Vol. 5 No. 1 (2026)

Pakistan Journal of Medical & Cardiological Review

https://pakjmcr.com/index.php/1/about

Online ISSN 3007-2387

Print ISSN 3007-2379

Emergence of High-Level Vancomycin Resistance in Staphylococcus aureus: Dissemination of vanA and vanB genes in Clinical MRSA and VRSA Isolates from Pakistan

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Pakistan Journal of Medical & Cardiological Review

https://pakjmcr.com/index.php/1/about

Online ISSN 3007-2387

Print ISSN 3007-2379

Author Details

Received on 12 Feb, 2026

Accepted on 26 March 2026

Published on 28 March 2026

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Abstract

Background: The emergence of vancomycin-resistant *Staphylococcus aureus* (VRSA) poses a significant threat to public health, severely limiting therapeutic options for life-threatening infections. This study aimed to determine the prevalence of VRSA among clinical isolates, characterize their antimicrobial resistance profiles, and detect the presence of key vancomycin resistance genes, *vanA* and *vanB*. **Methods:** A cross-sectional study was conducted on 203 non-duplicate *S. aureus* isolates collected from a tertiary care hospital in Islamabad, Pakistan. Isolates were identified using standard microbiological techniques (Gram stain, catalase, coagulase) and confirmed by 16S rRNA sequencing. Antimicrobial susceptibility testing (AST) was performed using the Kirby-Bauer disk diffusion method, with MRSA identified by cefoxitin resistance. Minimum inhibitory concentrations (MICs) for vancomycin were inferred from disk diffusion, and isolates showing resistance were further analyzed. Conventional PCR was employed to detect the *vanA* and *vanB* resistance genes. **Results:** Of the 203 *S. aureus* isolates, 41 (20.2%) were identified as methicillin-resistant *S. aureus* (MRSA). The MRSA isolates exhibited high levels of resistance to multiple antibiotic classes, including penicillin G (100%), erythromycin (87.5%), ciprofloxacin (75.7%), and co-trimoxazole (59.5%). Alarmingly, 2 (4.9%) of the MRSA isolates were confirmed as VRSA. Molecular analysis revealed a high prevalence of resistance determinants in the VRSA strains, with *vanA* detected in both isolates (100%), *vanB* in one isolate (50%), and one isolate harboring both genes. Among all MRSA isolates, the prevalence of *vanA* was 12.2% (5/41), *vanB* was 4.9% (2/41), and 14.6% (6/41) carried at least one of these genes. **Conclusion:** This study confirms the presence of VRSA in a major healthcare facility in Pakistan, with resistance mediated by the transferable *vanA* and *vanB* genes. The co-existence of multidrug resistance (MDR) in these isolates presents a critical clinical challenge. These findings underscore the urgent need for enhanced antimicrobial stewardship, routine surveillance for VRSA, and molecular characterization of resistance mechanisms to guide infection control policies and preserve the efficacy of last-resort antibiotics.

Keywords: VRSA, MRSA, Antimicrobial Resistance, *vanA*, *vanB*, Vancomycin Resistance, Pakistan

1. Introduction

Staphylococcus aureus remains a formidable opportunistic pathogen, capable of causing a spectrum of infections ranging from minor skin and soft tissue infections to life-threatening conditions such as bacteremia, endocarditis, and toxic shock syndrome (Cheung et al., 2021). The clinical management of *S. aureus* infections has been profoundly complicated by the organism's remarkable ability to acquire resistance to antimicrobial agents. The emergence of methicillin-resistant *S. aureus* (MRSA) in the 1960s, following the introduction of methicillin, marked a pivotal moment, rendering beta-lactam antibiotics largely ineffective (Lakhundi & Zhang, 2018). For decades, the glycopeptide antibiotic vancomycin served as the cornerstone of therapy for serious MRSA infections.

However, the extensive use of vancomycin has exerted selective pressure, leading to the emergence of strains with reduced susceptibility. This began with the report of vancomycin-intermediate *S. aureus* (VISA) in Japan in 1997 (Hiramatsu et al., 1997), followed by the first confirmed case of vancomycin-resistant *S. aureus* (VRSA) in the United States in 2002 (Centers for Disease Control and Prevention, 2002). VRSA poses a significantly greater threat, as it results from the horizontal acquisition of the *vanA* operon, most commonly from vancomycin-resistant enterococci (VRE). This operon, typically located on transposon Tn*1546*, encodes a set of enzymes that reprogram cell wall synthesis by replacing the terminal D-alanyl-D-alanine dipeptide of peptidoglycan precursors with D-alanyl-D-lactate. This modification reduces vancomycin's binding affinity by approximately 1000-fold, conferring high-level resistance (Cong et al., 2020).

While several VRSA cases have been documented globally, including in the United States, India, and Iran (Ziasistani et al., 2019), data on its prevalence and the molecular epidemiology of resistance genes in many regions, including Pakistan, remain sparse. Studies from various Pakistani cities have reported alarming rates of MRSA and have begun to document the emergence of VRSA, with prevalence ranging from 4.9% to 14% among MRSA isolates (Riaz et al., 2021; Saeed et al., 2019). The detection of *vanA* genes

in these isolates confirms the potential for horizontal gene transfer in local clinical settings (Saeed et al., 2019).

The dissemination of VRSA threatens to return healthcare to a pre-antibiotic era for these common infections. Therefore, continuous surveillance is critical to monitor the emergence and spread of VRSA, understand the resistance mechanisms at play, and inform evidence-based infection control and antimicrobial stewardship strategies. This study was designed to determine the prevalence of VRSA among clinical isolates from a major hospital in Islamabad, Pakistan, to characterize their antibiograms, and to molecularly detect the presence of the *vanA* and *vanB* resistance genes.

2. Materials and Methods

2.1. Study Design and Setting

A hospital-based, prospective cross-sectional study was conducted over a two-month period (January 1st to February 28th, 2023) in the Microbiology Department of the Pakistan Institute of Medical Sciences (PIMS), Islamabad. The study was approved by the institutional review board (or ethics committee) of Sarhad University of Science and Information Technology and PIMS.

2.2. Sample Size and Collection

A total of 203 non-duplicate clinical isolates of *S. aureus* were collected from various specimens, including pus (n=163), urine (n=26), and blood (n=14). Samples were collected from both inpatients and outpatients. Only samples collected using aseptic techniques, with complete patient data, and in sufficient quantity were included. Samples from patients on antibiotic therapy at the time of collection were excluded.

2.3. Phenotypic Identification and Biochemical Characterization

All samples were processed per standard operating procedures. Initial identification was based on colony morphology (size, pigmentation, hemolysis on blood agar) and Gram staining (Gram-positive cocci in clusters). Presumptive *S. aureus* isolates were subjected to a catalase test using 3% hydrogen peroxide and a tube coagulase test using rabbit plasma to differentiate from coagulase-negative staphylococci.

2.4. Antimicrobial Susceptibility Testing (AST)

Antimicrobial susceptibility was determined using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar (MHA) plates, following Clinical and Laboratory Standards

Institute (CLSI) guidelines (CLSI, 2022). A bacterial suspension equivalent to a 0.5 McFarland standard was inoculated onto MHA. The following antibiotic disks (Oxoid, UK) were tested: penicillin G (10 µg), cefoxitin (30 µg), erythromycin (15 µg), azithromycin (15 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), gentamicin (10 µg), chloramphenicol (30 µg), co-trimoxazole (25 µg), clindamycin (2 µg), and vancomycin (30 µg). Methicillin resistance was confirmed by resistance to cefoxitin. Vancomycin resistance was initially screened by disk diffusion, with isolates showing no zone of inhibition or zones smaller than the CLSI breakpoint being suspected as VRSA. The minimum inhibitory concentration (MIC) for vancomycin was not determined in this study due to resource constraints, with the primary focus on genotypic detection of resistance mechanisms.

2.5. Molecular Confirmation and Virulence Gene Detection

2.5.1. DNA Extraction

Genomic DNA was extracted from overnight cultures of all MRSA isolates using the GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA) according to the manufacturer's protocol for Gram-positive bacteria. The concentration and purity of extracted DNA were assessed using a NanoDrop spectrophotometer.

2.5.2. PCR Amplification of Resistance Genes

All 41 MRSA isolates were screened for the presence of *vanA* and *vanB* genes using conventional PCR. The primers and annealing temperatures used are detailed in Table 1. PCR reactions were carried out in a T-100 Thermal Cycler (Bio-Rad, USA) in a total volume of 20 µL, containing 8 µL of master mix, 1.5 µL each of forward and reverse primers (10 pmol/µL), 6 µL of nuclease-free water, and 3 µL of DNA template. The thermocycling conditions included an initial denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute, with a final extension at 72°C for 5 minutes. Positive controls (known VRSA strains) and negative controls (nuclease-free water) were included in each run.

2.5.3. Gel Electrophoresis

Amplified PCR products were resolved by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. A 100-bp DNA ladder (Thermo Scientific, USA) was used to

estimate fragment sizes. Gels were visualized under a UV transilluminator, and images were captured using a gel documentation system.

2.5.4. 16S rRNA Sequencing

To confirm species identification, selected isolates were sent to Macrogen Inc. (Seoul, South Korea) for 16S rRNA gene sequencing. Bacterial glycerol stocks were prepared and shipped. The resulting sequences were analyzed using BLASTn against the NCBI database to confirm species identity.

3. Results

3.1. Prevalence of MRSA and VRSA

Of the 203 *S. aureus* isolates, 41 (20.2%) were confirmed as MRSA based on cefoxitin resistance (Figure 1A). The remaining 162 (79.8%) were methicillin-susceptible *S. aureus* (MSSA). Among the MRSA isolates, two (4.9%) exhibited phenotypic resistance to vancomycin by disk diffusion, classifying them as VRSA. This corresponds to an overall VRSA prevalence of 0.99% (2/203) among all *S. aureus* isolates (Figure 1B). The majority of *S. aureus* isolates were from pus samples (80.3%), followed by urine (12.8%) and blood (6.9%).

3.2. Antimicrobial Resistance Profiles

The antimicrobial susceptibility patterns for the 41 MRSA isolates are detailed in Table 2. Resistance to multiple non-beta-lactam antibiotics was common. All MRSA isolates (100%) were resistant to penicillin G. High levels of resistance were observed for erythromycin (87.5%), ciprofloxacin (75.7%), co-trimoxazole (59.5%), azithromycin (62.5%), and levofloxacin (60%). Resistance to gentamicin and clindamycin was 30% and 22%, respectively. Notably, all isolates were susceptible to chloramphenicol (100%). The two VRSA isolates demonstrated an even more extensive resistance pattern, both showing resistance to penicillin, erythromycin, levofloxacin, and cefoxitin (Figure 2). One of the two VRSA isolates (50%) was also resistant to gentamicin, co-trimoxazole, and clindamycin.

3.3. Molecular Detection of *vanA* and *vanB* Genes

The *vanA* and *vanB* genes were detected in the MRSA isolates via PCR, with specific bands at 1030 bp and 635 bp, respectively (Figure 3). Among the 41 MRSA isolates screened, *vanA* was detected in 5 (12.2%), and *vanB* was detected in 2 (4.9%).

Cumulatively, 6 isolates (14.6%) harbored at least one of these vancomycin resistance genes (Table 3). In the two phenotypically VRSA isolates, *vanA* was present in both (100%), while *vanB* was present in one (50%). One of the VRSA isolates harbored both resistance genes (Table 4).

3.4. 16S rRNA Confirmation

16S rRNA sequencing of representative isolates confirmed their identity as *S. aureus* with >99% similarity to reference sequences in the GenBank database, validating the phenotypic identification methods.

4. Discussion

This study provides critical insights into the prevalence and molecular basis of vancomycin resistance in *S. aureus* from a major healthcare setting in Pakistan. The MRSA prevalence of 20.2% observed here is consistent with the lower end of the range reported in various Asian and Middle Eastern countries, but lower than several previous Pakistani studies which have reported rates as high as 65-76% (Riaz et al., 2021; Saeed et al., 2019; Ghahremani et al., 2018). This variation likely reflects differences in hospital settings, patient populations (e.g., ICU vs. general wards), infection control practices, and time periods of the studies. The high proportion of isolates from pus samples (80.3%) underscores the role of *S. aureus* in skin and soft tissue infections.

Of greater clinical concern is the identification of two VRSA isolates, representing 4.9% of MRSA and 0.99% of all *S. aureus*. This prevalence aligns with findings from Riaz et al. (2021) in Lahore (4.9%) and is lower than the 11.6% and 14% reported by Ghahremani et al. (2018) in Iran and Saeed et al. (2019) in Lahore, respectively. The presence of VRSA, even at this seemingly low level, is a sentinel event. It signals the establishment of high-level vancomycin resistance in a region already burdened with high MRSA rates, potentially compromising the efficacy of the last line of defense for these infections (Cong et al., 2020).

The AST results paint a worrying picture of MDR among the MRSA isolates. Resistance rates exceeding 50% for macrolides, fluoroquinolones, and sulfonamides severely limit empirical treatment options. This pattern is consistent with global trends for healthcare-associated MRSA clones (Kot et al., 2020; Hsu et al., 2021). The universal susceptibility to chloramphenicol is noteworthy, although its clinical use is often limited by its side effect

profile. The extensive resistance profile of the VRSA isolates, with one isolate showing resistance to 7 out of 8 non-chloramphenicol antibiotics tested, exemplifies the convergence of multiple resistance mechanisms in a single strain, creating a truly pan-resistant phenotype.

The molecular detection of *vanA* in 100% of VRSA isolates and *vanB* in 50% provides the genetic basis for the observed high-level resistance. This finding is comparable to studies from Baghdad (Basil et al., 2022), where all VRSA isolates were *vanA* positive, and from Egypt (Saber et al., 2022), which reported *vanA* in 64.7% of VRSA. The *vanA* gene, part of the Tn*1546* transposon, is the most common and clinically significant mechanism for high-level vancomycin resistance, as it confers resistance to both vancomycin and teicoplanin. Its presence in our isolates strongly suggests horizontal gene transfer from VRE, a phenomenon well-documented in the initial VRSA case in the U.S. and subsequently worldwide (Weigel et al., 2003). The detection of *vanA* and *vanB* in phenotypically MRSA isolates (12.2% and 4.9%, respectively) that were not VRSA is an intriguing finding. This could represent a "silent" reservoir of resistance genes, where the genes are present but not fully expressed, or where the vancomycin MIC is elevated but still below the resistance breakpoint (heteroresistance). This reservoir poses a significant risk for the future emergence of fully resistant strains under continued vancomycin selective pressure.

This study has several limitations. The sample size, while adequate for calculating prevalence, is relatively small, and the two-month collection period may not capture seasonal variations. The use of disk diffusion for initial VRSA screening, while following CLSI guidelines, is not the gold standard; vancomycin MIC determination by broth microdilution or E-test would have provided a more definitive phenotypic classification. Furthermore, we did not investigate the genetic environment of the *van* genes (e.g., presence of Tn*1546*) or perform molecular typing (e.g., *spa* typing, MLST) to understand the clonal relatedness of the isolates. Future studies should address these points.

5. Conclusion

This study confirms the presence of VRSA in a Pakistani tertiary care hospital, mediated by the acquisition of *vanA* and *vanB* genes. The co-resistance of these VRSA and other

MRSA strains to multiple antibiotic classes paints a concerning picture of MDR. The detection of *van* genes in phenotypically non-VRSA isolates highlights a silent threat of resistance gene dissemination. These findings underscore the critical need for robust antimicrobial stewardship programs, routine surveillance for vancomycin resistance using both phenotypic and genotypic methods, and stringent infection control measures to prevent the further emergence and spread of these dangerous pathogens. Continuous monitoring is essential to inform treatment guidelines and preserve the effectiveness of remaining therapeutic options.

6. Acknowledgments

The authors would like to thank the staff of the Microbiology Department at Pakistan Institute of Medical Sciences (PIMS), Islamabad, for their assistance with sample collection. We are also grateful to the Sarhad Institute of Allied Health Sciences for providing laboratory facilities and support.

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Tables and Figures

Table 1: Primers used for the detection of vancomycin resistance genes.

Gene	Primer Sequence (5' → 3')	Product Size (bp)	Reference
vanA	F: CATGAATAGAATAAAAGTTGCAATA R: CCCCTTTAACGCTAATACGATCA	1030	Kankalil et al., 2021
vanB	F: ATGGGAAGCCGATAGTC R: GATTTCGTTCCTCGACC	635	Kankalil et al., 2021

Table 2: Antimicrobial susceptibility profile of MRSA isolates (n=41).

Antibiotic Class	Antibiotic	Sensitive, n (%)	Resistant, n (%)
Penicillins	Penicillin G	0 (0)	41 (100)
Cephems	Cefoxitin	0 (0)	41 (100)
Macrolides	Erythromycin	2 (6.3)†	28 (87.5)
	Azithromycin	3 (37.5)†	5 (62.5)
Fluoroquinolones	Ciprofloxacin	8 (24.3)‡	28 (75.7)
	Levofloxacin	3 (30)‡	6 (60)
Aminoglycosides	Gentamicin	28 (70)	12 (30)
Sulfonamides	Co-trimoxazole	15 (40.5)	22 (59.5)
Lincosamides	Clindamycin	32 (78)	9 (22)
Glycopeptides	Vancomycin	40 (97.6)	1 (2.4)
Nitrobenzenes	Chloramphenicol	39 (100)	0 (0)

† Percentages based on total tested for that drug (n=32 for erythromycin, n=8 for azithromycin).

‡ Percentages based on total tested for that drug (n=37 for ciprofloxacin, n=10 for levofloxacin).

Table 3: Prevalence of *vanA* and *vanB* genes in MRSA isolates (n=41).

Gene Detected	Number of Isolates (n)	Percentage (%)
vanA only	5	12.2
vanB only	2	4.9

vanA + *vanB*	1*	2.4
Total (with at least one gene)	**6**	**14.6**

*This isolate is also counted in the *vanA* and *vanB* only categories, representing the one VRSA isolate with both genes. The "Total" row represents the cumulative number of unique isolates with any *van* gene.

Table 4: Prevalence of *vanA* and *vanB* genes in VRSA isolates (n=2).

Gene Detected	Number of Isolates (n)	Prevalence (%)
vanA	2	100
vanB	1	50
Both *vanA* and *vanB*	1	50

Figures

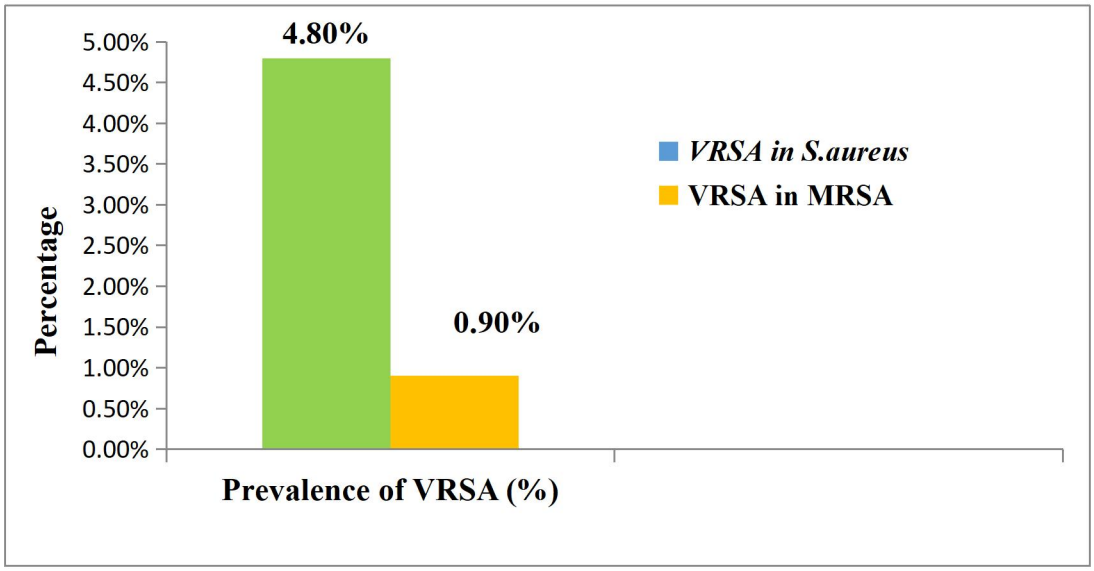
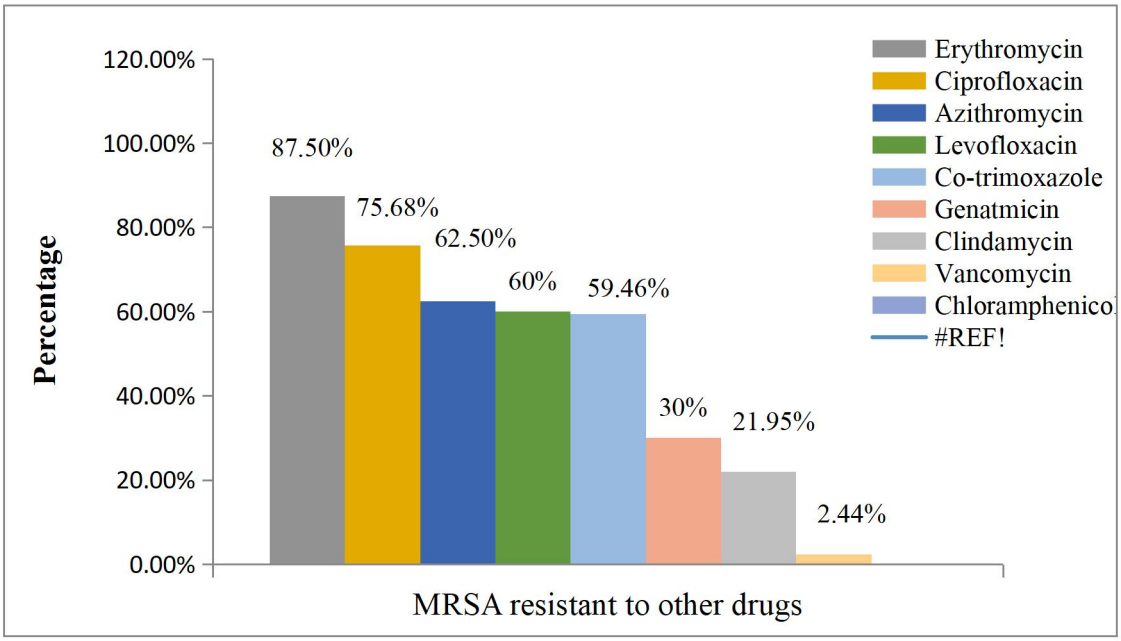


Figure 1: Prevalence of MRSA and VRSA. (A) Distribution of MRSA and MSSA among 203 *S. aureus* clinical isolates. (B) Prevalence of VRSA among MRSA isolates and the total *S. aureus* population.



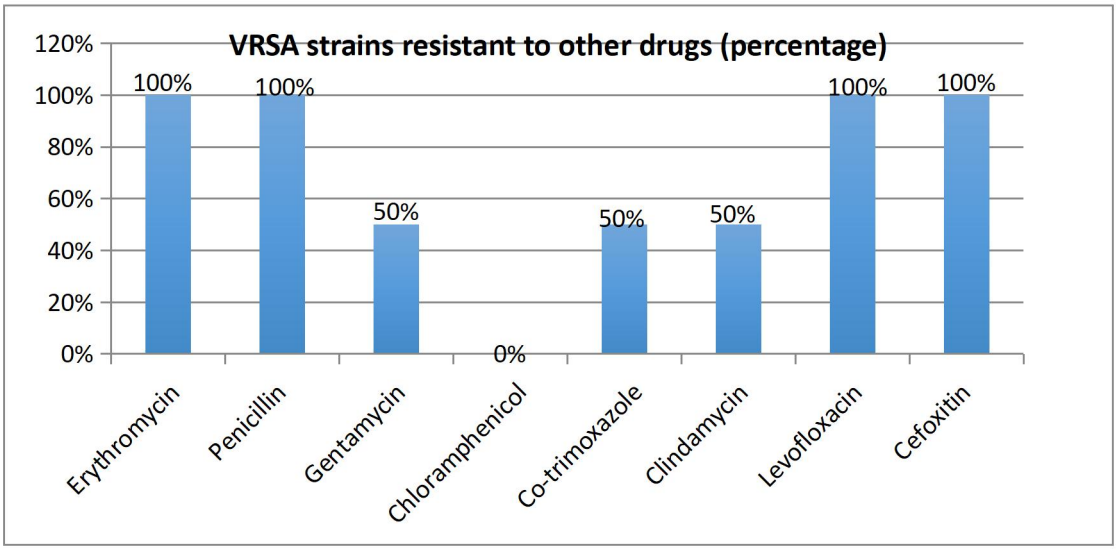


Figure 2: Antimicrobial resistance profile of the two VRSA isolates. Showing resistance (R) or susceptibility (S) to a panel of antibiotics. Pen: Penicillin G; Fox: Cefoxitin; E: Erythromycin; Levo: Levofloxacin; CN: Gentamicin; SXT: Co-trimoxazole; DA: Clindamycin; C: Chloramphenicol.

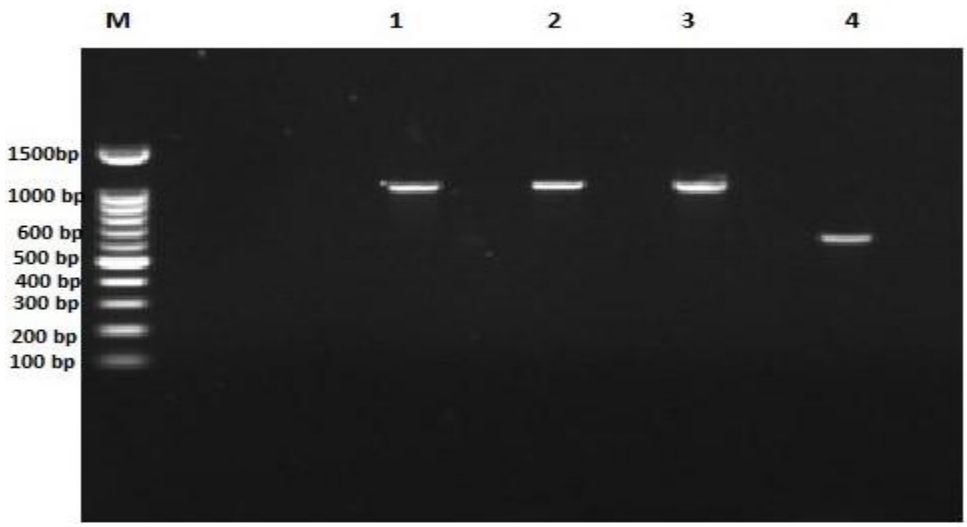


Figure 3: Detection of *vanA* and *vanB* genes by PCR. Agarose gel electrophoresis showing amplified products. Lane M: 100 bp DNA ladder; Lanes 1, 2, 6: Positive for *vanA* (1030 bp); Lanes 3, 4, 5, 7: Positive for *vanB* (635 bp); Lane P: Positive control for both genes.

Kingdom	Family	Genus	Species
Bacteria	Staphylococcaceae	Staphylococcus	Staphylococcus aureus

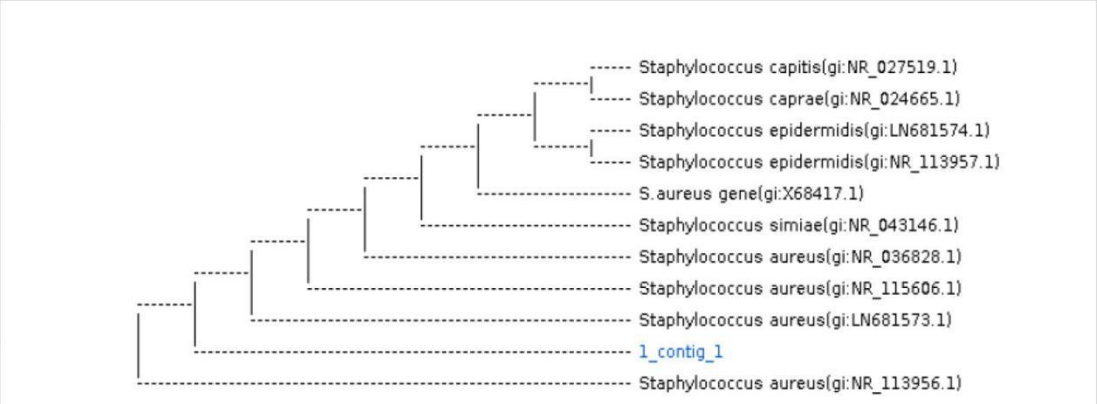


Fig.4: Phylogenetic tree of Staphylococci