Possible Horizontal Transfer of the vanB2 Gene among Genetically Diverse Strains of Vancomycin-Resistant Enterococcus faecium in a Korean Hospital

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A total of 25 isolates of vanB-containing Enterococcus faecium were recovered from patients in a single Korean hospital over a 20-month period. There were two distinct vanB2 patterns among the 11 pulsed-field gel electrophoresis types; 17 contained the prototype vanB2 and 8 contained a novel vanB2 with a 177-bp deletion in vanYB. Both vanB2 genes were transmissible in vitro at a mean frequency of $1.1 \times 10^{-8}$ transconjugants/donor. These results suggest the horizontal spread of vanB2 is occurring among genetically diverse strains of E. faecium in Korean hospitals.

Vancomycin-resistant enterococci (VRE) can be divided into five genetically distinct types, with VanA, VanB, VanD, and VanE (1, 8, 17) representing acquired phenotypes and VanC representing the intrinsic resistance phenotype (12). VanB-type resistance encoded by the vanB gene cluster is characterized by low-level vancomycin resistance (MICs = 8 to 16 μg/ml) and susceptibility to teicoplanin (MICs ≤ 1 μg/ml) (6, 7). The vanB gene cluster is divided into three subtypes based on DNA sequence data: vanB1, vanB2, and vanB3 (9, 16). The vanB determinants are commonly located on the enterococcal chromosome. Thus, dissemination of the resistance gene often is limited to either clonal spread of the host strain or through conjugative transposable elements (2, 18). Molecular epidemiologic techniques, such as pulsed-field gel electrophoresis (PFGE), do not detect the spread of resistance determinants during outbreaks. Therefore, it has been suggested that epidemiologic investigations of resistant organisms, such as VRE, should include analysis of the resistance determinant types in addition to the strain types (4, 10).

VRE were first detected in Korea in 1992 and have been recovered from patients in multiple hospitals ever since. The original VRE harbored vanA and showed high-level resistance (11; W. J. Kim, H. J. Cheong, D. R. Kim, and S. C. Park, Prog. Abstr. 1st Int. Conf. Enterococci, p. 51–52, 2000). The vanB-containing strains, on the other hand, did not appear in Korea until 1997. These strains showed moderate to high-level vancomycin resistance and diverse PFGE patterns. To better understand the spread of vanB-containing enterococci in Korea, all of the vanB VRE collected over a 2-year period from Ajou University Hospital in Korea were typed using PFGE, restriction analysis of the vanB determinant and the vanS\textsubscript{H}-vanY\textsubscript{H} intergenic area, and DNA sequence analysis of the vanS\textsubscript{H}-vanY\textsubscript{H} region.

Twenty-five VRE strains were obtained from patients in Ajou University Hospital from January 1997 to August 1998. The medical records of all 25 VRE patients were reviewed as part of an epidemiologic investigation. Twelve isolates were from urine cultures, eleven were from wounds, and two were from blood. Organisms were identified using conventional biochemical reactions, the Vitek system (bioMérieux, Hazelwood, Mo.), and the API 20 Strep system (bioMérieux). Disk diffusion susceptibility tests were performed using Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.) (14). Vancomycin, teicoplanin, and ampicillin MICs were determined by the agar dilution method using Mueller-Hinton agar (Difco Laboratories) (16); vancomycin-susceptible Enterococcus faecalis ATCC 29212 and vancomycin-resistant E. faecalis ATCC 51299 were used for quality control.

PFGE was performed with Smal (Gibco BRL, Gaithersburg, Md.) as described by Murray et al. (13), with pulse times beginning with 1 s and ending with 20 s at 6 V/cm for 24 h. Banding patterns were interpreted as previously described (20). Dendrograms based on Dice coefficients and clustered using an unweighted pair group method using arithmetic averages algorithm were generated using GelPrint AQ Software (Genomic Solutions Inc., Ann Arbor, Mich.).

Extraction of bacterial DNA was performed using a Dynabeads DNA DIRECT kit (Dynal, Oslo, Norway) as described by Haasheim and coworkers (10). The vancomycin resistance genotypes were determined using PCR with primers specific for sequences of the vanA, vanB, vanC1, and vanC2/C3 genes, as described previously (4, 5). The vanB subtypes were divided using PCR restriction fragment length polymorphisms. The 667-bp PCR fragment containing primers VanBF and VanBR was digested with HhaI (Gibco BRL) and analyzed via agarose gel electrophoresis. To perform polymorphism analysis of the vanS\textsubscript{H}-vanY\textsubscript{H} intergenic region, two different primer sets were used. Primer sequences and target locations used for specific
vanB gene cluster area are listed in Table 1. E. faecalis CDC (vanB1) (19) and E. faecalis SF300 (vanb2) (9) served as controls. Primers VanBF-VanBR and VanSY2F-VanSY2R were determined with the Oligo program (version 5.0; National Biosciences, Inc., Plymouth, Minn.). Amplification conditions were 94°C initially for 1 min; 94°C for 15 s, 60°C for 30 s, and 72°C for 1 min over 25 cycles; and a final 5-min extension period at 72°C. Long-distance PCR (L-PCR) covering 6,041 bp of the published vanB gene cluster from strain V583 was performed using primers VanBLF and VanBLR. The reactions were carried out in a 100-μl reaction volume including 1× Tricine buffer, 1.5 mM Mg acetate, a 0.2 mM concentration of each deoxynucleoside triphosphate, a 0.25 μM concentration of each primer, 2 U of Taq DNA polymerase (Perkin-Elmer, Norwalk, Conn.) and 1 μg of bacterial DNA. Using hot-start PCR, amplification conditions were 94°C for 1 min; 94°C for 15 s, and 68°C for 6 min for 16 cycles; 94°C for 15 s and 68°C for 6 min (with increments of 15 s per cycle for the 68°C step only) for 12 cycles; and a final 15-min extension period at 72°C. PCR products spanning the vanB gene cluster (6,041 bp) were digested with HinI and VanYB (position 2,375 to 2,551 [data not shown]). The 1,074-bp vanSB-vanYB gene amplification products from AJ01 and AJ07 were sequenced by using ABI Prism 377 (Perkin-Eimer) with the primers VanSY2F and VanYR.

Filter matings were performed by using E. faecalis JH2-2 as the recipient and seven strains (AJ01, AJ03, AJ05, AJ10, AJ23, AJ31, and AJ40) as the donors, as previously described (21). Transconjugants were selected on brain heart infusion agar plates containing rifampin (50 μg/ml), fusidic acid (20 μg/ml), and vancomycin (10 μg/ml). The transconjugants were examined for the presence of the vanB gene by restriction analysis of PCR products.

A total of 25 vancomycin-resistant E. faecium isolates were recovered from urine, wound, or blood cultures from 25 different patients over a 20-month period. Epidemiologic information did not suggest an outbreak but rather sporadic cross-transmission among patients. All isolates were resistant to ampicillin (MIC 32 μg/ml) and vancomycin (MIC ≥ 512 μg/ml), but only one isolate (AJ30) was resistant to teicoplanin (MIC = 64 μg/ml). PFGE revealed 11 major banding patterns and 10 subtypes among the isolates (Fig. 1). The isolates formed 7 clusters using a similarity cutoff of 85%. The first isolate was recovered in January 1997. Clusters 1, 2, 4, and 5 primarily represented isolates from the neurosurgery (NS) and rehabilitation medicine (RM) units. Clusters 3, 6, and 7 were primarily isolated from hematology (HE) and other surgical units, including orthopedic surgery, general surgery, and plastic surgery. No direct cross-transmission could be documented.

PCR analysis of the 25 isolates using the VanBF-VanBR primers demonstrated a product of the expected size (667 bp) from each of the isolates, indicating the presence of a vanB determinant. In addition, vanA was detected in isolate AJ30. Restriction analysis of the vanB PCR products in each case was consistent with that of vanB2 (Fig. 2A). Analysis of the vanSB-vanYB intergenic region using the VanSYF-VanSYR and VanSY2F-VanSY2R primer sets showed that 17 of 25 isolates contained a prototype vanb2 (9), designated type I, while 8 had a novel vanb2 pattern, designated type II (data not shown). This was confirmed by restriction analysis of the 6,041-bp long-range PCR product of the vanB gene cluster, which suggested that a deletion of a restriction site had occurred in the vanSB-to-vanYB gene region since the type II strains demonstrated one larger fragment (Fig. 2B, lane 7) than the type I control (Fig. 2B, lane 5). DNA sequence data of the 1,074-bp vanSB-to-vanYB gene region from AJ07 confirmed a 177-bp deletion in vanYB (position 2,375 to 2,551 [data not shown]). Representatives of the seven major PFGE patterns were mated with an E. faecalis recipient. All seven of the vanB2 donors transferred vancomycin resistance at a mean frequency of 1.1 × 10^-8 transconjugants per donor (range 4.8 × 10^-9 to 1.8 × 10^-8 transconjugants per donor). The vanB2 restriction profiles of transconjugants were indistinguishable from those of donors (data not shown).

The first vanB2 type I strain (AJ01) was isolated from a wound sample obtained from a 67-year-old female patient hospitalized on an NS intensive care unit. Ten weeks later, the second type I isolate (AJ03) was obtained from an NS patient; however, this isolate had a different PFGE type. No epidemiologic link could be established between the two patients. In April 1997, the first vanB2 type II VRE isolate was recovered from a wound sample from a 42-year-old male patient hospitalized on an OS unit. During the next 20 months, additional VRE with vanB2 types I and II were isolated; however, no clear epidemiologic links could be established among the patients. The strains showed a variety of PFGE types (Fig. 1).

### Table 1. PCR primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Specificity Location</th>
</tr>
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<tbody>
<tr>
<td>VanB1F</td>
<td>GTG ACA AAC CGG AGG CGA GGA</td>
<td>vanB1</td>
</tr>
<tr>
<td>VanB1R</td>
<td>CCG CCA TCC TCC TGC AAA AAA</td>
<td>vanB1</td>
</tr>
<tr>
<td>VanBF</td>
<td>CGC CAT ATT CTC CCC GGA TAG</td>
<td>vanB</td>
</tr>
<tr>
<td>VanBR</td>
<td>AAG CCC TCT GCA TCC AAG CAC</td>
<td>vanB</td>
</tr>
<tr>
<td>VanSYF</td>
<td>ATA TGC GCT GGA AAA AAC CTC</td>
<td>vanSP-YB</td>
</tr>
<tr>
<td>VanSYR</td>
<td>CCC CAG ATT GTT TCA TAT GCC</td>
<td>vanSP-YB</td>
</tr>
<tr>
<td>VanSY2F</td>
<td>TGG AAA ACA CCT CAG ATG GCG</td>
<td>vanSP-YB</td>
</tr>
<tr>
<td>VanSY2R</td>
<td>GGA TGG GTT TCT GCC TGT TCA</td>
<td>vanSP-YB</td>
</tr>
<tr>
<td>VanYB</td>
<td>GGAAGCGAAGCGGAGAAGCG</td>
<td>vanYB</td>
</tr>
<tr>
<td>VanBLF</td>
<td>ATG ATG ACA GCC CTG TCG GAT GA</td>
<td>vanB gene cluster</td>
</tr>
<tr>
<td>VanBLR</td>
<td>GGC ACC GTT GTG CCA TAA AAT CG</td>
<td>vanB gene cluster</td>
</tr>
</tbody>
</table>

* Primer described by Clark et al. (3).

* Primers described by Dahl et al. (4).
VRE with \( \text{vanB}_2 \) type I were primarily from patients from the NS and RM units, while the type II strains were isolated on the OS and HE units. The two exceptions were isolates AJ10 and AJ13, which were recovered from patients on an NS ward but had the type II L-PCR pattern. However, strain AJ10 was from a patient who was transferred from OS to the NS ward, and strain AJ13 was from the patient who occupied the bed next him. The \( \text{vanB}_2 \) VRE disappeared after implementation of infection control procedures including the isolation of infected patients.

The epidemiologic spread of VRE within a hospital is often investigated using PFGE (12, 13, 20). However, vancomycin resistance genes may move into previously susceptible enterococcal strains via plasmids or transposons (1, 2), particularly when VRE become endemic in a hospital (3). If this occurs, the differences in PFGE patterns among VRE isolates may suggest the influx of new VRE strains into the hospital rather than genetic exchange among endemic isolates. Such was the initial interpretation of the PFGE data from the VRE in this hospital, all of which carried a \( \text{vanB}_2 \) determinant. In this study, 17 strains carried a prototype \( \text{vanB}_2 \), while 8 contained a novel \( \text{vanB}_2 \) that harbored a 177-bp deletion in the \( \text{vanY}_{B} \) region and exhibited 47-bp changes (5.8%) and 19 amino acid changes compared to the \( \text{vanY}_{B} \) sequence of the reference strain V583. This difference provided useful epidemiologic information since most of the type I isolates were from patients on the NS...
and RM services and those of type II were from other surgical services. Our type II is different from the 789-bp insertion in vanB1 (E. faecalis V583); lane 2, isolate AJ01; lane 3, isolate AJ07. (B) Agar gel electrophoresis of the vanB L-PCR products and the restriction analysis of the vanB L-PCR products; lanes 2 and 3, vanB L-PCR products; lanes 4 to 7, HindII/NdeI-digested vanB L-PCR products; lane 2, isolate AJ01; lane 3, isolate AJ07; lane 4, E. faecalis V583 (vanB1); lane 5, E. faecalis SF300 (vanB2); lane 6, isolate AJ01; lane 7, isolate AJ07.

FIG. 2. (A) Restriction fragment analysis of the vanB PCR products. Shown are HhaI-digested vanB amplified fragments analyzed with agarose gel electrophoresis. Lanes 1 and 6, 100-bp DNA ladder (Promega, Madison, Wis.); lane 2, vanB1 quality control strain (E. faecalis V583); lane 3, vanB2 quality control strain (E. faecalis SF300); lane 4, isolate AJ01; lane 5, isolate AJ07. (B) Agar gel electrophoresis of the vanB L-PCR products and the restriction analysis of the vanB L-PCR products. Lanes 1 and 8, 1-kb ladder (Gibco BRL); lanes 2 and 3, vanB L-PCR products; lanes 4 to 7, HindII/NdeI-digested vanB L-PCR products; lane 2, isolate AJ01; lane 3, isolate AJ07; lane 4, E. faecalis V583 (vanB1); lane 5, E. faecalis SF300 (vanB2); lane 6, isolate AJ01; lane 7, isolate AJ07.

and RM services and those of type II were from other surgical services. Our type II is different from the 789-bp insertion in the vanS_F-vanY_H intergenic region recently reported by Dahl et al. (4). The continued appearance of the vanB2 type I determinant in unrelated isolates, such as AJ15 and AJ19, suggested that this gene was on a mobilizable element, a possibility which the in vitro mating studies have supported. We suspect that the novel vanB2 was derived from the prototype vanB2 gene and that both elements continue to be transmitted at low levels during ongoing cross-transmission of VRE at this hospital.

Nucleotide sequence accession numbers. The sequence data generated from vanS_F and vanY_H were submitted to GenBank and given the accession numbers AF302185 and AF302186.

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REFERENCES