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Major Article

The existence of only one haplotype of *Leishmania major* in the main and potential reservoir hosts of zoonotic cutaneous leishmaniasis using different molecular markers in a focal area in Iran

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ABSTRACT

Introduction: *Leishmania major* is the causative agent of zoonotic cutaneous leishmaniasis (ZCL), and great gerbils are the main reservoir hosts in Iran. Abarkouh in central Iran is an emerging focal point for which the reservoir hosts of ZCL are unclear. This research project was designed to detect any *Leishmania* parasites in different wild rodent species. **Methods:** All rodents captured in 2011 and 2012 from Abarkouh district were identified based on morphological characteristics and by amplification of the rodent cytochrome *b* (Cyt b) gene. To detect *Leishmania* infection in rodents, deoxyribonucleic acid (DNA) of each ear was extracted. Internal transcribed spacer-ribosomal deoxyribonucleic acid (ITS-rDNA), microsatellites, kinetoplast deoxyribonucleic acid (kDNA) and cytochrome *b* genes of *Leishmania* parasites were amplified by polymerase chain reaction (PCR). Restriction fragment length polymorphism (RFLP) and sequencing were employed to confirm the *Leishmania* identification. **Results:** Of 68 captured rodents in the region, 55 *Rhombomys opimus* were identified and nine *Leishmania* infections (9/55) were found. In addition, eight *Meriones libycus* and two *Tatera indica* were sampled, and one of each was confirmed to be infected. Two *Meriones persicus* and one *Mus musculus* were sampled with no infection. **Conclusions:** The results showed that all 11 unambiguously positive *Leishmania* infections were *Leishmania major*. Only one haplotype of *L. major* (GenBank access No. EF413075) was found and at least three rodents *R. opimus*, *M. libycus* and *T. indica*—appear to be the main and potential reservoir hosts in this ZCL focus. The reservoir hosts are variable and versatile in small ZCL focal locations.

Keywords: *Leishmania* parasite. Zoonotic cutaneous leishmaniasis. Rodents. Haplotype. Iran.

INTRODUCTION

Leishmaniasis is one of the nine emerging individual infectious diseases that have been largely neglected around the world and in the Middle East[1].

In Iran, leishmaniasis is observed in three clinical forms: zoonotic cutaneous leishmaniasis (ZCL), anthroponotic cutaneous leishmaniasis (ACL) and zoonotic visceral leishmaniasis (ZVL). ZCL caused by *Leishmania major* has a health as well as socioeconomic impacts in Iran. ZCL has been reported in rural regions of Iran in 15 of 31 Provinces,including: Bushehr, Hormozgan and Fars in the south[2,3]; Ilam and Khuzestan in the southwest and west[4,5]; Golestan, Khorasan and Semnan in the northeast and north[4-6]; and Isfahan in the central region[7].

During the life cycle of ZCL, which depends on the geographical location of the disease, sandflies act as vectors of *Leishmania*, *Bartonella bacilliformis* and some arboviruses, and wild rodents are considered to be the reservoir hosts[8]. Many investigations have been conducted on different aspects of ZCL in naturally important foci in Iran[9-11], although some areas of neighboring Provinces have been neglected for unknown reasons. Predisposing factors, such as increasing migration of patients from endemic foci to potential areas, irregular construction and urbanization and changing sandfly fauna in the region affect the distribution and survival of ZCL[9,12].

Yazd Province in central Iran is one of these regions, and the number of cases of ZCL has been increasing since 1981[10]. The official reports from the health center in Yazd Province has demonstrated that the number of cases of CL in the Ardakan area (which is an important potential focus in southwestern Yazd) increased from 1996 to 1997 (total: 372 cases), which

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may lead to monitoring and surveillance activities in this district[13]. A few studies have examined some cities in Yazd Province (Ardakan, Taft, Bafgh, and Khatam cities); however, no molecular-epidemiologic investigations have been performed in Abarkouh district yet. Abarkouh has many historical places (such as Abarkouh's Cedar) and tourism values therefore, tourists play an important role in spreading the infection to other Provinces. Thus, the isolation, detection and identification of *Leishmania* spp. in rodents are essential for disease prognosis, diagnosis methods, the monitoring of clinical outcomes, epidemiological perspectives and treatment program planning. Some reports have demonstrated that *Rhombomys opimus* and *Meriones libycus* are the most important reservoir hosts of ZCL in the neighboring Provinces of Isfahan and Shiraz[14,15]. The geographical distribution of reservoir hosts of ZCL in different regions of Iran is shown in **Figure 1**[11].

For population genetic studies and species identification, we utilized the cytochrome *b* gene of rodents, cytochrome *b* (Cyt b) of *Leishmania*, ITS1-5.8S ITS2 ribosomal deoxyribonucleic acid (rDNA), kinetoplast deoxyribonucleic acid (kDNA) and microsatellite deoxyribonucleic acid (DNA) genes were employed for detection of any *Leishmania* infection. Low intracellular polymorphism and readable sequences are important advantages of internal transcribed spacer-ribosomal deoxyribonucleic acid (ITS-rDNA) in molecular identifications[7,16-18]. The kinetoplast in Trypanosomatidae

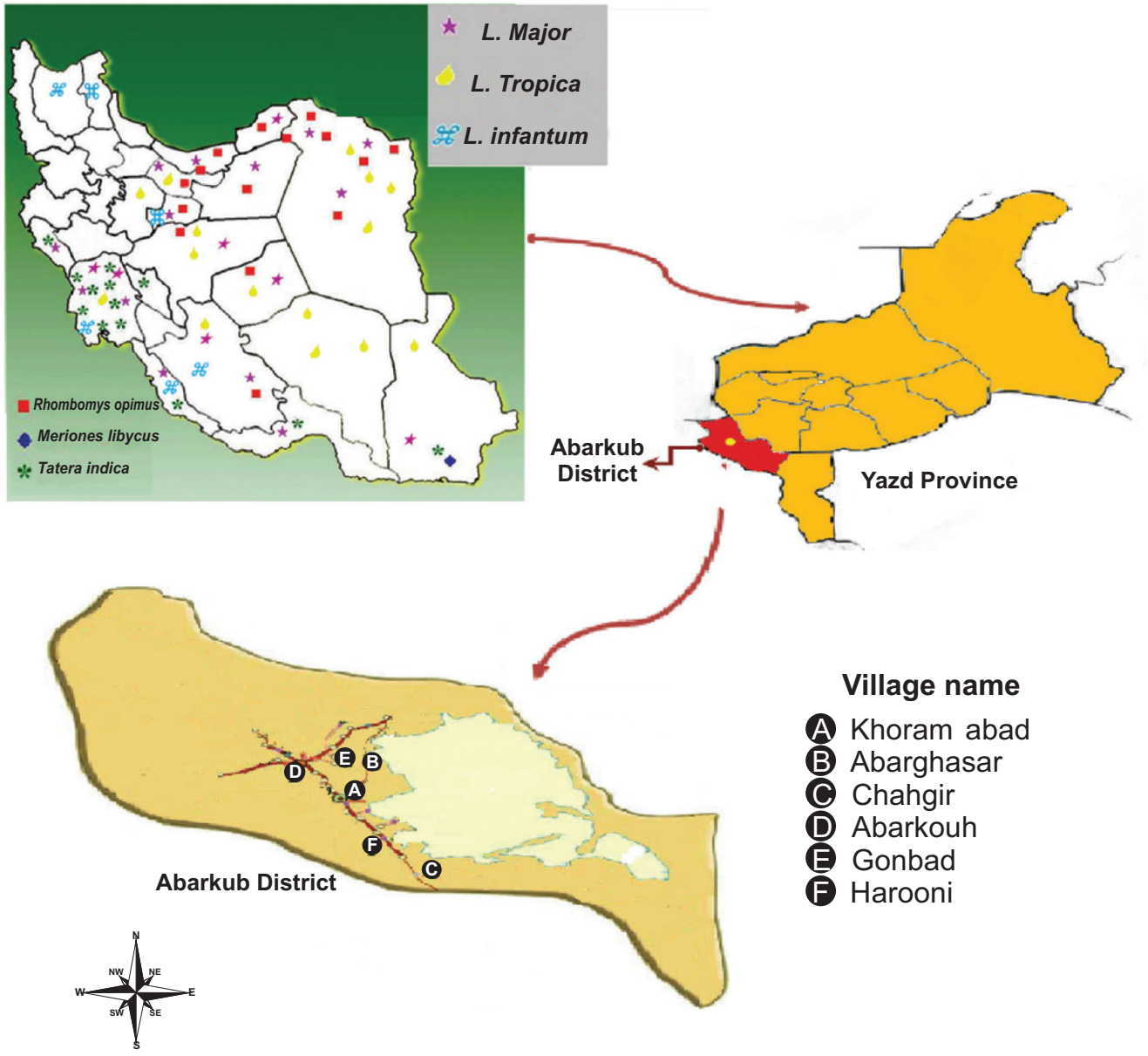

FIGURE 1 - Map of Abarkouh showing sampling regions and the geographical distribution of reservoir hosts of ZCL in different regions of Iran. *L:* *Leishmania;* **ZCL:** zoonotic cutaneous leishmaniasis.

contains nearly 10,000 small circular DNAs (kDNA minicircle). This minicircle comprises a variable region (600bp) and a conserved region (120bp). Microsatellite markers in *Leishmania* parasites are co-dominant and allelic and combine 1-7 nucleotide units into short, tandemly repeated DNA sequences[19,20]. Currently, multilocus microsatellite typing (MLMT) is being used widely in population genetic studies in different species of *Leishmania* parasites[21,22]. Minicircle kDNA and microsatellite ITS-rDNA are also well known as molecular markers for the detection of *Leishmania* infections[7,21].

The Cyt *b* gene encodes the central catalytic subunit of an enzyme present in the respiratory chain of mitochondria and exists in almost all organisms. This gene has been broadly used for phylogenetic studies and identification of animals and plants[23]. The Cyt *b* gene of the genus *Leishmania* consists of two regions: the edited region (the most 5′ region of 23bp), which undergoes ribonucleic acid (RNA) editing, and the non-edited region (the 3′ region of 1,056bp) [23].

Since the rodent fauna, the *Leishmania* species and their infection rate in Abarkouh district of Yazd Province, Iran, haves not been elucidated completely; we have designed this study in order to investigate these aspects of ZCL.

METHODS

Study area, sampling and laboratory methods

This cross-sectional/descriptive study was performed in 2011 and 2012, and rodent samples were obtained from 5 villages across Abarkouh district, Yazd Province, central Iran, including: Abarghasr, Haroni, Khorram abad, Gonabad and Chahgir.

Abarkouh district is situated between Fars (southern Iran) and Isfahan (central Iran) Provinces **(Figure 1)**. These Provinces are considered hyper-endemic regions that are important sites for ZCL[7]. Abarkouh district, with an altitude of 1,510 meters above sea level (a.s.l.) (4,954 feet), geographic coordinates of "31°07′44″N 53°16′57″E31.13°N 53.28°E" **(Figure 1)** and a population of approximately 21,818 people, is located in Yazd Province in central Iran. Due to its hot and dry climate and its proximity to Isfahan and Fars Provinces, Abarkouh is considered a new and emerging focus of ZCL as well.

The rodents were sampled in Abarkouh area 120km southwest of Yazd Province, using wooden and wire traps. To identify active colonies of rodents within a diameter of 1-1.5km around villages in Abarkouh, approximately 30-40 live traps were used, and the rodents were captured monthly by baiting with dates and cucumbers. The genus and species of each rodent were determined based on external features, including: ears, color, tail, body measurements, teeth, feet and cranium[15,24,25].

Each protocol and method applied in this survey was conducted according to the principles expressed in the Declaration of Helsinki and was approved by the Human and Animal Research Ethics Committee of the Pasteur Institute of Iran.

Two impression touch slides were obtained from both ears of each rodent by scratching. For brief microscopic observation, rodent samples were collected from the ears after removing the hair and making small scratches from which to extract serous fluid, which was then fixed on a microscopic slide with methanol and stained for 30min with Giemsa diluted 1:10. The slides were then observed under a light microscope to detect the presence of Leishman bodies.

Furthermore, serous fluid from the rodents' ears was injected into Balb/C mice to monitor for the appearance of *Leishmania* infection lesions. Prepared serous fluid from infected Balb/C mice accompanied by serous fluid from scratches from each ear of rodents was cultured in Novy-MacNeal-Nicolle (NNN) medium. Subsequently, the cultures were incubated at 22°C for 6 weeks. The cultures were checked at two-day intervals until they reached the growth phase (log phase) based on observation using an inverted microscope. Positive cultures were confirmed by the presence of promastigotes, which were sub-cultured into restriction fragment length polymorphism (RFLP) medium weekly.

The harvested promastigotes from the early stationary phase (approximately 2×10^6 promastigotes/ml) and serous fluid from each ear of the rodent were injected subcutaneously into the base tail of a Balb/C mouse. Inoculated Balb/C mice were examined weekly for the appearance of lesions at the injection site for 6 months. Samples from infected Balb/C mice with cutaneous lesions were used for DNA extraction.

Molecular methods

Smears prepared from infected Balb/C mice, with serous fluid and/or cuts from each rodent ear, were kept in separate 1.5ml microtubes containing 100µl phosphate-buffered saline (PBS) and then centrifuged briefly three times at 13,000rpm. The PBS was discarded. Each rodent ear was placed in a 1.5ml microtube and placed in liquid nitrogen 3 times for 3min each. The genomic DNA of each rodent and any parasite within was extracted using the ISH-Horovize method and a GeNet BIO kit (Global Gene Network South Korea); these procedures were carried out in the systematic molecular laboratory of the Pasteur Institute in a room where amplified and cloned DNAs were never processed[11,25].

The DNA samples extracted from rodent tissues were used in polymerase chain reaction (PCR) to amplify a 624bp fragment of the cytochrome b gene (Cyt *b*) from the mitochondrial DNA to accurately identify the rodent species. We followed the protocol of Kent and Norris **(Table 1)**[26].

The internal transcribed spacer-ribosomal deoxyribonucleic acid gene was amplified for the detection of *Leishmania* infection using ITS1-5.8SrRNA-ITS2 fragments, with ITS1F as the forward primer and ITS2R4 as the reverse primer **(Table 1)**[7].

To perform RFLP analysis, the PCR products were blunt digested using endonuclease *B*suR1 *(H*aeIII) (Fermentas, Life Sciences, Germany) in the recognition site pattern GG↓CC, as recommended by the manufacturer. Enzyme selection was performed by analyzing sequences of *Leishmania* reference species with CLC DNA Workbench 5.2 software (CLC bio A/s, Aarhus, Denmark)[27].

TABLE 1 - Primer sequences and conditions used for all employed genes for the identification of *Leishmania* parasites within rodents.

Genes	Primer name	Primer sequence	Fragment size (bp)	Annealing temperature & cycle number
ITS-rDNA	ITS1F (F)	5' GCAGCTGGATCATTTTCC 3'	462	58°C, 37
	ITS2R4 (R)	5' ATATGCA GAA GAGAGG AGG C 3'		
Microsatellite DNA	ITSmF1 (F)	5´ GTGTGGAAGCCAAGAGGAGG 3´	160	58°C, 37
	ITSmR2 (R)	5´ GCAAGCACCAGAGAGGAGT 3´		
kDNA	LINR4 (F)	5′ GGGGTTGGTGTAAAATAGGG 3′	650	Semi-nested PCR: 1st step: 52°C, 17
	LIN 17 (first-step R)	5′ TTTGAACGGGATTTCTG 3′		
	LIN19 (second-step R)	5′ CAGAACGCCCCTACCCG 3′		Semi-nested PCR: 2nd step: 58°C, 33
Leishmania Cytochrome *b*	LCBF1 (F)	5′ GGTGTAGGTTTTAGTTTAGG 3′	880	50°C, 39
	LCBR2 (R)	5′ CTACAATAAACAAATCATAATATACAATT 3′		
Rodent Cytochrome *b**	UNFOR403 (F)	5′ TGAGGACAAATATCATTCTGAGG 3′	624	58°C, 35
	UNREV1025 (R)	5′ GGTTGTCCTCCTCCAATTCATGTTA 3′		

Bp: Base pairs**; ITS-rDNA:** internal transcribed spacer-ribosomal deoxyribonucleic acid**; DNA:** deoxyribonucleic acid; **kDNA:** kinetoplast deoxyribonucleic acid**;** (F): Forward primer; (R): Reverse primer; *Rodent cytochrome *b* genes were not used for the detection of *Leishmania* infection but only for rodent species identification.

The primer sets LINR4 (forward), LIN17 (first-step reverse) and LIN19 (second-step reverse) were used in the semi-nested PCR for the minicircle kDNA gene[16]. The primers anneal within the conserved area of the minicircle and are based on the conserved sequence blocks recognized by Brewster and Baker **(Table 1)**[28].

The third method used for *Leishmania* infection identification was microsatellite ITS-rDNA analysis; the protocol used in this assay was designed by Parvizi et al.[25]. The primers were ITSMF1 (forward) and ITSMR2 (reverse) **(Table 1)**.

To detect *Leishmania* infection, we also used a fragment of the cytochrome *b* gene from mitochondrial DNA, and the primers used in this amplification were LCBF1 (forward) and LCBR2 (reverse) **(Table 1)**[17].

After amplification, the DNA samples were excised, purified and sequenced using an ABI PRISM TM310 automated sequencer (Applied Biosystems, USA). The sequences obtained were edited and aligned with database sequences using Sequencher™ v. 4.4 software to identify unique sequences (= haplotypes), which were analyzed phylogenetically using MEGA5.05 software[29,30].

Ethical considerations

This study was approved by the Department of Parasitology, Pasteur Institute of Iran, Tehran, Iran.

RESULTS

In total, 68 rodents were captured in five villages in Abarkouh district **(Figure 1; Table 2)**. Thirty-three of 68 rodents were alive and transported to the Pasteur Institute of Iran, Tehran, for further studies using conventional and molecular methods. Thirty-five rodents were dead after being caught by wire and wooden live traps at sampling sites. The ears of these dead rodents were used only for molecular methods. Based on morphological characteristics and rodent molecular markers (Cyt *b* sequences), five species were identified. The most abundant rodent was *R. opimus* (55/68: 81%). The frequencies and abundances of the other rodent species were as follows: *M. libycus* (8/68: 12%), *Meriones persicus* (2/68: 3%), *Tatera indica* (2/68: 3%) and *Mus musculus* (1/68: 1%), respectively **(Table 2)**.

Eleven of 68 (16%) rodents were found to be infected with *Leishmania* parasites using molecular methods (3/68 (4.4%) using ITS-rDNA, 10/68 (14%) using microsatellites, 3/68 (4.4%) using minicircle kDNA and 4/68 (5.8%) by amplifying Cyt *b* from *Leishmania* parasites). At least 3 of 5 rodent species had *Leishmania* infections **(Figure 2)**. Only two of 33 (6.06%) live rodents were found to be *Leishmania* positive using conventional methods, including impression touch smears from

TABLE 2 - *Leishmania* infections among different rodents captured in Abarkouh district in Yazd Province, Iran, using conventional and molecular methods.

Villages / Species	Frequency of captured rodents	Harooni	Abarghasr	Khoram abad	Gonbad	Chahgir	Conventional methods: Microscopic Observation of *Leishmania* parasites	Conventional methods: BALB/C Injection	Molecular methods: *Leishmania major* (+ve) by ITS-rDNA PCR	Molecular methods: *Leishmania major* (+ve) by microsatellite PCR	Molecular methods: *Leishmania major* (+ve) by kDNA PCR	Molecular methods: *Leishmania major* (+ve) by *Leishmania* Cyt *b* PCR	Total
Rhombomys opimus	81%	0/10	0/10	1/4	8/26	0/6	1/55	1/55	2*/55	8(3*)/55	2(1*)/55	3*/55	9* / 55 (13.04%)
Meriones libycus	12%	0	0	0/1	0/3	1/4	1/8	1/8	1/8	1**/8	1**/8	1**/8	1/8 (12.5%)
Mus musculus	1%	0	0	0	0	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1 (0%)
Meriones persicus	3%	0	0	0	0/1	0/1	0/2	0/2	0/2	0/2	0/2	0/2	0/2 (0%)
Tatera indica	3%	0	0	0	0	1/2	0/2	0/2	0/2	1/2	1*/2	0/2	1/2 (50%)
Total	100%	0/10 (0%)	0/10 (0%)	1/5 (20%)	8/30 (27%)	2/14 (14%)	2/68 (2.94%)	2/68 (2.94%)	3/68 (4.4%)	10 /68 (14%)	3/68 (4.4%)	4/68 (5.8%)	11 /68 (16%)

ITS-rDNA: internal transcribed spacer-ribosomal deoxyribonucleic acid**; kDNA:** kinetoplast deoxyribonucleic acid. **+ve:** Positive sample; **PCR:** polymerase chain reaction; **Cyt *b:*** Cytochorome *b.* *3 of 13 positive samples were also tested via one additional gene and were confirmed to have *Leishmania major* infection; **The positive microscopic and BALB/C injected samples also tested positive in molecular tests (ITS-rDNA, microsatellite and kDNA gene amplification).

the ear, light microscope observation, culturing in NNN and inoculating in Balb/C mice **(Table 2)**.

The most interesting result was that despite the low number of captured rodents, five different rodent species were collected and identified. *Leishmania* infection was detected from three of these species, and for the first time, *T. indica* was captured in Abarkouh district and identified both morphologically through diagnostic keys and molecularly by sequencing of the Cyt *b* gene. In addition, one of the two *T. indica* specimens was infected with *L. major*.

To find identify additional *Leishmania* parasite infections and molecular variation rates among collected samples, different genes were employed. Standard and semi-nested PCR were used to amplify ITS-rDNA, microsatellites, kDNA and Cyt *b* genes from *Leishmania* parasites **(Figure 2)**.

All 11 *Leishmania*-positive samples were analyzed using RFLP and sequencing to definitively identify *Leishmania* species **(Figure 2)**. With RFLP, which allows for the differentiation of each species unambiguously, two fragments of 120 and 310bp belonging to *L. major* were obtained **(Figure 2)**.

All sequences from positive samples by ITS-rDNA gene were blasted and confirmed to be most similar to *L. major*, and only *L. major* with one common haplotype (GenBank accession No. EF413075) was found after direct sequencing, editing, aligning and comparing with the sequences submitted to GenBank using Sequencher TM v. 4.4 and phylogenetic analysis by MEGA5.05 software.

DISCUSSION

In our study, only the *L. major* parasite with one common haplotype (GenBank access No. EF413075) was firmly identified in three rodent species. *Leishmania* parasites have been isolated from all three species in other ZCL foci in Iran[11,15,25,31]. This is the first report of *L. major* in only a small area of ZCL focus in Abarkouh. In our current publication, we also found *L. major*

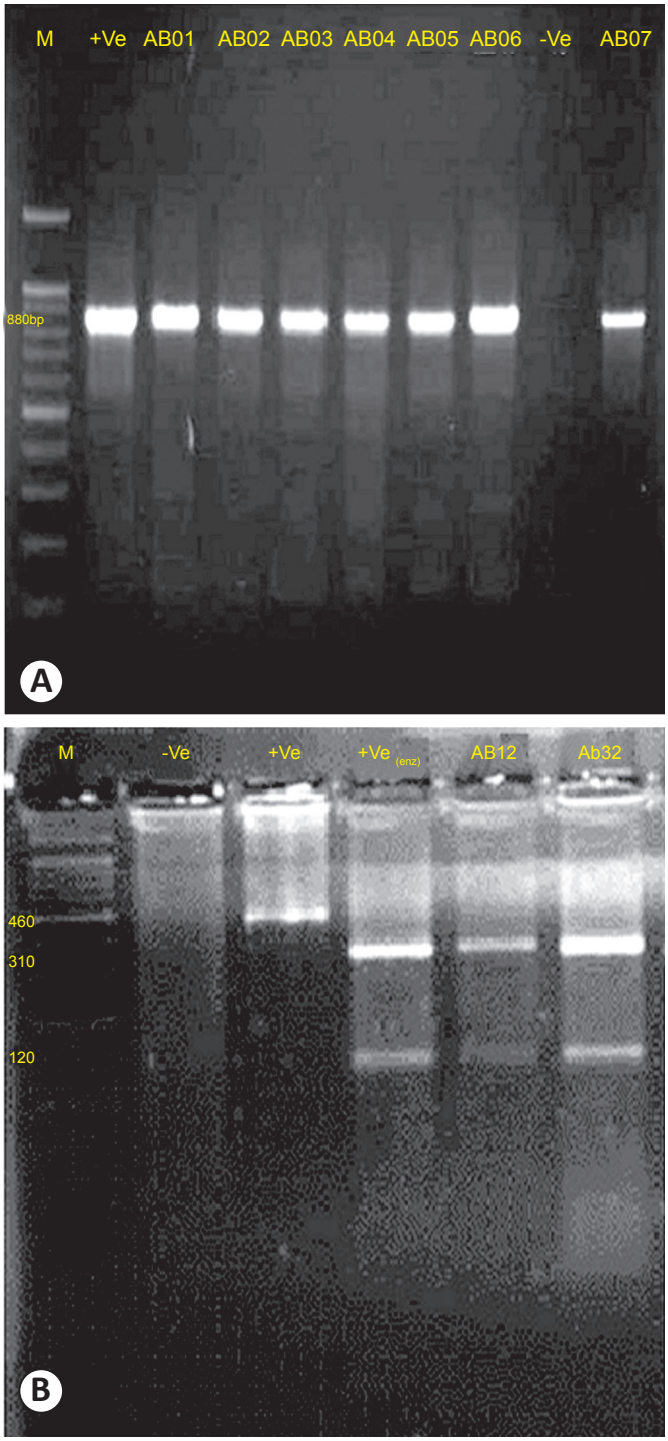

FIGURE 2 - A: Electrophoresis image of Cyt *b* gene amplification in *Leishmania* infection among different rodent species of Abarkouh district, Yazd Province, Iran. B: RFLP of ITS-rDNA gene electrophoresis image after digestion with BsuR1 (HaeIII) enzyme of PCR products using In-Silico software (CLC bio A/s, Aarhus, Denmark) of *Leishmania* infection among different rodent species of Abarkouh district, Yazd Province, Iran (Bordbar and Parvizi 2013) (+Ve contains *Leishmania major* parasite PCR product without the enzyme effect, and +Ve (enz) is a *Leishmania major* parasite PCR product with the enzyme effect). Cyt b: cytochrome *b*; **RFLP:** Restriction fragment length polymorphism; **ITS-rDNA:** internal transcribed spacer-ribosomal deoxyribonucleic acid; **PCR:** polymerase chain reaction. +ve: positive sample, enz: with enzyme.

in *P. papatasi*, a proven vector of ZCL in Iran, in the same area in Abarkouh where *L. major* was isolated in rodents[7,10,32].

Finding additional *Leishmania* infections in different rodent species compared with only one sandfly species can be explained by the fact that among sandflies, we mainly examined *P. papatasi*, and only a small number of other sandfly species were tested and found to be *Leishmania* negative, which did not provide sufficient for a precise result[10]. In addition, only *P. papatasi* is able to develop *L. major* in its midgut and transfer the parasite to salivary glands to cause ZCL[33]. However, we analyzed all the captured rodent samples from different species, and therefore, we were able to identify *Leishmania* infections in at least three rodent species. The *Leishmania* infection rate in rodents as the reservoir host of ZCL is much higher than in sandflies as vectors, and in some cases, more than 50% of the samples were found to be infected with *Leishmania* parasites[14].

Based on our experience in different ZCL foci in Iran, we expected to identify more *Leishmania* infections in reservoir hosts in Abarkouh district and to observe at least a small amount of variation in the ITS-rDNA gene of *L. major* in rodents. However, only one haplotype was found, and approximately 16% (11/68) of the tested rodents were infected with *Leishmania* parasites[11,15,25]. After sequencing, only one haplotype of *L. major*, which is also the common haplotype present in Iran, including Fars and Isfahan Provinces, was detected from Abarkouh rodent samples (GenBank accession No. EF413075).

The low density of sampled rodents as well as *Leishmania* parasites may be due to a control program of the health care authorities of Abarkouh district that uses zinc phosphate poison and the destruction of rodents' barrowers to control ZCL.

According to reports of different ZCL foci in Iran, many haplotypes of *L. major* have been identified in sandflies, rodents and humans[7,11,15,27]. The objective of the present study was to use molecular methods and different genes to identify additional *Leishmania* infections and various haplotypes; to this end, four different genes were employed to detect *Leishmania* infections in rodents and/or the numbers of haplotypes circulating in the area, but this method relies on a few sequences from all of the genes from our samples, and no variations were identified. We also used routine laboratory (conventional) methods, such as NNN cultures, microscopic observation and Balb/C mouse injection. Because most captured rodents died before being transferred to our lab, only a few live rodents were used for the conventional methods, and the infection rate was low. Only 2 infected samples were found by microscopic observation of the presence of amastigotes on slides and the appearance of a lesion after Balb/C mouse injection. Because the NNN cultures were prepared in the field and due to fungal infection in some cultures, no growth was shown in any of the cultures.

We employed five different genes during this investigation; the rodent Cyt *b* gene was amplified for accurate determination of the rodent's genus and species. For *Leishmania* infection, two mitochondrial genes (kDNA and *Leishmania* Cyt *b*) and two nuclear genes (ITS-rDNA and microsatellite ITS-rDNA) were used. In this investigation, the highest infection rate among

rodents (8/68) was detected using the microsatellite ITS-rDNA gene because of its short tandemly repeated DNA sequence fragments and because it is highly specific. A comparison of the rest of the genes demonstrated that Cyt *b* as a mitochondrial gene is more sensitive for *Leishmania* detection (4/68) because of its high copy numbers per cell; however, nuclear genes are more specific, and of those, the ITS-rDNA gene (3/68), because it is homogenous and highly conserved with few intracellular polymorphisms, a linear genome and has readable sequences, is considered a suitable gene for sequencing, genus, species, strain and/or even haplotype detection.

Our *Leishmania* infection data in rodents are similar to the results of a parallel study among sandflies and suspected patients that was carried out near the time of our investigation[10] (Parvizi P et al: unpublished data).

In previous investigations, reservoir hosts of ZCL have been distributed in different regions. *R. opimus* and *M. libycus* are dominant in the northeastern and central regions; *M. libycus* and *T. indica* in the central and southwestern regions and *T. indica* in southwestern and southern Iran[11,34]. *R. opimus* and *M. libycus* have previously been found to be infected with *L. major* parasites from Golestan and Isfahan Provinces[11,14,15,25]. In addition, *T. indica* was found to be infected with *L. major* in Fars Province, Iran[31]. Interestingly, we were able to identify *L. major* infections in all three of these rodents within Abarkouh district of Yazd Province in central Iran.

In this survey, *T. indica* was captured for the first time in Abarkouh district; the existence of this rodent in the area may be explained by the fact that Abarkouh neighbors Fars Province, which is a known habitat for *T. indica*[31], and the rodents can be transported and/or migrate to Abarkouh from Fars and vice versa. The simultaneous existence of *T. indica* along with *R. opimus* and *M. libycus* as main and potential reservoir hosts of ZCL in Abarkouh district and the fact that Abarkouh has been largely neglected as an important ZCL focus gives this district an important role in the ZCL life cycle, epidemiology, prognosis and disease-control programs.

Leishmania major was firmly identified in *R. opimus*, *M. libycus* and *T. indica*, which indicates that at least these three rodent species can be incriminated as reservoir hosts of ZCL in this location. *R. opimus* was abundant and had a greater rate of *L. major* infection and should be incriminated as the main reservoir host of ZCL[35].

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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