Convenient Selective Differential Broth for Isolation of Vancomycin-Resistant *Enterococcus* from Fecal Material

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Studies have shown that vancomycin broth enrichment is superior to direct plating for the detection of vancomycin-resistant enterococcus (VRE), but vancomycin selective broth is not generally commercially available. We developed an easy-to-prepare VRE selective differential broth and compared it to direct plating on bile esculin azide (BEA) agar for the isolation of VRE from fecal samples. A total of 528 consecutive rectal swabs and stools were inoculated onto BEA agar and into BEA broth with vancomycin at a concentration of 15 μg/ml (BEA VAN$_{15\mu g/ml}$ broth). After 1 to 2 days of incubation, broths were subcultured to BEA VAN$_{6\mu g/ml}$ agar. Bile esculin-positive colonies from the direct and broth subculture plates were evaluated for the presence of VRE by standard microbiological techniques. Addition of the broth enrichment step led to the detection of significantly more VRE isolates than did direct plating alone (28 versus 18 VRE isolates, respectively). In all, 30 VRE strains were isolated from 29 cultures, all of which were *Enterococcus faecium*. MICs of vancomycin ranged from 32 μg/ml ($n = 2$) to > 256 μg/ml ($n = 28$). Twenty-two VRE isolates were available for further testing: sixteen exhibited a VanA phenotype and six were of the VanB phenotype. *van* genotypes were in agreement with phenotypes for all VRE isolates except one, which could not be genotyped. The broth method also resulted in significantly fewer bile esculin-positive, non-VRE isolates requiring further workup. We have thus developed an easily prepared vancomycin selective differential broth that is significantly more sensitive and specific in the detection of VRE than is direct fecal plating to BEA agar.

The prevalence of vancomycin (VAN)-resistant *Enterococcus* (VRE) nosocomial infections has dramatically increased in recent years (14, 24). Current recommendations for hospital infection control include VRE fecal surveillance cultures, but the optimal methods for these cultures are unclear (6, 13). Nucleic acid amplification techniques for the identification of VRE in feces continue to gain acceptance. However, there is a continued need for an improved culture method of VRE detection (i) for the collection of viable isolates necessary for epidemiological and antimicrobial susceptibility studies and (ii) by those clinical microbiology laboratories that do not have access to nucleic acid amplification technology (8, 12, 17, 18). VAN broth enrichment has been shown to be superior to direct plating on VAN-containing media for VRE isolation, but VAN broth is not generally commercially available, and the in-house manufacturing of VAN selective broth is beyond the capability of many clinical microbiology laboratories (5, 7, 11, 22). Because of these problems associated with the use of VAN broth, commercially produced VRE selective differential agars containing VAN at concentrations of 6 to 8 μg/ml (e.g., bile esculin azide [BEA] agar with VAN) are often used. The use of

VAN at concentrations from 6 to 8 μg/ml is predicated upon the work of Swenson et al. and enables the reliable detection of *vanC* as well as *vanA* and *vanB* enterococcal strains (20). But, while high-level VAN resistance due to *vanA* or *vanB* in strains of *Enterococcus faecium* and *Enterococcus faecalis* clearly presents therapeutic difficulties, the clinical significance of *vanC* enterococci appears minimal (2, 9, 16, 23). We thus reasoned that increasing the level of VAN to 15 to 16 μg/ml would eliminate many of the false positives caused by *vanC*-containing non-*E. faecium*-*E. faecalis* enterococci (VAN MIC ≤ 16 μg/ml), while still detecting the majority of clinically important *vanA* and *vanB* strains. This level of VAN could theoretically be attained by placing a single 30-μg VAN disk into 2 ml of the enterococcus selective BEA broth (final VAN concentration, 15 μg/ml), both of which are commercially available. We report here the results of our evaluation of this selective differential medium.

(This work was presented in part at the American Society for Microbiology's 100th General Meeting in Los Angeles, Calif.)

MATERIALS AND METHODS

Members of our hematopoietic stem cell transplant population are routinely screened for gastrointestinal carriage of VRE. Over a 3-month period from October 1999 to March 2000, 528 consecutive fecal samples, consisting of rectal swabs or freshly passed stool, were received in the microbiology laboratory from 233 patients (median number of samples per patient, 2; range, 1 to 10). Each of these samples was cultured for VRE using both our standard direct plating method (referred to herein as the direct method) and the VAN broth enrichment method (referred to herein as the broth method). The direct method consisted

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of transferring stool or rectal swabs to BEA agar plates, incubating the plates at 35°C for 48 to 72 h in ambient air, and then selecting bile esculin-positive colonies of differing morphology for identification using standard microbiological techniques. Resistance to VAN was screened for using brain heart infusion (BHI) agar with VAN at a concentration of 6 μg/ml; VAN MICs were then determined by E-Test for all enterococcal isolates positive on the BHI screen. In the broth method, one 30-μg VAN disk designed for disk diffusion susceptibility testing was added to each tube containing 2 ml of BEA broth. The tubes were then briefly vortex mixed and allowed to elute overnight. Broths were then lightly inoculated by swabs dipped in stool or by rectal swab and were then briefly vortex mixed. After overnight incubation at 35°C in ambient air, the presence of growth and the bile esculin reaction were recorded for each broth tube. All broths were then subcultured to BEA agar with VAN at a concentration of 6 μg/ml (VAN was included to suppress the breakthrough growth of non-VRE bacteria). After incubation, the subculture plates were worked up for VRE as in the direct method.

vanA and *vanB* PCR analysis was performed using the method of Perez-Hernandez et al. (19). Briefly, DNA was extracted from pure cultures of each isolate with the Qiagen (Valencia, Calif.) DNeasy Tissue Kit. *E. faecalis* ATCC 51299 was used as a *vanB* positive control, *E. faecalis* 290-0583 (Via Christi Regional Medical Center, Wichita, Kans.) was used as a *vanA* positive control, and *E. faecalis* ATCC 29212 was used as a negative control. Primers used were A1 (5′-GGGAAAACGACAATTGC-3′) and A2 (5′-GTACAATGCGGCCGT TA-3′) for *vanA* and B1 (5′-ATGGGAAGCCGATAGTC-3′) and B2 (5′-GAT TTCGTTCCTCGACC-3′) for *vanB*. Separate 50-μl *vanA* and *vanB* reaction mixtures were performed with 1 μl of target DNA, 100 pmol of primers, and 2.5 U of *Taq* DNA polymerase (Stratagene, La Jolla, Calif.). Amplification was done on a Bio-Rad (Hercules, Calif.) I-cycler with a thermocycling profile of 94°C melting for 4 min, and then 30 cycles of 94°C melting for 30 s, 55°C annealing for 30 s, and 72°C extension for 60 s, with a final extension at 72°C for 10 m. PCR products of the expected size (*vanA*, 732 bp; *vanB*, 635 bp) were detected by gel electrophoresis in 1.5% agarose.

All media, VAN disks, and E-Test strips were purchased from Remel, Inc. (Lenexa, Kans.). Teicoplanin (TEC) MICs were determined by E-Test. VAN and TEC MICs were interpreted using NCCLS criteria (15). The level of VAN in our test broth was determined using the AxSYM vancomycin II fluorescence polarization immunoassay (Abbott Labs, Abbott Park, Ill.).

Statistical analysis was performed using logistic regression models with robust variance estimates calculated using the method of generalized estimating equations (26). This method appropriately accounts for the fact that multiple samples were utilized from the same subject in comparisons of proportions of VRE-positive tests, and the proportions of tests positive for any growth, between the two culture methods. All *P* values were two sided, and a *P* of <0.05 was considered to be statistically significant.

RESULTS

Six tubes of BEA broth with VAN at a concentration of 15 μg/ml (BEA VAN$_{15μg/ml}$) were analyzed and found to have a mean VAN concentration of 16.5 (range 15.4 to 17.9) μg/ml. Broth with this level of VAN was found to support the growth of *E. faecalis* ATCC 51299, a *vanB*-containing strain having a measured VAN MIC of 16 μg/ml, resulting in robust growth and a strongly positive bile esculin hydrolysis reaction after overnight incubation (21). In contrast, *E. faecalis* ATCC 29212, for which the MIC of VAN is 4 μg/ml, did not grow in this broth.

From 528 fecal cultures, a total of 29 cultures (5.5%) were positive for VRE by either method. Of these 29 cultures, 28 were detected by the broth method and 18 were detected by the direct method, a significant difference in favor of the broth method (Table 1). All VRE strains but one isolated by the direct method were also isolated by the broth method. All VRE were *E. faecium*; for 27 of these the MICs of VAN were >256 μg/ml and for 1 of them the MIC of VAN was 32 μg/ml. One culture yielded VRE isolates by both broth and direct methods for which the MICs of VAN were 32 and >256 μg/ml, respectively. The broth method produced significantly fewer

bile esculin-positive, non-VRE isolates (Table 1). The vast majority of nonenterococcal bile esculin-positive isolates were *Lactobacillus* sp. (data not shown). On average, colonies took 3 days to grow to sufficient size on the broth subculture plates, versus 2 days on the direct primary plates. In addition, the overnight incubation of the broth tubes added one additional day, for a total of 4 days before colony workup could begin for the broth method, versus 2 days for the direct method.

We also ascertained whether subculture of bile esculin-positive (i.e., black) broths only from the broth method would identify all VRE. During a 1-month period, 45 fecal samples were evaluated by the standard broth method (i.e., blind subculture of all broths after overnight incubation regardless of appearance). The colors of the broth tubes were also noted after overnight incubation. The tubes were then reincubated for 24 h, after which they were again subcultured, and their colors were noted. Two VRE isolates were recovered from these 45 samples: of these two, one broth had turned black after overnight incubation, and both were black by 48 h of incubation (Table 2). Using this modified broth approach resulted in 62% fewer broths worked up at 24 h, and 38% fewer broths at 48 h.

Of the original 30 VRE isolates, 22 were available for phenotypic and genotypic testing. Of the 22, 16 were found to be resistant to VAN and TEC, consistent with a VanA phenotype, while 6 were resistant to VAN but sensitive to TEC, indicating a VanB phenotype. PCR analysis for *van* genotype confirmed these findings for 21 or 22 isolates; one isolate that displayed the VanB phenotype could not be typed by PCR. Of the 15

TABLE 1. Comparison of BE-positive isolates as identified by broth and direct culture methods[a]

Isolate type	Number (%) of BE-positive isolates as identified by method		*P*
	Direct	Broth	
VRE	18 (3.4)	28 (5.3)	0.02
Non-VRE	426 (80.7)	193 (36.6)	<0.001
VSE	395 (92.7)	24 (12.4)	NC
Not *Enterococcus*	31 (7.3)	169 (87.6)	NC

[a] Abbreviations: BE, bile esculin; VSE, VAN-sensitive or -intermediate *Enterococcus*; NC, not calculated. Percentages based upon a total of 528 cultures for VRE and non-VRE isolates; non-VRE isolates are further broken down into VSE and non-enterococcal isolates.

TABLE 2. Subculture of bile esculin-positive and -negative BEA VAN$_{15μg/ml}$ broth cultures[a]

Subculture result[b]	No. of isolates with BEA VAN$_{15μg/ml}$ broth result after incubation of:			
	24 h		48 h	
	BE$^+$	BE$^-$	BE$^+$	BE$^-$
No growth	10	17	11	11
Growth				
Not VRE	6	10	15	6
VRE	1	1	2	0

[a] Abbreviations: BE$^+$ bile esculin-positive reaction (i.e., broth turns black) (*n* = 45); BE$^-$, bile esculin-negative reaction.
[b] Broth subcultured to BEA VAN$_{6μg/ml}$ agar after 24 and 48 h.

patients with at least one isolate available for phenotypic and genotypic analysis, ten patients were found to exclusively carry *vanA* strains, 4 carried *vanB* strains only, and 1 had both a *vanA* strain and a second strain with a VanA phenotype that could not be typed by PCR.

DISCUSSION

While the value of culture-based surveillance for VRE has been firmly established, the best method for carrying out this surveillance has not been determined. Various commercially available VAN-containing solid media, including BHI, Martin-Lewis, *Campylobacter*, and BEA agars, have been evaluated (4, 11, 20). While BEA agar with VAN is the only medium among this group to differentially identify VRE by virtue of the bile esculin hydrolysis reaction, it was found to be inferior to the others in its ability to cultivate VRE directly from feces by Landman et al. (11), a characteristic we have noted as well (unpublished observations). This may be due to both the highly selective nature of the BEA VAN agar as well as the anticipated complexities of *van* expression in the bacterial host. A number of studies have found that the inclusion of a VAN broth enrichment step provides sensitivity superior to that achieved by direct agar culture (5, 7, 11, 22). In spite of this, broth VRE selective media are not readily available from commercial suppliers. To address this, we compared the easy-to-prepare broth method with culture on BEA agar without VAN, the latter allowing us to comprehensively evaluate all enterococcal strains present in feces based upon morphological variations among strains. The sensitivity of the broth method was found to be superior to that of the direct method in its ability to detect VRE. A limitation of this study is that rectal swabs and stool were considered together, a situation common to many clinical laboratories. At least two studies have noted an apparent difference in the yield of VRE by culture between stool and rectal swabs, and further work will be needed to determine if such a difference in the sensitivity of the broth method exists when various sample types are used (3, 7).

While the VanA and VanB phenotypes typically demonstrate overt resistance as defined by the NCCLS (VAN MIC ≥ 32 μg/ml), VanB VAN MICs may occasionally be as low as 8 to 16 μg/ml (10, 15). The identification by the broth method of one *E. faecium vanB* strain for which the MIC of VAN was 32 μg/ml, as well as the method's ability to support the growth of the low-level-resistant (VAN MIC = 16 μg/ml) *E. faecalis* ATCC 51299 strain, suggests that this medium is capable of identifying low-level-resistant *vanB* VRE. Furthermore, the finding of only one low-level-resistant VRE strain is consistent with our past findings, where the vast majority of VRE at our institution have been *E. faecium* strains with high level VAN resistance (data not shown). Further studies will be necessary to confirm that the broth method can reliably identify enterococcal strains with low-level *vanB* resistance. However, it is clear that the broth method significantly reduced the number of VAN sensitive and intermediate enterococcal strains initially isolated.

We found all of our VRE to be *E. faecium* of both *vanA* and *vanB* genotypes, with the exception of one VanA strain of indeterminate *van* genotype. This latter strain came from a patient known to harbor a *vanA E. faecium* strain for which the

VAN and TEC MICs are identical to those of the untypeable strain; it is therefore possible that the latter strain was in fact a mutant of the coresident *vanA* strain in this patient. Other studies have found that the gut flora of study populations harbored various ratios of *vanA* and *vanB E. faecium* and/or *E. faecalis* strains, all of which were notably different from our population of predominantly *vanA E. faecium* (68%). The ratios of *van* type and enterococcal species (i.e., *E. faecium* or *E. faecalis*) which harbor these genetic determinants thus appear to be unique for a given population (1, 5, 22, 25).

While the broth method demonstrated superior sensitivity, it also required on average two more days to grow colonies of sufficient size for VRE workup versus the direct method (4 days versus 2 days, respectively). Aggressive infection control measures have been recommended for the control of VRE, and a delay of 2 days in making a final diagnosis of VRE may have an impact on control of VRE (6). The turnaround time of the broth method may be reduced by 1 day by eliminating the BHI VAN agar screening step and instead determining the MIC of each enterococcal strain isolated on the broth subculture plate. We have in fact adopted this modification in our laboratory.

In conclusion, we have identified a VRE broth enrichment culture method that is significantly more sensitive than direct agar culture, results in fewer VAN-sensitive and -intermediate enterococcal strains requiring full workup, and does not require the preparation and use of VAN stock solutions. While the turnaround time is increased with the broth method, advantages are gained with this method's superior sensitivity. Further studies will be necessary to more fully evaluate the performance of this method in comparison to direct plating on VAN containing media, in its utility with various fecal specimen types, and also in its ability to detect low-level *vanB* resistance.

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