ORIGINAL ARTICLE

Phenotypic and Genotypic Characterization of Macrolide, Lincosamide and Streptogramin Resistance among Nosocomial Staphylococci Isolated from Intensive Care Units

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ABSTRACT

Background: *The extensive misuse of antibiotics has increased cross-resistance to macrolide–lincosamide–streptogramin B (MLS_B) antibiotics among Staphylococci.* ***Objectives:*** *The study aimed to investigate the distribution of MLS_B resistance phenotypes and their encoding genes among Staphylococci isolated from the Intensive Care Units of Assiut University Hospitals.* ***Methodology****: A total of 243 nosocomial staphylococcal isolates were collected.* *MLS_B phenotypes were assessed by double disc diffusion method (D test) and the encoding genes (ermA, ermB, ermC, msrA, mphC and lnuA) were detected by PCR.* ***Results:*** *Of all isolates, 93.8% were resistant to erythromycin. MLS_B resistance phenotypes detected were the constitutive phenotype ($cMLS_B$) (56.8%), macrolide/macrolide–streptogramin B resistance (M/MS_B) (24.7%) and the inducible resistance ($iMLS_B$) (12.3%). The most prevalent MLS_B resistance genes were ermC in the $cMLS_B$, msrA in the M/MS_B and ermC and msrA in the $iMLS_B$ phenotype isolates. The most common gene combinations were either the msrA with erm genes or with both erm and mphC genes. Most of the strains harboring these combinations were of the $cMLS_B$ phenotype. The coexistence of the 4 gene groups was detected in 3.8% of the isolates; all of them were of the constitutive phenotype.* ***Conclusion:*** *A high percentage of erythromycin resistance and an alarming percentage of $iMLS_B$ phenotype were detected among our isolates. Routine D- test is mandatory to discover the inducible phenotype prone to acquire clindamycin resistance especially in patients with life threatening infections.*

INTRODUCTION

Staphylococci are of the most important pathogens causing nosocomial and community-associated infections worldwide. Lately, emerging resistance in this organism to many routinely used antibiotics has led to treatment failure of severe life threatening infections caused by Staphylococci [1,2].

Development of drug resistance in Staphylococci has guided the use of older compounds like macrolide, lincosamide, and streptogramin B (MLS_B) family of antibiotics as therapeutic alternatives [3]. However, the extensive and inappropriate use of these antibiotics has caused increased acquisition of cross-resistance to MLSB antibiotics among Staphylococci [4,5].

The mechanisms of resistance to MLS_B antibiotics are mainly related to the inhibition of protein synthesis. This can be mediated by several mechanisms: (a) ribosomal binding site modification (by methylation or mutation in the 23S rRNA gene) encoded by erythromycin resistance methylase (*erm*) genes (*ermA, ermB, ermC, ermY, and ermF*) "MLS-type" (b) active efflux mediated by methionine sulfoxide reductase (*msr) A/B* gene "M/MS_B type", and (c) enzymatic inactivation of antibiotics [5].

Target site modification mechanism is manifested as either constitutive ($cMLS_B$= erythromycin and clindamycin resistant) or inducible ($iMLS_B$= erythromycin resistant and clindamycin sensitive and D-test positive)[6-8]. In $iMLS_B$ phenotype, the bacteria produce inactive methylase mRNA, which becomes active only in the presence of a macrolide as an inducer (erythromycin) [9]. Treatment failures caused by iMLSB resistance have been reported [10].

The second mechanism is ATP dependent active efflux pump encoded by the *msrA* gene conferring cross-resistance only to 14- and 15-membered macrolides and streptogramin B except lincosamide (M/MS_B-type= erythromycin resistant and clindamycin sensitive and D-test negtive) [6-8].

The third mechanism of resistance depends on enzymatic inactivation of these antibiotic (e. g., macrolide phosphotransferase C encoded by *mphC* gene inactivating some macrolides or lincosamide nucleotidyl transferase encoded by *lnuA* gene inactivating lincosamides) [11-13].

A worrying trend is the possible spread of these resistance genes in health-care facilities and to the community with a potential risk of spread of multidrug resistant bacteria. Horizontal transfer was recorded recently for *erm* genes of staphylococcal clinical isolates [14-16].

Since the geographic location affects the frequency of the MLS_B resistance phenotypes even between different hospitals, the purpose of the study was to investigate the distribution of these phenotypes and their encoding genes among Staphylococci isolated from the intensive care units (ICUs) of Assiut University Hospitals, in South Egypt.

METHODOLOGY

Bacterial strains:

A total of 243 staphylococcal isolates were collected from different consecutive nosocomial clinical specimens sent to the Infection Control Research Laboratory of Assiut University Hospitals, in South Egypt over a period of six months (March 2017-August 2017). The study was approved by the Committee of Medical Ethics, Faculty of Medicine, Assiut University and was done in accordance with the latest revision of the Declaration of Helsinki. Informed consent was obtained from all patients.

Clinical specimens included 114 endotracheal aspirates, 36 sputum, 30 blood, 42 urine and 21 wound swabs. Strains were identified using conventional microbiological methods including Gram's stain, growth on mannitol salt agar and HiCrome Staph Selective Agar (HiMedia, Mumbai, India), colony morphology, catalase, tube coagulase, DNAse tests and API staph (Biomerieux, France).

Methicillin resistance in all staphylococcal isolates was determined by cefoxitin disc (30µg) (HiMedia, Mumbai, India) on Mueller Hinton agar plate[17], growth on Oxacillin Resistance Screening Agar Base (ORSAB, Oxoid Limited, UK) and verified by the molecular detection of *mecA* gene using primers in table (1).

Detection of MLS_B resistance phenotypes:

The double disc diffusion test (D test) was performed to identify different phenotypes of MLS_B resistance using erythromycin (15µg) and clindamycin (2µg) discs following CLSI recommendation [17]. Well isolated colonies of Staphylococci, prepared from an overnight growth of tested Staphylococcal isolates, were suspended in 0.85% sterile normal saline to achieve 0.5 McFarland turbidity (0.05 ml of 1.175% barium chloride dehydrate and 9.95 ml of 1% sulfuric acid) to yield a Staphylococci suspension of 1 x 10^6 to 5 x 10^6 cells/ml. The suspension was then spread on Mueller Hinton agar plates. An erythromycin disc and a clindamycin disc were placed 15 mm from each other on the inoculated plates and incubated at 37°C for 16-18 h. Interpretation of the test allowed the identification of the four different phenotypes[7,17]:

- The inducible MLS_B (iMLS) phenotype (D+): resistant to erythromycin and susceptible to clindamycin with a D-zone of inhibition around the clindamycin disc with flattening towards erythromycin disc.
- The M/MS_B phenotype (D-): resistant to erythromycin and susceptible to clindamycin without flattening towards erythromycin disc.
- The constitutive MLS_B (cMLS) phenotype: resistant to erythromycin as well as to clindamycin.
- The sensitive (S) phenotype: susceptible to both clindamycin and erythromycin.

Detection of genes encoding MLS_B resistance:

DNA was extracted from staphylococcal isolates by boiling method [18]. Detection of *ermA*, *ermB*, *ermC*, *msrA*, *mphC* and *lnuA* genes was done by PCR as previously described with a few modifications. Oligonucleotide primers used in this study (Eurofins Genomics, Germany) are shown in table (1). Amplification of these genes was performed in a Biorad T100 Thermocycler (Biorad, Edison, NJ.). PCR products were visualized in 2% agarose gel with ethidium bromide and analyzed using gel documentation EZ imager (Biorad, Edison, NJ.).

Table 1: Sequences of primers used for PCR

Gene	Sequence	conditions	Size (bp)	Reference
mecA	TGGCTATCGTGTCACAATCG/ CTGGAACTTGTTGAGCAGAG	35 (30 s, 95 °C; 30 s, 56 °C; 40 s, 72 °C)	310	[19]
ermA	GTTCAAGAACAATCAATACAGAG/ GGATCAGGAAAAGGACATTTTAC	30 (30 s, 94 °C; 30 s, 53 °C; 40 s, 72 °C)	421	[20]
ermB	CTATCTGATTGTTGAAGAAGGATT/ GTTTACTCTTGGTTTAGGATGAAA	30 (30 s, 94 °C; 30 s, 47 °C; 20 s, 72 °C)	142	[21]
ermC	ATCTTTGAAATCGGCTCAGG/ CAAACCCGTATTCCACGATT	30 (59 s, 94 °C; 59 s, 51 °C; 59 s, 72 °C)	295	[22]
msrA	GCAAATGGTGTAGGTAAGACAACT/ ATCATGTGATGTAAACAAAAT	30 (30 s, 94 °C; 10 s, 52 °C; 25 s, 72 °C)	400	[23]
mphC	GAGACTACCAAGAAGACCTGACG/ CATACGCCGATTCTCCTGAT	30 (30 s, 94 °C; 30 s, 53 °C; 40 s, 72 °C)	722	[24]
lnuA	GGTGGCTGGGGGGTAGATGTATTAACTGG/ GCTTCTTTTGAAATACATGGTATTTTTCGATC	30 (30 s, 94 °C; 30 s, 57 °C; 30 s, 72 °C)	323	[20]

RESULTS

A total of 243 *staphylococcal* spp. isolates were collected over the period of 6 months. Of which 210 (86.4%) were coagulase positive *Staphylococcus aureus* (CPS), [195/243 (80.2%) methicillin resistant *S. aureus* (MRSA) and 15/243 (6.2%) methicillin sensitive *S. aureus* (MSSA)], and 33 (13.6%) were coagulase negative Staphylococci (CNS); 17/33 (51.5%) *S. xylosus*, 12/33 (36.4%) *S. epidermidis*, 4/33 (12.1%) *S. hominis* [30/243 (12.3%) methicillin resistant CNS (MRCNS) and 3/243 (1.2%) methicillin sensitive CNS (MSCNS)].

Of all staphylococcal isolates 228 (93.8%) were resistant to erythromycin. The commonest MLS_B resistance phenotype detected was $cMLS_B$ [138/243 isolates (56.8%) (108 MRSA, 21 MRCNS and 9 MSSA)]. Sixty (24.7%) exhibited M/MS_B resistance (54 MRSA, 3 MSSA and 3 MRCNS). Moreover, 30 isolates (12.3%) exhibited $iMLS_B$ resistance (18 MRSA, 6 MRCNS, 3 MSSA and 3 MSCNS). Only 15 MRSA isolates didn't show resistance to erythromycin. Results are illustrated in table (2).

Table 2: MLS_B resistance phenotypes among staphylococci *isolates*

E	C	D-test	Phenotype	CPS		CNS		Total** (n=243)
				MRSA* (n=195)	MSSA* (n=15)	MRCNS* (n=30)	MSCNS* (n=3)	
R	R	-	$cMLS_B$	108(55.4%)	9(60%)	21(70%)	0 (0%)	138(56.8%)
R	S	-	M/MS_B	54(27.7%)	3 (20%)	3 (10%)	0(0%)	60 (24.7%)
R	S	+	$iMLS_B$	18 (9.2%)	3 (20%)	6 (20%)	3 (100%)	30 (12.3%)
S	S	-	S	15 (7.7%)	0	0	0	15 (6.2%)

CPS coagulase positive staphylococci, CNS coagulase negative staphylococci, MRSA methicillin resistant *Staphylococcus aureus*, MSSAmethicillin sensitive *Staphylococcus aureus*, MRCNS methicillin resistant coagulase negative staphylococci, MSCNS methicillin sensitive coagulase negative staphylococci

E erythromycin, C clindamycin, R resistant, S sensitive

MLS_B macrolide, lincosamide and streptogramin B, $cMLS_B$ constitutive phenotype,$iMLS_B$ inducible phenotype, M/MS_B macrolide/ macrolide streptogramin B, Ssensitive phenotype

*Percentages were calculated from the corresponding staphylococcal spp.

**Percentages were calculated from the total number of staphylococcal strains

Among all Staphylococci strains, the most commonly detected MLS_B resistance genes were *msrA* and *ermC* then *lnuA* (66.7%, 48.1%, and 44.4% respectively) as shown in table (3). Moreover, the predominating genes in isolates of the $cMLS_B$ phenotype were *ermC*, *msrA* and *ermB* (67.4%, 63%, and 43.5%, respectively). Similarly, *msrA* and *ermC* genes were dominating in the $iMLS_B$ phenotype (70% each), followed by *mphC* (40%). The prevailing gene in the M/MS_B phenotype was also *msrA* (85%) followed by *mphC* and *lnuA* (60% and 55%, respectively).

Table 3: Characterization of MLS_B resistance genes in relation to the different MLS_B phenotypes

Gene groups	$cMLS_B$* (n=138)	M/MS_B* (n=60)	$iMLS_B$* (n=30)	S* (n=15)	*p*-value	Total** (n=243)
erm A	3 (2.2%)	0 (0%)	0 (0%)	0 (0%)	0.2	3 (1.2%)
erm B	60 (43.5%)	12 (20%)	6 (20%)	3 (20%)	0.3	81 (33.3%)
erm C	93 (67.4%)	3 (5%)	21 (70%)	0	**0.001**	117 (48.1%)
msrA	87 (63%)	51 (85%)	21 (70%)	3 (20%)	**0.04**	162 (66.7%)
mphC	48 (34.8%)	36 (60%)	12 (40%)	0 (0%)	0.07	96 (39.5%)
lnuA	54 (39.1%)	33 (55%)	6 (20%)	15 (100%)	**0.02**	108 (44.4%)

MLS_B macrolide, lincosamide and streptogramin B, $cMLS_B$ constitutive phenotype,$iMLS_B$ inducible phenotype, M/MS_B macrolide/ macrolide streptogramin B, S sensitive phenotype

*Percentages were calculated from the corresponding MLS_B phenotype

**Total percentages were calculated from the total number of *staphylococcal* isolates

Different MLS_B resistance gene combinations were detected among the Staphylococci isolates, as presented in table (4). The most frequent MLS_B gene combination found among all isolates was that between *msrA, erm* and *mphC* genes (39 isolates, 61.5% of them exhibiting $cMLS_B$ phenotype) followed by *msrA-erm* genes combination (33 isolates, 72.7% of them exhibiting $cMLS_B$ phenotype). In addition, 24 isolates were having *lnuA* and *msrA genes* (50% of which were displaying M/MS_B phenotype). Eighteen isolates (all having $cMLS_B$ phenotype) had only *erm* resistance genes. A combination of the 4 genes, *erm +msrA+mphC+lnuA*, was found in 9 isolates, all were of the $cMLS_B$ phenotype. Notably, 3 isolates of the inducible phenotype were not carrying any of the MLS_B resistance genes. Moreover, no *erm* genes were detected in neither 24 nor 6 isolates of the $cMLS_B$ and $iMLS_B$ phenotypes, respectively. Likewise, *msrA* gene was not detected in 9 (15%) isolates of the M/MS_B phenotype. None of the isolates harbored *mphC* gene per se.

Table 4: Characterization of MLS_B resistance gene combinations in relation to the different MLS_B phenotypes

Gene groups	$cMLS_B$* (n=138)	M/MS_B* (n=60)	$iMLS_B$* (n=30)	S* (n=15)	Total** (n=243)
Erm	18 (100%)	0	0	0	18 (7.5%)
msrA	9 (60%)	6 (40%)	0(0%)	0	15 (6.2%)
mphC	0	0	0	0	0
lnuA	3 (16.7%)	3 (16.7%)	3 (16.7%)	9 (50%)	18 (7.5%)
erm+msrA	24 (72.7%)	0	9(27.3%)	0	33 (13.6%)
erm+mphC	9 (60%)	3 (20%)	3 (20%)	0	15 (6.2%)
erm+ lnuA	15 (83.3%)	0	0	3 (16.7%)	18 (7.5%)
msrA+mphC	0	12 (20%)	0	0	12 (5%)
msrA+ lnuA	9 (37.5%)	12 (50%)	0	3 (12.5%)	24(10%)
erm +msrA+mphC	24 (61.5%)	6 (15.4%)	9 (23.1%)	0	39 (16.2%)
erm+ msrA+ lnuA	12 (66.7%)	3 (16.7%)	3 (16.7%)	0	18 (7.5%)
erm+mphC+lnuA	3 (50%)	3 (50%)	0	0	6 (2.5%)
msrA+mphC+lnuA	3 (20%)	12 (80%)	0	0	15 (6.2%)
erm +msrA+mphC+lnuA	9 (100%)	0	0	0	9(3.8%)
No genes	0 (0%)	0	3 (100%)	0	3(1.2%)

MLS_B macrolide, lincosamide and streptogramin B, $cMLS_B$ constitutive phenotype,$iMLS_B$ inducible phenotype, M/MS_B macrolide/ macrolide streptogramin B, S sensitive phenotype

*Percentages were calculated from the total number of isolates having the corresponding gene/gene combination group

**Total percentages were calculated from the total number of *staphylococcal* isolates

DISCUSSION

Identification of MLS resistance phenotype is very crucial, since $iMLS_B$ phenotype transforms into $cMLS_B$ phenotype causing treatment failure in patients with life threatening staphylococcal infections [25].

In our study, 243 staphyloccocal isolates were collected over a 6 months period. Most of them were methicillin resistant and 93.8% of them were erythromycin resistant. Various patterns and frequencies of erythromycin resistance phenotypes were observed in different studies, where some investigators [26-28] reported that the constitutive phenotype is predominant over the inducible and M/MS_B phenotype. Consistent with Ghanbari et al. [29] most of our isolates which were MRSA showed high incidence of the constitutive phenotype followed by M/MS_B then $iMLS_B$ resistance phenotypes.

On the contrary, Mišić et al. [5] reported $iMLS_B$ the most prevalent resistance phenotype among staphylococcal isolates in Serbia, M/MS_B the second most common, followed by $cMLS_B$ phenotype (33.4%, 17.6% and 8.9%, respectively). Sasirekha et al. [30] reported higher percentages of constitutive, inducible and M/MS_B phenotypes among MSSA (7.84, 8.49 and 13.07 %, respectively) than MRSA (5.2, 0.65 and 5.88 %, respectively). Other studies [31,32] reported that M/MS_B phenotype was not detected among MRSA isolates. Regarding CNS, our results showed that $iMLS_B$ was the 2nd most detected phenotype in the MRCNS isolates followed by M/MS_B. On the other hand, Juda et al.,[33] found the M/MS_B phenotype predominating the *S. epidermidis* isolates, followed by $cMLS_B$ then $iMLS_B$

phenotype. Patterns of resistance vary by geographical region even among different hospitals, bacterial susceptibility and also difference in antimicrobial policies among healthcare settings[34].

The molecular analysis demonstrated that the most prevalent MLS_B resistance gene among our staphylococcal isolates was the *msrA* gene (66.7% of all isolates). Recent studies demonstrated similar results[5,34]. Consistent with recent reports [5, 33, 34] the *ermC* gene was the most predominant resistance gene in both the $cMLS_B$ and the $iMLS_B$ phenotypes. Our results showed that *mphC* was the 2nd prevalent gene after *msrA* gene in the M/MS_B phenotype isolates. Amongst them, 6 (10%) were msrA-negative but *mphC* positive .Juda et al.[33] reported that *mphC* was more prevalent than *msrA* gene among the M/MS_B phenotype with one (3%) *msrA*-negative *mphC* positive *S. epidermidis* strain. Whereas *msrA* gene encodes an ATP-dependent efflux pump, which actively removes 14-,15-membered MLS_B, the *mphC* gene encodes for a macrolide-modifying enzyme[35] which may explain the *msrA*-negative M/MS_B phenotype [33].

Some of the MLS_B resistance genes were detected in the erythromycin-clindamycin sensitive isolates. In agreement with these findings, Goudarzi et al. [36] reported 7.7% erythromycin susceptible staphylococcal strains harboring *ermB* or *ermC* gene. Mutation or down-regulation of the *erm* genes promoter region may explain these results [37,38]. These strains are prone to become resistant under intensive antibiotic selective pressure which necessitates performing phenotypic and genotypic tests to discover such isolates [39].

In this study, the most common gene combinations were either the *msrA* with *erm* genes or with both *erm* and *mph*C genes. Moreover, most of the strains harboring these combinations were of the $cMLS_B$ phenotype. The coexistence of the 4 gene groups was detected in 3.8% of the isolates; all of them were of the constitutive phenotype.

These results are consistent with those of the study by Mišic´ et al. [5] which reported that cMLSb phenotype was characterized by the presence of great number of gene combinations namely *ermB* + *lsaA*, *ermC* + *msrA*/*B*, and *ermB* + *msrA*/*B*. On the other hand, *ermC*, *mphC*, *linA*/*A'* combination was the most detected one and most of the strains carrying the 4 gene groups were of the M/MS_B phenotype in the study of Juda et al. [33].

Only 3 (1.2%) of our isolates showing $iMLS_B$ phenotype had no MLS resistance genes. Other studies reported more or less similar findings [5,40]. Also, in our study, 30 isolates (24 $cMLS_B$ and 6 $iMLS_B$) showed negative PCR results for *erm* genes. Earlier studies [8,36,41,42] reported comparable data. This may be explained by the possible presence of other variants of *erm* genes or efflux pump (*msrB*) that were not assessed in the study [38, 39,43].

CONCLUSION

A high percentage of erythromycin resistance and an alarming percentage of $iMLS_B$ phenotype are detected among our isolates. Routine D- test is mandatory to discover the inducible phenotype prone to acquire clindamycin resistance especially in patients with life threatening infections.

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