Influence of Technical Factor Variations during Inoculum Preparation on the Agar Dilution Plate-Count Method for Quantitation of *Staphylococcus aureus* Oxacillin Persisters

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The possible introduction of error into *Staphylococcus aureus* oxacillin intrinsic persister measurements by the agar dilution plate-count method resulting from technical factor variations associated with inoculum preparation was investigated. pH stability and exponential growth were shown to be present at the time of inoculum standardization. The use of glass or plastic tubes and the contamination of tube walls during inoculum broth culture produced no differences in test results. For *S. aureus* and oxacillin, the agar dilution plate-count method is a new and reliable approach to the quantitative study of bacterial inhibition and killing.

We recently described the agar dilution plate-count (ADPC) procedure for the quantitative measurement of bacterial inhibition and killing (14). For *Staphylococcus aureus* and oxacillin, we found the so-called tolerance phenomenon to be artifactual and to result from the detection of high-percentage persisters. For a given test system, isolate, and time of antimicrobial action, total persister percentage appears to represent the contribution of both intrinsic and adaptive persisters. Intrinsic persister percentage appears to be strain dependent, whereas adaptive persister percentage is highly influenced by technical factor variations, the latter probably accounting for the spurious results and inaccuracy associated with broth dilution plate-count methods. Factors known to promote cell dormancy (stationary-phase inoculum, lowering of pH, drying of bacteria), phenotypic changes (cell clumping, slime or capsule production), and physical separation of bacteria from the antimicrobial agent (adherence of bacteria to container walls above the antimicrobial broth) are probably the most important inducers of adaptive persisters, and for broth dilution plate-count methods they are probably operative during the entire broth dilution phase. In the ADPC method, except for the brief inoculum preparation step, bacteria are immobilized in a single-phase agar dilution system, a situation designed to prevent the development of adaptive persisters. This study examined the possibility that technical factor variations potentially inherent in the ADPC inoculation step promote the development of adaptive persisters, thereby introducing errors into intrinsic persister measurements.

S. aureus isolates were retrieved from -70°C stock storage by two 24-h passages on sheep blood agar in air at 35°C . The isolates, described in a previous study (14), had the following characteristics: R7, MIC = $0.5\text{ }\mu\text{g/ml}$, 24-h persister percentage at $8 \times \text{the MIC} = 0.5\%$; R4, MIC = $0.5\text{ }\mu\text{g/ml}$, 24-h persister percentage at $8 \times \text{the MIC} = 0.08\%$; and SPRMC (St. Paul-Ramsey Medical Center) C13, MIC = $0.5\text{ }\mu\text{g/ml}$, 24-h persister percentage at $8 \times \text{the MIC} = 1.2\%$. Material from three to five colonies on the day 2 retrieval plate was used to prepare common broth inocula as described below.

The potential decrease in antibiotic effect resulting from induction of cell dormancy by the possible lowering of pH (2, 4, 10, 13) and by the occurrence of nonexponential growth (1, 3, 5-9, 11, 12) during broth inoculum preparations was examined by serial pH determinations and growth studies using two methods of inoculum preparation. One inoculum preparation, as described in our original description of the ADPC method (14), was prepared by transferring material from several colonies on the second stock retrieval plate to 5 ml of Mueller-Hinton broth (MHB), which was then incubated for 4 h without agitation in air at 35°C . The pH was monitored at 15-min intervals by inserting the probe of a Beckman 20 pH meter (Beckman Instruments, Inc., Fullerton, Calif.) directly into the broth after thorough mixing. Growth-phase studies were performed at 15-min intervals by removing 100- μl samples of inoculum broth and diluting them 1:10, 1:100, or 1:1,000, as needed, with MHB for preparation of Mueller-Hinton agar pour plates, using 0.5 ml of the final dilution in 19.5 ml of molten Mueller-Hinton agar. The plates were incubated for 48 h in air at 35°C for colony counts. The second type of broth inoculum was prepared by a two-step procedure. An initial MHB inoculum culture was prepared as described above. After 2 h of incubation, the culture was thoroughly mixed, and 1 ml was transferred by serologic pipette to 200 ml of MHB. After thorough mixing, 15-ml samples were dispensed into tubes, which were then incubated in air at 35°C . At 15-min intervals, individual tubes were selected for pH measurement, after which 1:10, 1:100, and 1:1,000 dilutions in MHB were made for plate-count preparations as described above. Using isolates R7 and C13 for one-step inoculum preparation, pH values were found to range from 7.38 and 7.37, respectively, in a gradually declining fashion to 7.14 and 7.24 at 4 h. Viability counts became exponential at approximately 1.50 h and continued in exponential growth for the remainder of the test period. pH changes for the two-step inoculum preparation ranged from 7.28 and 7.30, respectively, to 7.23 and 7.25. Exponential growth began shortly after the start of the second broth phase and continued throughout the remainder of the incubation. These findings indicated that pH changes and nonexponential growth do not induce cell dormancy during ADPC inoculum preparation.

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The potential inadvertent inclusion of dormant bacteria produced by drying on container walls above the inoculum broth was studied by comparing the results of parallel ADPC tests using both one- and two-step inoculum preparations of isolate R7 with and without contamination of tube walls. For each trial, 10 ADPC tests with carefully prepared inoculum preparations and 10 ADPC tests with inoculum preparations with deliberately contaminated tube walls were performed in parallel. For the parallel tests, a 200-ml common inoculum was prepared as described above for the two-step inocula. Inocula without tube wall contamination were then made by carefully pipetting 5 ml of the common dilution into each of 10 test tubes, which were then incubated without mixing for 4 h. Inocula with deliberately contaminated tube walls were prepared from a common broth dilution by pipetting 5 ml into each tube and using the pipette to vigorously mix and thereby contaminate the walls above the broth surface. After 4 h of incubation, each inoculum broth was vigorously mixed, diluted appropriately, and used for preparation of 1 of the 20 parallel ADPC tests. For each ADPC test, 24-h persister plate counts were determined for each oxacillin concentration above the MIC. For each of the individual oxacillin concentrations, mean plate counts were determined for the 10 ADPC tests with carefully controlled inocula and for the 10 parallel tests with tube-wall-contaminated inocula. Standard statistical methods were then used to determine the probability that mean plate counts for each concentration would differ by more than what would occur by chance alone ($P = >0.05$). For both inoculum preparations, differences in mean plate counts for each oxacillin concentration above the MICs showed no statistical differences for tests originating from inocula with deliberate contamination of tube walls as compared with those that were carefully prepared.

The potential effect of using glass versus plastic tubes, as has been described for the broth dilution plate-count method (11), was studied by using a scheme similar to that just described for the tube wall contamination study. Samples (5 ml of a common two-step inoculum preparation) were pipetted carefully into 10 polypropylene test tubes (16 by 125 mm; Becton Dickinson, Cockeysville, Md.) and 10 borosilicate glass test tubes (16 by 125 mm; American Scientific Products, McGaw Park, Ill.). Inoculum broths were incubated for 4 h, and then they were diluted and used for the preparation of ADPC tests. With isolates R7 and R4, plate-count differences other than those expected by chance alone were not encountered for oxacillin concentrations above the MICs.

Common technical factor variations in the ADPC 4-h inoculum preparation step did not result in the production of significantly variable or increased numbers of adaptive

persisters which might contribute to and confuse the measurements of intrinsic strain-dependent persisters. With standardized and well-controlled conditions of inoculum preparation, the ADPC method should permit the accurate determination of intrinsic persisters in relation to various times of antimicrobial action and concentrations, an achievement which might considerably enhance our understanding of the persister phenomenon.

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LITERATURE CITED

1. Bigger, J. W. 1944. Treatment of staphylococcal infections with penicillin by intermittent sterilisation. *Lancet* ii:497-500.
2. Garrod, L. P. 1945. The action of penicillin on bacteria. *Br. Med. J.* 1:107-110.
3. Gunnison, J. B., M. A. Fraher, and E. Jawetz. 1964. Persistence of *Staphylococcus aureus* in penicillin *in vitro*. *J. Gen. Microbiol.* 35:335-349.
4. Horne, D., and A. Tomasz. 1981. pH-dependent penicillin tolerance of group B streptococci. *Antimicrob. Agents Chemother.* 20:128-135.
5. 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.
6. 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.
7. Kirby, W. M. M. 1945. Bacteriostatic and lytic actions of penicillin on sensitive and resistant staphylococci. *J. Clin. Invest.* 24:165-169.
8. Knox, R. 1945. Effect of penicillin on cultures in liquid and solid media. *Lancet* i:559-561.
9. Lee, S. W., E. J. Foley, and J. A. Epstein. 1944. Mode of action of penicillin. I. Bacterial growth and penicillin activity—*Staphylococcus aureus* FDA. *J. Bacteriol.* 48:393-399.
10. Parker, R. F. 1953. A paradoxical effect of penicillin. *J. Bacteriol.* 66:60-67.
11. Taylor, P. C., F. D. Schoenknect, 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.
12. Todd, E. W. 1945. Bacteriolytic action of penicillin. *Lancet* i:74-78.
13. 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.
14. Woolfrey, B. F., R. T. Lally, and M. N. Ederer. 1985. Evaluation of oxacillin tolerance in *Staphylococcus aureus* by a novel method. *Antimicrob. Agents Chemother.* 28:381-388.