

## Detection and typing of vancomycin-resistance genes of enterococci from clinical and nosocomial surveillance specimens by multiplex PCR

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### SUMMARY

Ninety-three clinical isolates of vancomycin-resistant enterococci (VRE) collected from nine hospitals in Taiwan were examined for the presence of *vanA*, *vanB*, *vanC1*, or *vanC2/vanC3* genes by a multiplex PCR. Forty-seven of these VRE isolates were *vanA* positive, 1 contained both *vanC1* and *vanA*, 40 harboured *vanB*, 2 were *vanC1*, and 3 were identified to be *vanC2/vanC3*. Twenty-four *vanA* isolates were sensitive to teicoplanin and thus did not have a typical VanA phenotype. Five isolates with the VanC phenotype harboured *vanB*. None of the 40 clinically isolated vancomycin-susceptible *E. faecium* or *E. faecalis* and the vancomycin-resistant *Leuconostoc* and *Pediococcus* isolates were positive for any of the *van* genes. While performing nosocomial surveillance, VRE were isolated from 47 of 467 rectal swabs by culture. Compared with the conventional culture method, the sensitivity and specificity of the multiplex PCR for detecting and identifying vancomycin-resistance genes in enterococci directly from culture-positive broth were 97.9% and 100%, respectively. The results suggest that genotypic characterization of vancomycin-resistance is necessary for all clinical VRE isolates and that the multiplex PCR assay can be an alternative method for this purpose.

### INTRODUCTION

Vancomycin-resistant enterococci (VRE) have emerged as important nosocomial pathogens. Two major types of acquired glycopeptide resistance designated VanA and VanB have been described [1]. They are encoded by two distinct gene clusters, *vanA* and *vanB*. VanA enterococci are typically resistant to high levels of vancomycin (MIC  $\geq$  128  $\mu$ g/ml) and teicoplanin (MIC  $\geq$  16  $\mu$ g/ml). VanB isolates exhibit various levels of resistance to vancomycin (MIC =

16–256  $\mu$ g/ml) but are susceptible to teicoplanin. Some VanA VRE have been shown to contain the *vanB* gene (2–4). Similarly, certain VanB VRE have been shown to harbor the *vanA* gene [2, 5]. A third type of low-level intrinsic vancomycin resistance, termed VanC, is found in motile enterococci *E. gallinarum*, *E. casseliflavus*, and *E. flavescens*, harboring *vanC1*, *vanC2*, and *vanC3* genes, respectively [6].

Since very few treatment options are available for VRE infections [7], rapid identification of VRE isolates or VRE-colonized patients is essential for preventing the spread of VRE. Certain infection control and VRE screening methods are recom-

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mended [8]. However, these microbiological methods are time-consuming and expensive [9] and are not capable of determining vancomycin-resistance genotypes (i.e. *vanA*, *vanB*, or *vanC*) [10–12]. In this study, we have developed a single-tube, multiplex PCR assay for the detection of VRE and identification of relevant vancomycin-resistance genotypes. We also compared the sensitivity and specificity of the multiplex PCR assay with those of conventional culture method for detecting VRE directly from culture broth of nosocomial surveillance specimens.

## MATERIALS AND METHODS

### Bacterial isolates

A total of 93 VRE (human-derived) isolates collected from 9 microbiology laboratories in Taiwan from May 1995 to October 1997 were studied. These VRE were isolated from blood, urine, abscess exudate, wound, stool or rectal swabs, drain tubes, ascites, or bile. All isolates were identified by conventional culture methods [13] and the Vitek GPC system (bioMérieux Vitek Inc., Hazelwood, MO, USA). All bacterial strains were stored at  $-70^{\circ}\text{C}$  in LB (Luria–Bertani) broth containing 15% glycerol until testing.

### Nosocomial surveillance specimens and culture conditions

During the 2-month surveillance period (April–May, 1997), a total of 467 rectal swabs were collected from patients in intensive care units of the Tri-Service General Hospital, Taiwan. All specimens were transported in Amies medium (Difco Laboratories, Detroit, MI, USA) and processed within 8 h of collection. The faecal material on each swab was suspended in 300  $\mu\text{l}$  of sterile water, and the mixture was vortexed vigorously for 5 s. One hundred microliters of the faecal suspension was inoculated into 3 ml of selective enrichment broth BEAB-V8 (BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD, USA), which is Enterococcosel broth containing 8  $\mu\text{g}$  vancomycin per ml. The culture was incubated at  $35^{\circ}\text{C}$  for 24–48 h and then subcultured on colistin-nalidixic acid (CNA) blood agar at  $35^{\circ}\text{C}$  for 24 h when it became dark and turbid. Enterococcus-like colonies on CNA blood agar plates were first screened with Gram stain and the pyrazinamidase (PYR) test. All Gram-positive and PYR-positive cocci were

further identified by conventional methods [13] and subcultured on Trypticase-based 5% sheep blood agar plates for vancomycin-susceptibility testing.

### Antimicrobial susceptibility testing

The MICs of vancomycin and teicoplanin for VRE were determined by the agar dilution method according to the current guidelines of the National Committee for Clinical Laboratory Standards [14]. Several quality control bacterial strains for the susceptibility test were applied including *Escherichia coli* ATCC25922, *Staphylococcus aureus* ATCC29213, and *Enterococcus faecalis* ATCC29212.

### DNA extraction

One hundred microliters of each positive BEAB-V8 culture were centrifuged at 13000 *g* for 5 min. The pellets were resuspended in 180  $\mu\text{l}$  of sterile distilled water, and, subsequent to the addition of 4 mg of lysozyme per milliliter of the suspension, the mixture was incubated at  $37^{\circ}\text{C}$  for 30 min. The cell lysate was then treated with 0.5 mg of proteinase K per ml at  $65^{\circ}\text{C}$  for 30 min and then incubated at  $95^{\circ}\text{C}$  for an additional 30 min. Three hundred microliters of ethanol were added to precipitate the DNA. The ethanol/lysate mixture was applied directly to a QIA amp Tissue Kit column (QIAGEN Inc., Chatsworth, CA, USA), and the DNA was purified as described by the manufacturer. The DNA was subsequently eluted with 50  $\mu\text{l}$  of TE buffer. The eluate was used in the multiplex PCR assay. For VRE grown on culture plates, bacterial DNA was extracted as described previously [15]. Briefly, a loop of bacterial cells were washed in lysis buffer (1% Triton X-100, 10 mmol/l Tris–HCl [pH 8.0], 1 mmol/l EDTA) and pelleted by centrifugation at 12000 *g* for 5 min. Each cell pellet was suspended in 100  $\mu\text{l}$  of lysis buffer and then incubated at  $100^{\circ}\text{C}$  for 30 min to release DNA. The cell lysates were centrifuged at 12000 *g* for 5 min to pellet cell debris. The supernatant was saved and stored at  $-70^{\circ}\text{C}$  until use.

### Multiplex PCR

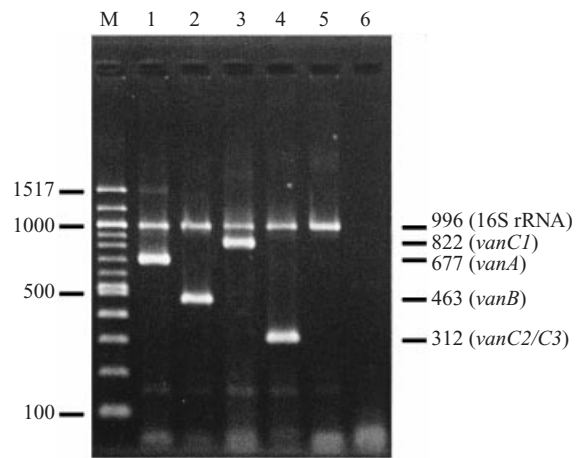
A multiplex PCR capable of identifying *vanA*, *vanB*, *vanC1*, and *vanC2/C3* genes [16] simultaneously was developed. The *vanC1* gene was amplified with primers 5'-GGTATCAAGGAAACCTC-3' and 5'-CTTCCG-

CCATCATAGCT-3' reported previously [17]. Three novel sets of primers were designed: (1) vanAF (5'-AATGTGCGAAAAACCTTGCG-3') and vanAR (5'-CCGTTTCCTGTATCCGTCC-3') for *vanA*, (2) vanBF (5'-CAAATCACTGGCCTACATTC-3') and vanBR (5'-TCTGCATCCAAGCACCCG-3') for *vanB*, and (3) vanC2/C3F (5'-TAAAGTCACCTGCGTTGAAG-3') and vanC2/C3R (5'-ATGCGAGCAAGACCTTTAAG-3') for *vanC2/C3*. The multiplex PCR mixtures consisted of buffer (10 mmol/l Tris-HCl, pH 8.8, 50 mmol/l KCl, 1.5 mmol/l MgCl<sub>2</sub>, and 0.1% Triton X-100), 200 µmol/l of each deoxynucleoside triphosphate, ten picomoles of each primer, one unit of DyNAzyme™II DNA polymerase (Finnzymes Oy, Espoo, Finland), and 5 µl of purified DNA in a total volume of 50 µl. A DNA thermocycler (Model 480, Perkin-Elmer Cetus, Emeryville, CA) was programmed according to the following: 5 min at 94 °C; 35 cycles of 1 min at 94 °C, 1 min at 58 °C, and 1 min at 72 °C; and 10 min at 72 °C. Amplified products were electrophoresed on a 1.5% [wt/vol] agarose gel at 100 V for 0.5 h and visualized by transillumination following ethidium bromide staining. *Enterococcus faecalis* AH803 (*vanA* gene) [18], *E. faecium* F901 (*vanB* gene), *E. gallinarum* CG-16 (*vanC1* gene), and *E. casseliflavus* L729 (*vanC2/C3* gene) and a vancomycin-susceptible *E. faecalis* strain (ATCC29212) were used as controls for the multiplex PCR. The conserved region of the 16S rRNA gene was amplified with universal primers U1 and U2 [19] to serve as the internal control.

## RESULTS

Of 93 VRE isolates, 57 (61.3%) were *E. faecium*, 30 (32.3%) were *E. faecalis*, 3 (3.2%) were *E. gallinarum*, and the remaining 3 (3.2%) were *E. casseliflavus*. The expected sizes of the multiplex PCR products were 677 bp for *vanA*, 463 bp for *vanB*, 822 bp for *vanC1*, and 312 bp for *vanC2/C3* (Fig. 1). All 93 isolates were positive for the multiplex PCR, generating at least one of the expected PCR products. The phenotypes and genotypes of these VRE isolates are presented in Table 1.

Thirty-eight (66.6%) of the 57 *E. faecium* isolates were determined to contain the *vanB* gene. The other 19 were found to harbour the *vanA* gene. All 19 *vanA*-containing *E. faecium* isolates were highly resistant to vancomycin (MIC ≥ 256 µg/ml), but 8 of these were susceptible to teicoplanin (MIC ≤ 8 µg/ml). Most (31/38, 81.6%) of the *vanB*-containing *E. faecium* were



**Fig. 1.** Amplification of DNA fragments from various VRE isolates by the multiplex PCR and the universal PCR [19] from BEAB-V8 culture positive broth. The PCR products were electrophoresed on 1.5% agarose gel. Lanes: 1, *E. faecalis* AH803 (*vanA*); 2, *E. faecium* F901 (*vanB*); 3, *E. gallinarum* CG-16 (*vanC1*); 4, *E. casseliflavus* L729 (*vanC2/C3*); 5, *E. faecalis* ATCC29212 (sensitive to vancomycin); 6, Reagent control; M, marker of 100-bp DNA ladder (New England Biolabs, Beverly, MA, USA). The predicted sizes (in base pairs) of the individual PCR product are indicated on the right.

highly resistant to vancomycin (MIC ≥ 64 µg/ml). Three *vanB*-containing *E. faecium* isolates exhibited a low-level resistance to vancomycin (MIC = 2–8 µg/ml).

Twenty-eight (93.3%) of the 30 *E. faecalis* isolates were determined to contain the *vanA* gene. The other two were found to harbour the *vanB* gene. All 28 *vanA*-containing *E. faecalis* isolates were highly resistant to vancomycin (MIC ≥ 64 µg/ml), but 16 of these were susceptible to teicoplanin (MIC ≤ 8 µg/ml). The two *vanB*-containing *E. faecalis* isolates had a low-level resistance to vancomycin (MIC = 4 µg/ml). The two *E. gallinarum* isolates were determined to contain *vanC1*. One of these two *E. gallinarum* isolates was highly resistant to vancomycin and was found to harbour both *vanA* and *vanC1*. Three *E. casseliflavus* isolates were determined to contain *vanC2*.

Two hundred and seventy-five of the 467 surveillance rectal swabs were positive for BEAB-V8 culture; 47 of these were identified as VRE by conventional culture methods (Table 2). Of these 47 VRE isolates, 38 were *E. faecium* and 9 were *E. faecalis*. Twenty-nine of the 38 *E. faecium* isolates contained the *vanB* gene and 9 contained the *vanA* gene. Of the 9 *E. faecalis* isolates, 8 were determined to contain *vanA* and 1 was shown to harbour *vanB*.

Table 1. Phenotypes and genotypes of VRE isolates

Gene(s) detected	Enterococcus spp. (no. of isolates)	MIC ( $\mu\text{g/ml}$ )		No. of isolates
		Vancomycin	Teicoplanin	
<i>vanA</i>	<i>E. faecium</i> (n = 19)	> 256	64	2
		> 256	48	2
		> 256	32	6
		> 256	16	1
		= 256	1-8	8
	<i>E. faecalis</i> (n = 28)	> 256	256	2
		> 256	64	1
		> 256	32	4
		> 256	16	5
		= 256	1-8	15
<i>vanB</i>	<i>E. faecium</i> (n = 38)	64	2	1
		> 256	1-2	28
		128	1	2
		64	4	1
		16-32	1	4
	<i>E. faecalis</i> (n = 2)	8	1	1
		4	1	1
		2	< 1	1
		4	1	2
		<i>vanC1 + vanA</i>	<i>E. gallinarum</i> (n = 1)	> 256
<i>vanC1</i>	<i>E. gallinarum</i> (n = 2)	2	1	2
<i>vanC2/C3</i>	<i>E. casseliflavus</i> (n = 3)	8	2	2
		4	2	1

Table 2. Vancomycin-resistance genotypes and vancomycin-teicoplanin susceptibility of VRE isolated from surveillance specimens

Gene detected	Species	MIC ( $\mu\text{g/ml}$ )		No. of isolates
		Vancomycin	Teicoplanin	
<i>vanA</i>	<i>E. faecium</i> (n = 9)	256	64	7
			32	2
	<i>E. faecalis</i> (n = 8)	256	64	7
<i>vanB</i>	<i>E. faecium</i> (n = 29)		32	1
		256	0.75-1	25
	<i>E. faecalis</i> (n = 1)	64	0.75-1	4
		256	1	1

n, number of isolates.

All but one of the BEAB-V8 culture-positive specimens were positive for the multiplex PCR, and all BEAB-V8 culture-negative specimens were negative for the multiplex PCR (Table 3). No vancomycin-resistant *E. gallinarum* or *E. casseliflavus* isolate was detected during the surveillance period. The sensitivity and specificity of the multiplex PCR for detection

and identification of VRE from BEAB-V8 culture-enriched specimens were 97.9% and 100%, respectively when compared with the conventional culture method. To determine the specificity of the multiplex PCR, 40 vancomycin-susceptible enterococci (20 strains of *E. faecalis* and 20 strains of *E. faecium* isolated from clinical specimens) and 3 each of

Table 3. Comparison between microbiology method and multiplex PCR in the detection of VRE from 275 BEAB-V8 culture positive specimens

	Microbiology method	
	Positive (n = 47)	Negative (n = 228)
Multiplex PCR		
DNA amplified	46	0
No amplification	1*	228

\* This isolate was identified as *E. faecium* containing the *vanB* gene. Vancomycin and teicoplanin MICs for this isolate were 64 and 1 µg/ml, respectively.

vancomycin-resistant *Leuconostoc* spp. and *Pediococcus* spp. were analysed. None of these microorganisms produced a PCR product.

## DISCUSSION

Since VRE are a significant nosocomial pathogen [20–22], it has become necessary to develop a simple, rapid, and cost-effective detection system to replace or complement the time-consuming conventional culture method. This study was intended to determine whether PCR could be used to accurately detect VRE in clinical and nosocomial surveillance specimens. The multiplex PCR assay developed in this study was found to be a convenient and rapid method for detecting and identifying VRE directly from presumptive culture-positive broth.

Similar PCR methods have been reported previously [23]. However, the PCR products amplified by these methods are not consistent with the estimated sizes, especially in the amplification of the *vanB* gene. The sizes of the *vanB* PCR products of these methods are variable due to the presence of three different *vanB* subtypes, B1, B2, and B3. This observation has also been reported by Dahl et al. [24]. In this study, we designed a new set of *vanB* primers capable of generating PCR products that are the same size from all three *vanB* subtypes. Our method also performs better on enrichment culture broth with a sensitivity of 97.9% (46/47) in the detection of VRE than other methods that detected only 85.1% (74/87) [25]. Our method also does not require restriction enzyme analysis in order to type vancomycin-resistance genes, whereas the method of Patel et al. [26] requires *MspI* digestion in order to distinguish *vanA* from *vanB* because the sizes of these two PCR products are identical.

Although the conventional culture method for detection and identification of VRE is quite sensitive, it requires at least 96 h to complete [25]. The turnaround time for the multiplex PCR developed in this study for detecting VRE starting from inoculation of BEAB-V8 enrichment broth was about 36 h. In addition, the cost for the multiplex PCR assay including labor was cheaper (US\$11.30 per assay) than that for the conventional culture method (US\$16.13 per assay). However, applying PCR method directly on every rectal swab specimens for detecting and identifying VRE is not cost-effective because the prevalence of VRE is usually quite low. In our hospital, it is only 17% of the presumptive growth in BEAB-V8 cultures of approximately 250 surveillance specimens per month. PCR would become cost-effective if it is applied to VRE selected by the BEAB-8 culture. Although the PCR method described in this study will yield vancomycin-resistance genotypes, it does not provide strain types that are normally determined by pulsed-field gel electrophoresis (PFGE). A separate PFGE typing would have to be performed.

The identification of vancomycin-resistance genotypes of VRE by the multiplex PCR worked well in this study. All VRE isolates from clinical or surveillance specimens produced a PCR product. The 40 clinically isolated vancomycin-susceptible *E. faecium* or *E. faecalis* and all vancomycin-resistant *Leuconostoc* and *Pediococcus* isolates examined in this study failed to generate a product of the expected size. One false-negative PCR result was observed from a BEAB-V8 culture positive broth. Since repeated multiplex PCR using DNA purified from the broth culture yielded the expected 996-bp product, this false-negative PCR result may be due to inhibition by certain components in the culture broth. Overall, results of this study suggest that the multiplex PCR assay has sufficient sensitivity and specificity to detect and identify VRE from clinical and nosocomial surveillance specimens.

Originally, we used Enterococcosel broth containing 6 µg/ml of vancomycin to screen VRE from rectal swabs and yielded results similar to those of previous reports [25, 27] in which approximately 30–40% of enterococci discovered were VanC VRE, such as *E. gallinarum* or *E. casseliflavus*. Although these low-level vancomycin resistance strains can be clinically important [28], they have not been associated with wide dissemination of vancomycin resistance. Therefore, a higher concentration of vancomycin was used.

With Enterococcosel broth containing 8 µg/ml (BEAB-V8) of vancomycin, all the VRE isolated in this study were either VanA or VanB VRE.

The *vanA* gene typically confers a high level of resistance to vancomycin (MIC ≥ 64 µg/ml) and teicoplanin (MIC ≥ 16 µg/ml), and the *vanB* gene normally results in a moderate level of resistance to vancomycin (MIC = 16–64 µg/ml) [1]. However, the *van* genotypes determined in this study by the multiplex PCR were not completely consistent with the vancomycin/teicoplanin-resistance phenotypes (Table 1). The discrepancies are summarized as follows: (1) 8 *vanA E. faecium* and 16 *vanA E. faecalis* isolates with the VanB phenotype, (2) 3 *vanB E. faecium* and 2 *vanB E. faecalis* isolates with the VanC phenotype, and (3) one *vanC1+vanA E. gallinarum* isolate with the VanA phenotype. These discrepancies have also been reported previously [2–5].

Recently, intrinsic vancomycin-resistant *E. gallinarum* and *E. casseliflavus* isolates have been found to exhibit a high-level of resistance to glycopeptide due to the presence of both *vanA* and *vanC1* genes or both *vanA* and *vanC2* genes [16, 29]. Thus, the vancomycin-resistance genotype of an isolate cannot always be inferred from its phenotype, and vice versa. These findings reaffirm the importance of routine vancomycin-resistance genotyping of VRE isolates, especially for investigation of outbreaks or for nosocomial surveillance [30]. Our multiplex PCR assay would be useful for these studies. Genotyping in combination with phenotyping of vancomycin resistance for VRE will provide useful information for patient treatment and for the implementation of appropriate infection-control measures.

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