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Incidence and Characteristics of Antibiotic-Tolerant Strains of *Staphylococcus aureus*

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Thirty clinical isolates of *Staphylococcus aureus* were tested for tolerance to oxacillin, cephalothin, and gentamicin by using killing curves and broth dilution susceptibility tests. Tolerance was defined as the presence of ≥100 colony-forming units per ml remaining after 24 h of incubation of the killing curve suspensions. Nineteen of 30 isolates were tolerant to oxacillin and cephalothin, but none was tolerant to gentamicin. Among the tolerant isolates, there was a wide spectrum of tolerance, with from 0.2 to 43% of the colony-forming units in the starting inoculum remaining after 18 to 24 h of incubation. Tolerance was unrelated to phage type or to any of several other characteristics studied.

Recent reports have described a phenomenon manifested by the failure of antibiotics that inhibit cell wall synthesis to eradicate infections caused by *Staphylococcus aureus* in spite of in vitro susceptibility data that indicate susceptibility of the organism to the antibiotic (9, 11, 12, 14). When tested by standard broth dilution susceptibility tests, the minimum inhibitory concentration (MIC) for these isolates is low, but the minimum bactericidal concentration (MBC) is high and clinically unachievable. Best et al. (2) referred to this phenomenon in *S. aureus* as drug "tolerance," similar to that observed by Tomasz (15) in pneumococci.

Unlike antibiotic resistance due to beta-lactamase production or intrinsic resistance, antibiotic tolerance cannot be detected by standard disk diffusion susceptibility tests. Best et al. (3), using broth dilution susceptibility tests to screen 60 clinical isolates of *S. aureus,* found only one isolate that exhibited tolerance to oxacillin. Mayhall et al. (12) screened 60 clinical isolates for tolerance to oxacillin and found 55% tolerant, with MICs of <1 μg/ml and MBCs of ≥50 μg/ml. Sabath et al. (14) described the characteristics of eight isolates of *S. aureus* tolerant to several antibiotics and stated that retrospective review of susceptibility data for blood isolates over a 12-month period in their hospital revealed that 44% were tolerant.

To further define the incidence and characteristics of tolerance among clinical isolates of *S. aureus,* we screened 30 isolates by using broth dilution tests and killing curves with oxacillin, cephalothin, and gentamicin. Additional studies were performed to examine other parameters which might differentiate tolerant from nontolerant isolates.

Isolates were identified as *S. aureus* by gross and microscopic morphology, by a positive coagulase test, and by mannitol fermentation. Oxacillin was provided as a standard powder by Bristol Laboratories; cephalothin was purchased as Keflin from Eli Lilly & Co., and gentamicin was supplied as a standard powder by the Schering Corp. Stock solutions of 200 μg/ml were prepared from each antibiotic and stored at −10°F (−23.3°C) in portions of 5 ml. Broth dilution susceptibility tests and killing curves were performed in duplicate in Mueller-Hinton broth (MHB) (Difco Laboratories). Inocula for these tests were prepared by incubation of each isolate in MHB at 35°C for 18 to 24 h. Colony counts indicated that this usually gave 10^9 colony-forming units (CFU)/ml. For broth dilution tests, the inoculum used was diluted 10^{-4} in MHB, and 0.5 ml was added to tubes containing 0.5-ml amounts of serial dilutions (200 to 0.2 μg/ml) of the antibiotic in MHB. After the tubes were incubated for 18 to 24 h and 42 to 48 h at 35°C, MICs were recorded for both sets in a duplicate series of dilutions and were defined as the lowest concentration of antibiotic showing no visible turbidity. MBCs were determined by subculture of the clear tubes to blood agar by using a 0.01-ml loop and taking care not to touch the side or shake the tube. For 8 of the 30 isolates, both sets of tubes without visible growth were subcultured at 24 h and, for the remaining 22 isolates, one set was subcultured at 24 h and one set was subcultured at 48 h. MBCs were recorded as the lowest concentration of

antibiotic which yielded no growth on subculture, i.e., >99.9% killing. For killing curves, an overnight broth culture of the strain to be tested was diluted 10^{-4} in MHB, and 2 ml of the inoculum suspension was added to each tube containing antibiotic in 2 ml of broth to give approximately 5×10^4 CFU/ml in 12.5 μg each of oxacillin and cephalothin for ml and 3.125 μg of gentamicin per ml. Tubes without antibiotic were included as controls, and tests and controls were incubated at 35°C and performed in duplicate. Samples (0.5 ml) were taken from the inoculum suspension at the beginning of each test and from the test and control suspensions after 6, 24, and 48 h of incubation. Samples were carried through serial 10-fold dilutions in sterile saline, and 0.1 ml from each dilution was spread on the surface of a blood agar plate for colony counts. Isolates were designated as tolerant when the 24-h sample from their killing curve had ≥100 CFU/ml (<99.9% killing).

The production of L-forms was determined by the method of Dalton et al. (7). The production of deoxyribonuclease was studied by using the techniques of DiSalvo (8), and penicillinase production was assayed by the method of Catlin (6). Phage typing was performed by the procedure recommended by the International Subcommittee of Phage Typing of Staphylococci (13). Disk diffusion susceptibility tests were performed by the method of Bauer et al. (1). Utilization of carbohydrate was tested by using media containing arabinose, raffinose, inositol, cellobiose, and inulin prepared according to the instructions of the manufacturers (Baltimore Biological Laboratory and Difco) in purple broth base (Difco). Esculin hydrolysis was determined by using selective enterococcus broth (Pfizer Inc.). Forty percent bile agar (Oxgall, Difco; Trypticase soy agar, BBL) and 15% NaCl broth (brain heart infusion, Difco) were used to test the ability of the isolates to grow in high concentrations of these substances.

Killing curves with the 30 isolates indicated that 19 were tolerant and 11 were nontolerant to both oxacillin and cephalothin (Fig. 1 and 2). Each isolate responded in the same way to both antibiotics. The differences between the percent decrease in colony counts from zero time, for tolerant and nontolerant isolates at 6, 24, and 48 h in killing curves with oxacillin and cephalothin, were tested for significance by the Wilcoxon

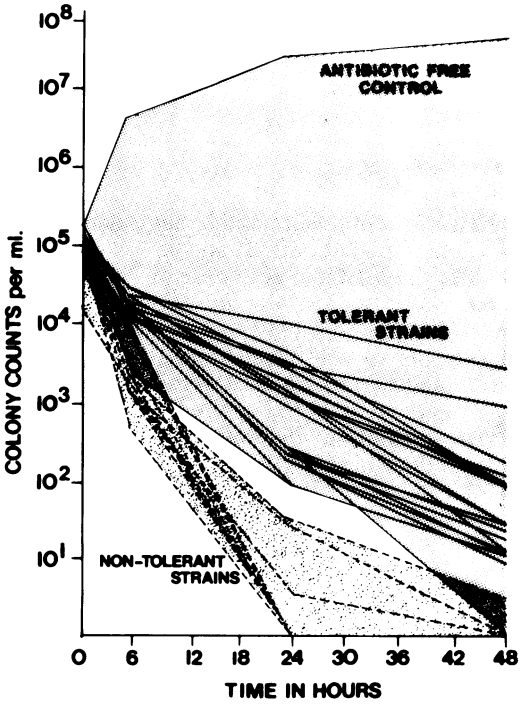

FIG. 1. *Killing curves of 30 clinical isolates of S. aureus incubated with 12.5 μg of oxacillin per ml. (——) Tolerant isolates; (- - -) nontolerant isolates.*

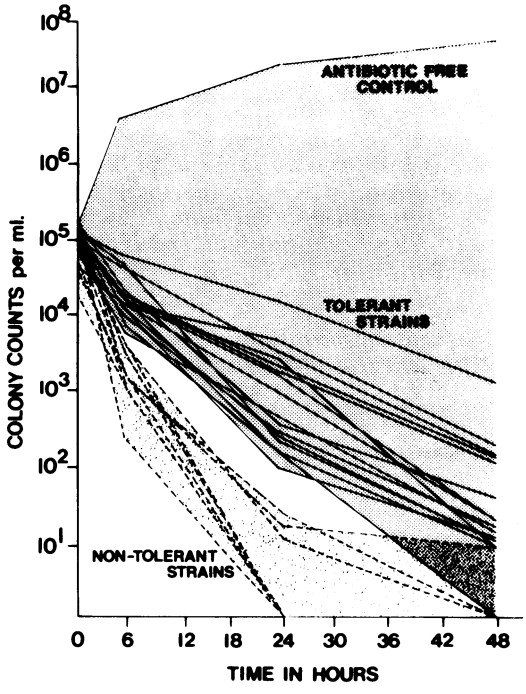

FIG. 2. *Killing curves of 30 clinical isolates of S. aureus incubated with 12.5 μg of cephalothin per ml. (——) Tolerant isolates; (- - -) nontolerant isolates.*

rank sum test. At each of these times, there was a significant difference between the percent decrease in colony counts for tolerant and nontolerant isolates ($P < 0.001$). Although all isolates tolerant to oxacillin and cephalothin had ≥100 CFU/ml at 24 h by definition, there was a spectrum of tolerance, with 0.2 to 43% of the initial CFU/ml remaining at 24 h. Killing curves performed with gentamicin indicated that it killed the isolates much more rapidly than did oxacillin or cephalothin. With two isolates, 10 CFU/ml remained viable at 24 h, whereas all CFU in the remaining isolates were killed within 24 h. Inoculum concentrations for all of the killing curves were similar (Fig. 1 and 2). Growth rates determined for each isolate from colony counts of antibiotic-free controls were similar for all isolates.

All isolates were susceptible to oxacillin, cephalothin, and gentamicin in disk diffusion tests. The MIC for each isolate was the same at 24 as at 48 h. MICs of all the antibiotics were uniformly low (≤1.5 μg/ml) for all isolates except for two with MICs of 6.25 and 3.1 μg of oxacillin per ml and two isolates with MICs of 3.1 μg of cepahlothin per ml (Table 1).

MBCs were determined for 16 of the 19 tolerant isolates after both 24 and 48 h of incubation by subculturing the tubes without visible growth from one series of dilutions in the duplicate set at 24 h and the other at 48 h. With each of the 16 isolates, at both 24 and 48 h, the MBCs were ≥25 μg of oxacillin per ml and ≥25 μg of cepholothin per ml, except for isolate 13, for which the MBC was 50 μg/ml at 24 h and 6.25 μg/ml at 48 h. MBCs for the three tolerant isolates, determined in duplicate at 24 h, were ≥50 μg/ml with both oxacillin and cephalothin (Tables 2 to 5).

TABLE 1. *Mean and range of MICs for 30 isolates of S. aureus tested against oxacillin, cephalothin, and gentamicin*

Antibiotic	MIC (μg/ml) for:	
	Tolerant isolates	Nontolerant isolates
Oxacillin		
Mean	1.48	0.51
Range	0.39–6.25	0.195–0.78
Cephalothin		
Mean	1.13	0.60
Range	0.39–3.125	39–1.56
Gentamicin		
Mean	0.57	0.41
Range	0.195–0.78	0.195–0.78

TABLE 2. *MBCs and total number of colonies from subcultures of tubes containing ≥12.5 μg of oxacillin per ml at 24 and 48 h for 16 tolerant and 6 nontolerant isolates of S. aureus*

Strain	24 h		48 h	
	MBC (μg/ml)	Total colonies recovered from ≥12.5 μg/ml	MBC (μg/ml)	Total colonies recovered from ≥12.5 μg/ml
Tolerant				
8	100	60	50	60
12	25	60	25	4
14	50	3	25	1
15	25	1	25	1
101	100	224	25	216
102	50	1	100	1
104	50	18	50	5
105	25	1	50	63
106	50	69	50	4
107	50	63	25	15
109	50	13	50	4
110	50	13	50	11
113	50	10	50	3
118	50	65	100	9
123	100	12	100	3
13	50	4	100 25	2
Nontolerant				
10	0.39	0[a]	3.125	0
11	0.78	0	1.56	0
103	0.78	0	25	1
111	12.5	0	3.125	0
119	6.25	0	25	11
121	0.78	0	0.78	0

MBCs for 6 of the 11 nontolerant isolates were also determined at both 24 and 48 h. For all six isolates, the MBCs of both oxacillin and cephalothin at 24 h were <25 μg/ml. MBCs for two of the six isolates (no. 103 and 119) were 25 μg of oxacillin per ml at 48 h and, for two other isolates (no. 10 and 111), the MBCs were 50 μg of cephalothin per ml at 48 h. For the five nontolerant isolates with MBCs determined in duplicate at 24 h, all had MBCs <25 μg/ml, except for two isolates (no. 4 and 7), for which the MBCs were 25 μg/ml for oxacillin (Tables 2 to 5).

Using our methods and inoculum concentrations, we found that survival of a single CFU in subcultures from tubes with no visible growth indicated <99.9% killing. Therefore, the MBC may be determined by the presence of a single colony on subculture of tubes without visible growth. However, as can be seen in Tables 2 and 3, for those isolates for which the MBCs were determined at both 24 and 48 h, MBCs of ≥25 μg/ml were seldom determined by a single col-

TABLE 3. *MBCs and total number of colonies from subcultures of tubes containing ≥12.5 μg of cephalothin per ml at 24 and 48 h for 16 tolerant and 6 nontolerant isolates of S. aureus*

Strain	24 h		48 h	
	MBC (μg/ml)	Total colonies recovered from ≥12.5 μg/ml	MBC (μg/ml)	Total colonies recovered from ≥12.5 μg/ml
Tolerant				
8	100	126	100	164
12	100	44	100	87
14	50	2	50	2
15	50	3	100	3
101	100	223	100	177
102	50	3	50	5
104	50	9	100	15
105	100	89	100	94
106	50	42	100	4
107	100	17	100	25
109	100	11	25	2
110	100	9	25	12
113	50	5	100	2
118	100	51	100	8
123	100	2	100	1
13	50	11	6.25	0
Nontolerant				
10	0.78	0	50	1
11	0.78	0	0.78	0
103	12.5	0	3.125	0
111	6.25	0	50	2
119	3.125	0	1.56	0
121	0.78	0	12.5	0

ony appearing on subculture. The third and fifth columns represent the total colonies achieved by adding together all colonies from subcultures of tubes containing 12.5, 25, 50, and 100 μg of oxacillin or cephalothin per ml for each isolate. Total colonies from tubes containing ≥12.5 μg/ml was usually ≥3 for the tolerant isolates and was always <3 for the nontolerant isolates.

The MBCs and mean total number of colonies subcultured from the four tubes with ≥12.5 μg/ml, for each isolate against which MBCs were determined in duplicate at 24 h, are shown in Tables 4 and 5. The numbers in the third column represent total colonies recovered from duplicate sets of tubes containing ≥12.5 μg of oxacillin or cephalothin per ml (eight tubes), and the numbers in the fourth column represent the mean total colonies for the two sets of tubes containing ≥12.5 μg/ml. Again, it can be seen that, in most cases, the total number (mean of two sets of dilutions) of colonies that appeared on subcultures of the tubes containing ≥12.5 μg/ml was ≥3 for tolerant isolates and <3 for nontolerant isolates. Thus, tolerance to oxacillin and cephalothin correlated well not only with an MBC ≥25 μg/ml but also with a total ≥3 colonies from subcultures of the four tubes containing ≥12.5 μg of oxacillin or cephalothin per ml. MBCs of gentamicin for the 30 isolates were all low; the highest was 3.1 μg/ml.

No relationship was found between phage type or L-form production and tolerance. Twenty-five of 30 isolates produced penicillinase. Seventeen of these isolates were tolerant and eight were nontolerant. All isolates elabo-

TABLE 4. *MBCs, total number of colonies from 24-h subcultures of a duplicate set of tubes containing ≥12.5 μg of oxacillin per ml, and mean total number of colonies from 24-h subcultures of tubes containing ≥12.5 μg/ml*

Strain	MBC (μg/ml)	Total colonies recovered from duplicate set of tubes with ≥12.5 μg/ml	Mean total colonies recovered from tubes with ≥12.5 μg/ml
Tolerant			
2	100	6	3
5	50	23	11.5
6	100	43	21.5
Nontolerant			
1	0.78	2	1
3	0.78	4	2
4	25	4	2
7	12.5	3	1.5
9	25	8	4

TABLE 5. *MBCs, total number of colonies from 24-h subcultures of a duplicate set of tubes containing ≥12.5 μg of cephalothin per ml, and mean total number of colonies from 24-h subcultures of tubes containing ≥12.5 μg/ml*

Strain	MBC (μg/ml)	Total colonies recovered from duplicate sets of tubes with ≥12.5 μg/ml	Mean total colonies recovered from tubes with ≥12.5 μg/ml
Tolerant			
2	100	4	2
5	100	7	3.5
6	100	22	11
Nontolerant			
1	0.39	0	0
3	0.78	1	0.5
4	0.78	0	0
7	0.39	0	0
9	6.25	0	

rated deoxyribonuclease. None of the 30 isolates could acidify any of the carbohydrates or hydrolyze esculin. All grew in 15% NaCl broth and on 40% bile agar. No relationship was observed between either production of pigment or hemolysis and tolerance. There was no relationship between clinical source of the isolates and tolerance.

Size and pigmentation of the colonies from tolerant isolates were observed throughout the study. After 24 h of incubation of subcultures for colony counts and MBC determinations, colonies ranged in size from large to pinpoint. The tiny colonies were usually less pigmented and showed less hemolysis than the larger colonies, regardless of parent strain morphology. After plates were incubated for an additional 24 h, the tiny colonies achieved the same size, pigmentation, and hemolytic characteristics as those of the parent strains, indicating that the undersized colonies were not members of a dwarf strain arising in the presence of antibiotics (5).

Gram stains were made of randomly selected colonies of various size and pigmentation to determine whether gross changes in cellular morphology could be observed. Only the normal variation in staphylococcal cell size and staining characteristics was seen.

In this study, 19 of 30 isolates (63%) were tolerant to the bactericidal effect of oxacillin and cephalothin, a frequency not too different from that reported by Mayhall et al. (12) and by Sabath et al. (14). Mayhall et al. used an MBC of $\geq$25 μg of oxacillin per ml to separate group 1 (tolerant) from group 2 (nontolerant) isolates of *S. aureus* in their study. Using an MBC of $\geq$25 μg of oxacillin or cephalothin per ml to define tolerance, one could predict that tolerant isolates would be separated from nontolerant isolates by killing curves carried out at a concentration of 12.5 μg of oxacillin or cephalothin per ml. Isolates for which the MBC is $\geq$25 μg/ml would be expected to have $\geq$100 CFU/ml remaining after 24 h of incubation (<99.9% killing), whereas those isolates for which the MBC was $\leq$12.5 μg/ml would be expected to have <100 CFU/ml at 24 h (>99.9% killing). This is what we in fact observed. MBCs for isolates identified as tolerant on killing curves were $\geq$25 μg/ml and, for those identified as nontolerant, the MBCs were <25 μg/ml. In addition, there was a statistically significant difference between tolerant and nontolerant isolates in the rate of killing at each point in time (6, 24, and 48 h). When clear tubes were subcultured to determine MBCs, a total of $\geq$3 colonies that were recovered from the four tubes containing 12.5, 25, 50, and 100 μg of oxacillin or cephalothin per ml correlated well with tolerance.

As observed by Mayhall et al. (12), only a minority of CFU in the tolerant isolates were not killed after 24 h of incubation with oxacillin or cephalothin. Although only a minority of the CFU in tolerant isolates were tolerant to the killing effect of oxacillin and cephalothin (Fig. 1 and 2), there was a spectrum of tolerance ranging from 0.2 to 43% survival of the CFU at 24 h. Mayhall et al. (12) and Sabath et al. (14) did not perform killing curves on their isolates from patients with infections that did not respond to therapy and, therefore, the killing kinetics of those strains are unknown. Since the majority of *S. aureus* infections respond to therapy, it is tempting to speculate that tolerant strains which cause failure of therapy would have killing curves similar to those among the uppermost curves in Fig. 1 and 2.

Although it was not the intention of this study to examine the mechanism of tolerance, we conclude from our data that the survival of CFU in tolerant isolates was not due to production of cell wall-deficient forms. As observed by Sabath et al. (14), phage typing of isolates in this study failed to differentiate between tolerant and nontolerant isolates. In a like manner, none of the other parameters we studied could separate tolerant from nontolerant isolates. Broth dilution susceptibility tests and killing curves remain the only modalities for detection of tolerance in *S. aureus*.

Mayhall et al. (12) suggested that the tolerant CFU in their group 1 isolates might be bacterial persisters similar to those described by Bigger (4) and further studied by Gunnison et al. (10). However, bacterial persisters are CFU that survive after at least 99.9% of the CFU in the original inoculum have been killed. It seems probable from our data that, with 0.2 to 43% of the CFU in the starting inoculum remaining viable after 18 to 24 h of incubation, the CFU from our isolates that are tolerant to the action of oxacillin and cephalothin are not the same as the bacterial persisters first described by Bigger (4). Further studies will be required to elucidate the nature and mechanism of antibiotic tolerance in isolates of *S. aureus*.

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