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Multiplex PCR Detection of *vanA*, *vanB*, *vanC-1*, and *vanC-2/3* Genes in Enterococci

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Vancomycin-resistant enterococci (VRE) are increasingly isolated from clinical specimens. One hundred clinical isolates of enterococci (*E. casseliflavus*/*E. flavescens* [*n* = 10], *E. faecalis* [*n* = 34], *E. faecium* [*n* = 43], *E. avium* [*n* = 1], *E. gallinarum* [*n* = 11], and *E. raffinosus* [*n* = 1]) were examined for the presence of *vanA*, *vanB*, *vanC-1*, and *vanC-2/3* genes by a single multiplex PCR performed directly with colonies from blood agar plates. Six previously characterized VRE strains which carry either *vanA*, *vanB*, *vanC-1*, or *vanC-2* genes were used as controls. To discriminate among *van* genes, the PCR amplicons were digested with *Msp*I and were electrophoresed on agarose gels. Because of significant sequence homology between *vanC-2* and *vanC-3* genes, this assay is unable to discriminate these genes from each other; therefore, these are referred to as *vanC-2/3* genes. PCR products were detected in 63 of the 100 clinical isolates. The restriction fragment length patterns were consistent with *vanA* for 10 strains, *vanB* for 30 strains, *vanC-1* for 12 strains, *vanC-2* for 6 strains, and *vanA* and *vanC-1* for 1 strain. The vancomycin MICs for the isolates with restriction fragment length patterns consistent with *vanA* and *vanB* were all ≥64 μg/ml. The vancomycin MICs for the isolates with restriction fragment length patterns consistent with *vanC-1* or *vanC-2* were 4 to 8 μg/ml. The vancomycin MICs for the isolates from which no PCR amplicons were produced were 2 to 4 μg/ml. A PCR product was produced in four isolates (vancomycin MICs, 4 to >256 μg/ml) with restriction fragment length patterns differing from those for the control *vanA*, *vanB*, *vanC-1*, and *vanC-2* isolates. DNA sequencing of these amplicons revealed that two of the four isolates had nucleic acid sequences which were closely related to the published sequence for the *vanB* gene and two had nucleic acid sequences which were closely related to the published sequence for the *vanC-2* and *vanC-3* genes. Multiplex PCR-restriction fragment length polymorphism appears to be a useful and convenient method for rapidly detecting and discriminating genotypes for vancomycin-resistant *Enterococcus* spp. in the clinical laboratory. In instances in which unusual restriction fragment patterns of PCR amplicons occur, DNA sequencing can be performed to discriminate *van* genotypes.

From 1989 through 1993, the percentage of nosocomial enterococcal infections reported to the Centers for Disease Control and Prevention's National Nosocomial Infections Surveillance system that were caused by vancomycin-resistant enterococci (VRE) increased from 0.3 to 7.9% (1). This increase poses important problems, including the lack of available antimicrobial therapy for these organisms and the possibility that the vancomycin resistance genes present in VRE can be transferred to other gram-positive bacteria, especially *Staphylococcus aureus*. Several recent nosocomial VRE outbreaks attest to the importance of early detection of VRE so that preventive measures including the isolation of infected patients can be instituted (10, 13, 14, 17). The Centers for Disease Control and Prevention has recently issued guidelines to prevent the spread of vancomycin resistance (12).

Three glycopeptide resistance phenotypes in enterococci can be distinguished on the basis of the level and inducibility of resistance to vancomycin and teicoplanin. The VanA type is characterized by acquired inducible resistance to both vancomycin and teicoplanin, and the VanB type is characterized by acquired inducible resistance to various concentrations of vancomycin but not to teicoplanin (16, 20, 23). VanA-type glycopeptide resistance has been described for *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus gallinarum*, *Enterococcus casseliflavus*, *Enterococcus durans*, *Enterococcus mundtii*, *Enterococcus raffinosus*, and *Enterococcus avium* (2). VanB-type glycopeptide resistance has been described for *E. faecalis* and *E. faecium*. VanC-type glycopeptide resistance is characterized by constitutive low-level resistance to vancomycin and is an intrinsic property of *E. gallinarum*, *E. casseliflavus*, and *Enterococcus flavescens* (7, 19). The genes associated with the VanA, VanB, and VanC phenotypes have been identified and are termed *vanA*, *vanB*, *vanC-1*, *vanC-2*, and *vanC-3* (7, 19). VanA and VanB types of glycopeptide resistance have been associated with outbreaks of vancomycin-resistant enterococci. This type of resistance is acquired and may potentially be associated with the transfer of resistance to other organisms, including *S. aureus*. VanC-type glycopeptide resistance, on the other hand, is constitutive; therefore, transfer to other organisms is not of so much concern.

We describe herein a convenient multiplex PCR-restriction fragment length polymorphism (PCR-RFLP) assay which can be performed directly with isolated colonies of *Enterococcus* spp. to detect and discriminate *vanA*, *vanB*, and *vanC-1* genes. The assay also detects DNA sequences of *vanC-2* and *vanC-3*, but because of significant sequence homology between these genes, DNA sequencing of PCR products is required to discriminate between them. Therefore, these are referred to here as the *vanC-2/3* gene.

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TABLE 1. Oligonucleotide primers used in the study

Gene	Primer name	Oligonucleotide sequence (5′ to 3′)	PCR product size (bp)	Sizes (bp) of *Msp*I restriction fragments
vanA	vanA-FOR	CATGACGTATCGGTAAAATC	885	231, 184, 163, 131/133
	vanAB-REV	ACCGGGCAGRGTATTGAC		
vanB	vanB-FOR	CATGATGTGTCGGTAAAATC	885	188/189, 160, 136
	vanAB-REV	ACCGGGCAGRGTATTGAC		
vanC-1	vanC123-FOR	GATGGCWGTATCCAAGGA	467	230/237
	vanC1-REV	GTGATCGTGGCGCTG		
vanC-2/3	vanC123-FOR	GATGGCWGTATCCAAGGA	429	338, 91
	vanC23-REV	ATCGAAAAAGCCGTCTAC		

MATERIALS AND METHODS

Bacterial isolates. One hundred clinical isolates of *Enterococcus* spp. identified by the Mayo Clinic Microbiology Laboratory (including referred specimens from Mayo Medical Laboratories) were studied. Sources included blood, urine, wounds, bile, abscesses, peritoneal fluid, throat swabs, stool, and other sterile and nonsterile body sources; isolates were collected from 1992 to 1995. Six previously characterized VRE strains were used as controls. These included *E. faecium* B7641 (*vanA*; vancomycin MIC, >256 μg/ml; teicoplanin MIC, >16 μg/ml), *E. faecalis* V583 (*vanB*; vancomycin MIC, 64 μg/ml; teicoplanin MIC, ≤8 μg/ml), *E. faecium* JB1 (*vanB*; vancomycin MIC, 256 μg/ml; teicoplanin MIC, ≤8 μg/ml), *E. casseliflavus* ATCC 25788 (*vanC-2*; vancomycin MIC, 4 μg/ml; teicoplanin MIC, ≤8 μg/ml), and *E. gallinarum* GS (*vanC-1*; vancomycin MIC, 4 μg/ml; teicoplanin MIC, ≤8 μg/ml) (all control strains were kindly provided by Daniel F. Sahm) (11, 22).

Identification and antimicrobial susceptibility testing of *Enterococcus* isolates. Enterococci were identified by 6.5% NaCl tolerance and growth on bile-esculin agar with esculin hydrolysis. Species-level identification of *E. faecium*, *E. faecalis*, *E. gallinarum*, *E. avium*, *E. raffinosus*, and the *E. casseliflavus*/*E. flavescens* group was based on the formation of acid in mannitol, sorbitol, sucrose, arabinose, raffinose, pyruvate and sorbose broths, arginine hydrolysis, motility, pigmentation, and growth on tellurite agar. Susceptibility testing was performed by an agar dilution method and by following the current guidelines of the National Committee for Clinical Laboratory Standards (18). Mueller-Hinton agar with vancomycin concentrations of 2, 4, 8, 16, 32, 64, 128, and 256 μg/ml and teicoplanin concentrations of 8 and 16 μg/ml were used, and MICs were determined after 24 h of incubation at 35°C in room air.

Oligonucleotide primers. Six oligonucleotide primers (Table 1) were synthesized and were used to amplify the *vanA*, *vanB*, *vanC-1*, *vanC-2/3* genes, based on the published DNA sequences for these genes (6–9, 19). Oligonucleotide primer pairs were checked for specificity by testing the control vancomycin-resistant enterococcus strains. The expected PCR product size and restriction fragment length pattern for each of the control strains were observed (Fig. 1).

Multiplex PCR-RFLP. For each reaction, a single bacterial colony taken from overnight growth on a blood agar plate was suspended in 50 μl of a PCR mixture containing 10 pmol of each of the oligonucleotide primers, 1.25 U of the enzyme *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), 200 μM (each) deoxynucleoside triphosphate (Boehringer Mannheim, Indianapolis, Ind.), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, and 5% glycerol. The tubes were overlaid with 2 drops of mineral oil, and the bacteria were lysed at 95°C for 10 min, followed by 36 cycles of amplification (94°C for 1 min, 56°C for 1 min, and 74°C for 1 min). One microliter of *Msp*I (10 U/ml) and 5 μl of 10× restriction enzyme buffer (Promega Corp., Madison, Wis.) were added to each PCR tube. The tubes were centrifuged at 13,200 × *g* for 20 s to drive the restriction enzyme into the PCR mixture, and then the mixture was incubated at 37°C overnight. The digested PCR products were electrophoresed on a 3% NuSieve agarose gel containing ethidium bromide and photographed.

PCR product sequencing. Six microliters of the PCR mixture, 1 μl of 1 U of shrimp alkaline phosphatase per μl, and 1 μl of 10 U of exonuclease I (United States Biochemicals) per μl were incubated at 37°C for 30 min, followed by incubation at 80°C for 15 min. One microliter of dimethyl sulfoxide and 1 μl of

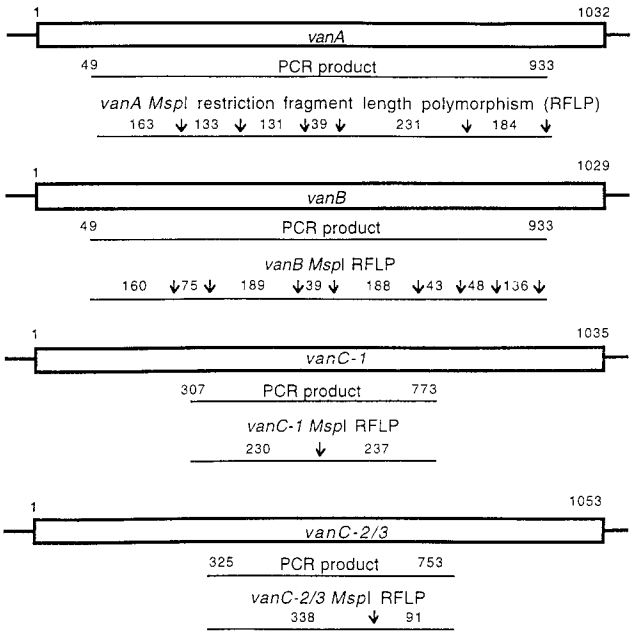

FIG. 1. DNA fragments from *Msp*I digestion of *vanA*, *vanB*, *vanC-1*, *vanC-2/3* gene PCR amplicons.

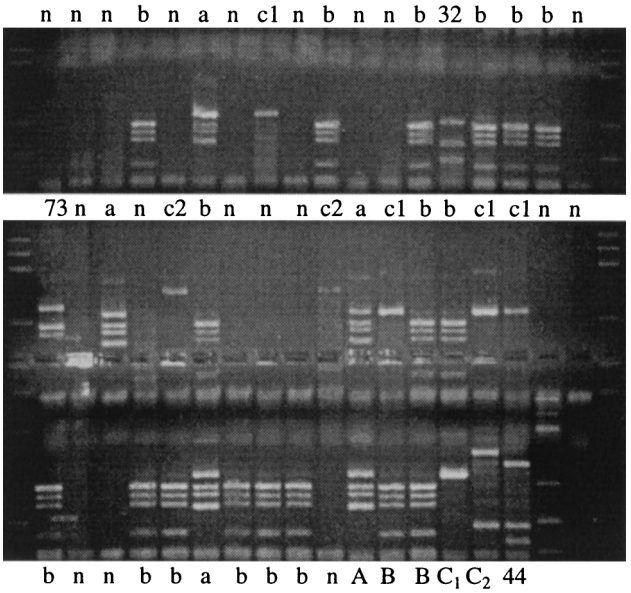

FIG. 2. Restriction fragment length patterns of selected study isolates. Lanes: a, *vanA*; b, *vanB*; c1, *vanC-1*; c2, *vanC-2*; *n*, no restriction fragment pattern; 32, isolate 32 (distinct restriction fragment pattern); 73, isolate 73 (distinct restriction fragment pattern); 44, isolate 44 (distinct restriction fragment pattern); A, control *vanA* isolate B7641; B, control *vanB* isolates V583 and JB1; C_1, control *vanC-1* isolate GS; C_2, control *vanC-2* isolate ATCC 25788.

TABLE 2. Identification and susceptibility results for clinical *Enterococcus* isolates

Gene(s) detected	No. of genes	*Enterococcus* spp. (no. of isolates)	MIC (μg/ml)	
			Vancomycin	Teicoplanin (no. of isolates)[a]
vanA	10	*E. faecium* (10)	>256	>16 (8)
			256	>16 (2)
vanA + *vanC-1*	1	*E. gallinarum* (1)	256	>16 (1)
vanB	30	*E. faecium* (28)	>256	>16 (2)
			>256	16 (1)
			>256	≤8 (12)
			256	≤8 (10)
			128	≤8 (3)
		E. faecalis (2)	128	≤8 (1)
			64	≤8 (1)
vanC-1	12	*E. gallinarum* (10)	8	≤8 (5)
			4	≤8 (5)
		E. faecium (1)	8	≤8 (1)
		E. faecalis (1)	4	≤8 (1)
vanC-2/3	6	*E. casseliflavus*/*E. flavescens* (6)	4	≤8 (6)
PCR amplicon produced, but with distinct restriction fragment length pattern	4	*E. faecalis* (2)	256	≤8 (1)
			>256	≤8 (1)
		E. casseliflavus/*E. flavescens* (2)	4	≤8 (2)
None	37	*E. casseliflavus*/*E. flavescens* (2)	4	≤8 (2)
		E. faecalis (29)	≤2	≤8 (27)
			4	≤8 (1)
			No growth	No growth (1)
		E. faecium (4)	≤2	≤8 (4)
		E. avium (1)	≤2	≤8 (1)
		E. raffinosus (1)	≤2	≤8 (1)

[a] Numbers in parentheses refer to the number of isolates for which the indicated vancomycin and teicoplanin MICs were obtained.

3.2 μM sequencing primer were then added. The DNA sequence was determined in both the 5′ to 3′ and the 3′ to 5′ directions with a *Taq* dideoxy terminator cycle sequencing kit and a 373A DNA sequencer (Applied Biosystems, Foster City, Calif.) by using a series of internal sequencing primers that provided appropriate coverage of the *van* genes. The sequence data were analyzed by using version 8 of the Genetics Computer Group sequence analysis software (3).

Nucleotide sequence accession numbers. The nucleotide sequences of isolates 45, 32, and 44 have been submitted to GenBank and have been given the accession numbers U72704, U72705, and U72706, respectively.

RESULTS

PCR products were detected for 63 of the 100 clinical *Enterococcus* isolates tested. The restriction fragment length patterns were consistent with *vanA* for 10 isolates, *vanB* for 30 isolates, *vanC-1* for 12 isolates, *vanC-2/3* for 6 isolates, and *vanA* and *vanC-1* for 1 isolate. Figure 2 shows a representative gel for 47 of these isolates. The vancomycin MICs for the isolates with restriction fragment length patterns consistent with *vanA* and *vanB* were all ≥64 μg/ml (Table 2). The vancomycin MICs for the isolates with restriction fragment length patterns consistent with *vanC-1* or *vanC-2/3* were 4 to 8 μg/ml. The vancomycin MICs for the isolates for which no PCR product was produced were 2 to 4 μg/ml.

A PCR product was produced for four isolates (vancomycin MICs, 4 to >256 μg/ml) with restriction fragment length patterns differing from those for the control *vanA*, *vanB*, *vanC-1*, and *vanC-2* isolates. This finding led us to compare the sequences of the amplicons of these isolates with those of the control organisms (Fig. 3). Isolate 45 had changes of 41 bp (5%) compared with the sequence of the control *vanB* strain, strain V583, which was used to determine the published sequence of the *vanB* gene (Fig. 3a) (8, 9). Isolate 44 had changes of 3 bp (1%) and isolate 32 had changes of 12 bp (3%) compared with the sequence of the control *vanC-2* strain, strain ATCC 25788 (Fig. 3b). Two of the changes in isolate 32 were also found in the published sequence of *vanC-3* (19). These changes accounted for the unique restriction enzyme patterns that were observed.

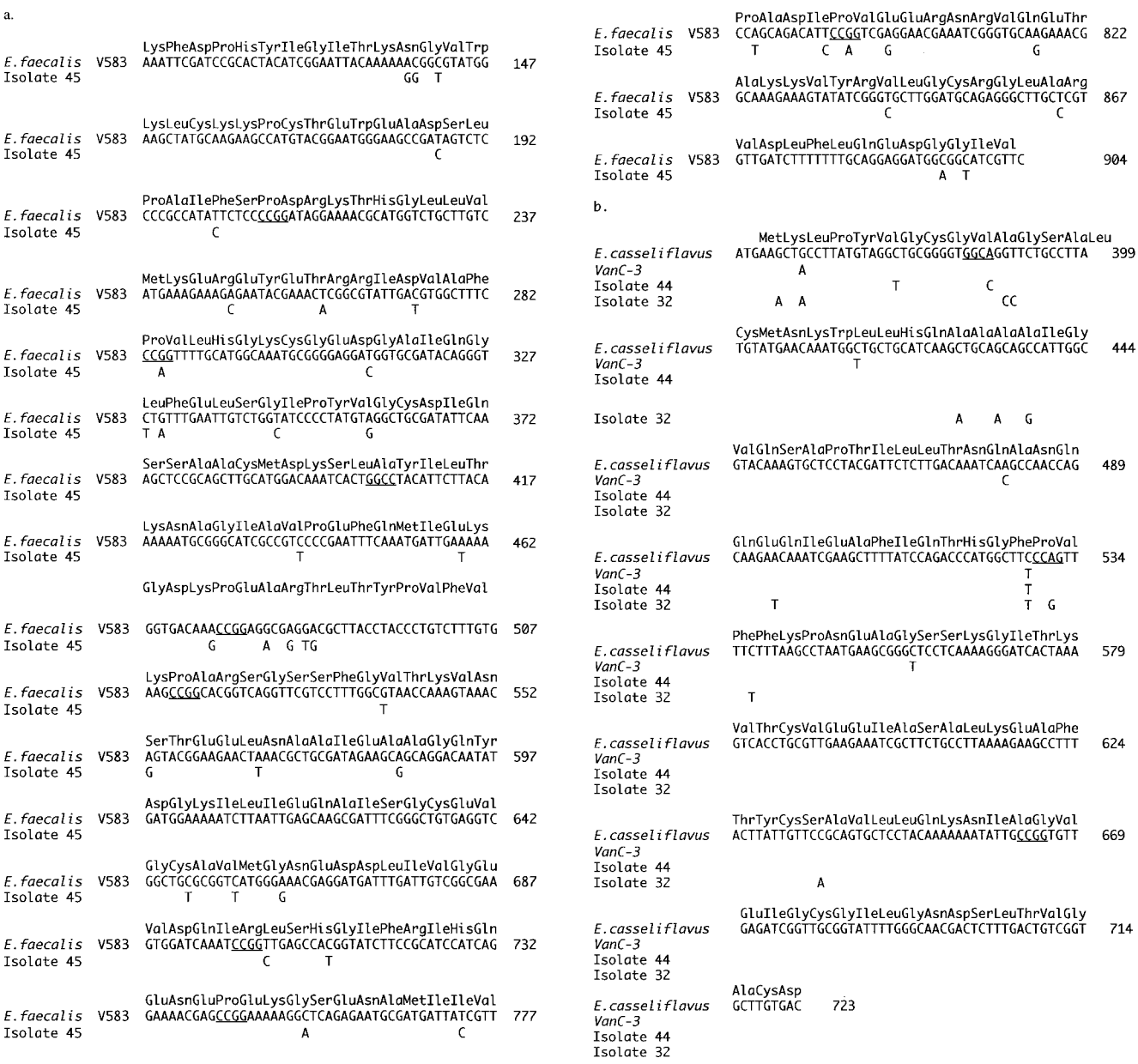

FIG. 3. DNA sequencing of isolates with distinct restriction patterns. (a) Isolates 45 and 73 were identical to each other (the sequencing of isolate 45 is shown) and resembled the sequence of control *vanB* isolate V583. (b) The sequences of isolates 32 and 44 resembled those of control *vanC-2* isolate ATCC 25788 and the published sequence of *vanC-3* (19). *Msp*I restriction enzyme sites are underlined. The sequences were submitted to GenBank (see text).

DISCUSSION

The multiplex PCR-RFLP assay in our hands is a convenient and rapid method for determining glycopeptide resistance genotypes for *Enterococcus* spp. in the clinical microbiology laboratory. The assay provides a more specific and rapid alternative to classical phenotypic methods for the detection of low-level glycopeptide resistance (MIC range, 4 to 8 μg/ml), as occurs with *vanC-1*-, *vanC-2*-, or *vanC-3*-associated resistance in *E. gallinarum*, *E. casseliflavus*, and *E. flavescens*. Current National Committee for Clinical Laboratory Standards interpretive breakpoints for susceptibility (MIC for susceptibility, ≤4 μg/ml) do not always allow for the discrimination of these genotypes. Nevertheless, the clinical relevance of *vanC* genotypes remains to be determined.

Our study confirms the work of Dutka-Malen and colleagues (5), who used a multiplex PCR to detect glycopeptide resistance genes in *Enterococcus* spp.; however, our study distinguishes itself in several ways. First, we demonstrated the feasibility of inoculating a single bacterial colony from a blood agar plate directly into the PCR mixture. Lysis is carried out by heating the mixture to 95°C for 10 min prior to cycling for amplification. This step saves time, making the assay more amenable to use in the clinical microbiology laboratory. Although we did not study this, it is likely that isolated colonies of *Enterococcus* spp. growing on primary isolation plates may be tested, with a result produced sooner than that produced by conventional susceptibility testing methods. This contrasts with DNA-DNA hybridization (probe) assays, which typically re-

quire subculturing of organisms in order to obtain adequate quantities for testing (15). The possibility also exists that our assay could be applied directly to clinical specimens, such as stool samples, in order to screen for vancomycin-resistant *Enterococcus* spp. Second, we added to the assay a restriction enzyme digestion step which confirms the expected PCR product and lessens the chances for contamination or amplicon carryover. As done in this study, the assay requires an overnight incubation because of this restriction enzyme digestion step. We have subsequently successfully carried out this assay using a 2-h digestion. Therefore, if our assay is applied directly to isolated colonies of *Enterococcus* spp. growing on a blood agar plate, results could be available the same day. Third, our study is different in that a large number of clinical *Enterococcus* spp. isolates were evaluated.

Four percent of the clinical *Enterococcus* isolates (four isolates) that we evaluated had restriction fragment length patterns distinct from those of the control organisms. DNA sequencing confirmed these differences. The sequence variability that we observed in these isolates is consistent with the hypothesis that the spread of vancomycin resistance among *Enterococcus* spp. not only may result from dissemination of a single clone but also may occur as the result of the horizontal transfer of resistance genes from as-yet-undefined organisms (21). This sequence variability indicates that caution is needed when applying stringent molecular approaches to the detection of vancomycin resistance genes in this group of microorganisms. That is, PCR primers which anneal to areas of *van* genes prone to sequence variability may fail to amplify these genes.

Our study isolates included one *E. gallinarum* isolate which carried both the *vanA* and *vanC-1* genes. This observation has previously been described for this organism (4). We detected the *vanC-1* gene in two enterococcal strains, which by phenotypic analysis appeared to be *E. faecalis* and *E. faecium*. To our knowledge, these findings have not previously been described. We recently confirmed the presence of the *vanC-1* gene in these isolates using previously described *vanC-1* gene primers (data not shown) (22). We determined the species of these isolates twice, with the following reproducible results. The *E. faecalis* isolate was nonmotile; arginine, mannitol, sorbitol, tellurite, sucrose, pyruvate, and bile esculin positive; and raffinose, arabinose, and sorbose negative. The *E. faecium* isolate was nonmotile; arginine, mannitol, arabinose, raffinose, sucrose, and bile esculin positive; and sorbitol, sorbose, tellurite, and pyruvate negative. Both isolates grew in 6.5% NaCl. Thus, these isolates were characterized as *E. faecium* and *E. faecalis* by standard phenotypic techniques, and both isolates carried the *vanC-1* genes by two different PCR assays (22). It is unknown whether, in these organisms, the resistance gene is plasmid mediated or transferable.

In conclusion, the multiplex PCR-RFLP method that we developed and that is described herein appears to be a convenient and rapid method for detecting glycopeptide resistance genes in *Enterococcus* spp. in the clinical microbiology laboratory. We feel that our multiplex PCR-RFLP assay might be useful for clinical microbiology laboratories interested in characterizing enterococci with questionable in vitro susceptibility results or for epidemiologic studies. Another potential use of this assay is to accurately identify *vanC* resistance and to study its clinical significance. One limitation of this and other, similar assays may be the sequence variability among *van* genes that we occasionally observed. DNA sequencing of PCR-generated amplicons from *Enterococcus van* genes that have unexpected restriction fragment length patterns will enhance our understanding of the sequence variability present among vancomycin resistance genes.

ADDENDUM IN PROOF

Following the acceptance of this article, we have reconfirmed the species identification of our *E. faecalis* isolate carrying the *vanC-1* gene by using previously described primers for identification of *E. faecalis* to the species level (5).

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