Characteristics of *Staphylococcus aureus* Isolated From Clinical Specimens in a Tertiary Care Hospital, Kathmandu, Nepal

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ABSTRACT

INTRODUCTION: Methicillin resistant *Staphylococcus aureus* (MRSA) is a major human pathogen associated with nosocomial and community infections. *mec*A gene is considered one of the important virulence factors of *S. aureus* responsible for acquiring resistance against methicillin. The main objective of this study was to explore the prevalence, antibiotic susceptibility pattern, and *mec* A gene.

METHODS: A total of 39 isolates of *S. aureus* were isolated from 954 clinical specimens processed in Microbiology laboratory of Himal Hospital, Kathmandu. Antimicrobial susceptibility test (AST) was performed by Kirby-Bauer disc diffusion method using cefoxitin, and performed Polymerase Chain Reaction (PCR) for amplification of *mecA* gene in MRSA isolates.

RESULTS: Out of 954 clinical samples, (16.2%; 153/954) samples had bacterial growth. Among 153 culture positive isolates, 25.5% (39/153) were positive for *S. aureus.* Among 39 *S. aureus* (61.5%; 24/39) were multiple drug resistant (MDR). On AST, amoxicillin was detected as the least effective while vancomycin was the most effective. The prevalence of methicillin resistance was 46% (18/39) of which 72.2% (13/18) were positive for *mecA* gene in PCR assay.

CONCLUSION: One in 4 culture positive isolates from the clinical specimens were *S. aureus*, of which almost two-thirds were MDR. Around half of the MDR showed MRSA and significant proportion of them were positive for *mecA* gene. This study concludes that the *mecA* gene is solely dependent for methicillin resistance in *S. aureus* but the presence of gene is not obligatory. PCR detection of the *mec*A gene is reliable, valid and can be suggested for the routine use in diagnostic laboratories.

KEYWORDS: *Staphylococcus aureus*, MRSA, cefoxitin, *mec*A gene

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Introduction

Methicillin Resistant *S. aureus* (MRSA) is a global threat causing serious infections in health facilities and the community, contributing 64% more deaths than the non-resistant form of the infections.[1] It is estimated that one-third of the general population is colonized by *S. aureus*, and the global pooled prevalence of MRSA colonization is 1.3%.[2] In 2014, the percentage of invasive MRSA isolates in Europe ranged from 0.9% in the Netherlands to 56% in Romania.[3] The prevalence of MRSA infections in countries of South and East Asia, and the Western Pacific region ranged from 2.3% to 69.1%.[4] MRSA led to adverse clinical outcomes compared with methicillin-sensitive *S. aureus* (MSSA).[5] It often causes metastatic infections such as infective endocarditis (IE), septic arthritis, and osteomyelitis.[3] More than 80 000 life-threatening infections are caused by MRSA and leads to 11 000 mortalities in a year.[6,7]

MRSA is generated when methicillin-susceptible *S. aureus* (MSSA) exogenously acquires a methicillin resistance gene, *mec*A, carried by a mobile genetic element known as staphylococcal cassette chromosome *mec (SCCmec)*, and is considered to be transmissible across staphylococcal species.[8] *mec*A gene is present in all MRSA strains that encodes penicillin binding protein 2a (PBP2a), which has a low tropism to all is β-lactam antibiotics, a cornerstone for producing MRSA phenomenon.[9]

MRSA are those strains of *S. aureus* that are resistant to the penicillinase-stable penicillin class of antibiotics such as methicillin, oxacillin, nafcillin, cloxacillin, and dicloxacillin by expression of *mec*A or other mechanisms, such as changes in affinity of penicillin binding proteins for oxacillin.[10,11] Laboratory

[†]Equally contributed.

identification requires culture (on Mannitol Salt agar) that takes between 48 and 72 hours followed by susceptibility testing for MRSA which takes another 16 to 24 hours.[12] The cefoxitin (30 µg) disk is used to detect MRSA by the disk diffusion method. *S. aureus* that are *mec*A positive should be considered as resistant to antibiotic oxacillin and other β-lactam group of antibiotics.[13]

Development of immunochromatographic test and molecular testing methods to detect MRSA have greatly reduced the time and labor required which can consequently help in improving infection control and reducing the cost.[14] MRSA detection by real-time polymerase chain reaction (PCR) tests are capable of detecting genes specific to *S. aureus*. To distinguish MRSA strains from methicillin sensitive *Staphylococcus aureus* (MSSA), PCR methods target a portion of DNA where the MRSA-specific SCC*mec* gene of *S. aureus* from the samples within 1 to 3 hours.[8] Molecular amplification of the *mec*A gene is recognized as a benchmark to diagnose MRSA in the community as these genes are highly conserved among staphylococcal species.[15]

Several studies in the past have reported the prevalence of MRSA infections in Nepal.[16-22] Past studies conducted at different settings in Nepal have shown the prevalence of MRSA ranging from 26.1% to 57.1%.[16-18,20,21,23] Increasingly in recent years, more studies have been carried out to explore the prevalence of MRSA infections in Nepal. Nevertheless, all these studies were mostly concentrated in phenotypic characterization with antimicrobial susceptibility test. This study explores the prevalence of MRSA infections, antimicrobial susceptibility patterns and detection of *mec*A gene by using molecular methods, PCR with objective to evaluate the usefulness of amplification of *mec*A gene and its reliability in the identification of MRSA strains.

Methods

Study area/collection of specimens

In the study period between September, 2017 and March, 2018, a total of 954 specimens such as Urine, Blood, and swabs from vagina, endo-cervix, intra-cervix, wound, ear, eye, semen; and other body fluids were collected from suspected patients in Himal Hospital, Kathmandu and were transferred to the laboratory for further processing.[24] Himal Hospital is a tertiary care health center located at the heart of Kathmandu valley. It is a 100-bedded hospital that provides services to 300 to 500 outpatients in a day.

Specimen processing/identification tests/biochemical tests

The colonies grown were identified based on the morphology, Gram's stain, and biochemical tests. *S. aureus* were confirmed using following tests: yellow colored colonies on mannitol salt agar, catalase positive, slide and tube coagulase positive, hydrolyzed gelatin, showed beta-hemolysis on blood agar, methyl red positive, Voges–Proskauer positive, nitrate reduction positive, fermentative, DNase producing, lactose, mannitol, maltose, mannose, sucrose and trehalose fermenting, and alkaline phosphatase positive.[22,25]

Antibiotic susceptibility testing (AST)

Antimicrobial susceptibility testing was performed by Kirby Bauer disc diffusion technique following clinical and laboratory standards institute (CLSI) guidelines, 2018.[11] The concentration of suspension of the test organism was made equivalent to 0.5 McFarland standards. Lawn culture was performed on Mueller–Hinton agar plate. Antibiotic discs were placed over the lawn culture and the plate was incubated aerobically at 35°C for 24 hours. Finally, the plate was observed for zone of inhibition and interpreted according to CLSI guidelines, 2018 The antibiotic discs used were amoxicillin, amikacin (30 µg), ampicillin (10 µg), cefoxitin (30 µg), ciprofloxacin (5 µg), clindamycin (2 µg), chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (10 µg), tetracycline (30 µg), cotrimoxazole (25 µg), vancomycin (30 µg), and meropenem (10 µg). Strains showing resistance to 3 or more than 3 different classes of antibiotics were considered multidrug resistant.[26] All confirmed isolates were stored 2 sets: 1 set at +4°C (later subcultured to carry out phenotypic characterization) and another set frozen in tryptic soy broth containing 10% glycerol and stored at −70°C for molecular analysis.

Identification of methicillin resistant Staphylococcus aureus *(MRSA) strains*

Methicillin resistant *S. aureus* (MRSA) was identified using cefoxitin (30 µg) disks. Plates were incubated at 35°C. for a 24-hour incubation period. The diameter of the zone of inhibition (ZOI) of growth was recorded and interpreted as susceptible or resistant based on the CLSI guideline. *S. aureus* isolates were deemed methicillin resistant when the ZOI was ⩽21 mm with the cefoxitin disk.[11]

Detection of mecA by PCR technique

DNA was extracted from the MRSA isolates by chloroform: phenol extraction method as described.[27] The primers used for *mec*A gene: forward primer of *mec*A gene (FP) 5′-ACT GCT ATC CAC CCT CAA AC-3′ and reverse primer (RP) 5′-CTG GTG AAG TTG TAA TCT GG-3′.[28] Final 10 µl solution which included master mix of 5 µl, forward primer 1 µl, reverse primer 1 µl, DNA 1 µl, nuclease free water 2 µl were utilized for PCR. Thermal cycling was conducted for 120 seconds at 95°C (initial denaturation); 30 seconds at 95°C (denaturation) and 30 seconds at 56.2°C (annealing), 20 seconds at 72°C (extension), 29 amplification cycles using primer and 5 min at 72°C and preserved at 4°C. After completion of PCR thermal cycle, gel band was observed under UV light, yielded a 163-bp DNA fragment that was visualized by electrophoresis

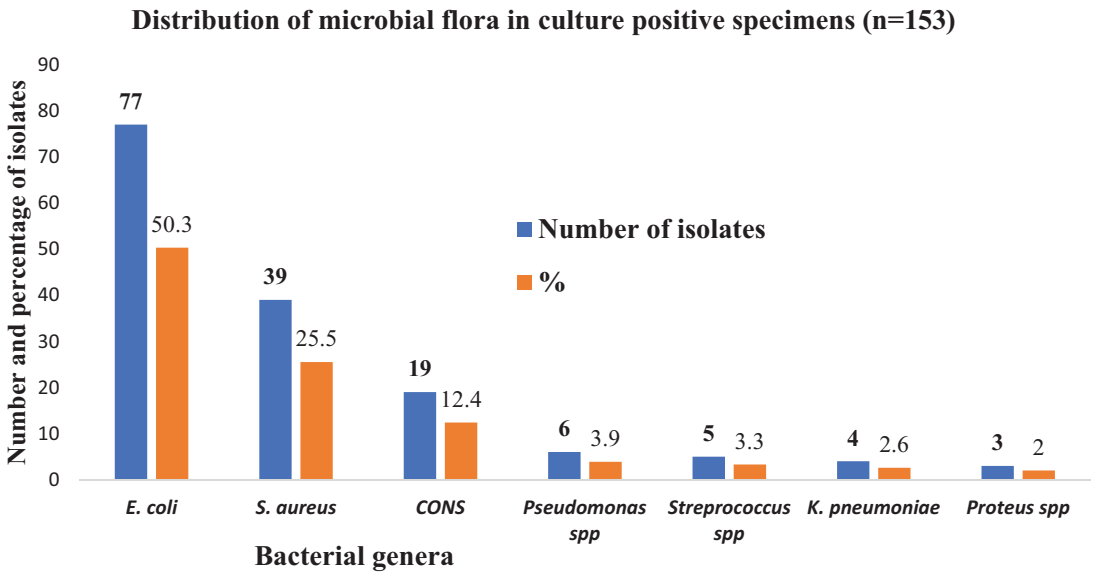

Figure 1. Distribution of bacterial genera among culture positive clinical specimens.

in 1.5% agarose gel stained with ethidium bromide staining and under UV light.[28,29]

Results

Distribution of bacterial genera, S. aureus, *and MRSA in clinical samples*

About 945 clinical specimens were examined for growth, among which 16.1% (153/945) samples had bacterial growth. Among 153 culture positive bacterial isolates (50.3%; 77/153) were *E. coli* followed by *S. aureus* (25.5%; 39/153), coagulase negative staphylococci (12.4%; 19/153), and *Pseudomonas* spp. (3.9%; 6/153) respectively (Figure 1).

The prevalence of *S. aureus* 25.5% (39/153). Among 39 *S. aureus*, (61.5%; 24/39) were multiple drug resistant (MDR). Out of 39 *S. aureus* isolated, 46.1% (18/39) were MRSA (Table 1).

Distribution of S. aureus *and MRSA according to age, gender, and type of patients*

Out of 39 *S. aureus* isolated, 53.8% (21/39) were isolated from age group: 15 to 45 years followed by age group <14 years (25.6%; 10/39) and age group >45 years 20.6%; 8/39) (Table 1).

Out of 18 MRSA, 66.7% (12/18) were isolated from the age group: 15 to 45 years followed by age group >45 (22.2%; 4/18) and age group <14 years (11.1%; 2/18) respectively. Male patients (61.1%; 11/18) had more MRSA than the female patients (38.9%; 7/18). Similarly, patients admitted in hospital (72.2%; 13/18) harbored more MRSA than outpatients (27.8%; 5/18) and found statistically significant ($P < .05$) (Table 1).

Antibiotic susceptibility patterns of S. aureus *and MRSA isolates*

All 39 *S. aureus* isolated were found 100% sensitive to vancomycin followed by amoxicillin (89.7%; 35/39) and cotrimoxazole (64.1%; 25/39) in decreasing order. All 18 MRSA isolates were found to be resistant to cefoxitin and amoxicillin followed by cotrimoxazole (83.3%; 15/18), and the prevalence of resistance was same across tetracycline, ciprofloxacin and erythromycin (72.2%; 13/18) (Table 2).

Gel electrophoresis and polymerase chain reaction (PCR)

Out of 18 MRSA isolates, 13 (72.2%) showed *mec*A gene on agarose gel containing extracted DNA, 5 µl EtBr, 1 kb marker, and on TAE buffer. Band was observed under UV light and the product size of *mec*A gene was 163 bp (Figure 2).

Discussion and Conclusion

Our study showed (16.2%) bacterial growth from different clinical specimens in culture. Among bacterial isolates, 1/4th of them were *S. aureus*. The findings of our study are consistent with other studies reported from *School of Health & Allied Sciences*, Pokhara University,[22] Chitwan,[16] B&B hospital, Lalitpur,[30] Manipal Teaching Hospital, Pokhara,[31] Lumbini Medical College and Teaching Hospital, Palpa,[20] Alka Hospital, Lalitpur,[23] and Birendra Military Hospital, Kathmandu.[32]

In our study, *S. aureus* were mostly sensitive to amikacin, meropenem, and gentamicin. The findings of our study resonate with previous studies reported from Nepal.[33] Antimicrobial susceptibility pattern of MRSA showed most of the tested antibiotics to be resistant except vancomycin, and amikacin. These findings are in line with few previous studies,[16,19,30,31] however, slightly contrasts with one past study, in which ciprofloxacin was sensitive to MRSA.[34] Antimicrobial resistance is a global threat and MRSA has emerged as an important human pathogen with wide range of antibiotic resistance.[6] Globally,

Table 1. Distribution of *S. aureus* and MRSA in clinical specimens and according to age group, gender, and type of patients.

CHARACTERS	BACTERIAL GROWTH	*S. AUREUS*	*P*-VALUE	MRSA	*P*-VALUE
Clinical specimens	*N* (%)	*N* (%)		*N* (%)	
Urine	90 (58.9)	9 (23. 1)		4 (22.2)	
Blood	20 (13.1)	4 (10.2)		2 (11.1)	
Wound swab	25 (16.3)	16 (41.1)		7(38.8)	
Pus	15 (9.8)	8 (20.5)		4 (22.2)	
Body fluid	3 (1.9)	2 (5.1)		1(5.5)	
Total	153	39		18	
Age group in years					
<14		10 (25.6)	.9	2 (11.1)	.31
(15-45)		21 (53.8)		12 (66.7)	
>45		8 (20.6)		4 (22.2)	
Gender					
Male		22 (56.4)	.08	11 (61.1)	.11
Female		17 (43.6)		7 (38.9)	
Type of patients					
Inpatients		23 (59)	.001	13 (72.2)	.002
Outpatients		16 (41)		5 (27.8)	

Table 2. Antibiotic susceptibility pattern of *S. aureus* and MRSA.

ANTIBIOTICS	*S. AUREUS* (N=39)		MRSA (N=18)	
	SENSITIVE N (%)	RESISTANT N (%)	SENSITIVE N (%)	RESISTANT N (%)
Gentamycin	28 (71.8)	11(28.2)	10 (55.5)	8 (44.5)
Amikacin	33 (84.6)	6 (15.4)	15 (83.3)	3 (16.7)
Amoxicillin	4 (10.3)	35 (89.7)	0 (0)	18 (100)
Cefoxitin	21 (53.9)	18 (46.1)	0 (0)	18 (100)
Meropenem	30 (76.9)	9 (23.1)	9 (50)	9 (50)
Vancomycin	39 (100)	0 (0)	18 (100)	0 (0)
Erythromycin	26 (66.7)	13 (33.3)	5 (27.8)	13 (72.2)
Chloramphenicol	31 (79.5)	8 (20.5)	8 (44.5)	10 (55.5)
Ciprofloxacin	25 (64.1)	14 (35.9)	5 (27.8)	13 (72.2)
Cotrimoxazole	14 (35.9)	25 (64.1)	3 (16.7)	15 (83.3)
Tetracycline	20 (51.3)	19 (48.7)	5 (27.8)	13 (72.2)

the prevalence of MRSA is highly heterogeneous. Past reports of MRSA from Nepal reported prevalence of 15.4% to 26.0%[17,35] and newer studies from various hospitals of Nepal reported higher prevalence: 26% to 69%.[36,37] Most of the MRSA related studies conducted in Nepal, used only cefoxitin and/or oxacillin for screening MRSA.

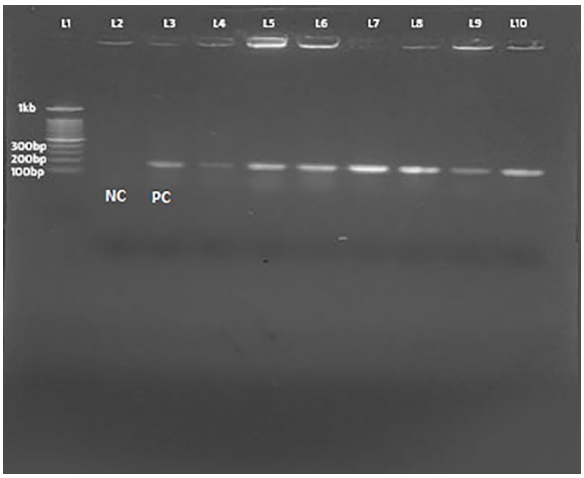

Figure 2. Visualization of amplified *mecA gene* under UV Trans-illuminator.
Abbreviations: L1, NEB 100 bp DNA marker; L2, Negative control *S. aureus ATCC* 29213; L3, Positive control *S. aureus ATCC* 49476; L4-L10, different clinical *S. aureus* isolates).

Detection of *mec*A gene is the major evidence for the detection of MRSA isolate. This statement was approved by many studies for example in Sudan,[38] Saudi Arabia,[39] Iraq,[40] Japan,[41] India,[42] Australia,[43] and USA.[44] However, our study showed low burden of the *mec*A gene (72.22%); this may raise questions to explore other intrinsic factors that may compete with *mec*A gene in producing resistance phenomenon in regions with high prevalence of MRSA. Nonetheless, the absence of *mec*A gene within resistant staphylococcal isolates have been reported globally.[45] Additionally, moderate methicillin resistance was observed in isolates that lacked the *mec*A gene mutations.[46]

In our study, 13 (72.22%) out of 18 MRSA show mecA gene while 27.78% showed absence of mecA gene. A previous study in Nigeria reported the complete absence of 5 major SCCmec types and *mec*A genes as well as the gene product of PBP2a in isolates which were phenotypically MRSA suggesting a probability of hyper-production of β-lactamase as a cause of the phenomenon.[47] Another study suggests that the specific alterations in different amino acids present in protein binding proteins cascade (PBPs 1, 2, and 3) could be the basis of resistance. These alterations were found to include 3 amino acid substitutions, which were identical and were present in PBPs 1, 2, and 3. Moreover, the same amino acid was found to have 2 other different substitutions in PBP1. Both the identical and different amino acid substitutions were observed in isolates from different multilocus types.[48] These findings provided clear evidence that there are mechanisms other than the presence of *mec*A gene responsible for beta-lactam resistance of MRSA and the molecular methods alone are not enough to confirm characterization of MRSA isolates, a point that should be considered by regional and reference laboratories.

High prevalence of MRSA reported in this study warrants an attention for rational and regulated use of antibiotics based on the laboratory confirmation of the disease and antibiotic susceptibility tests. In addition to phenotypic identification of MRSA, PCR-based detection of MRSA is essential to monitor its spread and characterization. However, this is a pilot study in a single hospital. Future studies with larger sample size and from multiple settings are essential to generate evidence on the characteristics and role of *mec*A gene.

Strengths and Limitations

This study will be a useful reference for future studies to explore and expand on the wider prevalence of MRSA organisms in hospitals, patients, and community people. Since our study was based on a phenotypic detection method and detection of *mec*A gene by conventional PCR, genotypic characterization of new homologues of *mec*A (*mec*B, *mec*C, and *mec*D) and whole genome sequencing of MRSA strains are helpful for future studies.

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Author Contributions

All the authors made substantial contribution to the study. SNK and NA conceived and designed the study. SNK collected samples, investigated, and recorded the laboratory findings. NA, GK, KBA, and UTS supervised the laboratory work. KRR, MRB, and PG advised and formulated the methodology for the study. SNK, BD, and KRR drafted the original draft. KRR and BA are responsible for reviewing several versions of manuscript. Others helped to review and amend this manuscript. All authors read and approved the final manuscript.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical Approval and Consent to Participate

The specimens and the data used in our study were derived from routine diagnostic database in Microbiology Laboratory of Himal Hospital, Naxal Kathmandu. Since the samples and data used in this study neither incurred direct involvement with the patients not interfered with the routine clinical care, formal ethics approval was deemed unnecessary complying with the guidelines of Nepal Health research Council.

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Appendix

Abbreviations

AST Antimicrobial susceptibility test
CLSI Clinical and laboratory standard institute
MRSA Methicillin Resistant *Staphylococcus aureus*
MSSA Methicillin sensitive *Staphylococcus aureus*
PCR Polymerase chain reaction
SCCmec Staphylococcal cassette chromosome *mec*
ZOI Zone of inhibition