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RESEARCH ARTICLE
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A NOVEL REAL-TIME PCR BASED APPROACH FOR THE DETECTION OF VAN GENE DETERMINANTS IN VANCOMYCIN RESISTANT ENTEROCOCCI FROM A TERTIARY CARE HOSPITAL SETTING IN INDORE, CENTRAL INDIA.

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Abstract-

Introduction- Vancomycin-resistant enterococci (VRE) are opportunistic pathogens capable of causing a broad spectrum of clinical infections, particularly in immunocompromised and hospitalized patients. To date, eight distinct phenotypic variants of acquired glycopeptide resistance have been identified in Enterococci: vanA, vanB, vanD, vanE, vanG, vanL, vanM, and vanN. In contrast, VanC represents an intrinsic resistance mechanism naturally occurring in Enterococcus gallinarum and Enterococcus casseliflavus. Among these, the vanA genotype is the most clinically relevant and is predominantly associated with E. faecium, accounting for the majority of VRE infections reported worldwide.

Aim- The objective of this study was to detect van gene determinants in Vancomycin Resistant Enterococci (VRE) isolated from both outpatient attendees and inpatients admitted to various wards of the hospital using Real-Time PCR based approach.

Material and Methods- A prospective cross-sectional study was carried out over a period of two years in the Department of Microbiology at Index Medical College and Research Centre, Indore, Madhya Pradesh. All Enterococcus isolates recovered from clinical specimens—including blood, urine, pus, sputum, wound swabs, catheter tips, and other body fluids—were included in the study. A total of 112 Enterococcus isolates were obtained using standard conventional culture techniques and confirmed through biochemical identification. Antimicrobial susceptibility testing was performed using the Kirby-Bauer disc diffusion method in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines. Minimum inhibitory concentrations (MICs) for vancomycin were determined using the broth dilution method.

Results-Among the 21 vancomycin-resistant isolates, Enterococcus faecium was the predominant species, representing 15 (71.4%) of the total isolates. This was followed by E. faecalis (23.8%) and E. gallinarum (4.8%), as identified using the disc diffusion method in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines. Confirmation of vancomycin resistance was further established through the broth microdilution method, with all isolates previously identified as resistant by disc diffusion showing elevated minimum inhibitory concentrations (MICs). Gender-wise distribution revealed that 9 (43%) of the VRE cases were from male patients, while 12 (57%) were

from female patients. Detection of vancomycin resistance genes was performed using real-time quantitative PCR (qPCR). Among the isolates, 13 (62%) carried the vanA gene, 5 (24%) harbored the vanB gene, and 3 isolates (14%) did not exhibit either vanA or vanB, indicating the possible involvement of other resistance mechanisms or gene variants not targeted by the assay.

Key words- VRE, NSS, CLED, CLSI, AST, Enterococcus faecalis, vanA, vanB, RT-PCR, MIC.

INTRODUCTION

The discovery and application of antibiotics represent a landmark in medical science, revolutionizing the treatment of bacterial infections. Despite their effectiveness, prolonged and widespread antibiotic usage has led to the emergence of resistant bacterial strains. These microorganisms have evolved various adaptive mechanisms to survive exposure to antimicrobial agents, primarily due to the selective pressure exerted by frequent antibiotic use. While such resistance provides a survival advantage to bacteria, it poses significant challenges for human health, including reduced treatment efficacy and a heightened risk of hospital-acquired infections [1,2].

Enterococcus species were independently identified in 1899 by MacCallum and Hastings in the United States and Thiercelin in France [3,4]. Over time, they have gained clinical significance, especially due to the emergence of glycopeptide-resistant strains. To date, eight phenotypic types of acquired vancomycin resistance have been characterized in Enterococci—vanA, vanB, vanD, vanE, vanG, vanL, vanM, and vanN—each exhibiting distinct genetic and phenotypic traits [5,6]. In addition to these acquired forms, VanC represents a naturally occurring resistance mechanism, confined to Enterococcus gallinarum and Enterococcus casseliflavus. Among the acquired resistance types, vanA is most commonly associated with E. faecium and is responsible for the majority of Vancomycin-resistant enterococcal (VRE) infections reported globally. The vanB genotype has also been identified in some isolates, contributing to vancomycin resistance through a different regulatory mechanism [7]. These resistance types can be differentiated based on their inducibility, transferability, and the level of resistance conferred to glycopeptide antibiotics such as vancomycin and teicoplanin.

In light of the significant clinical burden and increasing incidence of vancomycin-resistant enterococci (VRE), the World Health Organization has designated VRE as a high-priority pathogen, highlighting the urgent necessity for novel antimicrobial interventions and strengthened infection control practices (WHO, 2018) [8]. Globally, the prevalence of VRE among clinical isolates continues to rise at an alarming rate. This escalation is largely attributed to the inappropriate or excessive use of broad-spectrum antibiotics such as vancomycin, cephalosporins, and metronidazole, which disrupt the natural gut microbiota and create a selective environment conducive to the proliferation of VRE. The growing resistance is particularly worrisome because vancomycin remains a critical therapeutic agent for treating severe, life-threatening infections. A further concern is the potential for VRE to act as a reservoir of resistance genes, which could be horizontally transferred to other, more virulent gram-positive pathogens. Such genetic exchange could give rise to multidrug-resistant strains that are significantly more difficult to treat with existing antimicrobial therapies. At present, only a limited number of effective alternatives are available, with linezolid and pristinamycin among the few agents demonstrating reliable efficacy against VRE [9].

This study focuses on several key aspects: antibiotic susceptibility profiling of Enterococcus species, species-level identification of isolates, assessment of VRE prevalence, and molecular detection of vancomycin resistance genes. These investigations are especially pertinent to the Indian healthcare context, where VRE infections present a growing challenge. The outcomes of this research are expected to contribute meaningfully to infection control efforts and inform the development of antibiotic stewardship policies in hospitals across Indore and the broader region of Madhya Pradesh.

AIMS AND OBJECTIVES

The objective of this study was to detect van gene determinants in Vancomycin Resistant Enterococci (VRE) isolated from both outpatient attendees and inpatients admitted to various wards of the hospital using Real-Time PCR based approach.

MATERIAL AND METHODS

A prospective cross-sectional study was conducted in the Department of Microbiology, Index Medical College, Indore, M.P for a period of 2 years.

Inclusion Criteria: All Enterococcus isolates obtained from clinical specimens—including blood, urine, pus, sputum, wound swabs, catheter tips, and other body fluids—were included in the study.

Exclusion criteria: All commensal Enterococcus isolates obtained from anatomical sites such as the gastrointestinal tract, female genital tract, stool, and oropharyngeal (throat) swabs were excluded from the study.

SAMPLE COLLECTION AND PROCESSING

Clinical samples including pus, wound swabs, blood, urine, endotracheal aspirates, sputum, and other body fluids were collected from patients. Pus and wound discharge were obtained using sterile cotton swabs (HiMedia), while blood samples were collected aseptically and transported to the laboratory in brain heart infusion (BHI) broth. Clean-catch midstream urine samples were collected in sterile, wide-mouthed containers and transported to the laboratory immediately after collection. All samples were inoculated on appropriate culture media: blood agar and MacConkey agar for most specimens, while urine samples were specifically inoculated on cystine lactose electrolyte-deficient (CLED) agar. Enterococcus species were identified based on colony morphology, Gram staining, motility testing, and standard biochemical methods as described in established microbiological protocols [10,11,12,13].

Antimicrobial susceptibility testing: AST were performed by Kirby -Bauer disc diffusion method on MHA for all enterococci isolates result were interpreted according to the guideline laid down by the CLSI Guidelines 2024. Antimicrobial susceptibility testing in the presence of any potential growth was determined using the disc diffusion method according to the CLSI guidelines. The antimicrobial which was tested included: Discs of Ampicillin (10 µg), erythromycin (30µg), High Level Gentamicin (120µg), High Level streptomycin (300µg), Linezolid (10 µg), Teicoplanin (30 µg), Tetracycline (10µg), Tigecycline (15µg), Ciprofloxacin (5µg), Norfloxacin (10 µg), and Nitrofurantoin (300 µg) Vancomycin (30 µg), Levofloxacin (5µg) [14,15].

Detection of Vancomycin resistant enterococci: The broth dilution method was employed to determine the minimum inhibitory concentration (MIC) of vancomycin. This method is considered more accurate and reliable than the disc diffusion technique for assessing vancomycin susceptibility [16,17,18]. Mueller-Hinton agar plates were prepared with varying concentrations of vancomycin. A 10 µL aliquot of standardized bacterial suspension was inoculated onto each plate, followed by incubation at 37°C for 18–24 hours. The MIC was defined as the lowest concentration of vancomycin that completely inhibited visible bacterial growth, in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines, 2024. According to CLSI breakpoints, Enterococcus isolates were categorized as vancomycin-resistant if the MIC was ≥32 µg/mL, intermediately resistant if the MIC ranged from 8 to 16 µg/mL, and susceptible if the MIC was ≤4 µg/mL.

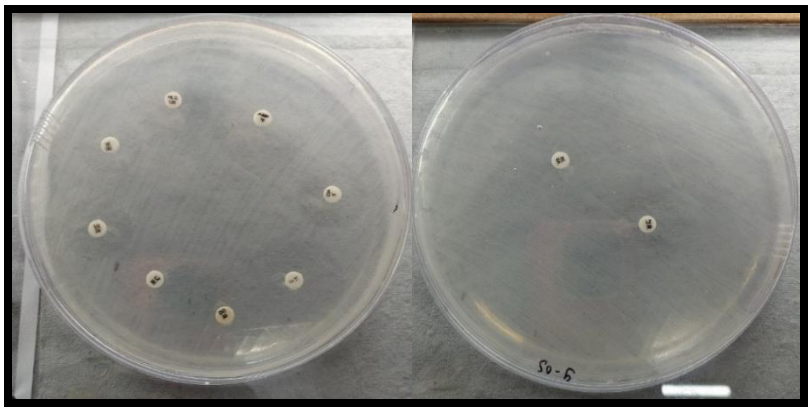

Fig 1: Vancomycin resistant and sensitive result by disc diffusion method

DNA extraction and characterization of van genes

All isolates that tested positive in the initial screening for vancomycin resistance were further analysed by real-time qPCR to detect the presence of vancomycin resistance genes [19,20]. A single colony of test isolates was selected from a fresh culture plate, then transferred into 5 ml of luria bertania broth and incubated overnight at 37ºC. The genomic DNA from each isolate was extracted using the DNA Extraction Kit (TRUPCR® Bacterial DNA Extraction Kit), following the manufacturer's instruction. Bacterial lysate prepared from Enterococcal isolates were subjected to Real time qPCR for the detection of van genes. Amplification of DNA was performed using CFX96 Real Time PCR system by Bio-Rad Laboratories, U.S.A.

Amplification

A highly standardized in vitro nucleic acid amplification assay Hi-PCR® Vancomycin Resistant Enterococci (VRE) (Multiplex) Probe PCR kit from HiMedia Laboratories Pvt. Ltd. was used for the detection of vanA, vanB genes. This method utilizes hydrolysis probes—short oligonucleotides labelled with a fluorescent reporter dye at the 5′end and a quencher dye at the 3′end— which enables fluorescence-based detection of target gene amplification. Initial denaturation step was done for 10 min at 95^0C followed by Denaturation step for 5 sec at 95^0C & Annealing/Extension for 1 min at 60^0C **(Table 1)**. The denaturation and annealing/extension step was repeated for 45 cycles and Acquisition on FAM, Joe, Cy5 & Cy5.5 was taken at annealing/extension step of each cycle **(Table 2)**.

Steps	Temperature	Time	Dye Acquisition	Cycles
Initial Denaturation	95°C	10 min	-	45
Denaturation	95°C	05 Sec	-	
Annealing/ Extension	60°C	1 min	yes	

Table 1: PCR Amplification and Cycling Conditions

Detection Channel				Result Interpretation
FAM (vanA)	**HEX (vanB)**	**Cy5 (Enterococcus spp.)**	**Cy5.5 (Internal Control)**	
+	-	+	+/-*	**vanA Positive: VRE**
-	+	+	+/-*	**vanB Positive: VRE**
+	+	+	+/-*	**vanA and vanB Positive: VRE**
-	-	+	+	**Negative for VRE**
-	-	-	-	**PCR inhibition or reagent failure.Repeat PCR or repeat extraction from original sample**

*The presence or absence of a signal in the Cy5.5 channel is not relevant for the validity of the test run due to competition between the test template and Internal Control template.

Table 2: RT-PCR Result Analysis

RESULTS

During the course of this prospective study, a variety of clinical specimens were collected from patients and processed in the Department of Microbiology, Index Medical College, Indore (Madhya Pradesh). A total of 112 Enterococcus isolates were recovered from these samples. Among the 21 vancomycin-resistant isolates, Enterococcus faecium was the predominant species, representing 15 (71.4%) of the total isolates. This was followed by E. faecalis (23.8%) and E. gallinarum (4.8%), as identified using the disc diffusion method in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines. Confirmation of vancomycin resistance was further established through the broth microdilution method, with all isolates previously identified as resistant by disc diffusion showing elevated minimum inhibitory concentrations (MICs). Gender-wise distribution revealed that 9 (43%) of the VRE cases were from male patients, while 12 (57%) were from female patients. Detection of vancomycin resistance genes was performed using real-time quantitative PCR (qPCR). Among the isolates, 13 (62%) carried the vanA gene, 5 (24%) harbored the vanB gene, and 3 isolates (14%) did not exhibit either vanA or vanB, indicating the possible involvement of other resistance mechanisms or gene variants not targeted by the assay.

Antibiotic agent	Sensitive No (%)	Intermediate sensitive No (%)	Resistant No (%)
Ampicillin	37(33%)	-	75(66.9%)
Erythromycin	29(26%)	-	83(74.1%)
Ciprofloxacin	31(27.6%)	-	81(72.3%)
Daptomycin	47(42%)	-	65(58%)
High level Gentamicin	73(65.1%)	-	39(34.8%)
High level streptomycin	82(73.2%)	-	30(26.7%)
Linezolid	109(97.3%)	-	3(2.6%)
Teicoplanin	97(86.6%)	-	15(13.3%)
Tetracycline	92(82.1%)	-	20(17.6%)
Tigecycline	78(69.6%)	-	34(30.3%)
Vancomycin	91(81.2%)		21(18.7%)
Nitrofurantoin	76(67.8%)	4(3.5%)	32(28.5%)
Norfloxacin	37(33%)	-	75(66.9%)
Levofloxacin	9(8%)	1(0.8%)	7(6.2%)

Table 3- AST pattern of Enterococci (n=112)

Name Of antibiotics	Sensitive-No.	Intermediate sensitive- No	Resistant No.
Ampicillin	1(4.8%)	-	20(95.2%)
Erythromycin	1(4.8%)	-	20(95.2%)
Ciprofloxacin	5(23.8%)	-	16(76.2%)
Daptomycin	5(23.8%)	-	16(76.2%)
High level Gentamicin	4(19.0%)	-	17(80.9%)
High level Streptomycin	19(90.4%)	-	2(9.5%)
Linezolid	7(33.0%)	-	14(66.6%)
Teicoplanin	7(33.0%)	-	14(66.6%)
Tetracycline	6(28.5%)	-	15(71.4%)
Tigecycline	6(28.5%)	-	15(71.4%)
Vancomycin	0(0%)	-	21(100%)

Table 4: AST pattern of Vancomycin Resistant Enterococci (n=21)

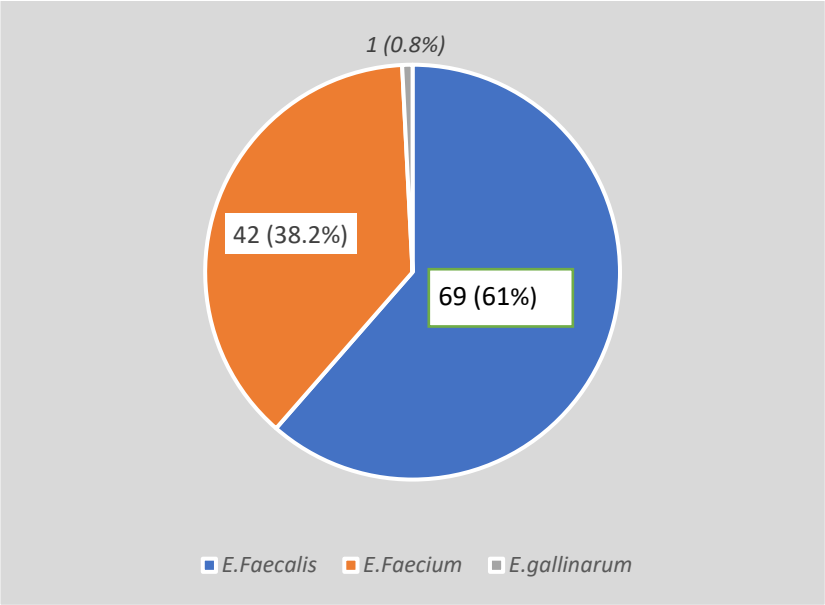

Fig 2: Distribution of Enterococcus species (n=112)

Species	Number	Percentage
E. feacium	15	71.4%
E. faecalis	5	23.8%
E. gallinarum	1	4.8%

Table 5: Distribution of VRE Species (n=21)

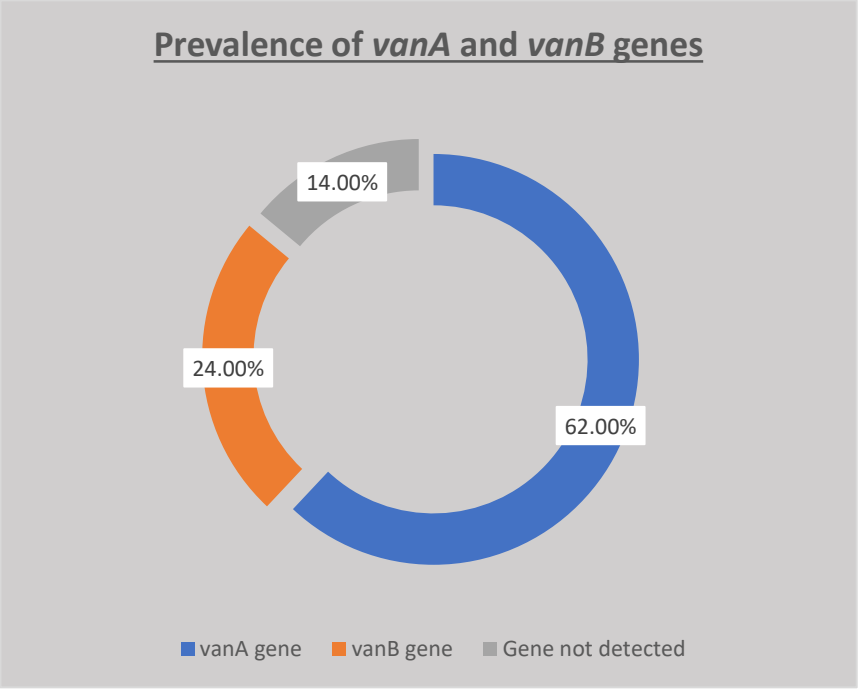

Fig 3: Distribution of vanA and vanB genes in VRE isolates (n=21)

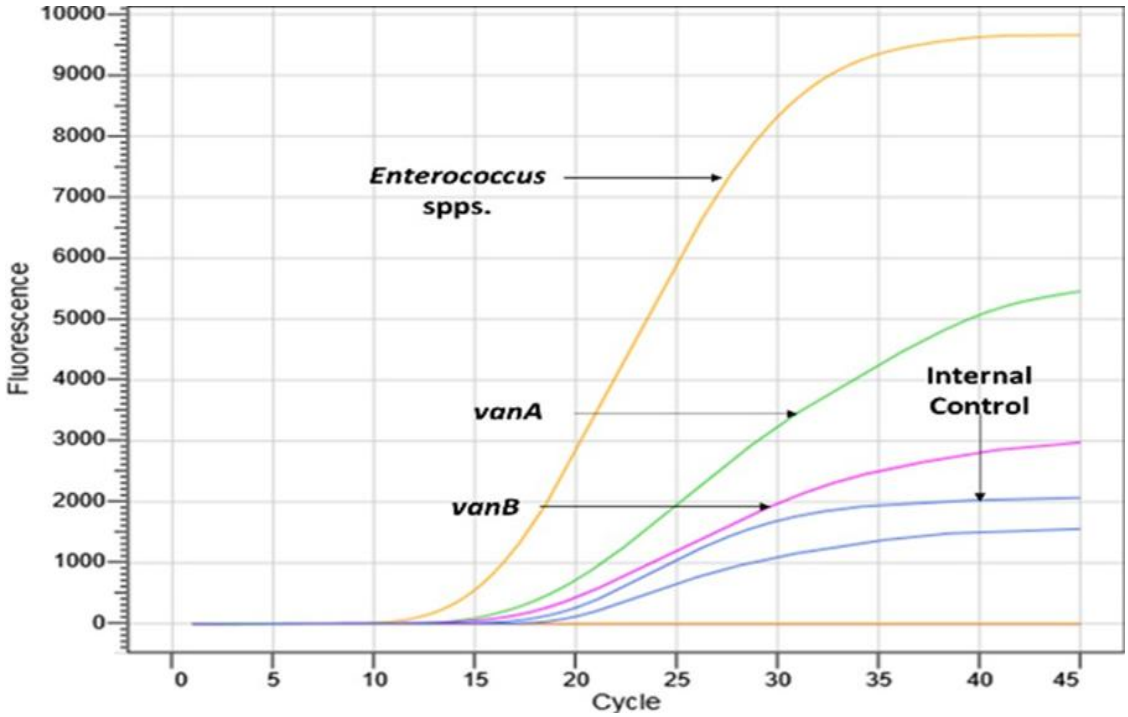

Fig 4: Real-time detection and typing of vanA & vanB genes in VRE isolates using FAM (Green), HEX (Purple), Cy5 (Yellow) & Cy5.5 (Blue) channels of Bio-Rad CFX-96 RT-PCR.

DISCUSSION

Enterococci are recognized as opportunistic pathogens capable of causing a wide range of infections, both in community and hospital settings. Among the various species, Enterococcus faecalis and Enterococcus faecium are the most frequently encountered in clinical specimens, accounting for approximately 85%–90% of all clinical Enterococcal isolates.

In the current study, 112 Enterococcus isolates were analyzed, of which 21 (18.7%) were identified as vancomycin-resistant enterococci (VRE). Species distribution among the VRE isolates revealed that E. faecium was more prevalent, comprising 15 (71.4%) of the isolates, which was followed by E. faecalis (23.8%) and E. gallinarum (4.8%). These findings are in line with the study conducted by Moosavian et al. (2018), who explored the molecular detection of vanA and vanB genes in VRE strains from ICU patients in Ahvaz, Iran. Their study examined 175 Enterococcal isolates, of which 129 (73.7%) were identified as either E. faecalis or E. faecium, and the remaining 46 (26.3%) as other Enterococcus species. Antimicrobial susceptibility testing showed high resistance rates to tetracycline, erythromycin, ciprofloxacin, and ampicillin. Among the 129 isolates, 56 (43.4%) exhibited resistance to both vancomycin and teicoplanin. Furthermore, molecular analysis demonstrated that 54 of the 59 vancomycin-resistant or intermediate isolates (91.5%) carried the vanA gene, while none harbored the vanB gene.

Supporting evidence was also provided by Gehan A. et al. (2018) in a study conducted at Zagazig University Hospitals, where vancomycin resistance was observed in 35.2% (57) of Enterococcal isolates. Among these, 38 isolates (66.7%) tested positive for the vanA gene, with minimum inhibitory concentrations (MICs) exceeding 64 µg/mL. Additionally, 12 isolates (21.05%) carried the vanB gene, and 5 isolates (8.8%) harbored the vanC gene, with MICs ranging from 4 to 16 µg/mL. The authors concluded that PCR targeting vanA offered the highest sensitivity for detecting vancomycin resistance compared to vanB and vanC PCR assays.

A more recent study by Sharma et al. (2024) reported similar findings. Out of 150 Enterococcal isolates screened in a tertiary care hospital, 11 were identified as vancomycin-resistant. The majority of VRE cases were found in male patients (63.6%), with E. faecalis accounting for 7 isolates and E. faecium

for 4. Genotypic characterization revealed that 9 (81.8%) of these isolates possessed the vanA gene, further reinforcing the predominance of vanA-mediated resistance in clinical VRE strains.

CONCLUSION

The findings of our study highlight the growing concern of Vancomycin resistant Enterococci (VRE) and the emergence of glycopeptide-resistant strains within our region. Enterococci have the potential to act as reservoirs for resistance genes, facilitating the spread of antimicrobial resistance. The rising trend suggests that infections caused by glycopeptide-resistant enterococci are likely to become more frequent in this geographical area. To effectively combat their spread, it is essential to adopt a multifaceted approach that includes the judicious use of antibiotics, strict implementation of isolation protocols in clinical settings, and the development of efficient, rapid surveillance systems for early detection and containment.

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