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Broth Microdilution Testing of *Pseudomonas aeruginosa* and Aminoglycosides: Need for Employing Dilutions Differing by Small Arithmetic Increments

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The use of dilutions differing by small arithmetic increments was studied as a means for improving the definition and measurement of minimum inhibitory concentrations and precision parameters for testing *Pseudomonas aeruginosa* versus the aminoglycosides by the broth microdilution test. For five strains of *P. aeruginosa* versus gentamicin, tobramycin, and amikacin, comparisons were made of minimum inhibitory concentrations which were replicated in parallel by using three microdilution systems: small increment panels prepared by us, modified twofold dilution panels prepared by us, and similar modified twofold dilution panels obtained commercially. The small increment dilutions were prepared to differ by concentrations of 1.0 μg/ml for gentamicin and tobramycin and by 2.0 μg/ml for amikacin. Use of the small increment dilutions resulted in the ability to measure minimum inhibitory concentrations at more closely spaced intervals than those dictated by modified twofold dilution schemes, and confidence limits were significantly improved. The average coefficient of variation for the small increment microdilution test results was 9.5%, with 99.5% of minimum inhibitory concentrations falling within ±2 small increment dilutions from their modal values.

The availability of broth microdilution panels from commercial sources in conjunction with the uncertainties about the usefulness of proposed inhibition zone diameter breakpoint schemes for the standardized disk agar diffusion test (3, 4, 6, 7, 10, 11, 12) have prompted many clinical microbiology laboratories to use microdilution panels for testing *Pseudomonas aeruginosa* versus the aminoglycosides. Currently available microdilution panels generally use either twofold dilution schemes or, as recently proposed (1, 2), modified twofold dilution schemes which utilize one or more intermediate dilution steps. The relatively large and exponentially related increments of such schemes do not permit a more refined and exact measurement of minimum inhibitory concentrations (MICs) and test precision parameters as are required for testing microorganism-antimicrobial agent combinations exhibiting low toxic to therapeutic ratios, such as *P. aeruginosa* versus the aminoglycosides. Because of this problem, the present study was designed to investigate the use of dilutions differing by small arithmetic increments as a possible means for refining the measurement of MICs and improving the definition and magnitude of test precision parameters.

MATERIALS AND METHODS

Experimental design. Three broth microdilution systems were used in parallel to determine 100 MIC measurements by each microdilution system on each of five *P. aeruginosa* stock strains versus gentamicin, tobramycin, and amikacin. The three microdilution systems consisted of one obtained commercially which used a modified twofold dilution scheme, one prepared by us with a similar modified twofold dilution scheme, and one prepared by us with dilutions which differed by small arithmetic increments. The microdilution panels for each set of three tests performed in parallel were inoculated by appropriate suspensions prepared from a common broth growth source which was individually prepared for each set of three tests. All parallel tests were inoculated and interpreted at approximately the same time. Single lots of reagents and media were used throughout the investigation. On completion of the experimental work, 100 triplicate MIC data sets were thus available for each *P. aeruginosa* strain-antimicrobial agent combination and were analyzed by conventional statistical methods.

***P. aeruginosa* strains.** Test strains consisted of *P. aeruginosa* ATCC 27853, and four strains, SPR-9, SPR-88, SPR-128, and SPR-287, which were obtained from the St. Paul-Ramsey Medical Center Clinical Microbiology Laboratory stock culture collection of recent clinical isolates. The *P. aeruginosa* strains were selected for their known MICs so as to provide values

TABLE 1. A comparison of MICs for gentamicin versus *P. aeruginosa* as determined by a microdilution test with dilutions differing by small arithmetic increments and microdilution tests with modified twofold dilution schemes

MIC (μg/ml)	No. of *P. aeruginosa* strain-microdilution test[a] combinations at each indicated MIC														
	ATCC 27853			SPR-88			SPR-128			SPR-287			SPR-9		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
>16											1	2	100	100	100
16						16		98	61		98	89			
15															
14															
13										1					
12										9					
11							1			38					
10							18			46					
9				1			47			4					
8				3	73	46	29	2	31	2	1	7			
7				63			5								
6				33	25	35			7			1			
5															
4	1	77	42		2	3			1			1			
3	79														
2	19	23	57												
1			1												
≤1	1														
≤0.5															

[a] A, Small increment microdilution test using 1.0-μg/ml increments for gentamicin; B, microdilution test prepared in our laboratory with a modified twofold dilution scheme; C, MMS microdilution test with a modified twofold dilution scheme.

ranging from susceptible to resistant for each antimicrobial agent. Strains were retrieved weekly from stock cultures by subculture to tubes of tryptic soy broth which were incubated for 3 to 5 h with mechanical agitation in air at 35°C, followed by two consecutive overnight 18- to 24-h subcultures on sheep blood agar plates in air at 35°C. Separate individual broth growth sources were prepared for each set of three parallel microdilution tests. These growth sources were prepared from either the second stock retrieval sheep blood agar plate or a daily prepared sheep blood agar purity plate by picking the center of 3 to 5 colonies into tubes of cation-adjusted Mueller-Hinton broth which were incubated 3 to 5 h in air at 35°C with mechanical agitation. Inoculum suspensions for each microdilution test system were prepared from the broth growth sources described below for each of the systems.

Small increment microdilution test. Small increment microdilution panels were prepared by the St. Paul-Ramsey Medical Center Clinical Microbiology Laboratory staff with the MIC-2000 dispenser (Dynatech Laboratories, Inc., Alexandria, Va.). Details of plate production, storage, inoculation, and incubation corresponded to those described by us previously (9). All dilutions were made with Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) that had been adjusted by us to provide calcium and magnesium concentrations of 5.5 ± 0.2 and 2.5 ± 0.2 mg/dl, respectively. For gentamicin (Schering Corp., Bloomfield, N.J.) and tobramycin (Eli Lilly & Co., Indianapolis, Ind.), microdilution panels were prepared so that dilutions differed by 1.0-μg/ml increments through 16.0 μg/ml. For amikacin (Bristol Laboratories, Syracuse, N.Y.), microdilution panels were prepared so that dilutions differed by 2.0 μg/ml and ranged from 2.0 through 32.0 μg/ml. Inoculum suspensions were prepared by diluting the growth sources with cation-adjusted Mueller-Hinton broth to the density of a 1.0 McFarland standard followed by an additional 1:10 dilution. The small increment microdilution panels were inoculated with the MIC-2000 inoculating apparatus, which was designed to produce a concentration of 8.5×10^5 colony-forming units per ml within the panel wells. The microdilution panels were incubated for approximately 18 h in air at 35°C before interpretation. MIC was defined as the minimum concentration of an antimicrobial agent which produced no visual turbidity, no clusters or clumps, and no focal opacity greater than 1 mm in diameter.

Commercial microdilution test with modified twofold dilution scheme. Gram-negative enteric microdilution panels (MMS, Micro-Media Systems, Potomac, Md.) were purchased as a single lot from a local supplier and stored at −20°C until used. The modified twofold dilution scheme for gentamicin and tobramycin provided concentrations ranging from 0.5 through 16 μg/ml, with the addition of a 6.0 μg/ml step. Similarly, concentrations for amikacin ranged from 2.0 through 32 μg/ml, with the addition of a 24.0 μg/ml step. The frozen microdilution panels were brought to room temperature before use. Inoculum suspensions were prepared by visually adjusting the broth growth source to the density of a 0.5 McFarland standard, which was then followed by a 1:50 dilution with a cation-adjusted 0.02% Tween-80 distilled water diluent, obtained from

TABLE 2. A comparison of MICs for tobramycin versus *P. aeruginosa* as determined by a microdilution test with dilutions differing by small arithmetic increments and microdilution tests with modified twofold dilution schemes

MIC (μg/ml)	No. of *P. aeruginosa* strain-microdilution test[a] combinations at each indicated MIC														
	ATCC 27853			SPR-88			SPR-128			SPR-287			SPR-9		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
>16															4
16														100	94
15															
14															
13															
12															
11													11		
10													34		
9													38		
8									1			7	12		2
7													3		
6									9		22	58	2		
5										8					
4						19	28	100	84	92	78	35			
3							72								
2				100	100	77			6						
1		41	57			4									
≤1	100														
≤0.5		59	43												

[a] A, Small increment microdilution test with 1.0-μg/ml increments for tobramycin; B, microdilution test prepared in our laboratory with a modified twofold dilution scheme; C, MMS microdilution test with a modified twofold dilution scheme.

the manufacturer, which was designed to produce calcium and magnesium concentrations of approximately 5.0 and 2.5 mg/ml in the panel wells. The microdilution panels were inoculated with disposable inoculating devices which were supplied as part of the MMS system and which were designed to produce final inoculum densities of 1×10^5 to 2×10^5 colony-forming units per ml in the wells.

Non-commercial microdilution panels with modified twofold dilution scheme. Microdilution panels with the same modified twofold dilution scheme as that described for the commercial microdilution panels were prepared by us by using the MIC-2000 apparatus as described for the small increment system, including the use of identical lots of media and reagents. Plates were sealed with tape, quick frozen at −70°C, and then stored at −20°C in plastic bags before use. Plates were inoculated with the MIC-2000 inoculating apparatus under conditions of inoculum preparation, incubation, and interpretation similar to those described previously for the small increment microdilution test system.

RESULTS

Tables 1 through 3 summarize the MIC results for the parallel tests performed by the three microdilution systems. Although the MMS and MIC-2000 systems used the same modified twofold dilution scheme, a considerably wider dispersion was noted for the MMS MICs. In this regard, from the combined MIC data from Tables 1 through 3, the percentages of MICs which were modal, ±1 modified twofold dilution step from the modal value, ±2 steps, and ±3 steps were 68.4, 30.0, 1.4, and 0.2%, respectively, for the MMS system, and 90.5, 9.1, 0.4, and 0%, respectively, for the MIC-2000 panels. In contrast, 99.5% of the MICs determined by the small increment microdilution test were within ±2 arithmetic increments from their modal values. Table 4 summarizes the coefficients of variation which were found for the small-increment microdilution test values at various levels of the MIC test dilution range. The average coefficient of variation was 9.5%.

DISCUSSION

The premise of this investigation is that currently used microdilution schemes provide MICs which are insufficiently exact for assessing the susceptibility of *P. aeruginosa* to the aminoglycosides. Our results demonstrate that MICs can be better defined and that precision parameters can be significantly improved by using dilutions that differ by small arithmetic increments rather than by twofold dilution steps or modifications thereof. MICs were definable by 1.0 μg/ml steps, with a coefficient of variation of 9.5%. Of replicated MICs, 99.5% were within ±2 small increment steps from their modal values. This improvement in MIC definition and precision parameters becomes particularly important when considering the validity of MICs

TABLE 3. A comparison of MICs for amikacin versus *P. aeruginosa* as determined by a microdilution test with dilutions differing by small arithmetic increments and microdilution tests with modified twofold dilution schemes

MIC (μg/ml)	No. of *P. aeruginosa* strain-microdilution test[a] combinations at each indicated MIC														
	ATCC 27853			SPR-88			SPR-128			SPR-287			SPR-9		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
>32												2	100	100	100
32									2		1	11			
30															
28															
26															
24								76	41		92	62			
22										5					
20							6			26					
18							41			54					
16					2	56	45	24	56	12	7	25			
14							2			1					
12							6			2					
10				5											
8		3	18	85	98	44									
6	1	1		1											
4	99	96	80	9					1						
2			2												
≤1															

[a] A, Small increment microdilution test with 2.0-μg/ml increments for amikacin; B, microdilution test prepared in our laboratory with a modified twofold dilution scheme; C, MMS microdilution test with a modified twofold dilution scheme.

which approach toxicity thresholds, since for twofold dilution schemes, MIC definition and confidence limits progressively worsen as dilution steps exponentially widen. Specifically, well-defined MICs are particularly important in the range of 6 to 12 μg/ml for gentamicin and tobramycin, and in the range of 12 to 24 μg/ml for amikacin. Correspondingly, the more exact determination of MICs in these ranges should augment and facilitate the use of pharmacokinetic dosing approaches for the aminoglycosides (5, 8, 13, 14), which permit a relatively exact adjustment of therapeutic peak levels. On the basis of these considerations and the results of this study, we suggest that dilutions differing by small arithmetic increments should be employed when testing *P. aeruginosa* versus the aminoglycosides by the microdilution test.

TABLE 4. Coefficients of variation for the small-increment microdilution test in relation to various average MICs representing a range from susceptible to resistant values for *P. aeruginosa* versus gentamicin, tobramycin, and amikacin

Aminoglycoside	*P. aeruginosa* strain	Mean MIC[a]	CV[b]
Amikacin	SPR-287	18.3	9.1
Amikacin	SPR-128	16.8	10.4
Gentamicin	SPR-287	10.5	8.2
Tobramycin	SPR-9	9.3	11.3
Gentamicin	SPR-128	8.8	9.5
Amikacin	SPR-88	7.7	16.2
Gentamicin	SPR-88	6.7	8.0
Amikacin	ATCC 27853	4.0	5.0
Tobramycin	SPR-287	4.0	6.8
Tobramycin	SPR-128	3.3	13.3
Gentamicin	ATCC 27853	2.8	16.0
Tobramycin	SPR-88	2.0	0

[a] Mean MIC, Average MIC in μg/ml for 100 MIC determinations for each *P. aeruginosa* strain-aminoglycoside combination.

[b] CV, Coefficient of variation; CV for all trials, 9.5%.

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