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Original paper

Identification and phylogenic analysis of *Leishmania* species among patients and reservoir hosts based on *N-acetylglucosamine-1-phosphate* transferase gene in Central Iran

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ABSTRACT. Leishmaniosis, a vector-born disease that infects humans and other vertebrates, is the result of infection with *Leishmania* species belong to the family Trypanosomatidae. The present study was performed to determine the status of cutaneous leishmaniosis in Isfahan province. Samples were taken from the margin of skin ulcers of patients with suspected CL referred to the medical health centers in Isfahan province. Also, ear and snout samples were taken from the rodents. In total, 85 parasitologically positive samples were subjected to the PCR-RFLP method based on the *nagt* gene for identification of *Leishmania* species, also 11 samples were subjected to sequencing and phylogenetic analysis. For all positive samples, a 1450–1460 bp band of the *nagt* gene was amplified in PCR method. The digestion pattern of *ACC1* enzyme in 79 of patients indicated *L. major* and in one sample was similar to *L. tropica.* Four rodent reservoirs distingue as L. *major* and one sample as *L. turanica.* Phylogenetic analysis confirmed the species identification and three haplotypes were reported. The results of the current study showed that *L. major* is the predominant species of *Leishmania* parasites in Isfahan province and the main reservoir of CL is *Rhombomys opimus*. Also, the *nagt* gene is a useful and practical marker for determining different species of *Leishmania* parasites as well as their phylogenetic analysis.

Keywords: *Leishmania,* zoonotic cutaneous leishmaniosis, *nagt* gene, Isfahan, Iran

Introduction

Cutaneous leishmaniosis (CL) is one of the most important known infectious skin diseases that caused by an intracellular protozoan parasite belonging to the genus *Leishmania*, which is transmitted to the reservoir and hosts by the bite of different species of sandflies preinfected with the *Leishmania* parasite [1]. CL causing various ulcerative lesions which has different clinical patterns, including typical and atypical forms [2,3].

The annual estimated incidence of CL cases is 0.7–1.2 million cases worldwide [4]. In recent years, although more than 20,000 cases of CL are reported annually in Iran, the actual number is probably four or five times higher, indicating Iran as a country at high risk of leishmaniosis [5]. Two main clinical forms of CL, have been reported in Iran, including anthroponotic cutaneous leishmaniosis (ACL) caused by *Leishmania tropica* (*L. tropica*), and zoonotic

Figure 1. The map of study areas in Isfahan province as an old endemic of ZCL in the center of Iran

cutaneous leishmaniosis (ZCL) due to *Leishmania major* (*L. major*) [6]. *Rhombomys opimus, Meriones lybicus* and *Tatera indica* were reported as main reservoirs of ZCL in different foci in Iran. Also humans are the main reservoirs of ACL, but sometimes dogs were suggested as animal reservoirs for this form of disease [6,7]. About 80% of CL cases in Iran include ZCL, which has been reported as a serious health problem in about 25 provinces of the country [6,8].

Isfahan province is located in a fertile plain region in the central part of Iran and it is one of the oldest and most important ZCL foci in the country. In 2012, this province was recognized as one of the centers with high prevalent ZCL cases [9]. The incidence of leishmaniosis has recently increased in this area which can be due to suitable climatic, environmental, and ecological conditions for the activity of reservoirs and vectors [10]. According to the latest studies, the variable trend of leishmaniosis in different years indicates that this disease is endemic in Isfahan province [5]. Based on research conducted in this area *R. opimus* and *M. lybicus* are the main primary and secondary reservoirs of *Leishmania* parasites, respectively [11].

Differentiation between *Leishmania* species is one of the main elements from the epidemiological point of view, as well as accurate prediction, control, and treatment of the disease [4,12]. Due to morphological similarity, *Leishmania* species cannot be differentiated by microscopic and culture methods. PCR-RFLP and sequence analysis of the genome is suitable for the differentiation of all *Leishmania* species. In recent years, the use of specific genetic markers by DNA-based methods, such as protein-coding genes such as *GP63*, *HSPs, CPB*, and *G6PD*, and ribosomal DNA (rDNA) genes such as *ITS, IGS*, and *ETS*, as well as mitochondrial genomes such as *KDNA*, *gRNA*, and *CSB*, have made it possible to identify *Leishmania* parasites and their species [13]. The present study was performed to determine the status of CL in the Isfahan province, central Iran, by determining the main species in patients and host reservoirs and research on the genetic diversity of *Leishmania* isolates using the *N-acetylglucosamine-1-phosphate* transferase (*nagt*) gene [4,14]. This gene is a single copy, functionally indispensable and highly conserved, which encodes the transmembrane protein of the endoplasmic reticulum, so the gene has become a suitable genetic marker for species identification and phylogenetic analysis [14,15]. This marker was used in several similar studies [4,16] and in the present study, for the first time, this marker was used to determine the species of *Leishmania* in Isfahan province.

Table 1. Access numbers of 11 *Leishmania* isolated from CL cases in Isfahan province that identified by *nagt*-PCR-RFLP

No	Isolation source	Disease	*Leishmania* spp.	Accession number
1	Human	CL	*L. major*	MW423814
2	Human	CL	*L. major*	MW423815
3	Human	CL	*L. major*	MW423816
4	Human	CL	*L. major*	MW423817
5	*M. lybicus*	CL	*L. turanica*	MW423818
6	*R. opimus*	CL	*L. major*	MW423819
7	Human	CL	*L. major*	MW423820
8	Human	CL	*L. major*	MW423821
9	Human	CL	*L. major*	MW423822
10	Human	CL	*L. major*	MW423823
11	Human	CL	*L. tropica*	MW423824

Materials and Methods

Study area

The present study was performed in Isfahan province (32.6539°N, 51.6660°E) with an area of 107045 km² and with a population of more than 5 million peoples in the central part of Iran. Several ZCL centers including Isfahan city, Natanz, Ardestan, Varzaneh, Khur o Biabanak Aran and Bidgol, and Kashan counties were monitored in this study [5] (Fig. 1).

Isolation of Leishmania from affected individuals

From September 2019 to February 2020, samples were collected from 97 patients with suspected CL referred to major health centers in Isfahan province. The patients were from different parts of the province where CL is endemic. These samples were taken by scraping the edges of skin ulcers after sterilizing them with 70% ethanol, then at the margin of the lesion, a small incision was made using a lancet and an impression smear was made on a microscopic slide. The exudate smears were dried at room temperature and fixed with methanol, staining was done with 10–15% Giemsa and the presence of amastigotes were examined by direct microscopy method. An aspirate from the same lesion was also cultured in the Novy-McNeal-Nicolle (NNN) and RPMI 1640 (Gibco, Life Technologies GmbH, Germany) supplemented with 15% heat-inactivated fetal bovine serum (FBS), plus 1% pen-strep (100 U/ml penicillin G, 100 µg/ml streptomycin) at the temperature of 24–25°C. Samples that were positive using the parasitological methods (microscopic examination and culture method) were intended for molecular identification.

The animal isolates were obtained from 15 hunted wild rodents as CL reservoir hosts in endemic areas of Isfahan province (Khor and Biabanak, Varzaneh and Borkhar). The rodents were caught alive with Sherman traps. The traps were installed before sunset next to active nests at a distance of 500 meters around the villages of endemic areas and were collected in the next morning. The genus and species of rodents, using external criteria based on morphological identification keys and according to the standard reference of Iranian rodents (body length, tail length, floor position, snout position, and color of different parts of the body, especially the lower part and abdomen) were determined [17]. To detect and culture isolates of *Leishmania* after anesthesia, parasites were obtained from the snouts and earlobes of the hunted rodents. Molecular evaluation of isolates was performed in rodents that were positive for the presence of amastigotes by parasitological methods.

Extraction of DNA

DNA was extracted using the Bioneer extraction kit (Bioneer Company, Korea) according to the manufacturer's instructions. Using a nanodrop spectrophotometer, the quality of the extracted

DNA was determined at 260 and 280 nm. Extracted DNA samples with a ratio of A260/A280, between 1.8 and 2 were selected and stored at –20°C until use.

PCR-RFLP

A partial sequence of the *nagt* gene was amplified, using the specific primers (L1, forward: 5'-TCA TGA CTC TTG GCC TGG TAG-3'; and L4, reverse: 5'-CTC TAG CGC ACT TCA TCG TAG-3'). Amplification was performed by PCR-ready pre-mix (Taq DNA Polymerase Master Mix RED, Denmark). Amplification reactions were adjusted for a total volume of 25 µl, containing 12.5 µl of premix, 1 µl of reverse and forward primer (10 pmol), 3 µl of DNA template, and 7.5 µl of double-distilled water. PCR amplification was programmed in a 96x thermocycler (Peq laboratory, Germany), with one initial denaturation step at 94°C for 1 minute, followed by 35 cycles at 94°C for 1 minute, 58°C for 1 minute then 72°C for 90 seconds, this process was continued by final extension for 5 minutes at 72°C [4,16]. Negative control and DNAs from standard reference strains of *L. turanica* (MRHO/IR/07/Kermanshah/Acc. No. EU395712), *L. major* [MRHO/IR/11/GOL-2 (incident number JN860745)] and *L. tropica* [MHOM/IR/02/Mash10 (incident number EF653267)] which were received as gifts from the School of Health, Tehran University of Medical Sciences, Tehran, Iran, were used as positive controls in all PCR reactions. About 3 µl of the amplicons were observed on the 1% agarose gel (Invitrogen, Life Technologies GmbH, and Germany) using safe nucleic acid staining. Primer sets (L1 and L4) were amplified approximately 1450–1460 bp fragments of *nagt* gene in *Leishmania* genus parasites. Identification of *Leishmania* species were performed using RFLP analysis. *Acetyl coenzyme A carboxylase 1* (*ACC1*) (Xmil) (Fermentas GmbH, Thermo Scientific, Germany) enzyme were created separable fragment sizes for different species of *Leishmania* genus parasites. For this purpose, 10 µl of the PCR product, 1 µl of the *ACC1* (Xmil) enzyme, 2 µl of the enzyme buffer, and 17 µl double distilled water were mixed. The prepared mixture was incubated for 12 hours at 37°C. These fragments were transported on 1.5% agarose gels and TAE (Tris, Acetate, and EDTA), and then their size was determined and visualized next to a marker and obtained bands were identified by a UV detector [14].

Sequencing and phylogenetic analysis of the nagt gene

The obtained PCR products from some patients and infected rodents, were sequenced by the Sanger method, with the same forward and reverse primers used for expansion by Genfanavaran company (info@genfanavaran.com). The sequences were edited manually using the Bio Edit software version 7.1.3.0. to find genetic similarities. Nucleotide sequences obtained from the current study were compared with similar previous DNA sequences deposited in the GenBank, using the Basic Local Alignment Search Tool (BLAST) analysis (http://blast. Ncbi.nlm.nih.gov). Accession numbers of the 27 *Leishmania nagt* sequences deposited in the GenBank that used in this study and out-group isolates are listed in table 2. The nucleotide sequences obtained from the present study were submitted to the GenBank using Sequin. Genetic distance between our sequences was calculated and phylogenetic tree were constructed by the kimura 2-parameter option of the Maximum likelihood method in a complete deletion procedure for sequences, by using Molecular Evolutionary Genetics Analysis Version X (MEGA-X) software. The bootstrap scores were set for 1000 replicates to estimate the robustness of the topologies [18]. The haplotype diversity and polymorphism analysis were calculated using the DNA sequence polymorphism software version6 (DnaSP v6). To investigate the haplotype, sequence alignment with a minimum length of 923 fragments was performed.

Statistical analysis

Demographic sociological data obtained from questionnaires in health centers were statistically analyzed by SPSS software version 16.0.

Ethical approval

The current study was approved by the Ethics Committee of Isfahan University of Medical Sciences, Isfahan, Iran (Approval Number: IR.MUI.MED.REC. 1398. 337, September 23, 2019).

Results

Eighty (80) samples out of 97 CL patients were positive for *Leishmania* parasite using at least one parasitological assay. In the current study, 29 out of 80 (36.25%) patients were females and 51 (63.75%) were males. The age ranged of the patients was from 2 to 92 years old, the patient was not observed for

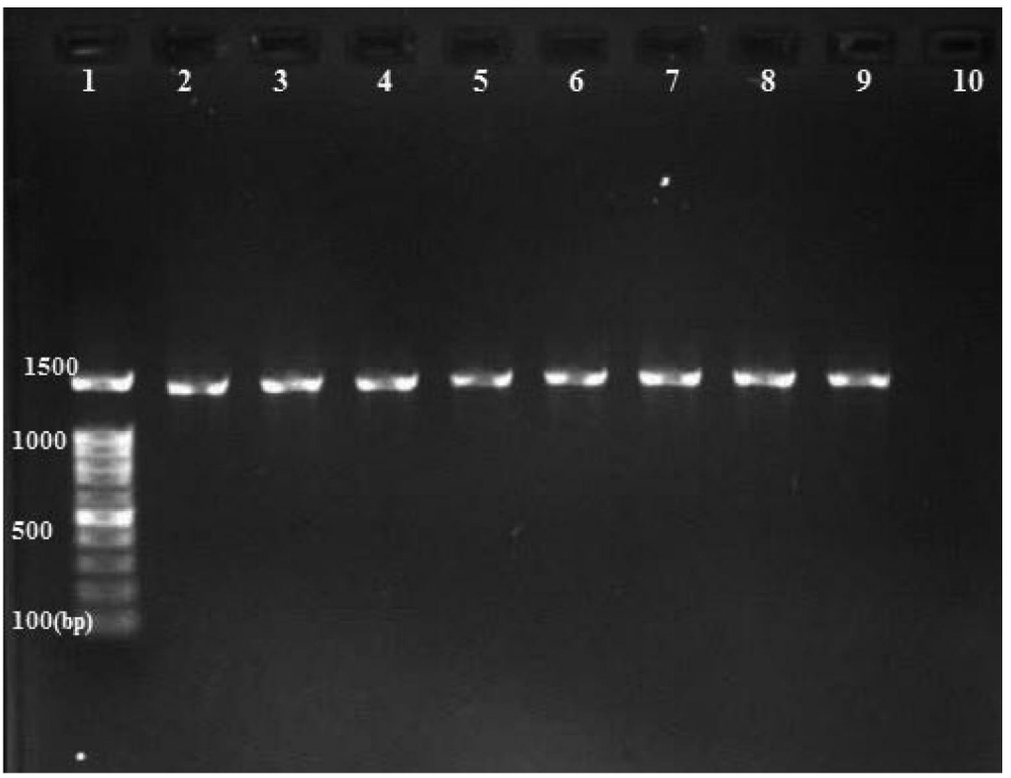

Figure 2. Gel electrophoresis of PCR product of *Leishmania* parasite *nagt* gene isolated from samples of patients with CL and reservoirs in Isfahan province. Line 1: 100 bp marker; Line 2, 3: positive control of *L. major*, *L. tropica*; Lines 4–7: samples of patients; Lines 8, 9: samples of reservoir rodents; Line 10: negative control

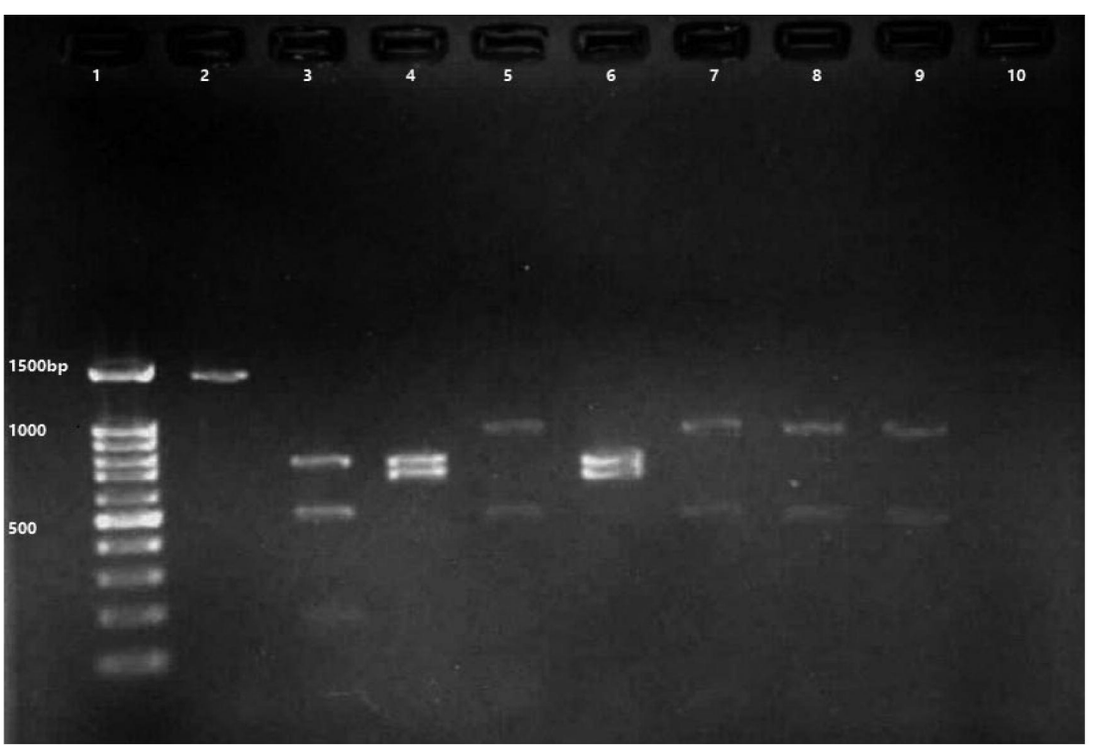

Figure 3. Gel electrophoresis of PCR-RFLP product of standard samples as well as samples of patients with CL and rodent reservoirs in Isfahan province. Line 1: 100 bp marker; Line 2: sample PCR product of patient without enzyme (current study); Lines 3–5: positive control of *L. infantum*, *L. tropica*, *L. major*; Line 6: *L. tropica*; Lines 7–9: *L. major;* Line 10: negative control

Table 2. Accession numbers of *Leishmania* isolates that registered in GenBank and used in this study

No	*Leishmania* spp./out group	Area	Accession number
1	*Leishmania major*	Iran	JX103536.1
2	*Leishmania major*	Iran	JX103519.1
3	*Leishmania major*	Iran	JX103529.1
4	*Leishmania major*	Iran	JX103549.1
5	*Leishmania major*	Iran	KF701205.1
6	*Leishmania major*	Iran	JX103550.1
7	*Leishmania major*	Iran	KU680837.1
8	*Leishmania major*	Iran	KX59012.1
9	*Leishmania major*	Iran	KF701199.1
10	*Leishmania major*	Tunisia	JN400235.1
11	*Leishmania major*	Tunisia	JN400215.1
12	*Leishmania major*	Iran	KX759009.1
13	*Leishmania tropica*	Iran	KU680841.1
14	*Leishmania tropica*	Iran	KU680840.1
15	*Leishmania tropica*	Iran	KF701207.1
16	*Leishmania tropica*	Tunisia	JN400198.1
17	*Leishmania tropica*	Iran	JX648327.1
18	*Leishmania tropica*	Iran	KU680838.1
19	*Leishmania infantum*	Iran	JX103539.1
20	*Leishmania infantum*	Iran	JX103538.1
21	*Leishmania infantum*	Tunisia	JN400203.1
22	*Leishmania infantum*	Iran	KT201383.1
23	*Leishmania gerbilli*	Iran	JX103548.1
24	*Leishmania gerbilli*	Turkey	DQ836158.1
25	*Leishmania turanica*	Turkey	DQ836157.1
26	*Leishmania turanica*	Iran	JX103553.1
27	*Leishmania turanica*	Iran	JX103530.1
28	*Trypanosoma cruzi*		XM807416.1

less than two years and there were only four over 60 years old. The disease was more common in patients between the ages of 1–10 (27.5%). 32 patients (40%) had only one lesion, 38 patients (47.5%) had 2–5 lesions and 10 patients (12.5%) had more than 5 lesions. In five cases out of 10 patients with more than five lesions, more than or equal to ten lesions were observed in different parts of the body. The most common part of the lesion was the hand (45/%), followed by the foot (18.75%), then the face (16/25%), jointly on the hands and feet (15 %) and other parts of the body 5%.

Among 15 captured rodents, one *M. lybicus* and 4 *R. opimus* were positive for *Leishmania* parasite.

Leishmania identification by RFLP analysis

Leishmania DNA was identified in the PCR assay in all 85 positive samples, CL patients (n = 80) and rodents (n = 5). For all positive samples as the positive control a 1450–1460 bp band of the *nagt* gene, were amplified (Fig. 2). *Leishmania* species were identified by the digestion pattern of

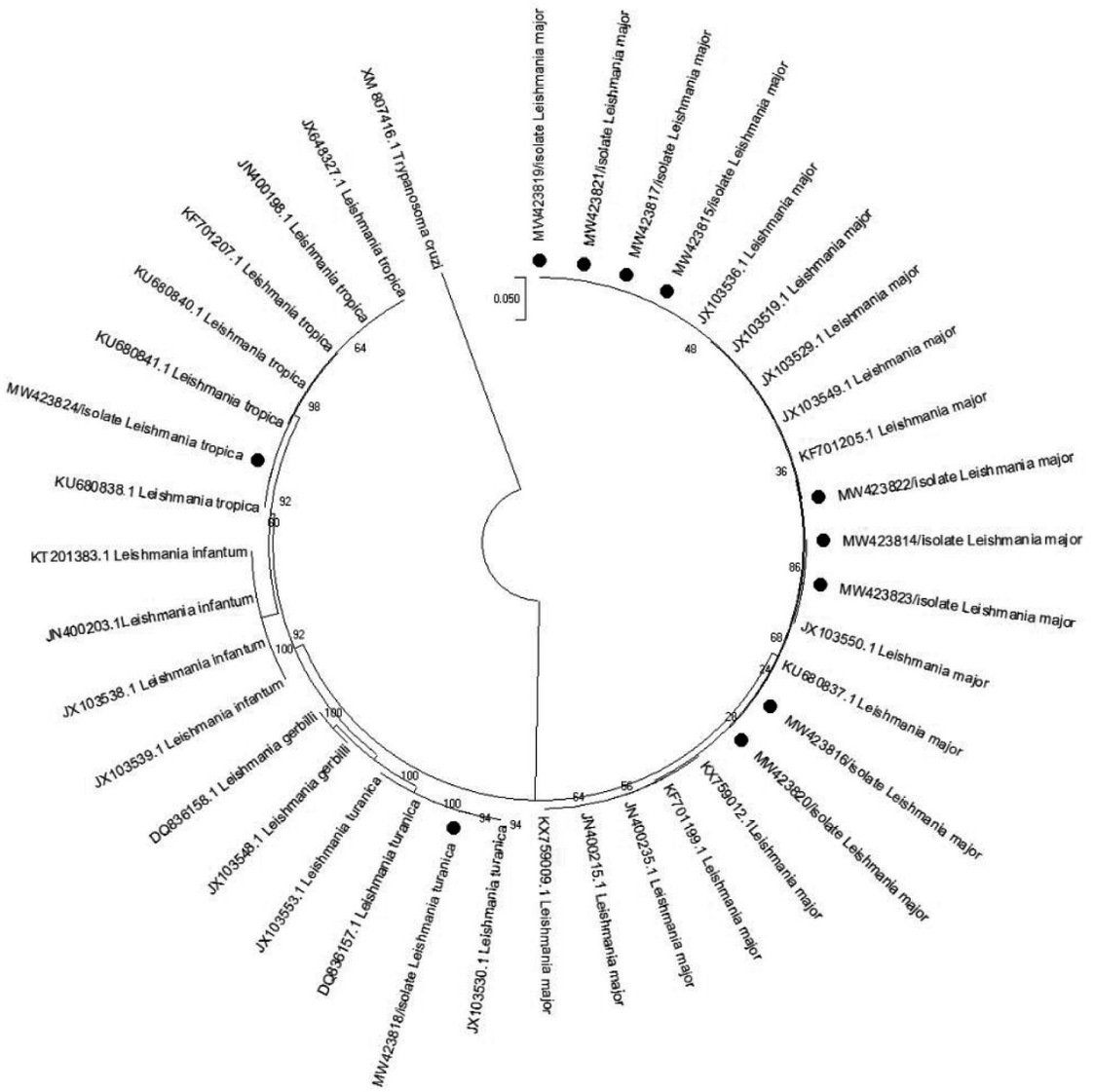

Figure 4. Phylogenetic tree inferred from 1450–1460 bp of *nagt* gene sequences of *Leishmania major* (n=8) *and L. tropica* (n=1) isolates from patients of the current study and *L. major* (n=1) and *L. turanica* (n=1) isolates from rodent reservoirs of the present study and other *Leishmania* species acquired from GenBank

ACC1 enzyme which are two bands of 680 and 780 bp for *L. tropica/L. turanica,* two bands of 500 and 950 bp for *L. major* and three bands of 780, 500, and 180 bp for *L. infantum* (Fig. 3) [4]. Compared with the patterns produced by the reference strains, one out of 80 isolates obtained from CL patients were identified as *L. tropica* and 79 as *L. major*, amongst 5 rodents' isolates, 1 and 4 isolates exhibited similar patterns to those of *L. turanica/L. tropica* and *L. major*, respectively.

Sequencing and phylogenetic tree

Eleven (11) samples including 2 isolates of rodents and 9 isolates of CL patients were selected for sequencing. The sequences of the present study were deposited in the GenBank using Sequin software (Accession numbers: MW423814-24) (Tab. 1).

The identities of all isolates of CL patients and 2 rodent isolates showed 95–100% similarity with *L. major, L. tropica,* and *L. turanica* species from Iran, Turkey, and Tunisia, which are deposited in the GenBank. The results of sequencing showed high agreement with PCR-RFLP method.

Genetic distance was calculated and a phylogenetic tree was constructed using the MEGA-X software (Fig. 4) All samples included *L. major*, *L. tropica,* and *L. turanica*, the *nagt* sequences of

Table 3. Genetic distance between the current *Leishmania* species based on *nagt* gene

	1	2	3	4	5
1. *L. major*					
2. *L. gerbilli*	0.033				
3. *L. tropica*	0.032	0.025			
4. *L. infantum*	0.046	0.038	0.028		
5. *L. turanica*	0.035	0.014	0.028	0.039	

some other species (*L. major, L. infantum, L. tropica, L. gerbilli*, and *L. turanica*) from the GenBank database were used to this purpose. Also, *Trypanosoma cruzi* was used as the outgroup (Tab. 2).

Sequences were grouped into four main separate clades, the first clade contained *L. major* species, the second contained *L. turanica* and *L. gerbilli* and third and four were markers of *L. infantum* and *L. tropica*, respectively. The intra-species similarity between present isolates of *L. major* was calculated to be 0.002. Inter-species diversity between *Leishmania* isolates in the current study and isolates registered in the GenBank based on *nagt* gene is presented in table 3.The lowest and highest inter-species similarity was found between *L. infantum-L. major* (0.046) and *L. turanica-L. gerbilli* (0.014), respectively.

In the current study, three haplotypes were reported among the *L. major* species that made up most of our sequences, including, [Hap-1: (Seq MW423814 Seq MW423823), Hap-2: (Seq MW423815 Seq MW423817 Seq MW423819 Seq MW423821 Seq MW423822) and Hap-3: (Seq MW423816 Seq MW423820)].

The haplotype diversity of *L. major* sequences in this study was calculated to be: Hd:0.6667. Among three haplotypes of *L. major* in the present study,

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MW423814      GTGCGTGTTCGGTCGCTTCGGTCTGCCCACCATGAACGTAACGAAGAAGTGGCTTCTTGG  539
MW423823      GTGCGTGTTCGGTCGCTTCGGTCTGCCCACCATGAACGTAACGAAGAAGTGGCTTCTTGG  540
MW423815      GTGCGTGTTCGGTCGCTTCGGTCTGCCCACCATGAACGTAACGAAGACGTGGCTTCTTGG  540
MW423817      GTGCGTGTTCGGTCGCTTCGGTCTGCCCACCATGAACGTAACGAAGACGTGGCTTCTTGG  540
MW423819      GTGCGTGTTCGGTCGCTTCGGTCTGCCCACCATGAACGTAACGAAGACGTGGCTTCTTGG  540
MW423821      GTGCGTGTTCGGTCGCTTCGGTCTGCCCACCATGAACGTAACGAAGACGTGGCTTCTTGG  540
MW423822      GTGCGTGTTCGGTCGCTTCGGTCTGCCCACCATGAACGTAACGAAGACGTGGCTTCTTGG  540
MW423816      GTGCGTGTTCGGTCGCTTCGGTCTGCCCACCATGAACGTAACGAAGACGTGGCTTCTTGG  540
MW423820      GTGCGTGTTCGGTCGCTTCGGTCTGCCCACCATGAACGTAACGAAGACGTGGCTTCTTGG  540
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MW423814      CAGCATCAACATTCTCGCCGGCGTCAATGGTGTGGAGGTGGGGCAGAGCATTGTGATCGC  779
MW423823      CAGCATCAACATTCTCGCCGGCGTCAATGGTGTGGAGGTGGGGCAGAGCATTGTGATCGC  780
MW423815      CAGCATCAACATTCTTGCCGGCGTCAATGGTGTGGAGGTGGGGCAGAGCATTGTGATCGC  780
MW423817      CAGCATCAACATTCTTGCCGGCGTCAATGGTGTGGAGGTGGGGCAGAGCATTGTGATCGC  780
MW423819      CAGCATCAACATTCTTGCCGGCGTCAATGGTGTGGAGGTGGGGCAGAGCATTGTGATCGC  780
MW423821      CAGCATCAACATTCTTGCCGGCGTCAATGGTGTGGAGGTGGGGCAGAGCATTGTGATCGC  780
MW423822      CAGCATCAACATTCTTGCCGGCGTCAATGGTGTGGAGGTGGGGCAGAGCATTGTGATCGC  780
MW423816      CAGCATCAACATTCTCGCCGGCGTCAATGGTGTGGAGGTGGGGCAGAGCATTGTGATCGC  780
MW423820      CAGCATCAACATTCTCGCCGGCGTCAATGGTGTGGAGGTGGGGCAGAGCATTGTGATCGC  780
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MW423814      CTTGAGCAGCGTCGACGCTGTGGCGGCAGATACACGCGACATGCGGAGCGATCATCGACT  899
MW423823      CTTGAGCAGCGTCGACGCTGTGGCGGCAGATACACGCGACATGCGGAGCGATCATCGACT  900
MW423815      CTTGAGCAGCGTCGACGCTGTGGCGGCAGATGCACGCGACATGCGGAGCGATCATCGACT  900
MW423817      CTTGAGCAGCGTCGACGCTGTGGCGGCAGATGCACGCGACATGCGGAGCGATCATCGACT  900
MW423819      CTTGAGCAGCGTCGACGCTGTGGCGGCAGATGCACGCGACATGCGGAGCGATCATCGACT  900
MW423821      CTTGAGCAGCGTCGACGCTGTGGCGGCAGATGCACGCGACATGCGGAGCGATCATCGACT  900
MW423822      CTTGAGCAGCGTCGACGCTGTGGCGGCAGATGCACGCGACATGCGGAGCGATCATCGACT  900
MW423816      CTTGAGCAGCGTCGACGCTGTGGCGGCAGATGCACGCGACATGCGGAGCGATCATCGACT  900
MW423820      CTTGAGCAGCGTCGACGCTGTGGCGGCAGATGCACGCGACATGCGGAGCGATCATCGACT  900
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Figure 5. Polymorphic loci of *L. major* sequences in the present study

polymorphisms were observed in nucleotide positions 527, 735, and 871 (Fig. 5).

Discussion

Leishmaniosis is one of the health problems in the world, CL is caused by different species of *Leishmania* spp. and is one of the vector-borne diseases. The distribution of a particular species of this parasite in each geographical area depends on several factors such as the presence of suitable reservoir and vector [19]. ZCL is among the endemic diseases of Iran [1]. Isfahan is one of the most important tourist, historical and religious provinces in Iran, that is one of the endemic areas of ZCL with the recent upward trend [5]. Migration and travel play an important role in prevalence of leishmaniosis, and in this regard, Isfahan province is a potential area for the spread of disease, which makes it necessary to comprehensive study of the spread causes of the disease in the region [7].

In the present study, the causative agents of leishmaniosis, as well as the genetic diversity of *Leishmania* isolates, were evaluated based on *nagt* gene. We showed *Leishmania* infection in 80 cases of 97 suspected patients in this study that all 80 exudates of patients and 5 samples from rodents were positive in both conventional and molecular technics, which can be attributed to the appropriateness of the genetic marker used and the molecular methods.

In the present study, among 80 positive patients, the proportion of female patients was lower than male, that could be due to their occupation and less exposure to sandfly bites, also more than half of the patients had more than or equal to two lesions which are agreed with the results of other studies [16,20]. The age group of 1–10 years in this study had the highest risk, this may be due to susceptibility to infection in children which was in line with the epidemiological study of Shirzadi et al. [9], but is not in line with previous studies in other endemic foci [16,21,22]. This high percentage may be due to children playing outdoors and being exposed to more sandfly bites.

Also, among 5 rodents that were positive for the presence of *Leishmania* parasite, one was *M. lybicus* and 4 *Rhombomys opimus*, which according to previous studies showed that the main reservoir of leishmaniosis in Isfahan province is *R. opimus* [11,23].

Accurate identification of *Leishmania* species in endemic areas is essential in epidemiological studies for control and prevention programs. Since parasitological methods are not able to identify *Leishmania* species, various molecular methods have recently been developed to determine the species and evaluate intraspecific variations [16]. The *nagt* gene is a fully conserved, single-copy gene, that is approximately 1450–60 bp per haploid genome, also encodes a microsomal transmembrane enzyme which enzymatic action in the first phase of N- glycosylation and encodes N-acetylglucosamine-1-phosphate transferase, hence a very suitable gene for phylogenetic analysis in *Leishmania* parasites [4,14,15].

The results of species identification in rodents (n=5) with *Leishmania* parasite showed that 4 isolates were *L. major* and one species was identified as *L. turanica.*

Our study showed that among 80 patients with CL, *L. major* was diagnosed in 79 patients and only in one patient *L. tropica* was the agent of leishmaniosis. The wet clinical appearance of lesions with secondary infections indicates a strong association between *Leishmania* species and the type of lesions in the present study. The findings of the present study are consistent with the results of previous studies in various ZCL centers in Iran [16,24,25]. Previous studies in Isfahan have shown the dominance of *L. major* as a causative agent of leishmaniosis [4,26].

The results of our study showed that *Leishmania* parasites can be well identified at the genus and species levels using the *nagt* gene with PCR-RFLP or sequencing method.

Hajjaran et al. [3] used this gene to study different isolates of *Leishmania* parasite isolated from different parts of Iran and observed 19 haplotypes among 49 samples. The researchers identified the *nagt* gene as an efficient gene for identifying the genetic relationships of the parasite. In a study conducted by Saberi et al. [15] in Ilam province using the nagt gene, no intraspecific differences were observed in *Leishmania* isolates and nucleotide sequences of samples. This could be due to the geographical proximity of the studied cities, the high relationship of the inhabitants of the studied areas with each other, or the presence of a fully protected *nagt* gene.

Phylogenetic analysis of the *nagt* gene demonstrates the efficiency of this gene in differentiating *Leishmania* species and distinguishes all species that isolates from human and rodents

[4,25]. All of the causative leishmaniosis agents of rodents and humans were grouped separately and distinctly in different clades. In terms of genetic variation about the patients isolates, the higher association between *L. tropica* and *L. infantum* were quite obvious, and the least association were observed between *L. tropica* and *L. major*, and in rodents' isolates, the highest association between *L. turanica* and *L. gerbilli* were observed, which was consistent with previous studies [4,16,25].

The phylogenetic tree analysis showed that the *Leishmania* sequences in this study were placed in a clade with other sequences isolated from Iran, Tunisia, and Turkey, it indicating that the *Leishmania* origin and place of isolation did not affect the resolution of the *nagt* gene. In this study, 3 haplotypes were reported among the *L. major* species, indicating diversity between these species that may make differences in terms of lesion formation, diagnosis, and treatment. Other Iranian researchers have reported intra-species diversity in *L. major* based on the *nagt* gene [4].

In conclusion, the obtained results, especially the dominance of *L. major*, show that Isfahan province is one of the endemic areas of ZCL in Iran. Given that the predominant and important species of *Leishmania* parasite in Isfahan province is *L. major*, this issue shows the importance of studying the status of rodents in this province. Therefore, it is recommended that rodents be prioritized for future research. It was also concluded that the *nagt* gene can be used effectively in the differentiation of *Leishmania* species.

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