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Lack of Reproducibility of Macrodilution MBCs for *Staphylococcus aureus*

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MBCs of methicillin, oxacillin, penicillin G, cephalothin, vancomycin, and gentamicin were determined by a standard broth macrodilution technique for 101 clinical isolates of methicillin-susceptible *Staphylococcus aureus*. Increased killing (more than 99.9%) was observed after 48 versus 24 h of incubation for many strains, and cross tolerance to antimicrobial bactericidal activity (less than 99.9% killing) was frequently observed among antimicrobial agents. However, these in vitro measurements of bactericidal activity against *S. aureus* were not reproducible.

Best and colleagues (4) described a strain of *Staphylococcus aureus* susceptible to oxacillin by MIC criteria but resistant to the bactericidal effect of oxacillin in killing-curve experiments. Strains tolerant to the bactericidal and lytic effects of oxacillin demonstrated oxacillin-induced inhibition of staphylococcal autolytic enzymes on the surface of the organism (3). From these studies, Best et al. concluded that the usual bactericidal effect of oxacillin was due to the combined effect of antimicrobial interference with cell wall synthesis and destruction of cell wall by endogenous autolytic enzymes. Mayhall et al. (25) found oxacillin tolerance in 33 of 60 clinical *S. aureus* isolates by MBC testing and log 5-CFU/ml killing-curve experiments with stationary-growth-phase inocula. Sabath et al. (33) defined "antimicrobial tolerance" as an MBC/MIC ratio of ≥32, after using standard techniques with log-growth-phase organisms to show cross tolerance among 28 of 63 blood culture isolates for the penicillins, cephalosporins, and vancomycin. Sabath and colleagues also confirmed impaired lysis and autolysin production in tolerant strains exposed to antimicrobial agents. Bradley et al. (5) found that 16 of 30 strains were tolerant to oxacillin and cephalothin with 0.2 to 43% of the initial log 5-cfu/ml stationary-phase inoculum surviving after 24 h of incubation in Mueller-Hinton broth. Goessens et al. (13) obtained similar results for 64 isolates, with 12.5% of the isolates showing more than 2% survivors after 24 h of incubation for a variety of antimicrobial concentrations, and termed this the tolerance percentage. Several retrospective clinical studies have suggested that the clinical response to beta-lactam therapy of antimicrobial agent-tolerant infections might be impaired (8, 17, 30), but tolerance did not affect the response to treatment in the rabbit model of staphylococcal endocarditis (14) or the rat model of staphylococcal pyelonephritis (16).

Numerous investigators have found the in vitro assessment of antimicrobial agent bactericidal activity against *S. aureus* very difficult to assess in a reproducible fashion. In vitro antimicrobial agent-induced staphylococcal killing has been reported to be influenced by the growth phase of the initial inoculum (2, 6, 20), type of media (29, 36), medium pH (7, 36), beta-lactam antimicrobial agent concentration with paradoxical reduction in killing at higher concentrations (10, 35), duration of incubation (18), in vitro inactivation of antimicrobial agents by beta-lactamases (6, 11, 21), emergence of drug-resistant mutants (26, 28), carry-over of active antimicrobial agent to subcultures (12, 18, 35), survival or organisms on the wall of test tubes above the fluid level (18, 25, 35), freezing of test organisms before testing (24, 33), and statistical considerations about sampling and counting survivors (27). A recent study that controlled most of these variables was unable to demonstrate the phenomenon of in vitro tolerance (35). Shanholtzer et al. (34), using macrodilution and microdilution broth techniques, were unable to obtain reproducible MBC endpoints for clindamycin, methicillin, cephalothin, gentamicin, and vancomycin with 67 *S. aureus* isolates. We report the inability to obtain reproducible macrodilution MBCs with 101 clinical isolates of *S. aureus* when a macrodilution technique similar to that used by Shanholtzer et al. was used (34).

MATERIALS AND METHODS

Organisms. One hundred and one fresh isolates of *S. aureus* were obtained from four Fargo community hospitals in 1978. All organisms were catalase, slide, and tube coagulase-positive, gram-positive cocci that formed clusters on gram stain and were susceptible to oxacillin and cephalothin by Kirby-Bauer disk diffusion tests (9, 23). Twenty-eight strains were susceptible to penicillin G; the remainder were penicillinase producers by the method of Rosenblatt and Neumann (31) or Lee and Komarmy (22).

Antibiotics. Standard powders were provided by the manufacturers: sodium oxacillin and sodium methicillin (Bristol Laboratories, Syracuse, N.Y.); penicillin G, sodium cephalothin, and vancomycin hydrochloride (Eli Lilly & Co., Indianapolis, Ind.); and gentamicin sulfate (Schering Corp., Bloomfield, N.J.).

MIC/MBC testing. MICs were determined in 1 ml of Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) in 10-ml glass test tubes with log 5-cfu/ml log-growth-phase inocula subcultured from the original isolation plates (1). MIC endpoints were read as the lowest antimicrobial concentration producing a clear tube. All inocula were quantitated and averaged to log 5.7 cfu/ml. Clear tubes were sampled after 24 h of incubation at 35°C with a calibrated 0.01-ml loop and streaked on quadrants of a sheep blood agar plate. CFUs were counted after 18 to 24 h of incubation at 35°C, and the MBC was read as the lowest antibiotic

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TABLE 1. MICs and MBCs after 24 and 48 h of incubation of log-growth-phase *S. aureus* strains at 35°C in Mueller-Hinton broth

Drug (no. of strains)	Range		MIC/MBC (μg/ml)			
			50%		90%	
	MIC	MBC	24 h	48 h	24 h	48 h
Methicillin (101)	1.56–12.5	1.56–>100	1.56	1.56	3.12	3.12
			12.5	1.56	>100	50
Oxacillin (21)	0.19–1.56	0.39–>100	0.19	0.39	0.39	0.78
			12.5	1.56	50	6.24
Penicillin G (28)	<0.19–0.39	0.19–50	<0.1	<0.19	0.19	0.19
			0.1	<0.19	6.24	0.39
Cephalothin (101)	0.19–12.5	0.19–>100	0.19	0.39	1.56	1.56
			1.56	1.56	50	12.5
Vancomycin (101)	0.19–6.24	0.78–>100	0.78	0.78	1.56	1.56
			12.5	6.24	50	25
Gentamicin (99)	0.19–12.5	0.19–>100	0.19	0.39	0.78	1.56
			3.12	1.56	25	12.5

concentration killing at least 99.9% of the initial inoculum. Tolerance is defined as an MBC/MIC ratio of ≥32:1 after 24 h of incubation at 35°C.

RESULTS

By MIC testing, all strains were susceptible to methicillin, oxacillin, cephalothin, and vancomycin (Table 1). Two gentamicin-resistant strains are omitted from Table 1. Seven strains with 48-h methicillin MBCs of >25 μg/ml and 14 strains with MBCs of <25 μg/ml were selected for oxacillin MIC and MBC testing. No differences between 24- and 48-h MICs were noted, although the 48-h MIC tended to be one tube higher than the 24-h MIC. In contrast, the proportions of strains tolerant to methicillin, oxacillin, penicillin G, vancomycin, and gentamicin were significantly reduced after 48 versus 24 h of incubation (Table 2). Cross tolerance between methicillin and oxacillin, penicillin G, cephalothin, and vancomycin is demonstrated by significantly higher 48-h MBCs for those agents against strains with methicillin MBCs of >25 μg/ml versus strains with methicillin MBCs of <25 μg/ml for oxacillin, penicillin G, and vancomycin but not cephalothin or gentamicin.

A control *S. aureus* strain (ATCC 25923) was included with each group of MIC/MBC determinations. The results of 17 to 20 repetitions for four antimicrobial agents are presented in Table 3. The MICs were reproducible within one tube dilution, but the MBCs were not. In addition, 24- and 48-h MBCs of oxacillin (18 strains), methicillin (14 strains), and penicillin G (20 strains) were determined in triplicate. Oxacillin MBCs were within one tube dilution for 17 of 20 (80%) triplicate sets after 24 h of incubation and for 18 of 24 (75%) sets after 48 h of incubation. The mean oxacillin MBC for triplicate sets varied as much as three tube dilutions on different days. Methicillin MBCs were less reproducible, but penicillin G MBCs were within one tube dilution for 17 of 20 (85%) triplicate sets after 24 h of incubation and for 19 of 20 (95%) after 48 h of incubation.

The same MBC methods produced clear endpoints for ampicillin and gentamicin versus *Escherichia coli* (ATCC 25922). Limited studies with 0.1-ml samples incorporated into agar pour plates rather than streaking 0.01 ml on the surface of agar plates resulted in more easily defined endpoints but did not improve the reproducibility of the results.

TABLE 2. Analysis of MBCs after 24 and 48 h of incubation in Mueller-Hinton broth at 35°C

Antibiotic (no. of strains)	% with MBC/MIC of ≥32 at:	
	24 h	48 h
Methicillin (101)	41	12[a]
Oxacillin (21)	13	1[a]
Penicillin G (28)	11	2[b]
Cephalothin (101)	30	14
Vancomycin (101)	46	16[a]
Gentamicin (99)	39	11[a]

[a] $P < 0.001$ by the chi-square test, comparing 28- and 48-h results.
[b] $P < 0.05$ by the chi-square test, comparing 28- and 48-h results.

DISCUSSION

From the beginning it has been recognized that the rate and magnitude of in vitro killing of *S. aureus* by beta-lactam antimicrobial agents is dependent upon test conditions and that quantitative measures of killing have been difficult to reproduce among different laboratories. Failure to agree upon uniform test conditions such as inoculum size, growth

TABLE 3. Reproducibility of *S. aureus* ATCC 25923 results after 24 and 48 h of incubation in Mueller-Hinton broth at 35°C

Antibiotic	Result	Mode (μg/ml)	Range (μg/ml)	Tube dilution variance	
				% ± 1	% ± 2
Methicillin	24-h MIC	1.96	0.78–3.12	100	
	48-h MIC	3.12	1.56–6.25	100	
	24-h MBC	6.25	1.56–>100	47	59
	48-h MBC	3.12	1.56–>100	71	76
Cephalothin	24-h MIC	0.19	0.19–0.39	100	
	48-h MIC	0.39	0.19–0.79	100	
	24-h MBC	0.39	0.19–50	58	68
	48-h MBC	0.39	0.19–3.12	74	95
Vancomycin	24-h MIC	1.56	0.78–6.25	90	100
	48-h MIC	3.12	0.78–12.5	82	100
	24-h MBC	12.5	1.56–>100	65	85
	48-h MBC	12.5	6.25–>100	90	95
Gentamicin	24-h MIC	0.19	0.19	100	
	48-h MIC	0.39	0.19–3.12	81	95
	24-h MBC	1.56	0.19–50	37	58
	48-h MBC	1.56	0.19–6.25	63	89

phase of the inoculum, media, pH, ion supplementation, addition of serum, method of inoculation and mixing, duration of incubation, subculturing techniques, and a definition of bactericidal endpoints have led investigators to use different techniques that produce results that are often at odds with the results of other researchers. Although progress has been made in reaching a concensus about test conditions, there still is no technique for the measurement of bactericidal antimicrobial activity that is reproducible in many laboratories and analogous to the inhibitory antimicrobial concentration measurements that have been established as reproducible and that correlate well with the outcome of treatment of infections in humans.

The current study is an evaluation of macrodilution broth MBCs for unselected clinical *S. aureus* isolates from community hospitals at a time when methicillin-resistant staphylococci were not endemic in the community. By using log-phase-growth organisms with a log 5-CFU/ml inoculum in Mueller-Hinton broth and Sabath's definition of antimicrobial tolerance as an MBC/MIC ratio of 32 or greater, we found that tolerance was frequently present, as others have found (13, 25, 33, 37), with cross tolerance for methicillin, oxacillin, cephalothin, penicillin G, and vancomycin but not gentamicin (25). Killing was increased by 48 h of incubation as compared with 24 h of incubation for methicillin, oxacillin, penicillin G, cephalothin, vancomycin, and gentamicin. Increased killing with longer incubation periods of incubation has been reported previously (18, 33). Our most significant finding was the lack of reproducibility of MBC measurements when a standard strain was used. This lack of reproducibility has been noted by others and has been attributed to the freezing of strains (24, 33) and the shaking of tubes (18, 25, 35). A recent, carefully done study trying to eliminate technical aspects that could lead to persistance of organisms failed to demonstrate antimicrobial tolerance in strains previously labeled as tolerant (35).

Editorials discussing staphylococcal antimicrobial tolerance have failed to stress the irreproducibility of this measurement (19, 32). From numerous and conflicting studies of this subject, a number of observations have emerged that may result in future agreement for a standard technique: (i) stationary-phase organisms are less susceptible to the bactericidal action of beta-lactam antimicrobial agents (2, 15), (ii) a reduction in killing occurs at higher concentrations of beta-lactam antimicrobial agents (10, 35) (iii) measures to reduce the survival of staphylococci above the fluid meniscus will improve apparent killing (18, 29, 35), (iv) inactivation of antimicrobial agents in vitro by staphylococcal beta-lactamases impairs killing by some beta-lactam agents (6, 11, 21), and (v) the emergence of antimicrobial agent-resistant mutants is a rare event seen mainly with gentamicin and rifampin (26, 28). Taylor and colleagues (35) have developed an MBC macrodilution procedure that seems to best address past problems by assaying the in vitro bactericidal activity of antistaphylococcal antimicrobial agents. Experience with this technique in other laboratories is needed to assess whether it is a reproducible measurement worth doing. Until that time, all measurements of bactericidal activity should be viewed with caution when considering the treatment of patients with staphylococcal infections.

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LITERATURE CITED

1. **Barry, A. L., and L. D. Sabath.** 1974. Special tests: bactericidal activity and activity of antimicrobics in combination, p. 431–435. *In* E. H. Lennette, E. H. Spaulding, and J. P. Truant (ed.), Manual of clinical microbiology, 2nd ed. American Society for Microbiology, Washington, D.C.
2. **Bigger, J. W.** 1944. Treatment of staphylococcal infections with penicillin by intermittant sterilization. Lancet **ii**:497–500.
3. **Best, G. K., N. H. Best, and A. V. Koval.** 1974. Evidence of participation of autolysins in bactericidal action of oxacillin on *Staphylococcus aureus*. Antimicrob. Agents Chemother. **6**:825–830.
4. **Best, G. K., A. V. Koval, and N. H. Best.** 1975. Susceptibility of clinical isolates of *Staphylococcus aureus* to killing by oxacillin. Can. J. Microbiol. **21**:1692–1697.
5. **Bradley, J. J., C. G. Mayhall, and H. P. Dalton.** 1978. Incidence and characteristics of antibiotic-tolerant strains of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **13**:1052–1057.
6. **Chapman, S. W., and R. T. Steigbigel.** 1983. Staphylococcal beta-lactamase and efficacy of beta-lactam antibiotics: in vitro and in vivo evaluation. J. Infect. Dis. **11**:21–26.
7. **Craven, N., and J. C. Anderson.** 1982. pH-dependent penicillin tolerance may protect intraleukocytic *Staphylococcus aureus* from killing by cloxacillin. Antimicrob. Agents Chemother. **21**:618–621.
8. **Denny, A. E., L. R. Peterson, D. N. Gerding, and W. H. Hall.** 1979. Serious staphylococcal infections with strains tolerant to bactericidal antibiotics. Arch. Int. Med. **139**:1026–1031.
9. **Drew, W. L., A. L. Barry, R. O'Toole, and J. C. Sherris.** 1972. Reliability of the Kirby-Bauer disc diffusion method for detecting methicillin-resistant strains of *Staphylococcus aureus*. Appl. Microbiol. **24**:240–247.
10. **Eagle, H., and A. D. Musselman.** 1948. The rate of bactericidal action of penicillin in vitro as a function of its concentration, and its paradoxically reduced activity at high concentrations against certain organisms. J. Exp. Med. **88**:99–131.
11. **Fong, I. W., E. R. Engelking, and W. M. M. Kirby.** 1976. Relative inactivation by *Staphylococcus aureus* of eight cephalosporin antibiotics. Antimicrob. Agents Chemother. **9**:939–944.
12. **Goessens, W. H. F., P. Fontijne, and M. F. Michel.** 1982. Factors influencing detection of tolerance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **22**:364–368.
13. **Goessens, W. H. F., P. Fontijne, M. van Raffe, and M. F. Michel.** 1984. Tolerance percentage as a criterion for the detection of tolerant *Staphylococcus aureus* strains. Antimicrob. Agents Chemother. **25**:575–578.
14. **Goldman, P. L., and R. G. Petersdorf.** 1979. Significance of methicillin tolerance in experimental staphylococcal endocarditis. Antimicrob. Agents Chemother. **15**:802–806.
15. **Gunnison, J. B., M. A. Fraher, and E. Jawetz.** 1964. Persistence of *Staphylococcus aureus* in penicillin in vitro. J. Gen. Microbiol. **35**:335–349.
16. **Guze, P. A., G. M. Kalmanson, and L. B. Guze.** 1982. The role of antibiotic tolerance in the response to treatment of pyelonephritis due to *Staphylococcus aureus* in rats. J. Infect. Dis. **145**:169–173.
17. **Hilty, M. D., J. S. Venglarcik, and G. K. Best.** 1980. Oxacillin-tolerant staphylococcal bacteremia in children. J. Pediatr. **96**:1035–1037.
18. **Ishida, K., P. A. Guze, G. M. Kalmanson, K. Albrandt, and L. B. Guze.** 1982. Variables in demonstrating methicillin tolerance in *Staphylococcus aureus* strains. Antimicrob. Agents Chemother. **21**:688–690.
19. **Kaye, D.** 1980. The clinical significance of tolerance of *Staphylococcus aureus*. Ann. Int. Med. **93**:924–925.
20. **Kim, K. S., and B. F. Anthony.** 1981. Importance of bacterial growth phase in determining minimal bactericidal concentrations of penicillin and methicillin. Antimicrob. Agents Chemother. **19**:1075–1077.
21. **Kono, M., M. Sasatsu, K. O'Hara, Y. Shiomi, and T. Hayasaka.** 1983. Mechanism of resistance to some cephalosporins in

Staphylococcus aureus. Antimicrob. Agents Chemother. **23**:938–940.

22. **Lee, W.-S., and L. Komarmy.** 1976. New method for detecting in vitro inactivation of penicillins by *Haemophilus influenzae* and *Staphylococcus aureus*. Antimicrob. Agents Chemother. **10**:564–566.
23. **Matsen, J. M., and A. L. Barry.** 1974. Susceptibility testing: diffusion test procedures, p. 418–427. *In* E. H. Lennette, E. H. Spaulding, and J. P. Truant (ed.), Manual of clinical microbiology, 2nd ed. American Society for Microbiology, Washington, D.C.
24. **Mayhall, C. G., and E. Apollo.** 1980. Effect of storage and changes in bacterial growth phase and antimicrobial concentrations on antimicrobial tolerance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **18**:784–788.
25. **Mayhall, C. G., G. Medoff, and J. J. Marr.** 1976. Variation in the susceptibility of strains of *Staphylococcus aureus* to oxacillin, cephalothin, and gentamicin. Antimicrob. Agents Chemother. **10**:707–712.
26. **Musher, D. M., R. E. Baughn, G. G. Templeton, and J. N. Minuth.** 1977. Emergence of variant forms of *Staphylococcus aureus* after exposure to gentamicin and infectivity of the variants in experimental animals. J. Infect. Dis. **136**:360–368.
27. **Pearson, R. D., R. T. Steigbigel, H. T. Davis, and S. W. Chapman.** 1980. Method for reliable determination of minimal lethal antibiotic concentrations. Antimicrob. Agents Chemother. **18**:699–708.
28. **Pelletier, L. L.** 1979. Virulent gentamicin-induced small colony variants of *Staphylococcus aureus*. J. Lab. Clin. Med. **94**:324–334.
29. **Peterson, L. R., D. N. Gerding, W. H. Hall, and E. A. Schierl.** 1978. Medium-dependent variation in bactericidal activity of antibiotics against susceptible *Staphylococcus aureus*. Antimicrob. Agents Chemother. **13**:665–668.
30. **Rajashekaraiah, K. R., T. Rice, V. S. Rao, D. Marsh, B. Ramakrishna, and C. A. Kallick.** 1980. Clinical significance of tolerant strains of *Staphylococcus aureus* in patients with endocarditis. Ann. Int. Med. **93**:796–801.
31. **Rosenblatt, J. E., and A. M. Neumann.** 1978. A rapid slide test for penicillinase. Am. J. Clin. Pathol. **69**:351–354.
32. **Sabath, L. D.** 1982. Mechanisms of resistance to beta-lactam antibiotics in strains of *Staphylococcus aureus*. Ann. Int. Med. **97**:339–344.
33. **Sabath, L. D., M. Laverdiere, N. Wheeler, D. Blazevic, and B. J. Wilkinson.** 1977. A new type of penicillin resistance of *Staphylococcal aureus*. Lancet **i**:443–447.
34. **Shanholtzer, C. J., L. R. Peterson, M. L. Mohn, J. A. Moody, and D. N. Gerding.** 1984. MBCs for *Staphylococcus aureus* as determined by macrodilution and microdilution techniques. Antimicrob. Agents Chemother. **26**:214–219.
35. **Taylor, P. C., F. D. Schoenknetcht, J. C. Sherris, and E. C. Linner.** 1983. Determination of minimum bactericidal concentrations of oxacillin for *Staphylococcus aureus*: influence and significance of technical factors. Antimicrob. Agents Chemother. **23**:142–150.
36. **Venglarcik, J. S., III, L. L. Blair, and L. M. Dunkle.** 1983. pH-dependent oxacillin tolerance of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **23**:232–235.
37. **Watanakunakorn, C.** 1978. Antibiotic tolerant *Staphylococcus aureus*. J. Antimicrob. Chemother. **4**:561–568.