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Original Article

Isolation and Molecular Identification of *Leishmania* spp. in Patients With Cutaneous Leishmaniasis in Golestan Province, Iran

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Abstract

Background and aims: Cutaneous leishmaniasis is a major health issue in many parts of Iran. Diagnosis of cutaneous leishmaniasis in a non-endemic area is not as simple as that in endemic foci. The management and treatment of this disease are global dilemmas. The purpose of this study was to identify Leishmania species isolated from human cutaneous leishmaniasis lesions in patients referred to health centers of Golestan province, Iran.

Methods: Cutaneous leishmaniasis patients with suspected lesions were clinically examined. History of journey to the endemic areas of zoonotic cutaneous leishmaniasis and/or anthroponotic cutaneous leishmaniasis and the characteristics of their lesion(s) were recorded. Diagnosis of the lesion was done by using direct smear microscopy and conventional polymerase chain reaction.

Results: Out of 360 patients, 202 (57.4 %) were male and 158 (42.8 %) were female. A total of 360 samples were selected from different cities. The number of infected samples in the cities is as follows: Azad Shahr [3 (1.5%)], Aq Qala [11 (5.7%)], Ali Abad [4 (2.1%)], Bandar Turkmen [3 (1.5%)], Bandar Gaz [2 (1%)], Ramian [3 (1.5%)], Kalaleh [23 (12.1%)], Kord koy [1 (0.5%)], Galikesh [7 (3.7 %)], Gomishan [12 (3.6 %)], Gorgan [13 (6.8 %)], Gonbad Qabus [99 (52.1 %)], Marave Tappe [7 (3.6%)] and Minoodasht [2 (1%)]. In the molecular investigations, 186 (96.8%) samples were observed to acquire rural cutaneous leishmaniasis, 4 (2.1%) to acquire urban cutaneous leishmaniasis and 170 (47.2%) to be uninfected.

Conclusion: Although history of visiting endemic areas is an important factor to be considered in diagnosis, parasitological confirmation is necessary for the initiation of treatment.

Keywords: Cutaneous Leishmaniasis, Golestan province, PCR, Iran.

Introduction

Leishmaniasis is a globally widespread infection for which no effective drug, vaccine, and pesticide have yet been provided and has no sterile immunity. Efforts in this field have not yet been successful. Leishmaniasis, according to the World Health Organization, is among the 6 leading infectious diseases in tropical regions across the world.[1] Leishmaniasis is endemic in 98 countries from 4 continents of the world (22 countries in Europe and America and 66 countries in Asia and Africa), and is regarded as the most important disease of tropical and subtropical regions after malaria.[2]

Different forms of the disease include self-limiting cutaneous leishmaniasis (CL), muco-cutaneous leishmaniasis (MCL), visceral leishmaniasis (VL) and diffuse CL (DCL). CL is the most common form and is caused by *Leishmania major, L. tropica,* and *L. aethiopica* as the main agents.[3] The common forms of CL in Iran are caused by *L. major* and *L. tropica* that are referred to as zoonotic CL (ZCL) and anthroponotic CL (ACL), respectively.[4] With an average incidence of over 150/100 000 population, CL had the highest level of increase in Iran from 2002 (n: 13729) to 2006 (n: approximately 24 000).[5] Although diagnosis of the typical form of CL in sporadic areas is not difficult, diagnosis of the atypical form of CL in sporadic areas is difficult. Atypical forms of the disease resemble various skin disorders such as ecthyma, tuberculosis, furuncle, atypical mycobacterium infections, deep mycosis, sarcoidosis, leprosy, syphilis, foreign body granuloma, and even some cases of malignant skin tumors, justifying the initiation of the treatment only when the lesion has been parasitologically confirmed. Diagnosis of CL is routinely made based on the presence of *L. major* amastigotes in a

direct smear prepared by scratching of the periphery of a suspected lesion. And research in endemic areas is usually carried out by using culture on Novy–Nicolle–McNeal (NNN) medium in addition to direct smear.[3,6] Surveys of molecular epidemiology suggest the sensitivity and specificity of the identification of CL forms. The purpose of this study was to identify *Leishmania* species isolated from patients with suspected diagnosis of CL referred to health centers in Golestan province using ITS1 PCR-RFLP.

Methods

Area of the Study

This study was conducted in Golestan province, northeast of Iran. Its area is approximately 20367.3 km^2, with the population of about 1 777 014. The capital of the province is Gorgan. The most important industries of the province are agriculture and animal husbandry (Figure 1).

Patients

A total of 360 patients with skin diseases and suspected diagnosis of CL were included in this study. The patients lived in one of the cities of Azad Shahr, Aq Qala, Ali Abad, Bandar Turkmen, Bandar Gaz, Ramian, Kaleh, Kordkuy, Galikesh, Gomishan, Gorgan, Gonbad Kavoos, Marave Tappeh and Minoo Dasht. All patients included in this study completed the consent form and were separately interviewed to fill out the checklist containing the demographic data and other information including the number of lesions, duration of the disease, and treatment(s) received. The procedure was based on National Protocol for Diagnosis and Treatment of CL, developed in accordance with the WHO recommendations. Each patient was separately interviewed and the characteristics of his/her lesion(s) were recorded.

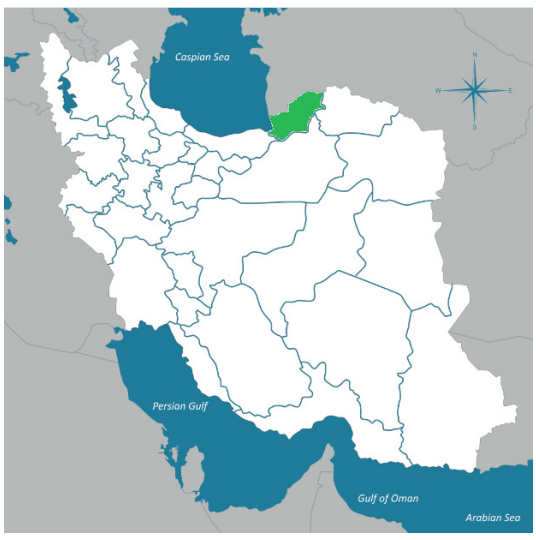

Figure 1. Location of Golestan Province in Iran.

Sample Collection

After cleaning and sterilizing of the skin using 70° ethyl alcohol, sample collection was done by scrapping of the margin lesion. Samples were used for slide examination and DNA extraction. From each patient's sample, three slides were prepared: one slide for Giemsa staining and microscopic examination, one for DNA extraction, and one for storage.

Slide Examination

One slide regarding to each patient was fixed with methanol and then stained with Giemsa (10%) and and examined microscopically for presence of amastigotes. Observing *Leishman* body or Amastigote in each slide showed the positive sample and reported CL.

DNA Extraction

All positive and negative slides were used for DNA extraction using the DNA GenAll Exgene Cell SV (#106-101, Gen All, Korea) according to the manufacturer's instructions. The extracted DNA was analyzed qualitatively and quantitatively by using spectrophotometer and agarose gel electrophoresis, respectively.

Detection and Identification

Genus detection was done using ITS1-PCR by the specific primers LITSR (5′-CTG GAT CAT TTT CCG ATG- 3′) and L58S (5′-TGA TAC CAC TTA TCG CAC TT-3′). Amplification reactions were performed in 25 µL containing the end concentration of 1x PCR buffer, 0.5 nM each primer, 0.2 mM dNTP, 1.5 mM MgCl2, and 5 mM DNA. The conditions of amplification were as follows: An initial denaturation at 94°C followed by 40 cycles of denaturation at 94°C, annealing at 50°C, and extension at 72°C, each for 45 seconds. The final extension was done at 72°C for 5 min. The PCR product was assessed using 1% agarose gel electrophoresis alongside 50 bp DNA ladder. The species identification was performed by RFLP analysis using *HaeIII* restriction enzyme at concentration of 10 U in each reaction solution of 20 µL volume. The digestive assessment was done using 3% agarose gel electrophoresis alongside 50 bp DNA ladder.

Statistical Analysis

The data were analyzed using the SPSS version 19. Chi-square test was used for statistical analysis of qualitative variables. Differences were considered to be statistically significant at P values of less than 0.05.

Results

To determine the species of *Leishmania*, PCR products were digested by the enzyme *Hae III* (Figure 2). In

addition to the standard samples of *L. major* and *L. tropica*, agarose gel electrophoresis was also used. After digesting the PCR products with the *Hae III* enzyme, 140 bp and 220 bp bands were observed in the *L. major,* and 80 bp and 200 bp bands were observed in the *L. tropica* (Figure 3).

A total of 360 samples were selected, including 3 (1.5%) samples from Azad Shahr, 11 (5.7%) from Aq Qala, 4 (2.1%) from Ali Abad, 3 (1.5%) from Bandar Turkmen, 2 (1%) from Bandar Gaz, 3 (1.5%) from Ramian, 23 (12.1%) from Kalaleh, 1 (0.5%) from Kord Koy, 7 (3.7%) from Galikesh, 12 (3.6%) from Gomishan, 13 (6.8%) from Gorgan, 99 (52.1%) from Gonbad Qabus, 7 (3.6 %) from Marave Tappe, and 2 (1%) from Minoodasht.

All 360 samples were collected from suspected patients referred to the health centers of the studied cities. The lesions of all patients were parasitologically examined. Microscopic observations showed that 180 (50%) of patients were negative and 180 (50%) were positive for CL; 170 (47.2%) were negative and 190 (52.7%) were positive for CL by the molecular method; 186 (96.8%) lesions of the patients were induced by *L. major,* and 4 (2.1%) lesions were induced by *L tropica*. The comparison of *L. major* and *L. Tropica* species in microscopic and molecular methods was conducted by the chi-square test which showed that $P=0.000$. A total of 190 samples were positive for CL by the molecular method, of which 118 (62.1%) cases were from the male patients and the rest from the female ones. The age range of the patients was reported to be from 6 months to 85 years. The number of wounds in patients varied from 1 to 30, and the number (percentage) of patients with 1-3 wounds was 145 (76.3%), 4-6 wounds 33 (17.3%), 7-9 wounds 6 (3.1%) 10-12 wounds 5 (2.6%) and more than 12 wounds 1 (0.5. %). Out of the 190 samples, 11 (5.8%) were related to the wounds of the face, 18 (5.9%) to the wounds of the hands and feet, 36 (18.9%) to the wounds of the hands, 78 (41%) to the wounds of the feet, and 47 (24.7%) to the wounds of the hands, feet and face ($P=0.209$) (Figure 4).

The studied diseases developed in July [1 (0.5%)], August [13 (6.8%)], September [49 (25.8%)], October [42 (22.1%)], November [47 (24.7%)], December [34 (17.9%)], January [2 (1%)], February [1 (0.5%)] and March [1 (0.5%)]. The size of the wounds varied from 2 mm to 50 mm, including the wounds of 1-3 mm in 91 (47.9%) samples, 4-6 mm in 30%, 7-9 mm in 11.6%, 10-12 mm in 2.6%, and larger than 12 mm in 15 (7.9%) samples. Regarding occupations, 43 (22.6%) cases were students, 30 (15.8%) were housewives, 30 (15.8%) were children, 23 (12.1%) were farmers, 15 (7.9%) were workers, 1 (0.5%) was employee, and the rest [30 (15.8%)] ($P=0.278$) (Figure 5).

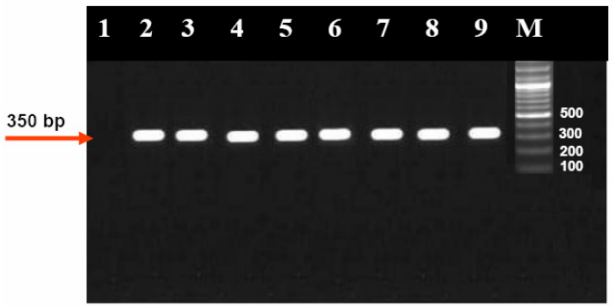

Figure 2. Agarose-Gel Electrophoresis for Evaluation of PCR Products; Lane 1. Negative control, Lanes 2-7. Isolated patient, Lane 8. *L. major,* Lane 9. *L. tropica,* Lane M. 50 bp DNA-ladder.

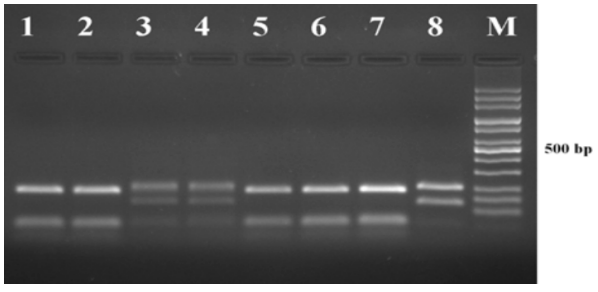

Figure 3. Restriction enzyme digestion profile of amplified ITS1 region with the restriction enzyme of *HaeIII*. Lanes 1 and 2: *L. major* isolated from patient, Lanes 3 and 4: *L. tropica* isolated from patient, Lanes 5 and 6: *L. major* isolated from patient, Lane 7: *L. tropica*, Lane 8: *L. major*, Lane M; 50 bp DNA-Ladder.

Discussion

Leishmaniasis is one of the important diseases many researchers have investigated from various aspects.[7] The persistence of this disease has been reported from various parts of the world due to immigration, population growth, infected *Phlebotomus* spp., and suitable environment.[8] *Leishmania* types have usually been diagnosed based on clinical symptoms and geographical regions, which is not reliable due to the variety of species in a particular geographical region. Direct microscopic examination has been a standard technique for diagnosis of CL in several years as a simple and inexpensive technique; however, it is not as effective as new molecular methods in terms of sensitivity. Further, this technique is not able to diagnose the different species of CL.[6,9] New parasites are different from those existing two or three decades ago because of drug resistance in some types of parasites. Determining the exact species of parasites plays an important role in correcting the epidemiological variety and in designing short-term and long-term strategies by planners and health policy makers to prevent, control and treat the parasitic infections. *Leishmania* includes a relatively large number of species and strains, which necessitates exploring its medical, veterinary and economic effects. In addition, it causes numerous diseases that are more challenging to control and prevent than those caused by other parasites. Given the difficulties facing the detection of parasite morphology, the present study was conducted to identify isolates using DNA-based techniques by means

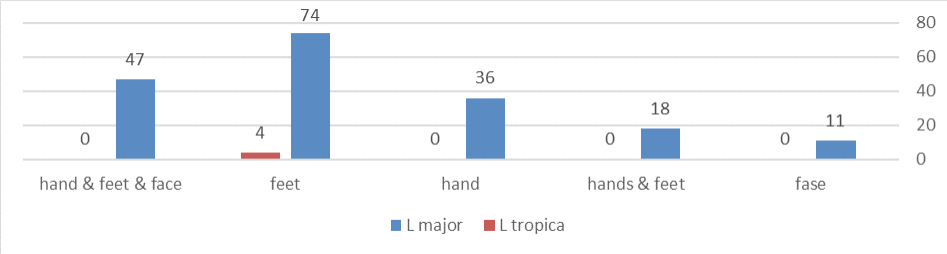

Figure 4. Frequency Distribution of *L. major* and *L. tropica* According to the Wound Site. *P*=0.209 (chi-square test).

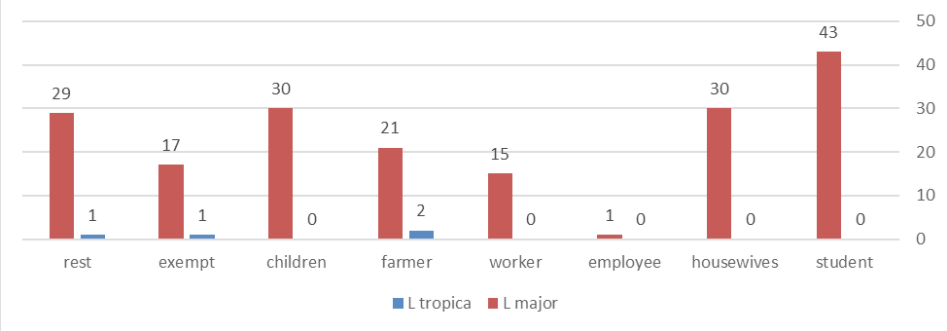

Figure 5. Frequency Distribution of L. major and L. tropica According to Patients' Jobs. *P*=0.278 (chi-square test).

of genetically engineered enzymes and genetic markers of ribosomal DNA *ITS1* sequences. Finally, the current study was carried out to identify the species of *Leishmania* parasite in stained slides of patients with suspected CL in Golestan province using the PCR-RFLP technique.[10,11] Given the PCR-RFLP technique results, from 360 studied isolates, 190 (52.7%) isolates were positive, of which 186 (97.8%) were *L. major* and 4 (2.2%) *L. tropica*. At present, *L. major* is considered the dominant species isolated from all isolates of Golestan province. In addition to the reported positive cases by direct smear microscopy, 32 cases that were previously diagnosed as positive using the PCR-RFLP technique were negative. The study of Spotin et al indicated that sensitivity of direct observation of parasites by means of theoptical microscope and PCR-RFLP technique was obtained 76.9% and 81.8%, respectively.[12] In agreement with our study, other investigations reported high sensitivity for the PCR-RFLP technique.[2,13] This technique is one of the best methods used in epidemiological and clinical studies to determine polymorphisms and identify microorganisms. In this technique, to extract sufficient DNA, first, the gene sequence is amplified, and then these parts are cut using the end nuclease enzymes and investigated by using specific probes. This method is one of the most successful, specific, sensitive and reliable techniques to determine and classify the parasite species that have been introduced as valid techniques in most of the diagnostic and epidemiological studies.[10,14,15] The advantage of the ITS1-PCR technique is that it enables researchers to diagnose all *Leishmania* types using the RFLP technique by means of only one enzyme. The aforementioned technique is a sensitive and valuable one in the chronic conditions in which the pathogens are not detectable using the simple microscope.[16] Ghasemloo et al[17] presented KDNA-PCR as a more sensitive method than ITS1 and microscopic methods. Additionally, Yehia et al compared three analyses of PCR for markers of ITS1, SLME and KDNA to diagnose CL, and reported sensitivity of 98.7% and 91% for KDNA-PCR and ITS1-PCR, respectively.[16] Although KDNA-PCR is one of the most sensitive methods, it is time-consuming and costly because it has 10 000 DNA minicircles in each parasite. The identification of each type requires separate primers of *Leishmania*.[16] In the study of Karimian et al,[19] 2 analyses of PCR were done for genetic markers of ITS1 and KDNA. Findings showed that the sensitivity of KDNA was far less than that of the ITS1.[18] Roelfsema reported that PCR was more sensitive by using ITS1 region than mini-exon.[19] Another objective of this study was to investigate the genetic differences between the *L. major* and *L. tropica* species. As already mentioned, genetic changes in parasitic protozoa are highly important in medicine.[15] In recent decades, various genetic methods using PCR techniques have been introduced that are efficient and sensitive means to identify species and strains of *Leishmania* parasites.[15,20-22] In the present study, the PCR-RFLP technique was utilized to identify the *Leishmania* type. First, the *ITS1* gene was multiplied using two primers called LITSr and L5.8s that were

classified as *Leishmania* when the isolate of 350 bp fragment was observed. Next, 2 types of *L. major* and *L. tropica* were isolated from the isolates using *Hae III* enzyme. In some studies, specimens that showed 220 and 140 bp fragments after digesting with the *Hae III* enzyme were considered *L. major*.[17,23] However, using the PCR-RFLP, LITSR and L58S primers, as well as applying *Hae III* enzyme on PCR products, Eslami et al[24] presented 210 and 140 bp fragments for *L. major*, which is consistent with this study.[24] In agreement with the present study, El-Beshbishy et al also reported 132 and 200 bp fragments for *L. major*.[25] According to the findings of Al-Nahhas, the existence of 60 and 200 bp fragments after digestion with *Hae III* on PCR products was considered *L. tropica*.[26] El-Beshbishy et al utilized the PCR-RFLP technique and *Hae III* enzyme on PCR products and identified 57 and 200 bp fragments as *L. tropica*, which is consistent with our study.[25] Considering the map of digestion of the *Hae III* enzyme, four fragments of 200, 64, 55 and 20 bp were produced likely by enzymatic digestion of *L. tropica*; however, only 60 bp fragment was detectable due to the small size of the first fragment (20 bp) and the overlapping of 2 fragments with molecular weights of 64 and 55 bp.[27] In conclusion, because of its high sensitivity and specificity, the molecular method seems to be useful to determine the epidemiology map for different species and to plan for controlling and preventing this disease. The history of visiting or living in known endemic areas is an important factor to suspect a lesion as CL. In order to better identify the pattern of transmission, molecular studies should be conducted quinquennially.

Ethical Approval

The study protocol was approved by the Research Ethics Committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran (code: IR.SSU.REC.1394.531).

Conflict of Interest Disclosures

None.

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References

1. Mirzaei F, Bafghi AF, Mohaghegh MA, Jaliani HZ, Faridnia R, Kalani H. In vitro anti-leishmanial activity of Satureja hortensis and *Artemisia dracunculus* extracts on Leishmania major promastigotes. J Parasit Dis. 2016;40(4):1571-4. doi: 10.1007/s12639-015-0730-9.
2. Hajjaran H, Vasigheh F, Mohebali M, Rezaei S, Mamishi S, Charedar S. Direct diagnosis of *Leishmania* species on serosity materials punctured from cutaneous leishmaniasis patients using PCR-RFLP. J Clin Lab Anal. 2011;25(1):20-4. doi: 10.1002/jcla.20377.
3. Mirzaie F, Eslami G, Yosefi MH, Pestehchian N. Molecular identification of Leishmania isolates obtained from patients suspected as having cutaneous leishmaniasis referred to reference laboratories from Yazd province in central Iran. Adv Biomed Res. 2013;2:92. doi: 10.4103/2277-9175.122525.
4. Nemati S, Fazaeli A, Hajjaran H, Khamesipour A, Anbaran MF, Bozorgomid A, et al. Genetic diversity and phylogenetic analysis of the Iranian *Leishmania* parasites based on HSP70 gene PCR-RFLP and sequence analysis. Korean J Parasitol. 2017;55(4):367-74. doi: 10.3347/kjp.2017.55.4.367.
5. Edrisiyan GH. Visceral Leishmaniasis in Iran and the role of serological tests in the diagnosis and epidemiological studies. Journal of Kerman University of Medical Sciences. 1996;3(2):97-108.
6. Khatami A, Firooz A, Gorouhi F, Dowlati Y. Systematic review of New World cutaneous leishmaniasis: several points. Int J Dermatol. 2009;48(2):201; author reply -2. doi: 10.1111/j.1365-4632.2009.03971.x.
7. Alvar J, Velez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and global estimates of its incidence. PLoS One. 2012;7(5):e35671. doi: 10.1371/journal.pone.0035671.
8. Yaghoobi-Ershadi MR, Hanafi-Bojd AA, Javadian E, Jafari R, Zahraei-Ramazani AR, Mohebali M. A new focus of cutaneous leishmaniasis caused by Leishmania tropica. Saudi Med J. 2002;23(3):291-4.
9. Tashakori M, Kuhls K, Al-Jawabreh A, Mauricio IL, Schonian G, Farajnia S, et al. *Leishmania major*: genetic heterogeneity of Iranian isolates by single-strand conformation polymorphism and sequence analysis of ribosomal DNA internal transcribed spacer. Acta Trop. 2006;98(1):52-8. doi: 10.1016/j.actatropica.2006.01.010.
10. Tesh RB, Modi GB. A simple method for experimental infection of phlebotomine sand flies with Leishmania. Am J Trop Med Hyg. 1984;33(1):41-6.
11. Lachaud L, Marchergui-Hammami S, Chabbert E, Dereure J, Dedet JP, Bastien P. Comparison of six PCR methods using peripheral blood for detection of canine visceral leishmaniasis. J Clin Microbiol. 2002;40(1):210-5.
12. Spotin A, Rouhani S, Ghaemmaghami P, Haghighi A, Zolfaghari MR, Amirkhani A, et al. Different morphologies of *Leishmania major* amastigotes with no molecular diversity in a neglected endemic area of zoonotic cutaneous leishmaniasis in Iran. Iran Biomed J. 2015;19(3):149-59.
13. Monroy-Ostria A, Nasereddin A, Monteon VM, Guzman-Bracho C, Jaffe CL. ITS1 PCR-RFLP diagnosis and characterization of *Leishmania* in clinical samples and strains from cases of human cutaneous leishmaniasis in states of the Mexican Southeast. Interdiscip Perspect Infect Dis. 2014;2014:607287. doi: 10.1155/2014/607287.
14. Rotureau B. Ecology of the leishmania species in the Guianan ecoregion complex. Am J Trop Med Hyg. 2006;74(1):81-96.
15. Schonian G, Nasereddin A, Dinse N, Schweynoch C, Schallig HD, 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.
16. Yehia L, Adib-Houreih M, Raslan WF, Kibbi AG, Loya A, Firooz A, et al. Molecular diagnosis of cutaneous leishmaniasis and species identification: analysis of 122 biopsies with varied parasite index. J Cutan Pathol. 2012;39(3):347-55. doi: 10.1111/j.1600-0560.2011.01861.x.
17. Ghasemloo H, Rasti S, Delavari M, Doroodgar A. Molecular Diagnosis of Clinical Isolates of Cutaneous Leishmaniasis

Using ITS1 and KDNA Genes and Genetic Polymorphism of Leishmania in Kashan, Iran. Pak J Biol Sci. 2016;19(3):136-42. doi: 10.3923/pjbs.2016.136.142.
18. Karamian M, Kuhls K, Hemmati M, Ghatee MA. Phylogenetic structure of Leishmania tropica in the new endemic focus Birjand in East Iran in comparison to other Iranian endemic regions. Acta Trop. 2016;158:68-76. doi: 10.1016/j.actatropica.2016.02.010.
19. Roelfsema JH, Nozari N, Herremans T, Kortbeek LM, Pinelli E. Evaluation and improvement of two PCR targets in molecular typing of clinical samples of Leishmania patients. Exp Parasitol. 2011;127(1):36-41. doi: 10.1016/j.exppara.2010.06.024.
20. Gasser RB, Stevenson LA, Chilton NB, Nansen P, Bucknell DG, Beveridge I. Species markers for equine strongyles detected in intergenic rDNA by PCR-RFLP. Mol Cell Probes. 1996;10(5):371-8. doi: 10.1006/mcpr.1996.0050.
21. Chargui N, Bastien P, Kallel K, Haouas N, Akrout FM, Masmoudi A, et al. Usefulness of PCR in the diagnosis of cutaneous leishmaniasis in Tunisia. Trans R Soc Trop Med Hyg. 2005;99(10):762-8. doi: 10.1016/j.trstmh.2005.06.002.
22. Jirku M, Zemanova E, Al-Jawabreh A, Schonian G, Lukes J. Development of a direct species-specific PCR assay for differential diagnosis of *Leishmania tropica*. Diagn Microbiol Infect Dis. 2006;55(1):75-9. doi: 10.1016/j.diagmicrobio.2005.12.001.
23. Behravan MR, Moin-Vaziri V, Haghighi A, Rahbarian N, Taghipour N, Abadi A, et al. Molecular identification of *Leishmania* species in a re-emerged focus of cutaneous leishmaniasis in Varamin district, Iran. J Arthropod Borne Dis. 2017;11(1):124-31.
24. Eslami G, Anvarii H, Ebadi M, Nabipour Y, Mirzaei F, Gholamrezaei M, et al. The changing profile of cutaneous leishmaniasis agent in a central province of Iran. Tanzan J Health Res. 2013;15(1):33-9.
25. El-Beshbishy HA, Al-Ali KH, El-Badry AA. Molecular characterization of cutaneous leishmaniasis in Al-Madinah Al-Munawarah province, western Saudi Arabia. Int J Infect Dis. 2013;17(5):e334-8. doi: 10.1016/j.ijid.2012.11.015.
26. Al-Nahhas SA, Kaldas RM. Characterization of *Leishmania* species isolated from cutaneous human samples from central region of Syria by RFLP analysis. ISRN Parasitol. 2013;2013:308726. doi: 10.5402/2013/308726.
27. Marfurt J, Nasereddin A, Niederwieser I, Jaffe CL, Beck HP, Felger I. Identification and differentiation of *Leishmania* species in clinical samples by PCR amplification of the miniexon sequence and subsequent restriction fragment length polymorphism analysis. J Clin Microbiol. 2003;41(7):3147-53.

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