

Susceptibility Testing of Clinical Isolates of *Enterococcus faecium* and *Enterococcus faecalis*

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We collected 103 clinical *Enterococcus faecium* isolates from across Canada, performed standard broth microdilution susceptibility testing, and compared these results with results from the MicroScan Pos MIC Type 6 panel (Baxter Health Care Corp., West Sacramento, Calif.) and the AMS-Vitek Gram-Positive Susceptibility card (Vitek Inc., St. Louis, Mo.). High-level aminoglycoside resistance to gentamicin and streptomycin was detected by a single-concentration agar method with 1,000 µg of each aminoglycoside per ml. In addition, we tested the effect of the lower calcium content in broth media as recommended in National Committee for Clinical Laboratory Standards (NCCLS) guideline M7-A2 on the activity of the highly calcium-dependent agent daptomycin. Of the 103 *E. faecium* isolates, there were 4 and 30 isolates with high-level gentamicin resistance (HLGR) and high-level streptomycin resistance (HLSR), respectively. An additional 39 (37 with HLGR and 36 with HLSR) *E. faecium* isolates were tested by both the MicroScan and the AMS-Vitek systems. The AMS-Vitek card demonstrated sensitivities of 95 and 82% for detecting HLGR strains and HLSR strains, respectively. The MicroScan panel demonstrated improved sensitivities for detecting HLGR (42 to 97%) and HLSR (64 to 84%) when readings were performed manually instead of being generated automatically. Ampicillin resistance (MIC, ≥ 16 µg/ml) was detected in 23 of the 103 *E. faecium* isolates. Only 14 and 20 of these were detected by the MicroScan panels and AMS-Vitek cards, respectively. β -Lactamase activity was not detected in any isolates. The lower calcium content in broth media recommended by NCCLS guideline M7-A2 markedly reduced the in vitro activity of daptomycin against *Enterococcus* spp.

Enterococci cause significant infections, including intra-abdominal sepsis, urinary tract infections, bacteremias, and endocarditis. These gram-positive organisms are inherently resistant to multiple antibiotics, including polymyxins, lincosamides, and trimethoprim-sulfamethoxazole, and have reduced susceptibility to cell wall-active agents such as β -lactams and vancomycin (16). Enterococci are also moderately resistant to aminoglycosides (MICs, 2 to 16 µg/ml). Nevertheless, enterococcal infections can be treated effectively with synergistic combinations of a cell wall-active agent plus an aminoglycoside (15, 27, 29). When enterococcal strains acquire aminoglycoside-inactivating enzymes, high-level aminoglycoside resistance (HLAR) (MICs, $\geq 2,000$ µg/ml) develops, and the synergism with a cell wall agent is lost (8, 14). High-level gentamicin resistance (HLGR) has been reported among clinical isolates of both *Enterococcus faecalis* and *Enterococcus faecium* (3, 9, 13, 28). In addition, β -lactamase-producing *E. faecalis* isolates (17, 20) and vancomycin-resistant enterococci (11, 24) have been reported. Non- β -lactamase-producing strains of enterococci that are highly resistant to ampicillin have also been reported (1, 22).

E. faecalis and *E. faecium* are the predominant enterococcal species associated with clinical infections in humans. In general, *E. faecium* strains are less susceptible to the β -lactams and aminoglycosides than are *E. faecalis* strains, and *E. faecium* strains are often more refractory to the synergistic effect of the antibiotic combination. Since few studies have specifically focused on the susceptibility patterns of a large number of clinical isolates of *E. faecium*, we carried out in vitro susceptibility testing of a large number of isolates

obtained from across Canada against standard and new antimicrobial agents and determined the prevalence of HLAR. We compared these results with the susceptibility test results generated by the MicroScan Pos MIC Type 6 panel (Baxter Health Care Corp., West Sacramento, Calif.) and the AMS-Vitek Gram-Positive Susceptibility card (Vitek Inc., St. Louis, Mo.). In addition, we determined the effect of the National Committee for Clinical Standards (NCCLS) guideline M7-A2 (19) calcium concentration recommendations on the in vitro activity of daptomycin, which has in vitro antimicrobial activity that is highly calcium dependent (2, 6, 10, 25).

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MATERIALS AND METHODS

Clinical isolates of *E. faecium* (103 strains) and *E. faecalis* (34 strains) were collected from nine major tertiary care centers from across Canada. Additional *E. faecium* isolates (39 strains) with known antimicrobial susceptibilities from the Public Health Research Institute of the State of New York were selected for HLAR to compare the accuracy of the MicroScan Pos MIC Type 6 panels and the AMS-Vitek Gram-Positive Susceptibility cards in detecting HLAR with that of single-concentration agar screen plates.

The identification of isolates was based on conventional methods (4, 5). All enterococcal isolates were stored in skim milk and glycerol at -70°C . Each isolate was subcultured twice on 5% sheep blood agar, and a fresh overnight subculture was used for testing.

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Susceptibility testing. Microdilution susceptibility testing was performed in accordance with NCCLS guideline M7-A2 (19). This guideline lowered the calcium and magnesium concentrations from the 50 and 25 mg/liter recommended in NCCLS guideline M7-A (18) to 25 and 12 mg/liter, respectively. Various concentrations of these two divalent cations were incorporated into media for the testing of daptomycin and teicoplanin. Testing of the remaining antimicrobial agents was done with Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) containing 25 mg of calcium and 12 mg of magnesium per liter. Microdilution trays were prepared by using the Quickspense II System (Sandy Springs Instrument Co., Belco, Inc., Vineland, N.J.). Fresh subculture isolates were adjusted to a 0.5 McFarland turbidity standard, and a final inoculum of 5×10^5 CFU/ml was obtained in each well. Trays were incubated at 35°C in atmospheric air for 18 to 24 h.

Susceptibility testing was also performed with the two semiautomated systems according to the instructions of the manufacturers. The MicroScan Type 6 panel is a reformulation of the original MIC Type 6 freeze-dried panel and contains glucose phosphate broth to enhance the growth of enterococci. The final inoculum for the MicroScan Pos MIC Type 6 panels was 1×10^5 to 4×10^5 CFU/ml, and the panels were incubated at 35°C in atmospheric air for 18 to 24 h. For the AMS-Vitek Gram-Positive Susceptibility cards, the final inoculum was 1.5×10^7 CFU/ml, and the cards were incubated under the same conditions for 6 h. The MicroScan panels and AMS-Vitek cards also contain wells with high concentrations of gentamicin and streptomycin. Growth in these wells is intended to predict HLAR, which indicates that synergy with β -lactams will not occur. For both the MicroScan Pos MIC Type 6 panels and the AMS-Vitek Gram-Positive Susceptibility cards, turbidity or any growth in the synergy wells was considered indicative of resistance. The synergy wells in the MicroScan panels were read by the automated Walk/Away system after 18 h of incubation and by visual inspection by two independent observers after 18 and 48 h of incubation. The AMS-Vitek cards were read by the Vitek Senior 240 and Information Management System after 6 h of incubation.

Daptomycin was provided by Eli Lilly Research Laboratories (Indianapolis, Ind.); teicoplanin was provided by Merrel Dow Pharmaceuticals, Inc. (Cincinnati, Ohio); imipenem was provided by Merck Frosst Canada (Mississauga, Ontario, Canada); and piperacillin was provided by Lederle Laboratories (Cynamid Canada Inc., Markham, Ontario, Canada). Vancomycin, ampicillin, streptomycin, and gentamicin were purchased from Sigma Chemical Co. (St. Louis, Mo.).

For the detection of HLAR, single-concentration agar screen plates were prepared with brain heart infusion agar (Oxoid, Basingstoke, England) with 1,000 μ g of gentamicin and 1,000 μ g of streptomycin per ml (7, 16). Colonies of each isolate were suspended in brain heart infusion broth (Difco) and incubated overnight to achieve an inoculum of 10^9 CFU/ml. Screen plates were inoculated with a Steers replicator to a final inoculum of approximately 10^6 CFU. The plates were incubated for 18 to 24 h at 35°C. Growth of two or more colonies was considered to indicate resistance.

β -Lactamase production was detected by using the chromogenic substrate in disks containing nitrocefin (BBL Microbiology, Cockeysville, Md.).

Control strains included *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, and *E. faecalis* ATCC 29212, HH22 (gentamicin and streptomycin resistant, β -lac-

TABLE 1. Comparative in vitro activities of antimicrobial agents against *E. faecium* and *E. faecalis* isolates^a

Organism (no. of isolates)	Antimicrobial agent	MIC (μ g/ml) ^b		
		Range	50%	90%
<i>E. faecium</i> (103)	Ampicillin	0.25->64	8	64
	Imipenem	2->64	32	>64
	Piperacillin	4->128	32	>128
	Vancomycin	0.5-16	2	8
	Daptomycin	1->64	32	32
	Teicoplanin	<0.06-2	0.5	1
<i>E. faecalis</i> (34)	Ampicillin	2-16	2	4
	Imipenem	4-64	8	16
	Piperacillin	4-64	8	16
	Vancomycin	1-4	2	4
	Daptomycin	2-64	8	32
	Teicoplanin	0.125-1	0.25	0.5

^a Microbroth dilution performed according to NCCLS guideline M7-A2.

^b 50% and 90%, MICs for 50 and 90% of isolates tested, respectively.

tamase positive), UWHC 1921 (gentamicin resistant and streptomycin susceptible), and UWHC 1936 (gentamicin susceptible and streptomycin resistant).

RESULTS

The in vitro activities of the antimicrobial agents against 103 clinical isolates of *E. faecium* and 34 isolates of *E. faecalis* in microbroth dilution tests are shown in Table 1. The MICs obtained for *E. faecium* were generally higher than those for *E. faecalis*. Twenty-two percent (23 of 103) *E. faecium* strains and 2.9% (1 of 34) *E. faecalis* strains were resistant to ampicillin (MIC, ≥ 16 μ g/ml). There were no enterococcal isolates resistant to vancomycin, but 22% (23 of 103) of the *E. faecium* isolates were considered intermediate (MIC, 8 to 16 μ g/ml), and 78% (80 of 103) were considered moderately susceptible (MIC, ≤ 4 μ g/ml). All 34 *E. faecalis* strains were moderately susceptible to vancomycin.

The agar screen method determined that 4 (3.9%) of 103 clinical isolates of *E. faecium* had HLGR, 30 (29%) of 103 clinical isolates had high-level resistance to streptomycin (HLSR), and 3 (2.9%) of 103 isolates had high-level resistance to both aminoglycosides. An additional 39 *E. faecium* isolates from New York with HLAR were evaluated. Of these, 37 had HLGR and 36 had HLSR. Thus, combining all clinical isolates and selected isolates with HLAR, a total of 41 HLGR *E. faecium* strains and 66 HLSR *E. faecium* strains were available for assessing the detection of HLAR by the MicroScan and AMS-Vitek systems. The automated results generated by the MicroScan Pos MIC Type 6 panel and AMS-Vitek Gram-Positive Susceptibility card when compared with the agar screen method indicated that specificity was nearly 100%; there was one false-positive result each for the MicroScan system and the AMS-Vitek system. One isolate was determined to be resistant to streptomycin with the MicroScan system, and one isolate was determined to be resistant to gentamicin with the AMS-Vitek, but these isolates were considered sensitive by the agar screen method. Visual readings of MicroScan panels at 18 and 48 h resulted in specificities of 99 and 96%, respectively, for detecting HLSR. The AMS-Vitek system was superior to the MicroScan system, with sensitivities of 95 versus 42% for detecting HLGR and 82 versus 64% for detecting HLSR (Table 2). However, visual inspection of MicroScan synergy

TABLE 2. Sensitivity of MicroScan Pos MIC Type 6 panel and AMS-Vitek Gram-Positive Susceptibility card for detection of HLAR in *E. faecium*

Aminoglycoside	Agar screen	No. resistant by:			
		MicroScan (sensitivity)		Vitek (sensitivity)	
		Automated readings ^a	Visual readings ^b at:		
			18 h	48 h	
Gentamicin	41	17 (42%)	33 (80%)	40 (97%)	39 (95%)
Streptomycin	66	42 (64%)	51 (77%)	56 (84%)	54 (82%)

^a Performed by Walk/Away at 18 h.

^b Performed by two independent observers each time.

wells improved sensitivities to 84 and 97% for detecting HLSR and HLGR, respectively.

With microbroth dilution techniques, we found 23 of 103 clinical isolates of *E. faecium* to be ampicillin resistant. To adequately compare the MicroScan Pos MIC Type 6 panel and the AMS-Vitek Gram-Positive Susceptibility card for the detection of ampicillin resistance, we included an additional 39 *E. faecium* isolates from New York, of which 37 were known to be highly ampicillin resistant. As shown in Table 3, the MicroScan system failed to adequately detect strains of *E. faecium* with low-level ampicillin resistance (MIC, 16 to 32 µg/ml) but was able to detect most isolates with higher-level ampicillin resistance (MIC, ≥64 µg/ml). The AMS-Vitek system performed similarly, but six susceptible isolates (MIC, 8 µg/ml) were found to be resistant by the AMS-Vitek system. When the chromogenic method with the nitrocefin disk was used, no β-lactamase activity was detected among any of these 142 *E. faecium* isolates.

Comparative in vitro activities of daptomycin and teicoplanin against enterococci with various concentrations of divalent cations according to NCCLS guidelines M7-A2 and M7-A indicated that there was a four- to eightfold increase in the daptomycin MICs for *E. faecium* when the calcium content was halved as proposed by NCCLS document M7-A2 (Table 4). Similarly, an 8- to 16-fold increase in daptomycin MICs was seen for the *E. faecalis* isolates. Magnesium content did not contribute to any significant changes (data not shown). Changes in cation content did not substantially affect teicoplanin activity against enterococci (data not shown).

DISCUSSION

The antimicrobial susceptibility test results for *E. faecium* in this study are comparable to the results reported by Bush et al. (1), who evaluated a smaller number of isolates (only

TABLE 3. Comparison of MicroScan Pos MIC Type 6 panel and AMS-Vitek Gram-Positive Susceptibility card for detection of ampicillin resistance in 142 strains of *E. faecium*^a

MIC (µg/ml) of resistant isolates (no. of isolates)	No. of resistant isolates by:	
	MicroScan	Vitek
<8 (66)	1	0
8 (16)	0	6
16 (7)	2	7
32 (5)	3	4
≥64 (48)	45	46

^a Resistance MIC, ≥16 µg/ml.

TABLE 4. Effect of calcium and magnesium concentrations on in vitro activity of daptomycin against clinical isolates of *E. faecalis* and *E. faecium*

Organism (no. of isolates)	Cation concn (mg/liter)		Daptomycin MIC (µg/ml) ^a		
	Calcium	Magnesium	Range	50%	90%
<i>E. faecium</i> (103)	50	25 ^b	<0.125-16	4	8
	50	12	<0.125-8	4	8
	25	25	0.25->64	32	64
	25	12 ^c	1->64	32	32
<i>E. faecalis</i> (34)	50	25 ^b	0.25-8	1	2
	50	12	0.25-8	1	2
	25	25	2-64	8	32
	25	12 ^c	2-64	8	32

^a 50% and 90%, MIC for 50 and 90% of isolates tested, respectively.

^b As in M7-A (previous NCCLS guidelines).

^c As in M7-A2 (current NCCLS guidelines).

30). The MICs for *E. faecalis* are severalfold lower than those for *E. faecium*, in keeping with the more-pronounced intrinsic resistance of *E. faecium*. Since the first report of plasmid-mediated HLGR in strains of *E. faecalis* (9), the frequency of HLGR has increased to between 4.5 and 55% (13, 28). Although reports of HLAR in *E. faecium* are few (1, 3), we found 3.9% of 103 clinical isolates of *E. faecium* to have HLGR, 29% to have HLSR, and 2.9% to have high-level resistance to both aminoglycosides. Since the prevalence of HLAR appears to be low among clinical *E. faecium* isolates collected from across Canada, we selected additional *E. faecium* isolates with known HLAR to better evaluate the accuracy of the MicroScan panels and AMS-Vitek cards to detect HLAR.

We used brain heart infusion agar screen plates containing 1,000 µg of gentamicin or streptomycin per ml. Previous studies have incorporated 2,000 µg of streptomycin per ml for testing; as in our previous study with *E. faecalis* (7), we found that results obtained with either 1,000 or 2,000 µg of streptomycin per ml were consistent and concordant when *E. faecium* was tested (data not shown). With the single-concentration agar screen plate method as the "gold standard", the AMS-Vitek Gram-Positive Susceptibility card was superior to the MicroScan Pos MIC Type 6 panel for detection of HLAR among isolates of *E. faecium*. The MicroScan panels demonstrated poor sensitivity for detection of HLAR when readings relied on the Walk/Away system. Detection of HLAR improved when readings were made by visual inspection, especially after 48 h of incubation. These findings are similar to those of Sahm et al. (21), who found that prolonging incubation from 24 to 48 h increased detection of resistance by microdilution tests, including MicroScan. Those authors stressed the difficulties of interpreting growth in the synergy wells. Our results are also comparable to those of Weissmann et al. (26), who reported sensitivities as high as 100% for detection of HLGR *E. faecium* strains and sensitivities of 94 to 98% for detection of HLSR strains with visual inspection of MicroScan synergy wells after 20 h of incubation. However, prolonged incubation and visual inspection make MicroScan panels less practical for routine detection of HLAR. Comparisons of the three methods for HLAR detection among *E. faecalis* have been reported elsewhere (7, 21, 23) and found to have similar specificities and sensitivities.

HLAR predicts loss of synergy with penicillin. Bush et al. (1) have also shown that the presence of high-level penicillin

or ampicillin resistance (defined as MICs of 200 and 100 $\mu\text{g/ml}$, respectively) predicts loss of synergy with an aminoglycoside. The mechanism of high-level penicillin or ampicillin resistance is unknown. This form of resistance is not associated with β -lactamase production. We found non- β -lactamase ampicillin resistance in 22% of clinical isolates of *E. faecium*. The MicroScan Pos MIC Type 6 panel failed to adequately detect ampicillin-resistant isolates with MICs between 16 and 32 $\mu\text{g/ml}$ but were able to detect most isolates with MICs ≥ 64 $\mu\text{g/ml}$. There was no difference in the number of ampicillin-resistant strains detected by manual and automated MicroScan readings (data not shown). The AMS-Vitek system is superior to the MicroScan system in detecting both lower and higher levels of ampicillin resistance among *E. faecium* isolates but erred in calling six isolates resistant when they were susceptible. Although this resulted in minor errors, difficulties in interpretation appear to center near the recommended interpretive breakpoint of the system. Most laboratories do not test beyond the concentration recommended by the NCCLS guideline. Further studies are needed to determine at what level ampicillin resistance reliably predicts loss of synergy with an aminoglycoside and whether current commercial susceptibility tests are adequate in detecting resistance at such a level (1, 22). Resistance to vancomycin and teicoplanin has been described (12) but was not detected in this study.

We found that lowering the calcium content from 50 to 25 mg/liter resulted in a significantly greater MIC of daptomycin for enterococci. Although this phenomenon is reported in other organisms, it is most evident in enterococci (2, 6, 10, 25). The critical calcium content in broth for maximal daptomycin activity was not determined. Broth microdilution susceptibility testing for daptomycin can lead to erroneous interpretations as either susceptible or resistant, depending on the concentration of calcium used. Reporting an organism as resistant to daptomycin when daptomycin may be of clinical value may be deleterious. The clinical significance of these observations needs to be clarified and correlated with clinical treatment studies. A consensus regarding acceptable MIC breakpoints for interpretation of microdilution broth susceptibility testing is necessary in light of these results.

Antimicrobial resistance among isolates of *E. faecium* has made treatment of infection due to these organisms an ongoing challenge. Routine susceptibility testing for enterococci should include determination of susceptibility to ampicillin and vancomycin in addition to screening for HLAR. At present, the MicroScan Pos MIC Type 6 panel and the AMS-Vitek Gram-Positive Susceptibility card are not accurate for these purposes, and alternative testing methods should be used.

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