Journal of Kerman University of Medical Sciences

JKMU

Journal of Kerman University of Medical Sciences, 2018; 25 (1): 67-76

Prevalence of *erm* Gene among Clinical Isolates of *Staphylococcus* aureus in Shahrekord, Iran

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Received: 28 August, 2017 Accepted: 15 February, 2018

ARTICLE INFO

Article type:
Original article

Keywords:
Staphylococcus aureus
D-test
Erm gene
Macrolide-lincosamid-streptogramin

Abstract

Background: Development of drug resistance to *Staphylococcus aureus (S. aureus)* has led to the use of older antibiotics such as macrolide-lincosamide-streptogramin B (MLSB) for the treatment of infections. MLS_B resistance can be caused by several mechanisms, however, one of the predominant reasons is target modification mediated by *erm* genes. The objective of this study is to determine the prevalence of *erm* genes and the frequency of constitutive MLS_B ($cMLS_B$), inducible MLSB ($iMLS_B$), and MS phenotypes using D-test and polymerase chain reaction (PCR) methods.

Methods: D-test was performed on 110 clinical specimens of *S. aureus* collected from Kashani and Hajar Hospitals in Shahrkord from October 2014 to May 2015. After sampling, DNA extraction was performed by simple boiling method and, in order to detect *erm* genes, multiplex PCR was carried out on erythromycin resistant isolates using specific primers.

Results: The result of this study revealed that among 110 *S. aureus* isolates examined, 35 (31.8%) were MRSA and frequency of $cMLS_B$, $iMLS_B$, and MS resistant phenotypes were 22 (20%), 9 (8.2%), and 2 (1.8%), respectively. The genes *ermA*, *ermB*, and *ermC* were detected in 27 (24.5%), 28 (25.4%), and 26 (23.6%) isolates.

Conclusion: This study demonstrated that $cMLS_B$ was the most common phenotype among isolated *S. aureus*. Moreover, another interesting point to notice in our study was the high frequency of the *ermB* gene in $iMLS_B$ resistant phenotypes.

Introduction

S. aureus is the leading causes of opportunistic and device-related infections and is responsible for a wide spectrum of human and animal diseases ranging from mild folliculitis to potentially fatal systemic illnesses, such as endocarditis and bacteremia (1-3). The prevalence of antibiotic-resistant bacteria in hospitals has strikingly increased in the past decades, leading to an increase in costs and the probability of problematic treatment of infections caused by this microorganism (4-6). Development of drug resistance of *S. aureus* has led to the use of older antibiotics such as macrolide-lincosamide-streptogramin B (MLSB) antibiotics (7). Clindamycin (a lincosamide) and erythromycin (a macrolide) represent two distinct classes of antimicrobial agents that cause protein synthesis inhibition by binding to 50S ribosomal subunits of bacterial cells (8). Resistance to both of these antibiotics

occurs through methylation of their ribosomal target site. Clindamycin and erythromycin resistance are typically mediated by *erm* genes (*ermA, ermB, ermC,* or *ermF*) that codes 23s rRNA methylase (7, 9). Clindamycin is often used as an antimicrobial therapeutic drug for the treatment of skin and soft tissue infections caused by *S. aureus* strains (10). Clindamycin resistance in *S. aureus* can be either constitutive or inducible. *S. aureus* isolates with inducible resistance are resistant to erythromycin but seem susceptible to clindamycin. In this position, treatments with clindamycin may be selective for constitutive *erm* mutants, which can lead to clinical treatment failure. whereas inducible resistance could not be detected by the usual antimicrobial susceptibility tests (11). The Clinical and Laboratory Standards Institute (CLSI) recommends a test for inducible clindamycin resistance of *staphylococci* isolates through using a D-zone test, which is an important test for optimal treatment of patients (12, 13). The current study was aimed to investigate the antibiotic susceptibility profile and the prevalence of the *ermA*, *ermB*, and *ermC* genes among *S. aureus* strains isolated from different clinical samples in Shahrekord.

Materials and Methods

Bacterial strains

This study was conducted from October 2014 to May 2015 at Kashani and Hajar Hospitals, Shahrekord, Iran. During the study, 110 clinical isolates of *S. aureus* were collected from various clinical specimens which were included wound, blood, urine, sputum and etc. Early identification was performed based on Gram-staining and biochemical tests such as catalase, coagulase, and DNase tests (14).

Phenotypic antimicrobial susceptibility testing

Antibiotic susceptibility testing was carried out using disk diffusion method (MAST Diagnostic, UK) according to guidelines of Clinical Laboratory Standards Institute (CLSI-2012) (15). The list of the antibiotics used in the test is presented in Table 1. Strain of *S. aureus* ATCC 25923 was used as control. D-test method was performed according to CLSI using clindamycin (2 µg) and erythromycin (15µg) disks. For this aim, suspensions of bacteria were prepared in the sterile saline (2 ml), equivalent to standard 0.5 McFarland. After culturing the bacteria, two antibiotic disks were placed on Muller-Hinton agar media in 15 mm distance (edge-to-edge) following this step the plates were incubated at 35°C for 24 h. Strains with D-shape of growth inhibition of clindamycin near the erythromycin disk were classified as resistant phenotypes to $iMLS_B$ (D-test positive), while those with a circular zone were classified as MS resistant phenotypes (D-test negative). Methicillin-resistant *S. aureus* (MRSA) strains were identified by assessment of cefoxitin susceptibility (30 µg) disk (Mast, UK) using disc diffusion method according to CLSI.

Table 1. Antimicrobial-susceptibility for S.aurus strains

Antibiotics	Susceptibilityof isolates					
	Susceptible		Intermediate		Resistant	
	No.	%	No.	%	No.	%
Penicillin (10 U)	13	11.81	0	0	97	88.2
Erythromycin (15 µg)	75	68.2	2	1.8	33	30
Gentamicin (10 µg)	91	82.7	1	0.9	18	16.4
Clindamycin (2 µg)	81	73.6	5	4.5	24	21.8
Tetracycline (30 µg)	62	21.8	5	35/4	83	55/3
Quinupristin/dalfopristin	110	100	0	0	0	0
Cephalexin (30 µg)	78	70.9	1	0.9	31	28.2
Cefoxitin (30 µg)	75	68.2	0	0	35	31.8

DNA extraction

DNA was extracted from 110 *S. aureus* isolates using simple boiling Method. 50 mg of bacterial biomass was suspended in 400 µl of TES (50 mM Tris hydrochloride [pH 8.0], 5 mM EDTA, 50 mM NaCl), and the suspension was heated at 95°C for 7 min and centrifuged at 10000 *g* for 10 min. The resulting supernatant was taken as DNA lysate and kept at -20°C for the polymerase chain reaction (PCR).

Molecular Detection of mecA Gene

PCR was performed for the detection of *mecA* gene using primers displayed in Table 2. PCR conditions for detection of *mecA* were as follows: initial denaturation at 94°C for 5 min,30 cycles of denaturation (94°C, 30 s), annealing (55°C, 30 s), extension (72°C, 30 s), and a final elongation at 72°C for 2 min.

Table 2. Primers used in this study

Target	Primer	Sequence	Product size (bp)	Reference
mecA	**F** **R**	AAAATCGATGGTAAAGGTTGGC AGTTCTGCAGTACCGGATTTG	583	(16)
ermA	**F** **R**	TATCTTATCGTTGAGAAGGGATT CTACACTTGGCTTAGGATGAAA	139	(17)
ermB	**F** **R**	CTATCTGATTGTTGAAGAAGGATT GTTTACTCTTGGTTTAGGATGAAA	142	(17)
ermC	**F** **R**	AATCGTCAATTCCTGCATGT TAATCGTGGAATACGGGTTTG	297	(17)

Multiplex-PCR for erm genes

Multiplex PCR was performed to detect *erm* genes in erythromycin resistance isolates using specific primers for the *ermA*, *B*, and *C* genes as exhibited in Table 2. Each PCR was performed in a final volume of 25 µL consisting 5 µL of DNA template, 2.5 µL of PCR buffer (×10), 1 µL $MgCl_2$ (50 mM), 0.5 µL of dNTPs (10 mM), 0.5 µL of each primer (2 µL totally), 0.25 µL of Ex-Taq

DNA polymerase (5 u/µL), and 11.25 µL distilled water. DNA was amplified on a thermocycler (Ependorf-Germany), and PCR conditions were as follows: initial denaturation at 94 °C for 10 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 60 s followed by a final extension at 72 °C for 5 min. The PCR products were electrophoresed on a 2% agarose gel (Sina gene, Iran) with TBE buffer and subsequently stained with DNA Green Viewer (Pars Toos, Iran) to see the amplified DNA fragments under gel documentation system (Trans Illuminator, Germany).

Statistical analysis

The data was analyzed by Chi-square test, using (SPSS) version v.15, and $p<0.05$was considered as significant.

Results

In this study, 110 isolates of *S. aureus* were collected from various clinical specimens, wound with 53 isolates (48.2%), blood with 23 (20.9%), sterile body fluids with 28 (25.4%), abscess with 2 (1.8%) and others with 4 cases (3.64%). Among the 110 *S. aureus* isolates, 64 (58.2%) were recovered from male patients and 46 (41.8%) from females. The patient's average age was 41.9 years. The antibiotic resistance profile for all isolates to macrolides as well as other tested antibiotics is listed in Table 1. The majority of isolates expressed resistance to penicillin 97 (88.2%). However, no resistance to Quinupristin-Dalfopristin was noted. 31.8% of isolates were resistant to cefoxitin. In disk diffusion testing, 33 (30%) strains showed resistance to erythromycin and were selected for further study. Furthermore, double disk diffusion test results revealed that 69 (62.7%) of the isolates were susceptible to both clindamycin and erythromycin and the prevalence of cMLSB, iMLSB and MS resistance phenotypes were 20%, 8.18%, and 1.8%, respectively. finally, 2 (1.8%) isolates were susceptible to erythromycin and resistant to clindamycin. Among nine isolates with $iMLS_B$ resistance phenotype, three isolates were MRSA. Thirty-three *S.aureus* isolates with erythromycin resistance phenotype were tested for the presence of the *erm* genes. In PCR testing, 5 (15.1%), 1 (3%) and 1 (3%) strains showed *ermB, ermA* and *ermC* genes amplification, respectively; 26 strains (78.7%) contained 2 or 3 of the studied *erm* genes. Both (*ermA* and *ermC*) genes were co-presented in 3 (9.1%) strains, (*ermA* and *ermB*) genes were in 1 (3%) strain and (*ermA*, B and C genes) were in 22 (66.7%) strains (Figure 1). Table 3 and 4 show the difference in MLS_B resistance phenotypes in relation to the presence of *ermA* and *ermC* genes. Most strains with the $cMLS_B$ resistance phenotype, 18 (81.8%), were MRSA. In addition, among 9 strains with $iMLS_B$ resistance phenotypes, 3 strains were MRSA, while all strains with the MS resistance phenotype were methicillin-susceptible *S. aureus* (MSSA). Of 35 MRSA strains, 1 strain contained the *ermB* gene, while in 75 MSSA strains 4 strains contained this gene. Furthermore, only 1 MSSA strain contained *ermC* gene. Prevalence of the *ermA* gene in MRSA and MSSA strains was 0 and 1, respectively. *ErmA, B* and *C* genes were co-presented in 18 of MRSA strains and 4 of the MSSA strains. Therefore, *ermA, B* and *C* genes were more common in MRSA erythromycin-resistant strains than the MSSA strains.

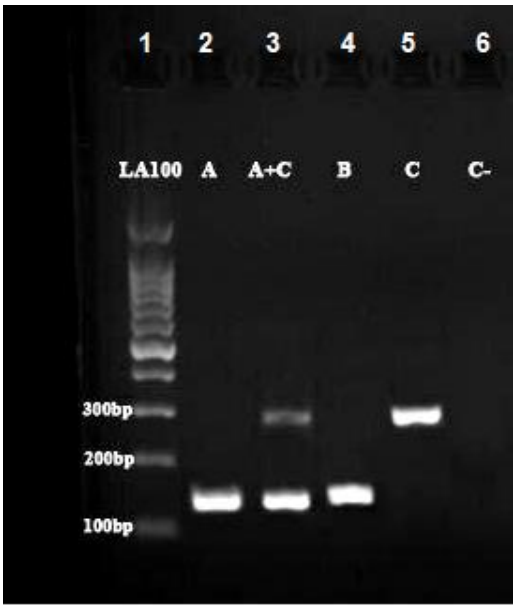

Figure 1. Gel electrophoresis of erm genes. Lane 1: ladder, Lane 2: ermA positive (139 bp), Lane 3 : *ermA*+C positive (139+297 bp), Lane 4: *ermB* positive (142 bp), Lane 5: *ermC* (297bp).

Table 3. Distribution of erm genes (*ermA*, B and C) based on resistanceto methicillin among *S. aureus* isolates

Gene	MRSA (n=)	MSSA (n=)	Total strains (n=33)
***erm*A**	0	1	1(3%)
***erm*C**	0	1	1(3%)
***erm*B**	1	4	5(15.1%)
***erm*A + C**	1	2	3(9.1%)
***erm*A + B**	1	0	1(3%)
***erm*B + C**	0	0	0(0%)
***erm*A + C+B**	18	4	22 (66.7%)

Table 4. Prevalence of ermA, B and C genes based on phenotipic test (D test)

Genotypes								
***erm*A+B+C**	***erm*B+C**	***erm*A+C**	***erm*A+B**	***erm*C**	***erm*B**	***erm*A**	**Gene**	
22	0	3	1	1	5	1	**NO**	
66.7%	0%	9.1%	3%	3%	15.1%	3%	**%**	
3	0	1	1	0	3	1	**NO**	**D^+**
33.3%	0%	11.1%	11.1%	0%	33.3%	11.1%	**%**	
				C	B	A	**Total**	
				26	28	27	**NO**	
				32.1%	34.5%	33.3%	**%**	

Discussion

S. aureus is responsible for an increasing number of serious hospital and community acquired infections. Recent studies have documented that *S. aureus* has become resistant to most available antibiotics. Additionally, antimicrobial resistance of *S. aureus* infections has changed, which in turn led to renewed interest in the use of MLS_B antibiotics, particularly clindamycin, for the treatment of these infections (18, 19). However, widespread use of these antibiotics without performing the D-test can lead to the emergence of resistant mutants to $cMLS_B$ from $iMLS_B$-resistant strains, which in turn results in failure of clindamycin therapy. Therefore, it is important for laboratories to declare the local prevalence of $iMLS_B$ isolates to ensure the determination of false susceptibility results of clindamycin and also to make accurate decision regarding the treatment based on the application of D-test (19-21). In the current study, we investigated the antibiotic resistance of *S. aureus* to penicillin, erythromycin, cephalexin, quinupristin/dalfopristin,tetracycline, cefoxitin, clindamycin and gentamicin through disk diffusion method. Results of resistance of *S. aureus* to antibiotics were as follows: penicillin 88.2%, erythromycin 30%, quinupristin/dalfopristin 0%, cephalexin 28.2%, tetracycline 55/3%, cefoxitin 31.8%, clindamycin 21.8% and gentamicin 16.4%. Results revealed that resistance to methicillin was lower in this study than those reported from shahrekord and other cities in Iran, This seems to be due to the variable drug administration and the different consumption of antibiotics in these hospitals (19, 22-25), but was comparable with a study conducted by Vivek *et al.* in India, in 2011 (4). Moreover, resistance to erythromycin 33(30%) was lower than the other studies from Iran and other countries (4, 7, 22, 26). Our results were in agreement with those studies conducted by Schmitz *et al.* on resistance to erythromycin in clinical isolates of *S. aureus* isolated from patients in 20 European university hospitals. They found that resistance to erythromycin was low (39%) (27). In the current study, most erythromycin-resistant strains showed resistance to clindamycin and were of MRSA 21 type (19%). These results suggested that there is probably a correlation between the mecA gene and macrolide-resistance genes, in *S. aureus* isolates in our study; similar data were reported by Fasihi *et al.* (28). The prevalence of $cMLS_B$, $iMLS_B$ and MS resistance phenotypes among erythromycin-resistant *S. aureus* were 20%, 8.2%, and 1.8%, respectively. Two isolates were also sensitive to both erythromycin and clindamycin. Three of MRSA isolates had a resistant phenotype to $iMLS_B$, which was lower than those reported by Moosavian *et al.* (19). In another study done by Naderinasab *et al*, 0.7% of methicillin resistant *staphylococci* isolates represented the $iMLS_B$ phenotype (23). The frequency of MLS_B resistance phenotypes has been reported variously in different countries, as some of these reports showed $iMLS_B$ rates ranging from 3.3% to 96.3% (4, 7, 19, 25, 29). For example, in a study conducted by Ghanbari *et al.* on 215 *S. aureus* strains isolated from Al-Zahra Hospital in Isfahan, the prevalence of $iMLS_B$, $cMLS_B$, and MS resistance phenotypes were estimated to be 9 (4.18%), 58 (26.9%), and 11 (5.1%), respectively (29). In Mashhad, Seifi *et al.* reported the prevalence of $iMLS_B$, $cMLS_B$ and MS resistance phenotypes as 11.37%, 26.07% and 12.32%, respectively (31). In India, the prevalence of

$iMLS_B$, $cMLS_B$, and MS phenotypes among *S. aureus* strains were 21%, 26.5%, and 12%, respectively; both the $iMLS_B$ and $cMLS_B$ resistant phenotypes were known to be significantly higher in MRSA isolates as compared to MSSA (32). Similar to our results, in a study conducted by Coutinho Vde L *et al.* the $cMLS_B$ resistant phenotype was the most prevalent phenotype (46.7%) and the $iMLS_B$ was found just in 3.3% of the isolates, indicating a difference in relation to $iMLS_B$ data in the present study (7). In our study, MS phenotype was found only among the MSSA isolates. Low prevalence of the MS resistant phenotype in the present study was similar to previous studies from Iran (18, 22, 30). Osman *et al.* reported a high prevalence of MS in Lebanon (33). The results of PCR in our study showed that *ermB* (77.8%), *ermC* (44.4%) and *ermA* (66.8%) were the most prevalent genes in erythromycin-resistant *S. aureus* isolates. An interesting point to notice in the present study was the high frequency of *ermB* gene which is in contrast with other studies from Iran and other countries (10, 22, 26, 29). According to the published literature, *ermB* is primarily originated from animal strains. It is the most prevalent gene among *enterococci* strains and is rarely identified in *staphylococci.* Shahrekord is a city with animal husbandry industry. Since the resistance to macrolides can spread from animals to human by plasmid, it seems that *ermB* has been transferred from animals to human in this region (22, 34, 35). Similar results demonstrated by Zmantar *et al.* (21) in two different studies revealed *ermB* (45.7%) was the most common gene during the evaluation of 81 *S. aureus* strains isolated from human auricular. These results are consistent with those reported by Goudarzi *et al.* that stated the most prevalent gene in *S. aureus* strains isolated from anterior nares was *ermC* (13.7%) (36). While Duran *et al.* reported that the *ermA* and *ermB* genes were more prevalent than the other erythromycin resistant genes in *S. aureus* isolates (5). In a study conducted by Emaneini *et al. ermC* (55%) gene was the prevalent gene and was predominant in MRSA, whereas *ermA* was common in MSSA (29). In another study conducted by Schmitz *et al, ermA* was more common in MRSA isolates and *ermC* was more common in MSSA (27). In addition, a notable finding of the present study was the coexistence of *ermA, B* and *C* genes in a considerable number of erythromycin-resistant *S. aureus* strains, including 22 cases (66.7%).

Conclusion

Our study is the first study to determine the phenotypic and genotypic characteristics of MLS_B resistant *S. aureus* isolates from Shahrekord area. The $cMLS_B$ resistance phenotype was the most common resistant phenotype and *ermB* gene was the most common gene among iMLS-resistant *S. aureus* and $iMLS_B$ phenotypes, with a low frequency. Since the rate of $iMLS_B$ resistance of *staphylococci* varies greatly in different geographical regions, it is required to perform the D-test for erythromycin-resistance, clindamycin-susceptible *S. aureus* isolates for appropriate therapy and wise use of antibiotics.

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