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Original Research Article

A Phenotypic and Genotypic Study of Prevalence of Inducible Clindamycin Resistance in *Staphylococcus Aureus* Strains Isolated from Clinical Samples at a Tertiary Care Centre

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Abstract:

Background: Clindamycin, a lincosamide represents a common choice of drug for skin and soft tissue infections as an alternative in case of resistance to methicillin. The routine invitro tests may fail to detect inducible clindamycin resistance resulting in therapeutic failure which can be detected by a simple D test routinely in laboratory.

Materials and Methods: 281 *Staphylococcus aureus* were isolated and subjected to phenotypic and genotypic detection of erm genes by antibiotic susceptibility testing with cefoxitin (30μg) and confirming its growth on MRSA screen agar. The isolates were subjected to double disc diffusion test with erythromycin and clindamycin discs placed adjacently at a distance of 15 to 20 mm and observed for the D zoning after overnight incubation. The ermA, ermB, ermC genes were detected from the isolates by PCR assay.

Results: Out of 281 *Staphylococcus aureus* isolates 93 (33%) were MRSA, 188 (66.9%) were MSSA. Inducible clindamycin resistance was found in 22 (23.6%) MRSA and 12 (6.3%) MSSA isolates. ermA, ermC genes are found more in MRSA when compared to MSSA (22.5%, 67.7% and 6.3%, 0.5% respectively).

Conclusion: This study shows the importance of D test to be used routinely to detect inducible clindamycin resistance. Since the isolates with inducible resistance have the ability to mutate and change to constitutive resistance, it may lead to treatment failure.

Keywords: D test, erm genes, constitutive MLSB phenotype, inducible MLSB phenotype, MRSA, MS phenotype.

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Introduction

Staphylococcus aureus is a ubiquitous microorganism attributing to diverse nosocomial and community acquired infections like skin and soft tissue infections to life threatening endocarditis.[1] The major concern about this bacterium is development of antibiotic resistance against penicillin called Methicillin Resistant *Staphylococcus aureus* which was first reported in 1961.[2] This resistance has increased the interest in use of MLSB antibiotics to treat such infections.

Among MLSB family clindamycin is considered the most ideal antibiotic due to its excellent pharmacokinetics.[3,4] Unfortunately their mistreatment has led to increased resistance in *Staphylococcus aureus* strains to MLSB antibiotics leading to their therapeutic failure.[5] Erythromycin and clindamycin belong to 2 specific classes of antibiotics which act on ribosomal subunit of microorganisms and inhibit protein synthesis. 3 mechanisms have been described for MLSB antibiotic resistance in Staphylococci: one, a methylase enzyme coded by erm gene which modifies the target site, two, an efflux pump coded by msr gene and third mechanism is a lincosamide nucleotidyl transferase enzyme coded by inu gene which inactivates lincosamides.[6,7] Erythromycin is a strong inducer whereas clindamycin is a slight inducer of resistance. Resistance against MLSB antibiotics is expressed as constitutive (cMLSB) or inducible (iMLSB) mediated through ermA and ermC genes which are the major determinants in Staphylococci.[8,9]

The inducible clindamycin resistance is responsible for treatment failure because such resistant isolates cannot be detected during routine antibiotic susceptibility testing if erythromycin and clindamycin are not kept adjacent to each other.[10] The CLSI recommends simple, easy, and doable double disc diffusion test for detection of such inducible clindamycin resistance isolates.[11] The prevalence of inducible clindamycin resistance varies with geographical

area; therefore the objective of this study is to provide the physicians with local data so as to prevent misuse of clindamycin and also help improve the clinical outcome in patients.

Materials and Methods

This is a cross-sectional study carried out between July 2023–December 2023 in the microbiology laboratory with a prior approval from the Institutional Ethics Committee.

The samples from both IP and OP patients and of all age groups and gender are included. Repeat samples and samples showing contamination were excluded from the study. All the samples were subjected to culture on routine media following standard microbiological procedures. Quality control was checked with *Staphylococcus aureus* ATCC 25923.

Phenotypic detection of MRSA was done using cefoxitin (Cx)30µg disc as per CLSI, and by using MRSA screen agar. Cx disc was used as surrogate marker for detection of mecA gene which mediates oxacillin resistance. The isolates showing zone size 21mm or less with cefoxitin were phenotypically confirmed as MRSA.

Figure 1: MRSA screen agar with blue colored colonies representing Methicillin resistant *staphylococcus aureus* colonies

Phenotypic detection of clindamycin resistance (D test): All isolates were subjected to D test on Muller Hinton agar plate as recommended by CLSI by placing erythromycin disk (15µg) and clindamycin disk (2µg) spaced 15-20 mm apart. Inducible clindamycin resistance is interpreted in 2 forms – one Flattening of the zone of inhibition adjacent to the erythromycin disk (D zone) and hazy growth within the zone of inhibition around clindamycin even if no D zone is apparent.[11] Isolates, which were erythromycin resistant and clindamycin sensitive, with no apparent D zone were interpreted as MS phenotype (D test negative). Isolates which were erythromycin resistant and clindamycin sensitive with apparent D zone were interpreted as Inducible clindamycin resistance phenotype (iMLS$_B$) (D test positive).

While, isolates which were resistant to both erythromycin and clindamycin interpreted as constitutive clindamycin resistance (cMLS$_B$). Isolates which were sensitive to both erythromycin and clindamycin were interpreted as susceptible phenotype.

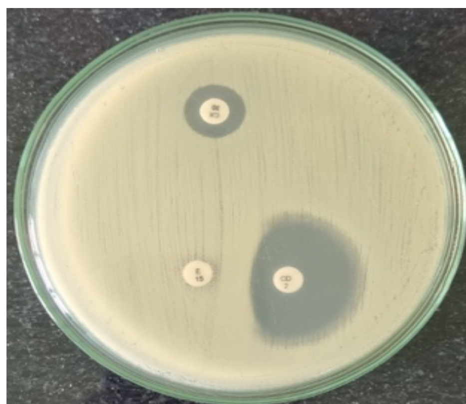

Figure 2: Muller-Hinton agar plate with *Staphylococcus aureus* showing cefoxitin resistance and inducible clindamycin resistance (iMLS$_B$)

Genotypic detection of erm genes

HiPurA Bacterial Genomic DNA Purification Kit was used for extraction of DNA from direct bacterial colonies followed by Polymerase Chain Reaction (PCR) assay for detection of ermA, ermB and ermC resistance genes. Primers were procured from Sigma-Aldrich, Missouri, United States.

Preparation of PCR mix for ermA, B and C

Genes for ermA, ermB, ermC were amplified using whole DNA extract from various isolates. Each PCR reaction tube held 12.5 µl of the 2X PCR master mix, 2 µl of DNA solution, 1 µl of the forward primer, 1 µl of the reverse primer, and 8.5 µl of nuclease-free water to complete the 25 µl reaction.

The PCR procedures for the erm genes were carried out according to the instructions provided by [2]. Amplification of ermA (139bp), ermB (142bp), ermC (190bp) genes was carried out according to previous studies [12]. Primers used for PCR were as follows:

- ermA/F:5'-TATCTTATCGTTGA-GAAGGGATT-3',
- ermA/R:5'-CTACACTTGGCTTAG-GATGAAA-3',
- ermB/F:5'-CTATCTGATTGTTGAA-GAAGGATT-3',
- ermB/R:5'-TTTACTCTTGGTTTAG-GATGAAA–3',
- ermC/F:5'-CTTGTTGATCACGA-TAATTTCC-3',
- ermC/R:5'-ATCTTTTAGCAAACCCG-TATTC-3'.

PCR conditions were as follows: Initial denaturation at 95°C for 3 minutes; followed by 35 cycles at 95°C for 30 seconds, various annealing temperatures (62.8°C for ermA, 59°C for ermB, 58°C for ermC) for 30 seconds, followed by extension at 72°C for 45 seconds and final extension at 72°C for 7 minutes. A reaction tube with all components except DNA template was used as a negative control. Known isolate with ermA, ermB, ermC genes was used as positive control.

Gel electrophoresis: Gel electrophoresis was done to separate amplicons and for detection of separated amplicons under ultraviolet light. Gene ruler-1kb DNA ladder was used as the marker (ThermoFischer Scientific, USA)

Statistical Analysis: The differences in the erythromycin resistance pattern between MRSA and MSSA was compared by Chi Square test using Graph pad Quick Calcs software and p value less than 0.05 was considered as significant.

Results

In our study a total of 281 *Staphylococcus aureus* were isolated, out of which 93 (33%) were MRSA and 188 (66.9%) were MSSA.

Table 1: Frequency of MRSA and MSSA isolates in different clinical samples

Samples	MRSA(n=93)	MSSA(n=188)	Total(n=281)
Pus/wound swabs	38 (40.8%)	64 (34%)	102 (36.2%)
Blood	28 (30.1%)	53 (28.1%)	81 (28.8%)
Urine	17 (18.2%)	32 (17%)	49 (17.4%)
Respiratory samples	7 (7.5%)	33 (17.5%)	40 (14.2%)
Others	3 (3.2%)	6 (3.1%)	9 (3.2%)

Maximum no. of samples were pus/wound swabs 102/281(36.2%), followed by blood 81/281(28.8%), urine 49/281(17.4%), respiratory samples and others 40/281(14.2%) and 9/281(3.2%) respectively.

Table 2: Comparison of Erythromycin resistance pattern with different phenotypes

Phenotype	MRSA	MSSA	Total	p-value
ER-R, CL-S, D^+ - iMLS$_B$	22 (23.6%)	12 (6.3%)	34 (12%)	0.08
ER-R, CL-R - cMLS$_B$	40 (43%)	19 (10.1%)	59 (20.9%)	0.006
ER-R, CL-S, D^- - MS	2 (2.1%)	35 (18.6%)	37 (13.1%)	< 0.0001
ER-S, CL-S- Susceptible	29 (31.1%)	122 (64.8%)	151 (53.7%)	< 0.0001
Total	93	188	281	

In the present study, inducible clindamycin resistance is shown in 22/93 (23.6%) MRSA isolates as compared to 12/188 (6.3%) MSSA isolates. Constitutive clindamycin resistance was also more 40/93 (43%) compared to 19/188 (10.1%) MSSA isolates. More no. of MSSA isolates 35/188 (18.6%), 122/188 (64.8%) had MS and susceptible phenotype when compared to 2/93 (93%), 29/93 (31.1%) MRSA isolates respectively.

Table 3: Distribution of erm genes among *staphylococcus* isolates

Genes	MRSA (n=93)	MSSA (n=188)
ermA	21 (22.5%)	12 (6.3%)
ermB	0 (0%)	0 (0%)
ermC	63 (67.7%)	1 (0.5%)

Most of the MRSA isolates were carrying ermC gene 63/93 (67.7%), followed by ermA gene 21/93 (22.5%). Among MSSA isolates ermA gene was predominant 12/188 (6.3%) followed by ermC 1/188 (0.5%), whereas no ermB genes were detected in MRSA as well as MSSA.

Discussion

One of the major global concerns is antimicrobial resistance especially in pathogens responsible for hospital acquired infections. *Staphylococcus aureus* are the commonest skin colonizers and a leading cause of community acquired and health care associated infection. [12-14] Our study revealed a higher percentage (36.2%) of *Staphylococcus aureus* isolates from pus/wound swab like study done in Nepal by Thapa et al.[4] In our study 33% of *Staphylococcus aureus* isolates were MRSA similar to studies done by Patel M et al and Lall M et al.[15,16]

In the present study the prevalence of iMLSB, cMLSB, MS, Susceptible phenotypes was 12%, 20.9%, 13.1%, 53.7% respectively and the percentage of inducible clindamycin resistance was in agreement with previous studies done in India by Patel M et al[15] while lower percentage of around 5% was also reported by Sasirekha B et al and Nafisi M et al.[17,18] Similar to studies done by Mansouri S and Moosavian M, our present study showed that the frequency of inducible clindamycin resistance was higher in MRSA phenotypes (23.6%) than MSSA phenotypes (6.3%) though there was no significant difference (p=0.08).[19,20] Among the ermA, ermB and ermC genes detected, ermC is found to be the predominant gene (67.7%) amongst MRSA followed by ermA (22.5%) in agreement with Saderi H et al.[21] Spiliopoulou I et al, Saderi H et al have shown no ermB gene in their MRSA isolates [22,23] similar to our study. In contrast, studies by Coutinho Vde L and Lina G have shown a frequency of 0.7%, 2.2% to 8.3%.[24,25]

Genotyping of *Staphylococcus aureus* isolates for detection of erm genes is considered as a superior tool for surveillance purpose but the continuous mutations in erm genes makes the use of genotyping difficult. Also, such a usage in resource poor setting is not possible for routine investigation. However, the phenotypic technique stipulates both the presence and expression of erm genes. Thus, the D-zone test is an easy and affordable method recommended by CLSI.

Conclusion

Clindamycin should be considered for the management of serious skin and soft tissue infections, taking into consideration the restricted range of antibiotics available for treatment of MRSA infections and the confines of Vancomycin. Double disc diffusion test is the simplest test which can be done to discriminate between inducible clindamycin resistance and susceptible clindamycin. The clinical microbiology labs should often use double disc susceptibility test as a standard practice in routine reporting. This early detection will enable the clinician to save time and consequently help in avoiding therapeutic failures.

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