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Original Article

Inducible clindamycin resistance in clinical isolates of *staphylococcus aureus* in Suez Canal University Hospital, Ismailia, Egypt

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Abstract

Introduction: The increasing incidence of methicillin resistance among *Staphylococci* has led to renewed interest in the usage of macrolide-lincosamide-streptogramin B (MLSB) antibiotics to treat *S. aureus* infections, with clindamycin being the preferable agent owing to its excellent pharmacokinetic properties. Inducible clindamycin resistance my lead to therapeutic failure.

Aim: Detection of the prevalence of constitutive and inducible clindamycin resistance in clinical isolates of *S. aureus* to improve the clinical outcomes in patients.

Methodology: A total of 176 non-duplicate staphylococcal isolates were isolated from different clinical samples. Methicillin resistance was detected using Cefoxitin disk diffusion (CDD) method. Phenotypic clindamycin resistance was performed for all isolates by D test. Polymerase Chain Reaction (PCR) assay were done for detection of *erm* resistance genes *(erm*A, *erm*B and *erm*C).

Results: Out of 176 strains of *S. aureus*, 108 isolates (61.3%) were identified as MRSA. Erythromycin and clindamycin resistance was detected in 96 isolates (54.5%) and 68 isolates (38.6%) respectively. Clindamycin resistance (cMLS$_B$) was significantly higher (p value < 0.001) in MRSA strains (56 isolates) compared to MSSA (12 isolates). Resistant genes were detected in 160 isolates (91%). The *erm*A gene was detected in 28 isolates (16%), the *erm*B gene was detected in 80 isolates (45.5%) ($p < 0.001$).

Conclusions and recommendations: The frequency of constitutive and inducible clindamycin resistance in MRSA isolates emphasizes the need to use D test in routine antimicrobial susceptibility testing to detect the susceptibility to clindamycin as the inducible resistance phenotype can inhibit the action of clindamycin and affect the treatment efficacy.

Key words: Clindamycin resistance; inducible; constitutive; MRSA; MLS$_B$; *erm* genes.

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Introduction

Staphylococcus aureus (*S.aureus*) infections are considered a common cause of both Health Care Associated Infections (HAIs) and Community Acquired Infections (CAIs) [1]. Increasing frequency of Methicillin Resistant Staphylococcus aureus (MRSA) and changing the pattern of antimicrobial resistance decreases the therapeutic options to treat these infections and force the physician to change the pattern of prescribed antimicrobials and use of macrolide-lincosamide-streptogramin group B (MLS$_B$) [2,3]. Clindamycin, one of the MLS$_B$ family of antibiotics, is the preferred agent in treatment of MRSA infections due to its excellent pharmacokinetics [4,5]. Though MLS$_B$ antibiotics are chemically different in structure they act by inhibition of bacterial protein synthesis through binding to 23 rRNA of 50S ribosomal subunit [4,6]. Widespread use of MLS$_B$ antibiotics had led to increased resistance to these antibiotics especially clindamycin [7-9]. Clindamycin resistance can be constitutive or inducible depending on the presence or absence of macrolide inducer [10]. Erythromycin is an inducer of clindamycin resistance by stimulating the production of erythromycin ribosome methylase (*erm*) that induce the expression of clindamycin resistance [11]. Constitutive clindamycin resistance isolates, where methylase is always produced, are resistant to both erythromycin and clindamycin due (*erm*) gene expression [12,13]. Inducible clindamycin resistance isolates display resistance to erythromycin but falsely susceptible to clindamycin on disc diffusion method [14]. Modification of the target site, efflux pump and drug inactivation are the main mechanisms of macrolide and lincosamide resistance in clinical isolates

[13]. Clinically, isolates with inducible clindamycin resistance are considered a serious problem due to difficulty in recognition of such infections by routine laboratory methods [15]. Failure to detect inducible clindamycin resistance *S. aureus* may lead to misuse of clindamycin and clinical therapeutic failure [15,16]. To overcome this problem, the Clinical and Laboratory Standards Institute (CLSI) recommend the disk diffusion induction test, erythromycin- clindamycin disk approximation test (D test) to detect clindamycin resistance inducible phenotype among *S. aureus* isolates [6]. Macrolide resistance can be induced by efflux pump mechanism, called Macrolides Streptogramin phenotype (MS phenotype), where the isolates are resistant to macrolides but clindamycin susceptible [17]. The pattern of clindamycin resistance among *S. aureus* varies from a region to another. Local data is important to improve antibiotic use and guide the empirical treatment (18). In our institution, there are no data published discussing the pattern of clindamycin resistance among clinical isolates of *S. aureus*. So, our aim of the study was to determine the prevalence of constitutive and inducible clindamycin resistance in *S. aureus* clinical isolates in Suez Canal University Hospital. This will help physicians to prevent the misuse of clindamycin and also improve the clinical outcomes in those patients.

Methodology

This cross-sectional descriptive study was carried out during the period between June 2018 and June 2019. Samples were processed in microbiology laboratory, Faculty of Medicine, Suez Canal University. The ethics committee of Faculty of Medicine, Suez Canal University had reviewed and approved the study.

Isolation and identification

A total of 176 non-duplicate staphylococcal isolates were isolated from different clinical samples. The isolated strains were identified by conventional laboratory methods such as colony morphology, catalase test, slide and tube coagulase test, growth on Mannitol Salt agar and novobiocin (5μg) disc susceptibility testing. The isolates were preserved in glycerol 15% in brain heart infusion broth (BHIB, Oxoid, Basingstoke, UK) at –80°C and then subculturing in BHIB at 37°C for 24 h.

Antibiotic susceptibility test

Antibiotic susceptibility testing was performed by Kirby Bauer disk diffusion method and interpreted according to CLSI guidelines [19]. The antibiotics tested were Azithromycin, erythromycin, clindamycin, cefoxitin (surrogate test for oxacillin), penicillin, trimethoprim-sulfamethoxazole, ceftaroline, linezolid, doxycycline, tetracycline, chloramphenicol and ciprofloxacin. (Oxoid, Basingstoke, UK).

Phenotypic detection of Methicillin resistance

Methicillin resistance was detected using Cefoxitin disk diffusion (CDD) method. Isolates with cefoxitin inhibition zone size ≥ 22mm were considered Methicillin susceptible and those with cefoxitin inhibition zone size ≤ 21 mm were considered Methicillin resistant.

Phenotypic detection of clindamycin resistance (D test)

All isolates were subjected to D test on Muller Hinton agar plate as recommended by CLSI [19] by using erythromycin disk (15 μg) and clindamycin disk (2 μg) spaced 15 -26 mm apart. Flattening of the zone of inhibition adjacent to the erythromycin disk (D zone) was interpreted as inducible clindamycin resistance. Hazy growth within the zone of inhibition around clindamycin was also interpreted as clindamycin resistance even if no D zone is apparent (19). Isolates, which were erythromycin resistant and clindamycin sensitive, with no apparent D zone were interpreted as MS phenotype (D test negative). Isolates which were erythromycin resistant and clindamycin sensitive with apparent D zone were interpreted as Inducible clindamycin resistance phenotype ($iMLS_B$) (D test positive). While, isolates which were resistant to both erythromycin and clindamycin interpreted as constitutive clindamycin resistance ($cMLS_B$). Isolates

Table 1. Primers sequence and PCR conditions for detection of *erm* genes.

Target gene	Primer sequence	PCR fragment size	No. of PCR cycles conditions
***erm*A**	F: GTTCAAGAACAATCAATACAGAG R: GGATCAGGAAAAGGACATTTTAC	421	30 (30 s at 94°C; 30 s at 52°C; 1 min at 72°C)
***erm*B**	F: CCGTTTACGAAATTGGAACAGGTAAAGGGC R: GAATCGAGACTTGAGTGTGC	359	30 (30 s at 94°C; 30 s at 55°C; 1 min at 72°C)
***erm*C**	F: GCTAATATTGTTTAAATCGTCAATTCC R: GGATCAGGAAAAGGACATTTTAC	572	30 (30 s at 94°C; 30 s at 52°C; 1 min at 72°C)

Table 2. Frequency of MRSA and MSSA according to different clinical samples.

	MRSA (n = 108)	MSSA (n =68)	Total (%) (n = 176)
Pus	69 (63.9%)	19 (27.9%)	88 (50%)
Blood	23 (21.3%)	15 (22.1%)	38 (21.6%)
Urine	5 (4.6%)	24 (35.3%)	29 (16.5%)
Tracheal aspirates	8 (7.4%)	4 (5.9%)	12 (6.8%)
Ascetic fluid	2 (1.9%)	4 (5.9%)	6 (3.4%)
Synovial fluid	1 (0.9%)	2 (2.9%)	3 (1.7%)
Total	108 (100%)	68 (100%)	176 (100%)

which were sensitive to both erythromycin and clindamycin were interpreted as susceptible phenotype.

Genotypic detection of erm genes

DNA was extracted from direct bacterial colonies using Qiagen DNA Mini kit 51304. Polymerase Chain Reaction (PCR) assay for detection of *erm*A, *erm*B and *erm*C resistance gene was carried out [8]. Primers sequence and PCR conditions were discussed in Table 1. Amplicons were analyzed by gel electrophoresis in 1.5 % agarose gel in 1X Tris-Borate-EDTA (TBE) buffer containing 0.1 µl/mL ethidium bromide compared to a 100 bp molecular size standard DNA ladder (Cleaver scientific, UK).

Statistical analysis

Statistical analysis was done using IBM Statistical Package of Social Sciences (SPSS) software version 22 for Windows ® software. Descriptive statistics: numerical presentation of data was done using frequency distribution tables. According to Kolmogorov-Smirnov normality testing, the data was non-parametric. Thus non-parametric data analysis was recommended; here Chi-squared test was used for study variables. p value was significant at < 0.05.

Results

A total of 176 strains of *S. aureus* were isolated from different clinical samples as shown in Table 2. Most strains of MRSA were isolated from pus specimens (63.9%), blood (21.3%), tracheal aspirates (7.4%), urine (4.6%), ascetic and synovial fluid (1.9% and 0.9% respectively). Otherwise, MSSA strains were commonly isolated from urine, pus, blood followed by tracheal, ascitic and synovial fluids (5.3%, 27.9%, 22.1%, 5.9%, 5.9% and 2.9 respectively) which was statistically significant ($p < 0.001$). All the strains were sensitive to linezolide. Most strains were sensitive to chloramphenicol, ciprofloxacin, ofloxacin and tetracycline representing 91%, 72.7%, 70.4% and 63.6% respectively.

Out of 176 isolated strains of *S. aureus*, 108 isolates (61.3%) were identified as MRSA and 68 isolates (38.7%) were MSSA. Resistance to erythromycin and clindamycin was detected in 96 isolates (54.5%) and 68 isolates (38.6%) respectively. Susceptibility to both erythromycin and clindamycin was found in 80 isolates. MS phenotype, $iMLS_B$ and $cMLS_B$ were seen in 4 (2.3%), 24 (13.6%) and 68 (38.6%) isolates respectively. Clindamycin resistance ($cMLS_B$) was significantly higher (p value < 0.001) in MRSA strains (56 isolates) compared to MSSA (12 isolates) as shown in Table 3.

All the 176 isolates were tested for the presence of MLS_B resistant genes and 160 (91%) contained one or more *erm* genes. The *erm*A gene was detected in 28 isolates (16%) (20 isolates MRSA and 8 isolates MSSA), the *erm*B gene was detected in 80 isolates (45.5%) (60 isolates MRSA and 20 isolates MSSA) which was statistically significant ($p < 0.001$) and the *erm*C was detected in 88 isolates (50%) (56 isolates MRSA and 32 isolates MSSA) as shown in Table 4. Combination of *erm* genes was detected in 104 isolates (59%) (68 MRSA and 36 MSSA) (Figure 1).

All *S.aureus* isolates with MS resistance phenotype (4 isolates) were MSSA and carried the 3 genes (*erm*A, *erm*B and *erm*B). Moreover, most of the isolates with $iMLS_B$ resistance phenotype carried both *erm*A and

Table 3. Distribution of clindamycin resistance phenotypes.

Phenotype	MRSA	MSSA	Total	P value
MS	0 (0.0%)	4 (5.9%)	4	
$iMLS_B$	20 (18.5%)	4 (5.9%)	24	
$cMLS_B$	56 (51.9%)	12 (17.6%)	68	< 0.001*
Sensitive	32 (29.6%)	48 (70.6%)	80	
Total	108 (100.0%)	68 (100.0%)	176	

* Statistically significant at p <0.05.

Table 4. Prevalence of *erm* genes in erythromycin resistant strains (MS phenotype, $iMLS_B$ and $cMLS_B$).

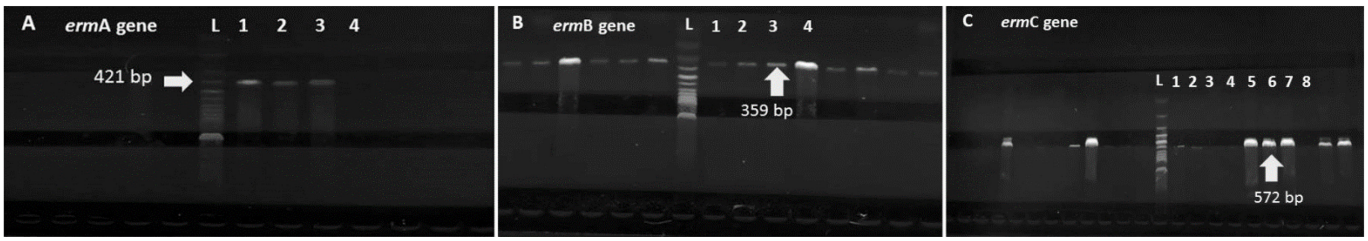
Genotype	MRSA	MSSA	P value	Relative risk	CI (95%)
*erm*A	20 (18.5%)	8 (11.8%)	0.234	1.201	(0.918-1.573)
*erm*B	60 (55.6%)	20 (29.4%)	< 0.001*	1.500	(1.839-1.901)
*erm*C	56 (51.9%)	32 (47.1%)	0.534	1.077	(0.851-1.362)
*erm*A+*erm*B	24 (22.2%)	8 (11.8%)	0.081	1.286	(1.008-1.640)
*erm*A+*erm*C	0 (0.0%)	0 (0.0%)	1.000	-	-
*erm*B+*erm*C	48 (44.4%)	28 (41.2%)	0.671	1.052	(0.832-1.331)
*erm*A+*erm*B+*erm*C	12 (11.1%)	8 (11.8%)	0.895	0.975	(0.668-1.423)

*erm*B genes (12 isolates, 8 MRSA and 4 MSSA), 8 isolates carried *erm*B and *erm*C genes and 4 isolates carry *erm*B gene only. We found that most of the isolates with $cMLS_B$ resistance phenotype were carrying both *erm*B and *erm*C (36 isolates, 24 MRSA and 12 MSSA) and 12 isolates was carrying *erm*B gene. Sixteen isolates were negative for the 3 genes by PCR, 12 of them were MRSA (8 were $cMLS_B$ resistance phenotype and 4 were sensitive phenotypically) and only 4 of them were MSSA of sensitive phenotype. Twenty isolates were detected to carry the 3 resistant genes, 12 of them were MRSA (all carrying the cMLSB phenotype) and 8 were detected as MSSA (4 of them were MS phenotype and the other were sensitive phenotype).

Discussion

Staphylococcus aureus is one of the most common organisms causing both hospital and community acquired infections [1]. Resistance to many antimicrobial agents signifies the problem and limits the treatment options. Last years, developing of new effective agents to treat such infections is considered an important new challenge in health care settings to overlap the changing pattern of resistance [20]. Clindamycin is considered an interesting alternative for treatment of staphylococcal infections as it is available for parenteral and oral use, in addition to its low cost, good tissue penetration and accumulation in abscesses[20]. MLSB antibiotics resistant strains emergence due to the misuse of these antibiotics has been reported as a new challenge in treating such infections [4,21]. Erythromycin resistance is typically related with clindamycin resistance. It is difficult to identify the inducible MLSB (iMLSB) phenotype in daily routine tests where isolates appear to be clindamycin-susceptible and erythromycin-resistant in laboratories in vitro. This false clindamycin susceptibility in iMLSB isolates may therefore lead to therapeutic failure if patients are treated with clindamycin [22]. In our study, out of 176 strains of *S. aureus*, 108 isolates (61.3%) were identified as MRSA and 68 isolates (38.7%) were MSSA. High rate of MRSA was reported in many studies in developed nations [23-26] and some studies reported a lower prevalence than our study (12,21, 27-30). Improper infection prevention and control practices, misuse of antibiotics, hospitalization in critical care units (as intensive care units) and use of devices (as urinary catheter, mechanical ventilation and intravascular catheters) may contribute in the emergence of MRSA. This discrepancy in the prevalence rate of MRSA among different regions and different countries emphasize the importance of developing local data for antimicrobial resistance to be a guide for empirical treatment and surveillance programs. In our study, resistance to erythromycin and clindamycin was seen in 54.5% and 38.6% respectively. A recent Indian study demonstrated that 39.14% of isolates were resistant to erythromycin [12]. Mansouri and Sadeghi reported a

Figure 1. Distribution of *erm* genes in clinical isolates.



(A) Distribution of *erm*A gene: lanes 1, 2 and 3 are *erm*A positive (421 bp), lane 4 is negative. Lane L is 100bp molecular size ladder. (B) Distribution of *erm*B gene: lanes 1, 2, 3 and 4 are *erm*B positive (359bp). Lane L is 100bp molecular size ladder. (C) Distribution of *erm*C gene: lanes 1, 2 , 3, 5 , 6 and 7 are *erm*C positive (572bp), lanes 3, 4 and 8 are negative. Lane L is 100bp molecular size ladder.

high prevalence of erythromycin resistance in Iran [31]. These variations may be due to the difference in antibiotic policies in different regions and the rates of macrolides and lincosamides consumption in our hospital. The incidence of $cMLS_B$ phenotype varies significantly from one country to another. Our study revealed higher constitutive resistance in comparison to inducible resistance (38.6% and 13.6% respectively). Clindamycin resistance ($cMLS_B$) was significantly higher (p value < 0.001) in MRSA strains (56 isolates) compared to MSSA (12 isolates). Fiebelkorn *et al* found that 34.12% of erythromycin resistant isolates showed constitutive resistance while 28.94% showed inducible resistance [21]. Mokta *et al* reported that 30% of *S.aureus* was clindamycin resistant with higher frequency of constitutive resistance compared to inducible resistance (17.14% and 13.71% respectively). Other studies reported higher prevalence of inducible resistance phenotypes than the constitutive phenotypes [5, 32,33]. In Europe, there is a high incidence of constitutive resistance in MRSA isolates (93%) where the inducible resistance phenotype was predominant in MSSA [34]. We reported a high incidence of constitutive resistance in MRSA (56%), however, the sensitive phenotype predominated among MSSA. Many studies reported increased incidence of constitutive phenotype resistance than inducible resistance among MRSA [12, 33, 35]. On the other hand, in 2005, Azap *et al*., reported higher incidence of inducible resistance among MRSA isolates than MSSA (5-7% vs 3.7%) [36]. Bottega *et al.*, in 2014 reported high prevalence of both constitutive and inducible resistance among MRSA than MSSA (68.9 vs 4.5% 10.3% vs 7.2%) [37]. This discrepancy between different studies can be due to the difference in bacterial susceptibility in different geographical regions. Also, the variation in antimicrobial prescriptions by physicians may affect the resistance pattern in different regions. This stresses on the significance of local surveillance in producing relevant local resistance data, for appropriate empiric treatment. Despite the fact that there is geographic variability among different resistance phenotypes, the prevalence of *erm* genes has been reported to be quietely similar in different contries. In our study, *erm*B gene was the most prevalent genotype among *S.aureus* isolates with significant difference between MRSA and MSSA. The most prevalent genotype among MRSA was *erm*B (55.6%) while *erm*C was the most prevalent among MSSA isolates (47.1%). Coutinbo *et al.,* reported that *erm*A was the most prevalent genotype in *S.aureus* with only 3 isolates (2%) carrying *erm*B gene [38]. In Canada, Martineau *et al*., reported a lower percentage of *erm*A in *S.aureus* [39]. In Europe, a multicenter study reported the high prevalence of *erm*A gene and the low prevalence of *erm*C and *erm*B genes [40]. This multicenter study was compatible with another study in denmak [41]. The presence of more than one *erm* gene was detected more in MRSA especially *erm*B and *erm*C (44.4%) followed by *erm*A and *erm*B (22.2%) while Coutinho *et al*., didn't detect any combination genes for resistance in *S.aureus* [38]. *erm*B was detected 1.5 more times in MRSA than MSSA and this difference was statistically significant (p < 0.001), however, no significant differences were found in the prevalence of *erm*A or *erm*C between MRSA and MSSA. Our results were not compatible with Jarajreh *et al.,* who reported that *erm*A was detected more than *erm*B and *erm*C [42].

We noticed that all *S.aureus* isolates with MS resistance phenotype (4 isolates) were MSSA and carry the 3 genes (*erm*A, *erm*B and *erm*C). These data suggest that MSSA strains had succeeded in acquiring the erm genes among *S. aureus* isolates. Moreover, most of inducible phenotype resistance was carrying both *erm*A and *erm*B genes, *erm*B and *erm*C genes or *erm*B gene only. While, most constitutive phenotype resistance was carrying both *erm*B and *erm*C or *erm*B gene. We found that 32 isolates were sensitive to both erythromycin and clindamycin phenotypically but carry *erm*B genes, 4 isolates carry both *erm*A and *erm*B genes, 32 carry both *erm*B and *erm*C. The presence of *erm* genes among isolates susceptible to erythromycin owing to the lack of expression of these genes causing down regulation of gene expression. This was already demonstrated in other studies [38,39]. Coutinho *et al*., found six *S.aureus* isolates resistant to both erythromycin and clindamycin but didn't carry any resistance genes [38]. Chung *et al.*, explained this finding by the presence of other genes as *msr*A and *msr*B [43]. In our study, all the isolates resistant to erythromycin and clindamycin carried at least one resistance gene alone or in combination with other genes. The prescence of *S. aureus* isolates with inducible resistance depend on difference in geographical areas, age of examined patients, bacterial species, difference in sample origin and strains source either community or nosocomial. The prevalence also differs from one hospital to another and even among patients [44,45].

Resistance to other antibiotics (non MLSB antibiotics) was higher in isolates carrying resistance genes in relation to isolates without *erm* genes. The resistance to chloramphenicol, tetracycline, ciprofloxacin, doxycycline, levofloxacin, gentamicin,

azithromycin, trimethoprime-sulfamethoxazole was significantly higher in isolates with *erm* genes than isolates without *erm* genes (p value < 0.005).

In conclusion, MRSA has became a major global health problem worldwide, both in community and hospitals. Because of their resistance to commonly used antibiotics, there is a need for the development of appropriate control policies in our hospital settings. Clindamycin resistance either iMLSB or cMLSB limits the therapeutic options for MRSA. In our study, we noticed a higher frequency of constitutive and inducible in MRSA isolates. MS phenotype, $iMLS_B$ and $cMLS_B$ were seen in 2.3%,13.6% and 38.6% respectively. Clindamycin resistance ($cMLS_B$) was significantly higher in MRSA strains compared to MSSA. The *erm*A , *erm*B and *erm*C genes were detected 16%,45.5% and 50% respectively. Combination of *erm* genes was detected in 104 isolates (59%). Sixteen isolates were negative for the 3 genes by PCR. Therefore, we recommended the use of D test in routine antimicrobial susceptibility testing to detect the susceptibility to clindamycin to help in guiding the clinicians regarding the judicious use of clindamycin.

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