Original Article

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Prevalence of MLSB Resistance and Observation of *erm* A & *erm* C Genes At A Tertiary Care Hospital

Microbiology Section

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ABSTRACT

Background: The increasing resistance to macrolide, lincosamide, streptogramin B (MLSB) agents among *Staphylococcus aureus* is becoming a challenge to microbiologist. Clindamycin has been a useful drug for treatment of infection caused by the *staphylococcus aureus*, but change in clindamycin sensitivity pattern due to various mechanisms is leading to therapeutic failure. One of the important mechanisms is mediation of resistance by *erm* genes. *Staphylococcus* strains which have *erm* genes show inducible clindamycin resistance that cannot be determined by routine disk diffusion test resulting in treatment failure.

Aim: This study was aimed to detect the prevalence of MLSBi and MLSBc resistance and observation of *erm* A & *erm* C genes among MLSBi isolates.

Materials and Methods: A total 500 *Staphylococcus aureus* were isolated; they were checked by disk induction test (D-Test). Those isolates which showed inducible clindamycin resistance were randomly selected and subjected to PCR for the observation of *erm* A and *erm* C genes.

Results: Prevalence of MLSBi and MLSBc isolates were almost similar that is 10.8% and 11.6% respectively. MLSBi isolates showed more resistance to drugs when compared to MLSBc isolates. Neither of MLSBi and MLSBc isolates was resistant to Vancomycin and Linezolid. Inducible clindamycin was mainly due to presence of *erm* A gene.

Conclusion: D- test should be mandatory at every microbiology laboratory and should be used in routine antibiotic procedure which will minimize the misuse of drug ultimately minimize the risk of treatment failure. PCR should be performed for the detection of genes responsible for erythromycin resistance as it is a quick and most sensitive method.

Keywords: D- test, *erm* genes, MRSA, PCR, *Staphylococcus aureus*

INTRODUCTION

Staphylococcus aureus is the reason of greatest apprehension as a pathogen because of its intrinsic virulence that it has ability to quickly adjust itself into environmental conditions. Severity and diversity of disease caused by *S. aureus* is the main reason of arrival of multi-drug resistance. One of the most common sorts of resistance is Methicillin resistance which has been threat to human wall fare for past 50 years.

A few options are available for the treatment of methicillin resistant (MRSA) staphylococcal infections, such as macrolides, lincosamides and streptogramin B (MLSB) with clindamycin being one of the good alternatives, particularly for skin and soft tissue infections and work as an alternative in penicillin allergic patients [1]. However, excess and inappropriate use of MLSB agents has led to an increase in number of *S. aureus* strains which are resistant to MLSB as well.

There are two primary mechanisms provides resistance to macrolide antibiotics [2]. Among *S. aureus* the gene *msr* A encodes efflux pump which is a primary mechanism of defense and quite common in some geographical areas [3]. The second mechanism includes modification of drug binding sites on the ribosomes that also enhances resistance to macrolides. These two mechanisms promotes resistance to macrolides, lincosamides and streptogramins B group of antibiotics and termed as MLSB resistance [4,5]. An *erm* gene usually *erm* A or *erm* C encodes methylation of 23S rRNA-binding which is shared commonly by these three drug classes [6].

In a previous study we had reported the prevalence of hospital and community associated MRSA along with antibiogram [7]. Now we undertook molecular studies for detection of *erm* A and *erm* C genes among inducible clindamycin resistant isolates, also illustrating the prevalence of MLSB resistance and antibiogram of inducible clindamycin resistance (MLSBi) and constitutive resistance (MLSBc) isolates.

MATERIALS AND METHODS

The present study was done for a period of 18 months (May 2013-October 2014). During this period a total of 500 *S. aureus* were isolated from different clinical samples such as pus, ear swab sputum, urine, blood, swabs from different sites etc, by the standard laboratory procedures [8] in the Department of Microbiology, National Institute of Medical Sciences, Jaipur, Rajasthan, India.

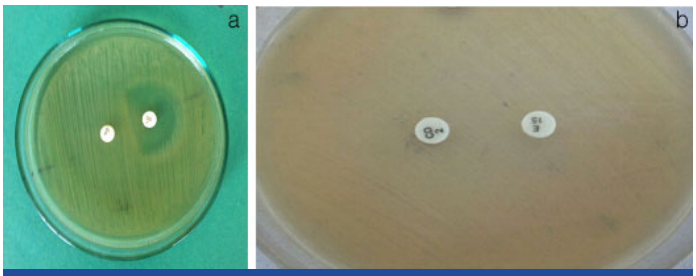
Detection of MRSA: MRSA detection was done by using cefoxitin 30µg (Hi Media, Mumbai). Those isolates showed zone of inhibition less than 21mm considered as MRSA.

Disk induction test: This test was performed to detect the presence of inducible clindamycin resistance among erythromycin resistant *S. aureus* isolated from clinical samples. A bacterial culture suspension was made equivalent to 0.5 McFarland's standard then a lawn culture is made on the Muller-Hinton agar plate on to which disc of clindamycin 2 µg (Hi Media, Mumbai) and erythromycin 15 µg (Hi Media, Mumbai) were placed at a distance of 15 mm edge to edge as per Clinical and Laboratory Standards Institute guidelines [9]. Four types of phenotypes were observed by the disk induction test (D-test).

1. Inducible MLSB phenotype: In this phenotype *S. aureus* isolates showed D shape zone around the clindamycin disk while resistant to erythromycin [Table/Fig-1a].
2. Constitutive MLSB phenotype: In this phenotype *S. aureus* isolates were resistant to both drugs clindamycin and erythromycin [Table/Fig-1b].
3. Moderate sensitive (MS) phenotype: *S. aureus* isolates exhibited resistance to erythromycin and sensitive to clindamycin.
4. Sensitive phenotype: Isolates of *S. aureus* sensitive to erythromycin and clindamycin.

Antibiotic susceptibility test of inducible and constitutive resistant isolates

Antibiotic susceptibility test was performed by Kirby- Bauer disk diffusion method. Twelve antibiotics were used (excluding Erythromycin (15µg) and Clindamycin (2 µg)) Ciprofloxacin (5µg), Cefoxitin (30µg), Tetracycline (30 µg), Amikacin (30 µg), Gentamicin (10µg), Co- trimoxazole (25µg), Norfloxacin (10µg), Chloramphenicol (30 µg), Teicoplanin (30 µg), Nitrofurontine (300 µg), Vancomycin (30µg) and Linezolid (30µg) (Hi media Mumbai) for observation of MLSBi and MLSBc isolates [Table/Fig-2].



[Table/Fig-1a,b]: Inducible & Constitutive MLSB

Antibiotics	*MLSBi	**MLSBc	p-value
Ciprofloxacin	72.22%	67.24%	0.67
Cefoxitin	85.18%	55.17%	0.011
Tetracycline	42.59%	53.44%	0.29
Amikacin	25.92%	39.65%	0.084
Gentamicin	51.85%	37.93%	0.14
Co- trimoxazole	37.03%	31.03%	0.46
Norfloxacin	25.92%	17.24%	0.17
Chloramphenicol	24.07%	12.06%	0.04
Teicoplanin	27.77%	10.34%	0.0035
Nitrofurontine	3.70%	3.44%	1
Vancomycin	0	0	-
Linezolid	0	0	-

[Table/Fig-2]: Antibiotic resistant pattern of MLSBi and MLSBc isolates
**MLSBc= Constitutive resistance, *MLSBi phenotype= Inducible clindamycin resistance

erm A and *erm* C genes detection

Detection of *erm* A and *erm* C genes was done by the method as described by Nizami Duran et al., [10]. Out of 54 MLSBi isolates, 24 isolates (44.44 %) were randomly selected for the observation of *erm* A and *erm* C genes which were statistically significant for evaluation of results. The genomic DNA of the selected strains was isolated by using KT- 03i (mercbioscience)

Primer designed for the study.

erm A (190 bp):

5'- AAG CGG TAA ACC CCT CTG A- '3

5'- TTC CGC ATT CCC TTC TCA AC- '3

erm C (299 bp) :-

5'- AAT CGT CAA TTC CTG CAT AT- '3

5'- TAA TCC TGG AAT ACG GGT TTG- '3

A total volume of 25 µl was taken for PCR amplification. It includes: 5 µl of genomic DNA sample which was added to 20 µl of PCR mixture. The PCR mixture consists of 20 mmol/l Tris-HCl, pH 8.4; 50 mmol/l KCl, 10 mmol/l $MgCl_2$, and 200 µmol/l each of deoxynucleoside triphosphates (dNTPs), 0.6 µmol/l each primers and 1 U Taq DNA polymerase [10].

The amplification process was started with an initial denaturation step (95°C, 3 min). Each PCR reaction consisted of 30 cycles of amplification (denaturation at 95°C for 30 sec, annealing at 54°C for 30 sec, and DNA chain extension at 72°C for 30 sec). A final extension cycle was performed at 72°C for 4 min [10].

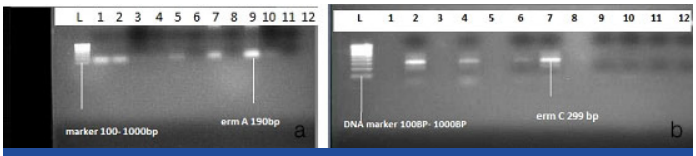
After amplification of the resistance genes, 10 µl of the PCR products were mixed with 3 µl of loading buffer and then loaded onto a two percent agarose gel and electrophoresis was performed in Tris-borate EDTA buffer containing 0.5 g of ethidium bromide per ml. Ethidium bromide stained DNA amplicons were visualized using a gel imaging system [10].

RESULTS

Antibiotic resistant pattern of MLSBi and MLSBc isolates is shown in [Table/Fig-2]. Distribution of MRSA and MSSA in co-relation with various phenotypes is given in [Table/Fig-3]. Gel electrophoresis image of *erm* A and *erm* C genes among MLSBi isolates is shown in [Table/Fig-4a&b].

Organism N= 500	ERN-S, CLN-S (%)	ERN–R , CLN-S MS Phenotype (%)	ERN-R, CLN-R MLSBc Phenotype (%)	ERN-R, CLN-D, MLSBi Phenotype (%)
MRSA N= 201	78 (38.80)	45 (22.38)	32 (15.92)	46(22.68)
MSSA N=299	221(73.91)	44 (14.71)	26 (8.69)	8 (2.67)
TOTAL	299 (59.8)	89 (17.8)	58 (11.6)	54(10.8)

[Table/Fig-3]: Distribution of MRSA and MSSA in Co-relation with various phenotypes
ERN=Erythromycin, CLN= Clindamycin, S= Sensitive, R= Resistant, D= D- Shape MS=Moderate Sensitive to both drugs, MLSBc= Constitutive resistance, MLSBi phenotype= Inducible clindamycin resistance



[Table/Fig-4a,b]: Electrophoresis image of *erm* A and *erm* C genes among MLSBi isolates

DISCUSSION

The D test was performed on erythromycin resistant isolates to detect the inducible phenotype. In our study we found high percentage of erythromycin resistant isolates 201(40.20%), out of them 89 (44.2%) exhibited MS phenotype, 58 (28.8%) exhibited MLSBc phenotype and 54 (26.8%) isolates were detected as inducible clindamycin resistant isolates [Table/Fig-3].

Overall prevalence of Inducible clindamycin resistance was 54 (10.8%). Kavita Prabhu et al., reported the similar results [11]. They reported 10.52% inducible clindamycin resistance among *Staphylococcus aureus*. Our results were also in accordance with N Seifi et al., who reported 11.37% of inducible clindamycin resistance [12]. In our study 46 (22.68%) MLSBi isolates were MRSA and only 8(2.67%) were MSSA, which is in agreement with Rahber M et al.,, who reported 22.6 % of Inducible clindamycin resistance isolates as MRSA and 4 % as MSSA [13].

We observed overall prevalence of constitute resistant isolates was 58 (11.6%) which is almost similar to the prevalence of inducible clindamycin resistant isolates. Urmi et al., reported 12% constitutive resistance among *S. aureus* that is similar results with our study [14]. In our study prevalence of constitutive resistant isolates among MRSA and MSSA was 32 (15.92%) and 26 (8.69%) respectively which is in accordance with K Prabhu et al., who reported 16.57 % constitutive resistance in MRSA and 6.2% in MSSA [11]. Gupta et al., reported MLSBc resistance in 19% of total isolates of which 46% were MRSA and 10% were MSSA [15].

Approximately 40 *erm* genes have been illustrated so far [5]. Among infection causing bacteria, *erm* genes are mainly borne by plasmids and transposons which are capable of being self-transferable. Therefore, a nomenclature system has been constructed to solve increasing complexity in designation. Twenty one classes of *erm* genes and as many corresponding *erm* proteins gets differentiated by this current nomenclature system. *erm* A, *erm* B, *erm* C, and

erm F are the four major classes that are seen in pathogenic microorganisms [5,16]. The *erm* A and *erm* C determinants are predominant in *staphylococci* [17]. The *erm* A genes are mainly spread in methicillin resistant strains which are borne by transposons related to Tn554, and *erm* C genes are frequently responsible for erythromycin resistance in methicillin-susceptible strains that are plasmids borne, Whereas *erm* B class genes are mainly restricted to *streptococci* and *enterococci*, and the *erm* F class genes to Bacteroides species and other anaerobic bacteria [5].

Although, reports are available in which *erm* B has been found to be associated with MLSBi resistance among *staphylococci* [10,17]. Each class is relatively specific, but not strictly confined to a bacterial genus and this reveals how easily they exchange their determinants.

For the detection of *erm* A and *erm* C genes 24 randomly selected MLSBi isolates were subjected to PCR. Of the 24 isolates, 7 (29%) isolates had *erm* A and 3 (13%) isolates possessed *erm* C genes. Fourteen (58%) strains gave negative results; did not have either *erm* A or *erm* C gene [Table/Fig-4]. We observed discordance among presence of *erm* genes and antibiotic susceptibility, similar observation was also reported by T Zmantar et al., and Sekiguchi et al., [18,19]. Mutation in the coding or promoter region of the PCR-detected genes could be a reason for this discordance. Moreover, this result can be explained by the location of these genes in small plasmids, which were occasionally lost. There could be possibility of other *erm* genes like *erm* B, *erm* F, etc. among 14 negative isolates.

The prevalence of *erm* genes may vary area to area and the population studied. We observed much less prevalence of *erm* A and *erm* C genes when compared to other studies. In various studies prevalence of *erm* A was reported more than *erm* C like Nizami duran et al., who reported 44 (52%) *erm* A and 24 (28%) *erm* C genes [10], Lim et al., reported 82.5% *erm* A and 2.6% *erm* C gene [20]. Similarly Gul et al., also reported high prevalence of *erm* A genes i.e. *erm* A and *erm* C were 55 (62%) and 23 (26%) respectively [21].

However few studies reported high prevalence of *erm* C genes than *erm* A genes among MLSBi strains. 54% *erm* C prevalence was reported by Thakker et al., [22]. In a study conducted by Fiebelkorn et al., *erm* C prevalence was depicted about 71% much higher when compared to our study [23].

CONCLUSION

In summary, 58 (28.8%) of erythromycin resistant isolates exhibited MLSBc phenotype and 54 (26.8%) isolates were detected as inducible clindamycin resistant isolates. This reveals that about one-fourth of the erythromycin-resistant would have been bewildered as clindamycin sensitive isolates causing therapeutic failure if D-test were not performed on erythromycin resistant isolates.

We observed that resistant to erythromycin was mainly due to presence of *erm* A genes and there was no correlation between genotype and antibiotic susceptibility. PCR definitely has advantages over routine disk diffusion test by reducing the time for detection of resistance genes, also it is highly sensitive than routine disk diffusion test with only limitation of being little expensive. In the lights of advantages we suggest that PCR should be performed for accurate detection of genes responsible for erythromycin resistance. This kind of studies is further required in various geographical areas to fill the gap of knowledge and proper implication of antibiotics.

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