ORIGINAL ARTICLE

Detection of Macrolide, Lincosamide and Streptogramin Resistance among Methicillin Resistant *Staphylococcus aureus* (MRSA) in Mumbai

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Abstract:

Background: The increase in incidence of Methicillin Resistant *Staphyloccocus aureus* (MRSA) and its extraordinary potential to develop antimicrobial resistance has highlighted the need for better agents to treat such infections. This has led to a renewed interest in use of new drugs for treatment with clindamycin and quinuprsitin-dalfopristin being the preferred choice for treatment. *Aim & Objectives*: This study was undertaken to detect the prevalence of Macrolide-Lincosamide-Streptogramin (MLS) resistance among clinical isolates of MRSA. *Material and Methods:* Two hundred and thirty clinical isolates of *S. aureus* were subjected to routine antibiotic susceptibility testing including cefoxitin, erythromycin and quinupristin-dalfopristin. Inducible resistance to clindamycin was tested by 'D' test as per Clinical and Laboratory Standards Institute (CLSI) guidelines. *Results:* Out of all *S. aureus* isolates, 93.91% were identified as MRSA. In the disc diffusion testing, 81.5% of isolates showed erythromycin resistance. Among these, the prevalence of constitutive ($cMLS_b$), inducible ($iMLS_b$) and MS-phenotype were 35.80%, 31.82% and 32.39% respectively by the D-test method. 77.8% of isolates were resistant to quinupristin-dalfopristin and the Minimum Inhibitory Concentration (MIC) ranged from 4–32 µg/ml. 89.20% of isolates were resistant to both quinupristin-dalfopristin and erythromycin of which 35.03%, 35.67% and 29.30% belonged to $iMLS_b$, $cMLS_b$ and MS phenotype respectively. *Conclusion:* The emergence of quinupristin-dalfopristin resistance and MLS_b phenotypes brings about the need for the simple and reliable D-test in routine diagnosis and further susceptibility testing for proper antimicrobial therapy.

Keywords: Clindamycin, D-test, Glycopeptides, Inducible Resistance, Quinupristin-dalfopristin

Introduction:

Antibiotics belonging to classes of Macrolide, Lincosamide and Streptogramin (MLS) are structurally unrelated; however, they have a similar mode of action. Erythromycin (ERY), a macrolide and clindamycin (CLI), a lincosamide represent two distinct classes of antimicrobial agents that inhibit protein synthesis by binding to the 50S ribosomal subunits of bacterial cells. Clindamycin is the most commonly used antibiotic to treat infections with Methicillin Resistant *Staphylococcus aureus* (MRSA). It is also the drug of choice for treating skin and soft tissue infections in patients allergic to penicillin [1].

In staphylococci, resistance to both of these antimicrobial agents has been observed [2]. The most common mechanism of Macrolide-Lincosamide-Streptogramin (MLS_b) resistance is by target-site modification typically mediated by products of ERY ribosome methylases (*erm*)

genes [3]. This resistance can either be constitutive ($cMLS_b$) or inducible ($iMLS_b$). In constitutive ($cMLS_b$) resistance the methylase enzyme is produced constitutively and hence the isolate is resistant to both ERY and CLI. In inducible ($iMLS_b$) resistance, the methylase enzyme is produced in presence of an inducer i.e. ERY and thus isolate are resistant to ERY but appear susceptible to CLI *in-vitro*. However, during the course of treatment with CLI, selection of *erm* mutants occurs which leads to development of resistance and subsequently to therapeutic failure [4]. On other hand, the *msrA* gene is responsible for active efflux of macrolides and streptogramin B antibiotics from bacterial cell, but has no effect on lincosamides. This is termed as MS phenotype which exhibits resistance to ERY and sensitivity to CLI *in-vitro* with successful treatment with CLI *in-vivo* [5].

Glycopeptides have been the drug of choice for the treatment of MRSA infections since a long time. However, emergence of reduced susceptibility to glycopeptides necessitated to scrutinize other treatment options such as quinupristin-dalfopristin (QD) and linezolid. Quinupristin-dalfopristin exhibits synergistic activity against most gram-positive bacteria and is approved globally for treatment of infections caused by vancomycin-resistant strains of *Enterococcus faecium*, MRSA, *Streptococcus pyogenes* and also in the treatment of nosocomial pneumonia [7]. Resistance to QD has a scarce worldwide with negligible rates reported in Europe, Latin America and North America. However, higher resistance rate (31%) has been reported in Taiwan even though this drug was not available for clinical use [8-10]. Although, this drug is not used clinically in India, varying resistance rates have been reported in the literature [6, 9, 11]. Hence, there is a need to monitor the prevalence of these resistant phenotypes which are widespread among MRSA strains for empiric therapy for MRSA infections [12].

Materials and Methods:

The present study was conducted from November 2013 to May 2014. A total of 230 clinical isolates of *S. aureus* from various clinical samples were included in the study. The source of clinical isolates were pus swabs, wound swabs, urine samples, tissues and blood samples. All the clinical isolates were identified for *S. aureus* on the basis of colony morphology, Gram staining, catalase test, tube coagulase and mannitol sugar fermentation.

Methicillin resistance was detected by the 30µg Cefoxitin disc diffusion test. Antibiotic resistance of 15µg ERY (Hi-Media Laboratories Pvt. Ltd., Mumbai) and 15µg QD (Hi-Media Laboratories Pvt. Ltd., Mumbai) was tested according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. The minimum inhibitory concentration (MIC) was carried out for QD resistant isolates by Epsilometer test (E-test) using HiMedia's Ezy MIC Strip (QD) (0.002-32µg/ml) [13]. The MIC interpretive criteria (µg/ml) for QD are $\leqslant$ 1 for susceptible, 2 for intermediate and $\geqslant$4 for resistance.

Erythromycin-resistant MRSA strains were selected for testing MLS_b by D-test as per CLSI guidelines [13]. A 0.5 McFarland suspension was prepared in normal saline for each isolate and inoculated on Mueller-Hinton agar (Hi-Media Laboratories Pvt. Ltd., Mumbai) plate. The D-test was performed by placing the 2µg CLI (Hi-Media Laboratories Pvt. Ltd., Mumbai) and 15µg ERY (Hi-Media Laboratories Pvt. Ltd., Mumbai) discs 15 mm apart edge to edge manually. The plates were incubated at 37°C for 24 hours. Three different phenotypes were observed after testing and then interpreted.

MS phenotype - isolates exhibiting resistance to ERY while sensitive to CLI and giving circular zone of inhibition around CLI have MS resistance.
$iMLS_b$ phenotype - Isolates showing resistance to ERY while being sensitive to CLI and giving a D-

shaped zone of inhibition around CLI with flattening toward ERY disc have $iMLS_b$ resistance. $cMLS_b$ phenotype - Isolates showing resistance to both ERY and CLI have $cMLS_b$ resistance. *S. aureus* ATCC 25923 was used as control for the disc diffusion tests. While *S. aureus* ATCC 29213 was used as a standard for MIC as recommended by CLSI guidelines.

Results:

During the study period, a total of 230 MRSA clinical isolates were collected and all 230 isolates were confirmed for *S. aureus* by microbiological methods. Further, the isolates were tested for methicillin resistant by cefoxitin disc diffusion test and 216 (93.91%) isolates were confirmed to be methicillin resistance (Fig.1).

In the disc diffusion testing, 176 (81.48%) isolates showed resistance to ERY while 40 (18.52%) isolates were sensitive to ERY. Among the 216 MRSA isolates, 168 (77.78%) were resistant, 1 (0.46%) was intermediate and 47 (21.76%) were sensitive to QD (Table 1).

The ERY resistant isolates were selected for further study. When the ERY resistant isolates were subjected to D test, 63 (35.80%) isolates showed $cMLS_b$ phenotype i.e. resistant to both ERY and CLI antibiotics. Out of the remaining 120 ERY resistant isolates, 56 (31.82%) isolates showed positive D-test indicating $iMLS_b$ phenotype, while 57 (32.39%) isolates showed true sensitivity to CLI i.e. D-test negative indicating MS phenotype (Fig.2).

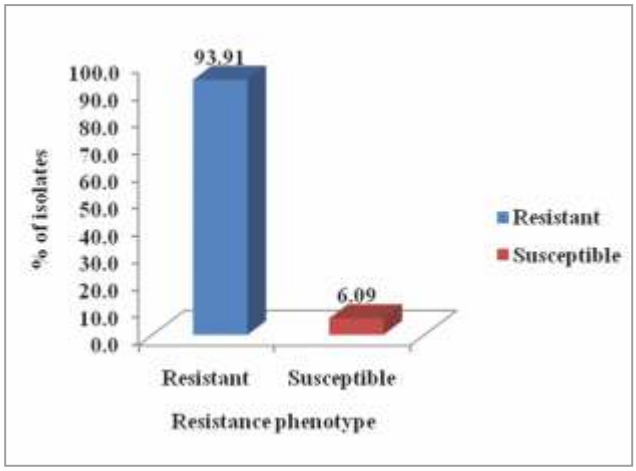

Fig.1: Percentage of Methicillin Resistance among *S. aureus* Isolates (N=230)

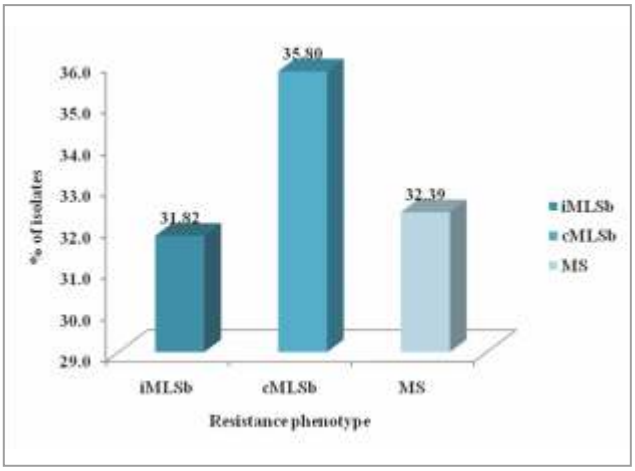

Fig.2: Result of D-Test for ERY-Resistant MRSA Isolates (N=176)

Table 1: Antibiotic Susceptibility Pattern against ERY and QD (N=216)

Antibiotic	% Resistant	% Intermediate	% Sensitive
ERY	81.48	0	18.52
QD	77.78	0.46	21.76

R = Resistant, I = Intermediate, S = Susceptible

The MIC range for QD resistant isolates was 4 – 32 µg/ml. A total of 147 (87.5%) isolates had MIC value of 32 µg/ml. The MIC_{50} and MIC_{90} were determined to be 32 µg/ml respectively. Out of 168 QD resistant isolates, 157 (89.20%) isolates were ERY resistant of which 55 (35.03%), 56 (35.67%) and 46 (29.30%) belonged to $iMLS_b$, $cMLS_b$ and MS phenotype respectively (Fig.3 and Fig.4). 11 (10.23%) isolates were ERY sensitive (Fig.3).

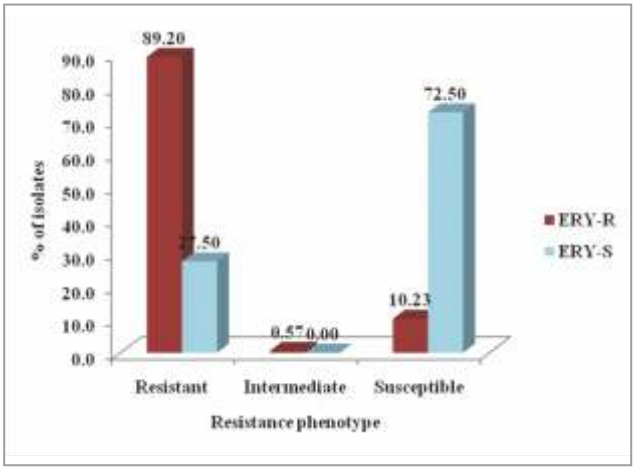

Fig.3: Distribution of QD Resistance against ERY Resistant MRSA Isolates (N=168)

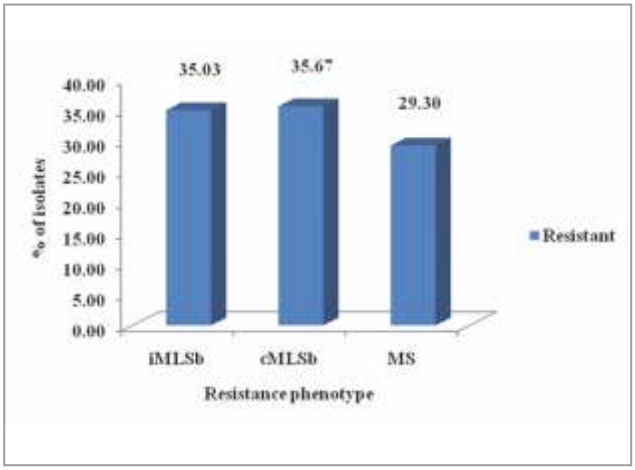

Fig.4: Distribution of MLS_b Phenotype against ERY Resistant MRSA Isolates with QD Resistance (N=157)

Discussion:

Macrolide-Lincosamide-Streptogramin (MLS_b) antibiotics can be used as alternative therapies for staphylococcal infections. These antibiotics have different structure, but similar mode of action. They inhibit bacterial protein synthesis by binding to 23S rRNA in 50S ribosomal subunits. However resistances to these drugs have already been observed. The main mechanisms responsible for MLS_b resistance in staphylococci are alterations in the 23S rRNA, encoded by ERY ribosome methylases (*erm*) genes, and an ATP-dependent efflux pump conferred by the *msr*(A) gene [14-15]. Methylation of the A2058 residue, located in the conserved domain V of 23S rRNA, takes place resulting in target-site modification and prevents the binding of MLS_b antibiotics to their ribosomal target. This phenomenon leads to cross-resistance to these antibiotics and produces the MLS_b phenotype, encoded by *erm* genes. Clindamycin is frequently used to treat skin and bone infections because of its tolerability, cost, oral form, and good tissue penetration [16]. However, resistance to ERY and CLI is increasing among clinical isolates of *S. aureus* worldwide [17].

Erythromycin resistance among MRSA isolates has been observed in various studies in India. In a study carried out by Indian Network for Surveillance of Antimicrobial Resistance (INSAR) group, India, the percentage of ERY-R was found to be 70.8% among a collection of 5864 MRSA isolates. However, the percentage of ERY-R in various regions varied from 28% to 67%. The percentage of ERY-R has been varying in different studies with Fomda *et al* reporting 94%, Pai *et al* reporting 80%, Arora *et al* reporting 61.7%, Rajaduraipandi *et al* reporting 60%, Saikia *et al* reporting 55.43%, Alvarez *et al* reporting 52.7% and Vysakh *et al* reporting 50% [18-22]. In the present study, the ERY-R has been observed in 176 out of 230 isolates which accounts to 81.5%.

Even though CLI remains a good alternative option for treating MRSA infections, due to its widespread use, resistance has been reported in the recent years with different mechanisms. Therefore, it is important to detect the type of resistance [1, 23]. Reporting *S. aureus* as susceptible to CLI without checking for inducible resistance may result in institution of inappropriate CLI therapy. On the other hand negative result for $iMLS_b$ resistance confirms CLI susceptibility and provides a very good therapeutic option [24].

There have been varying reports on the pattern of $iMLS_b$ resistance in staphylococci. Different regions show different pattern of resistance. Various studies carried out in India have reported higher percentage of $iMLS_b$ resistance as against $cMLS_b$ resistance [1, 25, 26]. In other studies higher percentage of $cMLS_b$ resistance has been reported [27-31].

In our study 31.82% and 35.80% of isolates tested positive for $iMLS_b$ and $cMLS_b$ phenotype respectively while 32.39% of isolates showed true sensitivity to CLI (MS phenotype). According to CLSI guidelines (2014), isolates with $iMLS_b$ phenotype can be reported as CLI-R. Thus the total population of CLI-R sums to about 67.61%. Our findings are in consistent with previous studies for both high percentages of $iMLS_b$ and $cMLS_b$ phenotypes. Hence, it is dangerous to use CLI when ERY testing shows a resistant phenotype. Routine D-testing might allow clinicians to retain confidence in CLI when ERY resistance is present [16]. The findings in this study suggest that the pattern of MLS_b phenotype differs across various geographical locations. The reason for such pattern could be to hospital environment, patient age, clinical samples and the antibiotic susceptibility profile of the bacteria [18]. This is where the D-test becomes significant.

Quinupristin-dalfopristin is a combination of streptogramin B i.e. quinupristin and streptogramin A i.e. dalfopristin in 30:70 ratio. These compounds are semisynthetic derivatives of naturally occurring pristinamycins, produced by *Streptomyces pristinaspiralis*. This is a novel drug for treatment of MRSA infections as emergence of intermediate susceptibility to glycopeptides has been reported [11, 32].

In our study, the resistance rate against this novel drug was determined to be 77.8%. The finding of this study is alarming and novel too as this is the first report of such high resistance rates in Mumbai. Our findings correlates with the findings of two studies carried out in India with resistance rates reported to be 79% and 87% respectively. However, in another study the findings were low to about 17.64% only [6, 9]. Kesari *et al* reported two cases of QD resistant MRSA, where disc diffusion results correlated well with MIC assays [11].

The findings of QD resistant MRSA suggest the presence of resistant genes for the components, Streptogramin A and B. When the 23S rRNA is methylated, resistance of the B component is achieved and confers MLS_b resistance with constitutive expression of the *erm* genes. This correlates with the findings as majority of $cMLS_b$ resistant isolates were also QD resistant in this study. This can be confirmed by carrying out molecular assays targeting the streptogramin resistance genes of component B i.e. streptogram in B resistance determinants *vgbA* and *vgbB* [33, 34].

Resistance of component A is mediated by two kinds of mechanisms. The first mechanism is by production of three acetyltransferase genes, *vatA*, *vatB*, and *vatC* which brings about acetylation of the component A. The second mechanism is mediated by efflux pumps (ABC porters), *vgaA* and *vgaB*. These resistance genes for

streptogramin A and B are often located on the same plasmids. However, few ERY sensitive isolates were QD resistant which indicates presence of other resistance mechanisms thus conferring streptogramin resistance which needs to be investigated [35].

In India, QD is used only as a research tool by procuring antibiotic discs from a commercial source. This drug is not available for *in vivo* patient management. However, the high resistance rates of QD resistance in MRSA brings out the need for more comprehensive region-wise laboratory work, before advocating this drug for treatment of multidrug–resistance MRSA infections [6, 11]. Thus, the emergence of the resistance to multiple antibiotics has left limited options for the clinicians. Therefore for an appropriate therapeutic decision, surveillance on the antimicrobial susceptibility patterns is important to understand new patterns in antibiotic resistance.

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