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Prevalence of induced clindamycin resistance in methicillin resistant *Staphylococcus aureus* from hospital population of coastal Andhara pradesh, South india

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Abstract

Clindamycin (Cd) is one of the important alternative antibiotics in the therapy of *Staphylococcus aureus*, particularly in methicillin resistant *Staphylococcus aureus* (MRSA). Without the double-disk test, all the *Staphylococcus aureus* isolates with inducible Cd would have been misclassified as Cd susceptible, resulting in an underestimated Cd resistance rate. We report a Cd clinical failure where resistance developed on therapy in D-test-positive MRSA strains. The D-test identifies inducible resistance that might presage mutational Cd resistance which can be either constitutive or inducible. The present study was aimed to know the prevalence and phenotypic characterization of induced Cd resistance in MRSA isolates from hospital patients of various medical wards, surgical wards, diabetic care centres and intensive care units (ICU) of different corporate hospitals of Coastal Andhra Pradesh, South India. The specimens were collected from various body fluids and swabs of patients;- blood (n=29), urine (n=50), pus(n=45), nasal swabs (n=40), respiratory tract swabs (n=40), eye swabs (n=50), ear swabs (n=89), and skin infection swabs (n=80). These samples were tested for the presence of MRSA and screening was done by Oxacillin discs. Out of the 153 coagulase positive *Staphylococcus aureus* isolates, 82 were MRSA. Erythromycin (Ery) resistance was observed in 23 isolates which expressed Cd inhibitory activity and one isolate was resistant to both Cd and Ery. The present study showed high level of multidrug resistance among MRSA and high incidence of Ery induced Cd resistance is also observed. Hence, it is advisable to include inducible Cd resistance testing as a part of routine antibiotic susceptibility as it may be missed in routine antibiotic testing to avoid treatment failure.

Key words: Clindamycin, erythromycin, Oxacillin, MRSA, D-zone test.

Introduction

Staphylococcus aureus has been reported as a major cause of community and hospital acquired infections [1]. The organism has a differential ability to spread and cause outbreaks in hospitals. Infections caused by *Staphylococcus aureus* usually respond to β-lactam and related group of antibiotics (macrolide-lincosamide-streptogramin (MLS) group). However, due to development of methicillin resistance amongst *Staphylococcus aureus* isolates (MRSA), treatment of these infections has become problematic. Indiscriminate use of multiple antibiotics, prolonged hospital stay, intravenous drug abuse, and carriage of MRSA in nose are few important risk factors for MRSA acquisition [2].

In 1966, when lincomycin was introduced clinically, erythromycin was no longer considered as a safe antianaerobic agent. Nevertheless, the obviously present "disappointed phenotype" (erythromycin resistant, clindamycin susceptible) was still ignored in the scientific literature [3]. Clindamycin (Cd), a lincosamide was widely used to treat *Staphylococcus aureus* in case of intolerance to penicillin or resistance to methicillin [4]. However, recent reports indicate that failure may occur in the case of inducible Cd resistance inspite of *invitro* susceptibility to clindamycin. Cd inhibits the production of toxins and virulence factors in Gram positive organism through inhibition of protein synthesis [5; 6]. Resistance mechanism to Cd in *Staphylococcus* is mediated by a methylase encoded by erythromycin resistant methylase (*erm*) gene and macrolides streptogramins resistance (*msrA*) genes.

Bacteria resist macrolide and lincosamide antibiotics in 3 ways: (1) through target-site modification by methylation or mutation that prevents the binding of the antibiotic to its ribosomal target, (2) through efflux of the antibiotic, and (3) by drug inactivation. In pathogenic microorganisms, the impact of the 3 mechanisms is unequal in terms of incidence and of clinical implications. Modification of the ribosomal target confers broad-spectrum resistance to macrolides and lincosamides, whereas efflux and inactivation affect only some of these molecules. Macrolides, lincosamides and group B streptogramins (MLS_B), have similar inhibitory effects on bacterial protein synthesis, but widely used in the treatment of Gram positive infections

The resistance mechanism is methylation of the 23s binding site. If this occurs then the bacteria are resistant to both the macrolides and the lincosamides. As a consequence of methylation, binding of erythromycin to its target is impaired. Expression of MLS_B resistance can be constitutive or inducible. Prevalence of induced clindamycin resistance in *S. aureus* was reported from many countries [7-10]. Many reports from India also recorded the emergence of induced clindamycin resistance in *S.aureus* [11-15].

The D-test identifies inducible resistance that might presage mutational clindamycin constitutive resistance. The D-test is performed by placing clindamycin and erythromycin disks at an edge-to-edge distance of 15 to 20 mm and looking for flattening of the clindamycin zone nearest the erythromycin disk [16]. A positive D-test suggests the presence of an *erm* gene that could result in inducible clindamycin resistance and clinical failure. There are few published clinical failures of clindamycin with emergence of resistance [17-21].

Erythromycin (Ery) induces the production of this methylase, which is why these strains are Ery-resistant, but mutations in the promoter region of *erm* allow_production of methylase without an inducer [22]. Methylation results in impaired binding of clindamycin that share this residue as a common binding site. MRSA are increasingly being reported as multidrug resistant with high resistance to macrolides and lincosamides leaving very few therapeutic options. Low levels of erythromycin are the most effective inducer of inducible macrolide, lincosamide and streptograminB (MLS_Bi) resistance. To detect MLS_Bi strains, there are special disk approximation tests that incorporate Ery induction of Cd resistance [22]. These strains involve the placement of an erythromycin disk in close proximity to a disk containing Cd or lincomycin. As the Ery diffuses through the agar, resistance to the lincosamides is induced, resulting in a flattening or blunting of the lincosamide zone of inhibition adjacent to the Ery disk, giving a D shape to the zone (D zone effect). In January 2004, NCCLS published a procedure for Cd induction testing in which Cd disks are placed 15 to 26 mm from an Ery disk either as part of a standard disk diffusion procedure or on an inoculum check agar plate [23].

In the present study, different phenotypic appearances of D-zone is demonstrated in hospital acquired-MRSA isolates from medical wards, surgical wards, diabetic care centres and intensive care units from different corporate hospitals of coastal Andhra Pradesh, South India.

Materials and Methods

Study group and samples

This study was conducted for a period of 11 months from August 2009 to June 2010. In this study a total of 478 samples were collected from 4 groups of patients; ~~of~~ medical wards (n=185), surgical wards (n=140), diabetic care centres (n=98) and intensive care units (ICU) (n=50) of various corporate hospitals from Coastal Andhra Pradesh, South India. Samples comprised of blood (=29), urine (n=50), pus (n=45), respiratory tract swabs (n=40), ear swabs (n-89), eye swabs (n=50), skin infection swabs (n=80), and anterior nasal swabs (n=95) from 4 groups of environments.

Culture

The swabs and body fluids of patient's samples were inoculated onto blood agar plates, each plate inoculated with sample of a single patient. These inoculated plates were incubated at 37° C for 18 – 24 hours. After inoculation on blood agar, the swabs were placed in brain heart infusion broth (BHI) with 7.5% sodium chloride, which were incubated at 37°C for 18- 24 hours. Inoculated BHI broth was sub cultured onto blood agar plates. From these blood agar plates, the colonies which were opaque, circular, pigmented with β hemolysis were identified as *S. aureus* by the Grams staining, Catalase and Coagulase (Slide and Tube) test [24]. Adequate controls were put-up at every stage. A total of 153 coagulase positive *S. aureus* strains were isolated and identified from the 478 clinical samples.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed for the antibiotics; Oxacillin (1µg), Gentamycin (10µg), Erythromycin(15µg), Cotrimoxazole(25µg), Vancomycin(30µg) (Hi-media) by Kirby-Bauer disc diffusion technique with quality control strain *S. aureus* ATCC25923 as per National Committee for Clinical Laboratory Standards [25]. Bacterial suspension matching 0.5 McFarland turbidity standards were inoculated on Muller-Hinton agar containing 4% NaCl and 6µg/ml oxacillin. Isolates showing visible growth after 24 h incubation at 33-35°C were identified as MRSA. Oxford strains of *S. aureus* NCTC 6571 sensitive to methicillin and *S. aureus* NCTC 12493 resistant to methicillin were used as control organisms. Final identification of MRSA was made on detection of mecA gene by PCR.

D-zone test

The Erythromycin and Clindamycin double disk susceptibility test (D-zone test) was performed as per NCCLS guideline

2004 [26]. All the isolates were subcultured on Muller-Hinton agar plates (Hi media, India). The clindamycin disk (2 µg) was manually placed approximately 12 mm from the erythromycin disk (15 µg) (Hi media, India) (measured edge to edge) [27]. The induction test results (D-shaped zone) were read at 16 to 18 hours by using transmitted and reflected light.

Results

Out of 478 clinical samples, 153 samples were found positive for *S. aureus*. Among 153 samples positive for *S. aureus,* 82 samples were identified as MRSA strains by testing the sensitivity to oxacillin. The total number of MRSA isolated along with their susceptibility pattern to various antibiotics is given in the **Table 1** and **Table 2**.

TABLE 1: Showing total no of isolates of Staphylococcus aureus and MRSA isolated from various samples of patients from 4 groups (Medical wards, surgical wards, diabetic care centres and Intensive care units (ICU).

Source of sample	Total No. of samples	Coagulase Positive S.aureus	MRSA
Blood	29	03	01
Urine	50	10	02
Pus	45	21	13
Nasal swabs	95	45	27
Respiratory tract swabs	40	02	00
Eye swabs	50	01	00
Ear swabs	89	30	13
Skin swabs	80	41	27
Total	478	153	82

TABLE 2: Susceptibility pattern of the coagulase positive *Staphylococcus aureus* isolated from four groups of environments

Antibiotic	Resistance %	Intermediate%	Sensitivity%	Total
Oxacillin	54	-	46	153
Gentamycin	-	-	100	153
Erythromycin	20	20	60	153
Co-trimoxazole	39	-	61	153
Vancomycin	-	-	100%	153

TABLE 2: Characteristics of clindamycin induction test phenotypes as tested by disk diffusion

Induction test	Resistance phenotype	Cd result	Ery result	Induction test description	No. of isolates
D	Inducible MLSB	S	R	Blunted, D-shaped clear zone around Cd disk proximal to the Ery disk	20
D+	Inducible MLSB	S	R	Blunted, D-shaped zone around Cd disk proximal to the Ery disk and small colonies growing to Cd disk in otherwise clear zone.	03
Neg	MSLB	S	R	Clear zone around Cd disk	04
HD	Constitutive MSLB	R	R	Two zones of growth appear around the Cd disk. One zone is a light, hazy growth extending from the Cd disk to the second zone where the growth is much heavier. The inner, hazy zone is blunted proximal to the Ery disk as in phenotype D	17
R	Constitutive MLSB	R	R	No hazy zone. Growth up to Cd and Ery disks	07
S	No resistance	S	S	Clear, susceptible zone diameters	31

Inducible clindamycin resistant-Phenotypes

Disk diffusion test yielded two distinct induction phenotypes and four non-induction phenotypes among the 82 MRSA isolates (**Table 2** and **Fig.1**). In the induction phenotype, the D-zone phenotype was observed among 20 isolates, showing blunt edge but an otherwise clear zone of inhibition around the Cd disk (at different distances **Fig. 2**). The second induction type i.e., D^+ phenotype, was observed in 3 isolates. These isolates showed blunting of zone of inhibition but also featured small colonies present between the edge of the zone of inhibition and Cd disk. Both D and D^+ results are considered positive for Cd induction (inducible MLS_B resistance). Four isolates showed Ery resistant and Cd susceptible zone diameters with no blunting of the zone. It is considered as negative phenotype. For 17 isolates growth was observed around both disks, although an inner zone of hazy growth, it is considered as hazy D (HD phenotype), which also showed some blunting. The HD

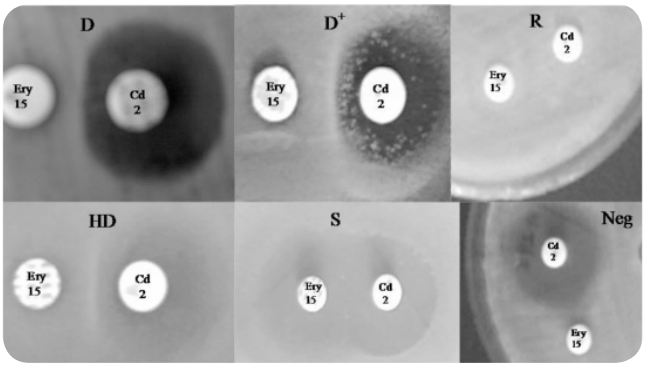

FIGURE 1. Showing the six phenotypes observed during Cd induction testing of *S.aureus* by disk diffusion. Ery (15 µg) and Cd (2 µg) disks.

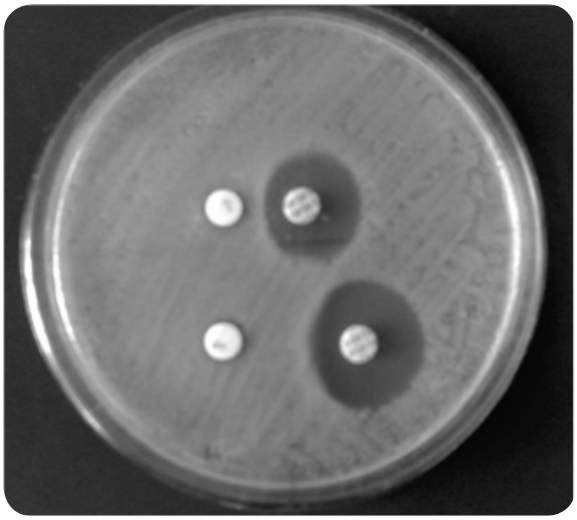

FIGURE 2. Inducible clindamycine resistance expressed by *S.aureus* at different distances.

phenotype was not considered indicative of induction since growth extended all the way to the edge of the disk (indicating Cd resistance). Seven isolates showed resistance against both Ery and Cd, and confluent growth was noted around both disks with no inner zone of inhibition considered as R phenotype. The HD phenotype and as R phenotype are considered positive for $cMLS_B$ resistance. Thirty one isolates showed large zones of inhibition around both the Ery and Cd disks i.e. S phenotype.

For isolates with D and D^+ phenotypes the ranges of the Ery and Cd zone diameter were similar. Although the inducible D zone was readily recognized at 16 to 18 hrs, for D^+ zone isolates, the ability to see the small colonies growing up to the Cd was more pronounced at 24 hrs particularly when using transmitted light rather than the reflected light. Simultaneously the isolates with Neg phenotype, the Cd and Ery zone diameters were similar to those of isolates with D and D^+ phenotype. For most of the isolates with HD phenotype, the hazy zone around the Cd disk was easy to distinguish from the solid growth in R phenotype.

Discussion

Infections caused by methicillin-resistant *S. aureus* have been associated with high morbidity and mortality rates. In Indian hospitals, MRSA is one of the common cause of hospital-acquired infections and different hospitals have reported about 30% to 80% methicillin resistance based on antibiotic sensitivity tests [28], whereas the present study showed 53.6% of MRSA strains. Microbial resistance to antibiotics mainly involves inactivation of inhibitors and/ or modification of targets (mutations of ribosomal proteins or rRNA genes). Resistance to MLS_B can occur by two different mechanisms: an active efflux mechanism encoded by the *msrA* gene and ribosomal target modification encoded by the *erm* gene (MLS_B resistance). MLS group of antibiotics exert their antibiotic effect by binding to the 23s portion of the 50S subunit of bacterial ribosomes to cause premature dissociation of the peptidyl-tRNA from the ribosome. The key reaction in protein synthesis, peptide bond formation, is promoted by the 23s portion of the 50S (the peptidyl transferase centre), and the growing peptide chain (peptidyl-tRNA) attached at the donor P site undergoes peptide linkage with an aminoacyl-tRNA at the acceptor A site. This reaction is inhibited by MLS group of antibiotics. The resistance mechanism is methylation of the 23s binding site. If this occurs then the bacteria are resistant to both the macrolides and the lincosamides. As a consequence of methylation, binding of erythromycin to its target is impaired. Expression of MLS_B resistance can be constitutive or inducible. In inducible resistance, the bacteria produce inactive mRNA that is unable to encode methylase. The mRNA becomes active only in the presence of a macrolide inducer. By contrast, in constitutive expression, active methylase mRNA is produced even in the absence of erythromycin, an inducer. The strains harboring an inducible erm gene are resistant to the inducers but remain susceptible to non inducer macrolides and lincosamides. Mutations in the promoter region of erm allow production of methylase without an inducer such as erythromycin, a macrolide [29; 30]. These mutants are stably erythromycin and clindamycin resistant. A wide range of microorganisms that are targets for macrolides and lincosamides, including gram-positive species, spirochetes, and anaerobes, express Erm methylases.

In this study, 28% of the MRSA were erythromycin inducible clindamycin resistant strains. Induced clindamycin resistant *S. aureus* tend to be multidrug resistant against a large number of currently available antimicrobial agents, because the overlapping binding sites of macrolides, lincosamides, and streptogramins B in 23S rRNA account for cross-resistance to the 3 classes of drugs, compromising treatment options and increasing the likelihood of inadequate antimicrobial therapy and increase in morbidity and mortality. The advance of In-

ducible clindamycin resistance has added a grave concern to the therapeutic dilemma caused by the presence of multidrug-resistant organisms in recent years, as clindamycin was an adequate therapy for skin and soft tissue infections caused by these strains, it was suggested not to use clindamycin in combination with erythromycin, which leads to induced clindamycin resistance [31].

This study demonstrated phenotypic appearances of the Cd zone adjacent to a standard 15 µg Ery disk in a conventional disk diffusion test. Flattening of the Cd disk diffusion zone in an Ery resistant isolate (D-zone effect) appears to be a reliable indicator of induced Cd resistant strains that harbour either the *ermA* or *ermC* gene constitutively. Cd resistant strains are easily recognized by a Cd zone shape with or without significant growth [32]. Positive disk diffusion induction results (D and D^+) could be read at 16 to 18 hrs using reflected light, however, transmitted light improved the ability to separate some non-inducible phenotypes, such as HD and R. Continued incubation of disk tests up to 24 hrs also helped to differentiate D from D^+ phenotypes, but the additional incubation time was not necessary to distinguish between Cd inducible and non-inducible isolates.

Most of the published induction test studies focus on identifying inducible Cd resistance among the isolates that are Ery resistant but Cd susceptible on routine testing [33]. In principle only Ery resistant but Cd susceptible isolates should be tested for inducible clindamycin resistance; however, some laboratories perform the D-zone test prospectively on susceptibility testing purity plates before the results of Ery and Cd resistance are known. Thus, number of isolates are tested that were either resistant or susceptible to both erythromycin and clindamycin to determine the phenotypes. Infact, the HD zone is a phenotype that may be confused with Cd induction. If the Cd test is not initially interpreted phenotypically, the predictions were not absolute due to the presence of multiple macrolide resistance determinants in our isolates. For a clinical laboratory, the differentiation of *erm*-mediated inducible MLS_B (D and D^+ phenotypes) isolates from isolates with *msrA* mediated $cMLS_B$ resistance is the critical issue because of the therapeutic implications of using Cd to treat a patient with an inducible Cd resistant *S. aureus* isolate. However, differentiating D from D^+ phenotype could also provide information to help in characterization of isolates for epidemic studies in health care and community setting.

Accurate susceptibility data are important for appropriate therapeutic decisions. If induced resistance can be reliably detected on a routine basis in clinically significant isolates, Cd can be safely and effectively used in those patients with true Cd-susceptible strains. In order to avoid the poor clinical outcomes but retain the usefulness of Cd, it would be helpful to know the prevalence of inducible resistance in clindamycin-erythromycin *S. aureus*.

D-zone test should be essentially carried out by clinical microbiology laboratories so as to differentiate inducible MLS_B resistance from that of constitutive MLS_B resistance, as in inducible resistance, the bacteria produce inactive mRNA that is unable to encode methylase. The mRNA becomes active only in the presence of erythromycin, a macrolide inducer. By contrast, in constitutive expression, active methylase mRNA is produced in the absence of an inducer Erythromycin. The strains harboring an inducible *erm* gene are resistant to the inducers but remain susceptible to noninducer macrolides and lincosamides. Mutations in the promoter region of erm allow production of methylase without an inducer such as erythromycin, a macrolide [30].

Due to the shared site of activity, these drugs can be antagonistic to each other and lincosamides should not be administered concurrently with erythromycin, chloramphenicol or most bactericidal agents.

Conclusion

This study emphasizes the prevalence of induced clindamycin resistance in MRSA from South India. Due to the shared site of activity, MLS_B group of antibiotics can be antagonistic to each other and lincosamides such as Clindamycin should not be administered concurrently with erythromycin, a Macrolide. Clindamycin-susceptible, erythromycin-resistant *Staphylococcus aureus* (clindamycin-erythromycin discordant) tend to develop clindamycin resistance, as erythromycin induces the production of methylase, which in turn inhibit the binding of clindamycin to 23s fraction of 50s ribosome. Hence, D-zone test should be essentially carried out by clinical microbiology laboratories so as to differentiate inducible MLS_B resistance from that of constitutive MLS_B resistance. The increasing prevalence of induced clindamycin resistance among MRSA and excessive use of antimicrobial agents has worsened the sensitivity, which call for further epidemiological studies.

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