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MBCs for *Staphylococcus aureus* as Determined by Macrodilution and Microdilution Techniques

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MBC testing of clindamycin, methicillin, cephalothin, gentamicin, and vancomycin with 67 clinical isolates of *Staphylococcus aureus* was examined by both standard macrodilution tubes and commercial microdilution trays. Standard macrodilution failed to give reproducible (99.9% killing) MBC results, even when a strictly defined protocol was followed. Continuous shaking during incubation resulted in regrowth of more colonies than did stationary incubation. Vortexing of incubated tubes before subculture resulted in regrowth of more colonies than did careful transfer of the contents to sterile tubes before vortexing and subculture. No significant difference in MBCs was demonstrated by the use of log-phase versus stationary-phase inocula. Use of the multiprong inoculator for subculture from commercial microdilution trays was unsatisfactory because, although antibiotics evaluated were inactivated by subculture to a pH 5.5 agar plate coated with a beta-lactamase solution, the volume of broth transferred by the prongs was small and inconsistent, ranging from 0 to 3 μl. Subcultures of commercial microdilution panels with a 1-μl loop, 10-μl pipette, and 100-μl pipette were also evaluated. Results of MBC testing were most reproducible when the entire 100-μl volume was aspirated from commercial microdilution wells after stirring and the contents of each well were spread over a separate sheep blood agar plate.

Determination of the bactericidal activity of an antimicrobial agent against an infecting organism may be useful in guiding therapy for serious infections, particularly in situations in which host defense mechanisms are limited. Before the usefulness of such data can be evaluated appropriately, a reproducible method for determining bactericidal activity must be devised.

Although no standard method has been established, MBC testing procedures involving subcultures from broth macrodilution (MACRO) tubes have been widely used. Results of such testing with some groups of organisms have been difficult to interpret and reproduce (2, 7, 11). Poor test reproducibility makes correlation with therapy of clinical infections exceedingly difficult. In addition, MACRO MBC testing is time consuming and cumbersome to perform. Objectives for this study were to (i) examine factors possibly related to the poor reproducibility of MACRO MBC test results, (ii) evaluate the usefulness of commercially available broth microdilution (MICRO) trays for MBC testing, and (iii) develop a reproducible method for in vitro evaluation of bactericidal activity.

MATERIALS AND METHODS

Inoculum preparation. Sixty-seven clinical isolates of *Staphylococcus aureus* recovered from blood and body fluid specimens were stored in tryptic soy broth (Difco Laboratories, Detroit, Mich.) plus 10% glycerine on glass beads at −70°C until tested. Before study, each organism was thawed and streaked onto a sheep blood agar plate and incubated overnight at 35°C. Five to ten isolated colonies from this plate were inoculated into broth and incubated in a 35°C water bath for 2 to 4 h until turbidity matched that of a 0.5 McFarland standard (log-phase growth). When the technique was modified to examine the effect of stationary-phase growth, incubation in broth was continued overnight; then turbidity was adjusted to match a 0.5 McFarland standard, and the suspension was immediately diluted and inoculated into the test systems.

MACRO MBC tests. Samples (1 ml) of Mueller-Hinton broth containing serial dilutions of an antimicrobial agent (clindamycin, methicillin, cephalothin, gentamicin, vancomycin) were used. The rack of inoculated tubes was gently shaken to mix the contents, and tubes were incubated in a 35°C air incubator for 22 to 24 h. Tubes were then individually shaken by hand to resuspend the contents, and a 0.01-ml calibrated loopful was streaked over a quadrant of a 90-mm blood agar plate (tryptic soy agar base with 5% sheep blood; GIBCO Diagnostics, Madison, Wis.). Colonies on subculture plates were counted after 24 h at 35°C. The MBC was defined as the lowest concentration of an antimicrobial agent which resulted in at least a 99.9% kill of the inoculum and was such that no higher concentration yielded less than a 99.9% kill. The initial inoculum concentration was determined for each test by plating a measured volume from the growth control tube at 0 time. Counts were 1×10^5 to 5×10^5 CFU/ml. The 99.9% kill calculated on that basis allowed from one to five colonies per 0.01-ml subculture. This MACRO MBC procedure was used for all 67 isolates. Modifications of this procedure which were performed in triplicate on two isolates in methicillin and cephalothin included vortexing rather than hand mixing just before subculture, carefully transferring tube contents to a sterile tube for vortexing just before subculture, and incubating tubes in a shaking water bath, followed by either hand mixing or vortexing just before subculture.

Inoculation of MICRO trays. Frozen broth microdilution Micro Scan trays (American Micro Scan, Campbell, Calif.) and Micro Media trays (Micro Media Systems, Inc., San Jose, Calif.) were inoculated with log-phase growth according to the instructions of the manufacturers, with a multiprong inoculator. This assured that the inoculum was intro-

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duced directly into the broth and did not contact the sides of the wells as did the inoculum in the MACRO tube tests. Colony counts at 0 time were determined by plating a measured volume from the seed tray. Trays were incubated for 24 h at 35°C. Various subculture procedures, described below, were then performed.

Multiprong inoculator MICRO MBC subcultures. The same type of multiprong inoculator used to inoculate trays was used to transfer samples from the wells, after mixing, to a rectangular tryptic soy agar (Difco) plate (40 ml of agar poured into a sterile seed tray). To inactivate aminoglycosides that might be carried over with the inoculum, the pH of some plates was lowered with NaH_2PO_4 to give a final agar surface pH of 5.5. To inactivate beta-lactam antibiotics, 1 ml of a sterile aqueous beta-lactamase (Whatman Biochemicals, Ltd., Maidstone, Kent, England) solution containing 48.5 ± 5 U of beta-lactamase I per ml and 6.8 ± 0.4 U of beta-lactamase II per ml was swabbed over the surface of the pH 5.5 agar and allowed to dry. Subcultures of 18 isolates with the multiprong inoculator were performed onto both plain pH 7.0 Trypticase soy agar plates and onto pH 5.5 beta-lactamase-coated plates. When the multiprong inoculator was found to be unsuitable for these subcultures because small and inconsistent volumes were transferred, this method was abandoned.

MICRO MBC subcultures (0.01 ml). Contents of wells showing no growth were stirred with the sterile tip of a semiautomated pipette (Medical Laboratory Automation, Inc., Mt. Vernon, N.Y.), and a 0.01-ml sample was removed and streaked over a quadrant of a sheep blood agar plate (a 0.01-ml calibrated loop was too large to fit into the wells). This pipette subculture procedure was performed for all 67 isolates.

Total-volume MICRO MBC subcultures. Contents of wells that did not show growth were stirred with a 100-μl semiautomatic pipette, and the entire contents of each well (100 μl) were aspirated and inoculated as a streak across the diameter of a 90-mm sheep blood agar plate. After allowing about 20 min for the liquid to be completely absorbed into the medium with the cover removed, the inoculum was spread over the entire surface of the plate with a bent glass rod that had been dipped in alcohol and flamed. Colonies on plates were counted after 24 h of incubation at 35°C. Preliminary testing confirmed that allowing the antibiotic to be absorbed minimized inhibition of the inoculum on the plate, compared with spreading the antibiotic solution without allowing it to absorb.

Four-hour subcultures. Immediately after inoculation, the growth well was stirred with a sterile 0.001-ml calibrated loop to mix the contents, and 0.001 ml was removed and plated onto a sheep blood agar plate to determine the 0 time inoculum concentration. Trays were incubated for 4 h in a 35°C air incubator and then removed, and the growth control well plus all wells containing antimicrobial agents of interest were stirred to mix, sampled with a sterile 0.001-ml loop, and streaked over quadrants of sheep blood agar plates. Trays were returned to the incubator for total-volume MICRO MBC subcultures after 24 h of incubation.

RESULTS

MACRO MBC tests. MACRO MBCs were not reproducible when testing was performed under these test conditions simultaneously by two workers or when performed multiple times by one worker. Table 1 summarizes the subculture results with cephalothin for one strain of *S. aureus* tested by two workers under our standard MACRO MBC test conditions and with several modifications of that protocol. The cephalothin MIC was 1 μg/ml or less. Vortexing after incubation, immediately before subculture, resulted in a slight increase in the number of organisms recovered compared with the somewhat more gentle hand mixing. Careful aspiration of tube contents, avoiding contact with the sides of the tube, and transfer to a sterile tube for vortexing and subculture resulted in the lowest numbers of organisms recovered, but triplicate results still did not agree well. Continuous shaking during incubation (shaking water bath) resulted in very heavy growth of organisms, particularly when followed by vortexing just before subculture. Cephalothin testing of another isolate and triplicate methicillin testing of the two strains showed equivalent results.

Growth-phase and stationary-phase inocula of five *S. aureus* strains were used to determine MACRO MBCs of methicillin and cephalothin by two workers. Although triplicate tests again failed to agree well, no difference between growth-phase and stationary-phase inocula was demonstrated. A total of 8 of 15 (53%) log-phase MBCs and 9 of 15 (60%) stationary-phase MBCs of methicillin were more than two doubling dilutions higher than the corresponding MIC. Cephalothin showed 11 of 15 (73%) log-phase and 9 of 15 (60%) stationary-phase inoculum MBCs more than two doubling dilutions above the corresponding MIC.

MICRO MBC testing. Preliminary work with a multiprong inoculator to transfer antibiotic solutions from the panels to a subculture plate seeded with *S. aureus* showed that the amount of antibiotic carried to the plate did produce some zones of inhibition. Identical testing performed on pH 5.5 plates coated with beta-lactamase solution to inactivate aminoglycoside and beta-lactam antibiotics showed no zones of inhibition, suggesting that these modifications were useful in limiting interference from antibiotic carry-over when this spot inoculation method was used.

Delivery of inoculum from the MICRO wells to the subculture plates, however, was found to be inconsistent. Upon visually examining the liquid on the prongs of the multiprong inoculator, it was apparent that the prongs held a larger volume when sampling from a seed tray than when sampling from the MICRO wells, presumably because of differences in the surface tension in the two types of containers. Estimates of volumes picked up by the multiprong inoculators under both testing conditions were made by visually comparing the size of the drop of liquid picked up by the prongs from seed trays and wells with that of measured volumes pipetted onto prongs of inoculators made by both companies (Micro Media and Micro Scan). Although inoculators from both manufacturers appeared to accurately pick up 5 μl of water with Tween from the seed tray, as they were designed to do, they picked up only 0 to 3 μl of liquid when sampling from the MICRO trays. MBC data from multiprong inoculator subcultures were therefore not evaluated.

Comparison of MACRO MBCs with 0.01-ml subculture MICRO MBCs. Much higher MBC:MIC ratios were seen with the conventional MACRO MBC procedure (10-μl loop subculture) when methicillin, cephalothin, gentamicin, and vancomycin were tested than with the MICRO MBC technique (10-μl semiautomatic pipette subculture), which was most similar of the MICRO subculture techniques evaluated. Clindamycin, not expected to be a bactericidal antibiotic, showed high MBC:MIC ratios with both methods. Data are summarized in Table 2.

Total-volume MICRO MBC subcultures. Twenty-three strains of *S. aureus* were tested with methicillin, cepha-

TABLE 1. Number of colonies from 0.01-ml MACRO subculture[a]

Cephalothin (μg/ml)	No. of colonies in:												
	Tubes stationary during incubation									Tubes incubated in shaking water bath			
	Hand mix			Vortex			Aspirate and then vortex in sterile tube			Hand mix		Vortex	
	A	A	B	A	A	B	A	A	B	A	A	A	A
64	3	16	0	>100	23	0	5	2	2	1	0	>100	>100
32	15	>100	0	40	6	0	13	1	2	>100	0	>100	>100
16	>100	>100	0	>100	6	8	22	2	3	>100	50	>100	>100
8	18	18	0	65	>100	3	19	2	8	>100	27	>100	>100
4	30	75	0	>100	82	3	13	5	4	50	78	>100	>100
2	14	56	0	67	52	2	18	2	13	>100	>100	>100	>100
1	11	>100	0	64	>100	3	13	2	62	>100	>100	>100	>100

[a] A, Test performed by worker A; B, test performed by worker B.

lothin, gentamicin, and vancomycin by this method. For methicillin, there was a clear break where growth on the subculture plate changed from >300 colonies to <10 colonies for 22 of 23 strains tested. This MBC was identical to the MIC for 8 of the strains and was one dilution above the MIC for 14 of the isolates. The remaining strain showed no growth from the well representing the MIC, or from the next two doubling dilutions, but the third well above the MIC grew 50 colonies on subculture. Gentamicin 24-h entire-well subcultures for the 23 strains uniformly failed to grow, with the exception of one colony from one concentration with one bacterial strain. Of the 23 isolates, 11 showed no growth on any of the vancomycin 24-h entire-well subcultures, whereas the remaining 12 strains grew 10 or fewer colonies per well. Four of the bacteria grew more than 10 colonies per well on cephalothin subcultures, and 12 isolates showed no growth from any of the cephalothin wells. By aspirating the contents of wells showing no growth and by allowing the streak of inoculum to be absorbed into the medium before spreading over the surface of the plate, inhibition resulting from antibiotic carry-over was minimized. Figure 1 demonstrates the difference in regrowth with cephalothin between spreading the inoculum over the plate immediately after it is deposited on the surface versus spreading the inoculum over the plate after the liquid has been absorbed as a streak. Figure 2 demonstrates the utility of this method for nafcillin.

TABLE 2. Number of 67 *S. aureus* strains showing MBC:MIC ratio

Antibiotic	Technique[a]	No. of strains with following MBC:MIC ratio					
		1:1	2:1	4:1	8:1	>8:1	NT[b]
Clindamycin	MACRO	3	0	0	0	62	2
	MICRO	2	0	0	0	63	2
Methicillin	MACRO	3	7	3	7	46	1
	MICRO	46	7	1	3	9	1
Cephalothin	MACRO	4	3	2	2	55	1
	MICRO	45	4	3	1	13	1
Gentamicin	MACRO	3	5	3	0	51	5
	MICRO	50	6	3	0	3	5
Vancomycin	MACRO	5	2	3	1	56	0
	MICRO	32	9	6	5	15	0

[a] MICRO, Micro Scan trays with 0.01-ml pipette subculture.
[b] NT, Not tested.

Four-hour MICRO MBC subcultures. The 23 strains of *S. aureus* on which total-volume subcultures were done were also tested by this method. Zero time colony counts from 0.001-ml growth well subcultures for the 23 isolates ranged from 28 to 132 colonies, with a mean of 74. Four-hour 0.001-ml subcultures from growth control wells of all 23 isolates showed >300 colonies. An additional isolate was excluded from the analysis because the 4-h 0.001-ml growth well subculture grew only 28 colonies.

Gentamicin 4-h 0.001-ml subcultures of only 3 of the 23 organisms showed any growth. In two cases this was a single colony from one concentration. The third isolate had growth which ranged from one to eight colonies from each of the 4-h wells. Cephalothin and vancomycin 4-h 0.001-ml subculture results were difficult to categorize, with growth of 4 to 50 colonies occurring from many wells.

For methicillin, when a breakpoint for 4-h subcultures was defined as the well in which growth from the 0.001-ml subculture changed from >100 colonies to <100 colonies, that MBC corresponded with the 24-h entire-well subculture MBC for 13 isolates, was one well lower for 8 isolates, and was two wells lower for 2 isolates. Methicillin results for three *S. aureus* strains tested in duplicate by total-volume and 4-h MICRO MBC tests are shown in Table 3. Methicillin, cephalothin, gentamicin, and vancomycin showed good agreement between duplicate counts at 24 h and showed low MBCs.

DISCUSSION

Factors that may contribute to the poor reproducibility of MBC tests have been addressed by numerous workers (2, 5, 7, 8, 11, 12). It is interesting to note that worker A (Table 1) used a semiautomatic pipette to introduce the inoculum into the tubes for all tests (apparently resulting in large numbers of organisms being deposited on the sides of tubes), whereas worker B added inoculum with a serological pipette, running inoculum along the side of each tube (apparently resulting in fewer organisms being left unexposed to the antimicrobial agent in the broth). The contrast in MACRO and MICRO MBC results (Table 2) may be evidence that the high MBCs obtained with our MACRO method are spuriously high because of organisms inadvertently inoculated onto the sides of tubes where they were not exposed to the antibiotic. This situation did not occur in the MICRO test since the multiprong inoculator introduces inoculum into the centers of the wells. Possible differences resulting from sampling MACRO tubes with a 10-μl loop and MICRO trays with a 10-μl pipette were not evaluated. If a MACRO MBC procedure is chosen,

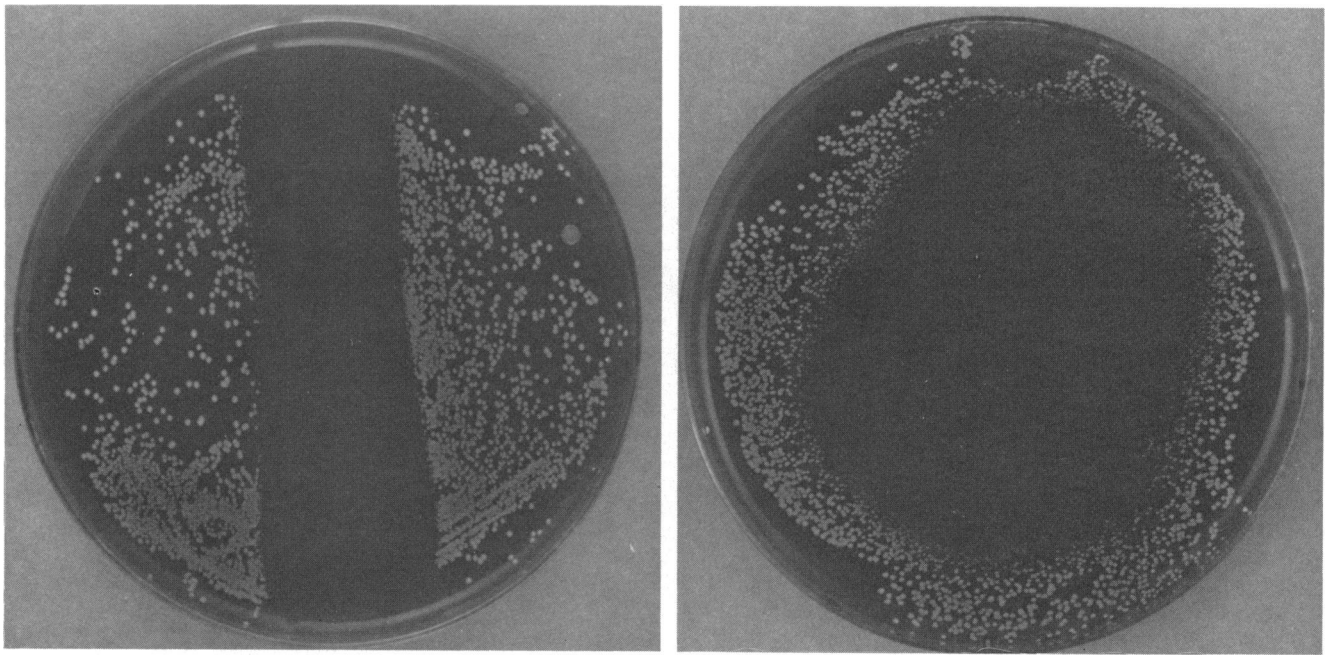

FIG. 1. Inoculum containing cephalothin at 16 μg/ml spread after allowing the streak of inoculum to be absorbed into the agar (A) or spread immediately without being absorbed (B). The effect of antibiotic carry-over is much more noticeable when the inoculum is spread immediately.

we concur that careful delivery of the inoculum beneath the broth surface of the antibiotic-containing tubes as recommended by Ishida and colleagues (7) and Taylor and colleagues (11) would minimize the number of organisms adhering to the tube sides. The aspiration technique we attempted would be expected to be equivalent to the careful sampling, without mixing, performed by Bradley and colleagues (3), but our colony counts were not reproducible on triplicate trials. Higher MBCs have been reported by Mayhall and Apollo (8) and Taylor and colleagues (11) with stationary-phase compared with log-phase growth. We did not demonstrate a difference in MBCs in tests inoculated with log-phase versus stationary-phase organism suspensions but had high percentages of organisms showing elevated MBCs by both methods.

Killing of more than 99.9% of the bacterial inoculum is a widely accepted definition of bactericidal activity. However, selection of this level of bactericidal activity is arbitrary, and

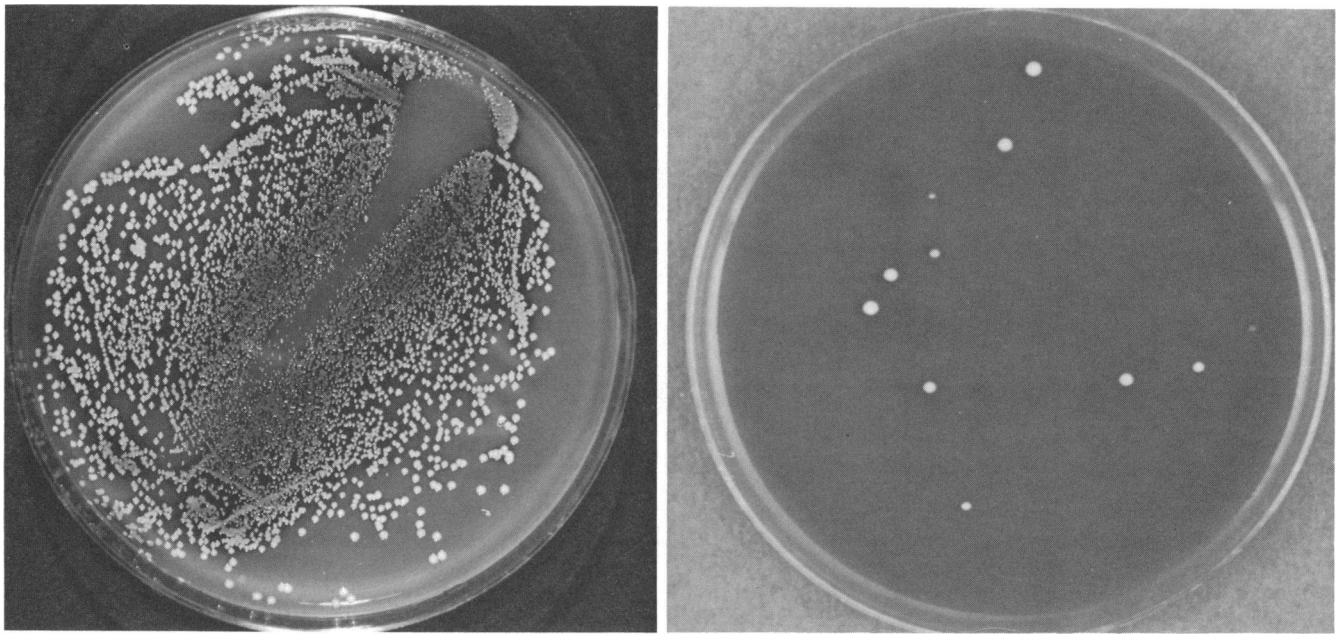

FIG. 2. Subcultures of nafcillin 8-μg/ml wells spread after drying. Wells were subcultured immediately after inoculation subcultured after 24 h of incubation in well to show potential inhibition from antibiotic carryovery (A) and (B). Although the antibiotic concentration was more than 10 times the MIC, growth is readily evident on the subculture plate, with little effect of antibiotic carry-over.

TABLE 3. Number of colonies from 0.001- and 0.1-ml MICRO subcultures[a]

Methicillin (μg/ml)	No. of colonies from: 0.001-ml 4-h cultures of strain						Total-volume 24-h subcultures of strain					
	1		2		3		1		2		3	
	A	B	A	B	A	B	A	B	A	B	A	B
Control	>300	>300	>300	>300	>300	>300	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000
0.25	250	>300	200	250	>300	>300	NT	NT	NT	NT	NT	NT
0.5	250	>300	200	250	156	200	NT	NT	NT	NT	NT	NT
1	200	208	69[b]	14[b]	>200	275	NT	1,000	0[b]	0[b]	NT	NT
2	13[b]	0[b]	2	2	110	8[b]	302	4[b]	0	1	1[b]	0[b]
4	1	1	0	0	1[b]	0	2[b]	2	0	1	1	1
8	7	6	1	0	1	2	0	3	0	0	1	0
16	26	17	5	0	24	9	1	1	0	0	0	0

[a] A, First test; B, duplicate tested another day. NT, Not tested (since well was cloudy).
[b] Well denoting MBC for that test.

the definition is difficult to apply in actual practice. This level of killing was suggested by Eagle and Musselman (4) when using an inoculum of 10^3 CFU/ml. They assumed that persistence of one organism implied persistence of infection; therefore, an initial inoculum of 10^3 CFU/ml would require a killing of >99.9% of the inoculum to achieve elimination of the organisms, and an initial inoculum of 10^6 CFU/ml would similarly require killing of >99.9999% of the organisms to ensure that none were viable. Although an inoculum concentration of 10^5 CFU/ml is widely used in susceptibility testing (1), a definition of bactericidal activity as >99.9% killing has often been retained since the volume of sample that can be plated and, therefore, the percentage of kill that can be determined when working with higher initial inoculum concentrations are limited. It is difficult to know what level of bactericidal activity is clinically important. A 1-log decrease in viable count after a short incubation may be as useful a starting point as a 99.9% reduction after 24 h.

For our standard MACRO and 0.01-ml subculture MICRO MBC testing, we chose to calculate the allowable number of colonies for subcultures of each organism based on the 0 time colony count in the growth control tube. Other workers have selected a number of colonies to represent allowable regrowth and applied this number to all tests. In either case, the error inherent in the sampling methods employed does not justify the weight given to colony counts near the cutoff point. Anhalt and colleagues (1) have addressed this problem and recommended a mathematical analysis as one way of evaluating the significance of colony counts slightly higher than the designated cutoff. Another problem is that of regrowth of more than the allowable number of colonies in concentrations higher than that which killed at least 99.9% of the inoculum. By requiring that no higher concentration than that designated as the MBC yield less than a 99.9% kill, we bias our MBCs toward much higher concentrations than do workers defining the MBC solely in terms of the lowest concentration to kill 99.9% of the inoculum. This probably accounts for the fairly large number of isolates shown by the MICRO 0.01-ml MBC procedure (Table 2) to have high MBCs even though inoculum was introduced directly into the broth without contacting the sides of the wells.

At least two techniques for performing efficient subcultures from MICRO trays have been reported by other workers. Harwick and colleagues (6) designed a device consisting of multiple horizontal loops, enabling simultaneous spot inoculation onto agar media from one row of a MICRO tray. Prober and colleagues (10), as well as Zinner and colleagues (12), have made use of multipoint inoculating devices. Although the inoculators we used did not perform the subcultures accurately or consistently, Prober et al. performed tests in triplicate with transfers to broth rather than agar media and Zinner et al. used a device different from the commercial inoculators we evaluated.

We examined several methods for mixing the contents of MICRO wells before pipette subculture. Although a vortex mixer has been used successfully by Prober and colleagues (10), we experienced problems with cross contamination from well to well even when a plastic seal strip was applied tightly to the tray. When, instead of removing the seal strip after vortexing, the strip was punctured over each well and the contents were sampled through the openings, the cross-contamination problem was eliminated based on comparison of prevortexing and postvortexing colony counts from the same wells, but the procedure was cumbersome and apparently unnecessary. Attempts at vortexing panels were therefore abandoned, and the well contents were stirred with the sterile pipette tip before aspirating the sample.

We favor further development of MICRO bactericidal tests since introduction of inoculum into broth without contacting the sides of the container is easily accomplished in MICRO trays but very difficult in MACRO tubes. Our current data indicate that MICRO MBCs with total-volume subculture yield reproducible results more easily than does the MACRO MBC test. Potential use of MICRO trays in determining killing rates also appears to merit further evaluation. Since a correlation between tolerance and rate of killing has been reported by Mayhall and colleagues (9), it may be preferable either to sample at one or more early timepoints, e.g., 4 h, when bacterial concentrations remain quite high or to sample a larger volume at a later time point as we did in the total-volume MICRO subcultures at 24 h or to do both. Even later sampling times may be indicated for antimicrobial agents that kill more slowly. The effect of lengthening the incubation time for subculture plates could also be examined.

We would caution other workers, however, to carefully evaluate the accuracy of using multiprong inoculators when sampling from the MICRO wells or to select an alternative subculture method such as the one found to be most reproducible in this investigation, which consisted of inoculating the entire contents of each well across a separate sheep blood plate, allowing the liquid to dry, and then spreading the sample over the entire plate surface.

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