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Authors: Nazanin Ahmad Khosravi, Azar Dokht Khosravi, Mehrandokht Sirous, Mohammad Hashemzadeh, Homayoun Amiri, Shokrollah Salmanzadeh, Seyed Mohammad Alavi, Zohreh Deilami, Maryam Mohsenpoor, Meisam Movahedi

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Molecular detection of isoniazid and rifampin-resistant *Mycobacterium tuberculosis* strains from southwest of Iran

Nazanin Ahmad Khosravi [1,2,3], Azar Dokht Khosravi [1,2,4], Mehrandokht Sirous [1,5], Mohammad Hashemzadeh [1,2], Homayoun Amiri [1], Shokrollah Salmanzadeh [2,6], Seyed Mohammad Alavi [1], Zohreh Deilami [3], Maryam Mohsenpoor [3], Meisam Movahedi [3]

[1] Infectious and Tropical Diseases Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

[2] Department of Microbiology, Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

[3] Khuzestan Tuberculosis Regional Reference Laboratory, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

[4] Iranian Study Group on Microbial Drug Resistance, Tehran, Iran

[5] Department of Microbiology and Parasitology, Faculty of Medicine, Bushehr University of Medical Sciences, Bushehr, Iran

[6] Tropical Medicine Ward, Razi Teaching Hospital, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

Corresponding author: Nazanin Ahmadkhosravi, e-mail: nazaninahmadkhosravi@gmail.com; Mehrandokht Sirous, e-mail: mehrandokht.sirous@gmail.com

ORCID

NA: https://orcid.org/0009-0007-2453-6351

ADK: https://orcid.org/0000-0002-7852-6868

MS: https://orcid.org/0000-0002-1509-9563

MH: https://orcid.org/0000-0002-8090-2332

HA: https://orcid.org/0009-0005-3960-7875

SS: https://orcid.org/0000-0002-5632-9425

SMA: https://orcid.org/0000-0003-4328-4747

ZD: https://orcid.org/0009-0002-4656-9107

MaM: https://orcid.org/0000-0002-1620-3625

MeM: https://orcid.org/0009-0009-5874-4743

ABSTRACT

Introduction and aim. The study of molecular mechanisms of resistance to first-line antibiotics for the treatment of tuberculosis is of great importance. Investigating these mechanisms provides valuable information to treatment policy makers. Therefore, this study aimed to identify mutations associated with resistance to rifampin (RIF) and isoniazid (INH) among drug-resistant *Mycobacterium tuberculosis* (MTB) isolates from a TB reference center in southwest Iran.

Material and methods. In this cross-sectional study (September 2014-February 2018), a total of 772 MTB isolates were confirmed by culture on Löwenstein-Jensen (LJ) medium and standard biochemical tests. Drug susceptibility testing of RIF and INH was determined by the proportion method on LJ medium. Mutations conferring resistance to INH and RIF were determined by polymerase chain reaction analysis and sequencing.

Results. In this study, 772 (22.68%) MTB strains were isolated from 3,404 TB-suspected patients, of whom 26.6% were women and 73.8% male. Of the 772 clinical strains, 8 (1.03%) were MDR (resistant to INH and RIF), 15 (1.94%) were resistant to RIF and 4 (0.5%) were resistant to INH. Of the 12 identified isolates identified (including both 4 isolates resistant to INH and 8 isolates resistant to MDR), 2 (16.6%) had a mutation at codon 315 of *katG*, 1 (8.33%) had a mutation at (-15) of *inhA*, and 9 (75%) did not show a detectable mutation. Regarding rifampin, the frequency of mutations in the *rpoB* gene was the following: codons 531 (n=7, 30.4%), 533 (n=5, 21.7%), 526 (n = 2, 8.69%) and 511 (n=1, 4.3%). Furthermore, no mutations were detected in 8 (34.7%) isolates.

Conclusion. The most prevalent mutation in INH resistant isolates was at codon 315 of *katG*. In RIF-resiatant isolates, the most prevalent mutation was at codon 531 of the *rpoB* gene. In a considerable number of isolates, no mutations in *katG*, *inhA*, and *rpoB* were found compared with deposited sequences available from NCBI GenBank.

Keywords. drug resistance, isoniazid, mutation, *Mycobacterium tuberculosis*, rifampin

Introduction

Tuberculosis (TB), an infection with *Mycobacterium tuberculosis* (MTB), continues to be a major global health burden and is one of the deadliest diseases worldwide.[1,2] According to the WHO (World Health Organization), approximately one-third of the world's population is infected with MTB. In 2023, the estimated number of patients was 10.8 million, resulting in 1.25 million deaths.[3] The development of drug-resistant tuberculosis is a considerable health challenge in the treatment of TB worldwide.[4] There has been a rapid increase in multidrug-resistant (MDR) strains of tuberculosis, defined as resistance to the first-line antibiotics isoniazid (INH) and rifampin (RIF).[4] A recent estimate showed 400,000 incident cases of MDR in 2023, with only a fraction receiving timely treatment.[3]

MTB can acquire resistance to anti-TB drugs through spontaneous mutations in genes encoding antibiotic targets, drug-activating enzymes, or drug efflux pumps. These genes, which encode drug metabolizing or drug-targeting enzymes, significantly affect the efficacy of anti-TB therapy.[5] Most INH-resistant strains (INH-R) have mutations in genes involved in cell wall synthesis, such as the *inhA* gene and its promoter, the *katG* gene (particularly codon 315), or the *oxyR-ahpC* region. The *katG* deletion mutants show greater resistance to INH than strains with mutations in *inhA* or its promoter.[6] Mutations occur predominantly occur in an ''hot spot'' 81 bp fragment of the *rpoB* gene, called the RIF resistance determining region (RRDR), which confers resistance to RIF.7 The *rpoB* gene encodes the β-subunit of RNA polymerase, and mutations are located at codons 531, 526, and 516.8.

Due to the varying frequencies across different geographical areas, it is essential to evaluate the prevalence of mutations.[9] Studying the molecular mechanisms of resistance to first-line antibiotics for tuberculosis treatment is of great importance. Investigating these mechanisms provides valuable information to treatment policy makers. The study of mutations related to resistance to first-line tuberculosis drugs in the southwestern region of Iran has rarely been evaluated. However, the pattern of these mutations may change over time.

Aim

Given the importance of first-line drugs in TB treatment, it is a need to constantly assess the frequency of resistance to RIF and INH and their distribution within the community. Therefore, this study aimed to identify mutations in *inhA*, *katG*, and *rpoB* genes associated with resistance to INH and RIF among MTB isolates from a TB reference center for TB in southwest Iran. The regional distribution of the mutation, the proportion of phenotypically resistant isolates without detectable mutations at the target loci, or its relationship to local molecular detection may be among the new findings that can be obtained through this study.

Material and methods

Ethical clearance

The ethical consideration of this research was confirmed by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (No: IR.AJUMS.REC.1396.208). All research was conducted in accordance with relevant guidelines/regulations. Written informed consent was received from all participants and/or their legal guardians.

Study design and bacterial isolates

In this cross-sectional study (September 2014-February 2018), MTB isolates were obtained from 3,404 samples of patients referred to the Ahvaz Regional TB Laboratory in southwest Iran. The Ahvaz Regional

Tuberculosis Laboratory in southwest Iran operates under the supervision of the World Health Organization and participates in regular quality assurance programs to further ensure consistency, reproducibility, and validity of culture and identification procedures. The sample size was determined using the G * Power software with the following parameters: power (1-β err prob)=0.95, α err prob = 0.05 and effect size = 0.12 Non-sterile respiratory specimens such as endotracheal aspirates, bronchoalveolar lavage (BAL), and sputum were sterilized using the NALC-NaOH method (standard N-acetyl-L-cysteine–sodium hydroxide) and then centrifuged at 3,000×*g* for 15 min. The resulting pellets were suspended again in sterile phosphate buffer (pH 6.8) and inoculated in LJ (Löwenstein-Jensen) medium. Body fluids or tissues were centrifuged at sterile sites and the pellets were inoculated directly into culture medium. Identification of MTB was confirmed using colony morphology, Ziehl-Neelsen acid fast staining and conventional biochemical tests, including catalase activity, nitrate reduction, and accumulation of niacin. The reference strain MTB H37Rv (ATCC 27294) was used as a quality control.[10] No isolates were excluded due to contamination or incomplete data from the study after confirmation. However, data on relapse, reinfection, or failure of treatment were unavailable for analysis.

Drug susceptibility testing (DST)

DST was done on LJ medium by the proportional method and according to WHO recommendations.[11] The critical concentrations of INH and RIF (Sigma Aldrich Co., St Louis, MO, USA) in the LJ medium were 0.2 μg/mL and 40 μg/mL, respectively.[11] Finally, a 1.0 McFarland suspension of bacterial colonies was prepared, and 10^{-2} and 10^{-4} dilutions were inoculated into LJ medium containing 0.2 μg/mL INH and 40 μg/mL RIF and LJ medium without antibiotics. The results were read after incubation for 28 days and 42 days at 36±1°C. Isolates were defined as drug-susceptible if the growth/colony count on antibiotic-containing LJ medium was ≤1% compared to the control medium without antibiotics. MTB H37Rv (ATCC 27294) was utilized as a quality control.

DNA extraction

Total DNA was extracted from drug-resistant isolates grown in LJ medium using the QIAamp Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, approximately one loopful of colonies was suspended in 180 μL of ATL buffer and heat-killed at 95 ° C for 30 min to ensure biosafety. The samples were incubated with 20 μL of proteinase K at 56 ° C for 1 h, followed by the standard binding, washing and elution steps. DNA was eluted in 200 μL of AE buffer and stored at -20 ° C until use. DNA purity and concentration were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA).

Polymerase chain reaction (PCR) amplification

The genes *katG*, *rpoB* and *inhA* genes were amplified by PCR by specific primers (Table 1).[12,13,14] The PCR reaction mixtures were obtained in a final volume of 50 µL, including 1.5 mM MgCl2, 5 µL of 10× PCR buffer, 0.2 µM of each primer, 0.2 mM of deoxynucleotide triphosphate (dNTP), 5 µL (10 ng) of genomic DNA, 2.5 U Taq DNA polymerase, and 35.4 µL of nuclease-free water. The amplifications were performed using a thermal cycler (Bio-Rad, Hercules, CA, USA) with the following cycling program: an initial 95 ° C denaturation step for 5 min, followed by 30 cycles of 95 ° C for 40 s, 64°C (*rpoB*, *inhA*) or 55 ° C (*katG*) for 1 min and 72°C for 40 s, and a final extension step of 72 ° C for 10 min.

DNA sequencing and analysis

The PCR products were sent to Bioneer Co., South Korea, for Sanger sequencing and purification using a 3730xl DNA analyzer (Thermo Fisher Scientific, USA). The sequences were trimmed and edited using Chromas software. To align the sequences obtained, ClustalW (https://www.genome.jp/tools-bin/clustalw) was utilized to specify the consensus sequences. The consensus sequences were subjected to nBLAST analysis (http://blast.ncbi.nlm.nih.gov) and compared with the H37Rv strain of MTB (ATCC 27294). For quality control, Bioneer Co., South Korea, considered the duplication rate, GC content distribution, nucleotide distribution, and base quality.

Table 1. Primers used in this research

Drugs	**Genes**	**Primer sequence (5′ to 3′)**	**Product size (bp)**	**Reference**
Isoniazid	*katG*	5'-CATGAACGACGTCGAAACAG-3' 5'-CGAGGAAACTGTTGTCCCAT-3	232	[12]
Rifampin	*rpoB*	5'-CGATCACACCGCAGACGTTG-3' 5'-GGTACGGCGTTTCGATGAAC-3'	318	[13]
Isoniazid	*inhA*	5'-ACATACCTGCTGCGCAAT-3' 5'-TCACATTCGACGCCAAAC-3'	400	[14]

Statistical analysis

The collected data were analyzed using the Statistical Package for the Social Sciences (SPSS) software version 22 (IBM Corporation, Armonk, New York, USA). The methods used included descriptive statistical tests (frequencies and percentages).

Results

In this study, 772 (22.68%) MTB strains were isolated from 3,404 TB-suspected patients, of whom 26.6% were women and 73.8% male. Most of the samples were sputum (59.84%), followed by BAL (37.82%), endotracheal tube samples (1.03%), and other sample types (eg, abscess, wound, tissue). The results of drug resistance tests using the proportion method in 772 isolates for RIF and INH indicated that 8 (1.03%) isolates were MDR, 15 (1.94%) were RIF monoresistant, and 4 (0.5%) were INH monoresistant. These 27 isolates were selected for gene sequencing. Amplification of the genes *katG*, *rpoB* and *inhA* genes in resistant MTB isolates produced products of 232 bp, 318 bp, and 400 bp, respectively. Sequencing of the *inhA* and *katG* genes of the 4 INH-monoresistant isolates revealed that 25% (1/4) harbored a mutation in the *katG* gene at codon 315. Among the RIF-monoresistant isolates, 26.6% (4/15), 20% (3/15), and 6.6% (1/15) carried mutations in the *rpoB* gene at codons 531, 533, and 511, respectively (Table 2).

Table 2. Mutation pattern of the promoter genes *rpoB*, *katG*, *mabA-inhA* in RIF and INH-resistant *Mycobacterium tuberculosis* with different drug resistance phenotypes *

Resistant isolate code	Resistant phenotype	Nucleotide change, *rpoB*	Amino acid change *rpoB*	Nucleotide change, *katG*	Amino acid change *katG*	Nucleotide change, *mabA-inhA* promoter
438	MDR	CAC→TAC	His526Tyr	_	_	_
701	MDR	CAC→CCC	His526Pro	AGC→ACC	Ser315Thr	_
577,646	MDR	CTG→CCG	Leu533 Pro	_	_	_
8,731	MDR	TCG→TTG	Ser531Leu	_	_	_
67	MDR	TCG→ TTG	Ser531Leu	_	_	(-15) C→T
86	MDR	_	_	_	_	_
10	RIF	CTG→CCG	Leu511Pro	_	_	_
39,292,550,841	RIF	TCG→TTG	Ser531Leu	_	_	_
108,341,924	RIF	CTG→CCG	Leu533Pro	_	_	_

108,198,274,281, 286, 410,860	RIF	_	_	_	_	_
851	INH	_	_	AGC→AAC	Ser315Asn	_
32,475,588	INH	_	_	_	_	_

* MDR multidrug resistance, RIF – rifampin, INH isoniazid

In one MDR-TB isolate, a combination of *katG* and *rpoB* mutations was determined, while in another isolate, a combination of *inhA* and *rpoB* mutations was identified. In seven MDR-TB isolates, only mutations in the *rpoB* gene at codons 531, 533, and 526 were observed. The reader is referred to Table 3 to see the details of the nucleotide / amino acid substitutions in the targeted genes (*inhA*, *katG*, and *rpoB*) of MTB isolates.

Table 3. Characteristics of RIF and INH resistant *Mycobacterium tuberculosis* isolates with *rpoB*, *katG*, *mabA-inhA* promoter mutations*

Phenotype	**Isolate code**	**Gender**	***RpoB***	***KatG***	***mabA-inhA***
MDR	438	Male	CAC→TAC (His526Tyr)	Wild type	Wild type
MDR	701	Female	CAC→CCC (His526Pro)	AGC→ACC (Ser315Thr)	Wild type
MDR	8	Male	TCG→TTG (Ser531Leu)	Wild type	Wild type
MDR	731	Male	TCG→TTG (Ser531Leu)	Wild type	Wild type
MDR	67	Male	TCG→TTG (Ser531Leu)	Wild type	(-15) C→T
MDR	577	Female	CTG→CCG (Leu533Pro)	Wild type	Wild type
MDR	646	Male	CTG→CCG (Leu533Pro)	Wild type	Wild type
MDR	86	Male	Wild type	Wild type	Wild type
RIF resistant	10	Female	CTG→CCG (Leu511Pro)	Wild type	Wild type
RIF resistant	39	Male	TCG→TTG (Ser531Leu)	Wild type	Wild type
RIF resistant	292	Male	TCG→TTG (Ser531Leu)	Wild type	Wild type
RIF resistant	550	Male	TCG→TTG (Ser531Leu)	Wild type	Wild type
RIF resistant	841	Male	TCG→TTG (Ser531Leu)	Wild type	Wild type
RIF resistant	108	Male	CTG→CCG (Leu533 Pro)	Wild type	Wild type
RIF resistant	341	Female	CTG→CCG (Leu533 Pro)	Wild type	Wild type
RIF resistant	924	Male	CTG→CCG (Leu533 Pro)	Wild type	Wild type
RIF resistant	106	Male	Wild type	Wild type	Wild type
RIF resistant	198	Male	Wild type	Wild type	Wild type
RIF resistant	274	Male	Wild type	Wild type	Wild type
RIF resistant	281	Male	Wild type	Wild type	Wild type
RIF resistant	286	Female	Wild type	Wild type	Wild type
RIF resistant	410	Male	Wild type	Wild type	Wild type
RIF resistant	860	Female	Wild type	Wild type	Wild type
INH resistant	851	Male	Wild type	AGC→AAC (Ser315Asn)	Wild type
	32	Female	Wild type	Wild type	Wild type
	475	Male	Wild type	Wild type	Wild type

INH resistant	588	Male	Wild type	Wild type	Wild type
INH resistant					
INH resistant					

* MDR multidrug resistance, RIF – rifampin, INH isoniazid

Discussion

In addition to the increasing global rate of antibiotic resistance among Gram-positive and Gram-negative bacteria, the spread of drug-resistant MTB poses a major problem for healthcare systems worldwide.[15-18] TB, especially drug-resistant TB, affects both individuals and countries.[19] In this study, the results of phenotypic DST revealed 8 (1.03%) MDR, 15 (1.94%) RIF-monoresistant and 4 (0.51%) INH-monoresistant MTB isolates. This is according to a recent report from Iran.[20] The prevalence of INH-resistant, RIF-resistant (RIF-R), and MDR-TB strains in various provinces of Iran has been reported as 0–16.7%, 0–16.1%, and 0–20%, respectively.[20] A considerable number of patients were male (n=570, 73.8%), whereas only 202 (26.16%) were female. In the investigations by Charan et al.[21] and Vilegas et al.[22], a high percentage of the patients were men (83.9% and 82.1%, respectively), consistent with our study. Although male patients constituted the majority of TB cases (73.8%), the number of MDR-TB cases was too small to determine any significant association between gender and resistance profile. In this research, amplification of *the katG, inhA, and rpoB* genes was performed using locus-specific primers. According to sequencing results, 16.6% (2/12) of INH-R isolates exhibited an amino acid variation in the *katG* gene, with 8.3% showing the S315T amino acid substitution. It is suggested that bacteria prefer the *katG*-S315T mutation because it reduces INH activation, while 30–40% of catalase-peroxidase activity required for virulence is preserved in these isolates.[23] These findings contrast sharply with other studies from different countries that demonstrated a higher percentage of the *katG*-S315T mutation, such as Southeast Asia (78.4%), Vietnam (85.3%), India (67.6%), Cameroon (64%), Romania (52.8%) and the United States (38%).[24-29] These differences may be explained by geographical variation, differences in study design, sample size, diagnostic methods, and circulating lineages.[30] In the current investigation, the mutation rate of the *inhA* gene was 8.3% (1/12), which is consistent with the study by Miotto et al.[31] However, it was higher than in previous research carried out by Hamed et al.[10] (3%), Solo et al.[23] (2%) and Diande et al.[32] (0%). Furthermore, the mutation rate of the *inhA* gene in this study was lower than that reported by Pinhata et al.[33] (33%), Ayalew et al.[34] (43%), Siddiqui et al.[35] (17%) and Zurita et al.[36] (20.2%). There were no combined mutations in *inhA* and *katG*, consistent with previous studies.[10,37] In total, 75% (9/12) of the INH-R strains did not show any variation in the *inhA* or *katG* genes. This may be explained by mutations in other genes, including those that control katG expression (*sigI* and *furA-katG* intergenic region), *ndh*, *oxyR*, *ahpC* and *kasA*, or by the efflux system.[34] In the present study, among 23 isolates of RIF-R, 30.4% of the *rpoB* gene mutations occurred at codon S531L, followed by 21.7% at codon L533P, 8.69% at codon H526T, and 4.3% at codon L511P. The presence of most mutations at codon 531 has also been reported in other studies,

consistent with our results.[10,37] Two possible mechanisms can explain this higher frequency: first, there may be a higher genetic alteration at this codon; secondly, the occurrence of other substitutions in this region of the β-subunit of polymerase is less successful.[2] In the current research, a comparison was made between MDR and RIF monoresistant strains, which demonstrated a higher incidence of the S531L mutation in the former strains (37.5 % to 26.6%), which is consistent with some studies.[38] Considering geographic regions, the prevalence of mutations in different codons of the *rpoB* gene varies in drug-resistant MTB isolates.[39] The observed differences may result from the administration of various drug analogues in different geographic regions, which provide a selective pressure for distinct genetic alterations.[39] In this study, no double mutations in genes related to RIF resistance, which was in contrast to previous studies.[2,32] Overall, there was no change in the sequenced region of 34.78% (8/23) of RIF-resistant isolates, most of which were related to RIF-monoresistant isolates (30.43%). It is necessary to conduct additional studies to identify genetic variation beyond the *rpoB* RDRR or in other associated genes, such as *rpoC* and *rpoA*, that may contribute to rifampin resistance in these isolates.[9] Our results revealed that although these tests target resistance-associated regions, a significant number of resistant isolates in our study lacked detectable mutations in the *inhA*, *katG*, and *rpoB* genes within these regions. This underscores the need to complement ongoing molecular diagnostics with expanded genomic panels in regions like southwest Iran. According to WHO guidelines, genetic diagnostic assays, including GeneXpert MTB/RIF and LIPA (Line Probe Assays), are recommended for the rapid detection of isoniazid and rifampin resistance.[40] GeneXpert MTB/RIF is also utilized to detect MTB and rifampicin resistance in Iran. DNA sequencing is highly accurate and can detect a wide range of mutations in antibiotic resistance genes across multiple pathogens.[41] Therefore, we used sequencing analysis for the *inhA*, *katG* and *rpoB* genes. The geographic variability of mutations underscores the importance of region-specific molecular surveillance to inform local TB control strategies and diagnostic practices. Furthermore, the lack of detected mutations in some INH-R isolates could be explained by mutations in genes such as *ahpC*, *ndh*, and *oxyR-ahpC*, which were not included in the current analysis. Future studies involving a broad range of genetic targets could better elucidate the complex mechanisms underlying isoniazid resistance.

Study limitations

This study had some limitations. These included a relatively small number of resistant isolates, the lack of evaluation of other resistance-associated genes (such as *ndh*, *oxyR-ahpC*, and *ahpC*), the lack of clonal relatedness analysis, and the lack of whole genome sequencing (WGS) for isolates without mutations in the genes studied. Furthermore, the lack of clinical data, such as a relapse or treatment failure, restricts the correlation of genetic mutations with phenotypic drug resistance. Another limitation was the lack of socio-demographic data.

Conclusion

The most prevalent mutation in INH resistant isolates was at codon 315 of *katG*. In RIF-resiatant isolates, the most prevalent mutation was at codon 531 of the *rpoB* gene. In a considerable number of isolates, mutations in *katG*, *inhA*, and *rpoB* were not found compared with deposited sequences available from NCBI GenBank. These results emphasize the significance of integrating regional mutation signatures into regional tuberculosis resistance monitoring initiatives and customizing molecular diagnostics to detect additional resistance markers apart from *katG*, *rpoB*, and *inhA* genes.

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Declarations

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Author contributions

Conceptualization, N.A.K. and M.S.; Methodology, N.A.K., M.S., and M.H.; Validation, A.D.K.; Formal Analysis, A.D.K. and H.A.; Investigation, S.S. and S.M.A.; Data Curation, N.A.K. and Z.D.; Writing – Original Draft Preparation, N.A.K. and A.D.K.; Writing – Review & Editing, M.S. and A.D.K.; Visualization, Ma.M.; Supervision, A.D.K. and Me.M.; Project Administration, A.D.K.

Conflicts of interest

We declare that there are no conflicts of interest on the part of any of the authors in this study.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical approval

Ethical consideration for this research was confirmed by the Ahvaz Jundishapur University of Medical Sciences Ethics Committee, Ahvaz, Iran (No: IR.AJUMS.REC.1396.208).

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