



SCREENING OF *ERM* GENE OF INDUCIBLE CLINDAMYCIN RESISTANT *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

Antibiotic resistance exhibited by *Staphylococcus aureus* is a growing global concern. This work was undertaken to determine the prevalence rate of inducible clindamycin resistant *S. aureus* in nasal sample and detect *ermB* gene in the isolates with inducible clindamycin resistance. Nasal swabs were collected from the school children and cultured on Mannitol Salt Agar (MSA) and Blood Agar (BA) for observation of colony morphology. Gram staining and biochemical test (catalase, oxidase, O-F and coagulase) were performed for further identification of the bacteria. The Kirby-Bauer disc diffusion method using a cefoxitin disc (30 µg) was used to detect methicillin resistant *S. aureus* (MRSA). All the MRSA isolates were tested for *ermB* gene by PCR amplification. Among 64 *S. aureus* isolates, 17 (26 %) were MRSA. The prevalence of Inducible clindamycin resistant *S. aureus* (iMLS_B) isolates was 23.4 % in the *S. aureus* isolates. All the isolates of MRSA were resistant to penicillin, while 88.2 % were sensitive to gentamicin. The prevalence of *ermB* gene was 3.1 % in the total *S. aureus* isolates and 11.7 % MRSA showed the presence of this gene. Routinely performing a D-test in laboratory will guide the clinicians on the rationale use of clindamycin and improving hygienic practices can reduce the spread of inducible clindamycin resistance.

Keywords: Methicillin resistant *S. aureus*, Coagulase test, *erm* gene, Inducible clindamycin resistant *S. aureus* (iMLS_B), D-test.

INTRODUCTION

Staphylococcus aureus (especially MRSA) can cause minor skin infections to life threatening infections. Decreased susceptibility to vancomycin among *S. aureus* is of great concern and challenge (Tiwari & Sen, 2006). This has encouraged physicians to prescribe alternative antibiotics such as macrolide-lincosamide-streptogramin (MLS) (Prabhu *et al.*, 2011). Clindamycin (CL) under lincosamide family is the best chosen to treat infection caused by both MRSA and MSSA because of its low cost, fewer side effects and high bioavailability (Baral & Khanal, 2017). MLS group of antibiotics exhibit three mechanisms of resistance which include: macrolide efflux pump, target site modification and enzymatic antibiotic inactivation (Kasten, 1999; Leclercq, 2002). Methylase enzyme produced by *erm* genes is responsible to modify the ribosomal target site, and as a result the antibiotic would not be able to bind target, which leads to constitutive and inducible resistance. Consistently expressing *erm* gene and exhibiting resistance to erythromycin, clindamycin and other members of MLS family are the features of a constitutive resistance phenotype. Inducible phenotype shows resistance to clindamycin in presence of erythromycin, a strong inducer of methylase enzyme. This isolate is resistant to erythromycin and susceptible to clindamycin in-vitro. Treatment with CL for this kind of phenotypes leads to treatment failure (Drinkovic *et al.*, 2001; Deotale *et al.*,

2010). Lack of identifying inducible clindamycin resistance leads to false reporting in lab, which could lead to treatment failure if CL is used therapeutically.

Phenotypic detection of this resistance is done by using disk diffusion test (especially D-test) because of being reliable, inexpensive and easy interpretation with high sensitivity and specificity (CLSI, 2015). To our knowledge, assessment of *erm* genes and their prevalence in *S. aureus* have not been done in Nepal. So, the objective of this study was to provide information on the prevalence of these genes in the community which would be a proper frame work for future researchers as well as health professional in prescribing antibiotics to address antibiotic resistance.

MATERIALS AND METHODS

A cross-sectional study was conducted to isolate *S. aureus* from nasal swab samples collected from two different schools of Kathmandu (Kirtipur Secondary and Mangal Secondary school). A total of 160 samples were collected for six months period (April-September 2018). A sterile cotton swab pre-matted with sterile saline was rotated 2 cm inside the vestibule of both anterior nares of the selected participant. The collected samples were then delivered in Nutrient broth within 4 hours of collection to the laboratory (Cheesebrough, 2006). With the permission of school principal, consent from school children as well as their immediate guardian was taken for the collection of nasal swabs. Ethical approval for this study was taken

from Nepal Health Research Council (NHRC), Nepal (Reg no.: 195/2018).

Isolation and identification of bacteria

Colony morphology and Gram staining were used as preliminary criteria for identification of *S. aureus*. The nasal swabs collected were streaked on Mannitol salt agar (MSA) and Blood agar (BA), incubated at 37° C for 24 hours. Beta hemolytic colonies on blood agar and typical mannitol fermenting colonies on MSA were observed. Mannitol fermenting colonies were sub-cultured on nutrient agar (NA) and incubated at 37° C for 24 hours. Pin-point size colonies on BA and golden yellow colony on NA having round, opaque, convex and smooth-glistening colonies with diameter 2-3 mm were indicative of *S. aureus*. Catalase, oxidase, O-F and coagulase (free and bound) test were performed for confirmation.

Antibiotic susceptibility testing

Antimicrobial susceptibility of the isolates was determined by Kirby-Bauer disk diffusion method following CLSI guidelines (CLSI 2015). After emulsifying 1-2 colonies in nutrient broth and incubating for 4 hours, the turbidity was compared with 0.5 McFarland standards. A sterile cotton swab was used to inoculate sample on Muller-Hinton agar and allowed to dry. The tested antibiotics were; penicillin (10 U), cefoxitin (30 µg), gentamycin (10 µg), erythromycin (15 µg), clindamycin (2 µg) and ciprofloxacin (5 µg). Results were interpreted based on the diameter of zone of inhibition.

Screening of MRSA

S. aureus isolated were screened for methicillin resistance by modified Kirby Bauer disc diffusion method using cefoxitin disc (30 µg) as per the standard guidelines. Then antibiogram of MRSA was done by Kirby Bauer disc diffusion method using Muller Hinton Agar (Nunes *et al.*, 1999). The standardized suspension of isolate was swabbed uniformly over entire surface of a sterile MHA plate. Using sterile forceps, selected antibiotics discs were placed on inoculated plate no closer than 15 mm from the edge and 24 mm from center of discs. The plates were then left at room temperature for 15 minutes for pre-diffusion and then incubated at (35-37 °C). On the basis of zone-size compared with that of control strains, the results were interpreted by standard interpretative guideline (CLSI, 2015).

Disk approximation test with erythromycin and clindamycin (D-zone test)

Inducible clindamycin resistance was determined using disk approximation test with erythromycin and clindamycin (D-zone test) as recommended by CLSI guidelines (CLSI, 2015). Briefly, 0.5 McFarland suspensions was prepared with organisms from an

overnight growth, inoculated and spread over the surface on Mueller-Hinton's agar plates. One erythromycin disk (15 µg) and one clindamycin disk (2 µg) were placed on the inoculated plates at the distance of 15 mm from each other and incubated at 35° C and read after 18 hr. Inducible clindamycin resistance was confirmed by forming of a flattening shape of the clindamycin inhibition zone (D shape) around the erythromycin disk which indicated that erythromycin have induced clindamycin resistance.

Amplification of *ermB* gene

DNA amplification was performed using specific primers for detection of *ermB* gene. Primers used for PCR were F: 5'-CATTTAACGACGAAACTGGC-3' and R: 5'-GGAACATCTGTGGTATGGCG-3' to amplify a 142 bp amplicon (Moosavian *et al.*, 2014). PCR amplification was performed according to conditions described in previous studies with some modification (Moosavian *et al.*, 2014). Each reaction was carried out in final volume of 25 µl with master mix (12.5 µL), forward primer (0.5 µL), reverse primer (0.5 µL), DNA template (4 µL) and 7.5 µL ddH₂O. Amplification conditions of initial denaturation (95° C /2 min) followed by, denaturation (95° C /30 sec), annealing (50.2° C /30 sec), extension at 72° C /30 sec for 30 cycles, final extension at 72° C /5 min. PCR products were analyzed on 1.5 % agarose gel by electrophoresis, stained with 0.5 µg/mL E thidium bromide solution and visualized under UV transilluminator (Moosavian *et al.*, 2014). A reaction containing all materials except DNA was used as a negative control. A native isolate harboring *ermB* gene was used as a positive control.

RESULTS

Among 160 isolates of nasal swab, 64 isolates were *S. aureus* out of which 26.56 % (n=17) were MRSA (Fig. 1). Among 64 *S. aureus* isolates, 23.4 % (15) was positive for inducible clindamycin resistance (Fig. 2).

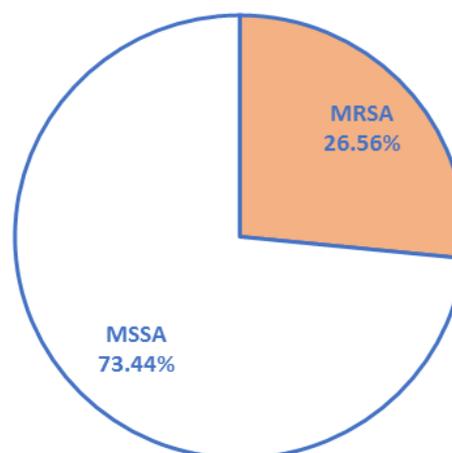


Fig. 1. Nasal carriage rate of MRSA and MSSA

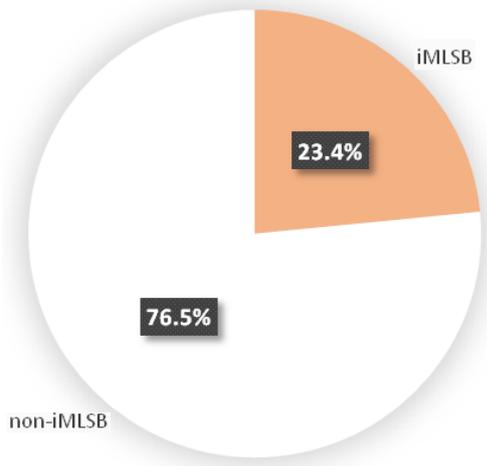


Fig. 2. Nasal carriage rate of iMLS_B

The results of antimicrobial susceptibility test showed that all isolates were resistant to penicillin and ceftazidime (100 %) and most of them were sensitive to gentamycin (88.2 %), as depicted in Table 1. Highest prevalence of MRSA and iMLS_B resistance was found in children of age group 13-15 (Table 2). Three MRSA isolates were inducible clindamycin resistant (Fig. 3). Similarly, 142 bp size *ermB* gene was amplified corresponding to the inducible clindamycin resistant strains (Fig. 4).

Table 1. Antibiotic susceptibility pattern of MRSA

Antibiotics used	Susceptibility patterns		
	Sensitive (%)	Intermediate (%)	Resistant (%)
Gentamycin (10 µg)	16 (94.1)	-	1 (5.8)
Erythromycin (15 µg)	4 (23.5)	3 (17.6)	10 (58.8)
Clindamycin (2 µg)	12 (70.5)	2(11.7)	3 (17.6)
Penicillin (10 units)	0	0	17 (100)
Ciprofloxacin (5 µg)	6 (35.2)	1 (5.8)	10 (58.8)

Table 2. Distribution of iMLS_B among age group

Age group	Female		Male	
	No. of MRSA	iMLS _B MRSA (%)	No. of MRSA	iMLS _B MRSA (%)
10-12	3	2 (66.6)	1	1 (100)
13-15	7	6 (85.7)	5	4 (80)
16	1	0	0	0



Fig. 3. D-test exhibiting inducible clindamycin resistant in *S. aureus* isolates

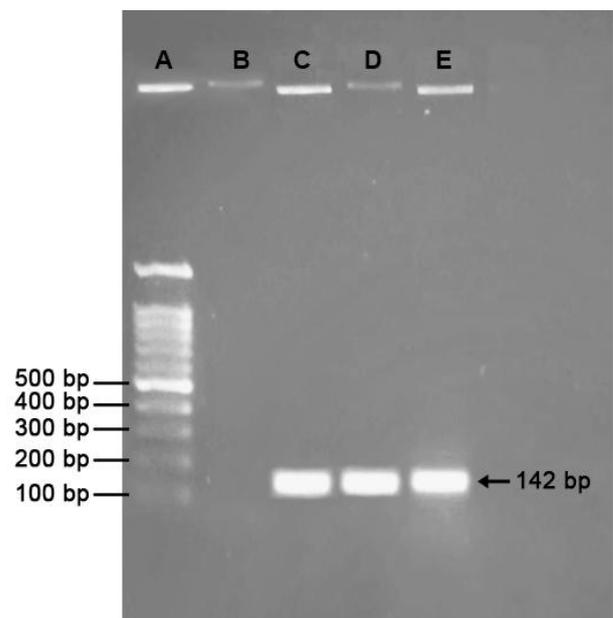


Fig. 4. PCR amplification of *ermB* gene in Staphylococcal isolates

Lane A- 100 bp DNA ladder, Lane B-negative control, Lane C- Positive control (142 bp), Lane D, E- samples positive for *ermB* gene

DISCUSSION

In our study, the prevalence of MRSA was 26.5 % which is consistent to recent studies (Pandey *et al.*, 2012; Sanjana *et al.*, 2010; Tiwari *et al.*, 2009) that showed the prevalence rate between 26-69 %. Also, the prevalence of iMLS_B resistance (23.45 %) was consistent to recent studies (Adhikari *et al.*, 2017; Baral & Khanal, 2017; Manandhar *et al.*, 2018) which showed the prevalence rate between 11.8-69.6 %. The iMLS_B resistance was found

higher in 13-15 years age group whereas none of the samples from age group 16 showed iMLS_B resistance. This difference in age group may be due to awareness of personal hygiene and rational use of antibiotics because they are living in the robust era of social media and internet. Studies have shown that social media and TV advertisements regarding infectious disease help eliciting behavioral change in people and control the spread of infectious disease (Misra *et al.*, 2018). Widespread use of social media among children of 16 or more has exposed them to such awareness campaigns about antibiotics. In the antibiotic sensitivity test, higher number of isolates were resistant towards penicillin (100 %) followed by erythromycin (58.8 %), ciprofloxacin (58.8 %), clindamycin (17.6 %) and gentamycin (5.8 %).

Furthermore, high prevalence of iMLS_B resistance among MRSA isolates denotes that antibiotic resistance is increasing at alarming rate. Low socio-economic condition, inadequate access to drinking water, crowding, overpopulation and uncontrolled air pollution can be the major cause for the elevation in resistance. The prevalence of *ermB* gene among *S. aureus* isolates was 3.1 % whereas the prevalence rate among iMLS_B isolates was 13.3 %. Previous studies are equivocal on the rate of prevalence where it ranged from none (Moosavian *et al.*, 2014) to 22.2 % (Havaei *et al.*, 2016). Several other studies (Aydeniz *et al.*, 2015; Fasihi *et al.*, 2016) including our study was in between this range of prevalence rate.

Since, researches on *erm* genes have not been reported in Nepal, this study may provide baseline data to design future studies aimed to determine the prevalence of different *erm* genes in different geographical locations of Nepal. Either MRSA or MSSA, approaches should be made to decolonize them from the carriers to prevent the spread and outbreak. Previous approaches include: screening, isolation and topical decolonization of patients (Melzer *et al.*, 2004). If we are able to control MRSA and MSSA spreads in community, inducible clindamycin resistance can also be prevented.

CONCLUSION

The present study showed that the rate of nasal carriage of MRSA was 26.5 % (17/64) and inducible clindamycin resistance prevalence rate was 23.4 % (15/64). Number of MRSA and iMLS_B colonization was higher in 10-15 years age group. This study also revealed that children of age 16 were not colonized with MRSA which can prove the effectiveness of media and TV. Since, *S. aureus* are resistant to the commonly used antibiotics, there is a need of effective control policies on antibiotic use. Diagnosis of this inducible clindamycin resistance type on routine basis is of clinical importance. Laboratory D-test should be made mandatory in hospitals so that D-test positive isolates can be avoided to be treated with clindamycin.

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