Original Article

# ITS2-rDNA fragments of Leishmania species isolated from the great gerbil in Iran, 2021

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## Abstract

**Background:** The great gerbil (*Rhombomys opimus*), is widely distributed in Asia and is a natural reservoir for zoonotic cutaneous leishmaniasis in many endemic areas, as well as Iran.

**Materials and Methods:** In this study, infection to *Leishmania* species was investigated by two methods, parasitological and molecular survey, in the small number of *R. opimus* collected from Jovain, a Zoonotic Cutaneous Leishmaniasis (ZCL) focus located in North East of Iran.

**Results:** Parasitological observation showed infection in only one of five rodents. But, *ITS2*-Nested-PCR revealed *Leishmania* infection in three out of 5 gerbils, including the parasitological positive one. Based on the PCR amplified size, two cases of infections were *Leishmania major* and one *Leishmania turanica*, their sequences are accessible in GenBank. The results of sequence analysis were consistent with the results obtained based on the size of the PCR.

**Conclusion:** These findings re-confirm the important role of *R. opimus* in the natural circulation of *Leishmania* spp and indicate the need to be concerned about the disease in the study area.

**Keywords:** Iran, *Leishmania turanica*, *Leishmania major*, Nested-PCR, *Rhombomys opimus*

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## Introduction

Leishmaniasis is a complex disease that is induced by obligate intracellular protozoa parasites belonging to more than 20 different *Leishmania* species and invades phagocytic host cells[1,2]. The parasite is transmitted to humans and other mammalian reservoir hosts by the infected female of a tiny (2–3 mm) blood-sucking female of Phlebotomine sand flies either anthroponotically or zoonotically through their bites[3]. Leishmaniasis has been reported as one of the most important neglected tropical diseases (NTDs) that affect more than 1 billion people living in endemic areas of the Mediterranean Basin, East Africa, the Americas, and Southeast Asia[4,5] . Phenotypes of the disease include three main clinical forms; visceral Leishmaniasis (VL) the most severe form, cutaneous Leishmaniasis (CL) the most common form and finally muco-cutaneous Leishmaniasis (MCL) the most disabling form[5,6]. According to the

latest report of the World Health Organization (WHO), an estimated of 30000 new cases of VL and more than 1 million new cases of CL occur annually[5]. Leishmaniasis is widespread in most countries in the Mediterranean basin, including Iran. Two main forms of Leishmaniasis have been observed in Iran; in fact, *Leishmania infantum* is responsible for VL and in the case of cutaneous disease, *Leishmania tropica* and *Leishmania major* are causative agents of Anthroponotic and Zoonotic Cutaneous Leishmaniasis, respectively[4].

Like other endemic areas, the most prevalent form of the disease in Iran is a cutaneous form (22000 cases annually) and the majority of these cases (80%) are ZCL form[7-9]. The causative agent of this form is *L. major* which transmits to humans via the infected bite of *Phlebotomus papatasi* (Diptera: Psychodidae) and Gerbillinae (Rodentia: Muridae) is the main reservoir host. This form was distributed in rural areas of 18 out of 31 provinces of Iran, with some main endemic foci located in northeast, central, and southwest parts of the country[8,10,11]. Wild rodents are considered the natural reservoir host of ZCL, the species of rodents differs in different geographical areas of disease distribution. The rodents *Meriones libycus*, *Tatera indica*, and *Meriones hurrianae* are considered the principal reservoir host respectively in some central and south foci, in the southwest and southeastern part of Iran. One of the most important rodents, the great gerbil *Rhombomys opimus*, is the main reservoir for ZCL in central and northeastern parts of Iran. Its infection rate to *L. major* was reported even more than 90% in some endemic foci of ZCL[12-16].

Khorasan-Razavi Province has common borders with Afghanistan and Turkmenistan in the east and Anthroponotic and Zoonotic Cutaneous leishmaniasis is endemic in many parts of the province[17]. One of the ZCL focus in Khorasan-Razavi Province is Jovain County which is located 75 km far from Sabzevar city. In an epidemiological survey which has been carried out in Jovain in 2001-2002, this county was introduced as a new focus of ZCL in Iran. The researchers resulted that *R. opimus* was the main reservoir of the disease since 15.6% of trapped species were found to be infected with *L. major*[18].

As a pilot study, the results of *Leishmania* infection of *R. opimus* collected from Jovain County will be presented here.

## Methods

**Ethical statements:** All procedures performed in this study involving animals were by the ethical standards of the Animal Ethics Committee of Shahid Beheshti University of Medical Sciences (Approval No. IR.SBMU.MSP.REC.1399.390). In this study, all efforts were made to minimize animal suffering within the course of our study.

**Study area:** The rodents were trapped in February 2020 in Jovain County (36.705230°N 57.417322°E), Khorasan-Razavi Province, Iran (Figure 1). Jovain city is located 75 km northwest of Sabzevar and 60 km of Esfarayen, both considered the main ZCL foci. The county with an area of about 1656.05 square kilometers, and an average altitude of 1,100 meters above sea level. The population of this county is more than 55 thousand people according to the last census in 2016. The climate is cool, and dry in the winter, and hot, and dry in the summer. The predominant vegetation included *Clostridium*, predominant crops include wheat, barley, watermelon, melon, sugar beet, alfalfa, cumin, rapeseed, cotton, and the predominant garden plants include grapes, almonds, pistachios a, and pomegranates[19].

**Rodent collection:** Active colonies of gerbils were identified and the great gerbils were caught in 5 of 10 Sherman live traps (30 cm × 15 cm × 15 cm wire mesh), which were placed near the entrances of active burrows and baited with bread and oil or butter and roasted walnuts. The trapped gerbils were transferred to the animal husbandry of the School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran. They were identified morphologically by reliable keys[20]. The presence of *Leishmania* parasites was investigated in rodents in captivity by microscopic and molecular examination.

**Examination of the natural infection of rodents to *Leishmania* spp**

**Direct parasitological test:** The rodents were anesthetized using an Isoflurane inhaler, then impression smears were prepared from the ears lobes of each rodent individually, fixed in methanol, and stained by the standard Giemsa method, and thereafter examined with light microscopically (1000×) for

detection of *Leishmania* amastigotes. Also, each ear lobe was transferred to a labeled microtube containing 500 µl of Ethanol 96% and kept at −20 °C until used for DNA extraction.

**DNA extraction, Nested-PCR assay, and Sequence analysis:** Whole genomic DNA containing ribosomal DNA of each lobe and in some cases from the slide smears were extracted by using phenol–chloroform method with slight modification[21]. Briefly, before DNA extraction, each ear lobe was washed three times with distilled water, and then lysed with 700 µl of lysis buffer added and ground with a pestle, and the remained steps were done according to the protocol. Extracted DNA was stored at −20 °C until use.

*Leishmania* DNA was amplified by nested PCR, according to Akhavan et al[13]. The external primers for the first run were Leish out F (5′-AAA CTC CTC TCTGGT GCT TGC-3′) and Leish out R (5′-AAA CAA AGG TTG TCG GGG G-3′), and for the second run internal primers were Leish in F (5′-AAT TCA ACT TCG CGT TGG CC-3′) and Leish in R (5′-CCT CTCTTT TTT CTC TGT GC-3′) were used the nested PCR conditions that set previously[22]. The amplification products were analyzed on 1.5% agarose gels, visualized under UV, and photographed. The DNA of reference strains of *L. major* (MRHO/IR/75/ER) and *Leishmania* turanica (MRHO/SU/1983/MARZ-051) were used to control the PCR condition as well the species identification. The expected amplified bands for three *Leishmania* spp are shown in table 1[13].

The PCR amplicons were finally sent to Pishgam Biotech Company, Tehran, Iran, for purifying and sequencing. Sequences were assembled and edited manually by Chromas program ver. 2.5.0.

The sequenced data were submitted to GenBank by using https://submit.ncbi.nlm.nih.gov/subs/genbank. For finding sequence similarity with GenBank sequences and for multiple sequence alignment, BLAST and online Multalin version 5.4.1 (multalin.toulouse.inra.fr/multalin/) were used, respectively.

# Results

**Rodents' species identification:** All five trapped rodents were identified as a great gerbil, *R. opimus* Lichtenstein, 1823, based on external morphological characters, mainly color, grooves on the incisor teeth, length of ears, and tail. The presence of two fine grooves on each incisor tooth is the most important characteristic of *R. opimus* identification (Figure 2).

***Leishmania* infection in *Rhombomys opimus*:** The rodents were screened for possible infection to the *Leishmania* parasite using the conventional parasitological method as well as the molecular survey by targeting *ITS2*. As figure 3 showed the amastigote form of the *Leishmania* parasite in Giemsa stained slide belonging to one of the *R. opimus*. Regardless of the results of the parasitological tests, the samples from all rodents were subjected to molecular analysis to trace the parasite DNA. Interestingly it was found that three rodents were infected with *Leishmania* spp. Based on the size of the amplified band in the first run (483 bp) and the second run of PCR (~245 bp), two of them including the positive parasitological rodent were diagnosed infected with *L. major*. In the third one, based on the size of the amplified product in the first run (399 bp) and second run of PCR (~141 bp) the species of the parasite was determined as *L. turanica* (Figure 4).

**Sequence analysis:** The PCR products were sequenced and deposited in GenBank (Accession Number ON721104-ON721106). BLAST analysis showed that 2 samples have 100% identity with those of *L. major* available in GenBank (MT497977, MT497955, and MN931859), whereas, sequence similarity with many *L. turanica* and *L. gerbilli* (AJ300486) were 85% and 86%, respectively.

As expected, sequence analyses revealed that the third positive isolates were *L. turanica*. BLAST analysis of *ITS2* partial sequences showed 100% homology with

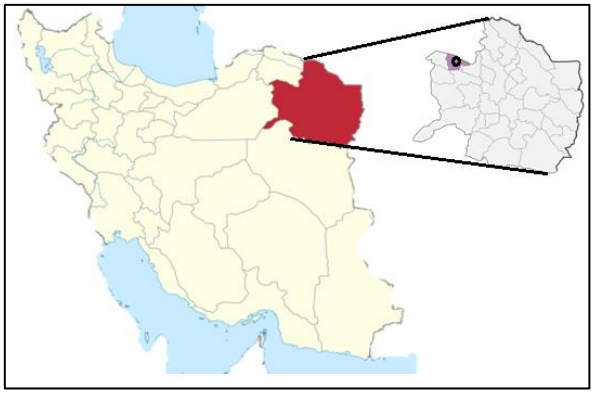

**Figure 1. Map showing the geographical location of Jovain (marked with black circle) and Khorrasan-Razavi Province in Iran.**


**Figure 2. *Rhombomys opimus* (Gerbillinae subfamily); a: photo showed great gerbil (*R. opimus*), b: distinctive character for *R. opimus* identification, two fine grooves on each incisor teeth (original photos).**

**Table 1:** Expected size (bp) of internal transcribed spacer *ITS2* products in the first and second round of PCR for different *Leishmania* spp using specific primers

| ***Leishmania* spp** | ***ITS2* products with external primers ( Leish out F & Leish out R)** | ***ITS2* products with internal primers ( Leish in F & Leish in R)** | Ref. |
|---|---|---|---|
| *L. major* | 483 bp | 245 bp | 13 |
| *L. gerbilli* | 441 bp | 206 bp | |
| *L. turanica* | 399 bp | 141 bp | |

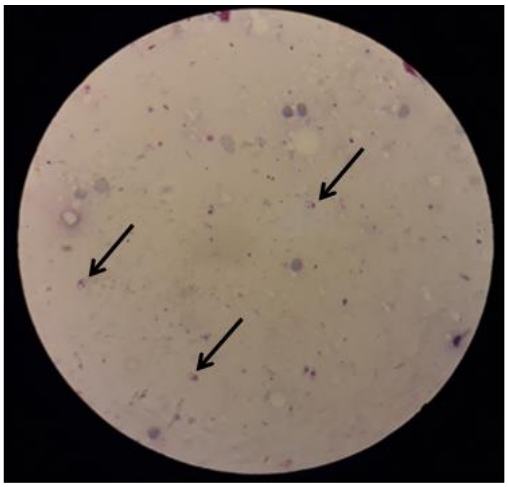

**Figure 3. Giemsa stain image showing the amastigote form of *Leishmania* spp isolated from ear lobe of *Rhombomys opimus*.**

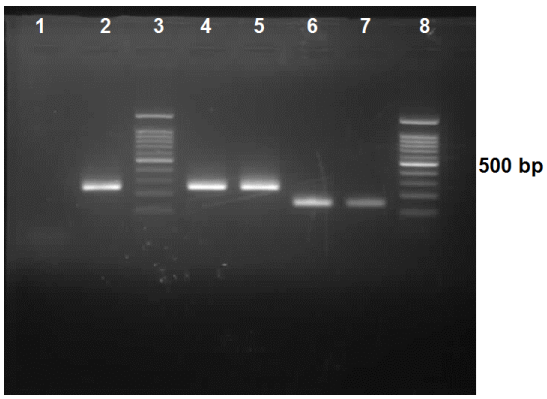


**Figure 4. Agarose gel image of amplified *ITS2* locus of *Leishmania* spp isolated from *Rhombomys opimus.* Lane1: Negative control, Lane 2: Standard strain of *Leishmania major* (MRHO/IR/75/ER), Lane 3: 100 bp ladder (Cinaclone, Iran), Lane 4 & 5: *Leishmania major* isolated in the current study, Lane 6: *Leishmania turanica* isolated in the current study, Lane 7: Standard strain of *Leishmania* turanica (MRHO/SU/1983/MARZ-051), Lane 8: 100 bp ladder (Cinaclone, Iran).**

those of *L. turanica* available in GenBank (MW228816- MW22881618), whereas identity with


**Figure 5. Nucleotide sequence diversities for *ITS2* gene (A): two *Leishmania major* (ON721104-ON721105) and (B): *Leishmania turanica* (ON721106) isolated from *R. opimus* in the current study and those available in GenBank.**

the *L. gerbilli* isolates (HQ830351, HF545839,) was 95-97%. Nucleotide sequence diversities for the *ITS2* gene in two *L. major* and the only *L. turanica* isolated in the current study are demonstrated in Figure 5.

## Discussion

Rodents belonging to the Gerbillinae subfamily are natural reservoir hosts of Cutaneous leishmaniasis caused by *L. major*, which *is* the predominant form of Leishmaniasis and was found in more than half of the provinces of Iran[14,22-24]. Jovain County is located in the endemic area of ZCL in Iran[18]. In this pilot study, five *R. opimus* were captured from Jovain, and their infection to *Leishmania* was investigated, the obtained results will be discussed here.

The rodent species was confirmed as *R. opimus* which is one of the most well-known main reservoir hosts for ZCL in central Asia, northern Afghanistan, and Iran (Central and north-east)[4,14,22-24]. The *Leishmania* infection of these rodents was tested by direct impression smear, also by *ITS2*-Nested-PCR. Based on the parasitological method, infection was found only in one rodent. Although the parasitological method is the gold standard for the detection of *Leishmania* infection, it is highly dependent on the expertise of the person in sampling, slide preparation, and degree of parasitism[25]. More importantly, species identification or detection of possible mix infection is not possible by this conventional method, so an alternative method such as using different molecular markers was proposed[26]. Here, we used nested PCR to amplify the Internal Transcribed Spacer 2 (*ITS2*) locus of the *Leishmania* genome. *ITS2* is a non-coding region of SSUrRNA, which separates coding regions. There are about 20-40 copies of the SSU rRNA gene per cell in *Leishmania* species[27]. In addition to the sample that was positive in terms of parasitological method, *ITS2*-Nested-PCR showed *Leishmania* infection in two other rodents. Despite the small number of rodents studied here, which can be the main drawback of this research, the results were interesting, three gerbils were infected among 5 trapped ones. One of the rodents was found infected with *L. turanica* a common *Leishmania* species found in *R. opimus*[4,22,28].

## Conclusion

Generally, having enough knowledge about different elements involved in ZCL (humans, reservoirs, vectors, and parasite) could be very useful in disease prevention or control. The earliest epidemiological work in Jovain was done in 2001-2002 which introduced this area as a new focus for ZCL and *R.*

*opimus* as a natural reservoir host. This area is industrial (industrial factories) and certainly, non-native workers were attracted, which can have an important impact on the epidemiology of ZCL. This fact highlights the need for up-to-date information on ZCL epidemiology elements. This study reaffirmed the results of the previous study and indicated the need to be concerned about disease circulation.

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## Declaration of Interest

We disclose any actual or potential conflict of interest including any financial, personal, or other relationships with other people or organizations.

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