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ORIGINAL ARTICLE

Frequency of genes encoding erythromycin ribosomal methylases among *Staphylococcus aureus* clinical isolates with different D-phenotypes in Tehran, Iran

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ABSTRACT

Background and Objectives: Macrolide, lincosamide and streptogramin type B (MLSB) antibiotics are important in the treatment of *Staphylococcus aureus* infections and existence of isolates with ability to resist against MLSB antibiotics is worrisome.

Materials and Methods: In this cross sectional study, 101 *S. aureus* isolates were collected from patients of five selected hospitals in Tehran over a period of five months. Disk diffusion tests and differentiation between constitutive and inducible resistances were carried out by D-test. The presence of *mecA, msrA, ermA* and *ermC* genes were detected using PCR or multiplex PCR.

Results: Out of 101 *S. aureus* isolates, 58 (57.4%) were methicillin resistant and 57 (56.4%) expressed resistance to erythromycin. The prevalence of constitutive MLSB (cMLSB), inducible MLSB (iMLSB) and MS (Negative) phenotype in all erythromycin resistant isolates were 71.9, 26.3 and 1.7%, respectively. Out of all the erythromycin resistant isolates, 57.8% harbored both *ermA* and *ermC* genes which possessed constitutive resistance. 8.7% of the isolates contained *ermA* gene alone which possessed inducible resistance with D phenotype and 5.2% of isolates just contained *ermC* gene which had inducible resistance with D^+ phenotype. *msrA* gene was detected in 3.5% of the erythromycin resistant *S. aureus* isolates with constitutive resistance. None of the genes were detected among MS phenotypes.

Conclusion: In this study, most of *S. aureus* isolates carried both *ermA* and *ermC* genes and there was a significant relationship (P value ≤ 0.05) between different resistance phenotypes and *erm* genes.

Keywords: *Staphylococcus aureus*, D-test, *Erm A, ErmC, MsrA*

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INTRODUCTION

Staphylococcus aureus has been recognized as a significant pathogen among infectious diseases of human. *S. aureus*, particularly methicillin resistant isolate (MRSA), is an important cause of hospital

and community acquired infection throughout the world.

The resistance to methicillin belongs to a penicillin-binding protein encoded by a mobile genetic element called the methicillin-resistant gene (*mecA*) (1). Increasing its resistance to antibiotics is limited to those available antibiotics which are prescribed for treatment of different infections caused by this bacterium (2).

Macrolide, lincosamide and streptogramin type B (MLSB) antibiotics such as erythromycin, clindamycin and streptogramin B are usually used in the treatment of infections, particularly skin and soft tissue infections. Clindamycin is a noteworthy choice in the treatment strategies for various reasons; 1) it has high tissue penetration (except for the central nervous system), 2) good oral absorption makes it appropriate for outpatient therapy, 3) clindamycin can be used as a choice antibiotic in patients suffer from allergy to penicillin. However, resistance to these antibiotics is significantly increasing all over the world (3, 4). Three different mechanisms of MLSB resistance have been described in *Staphylococcus* genus. One mechanism can occur through methylation of their ribosomal site which is mediated by the presence of erythromycin resistance methylase (*erm*) genes. Methylases reduce binding of MLSB antibiotics to the target site in the 50S ribosomal subunit. *ermA* and *ermC* are two common genes responsible for the MLSB resistance in *S. aureus*. Most of the bacteria targeted by macrolides and lincosamides, including Gram-positive species, spirochetes, and anaerobes, express Erm methylases. *erm(A)* genes are frequently spread in methicillin resistant isolates and are generated by Tn554 transposons while *erm(C)* genes are mostly responsible for erythromycin resistance in methicillin susceptible isolates and are carried by plasmids. The second mechanism can occur by drug efflux typically mediated by the ATP binding cassette (ABC) transporter *msrA* and the other mechanism is drug modification by two enzymes that confer resistance to macrolides and lincosamides such Mph(C) or Lnu(A), respectively (5-7). Despite the high incidence rate of MLSB resistant staphylococci, especially among MRSA (8-10), currently there is a little information available on the incidence and types of these resistant bacteria in Iran (11). The present study aimed to provide information regarding the prevalence of MLSB resistant *S. aureus* isolates in Tehran, Iran.

MATERIALS AND METHODS

Bacterial isolates. In this descriptive study, a total of 101 clinical isolates of *S. aureus* were collected from hospitalized patients in five hospitals in Tehran, Iran (December 2012 to April 2013). Isolates were obtained from different clinical specimens including wounds, respiratory tract, urine, blood, sterile body fluids and abscesses. Only one isolate per patient was included. An informed consent was obtained from all subjects enrolled in this study. Isolates were characterized as *S. aureus* by standard microbiological methods including Gram staining, catalase test, slide and tube coagulase test, growth on mannitol salt agar and deoxy ribonuclease test (12). All detected *S. aureus* isolates were stored in nutrient broth plus 20% glycerol at -70 °C until study time.

Phenotypic determination of antibiotic resistance. Determination of MLSB phenotypes was performed by the use of D-test as described previously (13) and according to the guidelines of Clinical and Laboratory Standards Institute (CLSI) (14). Three antibiotic resistance phenotypes were determined; isolates containing constitutive MLSB (cMLSB) which were resistant to both erythromycin and clindamycin, isolates containing inducible MLSB (iMLSB) which were resistant to erythromycin but sensitive to clindamycin and isolates containing the MS phenotype which showed resistance to merely macrolide and streptogramin B. A special disk diffusion procedure, D-test, was developed for discrimination of iMLSBs. In iMLSB resistant isolates, resistance to clindamycin was induced by diffusion of erythromycin through the agar which led to flattening of the clindamycin zone of inhibition closest to the erythromycin disk (A D-shaped zone) while MS phenotype contained isolates forming a circular zone around the clindamycin disk. Induction test in cMLSBs included two phenotypes; resistant (R) and hazy D zone (HD). In R phenotype, bacterial growth was seen in the presence of erythromycin and clindamycin. In contrast, in HD phenotype, in addition to the observed bacterial growth at the presence of erythromycin, two zones of growth could be observed around the clindamycin disk. Outer zone had a light and hazy growth extending to the clindamycin disk and a more dense bacterial growth in the inner zone which was blunted proximal to the erythromycin disk as observed in phenotype D.

Erythromycin resistant isolates with flattening of the inhibition zone around the clindamycin disk were considered as positive for iMLSB. Induction test description in iMLSB included two phenotypes; D and D^+. In D phenotype a D shaped clear zone was seen around the clindamycin disk proximal to the erythromycin disk while in D^+ phenotype, in addition to the observed D shaped zone around the clindamycin disk, small colonies were grown at the inhibition zone of the clindamycin disk. Bacterial growth at the presence of erythromycin and circular clear zone around clindamycin disk was considered as MS positive and negative phenotype in induction test (13). Detection of MRSA was performed by cefoxitine disk (30 µg) on the Mueller Hinton agar (Merck, England) plate according to the CLSI guidelines (14). All antibiotic disks were purchased from Mast Co, UK and *S. aureus* ATCC 25923 was used as a standard strain.

Amplification of *mecA, ermA, ermC, msrA* genes. Bacterial genomic DNA was extracted according to the method described previously (15). In Brief, five colonies from overnight incubated brain heart infusion agar plates (Merck, England) were suspended in 300 µl sterile distilled water and were heated at 100 °C for 15 min. After centrifugation at 14,000 rpm (10 min), the supernatant was used as the template DNA in PCR. Multiplex PCR was performed for detection of *ermA* and *ermC* genes whereas *mecA* and *msrA* genes were detected by single PCRs (15-17). Sequence of each primer and the reference for each PCR has been shown in Table 1. Amplification was performed in a final volume of 25 µl containing 0.5 µl of each primer (25 pmol), 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP Mix, 5 µl of template DNA and 1.5 U of Taq DNA polymerase. PCR products were electrophoresed on 1% agarose gel (Roche, Germany) at 100 volts and later stained with ethidium bromide solution to see the amplified DNA fragments under gel documentation system (UV Tech, UK) with a molecular size marker (100 bp ladders, Fermentas, Lithuania). Resistant *S. aureus* isolates (*ermA, ermC,* and *msrA* gene positive strains, courtesy of Dr. Fereshteh Jabalameli) were used as the positive controls.

Table 1. Primer sequences

Genes	Sequence (5'-3')	References
mecA	F: 5'-gtagaaatgactgaacgtccgataa-3'	15, 16
	R: 5'-ccaattccacattgtttcggtctaa-3'	
msrA	F: 5′-ggcacaataagagtgtttaaagg-3′	17
	R: 5′- aagttatatcatgaatagattgtcctgtt-3′	
*ermA**	F: 5'-gttcaagaacaatcaatacagag-3'	17
	R: 5'-ggatcaggaaaaggacattttac-3'	
*ermC**	F: 5'-gctaatattgtttaaatcgtcaattcc-3'	17
	R: 5'-ggatcaggaaaaggacattttac-3'	

*Multiplex PCR

Statistical analysis. Data were summarized using mean ± standard deviation (SD) and assurance intervals for the microbiological and demographic characteristics. All analyses were performed using SPSS (v. 20, Chicago, USA) using Fisher exact or Chi-square tests and P values ≤ 0.05 were considered statistically significant.

RESULTS

S. aureus isolates were isolated from wound (n=29), urine (n=25), respiratory tract (n=14), sterile body fluids (n=16), blood (n=11) and abscesses (n=6). These isolates were obtained from the patients admitted to internal medicine (48.0%), intensive care units (28.0%), infectious diseases (16.5%) and surgery (7.5%). Demographic characteristics showed that there were 64 males and 37 females with the mean age of 52.57 (± 21.15) years. The distribution of MLSB resistance phenotypes based on the studied genes is illustrated in Table 2. Among 101 *S. aureus* isolates, 58 (57.4%) were resistant to methicillin and 57 (56.4%) were reported as erythromycin resistant. Forty nine (84.4%) of MRSA isolates and 8 (18.6%) methicillin susceptible *S. aureus* (MSSA) isolates were resistant to erythromycin, respectively. The prevalence of cMLSB, iMLSB and MS resistance phenotypes among erythromycin resistant isolates were 71.9, 26.3 and 1.7%, respectively. *mecA* gene was detected in all MRSA isolates. Among 41 isolates with cMLSB resistance phenotype, 38 isolates were MRSA and 10 out of 15 isolates with iMLSB resistant phenotypes were MRSA. Thirty three isolates in the cMLSB group were positive for both *ermA* and *ermC* genes aside from the presence or absence of *msrA* gene. Eight isolates of iMLSB category contained one of *ermA* or *ermC* genes and none of them had *msrA* gene. MS phenotype was only found in one MRSA isolate and this isolate did not harbor

Table 2. Distribution of resistance phenotypes of MLSB based on the studied genes (*mecA, ermA, ermC* and *msrA*)

Genes	Resistance phenotypes of isolates (%)					
	R* (n=40)	HD (n=1)	D (n=11)	D+ (n=4)	N (n=1)	Total (n=57)
Negative PCR (for all 4 genes)	3 (7.5)	-	5 (45.4)	-	-	8 (14)
mecA	5 (12.5)	-	1 (9)	1 (25)	1 (100)	8 (14)
mecA + *ermA*	-	-	5 (45.4)	-	-	5 (8.7)
mecA + *ermC*	-	-	-	3 (75)	-	3 (5.2)
mecA + *ermA* + *ermC*	30 (75)	1 (100)	-	-	-	31 (54.3)
mecA + *ermA* + *ermC* + *msrA*	2 (5)	-	-	-	-	2 (3.5)

R*: resistant phenotype; HD: hazy D zone phenotype; D: D phenotype; D+: D+ phenotype; N: negative phenotype.

erm genes or *msrA* gene. The most common genes in erythromycin resistant isolates were *ermA* (66.6%), *ermC* (63.1%) and *msrA* (3.5%), respectively. None of the 8 MSSA erythromycin resistant isolates had *ermA, ermC* or *msrA* gene. Erythromycin susceptible isolates did not harbor *ermA, ermC* and *msrA* genes.

Among erythromycin resistant isolates, 40 isolates had constitutive resistance R phenotype and 32 of these isolates contained both *ermA* and *ermC* genes simultaneously with or without *msrA* gene. In this study only one isolate showed constitutive resistance HD phenotype which contained both *ermA* and *ermC* genes in the absence of *msrA* gene. The D-zone phenotype was observed in 11 isolates showing D-shaped clear zone around clindamycin disk. The D+ phenotype was observed in 4 isolates.

PCR amplifications (Fig. 1) revealed that five D phenotype isolates possess *ermA* gene alone and three isolates with D+ phenotype just harbored *ermC* gene. One negative phenotype isolate which showed resistance to erythromycin but was sensitive to clindamycin with a clear zone around clindamycin disk did not carry *ermA, ermC* or *msrA* genes (Table 2). The *mecA* gene was detected in all MRSA isolates. Among 41 cMLSB resistant phenotype isolates, 38 isolates were MRSA whereas 10 out of 15 iMLSB resistant phenotype isolates were MRSA. MS phenotype was only found in one MRSA isolate. None of 8 erythromycin resistant MSSAs had *ermA, ermC* or *msrA* genes. Erythromycin susceptible isolates did not harbor *ermA, ermC* or *msrA* genes.

In this study, most of the isolated *S. aureus* strains carried both of the *ermA* and *ermC* genes and a significant association was observed between different resistance phenotypes and *erm* genes ($P \leq 0.01$).

DISCUSSION

Resistance to antimicrobial agents is an important problem in clinical issues and MRSA is now one of the most common nosocomial pathogens in many countries (18). In this study the rate of methicillin resistance among clinical isolates of *S. aureus* was 57.4% which is higher than reports from Tehran (29.7-52%) (19-22), other cities in the country (44.4-56.8%) (23-26) or other countries including Pakistan (48%), Australia (33.6%) and Turkey (25.9%) (27-29). On the other hand, it was lower than reports from China (72.8%) and India (59.3%) (30, 31). Geographic vari-

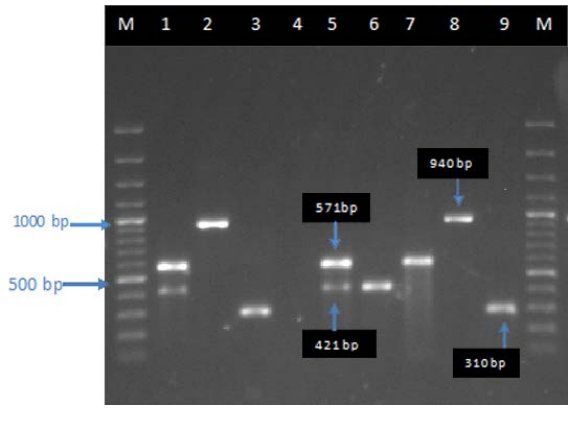

Fig. 1. Agarose gel electrophoresis patterns from multiplex and single PCR on *S. aureus* isolates. Lane M, 1000 bp DNA ladder; lane 1, positive control PCR for *ermA* (421 bp) and *ermC* (571bp) genes; lanes 2-3, positive control PCRs for *msrA* (940 bp) and *mecA* (310 bp) genes; lane 4, negative control PCR; lane 5, *ermA* and *ermC* positive MRSA (a R phenotype); lane 6, *ermA* gene positive MRSA (a D phenotype); lane 7, *ermC* gene positive MRSA (a D+ phenotype); lane 8, *msrA* gene positive MRSA (a R phenotype); lane 9, MRSA positive (a N phenotype).

ations in the prevalence rate of MRSA among Iranian *S. aureus* isolates and variations from one hospital to the others may be due to the various factors such as efficacy of practices in controlling the infection, healthcare facilities and antibiotic usages that vary in the hospitals (32). Due to the changing pattern of antibiotic resistance among *S. aureus* isolates, it would be wise to have periodical surveillance of these changes every 3 to 4 years (33).

In the present study, erythromycin resistance (56.4%) was higher than published reports from Belgium (37.4%) (7), Iran (42%) (21) or India (51.7%) (34) and lower than Turkish (60.4%) (29) or Korean reports (77.5%) (35). In this study, the rates of constitutive, inducible and MS phenotypes among 57 erythromycin resistant isolates were 71.9, 26.3 and 1.7%, respectively. The observed higher rate of cMLSB than iMLSB resistance phenotype among erythromycin resistant *S. aureus* isolates was in accordance to the other reports (11, 36, 37). Rate of inducible phenotype in the erythromycin resistant *S. aureus* isolates was higher than other Iranian studies (6.4%, 14%) (11, 23) and lower than studies from all over the world (6, 34, 37). In this study, the MS resistance phenotype was slightly higher than what reported by another study from Iran (11) and lower than other studies from Greece and the US (6, 13). Increased rate of inducible resistant isolates is attributed to the increased usages of macrolides and clindamycin. It is crucial to perform the D-test to determine the erythromycin resistance (3). In our study 57.8% of erythromycin resistant *S. aureus* isolates had a coexistence of *ermA* and *ermC* genes which was higher than the other one conducted study from Iran (48.4%) (11) and two published studies from Turkey (37.5 and 18.6%) (38, 39) but lower than Greece (0.5%) and Belgium (3%) (6, 7). *msrA* gene was detected in 3.5% of erythromycin resistant *S. aureus* isolates which was higher than reports from Canada (1%) (40) and lower than other reports from Belgium hospitals (5%) (7) and Turkey (9.5%) (29). Coexistence of *ermA* and *ermC* genes in constitutive resistance phenotype was similar to the reports in Turkey (38, 39). We found 10 MRSA isolates having inducible phenotype among which 5 isolates harbored *ermA* and 3 other isolates only consisted *ermC* gene. It is notable that the former group had D phenotype while the latter ones were D^+ similar to the findings of the study performed by Stward et al. (13). In our study *msrA* gene was seen in 2 constitutive resistant MRSA isolates in contrast with the study published by Spiloupoulou et al. (6) and Steward et al. (13) in which all *msrA* isolates represented MS resistance phenotype.

CONCLUSION

It appears that the high prevalence of inducible resistance in this study may be due to the variable use of erythromycin and clindamycin in Iran. This is the first report from Iran that shows differentiation of MLSB resistance phenotypes according to their corresponding genes which can provide information to help characterization of isolates for epidemiologic studies in the communities. D-test should be an obligatory test in routine disk diffusion methods to detect inducible antibiotic resistance in treatment of infections. On the other hand, increasing of (*ermA* + *ermC*) and *msrA* genes in the MRSA emphasizes on the accurate use of these antibiotics to prevent any treatment failure.

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