J Nepal Health Res Counc 2025 July-Sept; 23(68): 568-575

Prevalence of Inducible Clindamycin Resistance *Staphylococcus aureus* Associated with Wound Infection in Central Nepal

Aakriti KC,[1] Anil Pokhrel,[2] Binod Rayamajhee,[3,4] Sujan Khdaka,[5] Sanjeep Sapkota,[5] Alina Thapa,[5] Suprina Sharma,[2] Basudha Shrestha,[6] Pramod Poudel[7]

[1]Department of Microbiology, National College, Tribhuvan University, Kathmandu, Nepal, [2]Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal, [3]Department of Infection and Immunology, Kathmandu Research Institute for Biological Sciences, Lalitpur, Nepal, [4]School of Optometry and Vision Science, Faculty of Medicine and Health, UNSW, Sydney, Australia, [5]Department of Microbiology, Birendra Multiple Campus, Nepal, [6]Department of Microbiology, Kathmandu Model Hospital, Kathmandu, Nepal, [7]Central Department of Biotechnology, Tribhuvan University, Kritipur, Nepal.

ABSTRACT

Background: To determine the prevalence of inducible clindamycin resistance among methicillin-resistance *Staphylococcus aureus* (MRSA), and to detect the presence of *mecA* and *ermC* genes among MRSA recovered from hospital patients in central Nepal.

Methods: *Staphylococcus aureus* isolated from a total of 289 clinical specimens consisting of pus and wound swabs were analyzed and identified. The MRSA strains were screened using a cefoxitin (30 µg) disc following the CLSI procedure and a double-disc test (D-test) was applied to investigate iMLSB-resistant phenotypes among the MRSA isolates. The bacterial genomic DNA was extracted and *mecA* and *ermC* genes were detected using specific primer pairs.

Results: Among the 64 *S. aureus* strains, 39.1% of the isolates were MRSA. The prevalence of inducible clindamycin resistance among MRSA was observed to be 48%. All MRSA (100%) isolates were resistant to penicillin and amoxicillin, whereas all strains were susceptible to linezolid, vancomycin, teicoplanin, and tigecycline. Among MRSA isolates, 8% carried the *mecA* gene and 13.3% of iMLSB isolates were positive for the *ermC* gene.

Conclusions: A high rate of inducible clindamycin resistance among MRSA was observed. To identify the status of antibiotic resistance among *S. aureus*, further genomic-based studies are required.

Keywords: *Aureus*; *ermC* gene; inducible clindamycin resistance; *mecA* gene; methicillin-resistant *S.*

INTRODUCTION

Methicillin resistant *Staphylococcus aureus* (MRSA) is one of the leading causes of pyogenic wound infection in Nepal among hospitalized and non-hospitalized patients and treatment of wound infections is an emerging public health issue in Nepal, mainly due to rapidly growing bacterial antimicrobial resistance (AMR).[1] Changing patterns of antimicrobial resistance has led to renewed interest in the use of clindamycin[2] as an option for the treatment of MRSA-associated infections. On the same note, an increasing trend of inducible clindamycin resistance (iMLSB) among MRSA strains has been reported from Nepal.[3] However, there are no comprehensive studies reported correlating phenotypic resistance patterns with the genotypic resistance marker genes (*mecA* and *ermC*) in MRSA isolates from wound infections at a major tertiary care hospital in Nepal. This study was conducted to gauge the antibiotic susceptibility pattern of MRSA and the prevalence of iMLSB among MRSA strains, as well as the detection of *mecA* and *ermC* genes in clinical isolates of MRSA isolated from hospital patients attending a tertiary care hospital in central Nepal.

Correspondence: **Pramod Poudel Central Department of Biotechnology, Tribhuvan University, Kritipur, Nepal. Email: pramod.poudel@cdbt.tu.edu.np**

METHODS

A hospital-based prospective cross-sectional study was conducted to analyze aspirated pus and wound swabs collected from patients with wound infection attending a tertiary care hospital. This study was carried out between March to September 2019 at Kathmandu Model Hospital, Kathmandu, Nepal.

This study was reviewed and approved by Institutional Review Committee of Public Health Concern Trust PHECT, Nepal Kathmandu Model Hospital, Nepal, (IRC no. 005-2019).

Clinical and demographic data of each study patient with wound infection was retrieved from the hospital records and none of the medical identifiers of the study participants were transcribed into the study data file. Patients with known immunocompromised conditions such as AIDS, renal transplant, cancer, other malignancies, and chronic cardiovascular disease were excluded from the study.

All collected data were entered and analyzed using statistical software SPSS version 23.0 and R programming (version 1.2.5033). The p-value of <0.05 was considered statistically significant at 95% of the confidence level.

All the samples were processed and analyzed following the standard methods. Collected pus aspirates and wound swabs were inoculated on blood agar (BA) and mannitol salt agar (MSA), then incubated overnight aerobically at 37 ^{0}C, and sub-cultured on nutrient agar (NA) plates and incubated at 37 ^{0}C for 24 hours. Identification of *S. aureus* was done based on colony morphology on culture media and biochemical reactions such as catalase, coagulase, and DNase tests. All culture media were supplied by Himedia Laboratories Pvt. Ltd., Mumbai, India.

The antibiotic susceptibility test (AST) was performed by the modified Kirby-Bauer disk diffusion method on Mueller-Hinton agar (MHA) (Himedia Laboratories Pvt. Ltd, India) following the Clinical and Laboratory Standards Institute guidelines (CLSI, M100-S29). The antibiotic discs used for the antibiotic susceptibility test were: cefoxitin (CX/ 30 µg), amoxicillin (AMX/ 25 µg), ciprofloxacin (CIP/ 5 µg), cotrimoxazole (COT/ 25 µg), erythromycin (E/ 15 µg), gentamicin (GEN/ 10 µg), chloramphenicol (C/ 30 µg), amoxicillin/ clavulanic acid (AMC, 20/10 µg), doxycycline (DOX/ 30 µg), cephalexin (CN/ 30 µg), cloxacillin (COX/ 10 µg), linezolid (LZ/ 30 µg), penicillin (P/ 10 µg) and clindamycin (CD/ 2 µg). The minimum inhibitory concentration (MIC) values of the used antibiotics were unable to be determined due to the unavailability of the antibiotic powder during the study period. *S. aureus* ATCC 25923 was used as a reference strain for the AST.

MRSA was screened using a 30 µg cefoxitin (CX) disc and the results were interpreted following the CLSI M100-S29 cut-off values. *S. aureus* isolates with a zone of inhibition (ZoI) size ≤21 mm around the CX disc were confirmed as MRSA strains. The screening of inducible clindamycin was performed on MHA with clindamycin (2 µg) and erythromycin (15 µg) held 15 mm apart (edge to edge) with the same plate. Blunting of the circular ZoI around the clindamycin disc on the side facing the erythromycin disc was reported as an iMLSB isolate. [4]

Bacterial DNA was extracted following the protocol described by Lima De Castro Nunes et al [5] with no significant modification. PCR amplification of *mecA* [6] and *ermC* [7] genes was performed using specific primer pairs (Macrogen, S. Korea) as described previously.[6,7] The primer sequences with their respective amplicon size and amplification conditions are depicted in **Table 1.**

Table 1. Primers and amplification conditions for *mec*A and *erm*C genes.

Gene targeted	Primers used	Amplicon size (bp)	Amplification condition	
			Stage	Temperature, Time
***mec*A**	F 5'-AAA ATC GAT GGT AAA GGT TGG C-3'	533[6]	Initial denaturation	94°C, 5 min
			Denaturation	94°C, 30 sec
			Annealing	58°C, 30 sec 35cycles
	R 5'-AGT TCT GGA GTA CCG GAT TTG C-3'		Extension	72°C, 30 sec
			Final extension	72°C, 7 min
***erm*C**	F 5'- AGT ACA GAG GTG TAA TTT CG - 3'	520 [7]	Initial denaturation	94°C, 5 min
			Denaturation	94°C, 30 sec
			Annealing	53°C 30 sec 35cycles
	R 5'- AAT TCC TGC ATG TTT TAA GG - 3'		Extension	72°C, 30 sec
			Final extension	72°C, 7 min

A previously confirmed *S. aureus* isolates harboring *mecA* and *ermC* genes were used as positive control and molecular grade nuclease-free sterile water in the reaction mixture without extracted DNA template was used as a negative control in each PCR reaction. The amplified product was separated by agarose gel (1.5%) electrophoresis and visualized by ethidium bromide staining, then photographed under ultraviolet illumination. A DNA ladder of 100bp (Thermofisher, Massachusetts, United States) was used to compare the amplified bands on the agarose gel.

RESULTS

Among the 289 included patients in this study, 115 (60.2%) were female, where the mean age of the patients was 40.1±19.6 years. Out of 289 analyzed specimens (n=71 wound swabs and *n=218* aspirated pus), 172 (59.5%) specimens were positive for microbial growth, of which 126 growths were from pus aspirate and 46 were from wound swabs. Of the total bacterial growth, 64 (37.2%) were *S. aureus,* and CoNS constituted 20.9% of the isolates. *E. coli* (n=56), *Enterococcus faecalis* (n=9), *Acinetobacter calcoaceticus/baumannii* complex (ACBC) (n=7), and *Citrobacter freundii* (n=6) were common isolates, among others. The highest number of *S. aureus* was observed in patients of the age group of 20-29 years. A higher proportion of *S. aureus* (68.8%) was isolated from male patients compared to females. Statistically, the distribution of *S. aureus* among different age groups was significantly associated with the gender of patients (*p*-value= 0.027) (**Figure 1**).

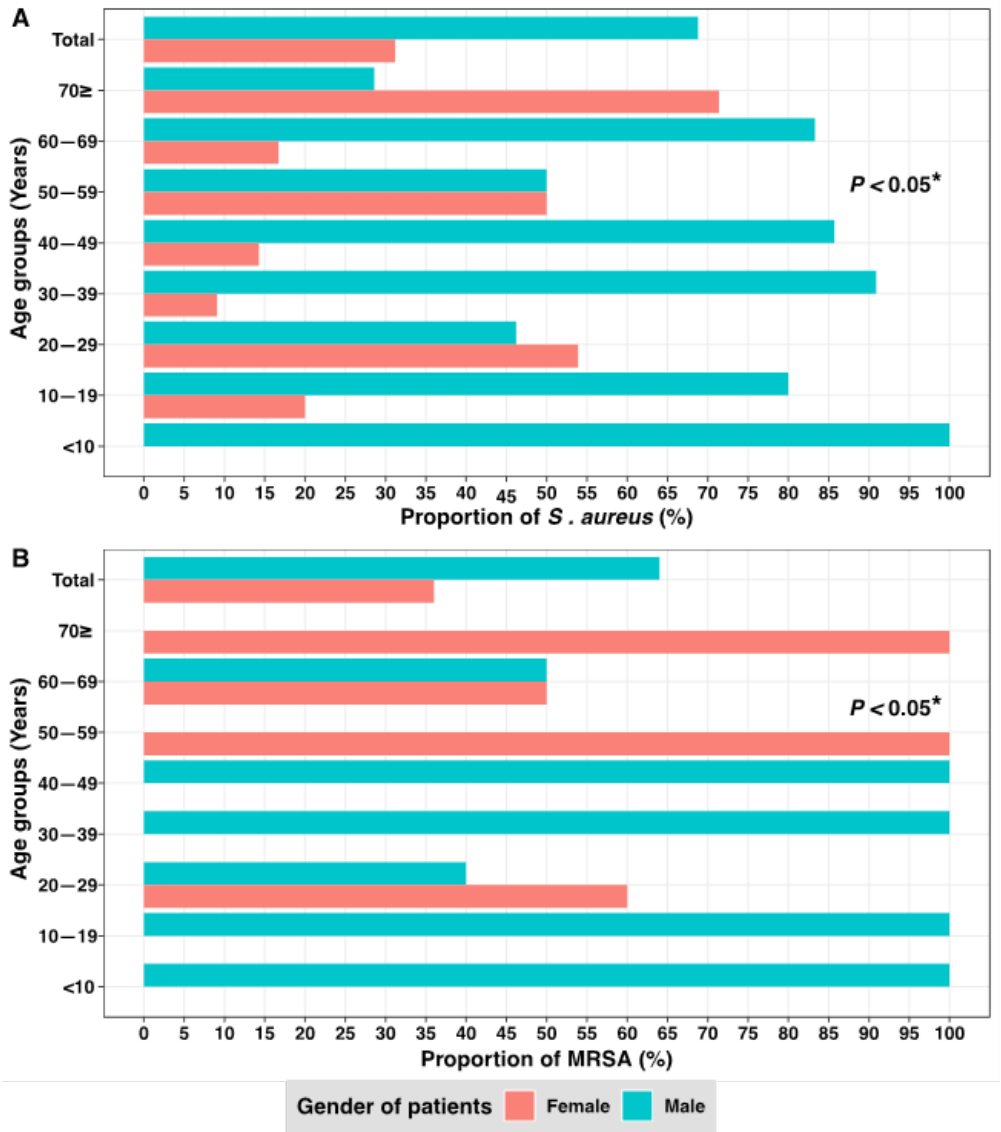

Figure 1.Proportions of S. aureus and MRSA among different age groups. Out of 172 bacterial growths recorded, 64 isolates were S. aureus and 36 isolates were CoNS. Specimens from males were more likely to have S. aureus growth.

Out of 64 *S. aureus* isolates, 25 (39.1%) were MRSA, and 39 (60.9%) isolates were MSSA. Among the 25 MRSA isolates, 16 (64.0%) isolates were from male patients, and 9 (36%) were isolated from female patients` specimens.

The highest percentage of MRSA was isolated from patients in the age group of 30-39 years (24%), whereas the lowest was observed in patients aged <10 years (4.0%). The AST pattern of MRSA isolates is depicted in **Table 2**. Out of 25 MRSA isolates, 36 CoNS and 39 MSSA isolates; 12 (48.0%), 1 (2.8%), and 2 (5.1%) isolates were found to be inducible clindamycin resistant, respectively.

Table 2.Antibiotic susceptibility patterns of *S. aureus* and MRSA isolates. *Neither of S. aureus isolates were resistant to Linezolid and no MRSA isolates were susceptible to cefoxitin, cloxacillin, and amoxicillin.* *:13(20.3%) *S. aureus* isolates and 3(12.0%) MRSA isolates showed 'intermediate' resistance to erythromycin disc-diffusion antibiotics sensitivity test.

Antibiotics	*S. aureus* isolates		MRSA isolates	
	Susceptible, *n* (%)	Resistant, *n* (%)	Susceptible, *n* (%)	Resistant, *n* (%)
Cefoxitin	40(62.5)	24(37.5)	0	25(100)
Erythromycin*	15(23.4)	36(56.3)	3(12)	19(76)
Clindamycin	40(62.5)	24(37.5)	7(28)	18(72)
Cloxacillin	40(62.5)	24(37.5)	0	25(100)
Penicillin	8(12.5)	56(87.5)	0	25(100)
Amoxycillin	8(12.5)	56(87.5)	0	25(100)
Amoxiclav	34(53.1)	30(46.9)	6(24)	19(76)
Ciprofloxacin	17(26.6)	47(73.4)	2(8)	23(92)
Cotrimoxazole	20(31.2)	44(68.7)	11(44)	14(56)
Gentamycin	56(87.5)	8(12.5)	20(80)	5(20)
Linezolid	64(100)	0	25(100)	0
Doxycycline	48(75)	16(25)	11(44)	14(56)
Cephalexin	37(57.2)	27(42.2)	7(28)	18(72)
Chloramphenicol	61(95.3)	3(4.7)	23(92)	2(8)
Vancomycin	nt	nt	25(100)	0
Teicoplanin	nt	nt	25(100)	0
Tigecycline	nt	nt	25(100)	0

Keys: nt: antibiotics not tested

Among all MRSA and CoNS, the *mec*A gene was detected in 3 (11.5%) isolates, of which 2 were MRSA and one was a methicillin-resistant coagulase-negative *Staphylococcus* (MRCoNS) isolate (**Figure 2A**). Fisher's exact test was performed, and no significant association was found between MRSA and the detection of the *mecA* gene (*p*-value = 0.115).

Fifteen isolates were inducible clindamycin resistant, among them *ermC* gene (**Figure 2B**) was detected in 2 (13.3%) isolates, one was MRSA and the other was MRCoNS. Fisher's exact test showed no relation between inducible clindamycin resistance and detection of the *ermC* gene (*p*-value= 0.143).

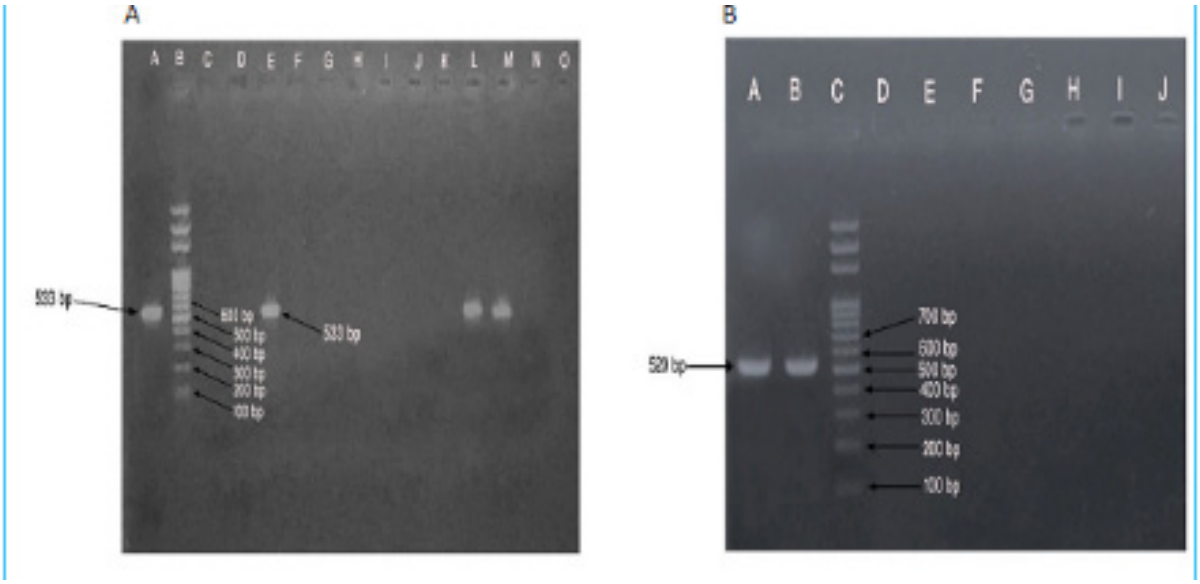

Figure 2.Photograph of agarose gel electrophoresis of amplified products of mecA and ermC genes. Among MRSA, 3 isolates harbored mecA gene but no significant association was found between MRSA and the detection of the mecA gene. Out of fifteen inducible clindamycin-resistant isolates, ermC gene was detected in 2 (13.3%) isolates.

DISCUSSION

A systemic review and meta-analysis from Nepal reported the pooled prevalence of MRSA to be 38.2%. [8] However, the reported prevalence of MRSA varies across the country. Such variation in incidence may be due to the use of different isolation techniques, such as the use of enrichment media, the use of oxacillin discs to screen for MRSA, the composition of MHA, and differences in the geographical distribution of the pathogen. [9] Moreover, the type of specimens taken also varies, we analyzed only pus aspirates and wound swabs for this study, while others have investigated blood, urine, nasal swabs, etc., along with pus and wound specimens, this selection of different specimens and proper specimen collection technique might influence the isolation of *S. aureus* and thus of MRSA. Hygienic conditions and health care facilities maintained in hospitals [10], the sensitivity of swabbing, and demographic characteristics of the study population [11] may also contribute to varying isolation rates of *S. aureus*. As documented in this study, previous studies have also found males at a higher risk of *S. aureus* and/or MRSA colonization and infection compared to females. [12] Although the underlying reason for the higher incidence of MRSA in males is not fully explored, personal hygiene is usually attributed as a factor for higher MRSA and/or *S. aureus* infection in males because, compared to females, males have a less positive attitude towards hygiene. Nowak et al. (2017) [13] attributed higher *S. aureus* carriage in males due to body fat, high body fat specifically visceral adipose tissue (VAT) which has a negative influence on the immune system [14], might be a risk factor for *S. aureus* colonization in men. [13]

S. aureus infection was more among patients in the age group of 20-29 years, while a higher prevalence of MRSA was observed in patients aged 30-39 years. Since different studies categorize age into groups into varying intervals, a proportionate comparison among them is not rational, furthermore, the association between age and *S. aureus* or MRSA colonization/infection is not well established.

The relatively low prevalence of the *mec*A gene among phenotypic MRSA in this study can be attributed to multiple factors. First, the MRSA isolates lacking the *mec*A gene may be because of the hyper-production of the ß-lactamase enzyme. [15] Second, apart from the *mec*A gene, two other genes homologous to *mecA*, called *mec*B and *mecC*, are also found to confer methicillin resistance in *S. aureus*. [16,17] Although rarely documented globally, but not yet detected in Nepal, this study suggests the possibility of *mec*B and *mec*C-mediated methicillin resistance in Nepal, further studies are anticipated to confirm the prevalence of *mec*B and *mec*C genes among *S. aureus* isolates in Nepal. Nevertheless, CLSI suggests reporting the isolates as MRSA when they are resistant to oxacillin or cefoxitin or both, regardless of the *mec*A gene. [18]

All MRSA isolates in our study were sensitive to vancomycin, suggesting vancomycin could be a drug of choice for the treatment of MRSA-associated infections in Nepal although a recent study from Nepal confirmed circulation of vancomycin-resistant *S. aureus* mediated by *vanB* gene. [19] The prevalence of iMLSB resistance among *S. aureus* isolates is inconsistent in Nepal. Such variation in iMLSB prevalence may be due to multiple factors, such as study population, geographical location, use of erythromycin for the therapy of *S. aureus*-associated infections, and type of clinical specimens processed. [20] Although the *erm*A gene was not detected in this study, this gene carried in *SCCmec* may mediate MLSB resistance in MRSA. [21] As observed in this study, high resistance to erythromycin (76%) and clindamycin (72%) implies wide consumption of these antibiotics, and increased use of these antibiotics may result in macrolide resistance due to selective pressure on bacteria. [22]

The overall prevalence of the *erm*C gene in this study (13.3%) is lower than reported by Nagarkoti et al. [23] from Nepal. The lower prevalence of the *erm*C gene in this study might be due to the iMLSB resistance mediated by *erm*A and/or *erm*B genes, which we were unable to detect due to limited availability of resources. The factors that downregulate the expression of *erm* genes [24,25] might also have contributed to the lower prevalence reported in this study.

Inducible clindamycin-resistant (iMLSB) strains appear to be erythromycin-resistant but clindamycin sensitive in routinely used standard disk-diffusion susceptibility testing [26] which may lead to wrong reporting of clindamycin susceptibility and thus the wrong prescription of clindamycin resulting in treatment failure. [27] For any erythromycin-resistant *S. aureus*, assuming clindamycin to be resistant solely based on erythromycin resistance could be fallacious, as it results in the elimination of clindamycin when it is actually sensitive, on the other hand, reporting clindamycin to be sensitive based on the standard AST without D-test could also be disastrous. The D-test assists laboratory personnel in determining whether clindamycin should be reported as susceptible or resistant. That eventually helps to reduce the burden of MRSA strains.

CONCLUSIONS

A higher prevalence of MRSA was observed in wound infections associated with *S. aureus* isolates. A higher prevalence of iMLSB resistance was observed among MRSA. Further molecular studies are required to better understand the roles of other genes in antibiotic resistance in MRSA and iMLSB in the Nepalese setting. Since vancomycin is sensitive to all MRSA isolates, it can be used to treat MRSA-associated infections.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

ACKNOWLEDGMENTS

The authors would like to thank all study participants and hospital staff of Kathmandu Model Hospital for their great support.

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