Phylogenetic study of treatment failure clinical isolates of Leishmania major

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Short report

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Abstract

Background: Molecular characteristics are necessary for designing programs for the control, prevention, and treatment against cutaneous leishmaniasis. In the present study, treatment failure (TF) clinical isolates of *Leishmania major* were phylogenetically analyzed using the barcode genes of *cytochrome oxidase* II (*COXII*) and 13A/B.

Methods: Samples were collected from 126 patients referred to the Diagnosis Laboratory Center from October 2017 to December 2019. All TF cases were assessed using *COXII* and 13A/B, and phylogenetic analysis was created using BLASTn, T-COFFEE, and MEGA 7.0.21.

Results: All 126 isolates were *L. major*, in which 5 isolates were TF. All isolates had successfully amplified by the specific primer for *COXII* region. The alignment analysis with all the 5 TF isolates and the standard Friedlin strain showed more than 98% similarity. Phylogenetic analysis with 13A/B showed that all 5 TF isolates are clustered in one group, although phylogenetic analysis showed different clustering after comparison of 5 TF isolates with 16 TR isolates and the same regions in other isolates in GenBank, NCBI.

Conclusions: All 21 isolates studied in this study, comprising TF and TR isolates, were clustered in near groups. However, 13A/B could differentiate the 5 TF isolates from selected 6 TR isolates, completely.

Background

Leishmaniasis is a common disease in the tropical and subtropical regions of the world that has a wide range of clinical symptoms caused by a protozoan from *Leishmania* spp. and transmitted by *Phlebotomus* spp. The symptoms vary from spontaneous cutaneous lesion to lethal visceral form [1, 2]. According to reports from the World Health Organization (WHO), more than 350 million people in over 98 countries are exposed to leishmaniasis. A total of 2 million new infections occur annually, with an estimated 0.7 to 1.5 million cases of cutaneous leishmaniasis [3]. CL is endemic in various parts of Europe, Africa, and Asian countries, particularly in Central Asia and the Middle East. Leishmaniasis is prevalent in many parts of Iran such as Tehran, Mashhad, Tabriz, Kerman, Isfahan, Neyshabur, Bam, and Yazd. CL mainly occurs in urban and rural forms, mainly caused by *L. tropica* and *L. major*, respectively.

Initially, *Leishmania* detection and identification were based on the size, the shape of the wound, geographic location, and injection to the laboratory animals [4]. These methods were unable to accurately identify the actual parasite species, so molecular techniques were used as a suitable alternative method. Molecular techniques also help to find out the genetic characterization of the parasite, which can be considered to result in precise and appropriate planning and control, as well as efficient vaccine and drug development [5]. Various target genes used in this field include a variety of mitochondrial and nucleus barcodes [6, 7].

Treatment of CL is considered one of the best strategies for control of the disease [8]. Recently, some cases of treatment failure (TF) have been reported from different foci of the world [9-11], which reported
different genetic analysis. It seems that molecular characterization and phylogenetic studies of TF isolates can help to find suitable strategies for vaccine development, prevention, and drug design. In this study, TF clinical isolates obtained from patients with CL were characterized molecularly and assessed phylogenetic analysis using one of the important mitochondrial barcodes named *Cytochrome oxidase II (COXII)* and the other important nucleous barcode gene named *13A/B*.

**Methods**

*Ethical statement*

The informed consent was completed by each patient participating in this study before recording the information and sampling based on the Helsinki declaration. This study was approved by the Ethics Committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

*Population study*

This study included a population of patients (126 cases) with zoonotic CL caused by *L. major* that five of them had no response to Glucantime, which referred to the laboratory of Isfahan Diagnosis Center, Iran, from October 2017 to December 2019. The patients with *L. major* were followed for three months. The cases with no responses to Glucantime treatment were considered as TF isolates. The cases with the response to Glucantime were considered as treatment response (TR).

*Sampling*

Samples were collected by scrubbing the lesion edge after disinfecting by using 70% alcohol. Two slides were prepared for either direct microscopic examination or molecular analysis for each patient.

*Detection and identification*

A microscopic examination was carried out to find the Leishman body. The DNA was extracted from stained smears using the DNA extraction kit (GeneAll, South Korea) based on manufacturer instructions. The quantity of the isolated DNA was evaluated using a spectrophotometer by nanodrop (ABI, USA). With the aim of verification of *Leishmania* spp., ITS1-PCR was used using LITSr-F 5'‐CTGGATCATTTTCCgATg-3' and L5.8s-R 5'‐TGATACCACTTATCGCACTT-3' [12]. Each amplification reaction mixture included 1x PCR buffer, 0.2 mM dNTP, 1.5 mM MgCl₂, 1.5 U *Taq* DNA polymerase, 0.5 µM each primer, and 100 ng DNA. The amplification temperature included the first denaturation 94 °C for 5 min that followed by 35 cycles of 94 °C for 45 s, 50°C for 45 s, and 72 °C for 45 s. The final elongation was done by 72 °C for 5 min. Positive and negative controls were considered in each run using *L. major* (MRHO/IR/75/ER) and ddH₂O, respectively. The amplification analysis was done using agarose gel electrophoresis (1%) alongside with 50 bp DNA ladder. The expected amplicon was about 300-350 bp for verification of *Leishmania* spp. detection. Molecular identification was performed on positive amplicons digested using *Hae III (Bsu RI)* for 3 h at 37 °C. The digestion banding pattern analysis was assessed using 3% agarose gel
electrophoresis alongside with 50 bp DNA ladder. *L. major* (MRHO/IR/75/ER) was considered as positive control. A banding pattern revealing two main bands of 220 and 140 bp was considered as *L. major*. All tests were done in triplicate.

*Molecular analysis of barcode gene of COXII*

The primer pair of *COXII* was COXII-F 5'-GCATAAATCCATGAAAACACCACA-3' and COXII-R 5'-TGGCTTTTATTTATCATTTTTGAATG-3' [13]. Each amplification reaction included 1x PCR buffer, 0.2 mM dNTP, 1.5 mM MgCl₂, 1.5 U Taq DNA polymerase, 0.5 µM each primer, and 100 ng DNA. The amplification protocol was the first denaturation at 94 °C in 1 cycle, followed by 35 cycles of 94 °C for 60 s, 57 °C for 20 s, and 72 °C for 30 s. The final elongation was done by 72 °C for 5 min. Positive and negative controls were considered in each run with *L. major* (MRHO/IR/75/ER) and ddH₂O, respectively. The amplification analysis was done using agarose gel electrophoresis (1.5%) alongside with 50 bp DNA ladder. The expected amplicon was 602 bp.

*Molecular analysis of barcode gene of 13A/B*

The primers of 13A/B and the reaction conditions were obtained from the study by Aghai-Maybodi et al. [14]. Positive and negative controls were considered in each run with *L. major* (MRHO/IR/75/ER) and ddH₂O, respectively. The amplification analysis was done using agarose gel electrophoresis (3%) alongside with 50 bp DNA ladder. The expected amplicon was 120 bp.

*Sequencing and molecular analysis*

The amplicons of the target barcode gene of *COXII* in 21 cases, including 5 TF and 16 TR cases were purified and sequenced. The amplicons of the target gene of 13A/B regarding 5 TF isolates and 6 TR isolates were purified and sequenced. The sequences were repaired and BLAST. All the studied sequences were submitted in the GenBank database using BankIt (NCBI). The related sequences were searched from GenBank (NCBI) using BLASTn, https://blast.ncbi.nlm.nih.gov/Blast.cgi [15]. Multiple alignments were done using T-COFFE. The phylogenetic analysis was done using MEGA 7.0.21 [16]. The evolutionary history was inferred using the Neighbor-Joining method [17]. The evolutionary distances were computed using the Maximum Composite Likelihood method [18] and were in the units of the number of base substitutions per site. All cases were eliminated if they had gaps and missing data. Bootstrap of 1000 was performed to estimate the node reliability for *COXII* and 500 for 13A/B. Sequences of *COXII* were compared to seven entries retrieved from GenBank, including KU680818.1, KU680819.1, KF815210.1, KF815211.1, EU140338.1, AF287688.1, and EF633106.1. The sequences of 13A/B regarding to 5 TF and 6 TR isolates were compared each other and phylogenetic tree was drawn.

**Results**

*Molecular analysis of COXII*
All 126 cases were successfully amplified using the primer pair of COXII (Supplementary file 1). The 21 isolates, including 5 TF and 16 TR, which were selected for sequencing, were sequenced successfully. All of them were submitted at GenBank, NCBI, using BankIt with the accession numbers of MH443402.2, MK972457.1, MH443393.2, MK972459.1, MH443400.2, MH443404.2, MH443399.2, MH443397.2, MH443396.2, MH443391.2, MH443398.2, MH443394.2, MH443403.2, MH443390.2, MH443401.2, MH443389.2, MK972460.1, MK972461.1, MH443395.2, MK972458.1, and MH443388.2. The five TF cases were MK972457-MK972461.

Phylogenetic tree analysis

Sequences of the COXII region from all 21 sequences were analyzed using nBLAST, T-COFFE, multiple alignments, and Mega 7.0.21 software. The phylogenetic trees of each isolate were drawn by the UPGMA method. The multiple alignments of five TF isolates' sequences were done with the Friedlin strain of L. major (Supplementary file 2). Accordingly, all five isolates have conserved areas in most sites compared with the European standard L. major (Friedlin), being significantly different from the standard European strain in several nucleotides except in terminal regions. Isolate 2 with the dispersed point in two nucleotides differs from the European standard strain and all isolates are dissimilar with the European standard strain in four nucleotides. Using the similarity matrix indicated that isolates 1-5 had the highest genetic similarity of nearly 100% (99.996%) with the European standard strain. The phylogenetic tree drawn based on the COXII locus shows this high genetic similarity by placing all the isolates in a single cluster (Supplementary file 2).

All 21 isolates were considered for phylogenetic tree analysis alongside the same region of COXII from some other isolates in the database of GenBank, NCBI, including KF815208.1, KY407539.1, KU680818.1, KU680819.1, KF815210.1, KF815211.1, EU140338.1, AF287688.1, and EF633106.1 (Figure 1).

Molecular analysis of 13A/B

Because COXII region could not differentiate the TF and TR isolates in this study, we used 13A/B analysis as one of the important nuclear barcode gene. For this reason, all 5 TE isolates were assessed with six TR isolates (Figure 2). None of the sequences related to 13A/B were deposited in GenBank, NCBI.

Discussion

This study evaluated molecular characterization of 21 isolates of L. major obtained from patients with CL using PCR assays by mitochondrion gene of COXII. All 126 isolates in this study were L. major and amplified COXII region successfully using the specific primer. Sequence analysis of the mentioned region in 21 isolates was clustered in a similar group in comparison with the same region in some other isolates from GenBank, NCBI.

Cytochromes are involved in the electron transport process of the mitochondrial respiratory chain. They are considered one of the most useful genes for molecular and phylogenetic studies [19, 20]. The COXII
has been sown a higher evolutionary rate in some other studies [6, 21]. Because of its multicopy nature, it can be used for clinical material and environmental samples, such as sandflies. Nevertheless, only a few phylogenetic studies of the genus Leishmania have included this marker and its discriminative power has not been established in detail [6, 19, 21]. Pilot studies have shown it to be useful for the discrimination of subgenera or species complexes, but its applicability for species delimitation has to be further studied. In our study, the region of COXII was used for amplification, and the results showed that this region had a suitable sequence for assessing the phylogenetic differences, in addition to being capable of detecting Leishmania with high sensitivity. It exhibited a good replication quality. This region is located on mitochondrial DNA maxicircles with different levels of conservation on DNA, which was also used to determine the heterogeneity of Leishmania and the phylogenetic relationship of Leishmania species [6].

After sequencing the amplified region with COXII in our study, it was found that all isolates had the highest similarity with the European standard strain, along with the identification of genus Leishmania. Isolates had many conserved sites with the European standard L. major and are ultimately conserved in most sites. Except in the terminal regions, all five TF isolates are significantly different from L. major (Friedlin) European standard strain in several nucleotides. The phylogenetic tree drawn based on the COXII locus shows this high genetic similarity by placing all the isolates in a single cluster. Although all isolates are placed in a single cluster suggesting more genetic similarity, T-COFFEE alignment analysis disclosed that these isolates contained many central conserved areas and little changes occurred in these areas during evolution. This finding is similar to that of Aghai-Maybodi et al. [14], who emphasized slight changes in the central conserved areas of Iranian isolates, as confirmed by its comparison with the standard strain. Although previous studies have further highlighted the ability of this primer pair to distinguish between species and subspecies and are used to confirm species differentiation, this was not achieved in this study. A possible reason could be the small size of the amplified sequence because species differentiation cannot be made in this area. Although the primer pairs revealed a high similarity between the amplified Leishmania isolates, it is used in studies on the differentiation between strains in different geographical regions [22]. But this barcode gene could not differentiate the TF and TR isolates in this study. However, the 13A/B region could differentiate them successfully (Figure 2). It seems that the barcode gene of 13A/B could be considered as one the important region for differentiation the isolates with different phenotypes. We recommended using this area for differentiation of TF isolates in some other different geography area.

**Conclusions**

The results showed that the region of COXII was suitable for the identification and phylogenetic analysis in Leishmania spp. All the 21 isolates studied in this study, comprising TF and TR isolates, were clustered in near groups
Abbreviations

Treatment failure – TF
Treatment response – TR
Leishmania - L.
cytochrome oxidase II – COXII
Cutaneous leishmaniasis – CL

Declarations

Ethics approval and consent to participate

The informed consent was completed by each patient participating in this study before recording the information and sampling based on the Helsinki declaration. This study was approved by the Ethics Committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran by the code of IR.SSU.MEDICINE.REC.1396.323.

Consent for publication

Consent form was filled out by all patients.

Availability of data and materials

All data are available.

Competing interests

The authors declare that they have no competing interests

Funding

We declared that the funding body was used for collection, analysis, and interpretation of data, and writing the manuscript.

Authors’ contributions

SH collected the samples, applied the molecular experiments, and wrote the draft of the manuscript, VR analyzed the patient data, MT interpreted the bioinformatics analysis, GE designed the study and contributed in major manuscript writing, SSH supervised the laboratory tests, MJB helped in manuscript writing. All authors read and approved the final manuscript.

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References

1. Eslami G, Hajimohammadi B, Jafari AA, Mirzaei F, Gholamrezai M, Anvari H, et al. Molecular identification of *Leishmania tropica* infections in patients with cutaneous leishmaniasis from an endemic central of Iran. Trop Biomed. 2014;31:592-9.

2. Feiz Haddad MH, Ghasemi E, Maraghi S, Tavala M. Identification of *Leishmania* species isolated from human cutaneous leishmaniasis in Mehran, Western Iran using nested PCR. Iranian J Parasitol. 2016;11:65-72.

3. Mouttaki T, Morales-Yuste M, Merino-Espinosa G, Chiheb S, Fellah H, Martin-Sanchez J, et al. Molecular diagnosis of cutaneous leishmaniasis and identification of the causative *Leishmania* species in Morocco by using three PCR-based assays. Parasit Vectors. 2014;7:420. doi: 10.1186/1756-3305-7-420.

4. Fotouhi-Ardakani R, Dabiri S, Ajdari S, Alimohammadian MH, AlaeeNovin E, Taleshi N, et al. Assessment of nuclear and mitochondrial genes in precise identification and analysis of genetic polymorphisms for the evaluation of *Leishmania* parasites. Infect Genet Evol. 2016;46:33-41. doi: 10.1016/j.meegid.2016.10.011.

5. Khosravi S, Hejazi S, Hashemzadeh M, Eslami G, Darani HY. Molecular diagnosis of old world leishmaniasis: real-time PCR based on tryptaredoxin peroxidase gene for the detection and identification of *Leishmania* spp. J Vector Borne Dis. 2012;49:15-8.

6. Lopes EG, Geraldo Junior CA, Marcili A, Silva RD, Keid LB, Oliveira TM, et al. Performance of conventional PCRs based on primers directed to nuclear and mitochondrial genes for the detection and identification of *Leishmania* spp. Rev Inst Med Trop Sao Paulo. 2016;58:41. doi: 10.1590/S1678-9946201658041.

7. Stevenson LG, Fedorko DP, Zelazny AM. An enhanced method for the identification of *Leishmania* spp. using real-time polymerase chain reaction and sequence analysis of the 7SL RNA gene region. Diagn Microbiol Infect Dis. 2010;66:432-5.

8. Hejazi H, Eslami G, Dalimi A. The parasiticidal effect of electricity on *Leishmania major*, both *in vitro* and *in vivo*. Ann Trop Med Parasitol. 2004;98:37-42.
9. Ahmadian S, Eslami G, Fatahi A, Hosseini SS, Vakili M, Ajamein Fahadan V, et al. J-binding protein 1 and J-binding protein 2 expression in clinical Leishmania major no response-antimonial isolates. J Parasit Dis. 2019;43:39-45. doi: 10.1007/s12639-018-1052-5.

10. Alijani Y, Hosseini SS, Ahmadian S, Boughattas S, Eslami G, Naderian S, et al. Molecular analysis of Aquaglyceroporin 1 gene in non-healing clinical isolates obtained from patients with cutaneous leishmaniasis from central of Iran. J Arthropod Borne Dis. 2019;13:145-52.

11. Eslami G, Zarchi MV, Moradi A, Hejazi SH, Sohrevardi SM, Vakili M, et al. Aquaglyceroporin 1 gene expression in antimony resistance and susceptible Leishmania major isolates. J Vector Borne Dis. 2016;53:370-4.

12. Eslami G, Salehi R, Khosravi S, Doudi M. Genetic analysis of clinical isolates of Leishmania major from Isfahan, Iran. J Vector Borne Dis. 2012;49:168-74.

13. Oliveira DM, Lonardoni MV, Teodoro U, Silveira TG. Comparison of different primes for PCR-based diagnosis of cutaneous leishmaniasis. Braz J Infect Dis. 2011;15:204-10.

14. Aghai-Maybodi M, Eslami G, Tohidfar M, Fattahi Bafghi A, Hosseini SS, Ahmadian S, et al. Molecular characterization of clinical Leishmania major isolates harboring ITS1 homology similar to the one in Crithidia spp. J Isfahan Med School. 2018;36:1261-6.

15. Altschul S, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 1997;25:3389-402.

16. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016;33:1870-4. doi: 10.1093/molbev/msw054.

17. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987;4:406-25.

18. Tamura K, Nei M, Kumar S. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci U S A. 2004;101:11030-5.

19. Ibrahim ME, Barker DC. The origin and evolution of the Leishmania donovani complex as inferred from a mitochondrial cytochrome oxidase II gene sequence. Infect Genet Evol. 2001;1:61-8.

20. Landis JR, Koch GG. The measurement of observer agreement for categorical data. Biometrics. 1977;33:159-74.

21. Cao DP, Guo XG, Chen DL, Chen JP. Species delimitation and phylogenetic relationships of Chinese Leishmania isolates reexamined using kinetoplast cytochrome oxidase II gene sequences. Parasitol Res. 2011;109:163-73. doi: 10.1007/s00436-010-2239-6

22. Martínez LP, Rebollo JA, Luna AL, Cocher S, Bejarano EE. Molecular identification of the parasites causing cutaneous leishmaniasis on the Caribbean coast of Colombia. Parasitol Res. 2010;106:647-52.

Figures
Figure 1

Phylogenetic tree of all 21 isolates mentioned in this study using the mitochondrion barcode gene of COXI. Five isolates with the accession number of MK972457-MK972461 are treatment failure isolates of Leishmania major obtained from patients with cutaneous leishmaniasis. Other 16 treatment response isolates in this study have the accession number of MH443389-MH443404. The other seven isolates were selected from the GenBank, NCBI.
Figure 2

Phylogenetic tree with the target of 13A/B from the five treatment failure isolate and the six treatment response isolates studied in this study. Isolates 1 to 5 were treatment failure and the others were treatment response.

Supplementary Files

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