Published in final edited form as:
Exp Parasitol. 2010 December ; 126(4): 552–556. doi:10.1016/j.exppara.2010.06.003.

Leishmania species: Detection and identification by nested PCR assay from skin samples of rodent reservoirs

Amir Ahmad Akhavan[a], **Hossein Mirhendi**[b], **Ali Khamesipour**[c], **Mohammad Hossein Alimohammadian**[d], **Yavar Rassi**[a], **Paul Bates**[e], **Shaden Kamhawi**[f], **Jesus G. Valenzuela**[f], **Mohammad Hossein Arandian**[g], **Hamid Abdoli**[g], **Niloufar Jalali-zand**[g], **Reza Jafari**[g], **Niloufar Shareghi**[g], **Maryam Ghanei**[g], and **Mohammad Reza Yaghoobi-Ershadi**[a,*]

[a] Department of Medical Entomology and Vector Control, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

[b] Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

[c] Center for Research and Training in Skin Diseases and Leprosy, Tehran University of Medical Sciences, Tehran, Iran

[d] Department of Immunology, Pasteur Institute of Iran, Tehran, Iran

[e] Division of Biomedical and Life Sciences, School of Health and Medicine, Lancaster University, Lancaster, UK

[f] Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institute of Health, Rockville, MD 20852, USA

[g] Esfahan Health Training and Research Center, Institute Public of Health Researches, Tehran University of Medical Sciences, Tehran, Iran

Abstract

Many rodent species act as reservoir hosts of zoonotic cutaneous leishmaniasis in endemic areas. In the present study a simple and reliable assay based on nested PCR was developed for the detection and identification of *Leishmania* parasites from rodent skin samples. We designed *Leishmania*-specific primers that successfully amplified ITS regions of *Leishmania major*, *Leishmania gerbilli* and *Leishmania turanica* using nested PCR. Out of 95 field collected *Rhombomys opimus*, 21 were positive by microscopic examination and 48 by nested PCR. The percentage of gerbils infected with *L. major*, *L. gerbilli* and *L. turanica* was 3.2%, 1.1% and 27.4%, respectively. In 15.8% of the rodents, we found mixed natural infections by *L. major* and *L. turanica*, 1.1% by *L. major* and *L. gerbilli*, and 2.1% by the three species. We concluded that this method is simple and reliable for detecting and identifying *Leishmania* species circulating in rodent populations.

Keywords

Leishmania major; *Leishmania gerbilli*; *Leishmania turanica*; Rodent; *Rhombomys opimus*; Cutaneous leishmaniasis; Nested PCR; Iran

*Corresponding author. Fax: +98 2188951393. yaghoobi.reza@gmail.com (M.R. Yaghoobi-Ershadi).

1. Introduction

Cutaneous leishmaniasis due to *Leishmania major* (CLM) is an increasing disease and a public health problem in many regions of the Old World. *L. major* is widely distributed in various rodent populations in arid and savannah regions (Gramiccia and Gradoni, 2005). Rodents belonging to the subfamily Gerbillinae are the main reservoir hosts of cutaneous leishmaniasis in Iran and other countries where CLM is endemic (Dubrovsky, 1979; Strelkova, 1996; Yaghoobi-Ershadi et al., 1996). Gerbils are the most abundant mammals in natural ecosystems of Old World deserts (Dubrovsky, 1979). Many rodent species act as reservoir hosts of CLM: *Rhombomys opimus* (great gerbil) in Central Asia, Northern Afghanistan and Iran; *Meriones libycus* (Libyan jird) in the Arabian Peninsula, Central Asia and Iran; *Meriones hurrianae* (Indian desert jird) in India and Iran; *Psammomys obesus* (fat sand rat) and *Meriones crassus* in Northern Africa and Middle East; *Ratus ratus* and *Arvicanthis niloticus* in Sudan (Abdalla et al., 2003) and *Tatera* spp. in subsaharan Africa and Iran (Gramiccia and Gradoni, 2005). *R. opimus* (Cricetidae: Gerbillinae) is the principal reservoir host of *L. major* over the vast territory of the Turan lowland (west and south Kazakhstan and Central Asia with adjacent parts in Afghanistan and Iran), Mongolia, and apparently, in some provinces of China. In the Turan lowland, naturally infected *R. opimus* were found in more than 200 places from where they were investigated and showed a higher infection rate than any other mammal (other rodents, insectivores, carnivores) investigated (Dubrovsky, 1979). All the proven sand fly vectors of CLM belong to the subgenus *Phlebotomus*, including *Phlebotomus papatasi*, the principal vector, and related species *Phlebotomus salehi* and *Phlebotomus duboscqi*. Well-described stable zoonotic cutaneous leishmaniasis (ZCL) foci are associated with *L. major* and *P. in North Africa and the Middle East, and with R. in central Asia, Afghanistan and Iran (*Gramiccia and Gradoni, 2005; Yaghoobi-Ershadi et al., 2003; Parvizi et al., 2005).

The geographic distribution and role of rodents as reservoir hosts of CLM in Iran are well known and *R. opimus* is considered the most important in Central and North East Iran (Yaghoobi-Ershadi and Javadian, 1996; Yaghoobi-Ershadi et al., 1996). The main foci of CLM in Iran are located in Esfahan Province, Central Iran, where the current study was carried out. Only single infection of *L. major* or *Leishmania turanica* has been detected and identified from rodents in Iran yet (Yaghoobi-Ershadi et al., 1996, 2004; Mohebali et al., 2004; Parvizi et al., 2008; Hajjaran et al., 2009). Recently, *L. major*, *L. turanica* and *Leishmania gerbilli* sensu lato were detected in sand flies in the northeast and centre of Iran (Parvizi and Ready, 2008).

One of the major obstacles for the control and understanding of this neglected disease is the detection and identification of *Leishmania* parasites in animal reservoirs. *L. major* infection is usually accompanied by non-pathogenic (sub-clinical) *L. turanic* or *L. gerbilli* (Strelkova, 1996; Strelkova et al., 2001) which are microscopy-confounding. Traditional techniques (direct examination and culture) commonly used for diagnosing leishmaniasis do not differentiate *Leishmania* species (Ben-Ismail et al., 1992; Shahbazi et al., 2008). Gold-standard isoenzyme characterization requires large scale parasite culture without contamination (Evans, 1989) and also it is possible that infections of *Leishmania* parasites will be missed due to disparate growth rates of different parasites in blood agar cultures (Ibrahim et al., 1994; Abdalla et al., 2003).

Nested PCR provides a rapid, sensitive, and specific alternative to traditional techniques. Moreover, diagnosis of *Leishmania* infection and species identification is done simultaneously. Here, we have developed a simple and reliable method to detect and identify *Leishmania* species from skin samples from naturally infected great gerbils using a nested PCR method and identified the parasites circulating in these animal reservoirs.

2. Materials and methods

2.1. Gerbil collection

The investigation was conducted over a period of 24 months from October 2006 to October 2008 in three rural districts (Borkhar, Sejzi and Badrood) of Esfahan Province, Central Iran where CLM is endemic. Active colonies of gerbils were identified and caught using 20–45 Sherman traps baited with cucumber. The trapped gerbils were transferred to the animal house facility at the Esfahan Health Research and Training Center, Institute of Public Health, Esfahan, Iran, and maintained for parasitological and molecular testing. The captured rodents were identified by morphological characters (Etemad, 1978) and only the great gerbils, *R. opimus*, were selected for the study.

2.2. Direct parasitological test

In the laboratory, the rodents were anaesthetized using intramuscular Ketamine hydrochloride (60 mg/kg) and Xylazine (5 mg/kg). Regardless of the presence of lesions, impression smears were prepared from the ear lobes of the animals (Edrissian et al., 1982) and stained by Giemsa. Samples were examined under the light microscope (1000×) for detection of *Leishmania* amastigotes.

After preparing direct smears, ear lobe samples were removed from anesthetized rodents and transferred to 500 μl of cold PBS (pH 7.4), thoroughly disrupted by grinding with a pestle and kept at −20 °C until use. The animals were nursed to recovery after these procedures. Animal procedures were approved by the Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran.

2.3. Polymerase chain reaction (PCR) assay

2.3.1. Deoxyribonucleic acid (DNA) extraction—Genomic DNA was extracted and purified using a conventional phenol–chloroform protocol (Sambrook and Russel, 2001) with slight modification. Briefly, a 100 μl of each disrupted tissue sample was transferred to a 1.5 ml microtube containing 200 μl of lysis buffer (100 mM Tris–HCl, pH 8; 10 mM EDTA, pH 8; 1% SDS; 100 mM NaCl; 2% Triton X-100) containing proteinase K (100 mg/ml), vortexed, and incubated at 56 °C for 1 h. Three hundred microliters of phenol–chloroform (1:1) were added, vortexed and centrifuged and chloroform extraction was performed again. An equal volume of isopropanol and 1/10 volume of 3 M sodium acetate (pH 5.2) were added to the supernatant and centrifuged for 15 min at 5000*g* and the precipitant washed with 70% ethanol and centrifuged for 5 min at 800*g*. The pellet was air dried and resuspended in 20 μl of distilled water and stored at −20 °C until use.

2.3.2. Primers design—To design universal primers for detecting and identifying the common *Leishmania* species found in gerbils (*L. major*, *L. gerbilli* and *L. turanica*) we focused on ribosomal DNA using GenBank sequences of internal transcribed spacer ITS2 (Table 1). The sequences from each species were aligned and compared using DNASIS software (Hitachi Software Engineering Co. Tokyo). The external primers, Leish out F (5′-AAA CTC CTC TCT GGT GCT TGC-3′) and Leish out R (5′-AAA CAA AGG TTG TCG GGG G-3′), and internal primers, Leish in F (5′-AAT TCA ACT TCG CGT TGG CC-3′) and Leish in R (5′-CCT CTC TTT TTT CTC TGT GC-3′) were selected to distinguish among the parasite species in a nested PCR system. Predicted PCR products are shown in Table 1.

2.3.3. PCR conditions—PCR amplification was carried out in an Applied Biosystems thermocycler. The initial PCR contained 0.6 μM of each forward (Leish out F) and reverse (Leish out R) external primers, 12.5 μl Taq DNA polymerase, 2X Master Mix Red (Amplicon, Denmark) and sterile distilled water to a final volume of 25 μl. An initial denaturation step at

95 °C for 5 min was followed by 30 cycles of: denaturation at 94 °C for 30 s, annealing at 60 °C for 45 s and extension at 72 °C for 1 min, with a final extension step of 72 °C for 5 min. The first round reactions were performed in duplicate with 1 and ½ μl volumes of DNA template. The second-round (nested) PCR was performed in a final volume of 20 μl containing 1 μl of a 1:10 dilution in distilled water of the first-round PCR product as template, 0.3 μM of each forward (Leish in F) and reverse (Leish in R) internal primers, 10 μl of Taq DNA polymerase and 2X Master mix Red. The reactions were cycled under the following conditions: 95 °C for 2 min, 25 cycles of 94 °C for 15 s, 62 °C for 30 s, 72 °C for 45 s followed by 72 °C for 5 min. PCR products were separated by 1.5% (w/v) agarose gel electrophoresis in TBE buffer (0.09 mM Tris, 0.09 mM boric acid and 20 mM EDTA, pH 8.3), visualized with ethidium bromide (0.5 μg/ml) and photographed.

Reference strains *L. major* (MRHO/IR/75/ER), *L. gerbilli* (MRHO/CN/60/GERBILLI) and *L. turanica* (MRHO/SU/1983/MARZ-051) were used as positive controls; samples of normal dermal tissues of naive gerbil (bred under laboratory conditions, without any exposure to *Leishmania*) and/or distilled water were used as negative controls. The PCR product of the negative control of the first-round PCR was used as the negative control in the second round and also the PCR product of the positive control of the first-round PCR was used as positive control in the second round. Necessary precautions such as adding mineral oil on the master mix, using filter pipette tips, sterilizing equipments with 10% sodium hypochlorite solution to avoid cross-contamination were taken.

3. Results

In the present study a simple and reliable assay based on nested PCR was developed for the detection and identification of *Leishmania* species involved in rodent leishmaniasis. The designed *Leishmania*-specific external and internal primers of the nested PCR successfully amplified the ITS2 region of the reference strains *L. major*, *L. gerbilli* and *L. turanica*, generating a single major product with a distinct size for each species (Fig. 1). The primers could detect and identify all three species by fragment size polymorphisms in both rounds of PCR but the sensitivity of the second round was augmented significantly (Table 2). The nested PCR assay tested on normal skin tissues of a naive gerbil and standard *Leishmania* strains showed no false negatives or false positives, respectively.

Specimens from 95 *R. opimus*, captured from CLM foci, were examined by two diagnostic techniques, direct (microscopic) examination and nested PCR. Out of 95 specimens, 21 specimens were positive by microscopic examination and 19 were positive by first-round PCR using external primers. Importantly, 48 specimens were positive after further testing with nested PCR (Table 2). Furthermore, compared to only 12 (57.1%) samples detected by the first-round PCR (of the 21 specimens positive by direct examination), all 21 (100%) specimens were positive by nested PCR. Out of 74 specimens negative by direct examination, 9.5% were positive by first-round PCR and 36.5% by nested PCR (Table 2). Out the 95 investigated gerbils, 3.2% were infected with *L. major*, 1.1% with *L. gerbilli* and 27.4% with *L. turanica*. We also found mixed natural infections with *L. major* and *L. turanica* in 15.8% of the rodents; *L. major* and *L. gerbilli* in 1.1% and 2.1% were infected with all three species. 94.6 of the infected gerbils showed no cutaneous leishmaniasis lesion on their ear lobes.

4. Discussion

Traditional techniques (direct examination and culture) commonly used for diagnosing leishmaniasis do not differentiate *Leishmania* species and their sensitivity is also less than molecular techniques (Ben-Ismail et al., 1992; Faber et al., 2003; Shahbazi et al., 2008). It is possible that infections of *Leishmania* parasites will be missed due to disparate growth rates

of different parasites in blood agar cultures (Ibrahim et al., 1994). Nested PCR provides a rapid, sensitive, and specific alternative to traditional techniques.

Moreover, diagnosis of *Leishmania* infection and species identification is done simultaneously. In this study a nested PCR method was successfully developed and applied for the detection and identification of rodent *Leishmania* infections commonly find in Iran and central Asia. Our results indicate that this method is sensitive and specifically distinguishes among *L. major*, *L. gerbilli* and *L. turanica*, the three most common parasites of *R. opimus*, the principal reservoir of *L. major* in Iran. The designed *Leishmania*-specific primers tested on DNA from pure standard strains of *L. major*, *L. gerbilli* and *L. turanica* generated a single major product different in size for each of the three parasite species. The size of these ITS2 ribosomal gene products corresponded to those predicted from GenBank sequence data. The second round PCR was much more sensitive than the first round. Both first and second primer pairs have been designed to identify all tested *Leishmania* species, therefore it is expected having more positive samples in the second round PCR than the first one.

Detection of *Leishmania* infection in over 50% (Table 2) of skin samples from field collected rodents attests to the sensitivity of the developed nested PCR. The specificity, sensitivity and rapid unambiguous distinction of mixed infections by nested PCR justifies its use as a diagnostic test for the three investigated *Leishmania* species.

The results of the current study show that *L. major*, *L. gerbilli* and *L. turanica* are circulating in *R. opimus* populations from Central Iran. It seems that there is some variation among *L. major* strains. The three parasite species *L. major*, *L. gerbilli* and *L. turanica* have been identified in naturally infected gerbils from Turkmenistan, Uzbekistan and Kazakhstan (Strelkova, 1996; Strelkova et al., 2001). *L. turanica* was reported as the dominant species in *R. opimus* populations in hypoendemic, mesoendemic and hyperendemic foci of ZCL in Turkmenistan and Uzbekistan (Strelkova et al., 2001). In vast territories of Central Asia, mixed infections of wild rodents with *L. major* and *L. turanica* are typical (Strelkova, 1996). In our study areas, *L. turanica* was the dominant species in *R. opimus* populations. We rarely found *R. opimus* infected with *L. major* or *L. gerbilli* alone and in most cases *L. major* infection was accompanied by *L. turanica*. In addition, this is the first report to our knowledge of a mixed natural co-infection with *L. major*, *L. turanica* and *L. gerbilli* in wild *R. opimus* populations from Iran or elsewhere.

In most of the previous studies conducted in Iran, only *L. major* has been isolated from great gerbils and characterized by using isoenzymes or DNA-based molecular techniques (Yaghoobi-Ershadi et al., 2003; Mohebali et al., 2004; Parvizi et al., 2008; Rassi et al., 2008). There are rare reports of *L. turanica* infection in rodents in Iran (Mohebali et al., 2004; Yaghoobi-Ershadi et al., 2004; Hajjaran et al., 2009). Identification of *Leishmania* species have mostly been based on culture and usually only one species of *Leishmania* is detected and identified by each examination (Yaghoobi-Ershadi et al., 2003; Mohebali et al., 2004; Hatam et al., 2005; Hajjaran et al., 2009), As the method presented in this study is not based on culture, it is simpler as well as more sensitive for detecting and identifying *Leishmania* parasites from skin samples in rodents. Gerbils infected by *L. major* are important in the transmission cycle of CLM (Gramiccia and Gradoni, 2005; Strelkova, 1996; Yaghoobi-Ershadi et al., 2003) and the distribution of *L. major*, the causative agent of ZCL in Central Asia, has been found to coincident with that of *R. opimus* (Strelkova, 1996). Therefore, it is important to accurately assess the rate of *L. major* infection in *R. opimus* and other important reservoirs.

In CLM foci where *L. major*, *L. gerbilli* and *L. turanica* circulate in the reservoir population of *R. opimus* and where *L. major–L. turanica* co-infections are common, the nested PCR method described in the current study is an reliable assay to distinguish the three species of

Leishmania and improve our estimates of reservoir infection rates with *L. major*, the human infecting species.

Acknowledgments

This research was supported by Research deputy of Tehran University of Medical Sciences, Project No. 3025. We are very grateful to Dr. P. Parvizi, Pasteur Institute of Iran, who provides us with the standard strains of *L. turanica* and *L. gerbilli* and also Mr. D. Iravani and Ms. M. Vaziri, Pasteur Institute of Iran for their technical assistance in this project. We also thank Dr. M. Jeddi-Tehrani, Avicenna Research Institute, ACECR, for his valuable comment on the molecular experiments.

Sincere thanks are also extended to head and staff of Esfahan Research and Training Center, Institute of Public Health Research for their assistance in the project. We are also grateful to Mr. E. Eskandar and Ms. A. Mirmohammadi, Center for Research and Training in Skin Diseases and Leprosy for their kind helps.

References

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.

Ben-Ismail R, Smith DF, Ready PD, Ayadi A, Gramiccia M, Ben-Osman A, Ben-Rachid MS. Sporadic cutaneous leishmaniasis in north Tunisia: identification of the causative agent as *Leishmania infantum* by the use of a diagnostic deoxyribonucleic acid prob. Transactions of the Royal Society of Tropical Medicine and Hygiene 1992;86:508–510. [PubMed: 1475816]

Dubrovsky, Y. WHO Traveling Seminar on Leishmaniasis Control. Ministry of Health; Moscow: 1979. Biology of Great Gerbil – The Principal Carrier of the Great of Zoonotic Cutaneous Leishmaniasis.

Edrissian, Gh; Zovein, Z.; Nadim, A. A simple technique for presentation of smears from the ear of *Rhombomys opimus* for the detection of leishmanial infection. Transactions of the Royal Society of Tropical Medicine and Hygiene 1982;76:706–707. [PubMed: 7179430]

Etemad, E. Mammals of Iran Rodents and Their Identification Keys. Vol. 1. National society of Guardian ship of Natural Resources and Human Environment; Tehran: 1978.

Evans, D. UNDP World Bank/WHO Special Programme for Research and Training in Tropical Diseases. World Health Organization; Geneva: 1989.

Faber WR, Oskam L, Van Gool T, Kroon NCM, Knegt-Junk KJ, Hofwegen H, Van Der Wal AC, Kager PA. Value of diagnostic techniques for cutaneous leishmaniasis. Journal of the American Academy of Dermatology 2003;49 (1):70–74. [PubMed: 12833011]

Gramiccia M, Gradoni L. The current status of zoonotic leishmaniases and approaches to disease control. International Journal for Parasitology 2005;35:1169–1180. [PubMed: 16162348]

Hajjaran H, Mohebali M, Alimoradi S, Abaei MR, Edrissian GhH. Isolation and characterization of pathogenic *Leishmania turanica* from *Nesokia indica* (Rodentia, Muridae) by PCR-RFLP and ITS1 sequencing in Iran. Transactions of the Royal Society of Tropical Medicine and Hygiene 2009;103:1177–1179. [PubMed: 18829057]

Hatam GR, Riyad M, Bichichi M, Hejazi SH, Guessous-Idrissi N, Ardehali S. Isoenzyme characterization of Iranian *Leishmania* isolates from cutaneous leishmaniasis. Iranian Journal of Science and Technology, Transaction A 2005;29:65–70.

Ibrahim ME, Smith AJ, Ali MH, Barker DC, Kharazmi A. The polymerase chain reaction can reveal the occurrence of naturally mixed infections with *Leishmania* parasites. Acta Tropica 1994;57:327–332. [PubMed: 7810388]

Mohebali M, Javadian E, Yaghoobi-Ershadi MR, Akhavan AA, Hajjaran H, Abaei MR. Characterization of leishmania infection in rodents from endemic areas of Islamic Republic of Iran. Eastern Mediterranean Health Journal 2004;10:591–599. [PubMed: 16335651]

Parvizi P, Mauricio I, Aransay AM, Miles MA, Ready PD. First detection of *Leishmania major* in peridomestic *Phlebotomus papatasi* from Isfahan province, Iran: comparison of nested PCR of nuclear ITS ribosomal DNA and semi-nested PCR of minicircle kinetoplast DNA. Acta Tropica 2005;93:75–83. [PubMed: 15589800]

Parvizi P, Ready PD. Nested PCRs and sequencing of nuclear ITS-rDNA fragments detects three *Leishmania* species of gerbils in sandflies from Iranian foci of zoonotic cutaneous leishmaniasis. Tropical Medicine and International Health 2008;13:1159–1171. [PubMed: 18631311]

Parvizi P, Moradi Gh, Akbari Gh, Farahmand M, Ready PD, Piazak N, Assmar M, Amirkhani A. PCR detection and sequencing of parasite ITS-rDNA gene from reservoir host of zoonotic cutaneous leishmaniasis in central Iran. Parasitology Research 2008;103:1289–1295. [PubMed: 18677623]

Rassi Y, Abai MR, Javadian E, Rafizadeh S, Imamian H, Mohebali M, Fateh M, Hajjaran H, Ismaili M. Molecular study on vectors and reservoir hosts of zoonotic cutaneous leishmaniasis in Central of Iran. Bulletin de la Societe de Pathologie Exotique 2008;101:425–428. [PubMed: 19192615]

Sambrook, J.; Russel, DW. Molecular Cloning: A Laboratory Manual. 3. Cold Spring Harbor Cold Spring Harbor Laboratory Press; New York: 2001.

Shahbazi F, Shahabi S, Kazemi B, Mohebali M, Abadi AR, Zare Z. Evaluation of PCR assay in diagnosis and identification of cutaneous leishmaniasis: a comparison with the parasitological methods. Parasitology Research 2008;103:1159–1162. [PubMed: 18651180]

Strelkova MV. Progress in studies on Central Asian foci of zoonotic cutaneous leishmaniasis a review. Folia Parasitologica 1996;43:1–6. [PubMed: 8682405]

Strelkova MV, Eliseev LN, Ponirovsky EN, Dergacheva TL, Annacharyeva DK, Erokhin PI, Evans DA. Mixed leishmanal infection in *Rhombomys opimus*: a key to the persistence of *Leishmanha major* from one transmission season to the next. Annals of Tropical Medicine and Parasitology 2001;95:811–819. [PubMed: 11784435]

Yaghoobi-Ershadi MR, Javadian E. Epidemiological study of reservoir hosts in an endemic area of zoonotic cutaneous leishmaniasis in Iran. Bulletin of the World Health Organization 1996;74:587–590. [PubMed: 9060218]

Yaghoobi-Ershadi MR, Akhavan AA, Mohebali M. *Meriones libycus* and *Rhombomys opimus* (Rodentia:gerbillidae) are the main reservoir hosts in a new focus of zoonotic cutaneous leishmaniasis in Iran. Transactions of the Royal Society of Tropical Medicine and Hygiene 1996;90:503–504. [PubMed: 8944255]

Yaghoobi-Ershadi MR, Akhavan AA, Zahraei-Ramazani AR, Abai MR, Ebrahimi B, Vafaei-Nezhad R, Hanafi-Bojd AA, Jafari R. Epidemiological study in a new focus of cutaneous leishmaniasis in the Islamic Republic of Iran. Eastern Mediterranean Health Journal 2003;9:816–826. [PubMed: 15748078]

Yaghoobi-Ershadi MR, Akhavan AA, Zahraei-Ramazani AR, Abai MR, Ebrahimi B, Vafaei-Nezhad R, Hanafi-Bojd AA, Jafari R. Letter to editor. Eastern Mediterranean Health Journal 2004;10:1.

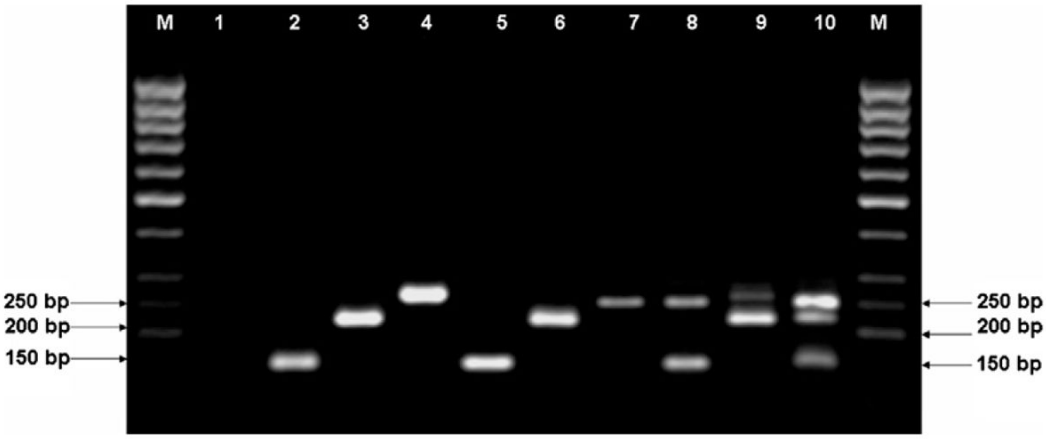

Fig. 1.

Agarose (1.5%) gel electrophoresis of nested PCR products. M, 50 bp ladder (Fermentas); lane 1, negative control (distilled water); lane 2, *L. turanica*; lane 3, *L. gerbilli*; lane 4, *L. major*; lanes 2–4 are reference strains; lane 5, *L. turanica*; lane 6, *L. gerbilli*; lane 7, *L. major*; lane 8, mixed infection of *L. major* and *L. turanica*; lane 9, mixed infection of *L. major* and *L. gerbilli*; lane 10, mixed infection of *L. major*, *L. gerbilli* and *L. turanica*. Lanes 5–10 are from skin samples of *Rhombomys opimus* rodents (field samples).

Table 1

Expected size (bp) of products following first round and second round PCR using GenBank sequences of internal transcribed spacer ITS2.

Leishmania species	ITS2 products with external primers	Predicted size of ITS2 products with internal primers	Genbank accession numbers of *L. major*, *L. turanica* and *L. gerbilli*
L. major	483	245 or 233*	FJ753394, FJ753393, FJ753392, FJ753391, DQ300195, AJ786166, AJ786165, AJ786164, AJ786163, AY260965, AJ300481, AJ272383, AJ000310
L. gerbilli	441	206	AJ300486
L. turanica	399	141	AJ272382, AJ272381, AJ272380, AJ272379, AJ272378, AJ000309, AJ000308, AJ000307

*This pattern was not observed in our standard strains or in samples from the gerbils.

Table 2

Detection of *Leishmania* parasites from rodent skin samples by direct examination, first-round PCR and nested PCR.

Diagnosis	No. of positive samples/no. of examined samples	% Positive
Clinical examination		
Direct examination	21/95	22.1
First-round PCR for samples positive by direct examination	12/21	57.1
First-round PCR for samples negative by direct examination	7/74	9.5
First-round PCR for all samples	19/95	20
Nested PCR for samples positive by direct examination	21/21	100
Nested PCR for samples negative by direct examination	27/74	36.5
Nested PCR for all samples	48/95	50.5