ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1980, p. 699-708
0066-4804/80/11-0699/10$02.00/0

Vol. 18, No. 5

Method for Reliable Determination of Minimal Lethal Antibiotic Concentrations

RICHARD D. PEARSON,[1]† ROY T. STEIGBIGEL,[1]* HENRY T. DAVIS,[2] AND STANLEY W. CHAPMAN[1]‡

Infectious Diseases Unit, Department of Medicine,[1] and Division of Biostatistics,[2] University of Rochester School of Medicine, Rochester, New York 14642

The lack of a standardized, statistically reliable method for in vitro determinations of the minimal lethal or bactericidal concentrations of antibiotics has complicated analyses of isolates of *Staphylococcus aureus* which appear to be inhibited but not killed by the usual concentrations of cell wall-active antibiotics. We describe a method which identifies some of the covariants involved in determinations of minimal lethal concentrations. Lethality was defined as a 99.9% reduction in the initial inoculum of bacteria after 24 h of incubation. We limited the sample volume to 0.01 ml to minimize the inhibitory effect of antibiotic carried over to the subculture plates. We provided a range for the initial inoculum and corresponding rejection values, which detected lethality with a high degree of sensitivity and specificity. When the number of colonies on subculture was equal to or less than the rejection value, the antibiotic was considered lethal for the test organism. Rejection values encompassed initial inocula from 10^5 to 10^7 colony-forming units per ml for single and duplicate samples and allowed for 1 or 5% variability in pipette volumes and errors in initial inoculum determinations. This method was used to determine the minimal lethal concentrations of semisynthetic penicillins for *S. aureus* isolates, one of which was tolerant to the killing action of penicillin.

Despite the widespread clinical application of broth dilution antimicrobial susceptibility testing, there are variations in the performance of tests to determine the minimal lethal (or bactericidal) concentration (MLC) of an antibiotic. Ericsson and Sherris (6) emphasized the importance of standardizing reagents and conditions for determinations of the minimal inhibitory concentration of an antibiotic. Unfortunately, there has been no uniform extension to MLC determinations. The variability in methods used to determine MLCs has become increasingly apparent in recent reports of tolerance of *Staphylococcus aureus* to the killing action of cell wall-active antibiotics (2, 4, 7-10). Although most investigators have defined the MLC as the lowest antibiotic concentration that kills 0.999 or more of the initial inoculum, there have been wide variations in the conditions of culture, the concentration of the initial inoculum, and the timing, number, and volume of samples subcultured to determine the number of surviving organisms. Even after the variability due to these covariant factors has been accounted for, intrinsic sampling variability due to the Poisson distribution of sample responses must be accommodated in any test procedure.

To determine reliably whether an antibiotic has produced a predefined reduction in the initial inoculum, a test procedure must be devised so that the number of colonies in a sample(s) is equal to or less than a specific value (hereafter termed the rejection value). Because of intrinsic sampling variability, it is not appropriate to interpret the rejection value as determining with certainty that a predefined kill has occurred. The sensitivity and specificity of the test procedure are used as indexes of the uncertainty of the procedure. The sensitivity of the test procedure is the true rate of correctly identifying antibiotic lethality, and the specificity is the true rate of correctly identifying nonlethality. A perfectly sensitive and specific procedure is clearly not possible. Furthermore, the sensitivity and specificity of a test procedure depends upon the choice (often not clearly stated) of experimental conditions. In this paper we (i) briefly outline the probability concepts that underlie every test for lethality, (ii) describe a model applicable to the determination of MLCs, which provides rejection values with a high degree of sensitivity and specificity, (iii) investigate the possibility of bias introduced by antibiotic transferred in sam-

† Present address: Department of Medicine, University of Virginia School of Medicine, Charlottesville, VA 22908.

‡ Present address: Department of Medicine, Veterans Administration Center, University of Mississippi Medical Center, Jackson, MS 39216.

ples to subculture plates, and (iv) give examples of the test procedure applied to MLC determinations for *S. aureus* isolates.

MATERIALS AND METHODS

Probability concepts. We use a standard definition of lethality. This definition states that if a proportion of 0.999 or greater of the initial inoculum has been killed by 24 h, the antibiotic is lethal; otherwise it is nonlethal. Hence, a test for antibiotic lethality is positive if it indicates that the antibiotic is lethal and negative if it indicates that the antibiotic is nonlethal. The sensitivity of the test is the true positive rate (that is, the percentage of positive samples from a population where lethality has occurred), and the specificity of the test is the true negative rate.

The test procedure which we used follows. We define j as the number of samples, each of volume v, 24 h after an antibiotic has been added to an initial inoculum. The total number (N) of viable colony-forming units (CFUs) in these j samples is then counted. We then compare N with the rejection value of the test (R). If $N \leq R$, the test is positive, and if $N \geq R$, the test is negative. If we let I denote the initial concentration of bacteria in CFU per milliliter, then the rejection point of the test (R) is a function of I, j, v, the definition of lethality, and the desired sensitivity of the test (in percent). The rejection value determines the specificity of the test. The calculation of the rejection value is based upon the probability distribution of N.

We let p denote the proportion of the initial inoculum remaining after 24 h. (If the antibiotic is lethal, we should have $p \leq 10^{-3}$.) It can be theoretically proven that if (i) each of the j samples is a random sample from the population and (ii) the samples are independent, then N follows a Poisson distribution with the mean $\theta = (j)(I)(v)(p)$.

Conversely, if N does not empirically follow a Poisson distribution, either the samples are not independent or, more likely, the sampling method does not yield random samples from the population. For samples to be representative, the population must be adequately mixed before sampling and each sample must be of the same volume.

Under the above-described Poisson model, the rejection value is defined to be a point such that a Poisson random variable with mean $\theta_L = (j)(I)(v)(10^{-3})$ is less than the rejection value in exactly β percent of the samples from that random variable. In probability notation, this is expressed, letting Y denote the Poisson random variable with mean θ_L, as: $\Pr\{Y \leq R\} = \beta/100$.

The exact value of the specificity of the test constructed above depends upon the alternative kill to which it is compared. For example, the test constructed above is more specific for an alternative of 0.995 kill than it is for an alternative of 0.998 kill. That is, the higher the proportion of initial inoculum remaining, the more specific the test is. We have used a 0.995 or less kill of the initial inoculum as a reasonable alternative kill to calculate specificity. The lowest specificity value would then occur when a proportion of exactly 0.995 of the initial inoculum has been killed. In that case, $P = 0.005$, and the total number of colonies in all samples follows a Poisson distribution with mean 5θ, where θ is the mean for a 0.999 kill. If γ equals the probability that $Z > R$, where Z is a Poisson random variable with mean 5θ, then the specificity of the test is $\gamma \times 100\%$.

Correction for error in sample volume. In making the calculations described above, we have assumed that the sample volume is exactly 0.01 ml. In practice the sample volume may vary due to variability in pipetting. The calculations described above are easily modified to incorporate such sampling error in determining the rejection point.

Suppose that the pipette sampling error is ±5%; that is, a 0.01-ml sample could be as small as 0.0095 ml or as large as 0.0105 ml. For sensitivity calculations, the worst case would be when all samples were 0.0105 ml (since this would lead to a value of N which is too large). To account for this in sensitivity, we used $v = 0.0105$ ml rather than $v = 0.01$ ml in the calculations. That is, if θ is the mean for a 0.999 kill, we used 1.05θ rather than θ in sensitivity calculations.

For specificity calculations, the worst case is when all samples are 0.0095 ml. Therefore, in these calculations $v = 0.0095$ ml should be used rather than $v = 0.01$ ml. Hence, for an alternative of 0.995 or less kill, we used $5 \times 0.95 \times \theta = 4.75\theta$ rather than 5θ in specificity calculations.

Abbreviated method for calculation of Poisson probabilities. For any probability β and any mean θ, we let $P_\beta(\theta)$ be defined by $\Pr\{Y \leq P_\beta(\theta)\} = \beta$, where Y is a Poisson random variable with mean θ. For the above-described sensitivity and specificity calculations, it was necessary to calculate $P_\beta(\theta)$ for varying values of β and θ. To find $P_\beta(\theta)$, the method described by Brownlee (5) was used. Instead of starting with the probability β, this method starts by choosing a value for $P_\beta(\theta)$, say the rejection value. If $\chi^2[2(R + 1)]$ is a chi-square random variable with $2(R + 1)$ degrees of freedom, then

$$\Pr\{Y \leq R\} = \beta = \Pr\{\chi^2[2(R + 1)] > 2\theta\} \quad (1)$$

(See below for justification.)

If the value of β calculated in formula 1 is too small, the rejection value is increased; if it is too large, the rejection value is decreased. This procedure is repeated until the desired value of β is obtained.

To illustrate, suppose that the initial concentration of bacteria (I) is 5×10^5 CFU/ml, double sampling is used ($j = 2$), and sample volumes are 0.01 ml ($v = 0.01$ ml). To construct a test of 99.95% sensitivity ($\beta = 0.9995$) for 0.999 kill ($p = 0.001$), we first note that $\theta = (j)(I)(v)(p)$ and thus that $\theta = (2)(5 \times 10^5)(0.01)(0.001) = 10$. Hence, $2\theta = 20$. Looking at table of chi-square percentage points, we see the following: $\Pr\{\chi^2_f > 20\} > 0.995$ if $f > 44$; $\Pr\{\chi^2_f > 20\} < 0.995$ if $f < 44$; and $\Pr\{\chi^2_{44} > 20\}$ is almost exactly 0.995. Therefore, $2(R + 1) = 44$, or $R = 21$.

That is, if we accept a 0.999 kill when $N \leq 21$, $R = 21$ is the most specific rejection point with a sensitivity of 99.95%.

Bacteria and antibiotics. *S. aureus* ATCC 25923, clinical isolates, and a tolerant *S. aureus* strain (Erickson strain) obtained from L. Sabath (University of Minnesota Medical School, Minneapolis) were studied. Nafcillin (sodium salt; Wyeth Laboratories, Philadelphia, Pa.), methicillin (sodium salt; Bristol Labo-

ratories, Syracuse, N.Y.), and cephalothin (sodium salt; Eli Lilly & Co., Indianapolis, Ind.) were prepared in distilled water at final concentrations of 100 μg/ml and stored at −70°C. Dilutions of these antibiotics in Mueller-Hinton broth (MHB) (lot 648383; Difco Laboratories, Detroit, Mich.) were prepared on the day of study.

S. aureus was grown overnight at 37°C in Trypticase soy broth (Difco Laboratories) and diluted to approximately 2×10^6 CFU/ml in MHB on the basis of optical density with a spectrophotometer (Spectronic 20; Bausch & Lomb, Inc., Rochester, N.Y.). Actual concentrations were determined by culturing 0.01-ml samples from serial 10-fold dilutions of the initial inocula on Trypticase soy agar (Difco Laboratories). Samples were spread with a glass rod on a rotating platform.

Goodness of fit to the Poisson distribution. Since the above-described calculations were based upon the assumption that N was Poisson distributed, several experiments were performed to determine the validity of the assumption. *S. aureus* ATCC 25923 was grown overnight in Trypticase soy broth and diluted to approximately 1×10^2, 5×10^3, or 1×10^4 CFU/ml in MHB on the basis of optical density; 25 consecutive 0.01-ml samples were taken from each of these bacterial concentrations with an automatic pipette (Oxford Laboratories, Foster City, Calif.) and spread onto Trypticase soy agar plates. The number of colonies on each plate was counted after 24 h of incubation in 5% CO_2–room air at 37°C. The data from these experiments (Table 1) were then analyzed in three different ways.

First, for each experiment an overall chi-square goodness of fit to the Poisson distribution was done. Second, estimating the concentration of inoculum from the mean number of colonies in the samples and assuming that this was the concentration for a just lethal kill (i.e., that the initial concentration was exactly 10^3 greater), the relevant rejection value was calculated by the procedure given above. The number of samples which was less than or equal to the rejection value was then compared with the sensitivity predicted by the Poisson model. Third, for the highest two concentrations the preceding procedure was repeated, assuming that we were observing the result of a 0.995 proportion nonlethal kill. In this case the number of samples less than or equal to the rejection value was compared with the specificity predicted by the Poisson model.

The last two analyses were done since important deviations from the Poisson model might affect sensitivity and specificity calculations.

Effect of antibiotic transferred in samples. To assess the effect of antibiotic transferred from the tubes to the subculture plates and to determine the optimum sample volume for MLC determinations, serial two-fold dilutions of nafcillin, methicillin, and cephalothin were prepared in MHB at concentrations ranging from 50 to 0.1 μg/ml; 2-ml volumes of each concentration were incubated in glass tubes (13 by 100 mm; Scientific Products, McGraw Park, Ill.) for 24 h at 37°C in room air. Automatic pipettes (Oxford Laboratories) were used to transfer 0.01 ml ± 1.5% (standard deviation; specifications of manufacturer), 0.05 ml ± 1.0%, and 0.1 ml ± 1.0% to the centers of Trypticase soy agar plates. The Erickson strain of *S. aureus* at a concentration of 2×10^2 bacteria per 0.01 ml of MHB was immediately added, and the sample was spread with a glass rod on a rotating platform. All samples were done in duplicate. Control plates to which no antibiotic was transferred were also prepared. All plates were incubated for 24 h at 37°C in 5% CO_2–room air before counting.

Broth dilution tests. Standard broth dilution susceptibility tests were performed by following the guidelines of Ericsson and Sherris (6). Serial twofold dilutions of the antibiotics were prepared in MHB; the highest concentration used was 100 μg/ml, and the lowest concentration used was 0.2 μg/ml. A 1-ml volume of each was placed in a glass tube (13 by 100 mm) to which 1 ml of MHB containing bacteria was added. The final concentrations of antibiotics ranged from 50 to 0.1 μg/ml, and the bacterial concentrations ranged from 3×10^5 to 1×10^6 CFU/ml. Duplicate tubes were incubated at 37°C in room air for 24 h. The minimal inhibitory concentration was the lowest antibiotic concentration which prevented visible growth.

The number of organisms surviving at 24 h was determined by agitating and sampling all tubes without visible growth and the first tube with growth. A 0.01-ml sample was plated as described above, incubated at 37°C in 5% CO_2–room air for 24 h, and counted.

RESULTS

Determination of rejection values. By employing the assumption that the number of colonies in a sample was Poisson distributed, the

TABLE 1. *Effect of sampling variability on the number of S. aureus colonies in 0.01-ml samples*[a]

Approximate concn (CFU/ml)	No. of colonies per plate[b]	No. of samples	Expected no. of samples if Poisson distributed
1×10^2	0 or 1	16	18.4
	2 or more	9	6.6
3×10^3	22 or fewer	1	0.44
	23–27	4	2.62
	28–32	5	6.77
	33–37	11	8.12
	38 or more	4	7.06
1×10^4	Less than 60	0	0
	60–79	6	0.02
	80–139	15	24.8
	140–159	3	0
	160 or more	1	0

[a] $n = 25$.

[b] With a concentration of 1×10^2 CFU/ml, the range was 0 to 6 colonies per plate, and the mean ± standard deviation was 1.28 ± 1.81 colonies per plate. With a concentration of 3×10^3 CFU/ml, the range was 21 to 56 colonies per plate, and the mean ± standard deviation was 34.24 ± 8.47 colonies per plate. And with a concentration of 1×10^4 CFU/ml, the range was 64 to 173 colonies per plate, and the mean ± standard deviation was 110.12 ± 30.97 colonies per plate.

rejection values were calculated by the method outlined above for initial inoculum values ranging from 10^5 to 10^7 CFU/ml. Using a sample volume (v) of 0.01 ml, we performed the calculations for both single samples ($j = 1$; Table 2) and double samples ($j = 2$; Table 3). Tables 2 and 3 provide rejection values which allow for 1% error in pipette volume, which is the level of error given by the manufacturer of the semiautomatic pipettes used in this study. In addition, a second set of rejection values is provided, which incorporate a 5% error in pipetting and the full sampling error inherent in the determination of the initial inoculum. The initial inoculum determination was subject to the same type of error as occurred in determinations of the number of CFU surviving after 24 h of incubation of the antibiotics with the bacteria. This error is incorporated in the second set of rejection values in Tables 2 and 3, along with their sensitivities and specificities for detection of lethality. The formulas used to calculate these rejection values are more complex than those given earlier and are shown below. Obviously, the choice of desired sensitivity affects the calculations for the tables. The rules which we employed in generating Tables 2 and 3 were as follows. (i) The sensitivity should be at least 70%. (ii) The specificity should be at least 99% without violating (i); otherwise it should be the specificity at the smallest attainable sensitivity above 70%. (iii) Subject to (ii), the sensitivity should be as large as possible unless both a sensitivity and specificity of 99% are simultaneously attainable. (iv) When a sensitivity and specificity of 99% are simultaneously attainable, the rejection value is chosen so that the sensitivity and specificity are roughly equal.

Applicability of the Poisson model. A chi-square goodness of fit test was applied to the sampling data in Table 1 to assess the applicability of the Poisson model as a probability model. The descriptive levels of significance for rejecting the Poisson model were $P > 0.01$ ($\chi^2 = 1.18$; df = 1) at 1×10^2 CFU/ml and $P > 0.10$ ($\chi^2 = 4.25$; df = 3) at 3×10^3 CFU/ml, but $P < 0.005$ ($\chi^2 > 20$; df = 3) at 1×10^4 CFU/ml. Thus, the Poisson model was a reasonable model for the lower two concentrations. The sensitivities of the rejection values calculated from the Poisson model appeared to be reasonably accurate for all three concentrations. For example, if the 1×10^2-CFU/ml concentration represented an exact 0.999 kill of a 1×10^5-CFU/ml initial concentration and a rejection point based on the Poisson model of one colony was used (Table 2), the predicated sensitivity was 75%. Empirically, 0.64 of the 25 samples gave a true positive result.

TABLE 2. *Rejection value and calculated sensitivity and specificity for each initial inoculum concentration on the basis of a single 0.01-ml sample*[a]

Initial inoculum (CFU/ml)	±1% Pipette error			±5% Pipette error with full sampling error for initial inoculum determination[b]		
	Rejection value[c]	Sensitivity (%)	Specificity (%)[d]	Rejection value	Sensitivity (%)	Specificity (%)
1×10^5	1	75	95	3	84	83
2×10^5	3	85	99	4	77	97
3×10^5	6	96	99	6	84	98
4×10^5	9	99	99	8	89	99
5×10^5	12	>99	>99	11	96	99
6×10^5	15	>99	>99	15	99	99
7×10^5	17	>99	>99	17	>99	>99
8×10^5	18	>99	>99	20	>99	>99
9×10^5	20	>99	>99	23	>99	>99
1×10^6	22	>99	>99	25	>99	>99
2×10^6	36	>99	>99	47	>99	>99
3×10^6	50	>99	>99	68	>99	>99
4×10^6	65	>99	>99	91	>99	>99
5×10^6	80	>99	>99	113	>99	>99
6×10^6	95	>99	>99	136	>99	>99
7×10^6	110	>99	>99	159	>99	>99
8×10^6	125	>99	>99	182	>99	>99
9×10^6	140	>99	>99	204	>99	>99
1×10^7	155	>99	>99	227	>99	>99

[a] When the number of colonies was equal to or less than the rejection value, the antibiotic was declared lethal (a 0.999 or greater reduction in the initial inoculum).

[b] Based on a single sample for the determination of the initial inoculum size.

[c] Number of colonies.

[d] Based on an alternative of 0.995 reduction in initial inoculum.

TABLE 3. *Rejection value and calculated sensitivity and specificity for each initial inoculum concentration on the basis of duplicate 0.01-ml samples*[a]

Initial inoculum (CFU/ml)	±1% Pipette error			±5% Pipette error with full sampling error for determination of initial inoculum[b]		
	Rejection value[c]	Sensitivity (%)	Specificity (%)[d]	Rejection value	Sensitivity (%)	Specificity (%)
1×10^5	3	85	99	4	77	97
2×10^5	9	99	>99	8	89	99
3×10^5	15	>99	>99	15	99	99
4×10^5	18	>99	>99	20	>99	>99
5×10^5	22	>99	>99	25	>99	>99
6×10^5	25	>99	>99	29	>99	>99
7×10^5	28	>99	>99	33	>99	>99
8×10^5	31	>99	>99	38	>99	>99
9×10^5	33	>99	>99	42	>99	>99
1×10^6	36	>99	>99	47	>99	>99
2×10^6	65	>99	>99	91	>99	>99
3×10^6	95	>99	>99	136	>99	>99
4×10^6	125	>99	>99	182	>99	>99
5×10^6	155	>99	>99	227	>99	>99
6×10^6	185	>99	>99	273	>99	>99
7×10^6	215	>99	>99	318	>99	>99
8×10^6	245	>99	>99	364	>99	>99
9×10^6	275	>99	>99	409	>99	>99
1×10^7	305	>99	>99	455	>99	>99

[a] When the sum of colonies from duplicate samples was equal to or less than the rejection value, the antibiotic was declared lethal.

[b] Based upon duplicate samples for determinations of the initial inoculum size.

[c] Number of colonies.

[d] Based on an alternative of 0.995 reduction in initial inoculum.

At 3×10^3 CFU/ml, which represented an exact 0.999 kill of 3×10^6 CFU/ml, with a rejection point of 50 colonies (Table 2), all of the samples (1.00) correctly indicated a lethal effect where the predicted sensitivity was 99%; and at 1×10^4 CFU/ml, which represented a 0.999 kill of 1×10^7 CFU/ml, with a rejection point of 155 (Table 2), 0.96 of the samples indicated lethality where the predicted sensitivity was 99%. There was also close agreement between the sampling data and specificities based on the Poisson model with an alternative 0.995 lethal effect. With the alternative of 0.995 lethal effect, the specificity is the proportion of samples greater than the rejection value for an initial inoculum 2×10^2 CFU/ml larger. Thus, for the 3×10^3-CFU/ml data, the rejection value based on 6×10^5 CFU/ml was 14. All 25 samples were greater, giving an empiric specificity of 1.00 with predicted >99%, and for the 1×10^4-CFU/ml data the empiric specificity was 1.00 with predicted >99%.

Antibiotic carry-over. As Fig. 1 shows, antibiotic was transferred in samples to subculture plates. The amount of antibiotic carried over to the plate was related to the volume of the sample and the antibiotic concentration in the tube. There was a more than 25% reduction in colonies on plates with 0.1-ml samples of nafcillin at concentrations of 3.1 μg/ml and above and with 0.05-ml samples at concentrations of 6.2 μg/ml and above. The effect was less with 0.01-ml samples, but there was still a reduction in colonies of more than 25% at a concentration of 50 μg/ml. Cephalothin produced reductions similar to those of nafcillin. The carry-over effect was less pronounced with methicillin, where 25% or greater reduction in colonies occurred only with 0.1-ml samples at antibiotic concentrations of 12.5 μg/ml and above. No reduction was observed with 0.01-ml samples. To minimize the effect of the antibiotic transferred to the subculture plates, only 0.01-ml samples were used for the MLC determinations.

Application of rejection values to MLC determination. The procedure described in this paper was used to determine the MLCs for three isolates of *S. aureus*, one of which was tolerant (Table 4). In each case, an overnight growth of bacteria was diluted on the basis of optical density to yield a concentration of approximately 5×10^5 CFU/ml. The actual concentration was determined by culturing duplicate samples from serial 10-fold dilutions of the initial inoculum. Tube dilution broth susceptibility testing was done as described above. The minimal inhibitory concentration was deter-

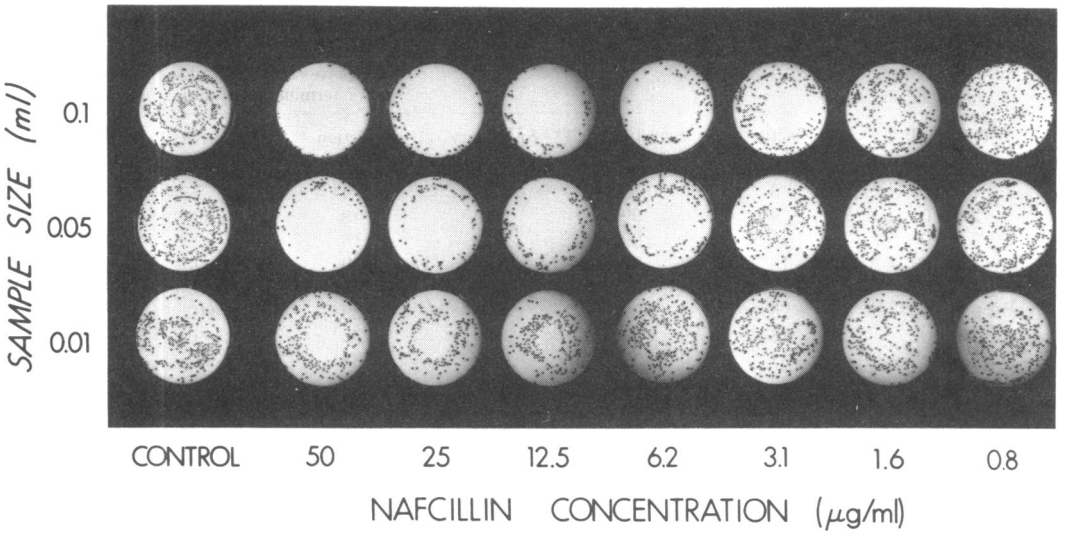

FIG. 1. *Effect of antibiotic transferred from tubes to subculture plates on the number of S. aureus colonies. Sample volumes of 0.1, 0.05, and 0.01 ml were obtained from tubes containing nafcillin serially diluted in MHB and incubated for 24 h at 37°C. The samples were placed onto the centers of tryptic soy agar plates along with 2×10^2 colonies of S. aureus and spread. Plates are arranged according to sample volume and nafcillin concentration. Colonies appear as black dots on the clear agar. The reduction in the numbers of colonies in the central portions of the plates is evident; the greatest effects were observed with the highest antibiotic concentrations and largest samples.*

mined at 24 h. The number of surviving CFU was then determined by taking 0.01-ml samples from tubes in which there was no visible growth and the tube with the next highest antibiotic concentration in which there was visible growth. Rejection values were obtained from Table 2 if a single sample was used or from Table 3 if duplicate samples were used. Rejection values for ±5% pipette error with full sampling error for initial inoculum determination were used. When the total number of surviving colonies in the sample(s) exceeded the rejection value, there was less than a 0.999 reduction in the initial inoculum, and the antibiotic was considered nonlethal. This was the case in examples 1 and 2 (Table 4). In examples 3 and 4, the number of colonies was less than the rejection value. The antibiotic had a lethal effect.

Although lethality can be assessed with single samples, as is illustrated in Table 4, example 4, we recommend that studies be done in duplicate because rejection values for duplicate samples are more sensitive and specific at lower initial inoculum concentrations. In addition, the use of duplicate tubes permits evaluation of a tube in one set which contains a number of CFU that is inconsistent with the numbers in adjacent tubes. In most instances there is a progressive decrease in the number of CFU with increasing antibiotic concentration. If the number of CFU from a single tube or two tubes ("skip tubes") in one set of duplicate tubes is inconsistent with this progression, the results of the other set can be used with rejection values for a single sample. We very infrequently encountered skip tubes with the same antibiotic concentration in both sets of duplicate tubes.

DISCUSSION

The variability in the methods used to determine MLCs has complicated the analyses of recent studies of tolerant *S. aureus* strains and confused attempts to characterize these strains and to determine their prevalence among clinical isolates. We have developed a standardized test procedure for MLC determinations which could provide the basis for comparison of data obtained in different laboratories and lead to a better understanding of discrepancies between the concentration of an antibiotic needed to inhibit growth and that needed to kill bacteria.

The concentration of the initial inoculum and the number and volume of samples subcultured are covariant parameters that affect the number of colonies in the sample(s) and, along with the definition of lethality, timing of subculture, and conditions of culture (i.e., temperature, atmosphere, growth phase, etc.), must be accounted for in a test procedure for MLCs. We have defined the MLC as the minimal antibiotic con-

TABLE 4. *Use of rejection values for MLC determinations*

Example no.	*S. aureus* isolate	Antibiotic	Initial inoculum (CFU/ml)	No. of colonies with an antibiotic concn (μg/ml) of:								No. of samples	Rejection value (no. of colonies)	MLC (μg/ml)	MIC (μg/ml)[a]
				50	25	12.5	6.2	3.1	1.6	0.8	0.4	0.2				
1	Erickson strain	Nafcillin	3.6×10^5	115[b]	165	84	74	416	TN[c]	TN	TN	TN	2	18[d]	>50	1.6
2	Erickson strain	Cephalothin	4.5×10^5	105[b]	197	160	173	500	TN	TN	TN	TN	2	23[d]	>50	0.8
3	Clinical isolate I	Nafcillin	7.5×10^5	6[b]	11	11	1	17	10	TN	TN	TN	2	36[d]	1.6	0.8
4	Clinical isolate II	Cephalothin	4.8×10^5	0[e]	0	2	3	6	0	0	TN	TN	1	10[f]	0.8	0.2

[a] MIC, Minimal inhibitory concentration.
[b] Total number of colonies in duplicate samples.
[c] TN, Colonies were too numerous to count or there were more than 500 colonies per plate.
[d] Rejection value extrapolated from Table 3.
[e] Number of colonies in a single sample.
[f] Rejection value from Table 2.

centration that produces a 0.999 reduction in the initial inoculum after 24 h of incubation at 37°C. The initial inoculum must be limited to a size that minimizes the effects of β-lactamase production and evolution of bacterial mutants on the minimal inhibitory concentration and MLC, but it must be large enough to allow a reliable determination of a 0.999 lethal effect. The range of the initial inoculum size is in part limited by the volume of the samples taken for subculture. In our experience and that of others (1), the volume of the samples must be 0.01 ml or less because of the antibiotic carry-over effect. Even then, the number of colonies at high nafcillin and cephalothin concentrations was reduced so that an antibiotic might appear lethal when it is not. The carry-over effect is dependent on the susceptibility of the bacteria to inhibition by the antibiotic and the degree of antibiotic inactivation that occurs during the initial 24-h incubation. β-Lactamase could be added to tubes before subculture to attempt inactivation of the antibiotic. This was not done because complete inactivation of the antibiotic could not be ensured, it would have increased the cost and time needed to perform the test, and it would have added the risk of contamination. Consequently, β-lactamase was not used in our study.

Intrinsic sampling variability makes it impossible to use as a rejection value the number of colonies that represents precisely a 0.999 lethal effect. It was necessary to develop a test procedure that took this into account and also allowed for variability in pipetting. The Poisson distribution, as assessed by a chi-square goodness of fit test, is an acceptable probability model for 0.01-ml samples taken from bacterial concentrations of 1×10^2 and 3×10^3 CFU/ml. The sensitivities and specificities based on the Poisson model proved to be reasonably accurate. Thus, the test procedure can be applied to initial inoculum concentrations of 1×10^5 to 3×10^6 CFU/ml when lethality is defined as a 0.999 reduction in initial inoculum. In practice, the initial inoculum is usually limited to 1×10^6 CFU/ml or less. Hence, the lack of fit to the Poisson distribution of samples from the 1×10^4-CFU/ml concentration would not be important in diagnostic laboratories, but even at that concentration sensitivities and specificities based on the Poisson model appeared to be reasonably accurate. As Tables 2 and 3 show, initial concentrations between 1×10^5 and 3×10^5 CFU/ml are associated with rejection values of reduced sensitivity and specificity. At concentrations above 3×10^5 CFU/ml, the rejection values are highly specific and sensitive. For these reasons we used initial inocula ranging from 3×10^5 to 1

$\times 10^6$ CFU/ml for MLC determinations and assumed ±5% variations in pipette volume and error in determining initial inoculum.

To obtain a very high degree of sensitivity and specificity in determining the MLCs of an antibiotic, the rejection values provided in Tables 2 and 3 should be adhered to closely, particularly when initial inocula in the preferred range of 5×10^5 to 1×10^6 cells per ml are used. The probability model used indicates that in most instances when lethal kills have occurred the results are much below the rejection value. At higher initial inocula (more than 10^6 bacteria per ml) there is a range close to the rejection value which could yield a relatively high sensitivity and specificity, but unless the sensitivity and specificity are recalculated, it is advisable to use the rejection values exactly as given; this will ensure a known high level of reliability.

Our studies were carried out with *S. aureus* in a macrotiter system. The method can also be applied to serum dilution MLCs, to other bacteria, and to broth dilution systems of different volumes as long as samples approximate a Poisson distribution. Preliminary studies in our laboratory indicate that this method is applicable to a microtiter system. It can also be used with kill curves to determine whether a 0.999 reduction in the initial inoculum has occurred at a specific time. Finally, it is possible to change the definition of lethality, the timing of subculture, and the covariant parameters of initial inoculum size, sample volume, and number of samples and generate a new set of rejection values with calculated specificity and sensitivity.

It is anticipated that the MLC varies as a function of multiple factors, including duration of incubation, growth phase of the organism, temperature, atmospheric gases, agitation, pH, and media. For example, it has been reported that the expression of tolerance varies as a function of the duration of incubation (7) and of the media used (8). Further study of these variables at multiple antibiotic concentrations with the application of covariant analysis could greatly enhance our understanding of in vitro drug-bacterium interactions and lead to identification of factors that affect the expression of tolerance in *S. aureus*.

Although the MLC may vary as a function of the factors discussed above, the procedure for MLC determinations can be standardized as follows. (i) Serial dilution broth susceptibility tests should be performed in duplicate, following the guidelines of Ericsson and Sherris (6). (ii) It is advisable to keep the initial inoculum in the range of 3×10^5 to 1×10^6 CFU/ml. The actual concentration of the initial inoculum is determined by duplicate sampling and serial 10-fold dilutions of the initial inoculum. Since the proportion of tolerant organisms within a resistant strain can be 7% or less, more than a few colonies should be selected for antibiotic susceptibility testing. Bradley et al. suggest removing all colonies from a subculture plate for preparation of the initial inoculum (3). Since initial inoculum determinations are also subject to error, the rejection values which allow for this error should be used. If multiple (10 or more) samples are taken to determine the initial inoculum concentration, the error is minimal, and the alternative set of rejection values given in Tables 2 and 3 could be used. (iii) The minimal inhibitory concentration should be determined at 24 h. (iv) The number of surviving organisms should then be determined by taking 0.01-ml samples from the tubes in which there is no visible growth and the tube with the next highest antibiotic concentration in which there is visible growth. (v) We have defined lethality as a 0.999 reduction in the initial inoculum. Rejection values which allow for 5% variability in pipette volume should be obtained from Table 3. When the total number of colonies on subculture is equal to or less than the rejection value, the antibiotic has had a lethal effect. The MLC is defined as the lowest antibiotic concentration that produces a lethal effect.

APPENDIX

Justification of formula 1. We want to show that if X is a Poisson random variable with parameter λ and Y is a chi-square random variable having $2(R + 1)$ degrees of freedom, then

$$\Pr\{X \leq R\} = 1 - \Pr\{Y < 2\lambda\} \quad (1)$$

We begin by noting that the density of a chi-square random variable with $2(R + 1)$ degrees of freedom is as follows:

$$f(x) = \{1/[2^{R+1}\Gamma(R + 1)]\}e^{-x/2}x^R$$

Therefore,

$$1 - \Pr\{Y < 2\lambda\} = \int_{2\lambda}^{\infty}\left[\frac{1}{2^{R+1}\Gamma(R + 1)}\right](e^{-x/2})(x^R)dx \quad (2)$$

Integrating by parts, it follows that if m is an integer, then

$$\int_{2\lambda}^{\infty} x^m e^{-x/2}dx = 2^{m+1}e^{-\lambda} + 2m\int_{2\lambda}^{\infty} x^{m-1}e^{-x/2}dx \quad (3)$$

Applying the equality of equation 3 repeatedly

to the right side of equation 2, we obtain

$$1 - \Pr\{Y < 2\lambda\} = \sum_{k=0}^{R} \frac{\lambda^k}{k!} e^{-\lambda}$$
$$= \sum_{k=0}^{R} \Pr\{X = k\}$$
$$= \Pr\{X \leq R\}$$

Incorporation of sampling error in initial inoculum determinations into rejection value calculations. If we let *IIC* denote the initial inoculum concentration and *FC* denote the final concentration, then $\rho = FC/IIC$. An antibiotic is defined to be lethal if $\rho \leq 1 \times 10^{-3}$.

Suppose *IIC* is determined as follows. First, a sample is taken from the initial inoculum, and this sample is diluted by a series of three 1:10 dilutions to create a solution whose concentration is $IIC \times 10^{-3}$. A 0.1-ml sample is taken from this latter solution and plated out, and the number of colonies in this 0.1-ml sample, which we define to be Y, is counted. It follows that Y should be a Poisson random variable with mean $\lambda_1 = IIC \times 10^{-4}$.

We assume that *FC* is determined as described above. That is, a 0.01-ml sample is taken from the final inoculum and plated out, and the number of colonies in this 0.01-ml sample, which we define to be X, is counted. As described above, it follows that X should be a Poisson random variable with mean $\lambda_2 = FC \times 10^{-2}$.

Clearly from the above, $\rho \leq 10^{-3}$ implies that $(\lambda_1/\lambda_2) \geq 10$.

As pointed out by Brownlee (5), using an argument similar to that described above, it can be shown that if Y and X are Poisson random variables with means λ_1 and λ_2, respectively, then

$$\Pr\{X \geq x \mid X + Y = x + y\} = \Pr\left\{F > \frac{x}{y+1} \cdot \frac{\lambda_1}{\lambda_2}\right\} \quad (4)$$

where F is an F random variable with degrees of freedom $2(y + 1)$ and $2x$; i.e., $F \sim F[2(y + 1), 2x]$.

Now suppose that we observe that $Y = y$ and therefore estimate that the *IIC* is $y \times 10^4$. We want to find a rejection point such that if $X > R$, we declare the antibiotic to be nonlethal, and if $X \leq R$ we declare the antibiotic to be lethal. Furthermore, we want the sensitivity of this procedure to be β percent. From equation 4 it follows that, by trial and error, we would choose R such that

$$\Pr\left\{F \leq \frac{R}{y+1} \times 10\right\} = \frac{\beta}{100} \quad (5)$$

where $F \sim F[2(y + 1), 2R]$.

When R is determined by equation 5, it follows that the specificity of the procedure against a kill which is at best 99.5% ($\rho \geq 5 \times 10^{-3}$) is γ percent, where

$$\frac{\gamma}{100} = \Pr\left\{F > \frac{R}{y+1} \times 2\right\} \quad (6)$$

with $F \sim F[2(y + 1), 2R]$.

However, formulas 5 and 6 do not allow for any error in pipetting. To be conservative, we chose to allow for a possible 5% error in pipetting (or possible slight departures from the Poisson distribution of either x or y). When this 5% error allowance is incorporated, formulas 5 and 6 become, respectively,

$$\Pr\left\{F \leq \frac{R}{y+1} \times 9.0\right\} = \frac{\beta}{100} \quad (7)$$

and

$$\frac{\gamma}{100} = \Pr\left\{F > \frac{R}{y+1} \times 2.2\right\} \quad (8)$$

where in both cases $F \sim F[2(y + 1), 2R]$.

This covers the case of single samples for both *IIC* and *FC* determinations. For double sampling, the formula may be used without modification if Y is taken to be the total number of colonies in both samples for *IIC* determinations and R is the rejection point for the total number of colonies in both samples for *FC* determinations.

ACKNOWLEDGMENTS

We are especially grateful to Charles L. Odoroff, Division of Biostatistics, University of Rochester School of Medicine and Dentistry, for his suggestions during the preparation of this manuscript, to Mary Karen Waenko for technical assistance, and to Margaret A. Christy, Margaret E. Aldrich, and Wendy H. Kurnath for secretarial assistance.

This work was supported by Public Health Service National Research Service Award 1F32 AI-06002-01 from the National Institutes of Health, by Public Health Service Training Grant 5T01 AI00028 from the National Institutes of Health, and by Public Health Service grant 2R01HL15790-06 from National Heart, Lung and Blood Institute.

LITERATURE CITED

1. **Barry, A. L., and R. A. Lasner.** 1979. *In vitro* methods for determining minimal lethal concentrations of antimicrobial agents. Am. J. Clin. Pathol. **71**:88-92.
2. **Best, G. K., A. V. Koval, and N. H. Best.** 1975. Susceptibility of clinical isolates of *Staphylococcus aureus* to killing by oxacillin. Can. J. Microbiol. **21**:1692-1697.
3. **Bradley, H. E., P. L. Weldy, and D. S. Hodes.** 1979. Tolerance in *Staphylococcus aureus*. Lancet **i**:150.
4. **Bradley, J. J., C. G. Mayhall, and H. P. Dalton.** 1978. Incidence and characteristics of antibiotic-tolerant strains of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **13**:1052-1057.
5. **Brownlee, K. A.** 1965. Statistical theory and methodology in science and engineering, 2nd ed. John Wiley & Sons, Inc., New York.
6. **Ericsson, H. M., and J. C. Sherris.** 1971. Antibiotic

sensitivity testing. Report of an international collaborative study. Acta Pathol. Microbiol. Scand. Sect. B Suppl. 217.

7. **Mayhall, C. G., G. Medoff, and J. J. Marr.** 1976. Variation in the susceptibility of strains of *Staphylococcus aureus* to oxacillin, cephalothin, and gentamicin. Antimicrob. Agents Chemother. **10**:707–712.
8. **Peterson, L. R., D. N. Gerding, W. H. Hall, and E. A. Schierl.** 1978. Medium-dependent variation in bactericidal activity of antibiotics against susceptible strains of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **13**:665–668.
9. **Sabath, L. D., N. Wheeler, M. Laverdiere, D. Blazevic, and B. J. Wilkinson.** 1977. A new type of penicillin resistance of *Staphylococcus aureus*. Lancet **i**:443–447.
10. **Watanakunakorn, C.** 1978. Antibiotic-tolerant *Staphylococcus aureus*. J. Antimicrob. Chemother. **4**:561–568.