Original Research Paper

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Phenotypic And Genotypic Study Of Macrolide, Lincosamide And Streptogramin B (Mlsb) Resistance In Clinical Isolates Of Staphylococcus Aureus

Medical Science

KEYWORDS : Clindamycin, D- Test, ermA gene, inducible MLSB

Umamageswari S.S.M — Associate Professor- Microbiology, Saveetha Medical College and Hospital

Anantharaman C — Associate Professor- Orthopedics, Thoothukudi Medical College

Shameem Banu A.S — Professor & HOD- Microbiology, Chettinad Health city

Kalyani M — Professor & HOD- Microbiology, Saveetha Medical College and Hospital

ABSTRACT

Emergence of Methicillin resistant Staphylococcus aureus (MRSA) emphasizes the need for alternative therapeutic options. So macrolide, lincosamide and streptogramin (MLSB) antibiotics are used to treat Staphylococcus aureus with preference to Clindamycin. In vitro routine tests for clindamycin susceptibility may fail to detect inducible clindamycin resistance due to erm genes resulting in treatment failure, thus necessitating the need to detect such resistance by a simple D test on a routine basis. The purpose of this study was to determine the frequency of cMLSB, iMLSB, and MS phenotypes using D-Test and PCR. Our study showed MRSA-33.1% and iMLSB - 14.11%. 9 strains of iMLSB had ermA gene detected by PCR. This study showed that D test should be used as a mandatory method in routine disc diffusion testing to detect inducible clindamycin resistance in Staphylococci for the optimum treatment of patients.

Introduction:

Infections caused by multidrug resistant *Staphylococcus aureus* still remain a problem in many healthcare setups [(1)].Emergence of *Methicillin resistant Staphylococcus aureus* (MRSA), *Vancomycin Intermediate staphylococcus aureus* (VISA) and *Vancomycin Resistant Staphylococcus aureus* (VRSA) emphasizes the need for alternative therapeutic options [(2)]. So macrolide, lincosamide and streptogramin B (MLS_B) antibiotics are used to treat *Staphylococcus aureus* with preference to clindamycin [(1, 3)]. However, widespread use of MLS_B antibiotics in all hospitals has led to increased number of resistant strain to clindamycin also.

Target site modification and active drug efflux are the mechanisms responsible for MLS_B resistance in *Staphylococcus aureus*. Target site modification is mediated by the presence of erm genes, predominantly ermA and ermC. Active drug efflux mechanism is mediated by msr (A)/ msr (B) gene.

Phenotypic expression of MLS_B resistance can be inducible MLS_B ($iMLS_B$) or constitute MLS_B ($cMLS_B$). In laboratory tests, *Staphylococcus aureus* with c MLS_B shows both erythromycin and clindamycin resistance but $iMLS_B$ shows erythromycin resistance and clindamycin sensitive. In $iMLS_B$ phenotype, treatment with clindamycin may lead to therapeutic failure due to emergence of constitutive resistance in vivo [(3, 4)]. For this reason screening of $iMLS_B$ resistance in laboratory and proper reporting is very important.

The aim of this study was to find out the percentage of *Staphylococcus aureus* having MLS_B using a standard test called D-Test and to do Polymerase chain reaction (PCR) to confirm the presence of erm gene in these clinical isolates.

Materials and Methods:

A total of 163 clinical isolates of *Staphylococcus aureus* were collected from tertiary care centre, Chennai from December 2014 to June 2015. *Staphylococcus aureus* were isolated from exudates (pus, wound swab, cerebrospinal fluid and synovial fluid), urine, blood and respiratory samples. *Staphylococcus aureus* was first identified by colony morphology, gram stain, catalase test and slide and tube coagulase test according to standard protocol [(5)].

Methicillin resistance was detected using cefoxitin disk diffusion test. According to the CLSI guidelines cefoxitin disk diffusion test was performed with 30microgram cefoxitin disk in Muller Hinton agar plate. This plate was incubated at 35^0c for 24 hours, after that reading done with transmitted light. Among *Staphylococcus aureus* if zone size is less than or equal to 21mm then mecA positive (MRSA).

Antibiotic susceptibility test of all *Staphylococcus* isolates were performed by Kirby Bauer disc diffusion method as per Clinical Laboratory Standards Institute (CLSI) guidelines[(6)], using Muller Hinton Agar .The following antibiotic discs, produced by Hi-media were used; Clindamycin(2µg), Erythromycin(15µg), tetracycline (30µg), Gentamycin(30µg), Ciprofloxacin(5µg), Cefazolin(30µg) ,Penicillin (10 units) , Cotrimoxazole(25µg), Vancomycin (E test), Linezolid (30µg) and Teicoplanin (30µg).

Inducible resistance to clindamycin was tested by 'D test' as per CLSI guidelines (Fig 1). In this test, erythromycin (15µg) disc was placed at a distance of 15 mm (edge to edge) from clindamycin (2µg) disc on a muller-hinton agar plate, previously inoculated with 0.5 McFarland standard bacterial suspensions. Following overnight incubation at 37°C, flattening of zone (D-shaped) around clindamycin resistance. [10] Three different phenotypes were found. *Staphylococcal* isolates which showed resistance to both erythromycin (zone size ≤13 mm) and clindamycin (zone size ≥14mm) were said to have constitutive MLSB phenotype. In inducible MLSB type *Staphylococcal* isolates show resistance to erythromycin (zone size ≤13 mm) and sensitivity to clindamycin (zone size ≥21mm) with D shaped zone of inhibition around clindamycin with flattening towards erythromycin disc. The last type is MS phenotype in which *Staphylococcal* isolates shows resistance to erythromycin (zone size ≤13 mm) and sensitivity to clindamycin (zone size ≥21mm) without D shaped zone of inhibition only circular zone of inhibition around erythromycin disc.

Quality control (QC) of erythromycin and clindamycin disc was performed with *Staphylococcus aureus* ATCC25923, according to standard disc diffusion QC procedure. Additional QC was performed with selected *Staphylococcus aureus* strains to demonstrate positive and negative D-test reactions.

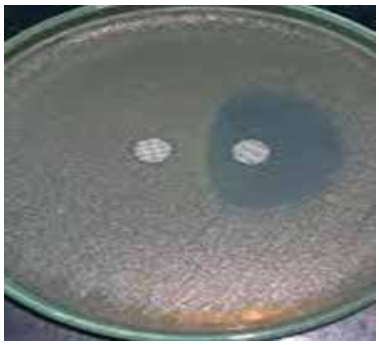


Fig 1: D-test

DETECTION OF ermA GENE:

DNA was extracted from *Staphylococcus aureus* which showed D –Test positive using Purefast bacterial DNA minispin purification kit in accordance with the manufacturer's protocol. PCR reaction was performed for the amplification of the 421 bp fragment of ermA gene using the following primer.

ermA "GTTCAAGAAC AATCAATACAGAG
GGATCAGGAA AAGGACATTTTAC"

PCR amplification reaction mixture (25µl) contained 10µl probe PCR master mix, 2.5µl of ermA primer probe mix, 2.5µl of internal control prime probe mix and 10µl of purified bacterial DNA. PCR conditions were as follows: Taq enzyme activation at 95^0 c for 15 min, 40 cycles of denaturation at 95^0 c for 20 sec, annealing at 56^0 c for 20 sec and final extension at 72^0 c for 20 sec.

Result:

Among the 163 *Staphylococcus aureus*, exudates samples 102 (62.6%), blood 23 (14.1%), urine 18 (11%) and 20 (12.3%) from respiratory samples. *Methicillin resistant Staphylococcus aureus* were found to be 54 (33.1%). (Table 1)

Table1: Presence of MRSA and MSSA in *Staphylococcus aureus*

Total samples	***Methicillin resistant Staphylococcus aureus* MRSA, n %**	***Methicillin sensitive Staphylococcus aureus* MSSA, n %**
163	54 (33.1%)	109 (66.9%)

Out of 163 Staphylococcus aureus isolates, 111 (68.09%) had erythromycin sensitive and clindamycin sensitive, 15 (9.20%) had $cMLS_B$ phenotype, 23 (14.11%) had $iMLS_B$ phenotype and 14 (8.58%) had MS phenotype (Table 2). Percentage of both inducible and constitutive resistance was higher among MRSA isolates shown in Table 3.

Table 2: susceptibility of erythromycin (E) and clindamycin (Cd) among all *Staphylococcus aureus* isolates.

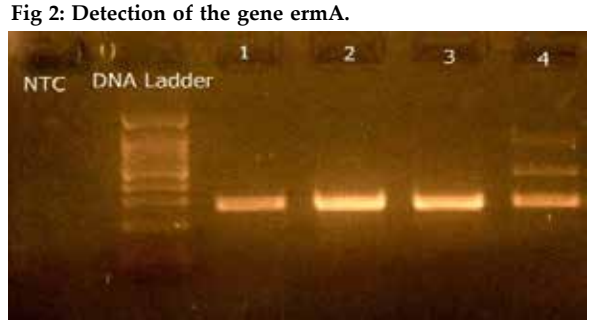
Susceptibility pattern (Phenotype)	Number of isolates	Percentage
E –sensitive, **Cd** – sensitive	111	68.09%
E- resistant, **Cd** – resistant Constitutive MLS_B ($cMLS_B$)	15	9.2%
E – resistant, **Cd** – sensitive Inducible MLS_B (MLS_B) **(D –Test Positive)**	23	14%
E – resistant, **Cd** – sensitive MS phenotype **(D –Test Negative)**	14	8.58%

Table 3: Association of clindamycin resistance with Methicillin resistance.

Clindamycin resistance	Methicillin resistance		TOTAL n = 163
	MRSA (n = 54)	MSSA (n =109)	
E –sensitive, **Cd** – sensitive	23 (42.59%)	88 (80.73%)	111(68.09%)
E- resistant, **Cd** –resistant Constitutive MLS_B ($cMLS_B$)	10 (18.51%)	5 (4.58%)	15 (9.20%)
E – resistant, **Cd** – sensitive Inducible MLS_B (MLS_B) **(D –Test Positive)**	14 (27.45%)	9 (8.25%)	23 (14.11%)
E – resistant, **Cd** – sensitive MS phenotype **(D –Test Negative)**	7 (12.96%)	7 (6.42%)	14 (8.58%)

Twenty three *Staphylococcus aureus* with iMLSB type were tested for the presence of ermA gene by PCR method, out of which 9 strains were detected to have ermA gene (Fig 2).

Fig 2: Detection of the gene ermA.



Discussion: Clindamycin is an excellent drug for *Staphylococcus* infection particularly skin and soft tissue infections. It has low side effect, low cost and good tissue penetration capacity. Clindamycin is used in out- patient treatment as a changeover drug after intravenous antibiotic for *Staphylococcus* infection because of its good oral absorption [7]. It is also better alternative drug for penicillin allergic patients [8]. However excessive use of this antibiotic also resulted in antibiotic resistance to clindamycin.

For appropriate antibiotic therapy for *Staphylococcus* infection, clindamycin sensitivity reporting is very important. Usually clindamycin resistance can be reported by doing regular antimicrobial testing but $iMLS_B$ resistance cannot be recognized by this regular methods. If a patient treated with clindamycin happens to be a case of $iMLS_B$ resistance then in vivo resistance will occur. D –test is the method to detect $iMLS_B$ resistance and can be done routinely in the lab [9].

In our study, erythromycin resistant *Staphylococcus aureus* were found to be 52 (31.90%). Among them 23 (14.11%) were found to be $iMLS_B$ phenotypic strain. This was detected by D- Test. Constitute resistant strain $cMLS_B$ was found to be 15 (9.20%) and 14 (8.58%) showed true sensitivity to clindamycin, that is MS phenotype. This study clearly shows that inducible MLS_B resistant is nearly 50% among the erythromycin resistance in *Staphylococcus aureus*. These findings correlate with the other studies [10]. But few studies show $iMLS_B$ resistance 15- 30% only [11, 12]. However D –Test is very important, otherwise erythromycin resistance would have been misidentified as clindamycin sensitive resulting in therapeutic failure.

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Comparing MRSA and MSSA, both $iMLS_B$ resistance and c MLS_B resistance were found to be more in MRSA. These findings were also similar with other studies [12]. There are also few studies that show higher percentage of $iMLS_B$ and $cMLS_B$ in MSSA [13, 14].

In our study we investigated ermA gene distribution among the 23 $iMLS_B$ phenotype Staphylococcus aureus. ermA genes were seen in 9 out of 23$iMLS_B$ phenotype. Among 9 strains, 7 strains were MRSA producers. It must be noted the frequency of erm genes is variable in different studies.

MRSA is a potent nosocomial pathogen. Clindamycin is a reserve drug and depending upon the severity of illness, it can be used among MRSA patient. Further, by using clindamycin, use of Vancomycin can be avoided [9]. But $iMLS_B$ resistance could limit the usage of clindamycin [15]. Proper reporting of inducible clindamycin resistance is possible with usage of simple, reliable D- Test routinely in the clinical laboratories.

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