

Molecular Analysis of *vanHAX* Element in Vancomycin Resistant Enterococci Isolated from Hospitalized Patients in Tehran

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Received 25 July 2007; revised 19 December 2007; accepted 16 January 2008

ABSTRACT

Background: Vancomycin (glycopeptide)-resistant enterococci (VRE or GRE) can cause serious problems for hospitalized patients due to the limited options for treatment of VRE infections. As infection with VRE increases in hospitals, further knowledge about vancomycin resistant genes is needed. **Methods:** Isolates of *Enterococcus* spp. were collected from hospitalized patients in Tehran (Iran) during 2006. Detailed molecular analysis was performed for vancomycin resistance genotype and *vanHAX* using conventional PCR and PCR-RFLP (restriction fragment length polymorphism), respectively. **Results:** out of 830 enterococci spp., 48 VRE isolates (5.8%) were obtained. All of VRE isolates carried *vanA* gene. *DdeI* digestion of *vanHAX* element showed the presence of point mutation at 8234 position. **Conclusion:** This study indicates that *vanA* is a predominant genotype in Iranian isolates. In addition, PCR-RFLP analysis revealed the presence of two types of *vanHAX* element in *vanA* harboring transposons. *Iran. Biomed. J. 12 (4): 223-228, 2008*

Keywords: Enterococci, *vanHAX*, Patients, Tehran

INTRODUCTION

Over the past decade, enterococci has become one of the leading causes of nosocomial infections [1]. Therapy of enterococcal infections is difficult because these pathogens show intrinsic resistance to many antibiotics including semisynthetic isoxazolyl penicillins, cephalosporins, low-level aminoglycosides, lincosamides, and cotrimoxazole. Moreover, in the clinical environment, enterococci become very easily resistant to penicillins, macrolides, tetracyclines, high-level aminoglycosides, as well as quinolones [2]. The ability of enterococci to acquire high-level resistance to the glycopeptide antibiotics: vancomycin and teicoplanin has exacerbated this problem and is of great clinical concern [3]. At present, vancomycin-resistant enterococci (VRE) have become major nosocomial pathogens worldwide [4].

The vancomycin resistance genes: *vanA*, *vanB*, *vanD*, *vanE*, and *vanG* are known to be acquired by various *Enterococcus* spp. [5]. VanA and VanB are common amongst enterococci isolates. The VanA phenotype is characterized by high-level inducible

resistance to both vancomycin and teicoplanin [3]. This type of resistance is determined by a highly conserved gene cluster, *vanRSHAX*, located on active Tn1546-type transposons [6]. Tn1546-type transposons contain two regulatory genes (*vanR* and *vanS*), three resistance genes (*vanH*, *vanA* and *vanX*) and two accessory genes (*vanY* and *vanZ*) that are not required for glycopeptide resistance. The minimal requirement for glycopeptide resistance is the expression of D-lactate dehydrogenase (*vanH*), D-Ala-D-Lac ligase (*vanA*) and DD-peptidase (*vanX*) genes, which results in the synthesis of peptidoglycan precursors with low affinity for glycopeptides and elimination of native (susceptible) precursors, therefore, conferring high-level resistance to glycopeptides [7]. The *vanHAX* region is a conserved region and the only reported variation in this region is a point mutation by *DdeI* which is a good approach for the study of variation of vancomycin resistance genes [8].

To date, a number of studies have been reported on VRE in Iran [9, 10]; however, there is no documented study on detailed molecular characterization of vancomycin resistance genes.

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The aim of the present study was to identify nosocomial enterococcal strains which are resistant to vancomycin and teicoplanin. Also, a detailed molecular characterization of DNA polymorphisms in the *vanHAX* gene cluster from hospitalized patients in Tehran (Iran) was performed using restriction fragment length polymorphism (RFLP) analysis.

MATERIALS AND METHODS

Patient population and bacterial strains. Samples were collected from 3 big hospitals (Amiraalam, Milad, Shariati) in Tehran (Iran), during 2006. All samples were collected from patients at least one week after hospitalization. The bacterial strains were obtained from patients with hospital infections defined by an infection control practitioner using the Centers for Disease Control and Prevention definitions for hospital infections [11]. The clinical samples were urine ($n = 700$), wound ($n = 45$), blood ($n = 39$), body fluid ($n = 20$), respiratory tract ($n = 16$) and abscess ($n = 10$). The isolates were initially characterized as enterococci, based on biochemical tests including Gram staining, presence of pyrrolidonyl arylamidase, catalase reaction, tolerance of 6.5% NaCl, the hydrolysis of esculin in the presence of bile, 0.04% telurite reduction, arabinose acidification and arginine decarboxylase activity. The species identification of *Enterococcus* was also confirmed by PCR using species-specific primers (Table 1).

Antimicrobial susceptibility tests. Susceptibility of enterococci was tested by the disk diffusion test for vancomycin (30 μ g). The susceptibility of VRE was

also determined for ampicillin (10 μ g), gentamicin (120 μ g), tetracycline (30 μ g), erythromycin (15 μ g), chloramphenicol (30 μ g), teicoplanin (30 μ g), dalfopristin-quinupristin (15 μ g) and linezolid (30 μ g) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (www.clsi.org). Disks were obtained from Bio-Rad (Hercules, CA, USA) except for dalfopristin-quinupristin and linezolid (Mast Diagnostics Ltd, Bootle, Mersey Side, UK). Minimum inhibitory concentrations (MIC) for vancomycin were determined using the micro dilution method according to the guidelines recommended by the CLSI document M7-A4 and the results were confirmed by Etest (AB Biodisk, Solna, Sweden) [12]. The MIC for teicoplanin was determined by the Etest according to manufacturer's instructions. Both antibiotics were tested in the range of 0.25-256 μ g/ml. *E. faecalis* ATCC 29212, and *E. faecalis* ATCC 51299 strains were used as quality control.

DNA preparation and PCR. All of vancomycin resistant isolates were tested for *van* genes using PCR. Extraction of bacterial DNA was performed using Qiagen DNeasy kit (Qiagen GmbH, Hilden, Germany). PCR reactions with specific primers were performed to identify *van* genotypes (*vanA* and *vanB*) of each VRE isolate (Table 1). PCR mix contained 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.2 mM of each dNTPs, 0.5 U of *Taq* DNA polymerase (HT Biotechnology, Cambridge, United Kingdom) and the specific primers (40 pmole) in total volume of 25 μ l. The PCR cycles were as follows: initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 1 min

Table 1. Species specific primers for *E. faecium* and *E. faecalis* and primers used for detecting *van* genes among enterococci isolates.

Gene	Sequence	product size	Reference
<i>E. faecalis</i>	5'-ATCAAGTACAGTTAGTCT 5'-ACGATTCAAAGCTAACTG	941	13
<i>E. faecium</i>	5'-TTGAGGCAGACCAGATTGACG 5'-TATGACAGCGACTCCGATTCC	658	13
<i>vanA</i>	5'-ATGAATAGAATAAAAAGTTGCAATA 5'-CCCCTTTAACGCTAATACGATCAA	1030	13
<i>vanB</i>	5'-GTGACAAACCGGAGGCGAGGA 5'-CCGCCATCCTCCTGCAAAAAA	433	13
<i>vanH1</i> <i>vanX2</i>	5'-ATGAATAACATCGGCATTAC 5'-TTATTTAACGGGGAAATC	2600	14

and a final extension at 72°C for 10 min [13]. A 100-bp DNA ladder was used as the molecular size marker. The gels were stained with ethidium bromide and photographed under UV light.

Amplification and characterization of vanHAX. Intergenic *vanHAX* amplification was performed using *vanH1* and *vanX2* specific primers. The reaction mix used at this step was the same as aforementioned reaction mix. The 2600 bp PCR amplicons from *HAX* region of the Tn1546-like elements were produced by an initial cycle at 94°C for 5 min, followed by 30 cycles denaturation at 94°C for 30s, annealing at 50°C for 30 s and extension at 72°C for 3.5 min and a final extension at 72°C for 10 min [14]. To compare the amplicons derived from *vanHAX*, PCR products were digested with 10 U *DdeI* restriction enzyme for 4 h (Roche Diagnostic GmbH, Mannheim, Germany). The resulting fragments were separated by electrophoresis through 1% agarose gels in 0.5 × TBE buffer. Sequences with mutation (G to T) at position 8324 in the *vanX* gene lacked a restriction site and therefore produced one larger fragment after digestion. The results of restriction analysis were confirmed by sequencing (Macrogen Research, Seoul, Korea). All of PCR-RFLP analyses were carried out using *E. faecium* BM4147 as the prototype.

RESULTS

VRE isolates and antibiotic susceptibility. A total of 830 *Enterococci* isolates were recovered from hospitalized patients. Among all isolates, 48 (5.8%) isolates (Milad hospital [$n = 33$], Shariati [$n = 13$], Amiraalam [$n = 2$]) were resistant to vancomycin and carried *vanA* genotype. Species distribution of VRE isolates was as follows: *E. faecium*, 47 strains (98%) and *E. faecalis*, 1 strain (2%). VRE isolates were collected from urine ($n = 41$), wound ($n = 2$), blood ($n = 1$), abscess ($n = 2$) and body fluid ($n = 2$) cultures. All of VRE isolates showed high-level resistance to vancomycin (MIC 128 mg/L) and teicoplanin (MIC 4-256 mg/L), which is characteristic of the *vanA* phenotype. In our collection, the percentage of resistance was, as expected, high for ampicillin (98%), erythromycin (92%), ciprofloxacin (92%), gentamicin (96%), and teicoplanin (92%), but relatively low for tetracycline (27%) and chloramphenicol (2%). All isolates were susceptible to linezolid and all

E. faecium isolates were susceptible to dalfopristin-quinupristin.

RFLP analysis of vanHAX. No size variation was observed in the amplicons of the *vanHAX* region (Fig. 1). The RFLP analysis revealed that 81% of the isolates produced identical pattern to the fragments obtained from the original Tn1546 element. The *vanHAX* amplified from 19% isolates were found to be of the other *DdeI* RFLP type, which is different from Tn1546. Amplicons with sequence identical to Tn1546 were expected to generate two distinct *DdeI* fragments: 850 and 600 bp and five faint fragments 350, 300, 250, 150 and 100 bp (Fig. 2a). However, sequences carrying a G-T mutation (GenBank accession No. M97297.1) at position 8234 generated one larger fragment of 950 bp and four fragments of 600, 350, 300, 250 and 150 bp (i.e. isolate 1, 5, 7 and 11) (Fig. 2b). These findings were confirmed with sequencing results. Out of 9 isolates harboring the mutation, 2 and the remaining 7 isolates were obtained from Shariati and Milad Hospitals (Tehran, Iran), respectively. The isolates were collected from surgical, internal, ICU and NICU wards.

DISCUSSION

Nosocomial infection with enterococci is a major concern at many hospitals throughout the world including Iranian hospitals [15, 16]. The epidemiology of enterococci is not fully understood since there are striking differences among different species of resistant isolates obtained from various geographic locations [17]. All of VRE in this study were isolated from patients with prolonged period of hospitalization, a well-known risk factor for nosocomial acquisition of VRE [18].

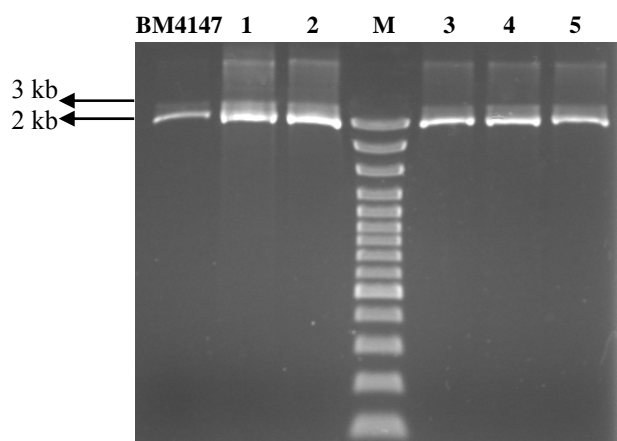


Fig. 1. The representative image shows 2600 bp amplicons of *vanHAX* gene.

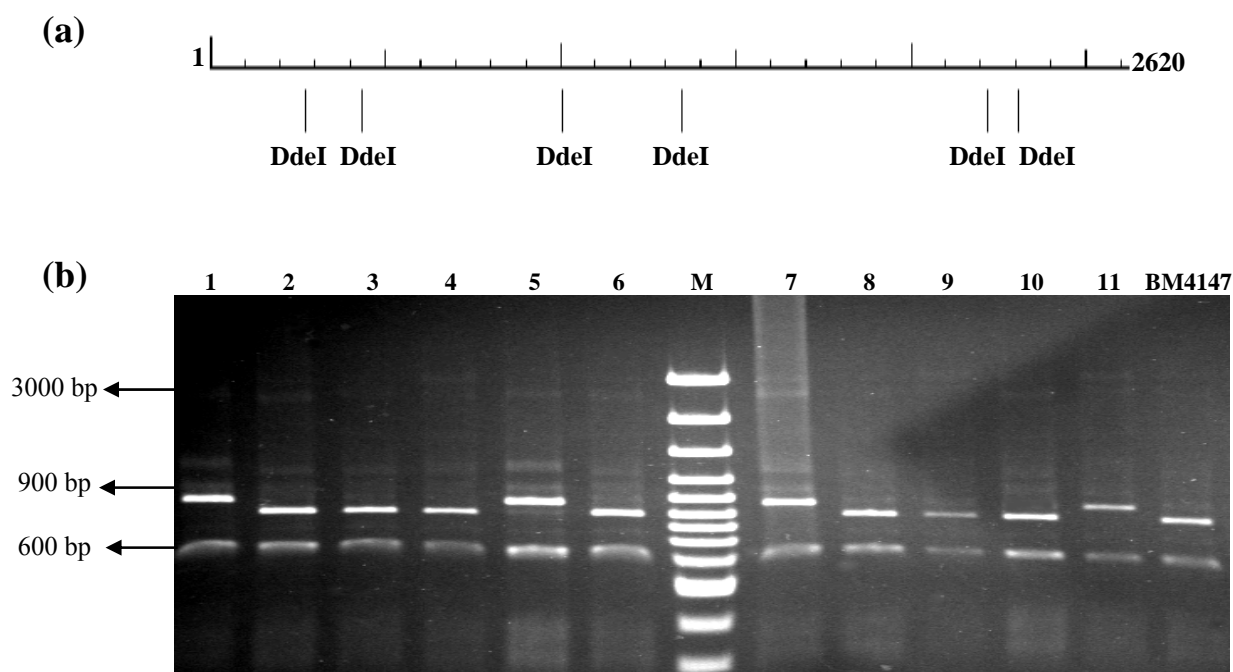


Fig. 2. (a) Proposed schematic *DdeI* restriction enzyme digestion for *vanHAX* element; (b) The *vanHAX* PCR-RFLP image using *DdeI* restriction enzyme and BM4147 as positive control.

The most active agents against all VRE isolates (*E. faecium* and *E. faecalis*) were linezolid and chloramphenicol, with no resistance. The excellent activity of linezolid against VRE has been previously reported [19]. Against vancomycin-resistant *E. faecium*, quinupristin/dalfopristin maintained activity in the majority of patients, without resistance, although inactivity of this antibiotic against *E. faecalis* resulted in resistance to dalfopristin-quinupristin. Ampicillin and ciprofloxacin, however, displayed almost universal resistance. The poor activity of fluoroquinolones and ampicillin against vancomycin-resistant *E. faecium* has been reported previously [19]. Nevertheless, *E. faecalis* showed susceptibility to ampicillin which is in accordance with other studies [20].

Furthermore, the ability of enterococci to acquire resistance to other agents like erythromycin and high concentrations of aminoglycosides is well recognized [21]. Most of isolates were susceptible to tetracycline and chloramphenicol. This might be resulted from the limited usage of these antibiotics. Consequently, treatment of VRE infections is a clinical challenge of great concern.

Among 830 isolates characterized in this study, 48 (5.8%) carried the *vanA* gene, however, no *vanB* gene was detected. All isolates shared a high level of vancomycin resistance characteristic; furthermore the majority of them were resistant to teicoplanin

showing *vanA* phenotype. Nevertheless, 8% of isolates having *vanA* genotype, were phenotypically teicoplanin susceptible. Our results present that there is 98% *E. faecium* and 2% *E. faecalis* among VRE isolates. A predominance of *E. faecium* with *vanA* genotype, as well as the predominance of *vanA* over *vanB* has been previously described in VRE and may be related to the less efficient mobilization of the *vanB* complex [21].

The *vanA* gene is the most prevalent gene among isolates causing VRE infections in humans. This gene is part of the transposable Tn1546 element, which was first characterized in 1993 by Arthur *et al.* [22]. Knowledge of Tn1546 diversity is important to distinguish between the dissemination of a single VRE clone and the transmission of a particular Tn1546 type through a genetically divergent population of enterococci [23]. The degree of diversity observed in the RFLP of the VanA element in enterococcal strains which have been studied worldwide is variable [14, 24]. In New Zealand, a single variant of the Tn1546-like element has spread among unrelated enterococcal strains [25]. However, it has been reported that heterogeneity of transposon loci RFLP patterns results from genetic events (deletion and insertion) within their less-conserved parts [23] or even in highly conserved regions as *vanRSHAX* [26].

In 19% of isolates, a single-base-pair difference was detected in the *vanX* gene. The detected difference at position 8234 in the *vanX* gene changed the amino acid at this position from a lysine to an asparagine but apparently did not affect the phenotype and did not have any effects on range of MIC. Taking together, there are two types of *vanHAX* elements in *vanA* transposons in VRE isolates collected from different hospitals wards, indicating that the presence of mutation is not related to the location where the organisms have been isolated.

Introduction of at least two transposon types in patients from different hospitals revealed the role of transposon in the spread of *vanA* VRE at Tehran hospitals. Thus, a genetic characterization of Tn1546 can provide information on dissemination of vancomycin resistance due to horizontal gene transfer. Even though, further studies are needed to clarify the different types of Tn1546 spreading among Iranian VRE.

In conclusion, *vanA* genotype is widely disseminated amongst VRE isolates in Tehran. Transposons harboring *vanA* gene are not homogeneous as shown by RFLP analysis. Furthermore, antibiotic susceptibility tests revealed that the VRE isolates were very susceptible to linezolid and chloramphenicol.

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