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**Original Article**

# Molecular Identification and Phylogenetic Analysis of *Leishmania major* Isolated from Zoonotic Cutaneous Leishmaniasis Foci in Northeast Iran

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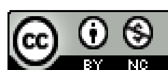
***Abstract***

***Background:*** Epidemiological studies, classification and genetic studies of *Leishmania* species are effective in treatment, control and prevention in endemic areas. We aimed to investigate the genetic diversity and phylogeny of *Leishmania* in Zoonotic foci located in northeastern Iran using *nagt* gene for the first time.

***Methods:*** DNA of 100 confirmed positive slides collected from the health centers of Sarkhes, Darghez, Fariman, Esfarayen, and Sabzevar were extracted during 2020-2021. The partial sequence of kDNA was amplified to identify the species. Twenty-five DNA samples were randomly subjected to amplify by *nagt* gene primes and were sequenced. The sequences were aligned with reference sequences in National Center for Biotechnology Information (NCBI). Then, the genetic similarities of the sequences were checked using Clustalx2.1 software and the phylogenetic tree was drawn by Mega 7 software.

***Results:*** All the positive samples were diagnosed as *L. major*. Approximately, half of the sequences of species were similar to two reference genes JX103550.1:404-712 *L. major* Esfahan and KX759012.1:568-807 *L. Major* Ilam (more than 90% similarity). According to the results of the phylogeny tree, the closest genotype to our study samples was JX103550.1:404-712 *L. major* Esfahan.

***Conclusion:*** The most causative agent CL in these areas was *L. major*. The genetic diversity of *L. major* was high such as other zoonotic foci in Iran. Due to the high similarity of the strains in the study areas with the strains of Isfahan and Ilam, similar control and prevention methods is suggested in these areas.



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## Introduction

Leishmaniasis is the third most significant arthropod-borne disease caused by different *Leishmania* species (1). Recent reports estimate more than 20000 annual leishmaniasis cases, making it a high-risk country for leishmaniasis. Among the three types of the disease (cutaneous, visceral and mucocutaneous), the cutaneous leishmaniasis (CL) is the most common form of the disease which includes about three-fourths of the total cases (2). Several contributing factors could be regarded as the main reasons for increasing trend, including ecological changes, post war disasters, population movement, poor garbage disposal system, insufficient vector and reservoir control (3).

CL is considered as a major public health problem in 88 countries throughout the world, including Iran. Two *Leishmania* species are the main casual agents of CL in Iran; *L. major* and *L. tropica*, which cause zoonotic (ZCL) and anthroponotic CL (ACL), respectively. ZCL is endemic in Esfahan Province, Semnan Province, Khuzestan, Ilam, Fars, Bushehr, Hormozgan Province and some parts of Razavi Khorasan, North Khorasan, South Khorasan. Whereas ACL is endemic in many large cities including Tehran, Shiraz, Kerman, bam and Mashhad (the capital of Razavi Khorasan) (4-6).

DNA sequences show genetic divergence more directly and precisely than traditional protein and DNA polymorphism-based methods for examining evolutional relationship amongst different related species (7). Molecular characterization of the causative agent of CL may facilitate the determining molecular epidemiology and hence can help in introducing taxonomic and population genetic diversity, the prognosis of the parasite, evaluation of a specific treatment, and effective control programs. PCR is a technique for identification and genetic characterization of *Leishmania* (8).

The gene encoding the first step of N-linked glycan biosynthesis, the microsomal membrane enzyme, N-acetylglucosamine-1-phosphate transferase (*nagt*), has been examined previously for species sequence variation in *Leishmania* (9). To evaluate further *nagt* in describing database has been expanded that were previously described (10, 11). This genetic marker has been applied by numerous researchers for comparable studies (12, 13). This gene is a single copy, highly conserved, and functionally indispensable, which make it a suitable marker for species identification and phylogenetic analysis (14).

Studies on phylogenetic analysis of *L. major* are rare in Iran despite the endemicity of the disease (15). The current study was conducted to determine the species in some cities identified as ZCL foci in Razavi and North Khorasan provinces located in northeast of Iran. Then, their relatedness to reference isolated from neighboring cities was evaluated by species identification, and investigation of the genetic diversity of *Leishmania* isolates using the N-acetylglucosamine-1-phosphate transferase (nagt) gene.

## Materials and Methods

### *Ethical statement*

This study was approved by the Ethical Committee of Mashhad University of Medical Sciences (IR.MUMS.MEDICAL.REC.1397.073) in accordance with the Helsinki Declaration and guidelines.

### *The study Population*

This study was conducted on 100 confirmed positive slides for CL. These slides were collected by passive sampling method in equal numbers from the health centers of Sarakhs, Dargaz, Sabzevar, Fairman (Razavi Khorasan province), and Esfarayen (North Khorasan province) located in zoonosis centers of CL in northeastern Iran during 2020-2021. The diagnosis of CL was based on detection of *Leishman* bodies in direct microscopic examination after Geimsa staining.

### *Leishmania gene amplification*

*Leishmania* DNA was extracted from direct slides using a ready commercial kit (Add-bio/Korea) to amplify partial sequence of KDNA to determine the species using forward (F):

(5' TCGCAGAACGCCCCTACC' 3') and reverse (R): (3' AGGGGTTGGTGTAAAATAGG 5' (Tuba Negin, Iran. The PCR products show 615 DNA base pairs (bp) for *L. major* and 744 bp for *L. tropica*. The sharp bands were observed at 60 °C and 2 µM MgCl2 for both species in a total reaction volume of 25 µM (5 µM DNA). Twenty-five DNA samples were randomly subjected to amplify by nagt gene primes for sequencing and phylogenic analysis. PCR technique was used for amplification of *nagt Leishmania* gene. The primers were F: 5'- TCATGACTCTTGGCCTGGTAG -3' and R: 5'-CTCTAGCGCACTTCATCGTAG-3. The amplification was carried out by PCR-ready pre-mix (Taq DNA Polymerase Master Mix amplicon. The PCR was carried out on 25 µl of primers, probe, and sample using BioRad (USA) according to the following instructions; a 5-minute cycle in 95 ºC, 35 final cycles consisting of three stages which are 40 second stage in 94 ºC, a 60 second stage in 58 ºC. This process is followed by final extension at 72 ºC for 60 second. About 5 µl of the products were visualized on the 1% agarose gel (Invitrogen, Life Technologies GmbH, and Germany). Negative and positive controls always monitored the reactions.

### *DNA Sequencing and Sequence Alignment*

To study the genetic diversity and phylogenetic relationship, PCR products of nagt gene were sent to codon company (Iran) for direct sequencing. The result of sequences was aligned with KU680845 references sequences in the National Center for Biotechnology Information (NCBI) using Basic local alignment search tool (BLAST) (http://blast.ncbi.nlm.nih.gov). The identity between the local and reference sequences was calculated using SIAS Sequence Identity And Similarity. Mega 7 software was used to construct the phylogenetic tree for *L. major* local isolates.

## Results

All 100 Giemsa-stained slides samples were positive for the presence of *Leishmania* by microscopy. The molecular analysis of all the samples showed positive results for *L. major* at the primary PCR. The partial sequence of nagt gene of 25 positive DNA samples were randomly amplified for further analysis.

### *Gel Electrophoresis*:

The PCR products were subjected to a 1% agarose gel electrophoresis and examined by exposure to ultra violate light (Fig. 1). *Leishmania* DNA was detected in all 25 microscopically positive samples in the PCR assay; a single 1450 bp band of the *nagt* gene was amplified in all the samples (Fig. 1), which was consistent with the product size of *Leishmania nagt* gene.

### *Sequencing and sequence analysis*

According to alignment results, more than of half of the *Leishmania* parasite isolated form these foci showed 96.37% and 96.45% sequence identity to reference gene from Esfahan (ACC. No. JX103550.1:404-712) and other reference gene from Ilam (ACC. No. KX759012.1:568-807).

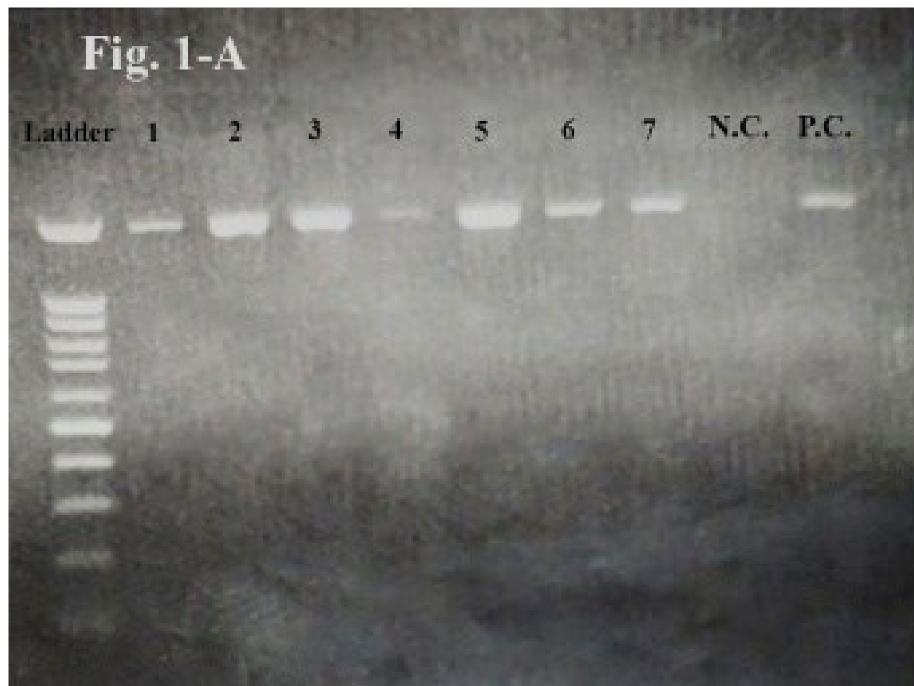


**Fig. 1:** PCR results of *nagt* amplicons of *Leishmania* spp. isolated from Giemsa-stained from ZCL foci in Razavi and North Khorasan provinces. The first line: 1500 ladder marker, Line 1-7: sample of patients. Line 8, 9: negative and positive controls, respectively

### *Phylogenetic Tree*

Mega 7 software was used to construct the phylogenetic tree of the local isolate of *L. major* in relation with four closest reference isolates (Fig. 2). The tree showed five main clades: on containing four reference isolates (from Esfahan and Ilam) and the other clade involving the other reference isolates (Edinburg). There was no clear grouping among the 25 isolates according to their geographical origin (Fig.2). Local isolates from Dargaz and Esfarayen had 99% identity to two reference isolates (Esfahan and Ilam) while other local isolates had 63% identity to reference isolates from Esfahan. (Fig. 2). Phylogenetic trees for Nagt gene constructed by the maximum likelihood method for local *L. major* and 5 reference isolates. Phylogenetic distance was estimated by the Tamura using the neighbor-joining two-parameter model. The tree was supported by bootstrapping with 1000 replicates (16).

According to the phylogenetic tree, the largest number of samples, which include 5 Sarakhs, 2 Sabzevar, 4 Fariman, 2 Esfarayen and 1 Dargaz, were placed in the first clade. A sample of *L. major*-72-nagt Sabzevar was placed in another clade, which was more closely related to the first clade. In the third clade, 2 samples of Esfarayen and 4 samples of Dargaz were placed, which were next to the samples of Ilam and Isfahan references indicating the great similarity of these strains to the mentioned cases. In the fourth clade, which showed more differences, 3 Sabzevar samples were placed (70, 73, 76-nagt Sabzevar). The highest diversity was seen in *L. major* Sabzevar species while the least intraspecies divergence was observed in Fariman and Sarakhs samples.

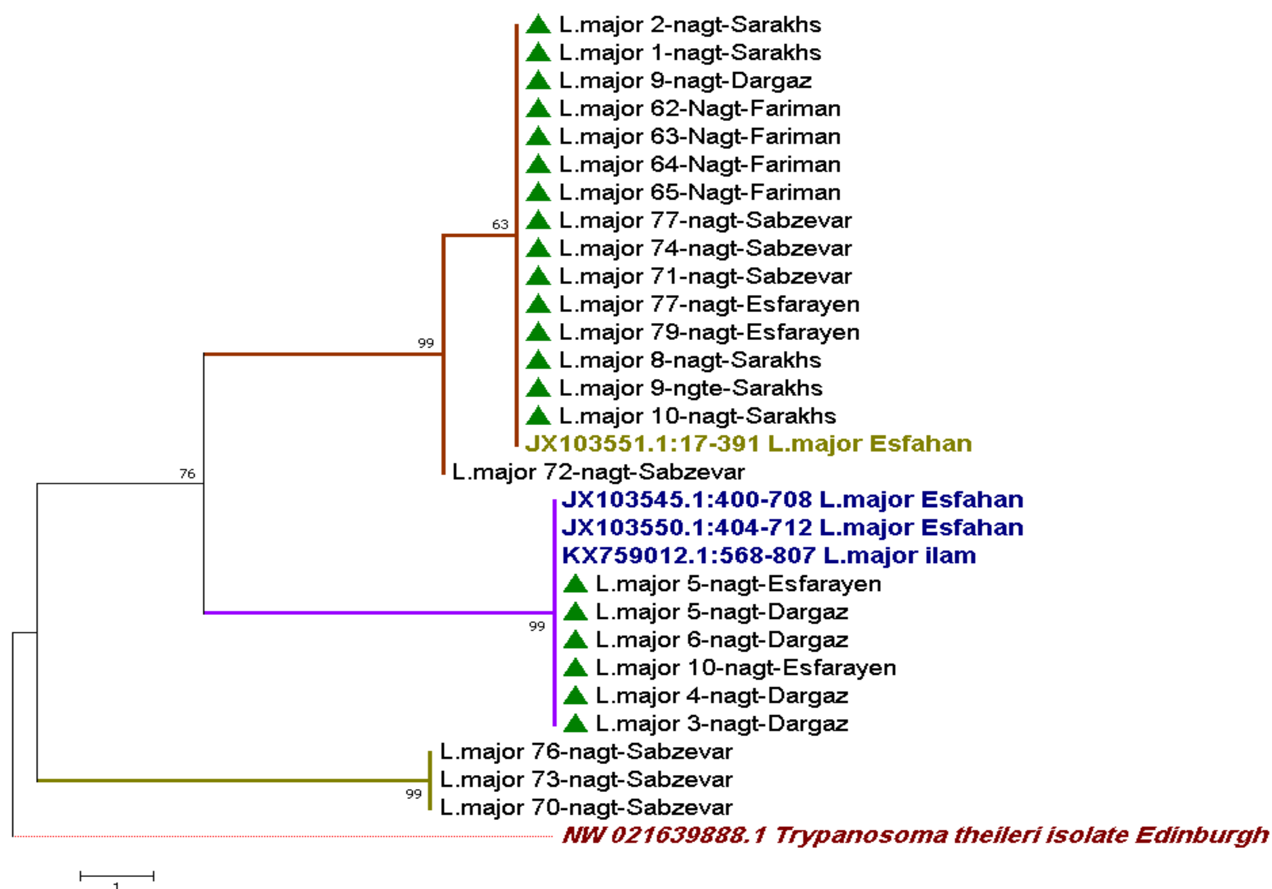


**Fig. 2:** Phylogenetic trees for Nagt gene constructed by the maximum likelihood method for local *L. major* isolates and five reference isolates. Phylogenetic distance was estimated using the maximum likelihood-parameter model. The tree was supported by bootstrapping with 1000 replicates. The numbers above the branches indicate the percentage of bootstrap samplings percentages

## Discussion

The Khorasan province has the highest prevalence of CL infection in Iran. As the province borders with other countries, changes in CL incidence and genetic variation may be associated with neighboring countries. (Change in epidemiology). Identification of *Leishmania* species usually relies on DNA amplification and sequence analysis. Conventional molecular methods using different molecular targets have been used to detect *Leishmania* spices and find genetic diversity in cases from Iranian leishmaniasis foci (17, 18).

In this study, we assessed the causative agents of CL in some ZCL foci of northeast of Iran based on kinetoplast gene. Recently, various molecular assays have been developed to identify species and even assess intraspecific variation. The *Leishmania* N-acetylglucosamine-1-phosphate transferase gene is a single-copy, highly conserved gene of approximately 1450–60 bp that encodes N-acetylglucosamine-1-phosphate transferase. We used nagt sequencing for the investigation of genetic diversity of *Leishmania* isolates in some endemic areas for ZCL located in Razavi Khorasan, and North Khorasan provinces in Iran (19).

The results obtained showed that the *Leishmania* species present in all 100 samples was *L. major*, which is not surprising. In previous

work at these regions, *L. major* was the dominant causative agent of leishmaniasis (20).

Based on phylogenetic tree, the *Leishmania* isolates were grouped into four main clades representing *L. major*. The highest intraspecies divergence was seen in *L. major* Sabzevar. However, Hajjaran et al. (17*)* no intraspecific differences were observed in *Leishmania* isolates in Ilam province using the nagt gene. This could be due to the less relationship of the inhabitants of Sabzevar and the presence of an interspecies divergence of nagt gene.

Different patterns of *L. major* isolates obtained from various endemic areas of Iran and previous reports suggesting heterogeneity of this species in Iran (21). In consistent with our phylogenetic tree shows levels of genetic heterogeneity between some isolates from different endemic areas. The smaller sizes of target fragments of selected genes to construct the phylogenetic tree by other researchers, nagt gene is longer containing fragments that are more different and this matter will be followed by more variation as seen in our phylogenetic tree. These findings are in agreeing with other studies (22).

## Conclusion

The main causative agent of leishmaniasis in Sarakhs, Sabzevar, Fairman, Esfarayen and Dargaz is *L. major,* which has been reported in other studies in Iran as well. The heterogeneity among *L. major* isolates have been observed in these areas and showed similarity with other isolates from ZCL foci in Iran. Hence, the *nagt* gene could be efficiently used to identify intraspecies divergence of *Leishmania* species. Further studies with greater number of samples are suggested to achieve a more comprehensive result on the genetic variation of *L. major* isolates in the study area.

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## Conflict of Interest

The authors declare that they have no conflict of interest

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