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PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF VANCOMYCIN RESISTANT ENTEROCOCCUS ISOLATES FROM CLINICAL SAMPLES AT A RURAL TERTIARY CARE HOSPITAL

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Abstract

Introduction: Vancomycin-resistant enterococcal (VRE) infections have become a significant concern in healthcare settings due to their emergence as a cause of nosocomial infections. These strains possess various resistance genes, leading to high-level inducible or low-level intrinsic resistance to vancomycin. Infections caused by VRE strains are associated with elevated mortality rates. This study aimed to characterize phenotypically and genotypically VRE strains isolated from diverse clinical samples.

Methods: One hundred enterococcal strains were isolated from various clinical samples between January 2015 and June 2016. Identification and speciation were performed using the Vitek ® 2 Compact system. Minimum inhibitory concentrations (MIC) of vancomycin and teicoplanin were determined using the E-test and agar dilution method. Genotypic characterization was conducted through multiplex polymerase chain reaction (PCR).

Results: Among the 100 Enterococcus isolates, six exhibited resistance to vancomycin by MIC testing. None of the isolates showed the VanA phenotype, while one demonstrated the VanB phenotype, speciated as E. faecalis. Three isolates displayed the VanC phenotype, identified as motile species E. gallinarum and E. casseliflavus. The three VanC phenotype isolates were positive for the vanC1 gene, indicating low-degree intrinsic vancomycin resistance. The VanB phenotype isolate positive for the vanB gene showed high-level inducible vancomycin resistance.

Conclusion: Although the VanA phenotype is commonly observed, this study did not find any VanA phenotype or genotype. These findings emphasize the need for strict protocols involving VRE screening and MIC testing.

Introduction

In recent years, Enterococci have garnered significant attention due to their emergence as a crucial cause of nosocomial infections, particularly in patients undergoing antimicrobial treatment. The rise of antibiotic resistance in enterococcal infections has posed considerable challenges to their management, often leading to increased difficulty and elevated mortality rates.[1] Vancomycin-resistant enterococcal infections have become a significant concern within the healthcare community. Three distinct glycopeptide resistance phenotypes (Van A, Van B, Van C) exist, and they can be differentiated based on the level and inducibility of resistance to vancomycin and teicoplanin. The VanA type has acquired inducible resistance to both vancomycin and teicoplanin, while the VanB type has acquired inducible resistance to vancomycin but not to teicoplanin. These distinctions play a crucial role in understanding and managing the resistance patterns in enterococcal infections.[2]

The VanC type exhibits constitutive low-level resistance to vancomycin and is observed in motile Enterococci, including E. gallinarum, E. casseliflavus, and E. flavescens. This inherent resistance characteristic in certain Enterococci strains further contributes to the complexity of managing vancomycin-resistant enterococcal infections. Understanding these distinctions is crucial for effective treatment strategies and infection control measures.[3] Various resistance genes are linked to the glycopeptide-resistant phenotypes of Enterococcus species, and they are identified as vanA, vanB, vanC1, vanC2/C3, vanD, among others. In India, infections caused by Vancomycin-resistant Enterococci (VRE) result in poor outcomes, presenting a significant challenge with mortality rates ranging between 60–70%. Effectively addressing and managing infections associated with these resistance genes is imperative for improving patient outcomes and public health.

The antibiotic resistance patterns of Enterococcus isolates have been thoroughly investigated; however, there is a lack of information regarding the phenotypic and genotypic characterization of vancomycin resistance among enterococcal isolates. This study aims to address this gap by detecting the antimicrobial resistance patterns among Enterococcus isolates obtained from clinical specimens in a rural tertiary care center in India. The study places special emphasis on the genotypic characterization of vancomycin resistance in enterococci. Through this research, a comprehensive understanding of the resistance profiles, particularly with regard to vancomycin, will be attained, contributing valuable insights to the field.

Material & Method

The present study was carried out from January 2015 to June 2016, in the Department of Microbiology, UP University of Medical Sciences Saifai, Etawah. Ethical clearance for the study was obtained from the Ethical Committee of the Institute, ensuring adherence to ethical standards and guidelines. A total of 100 enterococcal strains were isolated from diverse clinical samples, including urine, blood, pus, pleural fluid, CSF, high vaginal swab, ascitic fluid, etc. It's important to note that enterococci isolated from stool samples were excluded from the study. The identification and speciation of these isolates were conducted using the Vitek® 2 Compact system by BIOMERIEUX. All isolates were subsequently stocked for further testing and analysis.

Antibiotic susceptibility testing was conducted using the Modified Kirby Bauer disc diffusion method, following the guidelines outlined by the Clinical and Laboratory Standards Institute (CLSI). Antibiotic discs (Himedia) with specified concentrations were employed for testing, including penicillin (10U/disc), ampicillin (10µg), high-level gentamicin (120µg), high-level streptomycin (300µg), ciprofloxacin (5µg), vancomycin (30µg), teicoplanin (30µg), and linezolid (30µg). This standardized approach ensures consistency and adherence to established protocols in assessing the susceptibility of the isolates to various antibiotics.

The minimum inhibitory concentrations (MICs) of vancomycin were determined using both the E-test (Himedia) and the agar dilution method. As part of quality control measures, E. faecalis ATCC 29212 and E. faecalis ATCC 51299 were included as reference strains. These control strains ensure the accuracy and reliability of the MIC determinations, serving as benchmarks for the testing procedures. In the determination of the Minimum Inhibitory Concentration (MIC) of vancomycin against Enterococcus isolates using the agar dilution method, the process involves preparing a stock solution. The formula used for this preparation is:

$$1000/P \times V \times C = W$$

Where:

- P is the potency given by the manufacturer in relation to the base,
- V is the volume in milli-litters required,
- C is the final concentration of the solution,
- W is the weight of the antimicrobial to be dissolved in the volume V.

This formula aids in calculating the appropriate concentration and volume needed to create the stock solution for subsequent MIC testing. Muller Hinton agar plates were prepared with varying dilutions of vancomycin (2-1024µg/ml) and teicoplanin (0.25-512µg/ml). A direct colony suspension of 0.5 McFarland turbidity was created and then diluted 1/10th using normal saline. Subsequently, 1-10µl of this suspension were spotted on the agar surface. The plates were then incubated at 35°C in ambient air for 24 hours and read after this incubation period. A control plate without vancomycin and teicoplanin was also prepared. In the evaluation, the growth of 2 or more colonies at a specific antimicrobial dilution was considered indicative of resistance. Single colonies or faint hazes were disregarded in this assessment.[4]

Genotypic characterization of vancomycin resistance genes was conducted using Multiplex PCR. The aim was to detect the presence of genes encoding vancomycin resistance, specifically focusing on the most common ones: vanA, vanB, and vanC genotypes (either vanC1 gene or vanC2/C3 gene). DNA extraction was performed using the QIAamp DNA Mini Kit (50) from QIAGEN GmbH, located at Strasse 1, 40724 Hilden, GERMANY. The following PCR conditions and primers were employed for the genotypic characterization of vancomycin-resistant strains.

The PCR reaction mixture was prepared by combining the following components for each reaction (100µl mastermix):

- 10.0 µl of 10X buffer with MgCl2 (HIMEDIA)
- 0.5 µl of 100mM dNTPs (Sigma)
- 0.5 µl of Taq polymerase (HIMEDIA)
- 0.5 µl of forward primer (vanA, vanB, vanC1, vanC2/C3)
- 0.5 µl of reverse primer (vanA, vanB, vanC1, vanC2/C3)
- 10 µl of DNA template
- 75 µl of deionized water (PCR-grade water)

This mixture was subjected to 31 cycles of PCR using an XP thermocycler from Bioer. The primers were procured from Imperial Life Sciences Pvt. Ltd. Here are the primers for vanA, vanB, and vanC-1:

vanA:

- A1: 5’-GGGAAAACGACAATTGC-3’
- A2: 5’-GTACAATGCGGCCGTTA-3’

vanB:

- B1: 5’-ATGGGAAGCCGATAGTC-3’
- B2: 5’-GATTTCGTTCCTCGACC-3’

vanC-1:

- C1: 5'-GGTATCAAGGAAACCTC-3'
- C2 5'-CTTCCGCCATCATAGCT-3'

vanC2/C3

- D1 5'-CTCCTACGATTCTCTTG-3'
- D2 5'-CGAGCAAGACCTTTAAG-3

These primers were used for the genotypic characterization of vancomycin-resistant strains through Multiplex PCR.

Running Conditions of Thermal Cycler

Step 1	Initial Denaturation Temperature	94ºC	2min
Step 2	Denaturation Temperature	94ºC	1 min
Step 3	Annealing Temperature	54ºC	1min
Step 4	Extension Temperature	72ºC	1 min
Step 5	30times from step 2- step 4		
Step 6	Final extension Temperature	72ºC	10 min

The amplicons obtained from the PCR reactions were analyzed by electrophoresis using a 1.5% agarose gel (HIMEDIA, RM 273) containing ethidium bromide. The electrophoresis was conducted at 100 volts for 120 minutes in TAE buffer (HIMEDIA). A molecular marker (1000bp DNA Ladder, HIMEDIA MBT 049) was run alongside the samples for size reference. After electrophoresis, the gel was visualized under ultraviolet illumination, and the presence of bands was observed. The image of the gel, highlighting the bands, was captured using a GEL DOC TM XR+ Molecular Imager with Image Lab TM Software (BIO-RAD). This process allowed for the documentation and analysis of the PCR products based on their sizes and presence on the gel.

Result

In the study, a total of 100 isolates of Enterococcus species were obtained from diverse clinical samples. The distribution of isolates among different sample types is as follows:

- Urine specimens: 75%
- Blood specimens: 14%
- Exudates: 5%
- Vaginal specimens: 3%
- Other samples: 3%

The majority of Enterococcus isolates (67%) were derived from inpatients, while the remaining 33% were obtained from outpatients. This distribution provides insight into the prevalence and source of Enterococcus infections in the studied population.

Among the 100 isolates of Enterococcus, majority 63% showed resistance to ampicillin. The degree of resistance to ciprofloxacin was notably high, with 81% of all isolates being resistant. Only 1 isolate was found to be resistant to vancomycin by disk diffusion method. Regarding high-level gentamicin (HLG) resistance, 58 isolates were resistant by disk diffusion, and 60 were resistant by the screen agar method with 500 µg/ml of gentamicin. For high-level streptomycin (HLS) resistance, 36 isolates were resistant by disk diffusion, and 37 were resistant by the screen agar method with 2000 µg/ml of streptomycin. None of the Enterococcus isolates were resistant to teicoplanin and linezolid. This information provides a comprehensive overview of the antibiotic resistance patterns observed among the Enterococcus isolates in the study.

The study identified a Vancomycin-Resistant Enterococcus (VRE) isolate, specifically identified as E. faecium, with a minimum inhibitory concentration (MIC) of vancomycin ≥128 µg/ml. This isolate

demonstrated resistance to vancomycin by agar dilution, E-test, and disk diffusion methods. The VRE was recovered from the urine of an intensive care unit (ICU) patient who had been receiving vancomycin treatment for 10 days.
Additionally:

- Five isolates exhibited low-level resistance to vancomycin with an MIC ranging from 8-16 µg/ml.
- The majority of enterococcal isolates showed an MIC of vancomycin less than 2 µg/ml.
- In the study, the MIC50 and MIC90 for vancomycin were determined to be 2 and 4 µg/ml, respectively, by both E-test and agar dilution methods.

This information provides insights into the level of vancomycin resistance among Enterococcus isolates in the studied population and highlights the emergence of VRE in clinical settings.
In the study, no isolate demonstrated resistance to teicoplanin, as assessed by both the E-test and agar dilution methods. The Minimum Inhibitory Concentration (MIC) of teicoplanin in all isolates was found to be ≤ 0.5 µg/ml. This indicates a high susceptibility of the studied Enterococcus isolates to teicoplanin, suggesting its efficacy as an antimicrobial agent against these strains in the tested conditions. In the study, out of the 6 Vancomycin-Resistant Enterococcus (VRE) isolates:

- Two were obtained from urine samples.
- Two were obtained from pus and wound swabs.
- Two were obtained from blood samples.

All six of these VRE isolates showed resistance to high-level gentamicin (HLG) and high-level streptomycin (HLS) but were sensitive to linezolid. Additionally, it was observed that all VRE isolates were recovered from inpatients. The likelihood of obtaining VRE isolates was significantly higher in inpatients compared to outpatients, especially when compared to vancomycin-sensitive Enterococcus isolates ($P<0.001$). This finding emphasizes the association between VRE prevalence and inpatient status in the studied population.

Phenotypic characterization of VRE isolates: Among the Enterococcus isolates showing resistance to vancomycin two were identified as E. faecium. One was identified as E. faecalis, one as E.cassaliflavus & two as E. gallinarum.
The phenotypic characterization of Vancomycin-Resistant Enterococcus (VRE) isolates based on MIC testing revealed the following:

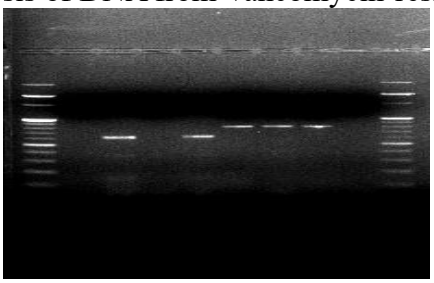
- No isolate was found to be resistant to teicoplanin, indicating the absence of the VanA type of phenotype (resistance to both vancomycin and teicoplanin).
- One isolate of E. faecium exhibited the VanB type of phenotype (resistant to vancomycin, sensitive to teicoplanin).
- Four isolates showed intermediate resistance to vancomycin (MIC=8µg/ml) but were sensitive to teicoplanin. Two of these isolates were identified as E. gallinarum, one as E. faecalis, and one as E. faecium.
- One isolate exhibited a vancomycin MIC of 16µg/ml but was sensitive to teicoplanin. This isolate was identified as E. casseliflavus.

Table1: Phenotypic and genotypic characterization of VRE isolates

Vancomycin resistance phenotype	Vancomycin resistance genotype (van gene clusters detected)	Speciation (phenotype)	No. of isolates (n=6)
VanA	VanA	Nil	Nil
VanB	VanB	E. faecium	01
VanC	vanC1	E. gallinarum	02
VanC	vanC1	E. cassaliflavus	01

In the genetic analysis of vancomycin resistance by polymerase chain reaction (PCR), out of the 6 Vancomycin-Resistant Enterococcus (VRE) isolates tested: Only four isolates were found to possess the vancomycin resistance genes, as detected through multiplex PCR. This suggests that vancomycin resistance in the studied VRE isolates is likely attributed to the presence of specific vancomycin resistance genes, as identified through genetic analysis. The figure (Fig. 1) likely provides a visual representation of the PCR results for the presence of these genes.

Fig.1: PCR analysis of DNA from Vancomycin-resistant enterococci



The genetic analysis results, as visualized in the gel electrophoresis image, indicate the presence of specific vancomycin resistance genes in the tested isolates. Here's a summary of the findings:

- **Lane 1:** M-100 base pair DNA ladder.
- **Lane 2:** Negative control.
- **Lane 3:** Positive control (E. faecalis ATCC 51299 with vanB gene).
- **Lane 4:** Sample 1 (E. faecalis; van gene absent).
- **Lane 5:** Sample 2 (E. faecium; vanB gene present).
- **Lane 6:** Sample 3 (E. gallinarum; vanC1 gene present).
- **Lane 7:** Sample 4 (E. casseliflavus; vanC1 gene present).
- **Lane 8:** Sample 5 (E. gallinarum; vanC1 gene present).
- **Lane 9:** Sample 6 (E. faecium; van gene absent).
- **Lane 10:** M-100 base pair DNA ladder.

This gel electrophoresis pattern provides a visual representation of the presence or absence of specific vancomycin resistance genes in each tested sample. The positive signals in Lanes 3, 5, 6, 7, and 8 indicate the presence of vancomycin resistance genes (vanB or vanC1), while the absence of a signal in Lanes 4 and 9 suggests the absence of these specific genes in those samples.

Discussion

In recent years, Enterococcus has emerged as a significant nosocomial pathogen. This is attributed to its intrinsic resistance to antibiotics such as cephalosporins and its capability to survive in adverse environmental conditions, as well as its tendency to colonize indwelling medical devices. The rise in antimicrobial resistance within Enterococcus is particularly notable in inpatient departments compared to outpatient settings. This trend underscores the importance of addressing and managing Enterococcus infections, especially in healthcare facilities where patients may be more vulnerable to these resilient bacteria.[5]

The prevalence of Vancomycin-Resistant Enterococcus (VRE) in India has historically been lower compared to Western countries. However, there has been an increasing trend in recent years. The first reported VRE isolate in India was documented in New Delhi by Mathur et al. in 1999.[6] Subsequent studies in different regions of North India have reported varying percentages of vancomycin resistance in Enterococcus isolates:

- A study from North India reported only 1% vancomycin resistance in Enterococcus.[7]
- Another study from Chandigarh showed only 5.5% VRE isolates from urine specimens. In this study, VRE isolates exhibited a low-degree vancomycin resistance ranging from 8-32 μg/ml.[8]
- Yet another study from North India indicated a prevalence of only 2% VRE.[9]

These findings suggest that while the prevalence of VRE in India is relatively lower than in some Western countries, there is a discernible upward trend, emphasizing the need for continued monitoring and research to understand and manage the emergence of antibiotic resistance in Enterococcus strains. In present study, the prevalence of vancomycin resistance among Enterococcus isolates was found to be 4%, which aligns with the findings of several other Indian studies. Among the VRE isolates identified, three exhibited a low-level vancomycin resistance, primarily observed in motile Enterococcus species such as E. casseliflavus and E. gallinarum. Additionally, one VRE isolate demonstrated high-level vancomycin resistance. Notably, all the VRE isolates in your study were obtained from patients who had been admitted to the Intensive Care Unit (ICU). This underscores the significance of monitoring and addressing vancomycin resistance, particularly in critical care settings where patients may be more susceptible to such infections.

The most common phenotype observed among Vancomycin-Resistant Enterococcus (VRE) strains is the VanA phenotype, and the second most common is the VanB phenotype. The characteristics of these phenotypes are as follows:

- **VanA Phenotype:** Shows high-level resistance to both vancomycin and teicoplanin (MICs ≥64 μg/ml).
- **VanB Phenotype:** Demonstrates resistance to vancomycin and sensitivity to teicoplanin.

In the present study, no VanA phenotype was identified, and only one VRE isolate exhibited the VanB phenotype. Additionally, the VanC phenotype, which indicates constitutive low-level vancomycin resistance and is mainly observed in motile species of Enterococcus like E. gallinarum and E. casseliflavus, was found in three isolates. These isolates were characterized as motile and speciated as E. gallinarum and E. casseliflavus.[10] These findings contribute to understanding the diversity of VRE phenotypes present in study population.

In the present study, it was observed that out of the six Vancomycin-Resistant Enterococcus (VRE) isolates, only one was identified as resistant by the disk diffusion method, while the remaining five showed low-level resistance to vancomycin by agar dilution and E-test methods. This indicates that the disk diffusion method might not have detected low-level resistance in these isolates. Furthermore, four out of the six isolates possessed vancomycin resistance genes, while two did not. This discrepancy may be attributed to the presence of uncommon genes that were not detected in the present study. The findings underscore the importance of routine monitoring of Minimum Inhibitory Concentrations (MIC) for critical antibiotics like vancomycin. This approach is crucial before categorizing isolates as resistant or intermediately sensitive. The emergence of VRE has been linked to the inappropriate use of vancomycin and poor compliance with infection control practices, emphasizing the need for vigilant monitoring and responsible antibiotic use.

In the present study, 4% of the isolates were identified as Vancomycin-Resistant Enterococcus (VRE) and possessed the vanB and vanC1 genes. This prevalence is in line with findings reported in other studies conducted in different parts of India, where the range of VRE isolates has been reported to be between 1-30%. It's noteworthy that while the vanA gene is commonly associated with VRE, none of the isolates in current study were found to harbor the vanA gene. This suggests that the resistance mechanisms observed in your study population may be different from those in regions where the vanA gene is more prevalent. Understanding the specific genetic basis of resistance is crucial for tailoring effective treatment strategies and infection control measures.

It's interesting to note that Enterococcus gallinarum and Enterococcus casseliflavus exhibit intrinsic vancomycin resistance that is not transferable. However, there is evidence suggesting that these strains can acquire additional resistance genes, such as vanA and vanB, which can then be disseminated to other Enterococcus strains. Reports have indicated the existence of VRE strains carrying both vanA/vanB and vanC1 genes. The presence of both vanA and vanC1 genes in a VRE isolate can alter its resistance pattern, emphasizing the complexity of resistance mechanisms in Enterococcus species. Understanding these dynamics is crucial for developing effective strategies to manage and control the spread of antibiotic resistance.[11]

The study detected vanC1 in two isolates of Enterococcus gallinarum and one isolate of Enterococcus casseliflavus. These findings align with the results reported by Descheemaeker et al.[12] and Salem-Bekhit et al.[13] In summary, the vancomycin resistance rate among Enterococcus isolates in this study was 4%, consistent with other studies conducted in India. The predominant phenotype of vancomycin resistance observed was the VanC phenotype, indicating low-level resistance to vancomycin. Additionally, the study identified the VanB phenotype (resistant to vancomycin, sensitive to teicoplanin), while the VanA phenotype (high-level resistance to both vancomycin and teicoplanin) was not observed.

The presence of vancomycin resistance in our isolates emphasizes the importance of strict enforcement of antibiotic policies and infection control measures. It highlights the need for vigilant measures to prevent the emergence and spread of multi-drug resistant bacteria. This underscores the significance of judicious antibiotic use and robust infection control practices in healthcare settings.

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