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Evaluation of 15 Motility Media and a Direct Microscopic Method for Detection of Motility in Enterococci

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Isolation of motile, *vanC* enterococci has yet to be a major infection control concern; however, rapid detection still is important. We evaluated 15 motility media from three manufacturers and a 2-h direct microscopic method for accurate detection of 89 enterococcal strains, including 72 *vanC* enterococcal strains. Resistance genes were confirmed by a multiplex PCR method with the *vanC* gene detected in all motile enterococci. Motility in the 72 *vanC* enterococci was detected at 30°C within 72 h in BD Biosciences motility nitrate medium and in Remel motility B medium, motility B medium supplemented with methyl-α-D-glucopyranoside (investigational), motility S medium, motility test medium, and motility test medium with tetrazolium indicator. Motility was also observed for all *vanC* enterococci with the 2-h direct (30°C incubation) microscopic detection method. All *Enterococcus faecalis* and *Enterococcus faecium* isolates were observed to be nonmotile in all media and by the direct microscopic method. Since differences between the various motility media tested were observed, the medium used for detection of enterococcal motility must be selected carefully.

Intrinsically vancomycin-resistant enterococci, which have *vanC* genes, have been reported to cause human clinical infection (10, 12) and can be recovered from surveillance cultures used to detect enterococci with acquired *vanA* and *vanB* vancomycin resistance (12, 14). The *vanC* enterococci, *Enterococcus gallinarum*, *Enterococcus casseliflavus*, and *Enterococcus flavescens*, have yet to be implicated in nosocomial outbreaks, but possibly could be transmitted from patient to patient without causing infection. Rapid, accurate identification of these enterococci is important in preventing the implementation of costly infection control measures. The reliability of automated identification systems to accurately identify the *vanC* enterococci has been a concern (2, 7, 11; K. Ramotar, W. Woods, L. Larocque, and B. Toye, Abstr. 98th Gen. Meet. Am. Soc. Microbiol., abstr. C359, 1998). Conventional motility and pigment production tests to help to identify the *vanC* enterococci have been described by Facklam and Collins (5) and are recommended by the National Committee for Clinical Laboratory Standards (9). However, the reliability of these tests has also been questioned (13, 15; Ramotar et al., Abstr. 98th Gen. Meet. Am. Soc. Microbiol.). Many formulations of motility media are available, and most were originally designed to detect motility in enteric gram-negative bacilli (1). Facklam and Collins (5) described a modified Difco motility medium that was used to characterize strains of motile enterococci. New identification tests to help identify the *vanC* enterococci include the methyl-α-D-glucopyranoside (MDG) test (4, 6, 7, 13) and gene amplification by PCR to detect the specific *vanC1*, *vanC2*, and *vanC3* genes (2, 3, 8, 12). The MDG test is currently available from a limited number of manufacturers, and the PCR method remains an investigational or research tool. Therefore, in this study, we determined the abilities of various motility media and a direct microscopic method to accurately detect motility in *vanC* enterococci. We also evaluated an investigational motility medium that included MDG. The *van* gene type was verified by PCR for all enterococci tested.

MATERIALS AND METHODS

Organisms. Eighty-nine enterococcal strains were tested, including 41 *E. gallinarum*, 31 *E. casseliflavus*, 11 *E. faecium*, and 6 *E. faecalis* strains. All strains were recent isolates obtained from diverse inpatient and outpatient populations, isolated from either clinical or surveillance specimens. All isolates were identified by conventional biochemical tests methods as described by Facklam and Collins (5). All isolates were freshly grown (18 to 24 h) on Trypticase soy–5% sheep blood agar (BD Biosciences, Cockeysville, Md.) prior to testing.

PCR detection of vancomycin resistance genes. Gene amplification by PCR of the resistance genes to confirm the identification of each isolate was performed as previously described (8; S. Elsayed, N. Hamilton, D. Boyd, M. Mulvey, R. Kariyama, R. Mitsuhata, and H. Kumon, Letter, J. Clin. Microbiol. **39:**2367-2368, 2001). A cell suspension was prepared in 50 μl of 7.5% Chelex 100 to achieve the turbidity of a no. 3 McFarland standard and heated for 10 min at 100°C, and 2.5 μl of a centrifuged supernatant was used for PCR. A novel primer combination with optimized concentrations of *vanA, vanB*, *vanC1*, *vanC2*/*C3*, *E. faecalis*-specific, *E. faecium*-specific, and *rrs* primers was added to the reaction mixtures (S. Elsayed et al., Letter). The multiplex PCR was performed in a total volume of 25 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate (dNTP [dATP, dCTP, dGTP, and dTTP]), and 0.625 U of *Taq* DNA polymerase (Takara Shuzo Co., Kusatsu, Japan). DNA amplification included thermal cycling with initial denaturation at 94°C for 5 min, 30 cycles of amplification (denaturation at 94°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 1 min), and a final extension at 72°C for 10 min in a GeneAmp PCR system 9700 (PE Biosystems, Tokyo, Japan). PCR products were analyzed on a 1.5% agarose gel stained with ethidium bromide.

Motility tests. Fifteen types of motility media from three manufacturers were tested along with a direct wet mount method described previously (14). The motility media included the following: from BD Biosciences, motility indole lysine sulfide (MILS), motility indole ornithine (MIO), motility nitrate (MN), motility test medium (MTM) with triphenyl-tetrazolium chloride indicator (MTM-TTC), and sulfide indole motility (SIM); from Remel (Lenexa, Kans.), motility B (MOTB), motility B with TTC (MOTB-TTC), motility B and MDG (MOTB-MDG [investigational]), motility GI (MOT GI), MIO, motility S

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TABLE 1. Detection of motility at 30°C in *E. casseliflavus* and *E. gallinarum* by 15 motility media and a direct microscopic method

Manufacturer and media	No. of strains with motility detection result							
	E. casseliflavus (n = 31)				*E. gallinarum* (n = 41)			
	24 h	48–72 h	Negative	%[a]	24 h	48–72 h	Negative	%[a]
BD								
Motility indole lysine H_2S	12	4	15	52	32	6	3	93
Motility indole ornithine	23	4	4	87	35	3	3	93
Motility nitrate	28	3	0	100	36	5	0	100
Motility test medium-TTC	25	5	1	97	37	4	0	100
SIM	29	1	1	97	41	0	0	100
PML								
Motility indole ornithine	29	0	2	94	41	0	0	100
Motility test medium-TTC	9	10	12	61	24	11	6	85
Remel								
Motility B	27	4	0	100	39	2	0	100
Motility B-TTC	29	1	1	97	41	0	0	100
Motility B-MDG[b]	31/29	0/2	0/0	100/100	41/41	0/0	0/0	100/100
Motility GI	20	10	1	97	25	12	4	90
Motility indole ornithine	4	3	24	23	21	11	9	78
Motility S	24	7	0	100	39	2	0	100
Motility test medium	30	1	0	100	41	0	0	100
Motility test medium-TTC	31	0	0	100	41	0	0	100
Direct 2-h microscopy	31[c]			100	41[c]			100

[a] Percent detected after 72 h.
[b] Motility B results/MDG results.
[c] Results after 2 h.

(MOTS), MTM, and MTM-TTC; and from PML (Tualatin, Oreg.), MIO and MTM-TTC. A heavy suspension of each strain was prepared in Trypticase soy broth. Each medium was inoculated with a single stab of an inoculating needle seeded with the organism suspension. Each medium type was inoculated in duplicate, with one tube incubated at 30°C and one tube incubated at 37°C. Tubes were examined independently by two microbiologists for motility according to the manufacturer's instructions at 24, 48, and 72 h. For the direct wet mount method, one drop from a Pasteur pipette (approximately 50 μl) of each organism suspension was added to duplicate Trypticase soy broth tubes: one incubated at 30°C and one incubated at 37°C for 2 h. *Enterobacter aerogenes* (motile) and *Klebsiella pneumoniae* (nonmotile) were used as controls for all motility tests. Following incubation, 50 μl from each tube was examined independently by two microbiologists via dark-field microscopy at magnifications of ×400 and ×1,000. Positive motility was identified by observing coccoid cells or chaining cocci that clearly exhibited rapid, directional, darting motility compared to the negative controls' nonmotile, vibrating, nondirectional motion of each cell, characteristic of Brownian movement. The positive control was observed to ensure that the Trypticase soy broth supported motility within the time course of incubation.

RESULTS

All 41 *E. gallinarum* strains tested were confirmed by PCR to have the *vanC1* gene. Two strains were found to additionally possess the *vanA* gene, and one strain was found to additionally possess the *vanB* gene. All 31 *E. casseliflavus* strains identified by a positive pigment test were confirmed by PCR to have the *vanC2/3* gene. All *vanC2/3* enterococci in this study fermented ribose and were considered to be *E. casseliflavus*. Of the 11 *E. faecium* strains, the *vanA* gene was detected in 9 and the *vanB* gene was detected in 2. Of the six *E. faecalis* strains, the *vanA* and *vanB* genes were detected in four and two strains, respectively.

The percentage of motile enterococci detected at 30°C by the 15 motility media and the microscopic method is listed in Table 1. The 11 *E. faecium* and 6 *E. faecalis* strains were identified as nonmotile by all 15 motility media and the microscopic method. The Remel MOTB-MDG and Remel MTM-TTC were the only medium-based methods to detect motility in all 72 PCR-confirmed *vanC* enterococcal strains after 24 h of incubation. Additionally within the 72-h incubation period, the Remel MOTB, Remel MOTS, Remel MTM, and BD MN detected motility in all 72 PCR-confirmed *vanC* enterococci. Motility was detected in 71 of the 72 (99%) PCR-confirmed *vanC* enterococci within 72 h in the Remel MDTB-TTC, BD SIM, and BD MTM-TTC. Several formulations of media tested from the various manufacturers were considered to be unreliable for detection of enterococcal motility. These included the Remel MIO, which detected motility in 39 of 72 (54%) *vanC* enterococci; the BD MILS, which detected motility in 54 of 72 (75%) *vanC* enterococci; and the PML MTM-TTC, which detected motility in 54 of the 72 (75%) *vanC* enterococci. (For these results, all detection was done at 30°C.) The direct microscopic wet mount detected all 72 motile enterococci after 2 h of incubation. Acid production from MDG in the MOTB-MDG was positive for all strains, with only 2 strains of *E. casseliflavus* requiring incubation longer than 24 h (one strain requiring 48 h, and one requiring 72 h), as shown in Table 1.

Two motility medium formulations, MTM-TTC and MIO, were available from all three manufacturers, but yielded varied results based on the manufacturer. The Remel MTM-TTC formulation yielded the best results, with motility detected in 100% of the PCR-confirmed *vanC* enterococci at 30°C after 24 h. The BD MTM-TTC formulation detected motility in 71 of the 72 (99%) PCR-confirmed *vanC* enterococci. The PML

MTM-TTC only detected motility in 54 (75%) strains at 30°C after 72 h of incubation. The second motility medium formulation, MIO, which was available from all three manufacturers, also yielded varied results. The PML MIO detected motility in 70 of the 72 (97%) PCR-confirmed *vanC* enterococci, and the BD MIO detected motility in 65 of the 72 (90%) PCR-confirmed *vanC* enterococci. The Remel MIO detected motility in only 39 of the 72 (54%) strains.

Incubation was performed for all tests at both 30 and 37°C. Our data (not shown) were virtually identical for the two incubation temperatures, showing no significant difference (Student's t test, $P = 0.994$) in overall detection of motility based on incubation temperature.

DISCUSSION

A multiplex PCR was used to detect resistance genes in the enterococci tested; however, the *vanC2* and *vanC3* genes are highly homologous and were not differentiated in this study. Fermentation of ribose was used to differentiate *E. casseliflavus* from *E. flavescens*. Although no typing methods, such as pulsed-field gel electrophoresis, were performed, the isolates tested were considered to be heterogeneous in origin due to the diverse patient populations from which they were isolated and the lack of reports of nosocomial spread or outbreaks of *vanC* enterococci.

All formulations of media except the PML MTM-TTC, BD MILS, and Remel MIO were able to reliably detect motility (>90% detected) in the *vanC* enterococci tested and were considered acceptable in this study for detection of motility in the *vanC* enterococci. These results correlate well with those obtained by Cartwright et al. (2), who used the Facklam method (5) and PCR for *vanC* confirmation.

Our data for the two motility media MTM-TTC and MIO obtained from all three manufacturers indicate that the manufacturing of the medium may play a major role in the ability or reliability of a particular medium to detect motility in *vanC* enterococci. The manufacturer's formulation may have contributed to the nonmotile strains previously reported (13, 15; K. Ramotar, W. Woods, L. Larocque, and B. Toye, Abstr. 98th Gen. Meet. Am. Soc. Microbiol., abstr. C359, 1998). These authors, citing the Facklam procedure (5), failed to detect motility in several strains and concluded that the motility characteristic was not stable and that testing with motility media was unreliable. It is possible that some of the nonmotile strains were tested in media obtained from a manufacturer whose medium we observed to be less reliable for the detection of enterococcal motility. Our data for the PML MTM-TTC are similar to those obtained by Ramotar et al. (Abstr. 98th Gen. Meet. Am. Soc. Microbiol.) and indicate that this medium formulation may be unreliable for the detection of motility in enterococci. In another report, an unspecified PML motility test medium was reported to detect all motile enterococci (12). The varied results reported with one manufacturer's product may be due to lot variations in the preparation of the medium. We observed during the inoculation step that the PML MTM-TTC product appeared thicker than the other motility medium products tested and may have contained excess agar or may have become dehydrated prior to our inoculation, each of which could adversely affect motility.

Rapid detection of the *vanC* enterococci is considered important in helping to prevent institution of expensive infection control practices associated with isolation of vancomycin-resistant enterococci. The 2-h microscopic method is a simple test to set up, and the motility of the enterococci is easily observable by a trained microbiologist. Combined with the detection of colony pigment, enterococcal isolates can be rapidly identified as the motile enterococci *E. casseliflavus* and *E. gallinarum*. One rapid agar-based method has been reported to yield reliable detection of motility in *vanC* enterococci in 4 h (6). All other agar-based methods required 24 to 72 h of incubation. In this study, our data confirm that the 2-h direct microscopic method is acceptable for rapid detection of motility in enterococci (14), especially where PCR may not be available to confirm identification.

The MDG test has also been reported to be a reliable method for detection of the *vanC* enterococci (4, 6, 13), but requires at least 24 h of incubation. Our data with Remel's investigational medium, motility B medium with MDG, support these previous data. Combined with the MOTB, a reliable motility test according to our data, this investigational medium may be more practical and reliable for detection of a wider variety of strains of *vanC* enterococci, including any strains that may exhibit true nonmotile characteristics.

All tests were incubated at both 30 and 37°C, since the MDG test had been incubated at 35 to 37°C in previous reports (4, 6, 13). Also, a rapid 4-h motility test that detected all but one motile strain when incubated at 35°C has been reported (6), and the data compare favorably with our data, in which there was no significant difference (Student's t test, $P = 0.994$) in overall detection of motility based on temperature of incubation. All *vanC* strains were MDG positive at both 30 and 37°C. Incubation temperature does not appear to play a major role in observation of motility in enterococci when a motility medium or direct microscopic method is used.

Various formulations of motility media from different manufacturers as well as the direct microscopic wet mount test are reliable for detection of motility in *E. gallinarum* and *E. casseliflavus*. The MDG test combined with a motility medium is also reliable for detection of *vanC* enterococci. Elucidation of true nonmotile enterococcal strains may require further investigations, such as flagellar studies.

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