Clinical implications of vancomycin-resistant *Enterococcus faecium* (VRE) with VanD phenotype and *vanA* genotype

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**Objectives**: To investigate the clinical implications of vancomycin-resistant *Enterococcus faecium* (VRE) with VanD phenotype and *vanA* genotype (VanD-vanA VRE).

**Methods**: We tested in vitro and in vivo efficacies of teicoplanin against VanD-vanA VRE strains. Change in teicoplanin MICs was monitored during incubation with teicoplanin. In vitro and in vivo time–kill assay and survival analysis using a mouse peritonitis model were performed.

**Results**: Teicoplanin MICs of VanD-vanA VRE strains increased to 128 mg/L within 48 h when they were cultured with 120 mg/L teicoplanin. In vitro and in vivo time–kill assay showed that VanD-vanA VRE strains were not eliminated by 120 mg/L teicoplanin in contrast to vancomycin-susceptible *E. faecium* and VanD-vanB strains. The survival rate of mice infected with VanD-vanA VRE strains treated with teicoplanin was comparable with that of untreated mice.

**Conclusion**: Data suggest that teicoplanin would fail in the treatment of VanD type VRE infections if the strains contained the *vanA* gene, which cannot be detected in the clinical microbiology laboratory.

**Keywords**: vancomycin-resistant enterococci, teicoplanin, VanD-vanA

**Introduction**

Enterococci have become a clinical concern with the emergence of vancomycin-resistant *Enterococcus faecium* (VRE).1,2 The importance of VRE has increased due to limited treatment options and increased morbidity and mortality.3,4 VanA and VanB are the most common phenotypes among six phenotypes of glycopeptide resistance in enterococci.5 The VanA phenotype, which is encoded by the *vanA* gene, is characterized by acquired inducible and high-level resistance to vancomycin and teicoplanin. In contrast, the VanB phenotype is characterized by variable levels of vancomycin resistance, and susceptibility to teicoplanin, and is encoded by *vanB*.5,6

Recent studies reported VRE strains from Korea, Japan and Taiwan with incongruence between phenotype and genotype, which were susceptible to teicoplanin by in vitro test, despite the presence of the *vanA* gene.7–12 It has been suggested that point mutations in the sensor domain of the *vanS* gene in Tn1546-like element (or *vanA* operon)7,8,10 or impairment of accessory proteins VanY and VanZ9,11 would be the reason for the loss of teicoplanin resistance in these strains. Our recent report showed that 15% of VRE isolates from Korean hospitals were teicoplanin-susceptible or -intermediate in spite of the *vanA* gene,13 which is defined as VanD phenotype as in Naas et al.14 Because these strains are reported to be susceptible or intermediate to teicoplanin based on in vitro tests in the clinical microbiology laboratory, clinicians would use teicoplanin for the treatment of patients. However, clinical efficacy of teicoplanin against these VanD-vanA VRE strains has not been investigated. Given the increasing frequency of VanD-vanA VRE strains and
the very limited therapeutic options for VRE infections, this
would be an important issue in clinical practice. We report here
on the \textit{in vitro} and \textit{in vivo} evaluation of teicoplanin against VRE
strains with VanD phenotype and \textit{vanA} genotype.

Materials and methods

Bacterial strains and antimicrobial susceptibility testing

\textit{Enterococcus faecium} strains used in this study were isolated from tertiary care hospitals in Korea (Samsung Medical Center, Kyungpook National University Hospital and Chungnam National University Hospital) (Table 1). Their genetic backgrounds were determined by multilocus sequence typing (MLST), as described elsewhere.\textsuperscript{9,15} The \textit{van} genotype for glycopeptide resistance was determined by the multiplex PCR method.\textsuperscript{16} They were tested for \textit{in vitro} vancomycin and teicoplanin susceptibilities by evaluating MICs using the broth microdilution method according to the CLSI.\textsuperscript{17} Six strains showed VanD phenotype (i.e. \textit{vancomycin-resistant and teicoplanin-susceptible or intermediate}) with \textit{vanA} gene. Although these six strains are classified as susceptible or intermediate to teicoplanin according to the CLSI,\textsuperscript{17} teicoplanin MICs of 8 and 16 mg/L could be classified as intermediate or even resistant, respectively, according to the European Committee on Antimicrobial Susceptibility Testing.\textsuperscript{18} As comparators, one strain with VanA phenotype and \textit{vanA} genotype, one strain with VanB phenotype and \textit{vanB} genotype, and one \textit{vancomycin-susceptible Enterococcus faecium} (VSE) strain were tested. \textit{Enterococcus faecalis} ATCC 29212 and \textit{Staphylococcus aureus} ATCC 29213 strains used were reference strains in \textit{in vitro} susceptibility testing.

Genetic analysis of \textit{Tn1546}-like element

Genetic variations among \textit{van} gene cluster (\textit{Tn1546}-like element) of VanD-\textit{vanA} VRE strains were investigated by the PCR assay and sequencing as in previous studies.\textsuperscript{11,19} Briefly, overlapping PCR amplification of internal regions of \textit{Tn1546}-like element was performed, and PCR fragments longer or shorter than that of the prototype \textit{vanA} gene cluster of BM4147 were sequenced. The genetic structures of \textit{Tn1546}-like elements of the strains after exposure to teicoplanin were also determined by PCR and sequencing of VanR, VanS, VanX and VanY genes.

\textbf{MIC changes after exposure to teicoplanin}

Changes in teicoplanin MICs after exposure to teicoplanin were determined for eight isolates of \textit{E. faecium} (#18, #44, #88, 01-182, 06-12, 06-16, 07-16 and VSE-6800). Isolates were cultured in Mueller–Hinton broth containing 120 mg/L teicoplanin and were harvested after 2, 4, 6, 8, 12, 24, 48 and 72 h of culture. Teicoplanin MIC for each isolate was determined using the broth microdilution method according to the CLSI.\textsuperscript{17}

\textbf{In vitro time–kill assay}

\textit{In vitro} time–kill curves were determined for eight isolates of \textit{E. faecium} (#18, #44, #88, 01-182, 06-12, 06-16, 07-16 and VSE-6800). Bacterial colonies were inoculated onto blood agar plates and were incubated at 37°C overnight. Each culture was suspended in saline solution (0.9% NaCl) and inoculated into freshly prepared Mueller–Hinton broth, which contained 4\textsuperscript{+} MIC, 8\textsuperscript{+} MIC and 120 mg/L teicoplanin, adjusted to a final concentration of 10\textsuperscript{6} cfu/mL. Viable counts were evaluated at 4, 8, 12, 24, 48 and 72 h with shaking incubation at 37°C including antibiotic-free control. All time–kill curve experiments were performed at least three times and were averaged.

\textbf{Establishment of mouse peritonitis model}

To establish the mouse peritonitis model, 4- to 6-week-old female outbred ICR mice with a mean weight of 20 g were purchased from Orient Co. Ltd (Gyeonggi, Korea) and adapted to standardized environmental conditions (temperature, 24 ± 2°C and humidity, 55 ± 10%) for 1 week before the experiments. All animal procedures were performed in accordance with the institution’s guidelines for the humane handling, care and treatment of research animals. Mice were administered intraperitoneally with 1 mL of an overnight \textit{E. faecium} culture suspended in saline solution (0.9% NaCl) containing 5% pig mucin (Sigma, St Louis, MO, USA) to a final cell density corresponding to the minimal lethal dose, which is shown in Table 2. If untreated, mice died by 48–72 h. A 0.3 mL

Table 1. Characteristics of \textit{E. faecium} strains used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Origin\textsuperscript{a}</th>
<th>Phenotype/genotype</th>
<th>Source</th>
<th>MIC (mg/L)</th>
<th>MLST\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSE-6800</td>
<td>SMC</td>
<td>—</td>
<td>blood</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>#18</td>
<td>SMC</td>
<td>VanA\textit{-}vanA</td>
<td>blood</td>
<td>&gt;64</td>
<td>64</td>
</tr>
<tr>
<td>#88</td>
<td>SMC</td>
<td>VanB\textit{-}vanB</td>
<td>pus</td>
<td>64</td>
<td>0.5</td>
</tr>
<tr>
<td>#44</td>
<td>SMC</td>
<td>VanD\textit{-}vanA</td>
<td>blood</td>
<td>&gt;64</td>
<td>4</td>
</tr>
<tr>
<td>#28</td>
<td>SMC</td>
<td>VanD\textit{-}vanA</td>
<td>rectal swab</td>
<td>&gt;64</td>
<td>4</td>
</tr>
<tr>
<td>01-182\textsuperscript{c}</td>
<td>SMC</td>
<td>VanD\textit{-}vanA</td>
<td>rectal swab</td>
<td>32</td>
<td>16</td>
</tr>
<tr>
<td>06-12\textsuperscript{d}</td>
<td>KNUH</td>
<td>VanD\textit{-}vanA</td>
<td>NA</td>
<td>64</td>
<td>8</td>
</tr>
<tr>
<td>06-16\textsuperscript{d}</td>
<td>KNUH</td>
<td>VanD\textit{-}vanA</td>
<td>NA</td>
<td>&gt;64</td>
<td>8</td>
</tr>
<tr>
<td>07-16\textsuperscript{d}</td>
<td>CNUH</td>
<td>VanD\textit{-}vanA</td>
<td>NA</td>
<td>&gt;64</td>
<td>16</td>
</tr>
</tbody>
</table>

\textsuperscript{a}SMC, Samsung Medical Center; KNUH, Kyungpook National University Hospital; CNUH, Chungnam National University Hospital.

\textsuperscript{b}Sequence type (ST, \textit{tphA}-\textit{ddl}-\textit{gdh}-\textit{parK}-\textit{gyd}-\textit{pat5}-\textit{adh}).

\textsuperscript{c}These strains were analysed in a previous study.\textsuperscript{7}

NA, not available.
dose of teicoplanin (40 mg/kg) was given subcutaneously to infected mice 3 h after infection. Mice were observed for 72 h following inoculation and the teicoplanin treatment. The concentration of teicoplanin was adjusted to 120 mg/L, which is the peak level of teicoplanin treatment in human serum based on previous experiments. When teicoplanin 40 mg/kg was injected into the mouse, the peak level of teicoplanin (120 mg/L) in mouse serum was achieved; teicoplanin concentrations were determined microbiologically by an agar-dilution method.

**In vivo time–kill assay**

The in vivo time–kill curves were determined by inoculating various enterococcal strains into mice in each teicoplanin treatment group. Mice were infected as in the mouse peritonitis model. Four enterococcal strains were used: VSE-6800, #18 (VanA–vanA), #44 (VanD–vanA) and #88 (VanB–vanB). Teicoplanin (40 mg/kg) was administered subcutaneously 3 h after infection to achieve the peak concentration of 120 mg/L. Untreated mice were used as controls. Blood samples were obtained from three infected mice by cardiac puncture at 0, 6, 12, 24, 48 and 72 h after treatment with teicoplanin. Samples were plated on blood agar with appropriate dilution for the determination of viable bacterial cell counts after overnight incubation in 5% CO₂.

**Survival analysis**

Survival of mice was evaluated for five groups with different enterococcal strains: VSE-6800, #18 (VanA–vanA), #28 (VanD–vanA), #44 (VanD–vanA) and #88 (VanB–vanB). Five to 21 mice were prepared as described earlier for each strain and controls. As for in vivo time–kill growth curve determination, 40 mg/kg of teicoplanin was administered subcutaneously 3 h after infection and re-administered once a day. Untreated mice were used as controls. The cumulative per cent survival (%) was estimated at 12, 24, 36, 48, 60 and 72 h after administration of teicoplanin. Evaluation of survival rate differences between the treatment group and the control group was performed using the Kaplan–Meier method. A P value of less than 0.05 was considered significant. SPSS for Windows (version 11.5 software package; SPSS Inc., Chicago, IL, USA) was used for the analysis. 

### Table 2. Results of survival analysis

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Inoculum dose (log cfu/mL)</th>
<th>No. of mice tested</th>
<th>Survival rate (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSE-6800</td>
<td>10.19</td>
<td>control (5)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>treatment (10)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>#88 (VanB–vanB)</td>
<td>9.70</td>
<td>control (14)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>treatment (15)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>#18 (VanA–vanA)</td>
<td>10.10</td>
<td>control (7)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>treatment (10)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>#44 (VanD–vanA)</td>
<td>10.20</td>
<td>control (15)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>treatment (14)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>#28 (VanD–vanA)</td>
<td>10.09</td>
<td>control (21)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>treatment (20)</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

*Kaplan–Meier method comparing between control and treatment groups.

**Results**

### Genetic analysis of Tn1546-like element of VRE strains

Genetic backgrounds of *E. faecium* isolates, which were determined using MLST, showed that they belonged to ST78 and its single locus variants (Table 1). ST78 is a single locus variant of ST17 and thus related to the internationally disseminated lineage, CC-17. Structures of Tn1546-like elements, which account for glycopeptide resistance of *vanA* genotype in *Enterococcus* species, are shown in Figure 1. All isolates except #28 and 06-12 showed different structures. This indicates that loss of teicoplanin resistance in VanD–VanA VRE strains did not occur by horizontal transfer of an impaired Tn1546-like element, but by independent deletion of *vanY* or *vanZ* associated with insertion of IS1216V. No point mutations in *vanS* were found in any strain, but an IS1216V element was inserted within *vanS* in strain 06-16.

### MIC changes after exposure to teicoplanin

Teicoplanin MICs did not change significantly in enterococcal strains with congruent phenotype and genotype. VSE and VanB–VanB VRE strains retained susceptibility to teicoplanin (MICs, both 1 mg/L) after exposure to 120 mg/L teicoplanin. Exposure to teicoplanin resulted in only a 2-fold increase in MIC, from 64 to 128 mg/L, in VanA–VanA VRE strain. However, significant increases in teicoplanin MICs were observed in VanD–VanA VRE strains after exposure to teicoplanin. Within 48 h of exposure to 120 mg/L teicoplanin, teicoplanin MICs increased from 4–16 to 128 mg/L. This suggests that VanD–VanA VRE strains could acquire high-level resistance to teicoplanin, if they are exposed to teicoplanin, or that teicoplanin resistance was preserved after removal of the selective pressure of teicoplanin.

### In vitro time–kill analysis

The in vitro time–kill curve showed that the growth of VSE and VanB–VanB VRE strains was inhibited by more than 2 logs by
120 mg/L teicoplanin, whereas Van-D-vanA and VanA-vanA VRE strains were not inhibited by this concentration of teicoplanin (Figure 2). Although a VanA-vanA strain was inhibited by 8× MIC of teicoplanin (512 mg/L), this concentration is more than four times the achievable peak concentration (120 mg/L) in humans.

**In vivo time–kill analysis**

The results of in vivo growth curve experiments were demonstrated in Figure 3. In vivo time–kill analysis showed that all mice infected with *E. faecium* strains died within 48 h and bacterial cell numbers did not decrease if they were not treated with teicoplanin. VSE and VanB-vanB VRE strains were eradicated by teicoplanin after 48 h of treatment, and the infected mice survived. However, VanA-vanA and VanD-vanA VRE strains were not eradicated from blood of the infected mice despite teicoplanin treatment. The mice infected with VanA-vanA or VanD-vanA VRE strains used in the in vivo time–kill experiments died within 48 h irrespective of treatment with teicoplanin.

**Survival analysis**

The Kaplan–Meier survival analysis showed that teicoplanin treatment has significantly increased the survival rate of the mice infected with VSE (*P* = 0.007) or VanB-vanB VRE strains (*P* = 0.001). However, the survival rate of mice infected with VanA-vanA or VanD-vanA VRE strains treated with 120 mg/L teicoplanin was comparable with that of untreated controls (Table 2).

**Discussion**

In clinical practice, only a few antimicrobial agents can be used for the treatment of VRE infections, and these include quinupristin–dalfopristin, linezolid and teicoplanin. Teicoplanin could be one of the therapeutic options for teicoplanin-susceptible VRE infections either alone or in combination with clindamycin, rifampicin and ampicillin. However, because it has been also described that VanS(B) gene mutations mediate constitutive or teicoplanin-inducible expression of resistance, combination therapy with teicoplanin and aminoglycopeptides is more highly recommended. In a surveillance study in Korean hospitals, 15.3% of VRE isolates showed teicoplanin susceptibility despite *vanA* genotype. Most of them are thought to emerge sporadically due to the different structure of Tn1546-like elements. However, it is possible that Van-D-vanA VRE strains disseminate clonally both between and within hospitals because they belong to the same clonal complex, CC-78. A nosocomial outbreak VRE showing susceptibility or intermediate resistance to teicoplanin associated with *vanA* genotype was reported in a French hospital.

Because Van-D-vanA VRE strains showed phenotype susceptibility to teicoplanin by in vitro tests, teicoplanin could be selected for use in the clinical practice. Data from this study, however, suggested that teicoplanin therapy will fail if VanB- or VanD-type VRE strains contain the *vanA* gene. According to our in vitro time–kill assay, teicoplanin was not active against Van-D-vanA VRE strains. These strains also acquired high-level resistance after exposure to teicoplanin without any structural change in the Tn1546-like element, which was determined by PCR and sequencing of VanR, VanS, VanX and VanY genes. In a mouse peritonitis model, teicoplanin failed to eradicate Van-D-vanA VRE strains. These results are consistent with the
previous studies using a rabbit endocarditis model, in which VanD-type \textit{E. faecium} with a \textit{vanD} glycopeptide resistance operon was not cured by teicoplanin and \textit{in vivo} emergence of teicoplanin-resistant mutants in VanB-type \textit{E. faecalis} occurred during teicoplanin treatment.\textsuperscript{24,25}

VanD-\textit{vanA} VRE strains may contain heterogeneous populations consisting of VanA-\textit{vanA} and vancomycin-susceptible or VanB-\textit{vanB} cells. Recently, Naas \textit{et al.}\textsuperscript{14} showed that the low teicoplanin MIC for VRE strains with the \textit{vanA} gene is due to insertion of IS16 in the \textit{vanY} gene, resulting in heterogeneous expression of vancomycin resistance. Heterogeneous expression of vancomycin resistance resulted from heterogeneous \textit{E. faecium} populations, which consisted of susceptible and highly resistant cells. In our study, no heterogeneous colonies showing teicoplanin and vancomycin resistances were detected, when they were tested using teicoplanin and vancomycin Etest strips (AB Biodisk, Solna, Sweden). In these strains, \textit{vanB} gene could not be amplified by multiplex PCR assay for the genotyping of

\textbf{Figure 2.} The results of \textit{in vitro} time–kill analysis in \textit{E. faecium} isolates. Open diamonds, 0 × MIC; open squares, 4 × MIC; open triangles, 8 × MIC; open circles, 120 mg/L.
glycopeptide resistance. In addition, genetic structures of Tn1546-like elements determined from colonies, which survived after exposure to teicoplanin, did not change. Thus, the VanD-vanA VRE strains included in our study may not contain heterogeneous populations.

Although the results in this study may be strain-dependent, data from the current study suggest that phenotypic characteristics based on in vitro susceptibility tests in VRE isolates cannot be the sole guide to the selection of antimicrobial agents in clinical practice. Genotypic characteristics of glycopeptide resistance in enterococci should also be considered in the treatment of VRE infection. Generally, however, genotyping of VRE strains is not routinely performed in most clinical microbiology laboratories. Given these in vitro and in vivo data about VanD-vanA VRE strains, genotyping for glycopeptide resistance would be required in the hospitals where VanD-vanA VRE strains emerge.

In summary, we showed that teicoplanin was not effective against VanD-vanA VRE strains both in vitro and in vivo, despite its phenotypic susceptibility to teicoplanin. When teicoplanin is considered to treat severe VanD-type VRE infections, genotyping of glycopeptide resistance is warranted.

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Transparency declarations
None to declare.

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