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Original Article

Inter- and Intraspecific Variations of *Leishmania* Strains Isolated from Patients with Cutaneous and Visceral Leishmaniases in Fars Province, South of Iran

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Abstract

Background: Cutaneous and visceral leishmaniases are present in Fars Province in the south of Iran. The current study aimed to evaluate the inter- and intragenic diversities of *Leishmania* species isolated from patients with leishmaniasis in Fars Province, using PCR-based analyses and DNA sequencing of the N-acetylglucosamine-1-phosphate transferase (*nagt*) gene.

Methods: Clinical samples were taken from the skin lesions of 120 individuals with clinical suspicion of cutaneous leishmaniasis (CL) referred to the major health centers of Shiraz. Along with microscopic examination, a part of each sample was used for *in vitro* cultivation. DNA was extracted from the cultured parasites and the *nagt* gene was PCR-amplified. For RFLP analysis, the PCR product of the *nagt* gene was digested with the *Acc1* restriction enzyme. Moreover, the PCR products of 23 isolates were sequenced and analyzed, using MEGA5.

Results: From the 120 patients with clinical suspicion of CL, 110 (91.7%) cases were found to be positive by direct microscopy while 77 (64.1%) of the cultures were positive. Digestion of the PCR product with the *Acc1* restriction enzyme detected *L. major* in 57 out of the 77 (74.1%) and *L. tropica,* in 20 out of the 77 (25.9%) cases with CL. Phylogenetic analysis grouped the *Leishmania* isolates into 3 main clades, representing *L. major*, *L. infantum*, and *L. tropica*,encompassing 2, 2, and 2 haplotypes, respectively. Within the clades, the *L. tropica* intraspecies divergence was more pronounced in *L. major*.

Conclusion: The findings of this study demonstrated that the causative agent of CL in Fars Province was mainly *L. major* and that there was considerable heterogeneity between the *Leishmania* species and also within the *L. major* isolates.

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Keywords ● Leishmaniasis ● Cutaneous ● Visceral ● Genetic variation ● *Nagt* gene ● Iran

What's Known

- Cutaneous and visceral leishmaniasis is present in Fars Province.
- *L. major*, *L. tropica*, and *L. infantum* are the causative agents of both cutaneous and visceral leishmaniasis in Fars Province.

What's New

- The causative agent of CL in Fars Province is mainly *L. major*, and there is considerable heterogeneity between the *Leishmania* species and also within the *L. major* isolates in the region.
- *L. major*, *L. tropica*, and *L. infantum* belong to different clades.

Introduction

Leishmaniasis is a vector-borne disease caused by an intracellular protozoan parasite species belonging to the genus *Leishmania* (*L.*).[1] The disease is transmitted to humans by the bite of infected female phlebotomine sandflies.[1] The disease

can present in 3 main forms, comprising visceral leishmaniasis (VL, often known as kala-azar), cutaneous leishmaniasis (CL), and mucocutaneous leishmaniasis. CL and VL are present in most of (14 of the 22) the countries of the Eastern Mediterranean Region of the World Health Organization.[2]

In Iran, leishmaniasis exists as a medical health problem almost in all provinces. CL has been reported from Khorasan Province (northeast), Isfahan, Kerman, Yazd (center), and Fars Province (south).[3,4] VL is a major health problem in the northwest and southern parts of the country. In Iran, *L. major* and *L. tropica* are the causative agents of zoonotic and anthroponotic cutaneous leishmaniases, respectively, while VL is caused by *L. infantum.*[4,5] Among the 30 provinces of Iran, Fars Province, located in the south of the country, is one of the most important foci of CL (both anthroponotic and zoonotic) and VL.[3,6-9]

Razmjou et al.[7] evaluated the prevalence of CL infection among 1,000 inhabitants of 3 villages around Shiraz and found that 23.2% of the inhabitants had a history of CL or were infected with *Leishmania*. The authors also identified the *L. major* strain in the majority of the patients. In another study in Jahroum district, Davami et al.[8] reported that the predominant species of *Leishmania* in the patients with CL was *L. major* (87.5%), while *L. tropica* was isolated from only 12.5% of the patients. In a recent study conducted By Oryan et al.[9] in Fars Province, from 98 patients with CL, *L. major* was isolated from 97 patients and only one of the patients was infected with *L. tropica*.

For unknown reasons, the incidence of CL has been on the increase during the last several years in this area. Canines (dogs) are the main reservoirs of VL, although feline leishmaniasis has also been reported from the region and cats may also be involved in the epidemiology of the disease.[10-12] Northwestern and some southern provinces, including Fars and Bushehr, are the main foci of VL and more than 90% of VL cases in Iran have been reported from these areas.[10] The main foci of VL in Fars Province are Kazerun, Nourabad, Firouzabad and Darab districts.[5,13] Based on a hospital's records, 260 cases of VL were registered between 2001 and 2009 from these regions.[13]

In Iran, rodents are the main reservoirs of zoonotic CL, while dogs and humans are considered the reservoirs of anthroponotic CL. In a study conducted by Mirzae et al,[14] approximately one-third of the rodents captured from the deserts in Fars Province and Turkmen Sahra tested positive for *Leishmania*, with *L. major* constituting the predominant infectious agent in these animals. In another study by Akhoundi et al.,[15] a total of 593 rodents were captured from 6 CL-endemic foci, including Fars province, and *L. major* was detected in 145 (24.4%) of the cases.

Sandflies are the vector of CL in Iran. In Fars Province, *Phlebotomus papatasi* and also *Phlebotomus salehi* have been reported as the vectors of *L. major*.[16,17]

The biochemical and molecular characterization of *Leishmania* parasites and the analysis of their genetic diversity are important not only for their diagnosis and control but also for relevant epidemiological and taxonomic surveys. Several DNA-based molecular methods have been developed to evaluate the genetic diversity within and between *Leishmania* species and strains.[4,18-22] The target DNAs for these studies have been kinetoplast DNA, rDNA (internal transcribed spacer [ITS]), repetitive nuclear DNA, mini-exon genes, and microsatellite DNA.[19-22] One of the target genes used to evaluate the genetic diversity of *Leishmania* parasites is the N-acetylglucosamine-1-phosphate transferase (*nagt*) gene, a single-copy and highly conserved gene of about 1.4 kb which codes the endoplasmic reticulum transmembrane protein.[23,24]

The current study aimed to evaluate the inter- and intragenic diversities of *Leishmania* species isolated from patients with leishmaniasis in the southern Iranian province of Fars using polymerase chain reaction (PCR)-based analyses and DNA sequencing of the *nagt* gene.

Subjects and Methods

The subjects of this study were 120 individuals with clinical suspicion CL referred to the major health centers of Shiraz (Valfajr and other university-affiliated health centers) between 2011 and 2013. These patients came from different parts of the province where CL is endemic. The study was approved by the Ethics Committee of Shiraz University of Medical Sciences, and consent was obtained from the participants.

Sampling and Culture

Clinical samples (smears) were taken from the skin lesion of each patient suspected of CL, stained with Giemsa stain, and examined via light microscopy. In addition to microscopic examination, a part of each sample was aseptically inoculated into tubes containing an NNN medium. The cultures were kept at 22-25 °C and sub-cultured in an RPMI-1640 (Gibco,

Life Technologies GmbH, Germany) medium supplemented with 15% fetal bovine serum (Gibco, Germany), 100 µg/mL of penicillin, and 100 µg/mL of streptomycin (Gibco, Germany). Promastigotes of *Leishmania* were seen in the culture of 77 patients, and parasites were harvested in the stationary phase of growth and used for DNA extraction. Moreover, 3 microscopic slides from patients with VL referred to the Department of Parasitology at Shiraz University of Medical Sciences (one sample from Kazerun and 2 samples from Lamerd) were included in the study and DNA was extracted from these slides.

DNA Extraction

DNA was extracted from the cultured parasites by using a High Pure PCR Template Preparation Kit (Roche Diagnostic GmbH, Germany) according to the manufacturer's instructions. DNA was extracted from the microscopic slides by washing the slides with absolute ethanol before covering them with 250 µL of lysis buffer (50 mM of Tris, 50 mM of NaCl, 10 mM of EDTA, pH 7.4, 1% v/v Triton X-100, and 100 µg of proteinase K per mL) for 1-2 minutes. The smears were removed from the slides and transferred to a 1.5 mL reaction tube. Cell lysis was concluded by incubation for at least 3 hours or overnight at 56 °C. The lysate was extracted by phenol-chloroform extraction, followed by ethanol precipitation. The DNA was re-suspended in 50 µL of double-distilled water and stored at 4 °C before use.

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (RFLP)

The *nagt* gene was PCR-amplified from genomic DNAs, using the primers L1 (Forward):(5′ TCA TGA CTC TTG GCC TGG TAG) and L4 (Reverse): (5′ CTC TAG CGC ACT TCA TCG TAG).[25]

PCR was carried out using a 96X thermocycler (Peqlab Biotechnologie, Germany) by one cycle of initial denaturation at 95 ºC for 5 minutes, followed by 30 cycles of denaturation at 94 ºC for 1 minute, annealing temperature at 55 ºC for 1 minute, extension temperature at 72 ºC for 90 seconds, and final extension at 72 ºC for 5 minutes.[25] The PCR products were separated by electrophoresis in 1.5% agarose gel and stained with safe stain. PCR amplification of *nagt* from all the *Leishmania* isolates produced fragments of about 1.4 kb. Next, 10 µL of the PCR products, containing the amplified *nagt* region (1.4 kb), was digested with the fast digestion *Acc1* (Xmi1) restriction enzyme (Fermentas GmbH, Thermo Scientific, Germany) in accordance with the manufacturer's instructions. The restriction products were subjected to electrophoresis in 3% agarose gels and visualized under UV light after staining in safe stain. DNAs from Iranian reference strains, *L. tropica* (Acc. # EF653267), *L. major* (Acc. # JN860745), and *L. infantum* (Acc. # EU810776), which were previously identified based on ITS1 gene analyses were included as positive controls in all the PCR assays.

DNA Sequencing and Phylogenetic Analysis

The PCR products of the *nagt* gene from 23 isolates were sequenced using the same forward and reverse primers employed for amplification with an ABI 3730 sequencer and via Sanger sequencing (Bioneer, Daejeon, South Korea).The sequences were aligned and compared with those of existing sequences related to *Leishmania species* available in GenBank, using Clustal X. Twenty-one multiple alignments were performed with data related to *Leishmania* species from Iran and other countries deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank). A phylogenetic tree was constructed via Tamura 3-parameter option of the neighbor-joining method using MEGA5 (http://www.megasoftware.net).

Results

Among the 120 individual suspected of CL, 110 (91.7%) were found to be positive by direct microscopy while 77 (64.1%) were positive according to the culture method. PCR and PCR-RFLP were performed on all of the 77 cultured isolates. Three VL cases had been previously confirmed by direct microscopy and were further confirmed by RFLP-PCR in this study. The *nagt* genes from the 80 selected isolates were amplified using the L1/L4 primer set for PCR. The PCR amplification of *nagt* from all the *Leishmania* isolates yielded fragments of about 1.4 kb. The digestion of the PCR product (the 1.4 kb *nagt* gene) with the *Acc1* restriction enzyme yielded 2 bands of 500 and 950 bp, indicative of *L. major* in 57 out of the 77 (74.1%) CL cases, and 2 bands of 680 and 780 bp, indicative of *L. tropica,* in 20 out of the 77 (25.9%) CL cases. The digestion of the PCR products of the samples obtained from the patients with VL produced 3 bands of 200, 500, and 780 bp, indicative of *L. infantum* in all the 3 cases. Figure 1 shows the RFLP pattern of the *Leishmania* isolates in this study.

The distribution of the *Leishmania* species in different areas of Fars Province was different

(table 1). The dominant species of *Leishmania* isolated from the patients with CL in all areas was *L. major*, while *L. tropica* was isolated from about 40% of the patients with CL from Fasa and Shiraz (capital of the province). Table 1 depicts the distribution of the *Leishmania* species in different areas of the province in the current study.

The alignment of the *nagt* gene sequences of the *L. major* isolates showed 5 DNA variable sites in which nucleotides at the positions of 357 A>G, 762 T>C, 970 C>A, 1050 C>G, and 1054 A>T were single-base substituted. In the *L. tropica* isolates, the only single-base substitution was at the nucleotide site of 313A>G.

The alignment of protein sequences showed a difference in the position of 352 threonine>serine in the *L. major* isolates and the substitution of leucine>valine in the position of 313 in the *L. tropica* isolates.

The sequence data for the 23 *nagt* sequences obtained in this study were deposited in GenBank with accession numbers of KF701191 to KF701213, as is reflected in table 2.

Phylogenetic Analysis

All of the identified *Leishmania* isolates were taxonomically grouped into 3 main clades: *L. tropica*, *L. major*, and *L. infantum*. All the sequenced cases were analyzed using MEGA5, and their phylogenetic tree was drawn for phylogenetic analysis. Within the clades, *L. tropica* was more associated with *L. infantum* and had less intra-divergence. Intraspecies divergence was more pronounced in *L. major*. Figure 2 shows the phylogenetic tree of the *nagt* region nucleotide sequences of the *Leishmania* isolates recovered from the patients with CL and VL. The numbers above the branches represent bootstrap percentages.

Table 1: Distribution of cutaneous *Leishmania* species in different areas of Fars Province, southern Iran, based on *nagt* -PCR-RFLP analysis

City name	*L. major*		*L. tropica*		Total	
	No	Percent	No	Percent	No	Percent
Kharameh	4	7	-	-	4	5.2
Fasa	11	19.3	8	40	19	24.7
Darioon	6	10.5	-	-	6	7.8
Sepidan-Beiza	2	3.5	-	-	2	2.6
Marvdasht	1	1.8	1	5	2	2.6
Shiraz	28	49	9	45	37	48
Larestan	1	1.8	-	-	1	1.3
Farashband	1	1.8	-	-	1	1.3
Ghir-Karzin	2	3.5	2	10	4	5.2
Firouzabad	1	1.8	-	-	1	1.3
Total	57	100	20	100	77	100

Discussion

In the current study, we applied PCR-RFLP based on the *nagt* gene for species identification and sequencing analysis to find out the inter- and intragenic diversities of *Leishmania* species isolated from patients with CL or VL in the southern Iranian province of Fars. We detected *L. major* and *L. tropica* in 57 (74.1%) and 20 (25.9%) of the patients with CL, respectively. The causative agent of VL in all the 3 VL cases was *L. infantum*. Based on the findings of this study, *L. major* was identified as the main causative agent of CL in this area. In previous studies in the region, *L. major*, *L. tropica*, and infrequently *L. infantum* were reported as the

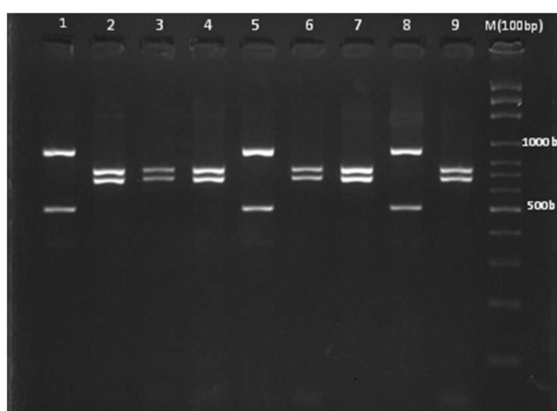

Figure 1: Restriction fragment length polymorphism (RFLP) patterns of the *Leishmania* isolates along with reference strain, digested with the *Acc1* restriction enzyme. Lanes 1, 5, and 8: *L. major*; Lanes 2, 3, 4, 6, 7, and 9: *L. tropica*.

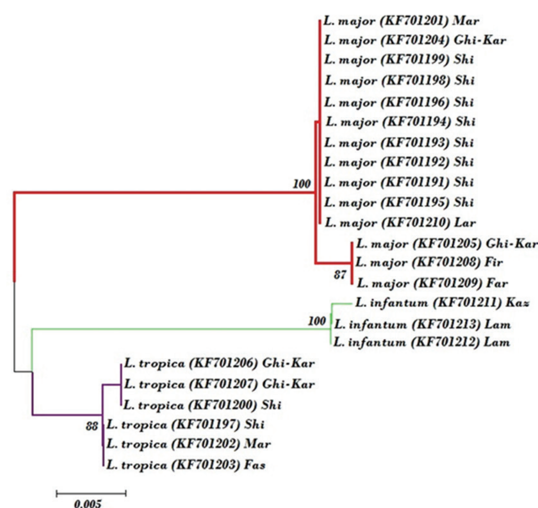

Figure 2: Phylogenic tree of the N-acetylglucosamine-1-phosphate transferase (*nagt*) region nucleotide sequences of the *Leishmania species* isolated from patients with cutaneous leishmaniasis (CL) or visceral leishmaniasis (VL) in Fars Province, southern Iran. The tree was constructed by using the neighbor-joining, Tamura 3-parameter model in MEGA5. The numbers above the branches indicate the percentage of bootstrap samplings. Branches without numbers have frequencies of less than 70%.

Table 2: Accession numbers of *Leishmania nagt* sequences in Iranian species in Fars Province deposited in GenBank

No.	Isolate code	Place of isolation city/province	Disease	RFLP/ SEQ	Species	Source of infection	Acc. No.
1	MHOM/IR/12/Shiraz1	Shiraz/Fars	CL	+/+	*L. major*	Human	KF701191
2	MHOM/IR/12/Shiraz2	Shiraz/Fars	CL	+/+	*L. major*	Human	KF701192
3	MHOM/IR/12/Shiraz3	Shiraz/Fars	CL	+/+	*L. major*	Human	KF701193
4	MHOM/IR/12/Shiraz4	Shiraz/Fars	CL	+/+	*L. major*	Human	KF701194
5	MHOM/IR/12/Shiraz5	Shiraz/Fars	CL	+/+	*L. major*	Human	KF701195
6	MHOM/IR/12/Shiraz6	Shiraz/Fars	CL	+/+	*L. major*	Human	KF701196
7	MHOM/IR/12/Shiraz7	Shiraz/Fars	CL	+/+	*L. tropica*	Human	KF701197
8	MHOM/IR/12/Shiraz8	Shiraz/Fars	CL	+/+	*L. major*	Human	KF701198
9	MHOM/IR/12/Shiraz9	Shiraz/Fars	CL	+/+	*L. major*	Human	KF701199
10	MHOM/IR/11//Shiraz10	Shiraz/Fars	CL	+/+	*L. tropica*	Human	KF701200
11	MHOM/IR/12/Marvdasht1	Marvdasht/Fars	CL	+/+	*L. major*	Human	KF701201
12	MHOM/IR/12/Marvdasht2	Marvdasht/Fars	CL	+/+	*L. tropica*	Human	KF701202
13	MHOM/IR/12/Fasa1	Fasa/Fars	CL	+/+	*L. tropica*	Human	KF701203
14	MHOM/IR/11/Ghir-Karzin1	Ghir-Karzin/Fars	CL	+/+	*L. major*	Human	KF701204
15	MHOM/IR/11/Ghir-Karzin2	Ghir-Karzin/Fars	CL	+/+	*L. major*	Human	KF701205
16	MHOM/IR/11/Ghir-Karzin3	Ghir-Karzin/Fars	CL	+/+	*L. tropica*	Human	KF701206
17	MHOM/IR/11/Ghir-Karzin4	Ghir-Karzin/Fars	CL	+/+	*L. tropica*	Human	KF701207
18	MHOM/IR/11/Firouzabad	Firouzabad/Fars	CL	+/+	*L. major*	Human	KF701208
19	MHOM/IR/11/Farashband	Farashband/Fars	CL	+/+	*L. major*	Human	KF701209
20	MHOM/IR/11/Larestan	Larestan/Fars	CL	+/+	*L. major*	Human	KF701210
21	MHOM/IR/11/Kazerun	Kazerun/Fars	VL	+/+	*L. infantum*	Human	KF701211
22	MHOM/IR/11/Lamerd1	Lamerd/Fars	VL	+/+	*L .infantum*	Human	KF701212
23	MHOM/IR/11/Lamerd2	Lamerd/Fars	VL	+/+	*L. infantum*	Human	KF701213

causative agents of CL.[3,9] The findings of the current study are consistent with the previous studies which considered *L. major* as the main agent for CL in this region.[3,7-9]

In a comprehensive study, Mahmoudzadeh–Niknam et al.[26] showed that from 341 *Leishmania* isolates collected from different CL-endemic areas of Iran, 283 and 58 isolates belonged to *L. major* and *L. tropica* species, respectively. The main prevalent species of *Leishmania* was found to be *L. major* in areas like Semnan, Golestan, North Khorasan, Isfahan, Ilam, Khuzestan, Fars, and Hormozgan provinces, while the isolates from Razavi Khorasan, Kerman, and Yazd were mainly *L. tropica*. Their findings also showed that both *L. major* and *L. tropica* were endemic in 11 provinces in Iran (including Fars).[26] The findings of that study are consistent with our study insofar as we demonstrated that the main causative agent of CL in Fars Province was *L. major.* In a recent investigation in Fars Province, Akhoundi et al.[15] found that 93% of the patients with CL in their study were infected with *L. major.* In a more recent study, the RFLP analysis of the *nagt* gene of *Leishmania* isolates from patients with CL obtained from different endemic areas of Iran (but not Fars Province) was used to evaluate the molecular epidemiology of CL.[25] The results of that study showed that about 53% of the CL cases were caused by *L. tropica* and that around 47% of the cases were due to *L. major*.[25] This is somewhat different from the pattern observed in our study, where most of the patients with CL were infected with *L. major*. The main reason for this difference is that in the previous study, the samples were collected from the provinces known as the endemic foci of anthroponotic CL caused by *L. tropica*, whereas in Fars Province (where our study was conducted), the dominant causative agent of CL has been reported to be *L. major*. Our findings further confirm this concept. The epidemiological pattern of CL in several areas of Iran appears to have changed in the last decade, from a predominantly urban disease to one that is most common in rural areas.[8,9] This is perhaps due to the expansion of the geographical distribution of *L. major* and also the urbanization of rural areas near the colonies of rodents.

In the present study, we assessed the inter- and intra-variations of the DNA polymorphisms of the *nagt* gene among the isolates of *L. major*, *L. tropica*, and *L. infantum* by sequence analysis and showed the highest difference of the *nagt* gene for *L. major* and the least difference for *L. tropica* in this region. The highest difference of the *nagt* gene between the *Leishmania* species was for *L. major* and *L. infantum* and the least difference was for *L. tropica* and *L. infantum*. An evaluation

of the genetic diversity of the *Leishmania* isolates of patients with CL from different provinces of Iran (but not Fars Province) by Hajjaran et al.[25] revealed the highest mean intraspecies diversity among *L. major* isolates. This is in keeping with our findings in as much as we showed that the highest intraspecies diversity was among the *L. major* isolates. Polymorphism within ITS or minicircle DNA has been previously reported within the different species of *Leishmania* in the region, using different molecular methods.[4,9,27] Moreover, the heterogeneity of the ribosomal DNA of *L. major* has been previously reported in Fars Province.[9] In a study by Oryan et al.[9] in Fars Province, the sequence analysis of kDNA from 21 *L. major* strains showed a high genetic polymorphism of *L. major*. Our findings further confirmed the heterogeneity of *L. major* and *L. tropica* in this region. The substantial genetic variability of *L. major* might be due to differences in reservoir hosts and diversity of vector populations (i.e. *Phlebotomus papatasi* and *Phlebotomus salehi* in Fars Province).[14]

In a recent study by Ghatee et al.[28] using ITS from the ribosomal DNA locus, the heterogeneity and phylogeny of *L. tropica* of 61 isolates in southern Iran revealed 4 haplotypes in the isolates from Kerman Province and 2 haplotypes among the isolates from the city of Shiraz. This is again somewhat in keeping with our findings insofar as we demonstrated a low number of haplotypes in the *L. tropica* isolates in comparison with the other isolates.

Our phylogenetic analysis of the 23 isolates in this study revealed that *L. major*, *L. tropica*, and *L. infantum* belonged to different clades. Each of the 3 species had a similar number (i.e. 2) of haplotypes. This is to a certain extent different from the results of a study by Tashakori et al.[27] in Iran, which reported a higher number of haplotypes for *L. tropica*. In our previous study, the *nagt* genes in *L. major* and *L. tropica* isolates collected from different areas of the country (apart from Fars Province) showed 5 and 8 haplotypes, respectively.[25] This can be explained by the diversity of the samples collected from different geographical areas. Support for this idea comes from a study by Mirzae et al,[14] who demonstrated that strains from Turkmen Sahra and from Fars Province were genetically different and that they belonged to different genetic groups, largely corresponding to their geographical origins.

Although phylogenetic trees derived from the *nagt* sequences support a clear divergence between *L. major* and *L. tropica*, there was no clear grouping between these 2 isolates according to their geographical origin. However, such divergence was seen in other species where *L. infantum* isolated from patients with VL from Kazerun sat in a different branch. This may suggest that the VL isolate from Kazerun is somewhat a different strain of *L. infantum*. Such assumption needs further research with a larger number of patients with VL from this region. The other *L. infantum* isolate, from Lamerd, sat in the same branch as the isolates from Meshkinshahr, Boomehen, and also from Turkey. A previous study reported a correlation between genetic polymorphism and the geographical location and clinical manifestation of *L. major*.[9] Nonetheless, such connection was not found either in our study or in other similar molecular studies in Iran.[4,25]

Conclusion

Our results demonstrated that the causative agent of CL in Fars province was mainly *L. major* and that there was considerable heterogeneity between the *Leishmania* species and also within the *L. major* isolates. A further study with more samples from patients with VL is suggested.

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Conflict of Interest: None declared.

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