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Rapid Detection of *vanA* and *vanB* Genes Directly from Clinical Specimens and Enrichment Broths by Real-Time Multiplex PCR Assay

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A real-time PCR assay previously developed for use on the Roche LightCycler platform was investigated as an alternative to culture for the direct detection of vancomycin-resistant enterococci (VRE) in clinical specimens. PCR primers and fluorescence resonance energy transfer hybridization probes specific for the *vanA* and *vanB* genes were combined in a multiplex real-time PCR assay performed directly with fecal material obtained by rectal swabbing and with enrichment broth samples. DNA was prepared from the rectal swabs and enrichment broths with a commercially available DNA preparation column designed specifically for use with fecal specimens. One hundred eighty duplicate rectal swabs were obtained from 42 patients who were previously found to be positive for VRE and who were being monitored for carriage of VRE. Direct and enrichment broth cultures were performed with one swab, while PCR was performed with the other swab as well as any corresponding presumptive positive enrichment broth. In total, 100 specimens from 30 patients remained positive for VRE by at least one method. The multiplex real-time PCR was positive for 88 enrichment broths of rectal swabs from 27 patients but for only 45 rectal swabs from 15 patients. Direct culture was positive for VRE for only 43 specimens from 11 patients, while enrichment broth culture was positive for VRE for 75 specimens from 22 patients. Inhibition studies for the multiplex real-time PCR assay, performed by spiking the DNA extracts from 50 negative rectal swabs and the corresponding enrichment broths with between 1 and 10 CFU of a VanB *Enterococcus faecium* strain, detected inhibition rates of 55.1 and 10%, respectively. PCR performed directly with enrichment broths was found to be significantly more sensitive than enrichment broth culture ($P < 0.025$). Negative samples were identified significantly earlier by PCR than by culture alone.

In recent years enterococci have increasingly become responsible for serious clinical and nosocomial infections, including endocarditis, bacteremia, and urinary tract infections (4). They are now recognized as the third most prevalent cause of nosocomial bacteremias (10). The increase in the incidence of enterococcal infections is partly a result of the increasing numbers of immunocompromised patients but is also a result of the spread of multiresistant enterococci. The emergence and spread of glycopeptide resistance in enterococci has become a significant clinical concern, and vancomycin-resistant enterococci (VRE) are now an increasingly important universal problem in hospitals worldwide.

Rapid and accurate identification of VRE is crucial in the management and treatment of both colonized and infected patients, to allow selection of appropriate antimicrobial treatment and to prevent the spread of VRE by implementing appropriate infection control procedures (7). Culture-based screening methods for VRE are typically time-consuming and can take from 1 to 5 days to complete (9, 11, 15, 16). The phenotypic methods used at present for the detection of glycopeptide resistance are also limited in their abilities to detect low-level glycopeptide resistance and to distinguish between the different Van types (3, 6, 17). Molecular methods based on PCR for the detection of glycopeptide resistance were first described in 1995 (3). PCR-based molecular methods performed with enterococcal isolates have been demonstrated to be feasible alternatives to phenotypic methods for the detection of glycopeptide resistance (3, 6, 9). Some investigators consider them to be superior, as they overcome the limitations of phenotypic methods, while they provide advantages in terms of the time taken to obtain a result (1, 2, 16).

Many hospitals now have surveillance programs for VRE. However, most use culture-based detection methods, which have inherent limitations. Delays of up to 5 days to obtain a result significantly affect the timely implementation of appropriate infection control procedures, including patient isolation and cohorting. Therefore, many microbiology laboratories have recently introduced PCR for confirmation of the presence of isolates of VRE to facilitate the rapid and accurate identification of these organisms. The application of PCR for the detection of VRE directly from clinical surveillance specimens or enrichment broths can further reduce the detection time (11, 15, 16, 18). Those studies that have used conventional PCR have reported various degrees of sensitivity and high degrees of specificity, thus providing encouraging results for the direct detection of VRE in these specimens.

The advent of real-time PCR technology offers the potential for confirmation of the presence of VRE more rapidly than is possible by either conventional PCR or phenotype-based methods. The LightCycler (Roche Molecular Biochemicals, Mannheim, Germany) is a commercially available instrument de-

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signed to rapidly perform both the PCR and the real-time fluorescence-based detection of the PCR product in a closed system. We have previously reported on the performance of a real-time PCR assay with the LightCycler system for the simultaneous detection of the *vanA* and *vanB* genes in enterococcal isolates (14). In this paper we describe the application of the real-time PCR assay directly with rectal swabs and enrichment broths for the detection of VanA and VanB VRE. The performance of the real-time PCR was evaluated with clinical surveillance specimens from a cohort of patients who were previously found to be positive for VRE and who were being monitored for carriage of VRE. The results of real-time PCR testing of these samples were compared to those of screening by traditional direct culture and enrichment broth culture combined with biochemical identification and antimicrobial susceptibility testing.

MATERIALS AND METHODS

Specimens. One hundred eighty duplicate rectal swab specimens were obtained from 42 patients who were previously found to be positive for VRE (VanA or VanB strains) and who were being monitored for carriage of VRE. Direct and enrichment broth cultures were performed with one swab, while PCR was performed with the other swab as well as any corresponding presumptive positive enrichment broth. The allocation of which swab in a set was tested by culture or PCR was random. The control strains of enterococci used in the real-time PCR assay were an *Enterococcus faecalis* strain containing the *vanB* gene (ATCC 51299) and a clinical isolate of *E. faecium* containing the *vanA* gene (ATCC 27637).

Culture media and conditions. Rectal swabs for culture were first inoculated directly onto CHROMagar Orientation medium agar plates (CHROMagar Microbiology, Paris, France) containing 6 mg of vancomycin per liter and 8 mg of gentamicin per liter (COVG) and were then placed into Enterococcosel enrichment broth (BBL Microbiology Systems, Cockeysville, Md.) containing 8 mg of vancomycin per liter (EV broth). The COVG plates were incubated at 35°C and examined after 24 and 36 h of incubation. The EV broths were incubated at 35°C and examined after 24 to 36 h of incubation. Brown or black EV broths were subcultured onto CHROMagar Orientation medium containing no antibiotics.

Conventional organism identification and susceptibility testing. Blue colonies resembling enterococci on the COVG plates or the CHROMagar subculture of the EV broth were further identified by conventional laboratory methods, including Gram staining and determination of colonial morphology and biochemical and growth characteristics (5) and were tested for the presence of the *vanA* and *vanB* genes by PCR (14). Isolates that were likely VRE were screened for vancomycin resistance by using brain heart infusion agar (BHIA) containing 6 mg of vancomycin per liter and BHIA containing 16 mg of vancomycin per liter. The vancomycin susceptibility of each isolate was confirmed by disk susceptibility testing by National Committee for Clinical Laboratory Standards (NCCLS) methods (13). The MICs of vancomycin and teicoplanin for the isolates were determined by the Etest (AB Biodisk).

DNA extraction. The fecal material from the swab used for PCR was suspended in 0.5 ml of sterile water by vortexing vigorously for 20 s. Aliquots (200 μl) of these suspensions and aliquots (500 μl) of the EV broths positive for VRE were stored at −20°C until they were ready for processing. Specimens were stored for no longer than 3 weeks, with the majority being processed within a few days. The QIAamp DNA Stool Mini kit (Qiagen Inc., Hilden, Germany) was used to extract the DNA from both specimens. Two hundred-microliter samples of the thawed rectal swab suspension or EV broth were processed according to the instructions of the manufacturer, except that the extracted DNA was eluted into 50 μl instead of 200 μl of Buffer AE (Qiagen Inc.).

Real-time PCR assay. A multiplex PCR suitable for use on the Roche LightCycler platform and capable of identifying the *vanA* and *vanB* genes simultaneously was developed. The oligonucleotide primers, hybridization probes, and reaction conditions used for this assay have been reported previously (14). NCCLS guidelines, which include contamination control measures and the use of DNA-negative samples as reaction controls in every run, were followed for all DNA manipulations (12).

Assessment of inhibition of real-time PCR assay. To estimate the underlying PCR inhibition rate, the DNA extracted from 50 negative rectal swabs and corresponding enrichment broths of specimens from a subset of patients determined to be negative for VRE by both PCR and culture were spiked with an amount of DNA from a VanB *E. faecalis* strain equivalent to 1 to 10 CFU per total reaction mixture. The inhibition rate was determined to be 55% for rectal swabs and 10% for EV broths (data not shown). This may be a very sensitive method for the assessment of inhibition, as 1 to 10 CFU was determined to be the lower limit of detection for the PCR assay (data not shown).

Resolution of discordant results. The performance of the real-time PCR was evaluated with clinical surveillance specimens from a cohort of patients who were previously found to be positive for VRE, as confirmed by culture, and who were being monitored for carriage of VRE. For the purposes of this study, a positive result by any of the methods under investigation was considered a true-positive result.

TABLE 1. Detection of VRE by PCR and culture in 180 specimens from 42 patients

Parameter evaluated	No. (%) positive	
	Specimens (n = 180)	Patients (n = 42)
Total positive by PCR or culture	100 (100)	30 (100)
EV broth PCR positive	88 (88)	27 (90)
Rectal swab PCR positive	45 (45)	15 (50)
EV broth culture positive	75 (75)	22 (73.3)
Direct culture positive	43 (43)	11 (36.7)
Direct or EV broth culture positive	77 (77)	22 (73.3)

RESULTS

Overall detection of VRE. In total, 100 specimens from 30 patients remained positive for VRE by at least one method (direct culture, enrichment broth culture, PCR directly with a rectal swab, or PCR with EV broth) during the evaluation period (Table 1).

Detection of VRE by culture. Seventy-seven (77%) specimens from 22 (73.3%) patients were positive for VRE by at least one culture method (either direct culture of a rectal swab on COVG or culture following enrichment in EV broth; Table 1). Seventy-three specimens from 21 patients were found to contain VanB isolates of VRE, and four specimens from 1 patient were found to contain VanA isolates of VRE. In our hands, the performance of direct culture of the rectal swab was poor compared with that of culture of the enrichment broth, with only 43 (43%) specimens from 11 (36.7%) patients being positive for VRE on the COVG plate used for direct culture but 75 (75%) specimens from 22 (73.3%) patients being positive for VRE by use of the EV broth (Table 1).

Detection of VRE by PCR. PCR with EV broth detected 88 (88%) of the 100 positive specimens from 27 (90%) of the 30 patients positive for VRE (Table 1). Four specimens from one patient were found to contain VanA isolates of VRE, and the remaining 84 specimens from 26 patients were found to contain VanB isolates of VRE. The sensitivity of the PCR performed directly with the rectal swab specimen for the detection of VRE was poor compared to that of the PCR with EV broth. This was not surprising, given the levels of inhibition identified for each specimen type (55 and 10%, respectively). Only 45 (45%) specimens from 15 (50%) patients were found to be positive for VRE by the PCR performed directly with rectal swab specimens (Table 1). However, the PCR with the rectal swab specimen identified an additional three (3%) positive swabs from three (10%) patients that were not detected by the

TABLE 2. Comparative performance of real-time PCR with EV broths for detection of VRE

Test and result	No. of specimens with the following result by real-time PCR with EV broth:	
	Positive	Negative
Culture methods combined		
Positive	68	9
Negative	20	83
Rectal swab PCR		
Positive	42	3
Negative	46	89

PCR with EV broth, direct culture, or enrichment broth culture (Table 2). VRE were not detected by any of the methods in three additional specimens from one of these three patients, while for the other two patients VRE were detected in only the one specimen.

Performance of PCR with EV broth compared to that of culture for detection of VRE. In total, 97 specimens were positive by either the PCR with EV broth, direct culture on COVG plates, or enrichment broth culture. Table 2 compares the performance of the PCR with EV broths with that of direct and enrichment broth (EV broth) culture combined for the detection of VRE in these specimens. Although the PCR with EV broth detected VRE significantly more often in specimens positive for VRE than both culture methods combined ($P < 0.025$; McNemar's test), nine specimens from six patients were negative by the PCR with EV broth but culture positive. However, other specimens that were positive by the PCR with EV broth were collected from all six patients. The 20 specimens that were positive by the PCR with EV broth but culture negative were from 10 different patients. For 6 of these 10 patients, from whom 8 of the 20 specimens were collected, at least one other specimen that was culture positive was collected during the study.

Time to result and cost of performing PCR and culture. As no culture was required, the most rapid results were available when PCR was performed directly with the rectal swab (between 3 and 4 h, depending on the number of specimens processed). The PCR with EV broth required an additional 24 h for incubation of the EV broth prior to performance of the PCR (total time, 24 to 28 h). The time to the retrieval of results for the two culture-only-based methods was between 24 h and 5 days, depending on what additional tests were required to exclude or include any presumptive strain of VRE isolated.

Based on the testing of a batch of 28 patient samples, the total cost of performing each of the PCR assays was $12.49 (all values are in Australian dollars) per sample for the PCR. This comprised the costs for the reagents ($9.12) and labor ($3.37). For the PCR with EV broth there was also the additional cost of the EV broth ($0.44). In comparison, the total cost of performing the combined culture method (direct plating plus culture in EV broth) for VRE was $15.41 per sample, which comprised the costs for the reagents ($6.43) and labor ($8.98). This was an average cost for the culture method and will vary greatly, depending on the degree of organism identification required and the susceptibility studies performed.

DISCUSSION

Molecular methods such as PCR have been shown to have advantages over phenotypic methods for the detection and identification of VRE by overcoming the limitations of phenotypic methods for the detection of low-level glycopeptide resistance and to distinguish between the different Van types, while they reduce the time required to detect and identify these organisms (1, 2, 3, 6, 9, 16). This has led to the increased use of PCR methods for confirmation of the presence of isolates of VRE.

The application of PCR for the detection of VRE directly in clinical or surveillance samples or enrichment broths can provide an even more rapid means of detection of VRE and allow the more timely implementation of infection control interventions. The few studies to date that have applied PCR for the detection of VRE in this manner have reported various results. The sensitivities of PCRs performed directly with enrichment broths of rectal specimens ranged from 85.1 to 97.9% and had 100% specificities (11, 16, 18). Studies of PCRs performed directly with fecal samples or rectal swabs have reported sensitivities ranging from 67.8 to 87.2% on initial testing of samples, with their specificities approaching 100% (15, 18). However, none of these studies used the real-time PCR technology, which is less labor-intensive than conventional PCR methods and which provides a rapid, sensitive, and specific means for the detection and identification of infectious agents while reducing the risk of contamination by a previously amplified product.

We have previously reported on the performance of a real-time PCR assay with the LightCycler system for the simultaneous detection of the *vanA* and *vanB* genes in enterococcal isolates (14). In this study we have assessed the performance of this assay directly with rectal swabs and enrichment broths as an alternative to culture for the detection of VanA and VanB isolates of VRE.

Only 55.8% (43 of 77) of all culture-positive specimens were detected by direct plate culture of the rectal swab, whereas 97.4% (75 of 77) of all culture-positive specimens were detected by enrichment culture with EV broth. The sensitivity of direct culture was relatively low compared to those reported elsewhere, in which the sensitivities of the direct culture method ranged from 63.4 to 89.1% (16, 18). However, the performances of direct and enrichment broth cultures for the detection of VRE vary greatly depending on the medium used and the load of VRE in the original sample. Other investigators have reported sensitivities as low as 53.4% (8).

Likewise, the sensitivity of the PCR performed directly with rectal swabs reported in this study (45% of all positive specimens) is lower than those reported in other studies, in which investigators have reported sensitivities ranging from 67.8 to 87.2% for PCRs performed directly with fecal or rectal swab specimens (15, 18). The relatively high level of PCR inhibition (55.1%) demonstrated for the rectal swab specimens appears to be a factor that contributes to the low sensitivity of the direct PCR reported in this study. However, the low yield of VRE obtained by direct culture compared to that obtained by en-

richment broth culture suggests that it is likely that a number of the patients in our study were excreting small numbers of VRE in their stool specimens during the time of the study and that this could also be a contributing factor to the low sensitivity of the direct PCR method compared to the sensitivities obtained in other studies.

In our study, the PCR with EV broth was the most sensitive method for the detection of VRE, detecting 88% of all positive specimens, whereas the next best method was direct and enrichment broth culture combined, which detected 77% of all positive specimens. Other studies have reported the sensitivity of PCR with enrichment broth samples to be comparable to that of culture for the detection of VRE (11, 15, 16, 18). This is the first report of the performance of a PCR method with enrichment broth samples which was found to be more sensitive than either direct or enrichment culture for the detection of VRE. Our findings are possibly a result of the use of the QIAamp DNA Stool Mini kit (Qiagen Inc.), which has been specifically designed to extract DNA from stool specimens. This system was not used in any of the other studies and may be better at extracting enteroccocal DNA and/or removing PCR inhibitors from enrichment broths than the DNA extraction methods described previously. However, from the results of our study, the Qiagen QIAamp DNA Stool Mini kit did not appear to extract enteroccocal DNA and/or remove PCR inhibitors from rectal swab specimens any better than other previously described extraction methods. This indicates that further development is required to improve the process of extracting enteroccocal DNA and/or removing PCR inhibitors from stool or rectal swab specimens.

Twenty specimens were positive by the PCR with EV broth but culture negative, and eight of these specimens were from patients for whom at least one other specimen collected during the time of the study was culture positive. All the PCR-positive specimens were considered true positive even if the results could not be confirmed by culture. This could be a potential criticism of the study, as the *vanA* and *vanB* genes have been detected in organisms other than *E. feacalis* and *E. faecium* (15). We considered this approach to be valid, as all the specimens tested were from patients who had previously (within 2 months of the study) been confirmed by culture to be positive for VRE. However, the clinical and infection control significance of these PCR-positive and culture-negative specimens remains problematic, as they are likely to be from patients with either a low burden of VRE or nonviable VRE.

Although the PCR performed directly with rectal swabs provided results the most rapidly, we believe that the low sensitivity of the method for the detection of VRE makes it unsuitable as an alternative to culture for the detection of VRE. However, this study does demonstrate that PCR with EV broth is a suitable alternative.

The PCR assay with EV broth had a sensitivity greater than that of culture. Furthermore, the results were available for the majority of specimens at least 2 days earlier, and there were significant savings in labor costs with the PCR method. A limitation of the PCR method is that strain typing studies cannot be conducted with samples positive for VRE. This can generally be overcome by subculturing PCR-positive broths to recover any VRE for further investigations.

The PCR assay with EV broth is now used as the screening assay in the Royal Perth Hospital's program for surveillance for VRE. The reduced time required to obtain a result was important in the management of a recent outbreak caused by VanB isolates of VRE, as it allowed the earlier implementation of infection control measures and improved the management of contact patients.

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