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Distribution of *Mec*A and *Erm* Genes among Methicillin-resistant *Staphylococcus Aureus* with Inducible Resistance to Clindamycin

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ABSTRACT

Background: The emergence of Methicillin-resistant *Staphylococcus aureus* and its ability to confer cross-resistance to macrolide-lincosamide-streptogramin B has complicated the treatment against it. Gene-based studies among phenotypic methicillin-resistant isolates with inducible resistance to clindamycin are less available in Nepal. This work was undertaken to detect the *mec*A and *erm* genes among such phenotypes isolated from clinical samples.

Methods: *S. aureus* isolated from different clinical samples was identified by standard microbiological procedures (Gram-staining, colony morphology, and different biochemical tests). Methicillin-resistant and inducible resistant to clindamycin phenotypes were detected by using cefoxitin disc (30 µg) and a double disk diffusion test according to the Clinical and Laboratory Standards Institute guidelines and *mec*A and *erm* genes were detected by polymerase chain reaction.

Results: Among 120 *S. aureus* isolates, 51.67% (n=62) were MRSA, and the prevalence of inducibly-resistant, constitutively-resistant and Macrolide-Streptogramin phenotypes were 15.83% (n=19), 28.33% (n=34) and 15.83% (n=19) respectively. While 35.84% (n=43) of isolates showed sensitivity to both antibiotics, erythromycin and clindamycin. Out of 14 inducibly-resistant phenotypes, 57.14% (n=8) were found carrying *erm*C and 28.57% (n=4) phenotypes contained both *erm*A and *erm*C. All phenotypes were positive for the *mec*A gene.

Conclusions: Macrolides-Lincosamide-Streptogramin B resistance was predominant among methicillin-resistant *S. aureus*. While all isolates with inducible clindamycin resistance harbored *mec*A gene, most of them also harbored *erm*C gene. The higher prevalence of inducible-resistant to clindamycin indicated the need for rational use of antimicrobial agents.

Keywords: *Erm* gene; iMLS$_B$ resistance; *mec*A; methicillin-resistance; MRSA

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is the leading cause of both healthcare and community-associated infections with significant morbidity and mortality.[1] The severity of MRSA infections ranges from mild skin infections to life-threatening fulminant infections like septicemia, pneumonia, meningitis, necrotizing fasciitis, and infective endocarditis.[1-3] The use of macrolide-lincosamide-streptogramin B (MLS$_B$) antibiotics is often considered an alternative approach to manage MRSA infection.[4-6] Clindamycin has been used as empiric therapy for the rising incidence of MRSA, particularly in Nepal. But, the rise in the rate of inducible resistance to clindamycin is a threat to infection management.[4,7,8] This study was undertaken to determine the frequency of *mec*A and *erm* genes among phenotypic MRSA with inducible resistance to clindamycin; the findings of the present study could prevent therapeutic failure in MRSA infection in hospital settings.

METHODS

This was a hospital-based cross-sectional and prospective

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study. This study was carried out at Om Hospital & Research Centre, Kathmandu from June 2018 to March 2019. Ethical clearance (Ref No. 1877) for this study was obtained from Nepal Health Research Council (NHRC). Written informed consent was taken from all the patients before sample collection. For children under 18 years of age, informed consent was obtained from a parent and/or legal guardian, and the study was performed according to the principles expressed in Declaration of Helsinki. Since this study did not use any identifying information or images of any patients, patients consent for publication is not applicable.

All the specimens (pus, blood, and urine) from all the patients who attended Om hospital during the study period, and provided a consent to be enrolled in the study were included in the study. Specimens from patients who denied to be enrolled, and improperly labeled specimen were excluded for further processing. A total of 1140 clinical specimens (pus from different body part, n=278; blood-n=384; and urine-n=478) were collected from in- and out-patients and processed within 1-2 hours. Specimens were collected by trained and experienced laboratory professionals of the hospital.

All the clinical specimens were streaked on Mannitol salt agar (MSA) and Blood Agar (BA), and incubated at 37° C aerobically for 24 hours. Mannitol fermenting colonies were sub-cultured on Nutrient Agar (NA) at 37° C aerobically for 24 hours for further identification. *S. aureus* was identified based on colony morphology, Grams staining, and biochemical tests such as catalase, coagulase (free and bound), oxidative-fermentative (O/F) test and DNase tests.

The antibiotic susceptibility pattern of isolates was determined by the modified Kirby-Bauer disc diffusion method in Mueller-Hinton Agar (MHA) according to the Clinical Laboratory Standard Institute (CLSI-2018). The tested antibiotics were ampicillin (10 µg), cefoxitin (30 µg), ciprofloxacin (30 µg), clindamycin (2 µg), cotrimoxazole (30 µg), erythromycin (15 µg), gentamicin (10 µg), levofloxacin (5 µg), nitrofurantoin (300 µg) and vancomycin (30 µg). MRSA phenotypes were screened using a cefoxitin disk (30 µg). Isolates with a diameter of the zone of inhibition ≤21 mm were considered methicillin-resistant.

The D-test method was performed using clindamycin (2 µg) and erythromycin (15 µg) disks. For this purpose, suspensions of bacteria were prepared in sterile saline (2 mL) equivalent to standard 0.5 McFarland, which was swabbed in an MHA plate using a sterile cotton swab, and then two antibiotics were placed on Muller-Hinton Agar (MHA) media at 15 mm distance (edge-edge). Isolates with a flat zone of growth inhibition to clindamycin near the erythromycin disk (D-shape) were classified as $iMLS_B$ (D-positive), while those with a clear zone were classified as MS resistant (D- test negative) based on CLSI-2018.

Amplification of *mec*A, *erm*A, *erm*B, and *erm*C genes

DNA amplification was performed using specific primers and conditions described in previous studies without significant modifications.[9] A summary of the PCR amplification of different genes is presented in table 1.

Table 1. PCR primers and amplification condition.

Gene targeted	Primers used	Amplicon size(bp)	Amplification condition		
			Stage	Temperature, Time	
mecA	F: 5'-ACT GCT ATC CAC CCT CAA AC -3' R: 5'-CTG GTG AAG TTG TAA TCT GG -3'	163	Initial denaturation	94°C, 2 min	
			Denaturation Annealing Extension	95°C, 30 sec 54.2°C, 30 sec 72°C, 20 sec	29 cycles
			Final extension	72°C, 5 min	
ermA	F: 5'- AAG CGG TAA ACC CCT CTG A- 3' R 5'-TTC GCA AAT CCC TTC TCA AC-3'	190	Initial denaturation	94°C, 4 min	
			Denaturation Annealing Extension	94°C, 30 sec 55°C 30 sec 72°C, 30 sec	30 cycles
			Final extension	72°C, 4 min	
ermB	F: 5'- CAT TTA ACG ACG AAA CTG GC- 3' R 5'-GGA ACA TCT GTG GTA TGG CG - 3'	405	Initial denaturation	95°C, 2 min	
			Denaturation Annealing Extension	95°C, 30 sec 50.2°C 30 sec 72°C, 50 sec	32 cycles
			Final extension	72°C, 5 min	

ermC	F: 5'- GGA ACA TCT GTG GTA TGG CG - 3' R 5'- TAA TCG TGG AAT ACG GGT TTG -3'	299	Initial denaturation Denaturation Annealing Extension Final extension	95°C, 2 min 95°C, 30 sec 52.4°C 30 sec 72°C, 30 sec 72°C, 5 min	29 cycles

PCR products were analyzed by separating them on 1.8% agarose gel by electrophoresis, stained with 0.5 µg/ mL Ethidium Bromide, and visualized under a UV transilluminator. [9] A reaction containing all materials except DNA templet was used as a negative control and multiple isolates harboring *mec*A, *erm*A, *erm*B, and *erm*C genes were used as a positive control for *mec*A and *erm* genes. A 100 bp ladder from GeneDireX, Inc. was used to identify the size of amplified products.

All the data obtained was analyzed using SPSS version 25 for Windows. Pearson Chi-square test was used for analyzing bi-variant association. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Among 1140 specimens processed, staphylococci growth was observed in 273 specimens (pus: 137, blood: 61, and urine: 75). Out of total staphylococci, 153 isolates (pus: 62, blood: 38, urine: 53) were coagulase-negative staphylococci (CoNS), and the rest 120 (pus: 75, blood: 23, urine: 22) were *S. aureus* isolates.

Among all the *S. aureus* isolates, most of the isolates (n=87) were ampicillin-resistant and 98 isolates were gentamicin sensitive. But all 120 isolates were susceptible to vancomycin (Table 2). Sixty-two of the total *S. aureus* isolates (pus: 37, blood: 14, urine: 11) were MRSA, and the others were MSSA. fourteen (pus: 11, blood: 2, urine: 1) MSSA isolates were MDR. Among all *S. aureus* isolates, 34 (pus: 17, blood: 8, urine: 9) were cMLSB, of which 24 were MRSA. Nineteen of the *S. aureus* isolates (pus: 15, blood: 2, urine: 2) were iMLSB, of which 14 were MRSA (Table 3). Statistically, MLS_B resistant phenotypes ($cMLS_B$ & $iMLS_B$) were significantly associated with MRSA (p=0.014 & p=0.04 respectively) (Table 3).

The PCR was performed on 14 iMLSB MRSA isolates, all of which were found to harbor the *mec*A gene. *erm*A, *erm*B, and *erm*C genes were detected in 4, 4, and 8 isolates, respectively. Two isolates harbored both *erm*A and *erm*B gene, 4 isolates harbored both *erm*A and *erm*C genes, and 4 isolates harbored both *erm*B and *erm*C genes.

Table 2. Antibiotic susceptibility pattern of S. aureus

Name of antibiotics, Potency (µg/disc)	Sensitive, N (%)	Resistant, N (%)
Erythromycin (15)	47 (39.17)	73 (60.83)
Clindamycin (2)	64 (53.33)	56 (46.67)
Cefoxitin (30)	58 (48.33)	62 (51.67)
Ciprofloxacin (5)	68 (56.67)	52 (43.33)
Ampicillin (10)	33 (27.50)	87 (72.50)
Gentamicin (10)	98 (81.67)	22 (18.33)
Levofloxacin (5)	91 (75.83)	29 (24.17)
Cotrimoxazole (25)	74 (61.67)	46 (38.33)
Vancomycin (5)	120 (100)	-
Nitrofurantoin* (300)	22 (100)	-

**For Isolates from Urine*

Table 3. Distribution of different resistant phenotypes among *S. aureus* isolates.

Phenotype	Phenotype			p-value
	MRSA, N (%)	MSSA, N (%)	Total, N (%)	
E-S, CD-S	14 (22.58)	29 (50.0)	43 (35.84)	0.002*
E-S, CD-R	2 (3.22)	3 (5.17)	5 (4.17)	0.672
E-R, C-R ($cMLS_B$)	24 (38.71)	10 (17.24)	34 (28.33)	0.014*
E-R, CD-S ($iMLS_B$)	14 (22.58)	5 (8.62)	19 (15.83)	0.046*
E-R, CD-S (MS)	8 (12.90)	11(18.96)	19 (15.83)	0.455
Total	62 (51.67)	58 (48.33)	120 (100)	

**significant (According to Chi-square test); E-Erythromycin, CD-Clindamycin, S-sensitive, R-resistant, $cMLS_B$-constitutively resistant to clindamycin, $iMLS_B$-inducibely resistant to clindamycin, MS-MS phenotype*

DISCUSSION

MRSA infections have become a major global health problem. A serious concern is the ability of *S. aureus* (especially MRSA) isolates to acquire resistance that has complicated medical care and caused important treatment challenges. Moreover, the ability of strains with an $iMLS_B$ phenotype to gradually change into a $cMLS_B$

phenotype during therapy leads to further difficulties in treatment therapy in hospital settings.

In this study, most *S. aureus* isolates were recovered from pus and then followed by blood and urine; nevertheless, the prevalence of isolates in pus was statistically insignificant (p=0.616). A high frequency of *S. aureus* isolates in pus samples has been reported in other studies in Nepal [10] and other parts of the world. [11] In this study, among the total of 120 *S. aureus* isolates, more than half (51.67%) of isolates were MRSA. Such a high prevalence rate of MRSA observed in this study might be due to different factors like prolonged hospitalization, [12] prolonged treatment in intensive care unit, [13] self-medication, and antibiotic abuse. [14] Most *S. aureus* isolates were found to be nonsusceptible to commonly used antibiotics like ampicillin and erythromycin but were sensitive to gentamycin and vancomycin. The high resistance to penicillin and high susceptibility to vancomycin is commonly noted for *S. aureus* isolates at different hospitals worldwide.[4] But in the case of vancomycin, CLSI (2018, M100) recommends minimum inhibitory concentration (MIC) test over disk diffusion, since disk-diffusion does not discriminate between vancomycin-intermediate isolates and vancomycin-susceptible isolates. Furthermore, disk-diffusion cannot distinguish between vancomycin-susceptible and vancomycin-resistant CoNS isolates. A variable incidence of MLS_B ($iMLS_B$ or $cMLS_B$) resistance has been reported in different geographical regions by different studies. In Europe, there is a high incidence of 93% of $cMLS_B$ resistant phenotype in MRSA, whereas the $iMLS_B$ is predominant in MSSA.[15,16] However, this study identified a significant presence of both $cMLS_B$ and $iMLS_B$ resistant phenotypes (p= 0.014 & p=0.046) among MRSA than MSSA phenotypes (22.58% versus 8.62%), which was similar to a study from Iran (26.9% versus 4.18%). [9] In contrast, Sasirekha et al (2014) [17] reported a higher frequency of $iMLS_B$ and $cMLS_B$ among the MSSA (0.65%, 5.22%) than MRSA (7.84%, 8.49%). The high occurrence of $iMLS_B$ and $cMLS_B$ resistant phenotypes among MRSA isolates could be attributed to the rapid emergence of clindamycin resistance in MRSA isolates.[18] Five isolates showed clindamycin resistance and erythromycin sensitivity, indicating E-S, CD-R phenotypes, most likely due to the insertion of the gene that encodes lincosamide nucleotide transferase that inactivates lincosamide (clindamycin).[19]

Among 14 iMLSB, all isolates harbored *mec*A and the most prevalent *erm* gene was *erm*C, similar to other previous studies,[15,20,21] but contradicting Coutinho et al who reported the least *erm*C compared to other *erm* genes.[19] Since there have been only a few reports describing the development of constitutive MLS_B resistance and the presence of clindamycin clinical failure among patients having *S. aureus* infection with $iMLS_B$ resistant strains,[8,22,23] this study may provide baseline data to design future studies aimed to investigate the relationship between $iMLS_B$ resistance and clindamycin clinical failure. The results of this study represent a single hospital and geographic area; the frequency of *mec*A and *erm* genes among $iMLS_B$ may differ in different regions. More studies are required to gain a better understanding of the relationship between *mec*A and *erm* genes. Furthermore, our finding further supports the importance of performing the D-test as a routine test if the local prevalence of $iMLS_B$ is found to be substantial.

CONCLUSIONS

The results of this study showed that MLS_B was significantly associated with MRSA phenotypes and *erm*C gene was the most widely distributed *erm* gene among $iMLS_B$ resistant MRSA phenotypes that all harbored *mec*A gene. There is a need for judicious use of antimicrobial agents to prevent the spread of $iMLS_B$-resistant strains.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

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