



Evaluation of drug sensitivity of *Enterococcus faecium* and *Enterococcus faecalis* Strains and Detection of *VanA/B* Genes in Vancomycin Resistance Isolated by PCR Method

Mohammad Reza khataminejad^{1*}, Zahra Ghanizadeh Dizaj¹, Saeed Zaker Bostanabad²

1. Department of medical Microbiology, Faculty of Medical Sciences, Islamic Azad University of Tonekabon Branch, Tonekabon, Iran.

2. Department of medical Microbiology, Faculty of Medical Sciences, Molecular and Cellular Biology, Islamic Azad University of Parand Branch, Parand, Iran.

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ABSTRACT

Enterococci, as the major cause of nosocomial infections, have been shown to have an increase in drug resistance during the past two decades. The Enterococcal emergence of Vancomycin-resistant is very important nowadays. The purpose of this study was to determine the drug sensitivity of *Enterococcus faecalis* and *Enterococcus faecium* species against Vancomycin, Tetracycline, Gentamicin, Erythromycin and the presence of *Van A / B* genes in resistant Enterococci to Vancomycin. A total of 189 samples, including urine, blood, wound and stool were collected from patients referred to Masoud Medical laboratory in Tehran, Iran, during years 2011-2012. Strains of Enterococci were identified by standard methods and antibiotic susceptibility testing was performed by disk diffusion according to standard methods (CLSI). The test was carried out on the samples resistant to Vancomycin for determining MIC levels. The frequencies of *VanA* and *VanB* were studied by using of PCR. A total of 89 *Enterococcus* were isolated in this study. In this study, 89 *Enterococcus* were obtained, of which 8 were resistant to Vancomycin based on the disk diffusion method. All Vancomycin resistant isolates showed *VanA* gene, while none of them (0%) carried *VanB* gene. The results obtained by this study indicated that Vancomycin-resistant Enterococci isolates did not commonly carry the *VanA* gene. However, *VanA* gene was fairly frequent as it was detected in 8 strains, while *VanB* gene was found in none of the strains.

1. Introduction

Enterococci are among 450 species of aerobic and anaerobic bacteria of the normal intestinal flora of humans and some animals. Before identification of multiple resistant Enterococcal strains in the late 1970s, they were regarded as relatively safe organisms. Enterococci, as the major cause of nosocomial

infections, have shown an increase in drug resistance during the past two decades. Enterococci are the matter of discussion as a major clinical challenge for physicians. Today, the emergence of Vancomycin-resistant Enterococcal is important in several ways such as Enterococcal treatment with acquired resistance to Vancomycin, and transfer of Vancomycin resistant gene to Vancomycin-susceptible

*Corresponding author: Dr. khataminezhad
E-mail address: mr_khataminezhad@yahoo.com

Staphylococcus aureus has been confirmed (Hayden, 2000; Alligent, 1993). Epidemiological studies in the United States and Europe concerning the result of inappropriate use of antibiotics and Vancomycin-resistant Enterococcal showed increasing efforts are needed in prevention and control of Vancomycin-resistant Enterococci and *Staphylococcus* (Harbarth et al., 2002).

The first Vancomycin-resistant Enterococci were reported in the kidney and urinary tract from the hospital in Paris and London in 1986 (Morrison et al., 1992). In 1988, the first report of Vancomycin-resistant Enterococci in America was released with a prevalence of 0.3-0.4%. However, the prevalence increased up to 7.9-10% in less than seven years (Leclercg et al., 1992) and Vancomycin-resistant Enterococci were generally found in the hospitals of the United States (Boyce, 1995). Although Vancomycin-resistant Enterococci were first identified in European countries, but the Prevalence of microorganisms in these countries has remained low at about 2% (Leclercg et al., 1992). *E.faecalis* and *E.faecium* are responsible for most infections in general hospitals, which in most cases are resistant to Vancomycin and Penicillin. *E.faecium*, as the cause of 10% Enterococcal infections, is more resistant to antibiotics as compared to *E.faecalis*. There are reports that in addition to Vancomycin, *E.faecalis* is resistant to other antibiotics too (Harbarth et al., 2002).

Achieving high levels of resistance to Aminoglycosides, Penicillin and Vancomycin cause problems in the treatment of infections caused by Enterococci, especially those caused by *E.faecium* (Cetikaya et al., 2000; Leclercg et al., 1992). Given the importance of VRE resistant *Enterococcus* isolates, the purpose of this study was to determine drug sensitivity of *E.faecalis* and *E.faecium* species against Vancomycin, Tetracycline, Gentamicin, Erythromycin and the presence of genes *VanA/B* in isolates resistant to Vancomycin.

2. Materials and Methods

2.1. Sample collection

In this study, 189 Clinical specimens including urine, blood, wound and stool samples

were collected from patients referred to the Maud clinical laboratory in Tehran, Iran, during years 2012-2013. The first example of suspicious wound, blood, urine, feces, abscesses and other specimens from inpatients and outpatients were prepared. Identification of strains to the genus level was separated by using the following characteristics: the samples culture medium on blood agar, Eosin Methylene Blue, gram-positive cocci and catalase-negative of strain. Then growth at 10-45°C, 60°C tolerance, hydrolysis of esculin, hippurate hydrolysis, determine the susceptibility of isolates to drive Optochin, tolerance of 1% methylene blue and Sodium azide 0.5%, growth in NaCl 6.5%, glucose fermentation and acid production of Sorbose, Raffinose, Sucrose, Mannitol, Sorbitol, Arabinose and Ribose. In this study *E.faecalis* ATCC29212 and *E.faecium* ATCC51559 was used as a positive control (Murray, 2000).

2.2. Antibiotic susceptibility

Susceptibility to antimicrobial agents was tested by disc diffusion (Kirby-Bauer) method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Assessment of drug sensitivity was performed towards some antibiotics such as Erythromycin (30 µg), Tetracycline (30 µg), Gentamicin (30 µg) and Vancomycin (30 µg). MICs of Vancomycin were determined by the E. Test (AB Biodisk, Solna, Sweden) method on Mueller-Hinton agar according to the manufacturer's instructions.

2.3. DNA extraction

Vancomycin resistant isolates of *E.faecalis* and *E.faecium* were cultured for 24 h in LB medium (yeast extract, tripton and sodium chloride salts). Then the extraction of DNA (Extraction DNA kits Sina Clon) from resistance isolates was performed according to the manufacturer's instructions.

2.4. Amplification with Specific primers of Van A and Van B

PCR reactions were Performed in a volume of 25 µl consisting of 3µl (10X PCR buffer), 1µl (1.5 mM MgCl₂), 2µl (25 pmolL of each primer)

(Table 1), 0.5µl (0.25 U Taq DNA polymerase), 0.5µl (0.2 mM dNTP Mix), 2µl of DNA template (0.4 µg/ml) and 16µl distilled water. The PCR conditions consisted of a Pre-denaturation step at 94°C for 5 min, followed by 30 cycles of 30 sec at 94°C, 30 sec at 50°C and 45 sec at 72°C. A final extension step was performed at 72°C for 5 min. Amplified products were analyzed by electrophoresis on 1.5% agarose. DNA bands were visualized by staining with Ethidium Bromide and Photographed under UV illumination (Mohamadi et al., 2012; Honarmand., 2012).

3. Results

In this study, 89 Enterococci isolates were detected, of which 81 (91%) and 8 (9%) were *E.faecalis* and Arabinose positive *E.faecium*, respectively. The highest number of *Enterococcus* was obtained from urine samples which was 60 (82.2%) (Table 2).

56 (76.7%) of the samples were *E.faecalis*, and 4 (5.5%) of them were *E.faecium*. The lowest number of *Enterococcus* was detected from fecal samples (32 samples, 17%), of which the number of 2 (6.2%) were *E.faecalis*. In this study, no *E.faecium* was isolated from the Feces. Of the 89 isolates, 8 (9.1%) Enterococci were resistant to Vancomycin based on disk diffusion method. According to the results obtained from disk diffusion method, the highest and the least rates of drug resistance were related to Gentamicin and Erythromycin with 75.6% and 46.3%, respectively (Table 3).

Also, among 89 *Enterococcus* isolates, 2 (2.5%) isolates of *E.faecium* showed multiple drug resistant (MDR). The results of MIC showed that 1 isolate (12.5%) was resistant to Vancomycin with MIC > 256 µg/ml, 2 isolates showed MIC > 32 µg/ml, and the remaining isolates were in the range of 4-32 µg/ml. The strains with high resistance to Vancomycin were *E.faecium* isolated from the Urine samples. 50% of Vancomycin-*Enterococcus* resistant was isolated from Urine samples. Also, no Vancomycin resistant Enterococci was obtained from stool samples. After extraction of DNA, PCR was performed

using specific Primers of *VanA* and *VanB*. Eight strains of *Enterococcus* in the Disk diffusion method showed resistance to Vancomycin. The *VanA* gene was the Glycopeptides resistance determinant found in all isolates. No *VanB* gene was detected. The highest and the lowest percentage of Vancomycin resistant Enterococci (VRE) were detected in Urine and Feces, with 50% (4 strains) and 0% (no strain), respectively. Also, of 4 remaining isolates that were carrying *VanA* gene, 3 (37.5%) were those isolated from blood and 1 (12.5%) from wound. 3 isolates of *E.faecalis* (3.7%) and 5 isolates of *E.faecium* (62.5%) showed the presence of *VanA* gene by PCR technique (Table 4).

After electrophoresis of PCR products, the 400 bp and 635 bp bands represented the presence of *VanA* and *VanB* genes (Figures 2 and 3).

4. Discussion

Vancomycin resistant Enterococci were first discovered in England in 1982, although their prevalence in European countries is lower than the USA. This difference could be justified by the fact that the use of Glycopeptide antibiotics such as Avoparcin in Livestock has been restricted in European countries (Cetikaya et al., 2000; Ruoff., 1990). In general, virulence factors in pathogenic *Enterococcus* are not complex, but their treatments are taken into consideration due to their wide variety of antibiotic resistance. The main risk factors causing resistance to high concentrations of Glycopeptide and Aminoglycoside include acquired infections, prolonged hospitalization, inappropriate use of antibiotics such as third-generation Cephalosporins, Metronidazole, Vancomycin, Avoparcin use in pet foods, transplantation particularly liver and kidney, diabetes, hematologic malignancy, and AIDS. Perez-Hernandez and colleagues (2002) through their study on 437 samples from the Canary Islands of Spain indicated that there were only three Enterococci samples. The phenotypic *VanA* was observed in one isolated *E.faecalis*. Jaewook Yang and colleagues in 2006 showed

that resistance to Vancomycin and Teicoplanin in Korea was shown to be 12-16%. In this study, 4 isolates showed phenotypic *VanA* Vancomycin resistance, while no sample was detected with phenotypic *VanB*. The samples comprised four *E.faecium* (Jaewook, 2007). In 1999, a researcher named HraKh investigated on 38 *E.faecalis* strains isolated from urinary tract and 18 *E.faecalis* strains isolated from rectal swabs using RAPD-PCR method, which resulted in detection of 4 Vancomycin resistant isolates. He also evaluated drug sensitivity to a number of antibiotics such as Ampicillin, Gentamicin, Vancomycin and Teicoplanin. All isolates, except 4, were sensitive to Vancomycin, and Ampicillin and Teicoplanin. All isolates from urinary tract, except 4, were resistant to Gentamicin too (Harakeh., 2000). Jung et al., in 2003 used PCR and PFGE to identify Enterococci Vancomycin resistant isolates. He also tested the drug susceptibility of Enterococci isolated from animals to various drugs such as Penicillin, Teicoplanin, Ampicillin, Chloramphenicol, Ciprofloxacin, Erythromycin, Gentamicin, Vancomycin and Tetracycline. The isolates were mostly were *E.faecalis*, *E.gallinarum* and *E.faecium*. Of 243 Enterococci isolates, 51 isolates of *E.faecium* and 144 isolates of *E.gallinarum* were carrying *VanA* and *VanC1* resistance genes, respectively (Jung et al., 2007). Malani et al. used PFGE method to identify *VanA* and *VanB* resistance genes to Vancomycin in *E.faecalis* isolates collected

from hospital clinical specimens during the years 1991-2000. (Malani, 2002). Of 189 clinical samples tested in this study, 81 (91%) *E.faecalis* and 8 (9%) *E.faecium* were detected. Most of Enterococci with 60 (82.2%) isolates were obtained from urine samples, of which 56 (76.6%) and 4 (5.5%) were *E.faecalis* and *E.faecium*, respectively. The minimum number of Enterococci was detected in stool samples with 32 (17%) isolates, of which 2 (2.6%) were *E.faecalis*. In this study, no (0%) *E.faecium* was isolated from the feces. The infectious diseases caused by Vancomycin resistant Enterococci are generally increasing nowadays. In this study, of 8 strains of Vancomycin resistant *Enterococcus* to detect by disk diffusion method, all (100%) were carrying *VanA* gene while none of them (0%) had *VanB* gene. There is a risk of transmission of resistance genes to other bacteria such as *Staphylococcal*. VRE outbreak should be restricted. Use of the drug should be administered with caution in animals and human. A permanent control of Glycopeptides resistant Enterococci outbreak strains in the hospital environment is essential.

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Table 1. PCR primers used for *VanA* and *VanB* detection

Genes	Sequence	PCR product (bp)
<i>VanA</i>	F(Forward): 5'-TCTGCAATAGAGATAGCCGC-3' R(Reverse): 5'-GGAGTAGCTATCCCAGCATT-3'	400
<i>VanB</i>	F (Forward) :5'-ATGGGAAGCCGATAGTC-3' R(Reverse): 5'-GATTTCGTTCCCTCGACC-3'	635

Table 2. Number (%) of *Enterococcus* isolated in various samples

samples	No. of samples (%)	No. of (%) <i>E. faecalis</i> isolated	No. of (%) <i>E. faecium</i> isolated	No. of (%) <i>Enterococcus</i> isolates
Urine	73(38.6)	56(76.7)	4(5.5)	60(82.2)
Blood	45(23.8)	10(22.2)	1(2.2)	11(24.4)
Wound	39(20.6)	13(33.3)	3(7.9)	16(41)
Stool	32(17)	2(6.2)	0(0)	2(6.2)
Total	189(100)	81(42.8)	8(4.2)	89(47)

Table 3. Antimicrobial profile in disk diffuse method

Bacteria	No. (%) <i>Enterococcus</i> isolates	No(%) resistance to disk strains			
		V	TET	G	E
<i>E. faecalis</i>	81(42.8)	3(3.7)	45(55.5)	67(82.7)	42(51.8)
<i>E. faecium</i>	8(4.2)	5(62.5)	4(50)	6(75)	4(50)

Table4. Distribution of *VanA* and *VanB* genes in *Enterococcus* isolated

Gene	No (%) of strains carrying the gene	Samples			
		Urine	Blood	Wound	Stool
<i>VanA</i>	8(100)	4(50)	3(37.5)	1(12.5)	0(0)
<i>VanB</i>	0(0)	0(0)	0(0)	0(0)	0(0)

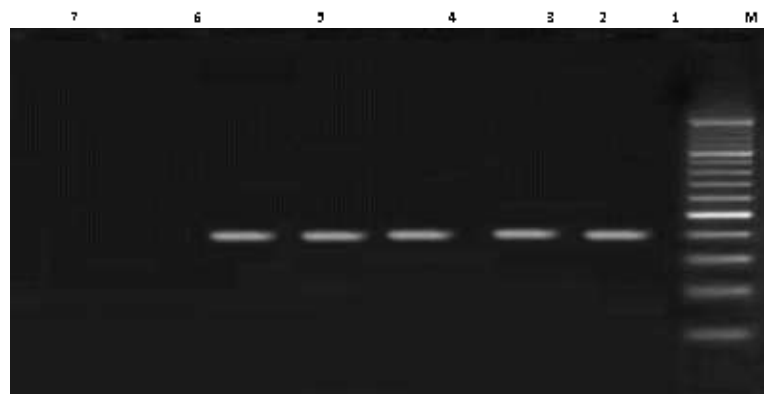


Figure1. PCR Products for *VanA* gene in 5 selective strains of *Enterococcus*. M: DNA marker (100 bp) Lane 1: *E.faecalis* ATCC51559. Lane 2: *E.faecium* isolated from urine sample. Lane 3: *E.faecium* isolated from wound sample. Lane 4: *E. faecalis* isolated from urine sample. Lane 5: *E.faecalis* isolated from blood sample. Lane 6: *S.aureus* ATCC 29213 (negative control). Lane 7: *S.pyogenes* ATCC 19615(negative control)

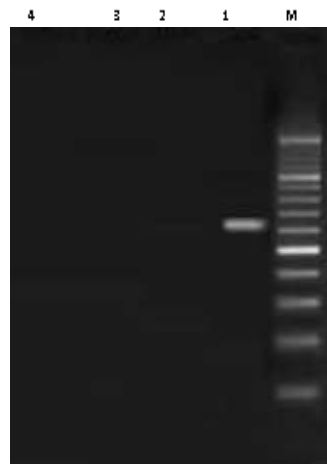


Figure 2. PCR products for *VanB* gene in Vancomycin resistance *Enterococcus* strains. M: DNA marker (100 bp). Lane 1: *E.faecium* ATCC29212 (Positive control). Lane 2: *E.faecium* isolated from Urine sample. Lane 3: *E.faecium* isolated from Wound sample. Lane 4: *E.faecalis* isolated from Urine sample

Refereces

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