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ORIGINAL ARTICLE

Faster and economical screening for vancomycin-resistant enterococci by sequential use of chromogenic agar and real-time polymerase chain reaction

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vanB

Abstract *Background/Purpose:* Screening for vancomycin-resistant enterococci (VRE) by culture takes days to generate results, while polymerase chain reaction (PCR) testing directly from clinical specimens lacks specificity. The aims of this study were to develop a real-time PCR to detect and identify *Enterococcus faecium*, *Enterococcus faecalis*, and *vanA* and *vanB* genes, and to evaluate the impact of this PCR on test-reporting times when performing it directly from suspect VRE isolates present on screening chromogenic media.
Methods: The tetraplex PCR primers were designed to amplify *E. faecium*, *E. faecalis*, and *vanA* and *vanB* genes, with melt-curve analysis of PCR products. Following analytical and clinical validation of the molecular assay, PCR testing was performed for target colonies present on VRE chromogenic media. PCR results were evaluated against conventional phenotypic identification and susceptibility testing, with the time to result being monitored for both modalities.
Results: A total of 519 colonies from clinical specimens were tested concurrently by real-time PCR and phenotypic methods. In all, 223 isolates were identified with phenotypic vancomycin resistance (*vanA*, $n = 108$; *vanB*, $n = 105$; non-*vanA*/*vanB* = 10), with complete agreement between PCR and phenotypic testing for vancomycin-resistant *E. faecium* and *E. faecalis*. The majority (88.6%) of PCR results were reported, on average, 24.8 hours earlier than those of phenotypic testing, with 68% reduction in total costs.
Conclusion: The use of culture on selective media, followed by direct colony PCR confirmation allows faster and economical VRE screening.

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Introduction

Since their emergence in the early 1980s, vancomycin-resistant enterococci (VRE) have become significant health care-acquired global pathogens. Asymptomatic colonization of patients and residents of long-term care facilities has been demonstrated to be frequent,[1] and this may result in widespread environmental contamination.[2] Active surveillance for VRE detects asymptomatic carriers, with the potential to reduce in-hospital transmission.[3] However, laboratory methods for the detection of VRE vary in performance. Routine culture on standard media with phenotypic identification and susceptibility testing takes 2—4 days for results to become available. Chromogenic selective media have improved the specificity and sensitivity of screening cultures,[4] but provide only presumptive identification of VRE, requiring confirmation by standard phenotypic methods.

Direct molecular detection of VRE from clinical specimens has been evaluated by multiple studies using conventional polymerase chain reaction (PCR),[5] real-time PCR,[6] and commercial molecular kits.[7,8] Although PCR assays have high clinical sensitivity, clinical specificity is lower and generally confounded by the presence of nonenterococcal enteric bacteria that possess the *vanB* gene.[5,7,8]

Combination of primary culture on chromogenic media and rapid confirmation of suspect colonies by PCR has the potential to provide both improved specificity and faster reporting times. The aims of this study were to develop a real-time PCR to detect and identify *Enterococcus faecium*, *Enterococcus faecalis*, and *vanA* and *vanB* genes from bacterial colonies, and to evaluate the impact of this PCR on test reporting times when performing it directly from suspect colonies present on chromogenic media.

Methods

This study was performed in an 800-bed tertiary referral hospital that provides acute medical and surgical services. VRE screening was routinely performed for inpatients on renal dialysis therapy and inpatients with a length of hospitalization exceeding 14 days. Inpatient contacts of known or identified carriers with VRE were also screened. Perianal or rectal swabs were plated onto chromogenic agar (Brilliance VRE agar; Oxoid, Basingstoke, UK) and enterococcal agar containing 6 mg/L of vancomycin (VRE Agar; Oxoid, Basingstoke, UK). Media were incubated at 35°C in ambient air for 40—48 hours, and visually inspected for suspected colonial growth after 18—24 hours and 40—48 hours of incubation, with subsequent confirmatory test performed at the first visible growth. Suspected VRE colonies on either medium were subcultured to nonselective media (Trypticase Soy agar with 5% Sheep Blood; BD, Franklin Lakes, New Jersey, USA) for phenotypic identification and susceptibility testing (Vitek ID-GP and AST-P67; BioMérieux, Marcy l'Etoile, France).

The real-time PCR assay was a multiplex reaction with primers targeted at the *ddl* gene in *E. faecium* and *E. faecalis*,[9] with a second primer set targeted at the *vanA* and *vanB* genes that mediate vancomycin resistance in enterococci.[10] PCR reaction was performed in 20 μL volume containing 10 μL of 2× MeltDoctor HRM Master Mix (with SYTO-9 dye; Life Technologies, Singapore) and primers (0.2 μm/L of *E. faecium* forward/reverse primer each, 0.3 μm/L of *E. faecalis* forward/reverse primer each, 0.3 μm/L of *vanA* and *vanB* common forward primer, 0.15 μm/L of *vanA* reverse primer, and 0.4 μm/L of *vanB* reverse primer). The PCR reaction was performed on a real-time thermocycler (CFX96; Bio-Rad, Hercules, California, USA) with the following cycling conditions: preheated at 95°C for 10 minutes and then 35 cycles at 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 45 seconds. The amplification cycles were followed by a melting cycle from 65°C to 90°C with incremental temperature escalation at a rate of 0.2°C every 5 seconds. The presence of targeted PCR products was determined by standard melt-curve analysis; presence of specific melt peaks indicated the presence of the targeted genes (Table 1).

The real-time PCR was developed in two phases. In the first phase, the analytical sensitivity and specificity of species component of the real-time assay were assessed against a test panel consisting of 35 bacterial strains (16 different bacterial genera, including 5 different enterococcal species). Analytical sensitivity of the *vanA* and *vanB* gene components was tested using four known strains of vancomycin-resistant *E. faecium* and *E. faecalis* from external proficiency testing schemes, with the resistance gene component confirmed by conventional PCR.[11] In the second phase, clinical specificity testing for the real-time PCR was evaluated using 203 bacterial isolates taken directly from primary VRE culture media. Speciation results from the real-time PCR were compared against phenotypic identification. The presence of the *vanA* or *vanB* gene confirmed by the real-time assay was interpreted as an indication of vancomycin resistance, and this was compared against phenotypic susceptibility testing, while the specific *van* genotype results were compared against the results of conventional PCR[11] (Figure 1).

Following analytical and clinical validation, the real-time PCR assay was introduced for routine clinical testing (Figure 1). PCR was performed directly from suspected colonies present on screening media. If only a single colony was present on primary culture, PCR testing was performed the next day from the subcultured isolate. A single bacterial colony was inoculated directly from culture media into 100 μL of molecular-grade water and vortexed. The suspension (2 μL) was used as the template for PCR testing. Conventional phenotypic testing was performed in parallel, as previously described. Molecular testing was available from Monday to Friday, while phenotypic testing was available from Monday to Saturday. The time to results for PCR and phenotypic testing were taken, respectively, as the time when the PCR result was available, and the time when phenotypic identification and susceptibilities were available.

The cost of VRE identification was based on the direct cost of consumables used for testing. The time to perform a single test was calculated by noting the actual hands-on time required for both phenotypic or molecular testing, and recording the number of isolates tested per batch of testing. Incubation time and PCR cycling time were not included for this calculation. The test time required for each isolate was the total hands-on time required for each

Table 1 Multiplex real-time PCR used for differentiation of *Enterococcus* species and *van* A/B genotypes.

Oligonucleotide	5′—3′ sequence	Target gene	Melting peak T_m (°C; ± 1 SD)
Van AB-Aus-F	GTAGGCTGCGATATTCAAAGC	*vanA/B*	NA
Van A-Aus-R	CGATTCAATTGCGTAGTCCAA	*vanA*	80.2 (± 0.15)
Van B-Aus-R	GCCGACAATCAAATCATCCTC	*vanB*	83.0 (± 0.17)
Enterococcus faecalis-HRM-F	GTGGCTTAAGTCGCTGTGAT	$ddl_{E.\ faecalis}$	73.2 (± 0.2)
E. faecalis-HRM-R	AGGCATGGTGTTCAATTCAT		
E. faecium-HRM-F	TTTACAAGCTGCTGGTGTGC	$ddl_{E.\ faecium}$	75.4 (± 0.19)
E. faecium-HRM-R	AACCCATATTCGCAGGTTTG		

NA = not available; PCR = polymerase chain reaction; SD = standard deviation.

batch of testing, divided by the number of isolates tested. Testing time was measured for five separate batches of testing, and the average testing time per isolate was calculated. For both test methods, the costs of reagents and consumables were calculated for each test, which included the cost of appropriate positive and negative controls for each batch of molecular test. Prices were estimated in Singapore dollars (SGD) and converted to United States dollars (USD), based on an exchange rate of 1 SGD = 0.78 USD.

Results

The analytical sensitivity and specificity of the real-time PCR assay for identification of *E. faecium*, *E. faecalis*, and *vanA* and *vanB* genes was 100%, when evaluated against the test panel. A total of 203 clinical isolates were phenotypically identified as *E. faecalis* (n = 38), *E. faecium* (n = 141), and nonenterococcal isolates (n = 24), with 55 isolates identified as VRE. The clinical sensitivity and specificity of the real-time PCR was 100% for the evaluated 203 clinical isolates, with no discordant results for either bacterial speciation or vancomycin resistance. Conventional PCR performed on the 55 VRE isolated demonstrated the presence of the *vanA* gene in 23 isolates and the *vanB* gene in 32 isolates. There was 100% agreement between conventional PCR and real-time PCR for differentiation of the *van* genotypes.

A total of 519 routine clinical specimens with suspect colonies growing on screening agar were tested concurrently with real-time PCR and standard phenotypic methods. Of these specimens, 296 were negative for VRE (vancomycin-susceptible *Enterococcus* spp., n = 295; *Streptococcus durans*, n = 1). The remaining 223 specimens were positive, by phenotypic testing, for vancomycin-resistant *Enterococcus* species (*E. faecium*, n = 208; *E. faecalis*, n = 4; *Enterococcus gallinarum*, n = 9; *Enterococcus casseliflavus*, n = 1; and *Enterococcus avium*, n = 1). There was complete concordance for speciation of *E. faecalis* and *E. faecium* when PCR results were compared with the results of phenotypic testing. *VanA/B* genes were present in 213 isolates. One enterococcal isolate was positive, by PCR, for the *vanA* gene only and negative for *E. faecium*/*E. faecalis* genes, and this was phenotypically identified as vancomycin-resistant *E. avium* by Vitek ID-GP (Table 2). The presence of phenotypic glycopeptide resistance was concordant with the presence of *vanA/B* genes by real-time PCR, while conventional PCR and real-time PCR showed complete agreement for the differentiation of the *vanA* and *vanB* genes.

For all tested samples, PCR results were available earlier than the phenotypic testing results (mean 21.6 hours; range, −31.2—91.2 hours). Of all PCR results, 460 (88.6%) were available sooner than phenotypic results, and these results were available, on average, 24.8 hours before phenotypic testing results were available. For the remaining 59 (11.4%) tests, PCR results were available later than phenotypic results by an average of 11.5 hours.

Technologist hands-on time for each tested isolate was 4.0 minutes for phenotypic testing and 3.6 minutes for molecular testing. The cost of consumables and reagents per test was 18.00 USD for phenotypic testing, compared with 5.80 USD for molecular testing.

Discussion

Screening for carriers of VRE has been demonstrated to both reduce cross-transmission within health-care institutions and improve identification of unrecognized carriers.[12] Culture methods perform well, but require relatively long testing and reporting times. PCR detection of *Enterococcus*-specific genes and *van* gene determinants directly from clinical specimens has been evaluated in multiple studies, with varying results. In general, detection of the *vanA* gene demonstrates good specificity for the presence of VRE, but specificity for the *vanB* gene is lower.[7,13,14]

Other studies have previously demonstrated the technical feasibility of performing VRE detection from colonial growth following standard culture. Palladino and colleagues[6,15] initially used fluorescence resonance energy transfer (FRET)-based probes from plate culture and then extended this to direct testing from enrichment broths. A second study performed DNA extraction from enrichment broths, followed by the use of a commercial kit.[16] This study differed from the preceding ones by the use of chromogenic agar for primary screening of VRE from clinical specimens, with suspect colonial growth confirmed by real-time PCR. Our aims were to design a real-time PCR method that was easy, quick to perform, and of low cost, and improved the time to result. We achieved this by using a direct colony extraction method (reducing the cost of extraction consumables and hands-on time, and negating the need for expensive automated DNA extraction technology) and melt-curve analysis rather than more expensive probe-based methodologies. The study methods used

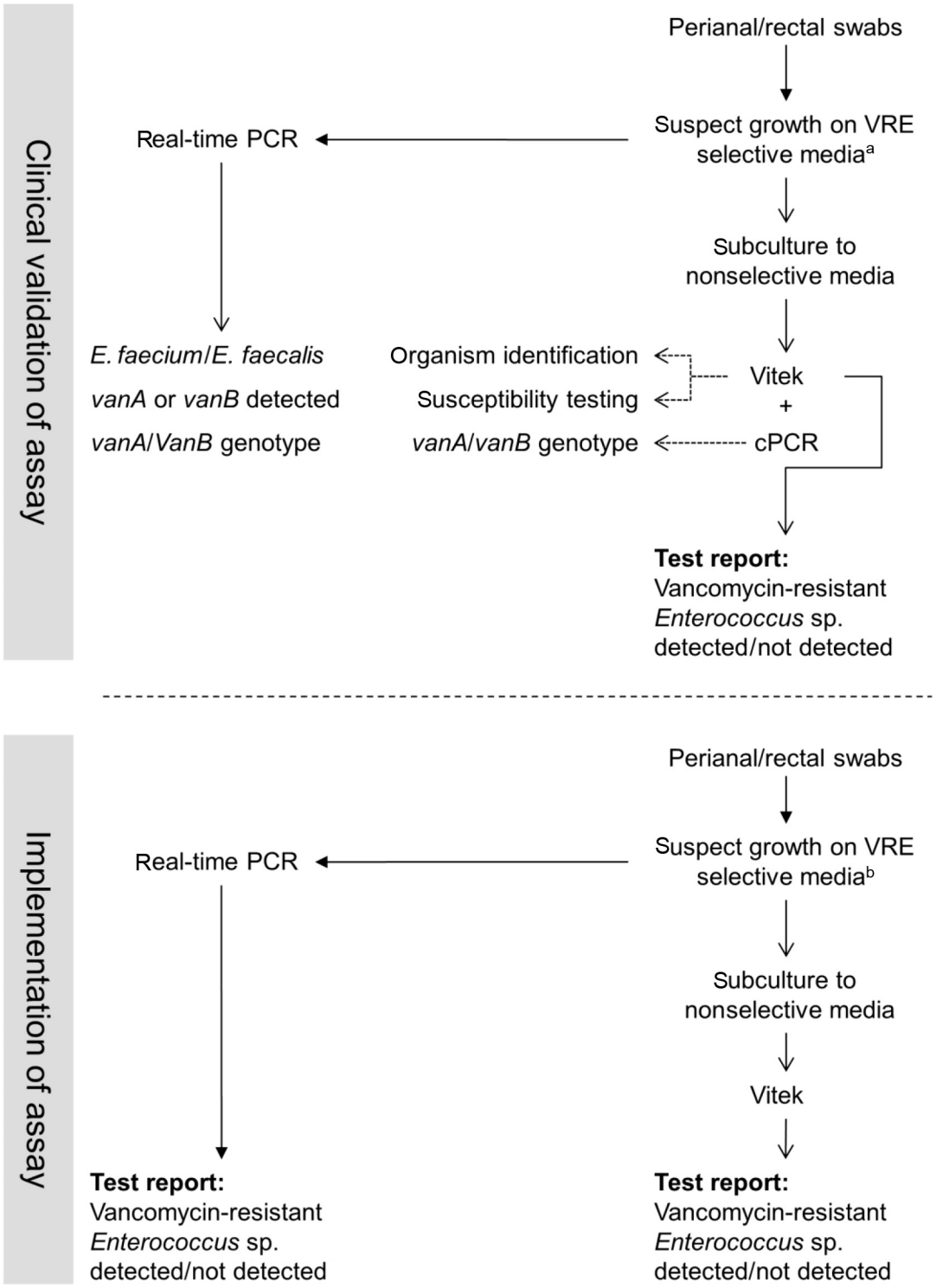

Figure 1. Validation and clinical implementation of real-time PCR in conjunction with culture. [a] Isolates tested: *Enterococcus faecium vanA* ($n = 22$), *E. faecium vanB* ($n = 32$), *E. faecalis vanA* ($n = 1$), vancomycin-susceptible *E. faecium* ($n = 87$), vancomycin-susceptible *E. faecalis* ($n = 37$), nonenterococci ($n = 24$). [b] Isolates tested: *E. faecium vanA* ($n = 103$), *E. faecium vanB* ($n = 105$), *E. faecalis vanA* ($n = 4$), *E. avium vanA* ($n = 1$), vancomycin-susceptible *E. faecium* ($n = 198$), vancomycin-susceptible *E. faecalis* ($n = 91$), other enterococci ($n = 16$), other streptococci ($n = 1$). cPCR = conventional polymerase chain reaction; PCR = polymerase chain reaction; VRE = vancomycin-resistant enterococci.

Table 2 Isolation of vancomycin-resistant *Enterococcus* species and detection of *vanA*/*vanB* genes by real-time PCR following implementation of assay.

Species	*Van* gene by real-time PCR		
	vanA	*vanB*	*vanA*/*vanB* not detected
Enterococcus avium	1		
Enterococcus casseliflavus			1
Enterococcus faecalis	4		
Enterococcus faecium	103	105	
Enterococcus gallinarum			9
Total	108	105	10

PCR = polymerase chain reaction.

combine the specificity of culture, while improving test reporting times and reducing test costs.

This method has some limitations. As PCR testing is not typically available on weekends, phenotypic testing may generate earlier results for a proportion of cases (11% of tests, in this study). The real-time PCR is restricted to the detection of the *vanA* and *vanB* genes, and does not detect the presence of either the intrinsic *vanC* gene of *E. casseliflavus* or *E. gallinarum*, or the other newly described transmissible *van* genes.[17] The significance of potentially missing non-*vanA*/*vanB* genes will depend on the local epidemiology of circulating genes in VRE. In general, the *vanA* and *vanB* determinants remain the most common causes of acquired vancomycin resistance in *E. faecium* and *E. faecalis*. Finally, analysis of cost is always subject to regional prices, and each individual laboratory needs to evaluate cost effectiveness of any test method based on local data.

Matrix Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) MS is potentially useful for rapid speciation. However, identification of the species may be dependent on the culture media from which the colony is tested,[18] and the accuracy of MALDI-TOF identification directly from chromogenic media would require validation. In addition, growth of *Enterococcus* species on VRE-selective chromogenic media still requires confirmation of vancomycin resistance, as most media lack sufficient specificity. For example, in the evaluation of VRE Brilliance media by Ongut and colleagues,[19] only 20 out of 52 isolates with positive suspect growth on the media were confirmed as true VRE.

In summary, VRE screening on selective media followed by PCR confirmation of suspect VRE isolates allows positive results to be available 24 hours earlier than conventional phenotypic testing methods.

Conflicts of interest

The authors declare no conflicts of interest.

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