

PCR-RFLP diagnosis and characterization of *Leishmania* species causing human cutaneous leishmaniasis and evaluation of treatment times with glucantime in these patients

Hashemi, N.¹, Mohaghegh, M.A.¹, Hashemi, M.², Azami, M.^{3,4}, Mortazavidehkordi, N.¹, Hashemi, C.⁵ and Hejazi, S.H.^{1,3*}

¹Department of Parasitology and Mycology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran

²Deputy of Research and Technology, North Khorasan University of Medical Sciences, Bojnurd, Iran

³Skin Diseases and Leishmaniasis Research Center, Isfahan University of Medical Sciences, Isfahan, Iran

⁴Department of Parasitology, Faculty of Veterinary Medicine, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran

⁵Dentist, North Khorasan, Social Security Organization, Bojnurd, Iran

*Corresponding author e-mail: hejazi@med.mui.ac.ir

Received 17 September 2015; received in revised form 3 March 2016; accepted 4 March 2016

Abstract. Antimony compounds are first line treatments for cutaneous leishmaniasis. The prognosis of the disease varies depending on the type of medicine and species. We aimed to determine the species responsible for cutaneous leishmaniasis in patients referred to Skin Diseases and Leishmaniasis Research Center in Isfahan and Bam Health Center (Kerman) in order to follow and assess the complete healing of the lesions. A total of 40 skin lesions samples were collected from patients with cutaneous leishmaniasis (CL) from January 2014 to 2015. Dermal scrapings were analyzed by examination of Giemsa-stained smears. Parasites were cultured and isolated in NNN and RPMI 1640 medium and DNA was extracted. We used PCR-RFLP assays of ITS1 genes for direct identification of *Leishmania* species. Treatment process was assessed after a treatment period with glucantime and healing of the studied cases was followed up. All the samples from Isfahan and Bam regions were *L. major* and *L. tropica* species respectively. In patients infected with *L. major* and *L. tropica* treated with glucantime, the shortest healing period was 40 days in 5(25%) and 60 days in 3(15.8%) patients, respectively and the longest healing period was 100 days in 1 (5%) and 160 days in 1 (5.3%) patient, respectively. The mean complete healing periods in patients with *L. tropica* and *L. major* were 100 and 58 days, respectively ($P < 0.001$). Average recovery period for people with dry cutaneous leishmaniasis is longer than average recovery period for people with wet cutaneous leishmaniasis.

INTRODUCTION

Leishmaniasis is a vector-born disease caused by *Leishmania* spp. and is transmitted through bite of an infected sand fly (Asilian & Davami, 2006; Cruz *et al.*, 2007; Minodier & Parola, 2007). Leishmaniasis is predominantly reported affecting humans in tropical and semi-tropical regions with a 90% prevalence reported from Iran, Afghanistan,

Al-Jazeera, Brazil, and Saudi Arabia (Sundar *et al.*, 2002).

After African trypanosomiasis, dengue and malaria, cutaneous leishmaniasis (CL) is the next important vector-borne parasitic infection with prevalence rate varying between 2-350 million humans in the world. In Iran, the anthroponotic type of CL, called urban CL, has been often reported from densely populated cities such as Tehran,

Shiraz, Mashhad, Khaf, Taybad and Kerman and its zoonotic type has been further reported from Isfahan, Khuzestan, Ilam, Bushehr and Semnan provinces (Tashakori *et al.*, 2003; Tashakori *et al.*, 2006; Mohaghegh *et al.*, 2013; Salehi *et al.*, 2014; Fata *et al.*, 2015).

Diagnosis based solely on previous studies and reports of various species in a specific region is not reliable (el Tai *et al.*, 2000). For years, the standard diagnostic method of CL was direct microscopic tests, which were relatively simple and inexpensive. However, the sensitivity and specificity of microscopic methods is considerably lower than that of molecular methods in differentiating between *Leishmania* species (Rotureau *et al.*, 2006; Yang *et al.*, 2007). Serological methods have been found not sensitive to differentiate *Leishmania* antigens from other pathogens that are not commonly used in CL diagnosis (Marfurt *et al.*, 2003). In recent years, novel molecular methods based on polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and nested PCR have been used to determine the species of *Leishmania* isolates. With the help of these techniques, higher sensitivity and specificity to parasitic species can be determined in a short time (Yehia *et al.*, 2012).

The treatment of *Leishmania* differs according to the species of parasite. Therefore, identification of parasite species is crucial to plan a program for control, prevention and treatment of the disease. Antimony compounds are the first line of treatment in patients with CL. Meglumine antimonite (Glucontime®) and sodium stibogluconate (Pentostam™) are two antimony compounds commonly used to treat CL in Iran and other countries. These compounds were initially used for treatment of schistosomiasis (Reithinger *et al.*, 2005; Minodier & Parola, 2007).

The aim of this study is to determine CL-causing *Leishmania* species in patients referred to the Skin Diseases and Leishmaniasis Research Center in Isfahan and Bam health centers (Kerman) in order to follow and assess the complete healing of the lesions.

Patients

This survey was conducted from January 2014 to January 2015 in hyperendemic cities of Isfahan and Bam, Iran. Informed consent was obtained from all individual participants included in the study. This study was approved by the university research ethics committee (UREC), Isfahan University of Medical Science. The samples were collected from patients referred to the Bam Health Center and the Skin Diseases and Leishmaniasis Research Center, Isfahan University of Medical Sciences.

Sampling

Forty positive CL cases obtained from two centers with 20 patients from each center. Samples taken from the margin of skin ulcer were spread on a glass slide, stained with Giemsa (Merck, Darmstadt, Germany) and confirmed under light microscope.

Culture of samples

Part of the samples taken from the margin of the lesion of each patient was transferred to Novy-MacNeal-Nicolle (NNN) medium (Difco, Detroit, MI) for primary isolation and were subcultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FCS, 100 IU/ml of penicillin, 100 µg/ml of streptomycin, and incubated at $25 \pm 1^\circ\text{C}$.

DNA extraction

In RPMI 1640 (Invitrogen, Carlsbad, CA) medium, amastigotes transformed to promastigotes. A specimen from the second subculture was used for DNA extraction. Promastigotes were three times washed with phosphate buffered saline (PBS; pH: 7.4) and DNA was extracted from the samples using High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany).

PCR

ITS1 region of the extracted DNA was amplified using PCR technique with the following primer pairs: LITSR 5'-CTGGATCATTTTCCGATG-3' and L5.8S 5'-TGATACCACTTATCGCACTT-3' in a thermo-

cycler (Corbett, USA). In addition, the extracted DNA of standard species *L. tropica* (MH/IR/99/yazd1) and *L. major* (MRHO/IR/75/ER) were used as positive controls. Ten microliters of PCR product was electrophoresed on 1% agarose gel (Sigma-Aldrich, Germany). The gel stained with ethidium bromide, and visualized on a UV transilluminator.

Digestion with HaeIII enzyme (RFLP)

HaeIII enzyme (Fermentas, MBI) was used to digest the amplified DNAs. The reaction mixture contained 5 µl of the amplified DNAs, 1.5 µl of 10X Buffer, 1 µl of HaeIII and 7.5 µl of ddH₂O in a microtube and was incubated at 37°C for 2 hrs. Then, 10 µl of the reaction solution was electrophoresed on 1.5% agarose gel (Sigma-Aldrich, Germany) and bands visualized by UV light after being stained with ethidium bromide (0.3 µg/mL).

Following the healing process of the lesions

In order to determine drug resistance in the species of *Leishmania*, the clinical healing process of the lesions was followed. The patients were examined by a dermatologist and the amount of healing after a course of treatment was evaluated and recorded for each patient. The initial treatment course comprised intramuscular injection of 20 mg/kg/day Glucantime® for 21 days in 2 phases at an interval of 14 days between phases. In addition to intramuscular injection, 0.5 ml Glucantime® was used for injection inside the lesion for 5-7 weeks. Patients were under traditional Glucantime® therapy and were followed up for 3 treatment phase to detect their cure courses.

RESULTS

As shown in Figure 1, PCR product electrophoresis on a 1% agarose gel revealed a 350 bp band for all of the *Leishmania* samples, as observed for the standard samples of *L. major* (MRHO/IR/75/ER) and *L. tropica* (MH/IR/yazd1). After digesting the PCR product for standard *L. major* samples (MRHO/IR/75/ER) with Hae²²² enzyme, two

bands 140 and 220 bp were observed on a 1.5% agarose gel. In addition, the digested amplicon of the PCR product for standard *L. tropica* samples (MH/IR/99/yazd1) showed two bands 60 and 200 bp. Compared to the standard samples, all of the samples in Isfahan region were the same that was for standard *L. major* samples (MRHO/IR/75/ER) (Figure 2). Moreover, all of the samples in Bam region showed a pattern similar to standard *L. tropica* samples (MH/IR/99/yazd1) (Figure 2).

Twenty out of the 40 assessed samples were related to CL caused by *L. tropica*, including 10 men and 10 women. The 20 other patients with CL were identified infected with *L. major*, 8 of which were women and the remaining were men. Nine (45%) of the patients infected with *L. major* had only one lesion, while 4 (20%), 4 (20%), 2 (10%), and 1 (5%) of them had 2, 3, 5, and 7 lesions, respectively. In the patients infected with *L. tropica*, 5 (25%), 11 (55%), 1 (5%), 2 (10%), and 1 (5%) of them had 1, 2, 3, 4, and 5 lesions, respectively. In the patients infected with *L. major*, 30% of the lesions were observed on the hands, while 10%, 25%, and 35% were on the feet, face, and other body parts, respectively. In the patients infected with *L. tropica*, 50% of the lesions were observed on the hands and followed by on the feet (15%), face (15%) and other regions (20%).

With respect to the type of Glucantim injection and treatment phase, we found that in patients infected with *L. major* exposed to intralesion injections (n=9), 7 (7.77%) and 2 (33.3%) patients were completely treated in the first and second treatment phase, respectively. In patients infected with *L. major* exposed to intramuscular injections (n=11), 2 (18.2%), 6 (54.5%), and 3 (27.3%) patients were completely treated in the first, second, and third treatment phase, respectively.

With respect to the method of Glucantime® injection and treatment phase, we found that in patients infected with *L. tropica* exposed to intralesion injections (n=12), 2 (16.7%), 6 (50%), and 4 (33.3%) patients were completely treated in the first, second, and third treatment phase, respectively. In patients infected with *L.*

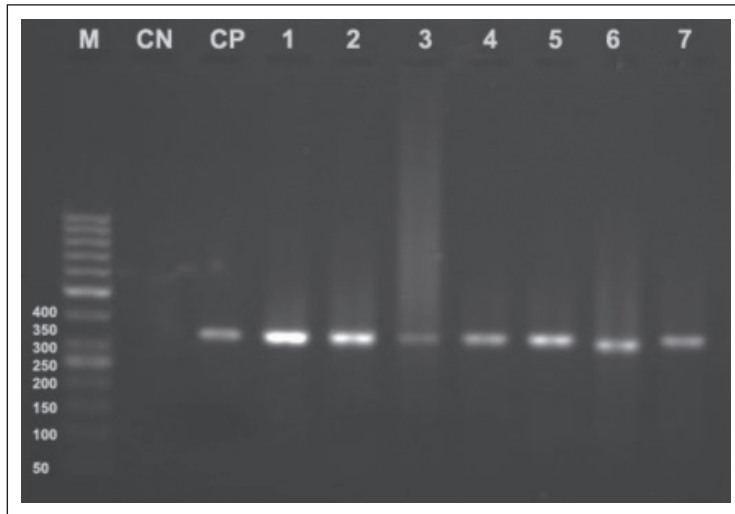


Figure 1. Electrophoresis results of ITS1-PCR from *Leishmania* human isolates. M, Molecular marker (50 bp); Lane CN, negative control; Lane CP, positive control of *Leishmania major* (MRHO/IR/75/ER); Lanes 1-7, human samples.

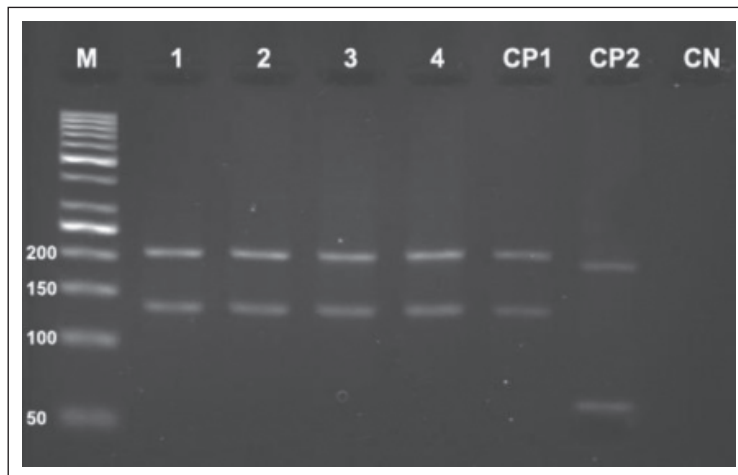


Figure 2. PCR-RFLP analysis of ITS1 region for identification of *Leishmania* species using HaeIII. Lanes 1-4, human samples (*L. major*); Lane CP1, positive control of *L. major* (MHOM/IR/75/ER); Lane CP2, positive control of *L. tropica* (MHOM/IR/99/YAZ1); Lane CN, negative control; Lane M, molecular marker (50 bp).

tropica exposed to intramuscular injections (n=7), 3 (42.9%) and 4 (57.1%) patients were completely treated in the second and third treatment phase, respectively. One patient lost to follow-up. The mean complete healing periods in patients with *L. tropica* and *L. major* were 100 and 58 days, respectively (P<0.001).

In the patients infected with *L. major* treated with Glucantime[®], the shortest healing period was 40 days in 5 (25%) patients and the longest healing period was 100 days in 1 (5%) patient. In addition, in the patients infected with *L. tropica* treated with Glucantime[®], the shortest healing period was 60 days in 3 (15.8%) patients and the longest

Table 1. Duration of treatment using Glucantime® in the patients infected with *L. major*

Duration of treatment (day)	Number of patients	Percentage
40	5	25
47	1	5
50	6	30
60	1	5
65	1	5
70	2	10
80	1	5
90	2	10
100	1	5
Total	20	100

Table 2. Duration of treatment using Glucantime® in the patients infected with *L. tropica*

Duration of treatment (day)	Number of patients	Percentage
60	3	15.8
70	1	5.3
80	3	15.8
90	2	10.5
100	3	15.8
120	3	15.8
130	1	5.3
140	1	5.3
150	1	5.3
160	1	5.3
Patients lost to follow-up	1	5.3
Total	20	100

healing period was 160 days in 1 (5.3%) patient (Tables 1 and 2). The mean complete healing period in the patients with *L. tropica* and *L. major* were 100 and 58 days, respectively ($P < 0.001$).

DISCUSSION

Leishmania species have variable responses to different treatment regimens, it is therefore important to determine the type of *Leishmania* species for more effective treatment options (Berman, 1997). Recent studies have shown that PCR technique has higher specificity relative to traditional methods, such as culture and microscopic examination, considering the extensive morphological similarities among species (Yehia *et al.*, 2012). Antimony compounds

were used initially to treat schistosomiasis (Beheshti *et al.*, 2007). Currently, these compounds are considered as first line treatments of CL. Glucantime® is a pentavalent antimony compound and can be administered in intramuscular and intralesional forms (Reithinger *et al.*, 2005; Minodier & Parola, 2007). Antimony compounds inhibit the glycolytic activity of parasites and fatty acid oxidation leading to reduced energy for the survival of parasites (Beheshti *et al.*, 2007), the best effect of which has been observed when the host immune system has normal functions. The known side effects of these medicines are nausea, vomiting, skin rashes, cardiac, renal, and hepatic complications (Falcoff *et al.*, 1994; Delgado *et al.*, 1999).

Bensussan *et al.* (2006) used PCR-RFLP (ITS) to determine *Leishmania* species and found that this method could identify the species in 74% of the positive samples. In a study in India, using PCR-RFLP on DNA obtained from the culture parasites, the results showed the amplification of TS1 ribosomal region with a 300-350 bp fragments. After digesting with HaeIII enzyme, *L. major* showed two bands 160 and 210 bp on an agarose gel (Kumar *et al.*, 2007).

In the present study, in patients infected with *L. major*, the mean treatment duration was 46 and 68 days among those who received intralesional and intramuscular injections, respectively. In a similar study in Brazil, patients with *L. braziliensis* treated with Glucantime® and Pentostam™ recovered after three months at a rate of 62% and 55%, respectively (Saldanha *et al.*, 1999).

In another study, three treatment regimens were compared for the intralesional injection of Glucantime® (daily, every other day, and weekly). The researchers concluded that the intralesional injection of Glucantime® is more effective on a weekly basis or every other day compared with its daily use (Tallab *et al.*, 1996). We used the weekly regimen for the injection of Glucantime® in the lesions and found that 77.7% and 22.2% of patients with *L. major* recovered in the first and second treatment phase, respectively.

In a study in Isfahan on patients with CL (*L. major*), the patients (n=32) were

divided into two treatment groups, either intramuscular injection of Glucantime® 20 mg/kg with pentoxifylline 400 mg three times a day or intramuscular injection of Glucantime® alone). The patients were followed for three months and the results showed that 81.3% and 12.5% of the patients completely recovered after three months in groups 1 and 2, respectively (Sadeghian & Nilforoushzadeh, 2006). In our study, all patients with CL (*L. major*) treated with intramuscular injections of Glucantime® were completely treated within three months. Firdous *et al.*, 2009 found that after 21 days of treatment, 81% of the patients with *L. major* receiving intramuscular Glucantime® recovered. However, in our study, only 18.2% of the patients with *L. major* recovered within a similar period. It should be noted that the sample size of the mentioned study was much larger than ours.

Antimony compounds have been widely used for nearly 60 years without any evidence of resistance, however, in the past 15 years, the increased level of resistance is alarming (Natera *et al.*, 2007). It is therefore important to produce novel medicines by combining currently available medicines and newly developed drugs to follow the course of treatment. It has been shown that the mechanism of drug resistance is related to drug metabolism and dissemination (Croft *et al.*, 2006).

Drug resistance is one of the main complications of infection control leading to failure in treating kala-azar, mucosal leishmaniasis, and CL in endemic regions (Ponte-Sucre, 2003). Drug resistance is a common reason for increased P-glycoprotein involved in membrane transport. Drug resistance is also related to physiological changes in infectious parasites, intracellular metabolism, host-parasite interaction, and reduced expression of virulence factors of parasites such as acid phosphatase. Changes in the activity of enzymes involved in intracellular metabolism are also related to drug resistance (Ponte-Sucre, 2003; Natera *et al.*, 2007).

Clinical trials have assessed factors that influence the effect of antimony compounds and azoles in treating leishmaniasis

including the sensitivity and innate differences of various *Leishmania* species. Moreover, factors such as a decrease in ability of the immune system and inadequate dosage could also contribute to treatment failure (Ponte-Sucre, 2003; Hadighi *et al.*, 2006).

CONCLUSION

In this study, we decided to use ITS1 PCR-RFLP technique for *Leishmania* species identification. Correct diagnosis and identification of *Leishmania* species is important to evaluate the prognosis and appropriate treatment of CL. This study suggests that PCR-RFLP assay in patients with CL using HaeIII enzyme is useful for the rapid identification of *Leishmania* species.

Acknowledgements. We would like to thank the personnel of the Parasitology Faculty of Isfahan University of Medical Sciences and Sedigheh Tahereh Skin Diseases and Leishmaniasis Research Center as well as Bam Health Center for their kind cooperation.

Conflict of interest

The authors have no financial or personal relationship with other people or organizations that could inappropriately influence or bias this paper.

Ethical approval

The presented study was conducted in accordance with Helsinki declaration (1974) and recommendation of the college of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran, an entity as associated with the International Council for Human Laboratory Sciences. This project was approved by the Human Research Ethics Committee of Isfahan University of Medical Sciences.

REFERENCES

Asilian, A. & Davami, M. (2006). Comparison between the efficacy of photodynamic therapy and topical paromomycin in the

- treatment of Old World cutaneous leishmaniasis: a placebo-controlled, randomized clinical trial. *Clinical and Experimental Dermatology* **31**: 634-637.
- Beheshti, M., Ghotbi, S. & Amirzade, S. (2007). Therapeutic and adverse effects of glucantime used for treatment of cutaneous leishmaniasis. *Shiraz E-Med Journal* **4**.
- Bensoussan, E., Nasereddin, A., Jonas, F., Schnur, L.F. & Jaffe, C.L. (2006). Comparison of PCR assays for diagnosis of cutaneous leishmaniasis. *Journal Clinical Microbiology* **44**: 1435-1439.
- Berman, J. D. (1997). Human leishmaniasis: clinical, diagnostic, and chemotherapeutic developments in the last 10 years. *Clinical Infectious Disease* **24**: 684-703.
- Croft, S. L., Sundar, S. & Fairlamb, A.H. (2006). Drug resistance in leishmaniasis. *Clinical Microbiology Review* **19**: 111-126.
- Cruz, A., Rainey, P.M., Herwaldt, B.L., Stagni, G., Palacios, R., Trujillo, R. & Saravia, N.G. (2007). Pharmacokinetics of antimony in children treated for leishmaniasis with meglumine antimoniate. *Journal Infectious Disease* **195**: 602-608.
- Delgado, J., Macias, J., Pineda, J. A., Corzo, J.E., Gonzalez-Moreno, M.P., de la Rosa, R., Sanchez-Quijano, A., Leal, M. & Lissen, E. (1999). High frequency of serious side effects from meglumine antimoniate given without an upper limit dose for the treatment of visceral leishmaniasis in human immunodeficiency virus type-1-infected patients. *American Journal of Tropical Medicine and Hygiene* **61**: 766-769.
- el Tai, N.O., Osman, O.F., el Fari, M., Presber, W. & Schonian, G. (2000). Genetic heterogeneity of ribosomal internal transcribed spacer in clinical samples of *Leishmania donovani* spotted on filter paper as revealed by single-strand conformation polymorphisms and sequencing. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **94**: 575-579.
- Falcoff, E., Taranto, N.J., Remondegui, C.E., Dedet, J.P., Canini, L.M., Ripoll, C.M., Dimier-David, L., Vargas, F., Gimenez, L.A., Bernabo, J.G. & *et al.* (1994). Clinical healing of antimony-resistant cutaneous or mucocutaneous leishmaniasis following the combined administration of interferon-gamma and pentavalent antimonial compounds. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **88**: 95-97.
- Fata, A., Salehi Sangani, G., Rafatpanah, H., Mousavi Bazzaz, M., Mohaghegh, M. & Movahedi, A. (2015). Identification of *Leishmania* species by kinetoplast DNA-polymerase chain reaction for the first time in Khaf district, Khorasan-e-Razavi province, Iran. *Tropical Parasitology* **5**: 50-54.
- Firdous, R., Yasinzai, M. & Ranja, K. (2009). Efficacy of glucantime in the treatment of old world cutaneous leishmaniasis. *International Journal of Dermatology* **48**: 758-762.
- Hadighi, R., Mohebbali, M., Boucher, P., Hajjaran, H., Khamesipour, A. & Ouellette, M. (2006). Unresponsiveness to Glucantime treatment in Iranian cutaneous leishmaniasis due to drug-resistant *Leishmania tropica* parasites. *PLoS Medicine* **3**: e162.
- Kumar, R., Bumb, R.A., Ansari, N.A., Mehta, R.D. & Salotra, P. (2007). Cutaneous leishmaniasis caused by *Leishmania tropica* in Bikaner, India: parasite identification and characterization using molecular and immunologic tools. *American Journal of Tropical Medicine and Hygiene* **76**: 896-901.
- Marfurt, J., Nasereddin, A., Niederwieser, I., Jaffe, C.L., Beck, H.P. & Felger, I. (2003). Identification and differentiation of *Leishmania* species in clinical samples by PCR amplification of the miniexon sequence and subsequent restriction fragment length polymorphism analysis. *Journal of Clinical Microbiology* **41**: 3147-3153.
- Minodier, P. & Parola, P. (2007). Cutaneous leishmaniasis treatment. *Travel Medicine Infectious Disease* **5**: 150-158.

- Mohaghegh, M., Fata, A., Salehi, G., Berenji, F., Bazzaz, M.M., Rafatpanah, H., Parian, M. & Movahedi, A. (2013). Molecular identification of *Leishmania* species using samples obtained from negative stained smears. *Iranian Journal of Parasitology* **8**: 337-341.
- Natera, S., Machuca, C., Padron-Nieves, M., Romero, A., Diaz, E. & Ponte-Sucre, A. (2007). *Leishmania* spp.: proficiency of drug-resistant parasites. *International Journal of Antimicrobial and Agents* **29**: 637-642.
- Ponte-Sucre, A. (2003). Physiological consequences of drug resistance in *Leishmania* and their relevance for chemotherapy. *Kinetoplastid Biology Disease* **2**: 14.
- Reithinger, R., Mohsen, M., Wahid, M., Bismullah, M., Quinnell, R.J., Davies, C.R., Kolaczinski, J. & David, J.R. (2005). Efficacy of thermotherapy to treat cutaneous leishmaniasis caused by *Leishmania tropica* in Kabul, Afghanistan: a randomized, controlled trial. *Clinical Infectious Disease* **40**: 1148-1155.
- Rotureau, B., Ravel, C., Couppie, P., Pratlong, F., Nacher, M., Dedet, J.P. & Carme, B. (2006). Use of PCR-restriction fragment length polymorphism analysis to identify the main new world *Leishmania* species and analyze their taxonomic properties and polymorphism by application of the assay to clinical samples. *Journal of Clinical Microbiology* **44**: 459-467.
- Sadeghian, G. & Nilforoushzadeh, M.A. (2006). Effect of combination therapy with systemic glucantime and pentoxifylline in the treatment of cutaneous leishmaniasis. *International Journal of Dermatology* **45**: 819-821.
- Saldanha, A.C., Romero, G.A., Mercham-Hamann, E., Magalhaes, A.V. & Macedo Vde, O. (1999). A comparative study between sodium stibogluconate BP 88R and meglumine antimoniate in the treatment of cutaneous leishmaniasis: The efficacy and safety. *The Revista da Sociedade Brasileira de Medicina Tropical* **32**: 383-387.
- Salehi, G., Fata, A., Mohaghegh, M., Mousavi Bazzaz, M., Rafatpanah, H. & Movahedi, A. (2014). Molecular identification of *Leishmania* species in Taybad district, Iran. *Asian Pacific Journal of Tropical Disease* **4**: S535-S539.
- Sundar, S., Jha, T.K., Thakur, C.P., Mishra, M., Singh, V.R. & Buffels, R. (2002). Low-dose liposomal amphotericin B in refractory Indian visceral leishmaniasis: a multicenter study. *American Journal of Tropical Medicine and Hygiene* **66**: 143-146.
- Tallab, T.M., Bahamdani, K.A., Mirdad, S., Johargi, H., Mourad, M.M., Ibrahim, K., el Sherbini, A.H., Karkashan, E., Khare, A.K. & Jamal, A. (1996). Cutaneous leishmaniasis: schedules for intralesional treatment with sodium stibogluconate. *International Journal of Dermatology* **35**: 594-597.
- Tashakori, M., Ajdary, S., Kariminia, A., Mahboudi, F. & Alimohammadian, M. (2003). Characterization of *Leishmania* species and *L. major* strains in different endemic areas of cutaneous leishmaniasis in Iran. *Iranian Biomedical Journal* **7**: 43-50.
- Tashakori, M., Kuhls, K., Al-Jawabreh, A., Mauricio, I.L., Schonian, G., Farajnia, S. & Alimohammadian, M.H. (2006). *Leishmania major*: genetic heterogeneity of Iranian isolates by single-strand conformation polymorphism and sequence analysis of ribosomal DNA internal transcribed spacer. *Acta Tropica* **98**: 52-58.
- Yang, Z.H., Huang, J.X. & Yao, Y.J. (2007). Autoscreening of restriction endonucleases for PCR-restriction fragment length polymorphism identification of fungal species, with *Pleurotus* spp. as an example. *Applied Environmental Microbiology* **73**: 7947-7958.
- Yehia, L., Adib-Houreh, M., Raslan, W.F., Kibbi, A.G., Loya, A., Firooz, A., Satti, M., El-Sabban, M. & Khalifeh, I. (2012). Molecular diagnosis of cutaneous leishmaniasis and species identification: analysis of 122 biopsies with varied parasite index. *Journal of Cutaneous Pathology* **39**: 347-355.