Characterization of resistance genes to macrolides, lincosamides and streptogramins (MLS) among clinical isolates of *Staphylococcus aureus* in North Lebanon

**Abstract**

**Objective:** *Staphylococcus aureus* is one of the most significant pathogens causing significant morbidity and mortality. Moreover, the incidence of MLS *S. aureus* (resistant at least for one macrolide) infections continues to grow globally. The aim of this study is to examine the expression of resistance of *S. aureus* isolates to MLS and the prevalence of genes involved in this resistance using PCR.

**Methods:** A total 38 strains of *S. aureus* MLS-resistant were isolated in the Microbiology Labs at Nini Hospital in North Lebanon. The disk diffusion method was used to determine the phenotype of the MLS resistance. The resistance genes involved were detected by PCR using specific gene primers for *ermA*, *ermB*, *ermC*, *msrA*, *linA*, *mefA*, *vat* and *vgb* genes.

**Results:** A total of 55.3% of the isolates were positive for inducible phenotype (iMLS$_B$), of these 15.8% were positive for the constitutive phenotype (cMLS$_B$), 23.7% for MS$_B$ phenotype and 5.2% for L phenotype. The *ermC* gene was the most prevalent (52.6%), while *ermA*, *ermB*, *msrA* and *linA* genes were observed with lower prevalence. A combination of several of these genes was detected, whereas the *vgb*, *vat* and *mefA* genes were not detected in any of the clinical isolates.

**Conclusion:** This study is the first investigated characterization of MLS resistance genes in clinical isolates of *S. aureus* in Lebanon according to
Introduction

Macrolides have been known for more than six decades, and, since the introduction of erythromycin molecule into therapy, a number of these antibiotics have been developed for clinical use. For years, these drugs have represented a major alternative of beta-lactams for the treatment of infections caused by gram-positive bacteria such as ß-hemolytic Streptococci, Streptococcus pneumoniae and Staphylococcus aureus. Macrolides, lincosamides and streptogramines (MLS) are related molecules, with similar antibacterial spectrum and mechanisms, but with different chemical structure. The use of these antibiotics has been accompanied by the rapid appearance of resistant strains in staphylococci [1,2]. Various resistance mechanisms have been described to these antimicrobials including ribosomal target modification by single mutation or methylation of 23SrRNA gene, modification of the drug, and efflux pumps [3]. However, the predominant mechanism in staphylococci resistance is target modification mediated by \( \text{erm}A \), \( \text{erm}B \) and \( \text{erm}C \) (erythromycin ribosome methylase genes) [1,3].

Several common genes responsible for resistance to macrolide, lincosamide and streptogramin B (MLS\(_B\)) antibiotics were reported. The \( \text{erm} \) genes encode enzymes that confer inducible (iMLS\(_B\)) or constitutive (cMLS\(_B\)) resistance to MLS\(_B\) antibiotics via methylation of a single adenine in the 23S rRNA gene, thereby reducing binding by MLS\(_B\) antibiotics to the 50S large subunit of the ribosome [4]. Another fairly common mechanism of macrolide resistance were reported in \( S. \) aureus and mediated by two important efflux genes, \( \text{msrA} \) (macrolide efflux) and \( \text{mefA} \) (macrolide efflux protein A) genes, which confer only resistance to macrolide and streptogramin B (MS\(_B\)) antibiotics [3,5]. The \( \text{msrA} \) gene encodes a 488-amino-acid ATP-binding cassette (ABC) transporter hydrophilic protein that contains two ATP-binding motifs characteristic of the ABC transporters. However, the \( \text{mefA} \) gene is a proton motive force-driven efflux pump involved in resistance to MS\(_B\) antibiotics [3,5,6]. Moreover, hydrolysis of antibiotics through the activity of esterases and/or phosphotransferases has only been reported in staphylococci [3].

Resistance to MLS\(_B\) among \( S. \) aureus is an increasing problem. The overlapping binding sites of MLS\(_B\) in 23S rRNA causes cross-resistance to the three classes of antibiotics. A wide range of bacterial pathogens that are targets for MLS\(_B\) express Erm methylases. The new nomenclature system distinguishes 21 classes of \( \text{erm} \) genes and as many corresponding Erm methylases. The new nomenclature system distinguishes 21 classes of \( \text{erm} \) genes and as many corresponding Erm methylases.

Keywords: \textit{Staphylococcus aureus}, Macrolides, Antimicrobial resistance, \( \text{erm}C \), \( \text{msr}A \), Lebanon
gene is mostly associated on transposon Tn551 and the penicillinase plasmid (pI258) [9,10]. However, the \textit{ermC} gene is normally found on small plasmids ranging in size from 2.4 to 5 kb [4].

Other resistance mechanisms to MLS were reported. The O-nucleotidyltransferases encoded in \textit{S. aureus} by lincosamide inactivation nucleotidylation (\textit{lin}) genes, confer resistance to lincomycin but not to clindamycin (L phenotype)[1,11,12]. The staphylococcal \textit{vat} genes confer resistance to streptogramin A and similar compounds by acetylation of the antibiotics. In addition, the staphylococcal \textit{vga} genes encode related ATP-binding proteins probably involved in the active efflux of A compounds [13,14].

This study aimed to investigate the epidemiology of MLS resistance in Lebanon, particularly prevalence of MLS resistance phenotypes and molecularly characterize the macrolide resistance genes in clinical strains of \textit{S. aureus} isolated from patients in North Lebanon.

Material and Methods

Sample collection

Thirty-eight \textit{S. aureus} MLS-resistant isolates were collected from patients in Nini hospital. All isolates were transported immediately to the Health and Environmental Microbiology Laboratory strain bank, in the AZM center for research in biotechnology, doctoral school, Lebanese University in Tripoli.

Antimicrobial susceptibility test

The susceptibility to antibiotics was performed by the disk diffusion method on Muller-Hinton agar (Bio-Rad, France) according to CLSI (Clinical and Laboratory Standards Institute) and CA-SFM 2015 (Comité de l’Antibiogramme de la Société Française de Microbiologie 2015) recommendations.

MLS resistance phenotypes were performed using D-test with the following antibiotics: Erythromycin (15UI), lincomycin (15 μg), spiramycin (100μg) and pristinamycin (15μg).

DNA extraction and molecular identification of resistance genes

All \textit{S. aureus} strains were tested for detection of macroline resistance genes. DNA was extracted using the GenElute™ Bacterial Genomic DNA (Sigma Aldrich®, England), according to the manufacturer’s recommended procedures. The DNA was eluted in 200 μl of elution buffer and stored at -20°C until use. All genes known to be responsible for \textit{S. aureus} resistance to beta-lactams (\textit{mecA}) and MLS (\textit{ermA}, \textit{ermB}, \textit{ermC}, \textit{msrA}, \textit{mefA}, \textit{vat}, \textit{vga}, and \textit{linA} genes) were detected using PCR and primers specific to each gene (Table 1). Purified bacterial DNA from control strains and deionized water were respectively used as positive and negative control in each PCR run.

Statistical analyses

Statistical analyses were performed with GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA) using the Fisher’s exact test to explore the association between resistance to MLS and resistance to beta-lactams. The general significance level was set at a P-value below 0.05.

Results

The percentage of susceptibility of all \textit{S. aureus} isolates is showed in Table 2. Resistance to beta-lactams was found in 28/ 38 (73.7%) strains using the disk diffusion method and PCR targeting \textit{mecA} gene. MLS resistance phenotypes were determined by with D-test showed that among 38 MLS-resistant isolates, 27 (71.1%) exhibited the MLS B phenotype: 6 (15.8%) belonged to the cMLS B, and 21 (55.3%) to the iMLS B phenotype. The remaining 11 isolates (28.9%) were confirmed as MS B (23.7%) and L (5.2%) phenotypes (Table 3). No significant association between resistance to MLS and resistance to beta-lactams was found (Fisher’s exact test, p-value = 0.6).

In addition, PCR analysis targeting macrolide resistance genes showed that all iMLS B and cMLS B
strains harbored at least the \textit{ermC} gene, except three \textit{iMLS} \textit{B} isolates that did not carry any of the tested genes. The \textit{MS} \textit{B} isolates carried \textit{ermA} gene (1/9), \textit{mrsA} gene (5/9), both \textit{ermA} and \textit{ermC} genes (1/9) and both \textit{ermC} and \textit{mrsA} genes (2/9). The two \textit{L} isolates carried \textit{linA} and both \textit{linA} and \textit{mrsA} respectively. On the other hand, \textit{mefA}, \textit{vat} and \textit{vgb} genes were not detected in any of the isolates (Table 4).

---

Table 1. Primers and PCR conditions used to detect the resistance genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’-3’)</th>
<th>Size (bp)</th>
<th>Reference</th>
<th>Protocol cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{mecA}</td>
<td>F. GTGAAAATGACTGAACGTCCGATAA &lt;br&gt; R. CCAATTCCACATTGTTGCTCAA</td>
<td>310</td>
<td>[15]</td>
<td>35 cycles (30 s at 95°C; 45 s at 50°C; 30 s at 72°C)</td>
</tr>
<tr>
<td>\textit{ermA}</td>
<td>F. AAGCCGTTAAAACCCCTCTGA &lt;br&gt; R. TTCGCAATCCCTCTCAAC</td>
<td>190</td>
<td>[16]</td>
<td>30 cycles (30 s at 94°C; 30 s at 52°C; 1 min at 72°C)</td>
</tr>
<tr>
<td>\textit{ermB}</td>
<td>F. CTATCTGTGTTGAAGAGGATT &lt;br&gt; R. GTTTACTCTGTGTTAGGATTGAAA</td>
<td>142</td>
<td>[16]</td>
<td>Same as \textit{ermA}</td>
</tr>
<tr>
<td>\textit{ermC}</td>
<td>F. AATCTGCAATCTGCTAGT &lt;br&gt; R. TAATCTGGAATACGGGTGTTG</td>
<td>299</td>
<td>[16]</td>
<td>Same as \textit{ermA}</td>
</tr>
<tr>
<td>\textit{linA}</td>
<td>F. GGTTGCTGGGGGGTAGATGTATTAACTGG &lt;br&gt; R. GCTTTCTTTTGAAATACATGGTATTTTTCGATC</td>
<td>323</td>
<td>[17]</td>
<td>30 cycles (30 s at 94°C; 30 s at 57°C; 1 min at 72°C)</td>
</tr>
<tr>
<td>\textit{Vat}</td>
<td>F. CAATGACCAGACCGCTGAC &lt;br&gt; R. CTCGACAATTGCTGATATCC</td>
<td>619</td>
<td>[16]</td>
<td>Same as \textit{ermA}</td>
</tr>
<tr>
<td>\textit{Vgb}</td>
<td>F. ACTAACCAAGATCACCGACC &lt;br&gt; R. TTATGCTTCTGACCTTGTT</td>
<td>734</td>
<td>[17]</td>
<td>30 cycles (1 min at 94°C; 1 min at 53°C; 2 min at 72°C)</td>
</tr>
<tr>
<td>\textit{msrA}</td>
<td>F. GGCACAATAAGGTGTTAAGG &lt;br&gt; R. AGGTTGATTGATGTTGTTTGGT</td>
<td>940</td>
<td>[17]</td>
<td>25 cycles (1 min at 94°C; 1 min at 50°C; 90 s at 72°C)</td>
</tr>
<tr>
<td>\textit{mefA}</td>
<td>F. AGTATCATTACACTAGTGC &lt;br&gt; R. TTCTTCGTTCTAAAGTGG</td>
<td>348</td>
<td>[18]</td>
<td>35 cycles (30 s at 94°C; 30 s at 50°C; 90 s at 72°C)</td>
</tr>
</tbody>
</table>

Table 2. Percentage of sensitivity of thirty-eight \textit{S. aureus} isolates

<table>
<thead>
<tr>
<th>Antibiotic (disk charge)</th>
<th>% of susceptible</th>
<th>Antibiotic (disk charge)</th>
<th>% of susceptible</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fusidic acid (10 µg)</td>
<td>76.3%</td>
<td>Novobiocin (5 µg)</td>
<td>89.2%</td>
</tr>
<tr>
<td>Amoxicillin/clavulanic acid (20/10 µg)</td>
<td>26.3%</td>
<td>Oxacillin (5 µg)</td>
<td>26.3%</td>
</tr>
<tr>
<td>Cefoxitin (30 µg)</td>
<td>26.3%</td>
<td>Ciprofloxacin (5 µg)</td>
<td>34.2%</td>
</tr>
<tr>
<td>Chloramphenicol (30 µg)</td>
<td>94.7%</td>
<td>Pristinamycin (15 µg)</td>
<td>97.4%</td>
</tr>
<tr>
<td>Erythromycin (15 µg)</td>
<td>5.2%</td>
<td>Rifampicin (30 µg)</td>
<td>94.6%</td>
</tr>
<tr>
<td>Fosfomycin (50 µg)</td>
<td>92.1%</td>
<td>Spiramycin (100 µg)</td>
<td>57.9%</td>
</tr>
<tr>
<td>Gentamicin (15 µg)</td>
<td>78.9%</td>
<td>Teicoplanin (30 µg)</td>
<td>100%</td>
</tr>
<tr>
<td>Kanamycin (30 µg)</td>
<td>71.1%</td>
<td>Tetracycline (30 µg)</td>
<td>78.9%</td>
</tr>
<tr>
<td>Lincomycin (15 µg)</td>
<td>76.3%</td>
<td>Tigecycline (15 µg)</td>
<td>97.4%</td>
</tr>
<tr>
<td>Linézolid (30 µg)</td>
<td>100%</td>
<td>Tobramycin (10 µg)</td>
<td>76.3%</td>
</tr>
<tr>
<td>Minocycline (30 µg)</td>
<td>97.3%</td>
<td>Trimethoprim/sulfamethoxazole (1,25/23,75 µg)</td>
<td>81.6%</td>
</tr>
<tr>
<td>Nitrofuran (300 µg)</td>
<td>100%</td>
<td>Vancomycin (30 µg)</td>
<td>100%</td>
</tr>
</tbody>
</table>

---

This article is available from: www.iajaa.org / www.medbrary.com
Discussion

The resistance to antibiotics among S. aureus is an increasing problem, both in hospitals and communities of most Arab Middle East countries. A recent review by Tokajian et al. [19] on the epidemiology of S. aureus in these countries showed wide genetic change due to the introduction of new clones from other countries. Moreover, a previous study conducted in the same geographic region, demonstrated that methicillin-resistant S. aureus (MRSA) strains colonizing infants carried 1-3 clinically important staphylococcal toxin genes [20]. In Lebanon, a recent study on antimicrobial susceptibility patterns of S. aureus clinical isolates revealed that only 56% were susceptible to all tested antibiotics [21]. In addition, the prevalence of MRSA infection are also increased in Lebanon in the last decade [22,23]. These reports have led to renewed interest in the usage of MLS to treat staphylococcal infections [24]. Clindamycin is now a good alternative in the treatment of infections due to S. aureus, particularly MRSA isolates and as an alternative in penicillin-allergic patients. However, clindamycin resistance can be developed rapidly in S. aureus strains, and constitutive resistant mutants have arisen both in vitro testing and in vivo clinical therapy [25,26].

The CLSI and CA-SFM 2015 guidelines for disk diffusion susceptibility recommend the use of D-test to detect the inducible clindamycin resistance (iMLSB phenotype), and suggest that S. aureus isolates with the iMLSB phenotype should be reported as clindamycin-susceptible, but associated with clinical failures possibility related to the selection of clindamycin-resistant mutants.

A total of 38 S. aureus clinical isolates were examined. Firstly, according to the D-test screening, the iMLSB phenotype was the most predominant resistant phenotype (55.3%). These findings were different from the results obtained in a study conducted among S. aureus isolates in Turkey, whereby the cMLSB phenotype was the predominant resistance phenotype (63%) [27]. Otherwise, we reported a similar distribution of MLS resistance phenotypes to that described in the UK by Hamilton-Miller et al. [28]. However, various studies conducted in Turkey [27], Europe [29], Japan

Table 3. The MLS phenotypes of resistance detected for S. aureus isolates.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>iMLS B (%)</th>
<th>cMLS B (%)</th>
<th>MS B (%)</th>
<th>L (%)</th>
<th>Total no. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRSA</td>
<td>16 (42.1%)</td>
<td>5 (13.2%)</td>
<td>5 (13.2%)</td>
<td>2 (5.2%)</td>
<td>28 (73.7%)</td>
</tr>
<tr>
<td>MSSA</td>
<td>5 (13.2%)</td>
<td>1 (2.6%)</td>
<td>4 (10.5%)</td>
<td>0 (0%)</td>
<td>10 (26.3%)</td>
</tr>
<tr>
<td>Total</td>
<td>21 (55.3%)</td>
<td>6 (15.8%)</td>
<td>9 (23.7%)</td>
<td>2 (5.2%)</td>
<td>38 (100%)</td>
</tr>
</tbody>
</table>

Table 4. Correlation between genotypes and phenotypes for MLS resistance for S. aureus isolates.

| Phenotype | Genotype  
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ermA</td>
<td>ermC</td>
<td>ermB/ermC</td>
<td>ermA/ermC</td>
<td>ermB/ermC/mrsA</td>
<td>mrsA</td>
<td>linA</td>
<td>linA/mrsA</td>
<td>NI</td>
</tr>
<tr>
<td>iMLSB</td>
<td>0</td>
<td>15</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>cMLSB</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>1#</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MSB</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

*aPristinamycin-resistant strain; No. I was non-identified genotype.
Colombia [31], and France [17], reported that the iMLS$_B$ and cMLS$_B$ phenotypes were predominant in methicillin-susceptible S. aureus (MSSA) and MRSA isolates, respectively. However, may be due to the low number of examined S. aureus strains in this study, we could not find any association between MLS-resistant phenotypes and resistance to beta-lactams.

Secondly, it has reported that accurate and rapid determination of antimicrobial resistance genes will help to select the proper the treatment of S. aureus infections and to avoid the spread of resistant genes [32,33]. Overall, 36 / 38 of the examined strains were resistant to erythromycin, of these, 91.6% had at least one of these genes:ermA, ermB, ermC and/or msrA. These results are similar to those reported by Martinau et al. [34] who demonstrated the same findings among S. aureus strains resistant to erythromycin. However, 3 of those isolates did not carry any of the tested genes. These results were probably associated with the presence of other genes with low frequency in S. aureus, which were not evaluated in our investigation [33]. Also, this study found that efflux genes such as msrA gene was present either alone or in association with other genes (23.7%), while the mefA gene was absent from all tested isolates. These results concur with those described by Zmantar et al. [35], who reported the same observation. Furthermore, we found a predominance of ermC gene (27/38) among MLS resistance genes. The ermC gene was described to be the predominant MLS resistance gene in S. aureus isolates in Greece [36]. On the other hand, the ermA gene was detected alone in one isolate, and in association with ermC gene in 3 isolates of S. aureus, while a Colombian investigation reported that 100% of MRSA resistant to erythromycin had the cMLS$_B$ resistance phenotype and harbored the ermA gene [31]. In the same context, recent data from a multicenter study in Europe confirmed the predominance of ermA gene among S. aureus, while ermC and ermB genes were rarely detected [37]. Additionally, two isolates in our study harbored the linA gene. These results are also supported by recent findings, which showed that the linA gene was rarely detected in S. aureus strains [17,38,39].

Finally, all tested S. aureus strains, including methicillin resistant and MLS-resistant were susceptible to linezolid and vancomycin. Our data accord with a previous study conducted by Kanj et al. [40], in which good activity of these antibiotics was described against most Gram-positive pathogens from the Middle East and Africa.

In conclusion, to our best knowledge, this study is the first investigation regarding characterization of MLS resistance genes in clinical isolates of S. aureus in Lebanon. Our data indicates a predominance of iMLS$_B$ phenotype and ermC gene in these isolates. Other genes (ermA, ermB, msrA and linA) were found with lower prevalence. The epidemiological significance of this study remains to be confirmed by further testing large number of S. aureus strains.

Acknowledgements

We would like to thank Taha Abdou and Mariam Yehya for their excellent technical assistance, Husam Khaled for the critical reading of the manuscript, and Pr. Ghassan Matar from American University of Beirut for kindly providing us control strains. Both Marwan Osman and Azza Al Nasbeh have equally contributed in this paper.

Funding

This study was financed by the AZM center for research in biotechnology and its applications, Doctoral School of Science and Technology, Lebanese University, Tripoli, Lebanon.
Competing Interests

The authors have declared that no competing interests exist.

References


28. Hamilton-Miller JM, Shah S. Patterns of phenotypic resistance


