# Genotypically Confirmed Vancomycin-Resistant *Staphylococcus aureus* With *vanB* Gene Among Clinical Isolates in Kathmandu

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**ABSTRACT**

**PURPOSE:** Methicillin-resistant *Staphylococcus aureus*, a common bacterial pathogen causes various infections. The acquisition of various antimicrobial-resistant genes in *S. aureus* has led to the transformation of this bacterium into a superbug. Vancomycin resistance among MRSA isolates is an emerging threat in empirical therapy of various infections. The study was hence aimed to find out the susceptibility status of *S. aureus* isolates toward vancomycin and detect *mecA, vanA*, and *vanB* genes among the isolates.

**METHODS:** A total of 1245 clinical samples from the participants attending a tertiary care hospital in Kathmandu were processed. *S. aureus* isolated from the samples were subjected to antibiotic susceptibility patterns using the modified Kirby-Bauer disk diffusion method. Agar dilution method was used to determine the minimum inhibitory concentration of vancomycin. The antibiotic-resistant genes such as *mecA, vanA*, and *vanB* among *S. aureus* isolates were screened by a conventional polymerase chain reaction.

**RESULTS:** Of 1245 samples, 80 *S. aureus* were identified. Out of which, 47.5% (38/80) were phenotypically confirmed MRSA isolates. *mec*A gene was detected in 84.2% (32/38) of MRSA isolates. 10.5% (4/38) were confirmed as vancomycin-intermediate *S. aureus* (VISA) by MIC determination. None of the isolates was positive for *the vanA* gene; however, 2 isolates were found to possess the *vanB* gene. The 2 isolates have vancomycin MIC breakpoints of 4 to 8 μg/mL.

**CONCLUSION:** There might be a spreading of vancomycin resistance among *S. aureus*, creating serious public health problems. Therefore, measures to limit vancomycin resistance should be considered in healthcare facilities as immediately as possible.

**KEYWORDS:** *Staphylococcus aureus*, Multidrug-resistant, MRSA, VISA, *mecA*, *vanB*

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## Background

*Staphylococcus aureus*, a Gram-positive bacterium, is both a human pathogen and part of the normal flora. *S. aureus* causes various infections, including skin and deep tissue infections (boils, carbuncles, furuncles, wound infections, folliculitis, bullous or pustular impetigo, scalded skin syndrome, cellulitis, necrotizing fasciitis),[1-5] urinary tract infections,[4] bacterial conjunctivitis, osteomyelitis and nosocomial infections such as surgical wound infections, lower respiratory tract infections and medical implant infections.[6] It can also cause toxin-associated poisoning through enterotoxin called staphylococcal food poisoning.[7,8] In addition, the toxins act as superantigens in toxic shock syndrome, eliciting inflammation by activating T-lymphocytes to release tissue necrosis factor α (TNFα) and interleukin-1. These cytokines activate other cells such as fibroblasts, epithelial cells, and endothelial cells leading to prolong inflammation followed by increased vascular permeability.[9,10]

The dissemination of methicillin-resistant *S. aureus* (MRSA) across developing countries has been a significant obstacle in implementing antibiotic treatment and the outcome of severe *S. aureus* infections.[4] Resistance to beta-lactam antibiotics by MRSA strains is associated with transferable bacterial genome islands (GI) known as staphylococcal chromosomal cassette *mec* (SCC*mec*).[11] The *mec* gene is responsible for methicillin resistance. These islands are evolving rapidly and contain a series of genomic elements named *mecA*, *mecB*, *mecC*, and so on.[12,13] These genes are not only associated with resistance of S. aureus to methicillin but also to other antibiotic categories such as macrolides, lincosamides, streptogramins B, tetracyclines, and aminoglycosides.[11] Two glycopeptides, namely vancomycin and teicoplanin have been introduced as

the drugs of choice to cure and prevent of MRSA infections.[7] However, the routine prescription of vancomycin in empirical treatment can lead to a rapid emergence of vancomycin-intermediate *S. aureus* (VISA) and vancomycin-resistant *S. aureus* (VRSA).[14] The resistance to vancomycin in *S. aureus* is due to the acquisition of the *vanA* operon from *Enterococcus* spp., which is carried in transposon Tn1546.[15] In 2002, the first VRSA strains were reported, possessing the VanA operon in transposon Tn1546.[16] A ligase encoded by *the vanA* gene alters the dipeptide residue that substantially lowers the affinity for the antibiotic resulting in the non-susceptibility of isolates toward the drug.[11,15] There are more operons such as VanA, VanB, VanC, VanD, VanE, VanG, VanL, VanM, and VanN named after the ligase they encode.[17]

The emergence of MRSA with MDR property has left us with very few therapeutic options for staphylococcal infections. Mainly, in the case of Nepal, very few studies have been conducted, and the prevalence rate of MRSA is inconsistent, ranging from 15.4% to 69%.[18-21] We hypothesized that the susceptibility of MRSA isolates toward vancomycin may have decreased over time. There might be a spreading of VRSA in healthcare facilities. There are limited studies from Nepal reporting on the vancomycin susceptibility patterns of MRSA isolates through the determination of MIC and detection of genes conferring vancomycin resistance. The genetic characterization of such strains is also lacking. Since the empirical use of vancomycin is quite high in our healthcare facilities, we believed that the spread of VRSA strains might have occurred among clinical *S. aureus* isolates. Therefore, our study was conducted to determine the current prevalence of MRSA among the clinical *S. aureus* isolates. We also assessed the vancomycin resistance of MRSA isolates through the screening of potential genes for methicillin and vancomycin resistance.

## Methods

### *Ethics approval and the patient's consent*

The study received approval from the Institutional Review Committee at the Institute of Science and Technology, Tribhuvan University, Kirtipur, Kathmandu, Nepal (Ethical Approval Ref. No.: IRC/IOST-4/2019). Prior to collecting samples from the participants, written informed consent was obtained. In the cases of children, consent was obtained from their parents.

### *Study design*

A descriptive and hospital-based cross-sectional study was carried out in Grande International Hospital, Dhapasi, Kathmandu, from December 2019 to June 2020. Specimens received from the participants suspected of bacterial infections attending the hospital were processed at the hospital's laboratory, and the molecular biology section was performed at the Central Department of Microbiology Laboratory, Tribhuvan University, Kirtipur Kathmandu.

### *Inclusion and exclusion criteria*

Participants of all age groups from both sexes were enrolled in the study. Participants with a recent history of antibiotic consumption at the time of enrollment were excluded from the study. Inadequate specimens or samples showing visible signs of contamination were not included.

### *Processing clinical samples*

A total of 1245 different clinical samples, including blood (n = 382), pus (n = 394), and wound (n = 469) swabs, were processed during the 6-month sample collection period. The samples were collected aseptically by experienced medical personnel, placed in a clean, leak-proof container, and labeled. The clinical signs and symptoms, previous infections, presence of any underlying diseases, and prior antibiotic administration were obtained at the time of sample collection.[22]

The collected blood samples were immediately inoculated in BACTEC 9050 culture vial (CAT no. 445800) and delivered to the microbiology laboratory. Wound and pus samples from the abscess, post-operative wound, deep-seated, superficial incisional, or organ/space surgical site infections were collected on a sterile cotton swab or aspirated into a syringe containing the innermost portion of the lesion or exudates.[23] All procedures were done following standard methodology.

### *Culture of the samples*

Blood samples were immediately processed in BACTEC 9050 following the company's standard operating procedure. Positive samples were sub-cultured on Mac Conkey Agar (MA) and blood agar (BA) and subsequently incubated at 37°C for 5 days. Pus or wound swabs were directly inoculated onto MA agar and BA agar and incubated for 24 hours at 37°C. The plates which showed no growth even after incubation at 37°C for up to 48 hours were discarded. The bacterial growth with isolated colonies was identified by microbiological procedures including colony characterization on selective media, biochemical characterization (including coagulase test, DNase test), and serotyping.[22,23]

### *Antibiotic susceptibility test*

Bacterial susceptibility to selected antibiotics was performed in vitro by using the modified Kirby-Bauer disk diffusion method following Clinical and Laboratory Standards Institute (CLSI) guidelines.[24] Antibiotics; cefoxitin (30 µg), chloramphenicol (30 µg), ciprofloxacin (30 µg), clindamycin (2 µg), erythromycin (15 µg), gentamicin (10 µg), nitrofurantoin (300 µg), tetracycline (30 µg), and penicillin (10 µg) were used. Bacteria that were resistant to 3 or more different classes of antibiotics were tagged as multidrug-resistant (MDR).[25]

### *Methicillin-resistant Staphylococcus aureus (MRSA)*

The screening of MRSA was performed using the antibiotics cefoxitin disk following CLSI guidelines.[24] The growth of *S. aureus* with a zone of inhibition (ZOI) of less than 22 mm was considered MRSA.

### *Determination of minimum inhibitory concentration (MIC) of vancomycin*

All *S. aureus* isolates were processed to determine the MIC for vancomycin drug by agar dilution method as described in CLSI guidelines[24] using a series of vancomycin concentrations ranging from 0.0625 to 32 µg/mL. The inoculums were applied onto Mueller-Hinton agar (MHA) plates and incubated for 18 hours at 37°C. *S. aureus* ATCC 25923 was included in each test as a control for antibiotic potency. The lowest concentration of vancomycin that completely inhibited the growth was considered as MIC value.[26]

### *Chromosomal and Plasmid DNA extraction*

All MRSA and 5 MSSA (10% of total MSSA isolates) were further selected for the screening of the *mecA* gene. 1.5 mL of bacterial culture grown in Luria-Bertani (LB) broth was taken into a microcentrifuge tube from each culture. Chromosomal DNA was isolated following the chromosomal DNA extraction method.[27] The plasmid DNA was isolated by the alkaline lysis method (using the mini-preparation method).[27,28] Similarly, van*A and vanB* genes were screened among the MRSA isolates. *S. aureus* ATCC 29213 (*mecA* negative) and *S. aureus* ATCC 700699 (*mecA* positive) were used in each step as quality control organisms.

### *PCR amplification of mecA, vanA, and vanB genes*

The amplification of targeted genes was performed on a thermocycler GeNei Polymerase Chain Reaction (PCR) machine. The reaction mixture contained a PCR master mix (200 µM of dNTPs, 120 nM of forward primer, 120 nM of reverse primer, 5 U/µL of Taq polymerase in 1x PCR buffer, 25 mM $MgCl_2$), 1 µL of template DNA, and final volume adjusted with PCR grade water to 25 µL.

Three sets of primers; forward 5′-ACTGCTATCCACCC TCAAAC-3′ and reverse 5′-CTGGTGAAGTTGTAATC TGG-3′ (Macrogen, Korea, Amplicon size-163 bp) for *the mecA* gene,[29] forward 5′- ATGAATAGAATAAAAGTTGC-3′ and reverse 5′-TCACCCCTTTAACGCTAATA-3′ (Macrogen, Korea, Amplicon size-1100 bp) for *the vanA* gene[22] and forward 5′-ACGGAATGGGAAGCCGA-3′ and reverse 5′-TGCACCCGATTTCGTTC-3′ (Macrogen, Korea, Amplicon size-647 bp) for *the van*B gene[30] were used. The thermal cycling conditions to amplify the *mecA* gene was set at an initial denaturation of 94°C for 3 minutes followed by denaturation at 94°C for 45 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 3 minutes for 35 cycles, and final extension at 72°C for 2 minutes. For *the vanA* gene amplification, an initial denaturation of 95°C for 2 minutes and then denaturation at 95°C for 1 minute, annealing at 56°C for 1 minute, extension at 72°C for 1 minute for 40 cycles, and final extension at 72°C for 5 minutes were used. The *vanB* gene was amplified with the similar PCR reaction condition of *vanA* gene amplification with primer annealing temperature at 58°C for 1 minute.[29,31] The post-PCR analysis was performed using 10 µL of each amplified product mixed with 2.5 µL of loading dye and electrophoresed on 1.5% agarose gel. The separated DNA bands were visualized with ethidium bromide (0.3 µg/mL) in a UV trans-illuminator (Cleaver Scientific Ltd).

### *Quality control*

All research works were performed to maintain aseptic conditions, and results were interpreted based on performance against standard American Type Culture Collection (ATCC) cultures. Positive control and negative control were used. The amplicons were compared with standards for analysis.

### *Statistical analysis*

The association between nominal data, antibiotic resistance, MDR, MRSA, and VISA was determined using the Chi-square test. A *P*-value of $<.05$ was considered significant. The data was analyzed using SPSS version 25.0 software.

## Results

A total of 80 *S. aureus* were isolated from 1245 clinical samples, out of which, 55% (n = 44) were from male participants and 45% (n = 36) from female participants. The highest percentage of *S. aureus* was found in pus samples (n = 42; 52.5%), followed by wound swabs (n = 21; 26.3%) and blood (n = 17; 21.3%). 62.5% (n = 50) were from inpatients and 37.5% (n = 30) isolates were from outpatients.

### *Antibiotic susceptibility profiles of* S. aureus *and MDR strains*

The highest levels of drug resistance were observed for penicillin (92.5%) followed by ciprofloxacin (83.8%) and erythromycin (62.5%). The lowest resistance levels were observed for chloramphenicol (6.3%) (Table 1). Likewise, 62.5% (50/80) were multidrug-resistant (MDR) strains. Eighty-three percent (n = 14/17) of isolates from blood samples were MDR, followed by wound swabs (n = 13/21; 61.9%), and pus samples (n = 23/42; 54.8%) (Figure 1). Cefoxitin, ciprofloxacin, clindamycin, and erythromycin-resistant isolates were significantly associated with MDR ($P < .001$). In addition, all isolates found

**Table 1.** Distribution of *mecA*, *vanA*, and *vanB* genes among *S. aureus* isolates.

| *S. AUREUS* ISOLATES | TOTAL NUMBER PROCESSED | POSITIVE NUMBER (%) |
|---|---|---|
| *S. aureus* | 1245 | 80 (6.4) |
| MSSA | 80 | 42 (52.5) |
| Phenotypic MRSA | 80 | 38 (47.5) |
| *mecA* gene among MRSA isolates | 38 | 32 (84.2) |
| *mecA* gene among 10% of MSSA isolates | 5 | 0 |
| *vanA* gene among MRSA isolates | 38 | 0 |
| *vanB* gene among MRSA isolates | 38 | 2 (5.3) |

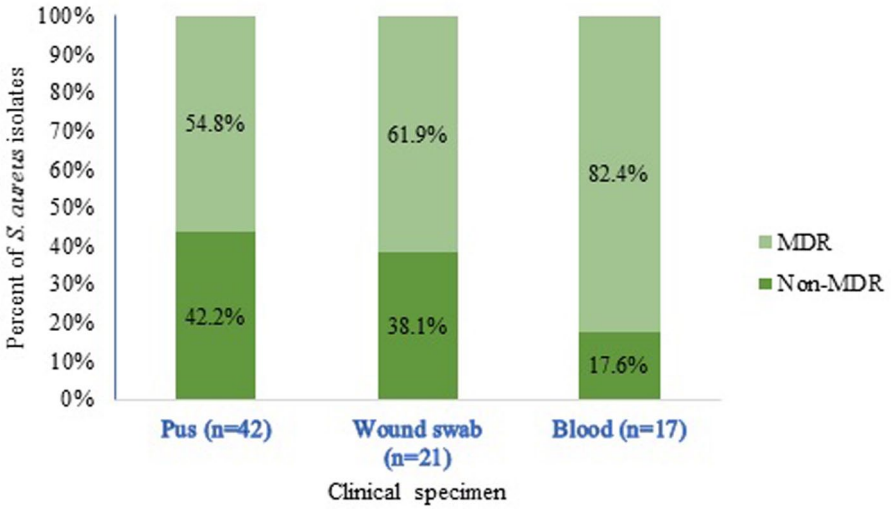


**Figure 1.** Distribution of MDR S. aureus from different clinical samples.

to be sensitive to penicillin were non-MDR; however, all MDR were resistant to tetracycline, chloramphenicol, and nitrofurantoin (Figures 2 and 3).

### *Methicillin and vancomycin resistance* S. aureus *and Minimum inhibitory concentration (MIC) of vancomycin*

A total of 38 (47.5%) isolates were phenotypically confirmed as MRSA by the cefoxitin disk diffusion method, out of which, 11% (n = 4) isolates were found to be intermediate resistant to vancomycin (MIC ≈ 8 µg/mL) (Figure 4).

### *Distribution of mecA and vancomycin-resistant genes in MRSA isolates*

Thirty-two isolates possessed *the mecA* gene among 43 isolates (38 MRSA and 5 MSSA) (Figure 5). Ten percent (n = 5/42) of MSSA were randomly selected to screen for the *mecA* gene. However, all *mecA* gene-carrying isolates were MRSA. Likewise, 2 MRSA isolates possessed the *vanB* gene, both of which are intermediate-resistant to vancomycin (8 µg/mL). None of the isolates possessed the *vanA* gene (Table 2, Figure 6).

### *Distribution of MRSA, MSSA, VISA, mecA, vanA, and vanB genes in different specimens*

The highest number of MRSA were isolated from pus samples, followed by an equal number of isolates from wound and blood samples. A higher number of MRSA isolates from pus samples possessed *the mecA* gene (n = 18/32; 56.3%), followed by blood samples (n = 9/17; 52.9%). Of 4 VISA isolates, 3 isolates were from pus samples, and 1 isolate was from a wound swab. On the other hand, among 2 isolates possessing the *vanB* gene, one was from a pus sample and the other from a wound swab (Table 2).

## Discussion

We detected MRSA in about 48% of *S. aureus* and among them 4 were VISA. Among MRSA, the *mec*A gene was detected in more than 80% of the isolates. *van*A gene was not detected from VISA but *van*B was present in half of the VISA. This suggests that there is a problem of MRSA in clinical settings and the emergence of VISA with the *van*B gene would further complicate the treatment of *S. aureus* infection.

Although the detection of *S. aureus* in this study was relatively less (6.4%) than in the studies conducted previously in

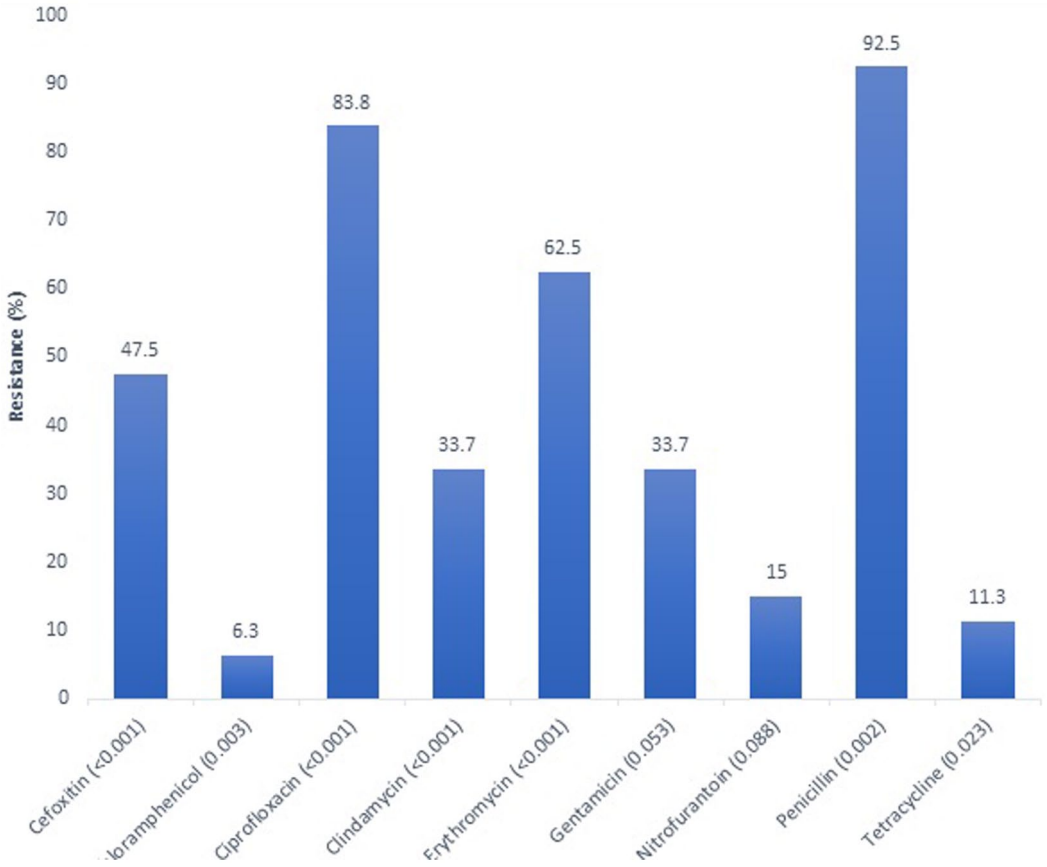


**Figure 2.** Antibiotic susceptibility patterns of S. aureus isolates. Y-axis shows resistance (%) while X-axis shows antibiotics used in the study with a *P*-value in parenthesis.


**Figure 3.** Antibiotic susceptibility pattern of MRSA; The isolate was resistant to Erythromycin (E 15 μg) and Ciprofloxacin (CIP 5 μg) while it was sensitive to Tetracycline (TEI 30 μg), Gentamicin (G 10 μg) and Nitrofurantoin (NIT 200 μg).

tertiary care hospitals in Nepal[20,32],*S. aureus* is still a significant bacterial pathogen isolated from pus, wound, and blood infections. The rate of *S. aureus* colonization between the inpatients and outpatients was statistically not significant. Among 9 different antibiotics used in this study as recommended by CLSI, the highest percentages of isolates were resistant to penicillin. The study, however, revealed the finding of a similar rate of MRSA as compared with previous or simultaneous studies at different tertiary hospitals in Nepal,[18-20,32-34] while other studies from Kathmandu, Nepal,[35] and Pakistan being reported at more than 50%.[36] MRSA causes significant effects on patients' health, prolonged hospital stays, and extended antibiotic therapy[37-39] making MRSA infections a significant threat in hospital settings for therapeutic management.

We focused on the detection of the *mecA* gene on both chromosomal DNA and plasmid DNA. Out of 38 MRSA isolates, 84.2% of isolates possessed the *mecA* gene by conventional PCR with specific primers. Kandel et al[34] reported 72.2% of 18 tested MRSA isolates were found to possess similar gene in Nepal. Our results agree with this report, further confirming that the *mecA* gene is present in most MRSA isolates in Nepal. The presence of the *mec*A gene in MRSA isolates has been documented in several previous studies.[36,40-43] These studies also pinpoint the *mec*A gene as the predominant gene among MRSA isolates. However, in a previous study, we also observed 6 isolates of phenotypic MRSA that were negative for the *mecA* gene, as seen in the other studies.[44,45] The presence of other intrinsic factors, other *mec* genes, mainly the *mecB* and *mecC*, and several allotypes might contribute to methicillin resistance despite lacking the *mecA* gene.[46] We observed that 84.2% of isolates possessing the *mecA* gene were resistant to methicillin. Therefore, all the genotypic possibilities should be analyzed for the phenotypic expression of methicillin resistance in *S. aureus*

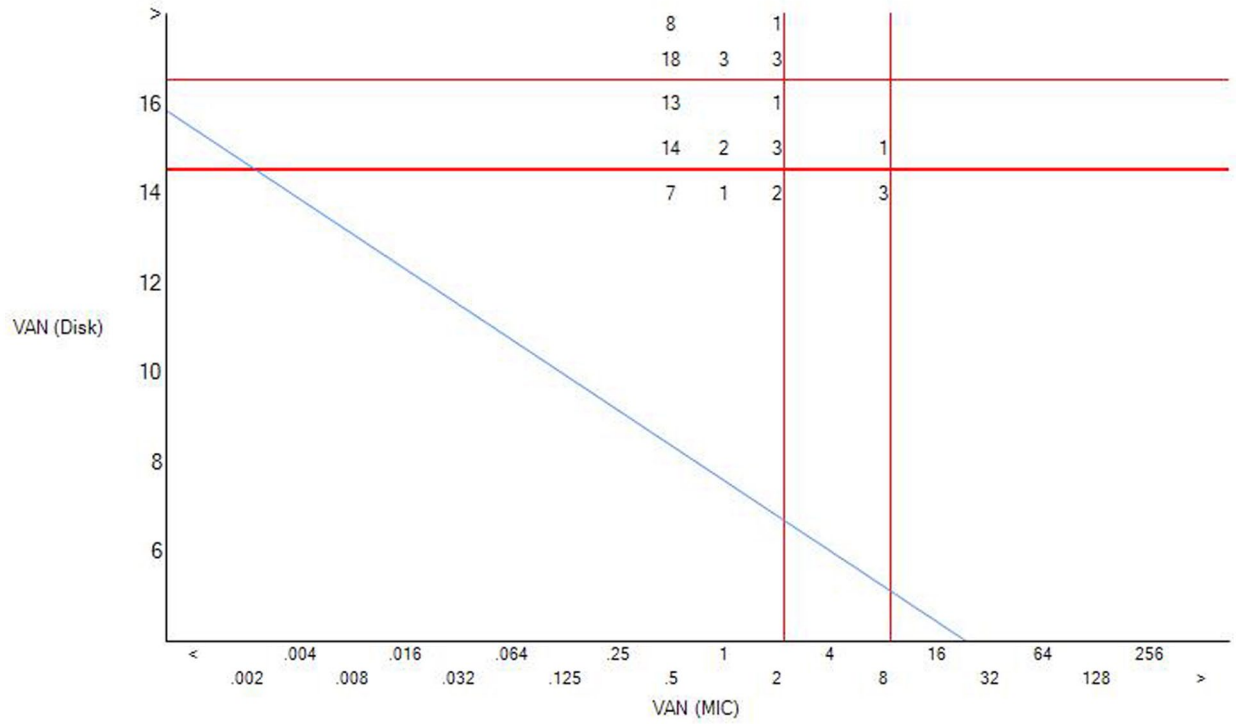


**Figure 4.** Scatter plot showing MIC breakpoint of vancomycin for *S. aureus* isolates. X-axis shows the MIC value for vancomycin while Y-axis shows the zone of inhibition (mm) for the vancomycin disk. The values on the top are the number of isolates.

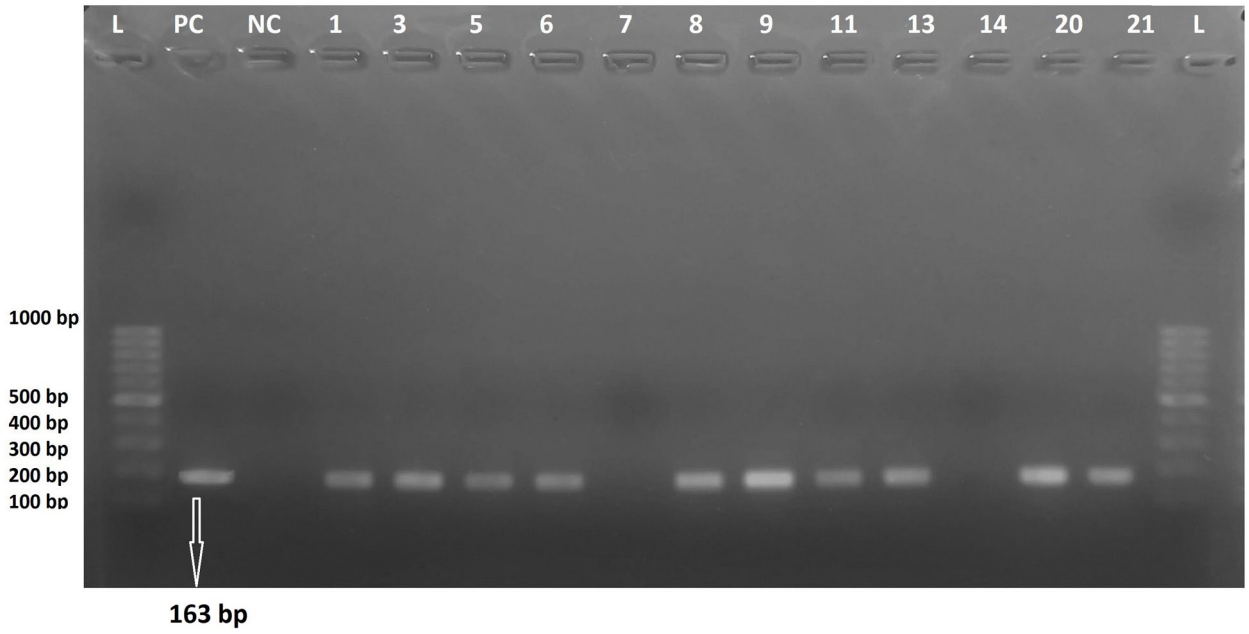


**Figure 5.** Amplification of mecA gene (163 bp) by conventional PCR method (L-DNA Ladder, PC-Positive control, NC-Negative control). mecA gene was detected from Isolates numbers; 1, 3, 5, 6, 8, 9, 11, 13, 20, and 21, while Isolate numbers 7 and 14 didn't possess the gene.

in order to determine the appropriate epidemiological marker of methicillin resistance.[47] However, as a study limitation, we couldn't detect all potential *mec* genes that might cause resistance.

All MRSA isolates were processed for determining MIC using a series of vancomycin concentrations ranging from 0.0625 to 32 μg/mL. Our results are comparable with previous studies from Nepal[32,35] and India.[48] In our current study, the *vanA* gene was not found but the *vanB* gene was detected in 2 MRSA isolates. However, in the previous study, we reported 2 VRSA isolates possessing the *vanA* gene.[49] The *vanB* gene harboring enterococci confers variable resistance against vancomycin ranging from 4 to 1000 μg/mL.[22,50] Similarly, the *vanA* gene is also responsible for the reduced susceptibility of *S. aureus*

**Table 2.** Distribution of MRSA, MSSA, VISA, *mecA*, *vanA*, and *vanB* in different clinical specimens.

| ISOLATES/GENES | PUS SAMPLE (%) | WOUND SWAB (%) | BLOOD (%) |
|---|---|---|---|
| MRSA (n=38) | 18 (47.4) | 10 (26.3) | 10 (26.3) |
| MSSA (n=42) | 24 (57.1) | 11 (26.2) | 7 (16.7) |
| VISA (n=4) | 3 (75.0) | 1 (25.0) | 0 |
| mecA gene (n=32) | 18 (56.3) | 5 (15.6) | 9 (28.1) |
| *vanA* gene (n=0) | 0 | 0 | 0 |
| *vanB* gene (n=2) | 1 (50.0) | 1 (50.0) | 0 |

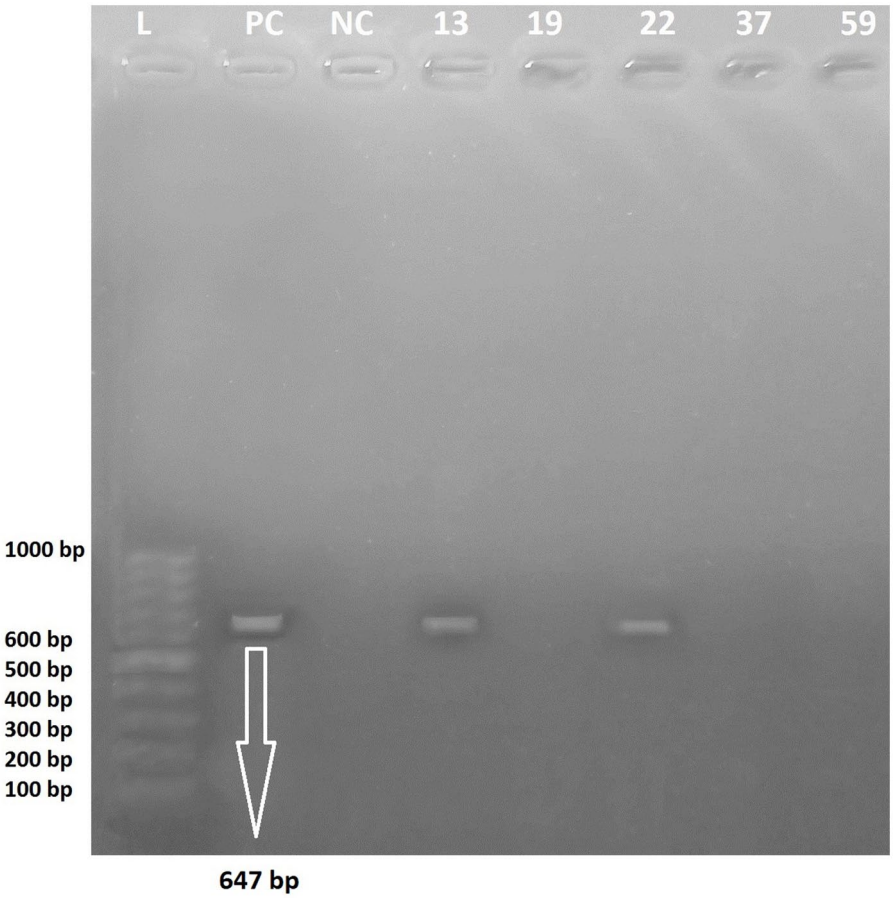


**Figure 6.** Amplification of vanB gene (647 bp) by conventional PCR method (L-DNA Ladder, PC-Positive control for vanB gene, NC-Negative control). vanB gene was detected from Isolates numbers; 13 and 22, while Isolate numbers 19, 37 and 59 didn't possess the gene.

toward vancomycin which has been reported to be transferred from *Enterococcus faecalis* and *E. faecium*.[50] Although *van* genes are responsible for conferring resistance to vancomycin, we only reported 2 VISA isolates possessing the *vanB* gene. The occurrence of those genes and the increasing resistance of *S. aureus* to vancomycin is a cause of concern due to the potential emergence of VRSA strains in our clinical settings. Globally, VRSA isolates have been reported from many countries.[50,51]

The infections caused by VISA/VRSA could be even higher as many clinical settings have limited availability of treatment options.[52] The genetic sequencing of these organisms would provide a deeper understanding of the identified resistance patterns. This, together with the limited time in which sample collection took place and the lack of funds for screening the series of *mec* and *van* genes are limitations of this study.

The findings of this study provide information regarding the continuous burden of MRSA isolates in hospital settings and the status of susceptibility of MRSA toward vancomycin. The information from this study may help in policy-making to prescribe antimicrobials for preventing the emergence of antimicrobial resistance, management of MRSA infections, and subsequent complexity by such MDR strains.

## Conclusions

Detection of the *vanB* gene among MRSA isolates from a tertiary care hospital in Kathmandu alerts to the potential spread of the vancomycin resistance genes among clinical *S. aureus* isolates and other clinically important bacterial species. Since vancomycin is one of the few antibiotics prescribed to cure MRSA infections, developing resistance against vancomycin poses a severe public health threat. Hence, the dispensation of antimicrobials should be supervised to prevent their inadvertent use, while therapeutic choices should be guided by Antibiotic Susceptibility testing results.

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## Author Contributions

All authors had substantially contributed in designing and conception of this study, data acquisition, or analysis and interpretation of results. All have actively played a role in drafting the manuscript or revising it. All authors approved the final version of the manuscript and gave consent to publish it in this journal.

## Ethical Approval and Consent to Participate

The study was approved by the Institutional Review Committee, Institute of Science and Technology, Tribhuvan University, Kathmandu, Nepal. Written informed consent was obtained from all the patients before collecting data and samples.

## Consent for Publication

Not applicable

## Availability of Data and Materials

The raw data of the study will be provided on request to the corresponding author at upendrats@gmail.com.

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