Incidence of clindamycin resistance among clinical isolates of Staphylococci in a tertiary care centre of Manipur, India
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Article History
Received: 12/09/2019
Accepted: 20/09/2019
Available online: 28/10/2019

Abstract:
Introduction: Therapeutic failure of clindamycin against both methicillin resistant and sensitive staphylococcal isolates has been documented as due to multiple mechanisms that confer resistance to macrolide, lincosamide and streptogramin B (MLSB) antibiotics which can be inducible or constitutive. Routine in-vitro antimicrobial susceptibility testing for clindamycin may fail to detect such inducible clindamycin resistance, thus necessitating the need to detect such isolates by employing a simple double disk approximation or D-test. The study was taken up with the objective of determining the incidence of inducible clindamycin resistance among staphylococcal isolates using D test.

Materials & Methods: Isolation, identification and antibiotic susceptibility testing of 751 S. aureus and 501 CONS clinical isolates obtained from January 2012 to December 2015 were performed using conventional standard protocol as per CLSI guidelines. Erythromycin resistant and clindamycin sensitive isolates were further subjected to D test for detecting inducible clindamycin resistance phenotype.
Results: A total of 385 isolates were found to be resistant to erythromycin and sensitive to clindamycin. 244 (19.49%) isolates were demonstrated as inducible clindamycin resistance (iMLSB) by the D test.

Conclusion: The study showed the presence of iMLSB resistance among staphylococcal isolates in our tertiary care hospital. Clinical microbiology laboratories should use the double-disc diffusion test (D test) as standard practice with all erythromycin resistant staphylococci to avoid treatment failures of clindamycin.

Key words: D-test, Inducible clindamycin resistance, staphylococci


Introduction:

Staphylococcus aureus and coagulase-negative staphylococci (CoNS) are increasingly reported for causing nosocomial and community-acquired infections worldwide. The increasing occurrence of methicillin resistance among staphylococci is another big problem. [1] The macrolide-lincosamide-streptogramin B (MLSB) family of antibiotics is commonly used in the treatment of staphylococcal infections. [2] These antibiotics are chemically distinct, but have similar inhibitory effects on bacterial protein synthesis. Clindamycin, a derivative of lincomycin, is a useful antimicrobial for the treatment of skin, soft tissue or bone infection caused by Staphylococcus spp. especially methicillin resistant S. aureus (MRSA) because of its excellent pharmacokinetic properties like good excellent tissue penetration except central nervous system, oral absorption and no need of renal dosing adjustment. [3] However, the widespread use of this antibiotic has been accompanied by increased numbers of resistant strains among staphylococci.

The MLSB family of antibiotics has three different mechanisms of resistance: target site modification, enzymic antibiotic inactivation and macrolide efflux pumps. [4] Target site modification is the most common mechanism of acquired resistance to macrolides, lincosamides, and streptogramin B (MLS) antibiotics in staphylococci and confers cross-resistance to the MLS antibiotics (the so-called MLSB phenotype). [5] Macrolide resistance due to ribosomal target modification affects the activities of both macrolides and clindamycin; it is mediated by erythromycin ribosomal methylases encoded by ermA/ermC genes. Such resistance may be inducible or constitutive. [6,7] Macrolide resistance due to active efflux is encoded by the macrolide-streptogramin resistance (msrA) gene in Staphylococcus spp. This energy-dependent pump effectively expels macrolides from the bacterial cell before they can bind to their target site on the ribosome. [8] It results in
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resistance to macrolides and streptogramin B antibiotics, but not to lincosamides (MS phenotype). Clindamycin is active against such isolates and there is no risk of therapy failure in such cases.[7]

Inducible MLSB resistance cannot be determined using standard antimicrobial susceptibility testing methods, including standard disk diffusion test, Etest, automated susceptibility-testing platforms, standard broth-based or agar dilution susceptibility tests.[2,6] In routine antimicrobial susceptibility tests, strains with inducible clindamycin resistance appear as erythromycin resistant and clindamycin susceptible. Strains expressing Inducible MLSB resistance can be detected by a simple test, known as Disk approximation test or D-zone test where a flattened inhibition zone around the clindamycin disk (positive ‘D-zone test’) when erythromycin and clindamycin disks are placed close to each other. [9]

Although the occurrence of inducible MLSB has been documented worldwide and other parts of India, there is paucity of data regarding this problem in the whole north-east India and no studies have been initiated to determine the magnitude of this antimicrobial resistance pattern so far in Manipur. Hence, the present study was undertaken to assess the incidence of inducible clindamycin resistance among Staphylococcus spp. using D test in our region.

Materials and methods:

A total of 751 S. aureus and 501 CoNS isolates from clinical specimens such as pus, wound swab, aural swab, blood, urine, sputum and central venous line catheter obtained during the period from January 2012 to December 2015 in the department of microbiology at a tertiary care hospital of Manipur, India were included in this cross-sectional study. All isolates were identified morphologically and biochemically by adopting standard laboratory procedures (10). The isolates were subjected to antimicrobial susceptibility testing determined by Kirby-Bauer disk diffusion method on Mueller Hinton agar plate, using commercially available 6mm disks (HIMEDIA,Mumbai,India) cefoxitin (30μg), Ampicillin (10 μg), Penicillin G (10 units), Cotrimoxazole(1.25/23.75μg), Ciprofloxacin (5 μg), Vancomycin (30μg), Erythromycin (15 μg), amikacin (30μg), Clindamycin (2 μg), Linezolid(30μg), teicoplanin(30μg) and tigecycline (15μg) following CLSI guidelines.

Methicillin resistance was identified by disk diffusion using cefoxitin (30μg). Diameter of the circular zone of inhibition ≥ 22mm (S. aureus) or ≥25mm (coagulase negative staphylococci) was interpreted as sensitive and ≤21mm (S. aureus) or ≤24mm (coagulase negative staphylococci) as resistant according to CLSI guidelines.[10]
The isolates that turned out to be erythromycin resistant (zone inhibition size ≤ 13mm) were further subjected to the double disk approximation test (D-test) as per CLSI guidelines for inducible clindamycin resistance. Herein, 0.5 McFarland’s standard suspension of organisms was plated onto an MHA plate. An erythromycin disk (15 μg) and a clindamycin disk (2 μg) were placed 15 mm apart edge-to-edge on the MHA plate. Plates were analyzed after 18 hours of incubation at 35°C. Flattening of the zone of inhibition adjacent to the erythromycin disk (referred to as a D-zone) or hazy growth within the zone of inhibition around clindamycin, even if no D-zone is apparent, was interpreted as inducible clindamycin resistance (iMLSB).[^11]

Isolates resistant to erythromycin (zone size ≤ 13mm) but sensitive to clindamycin (zone size ≥ 21mm) with a circular zone of inhibition around clindamycin was interpreted as MS phenotype.[^12]

Constitutive MLSB phenotype was considered when the isolates were resistant to both erythromycin (zone size ≤ 13mm) and clindamycin (zone size ≤ 14mm).[^12]

**Quality control:** Quality control of the erythromycin and clindamycin discs was performed with ATCC *Staphylococcus aureus* 25923.

**Statistical analysis:** Descriptive statistics like percentage and proportion were used to present the data. Bivariate analysis was done and the results were interpreted in terms of odds ratio with confidence interval of 95%. Analysis was done using Epi Info 7. $P < 0.05$ was considered significant.

**Results:** Of the total 1252 staphylococcal isolates (751 *S. aureus* and 501 coagulase negative staphylococci) included in the study, 382 isolates were methicillin resistant *Staphylococcus aureus* (MRSA), 369 were methicillin sensitive *Staphylococcus aureus* (MSSA), 269 were methicillin resistant coagulase negative staphylococci (MRCoNS) and 232 were methicillin sensitive coagulase negative *staphylococci* (MSCoNS). Among these, 607 staphylococcal isolates (48.48%) were found to be resistant to erythromycin. Positive D test or inducible MLSB was seen in 244 (19.49%) isolates and 141 (11.26%) isolates had MS phenotype (Fig.1 & 2). 222 (17.73%) isolates were resistant to both erythromycin and clindamycin determining cMLSB phenotype. Among MRSA isolates, constitutive resistance was found to be predominated over inducible and MS phenotypes. However, inducible resistance was more prevalent among MSSA, MRCoNS and MSCoNS isolates in compared to constitutive and MS phenotypes. (Table 1)

**Table 1: Distribution of MLSB resistance phenotypes among staphylococcal isolates**

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>MRSA (%)</th>
<th>MSSA (%)</th>
<th>MRCoNS (%)</th>
<th>MSCoNS (%)</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-S,CL-S</td>
<td>134(35.1)</td>
<td>285(77.2)</td>
<td>85(31.6)</td>
<td>141(60.8)</td>
<td>645(51.52)</td>
</tr>
<tr>
<td>E-R,CL-R</td>
<td>126(32.9)</td>
<td>33(8.9)</td>
<td>36(13.4)</td>
<td>27(11.6)</td>
<td>222(17.73)</td>
</tr>
</tbody>
</table>

[^11]: Author's citation
[^12]: Author's citation

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Inducible MLSB resistance phenotype levels among *S. aureus* and coagulase negative staphylococci (CoNS) isolates were 17.3% and 22.7% respectively. When the *S. aureus* and CoNS strains among all staphylococcal isolates were statistically compared, inducible clindamycin resistance and MS phenotype were determined to be respectively 29% and 76% more positive in CoNS strains than that in *S. aureus* isolates, and constitutive phenotype (cMLSB) was found to be 1.86 times more positive in *S. aureus* (Table 2).

### Table 2: Comparison of *S. aureus* and CNS against MLSB resistance phenotypes

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th><em>S. aureus</em> (%)</th>
<th>CONS (%)</th>
<th>P value</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-S,CL-S</td>
<td>419(55.8)</td>
<td>226(45.1)</td>
<td>0.0002</td>
<td>1.5357</td>
<td>1.2233-1.9278</td>
</tr>
<tr>
<td>E-R,CL-R</td>
<td>159(21.2)</td>
<td>63(12.6)</td>
<td>0.0000</td>
<td>1.8673</td>
<td>1.3602-2.5634</td>
</tr>
<tr>
<td>E-R, CL-S, D test +ve</td>
<td>130(17.3)</td>
<td>114(22.7)</td>
<td>0.0172</td>
<td>0.7107</td>
<td>0.5362-0.9419</td>
</tr>
<tr>
<td>E-R, CL-S, D test -ve</td>
<td>43(5.7)</td>
<td>98(19.6)</td>
<td>0.0000</td>
<td>0.2498</td>
<td>0.1710-0.3648</td>
</tr>
<tr>
<td>Total</td>
<td>751</td>
<td>501</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

E= erythromycin, CL= Clindamycin, CNS= Coagulase negative staphylococci, OR=Odds ratio, CI= confidence interval

When MRSA and MSSA strains among *S. aureus* were compared, inducible clindamycin resistance, clindamycin resistance and the MS phenotype were determined to be 2.64, 5.01 and 2.62 times more likely, respectively, in MRSA against MSSA isolates (Table 3).

### Table 3: Comparison of MRSA and MSSA against MLSB resistance phenotypes

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>MRSA (%)</th>
<th>MSSA (%)</th>
<th>P value</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-S,CL-S</td>
<td>134(35.1)</td>
<td>285(77.2)</td>
<td>0.0000</td>
<td>0.1593</td>
<td>0.1155-0.2196</td>
</tr>
<tr>
<td>E-R,CL-R</td>
<td>126(32.9)</td>
<td>33(8.9)</td>
<td>0.0000</td>
<td>5.0114</td>
<td>3.3048-7.5993</td>
</tr>
<tr>
<td>E-R, CL-S, D test +ve</td>
<td>91(23.8)</td>
<td>39(10.6)</td>
<td>0.0000</td>
<td>2.6460</td>
<td>1.7615-3.9747</td>
</tr>
<tr>
<td>E-R, CL-S, D test -ve</td>
<td>31(8.2)</td>
<td>12(3.3)</td>
<td>0.0041</td>
<td>2.6275</td>
<td>1.3279-5.1992</td>
</tr>
</tbody>
</table>
Comparison of MRCoNS and MSCoNS among CoNS isolates demonstrated that inducible clindamycin resistance, clindamycin resistance and the MS phenotype were determined to be 2.34, 1.7 and 2.41 times more likely, respectively, in MRCoNS against MSCoNS isolates (Table 4).

Table 4: Comparison of MRCoNS and MSCoNS against MLSB resistance phenotypes

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>MRCoNS (%)</th>
<th>MSCoNS (%)</th>
<th>p value</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-S, CL-S</td>
<td>85(31.6)</td>
<td>141(60.8)</td>
<td>0.0000</td>
<td>0.2981</td>
<td>0.2063-0.4308</td>
</tr>
<tr>
<td>E-R, CL-R</td>
<td>56(13.4)</td>
<td>27(11.6)</td>
<td>0.5569</td>
<td>1.1731</td>
<td>0.6883-1.9993</td>
</tr>
<tr>
<td>E-R, CL-S, D test +ve</td>
<td>79(29.4)</td>
<td>35(15.1)</td>
<td>0.0001</td>
<td>2.3403</td>
<td>1.4996-3.6524</td>
</tr>
<tr>
<td>E-R, CL-S, D test -ve</td>
<td>69(25.6)</td>
<td>29(12.5)</td>
<td>0.0002</td>
<td>2.4150</td>
<td>1.5008-3.8860</td>
</tr>
<tr>
<td>Total</td>
<td>269</td>
<td>232</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Discussion:

Clindamycin has become an important antimicrobial for the treatment of both methicillin susceptible and resistant staphylococcal infections as well as an alternative in penicillin-allergic patients. However, development of resistance to MLSB antibiotics with regard to clindamycin for staphylococcal infections is a big issue. Standard disk diffusion, Etest and broth microdilution fail to detect inducible clindamycin resistance. D-test becomes an imperative part of routine antimicrobial susceptibility test for all clinical staphylococcal isolates to identify clindamycin-resistant and truly clindamycin-sensitive isolates. Clinical and Laboratory Standards Institute (CLSI) has recommended the double disk diffusion or D-test to detect inducible clindamycin resistance on routine basis.

In the present study, the overall incidence of inducible MLSB resistance phenotype among the staphylococcal isolates was 19.49% (17.3% of S. aureus and 22.7% of CoNS). Reports on the occurrence of MLSB resistance pattern among Staphylococcus spp. showed wide region wise variations. In a study conducted by Van der Heijden et al. in Brazil, 11.3% of S. aureus and 13.7% of CNS isolates were found to possess MLSB resistance phenotype. Fiebelkorn et al demonstrated inducible clindamycin resistance in 29% of S. aureus and 30% of CoNS isolates. Yilmaz et al. reported the figures of 24.4% among MRSA isolates, 14.8% among MSSA isolates,
25.7% among MRCoNS isolates and 19.9% among MSCoNS isolates for inducible clindamycin resistance. \cite{4}

Levin \textit{et al.} (27.5%) reported high incidence pattern for inducible clindamycin resistance phenotype among \textit{S. aureus} while Azap \textit{et al.} (15-30%) and Schreckenberger \textit{et al.} (14-35%) obtained high figures for methicillin resistant and sensitive CoNS isolates. \cite{15,16,17} In Indian subcontinent, Deotale \textit{et al.}, Upadhya \textit{et al.}, Ajantha \textit{et al.}, Gadepalli \textit{et al.} and Banik \textit{et al.} reported an incidence of 14.5%, 20.2%, 7.2%, 21% and 10.4% respectively for the inducible resistance phenotype among \textit{S. aureus}. \cite{18,19,20,21,22}

Constitutive clindamycin resistance (cMLSb) in our study was found in 17.73% of all staphylococcal isolates, which was in concordance with the findings of Banik \textit{et al.} (16.88%), Juyal \textit{et al.} (14.64%) and Gade \textit{et al.} (12.4%). \cite{22,24} However, Gadepalli \textit{et al.} (26.5%), Yilmaz \textit{et al.} (28.3%), Azap \textit{et al.} (33.3%), and Schreckenberger \textit{et al.} (38.14%) documented higher occurrence of cMLSb. \cite{4,16,17,21}

Our study demonstrated clindamycin-sensitive strains (MS phenotype) in 11.26% of all isolates tested. This was correlated with the findings of Yilmaz \textit{et al.} (9%), Banik \textit{et al.} (11.52%), Gadepalli \textit{et al.} (12%) and Gade \textit{et al.} (12%). \cite{4,22,21,23} This fact implies that clindamycin can be safely and effectively employed as a therapeutic drug in such clinical scenarios despite macrolide resistance. Hence, labelling all erythromycin resistant staphylococci as clindamycin resistant or not reporting clindamycin resistant when erythromycin resistant is present will likely prevent the use of clindamycin in treating infections that would likely respond to clindamycin therapy.

In the present study, the three resistance phenotypes were found more often in methicillin resistant staphylococcal strains compared to methicillin sensitive strains. High occurrences of inducible resistance, constitutive resistance and MS phenotypes were observed among MRSA (23.8%, 32.9%, 8.2% respectively) and MRCONS (29.4%, 13.4%, 25.6% respectively) as compared to MSSA (10.6%, 8.9%, 3.3%) and MSCoNS (15.1%, 11.6%, 12.5%) isolates. Similar results were reported by Yilmaz \textit{et al.} and Azap \textit{et al.} \cite{4,16} However, Levin \textit{et al.}, Schreckenberger \textit{et al.} and Banik \textit{et al.} demonstrated higher percentages of inducible and constitutive clindamycin resistance in MSSA isolates compared to MRSA isolates. \cite{15,17,22}

In our study, vancomycin, teicoplanin and linezolid turned out to be the most effective antimicrobials against the erythromycin resistant isolates followed by tigecycline. Sasirekha \textit{et al.}, Nikam \textit{et al.} and Rahbar \textit{et al.} reported that all the erythromycin resistant isolates were found susceptible to vancomycin and linezolid. \cite{25,26,27}

Of the total 607 isolates of staphylococci with erythromycin resistant, a high percentage of 40.20% (244/607) isolates turned out to be inducible MLS\textsubscript{B} resistance by the D test. Our study demonstrated that if the D-
test had not been performed, almost half of the erythromycin-resistant isolates would have been identified incorrectly as clindamycin sensitive, leading to therapeutic failure. The double-disk diffusion or D test is needed to distinguish inducible clindamycin resistance from true clindamycin-sensitive strains among staphylococcal isolates with erythromycin resistant.

It is important for laboratories to be aware of the local prevalence data of inducible clindamycin resistance so that decisions can be made whether to perform the D-test routinely or whether to report all erythromycin-resistant Staphylococcus spp. isolates as also being clindamycin resistant. This prevalence may change over time with the emergence of strains with different sensitivity patterns, so periodic surveys should be performed if testing is not routine. [28]

A number of authors have suggested that a range of distances up to 28 mm may be used for performance of the D-test, and the CLSI states that edge-to-edge distances of 15 to 26 mm may be used. [10] The greater distance is more convenient since automated disk dispensers generally place disks 20 to 26 mm apart. For our study, we opted the minimum distance recommended by the CLSI (15 mm) since there is conflicting opinion about the accuracy of the D-test when distances above 20 mm are used. [2,29,30]

Clinical microbiology laboratories should employ the double-disc diffusion or D test as standard practice in all erythromycin resistant staphylococci as it is simple, easy to perform, interpret and reliable method of detecting inducible resistance to clindamycin. Furthermore, in applying the susceptibility test to staphylococcal isolates, clinical microbiology laboratories should place the erythromycin disk 15 mm apart from the clindamycin disk. Early detection of inducible MLSB resistance will certainly help the clinician to initiate appropriate antimicrobials at the right time and therapeutic failures using clindamycin unnecessarily can thus be avoided.
Fig 1: Positive D test showing blunting of clindamycin disk (iMLSB).

Fig 2: Negative D test (MS phenotype)

References:


