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Selective Isolation of Vancomycin-Resistant Enterococci

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Broth formulations of two media selective for enterococci, Enterococcosel and M-Enterococcus broths, were supplemented with 6 μg of vancomycin per ml and evaluated for isolation of vancomycin-resistant enterococci (VRE). Each broth was challenged with various concentrations of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and vancomycin-susceptible and vancomycin-resistant enterococci and with 193 perianal specimens obtained from patients at risk in our institution for VRE colonization. Both the Enterococcosel and M-Enterococcus broths with vancomycin detected as few as 1 to 9 CFU of VRE while inhibiting growth of the other organisms tested. *Enterococcus faecium organisms* (MIC, >256 μg/ml) were recovered from 66 perianal swab cultures in the Enterococcosel-vancomycin broth, and VRE were recovered from 62 perianal swab cultures in the M-Enterococcus-vancomycin broth. Enterococcosel-vancomycin broth detected VRE in perianal specimens 48 h earlier than did M-Enterococcus-vancomycin broth. Enterococcosel broth with 6 μg of vancomycin per ml can be used for the rapid and selective isolation of VRE from surveillance specimens.

In the United States, enterococci are the second most common cause of hospital-acquired infections, often causing urinary tract, surgical wound, and bloodstream infections (2, 8, 17). The emergence of enterococci resistant to vancomycin poses the particular dilemma of controlling transmission of these organisms from patient to patient. Nosocomial outbreaks involving vancomycin-resistant enterococci (VRE) have been reported (3, 4, 12, 13). Infection control measures often rely on the isolation and containment of patients who are colonized or infected with VRE (10). Although the manner of colonization with VRE remains unclear, rapid identification of VRE-colonized patients by means of surveillance cultures is often required.

Selective isolation of VRE has been reported for several commercially available and in-house-prepared agar and broth medium formulations (5, 8, 11, 18). M-Enterococcus agar and Enterococcosel broth are used for selective isolation of enterococci from water, sewage, and feces (16). Selective broth is recommended for isolation of group B streptococci from women colonized during pregnancy (1, 15). Therefore, we evaluated the broth formulations of M-Enterococcus medium and Enterococcosel broth, both supplemented with 6 μg of vancomycin per ml, for the isolation of VRE.

(A preliminary report of this work was presented previously [20].)

MATERIALS AND METHODS

Enterococcosel broth (B-D Microbiology Systems, Cockeysville, Md.), a selective broth with bile-esculin and sodium azide, was supplemented with 6 μg of vancomycin per ml (EBVA). M-Enterococcus broth (Difco Laboratories, Detroit, Mich.), a selective medium with sodium azide and triphenyltetrazolium chloride indicator, was prepared according to the manufacturer's instructions and was supplemented with 6 μg of vancomycin per ml (M-EVA). The vancomycin levels in EBVA and M-EVA were checked with the Abbott TDX (Abbott Laboratories, North Chicago, Ill.). Both EBVA and M-EVA were challenged with known bacterial suspensions which were prepared in sterile saline and which contained *Enterococcus faecium* (vancomycin-resistant strain WCMC123 [MIC, ≥32 μg/ml] and vancomycin-susceptible strain WCMCH3 [MIC, ≤2 μg/ml]), *Enterococcus faecalis* (vancomycin-susceptible ATCC 29212 and vancomycin-intermediate WCMC2F [MIC = 16 μg/ml]), *Enterococcus gallinarum* WCMC151 (MIC = 8 μg/ml), *Enterococcus casseliflavus* WCMC26287 (MIC = 4 μg/ml), *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 25923. Bacterial suspensions at concentrations of 0 to 10^5 CFU/ml were verified by inoculating Trypticase-based 5% sheep blood agar (TSBA) with each suspension with a calibrated loop or pipette. Each sample of EBVA and M-EVA was inoculated with 100 μl of bacterial suspension (total inoculum range, 10^{-1} to 10^4 total CFU for each organism) and incubated in ambient air at 35°C for up to 48 h, with observations being made after approximately 18, 24, 42, and 48 h. Each test was performed in duplicate.

One-hundred ninety-three perianal swabs were obtained from 107 patients at risk for VRE colonization (oncology and intensive care unit patients), placed into 1 ml of sterile saline, and thoroughly mixed to achieve an even inoculum suspension. Each EBVA, M-EVA, and Columbia colistin-nalidixic acid (CNA) sheep blood agar medium was inoculated with 100 μl of the perianal saline suspension. The CNA blood agar was streaked for isolation, and a 30-μg vancomycin disk was placed between the first and second quadrants (CNA-VA). All media were incubated in ambient air at 35°C for up to 48 h.

Broths from all tests, regardless of growth indications, were subcultured after 24 and 48 h of incubation by inoculating a TSBA plate with 100 μl of broth, streaking the plate for isolation, and incubating it in ambient air at 35°C for up to 48 h. Any distinct color change in EBVA broth from amber to brown-black because of esculin hydrolysis was recorded as positive. A direct broth Gram stain was performed with all esculin-positive EBVA broths inoculated with perianal specimens. All CNA-VA plates and TSBA subculture plates from EBVA and M-EVA broths were examined for colonies with enterococcal morphology, with the entire CNA-VA plate being examined, especially within the vancomycin disk zone of inhibition. All VRE were identified as catalase-negative, gram-positive cocci that were pyroglutamyl-*B*-naphthylamide test positive, with vancomycin resistance being confirmed by a vancomycin E test (AB Biodisk, Culver City, Calif.) performed according to the manufacturer's instructions. Briefly, a suspension equivalent to a McFarland number 1 standard was used to inoculate the entire surface of a Mueller-Hinton agar plate, a vancomycin E test strip was placed onto the plate, the plate was incubated in ambient air at 35°C for 24 h, and MIC results were obtained on the basis of the point where the ellipse of growth inhibition intersected the E test strip. Species level identifications of the VRE isolated from perianal specimens were obtained by conventional methods (6).

RESULTS

Organism growth from the known bacterial suspensions was suppressed or completely inhibited in the EBVA and M-EVA broths for each inoculum concentration of *E. coli*, *S. aureus*, *P. aeruginosa*, and vancomycin-susceptible enterococci (*E. faecalis* ATCC 29212, *E. faecium* WCMCH3, and *E. casseliflavus* WCMC26877) tested (Table 1). As few as 1 to 9 CFU of the VRE (*E. faecium* WCMC123, *E. faecalis* WCMC2F, and *E.*

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TABLE 1. Selectivity of EBVA and M-EVA challenged with bacteria

Organism	Vancomycin MIC	Recovery of bacteria (CFU/ml)[a]			
		EBVA		M-EVA	
		1–9[b]	10^4[b]	1–9[b]	10^4[b]
E. faecium WCMC123	>256 μg/ml	$>10^5$	$>10^5$	$>10^5$	$>10^5$
E. faecium WCMCH3	≤2 μg/ml	0	20	0	72
E. faecalis ATCC 29212	≤2 μg/ml	0	30	0	54
E. faecalis WCMC2F	16 μg/ml	$>10^5$	$>10^5$	$>10^5$	$>10^5$
E. casseliflavus WCMC26877	4 μg/ml	0	0	0	0
E. gallinarum WCMC151	8 μg/ml	10^5	$>10^5$	$>10^5$	$>10^5$
S. aureus ATCC 25923	≤2 μg/ml	0	0	0	0
E. coli ATCC 25922	>256 μg/ml	0	0	0	0
P. aeruginosa ATCC 27853	>256 μg/ml	0	25	0	40

[a] The figures are average recoveries after subculture.
[b] Total CFU in the initial inoculum.

gallinarum WCMC151) was recovered in both the EBVA and M-EVA broths, with $>10^5$ CFU of the VRE per ml being isolated from TSBA subcultures of all broths inoculated with at least 1 to 9 CFU. All EBVA broths inoculated with at least 1 to 9 total CFU of the VRE tested had a positive esculin reaction after a 48-h incubation. No VRE were recovered from TSBA subcultures of any esculin-negative EBVA broths (<10 CFU inoculated) or from M-EVA broths inoculated with less than 10 CFU. Growth indications, i.e., cloudy broth or triphenyltetrazolium reduction, were not clearly observed for M-EVA broths and were not recorded. Low numbers (<100 CFU/ml) of vancomycin-susceptible *E. faecalis*, vancomycin-susceptible *E. faecium*, and *P. aeruginosa* were recovered from subcultures of both EBVA and M-EVA broths that had been inoculated with higher concentrations (10^4 total CFU) of these organisms (Table 1). None of the EBVA broths from which these organisms were recovered had a positive esculin reaction. All EBVA broths inoculated with *E. casseliflavus* were esculin negative, and no organisms were isolated from TSBA subcultures.

VRE, all identified as *E. faecium* and for which MICs of vancomycin were >256 μg/ml, were recovered by at least one method from 66 of the 193 perianal specimens (Table 2). The 66 VRE were recovered from 38 of the 107 patients (36% colonization rate). All 66 VRE were grown with EBVA, 62 (94%) were grown with M-EVA, and 51 (77%) were grown with CNA-VA agar. There were 49 VRE identified by all three methods; 13 were grown with EBVA and M-EVA only, 2 were grown with EBVA and CNA-VA only, and 2 were grown with EBVA alone. Esculin hydrolysis was observed in 83 EBVA broths; VRE were isolated from 66 broths, and 17 were esculin positive, with no VRE being isolated from TSBA subcultures. These 17 cultures were also negative for VRE by the CNA-VA and M-EVA methods. Gram stains of the esculin-positive EBVA broths showed gram-positive cocci in all 66 VRE-positive cultures, but gram-positive cocci were seen in only 2 of the 17 VRE-negative, esculin-positive EBVA broths. Gram-positive bacilli, lactobacillus-like organisms isolated from TSBA subcultures, were seen in 10 VRE-negative, esculin-positive EBVA broths. No organisms were seen after the direct Gram staining of five VRE-negative, esculin-positive EBVA broths. Vancomycin-resistant cocci other than enterococci were recovered from 3 of the 17 broths, 2 with gram-positive cocci seen on direct Gram stains and one with no organisms seen. No VRE were isolated from TSBA subcultures of any esculin-negative EBVA broths. The sensitivity and specificity of the esculin reaction in EBVA as an indication of the presence of VRE were 100 and 87%, respectively. With the direct Gram stain results for VRE detection being considered along with the esculin reaction, the specificity increased to 98%, with gram-positive cocci being seen in only two esculin-positive EBVA broths that were negative for VRE. Low numbers ($<10^4$ CFU/ml) of vancomycin-susceptible enterococci were recovered from TSBA subcultures of 47 esculin-negative EBVA broths, 52 M-EBVA broths, and 89 of the 142 (63%) CNA-VA agar VRE-negative cultures.

TABLE 2. Selective isolation of VRE from perianal swabs by three media

Recovery of VRE[a]			No. of perianal specimens	Cumulative no. of positives
EBVA	M-EVA	CNA-VA		
+	+	+	49	49
+	+	−	13	62
+	−	+	2	64
+	−	−	2	66
−	−	−	127	
Total			193	

[a] +, confirmed VRE; −, no VRE.

TABLE 3. Time to detection of VRE by three media

Medium	Total no. of VRE-positive broths	No. of positives detected by:		% Detected by 24 h
		24 h	48 h	
EBVA	66	56	10	85
M-EVA	62	46	16	74
CNA-VA	51	45	6	88

Fifty-six of the 66 (85%) VRE isolated from esculin-positive EBVA broths and 46 of the 62 (74%) VRE isolated from M-EVA broths were recovered from broths incubated for 24 h (Table 3). Confirmation of VRE isolated from EBVA and M-EVA took an additional 48 h: 24 h for TSBA subcultures and 24 h for biochemical and susceptibility testing. On the basis of a positive esculin reaction and the presence of gram-positive cocci as determined with a Gram stain, EBVA detected the VRE 48 h earlier than the method involving subcultures and confirmatory testing of EBVA and M-EVA. Enterococcus-like colonies on CNA-VA were initially detected after 24 h of incubation for 45 of the 51 (88%) specimens subsequently confirmed as having VRE. All EBVA broths with a negative esculin reaction and CNA-VA plates with no enterococcus-like colonies were both considered negative after 48 h of incubation. All M-EBVA broths required an additional 24 h (total, 72 h) to determine a negative because of the requirement for TSBA subcultures.

DISCUSSION

In this study, we report the selective isolation of VRE from colonized patients by the use of two commercially available enterococcus-selective broths supplemented with the recommended 6 μg of vancomycin per ml (14). We evaluated enterococcus-selective broths for the isolation of VRE, since broth is recommended for the selective isolation of group B streptococci from colonized patients (1, 15). The only other broth to be evaluated for the selective isolation of VRE is

kanamycin esculin azide broth; however, 20 μg of vancomycin per ml was used, and the level of selectivity was not reported (11). Several agar-based media have been evaluated (5, 9, 18), but they often require extensive preparation prior to use. Enterococcosel broth is commercially available, and vancomycin can easily be aseptically added to achieve a final concentration of 6 μg/ml. The M-Enterococcosel broth formulation is not readily available, and the medium powder was obtained on a custom basis.

Both M-EVA and EBVA were selective for both high and low levels of VRE and were able to detect VRE in a broth inoculated with as few as 1 to 9 total CFU of VRE. Both broth methods also showed enhanced growth of VRE in broths inoculated with at least 1 to 9 total CFU, giving final TSBA subculture colony counts of $>10^5$ CFU/ml. Enhanced growth, with increased numbers of organisms recovered, might not be achieved with agar-based selective media, for which the number of colonies recovered is usually equal to or less than the number of CFU present in the initial inoculum. Thus, low numbers of VRE present in a specimen might be missed with agar-based VRE-selective media. No other tested bacteria from the known suspensions appeared to actively grow in M-EVA or EBVA, although some organisms did remain viable at the higher initial inoculum concentrations and were recovered from TSBA subcultures in decreased numbers. This result was particularly observed for vancomycin-susceptible enterococci, perhaps because vancomycin was not bactericidal.

Both the EBVA and M-EVA methods were superior to the CNA-VA method for the selective isolation of VRE from perianal specimens. The original inoculum density of VRE in the perianal specimens was not determined and could be considered a limitation of this study. The VRE not isolated by the CNA-VA method could have been due to the presence of low numbers of VRE in the perianal swab specimen that may have been overgrown by other fecal flora. Further studies to determine the number of VRE present in stool or other rectal surveillance specimens might be helpful. A CNA-based blood agar with vancomycin added has been shown to be reliable for the selective isolation of VRE (18), although the selectivity and sensitivity of the medium were not noted and the medium is currently not available commercially. The CNA-VA method described in this study is not recommended for the primary isolation of VRE from rectal specimens. Although VRE could have been missed by all three methods, it is unlikely that any VRE-colonized patients were missed by the EBVA method, since no VRE were recovered by the M-EVA and/or CNA-VA methods that were not recovered by the EBVA method.

Indicator systems that allow for the detection of enteroccal growth, a tetrazolium compound in M-EVA and esculin in EBVA, are present in both broth formulations (16). Although it did not appear to affect the recovery of VRE in this study, the tetrazolium compound, which in the agar formulation is reduced by enterococcal growth to yield red colonies, appeared to precipitate upon autoclave sterilization and was not observed to be a reliable indicator of growth in M-EVA. The tetrazolium indicator might have been more reliable if the medium was filter sterilized or if tubes were prepared and obtained from a commercial source. Growth of VRE in EBVA yielded a distinct color change to brown-black because of the hydrolysis of esculin. All EBVA broths inoculated with at least 1 to 9 total CFU of VRE showed a positive esculin hydrolysis reaction, presumably because of the growth of the VRE. No other organism tested during the bacterial challenge study, including the vancomycin-susceptible enterococci, had a positive esculin reaction in EBVA. This result indicates that the organisms recovered in low numbers from EBVA were inhibited and not actively growing in the broth. After being challenged with perianal specimens, 17 EBVA broths were esculin positive and VRE negative, with growth that was likely due to esculin-positive, nonenterococcal, vancomycin-resistant gram-positive organisms (6).

A direct Gram stain of all esculin-positive EBVA broths allowed for the rapid identification of VRE, since only 2 of the 17 VRE-negative, esculin-positive broths had gram-positive cocci by direct Gram stain. Gram-positive bacilli, presumably lactobacilli, appeared to be the most common cause (10 of 17) of VRE-negative, esculin-positive EBVA broths and were easily detected as gram-positive bacilli by direct Gram stains of the positive EBVA broths. Clindamycin has been added to other VRE-selective media to reduce any lactobacilli contamination (5). Clindamycin was not added to the broths in this study, since lactobacilli were not frequently isolated (10 of 193 perianal specimens). Nonenterococcal vancomycin-resistant cocci were rarely isolated (total, three isolates) from esculin-positive EBVA broths and were identified as nonenterococcal organisms by conventional tests (6).

Since rapid detection and confirmation of VRE infection may be important for the institution of appropriate infection control measures, the media tested in this study were also evaluated for the time each took to detect VRE. The time to detection of VRE-positive and -negative perianal cultures was most rapid for EBVA, with 56 (84%) positives being detected after overnight incubation (on the basis of positive esculin reactions and appropriate Gram stain results), while all esculin-negative broths were negative for VRE after 2 days of incubation. With EBVA, the identification of VRE on the basis of esculin-positive broth and the detection of gram-positive cocci by direct Gram stains was obtained 2 days earlier than that with M-EVA. One major problem with M-EVA was the lack of a good indicator system in the broths as prepared. These M-EVA broths required routine TSBA subcultures, and VRE confirmation took at least three days. Biochemical confirmation of all suspected enterococcal colonies isolated by TSBA subculture of EBVA resulted in increased times to confirm the isolate as VRE; however, infection control could be notified with a preliminary report of VRE on the basis of the detection of the esculin-positive EBVA and Gram stain results. This early notification, although preliminary, could result in the institution of appropriate infection control measures that might reduce the spread of VRE. Biochemical confirmation of enterococcus-like colonies isolated on any agar-based selective medium could also result in similar increases in time to confirm the presence of VRE, thus delaying notification of infection control. The times to the detection and confirmation of VRE have not been reported for agar-based selective media (5, 9, 11, 18), and further studies to determine VRE detection times may be helpful. There was additional technical time required to confirm negative perianal cultures for EBVA when there was a false esculin-positive broth; however, these results should not be reported to infection control, since the Gram stain for most of these broths (15 of 17) did not show gram-positive cocci. The M-EVA method was determined to be too labor-intensive and to result in unacceptable reporting delays because of the requirement for all M-EVA broths to be subcultured and any enterococcus-like colonies to be tested for VRE. No esculin-negative EBVA broths grew VRE on subculture; therefore, routine subcultures of these negative broth are not recommended, and negatives could be reported after the 2 days of incubation.

Isolation of suspected VRE from colonized patients by a screening method often poses an infection control problem during nosocomial outbreaks of vancomycin-resistant *E. fae-*

cium and *E. faecalis* infections (3, 4, 12, 13). Although only high-level vancomycin-resistant *E. faecium* was isolated from the perianal cultures, the data obtained with the challenge organisms indicate that the broths are capable of selective isolation of a variety of VRE, including low-level vancomycin-resistant *E. gallinarum*. Low-level vancomycin-resistant *E. casseliflavus* and *E. gallinarum* (*vanC* enterococci) have yet to be implicated in a nosocomial outbreak. The 66 perianal VRE were not tested for genetic relatedness or resistance markers; however, previous studies have shown our VRE population to be heterogeneous *vanA E. faecium* (13, 19). Although no *E. casseliflavus* or *E. gallinarum* was isolated during this study, the potential exists for their selective isolation in EBVA and M-EVA, since they have been shown to be colonizers of the gastrointestinal tract (7). Since appropriate infection control measures often include costly isolation procedures (10), identification of all VRE isolates to rule out low-level resistant *E. casseliflavus* and *E. gallinarum*, along with confirmatory vancomycin resistance testing, should be performed.

Both EBVA and M-EVA are selective for VRE. Enterococcosel broth with 6 μg of vancomycin per ml was preferred for the rapid selective isolation and detection of VRE from surveillance specimens. The esculin reaction and direct Gram stain are rapid, accurate indicators of VRE in EBVA. Confirmation by subculture, biochemical testing, and confirmatory vancomycin MIC testing of VRE recovered from esculin-positive EBVA should be performed.

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