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

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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

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our knowledge. The study revealed a high prevalence of the inducible resistance to lincosamides (iMLS_B phenotype) and the most prevalent resistance determinants was *ermC*.

Keywords: *Staphylococcus aureus*, Macrolides, Antimicrobial resistance, *ermC*, *msrA*, Lebanon

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 β -hemolytic *Streptococci*, *Streptococcus pneumoniae* and *Staphylococcus aureus*. Macrolides, lincosamides and streptogramins (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 23S rRNA gene, modification of the drug, and efflux pumps [3]. However, the predominant mechanism in staphylococci resistance is target modification mediated by *ermA*, *ermB* and *ermC* (erythromycin ribosome methylase genes) [1,3].

Several common genes responsible for resistance to macrolide, lincosamide and streptogramin B (MLS_B) antibiotics were reported. The *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, *msrA* (macrolide efflux) and *mefA* (macrolide efflux protein A) genes, which confer only resistance to macrolide and streptogramin B (MLS_B) antibiotics [3,5]. The *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 *mefA* gene is a proton motive force-driven efflux pump involved in resistance to MLS_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 *erm* genes and as many corresponding Erm proteins with predominance of *ermA*, *ermB* and *ermC* in staphylococcal gene classes [2,3]. In bacterial pathogens, these determinants are mainly carried by plasmids and transposons that are self-transferable. The *ermA* gene is often harbored on the transposon Tn554 which has been described as predominantly having a single specific site for insertion in the *S. aureus* chromosome [7,8]. Furthermore, the *ermB*

gene is mostly associated on transposon Tn551 and the penicillinase plasmid (pI258) [9,10]. However, the *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 *S. aureus* by lincosamide inactivation nucleotidylation (*lin*) genes, confer resistance to lincomycin but not to clindamycin (L phenotype) [1,11,12]. The staphylococcal *vat* genes confer resistance to streptogramin A and similar compounds by acetylation of the antibiotics. In addition, the staphylococcal *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 *S. aureus* isolated from patients in North Lebanon.

Material and Methods

Sample collection

Thirty-eight *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 *S. aureus* strains were tested for detection of macrolide 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 *S. aureus* resistance to beta-lactams (*mecA*) and MLS (*ermA*, *ermB*, *ermC*, *msrA*, *mefA*, *vat*, *vgb*, and *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 *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 *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

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

Gene	Primer sequence (5'-3')	Size (bp)	Reference	Protocol cycle
<i>mecA</i>	F. GTAGAAATGACTGAACGTCCGATAA R. CCAATTCCACATTGTTTCGGTCTAA	310	[15]	35 cycles (30 s at 95°C; 45 s at 50°C; 30 s at 72°C) ;
<i>ermA</i>	F. AAGCGGTAAACCCCTCTGA R. TTCGCAAATCCCCTCTCAAC	190	[16]	30 cycles (30 s at 94°C; 30 s at 52°C; 1 min at 72°C) ;
<i>ermB</i>	F. CTATCTGATTGTTGAAGAAGGATT R. GTTACTCTTGGTTAGGATGAAA	142	[16]	Same as <i>ermA</i>
<i>ermC</i>	F. AATCGTCAATTCCTGCATGT R. TAATCGTGGAATACGGGTTTG	299	[16]	Same as <i>ermA</i>
<i>linA</i>	F. GGTGGCTGGGGGGTAGATGTATTAAGTGG R. GCTTCTTTTGAAATACATGATTTTTCGATC	323	[17]	30 cycles (30 s at 94°C; 30 s at 57°C; 1 min at 72°C) ;
<i>Vat</i>	F. CAATGACCATGGACCTGATC R. CTCAGCATTCGATATCTCC	619	[16]	Same as <i>ermA</i>
<i>Vgb</i>	F. ACTAACCAAGATACAGGACC R. TTATTGCTTGTCAGCCTTCC	734	[17]	30 cycles (1 min at 94°C; 1 min at 53°C; 2 min at 72°C) ;
<i>msrA</i>	F. GGCACAATAAGAGTGTTTAAAGG R. AAGTTATATCATGAATAGATTGTCCTGTT	940	[17]	25 cycles (1 min at 94°C; 1 min at 50°C; 90 s at 72°C) ;
<i>mefA</i>	F. AGTATCATTAATCACTAGTGC R. TTCTTCTGGTACTAAAAGTGG	348	[18]	35 cycles (30 s at 94°C; 30 s at 50°C; 90 s at 72°C) ;

Table 2. Percentage of sensitivity of thirty-eight *S. aureus* isolates

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

strains harbored at least the *ermC* gene, except three iMLS_B isolates that did not carry any of the tested genes. The MS_B isolates carried *ermA* gene (1/9), *mrsA* gene (5/9), both *ermA* and *ermC* genes (1/9) and

both *ermC* and *mrsA* genes (2/9). The two L isolates carried *linA* and both *linA* and *mrsA* respectively. On the other hand, *mefA*, *vat* and *vgb* genes were not detected in any of the isolates (**Table 4**).

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

Phenotype	iMLS _B (%)	cMLS _B (%)	MS _B (%)	L (%)	Total no. (%)
MRSA	16 (42.1%)	5 (13.2%)	5 (13.2%)	2 (5.2%)	28 (73.7%)
MSSA	5 (13.2%)	1 (2.6%)	4 (10.5%)	0 (0%)	10 (26.3%)
Total	21 (55.3%)	6 (15.8%)	9 (23.7%)	2 (5.2%)	38 (100%)

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

Phenotype	Genotype									
	<i>ermA</i>	<i>ermC</i>	<i>ermB/ermC</i>	<i>ermA/ermC</i>	<i>ermB/ermC/mrsA</i>	<i>ermC/mrsA</i>	<i>mrsA</i>	<i>linA</i>	<i>linA/mrsA</i>	NI
iMLS _B	0	15	1	1	1	0	0	0	0	3
cMLS _B	0	5	0	1 ^a	0	0	0	0	0	0
MS _B	1	0	0	1	0	2	5	0	0	0
L	0	0	0	0	0	0	0	1	1	0

^aPristinamycin-resistant strain ; No.I was non-identified genotype.

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 (iMLS_B phenotype), and suggest that *S. aureus* isolates with the iMLS_B 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 iMLS_B 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 cMLS_B 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

[30]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 MLSresistance 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/ormsrA. 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.

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Competing Interests

The authors have declared that no competing interests exist.

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