

# Molecular detection of *vanA* and *vanB* genes among vancomycin-resistant enterococci in ICU-hospitalized patients in Ahvaz in southwest of Iran

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**Objective:** Nosocomial infections due to vancomycin-resistant enterococci (VRE) are known as a source of spreading these bacteria. The aim of this prospective study was molecular detection of *vanA* and *vanB* genes among VRE isolated from patients admitted to intensive care units (ICUs) in Ahvaz in southwest of Iran.

**Materials and methods:** Overall, 243 non-duplicate rectal swab specimens were collected from ICU-hospitalized patients in teaching hospitals affiliated to Ahvaz Jundishapur University of Medical Sciences, Iran. The specimens were inoculated on suitable culture media, and isolates were identified by standard biochemical tests. The susceptibility and resistance of enterococci to 10 antibiotics were determined based on the Clinical and Laboratory Standards Institute guidelines. Resistance to vancomycin was phenotypically detected by vancomycin screening test, and the *vanA* and *vanB* genes in vancomycin-resistant isolates were amplified by multiplex PCR method.

**Results:** Of 175 specimens containing enterococci, 129 (73.7%) isolates were detected as *Enterococcus faecium* and *Enterococcus faecalis* and 46 (26.3%) isolates as *Enterococcus spp.* The results of susceptibility test showed high rates of resistance to tetracycline, erythromycin, ciprofloxacin, and ampicillin. Moreover, based on this test, out of 129 *Enterococcus* isolates, 56 (43.4%) were resistant to vancomycin and teicoplanin. Also, among 59 vancomycin-resistant or semi-susceptible isolates, *vanA* gene was detected in 54 (91.5%) isolates, while none of the isolates had *vanB* gene.

**Conclusion:** According to the results of this study, to prevent the spread of vancomycin-resistant *Enterococcus* strains, especially in nosocomial infections, the susceptibility of isolates should be determined before vancomycin prescription.

**Keywords:** *Enterococcus faecalis*, *Enterococcus faecium*, multiplex PCR, vancomycin

## Introduction

Enterococci are gram-positive bacteria which are the natural flora of the human intestinal tract.<sup>1</sup> Although enterococci are commensal members of the intestinal tract, these organisms can cause nosocomial and community-acquired infections. Urinary tract infections are the most common infections caused by enterococci. These organisms could also be the causative agents of cholecystitis, peritonitis, bacteremia, endocarditis, meningitis, and wound infections.<sup>2</sup>

These organisms have been reported as the second and third etiologic agents of urinary tract infections and nosocomial bacteremia, respectively.<sup>3</sup> Based on a recent report, enterococcal infection occurs in 14.3% of patients admitted to intensive care units (ICUs) during a 24-month period.<sup>4</sup>

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The most common species isolated from clinical samples are *Enterococcus faecalis* and *Enterococcus faecium*.<sup>5</sup> One of the main reasons that enterococci can survive in hospitals is their inherent resistance to some antibiotics used in hospitals and their ability in acquiring resistance to these common antibiotics.

This resistance has probably been acquired by both mutation and/or receiving resistant genes. In the last decade, the acquisition of resistance to aminoglycosides and glycopeptide antibiotics, especially vancomycin and teicoplanin used in the treatment of enterococci infections, has been common. However, there have been no significant reports on resistance to these antibiotics.<sup>6</sup> Six genotypes of resistant enterococci to glycopeptide, including *vanA*, *B*, *C*, *D*, *E*, and *G* have been identified in recent years, the most important of which are *vanA* and *vanB*.

*VanA* and *vanB* genes are the most common phenotypes observed in hospital isolates. Enterococci containing *vanA* gene are highly resistant to vancomycin and teicoplanin antibiotics, while enterococci containing *vanB* gene show high resistance to vancomycin and susceptibility to teicoplanin.<sup>2</sup> The hospital prevalence of vancomycin-resistant enterococci (VRE) has been reported in association with the transfer of resistance genes horizontally among the epidemic clones of these bacteria.<sup>7</sup>

Failure of antibiotic treatments against VRE is a serious and growing problem, resulting in increased mortality and hospital costs.<sup>8</sup> For example, the cost of treatment of infections caused by VRE is estimated ten times more than the treatment of enterococci without this resistance.<sup>9</sup> A multitude of studies have shown that patients with bacteremia caused by VRE have a higher mortality rate compared with patients with enterococci susceptible to these antibiotics. Patients infected or colonized with VRE are a source for spread of these bacteria, and the presence of VRE in the digestive system of patients is associated with clinically serious infections such as gastrointestinal tract infections.<sup>10</sup>

Hospitalized patients colonized with VRE are a reservoir for the spread of these bacteria.<sup>11</sup> Among the problems caused by VRE are medical restrictions for treating these infections and the ability of these bacteria in the transfer of vancomycin-resistant genes to other gram-positive pathogens. If vancomycin-resistant genes be transferred to the methicillin-resistant *Staphylococcus aureus*, this pathogen is converted to vancomycin-resistant *S. aureus* that does not respond to conventional antibiotics.<sup>12</sup>

Infections caused by VRE in Iran, like other countries, have increased and are followed by mortality, especially in

people with weak immune systems.<sup>13</sup> This study aimed to evaluate the rate of intestinal colonization of VRE carrying *vanA* and *vanB* genes in patients admitted to ICUs in Ahvaz teaching hospitals, Iran.

## Materials and methods

In this cross-sectional study, during a period of 12 months, rectal swab specimens were collected from ICU-hospitalized patients at Emam Khomeini and Golestan teaching hospitals affiliated to Ahvaz Jundishapur University of Medical Sciences (AJUMS) in southwest of Iran. These two hospitals are the main hospitals of this city. Ahvaz is one of the major metropolises in Iran, which is considered to be the seventh most populated city in Iran.

Emam Khomeini and Golestan hospitals have 690 and 615 active beds, respectively. On a monthly basis, 20,000 patients are admitted to general clinics and 4,000 patients are hospitalized in these two hospitals.

The active units in these two big hospitals are as follow: ENT, Skin, Oral and Maxillo-Facial Surgery, Infertility, Women-Gynecology, Orthopedics, Urology, Nephrology, Cardiovascular and Pulmonary Section, General and Pediatric Surgery, Physiotherapy, Endoscopy, Angiography, NICU, CCU, ICU, Ophthalmology, Dentistry, Radiotherapy, Nuclear Medicine, MRI, Oncology, Neurology, and other units.

The inclusion criteria were at least one of the following conditions: 1) patients who have been hospitalized for more than 5 days; 2) patients who have received long-term antibiotic vancomycin; 3) patients who have undergone invasive procedures such as catheterization and shunt; and 4) patients with a history of surgery in the chest or abdomen.<sup>14</sup> The patients who did not sign the written consent forms or were in a coma were excluded from the study.

## Swab specimen collection

In this study, 243 non-duplicate specimens (one per patient) were collected. A sterile swab with a size of 3–5 cm was entered into the patient's rectum and stirred ~360°; then, the rectal swab was put into a tube containing bile esculin broth (Merck Co, Germany) as a transport medium. The swabs were immediately transferred to the microbiology laboratory of School of Medicine, AJUMS.

The specimens were inoculated on culture media such as blood agar, brain heart infusion (BHI) agar, and bile esculin agar/broth (Merck & Co., Inc.). The plates were incubated at 37°C for 18–24 hours, and the isolated colonies were examined by standard biochemical tests.

## Standard biochemical tests

After gram staining, all the isolates were identified by standard biochemical tests such as catalase, 6.5% NaCl, pyrrolidonyl arylamidase (PYR) test, carbohydrates fermentation, motility, pigment production, and vancomycin screening test.<sup>15</sup> The gram-positive diplococci, which were negative in catalase and motility tests and positive in esculin hydrolysis and PYR tests, were considered as *Enterococcus* isolates. Also, these isolates were identified as *E. faecalis* and *E. faecium* and other enterococci species based on pigment production on blood agar medium and carbohydrates fermentation such as arabinose, mannitol, sorbitol, sorbose, and lactose.

## Antibiotic susceptibility test

The susceptibility and resistance patterns of *E. faecalis* and *E. faecium* species to 10 antibiotics were evaluated on Muller Hinton agar (Merck) by the standard Kirby-Bauer disk diffusion method based on the Clinical and Laboratory Standards Institute (CLSI) guidelines.<sup>16</sup> In this test, each isolate concentration was equated to 0.5 McFarland, as a standard concentration and the antimicrobial disks (Rosco Diagnostica, Taastrup, Denmark) included: vancomycin (30 µg), teicoplanin (30 µg), ampicillin (10 µg), ciprofloxacin (5 µg), erythromycin (15 µg), tetracycline (30 µg), chloramphenicol (30 µg), fosfomycin (200 µg), linezolid (30 µg), and nitrofurantoin (300 µg).

## Vancomycin screening test

This test was performed in order to evaluate phenotypical vancomycin-resistant isolates using BHI agar (Merck) containing 6 µg/mL vancomycin (Sigma-Aldrich Co., St Louis, MO, USA) based on CLSI directions.<sup>16</sup>

## The multiplex PCR method

DNA extraction from *Enterococcus* isolates was carried out by the boiling method,<sup>17</sup> and DNA concentration was measured by biophotometer (Eppendorf, Hamburg, Germany). *VanA* and *vanB* genes were amplified using the following primers in a thermocycler (Eppendorf):

A1 5'-GGGAAAACGACAATTGC-3' and A2 5-GTACAATGCGGCCGTTA-3' with a product size of 732 bp for *vanA*; B1 5'-ATGGGAAGCCGATAGTC-3' and B2

5'-GATTCGTTCCCTCGA CC-3' with a product size of 635 bp for *vanB*.<sup>18</sup>

The PCR conditions consisted of a pre-denaturation step at 94°C for 5 minutes, followed by 30 cycles of 45 seconds at 94°C, 45 seconds at 59°C, and 45 seconds at 72°C. A final extension step was performed at 72°C for 5 minutes.

## Electrophoresis of PCR products

The PCR products were electrophoresed on 1.5% agarose. The voltage used for electrophoresis was 100 volts for 60 minutes.<sup>3</sup> The Gel Documentation System (ProteinSimple, San Jose, CA, USA) was used for viewing and imaging the PCR products on the gel. *E. faecium* ATCC 51559 and *E. faecalis* ATCC 51299 were used as positive controls for *vanA* and *vanB* genes, respectively. Also, *E. faecalis* ATCC 29212 was used as the negative control for lacking the vancomycin resistance genes.<sup>3,13</sup>

## Statistical analysis

To analyze the data, chi-squared test was run in SPSS version 16 (SPSS Inc., Chicago, IL, USA). *P*-value <0.05 was considered statistically significant.

## Ethical considerations

This study was confirmed by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (No. 1392.173).

## Results

In this study, 243 non-duplicate specimens collected from the hospitalized patients were examined. The culture results of 23 rectal swab specimens in esculin broth/agar were negative for *Enterococcus* growth. Although culture of specimens in esculin medium was positive for 220 rectal swab specimens, the standard biochemical tests confirmed *Enterococcus* growth only in 175 cases, 95 (54.3%) of which belonged to *E. faecium* (Table 1). The cases that were not confirmed as *Enterococcus* were omitted from the study.

Out of the 243 examined patients, 166 (68%) cases were male and 77 (32%) cases were female.

Antimicrobial susceptibility test was performed by the disk diffusion agar method according to the CLSI guidelines for *E. faecium* (95 isolates) and *E. faecalis* (34 isolates). Based on the results of antibiogram test, the most resistant

**Table 1** Frequency of 175 different species of *Enterococcus* isolates from hospitalized patients

Total number of species	<i>Enterococcus faecium</i>		<i>Enterococcus faecalis</i>		Other <i>Enterococcus</i> spp.	
	N	%	N	%	N	%
175	95	54.3	34	19.4	46	26.3

isolates to ciprofloxacin (75.8%) and erythromycin (76.8%) were *E. faecium* and the most resistant isolate to erythromycin and tetracycline was *E. faecalis* (52.9% and 73.5%, respectively, Table 2).

Vancomycin screening test was performed for all *Enterococcus* isolates that were resistant or semi-susceptible to vancomycin in the disk diffusion agar method. These results showed that out of 59 isolates that were inoculated on BHI agar containing 6 µg/mL vancomycin, 53 isolates grew, which indicated they were fully resistant to vancomycin. We also

**Table 2** The susceptibility test results of 129 enterococci isolates including 59 *Enterococcus faecium* and 34 *Enterococcus faecalis* isolates

Antibiotics	Resistant		Intermediate		Susceptible	
	N	%	N	%	N	%
Vancomycin	56	43.4	3	2.4	70	54.3
Teicoplanin	56	43.4	0	0	73	56.6
Ampicillin	68	52.7	0	0	61	47.3
Ciprofloxacin	76	58.9	23	17.8	30	23.3
Erythromycin	91	70.5	26	20.2	12	9.3
Tetracycline	94	72.8	2	1.6	33	25.6
Chloramphenicol	36	27.9	18	14.0	75	58.1
Fosfomycin	2	1.5	3	2.3	124	96.2
Linezolid	1	0.7	4	3.1	124	96.2
Nitrofurantoin	2	1.5	6	4.7	121	93.8

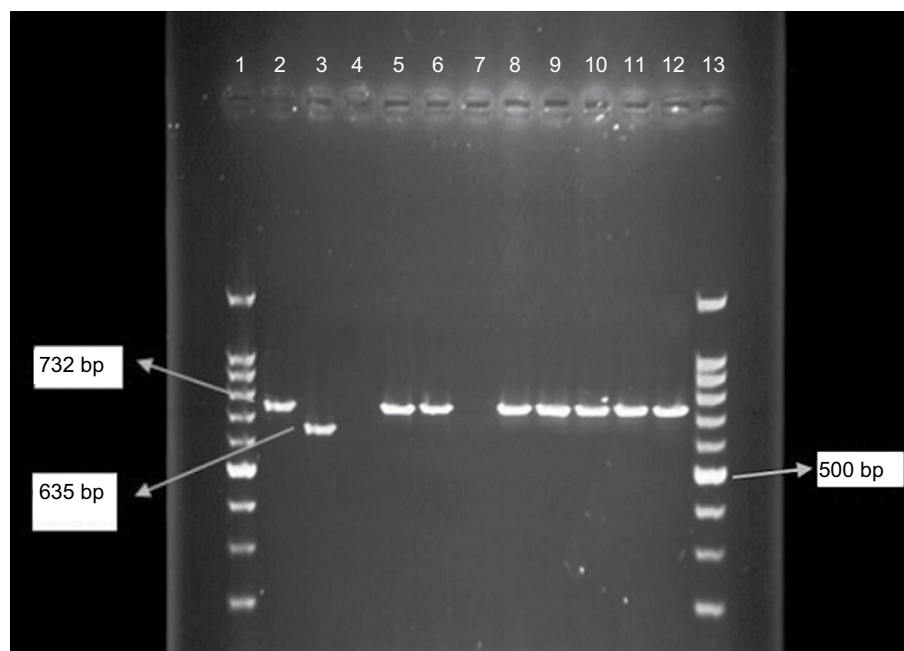
found that out of 59 resistant or semi-susceptible isolates to vancomycin, 56 and three isolates were identified as *E. faecium* and *E. faecalis*, respectively.

The *vanA* and *vanB* genes were amplified in all the resistant or semi-susceptible isolates to vancomycin and teicoplanin by the multiplex PCR method. The results showed that out of 59 isolates, 54 (91.5%) contained *vanA* gene, and 5 (8.5%) isolates were without *vanA* and *vanB* genes (Figure 1). Finally, among 129 patients who were positive for *E. faecium* and *E. faecalis* based on the presence of *vanA* gene in 54 isolates, VRE prevalence was 41.8%. Also, out of 56 *E. faecium* isolates, 54 contained *vanA* gene; however, two *E. faecium* isolates and three *E. faecalis* isolates did not have *vanA* gene ( $P<0.05$ ).

## Discussion

Enterococci are opportunist pathogens that could cause nosocomial and community-acquired infections. Among different species, *E. faecalis* and *E. faecium* are the most common ones that constitute about 85%–90% of all clinical isolates.<sup>19,20</sup> Based on a recent study, 14.3% of patients admitted to ICUs were affected by enterococcal infections.<sup>4</sup>

In the present study, out of 343 rectal swab specimens, 175 *Enterococcus* isolates including 95, 34, and 46 isolates of *E. faecium*, *E. faecalis*, and *Enterococcus* spp. were detected, respectively.



**Figure 1** The results of electrophoresis of multiplex PCR productions of *vanA* and *vanB* genes.

**Notes:** Lanes 1 and 13: The size marker (DNA ladder) (100–1000 bp); Lane 2: positive control of *vanA* gene with the size of 732 bp (*Enterococcus faecium* ATCC 51559); Lane 3: positive control of *vanB* gene with size of 635 bp (*Enterococcus faecalis* ATCC 51299); Lane 4: negative control of the genes *vanA* and *vanB* (*E. faecalis* ATCC 29212); Lanes 5 and 6, and 8–12: isolates containing *vanA* gene; and Lane 7: one isolate lacking *vanA* and *vanB* genes.

In our study, the prevalence rate of *E. faecium* (54.3%) was more than *E. faecalis* (19.4%), which is consistent with the study of Sharifi et al in northwest of Iran,<sup>21</sup> but it was different from the results of Emaneini et al in Tehran, the capital of Iran and Kafil et al in Tabriz, Iran.<sup>22,23</sup> They showed that the prevalence rate of *E. faecalis* (64.4% and 57%) was more than *E. faecium* isolates (35.6% and 43%). The reason for these differences in findings could be related to the types of specimens, for example, rectal swabs were used in our study, but other sorts of specimens such as urine, blood, or wound were used in their studies. Since colonization of enterococci in the digestive system is the most common source of spread of VRE,<sup>24</sup> we used rectal swabs for sampling in this study. However, the results of some studies in South Korea<sup>25</sup> and Switzerland<sup>11</sup> were in line with our findings regarding the higher frequency of *E. faecium* compared to *E. faecalis*.

Some studies in Iran<sup>22</sup> and China<sup>26</sup> have indicated that multidrug-resistant enterococci, especially vancomycin-resistant *E. faecium* isolates, have increased in recent years, which was confirmed in our study. However, among enterococci isolated from different kinds of clinical specimens such as urine, blood, and wound, the prevalence of *E. faecalis* still surpasses that of *E. faecium*.<sup>27</sup>

According to the antibiogram test, all *E. faecalis* isolates were susceptible to teicoplanin, ampicillin, fosfomicin, linezolid, and nitrofurantoin. A reason for high susceptibility to these antibiotics is probably less prescription of them by physicians. Our results were in agreement with those of Kafil et al in Iran who showed that VRE isolates were susceptible to linezolid, nitrofurantoin, and tigecyclin.<sup>23</sup>

Our study also revealed high resistance to tetracycline and erythromycin (Table 2), but the resistance rate to these antibiotics for *E. faecium* was more than that of *E. faecium* isolates. Also, out of 129 *Enterococcus* isolates, one isolate was linezolid-resistant and two isolates were resistant to fosfomicin and nitrofurantoin.

The results of our study indicated an increase in vancomycin-resistant *E. faecium* isolates compared to some studies in Iran, such as the study of Emaneini et al in Tehran.<sup>22</sup> The reason for this finding could be related to the studied cases. We studied patients hospitalized in ICUs for more than 5 days, and in some cases, they had received vancomycin for a long time, thus they were at high risk for infection with VRE. Some previous studies in USA<sup>28</sup> and Iran<sup>29</sup> also showed that the rates of vancomycin-resistant *E. faecium* isolates were 80% and 100%, respectively. In addition, a recent study in Iran indicated that *E. faecium* strains were varied among environmental and clinical VRE isolates.<sup>30</sup>

The phenotypic vancomycin screening test was performed for all enterococci isolates resistant or semi-susceptible to vancomycin in disk-diffusion agar test. Since according to the CLSI criteria, susceptibility of screening test using the BHI medium containing 6 µL/mL vancomycin is more than disk-diffusion agar test, the results of the screening test confirmed that 53 *E. faecium* isolates were resistant to vancomycin and three semi-susceptible isolates of *E. faecalis* were identified to be susceptible to vancomycin. However, three *E. faecium* isolates showed resistance in disk-diffusion agar test, but did not grow in screening test followed by PCR method.

The multiplex PCR assay showed that 54 (91.5%) of *Enterococcus* isolates contained *vanA* gene, and none of the isolates contained *vanB* gene. However, the prevalence of *vanA* gene among *E. faecium* and *E. faecalis* strains was significantly different ( $P < 0.05$ ). The phenotypical resistance of five *E. faecium* and *E. faecalis* isolates which had no *vanA* and *vanB* genes could be related to different reasons other than genetic agents.

Talebi et al in Iran also showed the presence of *vanA* gene and the absence of *vanB* among vancomycin-resistant *Enterococcus* isolates.<sup>8</sup> Similarly, the results of a study in northwest of Iran (2012) showed that 43 VRE strains contained *vanA* gene, three strains contained *vanB* gene, and two strains did not have any of the two genes.<sup>21</sup> These results were relatively consistent with the present findings, but the slight differences can partially be attributed to the type of specimens and the study period.

However, the findings of Karki et al, in Melbourne, Australia, were not consistent with our results.<sup>31</sup> Among 331 rectal specimens, they found 58 (17.5%) VRE containing *vanB* gene, but none of them had *vanA*. The reason expressed in their study was that *vanB* gene in VRE has been endemic in the region. Since unlike *vanA* gene, *vanB* is clustered and occupies a large area on the chromosome, the possibility of its transmission among strains is less. Furthermore, *vanB* gene is mainly associated with epidemics and food contamination, while *vanA* gene is associated with clinical strains,<sup>10</sup> and the fact that patients received vancomycin for a long time justifies the existence of *vanA* gene in VRE.

## Conclusion

Generally, the prevalence of VRE in our hospitals and studied area is increasing. Considering the importance of vancomycin as a bactericidal antibiotic in the treatment of bacterial infections, the outbreak of VRE may create many problems for patients' health. Through the detection of resistant strains and proper treatment of infected patients, resistance development to vancomycin can be prevented.

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## Disclosure

The authors report no conflicts of interest in this work.

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