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Erythromycin and Clindamycin Resistance in *Staphylococci* Isolated from Pediatric Hospital in Egypt



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Mona Mohiedden Abdelhalim¹, Sahar Tolba², Doha Mohamed Dawoud^{2*}, Mohamed Khaled Ibrahim²

¹Department of Clinical and Chemical Pathology,
Faculty of medicine - Cairo University

² Department of Microbiology, Faculty of science -Ain-shams University -- Cairo, Egypt.

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ABSTRACT

Erythromycin and clindamycin are major alternatives to β -lactam antibiotics. Bacterial resistance to macrolide and lincosamide antibiotics is increasing worldwide. *Erm* genes express either constitutive macrolide, lincosamide streptogramin B resistance phenotype (cMLS_B) or inducible phenotype (iMLS_B) and *msrA* gene expresses (MS_B) phenotype. The aim of this study was to determine the frequency of MLS_B resistance phenotypes and genotypes and correlation between them in erythromycin-resistant staphylococci. In present study, 154 staphylococcal isolates were collected from various clinical specimens from Cairo University Specialized Pediatric Hospital in Cairo, from September 2012- February 2014, and investigated for erythromycin resistance phenotypes by D-zone test and genotypes by multiplex PCR. Ninety 90/154 (58.4%) were erythromycin resistant. Erythromycin-resistant *S. aureus* (ERSA) were 20/69 (28.9%), while coagulase negative staphylococci (ERCoNS) were 70/85 (82.3%). Only 35.8% of methicillin-resistant *S. aureus* (MRSA) were (ERSA) and 89.3% of MRCoNS were ERCoNS. cMLS_B was 80%, 38.5%, in *S. aureus* and CoNS respectively, it was more common among MRSA (84.2%). By PCR, ER gene was mainly *ermC* (70% in SA, 37.14% in CoNS). In Conclusion, cMLS_B and *ermC* were the predominant and no complete correlation between phenotypic and genotypic methods was found.

INTRODUCTION

Macrolides, lincosamides and streptogramin B (MLS_B) are major alternatives to β -lactam antibiotics in the treatment of infections caused by *Staphylococcus spp.* especially during the increase of methicillin-resistance among staphylococci which is considered as a therapeutic threat. In addition, they are the choice for penicillin-allergic patients (1, 2, 3). However, the widespread use of MLS_B antibiotics has led to an increase in the number of staphylococcal resistant strains (4).

Macrolide resistance in *Staphylococcus aureus* (SA) and coagulase-negative staphylococci (CoNS) is suggested to be due to an active efflux mechanism encoded by *msrA* gene which confers resistance to 14- and the 15- membered macrolides and type B streptogramins only (MS_B phenotype) which are categorized as resistance to erythromycin, inducible resistance to streptogramin B, and susceptibility to clindamycin by efflux (5). Another mechanism of resistance is due to ribosomal target modification by *erm* genes which encode Erm-type methyltransferases that confer inducible or constitutive resistance to MLS_B agents via methylation of the 23S rRNA, thereby reducing binding by MLS agents to the ribosome (6, 7). Expression of MLS_B resistance can be constitutive or inducible. In inducible resistance (iMLS_B), bacteria produce inactive mRNA that is unable to encode methylase. The mRNA becomes active only in the presence of a macrolide inducer (Erythromycin). On contrast, in constitutive expression (cMLS_B), active methylase mRNA is produced in the absence of an inducer (8, 9). The treatment of patients harboring iMLS_B staphylococci with clindamycin leads to the development of constitutive resistance, subsequently leading to therapeutic failure (10). The Clinical and Laboratory Standards Institute (CLSI) has recommended the erythromycin-clindamycin disc approximation test (D- zone) to detect inducible clindamycin resistance (11). The aim of present study was to determine the frequency of MLS_B resistance phenotypes and genotypes and correlation between them in erythromycin-resistant staphylococci.

MATERIALS AND METHODS

Clinical samples

Two hundred clinical samples were collected from Microbiology lab at Cairo University Specialized Pediatric Hospital (CUSPH) in Cairo, Egypt, from September 2012 to February 2014. Samples were collected from different sources such as blood, sputum, wound, palate

swabs and endotracheal aspirate. All the samples were collected from neonates, infants and children with age range from <1 – 13 years. Samples were cultured on blood agar, chocolate agar and MacConkey agar. Colonies were microbiologically investigated; those suspected of being staphylococci were selected and screened for erythromycin resistance by disk diffusion test (D.D).

Identification of staphylococcal isolates

All isolates were identified by standard microbiological methods. Gram stain, catalase and coagulase tests were carried out according to reported methods (12). *Staphylococcus* species were identified on the basis of a variety of conventional phenotypic characters as described in standard methods (13).

Antibiotic susceptibility test

Antibiotic sensitivity tests were performed using Kirby Bauer's disc diffusion method according to the standards of CLSI. The antibiotics discs used represented different groups of antibiotics and they were the most prescribed by pediatricians for treatment of staphylococci. The following 8 antibiotics were used in this study: Vancomycin (VA), Cefoxitin (FOX), Clindamycin (CD), Erythromycin (E), Trimethoprim/sulfamethoxazole (TS), Gentamicin (GM), Doxycycline (DXT), Ciprofloxacin (CIP) (Mast Diagnostics). The inhibition zones were measured and were interpreted according to the CLSI recommendations (11).

Macrolide lincosamide streptogramin B (MLS_B) resistance phenotype:

Inducible clindamycin resistance was determined using disk approximation test (D-zone test) as recommended by CLSI (11).

Determination of minimal inhibitory concentration (MIC):

MIC of macrolides and lincosamides were assessed by E-test (LIOFILCHEM DIAGNOSTIC) according to the manufacturer's instructions and the results were interpreted according to CLSI recommendations (11). MIC tests were performed to determine the susceptibility of staphylococcal isolates to vancomycin by E-test (bioMérieux); the results were interpreted according to CLSI recommendations (11).

DNA extraction and PCR

Genomic DNA was extracted from the bacterial isolates by Gene JET Genomic DNA purification kit according to the manufacturer's instructions (Thermo SCIENTIFIC). PCR was performed in a final volume of 25 µl. DNA template (100 ng/ µl), 12.5 µl Dream Taq Green PCR Master Mix (Sigma) and 20 pmol of each primer. The primers used in this study are presented in Table (1). Two separate multiplex PCR were performed, the first utilizing 3 primers (*ermA*, *ermC*, *msrA*) and the second, utilizing (*ermB*, *mef*). PCR was performed in a DNA thermocycler (Seegene) with the following cycling conditions: an initial 3 min at 96°C, followed by 30 cycles of 30 sec at 95°C , 30 sec at 55°C and 2 minute at 72°C followed by a single elongation cycle for 10 min at 72°C (Table 1). PCR products were analyzed by electrophoresis in 1% agarose gel. Visualization and image acquisition was performed with Gel.Doc. (biometra) (14). Single PCR reactions were carried out for confirmation.

Table (1): Primers used in the multiplex PCR

Gene	Primer 5'-3'	amplicon size (pb)
<i>ermA</i>	F5'- TAT CTT ATC GTT GAG AAG GGA TT-3' R5'-CTA CAC TTG GCT TAG GAT GAA A-3'	139
<i>ermB</i>	F5'-CTA TCT GAT TGT TGA AGA AGG ATT-3' R5'-GTT TAC TCT TGG TTT AGG ATG AAA-3'	142
<i>ermC</i>	F5'-CTT GTT GAT CAC GAT AAT TTC C-3' R5'-ATC TTT TAG CAA ACC CGT ATT C-3'	190
<i>msrA</i>	F5'-TCC AAT CAT AGC ACA AAA TC-3' R5'-AAT TCC CTC TAT TTG GTG GT-3'	163
<i>mef</i>	F5'-AGTATCATTAACTACTAGTGC-3' R5'-TTCTTCTGGTACAAAAGTGG-3'	348

RESULTS

Identification of clinical isolates

Out of 200 clinical samples, a total of 154 staphylococcal isolates including 69 *S. aureus* and 85 CoNS were recovered and screened for erythromycin resistance.

Ninety isolates were resistant to erythromycin; twenty of them were identified as *S. aureus*. Seventy isolates were identified as coagulase-negative staphylococci (CoNS) (Table 2).

Based on novobiocin and polymyxin B susceptibility, growth on DNase agar media, urease test and ornithine decarboxylase test, the seventy CoNS isolates were subdivided into five species: *S. warneri*, *S. saprophyticus*, *S. lugdunensis*, *S. schleiferi* and *S. haemolyticus*. Eighteen isolates were resistant to novobiocin and were identified as *S. saprophyticus* (Table 2).

Susceptibility to antibiotics

All the isolates were susceptible to vancomycin (100%). However, 94.4% were multi-drug resistance (MDR) which showed resistance to 3 or more antibiotic classes. All the isolates were resistant to erythromycin (100%) followed by cefoxitin (95.5 %), clindamycin (71 %), gentamicin (67.7 %), ciprofloxacin (61 %), doxycycline (50%) and trimethoprim-sulfamethoxazole (45.5%) respectively (Table 3). All *S. aureus* isolates were resistant to erythromycin, clindamycin and they showed resistance to other antibiotics with ranges from 5 to 95%. The majority of CoNS isolates was MDR and showed resistance to most of the antibiotics used. The 4 isolates, of *S. haemolyticus*, were 100% resistant to four antibiotics, erythromycin, cefoxitin, trimethoprim/sulfamethoxazole and doxycycline, and the resistance was from 50 to 75% to the other 3 antibiotics. *S. warneri*, *S. saprophyticus*, *S. lugdunensis*, and *S. schleiferi* showed resistance with variable ranges however, the higher resistance recorded after erythromycin was to cefoxitin (Table 3).

Table (2): Identification tests for *S. aureus* and CoNS

Bacterial isolates	Differential test					
	Staphylo-coagulase	Heat stable nuclease	Ornithine decarboxylase	urease	Novobiocin resistance	PB resistance
<i>S. aureus</i> (20)	+	+	ND	ND	ND	ND
<i>S. haemolyticus</i> (4)	-	-	-	-	-	-
<i>S. lugdunensis</i> (18)	-	-	+	ND	-	-
<i>S. saprophyticus</i> (18)	-	ND	ND	ND	+	-
<i>S. schleiferi</i> (7)	-	+	ND	ND	-	-
<i>S. warneri</i> (23)	-	-	-	+	-	-

ND: Not Determined

Table (3): Percentage of the antibiotic susceptibility of the staphylococcal clinical isolates

Bacterial isolate	Vancomycin			Cefoxitin			Trimethoprim/sulfamethoxazole			Gentamicin			Doxycycline			Ciprofloxacin			Erythromycin			Clindamycin		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
<i>S. aureus</i> (20)	100	0	0	5	0	95	95	0	5	10	0	90	5	0	95	15	0	85	0	0	100	0	0	100
<i>S. warneri</i> (23)	100	0	0	4.3	0	95.6	30.4	13	56.5	39.1	4.34	56.5	69.5	4.3	26	52.1	0	47.8	0	0	100	26.1	0	73.9
<i>S. haemolyticus</i> (4)	100	0	0	0	0	100	0	0	100	25	25	50	0	0	100	25	0	75	0	0	100	25	0	75
<i>S. saprophyticus</i> (18)	100	0	0	11	0	88.8	27.7	5.5	66.6	50	0	50	50	5.5	44.4	61.1	0	38.8	0	0	100	50	0	50
<i>S. schleiferi</i> (7)	100	0	0	0	0	100	14.2	42.8	42.8	0	0	100	71.4	0	28.5	14.2	0	85.7	0	0	100	42.9	0	57.1
<i>S. lugdunensis</i> (18)	100	0	0	0	0	100	44.4	11.1	44.4	33.3	0	66.6	66.6	0	33.3	38.8	0	61.1	0	0	100	38.9	0	61.1

S: Sensitive I: Intermediate R: Resistance

Macrolide lincosamide streptogramin B (MLS_B) resistance phenotype

All the isolates were screened for MLS_B resistance phenotype by double disk diffusion test (D-zone test). All staphylococcal isolates showed extensive resistance to 7 antibiotic classes (9 isolates) were resistant to erythromycin with MIC value >256 µg/ml. Out of 20 *S. aureus* isolates resistant to erythromycin with MIC value >256µg/ml, 16 isolates (80%) exhibited cMLS_B resistance, in addition, they showed high MIC value for clindamycin >256 µg/ml, 4 isolates (20%) iMLS_B with that recorded very low MIC with clindamycin 0.064 µg/ml. No isolates had MS_B resistance phenotype. The results demonstrated that cMLS_B (80%) was the predominant phenotype among *S. aureus* isolates and the predominant phenotype among CoNS isolates was cMLS_B 27/70 (38.5%) followed by MS_B 26/70 (37.1%) phenotype (Table 4).

(MLS_B) resistance genotype

ermC was detected by PCR in 40 isolates, 29 isolates had cMLS_B phenotype, 9 were iMLS_B and 2 isolates had MS_B phenotypes (Table 4). *ermA* gene was detected in only 2 isolates, one

isolate was *S. aureus* exhibited iMLS_B resistance phenotype and the another isolate was *S. warneri* exhibited MS_B phenotype. Twenty two staphylococcal isolates had *msrA* gene, 8 isolates had cMLS_B phenotype and 14 isolates exhibited MS_B phenotype.

Table (4): Correlation between the MLS_B resistance phenotypes and presence of macrolide resistance genes

Bacterial isolate	phenotype	Genotype					
		<i>ermA</i>	<i>ermB</i>	<i>ermC</i>	<i>msrA</i>	<i>mef</i>	-ve PCR
<i>S. aureus</i> (20)	cMLS _B (16)	0	0	13	1	0	2
	iMLS _B (4)	1	0	1	0	0	2
	MS _B (0)	0	0	0	0	0	0
<i>S. warneri</i> (23)	cMLS _B (11)	0	0	8	2	0	1
	iMLS _B (6)	0	0	2	0	0	4
	MS _B (6)	1	0	1	2	0	2
<i>S. haemolyticus</i> (4)	cMLS _B (1)	0	0	0	1	0	0
	iMLS _B (2)	0	0	1	0	0	1
	MS _B (1)	0	0	0	1	0	0
<i>S. saprophyticus</i> (18)	cMLS _B (5)	0	0	3	1	0	1
	iMLS _B (4)	0	0	1	0	0	3
	MS _B (9)	0	0	0	5	0	4
<i>S. schleiferi</i> (7)	cMLS _B (4)	0	0	3	1	0	0
	iMLS _B (0)	0	0	0	0	0	0
	MS _B (3)	0	0	0	1	0	2
<i>S. lugdunensis</i> (18)	cMLS _B (6)	0	0	2	2	0	2
	iMLS _B (5)	0	0	4	0	0	1
	MS _B (7)	0	0	1	5	0	1

DISCUSSION

The present study revealed that the erythromycin resistance rate between the staphylococcal isolates was 58% and the erythromycin-resistant *S. aureus* (ERSA) were 28.9%. Erythromycin resistance among *S. aureus* isolates in present study was considerably low in comparison to previous studies (15- 19). In contrast to *S. aureus* isolates, data revealed that out of 85 CoNS, 70 isolates (82.3%) were erythromycin resistant (ERCoNS), the

erythromycin resistance among CoNS in present study was relatively high. These data coordinate with reported data (20, 21). Methicillin-resistant *S. aureus* (MRSA) were 53/69 (76.8%) and data revealed that only 19/53 (35.8 %) of MRSA isolates were ERSA, however it was higher than the values in MSSA (1/16 = 6.2%), this could be due to the abuse of erythromycin in patients infected with MRSA facilitating the development of erythromycin resistance in MRSA (22, 21). While in CoNS isolates, 75/85 (88.2%) were methicillin resistant (MRCoNS) from which 67/75 (89.3%) were ERCoNS, and it was higher than the values in MSCoNS (3/10=30%).

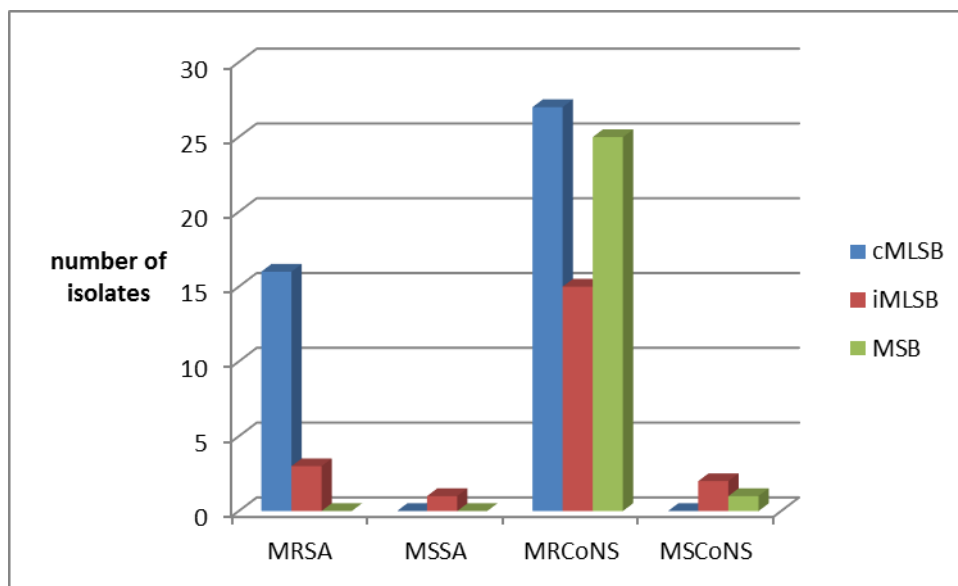


Fig (1): The relation between MR and MLS_B phenotypes in the staphylococcal isolates

The constitutive phenotype was the predominant among MRSA, as shown in Fig (1), these data are in agreement with reported results of (22-24, 3). The prevalence of the cMLS_B among MRS isolates than MSS indicated the selective pressure due to the failure of methicillin in treatment which forces the clinicians to use erythromycin and clindamycin.

In the present study, *ermC* was the most prevalent gene among ERSA (14/20=70%) (Fig 2), these results are congruent with reported reports (22, 25, 26), who reported that *ermC* was the predominant gene among SA isolates. It was also reported that *ermC* had replaced *ermA* as the dominant erythromycin resistance methylase gene among *S. aureus* (27). However, other studies demonstrated that *ermA* was the most frequent gene among SA isolates (16, 18, 20, 23, 28, 29, 30).

The most prevalent gene among ERCoNS was *ermC* (26/70=37.14%) followed by *msrA* gene (21/70=30%) in accordance with reported reports (26) (Fig 2). Many studies have reported the predominance of *ermC* gene among CoNS isolates (5, 16, 20, 21, 23, 30, 31). *ermB* and *mef* genes were not detected in any *staphylococcal* isolate. It was stated that *ermB* gene is generally found in animal *staphylococcal* strains (30, 32). *mef* gene was not identified in *S. aureus* and with less frequent distribution among CoNS (20).

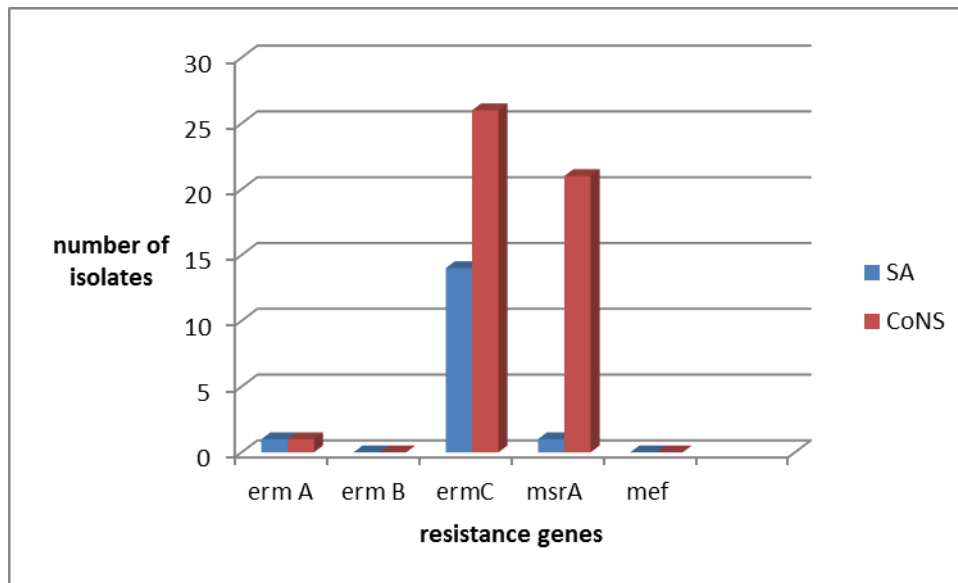


Fig (2): The distribution of resistance genes among SA and CoNS

Erythromycin resistance was detected phenotypically in 26 isolates while they exhibited negative PCR, in addition, 11 isolates demonstrated disagreement between phenotype and genotype and this could be due to the presence of another resistance mechanisms and as the result of coexistence of different genes in bacterial cells that exhibited the complexity of staphylococcal resistance to MLS_B antibiotics where the presence of some genes do not always lead to phenotypic expression of resistance (33). From the previous results, it can be stated that there was no full association found between genotype and phenotype patterns to detect macrolides resistance and this observation was also found in the earlier study (26).

CONCLUSION

$cMLS_B$ was the predominant phenotype especially among methicillin-resistant isolates and *ermC* gene was the most distributed gene among staphylococcal isolates but also we need complementary research to determine the probabilities of existence of other variants of *erm* genes or *msr* genes and the presence of other rare genes that are responsible for macrolides

and lincosamides resistance such as *mphC*, *ere*, *cfr*, *lsa*, *vga*, *lin* genes to complete the correlation between phenotypic and genotypic methods.

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