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**RESEARCH ARTICLE**

# Investigation of the Prevalence of vanA and vanB genes in vancomycin resistant enterococcus (VRE) by Taq Man real time PCR Assay

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**ABSTRACT**

**Objective:** Enterococci are important nosocomial agents and due to their potential antimicrobial resistance they have a significant role in the dissemination of resistance genes. Currently, these species are described as healthcare concern. The aim of this study was to determine *vanA* and *vanB* genes in vancomycin resistant enterococci (VRE) strains isolated from the various clinical samples in the hospitals in Iran.

**Methods:** Susceptibility of 235 strains to vancomycin was screened as minimum inhibitory concentration (MIC) by E-test. The genes encoding modifying vancomycin precursor's dipeptide termini named as *vanA* and *vanB* genes were targeted by Taq Man real time PCR assay in vancomycin resistant and vancomycin intermediate resistant *Enterococcus faecalis* and *Enterococcus faecium* strains.

**Results:** A total of 235 enterococci were isolated from the clinical specimens. One hundred and ninety three (82.1%) of them were defined as *E. faecalis*, 33 (14.0%) *E. faecium*, 1/235 (0.4%) *E. avium*, 1/235 (0.4%) *E. raffinosus* and 7/235 (3.0%) *E. galinarium*. The prevalence of vancomycin resistance was 13.6% (32/235) consisting of 18/235 (7.7%) *E. faecalis* and 6.0% (14/235) *E. faecium*. Among the 32 VRE strains, a total of 36 *vanA* and *vanB* genes were detected (some isolates had both *vanA* and *vanB* genes). These resistance genes were not detected in 5 out of 32 (15.6%) isolates.

**Conclusion:** *E. faecalis* was more common in clinical samples and *vanA* (58.3%) gene was the predominant gene among the VRE isolates. The current study showed that Taq Man real time PCR assay is the useful, precise and rapid detection of vancomycin resistance genes. *J Microbiol Infect Dis 2013;3(4): 192-198*

**Key words:** VRE, Taq Man real time PCR, *vanA*, *vanB*, *Enterococcus faecalis*, *Enterococcus faecium*

## Vankomisin dirençli enterokok'larda (VRE) *vanA* ve *vanB* gen prevalansının Taq Man real time PCR assay ile araştırılması

**ÖZET**

**Amaç:** Enterokoklar önemli hastane enfeksiyonu etkenidirler ve antimikrobiyal direnç potansiyelleri nedeniyle rezistans genlerinin yayılmasında önemli rolleri bulunmaktadır. Günümüzde bu mikroorganizmalar önemli bir halk sağlığı sorunu olarak tanımlanmıştır. Bu çalışmada İran'daki bazı hastanelerde değişik klinik örneklerden izole edilen vankomisin dirençli enterokok (VRE)'lar arasında *vanA* ve *vanB* genlerinin sıklığının araştırılması amaçlandı.

**Yöntemler:** Suşların vankomisin duyarlılıkları E test yöntemiyle araştırılarak minimum inhibitör konsantrasyon (MİK) değerleri belirlendi. Vankomisin dirençli ve vankomisine orta düzeyde dirençli *Enterococcus faecalis* ve *Enterococcus faecium* suşlarında Taq Man real time PCR assay ile vankomisin direncini kodlayan *vanA* ve *vanB* genleri hedeflendi.

**Bulgular:** Çalışma süresince klinik örneklerden toplam 235 enterokok suşu izole edildi. Bu şuşlardan 193'ü (% 82,1) *E. faecalis*, 33'ü (% 14,0) *E. faecium*, 1'i (% 0,4) *Enterococcus avium*, 1'i (% 0,4) *Enterococcus raffinosus* ve 7'si (% 3,0) *Enterococcus galinarium* olarak tanımlandı. Toplam 235 enterokok suşunda 32'si (% 13,6) VRE idi. Toplam 235 suştan 18 (% 7,7)'i *E. faecalis* ve 14'ü (% 6,0) *E. faecium* idi. Bu suşların 27 (% 84,4)'sinde *vanA* ve *vanB* genleri belirlendi (bazı izolatlar hem *vanA* ve hem *vanB* genlerine sahip idi). Suşların 5'inde (% 15,6) direnç geni belirlenemedi.

**Sonuçlar:** Araştırmamızda klinik VRE suşları arasında *E. faecalis* suşlarının sıklıkta olduğu ve *vanA* geninin vankomisin direncine sebep olan en sık gen olduğu gözlendi. Bu çalışma Taq Man real time PCR assay testinin vankomisin direnç genlerinin gösterilmesinde kullanılabilen, kesin ve hızlı bir test olduğunu göstermektedir.

**Anahtar kelimeler:** VRE, Taq Man real time PCR, *vanA*, *vanB*, *Enterococcus faecalis*, *Enterococcus faecium*

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## INTRODUCTION

Enterococci are Gram-positive and non-motile bacteria. They have some biochemical characteristics; oxidase and catalase negativity, 6.5% NaCl tolerance, and hydrolysis of bile in bile esculin agar.[1] Due to various different of antibiotic resistance genes, they are intrinsically resistant against aminoglycosides, macrolides, β-lactams and semisynthetic penicillins.[2] It has been shown in many studies that there was a trend in increasing of glycopeptides resistance in enterococci and vancomycin resistant enterococci (VREs) have gained much attention in the last decade.[3] Researchers have described mobile genetic elements (transposons and plasmids) as resistance genes transferring between enterococci.[4-6] One of the common resistance mechanisms to glycopeptides is vancomycin, dipeptide like termini (D-Ala- D-Lac) encoded by *vanA* and *vanB* clusters which, prompt to low affinity for glycopeptides.[3] More than 30 different species of enterococci have been identified but in Enterococcus faecalis and *Enterococcus faecium* are mostly isolated bacteria among them.[5,7]

After the first detection of vancomycin resistant enterococci (VREs) in 1980, they have emerged as important nosocomial pathogens in worldwide (4 and 8). Studies have shown that VRE were disseminated through the world. Currently, VREs is described as main concern in healthcare.[4,9] Findings concerning the prevalence of vancomycin resistant genes are necessary for controlling and proper treatment of enterococcal infections.[2] Many studies have mentioned that the resistance to vancomycin is complex process and needs to presence of multiple genes. Seven gene clusters were investigated in VRE including *vanA*, *vanB*, *vanC1*, *vanC*2, *vanD*, *vanE*, and *vanG*. Genes (*vanA* and *vanB*) encoding dipeptide like termini are responsible for high or moderate level vancomycin resistance.[3,7,9] The isolates having *vanA* are usually high resistant to vancomycin and teicoplanin due to *vanA* gene cluster. This resistance is mediated by *vanA* gene cluster by D-Ala-D-Lac encoded dipeptide like termini. *VanB* gene cluster produce D-Ala - D-Lac dipeptide like termini, and this protein provide moderate to severe vancomycin resistance but not to teicoplanin.[7]

Many studies have been conducted in different countries for the detection of *vanG*enotypes, prevalence and risk factors for VRE infections. For this reason, this study mainly was designed for the determination of VRE in clinical samples based on *vanA* and *vanB* genes. Rapid detection of VRE strains having *vanA* and *vanB* genes by Taq Man real time PCR assay is the main aim of this study.

## METHODS

A total of 235 enterococci were collected from different clinical samples of patients with suspected of clinical infection including urine, blood, sputum, CSF, pleura, fluid, and wound from June to November 2011 in 6 hospitals in Qazvin and Tehran, Iran. All of the enterococcal isolates were identified based on the following microbial tests consisting of growth in Azid Maltose Agar Medium, Gram stain, oxidase and catalase reaction, growth on bile esculin agar and 6.5% NaCl media as described elsewhere.[10] Differentiation test for enterococci was done by the scheme utilized in previously published for identity of enterococci including fermentation of carbohydrates (sorbitol, arabinose, raffinose, mannitole and sucrose), motility and pigmentation.[10,11]

### Susceptibility of microorganisms

Susceptibility test for 235 enterococci was performed by E-test according to the Clinical Laboratory Standard Institute (CLSI) guideline.[12] Suspensions (1.5 x $10^8$ cfu/ml) were prepared from the isolates and adjusted with 0.5 McFarland standard (measured by spectrophotometric analysis Gensyse 10 UV spectrum, USA) as for described in the CLSI guideline.[12] All isolates tested by vancomycin E-test strips (AB BIODISK, Solna, Sweden) on the Mueller-Hinton agar medium (Merck, Germany). Briefly 0.2 ml of bacterial suspension in normal saline was spreaded onto Muller-Hinton agar medium and the E-test strips were applied to the inoculated culture plates separately by using a template, as recommended by the manufacturer, and the plates were incubated at 35°C for 24 hours. The MICs breakpoints for vancomycin were determined by manufacturer's recommendation and the MICs were interpreted at the point of intersection between the inhibition zone and the E-test strip. A standart *E. faecalis* ATTC 29212 strain was used as a control.

### DNA Extraction

After performed E-test the isolates with 4<MIC<32 and MIC≥32 breakpoints were screened. Two pure colonies were emulsified in 10 ml of sterile Loria Bertani broth (Liofilchen, Italy) and incubated over night in shaken incubator (35°C for 24 hours) in ambient air to reach the turbidity equivalent to 0.5 McFarland standards. Cells harvested by centrifuge 8000 rpm for 5 minutes. The pellets consisting of bacterial cells were used for DNA extraction process. Following boil and freezing procedure (1 min 96°C and -20°C for 20 min, in this study) total DNA extraction was carried out by high pure DNA extraction kits (Roche, Mannheim, Germany). Dried

DNA was dissolved in 50 µl of distilled water. Consequently, quantity of DNA was measured by Nanodrope (Spectrophotometer 1000) and adjusted to 500 ng/µL.

### DNA confirmation test

Dried DNA was measured by PCR by utilizing rrs gene (16srRNA). Primer previously was utilized. Sequences of forward and reverse primer for 320 bp PCR product were 5′-GGATTAGATACCCTGGTAGTCC-3′ and 5′-TCGTTGCGCACCTTAACCAAC-3′ (4). The Amplification procedure was made by 20 µl as final volume containing 10 µl master amplicon, Primer Forward 2.5 µl, Primer Reverse 2.5 µl, and Template 5 µl. PCR process was optimized with *E. faecalis* V583 & *E. faecium* BM4147 as positive genotypes. Standardization of PCR performed by following condition: Denaturation 95°C for 30 sec (1 cycle), annealing 54°C for 1 min in 30 cycles. PCR products (320 bp) were electrophoresed and stained by gel red in 2% agarose gel (Max Pure Sgarose, Spain). Band of the amplicon was observed by Gel logic 212pro.

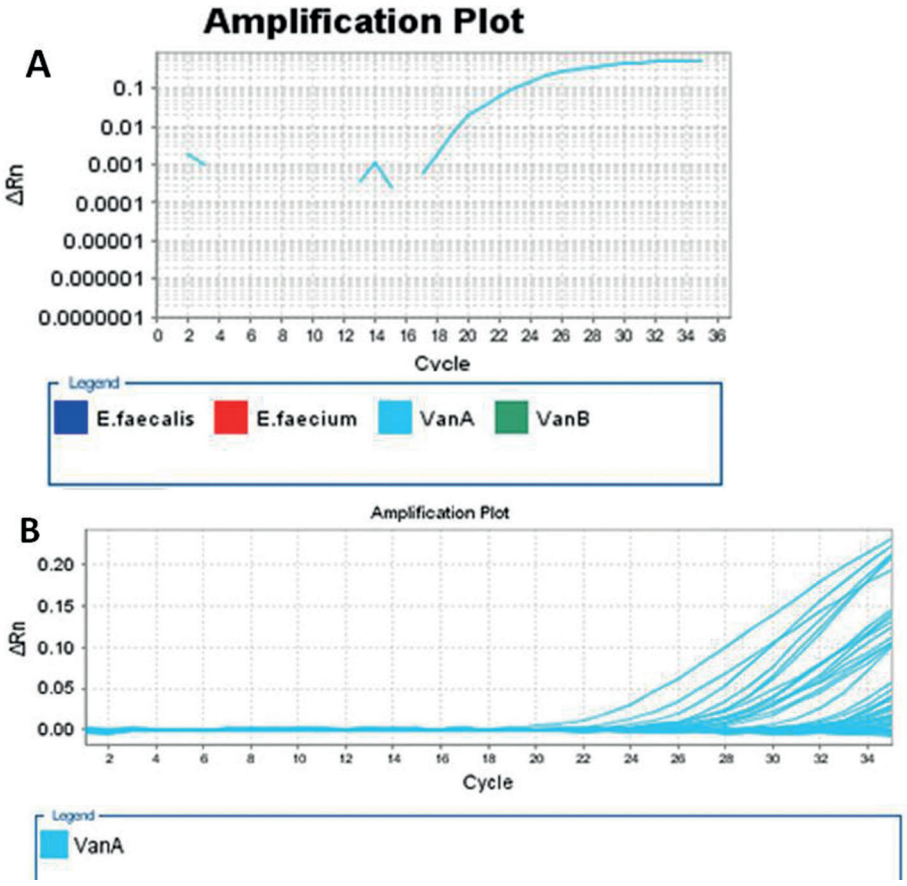


**Figure 1.** Amplification plots for standardization of *vanA* gene positive control BM4147 **(A)**, and for *vanA* positive in a clinical sample **(B)**

### Taq Man Real time PCR assays

For rapid detection of high and moderate vancomycin resistant enterococci (*vanA* and *vanB* genes in this study), vancomycin resistance determinants of *vanA* and *vanB* were targeted by real Time PCR. Detection of these genes was performed by an instrument (ABI7500, USA).

### Designation of primers and probes

Reference *vanA* and *vanB* sequences representing each of *vanA* and *vanB* (*vanB*1/*vanB*2/*vanB*3) were assembled from the GenBank data base (http://www.ncbi.nlm.gov/GenBank). Reference sequences with Accession number (M97297) and accession number (U00456.1) were used for *vanA* and *vanB* genes respectively. These sequences were aligned by using computer software and primer and probes were designed by beacon designer version 7 software. Two primers and taq man probes were designed specifically to detect genes encoding D-Ala-D-Lac dipeptide like termini. Probe reporters dyes (at 5′ position were either JOE or HEX) and quencher were (BHQ1 and Tamra). Sequences of primers and probes were described as *vanA* forward and reveres primer including 5′-ATCAACCATGTTGATGTAGC-3′ and 5′-AAGGGATACCGGACAATTCA-3′ for 136 bp amplicon. Sequence of *vanA* probe was

5′-TCCATCTTCACCTGACTTGCCA-3′. Sequence of designed forward reverse primer and probe for *vanB* gene including 5′-ACCCTGTCTTTGTGAAG-3′, 5′-GAAATCGCTTGCTCAAT-3′ for amplification of 121bp product and 5′-TCCATCATATTGTCCTGCTGCTTCTAT-3′. Taq Man real Time PCR optimized by reference strains as positive controls (isolates producing *vanA* BM4147 and *vanB* V583 as positive controls were obtained from microbial collection of the Pasteur Institute of Iran. Amplification was performed by final volume 23 µl consisting (10 µl ABI master mix, 2.5 pmol of each primer, 1.5 pmol of each probe and 5 µl template. Real time PCR cycle conditions were denaturation at 95°C for 15 sec, annealing at 52°C for 15 sec and extension at 72°C for 1 min (35 cycles). Amplification plot of positive controls and clinical isolates are showed in Figure 1 and 2.

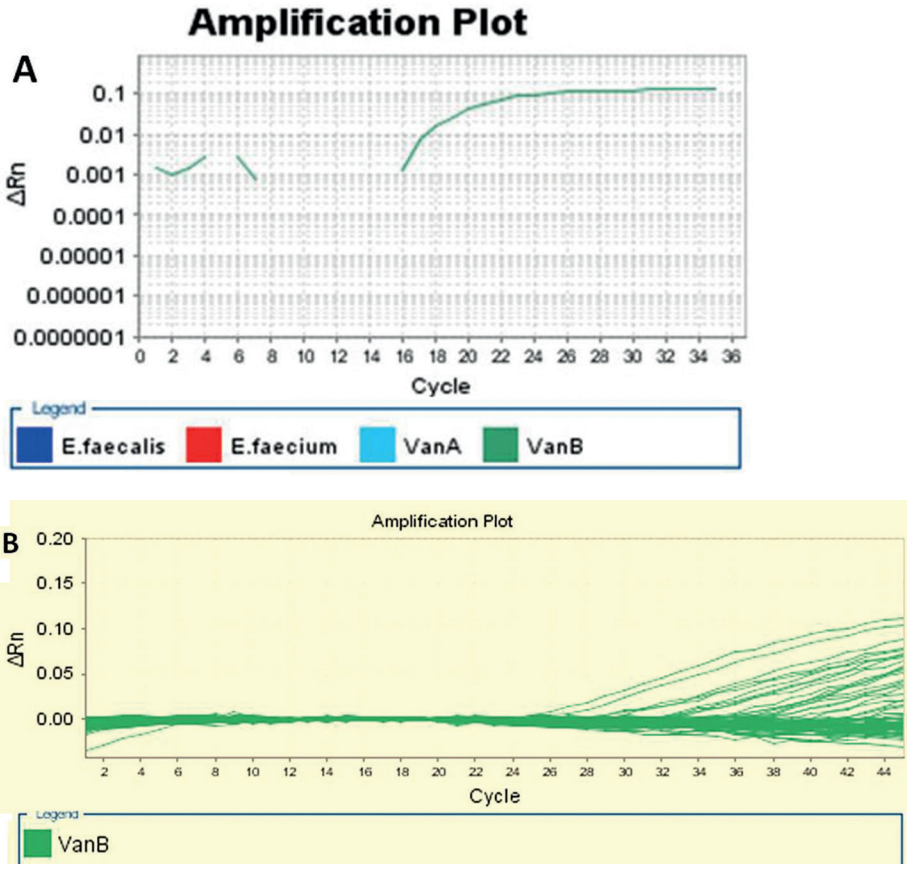


**Figure 2.** Amplification plots for standardization of *vanB* gene positive control BM4147 **(A)**, and for *vanB* positive in a clinical sample **(B)**

## RESULTS

We identified 193 strains (82.1%) as *Enterococcus faecalis*, 33 strains (14.0%) as *E. faecium*, 1 strain (0.4%) as *E. avium*, 1 strain (0.4%) as *E. raffinosus* and 7 strains (3.0%) as *E. galinarium* respectively. The isolates were obtained from CSF (5 strains), blood (21 strains), urine (165 strains), and other clinical samples (44 strains) respectively. Determined minimum inhibitory concentration (MIC) breakpoints in this study are listed in Table 1. Based on the E-test results, 32 (13.6%) out of 235 isolates were accepted as VRE. Fourteen (43.8%) of them were *E. faecalis* and 18 (56.2%) of them were *E. faecium*. Additionally, 28 (11.9%) enterococci were interpreted as intermediate resistant to vancomycin (Table 1).

**Table 1.** Results of vancomycin susceptibility test to the species level for the clinical isolates of enterococci by E-test (n=235)

| Vancomycin MIC (mcg/ml) | Susceptible (≤4) | Intermediate (8-16) | Resistant (≥32) |
|---|---|---|---|
| *E. faecalis* (n=193) | 153 (79.3) | 22 (11.4) | 18 (9.3) |
| *E. faecium* (n=33) | 18 (54.6) | 1 (3) | 14 (42.4) |
| *E. galinarium* (n=7) | 2 (28.6) | 5 (71.4) | |
| *E. raffinosus* (n=1) | 1 (100) | | |
| *E. avium* (n=1) | 1 (100) | | |

*Data presented as n (%).

Among the 32 VRE isolates, *vanA* and *vanB* genes were observed in 27 (84.4%). The only *vanA* gene was detected in 5 (27.8%), *vanB* gene was detected in 5 (27.8%) of 18 *E. faecalis* and the only *vanA* gene was detected in 7 (50%), *vanB* gene was detected in 1 (7.1%) of 14 *E. faecium* by Taq Man real time PCR (Table 2). Both *vanA* and *vanB* genes was detected in 6 (33.3%) of 18 *E. faecalis* and 3 (21.4%) of 14 *E. faecium* strains. We detected neither *vanA*, nor *vanB* gene in 2 of *E. faecalis* and 3 of *E. faecium* strains. The Taq man real time PCR assay disclosed as *vanA* is the predominant gene among the VRE strains (21 vs. 15). The *vanA* or *vanB* genes were not detected among the intermediate vancomycin resistant enterococci during the study (Table 2). The distribution of MIC breakpoint values determined by E-test for both species of *E. faecalis* and *E. faecium* to *vanA* and *vanB* genes are summarized in Table 3.

**Table 2.** Distribution of *vanA* and *vanB* genes to vancomycin susceptibility test results of enterococci (n=226)

| Vancomycin MIC (mcg/ml) | Susceptible (≤4) | Intermediate (8-16) | Resistant (≥32) |
|---|---|---|---|
| *Enterococcus faecalis* (n=193) | 153 (79.3) | 22 (11.4) | 18 (9.3) |
| *vanA* | 0 | 0 | 5 (27.8) |
| *vanB* | 0 | 0 | 5 (27.8) |
| *vanA* and *vanB* | 0 | 0 | 6 (33.3) |
| *Enterococcus faecium* (n=33) | 18 (54.6) | 1 (3) | 14 (42.4) |
| *vanA* | 0 | 0 | 7 (50) |
| *vanB* | 0 | 0 | 1 (7.1) |
| *vanA* and *vanB* | 0 | 0 | 3 (21.4) |

Data presented as n (%), MIC, Minimum inhibitory concentration.

**Table 3.** Distribution of minimum inhibitory concentration (MIC) breakpoint values to *vanA* and *vanB* genes by the E-test.

| Microorganism | MIC (µg/ml) | No. | Total, n (%) |
|---|---|---|---|
| *Enterococcus* faecalis (n=16) | | | |
| *vanA* | >256 | 1 | 5 (27.8) |
| | 256 | 1 | |
| | 128 | 3 | |
| *vanB* | 64 | 2 | 5 (27.8) |
| | 32 | 3 | |
| *vanA* and *vanB* | 256 | 1 | 6 (33.3) |
| | 196 | 1 | |
| | 64 | 4 | |
| *Enterococcus faecium* (n=11) | | | |
| *vanA* | >256 | 1 | 7 (50) |
| | 256 | 4 | |
| | 196 | 2 | |
| *vanB* | 96 | 1 | 1 (7.1) |
| *vanA* and *vanB* | 256 | 2 | 3 (21.4) |
| | 196 | 1 | |

## DISCUSSION

Enterococci as 1% of intestinal microflora in humans have been observed as more common nosocomial infection in worldwide. Among them *E. faecalis* and *E. faecium* are the most common isolated microorganisms from the human stool. In current study, our results for the frequency of enterococci in clinical samples in hospitals have similarity to other studies in this subject.[8,13,14] After identification of first VRE strain in early 1980, colonization and infectious diseases caused by these bacteria have been enhanced. Currently, these bacteria are important cause of nosocomial infections (NIs) and due to multidrug resistance features and treatment failures, the mortality rate in hospitalized patients are increased.[15,16] The results of the entire study show that the dissemination of VRE in hospitals in Qazvin and Tehran, Iran. The phenotypic methods such as E-test are the convenient and useful methods for determining of MIC values for vancomycin in enterococci. Our result are in accordance with the other studies which conducted by Sharifi et al. in Iran, but, in contrast to the results of the study which performed by Pérez-Hernández et al.[13,17]

Horizontal gene transfer has an important role for the dissemination of resistance genes in enterococci and transposon (Tn1546) also has an important role for the dissemination of vancomycin resistance genes. Although primarily *vanA* cluster (Tn1546 and Tn5482) was identified in *E. faecium*, it has also been detected in *E. faecalis*. The *vanB* gene cluster exists in Tn1546 and it has been identified in *E. faecalis* and *E. faecium* strains.[18] The real time PCR assay is rapid approach for the detection of vancomycin resistance genes, more specific than traditional phenotypic methods for identification of high level and intermediated vancomycin resistance. Several real time PCR protocols have been developed for the detection of resistance genes in

microorganisms.[7] The primer and probes used this study were designed by the Beacon Designer 7 software and concentration of primer and probes, annealing, temperature, amplification cycle with pure and defined concentration of template DNA were carefully adjusted in order to optimize to Taq Man real time PCR assay. Our optimized Taq Man real time PCR assay had 96% similarity of phenotypic methods including E-test for the description of VRE. Precise and rapid detection of vancomycin resistance genes enhances of the isolation accuracy of VRE from various clinical samples. Therefore molecular approaches such as Taq Man real time PCR assay are useful methods and then they can help to the clinicians for decision of appropriate antibiotics to the patients suffering from the infections caused by resistant microorganisms including VRE.

In current study 32 (13.6%) of the 235 clinical isolates were defined as vancomycin resistant by E-test. In addition, 27 (84.4%) out of 32 VRE strains had *vanA* or *vanB* or both and among them, 5 VRE isolates were negative for both *vanA* or *vanB* genes by Taq Man real time PCR assay (Table 1 and Table 2). The possible mechanism for emerging of vancomycin resistance among enterococci in the region probably occurred by high mutation frequency in ligase enzyme joining the two dipeptide termini such as D-Alanyl-D-Alanin.[19] Among the 32 VRE isolates, both *vanA* and *vanB* gene were determined in 3 strains of *E. faecium* and in 6 strains of *E. faecalis* respectively. The reason of this phenomena is unknown but instability in enterococci genome and transduction process or up and down regulation of genes probably can be considerable.[19] In this study, five VRE isolates had a MIC value of ≥32 mcg/ml by E-test but, neither *vanA* nor *vanB* genes were detected by real time PCR technique suggesting the presence of other *vanG*enes or other mechanisms responsible for the vancomycin resistance.[23]

In conclusion, due to the higher existence of vancomycin resistance related genes in VRE is necessitated to prevent of their dissemination in the hospital. We think the microbiology laboratories in the hospitals must be well equipped with the new and modern techniques for rapid detection of vancomycin resistance. The current study showed that Taq Man real time PCR assay is the useful, precise and rapid detection of vancomycin resistance genes in the clinical microbiology laboratory.

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