INDUCTION OF CLINDAMYCIN RESISTANCE IN CLINICAL ISOLATES OF *STAPHYLOCOCCUS AUREUS* FROM A TERTIARY CARE HOSPITAL.

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Abstract

Nowadays in *Staphylococcus aureus* isolates resistant to lincosamide, macrolide and streptogramin B (MLSB) group of antibiotics are expanded. Therefore, clindamycin is preferred drug for the treatment of infections caused by *S. aureus*, but due to change in sensitivity patterns of clindamycin it is leading to treatment failure. The three resistance phenotypes of MLSB antibiotics are iMLSB (inducible resistance) and cMLSB (constitutive resistance) that are resistant to macrolides, lincosamides and streptogramins B antibiotics, whereas MS resistance that is sole resistant to macrolides and streptogramins B antibiotics. Erythromycin ribosome methylase (*erm*) genes are responsible for expressing inducible clindamycin resistance among *S. aureus*. In the present investigation, a Double disc approximation/Disc induction test (D-test) and PCR were used. Out of 428 strains the prevalence of iMLSB, cMLSB and MS phenotypes were 36 (8.41%), 47 (10.98%) and 48(11.21%) respectively. It is concluded that D-test should be routinely done to avoid treatment failure due to clindamycin resistance. In addition, PCR is a simple, quick, reliable and sensitive method that could also be used in the detection of inducible clindamycin resistance. The reason for the lower prevalence of iMLSB phenotype in the present study could be due to the reason that samples included in this study were mostly from the rural areas as the exposure of antimicrobial agents is less.

Keywords: Clindamycin resistance, D-test, *ermA*, *ermC*, iMLSB, *S. aureus*

Introduction

Staphylococcus aureus causes serious and life threatening clinical infections therefore considered as most important pathogen.^[1] The emergence of resistance among *S. aureus* is an increasing problem nowadays, especially against methicillin.^[2] Methicillin resistant *S. aureus* (MRSA) is of great concern as it is not only resistant to methicillin but resistant to many other chemotherapeutic agents.^[3] Therefore, for the treatment of MRSA a renewed interest in macrolides, lincosamides and streptogramins B (MLSB) antibiotics therapy. The macrolides (erythromycin, clarithromycin, azithromycin), lincosamides (clindamycin) and streptogramins B (quinupristin) antibiotics are structurally unrelated but have a same mechanism of action, as the protein synthesis is hampered by binding to 50S ribosomal subunit 23S rRNA.^[4]

Clindamycin is the preferred therapy due to several reasons. Firstly, clindamycin has fine bioavailability with high oral absorption and used as outpatient therapy and also as follow-up therapy after intravenous administration. Secondly, it has elevated tissue penetration and activity, other than in CNS. Thirdly, the patients that are susceptible to penicillin are replaced with clindamycin treatment. Finally, it could be used to treat skin and soft tissue infections caused by MRSA.^[4,5] However, there has been an

increase in number of MLSB resistant *S. aureus* strains due to inappropriate and excessive utilization of MLSB group of antibiotics.^[2]

There are three different mechanisms of resistance of MLSB antibiotics in staphylococci. The first mechanism is modification of the ribosomal target and is encoded by erythromycin ribosome methylase (*erm*) gene which leads to the formation of enzyme methylase.^[6] The enzyme attaches one or two methyl group to the adenine residue in 23S rRNA component of the 50S ribosomal subunit and prevent binding of MLSB antibiotics to their ribosomal targets. The resistance in the *S. aureus* isolates are due to the MLSB antibiotics which are either inducible (iMLSB) or constitutive (cMLSB) constitution. In case of inducible MLSB resistance, the bacteria synthesized non-functional mRNA which is not capable for encoding methylase. Therefore, solely in the presence of macrolide inducer mRNA could be activated. On the contrary in cMLSB resistance, functional methylase mRNA is all the time synthesized even in the lack of inducer. The strains with cMLSB are non sensitive to erythromycin and clindamycin whereas strains with iMLSB phenotype are resistant to erythromycin and sensitive to clindamycin *in-vitro*.^[5,6] The treatment with clindamycin in such individuals results to clinical failure as low levels of erythromycin acts as inducer and therefore forms the basis of the D-test. The second

mechanism of resistance involves an efflux system which encodes macrolide streptogramin resistance (*msrA*) gene. The *msrA* gene gives rise to resistance to macrolides and streptogramin B antibiotics other than lincosamides which is responsible for MS resistance. Clindamycin is neither an inducer nor a substrate for the pump and the strains are fully sensitive to clindamycin.^[1] The third mechanism involves enzymes such as hydrolase, phosphotransferase, nucleotidyltransferase and lyases.^[7]

It is important to differentiate all the phenotypes, as it will help in guiding the therapy effectively.^[8] To screen the inducible clindamycin resistant strains, the CLSI has approve the phenotypic method by Double disk approximation/Disk induction test (D-test).^[9] A genotypic method was also carried out to confirm the existence of resistance genes through PCR. The present investigation was undertaken to establish the phenotypic and genotypic detection of inducible clindamycin resistance in *Staphylococcus aureus* isolated from various clinical samples.

Materials and Methods

Isolation and Identification of *S. aureus*

The present study was conducted at Centre for Interdisciplinary Biomedical Research and Department of Microbiology, AIMSR, Adesh University, Bathinda from January 2015 to December 2016. A total of 428 *S. aureus* (non-repetive) isolates from all clinical samples collected from the patients visiting IPD/OPD of Adesh Hospital. A written informed consent was obtained from patients before study. The research was started after getting the clearance from Thesis Research Committee and Ethical Committee of Adesh University, Bathinda. The specimens were collected under sterilized conditions after transportation all the samples were processed in Microbiology laboratory.

S. aureus was identified using standard microbiological procedures like colony and microscopic morphology on mannitol salt agar and biochemical reactions like catalase, slide and tube coagulase.^[10] As per the CLSI guidelines antibiotics resistance pattern was proceeded on Mueller Hinton Agar (MHA) by Kirby Bauer disc diffusion. 0.5 McFarland standard bacterial suspension was prepared and inoculated on the MHA plates.^[9]

Detection of MRSA

Methicillin resistance was investigated through phenotypic method by using cefoxitin (30 µg) and oxacillin (1 µg) discs diffusion technique with NaCl in media.

For the detection, the MH agar plate was inoculated with suspension of *S. aureus* with 0.5 McFarland turbidity. Cefoxitin and oxacillin discs were placed on the plates followed by incubation for 24 hrs. The results were reported based upon the criteria mentioned below.^[9]

Antibiotic	Sensitive	Intermediate	Resistant
Oxacillin	≥ 13 mm	11-12 mm	≤ 10 mm
Cefoxitin	≥ 22 mm	-	≤ 21 mm

Double disk approximation/Disk induction test (D-test)

Erythromycin resistant isolates of *S. aureus* were further examined for inducible clindamycin resistance by Double disk approximation investigations according to CLSI, 2015 guidelines. The 0.5 McFarland turbidity of overnight grown culture of erythromycin resistant *S. aureus* were processed and inoculated on MH agar plates. The disc of erythromycin and clindamycin were kept at a distance of 15 mm edge to edge from each other followed by incubation at 37°C. Three types of phenotypes were observed as follows^[11]:

1. Inducible MLS_B phenotype (iMLS_B)- *S. aureus* cultures showed zone size ≤13mm was resistance to erythromycin whereas zone size ≥21mm was sensitive to clindamycin and formed D shaped zone of inhibition around clindamycin and become flatter towards erythromycin disc were designated as iMLS_B phenotype.
2. Constitutive MLS_B phenotype (cMLS_B)- *S. aureus* isolates showed resistance for erythromycin (zone size ≤13mm) and clindamycin (zone size ≤14mm) were categorized as cMLS_B phenotype.
3. MS_B phenotype- *S. aureus* cultures displayed erythromycin resistance (zone size ≤13mm) whereas sensitive to clindamycin (zone size ≥21mm) were designated as MS_B phenotypes.

ermA and *ermC* gene detection

Out of 36 iMLSB phenotypic strains of *S. aureus*, 18 strains were randomly selected for detecting presence of *ermA* and *ermC* gene.

DNA extraction

The DNA was manually isolated by CTAB-NaCl modified method of Sharma et al.^[12] The stock bacterial culture were inoculated on blood agar plates and incubated for 24 hours at 37°C. Then the activated bacterial cultures were further inoculated in 5 ml Brain heart infusion broth and the cultures were incubated for overnight incubated at 37°C. The bacterial growth from each 5 ml broth was than divided into three eppendorf tubes. The eppendorf tubes were centrifuged at 8000 rpm for 10 minutes at 15°C and after the supernatant removal, the pellet was dissolve in 10 mM Tris EDTA with addition of lysozyme (20 mg/ml) and 10% sodium dodecyl sulphate. The mixture was vigorously mixed for 2 minutes followed by addition of 10 mg/ml Proteinase K and kept at 56°C for 1-3 hours. The incubation was preceded overnight if cells were not lysed. Then 30µl (per 50 ml mixture) 5M NaCl was added and mixed well. After the addition of 1% CTAB/0.7M NaCl the tubes were further kept at 65°C for 30 minutes. Further after cooling the tubes chloroform:isoamyl alcohol (24:1) was added to the tubes and mixed properly on the rocker for 2 min., followed by centrifugation at 5000 rpm at 25°C for 10 min. The aqueous phase was then transferred into a fresh eppendorfs and phenol:chloroform: isoamyl alcohol (25:24:1) was added to it. The samples were again

centrifuged at 5000 rpm at 25°C for 10 min. and the aqueous phase was shifted to a new tube. After the addition of 0.6 volume of isopropanol, the eppendorfs were incubated at -20°C for 2 hours or overnight followed by centrifugation at 7000 rpm for 5 min. The DNA pellets were washed with 70% ethanol twice and after evaporation of alcohol, the DNA was suspended in 10 mM Tris-EDTA buffer.

PCR amplification of *erm* genes

PCR reaction was carried out in a Biorad T100 Thermal cycler. The conserved primers for *ermA* and *ermC* genes were synthesized from Chromous Biotech (Bengaluru). The primers selected from the previous study^[6]: ***ermA* (190 bp)**: 5'- AAG CGG TAA ACC CCT CTG A- '3 and 5'- TTC CGC ATT CCC TTC TCA AC- '3. ***ermC* (299 bp)**: 5'- AAT CGT CAA TTC CTG CAT AT- '3 and 5'- TAA TCC TGG AAT ACG GGT TTG- '3. The amplification process was started with an initial denaturation of 94°C for 5 min. followed by 34 cycles of denaturation at 94°C for 1 minute, annealing at 52°C for 1 minute and extension at 72°C for 2 minutes. The final extension was conducted at 72°C for 8 minutes. Amplification was obtained after 2-3 hours and the PCR product of the respective isolates was detected by agarose gel electrophoresis.

Results

In the present research a total 428 *S. aureus* strains isolated from all clinical samples. The percentage of isolation of *S. aureus* among male patients was 235 (55.14%) and female patients were 192 (44.86%) as shown in Figure 1.

Figure 1: Percentage distribution of *Staphylococcus aureus* among male and female patients

The analysis of *S. aureus* was also done on basis of the age of the patients. The rate of isolation of *S. aureus* was 80 (18.69%) in the age group of 0-20 years, 177 (41.36%) in age group 21-40 years, 115 (26.87%) among patients from 41-60 years, 53 (12.38%) patients from 61-80 years and in patients of age more than 80 years the rate of isolation was 3 (0.70%) as shown in Figure 2.

Figure 2: Distribution of *Staphylococcus aureus* based on age and gender of patients

The detection of methicillin resistance among the *S. aureus* isolates was done using two methods. These methods were:

- **Cefoxitin disk diffusion method:** In this method by using cefoxitin disk the number of MRSA isolates was 191 (44.63%) and MSSA were 237 (55.37%).
- **Oxacillin disk diffusion method:** The number of MRSA isolates were 183 (42.76%) and MSSA were 245 (57.24%) by using oxacillin disk (Table 1).

Table 1: Distribution of methicillin resistant and sensitive *Staphylococcus aureus*

Isolate	Isolation of <i>S. aureus</i> using Cefoxitin disk	Isolation of <i>S. aureus</i> using Oxacillin disk
MRSA	191 (44.63%)	183 (42.76%)
MSSA	237 (55.37%)	245 (57.24%)

All the isolates were then subjected to identify erythromycin resistant strains using erythromycin disk. The D-test was also performed on isolates that were resistant to erythromycin. Among 428 strains, 36 (8.41%) strains were D-test positive i.e. they exhibited iMLSB phenotype, 47 (10.98%) strains exhibited cMLSB phenotype as they were resistant to both erythromycin and clindamycin and 48 (11.21%) strains were resistant to erythromycin but sensitive to clindamycin, thus designated that isolates were of MS phenotype. of the 36 iMLSB phenotypic strains, 21 (10.99%) strains were MRSA and 15 (6.32%) were MSSA, whereas in case of cMLSB phenotype out of 47 strains 34 (17.80%) were MRSA and 13 (5.48%) were MSSA and among 48 MS phenotypic strains 31 (16.23%) were MRSA and 17 (7.17%) were MSSA (Table 2).

Table 2: Distribution of MRSA and MSSA in co-relation with various phenotypes.

Organism	ERY-S, CLI-S	ERY-R, CLI-D iMLSB phenotype	ERY-R, CLI-R cMLSB phenotype	ERY-R, CLI-S MS phenotype
MRSA(n=191)	105(54.97%)	21(10.99%)	34(17.80%)	31(16.23%)
MSSA(n=237)	192(81.01%)	15(6.32%)	13(5.48%)	17(7.17%)
TOTAL	297(69.39%)	36(8.41%)	47(10.98%)	48(11.21%)

(ERY=Erythromycin, CLI=Clindamycin, S=Sensitive, R=Resistant, D=D shaped zone)

In PCR, 18 randomly selected iMLSB strains of *S. aureus* were investigated for existence of *ermA* and *ermC* gene. It was observed that *ermC* gene was present in all the isolates with single band of 299 bp, whereas *ermA* gene was not detected in any of the strain (Figure 3).

Figure 3: Agarose gel electrophoresis (1.2%) of PCR amplified product of *ermA* and *ermC* genes, Lane M and 7: 10,000 bp ladder, Lane 1-6: The PCR amplicon sizes for *ermA* (190 bp) and Lane 8-14: The PCR amplicon sizes for *ermC* genes (299 bp).

Discussion

In the current study, the percentage of isolation of *S. aureus* was higher among male patients (55.14%) than female patients (44.86%). Geeta and Rama^[13] also reported similar results i.e. 56.8% isolation of *S. aureus* from male patients and 43.2% from female patients. Another study conducted by Al-Zoubi *et al.*^[14] also investigated the *S. aureus* isolation higher in males patients (57%) than in the females patients (43%). The reason for high isolation of *S. aureus* from male patients might be due to the fact that in the study population maximum number of male patients visited the hospital with cases of roadside injuries and occupational injuries.

Based on age of patients, it was observed that maximum number of isolation of *S. aureus* was among age group 21-40 years i.e. 177 (41.36%) and least among patients of age more than 80 years (3) 0.70%. Al-Zoubi *et al.*^[14] observed that the maximum number of isolation of *S. aureus* was among the age group of 20-39 years i.e. 32.1% and it was also reported that percentage declined after 80 years of age, the finding of this study was similar to the results observed in the present study. This age group is a sexually active and considered as working group of the population. Therefore, this group will be more prone to trauma, abscesses and wound infections.

The methods used for the identification of methicillin resistance among *S. aureus* isolates were cefoxitin and oxacillin discs diffusion. It was observed that 191 (44.63%) MRSA and 237 (55.37%) MSSA isolates were detected by cefoxitin disc diffusion method whereas in case of oxacillin disc diffusion method 183 (42.76%) were MRSA and 245 (57.24%) were MSSA. There was dissimilarity between the

numbers of MRSA isolates identified by the both methods. As per the guidelines of CLSI, the cefoxitin is the indicator drug for methicillin resistant in *S. aureus* through disc diffusion method, therefore it could be used for identification of methicillin resistance in different isolates of *S. aureus*. Velasco *et al.*^[15] and Duran *et al.*^[16] also observed that disc diffusion test using cefoxitin disc is now an authentic method for the identification of MRSA and this is far more superior method than other phenotypic methods (such as oxacillin disc diffusion and oxacillin screen agar test) that are used currently.

Among the 428 *S. aureus* isolates 191(44.63%) were MRSA and 237(55.37%) were MSSA. The predominant isolates were MSSA. Similarly, Ajantha *et al.*^[17] reported 33.64% MRSA and 66.35% MSSA, Whereas Singh *et al.*^[18] reported prevalence of MRSA and MSSA as 37.8% and 62.2% respectively, indicating predominance of MSSA. Whereas, Velvizhi *et al.*^[3] and Mallick *et al.*^[4] reported lower prevalence of MSSA isolates i.e., 26% and 48.3% respectively as compared to the present investigation.

The prevalence of iMLSB phenotype was 8.41% in the present study and the result was in accordance with the earlier studies.^[3,11,16] Similarly, Al-Kasaby and El-Khier^[2], reported prevalence of iMLSB isolates as 14.2%, 10.52%, 7.94%, 10.8% and 12.17%, respectively. Lall and Sahini^[19] and Fiebelkorn *et al.*^[8] reported 20.32% and 25.38% of inducible resistance respectively which is higher as compared to the present study. In the present research it was observed that prevalence of iMLSB resistance among MRSA and MSSA were 10.99% and 6.32% respectively. Several studies from different parts of India reported that the prevalence of MRSA strains among iMLSB phenotype was higher.^[1] Schreckenberger *et al.*^[20] and Levin *et al.*^[21] described higher prevalence of inducible resistance in MSSA than the MRSA.

In the present study the prevalence of cMLSB phenotype was 10.98%. Lall and Sahini^[19] reported constitutive resistant as 10.16%, Abbas *et al.*^[6] (2015) reported 11.6% and Urmil *et al.*^[22] reported 12%. On the contrary, Velvizhi *et al.*^[3] reported high prevalence of 32%. In the present study the prevalence of constitutive resistant included in MRSA and MSSA was 17.80% and 5.48% respectively. Whereas, Abbas *et al.*^[6] and Mallick *et al.*^[4] reported prevalence similar to the present study.

In the current study the prevalence of MS phenotype was 11.21% which is almost similar to the prevalence of cMLSB phenotype. Velvizhi *et al.*^[3] and Prabhu *et al.*^[11] reported similar results in comparison to the present study. However, Fiebelkorn *et al.*^[8] reported high prevalence of 32.30%. While some studies reported lower prevalence of about 1.58%^[4] and 3.04%^[2]. The prevalence of MRSA and MSSA among MS phenotypic isolates was 16.23% and 7.17%, respectively, which is agreement with the previous study conducted by Prabhu *et al.*^[11]

In the present study, *ermC* gene was detected in all the isolates whereas presence of *ermA* gene was not reported in any of the isolates of iMLSB phenotypes (Fig. 1). Therefore, *S. aureus* isolated at Tertiary care hospital in Punjab having *ermC* prevalence. Similarly, the studies conducted in Greece^[23] and Turkey^[24] reported high prevalence of *ermC* gene. On the contrary studies conducted in Canada^[25] and Germany^[26] the most common gene reported was *ermA*. The frequency of *erm* genes was variable in different studies.

Conclusion

It is concluded that D-test should be routinely done to avoid treatment failure due to clindamycin resistance. In addition PCR being a simple, quick, reliable and sensitive method could also be used in detection of inducible clindamycin resistance. The reason of lower prevalence of iMLSB phenotype in the present study could be due to the reason that samples included were mostly from the rural areas as the exposure of antimicrobial agents is less.

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