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Original Article

Species Identification and Molecular Typing of *Leishmania* Spp. Using Targeting HSP70 Gene in Suspected Patients of Cutaneous Leishmaniasis from Sistan and Baluchestan Province, Southeast Iran

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Abstract

Background: Leishmaniasis is a sand fly-borne disease caused by the protozoan parasites belonging to the genus *Leishmania*. Because of the preventing and controlling methods, clinical course, prognosis and choice of treatment are differing from species; differentiation of species is critical. The present study was aimed to detect the parasite species using the PCR-RFLP method.

Methods: A total of 130 Giemsa-Stained slides from suspected Cutaneous leishmaniasis (CL) patients were examined under a light microscope at×1000. DNA from each slide was extracted PCR method was undertaken with HSP70 genes and the PCR products were digested with a restriction enzyme HaeIII (BsuR1). The study was conducted in the laboratory of Zahedan University of Medical Sciences in the Sistan and Baluchestan Province, southeastern Iran in 2015.

Results: From 130 suspected samples, 59 (45.3%) were positive by the microscopic examination, meanwhile 64 (49.2%) were positive by PCR-RFLP, *Leishmania* species were recognized, and *L. tropica* was introduced as predominant species in current study.

Conclusion: PCR-RFLP is a valuable technique for distinguish of *Leishmania* species. Furthermore, anthroponotic CL is the dominant cause of CL in Sistan and Baluchestan Province.

Introduction

Cutaneous leishmaniasis (CL) is known as an endemic disease in Iran and according to WHO, annually reports, nearly 20000 new cases occur per year in this country. Moreover, some researchers believe that it may be underestimating, and the actual rate is more than this (1, 2).

Leishmania major and *L. tropica* are the main cause of zoonotic CL (ZCL) and anthroponotic CL (ACL) in Iran, respectively (3). CL is reported in 17 out of 31 provinces of Iran and Sistan and Baluchestan is one of the important endemic foci of ACL and ZCL. In recent years, 3 cutaneous leishmainasis foci Chabahar, Konarak, and Mirjaveh were identified in Sistan and Baluchistan Province and CL cases reported progressively from these areas (4). Moreover, an outbreak of CL happened in Mirjaveh City for the first time in 1996 and this province is considered as a third focus of ZCL (5).

Differentiation of species is critical, because preventing and controlling methods, clinical course, prognosis and choice of treatment are different from species. Because of the morphological similarities, microscopic examinations are not capable to differentiate the species. Isoenzyme technique is considered as a golden standard method for characterization of especial species. This method requires an abundant number of parasites, is not cost effecting and is time-consuming (6).

Considering wide clinical spectrum of CL, conventional diagnostic methods such as morphological and parasitical methods are not appropriate methods anymore. In recent years application of high tech molecular methods, such as PCR- RFLP, RAPD-PCR, nested-PCR and Real time PCR are increasing sharply because of their high specificity and sensitivity in detection and identification (7, 8).

ITS, gp63, miniexon, kinetoplastic DNA and HSPs are the available genetic targets for molecular diagnosis of *Leishmania* species (9). Heat-shock proteins are highly conserved in all organisms. HSPs constitute a large family of proteins that are often categorized based on their molecular weight: hsp10, hsp40, hsp60, hsp70, hsp90, etc. (10). Hsp70 genes are sensitive enough for differentiation of *Leishmania* species. Because of the encoding for a major antigen, allow probing of the genetic variability of molecules and lower rate of genetic variation hsp70 genes are more priority than others (11). For future multigenic PCR-oriented approaches as well as studies involving direct populations, this new marker can be instrumental (9).

Sistan and Baluchestan is one of the endemic areas for CL but the species of *Leishmania* were identified. Therefore, the aim of present study was to detect the parasite species using the PCR-RFLP method.

Materials and Methods

Study Area

The province of Sitan and Baluchestan, is one of the 31 provinces of Iran, located in southeastern Iran, bordering Pakistan and Afghanistan with geographical coordinates between 29.4924°N 60.8669°E (Fig. 1). The capital is Zahedan. The most prominent climatologically phenomenon of the region is the heat. This province is characterized by long, hot and dry summers and short winters, whereas in coastal region, near the Oman Sea, the weather is warm with a high percentage of humidity.

Sample collection

In this experimental study, samples were collected from the individuals clinically suspected to CL and referred to Konarak and Chabahar health centers. A questioner was completed to record the essential information such as demographic information, sites of ulcer on the body and travel history.

Fig. 1: Map of Sistan and Baluchistan Province, Iran. Districts and geographical outlines are depicted

After obtaining a complete clinical history, finally 130 samples were taken for isolation of *Leishmania* species.

The study was conducted in the laboratory of Zahedan University of Medical Sciences in the Sistan and Baluchestan Province, south-eastern Iran in 2015. The study was approved by Ethics Committee of Zahehl University of Medic Sciences.

Samples Preparation

Skin suspected lesions were cleaned with 70% ethanol and the samples by injecting 0.2 ml of sterile saline into the dermis of the inside border of skin wounds with 1 ml of sterile insulin syringe were collected. Afterward suction, the serosity was moved to the sterile 1ml tube enclosing the 0.5 ml of ethanol 70%. The tubes were retained in freezer at _20 °C until molecular examination.

Microscopic examination

Direct smears for standard microscopically examination was prepared by aspiration of fluid from under the ulcer. After air-drying, slides were fixed with absolute methanol, stained with Giemsa 10%, and observed via optical microscopy (magnification ×1000) for the existence of amastigote forms of the parasite.

DNA extraction

DNA was extracted from Geimsa stained slides as declared by the manufacturer using the High Pure PCR Template Purification (Takapouzist, Iran DynaBio Blood/Tissue Genomic DNA Extraction Kit). The extracted DNA was stored at -20 ˚C until use.

PCR of the HSP70 gene

To detect and identification of the *Leishmania* species isolates, we used the HSP70 region, using the primers 70-IR-D (5'-CCAAGGTCGAGGAGGTCGAC TA- 3') and 70-IR-M (5'- ACGGGTAGGG GGAG-GAAAGA -3') (12). Amplification was done in 30 ml containing 3 ml of PCR buffer (Roche, Mannheim,Germany), 2.5 mM MgCl2 (25 mM; Roche), 1 ml Dntp (10 mM; Roche), 15 pMol of each primer, 10 ng of genomic DNA and 0.5 units of Taq polymerase (GeNet Bio, Korea). Amplification was done with 35 cycles, each of 30 s at 94 °C, 30-s at 49 °C, and 1 min at 72 °C in a thermocycler (Biometra, Goettingen, Germany). Then 5 mL of each PCR reaction was run on a 1% agarose gel and stained with ethidium bromide. Finally, reactions producing a product of about 750 bp were considered positive.

Insilico-RFLP for positions and RFLP of HSP 70 PCR products

The PCR product including the amplified HSP70 were digested with HaeIII (BsuR1) (Fermentas, Life Sciences, Germany) as commended by the producer. Digestion products were separated using 3% agarose gels in TAE buffer and visualized after staining by gel red.

Using software of CLCDNA in the insilico analysis and using Fasta format genes downloaded from GenBank and with BsuR1 enzymes, the number of regions in different species was identified. In HSP70 genes in the *L. major* using Bsur1enzymes and three sections and four sizes 70, 178, 183 and 323 bp were provided. Besides, *L. tropica* HSP70 gene was examined and two sections and three pieces with sizes 182, 243, 321bp were delivered.

DNA sequencing

PCR products of HSP70 from five isolates, 2 samples from *L. tropica* and three samples from *L. major* were purified from the agarose gel, by PCR purification kit (Bioneer, Korea), and sequenced from both directions (Applied Biosystems, DNA Analyzers Sequencing, Bioneer, Korea, Sanger method), using the same primers used in PCR. Multiple alignments were performed with data related to *Leishmania* spp. from Iran and other countries deposited in GenBank using BioEdit Sequence Alignment Editor Vr. 7.2.5 software. A Neighbor-Joining tree was constructed using MEGA6 software. Bootstrap analyses (using 1,000 replicates) were carried out to define the robustness of the finding.

Results

Suspected CL patients samples were microscopically surveyed at first. Out of 130 suspected patients, 59 (45.3%) were positive by the microscopic examination. DNA was extracted from all patients and 64 (49.2%) samples were positive. Twenty-four (37.5%) patients were from Konarak City and 40 (62.5%) were from Chabahar city (Fig.2) by PCR technique with HSP70 gene. For RFLP-PCR method, PCR products were digested by HAEIII as the restriction enzyme. The results of RFLP-PCR method indicated that 53 (82.8%) of samples patterns were identical to that of *L. tropica* and 11 (17.1%) to that of *L. major* (Fig. 3). The results of phylogenetic analysis of this study are shown in figure 4. Multiple lesions were seen in ZCL forms due to *L. major* and most ulcers were in hands and feet. However, ACL due to *L. tropica* usually accompanied with one lesion in face. The predominant species in these areas was *L. tropica.*

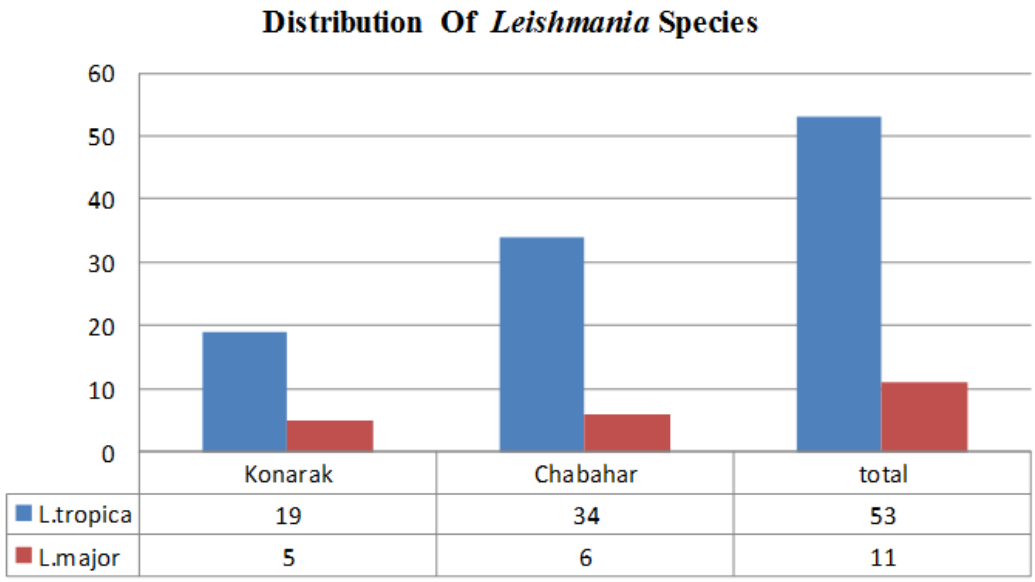

Fig. 2: Distribution of *Leishmania* species using PCR-RFLP based on patients infected study area in Konarak and Chabahar during 2014-2015

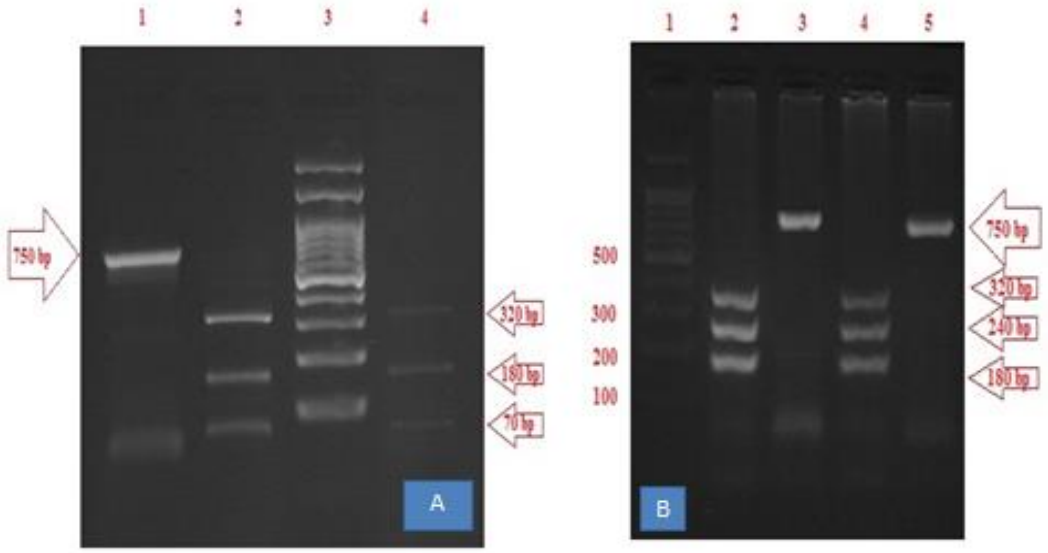

Fig. 3: HSP70-PCR and Restriction fragment length polymorphism (RFLP) patterns obtained from patient's samples.

A) Lane 1 HSP70-PCR banding patterns (750bp)
Lane 2, 4 *L.major* (72bp, 178bp, 183bp, 320bp)
Lane 3 100-bp size marker (Fermentas)

B) Lane 1 100-bp size marker (Fermentas)
Lane 2, 4 *L.tropica* (182bp, 240bp, 320bp)
Lane 5 HSP70-PCR banding patterns (750bp)

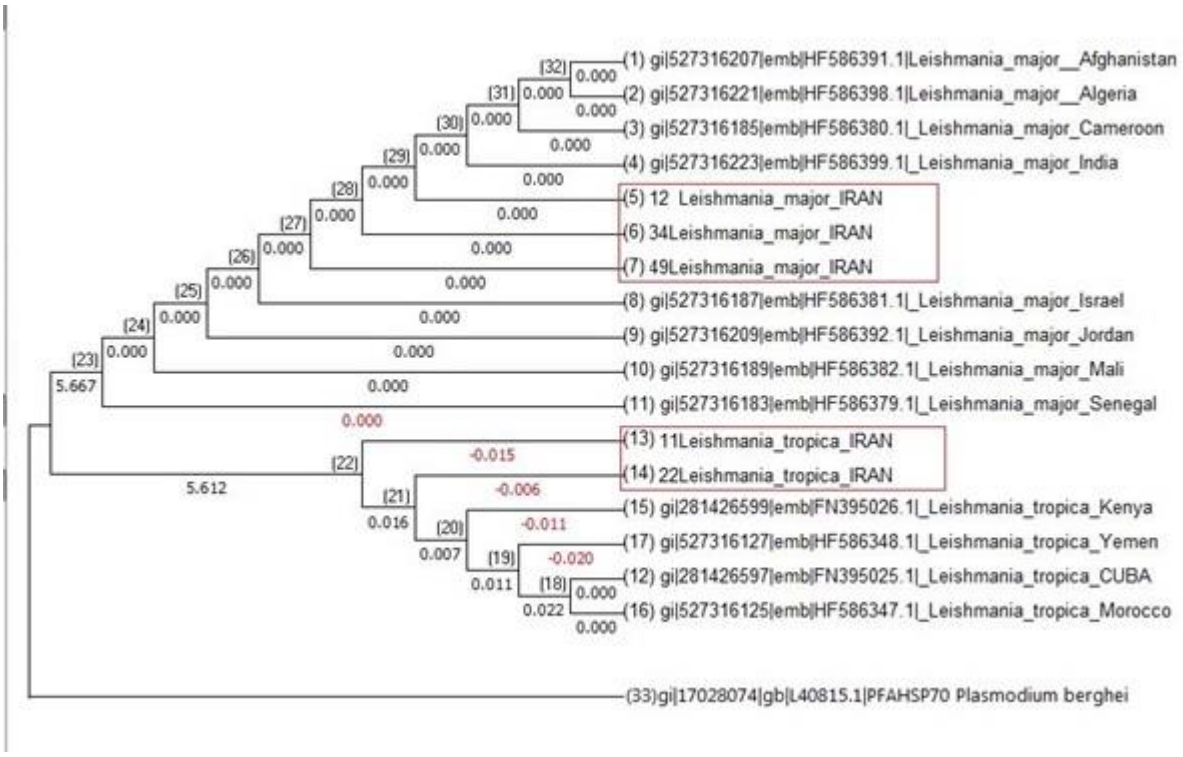

Fig. 4: Phylogenetic relationship of HSP70 sequences of isolates of *L.tropica* and *L.major* from Iran using Neighbor-Joining method. *Plasmodium berghei* (AN: L40815) was used as the out group

Discussion

Leishmaniasis is an important parasitic disease that is life threating for human in endemic areas (13). Cutaneous leishmaniasis is the most prevalent dermatological disease worldwide and considered as one of the challenging health problem in involved countries (14).

Afghanistan, Iran, Syria, Saudi Arabia, Algeria, Brazil and Peru are containing more than 90% of CL new cases, which occur annually (15). Iran is one of the endemic areas for CL so that in more than half of the population are at risk (16) and 20-40/100,000 cases are annually reported (17, 18). ACL (dry or urban) and ZCL (wet or rural) are two common forms of cutaneous leishmaniasis in Iran (19). Because of the different strategies of prevention and control, identification of the species is crucial (20).

CL has been emerged in recent years in Konark and Chabahar, Sistan and Baluchestan areas in southeast of Iran. These border regions have geographically important because of the business, tourism industry and exchanging visitors from neighboring countries. CL is an emerging disease in these areas, the main cause specie was not distinguished up to now, and medical doctors have relied on the clinical manifestations whereas the clinical symptoms are variable and may be confused with other etiological causes (20). Molecular-based techniques are valuable for identification of the species of *Leishmania* (21). PCR has been used widely for detection of infection, and identification of *Leishmania* species in CL, visceral leishmaniasis and other forms of the disease (22-24).

Phylogenetic studies in *Leishmania* needs more focusing on molecular markers; for this purpose (ITS) 1 and 2, cytochrome oxidase II gene, glycoprotein 63 gene, cysteine protease B genes, cytochrome B gene, heat shock protein genes and suchlike have been used for

analyzing phylogenetic issues in *Leishmania*. (25).

Heat shock proteins (HSPs) are likely to play important roles in the differentiation of the promastigotes in sandflies from to amastigotes in mammalian hosts (26-28). HSPs, which seem to be implicated in the adaptation and survival of the cell to heat and other stress conditions (29). Among HSPs, HSP70 is the most highly conserved in sequence and function in all organisms (30). Fraga et al. introduced an important and novel technique for identification of *Leishmania* species with sequencing of HSP70 regions (31). They are the best-conserved sequences along the evolutionary tree of life (32). The HSP70 gene is being widely used as a target for PCR-RFLP assays for *Leishmania* species differentiation because of its lower rate of genetic diversity than other markers for instance *gp63* or rDNA ITS genes (9, 33).

In several provinces of Iran CL is reported and to identification of the leishmaniasis species in several studies different laboratory methods have been performed (34, 35). In Mashhad Province, using ITS-PCR and PCR-RFLP, the dominant causative agent of CL was *L. tropica* (36-38). In Shush City in Khuzestan and in Shiraz Province by Nested PCR, the dominant species was *L. major* (35, 39).

In several studies, the molecular methods were used to identify *Leishmania* species in the patients, vectors and reservoirs. In Brazil recognition of *Leishmania* parasites showed PCR-RFLP profiling of the hsp70 gene as a valuable tool for recognizing *Leishmania* species related with human leishmaniasis (33). In Japan, *Leishmania* species was identified in the sand flies using PCR (40). In Spain, detection of *Leishmania* parasite in biopsies from 100 dogs using PCR was shown that in 63% of the dogs, *Leishmania* DNA could be detected by PCR in at least in one of the tissues studied (41). In France, *Leishmania* species was identified in the mouse tissues using Real-Time PCR (42).

For identification of *Leishmania* species isolates from Konark and Chabahar, Sistan and Baluchestan Province, we used HSP-70 as an ideal object for classification of different *Leishmania* species through the PCR-RFLP method. This method has been applied and showed to be valuable for diagnosis and identification of *Leishmania* (9, 12, 31, 33, 43-46).

In the current study, from 130 suspected CL patients infected, 59 (45.3%) were positive by the microscopic examination while using HSP70-PCR 64(49.2%) samples were positive. In a study, from 155 suspected CL patients, 69 (44.5%) cases were positive by microscopic examination and 86 (55.4%) were positive by ITS-PCR (47).

According to similar study conducted on samples from different parts of Iran, *L. major* is the most important causative agent of CL in Khuzestan, Ilam, Kermanshah and Semnan, however (48). *L. tropica* is reported from Kurdistan Province as the major cause of CL. Our study showed similar results; in Tehran, North Khurasan, Esfahan, Kerman, Fars and Razavi Khurasan both *L. tropica* and *L. major* were identified (48).

Additionally, there is not any report of *L. aethiopica*, *L. Mexicana* complex and *L. braziliensis* complex in human (48-50). Nevertheless in this area of study to identify the species of *Leishmania* parasite limited molecular approaches was carried out and there is not correct map for this issue so this study was conducted as a pilot.

Conclusion

PCR-RFLP is a valuable technique for distinguish of *Leishmania* species. Furthermore, Sistan and Baluchestan is one of the important foci of ACL and also ZCL and unlike to other study in these area, ACL is the dominant cause of CL. Free trading area, immigrants from Pakistan and Afghanistan country, Kerman and Yazd provinces (foci of ACL); are some reasons that *L. tropica* is the dominant

species. Additionally, dogs as a reservoir hosts *Phlebotomus sergenti* as a most prevalent sand fly in these areas are helping *L. tropica* to be a dominant species.

Meanwhile *L. tropica* (the dominant species.) is an anthroponotic parasite, therefore in order to control the disease, it is suggested to focus on chemotherapy of patients and for *L. major* should have to pay more attention on immigrants from rural areas and visitors from neighboring countries (Pakistan and Afghanistan) and rodents eradication.

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References

1. Nadim A JE, Mohebali M, Momeni AZ. *Leishmania* parasite and leishmaniasis. Tehran: Markaz Nashr Daneshgahi Press.; 2009.
2. Shirzadi M, Esfahani S, Mohebali M, Ershadi M, Gharachorlo F, Razavi M, Postigo J. Epidemiological status of leishmaniasis in the Islamic Republic Of Iran, 1983-2012. East Mediterr Health J. 2015;21:736-42.
3. Shirzadi M. Epidemiological information of cutaneous and visceral leishmaniasis in Iran. Report of the constructive meeting on cutaneous leishmaniasis. WHO, Geneva. 2008:7-8
4. Hamid K, SaiedReza N, Mehdi M, Ezat-Aldin J. Molecular characterization of *Leishmania* infection in sand flies from Sistan Va Baluchistan Province, Southeastern Iran. Jundishapur J Microbiol. 2012;2012:430-431.
5. Kassiri H. Bionomics of phlebotomine sand flies (diptera: Psychodidae) as vectors of leishmaniasis in the county of Iranshahr, Sistan-Baluchistan Province, southeast of Iran. Arch Clin Infect Dis. 2012;6:112-116.
6. Boggild AK, Miranda-Verastegui C, Espinosa D, Arevalo J, Martinez-Medina D, Llanos-Cuentas A, Low DE. Optimization of microculture and evaluation of miniculture for the isolation of *Leishmania* parasites from cutaneous lesions in Peru. Am J Trop Med Hyg. 2008;79:847-852.
7. Reithinger R, Dujardin J-C. Molecular diagnosis of leishmaniasis: Current status and future applications. J Clin Microbiol. 2007;45:21-25.
8. Yehia L, Adib-Houreih M, Raslan WF, Kibbi AG, Loya A, Firooz A, Satti M, El-Sabban M, Khalifeh I. Molecular diagnosis of cutaneous leishmaniasis and species identification: Analysis of 122 biopsies with varied parasite index. J Cutan Pathol. 2012;39:347-355.
9. Garcia L, Kindt A, Bermudez H, Llanos-Cuentas A, De Doncker S, Arevalo J, Tintaya KWQ, Dujardin JC. Culture-independent species typing of neotropical *Leishmania* for clinical validation of a pcr-based assay targeting heat shock protein 70 genes. J Clin Microbiol. 2004;42:2294-2297.
10. Li Z, Srivastava P. Heat-shock proteins. Current Protocols in Immunology. 2004:A. 1T. 1-A. 1T. 6.
11. Rico AI, Angel SO, Alonso C, Requena JM. Immunostimulatory properties of the *Leishmania infantum* heat shock proteins hsp70 and hsp83. Mol Immunol. 1999;36:1131-1139.
12. Requena JM, Chicharro C, García L, Parrado R, Puerta CJ, Cañavate C. Sequence analysis of the 3'-untranslated region of hsp70 (type i) genes in the genus *Leishmania*: Its usefulness as a molecular marker for species identification. Parasit Vectors. 2012;5:87.
13. Adegboye OA, Kotze D. Disease mapping of leishmaniasis outbreak in afghanistan: Spatial hierarchical bayesian analysis. Asian Pac J Trop Dis. 2012;2:253-259.
14. Ameen M. Cutaneous leishmaniasis: Advances in disease pathogenesis, diagnostics and therapeutics. Clin Exp Dermatol. 2010;35:699-705.
15. Gramiccia M, Gradoni L. The current status of zoonotic leishmaniases and approaches to

disease control. Int J Parasitol. 2005;35:1169-1180.
16. Mohebali M, Javadian E, Yaghoobi Ershadi M, Akhavan A, Hajjaran H, Abaei M. Characterization of *Leishmania infection* in rodents from endemic areas of the Islamic Republic of Iran. East Mediterr Health J. 2004;10:591-9.
17. Mohebali M. Zoonotic protozoa diseases. Tehran, Iran: Nadi Publication. 1996:60.
18. Yaghoobi-Ershadi M, Zahraei-Ramazani A, Akhavan A, Jalali-Zand A, Abdoli H, Nadim A. Rodent control operations against zoonotic cutaneous leishmaniasis in rural Iran. Ann Saudi Med. 2005;25:309-312.
19. Ready PD. Biology of phlebotomine sand flies as vectors of disease agents. Annu Rev Entomol. 2013;58:227-250.
20. Schönian G, Nasereddin A, Dinse N, Schweynoch C, Schallig HD, Presber W, Jaffe CL. Pcr diagnosis and characterization of *Leishmania* in local and imported clinical samples. Diagn Microbiol Infect Dis. 2003;47:349-358.
21. Murray HW, Berman JD, Davies CR, Saravia NG. Advances in leishmaniasis. Lancet. 2005;366:1561-1577.
22. Tavares R, Staggemeier R, Borges A, Rodrigues M, Castelan L, Vasconcelos J, Anschau M, Spalding SM. Molecular techniques for the study and diagnosis of parasite infection. J Venom Anim Toxins incl Trop Dis. 2011;17:239-248.
23. Yavar R, Abedin S, Reza AM, Ali OM, Sina R, Mehdi M, Reza Y-EM, Fatemeh M, Babak F. *Phlebotomus papatasi* and *Meriones libycus* as the vector and reservoir host of cutaneous leishmaniasis in Qomrood District, Qom Province, Central Iran. Asian Pac J Trop Dis. 2011;4:97-100.
24. Jombo G, Gyoh S. Unusual presentations of cutaneous leishmaniasis in clinical practice and potential challenges in diagnosis: A comprehensive analysis of literature reviews. Asian Pac J Trop Dis. 2010;3:917-921.
25. Fraga J, Montalvo AM, Van der Auwera G, Maes I, Dujardin J-C, Requena JM. Evolution and species discrimination according to the *Leishmania* heat-shock protein 20 gene. Infect Genet Evol. 2013;18:229-237.
26. Zilka A, Garlapati S, Dahan E, Yaolsky V, Shapira M. Developmental regulation of heat shock protein 83 in *Leishmania* 3' processing and mrna stability control transcript abundance, and translation is directed by a determinant in the 3'-untranslated region. J Biol Chem. 2001;276:47922-47929.
27. Bente M, Harder S, Wiesgigl M, Heukeshoven J, Gelhaus C, Krause E, Clos J, Bruchhaus I. Developmentally induced changes of the proteome in the protozoan parasite *Leishmania donovani*. Proteomics. 2003;3:1811-1829.
28. Krobitsch S, Clos J. A novel role for 100 kd heat shock proteins in the parasite *Leishmania donovani*. Cell Stress Chaperones. 1999;4:191-8.
29. Lindquist S. The heat-shock response. Annu Rev Phys Chem. 1986;55:1151-1191.
30. Gupta RS, Golding GB. Evolution of hsp70 gene and its implications regarding relationships between archaebacteria, eubacteria, and eukaryotes. J Mol Evol. 1993;37:573-582.
31. Fraga J, Montalvo AM, De Doncker S, Dujardin J-C, Van der Auwera G. Phylogeny of *Leishmania* species based on the heat-shock protein 70 gene. Infect Genet Evol. 2010;10:238-245.
32. Folgueira C, Requena JM. A postgenomic view of the heat shock proteins in kinetoplastids. FEMS Microbiol Rev. 2007;31:359-377.
33. da Silva LA, de Sousa CdS, da Graça GC, Porrozzi R, Cupolillo E. Sequence analysis and pcr-rflp profiling of the hsp70 gene as a valuable tool for identifying *Leishmania* species associated with human leishmaniasis in Brazil. Infect Genet Evol. 2010;10:77-83.
34. Hejazi H, Nasrifar P, Jamali S, Jahangirnezhad A, Khamesipour A. Identification of *Leishmania* species using monoclonal antibodies in Isfahan. Iran J Dermatol. 2000;1:7-11.
35. Maraghi S, Samarbaf Zadeh A. Identification of cutaneous leishmaniasis agents by nested-pcr in Shush City, Khozestan Province, Iran. Iran J Parasitol. 2007;12:13-15.
36. Vaeznia H, Dalimi A, Sadraei J, Pirstani M. Determination of *Leishmania* species causing cutaneous leishmaniasis in Mashhad by pcr-rflp method. Arch Razi Inst. 2009;64:39-44.
37. Reza MM, Masoud M, Jalil TA, Taghae SM, Javad YPM, Fariba B, Abdolmajid F. Molecular identification of *Leishmania* species

causing cutaneous leishmaniasis in Mashhad, Iran. Jundishapur J Microbiol. 2010;2010:195-200.
38. Pirstani M, Sadraei J, Dalimi A, Vaeznia H. Determination of *Leishmania* species causing cutaneous leishmaniasis in Mashhad by pcr-rflp method. Arch Razi Inst. 2016;64:39-44.
39. Razmjou S, Hejazy H, Motazedian MH, Baghaei M, Emamy M, Kalantary M. A new focus of zoonotic cutaneous leishmaniasis in Shiraz, Iran. Trans R Soc Trop Med Hyg. 2009;103:727-730.
40. Kato H, Uezato H, Katakura K, Calvopiña M, Marco JD, Barroso PA, Gomez EA, Mimori T, Korenaga M, Iwata H. Detection and identification of *Leishmania* species within naturally infected sand flies in the andean areas of ecuador by a polymerase chain reaction. Am J Trop Med Hyg. 2005;72:87-93.
41. Solano-Gallego L, Morell P, Arboix M, Alberola J, Ferrer L. Prevalence of *Leishmania infantum* infection in dogs living in an area of canine leishmaniasis endemicity using pcr on several tissues and serology. J Clin Microbiol. 2001;39:560-563.
42. Nicolas L, Prina E, Lang T, Milon G. Real-time pcr for detection and quantitation of *Leishmania* in mouse tissues. J Clin Microbiol. 2002;40:1666-1669.
43. Van der Auwera G, Maes I, De Doncker S, Ravel C, Cnops L, Van Esbroeck M, Van Gompel A, Clerinx J, Dujardin J. Heat-shock protein 70 gene sequencing for *Leishmania* species typing in european tropical infectious disease clinics. Euro Surveill. 2013;18:20543.
44. Montalvo A, Fraga J, Maes I, Dujardin J-C, Van der Auwera G. Three new sensitive and specific heat-shock protein 70 pcrs for global *Leishmania* species identification. Eur J Clin Microbiol Infect Dis. 2012;31:1453-1461.
45. Khanra S, Datta S, Mondal D, Saha P, Bandopadhyay SK, Roy S, Manna M. Rflps of its, its1 and hsp70 amplicons and sequencing of its1 of recent clinical isolates of kala-azar from India and Bangladesh confirms the association of *L. tropica* with the disease.Acta Trop. 2012;124:229-234.
46. Garcia A, Kindt A, Quispe-Tintaya K, Bermudez H, Llanos A, Arevalo J, Bañuls A, De Doncker S, Le Ray D, Dujardin J. American tegumentary leishmaniasis: Antigen-gene polymorphism, taxonomy and clinical pleomorphism. Infect Genet Evol. 2005;5:109-116.
47. 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. Parasitol Res. 2008;103:1159-1162.
48. Hajjaran H, Mohebali M, Mamishi S, Vasigheh F, Oshaghi MA, Naddaf SR, Teimouri A, Edrissian GH, Zarei Z. Molecular identification and polymorphism determination of cutaneous and visceral leishmaniasis agents isolated from human and animal hosts in Iran. Biomed Res Int. 2013;2013 :789326
49. Talari SA, Talaei R, Shajari G, Vakili Z, Taghaviardakani A. Childhood cutaneous leishmaniasis: Report of 117 cases from Iran. Korean J Parasitol. 2006;44:355-360.
50. Rahbarian N, Mesgarian A, Mahmoudi Rad M, Hajaran H, Shahbazi F, Mesgarian Z, Taghipour N. Identification of *Leishmania* species isolated from human cutaneous leishmaniasis using pcr method. J Res Health Sci. 2009;9:48-51.