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Research article

Evaluation of aminoglycoside modifying enzymes, SCCmec, *coagulase* gene and PCR-RFLP *coagulase* gene typing of *Staphylococcus aureus* isolates from hospitals in Shiraz, southwest of Iran

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ABSTRACT

Staphylococcus aureus is an important human pathogen that causes various infections. Aminoglycosides are broad-spectrum antibiotics used to treat methicillinresistant *S. aureus* (MRSA) infections. Typing of *S. aureus* isolates by coagulase gene typing and PCR-RFLP *coa* gene is a fast and suitable method for epidemiological studies. Aim of the present study was to evaluate the resistance to aminoglycosides, staphylococcal chromosomal cassette mec (SCCmec) types, coagulation typing and PCR-RFLP *coa* gene in clinical isolates of *S. aureus*. 192 *S. aureus* isolates were collected from Namazi and Shahid Faghihi hospitals. Antibiotic resistance was measured by disk diffusion method and MIC was determined for gentamicin. The presence of genes encoding aminoglycoside modifying enzymes (*AME*) and *mecA* gene were assessed by PCR. Also the coagulase typing, PCR-RFLP *coa* gene, and SCCmec typing were performed. Out of 192 isolated *S. aureus* isolates, 83 (43.2%) MRSA isolates were identified. In this study, a high resistance to streptomycin and gentamicin (98.7%) were observed. Among the *AME* genes, the *aac (6′)-Ie-aph (2″)* gene was the most common. Based on the SCCmec typing, it was determined that the prevalence of SCCmec type III (45.8%) was highest. From the amplification of the *coa* gene, 5 different types were obtained. Also, in digestion of *coa* gene products by *HaeIII* enzyme, 10 different RFLP patterns were observed. According to this study, aminoglycoside resistance is increasing among MRSA isolates. As a result, monitoring and control of aminoglycoside resistance can be effective in the treatment of MRSA isolates. Also, typing of *S. aureus* isolates based on *coagulase* gene polymorphism is a suitable method for epidemiological studies.

1. Introduction

Staphylococcus aureus is one of the most important Gram-positive bacteria that colonizes the skin, mucous membranes, and nose. About 20–40% of the healthy people in the community are carriers of *S. aureus* in their nose [1, 2]. This bacterium is one of the most important human infectious agents, especially in hospitals, and causes a wide range from mild skin infections to severe infections such as abscess formation, sepsis, endocarditis, osteomyelitis, urinary tract infections, and fatal necrotic pneumonia [3, 4]. Virulence factors (toxins and enzymes) and mechanisms of antibiotic resistance have made *S. aureus* an important pathogen [5]. Extensive use of antibiotics (beta-lactams, including penicillins and cephalosporins) has led to the spread of methicillin-resistant *S. aureus* (MRSA). MRSA isolates divided into two groups: Health care-Associated MRSA (HA-MRSA) and Community Acquired - MRSA (CA-MRSA) [6]. Resistant to a wide range of other antibiotics, including tetracycline and aminoglycosides is the main concern about MRSA (7). Aminoglycosides are broad-spectrum antibiotics used in combination with b-lactams for treatment of the *S. aureus*. These antibiotics by binding to the ribosomal 30S subunit, inhibit protein synthesis, so these antibiotics are bactericidal agents [8, 9]. *S. aureus* by producing aminoglycoside modifying enzymes (AME) is able to develop resistance to aminoglycosides. AMEs include aminoglycoside phosphotransferase (APH), acetyltransferase (AAC), and nucleotidyltransferase (ANT). The most common AME genes

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present in *S. aureus* are *aac (6′)-Ie-aph (2″), aph(3′)-IIIa*, *ant(4′)-Ia*, *aph(2″)-Ib*, *aph(2″)-Ic,* and *aph(2″)-Id*, which are mostly located on transposons or plasmids and have the ability to move between *S. aureus* [10]. The use of techniques that can rapidly detect and type bacteria is an essential requirement for epidemiological surveillance and hospital infection control [11]. Molecular typing techniques are the most important methods used to study epidemiology. Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) is a simple, accurate, reproducible, and easy method, which is widely used in typing *S. aureus* [12]. *Coagulase* gene typing can be used to *S. aureus* isolates typing. Since coagulase is produced in all *S. aureus* isolates and its size and restriction endonuclease site polymorphism are different in *S. aureus* strains, *Coagulase* gene typing by PCR-RFLP technique can be used as a practical method for typing of *S. aureus* [13, 14].

Therefore, the aim of this study was to evaluation of aminoglycoside modifying enzymes, SCCmec, coagulase gene and PCR-RFLP coagulase gene typing of *Staphylococcus aureus* isolates from hospitals in Shiraz, southwest of Iran.

2. Materials and methods

2.1. Isolation and identification of S. aureus

In this study, a total of 192 samples were collected during 2019–2020 from Nemazi (92 samples) and Shahid Faghihi (100 samples) hospitals in Shiraz from dermatology, emergency, ICU, internal medicine, surgery and neurology wards (skin, blood, wounds, fluids, nasal, sputum, eyes, and abscesses). The samples were identified using phenotypic and biochemical tests such as Gram staining, catalase, production of coagulase, DNase, and fermentation of mannitol [15].

2.2. DNA extraction

For this purpose, a DNA extraction kit, GeneAll Seoul Korea, was used to extract DNA from the isolated bacterial samples.

2.3. Detection of nuc gene

The strain identity of *S. aureus* was confirmed by PCR reaction for the heat-resistant nuclease (*nuc)* gene. Primers based on previous studies were used for this purpose [16]. PCR amplification was performed in a total volume of 25 μl containing 0.5 μl of each primer (10 pM), 12.5 μl of DNA polymerase master mix RED (Ampliqon Co, Inc, Denmark), 1 μl of DNA, and 10.5 μl of water (DNase and RNase free water). The PCR cycle consisted of denaturation at 94 °C for 5 min followed by 35 cycles at 94 °C for 30 s, annealing at 51–59 °C for 40 s, and extension at 72 °C for 40 s. Amplification products were analyzed using 1.5% agarose gel with KBC power load dye (CinnaGen Co. Iran) visualized through UV trans-illumination [17]. *S. aureus* ATCC 25923 was used as the control.

2.4. Antimicrobial susceptibility testing

To perform the antimicrobial susceptibility test, Clinical and Laboratory Standards Institute (CLSI) instructions was used. Antibiotic discs including Amikacin (30 μg), Gentamicin (10 μg), Tobramycin (40 μg), Kanamycin (30 μg), Netimicin (30 μg), Streptomycin (300 μg), and Spectinomycin (100 μg) were used on Mueller-Hinton agar. Also, the minimum inhibitory concentration (MIC) of gentamicin for *S. aureus* isolates was determined according to CLSI instructions [10].

2.5. Identification of methicillin resistant isolates

Methicillin resistance *S. aureus* isolates were primarily detected based on resistance to cefoxitin (30 μg) disk (Rosco, Denmark) by CLSI recommended disk diffusion method [17]; then, detection of *mecA* gene was performed by PCR method for final confirmation of methicillin-resistant isolates for cefoxytin. PCR amplifications were performed on a T100™ thermal cycler (Bio- Rad, Hercules, CA, USA). Final volume of 25 μl containing 12.5 μl Master mix (Amplicon, Denmark), 0.2 μl of each primer with concentration of 10 pmol/μl, and 2 μl of DNA template top up to 25 μl. The cycling condition was set up as follows: initial denaturation at 96 °C for 3 min, followed by 35 cycles of 30 s at 96 °C, annealing for 1 min at 55 °C and 2 min at 72 °C; and an extension for 10 min at 72 °C. Staining was performed with safe stain load dye (CinnaGen Co., Iran) and then observed under the UV trans-illuminator [18]. *S. aureus* ATCC 25923 was used as positive control for disk diffusion method.

2.6. Detection of AMEs genes

For this purpose, the presence of AME genes in *S. aureus* isolates was investigated. These genes include *aac(6′)-Ie-aph (2″)*, *aphz(3′)-IIIa*, *ant(4′)-Ia*, *aph(2″)-Ib*, *aph(2″)-Ic* and *aph(2″)-Id* [19]. The amplification conditions were as follows: In a total volume of 25 μl containing 0.5 μl of each primer (10 pM), 12.5 μl of DNA polymerase master mix RED (Ampliqon Co, Inc, Denmark), 1 μl of DNA, and 10.5 μl of water (DNase and RNase free water). The PCR cycle consisted of denaturation at 94 °C for 5 min followed by 35 cycles at 94 °C for 30 s, annealing at 51–59 °C for 40 s, and extension at 72 °C for 40 s. Amplified products were analyzed by electrophoresis on 1% agarose gel containing safe stain and photographed under UV illumination [20].

2.7. Detection of SCCmec types

Different types of SCCmec were carried out by the method described by Oliveira et al. [18]. Different types of SCCmec were studied by multiplex-PCR assay with specific primers for SCCmec types. Amplification of SCCmec genes were subjected to final volume of 25 μl containing 12.5 μl Master mix (Amplicon, Denmark), 0.2 μl of each primer with concentration of 10 pmol/μl, and 2 μl of DNA template top up to 25 μl. The PCR protocol comprised of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 60 s, annealing 55–59 °C for 60 s, extension at 72 °C for 1 min, and was followed by a final cycle of extension for 5 min at 72 °C. PCR products were detected by electrophoresis, using agarose 2% and stained with SYBER DNA safe stain, and then visualized under UV light.

2.8. Typing of S. aureus isolates based on coagulase typing method

The *coa* gene was amplified using specific primers and PCR reaction. Based on PCR amplification of the end region of the *coa* gene, 11 different types of PCR products are amplified, ranging in size from approximately 900–480 bp [10]. Restriction fragments length polymorphism (RFLP-PCR) technique was used for coagulase typing of *S. aureus* isolates. PCR amplification was performed in a total volume of 25 μl containing 0.5 mL of each primer (10 pM), 12.5 μl of DNA polymerase master mix RED (Ampliqon Co, Inc, Denmark), 1 μl of DNA, and 10.5 μl of water (DNase and RNase free water). The PCR cycle consisted of denaturation at 94 °C for 5 min followed by 35 cycles at 94 °C for 30 s, annealing at 51–59 °C for 40 s, and extension at 72 °C for 40 s [21]. The PCR product of the *coa* gene was digested using *HaeIII* endonuclease restriction enzyme (Fermentas). Briefly, 1 μl of *HaeIII* endonuclease enzyme, 10 μl of PCR product of the *coa* gene, 34 μl of distilled water and 5 μl of restriction buffer were mixed and incubated for 1 h at 37 °C. Finally, the obtained fragments were observed using electrophoresis in 1.5% agarose gel and under UV trans-illumination.

2.9. Statistical analysis

The statistical analysis was performed using SPSS, version 23.0 (SPSS IBM, New York, NY, USA).

3. Results

3.1. Identification and antibiotic resistance

192 strains were isolated (male: 104 and female: 88) from different samples (Table 1). The highest and lowest isolates were related to dermatology (99 isolates, 51.6%) and neurology (6 isolates 3.1%), respectively (Table 2). PCR test for *nuc* gene was used to confirm *S. aureus* isolates, and 192 isolates were positive for this gene. In the first stage, 90 strains were confirmed as MRSA by phenotypic methods (cefoxytin disk), and finally, among these strains, 83 strains were confirmed by genotypic method and the presence of *mecA* gene as MRSA strains. Resistance to aminoglycosides was also high among MRSA isolates. The highest and lowest resistance was related to Gentamicin- Streptomycin (82 isolates, 98.7%), and Netilmicin (50 isolates, 60.2%), respectively (Table 3). However, no significant association was found between *mec*-positive strains and aminoglycoside genes. Also, the MIC test was performed for Gentamicin, and the MIC ranged between 16-1024 mg/mL.

3.2. Coagulase typing method

Based on PCR amplification of the terminal region of the *coa* gene, 5 different regions were identified on Agarose gel electrophoresis (Genotype I 450 bp, Genotype II 500 bp, Genotype III 600 bp, Genotype IV 650 bp, and Genotype V 7500 bp) (Table 4). The highest and lowest PCR products were related to genotype IV(650 bp) (21 isolates) and (V 750 bp) (2 isolates), respectively.

3.3. HaeIII restriction enzyme digestion

The PCR products of the *coa* gene were affected by the *HaeIII* restriction enzyme, and between 80 bp to 500bp bands were observed. Based on the number and size of each of these bands, 10 distinct and recognizable patterns were observed (Figure 1). Some of these patterns included 0,2,3 and 4 bands, and the pattern containing 3 bands was more common among isolates. RFLP pattern E (7 isolates) and D (6 isolates) were found to be the most pattern (Table 4).

3.4. Detection of AMEs genes

Among the 83 MRSA isolates, the frequency of AMEs genes including *aac (6′)-Ie* (34 isolates, 41%), *ant (4′)-Ia* (7 isolates, 8.4%), *aph (3′)-IIIa* (22 isolates, 26.5%), and *aph (2″)* was not detected (Table 5) (there was no significant difference between antibiotic resistance and antibiotic resistance genes).

3.5. Detection of SCCmec types

Based on SCCmec typing, it was determined that out of 83 MRSA isolates, the highest SCCmec type was related to SCCmec type III (38

Table 1. Strains isolated from different samples patients.

Samples	Nemazi number	Shahid Faghihi number
Skin	33 (17.2%)	38 (19.8%)
Blood	25 (13%)	31 (16.2%)
Wounds	13 (6.7%)	10 (5.2%)
Fluids	7 (3.8%)	8 (4.2%)
Nasal	7 (3.7%)	6 (3.1%)
Sputum	4 (2.1%)	3 (1.6%)
Axillary	2 (1%)	2 (1%)
Eyes	1 (0.5%)	1 (0.5%)
Abscesses	0 (0%)	1 (0.5%)
Total	92 (48%)	100 (52%)

Table 2. Strains isolated from different wards of hospitals.

Wards	Nemazi number	Shahid Faghihi number
Dermatology	37 (19.2%)	62 (32.3%)
Emergency	14 (7.3%)	8 (4.2%)
ICU	10 (5.3%)	6 (3.1%)
Internal medicine	16 (8.4%)	14 (7.3%)
Surgery	11 (5.7%)	8 (4.1%)
Neurology	4 (2.1%)	2 (1%)
Total	92 (48%)	100 (52%)

Table 3. Frequency of aminoglycoside resistance among MRSA strains.

Antibiotics	Number
Gentamicin	82 (98.7%)
Streptomycin	82 (98.7%)
Kanamycin	81 (96.3%)
Tobramycin	78 (93.9%)
Amikacin	76 (91.5%)
Spectinomycin	75 (90.3%)
Netilmicin	50 (60.2%)

Table 4. *Coa* genotype, RFLP patterns and PCR product (bp) of MRSA.

Coa	RFLP (Pattern)	PCR Product (bp)	No
I (450 bp)	A	300 -400-500	3
II (550 bp)	B	200/300	4
III (600bp)	C	150/210/310	3
IV (650bp)	D	150/250/350	6
	E	200/300	7
	F	200/300/400	1
	G	150/210/310	3
	H	300/400	1
	I	300/400/500	3
V (750bp)	J	80/200/300/450	2

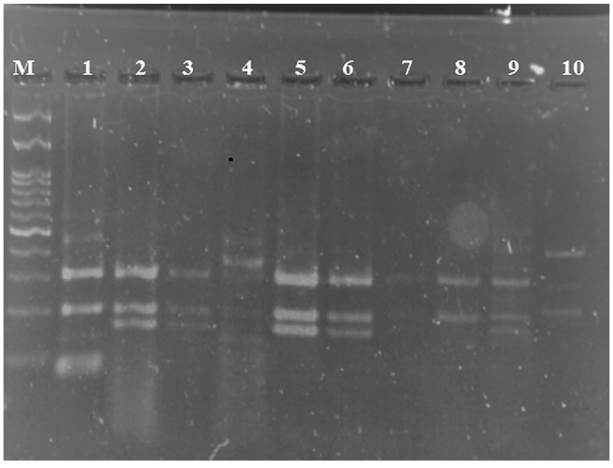

Figure 1. RFLP patterns of the *coa* gene amplicons following digestion with the restriction endonuclease (Electrophoresis in 1.5% agarose gel). *HaeIII*. M, 100-bp molecular size marker. Lane 1 (80, 200, 300, 450 bp), lane 2 (180, 200, 300 bp), lane 3 (180, 200, 300 bp), lane 4 (300, 400, 500 bp), lane 5 (180, 200, 300 bp), lane 6 (180, 200, 300 bp), lane 7 (no band), lane 8 (200, 300 bp), lane 9 (180, 200, 300 bp) and lane 10 (200, 400 bp).

isolates, 45.8%), IA (11 isolates, 13.2%), II (2 isolates 2.4%), and IV 2 isolates 2.4%) (Table 5).

4. Discussion

Staphylococcus aureus is one of the most important bacterial pathogens and is an important cause of nosocomial infections. In recent decades the antibiotic resistance including methicillin resistance *S. aureus* (MRSA) is increasing in the community and in the hospital [22]. *S. aureus* is able to survive in a variety of environmental conditions and is easily transmitted between hospital staff, patients, and different wards. On the other hand, uncontrolled and excessive use of antibiotics caused increases the prevalence of MRSA strains [23, 24]. For treatment of *S. aureus* infections, aminoglycosides are used in combination with beta-lactams and glycopeptides, However, the emergence of aminoglycoside-resistant isolates, which are mainly produced by AME enzymes, has affected the therapeutic effects of this group of antibiotics [9].

In this study, among 83 MRSA isolates, the highest resistance was related to Gentamicin and Streptomycin (82 isolates, 98.7%), while the lowest resistance was related to Netilmicin) 50 isolates, 60.2%). Seyedi-Marghaki et al. reported that the highest resistance to aminoglycosides was related to kanamycin (83.2%), tobramycin (76.2%), and gentamicin (71.4%), while netilmicin (23.8%) had the lowest resistance [10]. Mahdiyoun et al., reported that the highest resistance was related to erythromycin (84.4%) and gentamicin (71.7%) [22]. The results of these studies were in the same line with our study. The reason for the differences in antibiotic resistance between these studies can be attributed to the differences in antibiotics prescribed by the physician, arbitrary use, or overuse of antibiotics in different regions.

Also, the result of MIC test for gentamicin in our study was 1024–16 mg/mL, and in the study of Seyedi-Marghaki et al., 512 mg/mL was reported [10]. In the present study, the frequency of the genes encoding AME showed that the most common gene was *aac (6′)-Ie-aph (2″)* = 34 (41%), while the *aph (2″)-Id* gene was not identified in any of the isolates (there was no significant difference between antibiotic resistance and antibiotic resistance genes). Because aac (6′)-Ie-aph (2″) inactivates and is resistant to aminoglycosides [25], the high prevalence of this gene in our study is reasonable. In the study of Seyedi-Marghaki et al., the highest and lowest prevalence of aminoglycoside resistance among MRSA was related to *aac(6′)-Ie-aph(2″)* 19 isolates (45.2%) and *aph(2″)-Id 2* (4.8%) genes, respectively [10]. In the study of Mahdiyounet al., among the MRSA isolated from patients, the highest frequency was related to the *aac(6′)-Ie-aph(2″)* gene (77%) [22]. In another study, Perumal et al. reported that the highest and lowest frequencies of AME genes were related to *aac(6′)-Ie-aph(2″)* (55.4%) and *ant (4′) - Ia* (9%), respectively [26]. The results of these studies are very similar to our findings and show that *aac (6′)-Ie-aph (2″)* plays an important role in aminoglycoside resistance.

Because the presence of AME genes and *mecA* genes may be related, *SCCmec* typing was also performed in this study. The *SCCmec* typing is a useful and practical method for epidemiological studies in MRSA isolates [10]. In this study, among 83 MRSA isolates, *SCCmec* type III was the major type (38 isolates, 45.8%). Seyedi-Marghaki et al. and Pinheiro et al. reported that *SCCmec* type III (21 isolates 50%) and (53.2%, respectively was the major type of *SCCmec* [27]. The results of these studies are consistent with our study and show that *SCCmec* type III plays an important role in antibiotic resistance. Based on this study, which showed that SCCmec type III is the major type and the aac (6′)-Ie-aph (2″) gene was mainly associated with this cassette, it can be concluded that this resistance gene is carried by SCCmec type III and can spread antibiotic resistance.

Typing of *S. aureus* isolates based on *coa* gene and RFLP-coagulase is a suitable method for typing a large number of isolates in a short time. Compared to other typing methods such as Pulsed-field gel electrophoresis (PFGE), RFLP-coagulase typing is a better method because it is faster and can be used to study the prevalence of bacterial pandemics and ability to classify all *S. aureus* [28]. In the present study, the highest and lowest *coa* genotypes belonged to genotypes IV (21 isolates) and I (3 isolates), respectively. Following the effect of *HaeIII* digestion on the *coa* PCR product, 10 RFLP patterns were created. In a study, Abdulghany

Table 5. RFLP patterns, distribution of aminoglycoside-modifying enzyme-encoding genes, SCCmec type, and coa genotype of MRSA isolates.

No	RFLP (Pattern)	AME Genes	SCCmec Typing	Coa Typing
1	H	acc/ant/aph3	IA	IV
2	D	acc/aph3	III	IV
4	E	acc/aph3	III	II
10	G	acc/aph3	IA	IV
16	J	acc/ant	III	III
16	J	-	III	V
12	D	acc/aph3	III	IV
36	D	-	III	IV
23	E	aph3	IV	IV
21	D	-	III	IV
28	F	Acc	III	IV
28	G	Acc	III	IV
29	E	Acc	III	IV
65	I	acc/ant/aph3	IA	IV
70	R	acc/aph3	III	IV
100	T	Acc	III	IV
103	D	Acc	III	IV
111	D	-	III	IV
73	D	-	III	IV
74	D	-	III	IV
75	G	acc/ant/aph3	III	IV
76	A	aph3	IA	I
77	B	-	IA	II
78	B	aph3	III	II
79	C	-	IA	III
80	C	aph3	III	IV
81	D	acc/ant	IV	IV
82	D	-	III	IV
83	C	Acc	III	III
85	D	-	III	IV
86	I	Acc	III	IV
87	I	acc/aph3	III	IV
89	D	acc/aph3	III	IV
90	E	aph3	IA	IV
91	O	acc/aph3	III	IV
92	A	acc/aph3	IA	I
93	B	Acc	II	II
94	E	-	III	IV
95	D	acc/ant/aph3	III	IV
96	D	-	III	IV
97	D	Acc	III	IV
98	D	acc/aph3	IA	IV
99	D	Acc	III	IV
100	D	-	III	IV
101	D	Acc	III	IV
103	D	acc/aph3	III	IV
104	D	Acc	III	IV
108	D	Acc	IA	IV
110	A	acc/aph3	IA	I
111	D	-	III	IV
112	D	-	III	IV
118	E	acc/ant/aph3	III	II
39	E	Acc	II	III

et al. reported 10 distinct patterns out of 54 isolates [29]. In a study of Seyedi-Marghaki et al., 19 RFLP patterns were reported in 130 isolates [10]. Also, in the study of Mahmoudi et al., 8 patterns in 200 isolates were reported [30]. The reason for the difference between RFLP patterns in the present study and other studies is that the studied cities were different, and the samples and sampling warsd in each of these studies were different. PCR-RFLP typing has been shown to produce different patterns under the influence of different restriction enzymes [31, 32]. In our study, the highest PCR-RFLP pattern was related to patterns D (6 isolates, 150/250/350 bp) and E (7 isolates, 200/300 bp), which were present in genotype IV *Coa* (21 isolates, 650 bp).

5. Conclusions

The prevalence of MRSA isolates and resistance to antibiotics, including aminoglycosides are increasing. When phenotypic tests fail to the determination of resistance to antibiotics, we can use the genotypes test such as the identification of resistance genes method. According the results of this study, the *Iaac (6′) -Ie-aph (2″)* was the most common gene among the *S. aureus* isolates. Also, epidemiological studies on resistant bacteria and isolates by molecular typing methods are a suitable way to reduce resistant isolates and control infections. Among these methods, PCR-RFLP of the *coa* gene suitable method for epidemiological studies. Our results shown that RFLP pattern E and D were most pattern.

Declarations

Author contribution statement

Javad fathi: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Zahra Hashemizadeh: Conceived and designed the experiments; Performed the experiments.

Reza Solymani Dehkordi: Analyzed and interpreted the data; Wrote the paper.

Abdollah Bazargani: Analyzed and interpreted the data.

Kasra Javadi: Performed the experiments; Wrote the paper.

Hossein Hosseini-Nave: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Mahtab Hadadi: Performed the experiments; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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