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Original article

Have methicillin resistant *Staphylococcus aureus* clinical isolates to be also resistant to streptogramins?

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ABSTRACT

Background: Methicillin resistant *Staphylococcus aureus* (MRSA) is a significant pathogen causing high morbidity and mortality. The extensive misuse of antibiotics has led to the willing of using older compounds like macrolide, lincosamides, and streptogramin (MLS) antimicrobials family. This study aimed to detect phenotypic and molecular characterization of macrolide, lincosamides, and streptogramins resistance in MRSA clinical isolates. **Methods**: Antimicrobial susceptibility testing of 50 MRSA clinical isolates to MLS agents and quinupristin-dalfopristin (Q-D) was performed using disk diffusion method. Besides, polymerase chain reaction (PCR)was conducted for amplification of genes related to streptogramins resistance (ermA, ermB, ermC, msrA, vatA, vatB, vatC, vgaA and vgbA). **Results:** MLS_B resistance phenotypes detected were $cMLS_B$ 9(18%), $iMLS_B$ 5(10%), MS_B 8(16%), and 3(6%) isolates were LS_A phenotype. No resistance to Q-D was detected in any of the tested isolates .The most prevalent MLS_B resistance genes were *ermC* in the $cMLS_B$ and *msrA* in the MS_B. The 3 (6%) LS_A phenotype isolates expected to be S_A resistant, and the 22(44%) isolates expected to be S_B resistant, were sensitive to Q-D. Genes related to Q-D resistance (*vatA, vatB, vatC, vgaA* and *vgbA* genes) were not detected confirming the susceptibility of all the tested isolates to Q-D by disk diffusion method. **Conclusion**: Accurate identification of phenotypic and genotypic MLS_B resistance is a crucial approach to decrease the antibiotic resistance rates. The study revealed a high prevalence of the $cMLS_B$ phenotype and the most prevalent resistance determinants was ermC.

Introduction

The emergence of methicillin sesistant *Staphylococcus aureus* (MRSA) is a growing problem that led to the renewed interest regarding the use of older compounds like macrolide, lincosamides, and streptogramin (MLS) antimicrobials family of antibiotics as therapeutic alternatives to β-lactam antibiotics for the treatment of staphylococcal infections [1].

Streptogramins, dalfopristin (SA type) and quinupristin (SB type), represent one of the few potential antimicrobial agents for the treatment of infections caused by MRSA. They are a group of cyclic peptide antibiotics that interfere with protein synthesis by binding to the 50S subunit of the bacterial ribosome. Interestingly, the combination of both streptogramin antibiotics (SA+SB) leads to a strong synergistic activity, which is 100-fold higher than if both components act separately, and results

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in the bactericidal activity of the streptogramin mixture [2].

Due to their different chemical structure and drug binding sites, resistance against SA and SB type substances can be different. Resistance to SA antibiotics in staphylococci can be due to two mechanisms: first: the *vgaA*, *vgaB* and *vgaAv* genes encode ATP-binding proteins probably involved in active efflux of the A compounds. Second: the *vatA*, *vatB* and *vatC* genes encode acetyltransferases that inactivate the antibiotic. SA substances can show cross-resistance with lincosamides and pleuromutilins and were grouped to the lincosamide-streptogramin A-pleuromutilin (LSAP) antibiotics [3].

In contrast, resistance to SB antibiotics in staphylococci can be due to several mechanisms mediated by the *vgb* gene encoding a lactonase inactivates SB antibiotics, and another group of genes (*erm,* and *msr* genes) responsible for resistance to macrolide-lincosamide agents; *erm* genes act through ribosomal target site modification following methylation of the ribosome, while the *msr* genes mechanism is via the active efflux pumps. Although MLSB agents are chemically different, they show cross-resistance as they share similar drug binding site (23s rRNA in 50s ribosomal subunits), therefore the SB components were mapped to MLSB antibiotics [4].

Resistance to SB or SA can be predicted within the different phenotypic resistant patterns to macrolides and lincosamides (cMLSB, iMLSB, MSB, LSA). Altogether, only when SA resistance determinants are combined with SB resistance determinants, high level resistance to the synergic mixture SA+ SB is very likely [2].

The present study aimed to characterize phenotypic and molecular resistance to macrolide, lincosamides, streptogramins in MRSA isolated from different types of clinical samples.

Materials and methods

This is a descriptive cross-sectional study. Over a period of 3 months, a total of 50 non-repetitive MRSA isolates were collected anonymously in Microbiology Department in the Medical Research Institute hospital, Alexandria University from inpatients and outpatients, after approval of the ethical committee of Medical Research Institute.

Sample collection and identification

The 50 MRSA isolates were collected from different clinical samples (pus, nasal swab, sputum, blood, central venous catheter, ascitic fluid, pleural and synovial fluid). *Staphylococcus aureus* isolates were identified by their morphology and culture characteristics upon culturing on blood agar, using standard tests: catalase, and slide coagulase test and growth and fermentation of mannitol on mannitol salt agar. Methicillin resistant *Staphylococcus aureus* isolates were identified by the resistance of *S. aureus* to cefoxitin disc according to the recommendations of CLSI 2020 [5].

Antimicrobial susceptibility testing by disk diffusion method.

The antibiogram was performed by disk diffusion technique on Mueller–Hinton agar, using different classes of antimicrobial agents including quinupristin-dalfopristin (Q-D) (15µg), erythromycin (15µg), clindamycin (2µg), Oxacillin (1µg) cefoxitin (30µg), levofloxacin (5µg), linezolid (30µg), and teicoplanin 30µg. All the reagents used in antibiotic susceptibility tests were supplied by Oxoid Ltd, England. Regarding susceptibility testing to vancomycin, determination of MIC was done by broth dilution method. The results were interpreted according to the standards determined by CLSI 2020 [5].

Erythromycin-Clindamycin Double Disk Diffusion Test (D-Zone test) [5]

Inducible resistance to clindamycin using D test was performed by placing a disk of clindamycin (2 µg) at a distance of 15mm apart from an erythromycin disc (15 µg) on a Mueller Hinton agar plate streaked with confluent growth of the isolate. After incubation at 35 ˚C for 16–18 h, isolates that showed flattening of the inhibition zone around the clindamycin disk adjacent to erythromycin disk ("D" zone) indicated inducible clindamycin resistance (positive D test).

DNA extraction and molecular identification of resistance genes

DNA was extracted using boiling method [6]. *Staphylococcus aureus* resistance genes to streptogramins (*ermB, msrA, vatA, vatB, vatC, vgaA* and *vgbA* genes) were detected using polymerase chain reaction (PCR) while Multiplex PCR was used to identify *ermA* and *ermC* genes. The PCR was carried out by Veriti Thermal Cycler (Applied Biosystems). Primers specific to each gene and the annealing temperatures were listed in **table (1)**. Aliquots of amplified samples (10 µl) were analyzed by electrophoresis on a 1.7 % agarose gel and

stained with ethidium bromide and visualized by using UV transillumination.

Statistical analysis

Data analysis was performed using the Statistical Package for Social Sciences (SPSS ver.20 Chicago, IL, USA). Categorical data was described using frequency and percent chi-square and Fisher exact tests were used, with *p values <0.05* considered significant.

Table 1. Primers and the annealing temperatures for the detection of target genes.

Gene	Primer Nucleotide sequence	Amplicon size (bp)	Annealing temperature	Reference
ermA	Forward 5'- AAG CGG TAA ACC CCT CTG A -3' Reversed 5'- TTC GCA AAT CCC TTC TCA AC -3'	190	55℃	[7]
ermC	Forward 5´-AAT CGT CAA TTC CTG CAT GT-3´ ´ Reversed 5´-TAA TCG TGG AAT ACG GGT TTG-3´	299	55℃	[7]
ermB	Forward 5´-CCG TTT ACG AAA TTG GAA CAG GTA AAG GGC-3´ Reversed 5´-GAA TCG AGA CTT GAG TGT GC-3	335	55℃	[8]
vatA	Forward 5´-CAATGACCATGGACCTGATC-3´ Reversed 5´-CTTCAGCATT TCGATATCTC C-3´	619	52˚C	[9]
vatB	Forward 5´-CCCT GAT CCA AAT AGC ATA TAT CC-3´ Reversed 5´-CTA AAT CAG AGC TAC AAA GTG-3´	602	52˚C	[9]
vatC	Forward 5´-ATGAATTCGCAA-AATCAGCAAGG-3´ Reversed 5´-TCGTCTCGAGCT-CTAGGTCC-3´	580	55˚C	[10]
vgaA	Forward 5´-CCAGAACTGCTATTAGCAGATGAA-3´ Reversed 5´-AAGTTCGTTTCTCTTTTCGACG-3´	470	54˚C	[9]
vgbA	Forward 5´-ACTAACCAAGATACAGGACC-3´ Reversed 5´-TTATTGCTTGTCAGCCTTCC-3´	734	53˚C	[9]
msrA	Forward 5´-GGC ACA ATA AGA GTG TTT AAA GG-3´ Reversed 5´-AAG TTA TAT CAT GAA TAG ATT GTC CTG TT-3´	913	55℃	[11]

Results

The results of antibiotic susceptibility testing to different classes of antibiotics showed that the most active agents were vancomycin and linezolid (100%) followed by Q-D (88%) then teicoplanin (70%). On the other hand, the isolates exhibited moderate resistance to erythromycin (44%), levofloxacin (40%), clindamycin (28%). **(Table 2)**.

In this study, among the 50 MRSA isolates, the MLS resistant phenotypes were 9(18%) $cMLS_B$, 5(10%) $iMLS_B$, 8(16%) MS_B, and 3(6%) LS_A. On the other hand 25(50%) isolates were sensitive to the MLS_B agents (S/S) **(Figure 1)**.

The 3(6%) isolates having LS_A phenotype and expected to be S_A resistant, were sensitive to Q-D. On the other hand, there were 22(44%) isolates expected to be S_B resistant, they include 9(40.9 %), 5(22.5 %) and 8(36.5%) isolates had $cMLS_B$, $iMLS_B$ and MS_B resistant phenotypes respectively. Out of these 22 isolates, 19(86.4%) were Q-D sensitive and 3(13.6%) had intermediate resistance to Q-D **(Table3)**.

Among the 50 MRSA isolates, the *ermC* gene was the predominant gene (17/50) (34%) and there was a statistically significant ($p<.01$) association between *ermC* gene and $cMLS_B$ phenotype as it was detected in 8(47.2%) of them. On the other hand, *msrA* gene was found in 6(12%) isolates and there was statistically significant association between *msrA* gene and MS_B phenotype (FET 16.65, $p<.01$) as it was detected in 5(83.4%) of them. *ermC* and *msrA* was the only combination found. Whereas *ermB* gene wasn't detected in any of the tested MRSA isolates. Genes related to streptogramins resistance (*vatA, vatB, vatC, vgaA* and *vgbA* genes) which are required for full resistance to the streptogramin combination Q-D weren't detected in any of the tested MRSA isolates **(Table 4)**.

Out of 50 MRSA isolates, 44(88%) were sensitive to Q-D, of them only 20(45.5%) isolates carried resistant genes, where 2(4.5%) isolates were *ermA* gene positive, 12(27.4%) isolates were *ermC* gene alone positive, 3(6.8%) isolates were *msrA* gene alone positive and 3(6.8%) isolates *ermC+msrA* genes positive. On the other hand, out of the 6(12%) isolates with intermediate resistance to Q-D, 2(33.3%) isolates were *ermC* gene positive and the other 4(66.7%) isolates had no genes. All the 50 MRSA isolates were negative to *vatA*, *vatB*, *vatC*, *vgaA*, *vgaB* and *ermB* genes.

In this study, there were discrepancies between the genotypic and the phenotypic pattern of MRSA isolates when comparing the results of antibiotic susceptibility by disk diffusion method with gene analysis results. The *ermC* gene was detected in 3(6%) erythromycin sensitive *S. aureus* isolates in addition, 3(6%) isolates with resistance phenopattern (1 $cMLS_B$ and 2 MS_B) were PCR negative for MLS resistance genes **(Table 5)**.

Table 2. Susceptibility of the MRSA isolates to different classes of antibiotics by disc diffusion method.

Antibiotic	Sensitive		Intermediate		Resistant	
	No.	%	No.	%	No.	%
Vancomycin VA	50	100	0	0	0	0
Teicoplanin TEC	35	70	14	28	1	2
Linzolied LZD	50	100	0	0	0	0
Oxacillin OX	0	0	0	0	50	100
Cefoxitin FOX	0	0	0	0	50	100
Levofloxacin (LEV)	28	56	2	4	20	40
Erythromycin (E)	28	56	0	0	22	44
Clindamycin (DA)	33	66	3	6	14(9+5)*	28
Quinupristin-Dalfopristin (Q-D)	44	88	6	12	0	0

* 5 isolates showed D shape with clindamycin.

Table 3. Comparison between phenotypic result of susceptibility to combined streptogramin A & B (Q-D) by disk diffusion method and the expected resistance to streptogramin A or B of the 50 *MRSA* isolates.

Resistance phenotypic pattern n=50		**Phenotypic susceptibility to Quinupristin-Dalfopristin(Q-D)**		
		Sensitive n=44(88%)	**Intermediate n=6(12%)**	**Resistance n=0(0%)**
Expected streptogramin B resistance n=22(44%)	*$cMLS_B$ n =9(18%)	6(66.6%)	3(33.4%)	
	*$iMLS_B$ n =5(10%)	5(100%)		
	*MS_B n =8(16%)	8(100%)		
Expected streptogramin A resistance n=3(6%)	**LS_A n =3(6%)	3(100%)		
Expected streptogramin A and B Sensitive n=25(50%)	S/S n = 25(50%)	22(88%)	3	

Table 4. Association between MLS_B resistance phenotypes of MRSA isolates and the PCR results of the amplified genes.

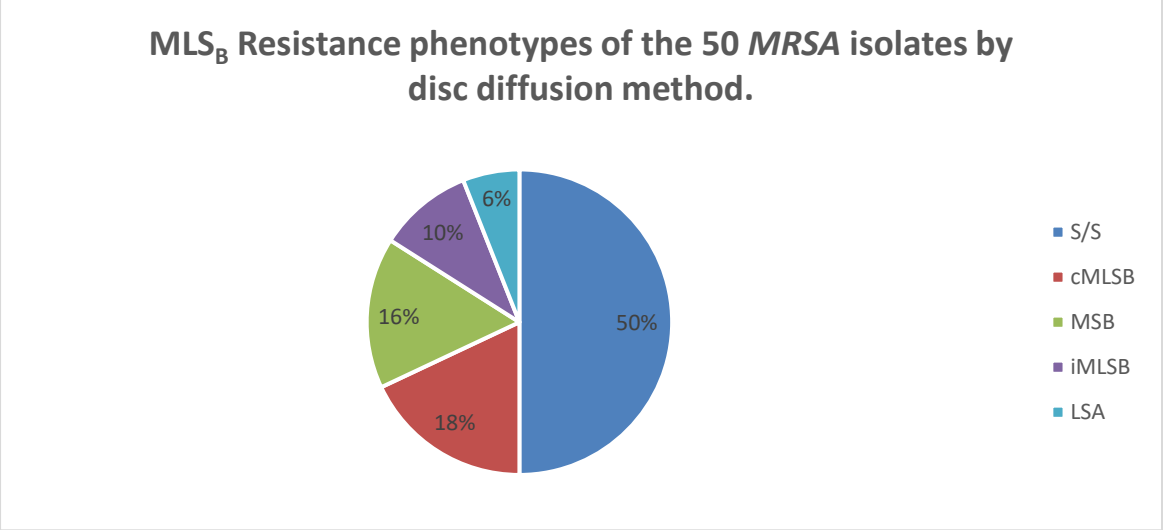
Resistance Phenotypes n=50	**Genotypes**										
	ermA **n=2 (4%)**	***ermB*** **n =0**	***ermC*** **alone n=14 (28%)**	***msrA*** **alone n=3 (6%)**	***ermC +msrA*** **n=3 (6%)**	***VatA*** **n =0**	***VatB*** **n =0**	***VatC*** **n =0**	***VgaA*** **n =0**	***VgbA*** **n =0**	**No genes n=28(56%)**
$cMLS_B$ n =9(18%)	-	-	8(88.8%)			-	-	-	-	-	1(11.2%)
$iMLS_B$ n =5(10%)	2(25%)	-	2(50%)	-	1(25%)	-	-	-	-	-	-
MS_B n =8(16%)	-	-	1(12.5%)	3(37.5%)	2(25%)	-	-	-	-	-	2(25%)
LS_A n =3(6%)	-	-	-	-	-	-	-	-	-	-	3(100%)
S/S n =25(50%)	-	-	3(12%)	-	-	-	-	-	-	-	22(88%)

Table 5. Comparison between phenotypic susceptibility results by disk diffusion method with the PCR results of the amplified genes among 50 MRSA isolates.

Antimicrobial agent		**Positive amplified genes**	**Negative amplified genes**	**P/G +/-**	**P/G -/+**	***P value***
Erythromycin n =50	Resistance n =22(44%)	19(86.4%)	**3(13.6%)**	3(13.6%)	3(10.7%)	*p<.01*
	Sensitive n= 28(56%)	**3(10.7%)**	25(89.3%)			
Streptogramins (Q-D) n =50	Intermediate n= 6(12%)	2(33.4%)	4(66.6%)	4(66.6%)	20(45.5%)	*P=.683*
	Sensitive n= 44(88%)	20(45.5%)	24(54.5%)			

P = phenotype G = genotype + = resistant - = sensitive

Figure 1. Distribution of 50 MRSA isolates according to their MLS_B resistance phenotypes by disc diffusion method.



Discussion

The streptogramins represent a unique class of compounds with synergistic modes of action that theoretically makes the emergence of resistance less likely than with other antimicrobial classes.

In this study, the most prevalent phenotype among the 50 MRSA was the cMLSB (18%), followed by MSB (16%), iMLSB (10%) and LSA (6%), The predominance of cMLSB phenotype was also reported by previous studies in Egypt (55.4%) [12], and in Greece (95 %) [13]. While a higher incidence of the iMLSB phenotype was reported among MRSA in Japan (38.7%) [14], also **Mišić et al.** reported its prevalence among staphylococcal isolates (33.4%) [11].

In this study, molecular analysis of (*ermA*, *ermB*, *ermC*, *vgbA* and *msrA*) was detected by PCR covering genes involved in resistance to SB. The results demonstrated that the *ermC* gene was the predominant among MRSA strains (34%, 17/50) with statistically significant association with cMLSB phenotype pattern (*p<.010*). The predominance of *ermC* among MRSA was also reported by previous studies [13,15-17]. On the other hand, in Japan the predominance of *ermA* gene was reported by **Otsuka et al.** [14], also Al-**Kasaby et al.** found the same result [18]. The second prevalent gene in this study was *msrA* (12%) and its association with MSB phenotype was statistically significant. (**p ≤ .010**). This association was also reported in the study of **El-Badawy et al.** but unlike

our results, they found that *msrA* gene was the most prevalent [12].

In this study the prevalence of *ermA* gene was (4%) while *ermB* gene, *and vgbA* gene were not detected. Although *ermB* gene was detected among the clinical isolates of *S. aureus* in the studies conducted in Egypt by **El-Badawy et al.** (33.3%) [12], and by **Al-Kasaby et a**l. (5.4%) [18] but its absence was also reported previously [15,19]. The absence of *ermB* gene was attributed to its dominance in animal *staphylococcal* strains [8].

In the current study, molecular analysis of (*vatA, vatB, vatC, vgaA*) was detected by PCR covering genes involved in resistance to SA, The results showed that these genes were not detected among the 50 MRSA isolates including those with different MLSB resistant phenotypes. This result is consistent with that in the study of **Osman et al.** who reported the absence of *vat* genes in their clinical isolates [7]. The association of SA and SB resistance genes with resistance to Q-D antibiotic was found in the study by **Adwan et al.** who found that out of the total 55 clinical MRSA isolates, 3 isolates (5.4%) were resistant to Q-D, two (3.6%) of them carried the *vat*(C) gene alone and one (1.8%) isolate carried both *vat*(A)/*vat*(C) genes [20].

It is worth remembering in the current work, all the 25 MRSA isolates expected to be resistant to SA (3 isolates) or SB (22 isolates) were sensitive to Q-D by disc diffusion method (only 3 with intermediate resistance), In addition none of them carried the resistance genes (*vatA, vatB, vatC, vgaA* and *vgbA*). This finding confirms the in-vitro susceptibility to Q-D of the constitutive and inducible MLSB-positive MRSA isolates included in the study, and also shows that in vitro resistance to Q-D requires its association with streptogramin resistance genes.

In this study, the discrepancies between the genotypic and the phenotypic resistance patterns were found in 6(12%) MRSA isolates when comparing their antibiotic susceptibility by disk diffusion method with gene analysis results. First, the *ermC* gene was detected in 3(6%) erythromycin sensitive MRSA isolates as previously mentioned in other studies [21,22]. Mutation or down-regulation of the *erm* genes promoter region may explain these findings [8]. The second form of a discrepancy was also detected where 3 (6%) phenotypically resistant isolates showed negative PCR results for MLS resistance genes. These results are consistent with previous studies [15,22]. This discrepancy could be due to the presence of another resistance mechanisms or due to the coexistence of different genes in bacterial cells that exhibited the complexity of staphylococcal resistance to MLSB antibiotics where the presence of some genes do not always lead to phenotypic expression of resistance [23]. Also may be attributed to either the location of these genes in small plasmids, which were occasionally lost, or presence of other variants of *erm* genes or efflux pump (*msrB*) that were not assessed in the study [8,22].

In this study, 45.5% (20/44) of the isolates sensitive to Q-D (P-/G+) carried the *ermA, ermC* & *msrA* genes but not *vga, vgb* or *vat* genes. On the other hand, 66.6% (4/6) of the MRSA isolates with intermediate resistance to Q-D were P+/G- and the concordant resistance P+/G+ was observed in 2(33.4%) isolates. The difference between number of isolates with intermediate resistance to Q-D with positive amplified genes and those with no amplified genes was not statistically significant (*p=0.683*) This finding could be explained by the fact that in all situations; (P+/G+, P+/G-, P-/G+) the isolates with positive phenotypic pattern (P+) has intermediate resistance and was not fully resistant to Q-D, on the other hand, the isolates with positive genotypic pattern (G+) the amplified genes (*erm* and/or *msr* genes) were those which are not needed to acquire full resistance to the streptogramin combination Q-D (*vga, vgb* or *vat* genes). Accordingly, we can consider that truly no discrepancy was found between phenotypic and genotypic results regarding susceptibility of the MRSA isolates to streptogramin combination.

According to the results of the present study, streptogramins are an effective therapy for MRSA infection. Because streptogramin resistance genes in staphylococci are carried on plasmid, therefore the wise use of Q-D is necessary to preserve their efficacy against it.

Conflict of interest

The authors declare that they have no conflict of interest.

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Ethical approval

The study was approved by the ethics committee of the Medical Research Institute – Alexandria University which is constituted and operating according to ICH GCP guidelines and applicable local and institutional regulations and guidelines which govern IRB operation.

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