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"Detection of Vancomycin Resistance in *Enterococcus* Species by Phenotypic and Genotypic Methods among Patients in a Tertiary Care Hospital in Indore, Central India"

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ABSTRACT

Background:
Enterococcus species are significant pathogens in hospital-acquired infections, largely due to their natural resistance to several commonly used antibiotics and their increasing ability to acquire resistance to newer therapeutic agents, including vancomycin.

Aim:
To detect vancomycin-resistant *Enterococcus* (VRE) in various clinical specimens from patients in a tertiary care hospital.

Objective: The primary objective of this study is to isolate *Enterococcus* species from various clinical samples, determine their antimicrobial susceptibility patterns, and identify vancomycin-resistant strains among them.

Material & Methods: This prospective cross-sectional study was conducted over a two-year period in the Department of Microbiology at Index Medical College and Research Centre, Indore, Madhya Pradesh. Clinical specimens such as blood, urine, pus, sputum, wound swabs, catheter tips, and other body fluids were processed for the isolation of *Enterococcus* species. A total of 112 *Enterococcus* isolates were obtained using standard culture methods and subsequently identified through conventional biochemical tests. Antimicrobial susceptibility testing was carried out using the Kirby-Bauer disc diffusion technique, following the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI). Among the 112 isolates, 21 were found to be resistant to vancomycin. To further evaluate resistance, the minimum inhibitory concentrations (MICs) of vancomycin were determined using the broth microdilution method.

Results: Out of the 112 *Enterococcus* isolates, 69 (62%) were obtained from inpatients, while 43 (38%) were isolated from outpatients. The majority of isolates were from female patients (65%), compared to males (35%). The highest incidence of infection was observed in individuals aged 31–40 years. Among the species identified, *E. faecalis* was the most prevalent, accounting for 61% of the total isolates. Regarding sample distribution, the majority of isolates were recovered from urine samples (51%), followed by pus (15%), blood (14%), body fluids (7%), wound swabs (3%), and high vaginal swabs (HVS) (2%). Vancomycin resistance was detected in 23.2% of isolates using the disc diffusion method, while the broth dilution method confirmed resistance in 18.75% of cases.

Conclusion: The findings of this study highlight the urgent need for stringent infection control practices and ongoing surveillance to monitor the spread of vancomycin-resistant *Enterococcus* (VRE). Given the observed prevalence, strict adherence to infection prevention measures is essential to limit transmission within healthcare settings. To curb the increasing incidence of VRE, it is vital for healthcare professionals to stay informed about emerging resistance patterns and to enforce robust antibiotic stewardship programs alongside comprehensive infection control strategies.

Key words- VRE, NSS, CLED, CLSI, AST, *Enterococcus faecalis*, *vanA, vanB.*

INTRODUCTION

Enterococcus species are part of the normal microbial flora of the gastrointestinal tract in both humans and animals. These organisms are facultative anaerobic, Gram-positive cocci and are capable of surviving in diverse environmental conditions. While they typically exist as harmless commensals, *Enterococci* have emerged as important opportunistic pathogens responsible for a variety of infections such as bacteraemia, urinary tract infections (UTIs), and endocarditis—occurring in both community and hospital settings. Among these, Vancomycin-Resistant *Enterococci* (VRE) have gained significant attention due to their role in healthcare-associated infections and their classification as a serious public health threat. Vancomycin, a glycopeptide antibiotic, exerts its bactericidal effect by disrupting bacterial cell wall synthesis. Specifically, it blocks the polymerization of peptidoglycan, a crucial component of the Gram-positive bacterial cell wall, by binding to the D-alanyl-D-alanine termini of peptidoglycan precursors. This inhibition prevents the formation and proper cross-linking of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), compromising cell wall integrity and ultimately leading to bacterial cell death through leakage of cytoplasmic contents.

However, VRE have developed mechanisms to evade this action. The two major resistance strategies include:

Alteration of the Target Site: VRE strains modify the terminal D-alanyl-D-alanine in the peptidoglycan precursor to either D-alanyl-D-lactate or D-alanyl-D-serine. These substitutions significantly reduce vancomycin's binding affinity, rendering the antibiotic ineffective.

Acquisition of Resistance Genes: VRE can acquire specific resistance genes, such as *vanA* and *vanB*, often through plasmid-mediated horizontal gene transfer from other resistant bacteria. These genes enable the production of altered peptidoglycan precursors, further contributing to high-level vancomycin resistance.

The prevalence of vancomycin-resistant *Enterococcus* (VRE) infections varies by geographic region and healthcare facility type. In hospital-acquired infections, *Enterococcus faecium* is the predominant species associated with VRE cases, while *Enterococcus faecalis* typically accounts for only 2% to 20% of VRE isolates. Critically ill patients, especially those admitted to Intensive Care Units (ICUs), are found to have the highest rates of VRE colonization and infection, even in cases presenting with mild clinical symptoms. Although *Enterococci* are commonly identified as colonizers rather than active pathogens in many clinical specimens, they are also capable of causing severe infections, such as endocarditis and meningitis.[1]

MATERIALS AND METHODS

Study population

The study population included patients of both sexes and all age groups attending the outpatient and inpatient departments of a tertiary care hospital in Indore M.P. Central India. A total of 112 strains of *Enterococci* were collected from various type of clinical samples submitted to the Microbiology Laboratory for culture and sensitivity. Clearance from Institutional Ethics Committee was obtained to carry out this study.

Isolation and identification: 112 *Enterococci* were isolated from various clinical samples (urine, pus, blood, catheter tip, and body fluid). 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. The isolates were identified to species level using standard procedures. [2-5]

Antimicrobial susceptibility testing: Antibiotic susceptibility test was done by Kirby-Bauer disc diffusion method on Muller-Hinton agar. Inoculum was prepared and adjusted to 0.5 McFarland's turbidity standard. Antibiotic disc was obtained from the Hi Media Laboratories (Mumbai).[6]

Determination of minimum inhibitory concentration by Broth micro dilution method: 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 [7-11]. 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, 2025. 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.

Genotypic method of VRE detection by Real Time quantitative qPCR

The genomic DNA from each isolate was extracted using the DNA Extraction Kit (TRUPCR® Bacterial DNA Extraction Kit). Real-time polymerase chain reaction, also called quantitative Polymerase Chain Reaction (qPCR) or kinetic Polymerase Chain Reaction, is a laboratory technique based on the principle of PCR. This technique is used to amplify a targeted DNA sequence by use of hydrolysis probes that are short oligonucleotides having fluorescent reporter dye attached to the 5' end and a quencher dye to the 3' end. All the isolates of *Enterococci* that were screen test positive were subjected to Real time qPCR to identify Vancomycin resistant genes.

Gene Amplification and Detection by Real time quantitative qPCR

A highly standardized in vitro nucleic acid amplification assay Hi-PCR vancomycin Resistance *Enterococci* (VRE) Multiplex prob. PCR Detection Kit (MBPCR134)' from HiMedia Laboratories Pvt Ltd., Thane West, Maharashtra INDIA was used for the detection *vanA*, *vanB* gene and *Enterococcus Spp*. Amplification of DNA was performed using CFX96 Real Time PCR system by Bio-Rad Laboratories, U.S.A.

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

Sampling: FAM/HEX/Cy5/Cy5.5

Hold: 4°C for ∞

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 this prospective study, various clinical specimens were collected and analysed in the Department of Microbiology at Index Medical College, Indore, Madhya Pradesh. A total of 112 *Enterococcus* isolates were obtained. Of these, 69 isolates (62%) were recovered from hospitalized patients (inpatients), while the remaining 43 (38%) were from outpatient samples. A higher isolation rate was observed in female patients (65%) compared to male patients (35%). The highest number of cases occurred in individuals aged between 31 and 40 years.

Species identification revealed that *E. faecalis* was the most commonly isolated species, accounting for 61% of the cases, followed by *E. faecium* (38.2%) and *E. gallinarum* (0.8%). Regarding the source of the isolates, urine samples contributed the majority (51%), followed by pus (15%), blood (14%), body fluids (7%), wound swabs (3%), and high vaginal swabs (2%). Vancomycin resistance was assessed using both the disc diffusion method and the broth microdilution technique. Resistance was observed in 26 isolates (23.2%) by disc diffusion, while 21 isolates (18.75%)

were confirmed as vancomycin-resistant using the broth dilution microtiter plate method. Among these 21 VRE isolates, 15 (71.5%) were identified as *E. faecalis* and 6 (28.5%) as *E. faecium*. Gender distribution among VRE cases showed that 9 (8.0%) were male and 12 (10.7%) were female. Molecular detection using real-time quantitative PCR (qPCR) revealed that among the 21 vancomycin-resistant isolates, 13 (61%) carried the *vanA* gene, 5 (23.8%) *vanB* gene, and in 3 isolates (14.2%), gene was not detected.

Sample	Number	Percentage
Urine	62	51%
Blood	17	14%
Pus	19	15%
Wound swab	03	3%
High vaginal swab	02	2%
Body Fluid	09	7%

Table 3: - Sample-wise prevalence of *Enterococci* (n=112)

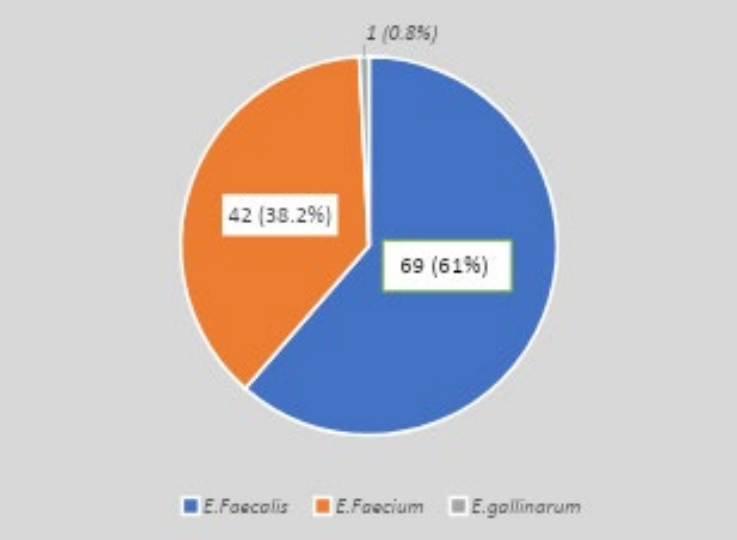

Fig 1: Distribution of *Enterococcus* species (n=112)

Method	Resistant no.	Percentage
Disc diffusion	26	23.2%
Broth Dilution Microtiter Plate	21	18.8%

Table 4: Comparison of Broth Micro-Dilution Method and Disc Diffusion Method for Vancomycin Resistance Detection in *Enterococcus* species

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)

Gender	Number	Percentage
Male	9	(42.8%)
Female	12	(57.2%)

Table 6: Gender wise distribution of VRE (n=21)

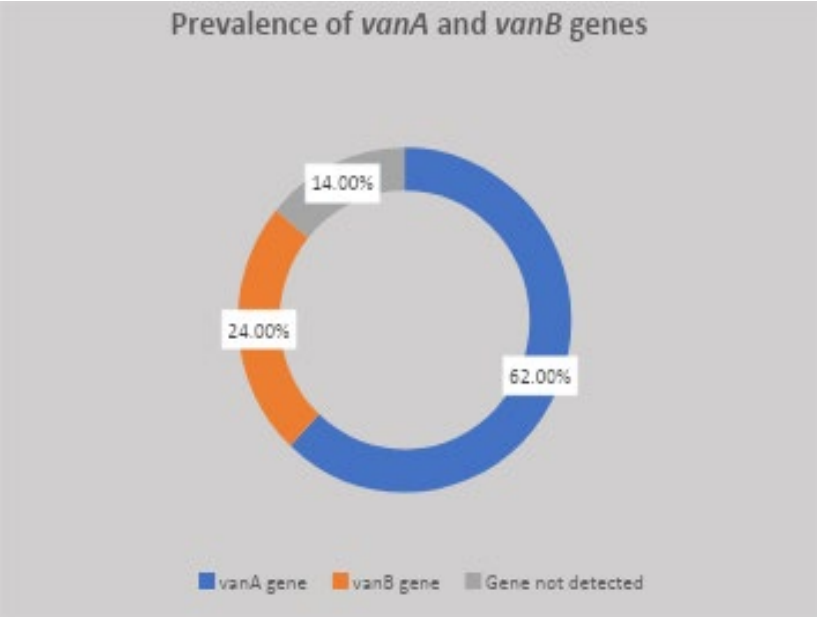

Fig2: Distribution of *vanA* and *vanB* genes among *VRE* isolate

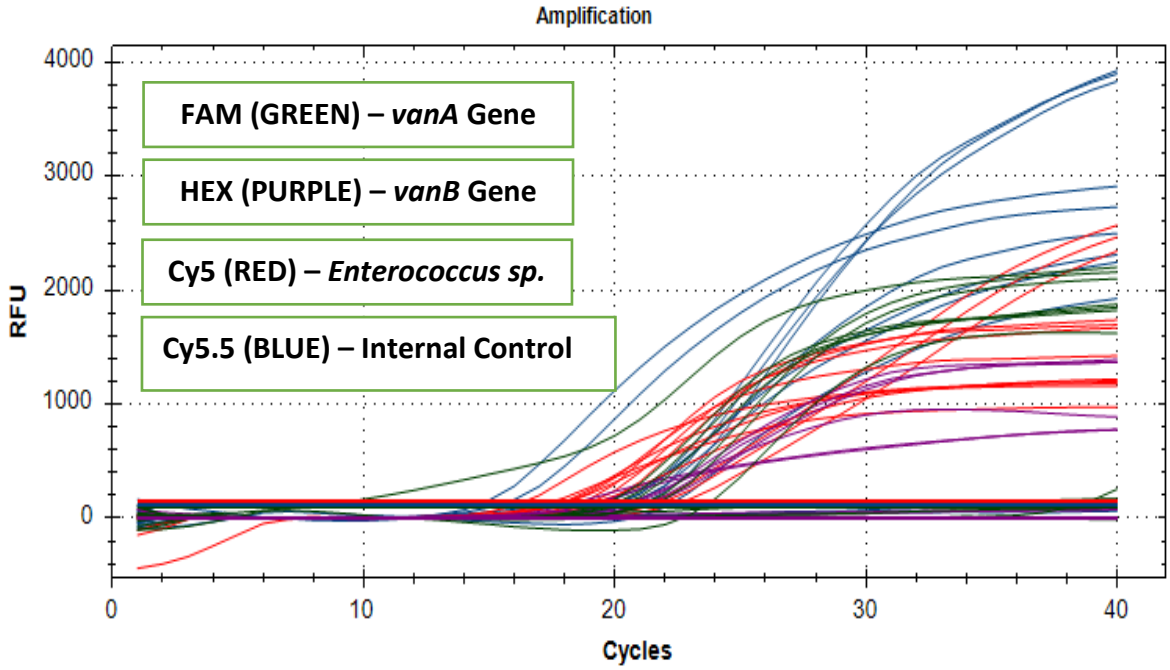

Fig 3: Real-time detection and typing of *vanA* & *vanB* genes in VRE isolates using FAM (Green), HEX (Purple), Cy5 (Red) & Cy5.5 (Blue) channels of Bio-Rad CFX-96 RT-PCR.

DISCUSSION

VRE has become an important nosocomial pathogen because of its rapid spread, high mortality rates associated with infections, limited option for treatment, and the possibility of transferring vancomycin resistance genes to other more virulent and more prevalent pathogens such as Staphylococcus aureus. In this study, a total of 112 *Enterococcus* isolates were recovered, with 69 (62%) obtained from inpatient (IPD) samples and 43 (38%) from outpatient (OPD) specimens. Species-level identification revealed *E. faecalis* as the predominant organism (61%), followed by E. *faecium* (38.2%) and *E. gallinarum* (0.8%). These findings are consistent with those reported by Verma B.S. et al. (2024), who documented *E. faecalis* as the most common species (64.2%), followed by E. *faecium* (23.58%), *E. durans* (13.2%), and *E. avium* (8.49%). In our study, 55% of the *E. faecalis* isolates were derived from urine samples. Vancomycin resistance was assessed using both disc diffusion and broth dilution methods. The disc diffusion technique identified 26 resistant isolates (23.2%), while the broth dilution method confirmed vancomycin resistance in 21 isolates (18.75%). Out of 21 VREs, 15 (71.5%) *E. faecalis* and 6 (28.5%) *E. faecium* were detected respectively. With respect to vancomycin resistance, E. *faecium* demonstrated the highest rate (13.3%), followed by *E. faecalis* (4.46%) and *E. gallinarum* (0.8%), resulting in an overall vancomycin-resistant *Enterococcus* (VRE) prevalence of 18.75%. These findings align with those reported by Shrestha et al. (2021). Additionally, our study confirmed that *E. faecium* exhibited higher resistance overall compared to *E. faecalis*. Compared other study showed Tripathti Sharma et al. Phenotypic Detection and Genotypic Characterization of Vancomycin-Resistant *Enterococci* in a Tertiary Care Hospital 2024 .out of 150 *Enterococcus* species isolated, were subjected to vancomycin resistance using conventional microbiological methods. The incidence rate of male VRE was 7/11 (63.6%) and female VRE was 4/11 (34.4%). Most VREs were from male patients of different age groups. Of these, 7 were *E. faecalis* and 4 were *E. faecium*. When these 11 strains of VRE were tested for the presence of the *vanA* gene, 9 (81.8%) were positive and 2 (18.2%) were negative. The present study showed the prevalence of *vanA* genes.

CONCLUSION

Our study reveals the problem of multiple drug-resistant *Enterococci* and emergence of glycopeptide resistant *Enterococci* in our geographical area. *Enterococci* may serve as reservoir of drug resistance gene. It is also probable that frequency of infection caused by glycopeptide resistant *Enterococci* will increase in our geographical area. To have an effective control of multidrug resistant *Enterococci*, prudent use of antibiotic, better isolation procedures in hospitals and other patient care environments and improved and rapid surveillance measures are required.

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