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Evaluation of Oxacillin Tolerance in *Staphylococcus aureus* by a Novel Method

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A novel agar dilution plate-count procedure for the quantitative measurement of bacterial inhibition and killing is described. For *Staphylococcus aureus* versus oxacillin, by the agar dilution plate-count procedure it was found that only 1 of 20 clinical isolates and 1 of 7 allegedly tolerant reference isolates met the conventional definition of tolerance. By using inocula of 10^5 CFU per plate, most isolates were demonstrated to have subpopulations of cells which, although inhibited, persisted for 24 h in oxacillin concentrations significantly above their MICs. The persister percentages at 24 h appeared to be strain dependent, and all persisters exhibited the paradoxical effect. For each isolate, the persister percentage markedly decreased after action by oxacillin for 48 h, and the paradoxical effect was greatly diminished. Our findings suggest that tolerance is an artificial and arbitrary concept that does not adequately characterize the inhibition and killing dynamics associated with the persister phenomenon.

The term tolerance is commonly used to describe the in vitro phenomenon in which, for a specific antimicrobial agent and microorganism combination, the MBC is found to greatly exceed the MIC when the latter would normally characterize the isolate as susceptible. In this sense, the term tolerance was first used by Tomasz and associates (37) in 1970 to describe what was thought to be a qualitatively new form of microbial resistance to penicillin which they observed for strains of *Streptococcus pneumoniae*. Their findings suggested that the tolerance phenomenon was due to a deficiency in autolytic enzyme activity resulting from the presence of potent enzyme inhibitors. The term was next used in 1975 by Best and co-workers (4), who reported that 1 of 60 *Staphylococcus aureus* clinical isolates exhibited oxacillin tolerance and accordingly emphasized the need to consider a microorganism as potentially resistant if a significant dissociation of MBC and MIC is observed. In 1976, Mayhall and associates (26) reported three cases of bacteremia caused by oxacillin-tolerant *S. aureus* which responded poorly to oxacillin therapy alone. They also found that 33 of 60 randomly selected *S. aureus* isolates exhibited significant oxacillin tolerance and noted a high degree of associated cephalothin and gentamicin tolerance. The report of Sabath and co-workers (34) in 1977 was largely responsible for alerting clinical microbiologists and clinicians to the concepts and potential clinical implications of the tolerance phenomenon. They described seven cases of serious *S. aureus* infection by oxacillin-tolerant isolates which did not respond to conventional therapy, and in a retrospective study they found that 28 of 63 *S. aureus* isolates from bacteremic patients exhibited tolerance to semisynthetic penicillins. Subsequently, other investigators (6, 16, 19, 25, 31, 33, 36) have reported different and widely varying incidences of tolerance of *S. aureus* to oxacillin or methicillin. Similarly, conflicting evidence as to the clinical significance of the tolerance phenomenon has been reported from both experimental animal and clinical studies. In the past several years evidence has gradually accumulated which questions the validity of MBCs as determined by broth dilution plate-count (BDPC) methods, the approach almost exclusively used in previous tolerance studies, and this evidence suggests that methodologic dependency may have been largely responsible for the divergent and conflicting results previously reported. As a consequence of such findings, it is apparent that the *S. aureus* oxacillin tolerance phenomenon should be re-evaluated by methods fundamentally different than the BDPC. The present report describes the development and standardization of such a method and its application to a study of *S. aureus* versus oxacillin, the results of which suggest that manifestations of high-percentage, strain-dependent persisters account for what has been arbitrarily and artifically defined as the tolerance phenomenon.

MATERIALS AND METHODS

Study design. An agar dilution plate-count (ADPC) method for the concurrent measurement of MIC and MBC was developed and standardized. As a basis for evaluating the performance of ADPC, a reference BDPC method was selected by testing 50 *S. aureus* clinical isolates in parallel by the standard broth macrodilution plate-count procedure and a recently proposed, carefully controlled broth macrodilution plate-count method. For evaluation of performance, the ADPC method was applied in parallel with the selected reference BDPC method to 20 *S. aureus* clinical isolates and to 7 allegedly oxacillin-tolerant *S. aureus* reference isolates. By using both log- (L) phase and stationary- (S) phase inoculum preparations, the results of the parallel tests were evaluated in relation to detection and expression of the tolerance phenomenon, the paradoxical effect, and the irregular or skip phenomenon.

Principles of ADPC. The resultant ADPC procedure is based on the following principles: (i) preparation of a carefully controlled broth inoculum by standard methods, (ii) use of the broth inoculum to prepare oxacillin agar dilution pour plates for quantitative colony counts, (iii) application of a thin agar overlay to the pour plates to prevent redistribution of surface colonies on subsequent manipulation, (iv) determination of MIC at 24 or 48 h by quantitative plate count, (v) application of a β-lactamase solution to MIC plates at the

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time of MIC determination to inactivate oxacillin, and (vi) further incubation of plates to determine MBC plate counts at 24 or 48 h after inactivation of oxacillin.

Development of ADPC. In accordance with precedence established by other investigators, L- and S-phase inoculum preparations were made from broth cultures incubated in air at 35°C for 4 and 24 h, respectively. All broth cultures and subsequent inoculum dilutions were made with cation adjusted (Ca, 5.5 ± 0.2 mg/dl; Mg, 2.5 ± 0.2 mg/dl) Mueller-Hinton broth (MHB). Inoculum preparations were targeted to deliver approximately 3.0×10^5 CFU to each 20-ml pour plate, thus providing a final inoculum density in agar of approximately 1.5×10^4 CFU/ml. With this arrangement, the breakpoint indicative of a 99.9% reduction in colony count would be approximately 3×10^2 CFU per plate, a situation designed to provide accurate colony counts and optimum counting statistics for evaluation of plate counts close to the breakpoint. Several dilution schemes for preparing appropriate inoculum suspensions were evaluated for accuracy and precision using pour-plate counts representing 20 or more primary broth cultures and pour-plate counts representing 20 or more replications from individual cultures. The method selected for preparing inocula and pour plates, as described in detail below, produced pour-plate counts at the 99.9% reduction breakpoint with the following statistical characteristics. For plate counts representing multiple broth cultures, each of which was derived from separate primary cultures of a single isolate, the range, mean, and coefficient of variation for plate counts at the 99.9% CFU reduction were 63 to 520 CFU per plate, 292 CFU per plate, and 12.8%, respectively, for L-phase preparations, and 161 to 572 CFU per plate, 329 CFU per plate, and 15%, respectively, for S-phase preparations. For plate counts representing multiple inoculum preparations, each derived from a single primary culture of the same strain, coefficients of variation for plate counts at the 99.9% CFU reduction breakpoint were 6.1 and 5%, respectively, for L- and S-phase preparations. These findings indicate that each ADPC test must include a reference plate count representing a 10^3 dilution of the inoculum preparation to establish the appropriate 99.9% CFU reduction breakpoint.

The following requirements were needed for the β-lactamase preparation to be used in the ADPC procedure: (i) activity in 1-ml aqueous solution sufficient to inactivate rapidly 128 μg of oxacillin per ml present in a 20-ml agar dilution plate, (ii) no inhibition of *S. aureus* by β-lactamase alone or by the products of its activity on oxacillin, and (iii) the ability to permeate a 20-ml agar dilution plate rapidly after application of 1 ml of a β-lactamase solution to the surface. After studying several commercial preparations, penase concentrate, provided as a sterile aqueous solution with an activity of 300 Levy units per ml for oxacillin, was purchased from Difco Laboratories, Detroit, Mich. When incorporated into either 20 ml of MHB or 20 ml of molten Mueller-Hinton agar (MHA) prior to inoculation with a susceptible *S. aureus* strain (MIC, 0.25 μg/ml), 1 ml of the β-lactamase solution was found to inactivate at least 128 μg of oxacillin per ml. Similarly, a 1-ml overlay of penase solution completely permeated uninoculated agar dilution plates and inactivated oxacillin concentrations of at least 128 μg/ml within 1 h at 25°C. This was demonstrated by removing agar casts from penase-treated dilution plates with subsequent inoculation of a highly susceptible strain of *S. aureus* to the exposed deep surface of the agar. Incorporation of penase alone or with oxacillin into MHA dilution plates prior to inoculation showed no alteration of plate count or colony morphology, and the pH remained stable in the range of 7.2 to 7.4. Application of the penase solution to 24-h pour plates produced considerable redistribution of surface microorganisms as judged by continued incubation of the plates. Surface redistribution of CFU was found to be completely prevented by overlaying each pour plate, at the time of preparation, with a thin layer of MHA containing oxacillin in the same concentration as that in the pour plate.

Outline of the ADPC method. Inoculum preparations for L- and S-phase cultures were initiated at 4 h and 18 to 24 h, respectively, prior to inoculum standardization, by transferring material from the centers of three to five well-isolated colonies on blood agar plates (BAP), prepared as described below for clinical and reference isolates, to 5 ml of MHB for incubation in air at 35°C. After appropriate incubation times, L- and S-phase cultures were diluted as needed with MHB to match the visual density of a 1.0 McFarland standard, a procedure expected to provide approximately 3×10^8 CFU/ml. Final inoculum preparations were then made with serologic pipettes to dispense 0.1 ml of the standardized broth culture into 9.9 ml of MHB, producing a 1:100 dilution with an expected density of 3×10^6 CFU/ml.

Oxacillin agar dilution plates were prepared as follows. A 1,280-μg/ml oxacillin stock solution, prepared biweekly in sterile deionized water, was stored in 10-ml fractions at −70°C. Immediately prior to preparation of pour plates, as indicated by the number of isolates to be tested, appropriate volumes of thawed stock oxacillin solution were diluted with sterile deionized water to prepare serial twofold dilutions ranging from 2.5 through 640 μg/ml. For each of these concentrations, 1.0 ml was transferred to a tube containing 19.0 ml of sterile molten MHA maintained at 50°C to yield a 20-ml agar dilution series (0.125 through 32 μg/ml) to be used for inoculation and dispensing into agar dilution pour plates. Similarly, for each concentration, 0.5 ml was transferred to a tube containing 9.5 ml of sterile molten MHA maintained at 50°C to prepare a 10-ml agar dilution series (0.125 through 32 μg/ml) to be used for overlaying the inoculated agar dilution pour plates with uninoculated agar. Each tube of the 20-ml molten agar oxacillin dilution series was then inoculated with 0.1 ml of a thoroughly mixed standardized inoculum preparation designed to deliver approximately 3×10^5 CFU to each tube. Each tube was thoroughly mixed and then poured into a sterile 100-mm petri dish and allowed to gel firmly. Tubes from the 10-ml dilution series were then used to pour an uninoculated agar overlay with an analogous oxacillin concentration onto the surface of each pour plate. Reference breakpoints indicating 99.9% colony reduction were determined for each inoculum suspension with 0.1 ml of a 10^3 dilution of the standardized inoculum suspension for preparation of a 20-ml pour plate. For each isolate, duplicate agar dilution test panels were prepared for each L- and S-phase preparation, and were incubated in air at 35°C. One panel was incubated for 24 h, at which time plate counts were made to determine the 24-h MIC. After the 24-h MIC plate counts were completed, 1 ml of penase was pipetted and spread onto the surface of each agar dilution pour plate. The plates were then allowed to stand at room temperature for 1 h prior to continued incubation at 35°C in air for subsequent determination of MBC plate counts at 24 and 48 h after the penicillinase application. The second agar dilution pour-plate panel was used to determine the MIC at 48 h of incubation, at which time 1 ml of penase was overlayed onto each plate. Plates were allowed to remain at room temperature for 1 h, and were then incubated at 35°C in air for determination of MBC counts at 24 and 48 h after inactiva-

TABLE 1. BDPC-1 and BDPC-2 applied in parallel to L- and S-phase cultures of 50 *S. aureus* clinical isolates

Method	Inoculum phase	MIC (μg/ml) at[a]:		MIC (μg/ml) range at:		No. of isolates exhibiting tolerance[b]	No. of isolates exhibiting paradoxical effect	No. of isolates exhibiting irregular effect
		24 h	48 h	24 h	48 h			
BDPC-1	L	0.52	1.75	0.125–2	0.25–16	7	1	19
	S	0.56	2.32	0.125–2	0.5–32	8	1	16
BDPC-2	L	0.54	0.97	0.25–1	0.5–2	0	1	5
	S	0.67	1.26	0.25–2	0.5–4	3	0	10

[a] Mean MIC in micrograms of oxacillin per milliliter.
[b] Tolerance is the number of isolates with MBC/MIC values ≥32.

tion of oxacillin. Colonies were counted with the unaided eye with pour plates being placed on a diffusely back-lighted grid pattern.

MIC was defined as the oxacillin concentration in the first plate of the ascending concentration series which exhibited a colony count at least two standard deviations below the reference 99.9% breakpoint. In actual application, colonies were usually absent at the MIC so that breakpoints were seldom needed. After application of β-lactamase and reincubation of pour plates for an additional 24 or 48 h, MBC was defined as the oxacillin concentration in the first plate of the ascending concentration series for which the plate count was at least two standard deviations below the reference plate count, with counts greater than the breakpoint being permitted for any or all of the higher concentrations. According to convention, isolates with MBC/MIC ratios ≥ 32 were scored as exhibiting oxacillin tolerance. The paradoxical effect, or Eagle effect (3, 7, 8, 35), was defined as the occurrence of progressively increasing plate counts for at least three consecutive oxacillin concentrations higher than the MIC. The irregular or skip effect (2) was defined as the presence of counts higher than the breakpoint occurring in an irregular or skipped fashion on plates with concentrations equal to or greater than the MIC and which showed no conformity with the paradoxical effect.

The BDPC reference test. The BDPC procedure (BDPC-1), as outlined in the *Manual of Clinical Microbiology* (2), has been the principal method used in previous studies of *S. aureus* oxacillin tolerance. Recently, Taylor and associates (36) have described modifications (BDPC-2) of the standard method which are said to improve MBC measurements. As a basis for selecting the BDPC test to be used as a reference method in the present investigation, parallel BDPC-1 and BDPC-2 procedures were performed on 50 randomly selected *S. aureus* fresh clinical isolates with both L- and S-phase inoculum preparations. BDPC-1 was performed in polypropylene tubes (12 by 75 mm; American Scientific Products, McGaw Park, Ill.) and MHB. BDPC-2, which we ultimately selected as the appropriate reference test, was performed as follows. On the day of use, 2-ml oxacillin twofold dilution panels, (0.125 through 32 μg/ml) were prepared with new borosilicate glass tubes (16 by 125 mm; American Scientific Products). Inoculum suspensions were prepared from an L- or S-phase culture by dilution to the density of a 0.5 McFarland standard with subsequent 1:20 dilution to achieve an expected inoculum density of 5×10^6 CFU/ml. After thorough mixing, 0.1 ml was inoculated into each dilution by carefully inserting the tip of the transfer serologic pipette beneath the broth surface without bubbling or splashing. Following inoculation, the tubes were carefully incubated in air at 35°C without shaking. Tubes were vortexed for 10 s after 20 h of incubation, and were again vortexed at the time of the 24-h MIC interpretation. From each tube, 0.1 ml was then transferred with a serologic pipette onto the surface of a BAP which was then incubated at 35°C in air for MBC determination at 48 and 72 h. Tubes were reincubated for 18 to 24 h for the 48-h MIC interpretations. MICs were determined visually as the lowest oxacillin concentration resulting in complete growth inhibition. For MBC determinations, colonies were counted with the unaided eye by using incident light. The tolerance phenomenon, the paradoxical effect, and the irregular effect were defined as previously described for the ADPC procedure.

***S. aureus* isolates.** *S. aureus* clinical isolates were randomly selected at the time of isolation in the St. Paul-Ramsey Medical Center Clinical Microbiology Laboratory. Each isolate was transferred from the primary culture to BAP for incubation at 35°C for 18 to 24 h. L- and S-phase cultures were prepared, as described above, from the cultures grown on BAP for 18 to 24 h. For selection of the reference BDPC test, 50 isolates were tested in parallel by the BDPC-1 and BDPC-2 tests. After selection of BDPC-2 as the reference test, 20 additional, randomly selected *S. aureus* clinical isolates and 7 reference isolates allegedly tolerant to oxacillin were tested in parallel by ADPC and BDPC-2 for evaluation of ADPC performance. Isolates R1, R2, and R3 were supplied by L. R. Peterson, Minneapolis Veterans Administration Medical Center, Minneapolis, Minn. Isolate R4 was obtained from R. J. Fass, Ohio State University Hospital, Columbus, Ohio. Isolates R5 and R6 were contributed by P. A. Guze, Wadsworth Veterans Administration Medical Center, Los Angeles, Calif. Isolate R7, the Evans strain, was donated by G. K. Best, Medical College of Georgia, Augusta, Ga. These reference isolates were received either on slants or as lyophilized preparations, and were retrieved by two passages for 18 to 24 h on BAP incubated in air at 35°C.

RESULTS

The results of the BDPC-1 and BDPC-2 tests performed in parallel on 50 *S. aureus* clinical isolates for selection of the reference BDPC test are summarized in Table 1. MICs at 24 and 48 h were similar for the two procedures and did not appear to be dependent on the inoculum phase. For BDPC-1, 48-h MICs were approximately four times greater than 24-h MICs. For BDPC-2, 48-h MICs were only two times greater than the 24-h MICs. For the two procedures, MIC ranges at 24 h were approximately the same, but at 48 h the MIC range was significantly greater for BDPC-1. Using BDPC-1, 14 and 16% of L- and S-phase preparations, respectively, were classified as exhibiting tolerance, whereas only 0 and 6%, respectively, were so designated by BDPC-2. Similarly,

TABLE 2. BDPC-2 and ADPC applied in parallel to L- and S-phase cultures of 20 *S. aureus* clinical isolates

Method	Inoculum phase	Oxacillin MIC (μg/ml) at:		Isolates exhibiting tolerance at[a]:				Isolates exhibiting paradoxical effect at:				Isolates exhibiting irregular effect at:			
		24 h	48 h	24/48 h	24/72 h	48/72 h	48/96 h	24/48 h	24/72 h	48/72 h	48/96 h	24/48 h	24/72 h	48/72 h	48/96 h
BDPC-2	L	0.51	0.70						C2			C11	C5		
												C12	C10		
	S	0.71	1.08	C2	C4			C4	C14			C10	C5		
				C9	C7							C11			
				C12								C12			
												C13			
ADPC	L	0.31	0.44		C5			C2	C1		C14				
					C13			C15	C5						
									C8						
									C14						
									C13						
	S	0.33	0.37	C9	C3			C15	C1						
					C7				C2						
					C11				C6						
					C13				C11						
					C-15				C12						

[a] Code numbers of isolates exhibiting MBC/MIC values of ≥32 at various times of MBC/MIC interpretations, e.g., 24/48 h indicated MIC determined at the time of 24-h β-lactamase application with MBC determined 48 h after initiation of the test and 24 h after application of β-lactamase.

BDPC-1 classified 38 and 32% of L- and S-phase preparations, respectively, as exhibiting the irregular effect, in contrast to 10 and 20% by BDPC-2. For BDPC-2, both the tolerance phenomenon and the irregular effect appeared to be dependent on the inoculum phase. The paradoxical effect was infrequently detected by either procedure. The similarity of these results with those reported by Ishida and associates (19) and Taylor and co-workers (36) prompted us to choose BDPC-2 as the reference BDPC procedure for the remaining portion of the study.

A comparison of results for ADPC and BDPC-2 applied to 20 *S. aureus* clinical isolates is presented in Table 2. ADPC MICs at 24 and 48 h were approximately equal, with L- and S-phase values also being similar. ADPC MICs were generally equal to or one twofold dilution step below the corresponding BDPC-2 value. ADPC and BDPC-2 each categorized several isolates as being oxacillin tolerant, particularly for S-phase preparations. Of special interest is the fact that, for ADPC, none of the isolates fulfilled the conventional definition of tolerance when oxacillin was permitted to act for 48 h. The paradoxical effect was observed more frequently by ADPC, particularly when oxacillin was permitted to act for only 24 h, and was observed for all isolates having sufficient colony counts on the dilution series plates, permitting valid assessment of the paradoxical effect. After oxacillin action for 48 h, the paradoxical effect was observed for only one isolate, with the paradoxical peak being significantly diminished as compared with that in the preparations

TABLE 3. BDPC-2 and ADPC applied in parallel to L- and S-phase cultures of *S. aureus* reference isolates previously described as oxacillin tolerant

Isolate no.	Method	MIC (μg/ml) applied to the following phase preparations[a]		MBC/MIC applied to the following phase preparations at the indicated times[b]:				Tolerance	Paradoxical effect	Irregular effect
				L		S				
		L	S	24/72 h	48/96 h	24/72 h	48/96 h			
R1	BDPC-2	0.25	0.5	4	4	2	2	−	−	+
	ADPC	0.25	0.25	2	2	1	1	−	+	−
R2	BDPC-2	0.5	0.25	4	4	8	8	−	−	−
	ADPC	0.5	0.5	1	1	1	1	−	+	−
R3	BDPC-2	0.5	0.5	1	1	2	2	−	−	−
	ADPC	0.5	0.5	1	1	1	1	−	+	−
R4	BDPC-2	1	1	1	1	2	2	−	−	−
	ADPC	0.5	0.5	1	1	1	1	−	+	−
R5	BDPC-2	0.5	0.5	2	2	2	2	−	+	−
	ADPC	0.25	0.25	2	2	2	4	−	+	−
R6	BDPC-2	0.5	0.5	2	2	1	1	−	−	−
	ADPC	0.25	0.25	1	1	1	1	−	+	−
R7	BDPC-2	1	2	32	32	16	16	+	+	−
	ADPC	0.5	0.5	>64	4	16	8	−	+	−

[a] Interpreted at 24 h.

[b] MBC/MIC ratios as determined for L- and S-phase preparations at various times for MIC and MBC intrepretations, e.g., 24/72 h indicates MIC determined at the time of 24-h β-lactamase application with MBC determined 72 h after initiation of the test and 48 h after application of β-lactamase.

TABLE 4. Triplicate ADPC L-phase test results for four allgedly oxacillin-tolerant reference isolates and two clinical isolates with high persister percentages

Isolate no.	Trial	MIC (μg/ml) at[a]:		MBC/MIC at[b]:		Tolerance at[b]:		Paradoxical effect at[b]:	
		24 h	48 h	24/72 h	48/96 h	24/72 h	48/96 h	24/72 h	48/96 h
R2	a	0.5	0.5	2	1			+	
	b	0.5	0.5	4	2			+	
	c	1	1	1	1			+	
R4	a	0.5	0.5	2	2			+	
	b	0.5	0.5	4	1			+	
	c	0.5	0.5	2	2			+	
R6	a	0.25	0.25	1	1			+	
	b	0.125	0.125	2	1			+	
	c	0.25	0.25	1	1			+	
R7	a	0.5	1	≥256	1	+		+	+
	b	0.5	1	≥256	2	+		+	+
	c	0.5	1	≥256	2	+		+	+
C5	a	0.25	0.25	2	2			+	
	b	0.25	0.25	4	2			+	
	c	0.25	0.25	2	1			+	
C13	a	0.5	0.5	128	1	+		+	+
	b	0.5	0.5	≥256	1	+		+	+
	c	0.5	0.5	≥256	1	+		+	+

[a] Micrograms of oxacillin permilliliter as determined at the designated time of β-lactamase application.
[b] See footnote *b* in Table 3.

at 24 h. The irregular effect was found with L- and S-phase BDPC-2 preparations, but was strikingly absent by ADPC. Of the two L-phase ADPC isolates which met the conventional definition of tolerance, both displayed the irregular effect by BDPC-2. Of the six S-phase ADPC isolates meeting the conventional definition of tolerance, two showed the irregular effect and two met the tolerance definition by BDPC-2.

The results of parallel BDPC-2 and ADPC testing of seven reference *S. aureus* isolates previously described as being oxacillin or methicillin tolerant are presented in Table 3. ADPC and BDPC-2 produced comparable MICs. MBC/MIC values tended to be somewhat larger for BDPC-2 than for ADPC. Only isolate R7, the Evans strain, showed distinctly high MBC/MIC values for both procedures, with ADPC results closely approximating the conventional definition of tolerance and BDPC values distinctly exceeding the requirements. The paradoxical effect was observed for all seven isolates by ADPC, but only for isolates R5 and R7 by BDPC-2. The irregular or skip effect occurred only with BDPC-2.

The results of triplicate ADPC retesting of four of the reference isolates and the two clinical isolates which were found to have high MBC/MIC values with L-phase preparations and ADPC are summarized in Table 4. For each of the four reference strains, the triplicate test results were in agreement and closely approximated the original results as presented in Table 3. The results for isolate R7 again met the conventional definition of tolerance with the 24/72 MBC/MIC results (24/72 indicates the MIC determined at the time of 24-h β-lactamase application with the MBC determined 72 h after incubation of the test and 48 h after β-lactamase application). However, when oxacillin was permitted to act for 48 h, plate counts were significantly decreased below the 0.1% breakpoint count, and the tolerance definition was not met. For isolate R7, colonies were extremely small and hardly detectable when the plates were examined 24 h following the β-lactamase solution application, a situation encountered only with isolate R4 among the other isolates. The paradoxical effect was observed in all three trials for each of the four reference isolates for the 24/72 plate counts but only for isolate R7 for the 48/96 plate counts. The 48/96 plate counts were one log or more lower than the 24/72 plate counts for all four isolates. The triplicate results for clinical isolate C5, although not meeting the conventional definition of tolerance, showed high plate counts approaching the 0.1% breakpoint on plates immediately above the MIC and paradoxical peak counts greatly exceeding the breakpoint. Triplicate retest results for clinical isolate C13 agreed with the initial ADPC results and met the conventional definition of tolerance, being strikingly similar to the results observed for isolate R7. After 48 h of oxacillin action, the percentages of persisting CFU fell distinctly below the 0.1% breakpoint, but a paradoxical effect was still detectable.

Figure 1 illustrates the averaged plate-count patterns from the triplicate retesting by ADPC for isolate R7. For concentrations higher than the MIC, 24/72 plate counts were not only greater than the 99.9% CFU reduction breakpoint but they also showed the paradoxical effect. After continued activity of oxacillin for 48 h, as indicated by the 48/96 plate counts, the paradoxical effect was again evident, but plate counts were significantly lower than the 24/72 counts. Figure 2 shows the plate-count patterns for isolate R4, which are similar to those found for the other reference isolates. The paradoxical effect was distinctly present for the 24/72 counts, with maximum counts at concentrations higher than the MIC rising to well above the 0.1% breakpoint. Again, continued action of oxacillin for 48 h diminished the counts significantly, and the paradoxical effect was barely evident.

DISCUSSION

Except for a few attempts to use agar dilution replicator methodology (9, 23, 24), previous investigations largely have used modifications of the BDPC approach for studying the quantitative dynamics of bacterial inhibition and killing. Evidence has gradually accumulated indicating a significant dependency of BDPC results, especially MBCs, on technical factor variations. It is not surprising that factors such as pH (18, 38), temperature (30), type of medium (22, 28, 32),

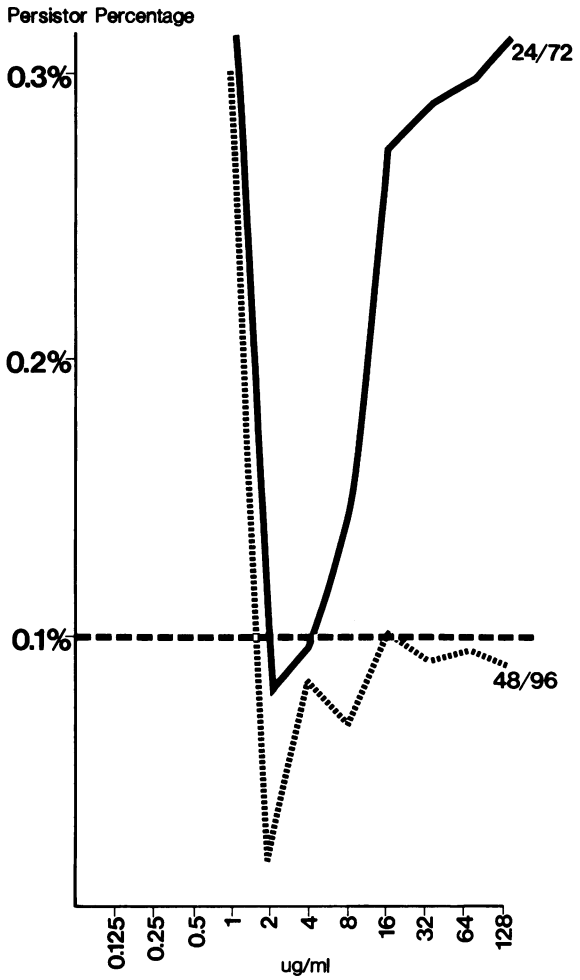

FIG. 1. Averaged plate-count patterns from the triplicate retesting by ADPC for isolate R7.

inoculum phase (5, 18, 20, 21, 25, 29), incubation time (1, 19, 22, 26, 34, 36), and carry-over of antimicrobial agents from MIC tubes to MBC plates (12, 19, 31, 33) have been shown to influence the measured incidence of tolerance and related phenomena for a variety of bacteria and antimicrobial agents. Less intuitively obvious has been the finding that bacteria subjected to broth dilution procedures may be inadvertently protected from the action of antimicrobial agents in broth cultures, thus causing tolerance interpretations to be spuriously high and often irregular in manifestation and reproducibility (15, 26, 39). In 1982, studying oxacillin tolerance in *S. aureus*, Ishida and associates (19) elegantly demonstrated and re-emphasized the need for meticulous attention to BDPC procedural details. They demonstrated the need to use L-phase inoculum preparations, careful introduction of inocula below broth surfaces to avoid contamination of the dilution tube wall, avoidance of mixing during MIC incubation, interpretation of MICs at 24 h, and the use of penicillinase to avoid carry-over of oxacillin onto MBC plates. By this approach, spurious tolerance interpretations were eliminated, and oxacillin tolerance was not found in L-phase cultures of 60 clinical isolates. However, all isolates could be made to appear tolerant if dilution tubes were vigorously mixed at the time of inoculation and a small incidence of tolerance was observed for carefully controlled S-phase preparations. In 1983, using *S. aureus* clinical isolates and allegedly oxacillin-tolerant reference strains, Taylor and associates (36) compared results obtained by the standard broth macrodilution BDPC method as outlined in the *Manual of Clinical Microbiology* with those of a carefully controlled procedure patterned along the lines recommended by Ishida and associates. For both L- and S-phase preparations, the majority of isolates in each group were interpreted as being oxacillin tolerant by the standard broth macrodilution method. In contrast, only a small incidence of tolerance was found for S-phase preparations, and no tolerance was found for L-phase preparations by the carefully controlled procedure. In the present study we also compared these two procedures to select and gain experience with the most appropriate BDPC method to be used as a reference in evaluating the new ADPC method. Our results were similar to those of Taylor and associates, and on this basis BDPC-2 was selected as the reference test for evaluation of ADPC.

Parallel tests by ADPC and BDPC-2 on clinical isolates and allegedly tolerant reference isolates showed the two procedures to produce comparable MICs. ADPC MIC plate counts fell dramatically at the MIC and were essentially absent on plates with concentrations above the MIC as long as oxacillin action was permitted to continue. ADPC MBC plate-count patterns showed significantly less variation and

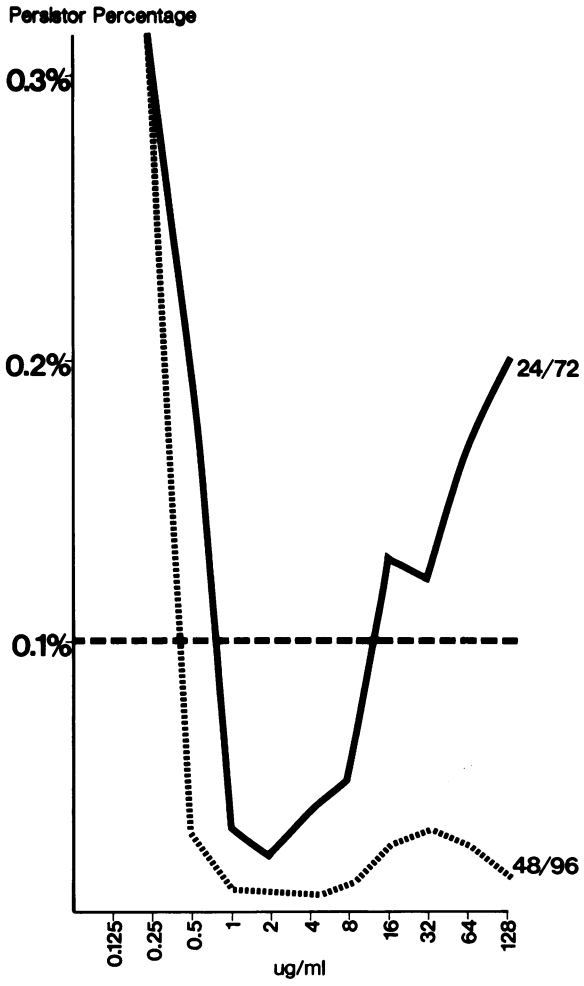

FIG. 2. Plate-count patterns for isolate R4.

irregularity than did BDPC-2 patterns, and various degrees of MBC-MIC dissociation were less frequently encountered. After inactivation of oxacillin, MBC plates showed distinctly larger colonies and slightly higher counts at 48 h than at 24 h. When oxacillin was permitted to act for 48 h, rather than 24 h, MBC plate counts were significantly lower, indicating a continued killing of the inhibited bacteria with oxacillin concentrations above the MIC, a finding also observed by others using BDPC methodology (1, 19, 22, 26, 34, 36). For ADPC, the paradoxical effect was observed for all isolates having survivors, or persisters, after exposure to oxacillin for 24 h, and this effect was not related to persister percentage. After oxacillin action for 48 h, the paradoxical effect was frequently absent or diminished in amplitude. The irregular or skip effect was not observed in the ADPC preparations. Of the allegedly oxacillin-tolerant reference isolates, only isolate R7 met the conventional definition of tolerance by BDPC-2 and was also the single reference isolate which closely approximated the definition by ADPC. For the six other reference isolates, although 24-h persister percentages were high, sometimes approaching 0.1%, none met the conventional tolerance definition.

The reproducibility of ADPC results was studied by performing triplicate retesting on four of the allegedly tolerant reference isolates and on two clinical isolates in which initial results met the conventional tolerance definition. Triplicate persister percentage results were comparable and matched those of the initial trial, except for one of the clinical isolates which had slightly lower persister percentages. Isolate R7 again showed >0.1% of the initial inoculum to persist in high concentrations of oxacillin for 24 h, but distinctly <0.1% persisted after 48 h of oxacillin activity. Our finding of a high persister percentage at 24 h for isolate R7 confirmed the findings of Best and associates (4), who reported it as the only 1 of 60 clinical *S. aureus* isolates to exhibit tolerance. The reproducibility of high persister percentages for isolate R7 and clinical isolate C13 along with reproducible lower persister percentages for other isolates suggests the existence of a spectrum of isolates, each of which is capable of producing a certain percentage of 24-h persisters as an intrinsic property. Additionally, although not studied for reproducibility, the other 18 clinical isolates showed persister percentages ranging from slightly below 0.1% to undetectable. The paradoxical effect was found for all detectable persisters and was demonstrated to be reproducible. For the low end of the persister percentage range, statistically valid counts were sometimes not present on one or more plates with oxacillin concentrations immediately above the MIC but were found to increase progressively on the plates with higher concentrations suggesting the emergence of paradoxical peaks. Our findings indicate that all *S. aureus* strains may produce subpopulations of 24-h oxacillin persisters, with quantitative expression of persister percentage being an intrinsic property of each strain. Accordingly, with high inoculum concentrations, 24-h persisters should be demonstrable for all isolates, with each exhibiting the paradoxical effect.

Of fundamental interest is the fact that, for ADPC, bacteria are immobilized in an agar gel matrix during the action of oxacillin and are therefore not subject to the technical factor variations which are inherent in the initial broth dilution incubation phase of BDPC methods. Factors influencing the production of persisters are therefore limited to conditions which are operative during preparation of the inoculum culture and to intrinsic properties of the microorganism. Culture conditions, such as the adherence to and drying of microorganisms on the culture tube walls above the broth surface, may produce artificial or adaptive mechanisms of persistence not related to intrinsic properties of the microorganism. Other culture conditions such as medium type, temperature of incubation, and time of incubation affect the proportion of cells in various life cycle phases within the culture, each phase of which may influence the expression of intrinsic persistence or act to promote adaptive persistence or both. In practice, persisters probably represent two populations, with one being related to intrinsic properties of the strain and the other being variously superimposed as the result of adaptive changes induced by technical factor variations. This situation, in combination with the use of various methodologies and arbitrary definitions, has no doubt been responsible for the conflicting results which have characterized previous investigations of the tolerance phenomenon.

Goessens and associates (10, 11, 12), using a carefully controlled BDPC method, recently introduced tolerance percentage to indicate the percentage of CFU surviving antimicrobial action. They found tolerance percentage to be strain dependent for *S. aureus* versus oxacillin and arbitrarily defined tolerance as a tolerance percentage of 2% or greater. On the basis of historical precedent, and in agreement with the original discussion of Mayhall and associates (26), we believe that persister percentage is a more appropriate term and that the so-called tolerance phenomenon should be attributed to the behavior and detection of persisters, as originally described by Hobby et al. (17) and Bigger (5) and later by others (13–15, 27). Accordingly, we believe that tolerance is not an appropriate term for characterizing microorganisms exhibiting high-level persister percentages defined by some arbitrary breakpoint. The term tolerance implies a uniform behavior for all cells of a population. In this sense, 100% of cells would be inhibited at the MIC, with 100% of cells remaining inhibited but viable for concentrations below the MBC. As used in the past, the term tolerance appears to be so ambiguous and arbitrarily defined as to preclude its usefulness. Rather than classifying microorganisms as tolerant or nontolerant by old definitions, it would seem that accurate measurement of persister percentages after various times of antimicrobial agent action would better serve to characterize the response of the isolate to the agent.

It is anticipated that ADPC methodology will prove useful for investigating many problems relating to bacterial inhibition and killing. The method should be readily adaptable for use with various antimicrobial agents and bacteria by substituting specific inhibitors of antimicrobial agents in place of β-lactamase and by modifying the agar medium. The standardization of ADPC methodology and its application to both clinical and experimental animal studies should help to provide new insight into the clinical significance of microorganisms with high persister percentages.

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