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Short Communication

# Detection of both *vanA* & *vanB* genes in *vanA* phenotypes of *Enterococci* by Taq Man RT-PCR

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## Abstract

Twenty seven isolates of vancomycin resistant *Enterococci* based on the disk diffusion and E- test have been screened; being found eight (0.3%) clinical isolates of *vanA* & *vanB* through Taq Man Real Time PCR assay. This study shows the presence of both *vanA* & *vanB* genotypes in *vanA* phenotypes clinical isolates in the three hospitals in Iran.

**Key words:** *vanA* & *vanB* genotypes, vancomycin, Taq Man Real Time PCR, *Enterococci*.

*Enterococci* as an opportunistic microbiota are one of the most important microorganisms found in the environment. In some condition they can become pathogen, causing urinary tract infection (UTI), skin infections, respiratory infections, endocarditis and sepsis (Emaneini *et al.*, 2008). This genus of bacteria due to various different of antibiotic resistance genes, intrinsically has shown resistance against some antibiotics including aminoglycosides, macrolids, β-lactams and semisyntetic penicillin's (Kacmaz *et al.*, 2009). Following the first isolated vancomycin resistant *enterococci* (VREs) in 1980; these microorganisms have emerged as important nosocomial pathogens in worldwide (Japoni *et al.*, 2009, Feizabadi *et al.*, 2008). Many studies have mentioned that the resistance to vancomycin is complex process and needs to presence of multiple genes (Malathum *et al.*, 2005, Kacmaz *et al.*, 2009). According to the Malathum (2005), seven genes clusters were investigated in vancomycin resistance *enterococci* including: (*vanA*, *vanB*, *vanc1*, *vanc2*, *vanD*, *vanE*, *and vanG*). Common resistance mechanism to glycopeptides such as vancomycin concerned with, dipeptide like termini (D-Ala-D-Lac) encoded by *vanA* and *vanB* clusters which, prompt to low affinity for vancomycin (Malathum *et al.*, 2005). Mobile genetic elements (transposons and plasmids), previously have been noticed as main resistance genes transferring among *Enterococci* spp (Palladino *et al.*, 2008, Emaneini *et al.*, 2008, Japoni *et al.*, 2009). Genes (*vanA* and *vanB*) encoding dipeptide like termini are responsible for high or moderate level vancomycin resistance (Malathum *et al.*, 2005, Palladino *et al.*, 2006, Arbeur *et al.*, 2008).

In this study, during 2011, one hundred eighty five strains of *enterococci* were isolated from clinical laboratories from three hospitals (Dr., Shariati, Sina, Masih Daneshvari) in Tehran, Iran. Isolated strains were identified using the conventional microbial tests according to the scheme utilized as previously described (Forbes *et al.*, 1998, Louis *et al.*, 2001). Following the incubation of inoculated isolates in Azid maltose agar culture medium (Himedia, India) at the 35 °C for 24 h, bacterial suspensions were prepared in sterile saline to obtain a turbidity of 0.5 MacFarland standard, equivalent to 1.5 x $10^8$ cfu/mL. Turbidity was measured by spectrophotometer instrument (Gensyse 10 UV spectrum, USA). Susceptibility testing for *Enterococci* isolates was performed by disk diffusion and E-test according to the clinical laboratory standard institute guideline (CLSI 2010). Disk diffusion method by utilizing disk containing 30 µg vancomycin (BD BBL™ Sensi Disc™) and E-test technology (AB BIODISK, Solna Sweden) as the recommended methods in CLSI 2010 were applied for detection of VREs strains. To carry out the tech-

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niques of disk diffusion and E-test Petri dishes with Mueller-Hinton (Merck, Germany) were applied. Bacteria were inoculated on the recommended media after dipped swabs in bacterial suspension, being implanted disks and E-test strips. Subsequently, plates were incubated in 35 °C for 24 h. Minimum inhibitory concentrations (MICs) breakpoints for vancomycin were determined by manufacturer's recommendation. *Enterococcus faecalis* ATTC 29212 and *Enterococcus faecium* BM4147 were used as sensitive and resistant strains, respectively. Both *vanA* & *vanB* positive genotypes underlined. See Table 1.

After susceptibility testing (E-test), *Enterococci* isolates with MIC ≥ 32 breakpoint were screened. Using a commercial kit (Roche, Diagnostics GmbH, Mannheim, Germany) genomic DNA from each VREs isolates was extracted. Purified DNA was dissolved in 50 µL distilled water. Quantity of extracted DNA was measured by NANODROP ®ND-1000 instrument (spectrophotometer 1000, USA) and adjusted to 500 ng.µL$^{-1}$.

Purified DNA was reconfirmed by polymerase chain reaction (PCR) by utilizing *rrs* gene (16s rRNA). Amplification protocol originally described by previously published study (Japoni *et al.*, 2009). PCR was performed utilizing 5 µL genomic DNA as template in total volume 25 µL containing; 10 µL master amplicon (Biolab, New England, UK), Forward primer (5'-GGATTAGATACCC TGGTGGTAGTCC-3') 1 µM, Reverse primer (5'-TCGTTGCGCACCTTAACCAAC-3') 1 µM and 8 µL mineral oil. PCR process was optimized with purified DNA

**Table 1** - Phenotype and genotype characteristics in VREs strains.

| *Enterococci* | Phenotypes | | Genotypes | | |
|---|---|---|---|---|---|
| VREs | Disc diffusion zone diameter | (MIC) µg/mL | *vanA* gene | *vanB* gene | *vanA* & *vanB* genes |
| *E. faecium* | 0(R) | > 256 µg/mL | P | N | N |
| *E. faecium* | 0(R) | 256 µg/mL | P | N | N |
| *E. faecium* | 1(R) | 256 µg/mL | P | N | N |
| *E. faecium* | 0(R) | 256 µg/mL | P | N | N |
| *E. faecium* | 0(R) | 256 µg/mL | P | N | N |
| *E. faecium* | 8(R) | 196 µg/mL | P | N | N |
| *E. faecium* | 8(R) | 196 µg/mL | P | N | N |
| *E. faecium* | 12(R) | 96 µg/mL | N | P | N |
| *E. faecium* | 0(R) | > 256 µg/mL | P | P | P |
| *E. faecium* | 0(R) | > 256 µg/mL | P | P | P |
| *E. faecium* | 1(R) | 256 µg/mL | P | P | P |
| *E. faecalis* | 0(R) | > 256 µg/mL | P | P | N |
| *E. faecalis* | 0(R) | 256 µg/mL | P | P | N |
| *E. faecalis* | 12(R) | 128 µg/mL | P | P | N |
| *E. faecalis* | 11(R) | 128 µg/mL | P | P | N |
| *E. faecalis* | 11(R) | 128 µg/mL | P | P | N |
| *E. faecalis* | 14 (R) | 64 µg/mL | N | P | N |
| *E. faecalis* | 14 (R) | 64 µg/mL | N | P | N |
| *E. faecalis* | 14(R) | 32 µg/mL | N | P | N |
| *E. faecalis* | 14(R) | 32 µg/mL | N | P | N |
| *E. faecalis* | 14(R) | 32 µg/mL | N | P | N |
| *E. faecalis* | 12 (R) | 96 µg/mL | N | P | N |
| *E. faecalis* | 8(R) | 196 µg/mL | P | P | P |
| *E. faecalis* | 0(R) | 256 µg/mL | P | P | P |
| *E. faecalis* | 0(R) | > 256 µg/mL | P | P | P |
| *E. faecalis* | 1(R) | 256 µg/mL | P | P | P |
| *E. faecalis* | 6(R) | 196 µg/mL | P | P | P |

R, resistant; VREs, vancomycin resistant *Enterococci*; MIC, minimum inhibitory concentration; µg/mL, microgram per milliliter; P, positive; N, negative.

of *Enterococcus faecalis* V583 and *Enterococcus faecium* BM4147 as positive genotypes. Amplification was performed on Gene Amp PCR system (Applied Biosystem, USA) using a program as follow: an initial cycle of denaturation 95 °C for 5 min followed by 35 cycles of 95 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, with final extension (Japoni *et al.*, 2009). Amplified products were visualized on 1.5% agarose gel (Max pure agarose, Spain) stained by etidium bromide by Gel logic 212 pro.

For precise and rapid detection of high and moderate vancomycin resistant *Enterococci* (*vanA* and *vanB* genes in this study), vancomycin resistance determinants *vanA* and *vanB* were targeted by Taq Man Real Time PCR assay. Determination of mentioned genes was performed using ABI 7500 USA instrument. (Figure 1). Specific primers and probes were designed as follow; reference *vanA* and *vanB* sequences representing each of *vanA* and *vanB* (*vanB1/vanB2/vanB3*) were assembled from the GenBank data base (http://www.ncbi.nlm.gov/GenBank). The utilized accession numbers were *vanA* (*M97297*) and *vanB* (*U00456*.1) respectively. Sequences were aligned using computer software beacon designer version 7. Sequence of designed primers and probes and Real Time PCR condition are listed in Table 2.

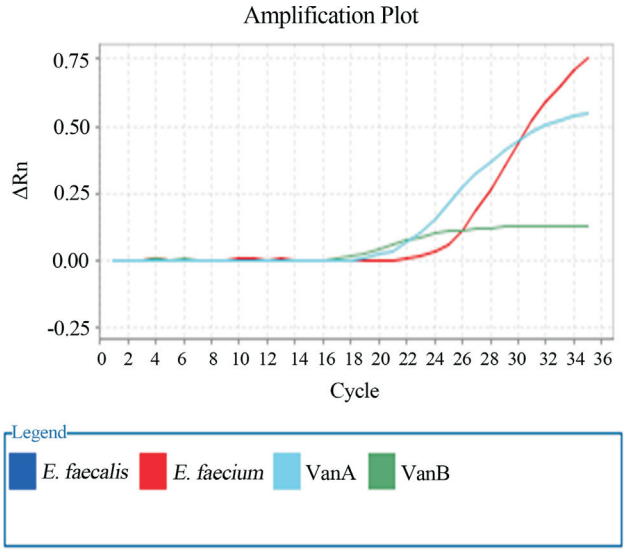


**Figure 1** - Standard linear amplification plot for *vanA* & *vanB* positive.

In this study statistical analyze was done by SPSS software version 16 by using Fisher exact test and $p < 0.05$ was considered significant.

Out of 185 *Enterococci* isolates 27 (0.14%) VREs strains consisting of 16/27 (0.59%) *Enterococcus faecalis* and 11/27 (0.41%) *Enterococcus faecium* were detected. Our study shows that *Enterococcus faecalis* and *Enterococcus faecium* were predominant isolates in VREs. Subsequently prevalence of *vanA* & *vanB* from *vanA* phenotypes were 3/27 (0.11%) *Enterococcus faecium* and 5/27 (0.18%) *Enterococcus faecalis.* Giridhara (2009) reported that, since 1980 (first report of isolated VREs) colonization and related infections by VREs as main causative nosocomial infections agents were enhanced (Giridhara *et al.*, 2009). Several Real Time PCR protocols have been developed for rapid and precise detection of resistance genes such as vancomycin resistance. According to the Mirzaei (2013) 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 (Mirzaei *et al.*, 2013). Our optimized Taq Man Real Time PCR assay had 96% similarity of phenotypic methods such as E-test and disk diffusion methods for a description of VREs.

The results of Taq Man Real Time PCR assay disclosed the isolates with *vanA* positive gene were predominant. *VanB* were detected, while *vanA* observed in eight (0.03%) high level resistant *Enterococci*. According to the Xiomara (2002) and Sharifi (2012) the phenotypic methods such as E-test are the convenient and useful methods for determining of minimum inhibitory concentration (MIC) for vancomycin in *Enterococci* (Xiomara *et al.*, 2002, Sharifi *et al.*, 2012). Our study affirmed mentioned studies and showed that Taq Man Real Time PCR assay is the useful methods for rapid and precise screening of VREs too. This method is more specific than traditional phenotypic methods for determination of both *vanA* and *vanB* genotypes in VREs phenotypes. Precise and rapid detection of vancomycin resistance genes lead to enhance the accuracy of VREs screening and consequently could help clinicians to timely administer appropriate antibiotics which may be life saving (Mirzaei *et al.*, 2013). According to the some mentioned studies dissemination of VREs due to *vanA* and *vanB* genes in Iran has presented serious challenges for the

**Table 2** - Sequence of designed primers and probes and Taq Man Real Time PCR assay condition.

| van primers | Forward (5'-3') | Reverse (5'-3') |
|---|---|---|
| *vanA* primer: | TGGAGCGACAGACATAACAGAT | ACACCTACGGGCGAGTTTC |
| *vanA* probe | JOE-TATTATTGCTCGTTTACCGTA -BHQ1 | |
| *vanB* primer: | TGATTGTCGGCGAAGTGGAT | GCGTGGATAGCGGCTGTA |
| *vanB* probe: | HEX- TCAGAGAATGCGATGATTATC-Tamra | |

Real Time PCR optimization: Denature: 95 °C for 15 s, Annealing: 55 °C for 15 s, Extension: 72 °C for 1 min, 35 cycles.
Real Time PCR condition: 10 µL Real Time PCR master Mix, 2.5 pmol of each primer, 1.5 pmol of each probe, 5 µL template, Final volume 23 µL.

medical community (Feizabadi *et al.*, 2008, Japoni *et al.*, 2009, Sharifi *et al.*, 2012, Mirzaei *et al.*, 2013) Current study reconfirmed previously published studies. After performing Taq Man Real Time PCR, three strains of *Enterococcus faecium* and five strains of *Enterococcus faecalis* were both *vanA* and *vanB* positive. According to the Wood ford (1997) during hospital outbreak of VREs, two *vanA* glycopeptides resistances Enterococcus *faecium* were isolated with plasmid mediated *vanB* resistance. Both were found to be identical to the *vanB* outbreak strain by pulsed field gel electrophoresis (PFGE). The genotype of this strain changed from *vanB* to *vanA* through an intermediate isolate that contained both the *vanA* and *vanB* genes clusters on distinct plasmids. In our study by using Taq Man Real Time PCR both *vanA* and *vanB* genes were detected too (Wood ford *et al.*, 1997). Michel (2001) reported that gene transferring in bacteria has an important role for the dissemination of resistance genes. Although primarily *vanA* cluster (in *TN1546* and *TN5482*) was identified in *Enterococcus faecium* also this cluster has been detected in *Enterococcus faecalis*. *VanB* gene cluster exist in *TN1546* and has been identified in *Enterococcus faecalis* and *Enterococcus faecium* strains. Instability in *Enterococci* genome and transduction process or up and down regulation of genes probably can be considered as main reasons (Michel *et al.*, 2001, Van den Braak *et al.*, 2000). According to the previously published study by Japoni (2009) *vanA* gene was detected as predominant determinant in VREs. Also, all of the isolates showed high level resistance to vancomycin itself (Japoni *et al.*, 2009). In this study, in addition to high levels of resistance to vancomycin, in eight of the samples both genes as the main determinants for resistance to vancomycin were observed. Due to the high sensitivity the utilized method in the current study, present of both associate indicators to resistance in some VREs are predictable. However, in most cases, only one of the genes for resistance is adequate (Malathum *et al.*, 2005).

The current study has shown; Taq Man Real Time PCR assay is the useful, precise and rapid method for detection of vancomycin resistance genes in the clinical microbiology laboratory. Although biochemical methods such as E-testing technology for the detection of antibiotics resistance could be more economically efficient, but given the time needed to identify the resistance, using molecular methods such as Taq Man Real Time assay can make a substantial contribution to save a patient's life.

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