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**Original Article**

# Identification of *Leishmania* Species Isolated from Human Cutaneous Leishmaniasis in Mehran, Western Iran Using Nested PCR

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*Received* 25 Jul 2015
*Accepted* 18 Sep 2015

***Keywords:***
Cutaneous leishmaniasis, Nested PCR, *Leishmania* parasites, Iran

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***Abstract***

***Background:*** The incidence of cutaneous leishmaniasis in the city of Mehran has risen sharply in recent years because the city borders Iraq, which has allowed entrance of different *Leishmania* strains. These strains have different shapes, periods of disease, and healing of lesions. The present study identified and determined cutaneous leishmaniasis species in this region.

**Methods**: This cross-sectional study was carried out by preparing slides from 92 patients with suspected cutaneous leishmaniasis lesions from Mehran during 2012-2013. Parasite genomic DNA was extracted and CSB2XF and CSB1XR primers were used to amplify the *Leishmania* minicircle kDNA regions. The parasite species were detected by specific 13Z and LIR primers by applying nested PCR technique.

**Results**: All banding patterns were diagnosed as *L. major* parasite by comparison of standard models with amplified fragments 560 bp in length from bands. The patients were 56.5% male and 43.5% female. The most frequently-infected age group was the 21-30 years group at a rate of 27.2%. About 56.3% of patients had a single lesion and a significant correlation was observed between age and number of lesions ($P > 0.05$).

**Conclusion**: The nested PCR technique was shown to be an effective method with high sensitivity and specificity for identification of human *Leishmania* parasites. Molecular analysis revealed that parasites isolated from Mehran were identified as *L. major* and the disease was rural in form.

## Introduction

Leishmaniasis is a very common parasitic disease in tropical and subtropical regions of the world. It has a wide range of clinical symptoms and is caused by a group of intracellular parasites of the genus *Leishmania* (1-2). The prominent characteristics of the disease include improved skin ulcers to fatal visceral forms (2, 3). The WHO considers the disease to be one of the most serious parasitic diseases globally (4-5). Leishmaniasis is classified as one of the top three parasitic diseases by the World Tropical Diseases Research Center (African trypanosomiasis, dengue fever, and leishmaniasis) (6). *Leishmania* infections have currently been reported by 98 countries in Asia, Europe, Africa, and America. Leishmaniasis is an endemic disease that has gradually increased in incidence in Iran (7) and is now is endemic to 15 provinces (8). The Disease Control Center reports that about 20,000 cases with different types of leishmaniasis occur annually in Iran. Undoubtedly, the actual number of cases is 4 to 5 times greater; about 80% of cases are detected as rural cutaneous leishmaniasis (9).

The high incidence of the disease in recent decades has imposed a considerable health and financial burden on the society. The presence of disease vectors and reservoirs throughout Iran mean that it has been reported throughout the country. Formerly, cutaneous leishmaniasis existed sporadically in specific areas. Today, it is endemic and has spread to disease-free regions (10). The existence of disease vectors in most regions of the country means that there is a risk of the disease becoming endemic in free regions.

Ilam Province is an endemic focus of leishmaniasis in Iran (11). The disease was introduced as a major health problem among soldiers and the military during the first Iran-Iraq war (1981-1983) (11, 12). Several foci have been identified for cutaneous leishmaniasis in Ilam Province (13), which had previously been limited to some extent. Demographic and environmental changes caused by war, instability in neighboring countries (especially Iraq), malnutrition, and possible immunodeficiency of patients have distorted the epidemiological aspects of the disease. These issues have increased the focus of the disease so that the cities of Mehran and Dehloran now face problematic health concerns (13).

Methods of combating this disease vary according to the biological characteristics of the parasite, its reservoirs, and vectors. Identifying different species and strains of parasite is essential to understanding the epidemiology and for treatment and control programs (10, 12). Parasitological methods only recognize parasite contamination; however; it is not possible to identify the exact species of parasite involved (14). The kDNA minicircle is one of the best parts of the parasite genome for sequencing to identify different *Leishmania* species (15).

A high incidence of cutaneous leishmaniasis in recent years in the city of Mehran and the war-torn border regions of Iraq (16-17), signifies the entrance of *Leishmania* strains exhibiting diverse behavior. The differences in shape and lesion type have prompted the design of the current study for detection and identification of the *Leishmania* species in Mehran using the nested PCR method.

## Materials and Methods

### *Collection, preparation, and microscopic specimen examination*

This cross-sectional study was carried out from August 2012 to August 2013 using stained slides from the isolated lesions of 92 patients with suspected cutaneous leishmaniasis. Two direct smears were prepared from each lesion and stained with Giemsa stain for laboratory purposes. Unstained smears were used for DNA extraction and molecular analy-

sis. Positive smears were searched and graded according to WHO laboratory methods under microscopic magnification (1000×) for *Leishmania* bodies both inside and outside the macrophages (18) as:

- 1-10 parasites/field means 4+
- 1-10 parasites/10 fields means 3+
- 1-10 parasites/100 fields means 2+
- 1-10 parasites/1000 fields means 1+

### DNA extraction

After cleaning the slides surfaces using xylol, they were covered with 250 µl lysis buffer to lyse the cells. Using a #12 scalpel blade, the slide contents were transferred completely and gently into 1.5 ml sterile Eppendorf microtubes (19) and washed three times with 200 µl sterile PBS. The microtubes were centrifuged at 8000 rpm for 5-10 min. DNA extraction was performed on the 92 isolate smears using Takapoozist Dynabio Blood/Tissue DNA Extraction Mini Kit according manufacturer instructions. The extracted DNA was preserved at -20 °C until PCR testing (20).

### Leishmania *reference strain*

Three standard isolated DNA references were collected from Pasteur Institute of Iran and examined along with clinical isolates of *L. major* (MHOM/IR/75/ER) and *L. tropica* (MHOM/IR/IR/99).

### Nested PCR

Nested PCR is a fast and reliable two-step PCR technique for product verification (21). The specific external CSB2XF primers (5′-ATTTTTCGCGATTTTCGCAGAAACG-3) and CSB1XR (5′-CGAGTAGCAGAAACTCCCGTTCA-3′) were used initially. In the second stage, specific internal 13Z primers (5′-ACTGGGGG-TTGGTGTAAAATAG-3′) and LiR (5′-TCGCA-GAACGCCCCT-3′) were applied (22). The specificity and sensitivity of this method is reported to be 92% and 100%, respectively (22). These primers were able to track and multiply the variable part of all forms of the *Leishmania* kDNA minicircle. Amplified fragments of *L. infantum* and *L. donovani* were 680 bp in length and fragments of *L. tropica* and *L. major* were 750 and 560 bp in length, respectively (22, 23).

Lyophilized premixes (PCR Premix; Bio-Neer; Korea) were applied. The prepared lyophilized premix volumes contained $MgCl_2$, KCL, Tris-HCL (pH 9.0), Taq DNA polymerase, and dNTP were adjusted to 2 µl. In addition, 1 µl of the first stage of each initial CSB1XR and CSB2XF primers at concentrations of 10 pmol (Bioneer; Korea) and 3 µl of genomic DNA were added to the complex. Finally, 13 µl of deionized water ($ddH_2O$) were added for a total volume of 20 µl for reaction. After pouring the material into the premixes, the contents were well vortexed and spin down for a few seconds. The container was then placed into a thermal cycler device (Eppendorf; Germany) and the following program was applied to amplify the purified DNA: an initial denaturation at 94 °C for 5 min, followed by 30 cycles each consisting of three steps: 30 s at 94 °C (denaturation), 60 s at 55 °C (annealing) and 90 s at 72 °C (extension). After the last cycle, the extension step was continued for a further 10 min, and then the reaction was held at 4 °C.

In the second step of PCR, the first stage product was diluted at a 1:10 ratio with sterile distilled water. In contrast to the previous step, the PCR product was used instead of the genomic DNA along with internal primer pairs of 13Z and LIR. The final volume of 20 µl for reaction was used as before with the same thermal program. The product was then electrophoresed on 2% agarose gel containing ethidium bromide at 80 V for 60 min. The gel was transferred into a UV transilluminator apparatus (TM-20). The bands generated by the samples were compared with standard and marker bands (100 bp DNA ladder marker, Fermentas) under UV light and the parasite species were determined using gel documentation.

To create an epidemiological map of the area, the results of the parasite species deter-

mined were recorded in forms. The results were then analyzed using chi-square testing with SPSS ver. 18 software (Chicago, IL, USA). A *P*-value < 0.05 was considered significant.

## Results

The *Leishmania* bodies of the 92 isolates were detected by microscopic observation. The samples were all tested by PCR technique. Electrophoresis of the nested PCR products and comparison of the banding patterns with those of the standard samples showed that the bands of all 92 isolates were 560 bp in length, which is standard for *L. major* (Fig. 1).

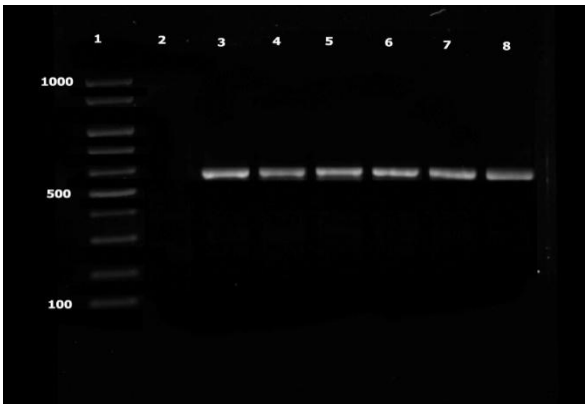


**Fig. 1**: Agarose gel electrophoresis of *Leishmania* isolates. Lane 1, DNA size marker 100 bp; lane 2, negative control; lane3, *L. major* (positive control 560 bp); lanes 4, 5, 6, 7 and 8, *L. major* isolates obtained from skin lesions of the patients in Mehran

**Table 1**: Frequency of cutaneous leishmaniasis in Mehran by gender and age group

| Age group (yr) | Gender | | | | Total | |
|---|---|---|---|---|---|---|
| | Male | | Female | | No | Percent |
| | No | Percent | No | Percent | | |
| <10 | 11 | 21.2 | 8 | 20 | 19 | 20.7 |
| 11-20 | 8 | 15.4 | 2 | 5 | 10 | 10.9 |
| 21-30 | 14 | 26.9 | 11 | 27.5 | 25 | 27.2 |
| 31-40 | 9 | 17.3 | 9 | 22.5 | 18 | 19.6 |
| >41 | 10 | 19.2 | 10 | 25 | 20 | 21.7 |
| Total | 52 | 56.5 | 40 | 43.5 | 92 | 100 |

Of the 92 patients, 52 (56.5%) cases were male and 40 (43.5%) were female. The most common frequency was observed in the 21-30 years age group (27.2%) and the lowest in the 11-20 years age group (10.9%) (Table 1). The data from current study showed no significant correlation for gender and age ($P = 0.572$).

Of the 92 patients, 50 (54.3%) had a single lesion, 24 (26.1%) had two, 12 (13%) had three, and 6 (6.5%) had more than three lesions. The data showed no statistically significant correlation ($P = 0.887$) for age and number of lesions. The anatomical location of each lesion was studied and it became clear that the hands ranked first (36%), followed by the legs (20.7%), face (15.2%), and other body parts (28.1%).

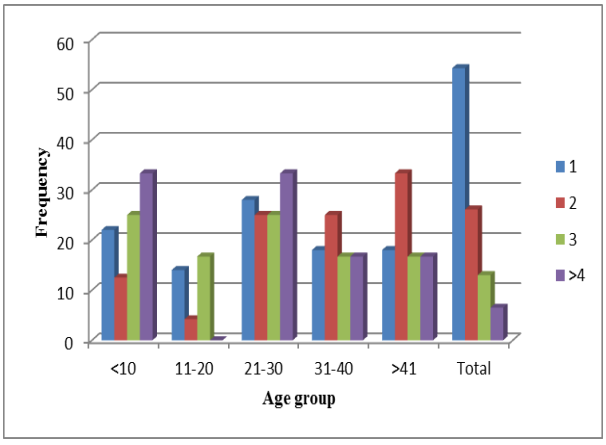


**Fig. 2**: Frequency of cutaneous leishmaniasis by number of lesions and age group in Mehran

No statistically significant association was found between lesion location and patient

gender. The duration of lesions was less than one month for 39 cases (42%) and one patient (1%) showed duration of about three months. All patients (100%) were confirmed to be Iranian nationals. The months showing the highest and lower rate of transmission were November (25%) and April (3.2%), respectively. About 44.5% of patients lived in rural settings and 54.5% of cases were suburban residents, workers, and military personnel working in the border regions.

## Discussion

Mehran is located at 33°7′15″ N latitude and 42°9′45″ E longitude at 155 m in elevation. The weather is very hot in the summer (+50°C) and the total annual rainfall is about 250 mm. The city is approximately 12 km from the zero point of the Iran-Iraq border. Significant changes in the epidemiology of the disease in Mehran relates to its geographic location, climate, the common border with Iraq (a focus of rural leishmaniasis) (16), environmental changes caused by the Iran-Iraq war, and conflict in the region in the past few years. Another cause is the presence of large numbers of troops from other parts of the country, especially Esfahan (a rural focus of cutaneous leishmaniasis). In recent decades, the incidence of the disease has increased and it is now considered to be a challenging health issue. It is necessary to select an appropriate method for the control of the disease to reduce the rate of people at risk by considering *Leishmania* foci, vectors, reservoir variety and, more importantly, parasite identification at the species level. Strategies for the control and prevention of rural and urban outbreaks differ. The results of the current study can be used to implement appropriate preventive strategies for disease control in Mehran.

The results showed that the age group most affected by disease was the 21-30 yr. This concurs with the results from Kasiri et al. for Ilam Province (13) and Ahmadi et al. for Borojerd (24) who both found the highest incidence in the 20-29 yr age group. By contrast, Maraghi et al. reported that in Shush, most cases appeared in patients of less than 10 years of age (25). Although the age pattern for cutaneous leishmaniasis varied, rural disease endemicity, parasite species, and host genome patterns increased in incidence in the younger age groups, which indicates high endemicity for this part of the country (26). Evidence suggests that the epidemiological focus in Mehran can be categorized as hyper endemic (Fig. 3).


**Fig. 3**: Map of Ilam and its counties in 2014 from Iran Meteorological Organization (13).

The data showed that 56.5% of patients were male and 43.5% female, which is consistent with previous studies from other parts of Iran (13, 26). Lesions were most frequently observed on the hands (35.9%), legs (20.7%), and face (15.2%). The results corresponded with those of previous studies from other parts of the country for a rural focus for cutaneous leishmaniasis (13, 15, 23). Lesions in rural areas are often facial and in urban areas more frequently appear on the hands and legs (9, 27, 28).

The present study showed that 54.3% patients had a single lesion, 26.1% had two, 13% had three, and 5.6% had multiple lesions. This agrees with results of studies from different parts of Iran (13, 23, 25, 29). Dedet et al. examined a select population and showed that

most patients recorded more than one lesion. This difference could probably be a result of the physiological characteristics of the vector with respect to feeding behavior (30). The incidence of cutaneous leishmaniasis in native residents with no travel history leads to the deduction that this disease is autochthonous in Mehran.

DNA-based techniques have extended cutaneous leishmaniasis diagnosis and parasite species identification. These methods have high sensitivity and specificity (31). The present study applied the nested PCR technique to examine 92 isolates and used standard samples to replicate parasite minicircle kDNA. PCR technique was performed on alleged *Leishmania* lesions samples and a correlation with the 560 bp band length of *L. major* was observed (32). The study indicates that *L. major* was the cause of all samples collected from Mehran for cutaneous leishmaniasis. Maraghi et al. also found *L. major* to be the predominant species for Shush (25). Cutaneous leishmaniasis in Fakeh and Musian that border the city of Mehran was caused by *L. major* (33-34). Tashakori et al. found in Dehloran, which is in the vicinity of Mehran, that *L. major* was the solitary agent for cutaneous leishmaniasis (32).

Localized conditions for leishmaniasis in this area are undoubtedly due to ecological characteristics of the disease vectors and reservoirs. Studies have documented that *Tatera indica* in the absence of *Rhombomys opimus* is the main reservoir of *L. major* (rural type) in Ilam (13). This rodent has been observed in the Iran-Iraq border areas, in all cities of Khuzestan province, and in the rural areas of Mehran (11). The present study showed that the main vector of cutaneous leishmaniasis in the region is *Phlebotomus papatasi* and the definitive reservoir of the disease in the absence of *R. opimus* is *T. indica*. Because the *Leishmania* species has been proven to cause the disease in this area, it is necessary to promote individual health by educating the community and implementing appropriate control and preventive measures. It is essential to implement measures for rodent control using rodenticides and to reduce the risk of vector contact with individuals. Bed nets impregnated with insecticide should also be distributed among military personnel, especially in the border areas.

## Conclusion

The cutaneous leishmaniasis found in Mehran was of the rural type. It was shown that 100% of isolate samples were caused by *L. major*, indicating the existence of a definite relationship between the disease vectors and reservoirs with transmission cycle elements in this area. The study also verified that nested PCR is an accurate and sensitive technique for detection and identification of *Leishmania* parasites.

## Acknowledgments

This study is part of M.Sc thesis for Ezatollah Ghasemi. It was financially supported by grant OG-92126 from the Vice Chancellor for Research Affairs of Ahvaz Jundishapur University of Medical Sciences. The authors express their appreciation and thanks to the Infectious and Tropical Diseases Research Center, Ahvaz Jundishapur University of Medical Sciences, and the staff of the Central Laboratory of the Prevention and Health Center for providing statistics and collecting samples from Mehran. Special thanks go to Dr. J. Mohammadi Asl and to Dr. A. Kaydani for their cooperation and guidance. Sincere thanks also go to Dr. K. Ahmadi Ankali for his helpful statistical consultation. The authors declare that there is no conflict of interests.

## References

1. Bates PA. Transmission of *Leishmania* metacyclic promastigotes by phlebotomine sand flies. Int J Parasitol. 2007; 37(10):1097–106.

2. Reithinger R, Dujardin JC, Louzir H, Pirmez C, Alexander B, Brooker S. Cutaneous leishmaniasis. Lancet Infect Dis 2007; 7: 581–9.
3. Croft SL, Coombs GH. Leishmaniasis– current chemotherapy and recent advances in the search for novel drugs. Trends Parasitol. 2003; 19(11):502–8.
4. González U, Pinart M, Firooz A, Enk C, Mendoza N, Vélez DI, et al. Preventive measures for leishmaniasis - The Cochrane Library. Cochrane Database Syst Rev. 2010; 10.
5. Piscopo T V, Azzopardi CM. Leishmaniasis. Postgr Med J. 2007; 83(976):649–57.
6. Desjeux P. The increase in risk factors for leishmaniasis worldwide. Trans R Soc Trop Med Hyg. 2001; 95(3):239–43.
7. Shirzadi M, Sharifian J, Zeinali M, Qarahgozloo F, Pourmozaffari J, Doosti S. Successful in zoonosis control programmes. 1st ed. Tehran: Ministry of Health and Medical Education publications; 2009.
8. Yaghoobi-Ershadi MR, Akhavan A a, Zahraei-Ramazani AR, Jalali-Zand AR, Piazak N. Bionomics of *Phlebotomus papatasi* (Diptera: Psychodidae) in an endemic focus of zoonotic cutaneous leishmaniasis in central Iran. J Vector Ecol. 2005; 30(1):115–8.
9. Yaghoobi-Ershadi M., Hanafi-Bojd A., Akhavan A., Zahrai-Ramazani A., Mohebali M. Epidemiological study in a new focus of cutaneous leishmaniosis due to *Leishmania major* in Ardestan town, central Iran. Acta Trop. 2001; 79(2):115–21.
10. Mohebali M, Javadian E, Yaghoobi-Ershadi MR, Akhavanaa, Hajjaran H, Abaei MR. Characterization of *Leishmania* infection in rodents from endemic areas of the Islamic Republic of Iran. East Mediterr Health J. 2004; 10(4-5):591–9.
11. Javadian E, Dehestani M, Nadim A, Rassi Y, Tahvildar-Bidruni G, SeyediRashti M, et al. Confirmation of *Tatera indica* (*Rodentia*: Gerbillidae) as the main reservoir host of zoonotic cutaneous leishmaniasis in the west of Iran. Iran J Public Health. 1998; 27(1-2):55–60.
12. Mehrabi Tavana A, Esfahani A. Cutaneous leishmaniasis in imposed war (Iraq against Iran) during 1980-1988. J ARMY Univ Med Sci Iriran. 2005; 3(9):507–11.
13. Kassiri H, Sharifinia N, Jalilian M, Shemshad K. Epidemiological aspects of cutaneous leishmaniasis in Ilam province, west of Iran (2000–2007). Asian Pacific J Trop Dis. 2012; 2:382–386.
14. Singh S. New developments in diagnosis of leishmaniasis. Indian J Med Res. 2006; 123(3):311–30.
15. 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. Appl Environ Microbiol. 2000; 66(5):1931–1938.
16. Centers for Disease Control and Prevention (CDC). Cutaneous leishmaniasis in U.S. military personnel-Southwest/Central Asia, 2002-2003. MMWR Morb Mortal Weekly Rep. 2003; 52(42):1009–1012.
17. Claborn D, Masuoka P, Morrow M, Keep L. Habitat analysis of North American sand flies near veterans returning from *Leishmania*-endemic war zones. Int J Health Geogr. 2008 Jan; 7:65. DOI: 10.1186/1476-072X-7-65.
18. WHO. Basic laboratory methods in medical parasitology. 1991.
19. Schönian G, Nasereddin A, Dinse N, Schweynoch C, Schallig HDF., Presber W, et al. PCR diagnosis and characterization of *Leishmania* in local and imported clinical samples. Diagn Microbiol Infect Dis. 2003; 47(1):349–58.
20. Khademvatan S, Neisi N, Maraghi S, Saki J. Diagnosis and identification of *Leishmania* spp. from Giemsa-stained slides, by real-time PCR and melting curve analysis in south-west of Iran. Ann Trop Med Parasitol. 2011; 105(8):559–65.
21. Kokozidou M. Evaluation of PCR methods for detection, species identification and determination of genetic variation in *L. infantum*. Giessen 2003.
22. Noyes HA, Reyburn H, Bailey JW SD. A nested-PCR-based schizodeme method for identifying *Leishmania* kinetoplast minicircle classes. J Clin Microbiol. 1998; 36(10):2877–81.
23. Mohammadi Azni S, Rassi Y, Oshaghi MA, Yaghoobi Ershadi MR, Mohebali M, Abaei MR, et al. Determination of parasite species of cutaneous leishmaniasis using Nested PCR in Damghan – Iran , during 2008. J Gorgan Univ Med Sci. 2011; 13(1):59–65.
24. Ahmadi N, Modiri M, Mamdohi S. First survey of cutaneous leishmaniasis in Borujerd county,

western Islamic Republic of Iran. East Mediterr Health J. 2013; 19(10):847–53.
25. Maraghi S, Mardanshah O, Rafiei A, Samarbafzadeh A, Vazirianzadeh B. Identification of Cutaneous Leishmaniasis Agents in Four Geographical Regions of Khuzestan Province Using Nested PCR. Jundishapur J Microbiol. 2013; 6(4):4–7.
26. Pagheh AS, Fakhar M, Mesgarian F, Gholami S, Badiee F, Sciences M, et al. Detection and Identification of Causative Agent of Cutaneous Leishmaniasis Using Specific PCR. J Maz Univ Med Sci. 2012; 22(Supple 1):85–92.
27. Fakhar M, F M, Hatam G, Habibi P, Karami M, Motazedian M, et al. Molecular epidemiology survey of cutaneous leishmaniasis in referral patients to Parasitology lab at Shiraz School of Medicine and importance application of PCR for diagnosis of disease. J Jahrom Univ Med Sci 2010; 8(1):1-5.
28. Ardehali S, Rezaei HR, Nadim A. *Leishmania* parasite and leishmaniasis. 2nd ed. Tehran: Iran University. Press; 1994: 3-11.
29. Ghasemian M, Maraghi S, Samarbafzadeh a R, Jelowdar A, Kalantari M. The PCR-based detection and identification of the parasites causing human cutaneous leishmaniasis in the Iranian city of Ahvaz. Ann Trop Med Parasitol. 2011; 105(3):209–15.
30. Dedet JP, Pratlong F. Leishmaniasis. Manson's Tropical diseases. 21st ed. Manson P, Cook G, Zumla A, editors. London: Saunders; 2003:1339-1364.
31. Oliveira JGS, Novais FO, de Oliveira CI, da Cruz Junior AC, Campos LF, da Rocha AV, et al. Polymerase chain reaction (PCR) is highly sensitive for diagnosis of mucosal leishmaniasis. Acta Trop. 2005; 94(1):55–9.
32. Tashakori M, Ajdary S, Kariminia A, Mahboudi F, Alimohammadian MH. Characterization of *Leishmania* species and *L. major* strains in different endemic areas of cutaneous leishmaniasis in Iran. Iran Biomed J. 2003; 7(2):43–50.
33. Nadim A, Javadian E. Key for species identification of sand flies. Iran J Public Health. 1976; 50(1):33–44.
34. Yaghoobi Ershadi MR, Akhavanaa, Mohebali M. *Meriones libycus* and *Rhombomys opimus* (*Rodentia*: Gerbillidae) are the main reservoir hosts in a new focus of zoonotic cutaneous leishmaniasis in Iran. Trans R Soc Trop Med Hyg. 1996; 90(5):503–4.