Rapid Detection of Vancomycin-Resistant Enterococci

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Campylobacter blood agar with clindamycin incubated in 6% CO2 served as a medium to both screen for vancomycin resistance and select for presumptive enterococci. Colonies that grew on the medium were specifically identified as enterococci within 30 min by the pyroglutamyl-β-naphthylamide and rapid bile esculin tests. The combination of a selective medium plus rapid enzyme substrate tests offered an inexpensive means to enumerate vancomycin-resistant enterococci from specimens by using readily available reagents.

Within a short time period enterococci not only have become resistant to vancomycin but have become major nosocomial pathogens (2, 4, 6, 9). Vancomycin resistance poses a particular dilemma because resistance to this antibiotic often accompanies resistance to other previously useful antibiotics such as ampicillin and the aminoglycosides. Resistance to vancomycin has been classified into three phenotypes: VanA, VanB, and VanC (1, 10). VanA is the most troublesome because bacteria possessing this trait are absolutely resistant to vancomycin and teicoplanin. Moreover, this trait is transferable by plasmids, unlike VanB and VanC, which are chromosomal traits (3, 11, 13).

Currently, many hospitals have been obligated to devote significant resources to the isolation and containment of patients with vancomycin-resistant enterococci (VRE). In addition to the diagnosis of patients infected with VRE, laboratories are faced with performing large numbers of surveillance cultures for these bacteria. The clinical microbiology laboratory of our 900-bed hospital is currently receiving approximately 50 specimens per week to rule out vancomycin-resistant enterococcal carriage in patients. The ability of the laboratory to rapidly identify carriage of VRE is particularly important since patients may be isolated, restricted from movement within the hospital and to extended-care facilities, and treated with a variety of antimicrobial agents until it can be demonstrated that they are free of this microbe.

A laboratory procedure was developed to meet the following needs: rapid elucidation of VRE from surveillance cultures, especially stool samples; utilization of readily accessible media and tests; and sensitivity and specificity of greater than 90%.

MATERIALS AND METHODS

Susceptibility testing of bacterial isolates. All antibiotic disk diffusion susceptibility tests of clinical isolates were performed by National Committee for Clinical Laboratory Standards methods (12). For each isolate, MICs were also determined in microdilution plates (Radiometer Inc., Westlake, Ohio).

Rapid determination of vancomycin resistance. In order to determine vancomycin resistance, media with a concentration of vancomycin equal to or greater than 10 μg/ml were considered optimum. At 10 μg/ml, enterococci expressing the clinically important VanA and VanB phenotypes should grow, whereas those expressing the clinically less significant VanC phenotype would not. Therefore, of the routine bacteriological media available, campylobacter blood agar (which contains 10 μg of vancomycin per ml) (Becton-Dickinson, Cockeysville, Md.) was the prime candidate. Martin-Lewis agar (which contains 4 μg of vancomycin per ml) (Becton-Dickinson) and Enterococcus (Difco, Detroit, Mich.) with 6 μg of vancomycin added per ml was also tested. A 5% Tryptase-based sheep blood agar plate was inoculated for use as a non-antibiotic-containing control plate. The media were incubated under the following atmospheric conditions: normal ambient air, 6% CO2, and 5% O2 (campylobacter atmosphere). In addition, plates were incubated at 35 and 42°C. Identification of a colony as enterococci required positive results of both of the following rapid (30-min) tests: the rapid bile esculin (RBE) test (5) and the pyroglutamyl-β-naphthylamide (PYR) test (8); it also required a Gram stain showing gram-positive cocci. Moreover, these results were confirmed by an extended series of biochemical tests (7).

Determination of efficiency of the rapid procedure. Vancomycin-susceptible and -resistant clinical enterococcal isolates, plus isolates that have been used as National Committee for Clinical Laboratory Standards biological standards, were diluted in normal physiological saline to approximately 102 and 103 CFU/ml to serve as controls. First, in order to determine percent recovery and quality of the recovered colonies, pure cultures of each isolate at each of the two concentrations were inoculated directly onto the surface of the test agar media. Second, to determine the percent recovery from stool samples and to assess the ability of the rapid method to discriminate between VRE and other enteric microorganisms, the test isolates and standards were mixed into stool surveillance specimens received by the clinical laboratory that were determined to be free of enterococci by prescreening. A small amount of stool material was added to 10 ml of each bacterial suspension, vigorously mixed, and then plated in the standard way onto the surfaces of the test agars. Lastly, 146 stool specimens from patients known to be carrying VRE were individually inoculated on the test media to assess which of the methods provided the best overall results in terms of utility and accuracy.

RESULTS AND DISCUSSION

It was found that VRE produced the largest and most distinct colonies on campylobacter blood agar incubated at 35°C in 6% CO2. Colonies were equivalent in size to those...
 grown on Trypticase-based 5% sheep blood agar. Growth was somewhat better on campylobacter blood agar than on Martin-Lewis agar. Vancomycin-susceptible enterococci did not grow on campylobacter blood or Martin-Lewis agar. However, on campylobacter, Martin-Lewis, and Enterococcosel-plus-vanco-
mycin agars, species of Lactobacillus that produced colonies similar in appearance to, although smaller than, enterococci 
were isolated from approximately 25% of the stool specimens. These lactobacilli also had positive bile-esculin, PYR, and 
RBE test results. Only lactobacilli and enterococci grew on 
the selective media; there were no other colonial types that resembled enterococci. Accordingly, susceptibility tests were 
performed for the lactobacilli to attempt to find an antibiotic 
to which the lactobacilli were susceptible and the enterococci 
were resistant. It was found that for all lactobacilli the MICs 
of clindamycin were ≤0.5 μg/ml, whereas all enterococci were 
highly resistant (MICs, >50 μg/ml). Therefore, all tests were 
repeated with two additions in parallel: on half of the plates a 
2-μg clindamycin disk was placed at the point of inoculation, 
and on the other half of the plates a solution of 75 μg of 
clindamycin per ml was rubbed on the surface of the agar with 
a sterile dacron swab, the liquid was allowed to dry, and the 
specimen was inoculated. As determined by calculation, a 
centration, at the surface, of approximately 8 μg/ml re-
resulted.

It was found that the addition of a clindamycin disk or 
clindamycin solution to the surfaces of the campylobacter 
plates inhibited the growth of lactobacilli while not affecting 
the growth of VRE. There were no colonial types other than 
those consistent with enterococci. The use of clindamycin 
obviated the need to perform numerous Gram stains to 
differentiate lactobacilli from enterococci. Although it was 
somewhat easier to place a clindamycin disk on the surface 
of an agar plate than to swab it with a solution, there were 
occasions in which there were too few colonies in the primary 
inoculum to demonstrate a distinct zone size. Therefore, it was 
decided that the clindamycin solution would be used routinely. 
It was found that this solution could be conveniently made by 
adding 2 ml of normal saline to a vial of 300 mg of injectable 
clindamycin obtained from the pharmacy. From this 150-mg/ml 
solution, a final concentration of 75 μg/ml was made in distilled 
water and frozen in 1-ml portions at −20°C until used. When 
needed, the clindamycin solution was brought to room 
temperature and 1 ml was spread across the surface of a plate 
and allowed to dry. The individual growth characteristics on 
the various media and results of biochemical tests are presented 
in Tables 1 and 2. While no Pediococcus or Leuconostoc species 
were recovered during this study, members of each of these 
genera possess characteristics that should allow distinction 
from enterococci (clindamycin susceptible, PYR test negative, 
Gram stain appearance).

Quantitative recovery of VRE from bacterial suspensions 
mixed with enterococci-free stool samples was greater than 
90% (Table 3). Enterococci colonies were distinct, and 
lactobacillus colonies caused no interference. Among the 146 
stool specimens from patients known to carry VRE, the rapid 
detection method found 143 positives, for a recovery of 98% 
on a presence-absence basis.

Therefore, the definitive procedure utilized campylobacter 
blood agar plates onto which 1 ml of a 75-μg/ml solution of 
clindamycin was spread over the surfaces and allowed to dry. 
The plates were incubated in 6% CO2 at 35°C and examined at 
18 h. Each colony was tested for its ability to produce positive 
RBE and PYR reactions. Colonies that were positive for these 
characteristics (growth on selective medium and positivity by 
the RBE and PYR tests) were identified as VRE. These results 
were confirmed by an extended range of biochemical and 
physiological tests (7).

The rapid elucidation of VRE is important for both labora-
tory practice and patient management. Hospitals are beginning 
to devote significant resources toward epidemiological surveil-
ance of these bacteria and, in many cases, are isolating

### Table 1. Characteristics of microbes from surveillance stool specimens on selective media

<table>
<thead>
<tr>
<th>Medium or plate and condition</th>
<th>Growth characteristica of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Enterococci</td>
</tr>
<tr>
<td>Enterococci + vancomycin (6 μg/ml)</td>
<td></td>
</tr>
<tr>
<td>Ambient air, 35°C</td>
<td>1+</td>
</tr>
<tr>
<td>Ambient air, 42°C</td>
<td>1+</td>
</tr>
<tr>
<td>CO2, 35°C</td>
<td>2+</td>
</tr>
<tr>
<td>Campy gas, 42°C</td>
<td>1+</td>
</tr>
<tr>
<td>Martin-Lewis</td>
<td></td>
</tr>
<tr>
<td>Ambient air, 35°C</td>
<td>3+</td>
</tr>
<tr>
<td>Ambient air, 42°C</td>
<td>3+</td>
</tr>
<tr>
<td>CO2, 35°C</td>
<td>4+</td>
</tr>
<tr>
<td>Campy gas, 42°C</td>
<td>3+</td>
</tr>
<tr>
<td>Campylobacter blood agar plate</td>
<td></td>
</tr>
<tr>
<td>Ambient air, 35°C</td>
<td>3+</td>
</tr>
<tr>
<td>Ambient air, 42°C</td>
<td>3+</td>
</tr>
<tr>
<td>CO2, 35°C</td>
<td>4+</td>
</tr>
<tr>
<td>Campy gas, 42°C</td>
<td>3+</td>
</tr>
<tr>
<td>Campylobacter blood agar plate + clindamycin (8 μg/ml)</td>
<td></td>
</tr>
<tr>
<td>Ambient air, 35°C</td>
<td>3+</td>
</tr>
<tr>
<td>Ambient air, 42°C</td>
<td>3+</td>
</tr>
<tr>
<td>CO2, 35°C</td>
<td>4+</td>
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<tr>
<td>Campy gas, 42°C</td>
<td>3+</td>
</tr>
<tr>
<td>Blood agar plate</td>
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<tr>
<td>Ambient air, 35°C</td>
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<tr>
<td>Ambient air, 42°C</td>
<td>3+</td>
</tr>
<tr>
<td>CO2, 35°C</td>
<td>4+</td>
</tr>
<tr>
<td>Campy gas, 42°C</td>
<td>3+</td>
</tr>
</tbody>
</table>

a Growth characteristics: 4+, largest colonies; 3+, moderately sized colonies; 2+, colonies reduced in size; 1+, colonies minimally visible; 0, no growth.

b Campy gas, 5% O2–10% CO2–85% N2.

### Table 2. Reactions of enterococci and lactobacilli isolated on vancomycin-containing selective media

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Result or characteristic</th>
<th>Gram stain</th>
<th>Susceptible to clindamycin (8 μg/ml)</th>
<th>RBE test</th>
<th>PYR test</th>
<th>Growth on 6% NaCl</th>
<th>Bile-esculin test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterococci</td>
<td></td>
<td>Positive, cocci</td>
<td>No</td>
<td>Strong positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Lactobacilli</td>
<td></td>
<td>Positive, rods</td>
<td>Yes</td>
<td>Weak positive</td>
<td>Positive</td>
<td>Weak positive</td>
<td>Positive</td>
</tr>
</tbody>
</table>
patients who harbor the organisms. Moreover, patients often remain in isolation until the microbes can no longer be isolated from stool samples and body sites. In response to this need, a rapid method to isolate VRE from clinical specimens by using readily available reagents was developed.

ACKNOWLEDGMENTS

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REFERENCES


