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Original Article

Detection of various types of resistance patterns and their correlation with minimal inhibitory concentrations against clindamycin among methicillin-resistant *Staphylococcus aureus* isolates

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Abstract

Purpose: The macrolide lincosamide streptogramin B (MLS_B) family of antibiotics serves as an alternative for the treatment of skin and soft tissue infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA). However, resistance to clindamycin too has emerged, which is of two types, inducible and constitutive. Therapeutic failure is common with inducible type of clindamycin resistance. This study was done to determine the various clindamycin resistance patterns in MRSA isolates and to compare them with minimal inhibitory concentration (MIC) of clindamycin. **Materials and Methods:** Fifty MRSA isolates were studied by disc approximation test (D test) to detect inducible $iMLS_B$ resistance and MIC by agar dilution technique. **Results:** Of the 50 isolates, 34 were sensitive to both clindamycin and erythromycin. 16 isolates showed different sensitivity patterns; nine of these were positive for D zone indicating inducible $iMLS_B$ resistance, five were positive for constitutive MLS_B resistance and two showed possible efflux mechanism for macrolide resistance. Out of the 34 sensitive isolates, 5 showed isolated colonies (subpopulation) inside the clindamycin-sensitive zone. When these sub-populations were tested further, two were constitutive MLS_B phenotypes, two were inducible $iMLS_B$ and one was HD (hazy D zone), which is D^+ with growth up to clindamycin disc (which is also considered as constitutive MLS_B phenotype). Seven isolates showed an MIC of ≥4 μg/ml to clindamycin in spite of being susceptible to both erythromycin and clindamycin by Kirby Bauer disc diffusion technique. Out of these seven isolates, five were those which grew as subpopulation inside the clindamycin-sensitive zone. **Conclusion:** Detection of $iMLS_B$ resistance among MRSA helps to avoid treatment failure with clindamycin. Studying the subpopulation inside the clindamycin-sensitive zone raises the question of existence of hetero-resistance or some other mechanism, which needs further study.

Key words: *D test, inducible clindamycin resistance, methicillin-resistant Staphylococcus aureus,minimal inhibitory concentration*

Introduction

Methicillin resistant *Staphylococcus aureus* (MRSA) has been recognized as an important nosocomial pathogen worldwide and such MRSA strains are known to be multidrug resistant. The macrolide lincosamide streptogramin B (MLS_B) family of antibiotics serves as an alternative therapeutic agent, with clindamycin being the preferred agent due to its excellent pharmacokinetic properties and good penetration into various tissues including bones, except cerebrospinal fluid.[1] However, widespread use of MLS_B antibiotics has led to an increase in the number of staphylococcal strains acquiring resistance to MLS_B antibiotics as well.[2] Common mechanism of resistance to MLS_B by staphylococcal strains is of three types. The first mechanism is target site modification by *erm* gene, which results in rRNA methylase production that can be either constitutive (constitutive MLS_B) or inducible ($iMLS_B$ phenotypes) where methylase is produced only in the presence of an inducer like erythromycin. The second mechanism of resistance is by efflux of antibiotic by *mrs A* gene (MS phenotype) and the third mechanism is by inactivation of lincosamides by chemical alteration mediated by the *inu A* gene, which appears to be rare.[3,4]

When tested *in vitro,* constitutively expressed MLS_B phenotypes are found to be resistant to both erythromycin and clindamycin. Inducible phenotypes ($iMLS_B$) are resistant to erythromycin and sensitive to clindamycin in the absence of an inducer. These $iMLS_B$ phenotypes, when tested in the presence of an inducer (erythromycin), show D shape zone of inhibition indicating clindamycin resistance.

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In contrast, MS phenotypes are resistant to erythromycin and sensitive to clindamycin without D zone, indicating efflux of macrolide antibiotic.[1]

When testing *in vitro,* if not looked for inducible type of resistance, clindamycin may appear to be sensitive in both $iMLS_B$ and MS phenotypes. In such cases, *in vivo* therapy with clindamycin may select constitutive *erm* mutants in $iMLS_B$ phenotype, leading to clinical therapeutic failure, which is not so in MS phenotype.[5,6] So, *in vitro* induction test (D test) is useful in distinguishing staphylococci that have inducible *erm* mediated resistance from those with efflux pump mediated resistance.

Thus, strains which are clindamycin sensitive may have two properties. One is exhibiting sensitive results in spite of possessing $iMLS_B$ gene and the other is exhibiting sensitive result in spite of possessing efflux mechanism which is reported only for erythromycin among the MLS_B group of antibiotics. Infections due to $iMLS_B$ strains are likely to fail to respond to clindamycin therapy since the methylase enzyme secretion gets activated which results in inactivation of the drug. On the other hand, infections due to MS type strains do not lead to failure in therapy. Hence, it becomes important to differentiate the $iMLS_B$ strains and MS phenotypes strains.[7,8]

Materials and Methods

A total of 50 MRSA isolates from skin and soft tissue infections, over a period of 9 months, from both inpatients and outpatients were included in the study. MRSA screening was done as per the CLSI guidelines by Kirby Bauer disc diffusion technique using oxacillin disc (1 μg).[9]

Erythromycin and clindamycin disc approximation test (D-test)

The isolates were subjected initially to "D test" as per CLSI guidelines.[10] The test was done by placing clindamycin disc (2 μg) and erythromycin disc (15 μg) (BD BBL™,USA) at a distance of 20 mm (edge to edge) on Mueller Hinton agar plate inoculated with 0.5 McFarland suspension of MRSA isolate. These plates were incubated at 37°C for 24 hours. A flattening of the zone of inhibition around clindamycin disc proximal to erythromycin disc (producing a zone of inhibition shaped like the letter D) was looked for, which was designated as D test positive, indicating inducible clindamycin resistance.[6]

Determination of minimum inhibitory concentration of clindamycin by agar dilution method

Minimum inhibitory concentration (MIC) to clindamycin (commercially available drug vial) was determined by agar dilution technique as per CLSI guidelines. The various concentrations of clindamycin tested were 0.25 to 8 μg/ml. An MIC of ≤0.5 μg/ml was considered as sensitive and MIC of ≥4 μg/ml was taken as resistant.[10]

Results

Different phenotypes were noticed among the strains tested. Induction phenotypes are the ones where D zone was positive. Induction phenotypes were further divided into D with clear zone of D around clindamycin disc [Figure 1] and D^+ where small colonies grew towards the clindamycin disc inside the D zone [Figure 2].

Non-induction phenotypes were D zone negative and are further divided into four types: First, as MS phenotype (erythromycin resistant and clindamycin sensitive without any D zone); secondly, as HD phenotype (hazy D zone), with two zones of growth around clindamycin disc, one zone is a light, hazy growth up to clindamycin disc and the second zone is heavy growth and showing "D" [Figure 3]; thirdly, as R phenotype, which is resistant to both clindamycin and erythromycin and fourthly, as S phenotype, which is sensitive to both clindamycin and erythromycin.

Out of the 50 isolates, 34 were S phenotypes, 5 were R (constitutive MLS_B) and 11 were susceptible to clindamycin and resistant to erythromycin. Out of these 11 isolates, 9 were D test positive ($iMLS_B$) and 2 were MS (efflux) phenotype. We did not find any D^+ or HD phenotype in these 11 isolates [Table 1].

Of the above 34 sensitive phenotypes, five isolates showed subpopulations inside the clindamycin zone of inhibition [Figure 4]. These subpopulations were subcultured and further tested for various induction and non-induction phenotypes. Interestingly, out of these five, two were found to be R phenotypes, one was D phenotype, one was D^+ phenotype and one was HD phenotype [Table 2].

MIC to clindamycin was detected using agar dilution technique. Of the 50 isolates tested, the 5 R phenotype strains showed a very high MIC value of >128 μg/ml. The two isolates with MS phenotype, which were resistant to erythromycin and sensitive to clindamycin without D zone (efflux), also had an MIC of >128 μg/ml. Of the nine isolates with D test positive phenotype, three had an MIC of ≥4 μg/ml and the remaining five isolates were in the

Table 1: Susceptibility pattern of the clinical MRSA isolates to erythromycin and clindamycin

No of strains	S phenotype	R phenotype	Resistant to erythromycin and sensitive to clindamycin	
50	34	05	11	
			D phenotype 09	MS phenotype 02

S - Sensitive to both erythromycin and clindamycin, R - Resistant to both erythromycin and clindamycin, D - D zone positive; MS - Resistant to erythromycin and sensitive to clindamycin without D zone, MRSA: Methicillin-resistant Staphylococcus aureus

Figure 1: D phenotype showing D-shaped zone of inhibition around clindamycin disc

Figure 2: D^+ phenotype with small colonies growing towards the clindamycin disc inside the D zone

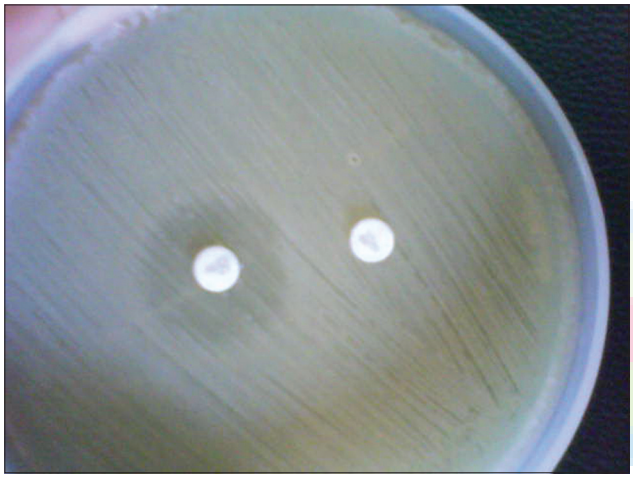

Figure 3: HD phenotype, with two zones of growth around clindamycin disc

Figure 4: Subpopulation inside the clindamycin-sensitive zone

Table 2: Different types of resistance patterns and their MIC values shown by subpopulation (growing in zone of inhibition around clindamycin) of five MRSA clinical isolates

Phenotype	No of strains	Sensitivity to erythromycin	Sensitivity to clindamycin	MIC to clindamycin (µg/ml)
R	2	R	R	>128
D	1	R	S	>128
D^+	1	R	S	>128
HD	1	R	S	>128

R - Resistant to both erythromycin and clindamycin; D - D zone positive; D^+ - Small colonies growing towards the clindamycin disc inside the D zone; HD - Two zones of growth around clindamycin disc, MIC: Minimal inhibitory concentration; MRSA: Methicillin-resistant Staphylococcus aureus

susceptible range. Of the remaining 34 isolates, 27 were in the susceptible range and 7 were resistant showing an MIC of >128 µg/ml [Table 3].

Table 3: Correlation between various phenotypic patterns of clinical MRSA isolates and their MIC values against clindamycin

No of strains	Phenotype	Resistant mechanism	MIC in mg/ml to clindamycin
34	S type (sensitive to both erythromycin and clindamycin)	-	27 isolates <2 07 isolates ≥4
05	R type (resistant to both erythromycin and clindamycin)	Constitutive (MLS_B)	>128
09	D positive (resistant to erythromycin and sensitive to clindamycin, with D zone)	$iMLS_B$	6 isolates <2 3 isolates ≥4
02	MS (resistant to erythromycin and sensitive to clindamycin, without D zone)	Efflux	>128

Discussion

Macrolides, lincosamide and streptogramin B (MLS_B) belong to a distinct class of antimicrobial agents that inhibit protein synthesis by binding to the 50S ribosomal subunits of bacterial cells. In staphylococci, resistance to these antimicrobial agents can occur by different mechanisms.

The first type of mechanism is methylation of the ribosomal target site. Resistance to MLS_B antibiotics most commonly results from acquisition of erythromycin-resistant methylase genes (*erm* gene-erythromycin ribosome methylation) which encode enzymes (methylases) that add one or two methyl groups (methylate) to a specific adenine residue (A2050) in the 23S rRNA within the 50S ribosomal subunit. The overlapping binding sites of MLS_B in 23S rRNA account for cross resistance to the three classes of drugs. The genes conferring MLS_B resistance typically found in *S.aureus* are *erm*(A), *erm*(B), *erm*(C) and *erm*(Y), which are usually plasmid mediated. Expression of MLS_B resistance can be inducible or constitutive. In inducible resistance, the bacteria produce inactive mRNA that is unable to encode methylases. The mRNA becomes active only in the presence of a macrolide inducer. Of all the macrolides, erythromycin is an effective inducer. By contrast, in constitutive expression, active methylated mRNA is produced even in the absence of an inducer.[5,11] The strains harboring an inducible *erm* gene are resistant to the inducer but remain susceptible to the non inducer macrolide and lincosamide; but the use of non inducer antibiotics such as clindamycin, can lead to selection of constitutive mutants at frequencies of 10^7 cfu.[12,13] The second type of mechanism is active efflux of macrolides encoded by plasmid borne *msr*(A) gene which has specificity for macrolides and type B streptogramin. Clindamycin is neither an inducer nor a substrate for the pump, and thus the strains remain fully susceptible to this antimicrobial and hence clindamycin can be an option for treatment.[11]

Prevalence of various phenotypes

In the present study, the prevalence of constitutive MLS_B, $iMLS_B$ and MS phenotypes in *S. aureus* isolates tested was found to be 10, 18 and 4%, respectively. The incidence of constitutive and $iMLS_B$ resistance varies by geographical region and even from hospital to hospital. The frequency of $iMLS_B$ resistance ranges from 7 to 94%.[14,15]

Phenotypic pattern of subpopulation

In the present study, five isolates showed subpopulation inside the zone of inhibition around the clindamycin disc. These individual colonies were tested to find the type of clindamycin resistance. Interestingly, it was found that these five isolates turned out to be of different phenotypes. Two isolates were constitutively MLS_B, one was $iMLS_B$, one was D^+ phenotype and one was HD phenotype which is also considered as constitutive MLS_B. While there is no clinical significance between D and D^+ phenotypes, it is critical that microbiologists recognise that both phenotypes are to be considered positive for D zone test results.[16,17]

Phenotypic correlation with MIC values

Both erythromycin and clindamycin-sensitive isolates along with subpopulation

Of the 50 strains tested, 34 were sensitive to both erythromycin and clindamycin by Kirby Bauer disc diffusion technique. Out of these 34 sensitive strains, 27 were sensitive to clindamycin by agar dilution technique with an MIC of <2 μg/ml. The remaining seven isolates showed an MIC of >4 μg/ml. Of these seven strains, five were those which showed subpopulation in clindamycin-sensitive zone. MIC of this subpopulation when tested separately was very high (>128 μg/ml). Since these five isolates had mixed population of both sensitive and resistant organisms, probably the resistant organisms grew in the higher drug concentration plates. The remaining two isolates did not show any subpopulation, but still had an MIC of >4 μg/ml, probably indicating other mechanisms of resistance.

Both erythromycin and clindamycin-resistant isolates

Five isolates which were resistant to both erythromycin and clindamycin (constitutive MLS_B) showed an MIC of >128 μg/ml.

MS phenotypes

Of the 11 isolates which were erythromycin resistant and clindamycin sensitive, two isolates which were D negative (MS efflux) showed an MIC of >128 μg/ml. This efflux mechanism is known to result in resistance to macrolides and streptogramin B antibiotics, but not to lincosamides. However, in the present study, these two isolates showed high MIC.

MIC in D phenotypes

Of the nine D strains tested for MIC, six showed an MIC of ≤2 μg/ml. However, three strains showed an MIC of ≥4 μg/ml. Since there is no inducer in MIC testing, it is possible that these three strains have a different type of resistance mechanism for this discrepancy. It needs further study to understand the possible mechanism of resistance in these strains.

For the clinical laboratory, the identification of inducible MLS_B resistance is the critical issue because of the therapeutic implications in using clindamycin to treat a patient with an inducible clindamycin-resistant *S. aureus* isolate.[18] Clindamycin is a useful drug in the treatment of skin and soft tissue infections and serious infections caused by staphylococcal species as well as anaerobes. It has excellent tissue penetration (except for the central nervous

system) and accumulates in abscesses, and no renal dosing adjustments are needed.[19] Good oral absorption makes it an important option in outpatient therapy or as follow up after intravenous therapy. Clindamycin is also of particular importance as an alternative antibiotic in the penicillin allergic patient.

However, if clindamycin is used for treatment of an isolate with $iMLS_B$ resistance, selection for a mutation in the macrolide responsive promoter region upstream of the *erm* gene may occur, leading to constitutive clindamycin resistance and treatment failure.[12]

There have also been a number of reported clindamycin or lincomycin therapy failures in serious infections due to staphylococci with inducible MLS_B resistance, indicating that it is not uncommon.[2] This has led to questioning the safety of clindamycin use against any erythromycin-resistant staphylococci. Because of the high reported incidence of inducible MLS_B resistance, particularly in *S. aureus,* it has been suggested that *in vitro* erythromycin resistance could serve as a surrogate for all MLS agents, regardless of susceptibility test results.

However, the present study showed few strains with high MIC values without an inducer. There are also few strains which showed higher MIC in spite of being sensitive to both erythromycin and clindamycin in disc diffusion technique, raising the possibility of hetero-resistance or other mechanism of resistance which needs further studies.

Thus, the possibility of hetero-resistance or other mechanism needs to be studied along with testing for subpopulations in zone of inhibition around clindamycin in MS strains and $iMLS_B$ strains.

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