Rapid Characterization Schemes for Surveillance Isolates of Vancomycin-Resistant Enterococci

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Surveillance cultures for vancomycin-resistant enterococci (VRE) and subsequent characterization of the isolates can be extremely burdensome and difficult. Therefore, efficient and reliable schemes for the characterization of surveillance isolates are needed. In this study, a commercial agar (bile esculin azide agar with 6 μg of vancomycin per ml [BEAA]; Remel, Lenexa, Kans.) was used in the initial screening step to establish relatively rapid (i.e., in ≤24 h from the time of isolation) phenotype-based and PCR-based schemes for the detection and characterization of VRE. The phenotype-based scheme included Gram staining of growth on BEAA and subculture of cocci on sheep blood agar plates for vancomycin disk diffusion and pyrazinamidase (PYR) testing. For the PCR scheme, inocula for \( \text{van} \) gene detection were taken directly from the BEAA plates. The phenotypic approach was applied to 378 surveillance cultures that yielded growth on BEAA. Gram staining quickly eliminated gram-positive bacilli from further testing, and a negative PYR test classified 25 additional isolates as probable pediococci. A positive PYR test reliably identified single-patient VRE isolates that included 83 Enterococcus faecium, 33 E. gallinarum, and 5 E. casseliflavus strains. The vancomycin inhibition zone size clearly distinguished VanA and VanB strains from VanC strains within 24 h of BEAA isolation. All VanA and VanB strains failed to produce zones of >6 mm, while only one VanC strain produced a zone of <15 mm. Challenging this phenotypic scheme with 47 stock VRE isolates produced similar findings. In direct PCR analyses, false-negative \( \text{van}A \) and \( \text{van}B \) results occurred with 12% of the specimens. Many of the false-negative reactions also failed to produce an internal control product, which underscores the need for including control primers when a PCR scheme is used. In summary, the phenotype- and the PCR-based schemes provide efficient methods for characterizing VRE within 24 h of isolation.

The emergence and dissemination of vancomycin-resistant enterococci (VRE) has prompted recommendations for surveillance and rapid detection of these organisms (5, 10). However, screening methods to detect VRE usually do not allow the type of VRE (i.e., VanA, VanB, or VanC) to be rapidly determined. As reported by Morris et al. (12), VanC strains may be frequently detected by surveillance screens, but they rarely cause the clinical or epidemiologic problems associated with VanA or VanB strains.

Therefore, while sensitive methods for detecting VRE are needed, these methods must be coupled with procedures that allow the laboratory to rapidly determine the likely Van type. This is essential for ensuring that appropriate infection control measures be implemented in a timely fashion but only when they are truly needed. Several papers have described the utility of bile esculin and azide-based media for the isolation of VRE (3, 7, 11, 17, 18). However, strategies for incorporation of these media into protocols that allow accurate, efficient, and rapid characterization of VRE have not been established.

The purpose of this study was to develop phenotype-based and PCR-based schemes that incorporate the use of commercially available bile esculin azide agar supplemented with vancomycin for the rapid (i.e., in ≤24 h from time of isolation) characterization of VRE.

Materials and Methods

Surveillance cultures. During a 1-month surveillance period, 2,264 urine specimens, submitted for routine bacteriologic culture, and 513 stool samples, submitted for Clostridium difficile toxin assay, were inoculated onto plates containing bile esculin azide agar supplemented with 6 μg of vancomycin/ml (BEAA plates; Remel, Lenexa, Kans.). BEAA plates were incubated aerobically, at 35°C, for up to 72 h and were examined daily for growth. All organisms that grew produced colonies that exhibited a brown to black halo. These isolates were subjected to further testing for detection and characterization of VRE.

Phenotypic characterization of isolates. Growth on BEAA was initially screened with Gram stain, and gram-positive bacilli were not identified further. Gram-positive cocci were subcultured on Trypticase-based 5% sheep blood agar (SBA), and a 30-μg vancomycin disk was placed in the first quadrant. After 18 to 24 h of incubation at 35°C, the vancomycin inhibitory zone size was determined. Growth on the SBA also served as the inoculum source for pyrazinamidase (PYR) and leucine aminopeptidase (LAP) disk tests (Remel).

Organisms that gave no zone of inhibition around the vancomycin disk and were PYR negative and LAP positive were recorded as presumptive pediococci. No presumptive Leuconostoc spp. (i.e., vancomycin resistant, PYR and LAP negative) were encountered.

All suspected enterococcal isolates (i.e., PYR and LAP positive) were subcultured and identified to the species level by conventional methods as previously described (4). Antimicrobial susceptibility profiles for enterococcal isolates were established by disk diffusion and agar screen methods recommended by the National Committee for Clinical Laboratory Standards (13, 14). MICs of ampicillin and vancomycin were determined by the E test (AB Biodisk, Solna, Sweden) as previously described (16).

Characterization of isolates by PCR. Direct PCR analysis was performed by using a swab to obtain inoculum from the surface of BEAA plates and prepare a suspension in TE buffer (pH 7.5). The resulting suspension either was used for immediate PCR analysis or was frozen at −70°C for future PCR analysis. Multiple PCR primers for vanA and vanB, preparation of reaction mixtures and conditions, and detection of amplicons by gel electrophoresis were done as previously described (6, 15). As internal controls for PCR conditions, universal bacterial primers D74 and RW01 for a conserved region of the 16S rRNA gene were included in each reaction mixture (9). Besides being subjected to direct PCR after growth on BEAA, all enterococcal isolates, including stock

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culture strains, were subjected to analysis for vanA, vanB, vanC1, and vanC2 by using a subculture of each isolate as previously described (6).

**Results of direct inoculation onto SBA of cultures grown on BEAA.**

One isolate produced a zone of 12 mm, three produced zones of 15 mm, and the remainder produced zones of >15 mm. One isolate produced a zone of 15 mm. For VanC1 (E. gallinarum) and vanB-containing strains failed. For all specimens tested, there was a total of 37 false-negative vanA, vanB, and internal control reactions. Although urine specimens were submitted four times more frequently than stool specimens, 30 (81%) of the false-negative reactions occurred with BEAA plates inoculated with stool specimens. The remaining seven occurred with plates inoculated with urine specimens. False-negative vanA or vanB PCR results occurred with plated specimens that contained E. gallinarum, E. casseliflavus, pediococci, or gram-positive bacilli.

**False-negative internal control reactions were also noted with specimens that yielded E. gallinarum and gram-positive bacilli. For all specimens tested, there was a total of 37 false-negative vanA, vanB, and internal control reactions. Although urine specimens were submitted four times more frequently than stool specimens, 30 (81%) of the false-negative reactions occurred with BEAA plates inoculated with stool specimens. The remaining seven occurred with plates inoculated with urine specimens. False-negative vanA or vanB PCR results occurred with plated specimens that contained E. gallinarum, E. casseliflavus, pediococci, or gram-positive bacilli.**

**For further evaluation of the vancomycin disk and PCR characterization schemes, 47 enterococcal stock culture strains that were obtained from other geographic locations and whose van gene contents had been previously established were used (Table 3). PCR profiles produced with inocula taken directly from BEAA plates were 100% correct for all VanA, VanB, and VanC strains tested; no false positives or false negatives occurred, and the internal control amplicon was consistently obtained.**

**By vancomycin disk testing on SBA, all VanA and VanB strains gave inhibitory zones that were ≤13 mm, and all but three had zones of 6 mm. For VanC1 (E. gallinarum) and**

**Table 1. Characterization of surveillance enterococcal isolates.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Gene content</th>
<th>No. of strains (%) susceptible to:</th>
<th>Vancomycin disk zone of inhibition (mm)</th>
<th>Vancomycin MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ampicillin</td>
<td>Gentamicin</td>
<td>Streptomycin</td>
</tr>
<tr>
<td>E. faecium</td>
<td>vanA</td>
<td>68</td>
<td>1 (2)</td>
<td>66 (97)</td>
</tr>
<tr>
<td></td>
<td>vanB</td>
<td>9</td>
<td>0 (0)</td>
<td>9 (100)</td>
</tr>
<tr>
<td>E. gallinarum</td>
<td>vanC1</td>
<td>33</td>
<td>29 (89)</td>
<td>33 (100)</td>
</tr>
<tr>
<td>E. casseliflavus</td>
<td>vanC2</td>
<td>5</td>
<td>5 (100)</td>
<td>5 (100)</td>
</tr>
</tbody>
</table>

* Based on 121 single patient isolates.
* After 18 to 24 h of incubation with inoculum taken directly from BEAA plate.
* One isolate produced a zone of 12 mm, three produced zones of 15 mm, and the remainder produced zones of >15 mm.
* One isolate produced a zone of 15 mm, and the remainder produced zones of >15 mm.

**Table 2. Direct PCR characterization of isolates grown on BEAA plates.**

<table>
<thead>
<tr>
<th>Species identified in specimen</th>
<th>Gene content</th>
<th>No. of specimens (%)</th>
<th>No. of specimens (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>vanA</td>
<td>vanB</td>
</tr>
<tr>
<td>E. faecium</td>
<td>vanA</td>
<td>67</td>
<td>59 (88)</td>
</tr>
<tr>
<td></td>
<td>vanB</td>
<td>8</td>
<td>0 (0)</td>
</tr>
<tr>
<td>E. gallinarum</td>
<td>vanC1</td>
<td>5</td>
<td>0 (0)</td>
</tr>
<tr>
<td>E. casseliflavus</td>
<td>vanC2</td>
<td>4</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Pediococcus</td>
<td>NA</td>
<td>16</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Gram-positive bacillus</td>
<td>NA</td>
<td>103</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

* Based on testing growth obtained from 237 individual BEAA plates.
* NA, not applicable.
VanC (E. casseliflavus) strains, vancomycin inhibition zones ranged from 14 to 18 mm, two strains produced zones of 14 mm, and four others produced zones of 15 mm.

**DISCUSSION**

Several previous publications have described the utility of bile esculin and azide-based media supplemented with vancomycin for the isolation of VRE (3, 7, 11, 17, 18). Our results with BEAA are consistent with those of previous studies and demonstrate that this medium is highly selective for vancomycin-resistant gram-positive bacteria. As such, BEAA provides a reliable starting point for the laboratory detection of resistant enterococci. However, growth of colonies with black halos on BEAA must be monitored by accurate and efficient methods for characterizing the organisms isolated.

Our data emphasize the importance of developing an effective characterization scheme for quickly identifying VanA and VanB VRE. In all, 46% of the surveillance specimens that yielded growth on BEAA contained gram-positive bacilli and/or probable pediococci. Furthermore, of the 121 single-patient enterococcal isolates found during surveillance, 31% were either E. gallinarum or E. casseliflavus (i.e., VanC strains). These results are similar to those reported by Morris et al. (12), in whose study approximately 40% of the enterococci discovered during surveillance were E. gallinarum. Although VanC strains can be clinically important (8), the intrinsically low level of vancomycin resistance these organisms exhibit has not been associated with wide dissemination of vancomycin resistance. In addition, they do not present the therapeutic dilemma so frequently characteristic of VanA and VanB strains of E. faecium and E. faecalis.

In the study by Morris et al. (12), no VanC strains were isolated from clinical specimens, even though these organisms were commonly isolated from surveillance cultures. Our experience was the same. Although VanC enterococci were isolated from 31% of the surveillance specimens, no bacteremia with VanC enterococci was detected during the surveillance period. In contrast, during the same time period, seven patients suffered bacteremia with VanA E. faecium. These findings substantiate the hypothesis that the isolation of VanC strains from surveillance cultures is not likely to be of immediate importance to infection control and infectious disease personnel, especially if these personnel are already heavily burdened with responses to patients infected with or carrying VanA or VanB strains. This underscores the importance of a laboratory’s ability to rapidly and reliably distinguish VanA and VanB enterococci from other less significant enterococcal isolates. Furthermore, because these more potent resistance genes can occur in enterococcal species other than E. faecium or E. faecalis, it is important that detection schemes not heavily rely on species identification (1, 2).

As pointed out by Van Horn et al. (18), confirmation of VanA or VanB enterococci isolated from bile esculin and sodium azide selective broth requires 48 h from the time growth is detected, and studies that establish the time needed to characterize strains isolated on agar-based selective media have not been reported. The results of the present study have allowed us to develop reliable and relatively rapid (≤24 h) schemes for identification of VanA and VanB enterococci isolated on BEAA plates. One scheme is based on phenotypic analysis, and the other is based on PCR analysis (Fig. 1).

The scheme begins with the duration of BEAA plate incubation. Although most (85%) of our enterococcal isolates were detected on BEAA within 48 h of inoculation (data not shown), holding the plates for a full 72 h increases the sensitivity. When growth is noted, a Gram stain is used to quickly distinguish between interference by gram-positive bacilli and the presence of gram-positive cocci that could be VRE. When the presence of gram-positive cocci is confirmed, either the phenotypic or the PCR analysis options are possible.

In the phenotypic analysis, isolates are subcultured on SBA to which a 30-μg vancomycin disk is added (Fig. 1). After overnight incubation, the vancomycin inhibition zone size is noted and a PYR disk test is performed. A negative PYR test indicates the isolate is not an Enterococcus sp., while a positive test, coupled with the organism’s ability to grow on a BEAA plate containing 6 μg of vancomycin/ml, reliably classifies the organism as an enterococcal isolate. Using these criteria, we found that the LAP test is not a necessary part of the phenotypic scheme. The vancomycin inhibition zone size is then used to categorize VRE as VanA, VanB, or VanC enterococcal isolates.

Although none of the VanA and VanB surveillance isolates produced inhibition zones of >6 mm, an overlap or near overlap in zone sizes produced by some VanB stock strains and VanC strains was noted (Tables 1 and 3). However, no VanA or VanB strains produced zones of ≥15 mm. Therefore, a buffer inhibition zone from >6 to ≤15 mm is recommended. The Van status of isolates that fall within this inhibitory zone range require confirmation based on phenotypic criteria such as vancomycin MIC determination. The MICs for VanA and VanB strains, which typically have inhibitory zones of ≤14 mm, are >16 μg/ml, whereas the MICs for VanC strains, which usually have inhibitory zones of ≥16 mm, are between 2 and 16 μg/ml. Other supportive criteria include ampicillin or high-level aminoglycoside resistance. VanC strains are not commonly resistant to either ampicillin or aminoglycosides (Table 1). Identification of the species by using recommended criteria may also be helpful (2). However, the ability of vanA to occur in enterococcal species other than E. faecium or E. faecalis must be kept in mind as a limitation of these criteria (1, 2). Alternatively, the status of such strains could be definitively established by PCR analysis, as described in the present study.

The phenotypic analysis scheme has been in place at our institution since the completion of the present study. Retrospective PCR analysis has shown that the phenotypic scheme correctly categorized 125 of 126 isolates (99%) as VRE. The single misidentified isolate resulted from a false-negative PYR test. Approximately 1% of the isolates encountered produced zones of >6 to ≤15 mm and required further analysis as recommended in Fig. 1.

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**TABLE 3.** Characterization of stock enterococcal isolates based on PCR and vancomycin inhibition zones on SBA

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Vancomycin MIC (μg/ml)</th>
<th>No. of strains (%)</th>
<th>Vancomycin inhibition zone (mm) on SBA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Vanomycin disk zone of inhibition (mm) on SBA&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>vanA (5)</td>
<td>512</td>
<td>5 (100)</td>
<td>5 (100)</td>
<td>6</td>
</tr>
<tr>
<td>vanB (15)</td>
<td>≧4</td>
<td>0 (0)</td>
<td>15 (100)</td>
<td>15 (100)</td>
</tr>
<tr>
<td>vanC1 (11)</td>
<td>4–16</td>
<td>0 (0)</td>
<td>11 (100)</td>
<td>14–18</td>
</tr>
<tr>
<td>vanC2 (10)</td>
<td>4–8</td>
<td>0 (0)</td>
<td>10 (100)</td>
<td>14–18</td>
</tr>
</tbody>
</table>

<sup>a</sup> The genotypes comprised strains as follows: vanA, E. faecium and E. faecalis; vanB, E. faecalis and E. faecium; vanC1, all E. gallinarum; vanC2, all E. casseliflavus.

<sup>b</sup> Following 18 to 24 h of incubation.

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*VanC2 (E. casseliflavus)* strains, vancomycin inhibition zones ranged from 14 to 18 mm, two strains produced zones of 14 mm, and four others produced zones of 15 mm.
The PCR approach investigated would allow the Van status of an isolate to be established without the need for subculture, thus providing a quicker turnaround than can be achieved with the phenotypic scheme. Although 100% accuracy was obtained with stock culture inocula taken directly from BEAA plates (Table 3), false-negative PCR results were noted with inocula obtained directly from surveillance plates, especially those that had been inoculated with stool specimens. These results suggest that interfering substances from the specimen may be introduced into the PCR mixture along with the inoculum. In most instances, the false-negative results were detected by the concomitant failure of the internal control product to amplify. Based on these findings, direct PCR testing with inoculum from BEAA plates appears to be a useful approach. However, inclusion of an internal control in the PCR mix is essential. When uncertainty about the results is encountered, repeat PCR analysis from a subculture or phenotypic analysis is warranted.

Because of the financial challenges facing clinical microbiology laboratories, the cost of performing these screens should be considered. At our institution, the phenotypic approach (including the BEAA plate, PYR disk, blood agar plate, and 30-μg vancomycin disk) costs approximately $2.15 per isolate, and materials for the PCR scheme cost approximately $3.00 per isolate. In comparison, the conventional identification scheme used to identify enterococcal species costs approximately $5.15 per isolate. Therefore, the costs of performing these schemes are reasonable; especially if one considers the extraordinary cost to an institution that can result from inaccurate or misleading information about the isolation of VRE.

In summary, the need to perform surveillance for detection of VRE can be extremely burdensome for clinical microbiology personnel and many other health care professionals. Protocols that allow for the most efficient use of resources and provide the most clinically relevant information are needed. The characterization schemes developed in this study employ a commercially available selective and differential medium as the springboard for rapid detection and characterization of VRE. However, while results obtained with these schemes provide quick and reliable information on which infection control and infectious disease specialists can act, more-thorough molecular and phenotypic follow-up analyses of isolates are often necessary to fully support epidemiologic and infection control efforts.

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REFERENCES


