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Variables in Demonstrating Methicillin Tolerance in *Staphylococcus aureus* Strains

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Certain technical considerations which affected the status of methicillin tolerance in *Staphylococcus aureus* strains were studied. Methods which consistently demonstrated tolerance or intolerance of a given strain were avoidance of inoculum splashing, use of a stationary-phase inoculum, 24-h tube incubation, and minimization of antibiotic carry-over. These studies suggested a need for the establishment of a standardized reference procedure for the determination of tolerance.

The term drug tolerance, meaning a marked discrepancy between minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), was first applied in 1970 to the action of penicillin against pneumococci by Tomasz et al. (16). Since then, tolerance has been described with a variety of other bacteria, including *Staphylococcus aureus* (1–4, 8, 9, 12–15), group D streptococci (7, 10), and group B streptococci (6). Bacterial strains have been considered to be tolerant if they exhibit an MBC 8- to 100-fold greater than an MIC in the usual susceptible range (1, 3, 13, 15).

Because the results of our initial attempts to classify *S. aureus* isolates from clinical material as tolerant or nontolerant to methicillin were inconsistent and frequently difficult to interpret, we have studied the effects of certain technical variations on tolerance and repeated each study three times.

Twenty-eight *S. aureus* strains obtained from clinical laboratories at the Harbor-UCLA Medical Center and the Veterans Administration Wadsworth Medical Center were studied. Sodium methicillin reference standard, provided by Bristol Laboratories (Syracuse, N.Y.), was prepared fresh for each experiment. Penicillinase (Penase; Difco Laboratories, Detroit, Mich.) was added to Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) plates at 10,000 U/ml when needed. The sterility of this enzyme was checked by incubating uninoculated plates as well as by culturing 1-ml portions in Trypticase soy broth (BBL Microbiology Systems). Its activity at 10,000 U/ml against 100 μg of methicillin per ml was found to be ≧96.9% in 1 min.

The MIC was determined in Mueller-Hinton broth by the macro-broth dilution procedure described by Washington and Sutter (17) and defined as the lowest concentration of antibiotic at which there was no visible turbidity after 24 or 48 h of incubation at 35°C.

Stationary-phase bacteria were generated overnight at 35°C in Trypticase soy broth. Logarithmic-phase bacteria were started from the overnight stationary phase and grown until the spectrophotometric reading reached an optical density of 0.4 or 0.5 at 540 nm (≅4 h).

The average MIC of methicillin for all but one strain was ≤3.12 μg/ml upon three repeated testings. The MIC of methicillin for the strain that was the exception was 6.25 μg/ml. At 24 or 48 h, duplicate 0.1-ml samples from tubes with no visible growth were subcultured by streaking onto Trypticase soy agar plates. The MBC was defined as ≧99.9% killing of the original inoculum after either 24 or 48 h.

Shaking was accomplished by vigorous mixing with a Vortex mixer for 3 s when bacteria were added to the antibiotic dilutions and when sampling was done. Mixing without shaking was accomplished with the use of a Gilson Pipetman 200D. The inoculum was added to the bottom of the tube and mixed by gently expelling the contents of the 0.1-ml tip five times.

The effects of vigorous shaking at various times on three strains are shown in Table 1. The most critical manipulation occurred at the time of bacterial inoculation. If shaking was done at the time of inoculation, all three strains could be made to appear tolerant by shaking at sampling. If mixing was done gently at the time of inoculation to avoid splashing the inoculum onto the walls of the vials, no tolerance was evident, regardless of whether shaking was done at sampling.

The effects of different inoculum growth

TABLE 1. Effects of shaking[a] on the status of tolerance[b] of three *S. aureus* strains

Shaking at:		Strain 8		Strain 9		Strain 10	
Inoculation	Sampling (24 h)	MBC/MIC ratio	Tolerant	MBC/MIC ratio	Tolerant	MBC/MIC ratio	Tolerant
Yes	No	2	No	2	No	2	No
	Yes	≧100	Yes	≧100	Yes	32	Yes
No	No	2	No	2	No	2	No
	Yes	2	No	2	No	2	No

[a] Shaking was accomplished by vigorous mixing with a Vortex mixer for 3 s.
[b] As defined by an MBC/MIC ratio of ≧16 determined with a stationary-phase inoculum.

phases and durations of MIC incubation times on nine strains were evaluated with two MBC/MIC ratios. The tolerance assays were carried out with the nonshaking technique. We did not demonstrate tolerance when a logarithmic-phase inoculum was used, regardless of the length of time of incubation. When stationary-phase inoculum and 24 h of incubation were used, we demonstrated tolerance in five of the nine strains by using an MBC/MIC ratio of ≧16 but in only two of these five strains by using an MBC/MIC ratio of ≧100. After 48 h of tube incubation, the phenomenon of tolerance disappeared.

The effects of methicillin carry-over on the status of tolerance of 16 strains, evaluated with MBC/MIC ratios, are summarized in Table 2. In this aspect of the study, subcultures to Trypticase soy agar plates were done in quadruplicate; two plates contained 10,000 U of penicillinase per ml, and two plates contained no penicillinase. To allow partially injured bacteria to grow out, we incubated the plates for 4 days. Colonies were confirmed as staphylococci by colonial morphology and coagulase production.

Tolerance was defined with two different criteria, an MBC/MIC ratio of ≧16 and an MBC/MIC ratio of ≧100. On plates without penicillinase, eight strains were tolerant as defined by an MBC/MIC ratio of ≧16, whereas only one strain was tolerant as defined by an MBC/MIC ratio of ≧100. However, when penicillinase was added to the agar, seven strains were tolerant as defined by an MBC/MIC ratio of ≧100, whereas one additional strain became tolerant as defined by an MBC/MIC ratio of ≧16.

We have shown that even a technical procedure as simple as mixing, if not properly executed, can result in a spuriously high MBC and produce "false" tolerant strains. This is presumably due to contamination of the walls of the culture tubes with bacteria that are not killed because they are not in contact with the antibiotic. It may also be due to the formation of bacterial aggregates, as shown by Gwynn et al. (4).

We have failed to demonstrate tolerance with a logarithmic-phase inoculum, confirming the observations of Mayhall and Apollo (8) and supporting the observations of Kim and Anthony (5), who demonstrated that the MBCs were higher with stationary-phase bacteria than with logarithmic-phase bacteria, resulting in a higher MBC/MIC ratio. These observations are in conflict with those of Sabath et al. (15) and Raynor et al. (14). We also failed to demonstrate tolerance in the nine strains studied when the tubes were incubated for 48 h and subcultured, a result which is not in agreement with the observations of Bradley and associates (2).

Using penicillinase, we have clearly shown the effect of antibiotic carry-over. This antibiotic carry-over effect in relation to tolerance was previously reported by Rajashekariah et al. (13) and well defined by Pearson et al. (11).

Finally, we have shown that the definition of tolerance of a given strain can be influenced by

TABLE 2. Effects of methicillin carry-over on the status of tolerance of 16 *S. aureus* strains as determined with two MBC/MIC ratios[a]

Plate	No. of strains that were			
	Tolerant		Nontolerant	
	MBC/MIC ratio of ≧16	MBC/MIC ratio of ≧100	MBC/MIC ratio of ≧16	MBC/MIC ratio of ≧100
Without penicillinase	8	1	8	15
With penicillinase	9	7	7	9

[a] Sixteen strains were studied with a stationary-phase inoculum and the nonshaking technique throughout. For determining MBCs, subcultures were made onto plates with and without penicillinase.

the arbitrary selection of an MBC/MIC criterion. The clinical relevance of the magnitude of the MBC/MIC ratio is not clear. We have demonstrated that tolerance (as defined by the MBC/MIC ratio of ≧32 determined with a stationary-phase inoculum, gentle mixing, and the use of penicillinase) does not influence clinical response to methicillin treatment in experimental staphylococcal pyelonephritis in rats (3a).

We have found the optimal conditions for the most reproducible demonstration of tolerance to be avoidance of splashing bacteria upon inoculation, the use of a stationary-phase inoculum, subculturing of MIC tubes for MBC determinations after 24 h of incubation, and the use of penicillinase to counteract antibiotic carry-over.

It is clear from our studies and from those of others (1, 3, 5, 6, 8, 12) that because there is a multiplicity of methodological determinants which affect the status of a given strain, a standardized reference procedure for the determination of tolerance needs to be established.

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