

## Research Article

# Detection of Methicillin Resistant *Staphylococcus aureus* and Determination of Minimum Inhibitory Concentration of Vancomycin for *Staphylococcus aureus* Isolated from Pus/Wound Swab Samples of the Patients Attending a Tertiary Care Hospital in Kathmandu, Nepal

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The present study was conducted to evaluate the performance of cefoxitin disc diffusion method and oxacillin broth microdilution method for detection of methicillin resistant *S. aureus* (MRSA), taking presence of *mecA* gene as reference. In addition, inducible clindamycin resistance and beta-lactamase production were studied and minimum inhibitory concentration (MIC) of vancomycin for *S. aureus* isolates was determined. A total of 711 nonrepeated pus/wound swab samples from different anatomic locations were included in the study. The *Staphylococcus aureus* was identified on the basis of colony morphology, Gram's stain, and biochemical tests. A total of 110 (15.47%) *S. aureus* isolates were recovered, of which 39 (35.50%) isolates were identified as MRSA by cefoxitin disc diffusion method. By oxacillin broth microdilution method, 31.82% of the *Staphylococcus aureus* isolates were found to be MRSA. However, *mecA* gene was present in only 29.1% of the isolates. Further, beta-lactamase production was observed in 71.82% of the isolates, while inducible clindamycin resistance was found in 10% of *S. aureus* isolates. The MIC value of vancomycin for *S. aureus* ranged from 0.016 µg/mL to 1 µg/mL. On the basis of the absolute sensitivity (100%), both phenotypic methods could be employed for routine diagnosis of MRSA in clinical microbiology laboratory; however cefoxitin disc diffusion could be preferred over MIC method considering time and labour factor.

## 1. Introduction

Although *Staphylococcus aureus* is a commensal of humans [1], it is also a frequent cause of human infections which may become serious if caused by antimicrobial resistant strains [2]. Antibiotic resistant *S. aureus*, especially MRSA, are equally adopted to hospitals and outer environments evolving as major pathogens of public health concern [3, 4].

Shortly after the introduction of methicillin in clinical world to treat infections caused by penicillinase producing *S.*

*aureus* in 1960, MRSA emerged and spread worldwide [5, 6]. The high rate of methicillin resistance among *Staphylococcus aureus* has resulted into the increased interest for the use of clindamycin for treatment of infections caused by *S. aureus* [7]. But recently, increasing numbers of strains of *S. aureus* are acquiring resistance toward clindamycin [7].

Vancomycin is regarded as the drug of choice for treatment of infections caused by MRSA [8]. But emergence of VISA and VRSA has been reported by many authors [8]. Further, there are reports of treatment failure of the infections

caused by MRSA having MIC of vancomycin just below cutoff value [8]. High vancomycin MIC for MRSA which are susceptible to vancomycin may indicate the drug resistance to many antibiotics [8].

MRSA is resistant to entire classes of  $\beta$ -lactams including cephalosporins and carbapenems and has higher risk of development of resistance to quinolones, aminoglycosides, and macrolides [9–12].

Methicillin resistance in *S. aureus* is mediated through an altered protein called low-affinity penicillin binding protein (PBP2a). PBP2a is encoded by *mecA* gene which is present in chromosomal mobile genetic element called Staphylococcal cassette chromosome *mec* (SCC*mec*) [13, 14]. Due to possible association of MRSA with multiple antibiotic resistance and relatively difficult and higher cost of treatment, the accurate and rapid identification of MRSA is crucial in clinical world for timely management of the infections caused by this superbug [15]. Detection of methicillin resistance in Nepal is based on cefoxitin and oxacillin disc diffusion methods with limited reports on MIC determination and detection of *mecA* gene by polymerase chain reaction (PCR) [16, 17]. In present study, we evaluated the performance of cefoxitin disc diffusion and oxacillin broth microdilution methods for detection of MRSA taking presence of *mecA* gene as reference. Further, we also studied the rates of inducible clindamycin resistance and beta-lactamase production among the strains of *S. aureus* and we determined the minimum inhibitory concentration of vancomycin for *S. aureus* isolated from pus/wound swab samples.

## 2. Materials and Methods

**2.1. Study Site and Population.** The present study was carried out among the patients (inpatients and outpatients) attending Shree Birendra Hospital, Kathmandu, Nepal, from July 2013 to January 2014. A total of 711 nonrepeated pus/wound swab samples from different anatomic locations received from the patients for bacteriological culture were included in the study.

**2.2. Isolation and Identification of *Staphylococcus aureus*.** The specimens were inoculated on blood agar and mannitol salt agar (HiMedia laboratories private limited, India) and incubated aerobically at 37°C for 48 hours. The strains of *Staphylococcus aureus* were identified on the basis of colony morphology, Gram's stain, and different biochemical tests [18].

**2.3. Antimicrobial Susceptibility Testing.** The antimicrobial susceptibility testing was performed by modified Kirby-Bauer disc diffusion technique using Mueller-Hinton agar (HiMedia laboratories private limited, India) following Clinical and Laboratory Standards Institute (CLSI) guidelines [19]. Antibiotic discs used were ciprofloxacin (5  $\mu$ g), clindamycin (2  $\mu$ g), chloramphenicol (30  $\mu$ g), erythromycin (15  $\mu$ g), gentamicin (10  $\mu$ g), tetracycline (30  $\mu$ g), cotrimoxazole (25  $\mu$ g), rifampin (5  $\mu$ g), mupirocin (200  $\mu$ g), and penicillin G (10 units).

**2.4. Detection of Strains of MRSA by Cefoxitin Disc Diffusion Method.** Susceptibility of *Staphylococcus aureus* isolates to cefoxitin (30  $\mu$ g) was determined by modified Kirby-Bauer disc diffusion method following CLSI guidelines [19]. The strains of *Staphylococcus aureus* which were found to be resistant to cefoxitin were screened as MRSA (Table 1).

**2.5. Determination of Minimum Inhibitory Concentrations (MICs) of Oxacillin and Vancomycin.** MICs of oxacillin (Table 1) and vancomycin for all isolates of *Staphylococcus aureus* were determined by broth microdilution method as described by Andrews [20] and CLSI M07-A9 guidelines [21]. The results were interpreted according to CLSI guidelines [19]. The concentrations of oxacillin used were 0.0125  $\mu$ g/mL to 128  $\mu$ g/mL and the concentrations of vancomycin used were 0.06  $\mu$ g/mL to 32  $\mu$ g/mL.

**2.6. Detection of  $\beta$ -Lactamase Production.**  $\beta$ -lactamase production in isolated *S. aureus* was detected by iodometric method as described by Samant and Pai [22].

**2.7. Detection of Inducible Clindamycin Resistance.** Erythromycin resistant isolates were tested for inducible clindamycin resistance by *D*-test as per CLSI guidelines [19].

**2.8. Detection of *mecA* Gene by Polymerase Chain Reaction (PCR).** Conventional phenol: chloroform method [23] was employed for extraction of chromosomal deoxyribonucleic acid (DNA) from the isolates. After optimization, the extracted DNA was subjected to PCR (Figure 1) for detection of *mecA* gene using PCR profiles described by Abu Shady et al. [24] (Table 1). The primer *mecAF* (5'-aaaatc gatg gtaaggttggc-3') and the reverse primer *mecAR* (5'-agttctggagtaccggatttgc-3') supplied by Eurogentec were used.

**2.9. Quality Control.** For quality control, *Escherichia coli* ATCC 25922, *S. aureus* ATCC 25923, *S. aureus* ATCC 29213 (*mecA* negative), and *S. aureus* ATCC 700699 (*mecA* positive) were used.

**2.10. Data Analysis.** The data obtained were analyzed with the help of statistical package for social sciences version 16.0. Chi-square test was used to analyze association between two variables and *P* value less than 0.05 was considered statistically significant.

## 3. Results

Among 711 pus/wound swab samples processed during the study, 110 (15.47%) showed culture positivity for *S. aureus*. Out of 110 *S. aureus*, 39 (35.50%) isolates were MRSA by cefoxitin disc diffusion method.

**3.1. Antibiotic Susceptibility Patterns of *S. aureus*.** Among the methicillin resistant strains, highest rate of susceptibility was seen toward chloramphenicol (100%) followed by mupirocin (97.40%). Similarly, among methicillin sensitive *S. aureus*

TABLE 1: Comparison of the phenotypic and genotypic methods for detection of MRSA.

Methods to identify MRSA strains	Different methods used for detection of MRSA		
	Cefoxitin disc diffusion	Oxacillin broth microdilution	Polymerase chain reaction
	Strains of <i>S. aureus</i> having zone of inhibition of $\leq 21$ mm to cefoxitin disc (30 $\mu\text{g}$ )	Strains of <i>S. aureus</i> having oxacillin MIC of $\geq 4$ $\mu\text{g}/\text{mL}$	Strains of <i>S. aureus</i> harboring <i>mecA</i> gene

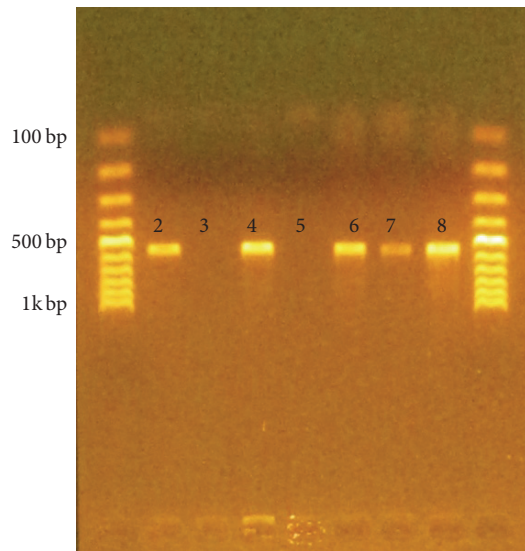


FIGURE 1: Gel electrophoresis showing the PCR products (lane 1 and lane 9: DNA ladder, lane 2: positive control, lane 3: negative control, lane 4: P18, lane 5: P36, lane 6: P53, lane 7: P78, and lane 8: P104).

(MSSA) strains, highest rate of susceptibility was seen to rifampin and tetracycline (100%) followed by chloramphenicol and mupirocin (98.60%) (Table 2).

**3.2.  $\beta$ -Lactamase Production among MRSA and MSSA.** Beta-lactamase production was observed in 79 (71.82%) isolates of total 110 *S. aureus*. Of which 52 (65.82%) isolates were MSSA and 27 (34.18%) isolates were MRSA. Statistically, there was no significant association between methicillin resistance and  $\beta$ -lactamase production ( $P$  value  $> 0.05$ ).

**3.3. Inducible Clindamycin Resistance among MSSA and MRSA.** The inducible clindamycin resistance was observed in 11 isolates. Among which, 6 were MSSA and 5 were MRSA. Statistically, there was no significant association between methicillin resistance and inducible clindamycin resistance ( $P$  value  $> 0.05$ ).

**3.4. Minimum Inhibitory Concentration of Oxacillin and Vancomycin.** A total of 35 (31.82%) *S. aureus* isolates were found to be MRSA by broth microdilution method with MIC cutoff value of 4  $\mu\text{g}/\text{mL}$ . Among them, 11 (31.43%) isolates had MIC of  $>128$   $\mu\text{g}/\text{mL}$  (high level oxacillin resistant strains). The MIC of oxacillin for *S. aureus* isolates ranged from 0.032  $\mu\text{g}/\text{mL}$  to 256  $\mu\text{g}/\text{mL}$ . Only 4 out of 39 MRSA screened

TABLE 2: Antibiotic susceptibility patterns of MSSA and MRSA.

Antibiotics	MSSA	MRSA	$P$ value
	Susceptible (%)	Susceptible (%)	
Erythromycin	33 (46.5)	7 (17.9)	0.003
Clindamycin	57 (80.3)	25 (64.1)	0.062
Gentamicin	64 (90.1)	14 (35.9)	0.000
Ciprofloxacin	37 (52.1)	9 (23.1)	0.003
Chloramphenicol	70 (98.6)	39 (100)	0.457
Cotrimoxazole	30 (42.2)	12 (30.8)	0.236
Mupirocin	70 (98.6)	38 (97.4)	0.664
Rifampin	71 (100)	35 (89.7)	0.006
Tetracycline	71 (100)	34 (87.2)	0.002
Penicillin G	19 (26.8)	0 (0)	0.000

by cefoxitin disc diffusion method were found to be susceptible to oxacillin by broth microdilution method. Spearman's correlation between the two phenotypic methods was significant (0.922) at the 0.01 level (2-tailed). Similarly, all *S. aureus* had MIC of vancomycin below 2  $\mu\text{g}/\text{mL}$  (0.016  $\mu\text{g}/\text{mL}$  to 1  $\mu\text{g}/\text{mL}$ ) that is susceptible to vancomycin irrespective to methicillin resistance.

**3.5. Detection of *mecA* Gene.** A total of 32 (29.1%) *S. aureus* isolates were found to contain *mecA* gene. All of the *mecA* containing strains of *S. aureus* were MRSA by both phenotypic methods, that is, cefoxitin disc diffusion method and oxacillin broth microdilution method. Four out of 39 MRSA screened by cefoxitin disc diffusion method, which were found to be susceptible to oxacillin by broth microdilution method, were not found to contain *mecA* gene. Further, the gene was found absent on MSSA detected by any of two phenotypic methods.

**3.6. Evaluation of Cefoxitin Disc Diffusion and Oxacillin Broth Microdilution Methods in Reference to Presence of *mecA* Gene.** *MecA* gene was found to be absent in 7 of the MRSA detected by cefoxitin disc diffusion method and 3 of the MRSA detected by oxacillin broth microdilution method. The sensitivity of both methods was 100% but the specificity of oxacillin broth microdilution method was greater (96.15%) than that of cefoxitin disc diffusion method (91.03%).

## 4. Discussion

In our study 35.50% of the isolates were found to be MRSA by cefoxitin disc diffusion method, which was comparable with the findings by Kshetry et al. (37.6%) [8] and Sanjana et al.



(39.6%) [25]. But lower prevalence was reported by Subedi and Brahmadathan (15.4%) [26] and Baral et al. (26%) [27] and higher prevalence was reported by Khanal and Jha (68%) [16] and Tiwari et al. (69.1%) [28]. The difference in rates of isolation of MRSA in different studies might be due to the difference in locations and time periods of the studies, difference in hygienic conditions maintained in different hospitals [8], healthcare facilities provided by the hospital, implementation of infection control program, and rational use of antibiotics, which may vary from hospital to hospital [29].

No resistance of MRSA to older drug, chloramphenicol, in our study indicates routine exposure of bacteria to newly developed antibiotics and reversal of susceptibility to outdated antibiotic [30]. The low incidence of mupirocin resistance signifies low usage of the antibiotic [31].

In the present study, inducible clindamycin resistance was found in 10% of *S. aureus* isolates, which was in agreement with the result reported by Ansari et al. (12.4%) [32]. In our study, the occurrence of inducible clindamycin resistance was not significantly different among MRSA and MSSA. However, differentiation of inducible clindamycin resistant phenotypes from others is crucial for therapeutic implication of clindamycin. As use of clindamycin for treatment of the infections caused by such bacteria may result into treatment failure [7], clindamycin should not be used for treatment of such infections; rather it should be used only for the treatment of the infections caused by bacteria which are negative for inducible clindamycin resistance. Clindamycin susceptible strains which are erythromycin resistant may show inducible clindamycin resistance (*D*-test positive) and it has been suggested that inducible clindamycin resistant strains should be reported as clindamycin resistant [19]. Avoiding the use of clindamycin for the treatment of infections caused by erythromycin resistant strains also omits the chances of treatment failure [33].

In the present study, 71.1% of isolates were beta-lactamase producers by iodometric method. This is low in comparison to finding by Shrestha and Rana in nosocomial *S. aureus* isolates in Kathmandu and Lalitpur based hospitals [34]. This may be due to high rate of drug resistance among nosocomial isolates. Globally, beta-lactamase production rate lies between 55.7% and 92.6% for Staphylococci [22]. In our study, all the beta-lactamase producers were also resistant to penicillin G.

In case of MSSA, penicillin is considered superior to oxacillin to treat *S. aureus* infections if they are penicillinase nonproducers [35]. Since most of the resistance in *S. aureus* is secondary to beta-lactamase production and high level production of the enzyme results in development of borderline methicillin resistant *Staphylococcus aureus*, detection of beta-lactamase in *S. aureus* is always crucial [36].

In this study, the sensitivity of both the cefoxitin disc diffusion method and oxacillin broth microdilution method was found to be 100% but specificity of oxacillin broth microdilution method was found to be better. However, cefoxitin disc diffusion is preferred over MIC determination because it is easy to perform and requires no special equipment [37]. *MecA* gene was not present in some of the strains of MRSA screened by cefoxitin disc diffusion method or oxacillin broth

microdilution method. But CLSI guidelines regard the isolates as MRSA if they are found resistant to either cefoxitin or oxacillin or both regardless of the presence of *mecA* gene [19].

Interestingly, isolates ( $n = 7$ ) which had no *mecA* gene but were found to be methicillin resistant by phenotypic methods were observed to be beta-lactamase producers. Those isolates ( $n = 4$ ) which were MRSA by cefoxitin method, but MSSA by oxacillin MIC method, had MIC value of 2  $\mu\text{g}/\text{mL}$ . However, the oxacillin MIC value of isolates ( $n = 3$ ) which were MRSA by both phenotypic methods but had no *mecA* gene was 4  $\mu\text{g}/\text{mL}$ . The possible reason for methicillin resistance in absence of *mecA* gene may be hyperproduction of  $\beta$ -lactamase [38, 39]. Besides, in a recent study by Ballhausen et al. [40], *mecC*, a *mecA* homologue, has also been found to confer methicillin resistance in *S. aureus* in which *mecA* gene was absent. Though more research is needed, questions can be raised in considering *mecA* as sole genetic marker for methicillin resistance. But we could not check the presence of *mecC* as a possible reason for the phenotypic expression of methicillin resistance in absence of *mecA* gene. The presence of *mecA* gene in plasmid of *S. aureus* isolates has also been reported [41]. Since our study was completely dependent on the detection of *mecA* on chromosomal DNA, plasmid encoded *mecA* may have contributed for methicillin resistance in phenotypic tests. Therefore, all the genotypic possibilities should be analyzed for the phenotypic expression of methicillin resistance in *S. aureus* in order to discover appropriate epidemiological marker of methicillin resistance [42].

In the global scenario, 13 VRSA isolates have been isolated since its first detection in 2002 in USA with scanty reports from India and Iran [43, 44]. The *vanA* gene responsible for reduced susceptibility of *S. aureus* toward vancomycin has been found to be transferred from *Enterococcus faecalis* and *E. faecium* [44].

In Nepal, there are limited literatures regarding MIC of vancomycin for *S. aureus* isolated from clinical samples. We reported the MICs of vancomycin for *S. aureus* to be 0.016  $\mu\text{g}/\text{mL}$  to 1  $\mu\text{g}/\text{mL}$ . Similarly, Kshetry et al. reported the MICs of vancomycin to MRSA to be 0.125  $\mu\text{g}/\text{mL}$  to 1  $\mu\text{g}/\text{mL}$  [8]. Slightly higher MICs were reported by Amatya et al. (i.e., 0.5  $\mu\text{g}/\text{mL}$  to 2  $\mu\text{g}/\text{mL}$ ) [45]. Till now no strains of *S. aureus* resistant to vancomycin have been reported from Nepal [46]. However, four VISA isolates have been reported by Pahadi et al. with MICs of vancomycin to MRSA ranging from 0.5  $\mu\text{g}/\text{mL}$  to 4  $\mu\text{g}/\text{mL}$  [46]. VISA and VRSA have been reported by many other authors from different countries [8]. Exposure of the *S. aureus* to vancomycin may be responsible for its reduced susceptibility to the reserve drug and it is attributed to the selective pressure [8]. It is difficult to treat the infections caused by VRSA due to limited antibiotics available for its treatment [8] and it is emerging as a serious public health problem.

## 5. Conclusions

On the basis of our findings, both phenotypic methods (cefoxitin disc diffusion and oxacillin broth microdilution)

could be used for routine diagnosis of MRSA; however cefoxitin disc diffusion might be preferred over MIC method considering time and labour factor. MRSA and inducible clindamycin resistant *S. aureus* are emerging as a serious threat to public health in Nepal. Vancomycin can still be used as the drug of choice for treatment of infections caused by MRSA.

## Abbreviations

MRSA:	Methicillin resistant <i>S. aureus</i>
MIC:	Minimum inhibitory concentration
VRSA:	Vancomycin resistant <i>S. aureus</i>
VISA:	Vancomycin intermediate sensitive <i>S. aureus</i>
PBP2a:	Low-affinity penicillin binding protein
SCCmec:	Staphylococcal cassette chromosome mec
PCR:	Polymerase chain reaction
CLSI:	Clinical and Laboratory Standards Institute
ATCC:	American type culture collection
MSSA:	Methicillin sensitive <i>S. aureus</i>
DNA:	Deoxyribonucleic acid.

## Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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