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Research Paper

Nasal Carriage of Methicillin Resistant Staphylococci with Inducible Clindamycin Resistance and *Pvl* Gene.

Microbiology

KEYWORDS : Nasal colonisation, Healthy children, Inducible clindamycin resistance, *pvl* gene.

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ABSTRACT

Anterior nares act as endogenous reservoir of S.aureus for clinical infections in the colonized individual and also as a source of cross-colonization for community spread in children. Clindamycin is used to treat staphylococcal skin and bone infections. Clinical failure of clindamycin therapy has been reported due to multiple mechanisms that confer resistance to macrolide lincosamide and streptogramin antibiotics. Hence, this study was taken up to screen for inducible clindamycin resistance among carrier isolates of staphylococci from orphanage children. A total of 222 samples were collected from the anterior nares of asymptomatic orphanage children in the community from which 92 S.aureus and 48 coagulase negative staphylococci (CoNS) were isolated. 26/92 (28%) S.aureus were MRSA and 15/48 (31%) CoNS were resistant to methicillin. pvl genes were detected in 29 isolates of which 10 were from MRSA and 19 were from MSSA. 24/140 (17%) were of the inducible MLSB (iMLSB) phenotype. All the isolates showing D-positive were found harbouring ermC gene.

Introduction

Staphylococcus aureus is a common pathogen responsible for community as well as hospital-associated infections. Community-Associated Methicillin-Resistant *Staphylococcus aureus* (CA-MRSA) has emerged as an important human pathogen. With a tropism for skin and soft tissue infections, osteomyelitis and lower respiratory tract disease, CA-MRSA now cause >50% of all community-acquired *S. aureus* infections (1). The anterior nares have been shown to be the main reservoir of *S. aureus* in both adults and children. *S. aureus* is transmitted to nares by contaminated hands and from surfaces where it can survive for months (2). The spread of colonization occur especially in close contact areas like schools, pre-schools or households (3) probably by the contaminated hands and surfaces. Nasal carriage is a significant risk factor for staphylococcal infection, with >80% of infecting isolates originating from the nose (4, 5).

Clindamycin is one of the important alternative antibiotics in the therapy of *S.aureus* infections. Clinical failure of clindamycin therapy has been reported due to multiple mechanisms that confer resistance to macrolides, lincosamides and Streptogramin B (MLSB) antibiotics. In vitro routine tests for clindamycin susceptibility may fail to detect inducible clindamycin resistance due to *erm* genes resulting in the treatment failure (6). This study was therefore taken up to screen for inducible clindamycin resistance amongst carrier isolates of staphylococci and molecular detection of *erm* genes.

Materials and Methods

Samples were collected from the anterior nares of healthy children belonging to orphanage of low socio-economic status with the help of a sterile cotton swab by swabbing of their anterior nares. The swabs were rubbed well by rotating five times over the inner wall of the nasal septum and were transported in salt nutrient broth (7.5%) to the laboratory and were processed. Based on the colony morphology and gram staining and mannitol fermentation, the gram-positive cocci in clusters were further identified based on the biochemical methods as per standard protocols.

Screening for methicillin resistance

1) Phenotypic Method

a) Cefoxitin disc diffusion method

All the isolates were subjected to cefoxitin disc diffusion test using a 30 µg disc. A 0.5 Mc Farland standard suspension of the isolate was made and lawn culture was done on Mueller-Hinton agar (MHA) plate. Plates were incubated at 37^0C for 24hrs and zone diameters were measured. An inhibition zone diameter of ≤ 19 mm was reported as methicillin resistant and ≥ 20 mm was considered as methicillin sensitive (7).

b) Oxacillin agar screening method

MHA plates containing 4% NaCl and 6 µg/ml of oxacillin were prepared. Plates were inoculated with 10 µL of 0.5 Mc Farland suspension of the isolate by spot inoculum and were incubated at 35^0C for 24hrs. Plates were observed carefully in transmitted light for any growth. Any growth after 24 hrs was considered methicillin resistant (8).

2) Molecular detection of *mecA, femA* and *pvl* genes

MRSA isolates were detected by multiplex PCR using *mecA* and *femA* by the method of Kondo et al., 2007 (9) and Berger et al., 1989 (10) along with the detection of *pvl* gene by the method of Lina et al., 1999 (11). The following were the primer sequences used in the study-

Target Gene	Oligonucleotide (primer) Sequence	Product Size
mecA Forward	F: 5' – TGC TAT CCA CCC TCA AAC AGG – 3'	286 bp
mecA Reverse	R: 5' – AAC GTT GTA ACC ACC CCA AGA – 3'	
femA Forward	F: 5' – AAA AAA GCA CAT AAC AAG CG – 3'	132bp
femA Reverse	R: 5' – GAT AAA GAA GAA ACC AGC AG – 3'	
pvl Forward	F: 5' – ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC A – 3'	433bp
pvl Reverse	R: 5' – GCA TCA AST GTA TTG GAT AGC AAA AGC – 3'	

PCR was performed in a 25µl reaction with 10X standard PCR buffer {100 mM Tris-HCl pH 8.3, 500 mM KCl; 1.5 mM MgCl2}, 200mM dNTP mix (Sigma), 25pmol of each primer (Sigma), 2.5U of Taq DNA polymerase and 1µL template DNA. Amplification was performed with initial denaturation at 94°C for 5 min, followed by denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 5 mints. The PCR products were analyzed in a 2% agarose gel in 1xTBE buffer. Ethidium bromide stained DNA amplicons were visualized using a gel imaging system.

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Erythromycin induced clindamycin resistance (D-Test)
Isolates that were erythromycin resistant were tested for inducible resistance by the 'D test' as per CLSI guidelines. Erythromycin (15 µg) disc was placed at a distance of 15 mm (edge to edge) from clindamycin (2 µg) on MHA plates inoculated with 0.5 Mc Farland bacterial suspensions. Plates were analyzed after 24 hrs of incubation at 37^0C. Interpretation of the inhibition zone diameters was as follows: If an isolate was erythromycin resistant and clindamycin susceptible, with a D-shaped inhibition zone around the clindamycin disc, it was considered to be positive for inducible resistance (D test positive, iMLSB phenotype). If the isolate was erythromycin resistant and clindamycin susceptible, with both zones of inhibition showing a circular shape, the isolate was considered to be negative for inducible resistance (D test negative, MS phenotype), but to have an active efflux pump. If the isolate was erythromycin resistant and clindamycin resistant, the isolate was considered to have the Macrolide–Lincosamide–Streptogramin B constitutive (cMLSB phenotype) resistance (12).

Molecular detection of *erm* genes
Genotypic detection of clindamycin resistance was carried out by using *ermA*, *ermB*, and *ermC* gene by the method of Sidhu et al.,2002(13). The following were the primer sequences used in the study-

Target Gene	Oligonucleotide (primer) Sequence	Product Size
ermA Forward	5'-TAT CTT ATC GTT GAG AAG GGA TT-3'	139bp
ermA Reverse	5'-CTA CAC TTG GCT TAG GAT GAA A-3'	
ermB Forward	5'-CTA TCT GAT TGT TGA AGA AGG ATT-3'	142bp
ermB Reverse	5'-GTT TAC TCT TGG TTT AGG ATG AAA-3'	
ermC Forward	5'-CTT GTT GAT CAC GAT AAT TTC C-3'	190bp
ermC Reverse	5'-ATC TTT TAG CAA ACC CGT ATT C-3'	

PCR was performed in a 25µl reaction with 10X standard PCR buffer {100 mM Tris-HCl pH 8.3, 500 mM KCl; 1.5 mM MgCl2}, 200mM dNTP mix (Sigma), 25pmol of each primer (Sigma), 2.5U of Taq DNA polymerase and 1µL template DNA. Amplification was performed with initial denaturation at 94°C for 5 min, followed by denaturation at 94°C for 1 min, annealing at 55°C for 30 sec, extension at 72°C for 1 min and final extension at 72°C for 5 mints. The PCR products were analyzed in a 2% agarose gel in 1xTBE buffer. Ethidium bromide stained DNA amplicons were visualized using a gel imaging system.

Results
A total of 222 samples were collected from the anterior nares of healthy children (orphanage) in the community. 92 were found to be *S.aureus* and 48 were found to be CoNS.

Screening for methicillin resistance:
Phenotypic Method
Screening for methicillin resistance by cefoxitin disc diffusion method and oxacillin agar screening method showed that 26/92 (28%) *S.aureus* and 15/48 (31%) coagulase negative staphylococci were found to be methicillin resistant.

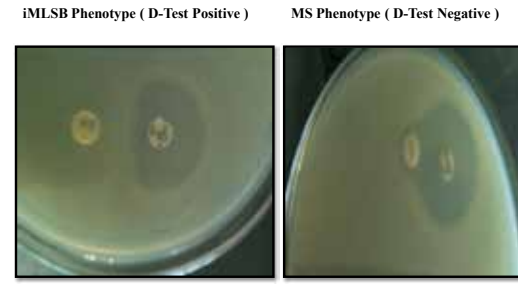
D-Test
Among the 140 staphylococcal isolates which were studied, 72/140 (51%) were erythromycin resistant. These isolates were subjected to the D test. 24/140 (17%) were D-zone positive i.e. of the inducible MLSB (iMLSB) phenotype which were resistant to erythromycin and sensitive to clindamycin, while 48/140 (34%) were negative for the D test, thus indicating that they were of the MS phenotype (Table 1 and Fig 1).

Table 1: Prevalence of erythromycin induced clindamycin resistance in staphylococcal isolates.

Type of organism	Total	ER-S CL-S	iMLSB phenotype (D positive)	cMLSB phenotype	MS phenotype (D negative)
MRSA	26	8 (31%)	7 (27%)	-	11 (42%)
MSSA	66	40 (61%)	8 (12%)	-	18 (27%)
MRCoNS	15	5 (33%)	3 (20%)	-	7 (47%)
MSCoNS	33	15 (46%)	6 (18%)	-	12 (36%)

Legend: ER-S - Erythromycin Sensitive, CL-S - Clindamycin Sensitive, iMLSB- Inducible Macrolide–Lincosamide–Streptogramin resistance (D-positive), cMLSB- Constitutive Macrolide–Lincosamide–Streptogramin resistance, MS- erythromycin resistant and clindamycin sensitive (D- negative).

Fig: 1 D-Test

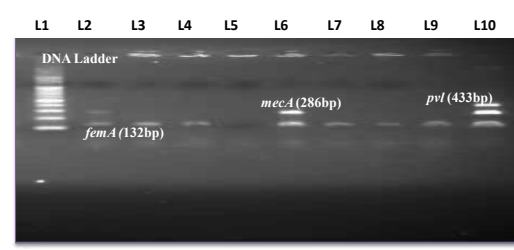


Erythromycin resistant and clindamycin sensitive staphylococcal isolate giving D shaped zone of inhibition around clindamycin with flattening towards erythromycin disc.

Erythromycin resistant and clindamycin sensitive staphylococcal isolate with circular zone of inhibition around clindamycin.

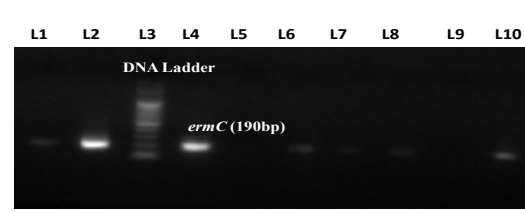
Genotypic Method:
A total of 26/92 (28%) and 15/48 (31%) staphylococci were found to harbour *mecA* gene confirming them as MRSA and methicillin resistant coagulase negative staphylococci (MR-CoNS). 92/140 staphylococci were found to be positive for the presence of *femA* gene. 29/140 (21%) showed the presence of *pvl* genes- 10 of which were from MRSA and 19 were from MSSA (Fig-2). All the isolates showing D-positive were found harbouring *ermC* gene (Fig-3). The results correlated with the findings of the phenotypic methods.

Fig: 2 Gel picture showing *femA*, *mecA* and *pvl* genes



L1- 100 bp DNA Ladder, L2- MRSA, L3-MSSA, L4-MSSA, L5-MSCoNS, L6-MRSA, L7-MSSA, L8-MSSA, L9-MSSA, L10-*pvl* MRSA

Fig:3 Gel picture showing *ermC* gene



L1, L2, L4, L6, L7, L8 and L10- *ermC* gene positive, L5 and L9-*ermC* gene negative, L3-100 bp DNA Ladder

Discussion:

S.aureus is an important pathogen associated with nosocomial and community-acquired infection. Several reports have documented an increase in infections caused by methicillin resistant *S.aureus*, mostly affecting children in several geographic regions. Anterior nares are the best ecological niches for *S.aureus* (14) and *S.aureus* nasal carriers may transmit the pathogen to others. It subsequently causes infections in susceptible hosts (15, 16).

In the present study, 222 nasal swabs were collected from inmates of orphanage. A total of 92 were found to be *S.aureus* and 48 were found to be coagulase negative staphylococci. 26/92 (28%) *S.aureus* were MRSA and 66/92 (72%) were MSSA. 15/48 (31%) coagulase negative staphylococci were resistant to methicillin and 33/48 (69%) were sensitive to methicillin.

pvl gene was detected in 29/92 (31%) *S.aureus* isolates in this study. 10 were from MRSA and 19 were from MSSA. Nasal carriage of *PVL* MRSA and MSSA found in this study may represent a risk of skin and soft tissue infections for colonized children. These strains can cause skin and soft tissue infections. Studies have suggested that environmental factors like prior antibiotic use, contact with a healthcare facility, poor socioeconomic conditions and overcrowding are involved in the increase of CA-MRSA nasal carriage (17, 18). Our study shows 38% (10/26) of nasal MRSA isolates were found to be positive for *pvl* gene which was lower than 58% *pvl* gene in nasal MRSA reported by Ellis et al.,2004.

Fiebelkorn et al., 2003 reported 28% (19) and Dizbay et al., 2008 reported 90% (20) of their staphylococcal strains were of the iMLSB phenotype. In our study, 24/140 (17%) were D-zone positive i.e. of the inducible MLSB (iMLSB) phenotype, while 48/140 (34%) were negative for the D test, thus indicating that they were of the MS phenotype. In our study, out of the 26 MRSA, 7 (27%) were of the iMLSB phenotype, lower than several studies from different parts of India reporting 30% to 64% of their MRSA strains to be of iMLSB phenotype (21). Out of 66 MSSA, 8 (12%) showed iMLSB phenotype while other researchers found that 4% to 15% of their MSSA strains were of the iMLSB phenotype. It was also observed that percentages of inducible clindamycin resistance were higher amongst methicillin resistant staphylococci as compared to methicillin sensitive staphylococci. This was in concordance with studies reported by Yilmaz et al., 2007 (22). In our study, none of the staphylococcal isolates showed cMLSB phenotype which was found to be in agreement with that of Angel et al.,2008(23).

Among the CoNS, out of 15 MRCoNS, 3 (20%) showed iMLSB phenotype which was much lower than 35% as reported by Paul (24). Out of 33 MSCoNS, 6 (18%) showed iMLSB phenotype which was found to be in agreement with findings of Yilmaz et al., 2007. All the isolates showing D-positive were found harbouring *ermC* gene. This was found to be in agreement with Spiliopoulou et al's.,(2004) findings (25) who reported *ermC* gene to be more prevalent among D-positive isolates. To our knowledge, this is the first study among orphanage population to screen for inducible resistance and *pvl* gene.

Conclusion

The present study shows high degree of nasal carriage of methicillin resistant staphylococci which are also showing inducible clindamycin resistance (both phenotypically by D-test and by the presence of *ermC* gene) and were also positive for *pvl* gene indicating risk of infection by resistant organisms potentially more virulent due to presence of *pvl* gene. To our knowledge, this is the first study of carrier staphylococcal isolates for inducible clindamycin and *pvl* gene detection from South India.

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