Detection of Vancomycin-Resistant Enterococci in Fecal Samples by PCR

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Surveillance cultures for vancomycin-resistant enterococci (VRE) are time-consuming and expensive for the laboratory to perform. Therefore, we investigated the use of PCR as an alternative method of detecting and identifying VRE directly in fecal samples. PCR primers directed to vanA, vanB, vanC1, and vanC2, and enterococcal ligase genes were used to detect and identify VRE in fecal material obtained by rectal or perirectal swabbing. Although PCR-inhibitory substances were present in DNA prepared directly from the swabs, the inhibitory substances could be reduced by processing the nucleic acid with two commercially available DNA preparation columns. Fecal material from 333 swabs was cultured on several selective agar media before and after broth enrichment. DNA was extracted from the fecal material and was analyzed by PCR. By using all four primer sets, only 59 (67.8%) of the samples were positive for vanA. However, after retesting the negative samples with only the vanA primer set, 77 (88.5%) of 87 specimens that were culture positive for Enterococcus faecium containing vanA were positive by PCR. One specimen was PCR positive for the vanA gene but culture negative for enterococci. The specificity of the vanA assay was 99.6%. PCR analysis of enrichment broth samples with all four primers sets after 15 to 18 h of incubation detected 74 (85.1%) of the 87 culture-positive specimens. The specificity of the vanA assay after the enrichment step was 100%. No vanB-containing enterococci were recovered by culture. Since 16 samples can be tested by PCR in 4 h (including electrophoresis), identification of VRE is possible within 8 h of specimen submission at a cost of approximately $10.12/assay. Thus, PCR may be a cost-effective alternative to culture for surveillance of VRE in some hospitals.

Vancomycin-resistant enterococci (VRE) have emerged as important nosocomial pathogens in the United States and elsewhere (2, 7, 15, 19, 21, 27). These organisms are often resistant to multiple antimicrobial agents limiting the number of therapeutic options available to the physician (10, 20). Many hospitals have initiated surveillance programs for VRE; however, these programs have proven to be time-consuming for infection control personnel and expensive for the microbiology laboratory to conduct (6, 21). Several different selective media for enhancing the recovery of VRE from stool or rectal samples have been proposed, but no single medium has proven to be universally acceptable (6, 12, 17, 21, 27, 31). Nonetheless, surveys of stools or rectal swabs are recommended by the Hospital Infection Control Practices Advisory Committee to allow for the early identification of colonized patients so that infection control measures to prevent person-to-person transmission of VRE can be instituted (11).

Vancomycin is a member of a broader class of antimicrobial agents referred to as glycopeptides (1). Glycopeptide resistance genotypes in enterococci include vanA (high-level resistance); vanB, vanB2, and vanD (moderate to high-level resistance); and vanC1, vanC2, and vanC3 (intrinsically low-level resistance) (1, 5, 24–26). While the vanA resistance gene has been detected in a wide variety of enterococcal species (1, 3, 4), vanC1, vanC2, and vanC3 have been recognized only in Enterococcus gallinarum, Enterococcus casseliflavus, and Enterococcus flaveescens, respectively (1, 18, 24). There is little information in the literature regarding the occurrence of vanB outside of Enterococcus faecalis and Enterococcus faecium (1, 13, 26), and only a single isolate of vanD has been reported (25). PCR assays have been devised to recognize the vanA, vanB, and vanC1 genotypes (3, 5) and have demonstrated that enterococci may contain more than one of these determinants (4).

Detection of glycopeptide resistance in enterococci by either disk diffusion or automated susceptibility testing methods has been shown to be less sensitive than that by broth microdilution methods (13, 28, 30). However, the broth methods require a full 24 h of incubation to detect strains with low-level resistance (23). Thus, the detection of VRE, particularly in rectal surveillance cultures where vancomycin-susceptible enterococci are often present, has been a challenge. In this study, we compared the sensitivities and specificities of PCR assays for vanA, vanB, vanC1, vanC2, and the enterococcal ligase primers (5) with the sensitivity and specificity of culture with and without broth enrichment, for the detection of VRE in clinical specimens.

MATERIALS AND METHODS

Selective media and culture condition. This study was carried out in three phases, each of which used a slightly different set of selective media. Five different selective agars and three different selective broths were used to screen for growth of VRE (Table 1). Rectal or perirectal swabs were collected from 333 patients from two hospitals in the metropolitan Atlanta area, placed in Amies transport medium, and processed within 8 h of collection. The fecal material from the swabs was suspended in 350 μl of sterile water, and the mixture was
TABLE 1. Selective media and enrichment broths used in this study

<table>
<thead>
<tr>
<th>Phase</th>
<th>No. of samples</th>
<th>Enrichment</th>
<th>Temp (°C)</th>
<th>Direct</th>
<th>Agar plate</th>
<th>Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>82</td>
<td>CNA + G20</td>
<td>42 or 45</td>
<td>CNA + G20</td>
<td>CNA + G20</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>175</td>
<td>CNA + G10 + A1</td>
<td>35</td>
<td>CNA + G10</td>
<td>CNA + G10 + A1</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>76</td>
<td>G2 + A1 + Z1 + V1b</td>
<td>35</td>
<td>E + V6</td>
<td>E + V6</td>
<td></td>
</tr>
</tbody>
</table>

a) Trypticase soy broth with 15 μg of colistin per ml, 15 μg of nalidixic acid per ml, and 20 μg of gentamicin per ml.

b) Columbia CNA agar with 20 μg of gentamicin per ml.

c) Columbia CNA agar with 20 μg of gentamicin per ml and 6 μg of vancomycin per ml.

d) Trypticase soy broth with 15 μg of colistin per ml, 15 μg of nalidixic acid per ml, 10 μg of gentamicin per ml, and 1 μg of amphotericin B per ml.

e) Columbia CNA agar with 10 μg of gentamicin per ml and 1 μg of amphotericin B per ml.

f) Columbia CNA agar with 10 μg of gentamicin per ml, 1 μg of amphotericin B per ml, and 6 μg of vancomycin per ml.

| Enterococcosel agar with 6 μg of vancomycin per ml.
| Trypticase soy broth with 2 μg of gentamicin per ml, 1 μg of amphotericin B per ml, 1 μg of aztreonam per ml, and 1 μg of vancomycin per ml.}

Detection of VRE by culture methods. Ninety-nine VRE isolates were recovered from 97 of 333 rectal specimens by at least one culture method (i.e., direct plating on agar or after enrichment broth; Table 2). Of the 99 isolates, 87 were identified biochemically as E. faecium and were shown by PCR to contain vanA, 6 were identified as E. gallinarum and contained vanC1, and another 6 were identified as E. casseliflavus and contained vanC2. One specimen contained both E. gallinarum and vanA-containing E. faecium, and another specimen contained both E. gallinarum and E. casseliflavus. No vanB-containing enterococci were isolated. VRE were detected in 6 specimens by direct plating only (vanA-containing enterococci plus vanC-containing enterococci; Table 2), in 16 specimens by the use of enrichment broth only, and in 76 specimens by both techniques. Many of the specimens positive only by broth en-
The enrichment broth used in each phase of the study (Table 1). In phase I several of the E. faecium isolates did not grow when the broth was incubated at 45°C, even though this species should thrive at this temperature (9). Subcultures of the organisms which were recovered by direct plating initially detected by PCR.

### Table 2. Detection of VRE in 333 fecal samples by PCR

<table>
<thead>
<tr>
<th>Samples detected by culture (no. of samples)</th>
<th>No. (%) of samples initially detected by PCR&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Enrichment broth</th>
<th>Extracted DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>vanA-containing enterococci</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Detected by direct plating only (3)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Detected by enrichment broth only (12)</td>
<td>3 (25.0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Detected by both methods (72)</td>
<td>71 (98.6)</td>
<td>59 (81.9)</td>
<td></td>
</tr>
<tr>
<td>Total detected by culture (87)</td>
<td>74 (85.1)</td>
<td>59 (67.8)</td>
<td></td>
</tr>
<tr>
<td><strong>vanC-containing enterococci</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Detected by direct plating only (3)</td>
<td>1 (33.3)</td>
<td>3 (100)</td>
<td></td>
</tr>
<tr>
<td>Detected by enrichment broth only (4&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>3 (75.0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Detected by both methods (4)</td>
<td>4 (100)</td>
<td>1 (25.0)</td>
<td></td>
</tr>
<tr>
<td>Total detected by culture (11&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>8 (72.7)</td>
<td>4 (36.4)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Does not include repeat testing of negative samples with vanA primers only.

### Table 3. Adjusted sensitivity and specificity of PCR assay with vanA primers compared with those of culture

<table>
<thead>
<tr>
<th>PCR result in assays with the indicated source of DNA</th>
<th>No. of samples with following result by culture:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enrichment broth culture&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Positive (n = 87)</td>
</tr>
<tr>
<td></td>
<td>Negative (n = 246)</td>
</tr>
<tr>
<td>Positive</td>
<td>74</td>
</tr>
<tr>
<td>Negative</td>
<td>13</td>
</tr>
<tr>
<td>DNA extracted from rectal swab&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Positive (n = 77)</td>
</tr>
<tr>
<td></td>
<td>Negative (n = 245)</td>
</tr>
<tr>
<td>Positive</td>
<td>77</td>
</tr>
<tr>
<td>Negative</td>
<td>10</td>
</tr>
</tbody>
</table>

<sup>a</sup> Sensitivity, 74 (85.1%) of 87 samples; specificity, 246 (100%) of 246 samples.

<sup>b</sup> Sensitivity, 77 (88.5%) of 87 samples; specificity, 245 (99.6%) of 246 samples.

The detection of VRE by PCR. Of 327 enterococci isolated during the course of the study from all selective media (which included multiple isolates of the same organism from agar and broth), 325 (99.4%) could be unambiguously identified by PCR with the ligase gene primers. This included 240 E. faecium and 61 E. faecalis isolates. In addition, using the vanC1 and vanC2 primers, we confirmed the biochemical identifications for 11 E. gallinarum and 13 E. casseliflavus isolates. Two of 63 strains identified biochemically as E. faecalis consistently failed to produce a PCR product with ddl<sub>E. faecalis</sub> gene primers. Two additional strains of vancomycin-susceptible E. faecalis, both isolated from the same specimen but on different selective media, were positive for the vanA gene by PCR. The presence of vanA gene sequences was confirmed by Southern blotting DNA hybridization with a vanA-specific DNA probe (3). In addition, two strains of vancomycin-resistant E. faecium isolated from the same specimen were positive for both the vanA and vanB genes by PCR, while a third strain, this one vancomycin susceptible, also was positive for vanB by PCR (data not shown). The presence of vanB sequences in the isolates was confirmed by using a vanB-specific DNA probe and an alternate set of PCR primers (3). All 206 strains showing high-level resistance to vancomycin by broth microdilution produced vanA products by PCR. The sensitivities of the assays with vanA, vanC1, and vanC2 primer sets compared with those of the reference broth microdilution MIC method were all 100%, while the sensitivities of the assays with ddl<sub>E. faecalis</sub> and ddl<sub>E. faecium</sub> primer sets compared with the sensitivity of biochemical identification were 96.8% and 100%, respectively. The specificities were 98.4% for vanA, 99.1% for vanB, and 100% for vanC1, vanC2, ddl<sub>E. faecalis</sub>, and ddl<sub>E. faecium</sub>

**Detection of the vanA and vanC genes in fecal material by PCR.** The sensitivity of the PCR assay for detecting the vanA determinant in organisms grown on agar plates by using only the vanA primer pair was determined to be between 1 and 10
CFU (data not shown). When combined with the other three primer sets in the multiplex assay, the sensitivity of the \( \text{vanA} \) primer set dropped to approximately 10 to 100 CFU (data not shown). This change in sensitivity was also reflected in the results obtained with clinical samples.

The sensitivity of PCR for detecting the \( \text{vanA} \) and \( \text{vanC} \) genes in nucleic acid extracted directly from the fecal suspensions varied according to the extent to which the nucleic acid was purified. For example, in \( \text{vanA} \) detection by PCR for three samples in which the DNA was purified only with a QIAamp column (Fig. 2A) and the results obtained after the nucleic acid underwent an additional purification step using Centrisep columns (Fig. 2B). The results indicate that three samples would have been classified as negative if only the QIAamp column had been used. Thus, inhibitors can reduce the sensitivity of the direct PCR assays. When a 2-\(\mu\)l sample was used with all four primer sets, the \( \text{vanA} \) PCR assay detected 59 (67.8\%) of 87 specimens that were positive for \( E. \text{faecium} \) containing \( \text{vanA} \) by at least one culture method. Of 28 false-negative specimens, 3 were positive for \( E. \text{faecium} \) containing \( \text{vanA} \) by direct plating, 12 were positive by broth enrichment, and 13 were positive by both direct plating and broth enrichment. One specimen that was PCR positive for \( \text{vanA} \) was negative by all culture methods. The \( \text{vanA} \) gene was detected with 2- and 4-\(\mu\)l samples, respectively, after purifying DNA only with QIAamp spin columns; lane 7, \( E. \text{faecalis} \); lane 8, \( E. \text{faecium} \); lane 9, \( E. \text{faecalis} \); lane 10, \( E. \text{gallinarum} \); lane 11, \( E. \text{faecalis} \); lane 12, no sample DNA. (B) Lanes 1 to 6, PCR products obtained from specimens 289 to 294, respectively, after purifying DNA with QIAamp and Centrisep spin columns; lanes 7 to 12, as described above for panel A. The faint band in lane 5 did not match the size of any of the controls and was not reproducible. It was assumed to be nonspecific.

The study focused on whether PCR could be used in the laboratory to replace more traditional surveillance culture methods for the detection of VRE in colonized patients. PCR has the potential to reduce both the time and cost of detecting VRE and can provide information on the vancomycin resistance genotype, which may be useful for epidemiologic studies (3). The cost of the PCR assay was less than the cost of the two-column method depicted in Fig. 1 including four primer sets (\( \text{vanA}, \text{vanB}, \text{vanC1}, \text{vanC2} \)) was $10.12 per reaction. The cost of the reagents ($1.62/reaction including the primers), the columns (QIAamp $1.10 and Centrisep $2.40), and labor ($15.00/h \times 20 \text{ min} or $5.00 for technologist time to process 16 samples plus 4 controls) will vary significantly on the basis of the volume of assays performed and the salary of the individual performing the test. By contrast, the cost of the culture method for VRE with broth enrichment ($0.75), a single selective CNA agar plate and a nonselective CNA agar plate ($1.05), biochemicals for identification ($2.30), and labor ($15.00/h \times 17 \text{ min} = $4.25) was $15.77.

**DISCUSSION**

This study focused on whether PCR could be used in the laboratory to replace more traditional surveillance culture methods for the detection of VRE in colonized patients. PCR has the potential to reduce both the time and cost of detecting VRE and can provide information on the vancomycin resistance genotype, which may be useful for epidemiologic studies (3). The cost of the PCR assay was less than the cost of the two-column method depicted in Fig. 1 including four primer sets (\( \text{vanA}, \text{vanB}, \text{vanC1}, \text{vanC2} \)) was $10.12 per reaction. The cost of the reagents ($1.62/reaction including the primers), the columns (QIAamp $1.10 and Centrisep $2.40), and labor ($15.00/h \times 20 \text{ min} or $5.00 for technologist time to process 16 samples plus 4 controls) will vary significantly on the basis of the volume of assays performed and the salary of the individual performing the test. By contrast, the cost of the culture method for VRE with broth enrichment ($0.75), a single selective CNA agar plate and a nonselective CNA agar plate ($1.05), biochemicals for identification ($2.30), and labor ($15.00/h \times 17 \text{ min} = $4.25) was $15.77.
culture method used in the study. A survey of three microbiology laboratories in the Atlanta area suggested that the cost of VRE screening per specimen ranged from $9.55 to $19.80, depending on the degree to which the organisms were identified and whether an MIC test or simply a vancomycin agar screening plate was used to confirm the resistance profile. While the PCR assay may be cost-effective, one potential drawback to this approach is that it precludes strain typing studies, which are integral to many infection control investigations. While it would be possible, especially if an enrichment broth was used, to culture those specimens that are positive by PCR to recover enterococci for strain typing, this would increase the overall cost of the surveillance system.

Stool is the most difficult specimen on which to perform PCR due to the presence of multiple substances that inhibit the polymerase enzyme (14, 16). Because of this inhibition, the PCR assays initially proved to be less sensitive than the culture techniques for the detection of VRE. However, the sensitivity of the assay increased when only the vanA primer was used with an increased amount of target DNA. Although the primer sequences were screened for the presence of possible hairpin and dimer formation, it is likely that there are unforeseen interactions among the eight primers when used together in a single tube. The amount of fecal material used for culture also was 1 to 2 orders of magnitude higher than that used in the PCR assays, which probably contributed to the overall lower sensitivity of the PCR assay. Increasing the amount of target DNA did not significantly increase the sensitivity of the assay, probably because too much target nucleic acid is also known to inhibit PCR.

The results of our PCR assays for VRE were compared with the results of culture tests with several different selective media and with and without the aid of broth enrichment. Several studies have advocated the use of broth enrichment for surveillance of VRE (12, 31). Enterococcosel-V agar and CNA-VGA agar showed equivalent sensitivities for the recovery of vanA-containing enterococci from rectal and perirectal samples (no attempt was made in this study to assess the differences between the recovery of VRE from perirectal versus rectal swabs); however, the Enterococcosel-V agar required at least 48 h of incubation to optimize recovery of VRE. Colonies were often only pinpoint at 24 h, and additional colonies were frequently recognized at 48 h; however, prolonged incubation for up to 5 days yielded relatively few additional isolates. While colonies on CNA-VGA were often large enough to be subcultured at 24 h, this medium was more likely to show growth of yeast and occasional gram-negative organisms than was Enterococcosel-V agar. Two formulations of CNA-VGA agar, i.e., those containing 20 or 10 μg of gentamicin per ml inhibited the growth of both E. faecalis N3, which harbors a vanB gene, and the vanC2-containing E. casseliflavus strain; however, these strains grew well on Enterococcosel-V agar. Vancomycin and gentamicin probably have a synergistic effect that inhibits the growth of both vanB- and some vanC-containing enterococci. Because of the additional cost of the CNA-VGA medium and its suppression of some vanB and vanC isolates, Enterococcosel-V agar or similar bile-esculin azide agar formulations may be a better choice for surveillance cultures. We did not evaluate Enterococcosel broth; however, the CNA-based enrichment broths used in this study, which were incubated for 15 to 18 h before subculture, did enhance the recovery of VRE, although it added approximately $1.50 to the cost of culture due to the additional supplies and technologist time required for processing.

PCR assays were performed with samples taken from the enrichment broth after 15 to 18 h of incubation and with nucleic acid extracted from fecal material from rectal swabs suspended in distilled water. We had presumed that the dilution of the inhibitory substances in water would allow us to use a simple and inexpensive column purification system for the isolation of nucleic acid. However, a single column did not yield sufficiently pure DNA for amplification. Inhibition was consistently eliminated only by processing the nucleic acid with an additional commercially available DNA preparation column. Nonetheless, the cost of processing samples through the two columns at $10.12 per specimen, including all PCR-related reagents and labor, was still less than the cost of culture. Sixteen samples could be completed in 8 h from the time of receipt of the specimens. If PCR reagents are already in use in the laboratory, this cost could be further reduced.

We examined the effect of using all four primer sets (vanA, vanB, vanC1, and vanC2) versus just the single vanA primer set in the PCR assays. The use of four primer sets clearly reduced the sensitivity of the PCR assay for detecting vanA-containing enterococcal strains both with purified organisms and with stool specimens. Thus, laboratories should consider using only vanA primers, or perhaps the combination of vanA and vanB primers, and forego the detection of the vanC isolates. The significance of detecting vanC-containing VRE from rectal samples remains unclear; however, nosocomial infections caused by E. gallinarum and E. casseliflavus appear to be rare (3, 15, 21). We could not adequately evaluate the detection of vanB-containing enterococci because they occurred infrequently in the hospitals participating in this study. Given that a non-vancomycin-containing CNA agar medium was included in the protocol and all enterococci including vancomycin-susceptible organisms were identified, we feel confident that vanB-containing strains would have been recovered had they been present in the patient population sampled. In addition, the vanB primer set used has successfully been used in our laboratory and other laboratories for the past 2 years (5).

A 15- to 20-h enrichment step in selective broth incubated at 35°C enhanced the sensitivity of the culture method. Incubation of the broth at 45°C, which is considered a differential test for enterococci, inhibited the growth of many of the E. gallinarum and vanA-containing E. faecium strains recovered in this study. The enrichment broth with 20 μg of gentamicin per ml and 6 μg of vancomycin per ml was too inhibitory for vanC-containing species. In phase II, a different enrichment broth, in which the concentration of gentamicin was reduced to 10 μg/ml and to which 1 μg of amphotericin B per ml was added, incubated at 35°C was used. This improved the recovery of vanC-containing species, but some VRE were still inhibited. In phase III, the concentrations of both gentamicin and vancomycin were reduced to 1 μg/ml and 1 μg of aztreonam per ml was added. This change allowed for the growth of the vanB and vanC control organisms but did not significantly improve the overall recovery of VRE and was very expensive. Given the results of this study and those of several other investigators (12, 31), Enterococcosel broth containing 6 μg of vancomycin per ml or a similar bile-esculin azide formulation may be the best enrichment broth for use in maximizing the detection of VRE from fecal samples.

Identification of VRE by PCR worked well. Only two isolates of E. faecalis failed to produce a PCR product of the appropriate size. Biochemically, these two strains were typical of E. faecalis. Of the three vancomycin-susceptible strains that were positive by PCR for vanA or vanB, all three were also positive in assays with different PCR primers, and each hybridized with a vanA or vanB gene probe, suggesting that the genes were present in the isolates but were nonfunctional (1). (We did not test PCR primer sets to the other genes in the vanco-
mycin operon to determine whether each component of the van gene cluster was present.) This suggests that the PCR assay does have sufficiently high specificity for use with fecal samples, although some vancomycin-susceptible organisms (3 [0.9%] of 333) will be falsely classified as VRE.

In summary, culture of rectal or perirectal samples with Enterococcosel-V agar after enrichment in supplemented CNA broth at 35°C followed by biochemical species identification and broth microdilution susceptibility testing was the most sensitive method in this study for detecting all types of VRE. However, completion of this method required at least 5 days, and additional tests were needed to distinguish VRE from *Leuconostoc* and *Pediococcus*, which can be vancomycin resistant and esculin hydrolysis positive. Since 16 samples can be tested by PCR in 4 h (including electrophoresis), identification of VRE is possible within 8 h of specimen submission by PCR of DNA extracted from a clinical specimen. If enrichment broth is used, the costly step of DNA extraction is eliminated but the time to the final results is extended to approximately 30 to 36 h, although this may make it easier for laboratories to batch process specimens that arrive in the laboratory throughout the day. Thus, PCR may be an attractive alternative to VRE. However, clinical laboratories that do not have sufficiently high specificity for use with fecal samples, although some vancomycin-susceptible organisms (3 [0.9%] of 333) will be falsely classified as VRE.

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REFERENCES


