

# Emergence of Vancomycin-Resistant Enterococci in Australia: Phenotypic and Genotypic Characteristics of Isolates

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**Enterococci with resistance to glycopeptides have recently emerged in Australia. We developed multiplex PCR assays for *vanA*, *vanB*, *vanC1*, and *vanC2* or *vanC3* in order to examine the genetic basis for vancomycin resistance in Australian isolates of vancomycin-resistant *Enterococcus faecium* and *E. faecalis* (VRE). The predominant genotype from human clinical *E. faecium* isolates was *vanB*. The PCR *van* genotype was consistent with the resistance phenotype in all but six cases. One *vanA* *E. faecalis* isolate had a VanB phenotype, one *vanB* *E. faecium* isolate had a VanA phenotype, and four *E. faecalis* isolates were consistently negative for *vanA*, *vanB*, *vanC1*, and *vanC2* or *vanC3*, even though they exhibited a VanB phenotype. These four isolates were subsequently examined for the presence of *vanD* by published methods and were found to be negative. No vancomycin-susceptible strains produced a PCR product. On the basis of our findings the epidemiology of VRE in Australia appears to be different from that in either the United States or Europe. Our multiplex PCR assays gave a rapid and accurate method for determining the genotype and confirming the identification of glycopeptide-resistant enterococci. Rapid and accurate methods are essential, because laboratory-based surveillance is critical in programs for the detection, control, and prevention of the transmission of glycopeptide-resistant enterococci.**

Vancomycin-resistant *Enterococcus faecium* and *E. faecalis* (VRE) were first described in Britain in 1988 (22) and soon afterward were reported from other European countries and the United States (14). In the United States they have become major nosocomial pathogens, rising in incidence from 0.3% in 1989 to 7.9% in 1993, as reported by the Centers for Disease Control and Prevention (5), and among patients in intensive care units they now represent 14% of isolates of enterococci retrieved from cultures of blood (5). Resistance to multiple antimicrobial agents as well as vancomycin, especially ampicillin and high levels of aminoglycosides, is typical of these isolates (15).

Two principal phenotypes of acquired vancomycin resistance have been described, VanA and VanB, encoded by two distinct gene clusters, the *vanA* and *vanB* clusters, respectively, which are carried on transposons Tn1546 and Tn1547, respectively (3). The VanA phenotype confers high-level resistance to both vancomycin and teicoplanin, while the VanB phenotype confers moderate to high-level resistance to vancomycin only. A third type of vancomycin resistance, termed VanC, has been known for many years to be a natural (intrinsic) vancomycin resistance found in the motile enterococci *E. casseliflavus*, *E. gallinarum*, and *E. flavescens* (3). VanC confers low-level resistance to vancomycin only. Unlike *E. faecalis* and *E. faecium*, the motile enterococci are infrequent pathogens in humans.

The troublesome features of enterococci with the VanA and VanB phenotypes include their high propensity for cross-infection and the resistance of many strains to all conventional agents. The demonstration that the *vanA* gene cluster can be transferred to *Staphylococcus aureus* in vitro and in vivo is further cause for concern (18).

The rapid emergence of VRE in the United States has been

attributed to the intensive clinical use of vancomycin in both parenteral and oral forms in that country (13) on a background of high-level usage of cephalosporins, which promote enterococcal superinfection (16, 23). In Europe, investigators have postulated an additional role for the use of the glycopeptide avoparcin as a growth promoter in intensive animal industries, resulting in colonization with VanA *E. faecium* and subsequent transmission to humans via the food chain (1).

The first vancomycin-resistant *E. faecium* isolate in Australia was isolated from a liver transplant recipient in Melbourne in 1994 (12). Since March 1996 multiple isolates of vancomycin-resistant *E. faecium* and vancomycin-resistant *E. faecalis* have occurred throughout Australia. Only a few of these strains have been reported in the literature (4, 8, 9, 19).

The National Antimicrobial Resistance Surveillance Program at the Women's and Children's Hospital in Adelaide is a referral center for antimicrobial resistance in Australia, and we have collected isolates from virtually all patients known to have VRE infections that have occurred since 1994. In order to characterize these strains further we have developed multiplex PCR assays for *vanA*, *vanB*, *vanC1*, and *vanC2* or *vanC3* and have used these to examine the genetic basis for vancomycin resistance in Australian isolates of VRE. The results have been compared to those obtained by conventional susceptibility testing with glycopeptides.

## MATERIALS AND METHODS

**Bacterial strains.** Two hundred forty-eight isolates of *Enterococcus* spp. referred to the National Antimicrobial Resistance Surveillance Program were studied. Previously characterized VRE strains were used as controls. These included *E. faecalis* ATCC 51299 (*vanB*; vancomycin MIC, 6 µg/ml; teicoplanin MIC, 0.5 µg/ml), *E. casseliflavus* ATCC 25788 (*vanC2*; vancomycin MIC, 6 µg/ml; teicoplanin MIC, 0.75 µg/ml), *E. gallinarum* NCDO 2313 (*vanC1*; vancomycin MIC, 12 µg/ml; teicoplanin MIC, 1 µg/ml), *E. faecalis* ATCC 19433 (vancomycin MIC, 1 µg/ml; teicoplanin MIC, 0.19 µg/ml), *E. faecium* ATCC 19434 (vancomycin MIC, 1 µg/ml; teicoplanin MIC, 0.75 µg/ml), and *E. faecalis* E19 (12) (*vanA*; vancomycin MIC, >256 µg/ml; teicoplanin MIC, >256 µg/ml).

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TABLE 1. PCR primer sequences

Primer	Sequence	Specificity	Location within gene (strand) <sup>a</sup>
VanABF	GTAGGCTGCGATATTCAAAGC	<i>vanA</i>	358–378 (+)
VanAR	CGATTCAATTGCGTAGTCCAA	<i>vanB</i>	355–375 (+)
VanBR	GCCGACAATCAAATCATCCTC	<i>vanA</i>	568–588 (–)
VanC1F	TGGTATTGGTATCAAGGAAACC	<i>vanB</i>	664–684 (–)
VanC1R	AGATTGGAGCGCTGTTTTGTC	<i>vanC1</i>	139–160 (+)
VanC23F	CAGCAGCCATTGGCGTACAA	<i>vanC1</i>	565–585 (–)
VanC23R	CAAGCAGTTTTTGTAGTAGTTC	<i>vanC2</i> and <i>vanC3</i>	431–450 (+)
		<i>vanC2</i> and <i>vanC3</i>	1006–1027 (–)

<sup>a</sup> Position in nucleotide sequence relative to the initiation codon. +, positive strand; –, negative strand.

**Identification and antimicrobial susceptibility testing.** Isolates were identified by a conventional test scheme (7). A multiplex PCR assay based on the specific detection of genes encoding D-alanine:D-alanine ligases (*ddl*) (6) was used to confirm the identification of *E. faecalis* and *E. faecium*.

The MICs of vancomycin and teicoplanin were determined for each isolate by the Ettest (AB Biodisk, Solna, Sweden) method on Mueller-Hinton agar (2, 11). The interpretative criteria of the National Committee for Clinical Laboratory Standards (17) were used to determine the susceptibilities of the isolates.

**Vancomycin resistance gene typing by PCR.** Enterococci were first grown overnight at 37°C in Todd-Hewitt broth, and then 1-ml volumes were microcentrifuged and the pellet was resuspended in 200 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). The suspensions were heated at 95°C for 20 min and then microcentrifuged for 2 min. Five-microliter volumes of the supernatant were subjected to PCR amplification in 50-µl reaction mixtures containing each deoxynucleoside triphosphate at a concentration of 200 µM, each primer at a concentration of approximately 1 µM, and 1 U of *Taq* polymerase (Boehringer Mannheim) in 10 mM Tris-HCl (pH 8.3)–50 mM KCl–2 mM MgCl<sub>2</sub>–0.1% gelatin–0.1% Tween 20–0.1% Nonidet P-40. The samples were subjected to 35 PCR cycles, each consisting of 1 min of denaturation at 94°C, 2 min of annealing at 60°C, and 2 min of elongation at 72°C. PCRs were analyzed by electrophoresis on 2% agarose gels and were stained with ethidium bromide. The oligonucleotide primers used for detection of *vanA*, *vanB*, *vanC1*, and *vanC2* or *vanC3* sequences were designed with reference to the sequences deposited in GenBank by P. Courvalin and colleagues under accession numbers X56895, L06138, M75132, L29638, and L29639, respectively. Primer sequences and specificities are presented in Table 1. For each sample, two PCRs were set up. One contained primers VanABF, VanAR, and VanBR, which direct amplification of 231- and 330-bp fragments from the *vanA* and *vanB* genes, respectively. The other contained primers VanC1F, VanC1R, VanC23F, and VanC23R, which direct amplification of 447- and 597-bp fragments from the *vanC1* gene and either the *vanC2* or the *vanC3* gene, respectively. Multiplex PCRs were tested in duplicate. Known positive and negative controls were also included with each PCR run.

Genotype-negative VRE isolates, for which vancomycin MICs were >4 µg/ml, were also tested for the presence of *vanD* by using the primers described by Perichon et al. (20).

## RESULTS

**Characterization of multiplex PCR assays for *vanA*, *vanB*, *vanC1*, and *vanC2* or *vanC3*.** In order to confirm the specificities of the various PCR primers listed in Table 1, representative reference VRE isolates and vancomycin-susceptible enterococci were analyzed as described in Materials and Methods (Fig. 1). Clear PCR products of the expected size (231, 330, 447, and 597 bp for *vanA*, *vanB*, *vanC1* and *vanC2* or *vanC3*, respectively) were obtained. There was 100% agreement between the PCR results and the previously published genotypes and phenotypes.

**PCR analysis of Australian enterococci.** A total of 139 VRE isolates from 14 institutions in seven cities (Adelaide, Brisbane, Darwin, Melbourne, Newcastle, Perth, and Sydney) throughout all mainland states of Australia were obtained. The VRE were either human clinical isolates ( $n = 41$ ) or isolates from the contacts of index patients ( $n = 63$ ), the environment ( $n = 33$ ), or animals ( $n = 2$ ). Other referred *Enterococcus* spp. were clinical isolates ( $n = 89$ ) or were from animals ( $n = 20$ ). The results of PCR analysis of the *van* genotype are presented in Table 2. PCR *van* genotype results for *E. faecalis* and *E.*

*faecium* isolates were consistent with the resistance phenotype for all but six isolates. The discrepancies were as follows: one *vanA* *E. faecalis* isolate with the VanB phenotype, one *vanB* *E. faecium* isolate with the VanA phenotype, and four *E. faecalis* isolates consistently negative for *vanA*, *vanB*, *vanC1*, *vanC2* or *vanC3*, and *vanD*, even though they exhibited a VanB phenotype. No vancomycin-susceptible strains produced a PCR product. All *E. faecium* ( $n = 133$ ) and *E. faecalis* ( $n = 60$ ) isolates were correctly identified by PCR with the *ddl* primers. Using the *vanC1* and *vanC2* or *vanC3* primers, we further confirmed the identification of 42 *E. gallinarum* and 9 *E. casseliflavus* isolates. Of the 41 VRE isolates noted as index isolates by the sending institution, 23 (56%) were *E. faecium* *vanB*, 7 (17%) were *E. faecium* *vanA*, 6 (15%) were *E. faecalis* *vanB*, 3 were *van*-negative *E. faecalis*, and 2 were *E. faecalis* *vanA*.

The vancomycin and teicoplanin susceptibility results are presented in Table 3. Interestingly, one *E. faecium* isolate and three *E. faecalis* isolates with intermediate resistance to vancomycin (MICs, 8 to 16 µg/ml) were PCR positive for *vanB*. For the *E. faecalis* *vanA* isolate with the VanB phenotype

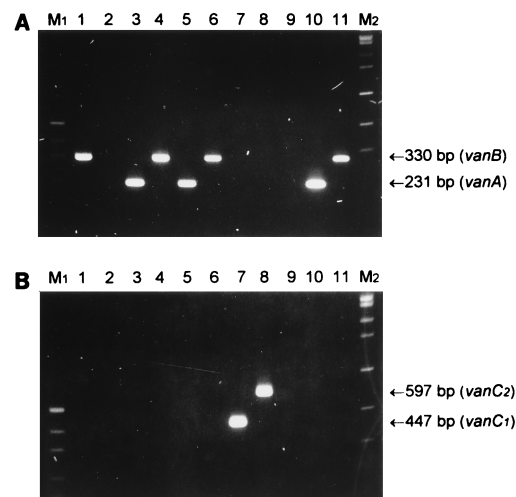


FIG. 1. PCR analysis of VRE. Enterococci were subjected to PCR analysis, as described in Materials and Methods, with primers VanABF, VanAR, and VanBR (A) or VanC1F, VanC1R, VanC23F, and VanC23R (B). The PCR mixtures were electrophoresed on 2% agarose gels and stained with ethidium bromide. Lanes: 1, *E. faecalis* ATCC 51299; 2, *E. faecium* ATCC 19434; 3, *E. faecalis* 91; 4, *E. faecalis* 3; 5, *E. faecium* 143; 6, *E. faecium* 135; 7, *E. gallinarum* 129; 8, *E. casseliflavus* 38; 9, *E. faecalis* 26; 10, *E. faecalis* 21; 11, *E. faecium* 30. Lane M<sub>1</sub>, pUC19 DNA digested with *Hpa*II (fragments of 501 and 489, 404, 331, 242, 190, and 147 bp are visible); lane M<sub>2</sub>, bacteriophage SPP1 DNA digested with *Eco*RI (fragments of 1,950, 1,860, 1,510, 1,390, 1,160, 980, 720, 480, and 360 bp are visible).

TABLE 2. Characteristics of Australian enterococci<sup>a</sup>

Species	Phenotype	No. of isolates with the following <i>van</i> genotype:					
		<i>vanA</i>	<i>vanB</i>	<i>vanC1</i>	<i>vanC2</i> or <i>vanC3</i>	Negative	Total
<i>E. faecalis</i>	Sensitive <sup>b</sup>					44	44
	VanA	1					1
	VanB	1	10			4	15
<i>E. faecium</i>	Sensitive <sup>b</sup>					10	10
	VanA	82	1				83
	VanB		40				40
<i>E. gallinarum</i>	VanC			42			42
<i>E. casseliflavus</i>	VanC				9		9
Other species <sup>c</sup>						4	4

<sup>a</sup> A total of 248 isolates were tested; VRE ( $n = 139$ ) comprised human clinical isolates ( $n = 41$ ) and isolates from contacts of index patients ( $n = 63$ ), the environment ( $n = 33$ ), and animals ( $n = 2$ ); other *Enterococcus* spp. ( $n = 109$ ) included clinical isolates ( $n = 89$ ) and isolates from animals ( $n = 20$ ). Refer to text for discussion on the prevalence of VRE from clinical isolates.

<sup>b</sup> Vancomycin MIC,  $\leq 4$   $\mu\text{g/ml}$ .

<sup>c</sup> *E. durans* ( $n = 2$ ) and *E. hirae* ( $n = 2$ ).

referred to above, the teicoplanin MIC was 4  $\mu\text{g/ml}$ . For the VanA *E. faecium* isolate which was *vanB* positive, the teicoplanin MIC was  $\geq 256$   $\mu\text{g/ml}$ . For the four VRE isolates which were *van* negative, the vancomycin MICs were in the range of 12 to 16  $\mu\text{g/ml}$ .

## DISCUSSION

The VRE isolated in Australia to date show considerable diversity in their phenotypes, genotypes, and geographic locations. All four combinations of genotype and species have been found, with the commonest being *E. faecium vanB*. While the clinical profiles of VRE-affected patients appear to be similar to those recorded in the United States and elsewhere (13), the predominance of *E. faecium vanB* rather than *E. faecium vanA* suggests an epidemiology different from that in either Europe or the United States.

The origin of VRE in Australia remains unclear. No strains appear to have been imported, although one occurred in a liver transplant recipient who was a New Zealand-born resident of Taiwan. This patient had entered Australia specifically for transplantation a few days prior to the procedure. *E. faecalis* of the VanB phenotype was initially isolated from blood cultures after surgery. The patient was treated with teicoplanin, but several days later a vancomycin-resistant enterococcus was again isolated from blood cultures, with the isolate identified as *E. faecalis* of the VanA phenotype. Genotyping showed that both isolates possessed the *vanB* gene, and subsequent ribotyping confirmed that the strains were identical. The emergence of resistance to teicoplanin has been recorded previously, albeit rarely (10).

The level of vancomycin use in Australia is relatively high and has been increasing over the last decade. There is significant regional variation in its use due to the variation in prevalence of multidrug-resistant *S. aureus* (21). Australia is also a high-level user of avoparcin as a growth promoter in the intensive animal industries. It is possible that the novel epidemiology of VRE in Australia may result from a combination of the high rates of use of vancomycin and avoparcin in humans and animals, respectively.

TABLE 3. Glycopeptide susceptibility

Gene	Species	MIC ( $\mu\text{g/ml}$ )		No. of isolates		
		Vancomycin	Teicoplanin			
<i>vanA</i>	<i>E. faecium</i> ( $n = 82$ )	256	>32	53		
		256	32	19		
		256	24	7		
		256	16	2		
		256	12	1		
	<i>E. faecalis</i> ( $n = 2$ )	256	256	1		
		256	4	1		
		<i>vanB</i>	<i>E. faecium</i> ( $n = 41$ )	256	256	1
				256	0.25–1.5	33
				128	1	1
64	0.75–1			3		
24	0.5–0.75			2		
12	0.5		1			
<i>E. faecalis</i> ( $n = 10$ )	256		0.25	1		
	128		0.38	1		
	48		0.5–0.75	2		
	32		0.5	2		
	24	0.38	1			
16	0.5	1				
12	0.25	1				
8	0.25	1				
<i>vanC1</i>	<i>E. gallinarum</i> ( $n = 42$ )	12	1	1		
		8	0.5–1.5	20		
		6	0.38–1	18		
		4	0.75–1	2		
		3	0.5	1		
<i>vanC2</i> or <i>vanC3</i>	<i>E. casseliflavus</i> ( $n = 9$ )	6	0.5–1	4		
		4	0.5–1	5		
None	<i>E. faecium</i> ( $n = 10$ )	1.5	0.75–1	3		
		1	0.75–1	5		
		0.75	0.125–1	2		
	<i>E. faecalis</i> ( $n = 48$ )	16	0.38	2		
		12	0.5	2		
		4	0.38–0.75	4		
		3	0.25–1	13		
		2	0.25–0.75	19		
	1.5	0.125–0.5	8			
	<i>E. durans</i> ( $n = 2$ )	6	0.38	1		
4		0.125	1			
<i>E. hirae</i> ( $n = 2$ )	1	0.094	1			
	0.75	0.094	1			

PCR methods have previously been used for the rapid identification of the vancomycin resistance genotype (6). In the present study we designed a set of PCR primers that provides for the simultaneous identification of all the major *van* genotypes under identical amplification conditions. Our multiplex *van* PCR assays were rapid and simple, giving clear-cut answers within 6 h. On the basis of phenotypic analysis, no false-positive results were generated by this test. PCR analysis also indicated that MIC determination alone is not sufficient for the

unambiguous classification of isolates of VRE. Also, difficulties continue to occur with commercial identification systems, and the not infrequent occurrence of nonmotile *E. gallinarum* and *E. casseliflavus* isolates and nonpigmented *E. casseliflavus* isolates compounds the problem. The *ddl* PCR was extremely useful for the identification of *E. faecalis* and *E. faecium* and, in combination with *vanC1* and *vanC2* or *vanC3* PCR, for the identification of *E. gallinarum* and *E. casseliflavus*. It is essential to have a rapid and accurate method for determination of the genotype and for confirmation of the identification of glycopeptide-resistant enterococci, especially during an outbreak or when performing surveillance for VRE. It is unlikely that the four strains with the VanB resistance phenotype that appeared to lack *vanA*, *vanB*, *vanC1*, and *vanC2* or *vanC3* or even the recently described *vanD* (20) were false-negative isolates (e.g., had the VanB resistance phenotype due to minor sequence variations in the primer annealing sites), because PCR analysis with independent *vanA* and *vanB* primers (6) was also negative. All four strains (from three patients) came from a single institution and gave two distinct pulsed-field gel electrophoresis patterns. Our results are consistent with either the existence of a significant variant of a current *van* genotype or a novel one. The *van* loci of these strains are undergoing further analysis.

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