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Original Article

Detection of glycopeptide resistance genes in enterococci by multiplex PCR

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ABSTRACT

Background: Vancomycin Resistant Enterococci (VRE) are a major cause of nosocomial infections. There are various phenotypic and genotypic methods of detection of glycopeptide resistance in enterococci. This study utilizes multiplex PCR for reliable detection of various glycopeptides resistance genes in VRE.

Method: This study was conducted to detect and to assess the prevalence of vancomycin resistance among enterococci isolates. From October 2011 to June 2013, a total of 96 non-repetitive isolates of enterococci from various clinical samples were analyzed. VRE were identified by Kirby Bauer disc diffusion method with Clinical and Laboratory Standards Institute (CLSI) guidelines. Minimum inhibitory concentration (MIC) of all isolates for vancomycin and teicoplanin was determined by E-test. Multiplex PCR was carried out for all enterococci isolates using six sets of primers.

Results: Out of 96 isolates, 14 (14.6%) were found to be resistant to vancomycin by vancomycin E-test method (MIC ≥ 32 $\mu\text{g/ml}$). Out of these 14 isolates, 13 were also resistant to teicoplanin (MIC ≥ 16 $\mu\text{g/ml}$). *VanA* gene was detected in all the 14 isolates by Multiplex PCR. One of the PCR amplicons was sent for sequencing and the sequence received was submitted in the GenBank (GenBank accession no. KF181100).

Conclusion: Prevalence of VRE in this study was 14.6%. Multiplex PCR is a robust, sensitive and specific technique, which can be used for rapid detection of various glycopeptide resistance genes. Rapid identification of patients infected or colonized with VRE is essential for implementation of appropriate control measures to prevent their spread.

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Introduction

Enterococci have evolved over the past century from being an intestinal commensal organism of little clinical significance to becoming one of the most common nosocomial pathogens associated with significant morbidity and mortality.^{1,2} Enterococci are important because of their role as the leading cause of nosocomial infections which have a significant role in dissemination and persistence of drug resistance genes.³

Vancomycin is a member of a class of antibiotics referred to as glycopeptides therefore vancomycin resistant enterococci (VRE) can also be referred to as Glycopeptide resistant enterococci (GRE). Six types of glycopeptides resistance have been described in enterococci that can be distinguished on the basis of the sequence of the structural gene for the resistance ligase (*vanA*, *vanB*, *vanC*, *vanD*, *vanE*, *vanG*).⁴

VanA type resistance is characterized by high-level resistance to vancomycin and teicoplanin, whereas *VanB* type strains are resistant to variable levels of vancomycin, but susceptible to teicoplanin.⁵ *VanD* type strains are resistant to moderate level of vancomycin and low level of teicoplanin.⁶ There are three *VanC* genes namely *Van C1*, *Van C2* and *Van C3*. These genes are specific to the motile VRE species *Enterococcus gallinarum*, *Enterococcus casseliflavus* and *Enterococcus flavescens* respectively.⁷ The *Van C2* and *Van C3* genotypes are closely related and are usually referred to as *Van C2/C3*. The *VanE* resistant phenotype is an inducible type of resistance to low levels of vancomycin that exhibit full susceptibility to teicoplanin.

Resistance to glycopeptides is disseminating rapidly and has spread to methicillin resistant *Staphylococcus aureus*.⁸ Rapid identification of VRE colonized patient is thus essential for detection of such clinical isolates to prevent the spread of VRE.

The aim of the study was to identify enterococci at species level by phenotypic methods and detect vancomycin resistance genes (*van* alphabet) by multiplex PCR.

Materials and methods

A cross-sectional study was conducted from October 2011 to June 2013. A total of 96 non-repetitive clinical isolates of enterococci were identified from various clinical samples. Detailed history of the patients was recorded which included age, sex, location, diagnosis, duration of hospitalization and exposure to antibiotics.

Identification of enterococci: The isolates were identified at the species level with the help of conventional phenotypic methods which included Gram's stain, colony morphology, catalase test, bile esculin test, growth in 6.5% NaCl, mannitol fermentation, arginine dihydrolase test, motility test, arabinose fermentation, lactose fermentation and sucrose fermentation.

Antibiotic susceptibility testing: Antibiotic susceptibility testing was carried out using Vancomycin disc (30 µg, HiMedia) and the results were interpreted according to CLSI guidelines. Zone size <14 mm was considered resistant and zone size >17 mm considered sensitive.

Minimum inhibitory concentration (MIC) determination: Minimum inhibitory concentration (MIC) of vancomycin and teicoplanin for all 96 isolates was determined by E-test (bio-Merieux) and the results were interpreted according to CLSI guidelines. MIC ≥32 µg/ml for vancomycin and teicoplanin was considered resistant.

Multiplex PCR

DNA extraction: DNA extraction was done from all isolates of enterococci using the QIAamp DNA mini kits from QIAGEN, Germany. Manufacturer's instructions were followed for extracting DNA from the fresh cultures.

Primers: Primers were procured from Integrated DNA Technologies (IDT), Inc. and are shown in Table 1.⁴

PCR reaction mixtures: PCR reaction mixtures were prepared under laminar flow under strict precautions to prevent cross contamination. For a single sample reaction of

Table 1 – Primers used in the multiplex PCR.

Primer ^a	Sequence (5' → 3')	Gene	Position ^b	Size of PCR product
vanA(+)	GGGAAAACGACAATTGC	van A	176–192	732
vanA(–)	GTACAATGCGGCCGTTA		907–891	
vanB(+)	ACGGAATGGGAAGCCGA	van B	169–185	647
vanB(–)	TGCACCCGATTTTCGTTC		815–799	
vanC(+)	ATGGATTGGTAYTKGTAT ^c	van	133–150/	815/827
vanC(–)	TAGCGGGAGTGMCYMGTAAC ^c	C1/2	142–159 947–929/ 968–950	
vanD(+)	TGTGGGATGCGATATTCAA	van D	357–375	500
vanD(–)	TGCAGCCAAGTATCCGGTAA		856–837	
vanE(+)	TGTGGGATCGGAGCTGCAG	van E	364–382	430
vanE(–)	ATAGTTTAGCTGGTAAC		793–777	
vanG(+)	CGGCATCCGCTGTTTGTGA	van G	68–86	941
vanG(–)	GAACGATAGACCAATGCCTT		1008–989	

^a +, Sense primer; –, antisense primer.

^b Nucleotide numbering begins at the initiation codon of the gene.

^c K = G or T; M = A or C; Y=C or T.

50 µl volume, various reagents were used as mentioned in Table 2.

Amplification was carried out with the following thermal cycling profile: initial denaturation for 3 min at 94 °C, 35 cycles of amplification consisting of 1 min at 94 °C, 1 min at 45 °C, and 1 min at 72 °C, with 7 min at 72 °C for the final extension.

Gel electrophoresis: DNA fragments were analyzed by electrophoresis in 0.5× Tris-borate-EDTA on a 1% agarose gel stained with ethidium bromide.

Sequencing: One of the PCR amplicons was sent for sequencing and was submitted in GenBank (Accession number – KF181100).

Results

Out of 96 isolates of enterococci, 70 (~73%) were *Enterococcus faecalis* and 26 (~27%) were *Enterococcus faecium*. A total of 14 isolates were found to be vancomycin resistant by Kirby–Bauer disc diffusion method and E-test. 13 isolates showed high level resistance to both vancomycin and teicoplanin (MIC > 256 µg/ml) by E-test (Fig. 1). One isolate showed high level resistance to vancomycin (MIC > 256 µg/ml) only and susceptibility to teicoplanin (MIC 2 µg/ml). 11 out of 26 isolates (42.3%) of *E. faecium* and 3 out of 70 (4.3%) isolates of *E. faecalis* were found to be resistant to vancomycin. All the 96 isolates were subjected to Multiplex PCR using 6 sets of primers as already described. *VanA* gene (bp size 732) was found to be present in 14 isolates (Fig. 2).

One of the PCR products isolated from urine sample from a patient of ICU (Medical) suffering from Chronic lymphoid leukemia was sent for sequencing. The sequence obtained was submitted to the GenBank, Bethesda, Maryland, USA, and the accession number provided was KF181100.

Table 2 – Various reagents used in PCR reaction mixture.

Reagent	Volume (µl)	Final concentration
Autoclaved Distilled water	19.35	—
PCR buffer (10×) with MgCl ₂ (15 mM)	5	1×, 1.5 mM
dNTP (10 mM)	0.25	50 µM
Primer <i>vanA</i> (forward)	1.25	0.5 µM
Primer <i>vanA</i> (reverse)	1.25	0.5 µM
Primer <i>vanB</i> (forward)	1.25	0.5 µM
Primer <i>vanB</i> (reverse)	1.25	0.5 µM
Primer <i>vanC</i> (forward)	1.25	0.5 µM
Primer <i>vanC</i> (reverse)	1.25	0.5 µM
Primer <i>vanD</i> (forward)	1.25	0.5 µM
Primer <i>vanD</i> (reverse)	1.25	0.5 µM
Primer <i>vanE</i> (forward)	1.25	0.5 µM
Primer <i>vanE</i> (reverse)	1.25	0.5 µM
Primer <i>vanG</i> (forward)	1.25	0.5 µM
Primer <i>vanG</i> (reverse)	1.25	0.5 µM
Taq DNA Polymerase (250U/µl)	0.4	2U/µl
DNA Template	10	—
Total	50	—

Fig. 1 – E-test showing MIC of vancomycin and teicoplanin against vancomycin-resistant strain of *Enterococcus faecium* (MIC > 256 µg/ml).

The most common clinical sample from which enterococci were isolated was urine. The distribution of various clinical samples is given in Fig. 3.

The average duration of hospitalization of all the patients was 9.04 days whereas that of patients with VRE and non-VRE was 13.42 days and 7.3 days respectively.

Out of 96 isolates, 26 were from ICU, 40 from various wards and 30 were from OPD. Ten isolates from ICU (38.5%) and 04 isolates from various wards (10%) were found to be VRE, whereas no VRE was detected from OPD patients.

A total of 24 patients were suffering from one or the other malignancy and were on immunosuppressive drugs or were neutropenic, 16 had surgical site infection (SSI) and 10 had

Fig. 2 – Analysis of PCR product by gel electrophoresis showing band at 732 base pair denoting *vanA* gene. A – positive control of *vanA* gene, band at 732 bp (*E. faecium* ATCC 51559) B – negative control D, N, Q – clinical isolates showing band at 732 bp denoting *vanA* gene J – 100 bp molecular marker.

Fig. 3 – Distribution of enterococcal isolates from various clinical samples.

CKD. Ten isolates from patients with malignancy (41.7%), 2 isolates from patients with CKD (20%) and 1 isolate from patients with SSI (6.25%) were vancomycin resistant.

Discussion

Enterococci colonize the bowels of more than 90% of healthy humans. *E. faecalis* accounts for more than 80% of the enterococcal isolates in clinical samples, but in recent years *E. faecium* has become more common, probably because of its greater antibiotic resistance particularly to vancomycin.^{9–11} For the last two decades, enterococci have been the 3rd most common cause of hospital-acquired infections (HAI) after *E. coli* and *S. aureus* and ahead of *Pseudomonas aeruginosa*.^{12,13} The most common nosocomial infection caused by enterococci are urinary tract infections (associated with instrumentation and antimicrobial administration) followed by intra-abdominal and pelvic infections. They also cause surgical site infections, bacteremia, endocarditis, neonatal sepsis and rarely meningitis.^{9,14}

In this study of 96 enterococcal isolates, the prevalence of vancomycin resistance enterococci was found to be 14.6%, which is very high. In the study of 291 enterococcal isolates by Deshpande et al, the prevalence of vancomycin resistance was found to be 19.6%.¹⁵ All the other studies from India have reported a prevalence of less than 10%. The high prevalence of vancomycin resistance among enterococci in the present study might be because of the increased vancomycin selection pressure, as this being a tertiary care centre, most of the patients are referred and have already been exposed to various antibiotics including vancomycin and cephalosporins. The vancomycin resistance was more prevalent in *E. faecium* (42.3%) than in *E. faecalis* (4.3%). Overall the vancomycin MIC levels of the 14 VRE isolates were very high (>256 µg/ml) indicating high-level resistance. The MIC of teicoplanin of 13 isolates was >256 µg/ml indicating high-level resistance to teicoplanin. As only 13 isolates showed high level resistance to both vancomycin and teicoplanin, it seemed that *vanA* gene was present in these 13 isolates. However, the results of multiplex PCR were only slightly different as *vanA* gene was detected in all the 14 isolates. Thus multiplex PCR is a better,

sensitive and specific technique for detection of glycopeptide resistance genes.

The patients from whom vancomycin resistant enterococci were isolated had increased duration of hospitalization (average duration 13.4 ± 2.3 days) as compared to patients from whom vancomycin sensitive enterococci were isolated (average duration 7.3 ± 4.83 days). Moreover all the patients from whom VRE was isolated had recent exposure to antibiotics during their hospitalization suggesting that prolonged hospitalization and prior exposure to antibiotics increases the risk of VRE.

Vancomycin resistant isolates were most commonly isolated from ICU (38.5%) followed by various wards (10%). All isolates of enterococci isolated from OPD were vancomycin sensitive. This suggests that the risk of VRE increases with the severity of the disease condition.

Most of the patients from whom VRE was isolated were suffering from malignancy and were on immunosuppressives or were severely neutropenic. This indicates that the risk of VRE increases with the degree of immunosuppression.

Conclusion

The prevalence of vancomycin resistance among enterococci isolates in this study is 14.6%, which is quite high. The resistance was found to be more common in *E. faecium* than in *E. faecalis*. Multiplex PCR can detect glycopeptide resistance genes with high sensitivity and specificity with a short turnaround time. The study shows that *vanA* gene is prevalent in this part of the country. This study also brings out the fact that the risk factors for vancomycin resistant enterococci were prolonged hospitalization, prior exposure to antibiotics, severity of disease condition and degree of immunosuppression.

Conflicts of interest

All authors have none to declare.

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