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DISTRIBUTION OF GENES ENCODING RESISTANCE TO MACROLIDES, LINCOSAMIDES, AND STREPTOGRAMINS AMONG METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* STRAINS ISOLATED FROM BURN PATIENTS

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The increasing resistance to macrolide, lincosamide, and streptogramin B agents among methicillin-resistant *Staphylococcus aureus* (MRSA) is a worldwide problem for the health community. This study aimed to investigate the prevalence of *ermA, ermB, ermC*, and *msrA* in MRSA strains isolated from burn patients in Ahvaz, southwest of Iran. A total of 76 isolates of *S. aureus* were collected from January to May 2017 from Taleghani Burn Hospital in Ahvaz. Among 76 *S. aureus* strains collected, 60 (78.9%) isolates were MRSA. The antimicrobial susceptibility testing for MRSA showed extreme high resistance rate to clarithromycin (100%) and azithromycin (100%), followed by erythromycin (98.3%). The PCR assay revealed that the frequency rates of *msrA, ermA*, and *ermC* genes were 23 (38.3%), 28 (46.7%), and 22 (36.7%), respectively. In addition, none of the MRSA isolates had the *ermB* gene. Because of the high prevalence of macrolide and lincosamide resistance found in MRSA isolates from infections of burn patients in Ahvaz, southwest of Iran, it is recommended that local periodic survey be performed for controlling the dissemination of antimicrobial resistance.

Keywords: *Staphylococcus aureus*, *erm* genes, MRSA, clindamycin, Iran

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Introduction

Infection is the most serious complication among burned patients, which is difficult to control and remains to be the leading cause of morbidity and mortality in these patients. In addition, invasive infections caused by antibiotic-resistant bacteria, which are responsible for 28%–65% of burn deaths globally, should be considered as a potential risk and their resistance pattern must be identified as soon as possible [1, 2].

Staphylococcus aureus is known to be one of the most common burn wound pathogens worldwide. Colonization of *S. aureus* on the surface of burn wounds could be associated with delayed wound healing, increased treatment costs through the need for expensive antibiotics, prolonged duration of stay at burn centers, and increased need for surgical interventions [3, 4].

Since the discovery of the first effective antimicrobials in medical science, *S. aureus* has demonstrated rapid development of antibiotic resistance, as well as developed resistance to the most variety of antibiotics [5]. Although β-lactam antibiotics are the main compounds utilized to treat staphylococci-related infections, the emergence of methicillin-resistant *S. aureus* (MRSA) and alterations in antimicrobial resistance pattern has caused renewed interest in the use of antibiotics, such as macrolide, lincosamide, and streptogramin B (MLSB), for the treatment of these infections [6].

This group of antibiotics, in spite of their different chemical structure, has a similar mode of action and has been classified in the same group. They inhibit protein synthesis by binding to the subunit 23S rRNA of the bacterial 50s ribosomal subunits [7]. Among MLSB, clindamycin, due to its pharmacokinetic properties such as good oral absorption, excellent penetration in the skin, and tolerability, is a frequent choice for some staphylococcal infections, particularly skin and soft-tissue infections. However, extensive use of these antibiotics has led to the emergence of resistance to them [8].

Resistance to MLSB antibiotics among staphylococci more often involves the following two mechanisms, such as the active efflux of the antimicrobial agent by an ATP-dependent pump encoded by *msrA* gene and the ribosomal binding site modification by 23S rRNA methylases mediated by one or more *erm* genes (*ermA, ermB, ermC*, and *ermF*) among which *ermA* and *ermC* are predominant genes [9].

Mechanism of ribosomal target site modification can be either constitutive or inducible. *S. aureus* isolates with constitutive resistance show resistance to erythromycin and clindamycin on *in vitro* testing, whereas isolates with inducible resistance show resistance to erythromycin but appear sensitive to clindamycin on disk diffusion testing [10, 11]. The aim of this study was to investigate the molecular detection of MLSB resistance genes (*ermA, ermB, ermC, mecA*, and

msrA) and antibiotic resistance profiles in MRSA strains isolated from burn patients using polymerase chain reaction (PCR) technique in southwest Iran.

Materials and Methods

Bacterial isolates

In this cross-sectional study, clinical samples were collected from burn patients, admitted to the Taleghani Burn Hospital, Ahvaz, Iran, from January to May 2017. The research was approved by the ethical committee of Ahvaz Jundishapur University of Medical Sciences, Khuzestan, Iran. As a part of the Ahvaz Jundishapur University of Medical Sciences policy, written informed consent was obtained from all patients. The study was conducted in accordance with the Declaration of Helsinki. The specimens included were urine, blood, abscess, deep wound, and endotracheal secretion. The samples were cultured on 10% sheep blood agar and Mannitol salt agar (Merck, Darmstadt, Germany). Presumptive staphylococcal colonies (growth on mannitol salt agar, Gram-positive, and catalase-positive cocci) were tested for production of DNase and coagulase. Isolates with positive reactions (DNase-positive and coagulase-positive) were considered as *S. aureus* [12].

Cefoxitin and oxacillin disk diffusion method

Susceptibility tests for *S. aureus* isolates were performed by the Kirby–Bauer disk diffusion method as recommended by Clinical and Laboratory Standards Institute (CLSI) using oxacillin (1 μg) and cefoxitin (30 μg) disks. The inhibition zones for the oxacillin disk with diameter ≤10 mm for *S. aureus* were considered to be resistant and the inhibition zone for cefoxitin with diameters of ≥20 and ≤19 mm were considered susceptible and resistant, respectively [13].

Epsilometer test

The Epsilometer test (E-test) was conducted for quantitative antimicrobial susceptibility testing using E-test strips (Liofilchem, Italy). A standard bacterial suspension equal to 0.5 McFarland inoculated on Mueller–Hinton agar (MHA) plates; then, E-test strips of tigecycline, linezolid, teicoplanin, vancomycin, and quinupristin/dalfopristin were placed on the medium surface and incubated at 35 °C for 24 h for detection of minimum inhibitory concentration (MIC). The MIC was

read at the lowest concentration at which the border of the elliptical inhibition zone intercepted the scale on the strip.

Oxacillin–salt agar screening

The presence of MRSA was confirmed by oxacillin–salt agar screening test. This test was performed according to CLSI recommendations [13]. For each isolate, 1 ml of standard 0.5 McFarland suspension was cultured on an MHA medium containing oxacillin (at a concentration of 6 μg/ml of media) and 4% NaCl. The plates were incubated in ambient air at 35 °C for 24 h. Any growth on the plate was indicated as oxacillin resistance.

MRSA antibiotic susceptibility pattern

Susceptibility testing of MRSA isolates against erythromycin (15 μg), clarithromycin (15 μg), azithromycin (15 μg), vancomycin (30 μg), clindamycin (2 μg), linezolid (30 mg), teicoplanin (30 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg), quinupristin/dalfopristin (15 μg), tigecycline (15 μg), gentamicin (10 μg), and rifampin (5 μg) disks (Mast, Merseyside, United Kingdom) was determined by the Kirby–Bauer disk diffusion method on MHA, according to the procedures described by the CLSI guidelines. On following this, inducible clindamycin resistance was determined using the D-zone test according to these guidelines [13]. *S. aureus* ATCC 25923 was used as the reference strain.

Amplification of 16S rRNA gene specific for S. aureus *and* ermA, ermB, ermC, mecA*, and* msrA *genes*

DNA was extracted from bacterial colonies by the simple boiling method as previously described [4]. In brief, a few bacterial colonies were suspended in 400 ml of tris ethylene diaminetetraacetic acid buffer (pH 8.0), and the solution was heated at 100 °C for 10 min and then centrifuged at 15,000 rpm for 15 min. The supernatant was used as template DNA in PCR.

The PCR assay was performed in 25 μl contained a DNA template (50 ng), 100 μM concentrations (each) of the four dNTPs, 1 U of Taq DNA polymerase (Cinnagen, Iran), 5 μl of Taq buffer (5×), 25 pM of each of forward and reverse primers *ermA, ermB, ermC*, and *msrA* (Table I). The PCR mixtures were subjected to thermal cycling (4 min at 94 °C, followed by 30 cycles of 30 s at 94 °C for denaturation, 30 s for annealing extension, and extension at 72 °C for 30 s). A final elongation at 72 °C for 5 min was achieved in a DNA thermal cycler [14].

Table I. Primers and their target genes used in this study

Primer	Sequence (5′–3′)	Product size (bp)	Reference
16S rRNA	F: GAA AGC GTG GGG ATC AAA CA R: TTG CGG GAC TTA ACC CAA CA	340	[15]
ermA	F: GAT TTC GTT CCT CGA CC R: TAT CTT ATC GTT GAG AAG GGA TT	139	[15]
ermB	F: CTA TCT GAT TGT TGA AGA AGG ATT R: TTT ACT CTT GGT TTA GGA TGA AA	142	[15]
ermC	F: CTT GTT GAT CAC GAT AAT TTC C R: ATC TTT TAG CAA ACC CGT ATT C	190	[15]
msrA	F: TCC AAT CAT TGC ACA AAA TC R: AAT TCC CTC TAT TTG GTG GTC	163	[16]
mecA	F: ACGGTAACATTGATCG-CAACG R: GGCCAATTCCACATTGTTTCG	176	[15]

PCR products were analyzed by 1% agarose gel electrophoresis in 1× tris-borate-EDTA buffer at pH 8.3. The amplification products were photographed and their size was determined using a 100-bp molecular size marker [14].

Statistical analysis

Descriptive data were analyzed using SPSS v.22.0 statistics software (IBM Corporation, Armonk, NY, USA). χ^2 and *t*-tests were used to analyze intergroup significance. In addition, $p < 0.05$ was considered statistically significant.

Results

Bacterial isolates

From the total screened samples during 6 months, 76 *S. aureus* were isolated by biochemical tests and *16S rRNA* gene PCR. Using cefoxitin and oxacillin disk diffusion and oxacillin–salt agar screening and PCR for the *mecA* gene, 60 (79%) of *S. aureus* were identified as being methicillin-resistant. Out of 60 MRSA isolates studied, 34 (56.7%) and 26 (43.3%) strains were collected from male and female patients, respectively. The sample sources according to the hospital wards were as follows: the internal women, internal men, pediatric, intensive care unit, and surgery, repair, and outpatient department (OPD), with proportions of 6 (10%), 12 (20%), 9 (15%), 26 (43.3%), 2 (3.3%), 4 (6.7%), and 1 (1.7%), respectively. About 88.3% (53/60) of isolates were obtained from wound culture specimens, 1 (1.7%) from urine culture, 3 (5%) from blood culture, and 3 (5%) from endotracheal secretion culture.

Antibiotic resistance pattern

According to disk diffusion results, all MRSA strains were resistant to clarithromycin and azithromycin. In addition, the majority of the strains was resistant to erythromycin 59 (98.33%), clindamycin 56 (93.3%), and trimethoprim/sulfamethoxazole 58 (96.67%), whereas, using the E-test, all MRSA isolates were susceptible to teicoplanin with a maximum range 0.25 (MIC ≤ 4 mg/ml), linezolid = 0.19 (MIC ≤ 4 mg/ml), vancomycin = 0.5 (MIC ≤ 2 mg/ml), and tigecycline = 0.25 (MIC ≤ 4 mg/ml) and showed resistance to oxacillin (MIC ≥ 4 mg/ml). Besides, the D-test results showed that 30 (50%) of the MRSA isolates have the inducible clindamycin resistance phenotype. The resistance profile for all isolates to macrolides and other tested antibiotics is listed in Table II. Fifty-nine (98.3%) isolates were simultaneously resistant to erythromycin, azithromycin, and clarithromycin (cross-resistance); whereas only 1 (1.7%) isolate had various macrolide susceptibility pattern. This isolate was susceptible to erythromycin and was resistant to azithromycin and clarithromycin. The highest antimicrobial resistance was related to wound specimens with 100% resistance to erythromycin, clarithromycin, and azithromycin and 93.3% resistance to clindamycin.

MDR profiles

The results of the susceptibility testing showed that all 60 MRSA isolates were resistant to at least two antibiotics, and the majority of isolates ($N = 58$, 96.6%) was multidrug-resistant (MDR) with five diverse patterns (Table III).

Table II. Prevalence of resistance to the tested antibiotics among MRSA isolates using the disk diffusion and *t*-test methods

Antibiotics	Resistant no. (%)	Intermediate no. (%)	Susceptible no. (%)
Erythromycin	59 (98.33)	0	1 (1.67)
Clarithromycin	60 (100)	0	0
Azithromycin	60 (100)	0	0
Vancomycin	0	0	60 (100)
Clindamycin	56 (93.3)	0	4 (6.67)
Linezolid	0	0	60 (100)
Teicoplanin	0	0	60 (100)
Trimethoprim/sulfamethoxazole	58 (96.67)		2 (3.33)
Quinupristin/dalfopristin	0	0	60 (100)
Tigecycline	0	0	60 (100)
Rifampin	9 (15)	0	51 (85)
Gentamicin	40 (66.6)	0	20 (33.3)

Note: MRSA: methicillin-resistant *Staphylococcus aureus.*

Table III. Multidrug-resistant profiles of methicillin-resistant *Staphylococcus aureus* isolates

Multidrug-resistant profile	Phenotypic resistance	Number of isolates (%)
I	ERY–CLR–AZM–SXT	2 (3.3)
II	ERY–CLR–AZM–CLI–SXT	16 (26.6)
III	ERY–CLR–AZM–CLI–SXT–GEN	30 (50.0)
IV	ERY–CLR–AZM–CLI–SXT–RIF–GEN	9 (15.0)
V	CLR–AZM–CLI–SXT–GEN	1 (1.6)

Note: ERY: erythromycin; CLR: clarithromycin; AZM: azithromycin; CLI: clindamycin; SXT: trimethoprim–sulfamethoxazole; RIF: rifampicin; GEN: gentamicin.

Most isolates (50%) had an antibiotic resistance profile of number III (erythromycin–clarithromycin–azithromycin–clindamycin–trimethoprim–sulfamethoxazole–gentamicin).

PCR

MRSA isolates were screened for the presence of *ermB, ermA, ermC*, and *msrA* genes as the main causative agents of resistance to macrolides. The frequency rates of *msrA, ermA*, and *ermC* genes in MRSA isolates were 23 (38.3%), 28 (46.7%), and 22 (36.7%), respectively. In addition, 32 (53.3%) MRSA isolates harbored at least one of the four investigated genes. Seventeen (28.3%) macrolide-resistant MRSA harbored *ermA, msrA*, and *ermC* genes simultaneously.

In contrast, the *ermB* gene was absent in all MRSA isolates. All (100%) *msrA-, ermA-*, and *ermC*-positive isolates were resistant to clarithromycin, azithromycin, and erythromycin and 98.33% of clindamycin-resistant isolates harbored genes of *ermA* and *msrA*. Statistical analyses showed that among the MRSA isolates, difference in prevalence of *ermA, ermC*, and *msrA* genes was significant in clarithromycin- and clindamycin-resistant MRSA isolates.

Discussion

S. aureus remains a major cause of wound infection in patients with burn injuries [17]. Infection by MRSA has been observed to be higher than 50% in burn units. The increase in antibiotic resistance of this pathogen involved in wound infections is a great therapeutic problem and worsens the prognosis of burn patients [18]. The high frequency of infections caused by MRSA and its diverse antimicrobial resistance patterns had led to the use of MLSB antibiotics in the

treatment of these infections [19]. At present, the widespread use of these antibiotics in treatment of infections caused by *S. aureus* has led to the emergence of MLSB-resistant strains [19, 20].

In this study, 79% of the isolated *S. aureus* strains was identified as MRSA by the application of the cefoxitin disk and oxacillin–salt agar screening, which is comparable to the 77.9% prevalence of MRSA in Iranian burn patients [21]. In a study from capital of Iran, Abbasian et al. [22] reported prevalence rate of 64.2% for MRSA in a burn hospital. The prevalence rate of MRSA in Iranian burn centers is different in various regions. However, several studies revealed the increasing prevalence of MRSA in our country [21]. These inconsistencies in the prevalence of MRSA among various regions might be due to the different antibiotic use patterns and dissimilar infection control strategies [22].

In this study, according to results of disk diffusion testing, most of the MRSA isolates showed high resistance rate to macrolide antibiotics including 100% resistance against clarithromycin and azithromycin and 98.3% against erythromycin, respectively. In a previous study performed by Seifi et al. [23], lower resistance rate (88.6%) was reported for erythromycin in clinical isolates of MRSA. In this study, similar to the previous report by Goudarzi et al. [14], the majority of the erythromycin-resistant isolates had cross-resistance to other macrolides. This study revealed a high level of resistance to clindamycin (93.3%) that was similar to the study from a regional burn center in Southeastern China [24]. Furthermore, this study indicated that more than 90% of MRSA isolates were MDR (resistance to three or more unique antimicrobial drug classes), which were in accordance with the results of another investigation from Iran carried out by Goudarzi et al. [25].

Moreover, our finding revealed that the vancomycin, linezolid, teicoplanin, and tigecycline were the most effective antibiotics against MRSA that was parallel with the findings reported by Amissah et al. [1] and Ohadian Moghadam et al. [26]. Therefore, the mentioned antibiotics can still be used for treatment of the infections caused by MRSA in burn patients in our region. In contrast to another report from Iran [25], our results showed low frequency of resistance to quinupristin/dalfopristin (10%) and rifampicin (15%) in MRSA isolates that is probably due to the low prescribing of these antibiotics in our region.

In this study, the molecular assay identified the *ermA* gene as the most frequent (46.7%) resistance gene in the MRSA strains isolated from burn patients. In addition, none of the MRSA isolates had the *ermB* gene that was in line with the report by Lina et al. [27]. These findings were in disagreement with the study by Fasihi et al. [28] performed in Kerman, Iran, in which an incidence of 11% and 3.5% was reported for *ermA* and *ermB* genes, respectively. It has been reported

that the prevalence of the *ermB* in staphylococci isolated from animal sources is higher than those isolated from human specimens [29].

Furthermore, the *erm* and *msr* genes have been reported in Denmark, the United Kingdom, and Tunisia. In Tunisia and Denmark, *ermB* and *ermA* genes were the most common clindamycin- and erythromycin-resistant genes, respectively, but in this study, *ermC* was the most common [28]. In this study, according to the results of PCR, the prevalence of *ermC* gene was lower than that of the *ermA* and *msrA* genes, whereas most studies report *ermC* as the most frequent genetic determinant [30, 31]. Regarding *msrA*, we found the incidence rate of 38.3% in MRSA isolates. In a study from Serbia, the *msrA* was the most common resistance gene [32].

The dissimilarities in the prevalence rate of MLSB resistance genes in different studies may be explained by the heterogeneous nature of erythromycin resistance, or it may be due to the loss of small plasmids that carry *erm* and *msr* genes [28]. We identified the $ermA + msrA + ermC$ gene combinations in 28.3% of the MRSA isolates. Similarly, the simultaneous presence of two or more MLSB resistance genes has been reported in previous studies from different countries [33, 34].

Conclusions

This study has investigated the frequency of MLSB resistance genes in MRSA strains isolated from burn patients using PCR method. This was the first study to investigate the frequency of these genes in MRSA isolated from burn patients in our region, which demonstrated the *ermA* gene as the most common MLSB resistance gene among erythromycin-resistant isolates.

Because of the high prevalence of macrolide and lincosamide resistance found in MRSA isolates from infections of burn patients in Ahvaz, Iran, a knowledge about susceptibility patterns may provide crucial information for controlling the dissemination of antimicrobial resistance and it is recommended that local periodic survey be performed.

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Conflict of Interest

The authors declare no competing interests.

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