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Antibiotic *Susceptibility* of Staphylococcus Aureus with VanA and MecA Genes

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ABSTRACT

Background: Staphylococcus aureus (S.aureus) is an emerging antibiotic resistant bacterium responsible for various infections in human. Resistance to methicillin and vancomycin are of prime concern in S. aureus. The study aims to determine the minimum inhibitory concentration (MIC) of Vancomycin and evaluate the existence of mecA and vanA genes, associated with antibiotic resistance.

Methods: Clinical specimens from three Kathmandu hospitals were processed and S. aureus was identified using conventional microbiological procedures. MRSA was phenotypically identified with cefoxitin (30µg) disc diffusion, while vancomycin susceptibility was assessed using the Ezy MICTM stripes. The mecA and vanA genes were detected by polymerase chain reaction (PCR).

Results: Out of 266 S. aureus samples from various clinical specimen subjected for analysis, 77 (28.9%) were found methicillin-resistant (MRSA) and 10 (3.8%) were observed vancomycin-resistant (VRSA). Vancomycin resistant isolates showed a significant correlation between resistance to ampicillin, chloramphenicol, and cefoxitin. The mecA gene was found in 39 of the MRSA isolates, having 50.64% of MRSA cases, while the vanA gene was detected in 4 of the VRSA cases, constituting 40% of VRSA occurrences.

Conclusions: The strains with higher vancomycin minimum inhibitory concentration values (≥ 1.5 µg/ml) displayed increased resistance rates to various antibiotics compared to strains with lower minimum inhibitory concentration values (< 1.5 µg/ml). The presence of vanA genes was strongly associated (100%) with vancomycin resistance, while the 10.3% mecA gene was identified from MRSA having resistance towards vancomycin also.

Keywords: Methicillin resistance; minimum inhibitory concentration; staphylococcus aureus; vancomycin resistance.

INTRODUCTION

Staphylococcus aureus can exist as commensal bacteria in the human body, comprising up to 20-30% of the microbial population. However, they can act as an opportunistic pathogen, causing severe diseases.[1] Methicillin-resistant S. *aureus* (MRSA) and multidrug-resistant strains pose a global threat due to their resistance to commonly used antibiotics.[2]

The emergence of multidrug resistant bacteria and MRSA have been reported in different hospitals of Nepal escalating challenge of antimicrobial resistance in the country's healthcare institutions.[3]

In Nepal, phenotypic studies on S. *aureus* have reported the presence of vancomycin-intermediate and vancomycin-resistant strains, yet data on vancomycin minimum inhibitory concentration (MIC) and *vanA* genes remain limited.[4]The rationale for this study is to determine the MIC and identify *mecA* and *vanA* genes in S. *aureus*, shedding light on antibiotic resistance mechanisms. The study aims to enhance knowledge of antibiotic resistance in S. *aureus*, aiding the development of effective treatment and control strategies.

METHODS

The study was a cross-sectional study performed during March 2020 to May 2021. The research was conducted in the samples collected from the patients visiting three

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tertiary care hospitals of Kathmandu. The specimen included swabs/pus from wound, burn, bed sore, lesions, urine, blood, body fluids and throat swabs. The samples were processed in the laboratory to isolate *S. aureus*. The sample collection was based on the patients of all age group and gender.

The hospitalized and outdoor patients seeking laboratory investigation in the targeted three tertiary care hospitals were included in the study. The samples were collected in the laboratory of the hospital. The microbiological and the biochemical tests were performed for the identification of bacteria which gone through Gram's staining, catalase test, oxidase test, coagulase test, and Oxidative-Fermentative (OF) test for the identification of *S. aureus*.[5,2] The *S. aureus* isolates were further tested for antibiotic susceptibility using Kirby Bauer Disc Diffusion method following the Clinical and Laboratory Standards Institute (CLSI) guideline.[6] *S. aureus* American Type Culture Collection(ATCC)25923 was used as the control strain and purity plate was maintained.[6]

Disk diffusion test of Ampicillin (10µg), Cotrimoxazole (25µg), Gentamicin (10µg), Ciprofloxacin (5µg), Chloramphenicol (30µg), Erythromycin (15µg), Cefoxitin (30µg), Tetracycline (30µg), and Vancomycin (30µg) was carried out using Kirby-Bauer disc diffusion method following the CLSI guidelines 2020.[6] The inoculums (turbidity equivalent to that of a 0.5 McFarland Standard) of the *S. aureus* clinical isolates was cultured on Mueller-Hinton agar plates and after 24hr incubation at 37^{0}C, the zone diameters were measured and interpreted according to CLSI guidelines. For the identification of MRSA strain, cefoxitin was used. Vancomycin Ezy MIC™ stripes were used to perform antimicrobial susceptibility testing against vancomycin. The critical minimal inhibitory concentration (MIC) of Vancomycin for susceptible *S. aureus* strains has been ≤2 µg/ml according to the Clinical and Laboratory Standards Institute guidelines.[6] The *S. aureus* isolates for which MICs of Vancomycin were 4-8 µg/ml and ≥16 µg/ml has been classified as Vancomycin Intermediately Sensitive *Staphylococcus aureus* (VISA) VISA, and Vancomycin-resistant *S. aureus* (VRSA), respectively.[4,6]

The isolates resistant to three or more than three different class of antibiotics were considered as the multi drug resistant (MDR) strain. The chromosomal and plasmid Deoxyribonucleic Acid (DNA) DNA from the *S. aureus* were extracted following the protocols of Qiagen test kit. The extracted DNA was quantified using the Nanodrop. The Polymerase Chain Reaction (PCR) PCR was done for the amplification and detection of *mecA* and *vanA* genes.

The PCR protocol described by Zhang et al. in 2004 was employed to detect the *mecA* genes associated with MRSA and the *vanA* genes related to VISA and VRSA.[7] Using the specific primer pairs, both the genomic and plasmid DNA were used as template and amplified. Positive control templates were also included for amplification. The oligonucleotide primers used for the PCR of *mecA were* TAG AAA TGA CTG AAC GTC CGA TAA (Forward) and CCA ATT CCA CAT TGT TTC GGT CTAA (Reverse). The amplicon size was 310 base pair.[7] The oligonucleotide primers used for the PCR of *vanA* were GGG AAA ACG ACA ATT GC (Forward) and GTA CAA TGC GGC CGT TA (Reverse). The amplicon size was 732 base pair.[8]

The PCR mixture was prepared in a final volume of 25 µℓ. The amplification mixture consisted of 3 µℓ template DNA(10 ng/ µl), 0.5 µℓ each forward(10 µM) and reverse primers(10 µM) and 21µℓ 1X mastermix (Solis Biodyne). The PCR was carried using the thermal cycle as below (Table 1).

Table 1. Thermal cycle program for *mecA* and *vanA* gene amplification.

PCR steps	*mecA* (310bp)		*vanA* (732bp)		
Initial denaturation	95°C	4 min	95 °C	5min	Single step
Denaturation	95°C	30 sec	94°C	45sec	
Annealing	53 °C	45sec	54 °C	45 sec	30 cycles
Extension	72°C	1min	72 °C	7 min	
Final extension	72°C	10 min	72°C	10 min	Single step

The PCR products were analyzed by electrophoresis on 2% agarose gel (MBI Fermentas) containing 0.4 ml/ml of ethidium bromide and visualized and pictured using UV transilluminator.

The study received ethical approval from the Nepal Health Research Council (Regd. No.114/2020). The patients were included only after getting their written consent for demographic data collection and sample collection. Uninterested patients or those who were taking the antibiotics for 48 hours were not included in the study.

The data collected was analyzed using the Statistical Package of IBM SPSS software version 20 (SPSS INC. Chicago, IL). Chi-square test was used for the comparison of categorical variables. The p-value less than 0.05 was considered statistically significant.

RESULTS

A total of 2004 specimens from three different referral hospitals of Kathmandu were processed during March 2020 to May 2021 and 266 *S. aureus* were isolated for phenotypic and genotypic characterization. The heat map reveals that maximum proportion of MRSA, VRSA were isolated from urine samples and the least were from blood (Fig.1).

	Sample type					Total
	Urine	Pus	Blood	Throat swab	Body fluid	
MSSA count (%)	53(28.0)	41(21.7)	17(9.0)	54(28.6)	24(12.7)	189(100.0)
MRSA count (%)	24(31.2)	24(31.2)	6(7.8)	18(23.4)	5(6.5)	77(100.0)
Vancomycin Sensitive based on MIC (%)	74(28.9)	62(24.2)	22(8.6)	70 (27.3)	28(10.9)	256(100.0
Vancomycin Resistant based on MIC(%)	3(30.0)	3(30.0)	1(10.0)	2(20.0)	1(10.0)	10(100.0)
Total	77	65	23	72	29	266
	28.9%	24.4%	8.6%	27.1%	10.9%	100.0%

Figure 1. Heat map of distribution of S. aureus, MRSA and VRSA in clinical specimens

Among 266 *S. aureus* isolates, 77 were MRSA. The MRSA strains exhibit higher percentage of resistance to vancomycin compared to MSSA strains (11.7%).The low p-value indicates that this difference is statistically significant.(Table1)

Table 2. Vancomycin resistant *S. aureus* among MRSA and MSSA.

S. aureus	Vancomycin susceptibility		p-value
	Sensitive	Resistant	
MSSA	188(99.5%)	1(0.5%)	<0.001
MRSA	68(88.3%)	9(11.7%)	
Total	**256(96.3%)**	**10 (3.8%)**	

In molecular analysis, all isolates with *vanA* genes were vancomycin resistant. Among *S. aureus* isolates without *vanA* gene, 2.3% of the isolates were resistant to vancomycin. Likewise, 6(2.6%) isolates without *mecA* gene were found vancomycin resistant and only 10.3% *S. aureus* with *mecA* gene were vancomycin resistant (Table 2).

Table 3. *vanA* and *mec A* genes among vancomycin resistant isolates.

Genes	Vancomycin resistant status		p-value
van A	Yes	No	
Detected	4 (100%)	0 (0%)	<0.001
Not detected	6 (2.3%)	256 (97.7%)	
mecA			
Detected	4 (10.3%)	35 (89.7%)	0.043
Not detected	6 (2.6%)	221(97.4%)	

The following figure of gel image depicts PCR screening for the *mecA* gene, specifically targeting a 310bp fragment. A 100 base pair ladder is loaded in the first lane as the marker, providing a size reference for the DNA fragments. The

positive control in the second lane is the benchmark for the presence of the *mecA* gene. Subsequent lanes exhibit PCR products, each originating from distinct DNA samples of Multi-Drug Resistant *S. aureus* (MDRSA) isolates.

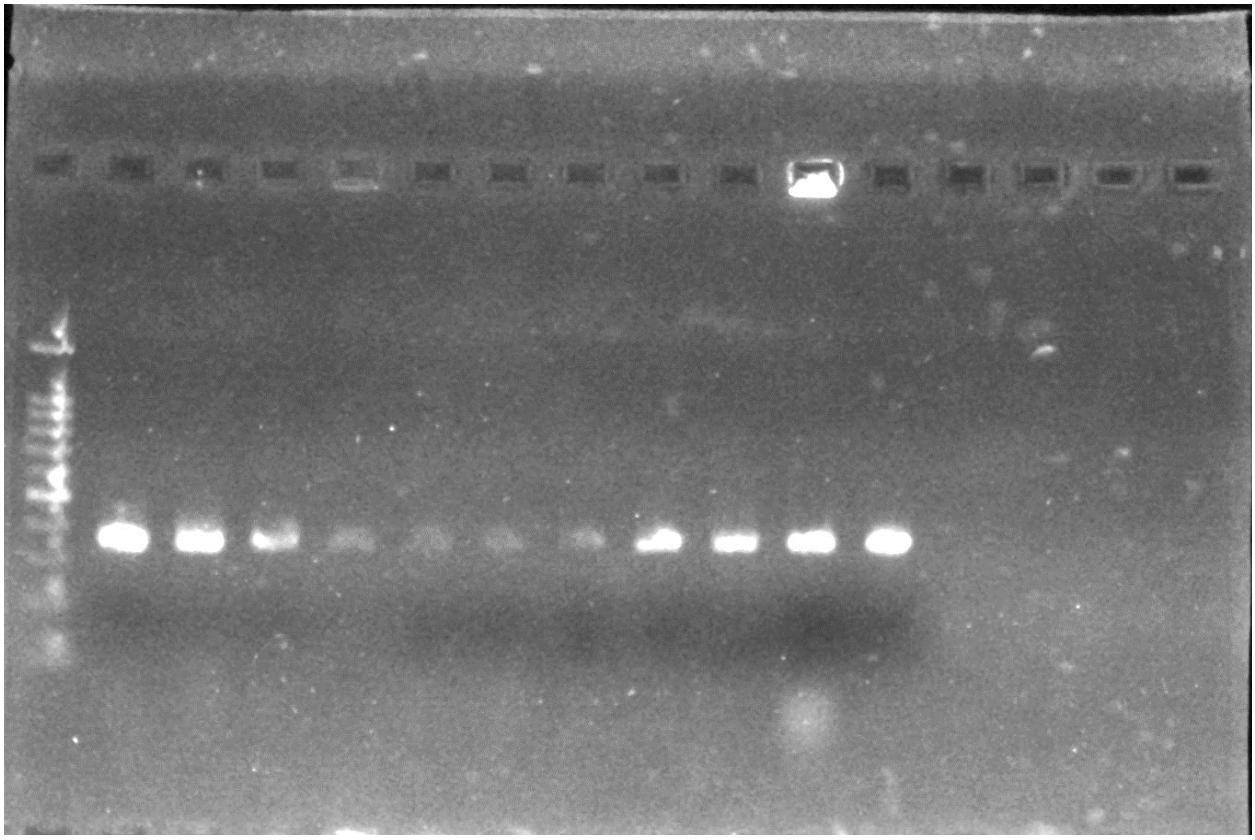

Figure 2. Representative gel image of PCR screening for mecA gene (310bp). From left to right, Lane 1- 100 bp ladder(Marker), Lane 2 - positive control, Last lane- Negative control, Remaining lane - PCR product of different DNA samples.

A strong association between resistance to Vancomycin, the presence of the *vanA* gene, and higher MIC values was observed. Individuals with the *vanA* gene or classified as resistant to Vancomycin tend to have significantly higher MIC values compared to those without the gene or classified as sensitive to Vancomycin. The p-values suggest that these differences are statistically significant (Table 3).

Table 4. Difference in Vancomycin MIC between resistant and sensitive isolates.

Particulars	Number (%)	MIC mean ± SD	p-value
Vancomycin			
Resistant	10 (3.8%)	2.5 ±0.527	<0.001
Sensitive	256 (96.2%)	1.085±0.199	
van A gene			
Detected	4 (1.5%)	2.875±0.478	<0.001
Non detected	262 (98.5%)	1.112±0.269	

The table 4 demonstrates a significant association between the Vancomycin MIC values and the susceptibility to the antibiotics Chloramphenicol, Cefoxitin, Tetracycline, Erythromycin, and Nitrofurantoin. Sensitive individuals tend to have lower MIC values, while Resistant individuals tend to have higher MIC values, and the p-values indicate the strength of these associations.

Table 5. Comparison of Vancomycin MIC with susceptibility pattern of Chloramphenicol, Cefoxitin, Tetracycline, Erythromycin and Nitrofurantoin.

Antibiotic	Susceptibility	Vancomycin MIC		p-value
		< 1.5	≥ 1.5	
Chloramphenicol	Sensitive	182 (91%)	18 (9%)	<0.001
	Intermediate	8 (72.7%)	3 (27.3%)	
	Resistant	24 (43.6%)	31 (56.4%)	
Cefoxitin	Sensitive	178 (94.2%)	11 (5.8%)	<0.001
	Intermediate	5 (71.4%)	2 (28.6%)	
	Resistant	31(44.3%)	39 (55.7%)	
Tetracycline	Sensitive	204 (85%)	36 (15%)	<0.001
	Intermediate	3 (37.5%)	5 (62.5%)	
	Resistant	7 (38.9%)	11 (61.1%)	
Erythromycin	Sensitive	180 (87.4%)	26 (12.6%)	<0.001
	Intermediate	14 (73.7%)	5 (26.3%)	
	Resistant	20 (48.8%)	21 (51.2%)	
Nitrofurantoin	Sensitive	47 (90.4%)	5 (9.6%)	0.004
	Intermediate	4 (66.7%)	2 (33.3%)	
	Resistant	11 (55%)	9 (45%)	

DISCUSSION

In our study, the majority of both MSSA and MRSA strains were sensitive to Vancomycin. However, MRSA strains showed a higher rate (4.7%) of resistance (9/189) compared to 1.29% MSSA (1/77) strains indicating that statistically significant association between vancomycin susceptibility and methicillin resistance status.

Regarding susceptibility to different antibiotics among vancomycin-sensitive and vancomycin-resistant strains, significant association was observed with susceptibility to Ampicillin, Chloramphenicol and Cefoxitin. A similar study in Turkey reported the similar findings with high percentage of VISA in MRSA isolates.[9] The *vanA* genes were isolated in 4 (40%) out of 10 vancomycin resistant isolates and *mecA genes in 4* (10.25%). A similar finding was reported in 2020, with a 33.3% detection rate for the *mecA* gene and 30% detection rate for the *vanA* gene.[10]

Our data indicates that the presence of the *van A* gene is absolute with Vancomycin intermediately sensitive isolates. A study in Bagdad in 2013 reported the similar finding. All *van A* genes were isolated from the Vancomycin resistant strain and the MIC results and PCR findings were similar.[8] However, in our finding all *van A* belong to VISA strain but all VISA do not contain *van A*. The data shows that vancomycin-resistant strains accounted for 3.8% of the total, with an average MIC of 2.5 ± 0.527. Vancomycin-sensitive strains comprised 96.2% of the total, with an average MIC of 1.085 ± 0.199. The presence of the *van A* gene was detected in 1.5% of the strains, with an average MIC of 2.875 ± 0.478, while it was not detected in 98.5% of the strains, with an average MIC of 1.112 ± 0.269. The differences in vancomycin susceptibility and the presence of the *van A* gene were highly significant ($p < 0.001$).

There are notable differences in antibiotic susceptibility based on vancomycin MIC thresholds. In general, strains with higher vancomycin MIC values (≥ 1.5mg/ml) tend to show higher resistance rates for Chloramphenicol, Cefoxitin, Tetracycline, Erythromycin, and Nitrofurantoin compared to strains with lower vancomycin MIC values (< 1.5 mg/ml). Saeed et al reported the similar findings with high prevalence of methicillin resistance among vancomycin resistant *S. aureus*.[10]

The observed association between higher vancomycin MIC values (≥ 1.5 mg/ml) and increased resistance rates to antibiotics like Chloramphenicol, Cefoxitin, Tetracycline, Erythromycin, and Nitrofurantoin in *S. aureus* may be related to several molecular mechanisms. Some bacteria have efflux pumps that can actively expel antibiotics from the cell. High vancomycin MIC values might indicate an upregulation of efflux pumps, leading to increased resistance to other antibiotics[11] . Vancomycin primarily targets bacterial cell walls. Strains

with higher vancomycin MIC values may have altered cell wall composition as a resistance mechanism. These alterations could impact the entry of other antibiotics into the cell, making the bacteria more resistant.[12]

In some cases, resistance genes for multiple antibiotics are found in close proximity on plasmids or other genetic elements. Strains with high vancomycin MIC values may carry such genetic clusters, leading to resistance against other antibiotics.[13] The molecular mechanisms involved in these findings may be complex and multifactorial. It is crucial to conduct further research to understand the specific genetic and molecular determinants responsible for the observed resistance patterns in clinical strains of *S. aureus*. This knowledge is essential for developing effective strategies to combat antibiotic resistance.[4,11,12,13]

CONCLUSION

Vancomycin resistant organisms were resistant to most of the other antibiotics. The findings revealed significant associations between vancomycin resistance and the presence of *mecA* and *vanA* genes. Notably, strains with higher vancomycin MIC values (≥ 1.5mg/ml) displayed increased resistance rates to various antibiotics, such as Chloramphenicol, Cefoxitin, Tetracycline, Erythromycin, and Nitrofurantoin, compared to strains with lower MIC values (<1.5 mg/ml)). The presence of *vanA* genes was strongly associated with vancomycin resistance, while the *mecA* gene was also linked to resistance. The Methicillin and Vancomycin resistance among *S. aureus* isolates showed that there should be continuous monitoring of antibiotic susceptibility of *S. aureus* to control further development of resistance.

CONFLICT OF INTEREST

None.

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