Further analyses showed that they were positive against ITS1 locus and produced a band of ~340 bp in gel electrophoresis. Also, ITS1 PCR–RFLP analysis by HaeIII revealed the fragments of 220 and 140 bp for infected rodents which are characteristic of *L. major*. The diagnostic fragments are 200 and 60 bp for *L. tropica* and 200, 80 and 60 bp for *L. infantum/L. donovani* (Figures 2, 3). Some of the positive PCRs against ITS1 region detected in *R. opimus* were successfully sequenced and submitted to GenBank (accession nos. KJ577703, KJ577704, KJ577705, KJ577706, KJ577707 KJ577708). All prepared samples from *M. libycus* were negative for *Leishmania* infection.
This is the first attempt on molecular detection of *Leishmania* infection among rodents as the reservoir host of disease in Esfarayen foci, north east of Iran.

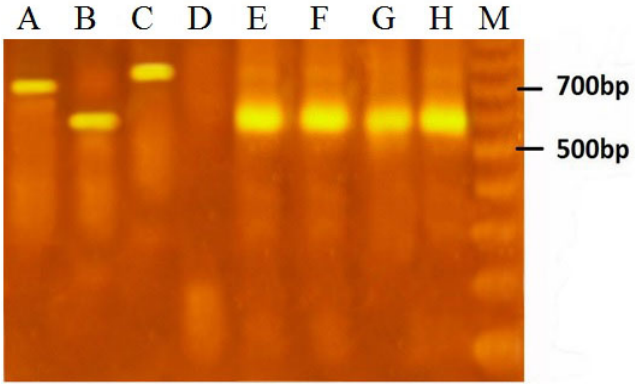

Fig 1: kDNA nested PCR amplification (560 bp). *L. major* in *R. opimus* (Lanes E,F,G,H); Positive control of *L. tropica* (Lane C, 720 bp); Positive control of *L. major* (Lane B); Positive control of *L. infantum*(680 bp, A); Negative control (Lane D) and (M)100 bp molecular weight marker (Fermentase).

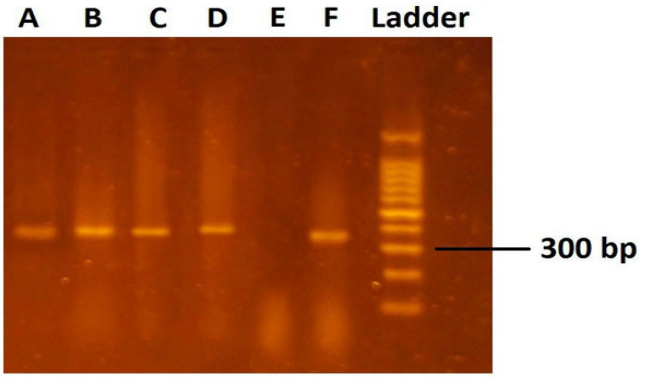

Fig 2: ITS1 amplification of *L. major* in *R. opimus* (Lanes A, B, C, D); Negative control (Lane E); positive control of *L. major* (Lane F) and (Ladder) 100 bp molecular weight marker (Fermentase).

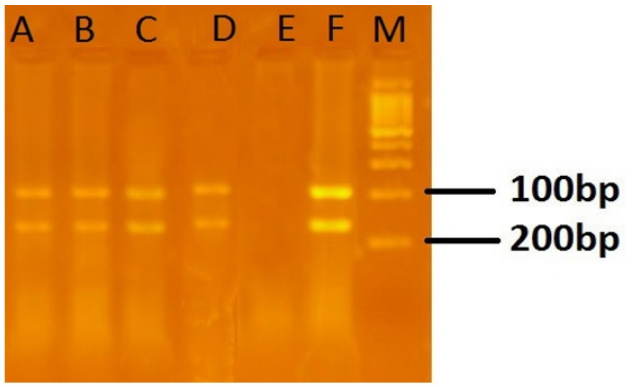

Fig 3: PCR-RFLP analysis of ITS1 region for identification of *Leishmania* species using HaeIII. (Lanes A,B,C,D) samples of infected *R. opimus* to *L. major* ; (Lane E) negative control ; (Lane F) positive control of *L. major* ; (Lane M) 100 bp molecular weight marker

4. Discussion

In endemic areas, the effective control of the human leishmaniasis requires a thorough knowledge of the ecology and epidemiology of the parasites causing the diseases, and their vertebrate and invertebrate hosts [18]. In many areas, however, despite considerable research on these diseases, the main 'reservoir' hosts and the species of sand fly responsible for most transmission have still to be identified. In many foci of ZCL there is at least one species of rodent that is sufficiently common and to be considered as a principal and main reservoir host of causative agent of disease.

Direct examination and culture of *Leishmania* parasites (traditional techniques) are insufficient for diagnosing of leishmaniasis [19, 20]. Furthermore, mixed infections of *Leishmania* parasites will be missed due to different growth rates of the different parasites in blood agar cultures [21]. Currently, the use of molecular methods in terms of sensitivity, speed and specificity are an interval to traditional methods.

In this survey we isolated some *L. major* parasites from *R. opimus* where its sequences were 100% identical to that of isolates from several *L. major* sequences deposited in Genbank, including isolates from Iran (Accession Nos. EF413075, KJ194178, JN860713, AY573187), Sudan (Accession No. AJ300481), Uzbekistan (Accession No.FN677357). Furthermore, We found that, our specimens were 99% similar to *L. major* from Turkey (Accession No.KJ002553) as well as *L. mexicana braziliensis* from Mexico (Accession No.AF339752).

This rodent has been also reported as the main reservoir in other foci of the disease in central of Iran [5, 7, 8]. This great gerbil, a colonial, burrowing rodent, is a common species in the arid desert and steppe regions of Central Asia. This species also exist in the southern territories of the former U.S.S.R. (i.e., Turkmenistan, Uzbekistan, Kazakhstan, and Tajikistan) and neighboring countries where ZCL is endemic and considered as an important public health problem, where *R. opimus* is considered the principal mammalian host of the parasite [22].

As a final conclusion, based on high density of *R. opimus* in the study areas, its natural infection with *Leishmania major*, Proximity of its colonies to human dwellings (< 200 m), this rodent is introduced as the main and principal reservoir host of disease in transmission to human by sand flies vectors. Although, all specimens of *M. libycus* were free of *leishmania* parasites, but considering the role of this rodent in other foci of ZCL in Iran, further investigation is needed.

5. Acknowledgement

The authors gratefully thank Esfarayen Health Center, Northern Khorasan province, Northeast of Iran for its field assistance. This study was financially supported by the School of Public Health, Tehran University of Medical Sciences (Project No. 24217) and Qazvin University of Medical Sciences.

6. References

1. Gramiccia M, Gradoni L. The current status of zoonotic leishmaniases and approaches to disease control. International Journal for Parasitology 2005; 35:1169–1180.
2. Dubrovsky Y. Biology of Great Gerbil – The Principal Carrier of the Great of Zoonotic Cutaneous Leishmaniasis. WHO Traveling Seminar on Leishmaniasis Control. Ministry of Health, Moscow, 1979.
3. Strelkova MV. Progress in studies on Central Asian foci of zoonotic cutaneous leishmaniasis a review. Folia Parasitologica 1996; 43:1–6.
4. Rassi Y, Javadian E, Amin M, Rafizadeh S, Vatandoost H, Motazedian H. Meriones libycus is the main reservoir of zoonotic cutaneous leishmaniasis in South Islamic Republic of Iran. East Med Hlth J 2006; 12(3-4):475-477.
5. Rassi Y, Abai MR, Javadian E, Rafizadeh S, Imamian H, Mohebali M *et al.* Molecular data on vectors and reservoir hosts of zoonotic cutaneous leishmaniasis in central Iran. Bull Soc Pathol Exot 2008; 101(5):425-428.
6. Abdalla NM, Eldosh AA , Abdulgani AM , Yusif BE, Magzoub MM. Typing and Characterization of leishmania sub-clinical isolates from Nuba Mountain, West of Sudan. Infection Genetic and Evolution 2003; 2(4):277.
7. Seyedi-Rashti MA, Nadim A. Epidemiology of cutaneous leishmaniasis in Iran. B. Khorassan area, part I; the reservoir. Bull Soc Pathol Exot 1967; 60:510–518.
8. Javadian E, Nadim A, Tahvidare-Bidruni GH, Assefi V. Epidemiology of cutaneaous leishmaniasis in Iran. B: Khorassan area, part V; report on a focus of zoonotic cutaneous leishmaniasis in Esferayen. Bull Soc Pathol Exot 1976; 69:140–143.
9. Rassi Y, Oshaghi MA, Mohammadi Azani S, Abaie MR, Rafizadeh S, Mohebali M *et al.* Molecular detection of leishmania infection dueto leishmania major and leishmania turanicain the vectors and reservoir host in Iran. Vector-borne and zoonotic diseases 2011; 11(2): 145-150.
10. Rassi Y, Gassemi MM, Javadian E, Rafizadeh S, Motazedian H, Vatandoost H. Vectors and reservoirs of

cutaneous leishmaniasis in Marvdasht district, southern Islamic republic of Iran. East Med Hlth J 2007; 13(3):686-693.
11. Seyedi-Rashti MA, Nadim A. Cutaneous leishmaniasis in Baluchistan, Iran. Abstracts of Poster Vol XI, International Congress for Tropical Medicine and Malaria, Calgary, Canada, 1984, 124.
12. Boitani L, Bartoli S. Macdonald Encyclopedia of Mammals, 1st edition. London: Macdonald & Co., 1980, 512.
13. Ziaei HA. Field Guide for Identifying of Iranian Desert Mammalians, 1st edition. Tehran: Iranian Environment Organization, 1996, 129-187.
14. Noyes HA, Reyburn H, Bailey JW, Smith D. A nested-PCRbased schizodeme method for identifying Leishmania kinetoplast minicircle classes directly from clinical samples and its application to the study of the epidemiology of Leishmania tropica in Pakistan. J Clin Microbiol 1998; 36:2877–2881.
15. El Tai N, Osman O, El Fari M, Presber W, Schönian G. Genetic heterogeneity of ribosomal internal transcribed spacer in clinical samples of Leishmania donovani spotted on filter paper as revealed by single-strand conformation polymorphisms and sequencing. Trans Royal Soc Trop Med Hyg 2000; 94(5):575-9.
16. Oshaghi MA, Ravasan NM, Javadian E, Mohebali M, Hajjaran H, Zare Z *et al.* Vector incrimination of sand flies in the most important visceral leishmaniasis focus in Iran. The American J Trop Med Hyg 2009; 81(4):572-577.
17. Hide M, Bañuls AL. Species-specific PCR assay for L. infantum/L. donovani discrimination. Acta Trop 2006; 100:241–245.
18. Aransay AM, Scoulica E, Tselentis Y. Detection and identification of Leishmania DNA within naturally infected sand flies by semi nested PCR on mini circle kinetoplastic DNA. Applied and Environmental Microbiology 2000; 66:1933–1938.
19. Ben-Ismail R, Smith DF, Ready PD, Ayadi A, Gramiccia M, Ben-Osman *et al.* Sporadic cutaneous leishmaniasis in north Tunisia: identification of the causative agent of Leishmania infantum by the use of a diagnostic deoxyribonucleic acid prob. Trans R Soc Trop Med Hyg 1992; 86:508-510.
20. Shahbazi F, Shahabi S, Kazemi B, Mohebali M, Abadi AR, Zare Z. Evaluation of PCR assay in diagnosis and identification of cutaneouse leishmaniasis: a comparison with the parasitological methods. Parasitol Res 2008; 103:1159–1162
21. Ibrahim ME, Smith AJ, Ali MH, Barker DC, Kharazmi A. The polymerase chain reaction can reveal the occurrence of naturally mixed infection with Leishmania parasites. Acta Trop 1994; 57:327-332.
22. Strelkova MV, Eliseev LN, Ponirovsky EN, Dergacheva TI, Evans DA. Mixed leishmanial infections in Rhombomys opimus: a key to the persistence of Leishmania major from one transmission season to the next. Ann Trop Med Parasitol 2001; 95:811-819.