

## MINIREVIEW

# Problems in In Vitro Determination of Antibiotic Tolerance in Clinical Isolates

JOHN C. SHERRIS

*Department of Microbiology and Immunology, University of Washington, Seattle, Washington 98195*

This minireview is the second of a two-part series which summarizes the major points of a symposium and roundtable discussion held at the 25th Interscience Conference on Antimicrobial Agents and Chemotherapy in Minneapolis in 1985. Part one considered phenotypic tolerance, tolerance in animal models, and controversies as to the definition and mechanisms of genotypic tolerance (60), and this part focuses on the techniques used to detect genotypic antibiotic tolerance in clinical isolates of bacteria.

Antibiotic tolerance, as first described by Tomasz et al. (57) in autolysin-deficient mutants of *Streptococcus pneumoniae*, has been the subject of many publications dealing with its detection, its occurrence among clinical isolates of various genera and species, and its therapeutic significance. Despite this activity, no clear consensus has emerged as to the incidence and medical implications of tolerance, and the greatest discrepancies in results have been in studies on *Staphylococcus aureus* (5, 7, 11, 13, 18, 19, 25, 39, 44, 49, 51-56, 62, 63). Likewise, studies on the significance of *S. aureus* tolerance in experimental infections have yielded variable results (17, 19, 20, 59). Concern with the topic was stimulated by observations of the poor reproducibility of measurements of antibiotic MBCs for this organism and thus of the impact of these observations on the detection and occurrence of tolerance as determined in clinical laboratories.

### DEFINITIONAL PROBLEMS

The detection of tolerance has been complicated by definitional inconsistencies. In the broadest sense, the term tolerant has usually been applied to bacterial strains or mutants which are inhibited by concentrations of a bactericidal antibiotic similar to those inhibiting the majority of susceptible wild-type isolates but that show unusual numbers of survivors in the presence of concentrations that are lethal to most other members of the species. In some cases, the definition has been extended to apply to the behavior of essentially all members of a species or group of organisms that are relatively resistant to the killing action of a bactericidal antibiotic, such as enterococci with penicillin (32), and to some pneumococci with unusually high penicillin MICs which also resist killing by even higher concentrations (34).

The earliest reports of tolerance were documented by kinetic studies (5, 29, 39, 50) that compared rates of killing of the test strain with those of control strains of the same species. However, the relationship of the concentration(s) of antibiotic tested to the MIC for the organism varied in different reports. Subsequently, in attempts to develop detection procedures that could be undertaken in any laboratory, criteria for specific numbers of survivors after overnight incubation were proposed for determining the MBC of

an antibiotic for an organism, and ratios of MBC to MIC were used to define an organism as tolerant. However, different ratios have been used by different workers; some examples are given in Table 1. Furthermore, as is discussed below, there were considerable variations in the techniques and conditions used to determine MICs and MBCs.

These definitional inconsistencies are complicated further by the fact that an unusually large number of survivors after, say, overnight incubation can be the result of several factors. First, the rate of exponential killing can be considerably slower than that of most strains of the species, which conforms to the definition of tolerance used in these minireviews. A second cause of an unusual number of survivors is the persister phenomenon first described by Bigger (6), in which a small proportion of the inoculum is in a physiological state that escapes killing. A third phenomenon is the paradoxical effect first described by Kirby (31), Garrod (15), and Eagle and Musselman (14), in which the proportion of survivors of some strains increases in higher concentrations of beta-lactam antibiotic, possibly because of a secondary inhibition of RNA or protein synthesis (41). These phenomena probably involve different processes but are not mutually exclusive. Some workers have applied the term tolerance or persister under conditions in which more than one of them may be major operative factors (18, 63).

The most commonly used methods for detecting tolerance in clinical laboratories are based on measurement of the MIC after overnight incubation followed by subcultures from tubes showing inhibition to determine the MBC. The MBC is usually defined as the lowest concentration that yields fewer than 0.1% survivors, and an organism is defined as tolerant when the MBC is  $\geq 32$  times the MIC. This definition of MBC uses the least accurate portion of the killing curve, may fail to distinguish strains with slower rates of killing from persisters, and is most subject to technical artifact because of the small number of colonies representing the breakpoint. Anhalt et al. (2) described endpoint criteria to prevent the paradoxical phenomenon or aberrant results from single tubes (skip tubes) from influencing MBC test results, and Pearson et al. (43) defined statistical parameters and rejection limits for MBC tests; however, published reports on tolerance have usually not indicated the application of such criteria.

### TECHNICAL FACTORS INFLUENCING MBC TEST RESULTS AND PROCEDURAL VARIATIONS

Variability of MBC test procedures within and between laboratories (40) has probably been the major factor in differences in the proportions of strains reported as tolerant (21, 25, 44, 54, 56). For example, most studies on the effect of the growth phase of the inoculum have shown substan-

TABLE 1. Examples of different definitions of *S. aureus* tolerance

Criterion	Reference(s)
MBC/MIC ratio	
≥100	.61
>32	.53
≥32	.2, 20, 44, 56
≥16	.29, 49
≥8	.13
≥2% Survivors after 24 h of exposure to	
≥64 µg of methicillin per ml	.17

tially more survivors after overnight incubation in the presence of a cell-wall-active bactericidal agent if the inoculum is in the stationary rather than the logarithmic phase (25, 27, 28, 38, 56). This effect is to be expected on the basis of the mode of action of these antibiotics. Inocula in both stages have been used in published descriptions of the occurrence of tolerance, and Mayhall and Apollo detected tolerance only when stationary-phase inocula were used (38).

Technical artifact can also lead to apparent tolerance. In the case of *S. aureus* and *Pseudomonas aeruginosa*, adherence to the walls of test tubes or growth in condensate above the meniscus of antibiotic-containing broth have been identified as major sources of difficulty, because some of these organisms may avoid exposure to the antibiotic (25, 33, 39, 54, 56). This may be manifested not only as high MBC test results but also as skipped tubes in which aberrant numbers of survivors are found from individual concentrations of antibiotic when adjacent concentrations show considerable lethality. This problem can be prevented by taking special care in placing the inoculum below the surface of the medium while avoiding letting it come in contact with test tube walls and by ensuring the exposure of all organisms to the antibiotic by vortexing the tube and reincubating for 2 or more h before sampling. The major problem resulting from this source of artifact occurs when the endpoint for the MBC test is at 0.1% survivors, because minimal contamination can lead to spuriously high results.

Incubation time before sampling for survivors obviously influences the residual number of viable organisms when killing is approximately exponential, and several workers have noted that tolerance seen after overnight incubation is not detected after 48 h (39, 53). Other variables, such as medium content (30, 42, 46) and pH (61), can influence the speed of killing by some bactericidal antibiotics. Such phenotypic tolerance was fully reviewed by Handwerger and Tomasz (21) and in the preceding minireview (60) and probably contributes to discrepancies in reports on the incidence of tolerance among clinical isolates.

Another potential source of technical artifact is carry-over of antibiotic from the test system to recovery plates in a concentration sufficient to inhibit surviving organisms. This problem is enhanced if large volumes (and, thus, more antibiotic) are transferred, when higher concentrations of antibiotic are involved, and when recovery plates are seeded to a small area, because this effectively increases the concentration of antibiotic at the site of the inoculum (3, 16). Under such conditions, it has been found essential to add antibiotic-inactivating enzymes, when available, to the reaction mixtures or to the recovery plates to avoid spuriously low counts (3). I have found that this is not necessary with beta-lactam antibiotics in concentrations of up to 16 to 32 times the MIC when volumes of 100 µl or less are spread

over whole recovery plates. This procedure can avoid the complexity and expense of adding high concentrations of beta-lactamases when beta-lactam antibiotics are being tested and makes the technique applicable to antibiotics for which inactivating conditions are not available.

Because of the problems of adherence to glassware in the macrobroth dilution procedure, several workers have used techniques based on agar dilution procedures for MBC tests with beta-lactam antibiotics. The number of survivors after overnight incubation has been determined after the inactivation of antibiotic with beta-lactamases (36, 37, 63) or by replica plating techniques (10). Fully quantitative results can be obtained, and Woolfrey et al. (63) found generally good comparability with a broth dilution method. Thus far, however, these procedures have been applied only under conditions involving overnight exposure to antimicrobial agent. Several workers have described modifications of diffusion susceptibility tests for detecting strains with high tolerance. These involve the replacement of disks of beta-lactam antibiotics after overnight incubation with disks containing an effective beta-lactamase (12, 45) or spot inoculation of a beta-lactamase-producing *Enterobacter* sp. in the center of zones of inhibition resulting from beta-lactam antibiotics (58). In each case, values are given for significant numbers of colonies corresponding to tolerance as determined by broth dilution methods. Another approach was used by Kim and Anthony (29), who reported the successful use of a combination of gradient plates and replica plating in detecting penicillin tolerance in group B and group D streptococci. Microdilution procedures have been modified for detecting MBCs but appear to have been subject to the same difficulties and variables that apply to macrobroth tests, with the added complication of the smaller volumes used. One procedure has been described in which good reproducibility was obtained when the entire contents of the wells were used as a source of inoculum for the subculture plates (54).

Thus, published descriptions of MBC test procedures have varied substantially in the techniques and conditions used, and this has certainly contributed to the wide discrepancies in proportions of *S. aureus* strains described as tolerant when counts of the survivors were made only after overnight incubation. Those studies in which stringent procedures were applied to ensure the exposure of all organisms in the inoculum to antibiotic (25, 54, 56, 63) found that *S. aureus* strains with MBC/MIC ratios of ≥32 after overnight incubation were rare when the MBC was based on ≤0.1% survivors. The Evans strain reported by Best et al. (4, 5) appears to be an example of a strain that has exhibited tolerance under these conditions in the hands of other investigators (63), although some workers have reported tolerance to be markedly unstable in strains held in stock culture (38, 52).

#### PROCEDURES FOR DETECTING *S. AUREUS* STRAINS WITH REDUCED KILLING RATES

The failure to detect tolerance to beta-lactam antibiotics when the most common definition is used and when stringent technical precautions are taken does not exclude the possibility that a particular *S. aureus* strain is killed substantially more slowly than others. Killing rates are closely exponential after the first 2 h of exposure to antibiotic in broth (4, 5, 7, 19, 23, 39, 50) and can be expressed as decimal reduction times, which are the periods required for one log 10 decrease in viability. Strains with decimal reduction times as long as 7 h would still yield fewer than 0.1% survivors after 24 h of

incubation and appear nontolerant in overnight tests even though most wild-type strains of *S. aureus* have decimal reduction times of only 1 to 2 h. Other procedures are therefore needed to detect such differences.

The only reliable method for detecting reduced killing rates involves the quantitative measurement of survivors at selected intervals during the period of interaction between the antibiotic and the organism, and studies using this procedure have yielded the best evidence of tolerance in clinical isolates of *S. aureus*. Killing curves which require the testing of multiple samples at intervals with several concentrations of antibiotic are obviously impractical as routine procedures in the clinical laboratory, and single measurements after overnight incubation have all the defects discussed above. Strains with slower than normal rates of exponential killing may, however, be detected by comparing the numbers of survivors after 5 or 6 h of exposure to antibiotic with those at 2-h or 0-time readings. Such an approach has been suggested by others (5, 21) and is a feasible undertaking for a skilled routine diagnostic laboratory. Organisms could be grown overnight at room temperature, diluted, briefly incubated at 35°C to ensure that they are in the exponential phase of growth, and adjusted to an inoculum of  $10^5$  to  $10^6$  bacteria per ml. A single antibiotic concentration of approximately eight times the estimated MIC for the organism can be used, and in this way the major confounding effects of antibiotic carry-over and of the paradoxical phenomenon can be avoided. The kill rate can be determined by comparing the early count with that after 5 or 6 h of exposure, and the test can be performed within a normal workday. The detection of survivors after further incubation for a total of 24 h should permit the detection of persisters as manifested by the survival of a subpopulation of the inoculum. We tested such an approach, found it to be adequately reproducible, and detected a small proportion of *S. aureus* strains which are killed much more slowly than most but which still do not meet the conventional definitions of tolerance in tests after 24 h of incubation (J. C. Sherris, F. D. Schoenknecht, and S. R. Swanzy, unpublished data). We believe that only such procedures can characterize the in vitro behavior of a number of *S. aureus* strains sufficient to allow a meaningful comparison with the in vivo response to therapy and thus determine the clinical significance of delayed killing and of the persister phenomenon should it occur in such a system. The detection of the paradoxical phenomenon requires the use of several concentrations of antibiotic and requires a separate study to determine its clinical meaning, if any.

### TOLERANCE IN OTHER SPECIES OF BACTERIA

Tolerance in other bacterial species is fully covered in a review by Handwerger and Tomasz (21). It is clear on the basis of the literature, and my experience has confirmed, that strains of several species of streptococci show tolerance of a degree that can be detected by the widely used 24-h test and the MBC/MIC ratio of  $\geq 32$  (1, 22, 24, 35, 47). The in vivo significance of these strains has been demonstrated in experimental endocarditis models (8, 9, 22), and there is strong evidence that they also influence the treatment of human endocarditis (1, 48). Other strains of streptococci which fail to achieve this definition and yet are killed more slowly than many wild-type strains also appear to exist, but their significance remains to be assessed.

### CONCLUSION

Tolerant strains showing an unusually slow rate of exponential killing have been unequivocally demonstrated among clinical isolates of a number of species. However, the status of tolerance among *S. aureus* strains remains somewhat elusive because of the definitional and technical variations used by different workers. Characterization of MBCs using the criterion of a 99.9% kill after 24 h of incubation is based on a measurement at the least accurate part of the killing curve and is subject to spuriously high values from adhesion to glassware unless special precautions are taken. When carefully determined with logarithmic-phase cells, tolerance as defined by MBC/MIC ratios of  $\geq 32$  is unusual in *S. aureus*; however, such measurements can fail to detect staphylococcal strains which are killed much more slowly than others of the same species.

Tolerance is best detected by quantitative killing curve procedures, and abbreviated and simplified techniques for achieving them appear feasible for the clinical laboratory but require further evaluation. There is a definite need for agreed-upon criteria for designating a strain as tolerant, for developing and evaluating a standard procedure for detecting such tolerance, and for selecting reference strains to ensure better intra- and interlaboratory reproducibility.

The therapeutic significance for humans of tolerance, of persisters, and of the paradoxical phenomenon in *S. aureus* remains largely uncertain, and the situation is little changed since it was described by Kaye (26) in 1981. Studies using standardized reproducible techniques and precise agreed-upon definitions are needed to resolve this uncertainty (25, 56).

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