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# Phenotypic and genotypic identification of *Staphylococcus aureus* resistant to clindamycin in Mansoura University Children Hospital, Egypt

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## Abstract:

**Background**: Clindamycin has been a good alternative drug to penicillins in the treatment of infections caused by *Staphylococcus aureus* but resistance to this agent has led to therapeutic failure. Inducible clindamycin resistance in staphylococci carrying *erm* genes may not be detectable by routine disk diffusion test. The objective of this study is to phenotypically detect clindamycin resistance in clinical *S. aureus* isolates and determine the prevalence of *erm*A, *erm*B and *erm*C carriage among these isolates.
**Methodology**: A total of 230 non-duplicate *S. aureus* were isolated from children admitted to Mansoura University Children Hospital during the period January 2016 and June 2017 by conventional microbiology method. *Invitro* antibiotic susceptibility to selected antibiotics including erythromycin was performed by the disk diffusion technique. The 'D-zone' test was used to phenotypically detect inducible clindamycin resistance. The presence of *erm*A, *erm*B and *erm*C genes was confirmed by multiplex polymerase chain reaction (m-PCR) assay.
**Results**: One hundred and seven (46.6%) isolates were phenotypically resistant to erythromycin while 109 (47.3%) were methicillin (cefoxitin) resistant *S. aureus* (MRSA). The macrolide-lincosamin-streptograminB ($MLS_B$) phenotypes among the erythromycin resistant isolates were 47 (44%) inducible $MLS_B$ and 46 (43%) constitutive $MLS_B$, while 14 (13.0%) were MS phenotype. Although, the $MLS_B$ phenotype was more predominant in MRSA (n=60, 56.1%) than MSSA (n=33, 30.7%) while the MS phenotype was more predominant in MSSA (n=9, 8.4%) than MRSA (n=5, 4.6%) isolates, the difference was not statistically significant ($p$=0.0777). The *erm*A (29.0%, n=31) and *erm*C (18.7%, n=20) were the most prevalent genes carried by the isolates while *erm*B was carried by a few (4.7%, n=5). Forty six (43%) isolates did not carry any detectable *erm* gene.
**Conclusion**: In this study, both inducible and constitutive clindamycin resistance phenotypes were common among *S. aureus* isolates. Although the genetic basis for this may be attributed to carriage of *erm*A, *erm*B and *erm*C genes, a number of the resistant isolates did not carry any of these genes.

**Keywords:** phenotypic, genotypic, macrolide-lincosamide-streptogramin B, *S. aureus*, children,

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# Identification phénotypique et génotypique de *Staphylococcus aureus* résistant à la clindamycine à l'Hôpital Universitaire Mansoura, Egypte

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## Abstrait:

**Contexte:** La clindamycine était un bon médicament alternatif aux pénicillines dans le traitement des infections causées par *Staphylococcus aureus*, mais la résistance à cet agent a entraîné un échec thérapeutique. La résistance inductible à la clindamycine chez les staphylocoques porteurs du gène erm peut ne pas être détectable par le test de diffusion systématique sur disque. L'objectif de cette étude est de détecter phénotypiquement la résistance à la clindamycine dans les isolats cliniques de S. aureus et de déterminer la prévalence du portage d'*erm*A, d'*erm*B et d'*erm*C parmi ces isolats.
sélection d'antibiotiques, notamment à l'érythromycine, a été réalisée par la technique de diffusion sur disque. Le test de la «zone D» a été utilisé pour détecter phénotypiquement la résistance inductible à la clindamycine. La présence des gènes *erm*A, *erm*B et *erm*C a été confirmée par un test de réaction en chaîne de la polymérase multiplexe (m-PCR).
**Résultats:** Cent sept isolats (46,6%) étaient phénotypiquement résistants à l'érythromycine, tandis que 109 (47,3%) étaient résistants à la méthicilline (céfoxitine) *S. aureus* (MRSA). Les phénotypes macrolide-lincosamin-streptogramine B ($MLS_B$) parmi les isolats résistants à l'érythromycine étaient 47 (44%) $MLS_B$ inductibles et 46 (43%) $MLS_B$ constitutifs, alors que 14 (13,0%) étaient des phénotypes MS. Bien que le phénotype $MLS_B$ soit plus prédominant dans le SARM (n=60, 56,1%) que le MSSA (n=33, 30,7%), le phénotype MS était plus prédominant dans le MSSA (n=9, 8,4%) que le SARM (n=5, 4,6%), la différence n'était pas statistiquement significative (p=0,0777). *Erm*A (29,0%, n=31) et *ermC* (18,7%, n=20) étaient les gènes les plus prévalents portés par les isolats, tandis que ermB était porté par quelques-uns (4,7%, n=5). Quarante-six (43%) isolats ne portaient aucun *erm* gène détectable.
**Conclusion:** Dans cette étude, les phénotypes de résistance à la clindamycine tant inductibles que constitutifs étaient courants parmi les isolats de *S. aureus*. Bien que la base génétique de ceci puisse être attribuée au portage des gènes *erm*A, *erm*B et *erm*C, un certain nombre des isolats résistants ne portaient aucun de ces gènes.


**Mots-clés:** phénotypique, génotypique, macrolide-lincosamide-streptogramine B, *S. aureus*, enfants

## Introduction:

*Staphylococcus aureus* is a common bacterial pathogen responsible for both community and hospital acquired infections. The infection caused by *S. aureus* ranges from mild skin and soft tissue to life threatening infections such as septicemia, meningitis and endocarditis (1). The major concern about this pathogen is its ability to acquire resistance to multiple antibiotics. Resistance to penicillin and methicillin has led to the use of alternative antibiotics especially macrolides and lincosamides such as clindamycin. Clindamycin can be given both orally and intravenously and it is effective against soft tissue infections as well as deep penetrating infections such as sepsis (2).

Resistance to clindamycin is usually associated with macrolides resistance especially erythromycin (3-7). The respon-sible mechanisms for macrolide and clindamycin resistance are attributed to two mechanisms (8). The first mechanism is due to the efflux of antibiotics outside the bacterial cell wall before its binding to ribosome. This mechanism is commonly reported in *S. aureus* and is controlled by *msr*A gene (9). The other mechanism is called $MLS_B$ (macrolide, lincosamide, streptogramin B) which is due to changes on the ribosome affecting the binding site as a result of methylation of the 23S rRNA–binding site of macrolides. This results in resistance to macrolides and is controlled by the *erm*A, *erm*B and *erm*C genes (8, 9).

Clindamycin resistance can be detected phenotypically by two methods. The first method detects constitutive clindamycin resistance ($cMLS_B$) phenotype by the disk diffusion technique. The other method called the "D-zone" test detects inducible clindamycin resistance ($iMLS_B$) in susceptible isolates by adding erythromycin (which is a known strong methylase inducer in susceptible strains) to clindamycin disks, which results in appearance of a blunting of the clindamycin inhibition zone (appearing like a letter D) on the margin closest to the erythromycin disk (10).

Published data on the presence of *S. aureus* strains resistant to clindamycin in Egypt are lacking, however, information on this could have important implications for treatment protocols against staphylococcal infections in the country. The aim of this research therefore is to determine the carriage rate of *erm* genes responsible for inducible clindamycin resistance among clinical *S. aureus* isolates in Egypt.

## Materials and method:

### Study design and subjects

This cross sectional study was conducted on randomly selected children admitted to Mansoura University Children Hospital, Egypt, between January 2016 and June 2017, from whom 230 non-repetitive *S. aureus* isolates were obtained. The study was approved by Mansoura Faculty of Medicine Institutional Ethical Committee

### Specimen collection

The samples for *S. aureus* isolation were from various clinical specimens such as

blood cultures (n=157), wound samples (n=41), and broncho alveolar lavage (n=32).

**Isolation and identification of *S. aureus***

All specimens were inoculated on Sheep blood and MacConkey agar plates, and incubated at 37°C aerobically for 24 hours. Identification of *S. aureus* was first done by colony morphology on plates, followed by conventional identification methods of Gram stain, catalase activity, and slide and tube coagulase tests.

**Antibiotic susceptibility test**

Antibiotic susceptibility test (AST) was performed on all identified *S. aureus* isolates (n=230) in accordance with the Clinical and Laboratory Standards Institute (CLSI) agar disk diffusion method on Mueller-Hinton (MH) agar (11) with the following disks (Oxoid, England); erythromycin (15µg), ampicillin-sulbactam (20 µg / 20 µg), amoxicillin-clavulanic acid (20 µg / 10 µg), piperacillin (30µg), ciprofloxacin (5µg), ceftriaxone (5µg), cephradine (30µg), cefotaxime (30µg), ceftazidime (30µg), and cefoperazone (75µg).

**Detection of methicillin resistance**

Methicillin resistance was detected by the cefoxitin (30µg) disk diffusion test on MH plates which were inoculated with the standardized inoculum of the *S. aureus* isolates (n=230), and incubated at 37°C for 24 hours. *S. aureus* with cefoxitin inhibition zone ≤ 21mm were considered MRSA (10).

**Detection of clindamycin resistance**

All *S. aureus* isolates resistant to erythromycin in the AST (n=107) were evaluated for clindamycin resistance by the disk diffusion method as previously described (10). The isolated colonies were diluted in sterile normal saline to obtain 0.5 McFarland's standard suspension. The inoculum was aseptically plated over MH agar plate, with both erythromycin (15µg) and clindamycin (2µg) disks placed ~15 mm apart edge to edge, and incubated aerobically at 37°C for 24 hours. Isolates resistant to erythromycin (zone of inhibition <13mm) with flattening of zone (D-zone) around the clindamycin disk (zone of inhibition≥21mm) were identified as inducible resistance phenotype (Fig 1).

Isolates resistant to both erythromycin and clindamycin (zone of inhibition<21mm) were identified as constitutive resistance phenotype ($cMLS_B$) and isolates resistant to erythromycin but susceptible to clindamycin (zone of inhibition ≥21mm) with no D-zone were identified as MS phenotype (10).

**Multiplex PCR for macrolide genes:**

DNA extraction of purified colonies of all *S. aureus* resistant to erythromycin in the AST (n=107) was done by Qiagen extraction kit and kept frozen at -20°C until use. Multiplex PCR was performed for the isolates for detection of *erm*A, *erm*B and *erm*C genes using primers with sequences as shown in Table 1 (9). Amplification was performed in a 25µL PCR mixture (Qiagen) with the use of 50ng DNA template, and 25 pmol/L each, of forward and reverse primers. Thermal cycling was performed as follows; 5 min at 94°C, then 1min at 95°C, 30 sec at annealing temp of 55°C, and 1min at 72°C for 30 cycles, with a final extension at 72°C for 5min.

Ten µL of PCR product was resolved on 2% agarose gel containing 0.5mg/mL ethidium bromide at 90V for 1h and visualized in a gel documentation system. The reaction mix containing all materials except DNA was used as negative control.

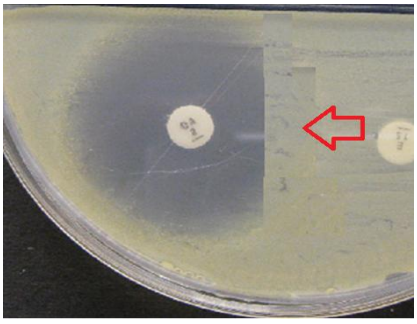


Fig 1: Mueller Hinton agar plate showing positive D-zone test in a *Staphylococcus aureus* isolate with inducible $MLS_B$ phenotype

Table 1: Genes and the primers sequences used in the study

| Target gene | Primer sequence | Product size, bp | Reference |
|---|---|---|---|
| *ermA* | F 5´-TAT CTT ATC GTT GAG AAG GGA TT-3 | 139 | 8 |
| | R 5´-CTA CAC TTG GCT TAG GAT GAA A-3´´ | | |
| *ermB* | F 5´-CTA TCT GAT TGT TGA AGA AGG ATT-3´ | 142 | 8 |
| | R 5´-GTT TAC TCT TGG TTT AGG ATG AAA-3´ | | |
| *ermC* | F 5´-CTT GTT GAT CAC GAT AAT TTC C-3´ | 190 | 8 |
| | R 5´-ATC TTT TAG CAA ACC CGT ATT C-3´ | | |

## Results:

A total of 230 non-repetitive *S. aureus* were isolated from the subjects, mainly from blood cultures (n=157, 68.4%), wound samples (n=41, 17.9%) and broncho-alveolar lavage (n=32, 13.7%). All isolates were resistant to the tested antibiotics to varying degrees, with 109 resistant to cefoxitin, representing MRSA rate of 47.3%, and 107 (46.6%) resistant to erythromycin (Table 2).

Table 2: *Invitro* antibiotic resistance of *Staphylococcus aureus* in Mansoura University Children Hospital, Egypt

| Antibiotic disk | Frequency of resistance (%) |
|---|---|
| Ciprofloxacin | 64 (27.8) |
| Ceftazidime | 44 (19.1) |
| Ceftriaxone | 62 (26.9) |
| Ampicillin/Sulbactam | 62 (26.9) |
| Cefoperazone | 38\| (16.3) |
| Cefotaxime | 52 (22.6) |
| Cephradine | 56 (24.3) |
| Piperacillin | 32 (13.9) |
| Amoxicillin/Clavulanic acid | 120 (52.1) |
| Erythromycin | 107 (46.6) |
| Cefoxitin | 109 (47.3) |

The result of the D-zone test shows that 93 of the 107 (86.9%) erythromycin resistant *S. aureus* isolates were $MLS_B$ phenotype ($iMLS_B$, 43.9% and $cMLS_B$, 43.0%) while 14 (13.1%) were MS phenotype (Table 3).

Although, the $MLS_B$ phenotype was more predominant in MRSA (n=60, 56.1%) than MSSA (n=33, 30.8%) while the MS phenotype was more predominant in MSSA (n=9, 8.4%) than MRSA (n=5, 4.7%) isolates, the difference was not statistically significant ($X^2 = 3.112$, $p = 0.0777$).

Table 4 shows the carriage rates of *erm* genes among the 107 erythromycin resistant *S. aureus* isolates, with *erm*A (29.0%, n=31), *erm*C (18.7%, n=20) and *erm*B (4.6%, n=5). One isolate (1.0%) carried both *erm*A and *erm*C genes while 4 (3.7%) isolates each carried *erm*A, *erm*B and *erm*C genes. A total of 46 (43.0%) isolates did not contain any of the *erm* genes. Fig 2 shows gel electrophoresis of isolates with amplified *erm*A and *erm*C genes, and others with no amplified gene product.

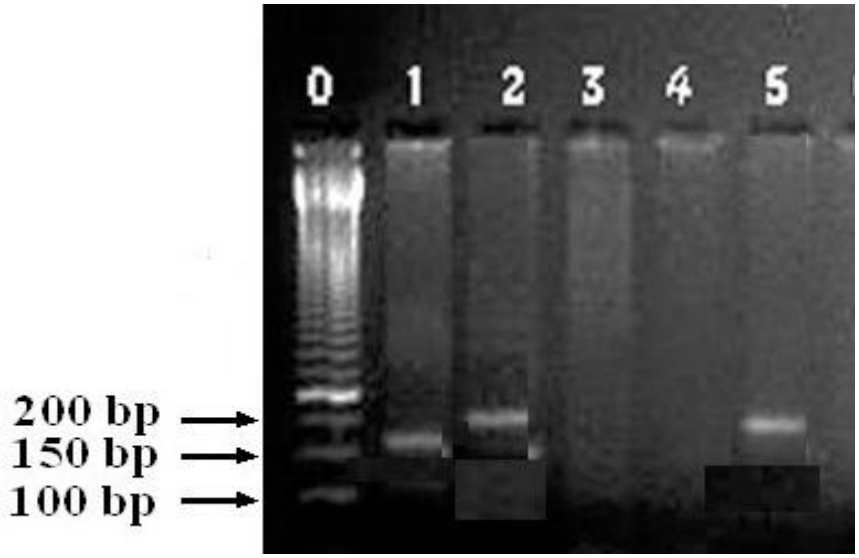
Table 4: Frequency of *erm* genes in phenotypic macrolide resistant *Staphylococcus aureus* isolates in Mansoura University Children Hospital, Egypt

| Macrolide resistance genes | Frequency (%) |
|---|---|
| *ermA* | 31(29.0) |
| *ermB* | 5 (4.6) |
| *ermC* | 20 (18.7) |
| *ermA/ermC* | 1 (1.0) |
| *ermA/ermB/ermC* | 4 (3.7) |
| No gene | 46 (43.0) |
| Total | 107 |

Table 3: Distribution of macrolide resistance phenotypes among *Staphylococcus aureus* isolates in Mansoura University Children Hospital, Egypt

| Macrolide resistance phenotype | *Staphylococcus aureus* isolates | | Total (%) | $X^2$ | *p* value |
|---|---|---|---|---|---|
| | MRSA (%) | MSSA (%) | | | |
| Inducible $MLS_B$ | 27 (25.2) | 20 (18.7) | 47 (43.9) | 3.112 | 0.0777 |
| Constitutive $MLS_B$ | 33 (30.8) | 13 (12.2) | 46 (43.0) | | |
| Sub-Total | 60 (56.1) | 33 (30.8) | 93 (86.9) | | |
| MS (ERY-R/CLI-S) | 5 (4.7) | 9 (8.4) | 14 (13.1) | | |
| Total | 65 (60.8) | 42 (39.2) | 107 (100) | | |

Ery-R = erythromycin resistant; CLI-S = clindamycin sensitive; MRSA = methicillin resistant *Staphylococcus aureus*; MSSA= methicillin sensitive *Staphylococcus aureus*; MLSB = macrolide-lincosamide-streptograminB




0 = molecular weight marker; 1 = isolate with amplified *erm*A gene; 2 & 5 = isolates with amplified *erm*C gene; 3 & 4 = isolate with no gene amplified

Fig 2: Gel electrophoresis of amplified *erm* genes in some macrolide resistant *Staphylococcus aureus* isolates

## Discussion:

The prevalence of MRSA has been increasing worldwide since last decades. This prevalence varies widely across different countries and also in hospitals of same country. In this study, isolated *S. aureus* strains showed high resistance to cefoxitin, representing MRSA rate of 47.3%, compared to a lower rate of 24% previously reported in a study from Minia, Egypt. However various studies have reported MRSA rate ranging from 50% to 68% (10,12,13).

There was also relatively high resistance to other beta lactam antibiotics such as ampicillin-sulbactam (26.9%), amoxicillin-clavulanic acid (52.1%), piperacillin (13.9%), ceftriaxone (26.9%), ceftazidime (19.1%), cefotaxime (22.6%), cephradine (24.3%), and cefoperazone (16.3%) in this study. This multidrug resistance among *S. aureus* can be explained by the common association of *mec*A (methicillin gene) with other resistant genes for beta lactamase production, leading to cross resistance to other beta lactam antibiotics. Previous studies have shown such association (14, 15).

Phenotypic resistance to erythromycin was 46.6% in this study. Similar rates of resistance to erythromycin were reported previously ranging from 46% to 51.7% (16-18) whereas lower rates ranging from 15.7% to 28.4% were reported by others (19,20). The MRSA isolates is our study had higher rate for both inducible $MLS_B$ (25.2%) and constitive $MLS_B$ (30.8%) phenotypes than the MSSA isolates (though not statistically significant), which agrees with the study of Prabhu et al., who reported 16.6% $cMLS_B$ phenotype in MRSA and 6.2% in MSSA (10). Also Gupta et al., reported $cMLS_B$ phenotype in 19% of the total isolates of which 46% were MRSA and 10% were MSSA (8). The phenotype of $MLS_B$ (either constitutive or inducible) may show great variations based on the patient groups in different hospitals and geographical regions. This variation may be attributed to several factors such as usage rate of macrolide antibiotics in different hospitals, age of patients, and source of tested isolates (15).

Clindamycin resistance is usually attributed to the carriage of any of *erm*A, *erm*B or *erm*C genes. The association of MRSA with clindamycin resistance can be attributed to acquisition of one of these genes via transposition to become part of the SCC*mec* cassette chromosomes containing the methicillin resistant (*mec*A) genes (16). In our study, *erm*A and *erm*C genes were the two genes frequently carried by the *S. aureus* isolates, while a few isolate carried the *erm*B gene. This is similar to the report of a study in Denmark over a 30 year period (1959–1988) by Westh et al., which showed high carriage rates of *erm*A and *erm*C genes among the *S. aureus* isolates but no *erm*B gene detected (21). Similarly, a multicenter study of 24 European University Hospitals in the year 2000 reported high prevalence of *erm*A (64.0%) and low prevalence of *erm*C

(19.5%) but much lower prevalence of *erm*B (0.6%) genes among 851 *S. aureus* isolates (22). A more recent study on animal staphylococci showed a similar pattern with carriage rate of 55.6% *erm*A, 30.6% *erm*C and 11.1% *erm*B genes (23). These are similar to the findings in our study.

## Conclusion:

From this study, we can conclude that clindamycin resistance is common among *S. aureus* isolates in Mansoura Children University Hospital, Egypt both inducible and constitutive types carrying mostly *erm*A and *erm*C genes that may be partly responsible for these phenotypes. Although, we did not find statistically significant association of clindamycin with methicillin resistance, nevertheless, we recommend routine mandatory testing of all *S. aureus* isolates for inducible clindamycin resistance in this hospital.

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