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Phylogenetic Evaluation and Molecular Variation of *Leishmania major* and *Leishmania tropica* Isolated from Different Parts of Iran

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ABSTRACT

Aims The aim of this study was species identification and phylogenic analysis of species of *Leishmania* isolated from clinical samples.

Materials & Methods The samples were collected from patients that were infected from different parts of Iran. After microscopic examination, we used PCR method for amplify the ITS1 (internal transcribed spacer 1) gene. RFLP method (digestion with Apo1 restriction enzyme) and for phylogenetic construction, DNA sequencing of PCR product were used.

Findings Two samples from Khorasan province (Mashhad) were Leishmania tropica (L. tropica), while others were *Leishmania major* (*L. major*). *L. tropica* samples are more variable compared with L. major. The molecular sequencing differences between L. major was related to geographical distribution. Based on the results of PCR product in the gel electrophoresis and DNA sequencing for *L. tropica* and *L. major*, the DNA sizes were between 350 and 369bp. The RFLP for L. major and L. tropica showed two and one bands, respectively. The sequences for all samples from central parts are the same, but there is difference with the samples isolated from North-East part of Iran.

Conclusion The sequences of ITS1 gene of Leishmania major separated from Damghan and Esfarayen are different from other samples. Similarity of DNA sequences of North-East part of Iran of *L. major* with samples from central parts was 99%. The similarity of two isolates of *L. tropica* was 96%. The most similarity of *Leishmania tropica* isolated was 95% with Indian isolate and the most similarity for Leishmania major was 99% with Friedlin strain

Keywords *Leishmania Major*; PCR; RFLP; Sequencing; Phylogenetic Analysis; Iran

CITATION LINKS

[1] The leishmaniases and Leishmania/HIV co-infections [2] Molecular detection and conventional identification of leishmania species in reservoir hosts of zoonotic cutaneous leishmaniasis in Fars province, South of Iran [3] Reactive nitrogen and oxygen intermediates in patients with cutaneous leishmaniasis [4] Leishmaniasis in the old world [5] Evolution, classification and geographical distribution [6] Clinical diagnosis of cutaneous leishmaniasis: A comparison study between standardized graded direct microscopy and ITS1-PCR of Giemsa-stained smears [7] Short report: ITS-1 DNA sequence confirmation of Leishmania major as a cause of cutaneous leishmaniasis from an outbreak focus in the Ho district, Southeastern Ghana [8] PCR diagnosis and characterization of Leishmania in local and imported clinical samples [9] Detection and species identification of Old World Leishmania in clinical samples using a PCR-based method [10] Diagnostic genotyping of Old and New World Leishmania species by PCR-RFLP [11] Diagnosis and characterization of Leishmania species in Giemsa-stained slides by PCR-RFLP [12] Determination of Leishmania species causing cutaneous leishmaniasis in Mashhad by PCR-RFLP method [13] Identification of Tunisian Leishmania spp. by PCR amplification of cysteine proteinase B (cpb) genes and phylogenetic analysis [14] Molecular characterization of Leishmania species isolated from cutaneous leishmaniasis in Yemen [15] Identification and phylogenetic relationship of Iranian strains of various Leishmania species isolated from cutaneous and visceral cases of leishmaniasis based on N-acetylglucosamine-1-phosphate transferase gene

Introduction

Leishmaniasis is a zoonotic and anthroponotic disease (WHO, 2000), which is endemic in some parts of Iran [1, 2]. It is expressed as a wide spectrum, from self- healing cutaneous lesion to serious and cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), or visceral leishmaniasis (VL) [3]. *L. major* and *L. tropica* are common agents of CL [4].

Laboratory diagnosis of cutaneous leishmaniasis is based on direct smear of lesion exudates and showing the parasites by microscopic examination of stained specimens. When the result of direct method is negative, the specimens are cultured. Since the serological methods have no sensitive response, especially in suppressed individuals like HIV patients, polymerase chain reaction (PCR) method is applied as a sensitive method. Besides, PCR and PCR-RFLP methods are used to identify species.

Characterization and identification of *Leishmania* species are absolutely important for prevention, controlling programs, and treatment [5]. In the present study, PCR and sequencing of ITS1 were employed to study *Leishmania*.

We selected ITS1 as the target to be studied because (i) ITS1 sequences are adequately polymorphic to detect different strains [6, 7]; (ii) Many of *Leishmania* ITS1 sequences are laid in sequences data banks, involving the pathogenic species of the genus [8]; ITS1 is a suitable target for both detection and identification of *Leishmania* spp. Evaluating the phylogeny in CL and finding the phylogenetic position can help us in controlling and therapeutic programs.

Materials and Methods

This project was approved by the Medical Ethics Committee of Tarbiat Modares University according to Declaration of Helsinki.

Clinical Samples and Reference Strain: In this study, we collected the samples from Razi Hospital in Tehran. After direct microscopic examination of the clinical samples collected from 70 patients from different parts of Iran that had come to the Razi Hospital, these specimens were cultured in Novy-MCNeal-Nicolle (NNN) media aseptically and transferred to the laboratory in Parasitology and Entomology Department of Tarbiat Modares University. The reference strain was *L. major* (MRHO/IR/75/ER). This research was confirmed by Ethics Committee of Tarbiat Modares University.

DNA Extraction: About 2×10^6 of *L. major* promastigotes were initially washed with phosphate buffered saline (PBS). It was, then, lysed in lysing buffer (0.1M Tris-HCl pH 8.0 containing 1% sodium dodecyl sulphate, 0.1M NaCl, and l0mM EDTA) and treated with proteinase K (100µg/ml) at 60°C for 2 h. The lysate was added to an equal volume of phenol/chloroform (25:25). The DNA extraction products were detected in 0.8% agarose gel and photographed.

PCR Amplification and Gel Electrophoresis: Genomic DNA isolated from promastigotes was used as a template to amplify the ITS1 gene by PCR. The reaction was performed in 25µl of the solution containing 3µl of template DNA, 0.5µl of dNTP (with concentration of 10mM and final concentration of 200µM), 0.5µl of *Taq* DNA polymerase (with concentration of 5 Unit/ml), 2.5µl of 10X PCR buffer, 0.75µl of $MgCl_2$ (50mM), 15.75µl of distilled water, and 1µl of each of the primers (10pmol/µl).

The primer sequences, which are used in this study, were as follow [9]:

LeF, 5'-TCCGCCCGAAAGTTCACCGATA-3'

LeR, 5'-CCAAGTCATCCATCGCGACACG-3'.

The PCR conditions were 5 min at 94°C for initial denaturation step followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 30 s, an extension at 72°C for 45 s, and a final extension at 72°C for 5 min.

The PCR products were analyzed by electrophoresis on 1.5% agarose gels, visualized by UV illumination and photographed.

Selection of the Restriction Endonuclease and RFLP: Based on the sequences of *L .major* and *L. tropica* retrieved from GenBank, we expected *L. major* to produce amplicons longer than those of *L. tropica* (at least 13bp). Therefore, the difference in amplicon size between *L. major* and *L. tropica* strains can be differentiated after electrophoresis on 3.5% agarose gel. *ApoI* was, thus, selected as a suitable enzyme because all of *L. donovani* complex and *L. major*are were digested by this enzyme, whereas it digests no *L. tropica* sequence [9].

Restriction digestion was performed according to methods described by Marfurt *et al.* [10]. We provided 10µl of the PCR products (0.1-0.5µg of DNA), 18µl of nuclease-free water, 2µl of 10X buffer Tango, and 1µl of *ApoI* enzyme (Fermentas) at 37ºC water bath overnight (16 h). The digest was, then, used for electrophoresis on 3.5% agarose gel. Fragment sizes were estimated by comparing the length of DNA bands with standard ladder (100bp).

DNA Sequencing and Phylogenetic Tree Construction: Sequencing was performed by Fazapajouh Company by using the primers

employed in the PCR according to the manufacturer's instructions.
Ten PCR products (10 samples) were sequenced with dideoxy method (Fazapajouh Company), using the PCR primers. Using the ClustalW 1.8 software package, the nucleotide sequences of *Leishmania* strains were aligned with a reference panel of sequences retrieved from the GenBank database. Phylogenetic analysis was conducted by using the neighbor-joining method for tree drawing. The reliability of phylogenetic classification was evaluated by a 1000-cycle bootstrap test.

Findings

Evaluation of the Method: After using direct and culture methods, all of the samples were positive. All samples were examined for the presence of *Leishmania* by using direct analysis (microscopic examination of Gimsa-stained skin lesion smears) and culture in NNN media. All collected samples were positive with PCR method.
Based on the results of PCR product in the gel electrophoresis and DNA sequencing for *L. tropica* and *L. major*, the DNA sizes were between 350 and 369bp (Figure 1).

Sequencing: Ten PCR products containing clinical samples (*L. tropica* and *L. major*) and reference strain of *L. major* were sequenced according to F and R primers. The sequenced genes are under registration in GenBank with following accession numbers: JN005821- JN005830.
The results of sequencing indicated that the length of fragments for *L .tropica* and *L. major* was 350bp and 369bp, respectively. It was additionally found out that there is restriction site of *ApoI* (AATT) for *L. major*, while there is no restriction site for *L. tropica* (Figures 2 and 3).

The alignments of the two *L. tropica* isolates from Mashhad (Khorasan Province) are shown in Figure 2.

RFLP: RFLP produced the DNA fragments that we expected. The resultant restriction fragments of samples were separated on a 3.5% agarose gel and we observed a difference in amplicon size between *L. major* and *L. infantum* or *L. tropica* (Figure 4). The reference strains showed the same results.
As *L. tropica* has no AATT sequence, we observed no digestion in its PCR products after the restriction enzyme was added (Figure 4). On the contrary, after digestion of *L. major*, we showed two bands with lengths of 192 and 177bp.

Phylogenetic Tree Construction: The results of phylogenetic tree, according to the isolates in this study and some GenBank sequences, are provided in Figure 5. Numbers on the branches indicate the repeated number that occurred among the trees by a 1000 cycle bootstrap test.

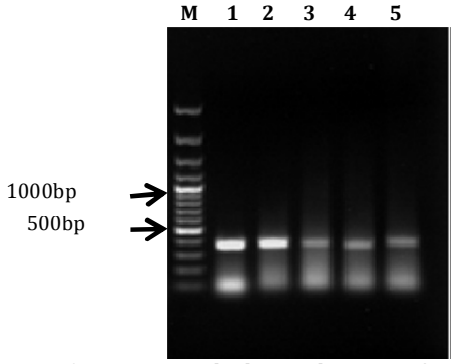

Figure1) Agarose gel electrophoresis of PCR products. Lanes 1 and 4: *Leishmania tropica*; Lanes 2, 3, and 5: *Leishmania major* (MRHO/IR/75/ER). The marker (Lane M) is a 100 bp ladder

```
Mashhad1    CAGCTGGATCATTTTCCGATGATTACACCCCC-CCAAAAAAACATATCCAAAACTCGGGG
Mashhad2    --------------------------------A------------T--------------
Mashhad1    AGGCCTATTATATACATTATAGGCCTTTCCCACATACACAGCAAACTTTTATCA-TCAAA
Mashhad2    ------------C-----------------C-T---C--------------x-C-----
Mashhad1    GTTTGCAGTAAACAAAAGGCCAATCGACGTTATAACCCACCCCCTATACCCAAAAGCAA
Mashhad2    ------------------------------------------------------------
Mashhad1    AAATGTCCGTTTATACAAATATACGGCGTTTCGGTTTTGTTGGCGGGGGGGGCGTGGGGGG
Mashhad2    ------------------------------------------------------------
Mashhad1    GATAACGGCTCACATAACGTGTCCCGATGGA-TGACTTGG
Mashhad2    ----------------------G-------A--------
```

Figure 2) Comparison of alignment of the two *L. tropica* isolates from Mashhad (Khorasan Province) that show 96% similarity between them

Figure 3) Alignment of ITS1 gene sequence of *L. major* isolates in comparison with reference/the standard strain (MRHO/IR/75/ER)

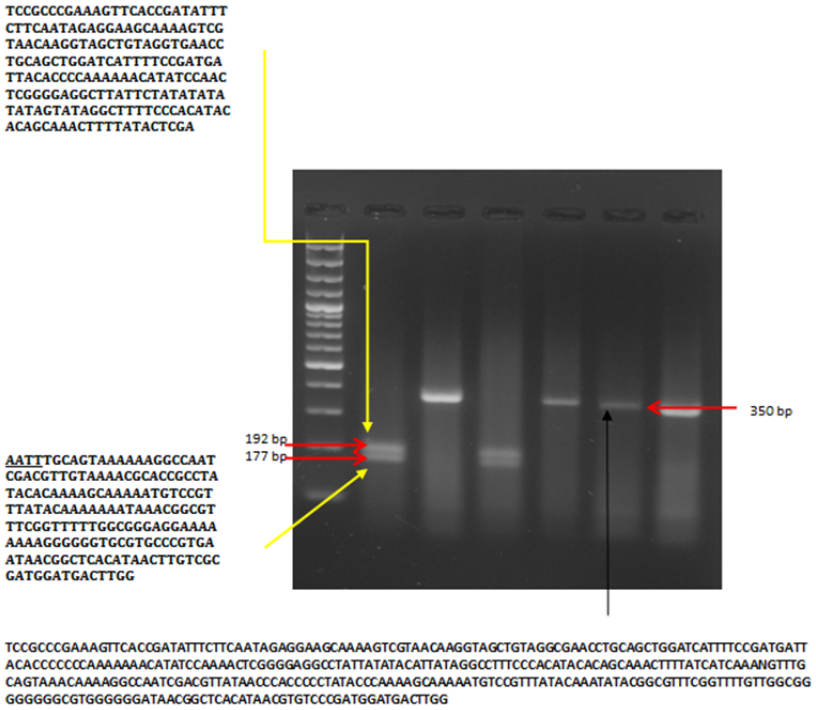

Figure 4) Agarose gel electrophoresis of PCR products and after digestion with *Apo*I. The marker (lane M) is a 100 bp ladder. Lane 1, *Leishmania tropica,* Lane 3, and 5, *Leishmania major.* Line 2, *Leishmania tropica* after digestion with *APO*I enzyme, lines4 and 6, *Leishmania major* after digestion with *APO*I enzyme

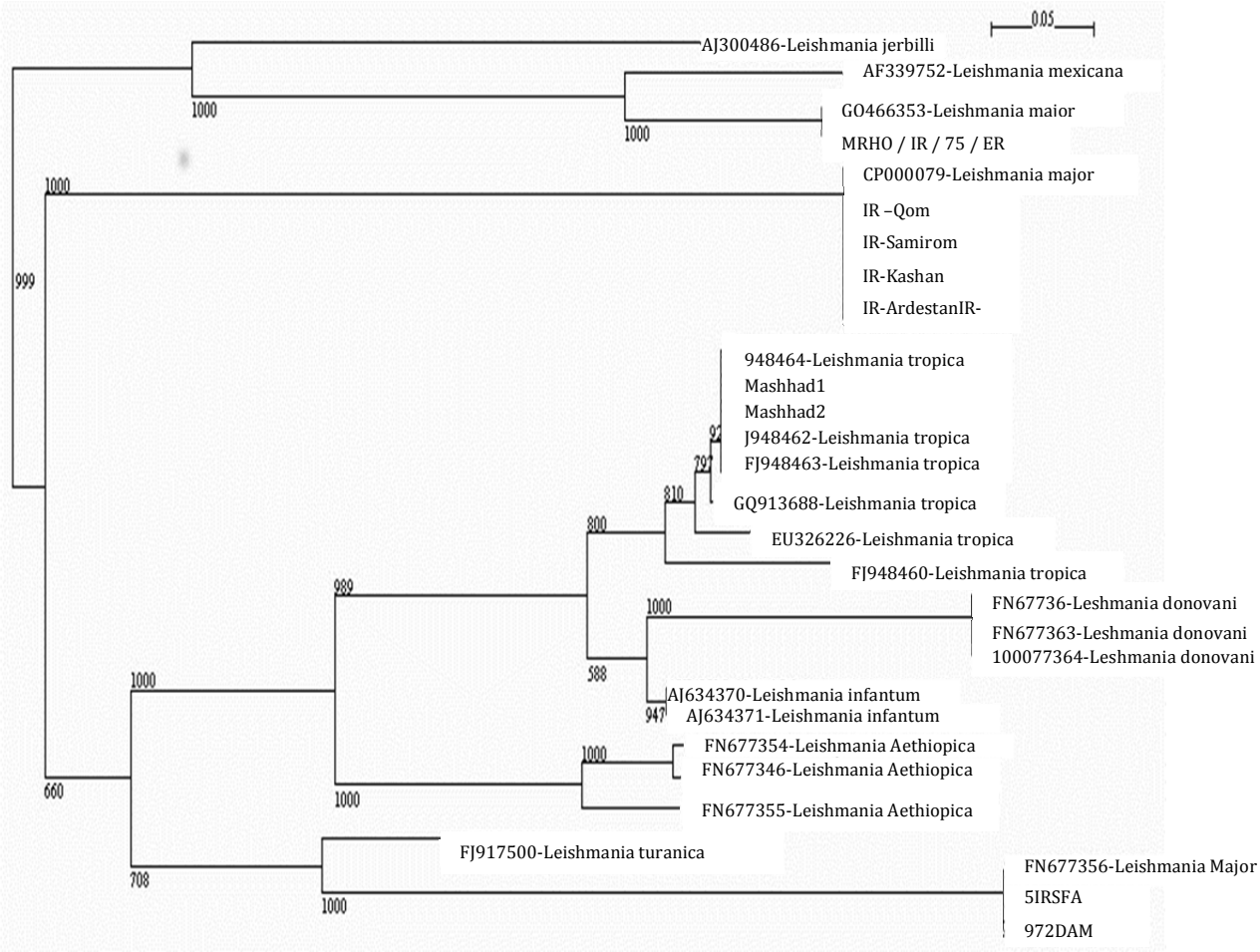

Figure 5) The tree constructed by using representative *Leishmania* sequences of Iranian isolates obtained in this study together with some GenBank records (with their accession numbers). The reliability of phylogenetic classification was evaluated by a 1,000-cycle bootstrap test

Discussion

Leishmaniasis is highly important because of patients' disability after complete treatment by the existing methods, relapse of the disease, secondary bacterial infection, its side effects, resistance, high treatment cost, being an opportunistic infection at patients with low immunity, high prevalence, spreading all over Iran (cutaneous type of the disease), and high mortality rate if it is not treated (visceral type of the disease). The group of diseases, like AIDS, that suppress the immunity system causes the relapse of this disease. Therefore, new methods and effective strategies are necessary to be developed in order to control and treat the disease.

L. tropica and *L. major* are causative agents of anthroponotic and zoonotic cutaneous leishmaniasis, respectively. The recognition of anthroponotic or zoonotic forms is helpful for selection the type of treatment and controlling programs.

The methods usually used for direct diagnosis of the parasite include microscopic test (Gimsa-stained) and *in vitro* culture. The latter has low sensitivity because there is small number of parasites in some species. Also, during culturing the parasites, some problems such as contamination of the *in vitro* cultures may take place.

Kazemi Rad *et al.* in 2008 used the ribosomal internal transcribed spacer 1 (ITS1) and amplified it with specific primers. They digested the PCR products with a restriction enzyme (HaeIII) and concluded that PCR-RFLP seems to be an effective method to identify *Leishmania* species from Gimsa-stained smears collected from both infected humans and animal reservoir hosts in Iran [11]. In our study, we used *ApoI* enzyme for PCR-RFLP method.

In 2008, Spanakos *et al*. obtained similar bands for *L. major* and *L. tropica* by using this gene and the enzyme [9].

By using PCR-RFLP in Mashhad samples, Vaeznia *et al*. in 2009 assessed 34% of the infected persons as *L. major* and 66% of the mas *L. tropica* [12].

Chaouch *et al*. used PCR technique of cystein proteinase B gene to discriminate between *L. major, L. tropica*, and *Leishmania infantum* Tunisian species. They found by phylogenetic analysis that there are different strains in Tunisian samples [13].

In a research in Yemen, Mahdy *et al*. by using ITS-1 in molecular characterization of *Leishmania* species isolated from cutaneous leishmaniasis showed two sequence types of *Leishmania tropica* [14].

Hajjaran *et al*. found no correlation between intra-species divergence and geographical distribution by using N-acetylglucosamine-1-phosphate transferase gene, but in this study, we showed differences with *Leishmania major* samples isolated from North-East of Iran with other parts by using ITS1 gene [15].

The results of sequencing different species and comparing them are as follow:

Similarity of DNA sequences of Damghan and Esfarayen isolates (North-East part of Iran) of *L. major* is 100%. Also, similarity of DNA sequences of Kerman, Ardestan, Kashan, Samirom, and Qom isolates (central part of Iran) is 100%. In addition, the similarity of these two groups is 99%. Moreover, the similarity among reference/standard group (MRHO/IR/75/ER) and North-East group and central group is 95%. The results showed that the sequences for all samples from central parts (Kerman, Ardestan, Kashan, Samirom, and Qom) are the same, but there is difference with the samples isolated from North-East part of Iran (Damghan and Esfarayen). The situation of these two samples in phylogeny tree showed the separation branch from the other samples and near to branch *Leishmania turanica* (Figure 6).

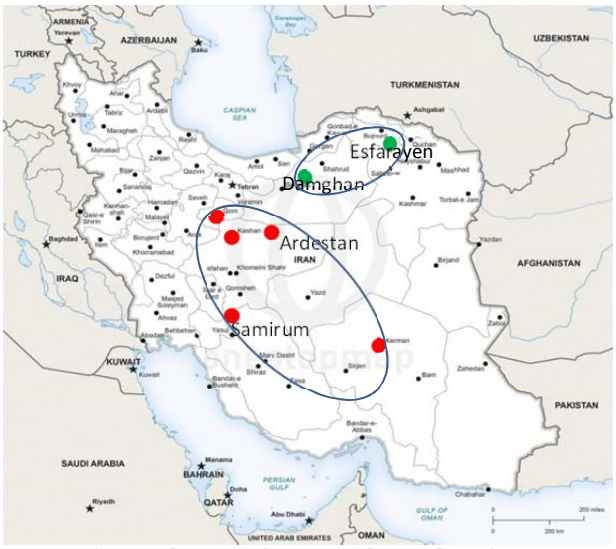

Figure 6) Distribution of separated samples of cutaneous leishmaniasis caused by *Leishmania major* in central parts of Iran (●), North-East part of Iran (●), and caused by *Leishmania tropica* (●)

The two *L. tropica* isolates had 96% similarity with each other. The similarity of one isolated of *L. tropica* was 95% with India isolated, 94% with Sudan isolate, and 92% with Shiraz (1, 2, and 3) isolates.

Another isolated of *L. tropica* has similarity of 94%, 91%, and 91% with India, Shiraz (1, 2, and 3), and Mashhad isolates, respectively.

The isolates of central group have the most similarity of 99% with Friedlin strain.

Also, the highest similarity of the isolate derived from Esfarayen was 99% with IPAP/IR/2008/DamgP2 strain.

Although the *L. major* samples were from different geographical areas, when compared to each other they showed less difference than *L. tropica* isolates.

It is recommended to use ITS1 gene to employ PCR method as an immediate way of leishmaniasis diagnosis, and to use RFLP method for recognizing

the leishmaniasis type.

The ITS1 gene of Iranian strain of *L. infantum* (MCAN/IR/97/LON) has 3 sites for *ApoI* restriction enzyme and will produce 4 bands with lengths of 390, 112, 72, and 19bp as a result of applying *ApoI* enzyme.

The results showed that Iranian isolates could discriminate by using the ITS1 gene and *Apo1* restriction enzyme.

Conclusion

Leishmaniasis is distributed in many parts of Iran. Finding an accurate method for diagnosis and identifying the species of Leishmania is very important. The results of this study showed that the gene and primers used for PCR and the enzyme used for RFLP are well selected.

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Ethical Permissions: This project was approved by the Medical Ethics Committee of Tarbiat Modares University according to Declaration of Helsinki.

Conflict of interests: No conflict of interest was reported by the authors.

Authors' Contribution: Nasibeh Beheshti (First author), Original researcher (30%); Fatemeh Ghaffarifar (Second author), Methodologist/ Discussion author (20%); Zohreh Sharifi (Third author), Assistant (5%); Zahra Eslamirad (Fourth author), Assistant (5%); Mohammad Farivar Sadri (Fifth author), Assistant (10%); Mohammad Saaid Dayer (Sixth author), Assistant (5%); Vahid Nasiri (Seventh author), Statistical analyst (5%); Parisa Ebrahimisadr (Eighth author), Introduction author (5%); Najla Hamidianfar (Ninth author), Assistant (5%); Sara Bayesh (Tenth author), Assistant (5%); Ogholniaz Jorjani (Eleventh author), Assistant (5%).

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