Comparison of Agar Dilution, Tube Dilution, and Broth Microdilution Susceptibility Tests for Determination of Ramoplanin MICs

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Standard broth microdilution (with and without bovine serum albumin [BSA] supplementation), tube dilution, and agar dilution susceptibility tests were compared for determining ramoplanin MICs. With a data base of 246 clinical isolates of gram-positive bacteria from 33 U.S. sites, it was shown that (i) agar and tube dilution susceptibility tests gave essentially the same results (93.9% of the test results were within 1 doubling dilution of equivalence), (ii) broth microdilution susceptibility tests gave results up to 5 doubling dilutions higher than agar or tube assays, and (iii) this data skewing could be reversed by BSA supplementation (final concentration, 0.02%) of the broth microdilution test medium.

Ramoplanin (MDL 62,198) is a glycolipodepsipeptide complex with high in vitro activity against gram-positive but not gram-negative bacteria (2–5). In this study, we directly compared the National Committee for Clinical Laboratory Standards agar dilution, tube dilution, and broth microdilution susceptibility tests for the measurement of ramoplanin MICs. Because of the reported enhancement of broth microdilution MICs by bovine serum albumin (BSA) supplementation (7), we also added this comparator to our study.

The assay quality control organisms *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 were purchased from Difco Laboratories, Detroit, Mich. Two hundred forty-six clinical isolates were obtained from 33 U.S. sites. These included 68 *S. aureus* isolates (32 oxacillin resistant), 19 *Staphylococcus epidermidis* isolates (15 oxacillin resistant), 26 *Staphylococcus haemolyticus* isolates (20 oxacillin resistant), 24 miscellaneous coagulase-negative staphylococci (6 *Staphylococcus hominis*, 4 *Staphylococcus saprophyticus*, 4 *Staphylococcus capitis*, 1 *Staphylococcus cohnii*, 2 *Staphylococcus sciuri*, 1 *Staphylococcus xylosus*, 3 *Staphylococcus warneri*, and 3 *Staphylococcus simulans* isolates), 20 *E. faecalis* isolates, 29 *Enterococcus faecium* isolates (7 Van A and 15 Van B), 2 *Enterococcus durans* isolates, 21 *Streptococcus pyogenes* isolates, 14 *Streptococcus agalactiae* isolates, 11 viridans streptococci, 3 group G streptococci, 3 *Micrococcus* spp., and 6 *Leuconostoc* spp. The identities of all isolates were confirmed in our laboratory.

Agar dilution, tube dilution, and broth microdilution (with and without 0.02% [final concentration] BSA; Fraction V, Miles, Kankakee, Ill.) were performed in accordance with the procedures outlined by the National Committee for Clinical Laboratory Standards (6). For each test, isolates were tested simultaneously by the four test formats with ramoplanin test dilutions freshly prepared from common master stock solution (0.1 M potassium phosphate buffer, pH 4.5).

The data from the comparative susceptibility tests are summarized in Table 1. The agar and tube dilution tests produced essentially identical results. Ramoplanin MICs for 231 (93.9%) of the 246 test organisms were within 1 doubling

dilution of equivalence. In contrast, broth microdilution MICs were much higher, with 175 (71.1%) and 156 (63.4%) of the values being 2 or more doubling dilutions above equivalence compared with tube and agar dilution values, respectively. This data skewing was observed across all species evaluated except for those organisms with which the broth dilution (micro- and tube) assays required fetal bovine serum (5%) supplementation (9 of 11 viridans streptococci and all *Leuconostoc* spp.). Substitution of 2% lysed horse blood for the fetal bovine serum did not produce the same effect. This skewing was also found to be independent of the β-lactam and glycopeptide resistance phenotype of the test organism. The significant variance of ramoplanin broth microdilution and plastic tube MICs from those obtained by glass tube or agar dilution assays has been ascribed to adherence of the antibiotic to plastic, has been shown to be independent of the type of microtiter tray employed, and could be obviated by protein supplementation of the medium (7, 8). Table 2 shows that 232 (94.3%) of the broth microdilution MICs were 2 or more doubling dilutions lower when the test medium contained a final concentration of 0.02% BSA. The only equivalent MICs in this comparison represented those organisms tested in the presence of 5% fetal bovine serum but not in the presence of 2% lysed horse blood. In contrast, 37.4 and 51.2% of the BSA-broth microdilution MICs were 2 or more doubling dilutions lower than those

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TABLE 1. Comparison of four MIC test formats for determination of ramoplanin susceptibilities

Comparison	No. of MICs at a doubling dilution difference of[a]:								
	≤−4	−3	−2	−1	0	+1	+2	+3	≥4
Broth vs:									
Tube				2	11	58	109	45	21
Agar				2	18	70	93	43	20
Agar vs tube			3	45	129	57	11	1	
BSA-broth vs:									
Broth	106	110	16	8	6				
Tube	1	19	72	121	30	3			
Agar		10	116	95	23	2			

[a] *n* = 246. Equivalent results are boxed.

TABLE 2. Quality control for the 16 assays used to generate the comparative MICs

Organism and MIC (μg/ml)	No. of positive assays			
	Agar	Tube	Broth	BSA-broth
S. aureus ATCC 29213				
2			13	
1	2		3	
0.5	14	1		1
0.25		12		6
0.125		3		9
E. faecalis ATCC 29212				
2			13	
1	7	5	3	
0.5	9	11		4
0.25				11
0.125				1

obtained by the tube and agar dilution tests, respectively. This skewing may indicate a requirement for BSA supplementation in the latter assay formats. The MIC susceptibility test format-related differences are also reflected in the limited amount of

TABLE 3. Effects of ramoplanin susceptibility test format on susceptibility test patterns

Organism(s) (*n*) and assay	MIC (μg/ml)[a]		
	Range	50%	90%
S. aureus (68)			
Broth	1–4	2	2
BSA-broth	0.06–0.5	0.125	0.25
Tube	0.25–1	0.5	1
Agar	0.25–1	0.5	1
Coagulase-negative staphylococci (69)[b]			
Broth	0.5–2	1	2
BSA-broth	0.015–0.25	0.125	0.25
Tube	0.06–1	0.25	1
Agar	0.06–1	0.5	0.5
Enterococci (51)[c]			
Broth	0.5–2	1	2
BSA-broth	0.06–0.25	0.125	0.25
Tube	0.03–1	0.25	0.5
Agar	0.125–1	0.25	1
Streptococci (40)[d]			
Broth	0.125–2	1	1
BSA-broth	0.0075–0.125	0.03	0.06
Tube	0.03–0.5	0.06	0.125
Agar	0.03–0.125	0.06	0.125

[a] 50% and 90%, MICs for 50 and 90% of isolates tested, respectively.
[b] Numbers of isolates: *S. epidermidis*, 19; *S. haemolyticus*, 26; *S. hominis*, 6; *S. saprophyticus*, 4; *S. capitis*, 4; *S. cohnii*, 1; *S. sciuri*, 2; *S. xylosus*, 1; *S. warneri*, 3; *S. simulans*, 3.
[c] Numbers of isolates: *E. faecalis*, 20; *E. faecium*, 29; *E. durans*, 2.
[d] Numbers of isolates: *Streptococcus pyogenes*, 21; *Streptococcus agalactiae*, 14; viridans streptococci, 2; group G streptococci, 3. Seven viridans streptococci were not included here because they were tested in the presence of 5% bovine serum and in the presence of 2% lysed horse blood.

test quality control data presented here (Table 2) and in compilations of MICs for 50 and 90% of strains tested (Table 3).

In summary, this study confirms and extends earlier observations (7) that showed a marked decrease in ramoplanin broth microdilution MICs when assays were run in protein-supplemented media. In contrast to these findings, Barry et al. (1) were unable to find such dramatic differences between BSA-broth microdilution and broth microdilution MICs. Such observations have important implications in establishing susceptibility breakpoints for ramoplanin and other antibiotics that may have a propensity for plastic adherence. In the study reported by Barry et al. (1) a tentative MIC breakpoint of ≤2.0 μg/ml was set for ramoplanin broth microdilution assays run without BSA supplementation while a tentative breakpoint of ≤1.0 μg/ml was set for BSA-supplemented broth microdilution and for agar dilution assays. Those data were based on the test population distribution characteristics with susceptibility breakpoints set to include isolates for which the MIC could be 1 doubling dilution greater than that for 99% of the tested strains. In our study, the unsupplemented broth microdilution MICs were 2.0 μg/ml for 69.1% of the *S. aureus* isolates, 40.6% of the coagulase-negative staphylococci, and 30.0% of the enterococci (for one *S. aureus* isolate, the MIC was 4.0 μg/ml). In addition, the tube dilution and agar dilution MICs were 1.0 μg/ml for 6.9 and 10.2% of our study population. For none of the test isolates were the MICs above 1.0 μg/ml with these techniques, and all BSA-supplemented broth microdilution MICs were ≤0.5 μg/ml. On the basis of these findings, we suggest raising the tentative ramoplanin susceptibility breakpoint to ≤4.0 μg/ml for broth microdilution assays performed without BSA supplementation and to ≤2.0 μg/ml for the remaining susceptibility tests.

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