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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 $vanY_B$. Both *vanB2* genes were transmissible in vitro at a mean frequency of 1.1×10^{-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 $vanS_B$-$vanY_B$ intergenic area, and DNA sequence analysis of the $vanS_B$-$vanY_B$ 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 *Sma*I (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 Haaheim 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 using primers VanBF and VanBR was digested with *Hha*I (Gibco BRL) and analyzed via agarose gel electrophoresis. To perform polymorphism analysis of the $vanS_B$-$vanY_B$ intergenic region, two different primer sets were used. Primer sequences and target locations used for specific

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TABLE 1. PCR primers used in this study

Primer	Sequence	Specificity	Location
VanB1F[a]	GTG ACA AAC CGG AGG CGA GGA	*vanB1*	5,434–5,454
VanB1R[a]	CCG CCA TCC TCC TGC AAA AAA	*vanB1*	5,866–5,846
VanBF	CGC CAT ATT CTC CCC GGA TAG	*vanB*	5,165–5,185
VanBR	AAG CCC TCT GCA TCC AAG CAC	*vanB*	5,831–5,811
VanSYF[b]	ATA TGC GCT GGA AAA CAC CTC	$vanS_B$-Y_B	2,114–2,134
VanSYR[b]	CCC CAG ATT GTT TCA TAT GCC	$vanS_B$-Y_B	2,422–2,402
VanSY2F	TGG AAA ACA CCT CAG ATG GCG	$vanS_B$-Y_B	2,122–2,142
VanSY2R	GGA TGG GGT TCT GCC TGT TCA	$vanS_B$-Y_B	2,666–2,646
VanYR	GGAAGCAGGAACGGGAAGCG	$vanY_B$	3,226–3,207
VanBLF	ATG ATG ACA GCC CTG TCG GAT GA	*vanB* gene cluster	409–431
VanBLR	GCG ACC GTT CGT CCA TAA AAT CG	*vanB* gene cluster	6,449–6,427

[a] Primer described by Clark et al. (3).
[b] Primers described by Dahl et al. (4).

vanB gene cluster area are listed in Table 1. *E. faecalis* V583 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 r*Tth* 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 *Hin*fI/*Nde*I (Gibco BRL) and analyzed via agarose gel electrophoresis. The 1,074-bp $vanS_B$-$vanY_B$ gene amplification products from AJ01 and AJ07 were sequenced by using ABI Prism 377 (Perkin-Elmer) 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 isolates 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* (9) (Fig. 2A). Analysis of the $vanS_B$-$vanY_B$ 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 $vanS_B$-to-$vanY_B$ 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 of the 1,074-bp $vanS_B$-to-$vanY_B$ region from AJ07 confirmed a 177-bp deletion in $vanY_B$ (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).

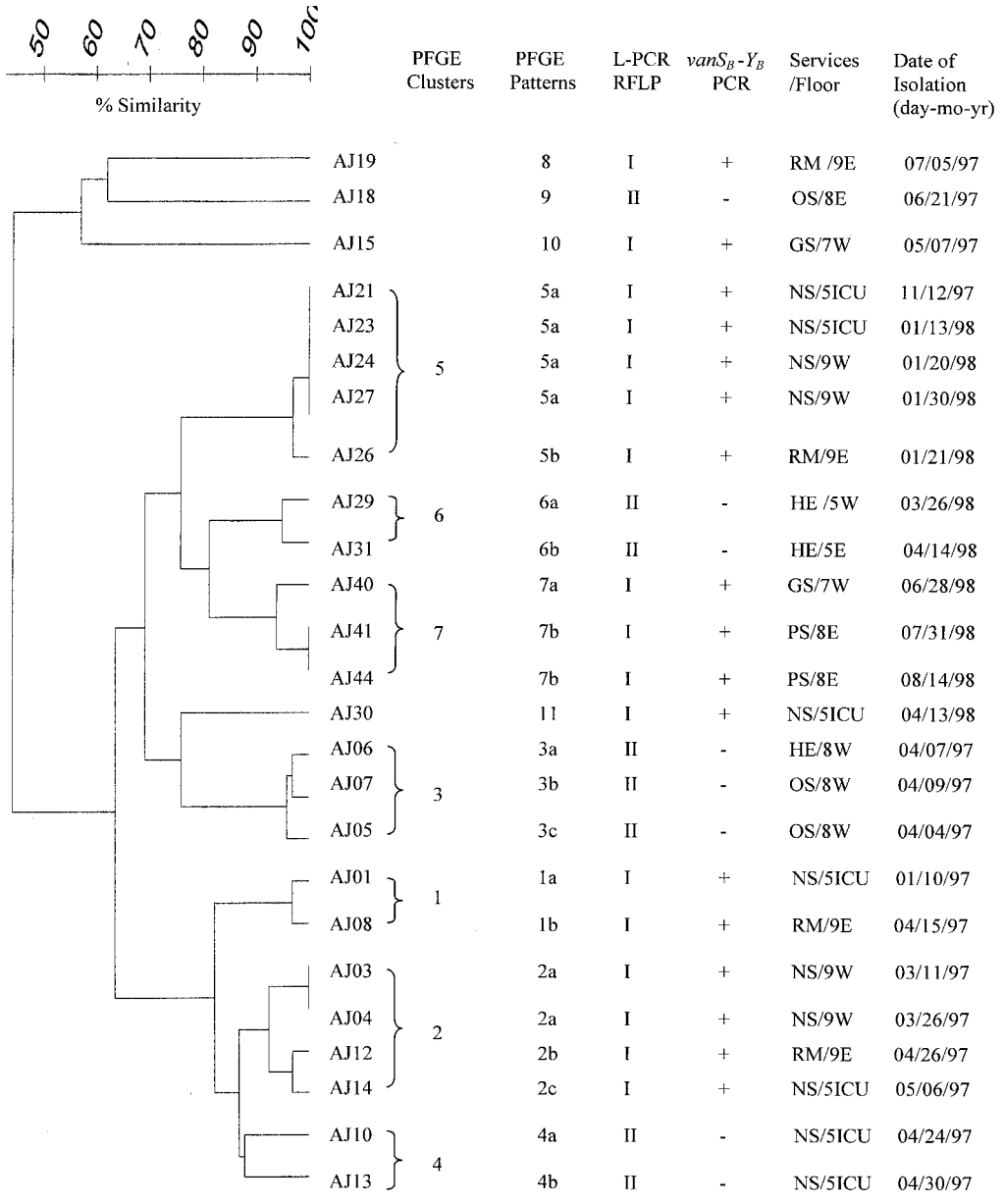

FIG. 1. Characteristics of *E. faecium vanB2* strains and results of structural data on *vanB* gene cluster and PFGE. Abbreviations: 9E, the ninth floor east ward; 7W, the seventh floor west ward; 5ICU, the fifth floor intensive care unit; OS, orthopedic surgery unit; GS, general surgery unit; PS, plastic surgery unit.

VRE with *vanB2* 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 *vanB2* 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 *vanB2* determinant. In this study, 17 strains carried a prototype *vanB2*, while 8 contained a novel *vanB2* that harbored a 177-bp deletion in the $vanY_B$ region and exhibited 47-bp changes (5.8%) and 19 amino acid changes compared to the $vanY_B$ 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

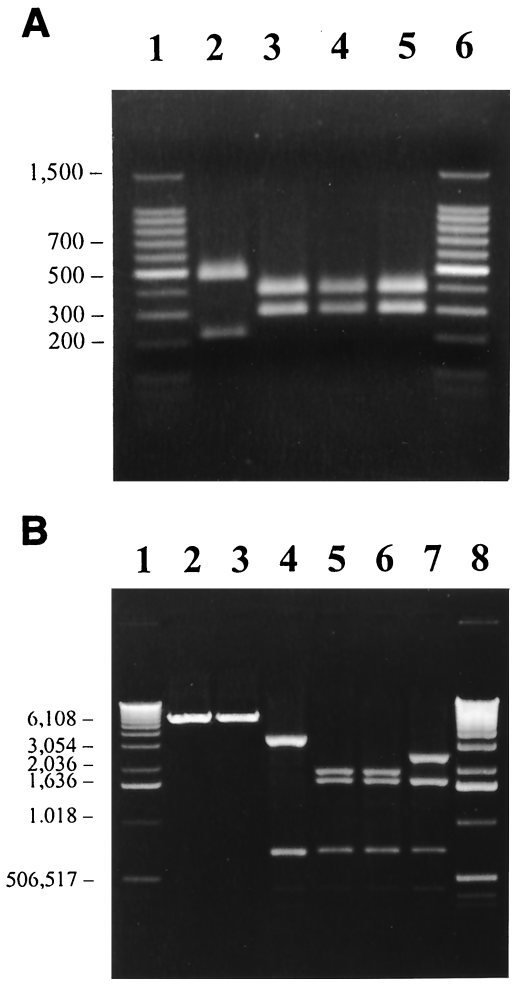

FIG. 2. (A) Restriction fragment analysis of the *vanB* PCR products. Shown are *Hha*I-digested *vanB* amplified fragments analyzed with agarose gel electrophoresis. Lanes 1 and 6, 100-bp DNA ladder (Promega, Madison, Wis.); lane 2, *vanB1* quality control strain (*E. faecalis* V583); lane 3, *vanB2* 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, *Hin*fI/*Nde*I-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 $vanS_B$-$vanY_B$ 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 $vanS_B$ and $vanY_B$ were submitted to GenBank and given the accession numbers AF302185 and AF302186.

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