Phenotypic Detection and Molecular Characterization of Vancomycin-Resistant Enterococci

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Abstract

Background: The emergence of resistance among enterococci threatens to make vancomycin obsolete in the treatment of infections caused by these bacteria. Resistance is observed most commonly in Enterococcus faecium, which is often resistant to aminoglycosides and β-lactams and to a lesser extent in other Enterococcus species, including Enterococcus faecalis. Aim: The aim of this study is to estimate the prevalence of vancomycin-resistant enterococci and study types of glycopeptide resistance genes. Materials and Methods: A total of 100 nonrepeat isolates of enterococci from various clinical samples were analyzed. As per the Clinical and Laboratory Standards Institute guidelines enterococci were screened for vancomycin resistance by Kirby–Bauer disc diffusion method. The minimum inhibitory concentration of all isolates for vancomycin was determined by Epsilometer test. Multiplex polymerase chain reaction (PCR) was carried out for vancomycin-resistance enterococcal isolates using six sets of primers to identify \( \text{vanA} \) genes responsible for resistance. Results: Twelve percent isolates were found to be vancomycin resistant. By multiplex PCR 100% vancomycin-resistant isolates carried \( \text{vanA} \) gene. However, \( \text{vanB}, \text{vanC}, \text{vanD}, \text{vanE}, \text{vanG} \) genes which encode other resistance ligase were not detected. Amplicons were sent for sequencing and the sequence received showed 100% identity with \( \text{vanA} \) gene. Conclusion: The prevalence of vancomycin resistance among enterococci isolates in this study was 12%. Multiplex PCR can detect \( \text{vanA} \) genes with high sensitivity and specificity responsible for vancomycin resistance. Treating serious infections caused by vancomycin-resistant enterococci has emerged as one of the leading clinical challenges for physicians because of limited therapeutic options.

Keywords: Minimum inhibitory concentration, multiplex polymerase chain reaction, \( \text{vanA}, \text{vanB}, \text{vancomycin-resistant enterococci} \)

Introduction

Enterococci are part of the normal human fecal flora. In the hospitalized patients, soft-tissue wounds, ulcers, and the gastrointestinal tract are the main sites of colonization.\(^1\) Whereas, oral cavity, genitourinary tract, and skin are sites less often colonized by enterococci. Vancomycin-resistant enterococci (VRE) as hospital-associated pathogens were first identified during the mid-1980s in Europe and have ever since spread rapidly worldwide.\(^2\)

In enterococci six types of glycopeptide resistance have been described – \( \text{vanA}, \text{vanB}, \text{vanC}, \text{vanD}, \text{vanE}, \text{vanG} \).\(^3\) \( \text{vanA} \) and \( \text{vanB} \) type resistance are characterized by high-level and variable levels resistance to vancomycin.\(^4\) There are three \( \text{vanC} \) genes: \( \text{vanC1}, \text{vanC2}, \text{vanC3} \), specific to the motile Enterococcus gallinarum, Enterococcus casseliflavus, and Enterococcus flavescens, respectively. Inducible low levels of vancomycin resistance are shown by the \( \text{vanE} \)- and \( \text{vanG} \)-resistant phenotype.\(^5\) The aim of the study is to estimate the prevalence of vancomycin-resistant enterococci and study types of glycopeptide-resistance genes.

Materials and Methods

Study design

The cross-sectional study was carried out in a tertiary care center between January 2013 and January 2016. With 95% confidence interval, \( \alpha \) of 5%, precision of 10%, and considering the prevalence of vancomycin-resistance enterococci in India, the required sample size was calculated to be 100 clinical isolates of Enterococci. This study was carried out on 100 consecutive, non-repeat isolates of enterococci isolated from

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various clinical samples from hospitalized patients received in the microbiology laboratory in a tertiary care center. Specimens such as pus, blood, urine, central line tip, and various others were collected aseptically and transported as per the standard protocol.

**Phenotypic identification**

The isolates of enterococci were identified and speciated on the basis of colony morphology, Gram stain, and various biochemical reactions such as catalase test, bile–esculin test, growth in 6.5% NaCl, pyrrolidonyl aminopeptidase test, mannnitol fermentation, arginine dihydrolase test, sucrose fermentation, arabinose fermentation, growth in pyruvate, lactose fermentation, and pigment production. *Enterococcus faecium* and *Enterococcus faecalis* were further confirmed by PCR analysis using specific *ddl* E. faecium and *ddl* E. faecalis genes, respectively.[6]

As per the Clinical and Laboratory Standards Institute (CLSI) guideline 2013, all enterococcal isolates were tested for their susceptibility by Kirby–Bauer method to various antibiotics. Apart from vancomycin (30 g), these 100 isolates were tested with antibiotics, namely, ampicillin (10 g), ciprofloxacin (5 g), high-level gentamicin (120 g), and high-level streptomycin (300 g) as per the CLSI guidelines.

**Testing for vancomycin-resistant enterococci**

Screening of VRE with vancomycin 30 µg disc was done. The CLSI recommended quality control strains, *E. faecalis* ATCC 29212 susceptible strain, and *E. faecalis* ATCC 51299-resistant strain. Minimum inhibitory concentration (MIC) of VRE isolates was determined by Epsilometer test (E-test – 0.016–256 µg/ml).

**van** genes characterization

QIAamp DNA mini kits from QIAGEN, Germany was used for DNA extraction. All VRE isolates were subjected to Multiplex PCR using 6 sets of primers.[7] [Table 1]. The genes analyzed in the present study were *vanA*, *vanB*, *vanC*, *vanD*, *vanE*, and *vanG* responsible for high-level vancomycin resistance in enterococci. PCR reactions were performed in a volume of 50 µl with the following in a reaction tube: 5 µl of DNA template, 1.5 mM MgCl₂, 0.1 mM (each) deoxynucleoside triphosphate, 1× PCR buffer, 2.5U of Taq DNA polymerase, and the amount of each primer in the PCR was as follows: 0.5 µM for *vanA*, *vanB*, *vanC*, *vanD*, *vanE*, and *vanG*. PCR was performed in a (Perkin–Elmer Gene Amp 2400) thermal cycler with an initial denaturation step of 3 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 45°C, and 1 min at 72°C; and a final extension step of 7 min at 72°C. PCR products were analyzed by electrophoresis at 100 V for 1–1/2 h on a 1% agarose gel stained with etidium bromide.

Postamplification analysis is done with gel electrophoresis with a 100-base pair molecular weight marker. The gel was viewed under UV transilluminator and was documented with the help of digital camera attached with the transilluminator and to the computer [Figure 1].

After multiplex PCR, the amplicons were sent for confirmation by sequencing. The sequencing method employed was Sanger’s capillary sequencing. The sequence was analyzed with the BLAST program from the National Center for Biotechnology Information (NCBI).

**Results**

Out of the 100 isolates, 52 (52%) were *E. faecalis* and 48 (48%) were *E. faecium* by conventional phenotypic and PCR analysis. The most common clinical sample from which enterococci were isolated was urine (60%) followed by pus (17%) and blood (15%), and tracheal aspirate (four percent), semen (two percent), and drain fluid (two percent).

A total of 12 isolates were found to be vancomycin resistant using 30 µg disc by Kirby–Bauer disc diffusion method as per the CLSI guideline. Nine (19%) out of 48 isolates of *E. faecium* and three (06%) out of 52 isolates of *E. faecalis* were found to be resistant to vancomycin. All 12 isolates those were found to be vancomycin resistant by Kirby–Bauer disc diffusion method were showing MIC >256 µg/ml by E-test.

| Table 1: Primers used in the multiplex polymerase chain reaction |
|------------------|-----------------|-----------------|-----------------|-----------------|
| Gene  | Primer* | Sequence (5′→3′) | Position† | Size of PCR product |
| vanA  | *vanA* (Fw) | GGGAAGAAGCAATTGC | 176–192 | 732 |
|  | *vanA* (R) | GTACAATGCAGCGCCGTTA | 907–891 |
| vanB  | *vanB* (Fw) | ACGGAATGGGAAGCCGA | 169–185 | 647 |
|  | *vanB* (R) | TGCAACCAGATTCGTC | 815–799 |
| vanC/2 | *vanC* (Fw) | ATGGATTTGATYTKTAT | 133–150/142–159 | 815/827 |
|  | *vanC* (R) | TAGCAGAGGTGMCYGTTAA | 947–929/968–950 |
| vanD  | *vanD* (Fw) | GTGGGGATGCGATATCCAA | 357–375 | 500 |
|  | *vanD* (R) | TGGACCCAAGTGACGCGTAA | 856–837 |
| vanE  | *vanE* (Fw) | GTGCGGATGCGGTATCCAG | 364–82 | 430 |
|  | *vanE* (R) | ATAGTTTAGCTGTAAC | 793–777 |
| vanG  | *vanG* (Fw) | CGAGACTCGGGTCTTTTGA | 68–86 | 941 |

*Fw, R: Nucleotide numbering begins at the initiation codon of the gene. K=G or T; M=A or C; Y=C or T. PCR: Polymerase chain reaction, FW: Forward primer, R: Reverse primer.

*Figures and tables have been adjusted for natural readability.*
Figure 1: Postamplification gel electrophoresis showing bands. vanA gene shows band at 732 bp

Twelve out of 12 VRE, that is, (100%) isolates carried vanA gene. However, vanB, vanC, vanD, vanE, and vanG genes were not detected in our study. Result of sequence blast on NCBI site showed 100% identity with vanA gene.

**Discussion**

In the present study, 12 (12%) isolates were found to be vancomycin resistant by disc diffusion method. MICs of vancomycin for all isolates were determined by the E-test method. All the 12 isolates showed resistance to vancomycin (MIC ≥256 µg/ml) by E-test.

All the 100 enterococcal isolates were subjected to multiplex PCR using six sets of primers as already described. All the 12 isolates which were found to be vancomycin resistant by phenotypic methods showed the presence of vanA gene by multiplex PCR, (band size – 732 bp on gel electrophoresis). This vanA gene is responsible for the high-level resistance to vancomycin and teicoplanin (MIC ≥256 µg/ml). Thus, the prevalence of vancomycin resistance among enterococcal isolates in the present study is 12%. 100% VRE isolates carried vanA gene. The result of sequence blast on NCBI site showed 100% identity with vanA gene.

Edet et al. detected 11 (2.6%) isolates of vancomycin-resistant enterococci from various clinical specimens consisting of four (12.5%) *E. faecium* and seven (1.9%) *E. faecalis*. Karmarkar et al. in their study of 52 enterococcal isolates, reported 12/42 (28.57%) isolates of *E. faecium* resistant to vancomycin with MIC ≥4 µg/ml but sensitive to teicoplanin. Similarly, Deshpande et al. reported 19.6% enterococcal isolates to be resistant to vancomycin.

However, Mathur et al. reported 1% isolates of *E. faecalis* resistant to vancomycin by disc diffusion and agar screening method. In the study of Taneja et al., eight (5.55%) VRE were detected by E-test and agar dilution method. In a study of 250 enterococcal isolates in Western India, Modi et al. found a prevalence of VRE to be only 4%. Similarly, Fernandes and Dhanashree in a study of 150 enterococcal isolates reported a prevalence of vancomycin resistance of 8.6%. Shafiyabi et al. also reported a prevalence of 5% of vancomycin resistance among enterococci isolates in a tertiary care center in South India. Compared to Western countries, vancomycin resistance is low in India, although its rising rate is concerning.

Nine out of 48 isolates (19%) of *E. faecium* and three out of 52 isolates, that is, 6% of *E. faecalis* were found to be resistant to vancomycin. In various studies, it has been found that *E. faecium* accounts for far fewer clinical enterococcal isolates than *E. faecalis*, but it is far more resistant to vancomycin. In a study conducted by Deshpande et al., <2% of *E. faecalis* were found to be resistant to vancomycin, whereas 52% of the *E. faecium* isolates were resistant to vancomycin.

In the present study, it has been seen that the results of disc diffusion method, MIC determination by E-test, and vancomycin resistance gene detection by multiplex PCR for enterococcal isolates have given similar results. Thus, vancomycin disc diffusion method and E-test method, which are simple and economical, can be used as useful screening test for detection of glycopeptide resistance in enterococcal isolates in clinical microbiology laboratory because agar dilution is labor-intensive and time-consuming. However, in resource-rich settings, multiplex PCR can be a time-saving, sensitive, and confirmatory technique for detection of specific vancomycin resistance genes in enterococci.

The epidemiology of VRE has not been elucidated completely. However, it is known that certain patient populations such as elderly patients or critically ill patients or immunosuppression such as ICU patients or patients in oncology or transplantation wards, those with an indwelling urinary or intravenous catheter and those who have had a prolonged hospital stay or received multiple antimicrobial agents are at increased risk for VRE infection. Treatment of infections caused by VRE is extremely challenging because of the limited therapeutic alternatives. While treating VRE infections an alarming problem is emergence linezolid resistance, even though linezolid has demonstrated good antienterococcal activity.

This study illustrates the increased prevalence of vancomycin resistance among enterococcal isolates. To establish a rational antibiotic policy for the better management of enterococcal infections efficient infection control and regular surveillance of antimicrobial resistance of enterococci should be done.

**Conclusion**

Enterococci in recent years have emerged as pathogens in association with serious nosocomial infections. Urinary tract infections are the most frequent infections caused by enterococci followed by wound infections and bloodstream infections. Either *E. faecalis* or *E. faecium* cause most of the clinical infections. For β-lactam- and aminoglycosides-resistant, Gram-positive bacteria vancomycin is generally used. Treating
serious infections caused by vancomycin-resistant enterococci has emerged as one of the leading clinical challenges for physicians because of limited therapeutic options. In this study, Kirby–Bauer disc diffusion method showed 12 isolates to be vancomycin resistance. These 12 isolates showed high-level resistance to vancomycin by E-test method, which was corroborated by the finding of vanA gene in these isolates by multiplex PCR.

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Conflicts of interest
There are no conflicts of interest.

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