JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1982, p. 784–793
0095-1137/82/110784-10$02.00/0

Vol. 16, No. 5

Analysis of a Single Reference Strain for Determination of Gentamicin Regression Line Constants and Inhibition Zone Diameter Breakpoints in Quality Control of Disk Diffusion Antibiotic Susceptibility Testing

GÖRAN KRONVALL

Department of Medical Microbiology, University of Lund, S-22362 Lund, Sweden

Received 16 February 1982/Accepted 2 August 1982

An equation was derived from known formulas to express the size of the inhibition zone diameter in the disk diffusion antibiotic susceptibility test as a function of the disk content of antibiotic. The equation permitted a calculation of regression line constants for the correlation between zone diameter and the minimum inhibitory concentration (MIC) with a single reference strain. Analysis of reference strains *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853, as well as 12 clinical isolates belonging to these species, showed a linearity between zone size squared and the logarithm of disk content in tests with 10-, 30-, and 100-μg gentamicin disks. All three species, however, gave regression line constants which were characteristic for the individual bacterial species. Calculations of zone diameter breakpoints corresponding to recommended MIC limits with *E. coli* and *P. aeruginosa* reference strains gave an accurate prediction of gentamicin susceptibility. Histogram analysis of 48 strains of *Streptococcus faecalis* from clinical specimens showed a distribution of zone diameter values which would result in false classification of susceptibility with breakpoints calculated for the other bacterial species studied. Single reference strain analysis of *S. faecalis* ATCC 29212 (gentamicin MIC, 8 μg/ml) permitted the calculation of breakpoints which accurately assigned the strains tested to the intermediate category of susceptibility. Single reference strain analysis offers a quality control method for individual laboratories that allows the determination of inhibition zone diameter breakpoints corresponding to recommended MIC limits with no MIC determinations required.

Determination of the antibiotic susceptibility of bacteria isolated from clinical specimens represents an important test in clinical microbiology laboratories. Despite recent technological advances in minimum inhibitory concentration (MIC) assays, the most widespread method used is still the disk diffusion test (1, 6, 8, 14–16, 27, 33). The advantages of this method over dilution tests are found in simplicity and ease of performance, direct control of purity of growth, and simple reading of results as well as interpretation of susceptibility. Furthermore, the reproducibility in individual laboratories is better for the disk diffusion method as compared with dilution tests (14). One serious drawback of the diffusion test, however, is still unsolved: there is a wide variation in inhibition zone results obtained in different laboratories with seemingly identical techniques. Despite several measures to standardize the test, this variability is not yet adequately controlled (4, 6, 8, 14, 23, 27).

Interpretive breakpoints for the different categories of susceptibility, susceptible (S), intermediate (I), and resistant (R), are presently set by translating MIC limits into inhibition zone diameters in millimeters (2, 6, 14, 27). The underlying regression analysis is based on studies of a large number of strains and is therefore usually performed only by reference laboratories (1, 14). From the known variation in disk diffusion results among different laboratories, it is apparent that the zone limits set might not represent correct MICs in individual laboratories (4, 19, 23). To minimize the resulting errors in interpretations, the I group of susceptibility has been transformed into an indeterminate group and considered a buffer zone (27, 32).

Factors governing the formation of zones of inhibition have been studied extensively (5, 9, 10–13, 21). From equations described in these early investigations, an alternative mathematical expression was derived in the present studies for

the correlation between the MIC of an antibiotic and the inhibition zone diameter. Only one reference strain with a known MIC is required in disk diffusion tests in which two or more different contents of antibiotic in the disks are used to determine the constants of the equation. The regression line constants obtained will include all the parameters unique to the method in individual laboratories and will therefore more accurately permit the calculation of interpretive zone diameter breakpoints corresponding to internationally accepted MIC limits.

MATERIALS AND METHODS

Bacterial strains and growth media. International control strains, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Streptococcus faecalis* ATCC 29212, and *Pseudomonas aeruginosa* ATCC 27853, were obtained from the National Bacteriological Laboratory, Stockholm, Sweden (4, 6, 27, 32). Five strains of *S. aureus*, five strains of *E. coli*, and two strains of *P. aeruginosa* were obtained from routine cultures at the Clinical Microbiology Laboratory, University Hospitals, Lund, Sweden. All strains were stored as stock cultures at −80°C in tryptone broth supplemented with 50% fetal calf serum. Subcultures were grown on 2% blood agar medium. In addition, 140 strains of *E. coli*, 46 strains of *P. aeruginosa*, and 48 strains of *S. faecalis* obtained from routine cultures were used in studies of zone size distributions.

Three different bacteriological culture media recommended for susceptibility testing were included in the studies. Medium 1 was Mueller-Hinton agar (batch no. 201-13443; Oxoid Ltd., Basingstoke, England). Medium 2 was antibiotic medium no. 2 (batch no. 282-17658; Oxoid Ltd.). Medium 3 was PDM antibiotic sensitivity medium (composition similar to Wellcotest agar; lot no. 91; AB Biodisk, Solna, Sweden).

Antibiotic disks. Paper disks containing different amounts of gentamicin were used in the studies. Disks containing 10 and 30 μg of gentamicin were purchased from AB Biodisk. Disks containing 1 and 100 μg of gentamicin were kindly supplied by I. Mortensen, Antibiotic Department, State Serum Institute, Copenhagen, Denmark.

Antibiotic susceptibility testing. The disk diffusion method used for antibiotic susceptibility testing followed recommended procedures described elsewhere (6, 14, 27). Plates were inoculated by the cotton swab method (14). Zone diameters were measured with a pair of calipers. All repeated tests were performed on consecutive days to include normal variability of an individual laboratory.

MIC determinations. Determination of MICs was performed by the agar plate dilution method (14, 34). Gentamicin was incorported in twofold dilution steps in PDM antibiotic sensitivity medium. Four-hour cultures of strains in tryptone broth were diluted and inoculated with a multipoint Steers replicator delivering approximately 4×10^4 organisms.

Computer calculations. Mathematical and statistical calculations were performed with a microcomputer, Metric model M85-T (Compucorp series 600, model 655; Scandia Metric AB, Solna, Sweden), with a 64-kilobyte RAM and two 640-kilobyte floppy disks. The software was obtained from Bioscand HB, Lund, Sweden. Computer aid was found necessary to minimize the work required for calculations in single strain regression line determinations and computation of laboratory-specific breakpoints. Mean values, standard deviation, and coefficients of variation were determined for homogeneous zone diameter populations and histograms plotted. Regression line constants and product moment coefficients of correlation were calculated according to the least-squares method and were used for MIC-to-zone diameter calculations.

RESULTS

Theoretical derivation of equations. Factors influencing the formation of zones of inhibition by antibiotics diffusing into a solid medium have been studied extensively, both theoretically and experimentally (9–13, 21). The mathematical models obtained take into account the diffusion constant of the antibiotic (D), the critical time at which the zone is formed (T), the distance between the antibiotic disk and the edge of the zone (X), the amount of antibiotic in the disk (m), and the critical concentration at which growth is no longer inhibited (m'). This classical relationship is shown in formula (1).

$$\ln m' = \ln m - (X^2/4DT) \quad (1)$$

In individual microbiological laboratories in which antibiotic susceptibility testing is performed routinely, several parameters are relatively constant (11). It is therefore possible to rewrite formula (1) using two constants, k and K:

$$\log \text{MIC} = \log Q - (Z^2/k) - K \quad (2)$$

or

$$Z^2 = A \log Q - A \log \text{MIC} + B \quad (3)$$

In formula (3), the direct correlation between m' and MIC is included (31), and the log base is changed to decimal. The inhibition zone (Z) is expressed as the square of the diameter in millimeters, which gives the best linearity, except at small zone values (11). Disk content (Q) is expressed in micrograms, and MIC is expressed as micrograms per milliliter. Constants A and B in formula (3) vary with the medium used, preincubation procedures, inoculum size, and minor modifications which are characteristic for an individual laboratory. These constants therefore include all those minor variations which give rise to interlaboratory differences in zone diameter results. From formula (3), it is clear that the calculation of these constants can be performed by using disk diffusion tests with two or more disk contents of each antibiotic together with a well-defined strain with known MICs.

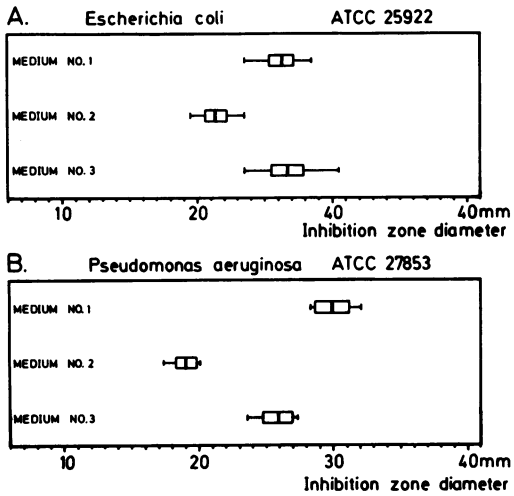

FIG. 1. Antibiotic susceptibility testing of reference strain *E. coli* ATCC 25922 (A) and *P. aeruginosa* ATCC 27853 (B) against gentamicin (disk content, 30 μg) with three different growth media. The inhibition zone diameters of 20 tests (A; media 1 and 2), 60 tests (A; medium 3) , and 12 tests (B) are shown as mean values (center bar), 1 standard deviation (open rectangle), and range.

Let $y = Z^2$, $x = \log Q$, and $C = -A \log \text{MIC} + B$

Then

$$y = Ax + C \tag{4}$$

and

$$A = \frac{N \sum xy - \sum x \sum y}{N \sum x^2 - (\sum x)^2} \tag{5}$$

Calculations with the least-squares method according to formulas (4) and (5) give constant A. Constant B is calculated from formula (3) with mean values of y and x.

Inhibition zones with different growth media. Three different growth media, all designed by manufacturers for disk diffusion susceptibility testing, were used in preliminary tests. Reference strains *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were tested on media 1, 2, and 3 with gentamicin disks containing 30 μg. This amount represents the standard disk content of gentamicin for susceptibility testing in Scandinavia (2). The inhibition zones recorded are summarized in Fig. 1. Different growth media gave different zone sizes for the two strains. Tests on media 1 and 3 showed larger inhibition zones, which were relatively similar for *E. coli* (Fig. 1A). Medium 2 gave the smallest zone diameter values. The differences between media 1, 2, and 3 were most marked for *P. aeruginosa*. These three media therefore seemed suitable for testing the capacity of single reference strain analysis to determine zone diameter breakpoints.

Single strain regression analysis. To test the validity of the calculation of regression line constants with equation (3), the linearity of experimental results was studied by using the three growth media. Five strains of *S. aureus*, five strains of *E. coli*, and two strains of *P. aeruginosa* were obtained from routine cultures and tested six times each against gentamicin-containing paper disks in disk diffusion susceptibility tests. The gentamicin contents of the disks were 10, 30, and 100 μg. Figure 2 summarizes the results of these experiments. The curves obtained showed good linearity over the whole range of disk potencies for all strains tested. The product moment coefficient of correlation (r) for *S. aureus* was 0.91 to 0.78 on medium 1, 0.97 to 0.92 on medium 2, and 0.93 to 0.76 on medium 3. For *E. coli*, the coefficients of correlation were 0.91 to 0.81, 0.96 to 0.87, and 0.89 to 0.82, respectively, and for *P. aeruginosa* they were 0.95 to 0.90, 0.98 to 0.97, and 0.96, respectively, on the three media. Disks containing 1 μg of gentamicin were also tested but gave zone sizes which did not fit the linear pattern, inhibition zone diameters being larger than expected.

A numerical expression of one curve parameter was obtained by calculating regression line constant A in formula (3) for the strains analyzed. Constant A is independent of the MIC for the individual strain and is a measure of the slope of the curve. The results are shown in Table 1. Calculated constants for strains on different media were similar within the *E. coli* and *P. aeruginosa* species. On the other hand, constant A for these two bacterial species was on completely different levels, indicating that the regression lines for *E. coli* and *P. aeruginosa* were completely different. Constant A values calculated for *S. aureus* strains on media 1 and 2 showed similarity to constants for *E. coli* and on medium 3 to the slope constant for *P. aeruginosa*. These experiments show that one gentamicin regression line is not applicable to all bacterial species.

Comparison between experimental and calculated MICs. MICs of gentamicin for the four reference strains as well as for five strains of *S. aureus*, five strains of *E. coli*, and one strain of *P. aeruginosa* were determined by the agar dilution method. Each strain was tested a minimum of six times, and a geometric mean value was calculated. The reference strains showed MICs in good agreement with data given in the literature (4, 18, 30, 34): *S. aureus* ATCC 25923, 0.25 μg/ml; *E. coli* ATCC 25922, 0.5 μg/ml; *S. faecalis* ATCC 29212, 8.0 μg/ml; and *P. aeruginosa* ATCC 27853, 1.0 μg/ml. All *S. aureus* and *E. coli* strains included from routine cultures

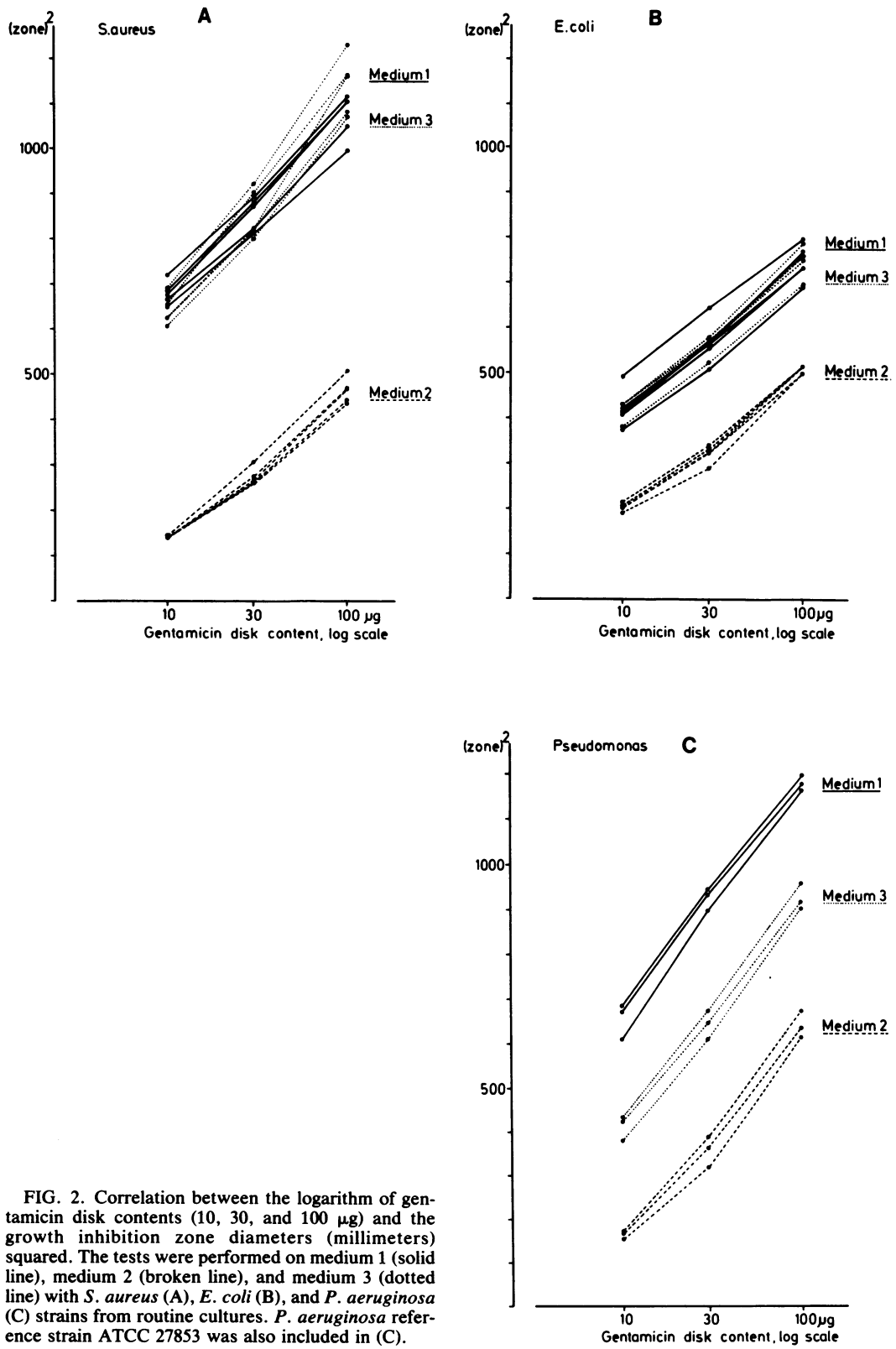

FIG. 2. Correlation between the logarithm of gentamicin disk contents (10, 30, and 100 µg) and the growth inhibition zone diameters (millimeters) squared. The tests were performed on medium 1 (solid line), medium 2 (broken line), and medium 3 (dotted line) with *S. aureus* (A), *E. coli* (B), and *P. aeruginosa* (C) strains from routine cultures. *P. aeruginosa* reference strain ATCC 27853 was also included in (C).

TABLE 1. Determination of gentamicin regression line constant *A* for different bacteria on different growth media[a]

Medium	*A* value (mean ± SD)		
	S. aureus (five strains)	*E. coli* (five strains)	*P. aeruginosa* (three strains)
1	391 ± 33	328 ± 23	522 ± 38
2	317 ± 18	314 ± 23	476 ± 22
3	496 ± 40	342 ± 21	512 ± 16

[a] Constant *A* in equation (3) was calculated for different bacteria tested on different growth media with inhibition zone values shown in Fig. 1.

were homogeneous within each species regarding MICs. The MIC means were 0.25 μg/ml for *S. aureus*, 0.64 μg/ml for *E. coli*, and 1.5 μg/ml for the *P. aeruginosa* strain.

To test the predictive value of the regression lines obtained with single strain analysis, the theoretical MICs were calculated. In one series of calculations, the regression line used was based on *E. coli* reference strain ATCC 25922 results, and in another series on *P. aeruginosa* ATCC 27853 results. The MICs obtained are shown in Table 2. The prediction of MICs for *S. aureus* and *E. coli* strains were most accurate with the *E. coli* reference strain. Conversely, prediction of a MIC for *P. aeruginosa* was only possible with the *P. aeruginosa* reference strain regression line. The fact that *S. aureus* values with medium 3 were closer to the true MIC with *E. coli* as the reference and not *P. aeruginosa*, as expected from constant *A* values in Table 1, further indicates that *S. aureus* also shows a species-specific gentamicin regression line.

Species-related interpretive breakpoints. The capacity of single strain regression line analysis to determine accurately the breakpoints for gentamicin was tested by using different substrates and different bacterial species. Breakpoints were calculated with two different gentamicin regression lines, one based on single strain analysis of reference strain *E. coli* ATCC 25922, and the other on *P. aeruginosa* ATCC 27853 (Table 3). The MICs used for the calculations represented recommended breakpoint levels and included internationally accepted limits, ≤4 and ≥8 μg/ml, as well as Scandinavian limits, ≤4 and >16 μg/ml, for the S and R categories, respectively (2, 6, 27). From Table 3 it is apparent that currently recommended zone diameter breakpoints for Scandinavian limits, −21, 20 to 16, and <16 mm for the S, I, and R categories, respectively, using medium 3, do not conform to breakpoints calculated for the susceptibility test as performed in our laboratory. It should be pointed out that the zone diameter values refer to the use of 30-μg gentamicin disks. Regression line equation (3) does permit a simple calculation of breakpoint values for other disk potencies as well.

TABLE 2. Gentamicin MICs for bacterial strains calculated from regression lines obtained by using single strain analysis[a]

Reference strain and medium	MIC (μg/ml)		
	S. aureus (0.25 μg/ml)[b]	*E. coli* (0.64 μg/ml)[b]	*P. aeruginosa* (1.5 μg/ml)[b]
E. coli ATCC 25922			
Medium 1	0.16	0.99	0.09
Medium 3	0.15	0.88	0.64
P. aeruginosa ATCC 27853			
Medium 1	1.4	5.4	1.0
Medium 3	0.42	1.6	1.2

[a] Zone diameters used for the calculation of MICs for the test strains correspond to data in Fig. 2. Mean values are given for *S. aureus* (five strains) and *E. coli* (five strains).

[b] True MICs are in parentheses.

TABLE 3. Breakpoints calculated from gentamicin regression lines obtained by using single reference strain analysis[a]

Reference strain and medium	Zone diameter breakpoints (mm) for MICs (μg/ml) of:			
	4.0	6.0	8.0	16.0
E. coli ATCC 25922				
Medium 1	18.5	16.6	15.2	11.1
Medium 3	17.7	15.8	14.2	9.5
P. aeruginosa ATCC 27853				
Medium 1	25.0	23.2	21.8	17.9
Medium 3	18.9	16.4	14.4	7.7

[a] Currently accepted MIC limits for gentamicin susceptibility converted into inhibition zones (in millimeters) for media 1 and 3 as calculated from regression lines obtained by using two different reference strains.

The relevance of the calculated breakpoints was analyzed in relation to individual inhibition zone diameters for different bacterial species. Histograms of zone values in millimeters were plotted for the routine strains included. Figure 3A and B shows histograms of *E. coli* tested against 30-μg gentamicin disks on media 1 and 3, respectively. From the data in Table 3, the lower limit for the S category on these two media was set to 18 and 17 mm, respectively. In Fig. 3C and D, histograms of *P. aeruginosa* on media 1 and 3 are shown, together with the lower limits for S, 24 and 18 mm, respectively. The large differences in zone diameter distributions seen with *P. aeruginosa* on the two media would normally make any interpretation difficult. With the breakpoints calculated by using single strain

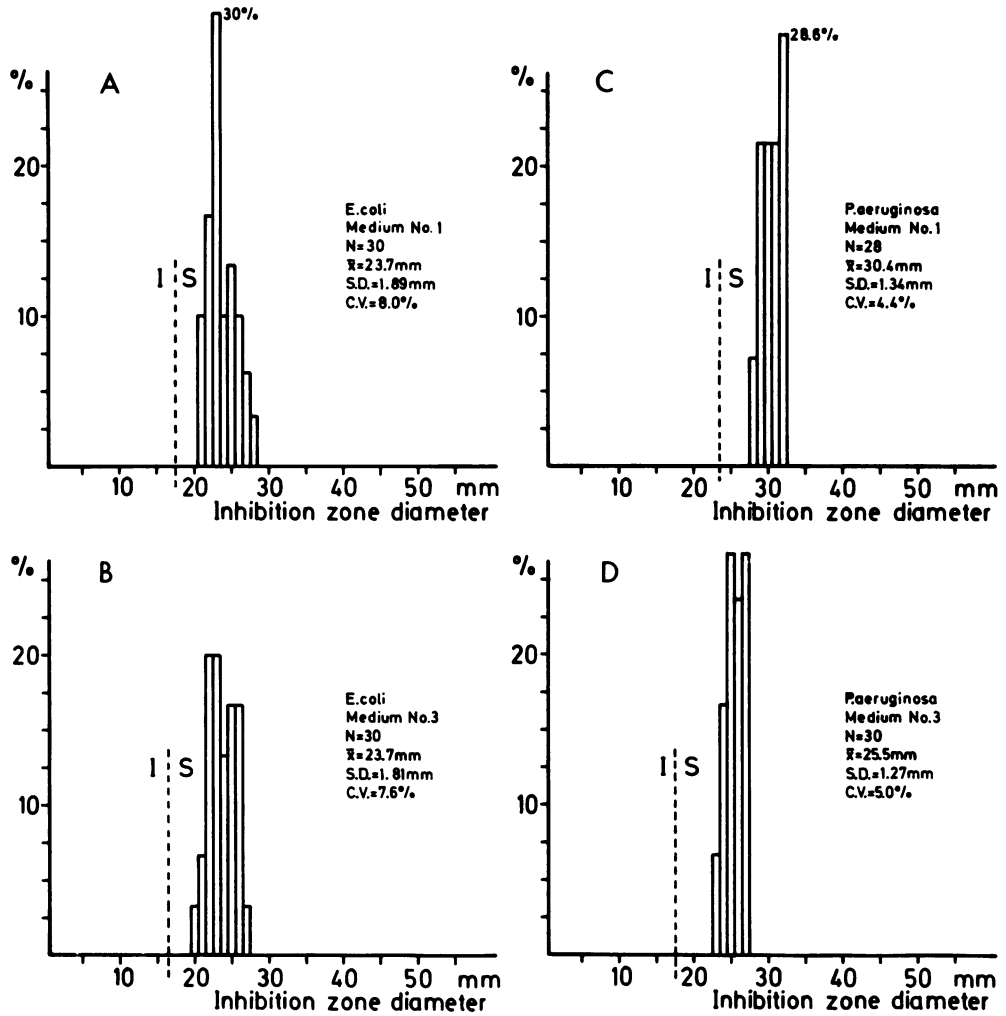

FIG. 3. Histograms of individual zone diameter values recorded for strains of *E. coli* (A and B) and *P. aeruginosa* (C and D) with 30-μg gentamicin disks on medium 1 (A and C) and medium 3 (B and D). Calculated mean values of the populations were 23.7 ± 1.9 mm (A), 23.7 ± 1.8 mm (B), 30.4 ± 1.3 mm (C), and 25.5 ± 1.3 mm (D). Species-related breakpoints corresponding to S ≤4 μg/ml were calculated by using single reference strain analysis.

regression analysis in the present studies, the correct limits for the different susceptibility categories obtained provided a basis for accurate interpretations of the zone diameters recorded.

Inhibition zone diameters for 140 routine isolates of *E. coli* and 46 strains of *P. aeruginosa* with 30-μg gentamicin disks were also recorded, and the histograms were plotted (Fig. 4). The calculated breakpoints permitted the proper designation of these populations to the S category.

Gentamicin susceptibility of *S. faecalis*. Routine strains of *S. faecalis* tested on medium 3 with 30-μg gentamicin disks gave an inhibition zone diameter distribution as shown in Fig. 5C, with a mean value of 19.2 mm. Strains belonging to this population show MICs around 8 μg/ml and therefore should be assigned to the I category. However, breakpoints calculated with the *E. coli* reference strain would falsely give the S designation to 96% of the *S. faecalis* strains.

Since a series-specific single strain regression analysis apparently was required, the reference strain, *S. faecalis* ATCC 29212, was tested on medium 3 with 10-, 30-, and 100-μg gentamicin disks. The correlation between squared zone diameters and log disk contents was linear, confirming that regression line equation (3) was valid (Fig. 5A). With the constants thus known, the corresponding MIC-zone correlation was plotted (Fig. 5B). From this curve, the mean zone diameter value of the *S. faecalis* population, 19.2 mm, was found to correspond to an

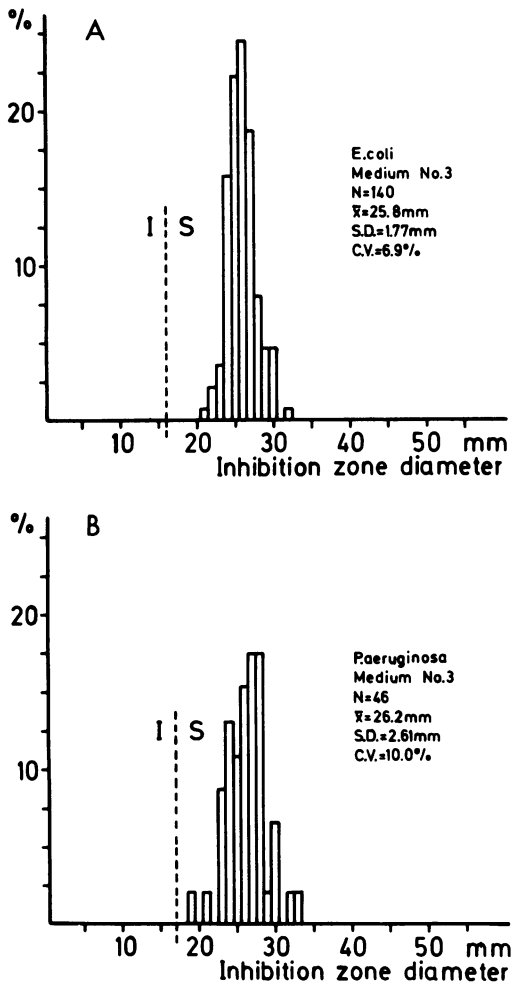

FIG. 4. Histograms of zone diameter values obtained from routine antibiotic susceptibility tests of 140 strains of *E. coli* (A) and 46 strains of *P. aeruginosa* (B) with 30-μg gentamicin disks. Mean values for the populations were calculated as 25.8 ± 1.8 and 26.2 ± 2.6 mm, respectively. Species-related breakpoints are indicated.

MIC of 7.0 μg/ml, in agreement with the dilution test MIC. The breakpoints corresponding to Scandinavian limits were also calculated, 4 μg/ml = 21.8 mm and 16 μg/ml = 14.6 mm. By using these breakpoints, the population peak was assigned to the I category (Fig. 5C). The single strain regression line analysis described therefore seems to be capable of defining accurately the inhibition zone diameter breakpoints in disk diffusion antibiotic susceptibility tests.

DISCUSSION

Quality control procedures currently recommended for analysis of disk diffusion antibiotic susceptibility tests are capable of measuring both accuracy and precision (4, 6, 27). Despite these established procedures, the results of susceptibility tests are still far from satisfactory (19, 23, 24). The major problem encountered is the wide variability in inhibition zone diameter results in different laboratories (14, 23). This variability gives very poor accuracy if the millimeter breakpoints used to determine susceptibility are calculated from regression lines obtained in one laboratory. A major defect of present quality control procedures is the inability to provide guidelines for correct setting of breakpoints when the accuracy has been determined. The quality control limits set by the National Committee for Clinical Laboratory Standards are therefore quite generous (1, 4, 6, 23, 27, 32). *E. coli* ATCC 25922, when tested against gentamicin, has been given an accepted range of 19 to 26 mm for a mean of five determinations in an individual laboratory. This range corresponds to MICs between 0.31 and 1.6 μg/ml, as calculated from published regression lines. The MIC for the reference strain is 0.5 μg/ml. The range given for the means therefore permits a large degree of inaccuracy among different laboratories. Other strain-antibiotic combinations show an even worse picture. In a recent report, the present situation with such poor accuracy was described as "disquieting" (19).

From theoretical aspects of the formation of inhibition zones (9–13, 21), an alternative equation was derived in the present study which permitted the determination of regression line constants with one single strain with a known MIC in tests with disks containing different amounts of antibiotic. The linearity expressed by equation (3) for single strain analysis was experimentally confirmed (Fig. 2 and Table 2). The results indicated that gentamicin regression lines determined on the same growth medium differ considerably among different bacterial species (Table 1). Constant *A*, determining the slope of the regression line, was different for *E. coli* and *P. aeruginosa* on the three growth media used (Table 1). The current use of one single regression line for all bacterial species is therefore not valid. In the case of *P. aeruginosa*, this is a well-known phenomenon (7). The single strain regression line analysis described was capable of determining correct gentamicin breakpoints for *E. coli* and *P. aeruginosa* on two different growth media (Fig. 3 and 4). Species-specific regression line analysis was also applied to *S. faecalis*. From single reference strain analysis, the relevant breakpoints could be determined (Fig. 5). The method described is simple and does not require any MIC determinations; it is therefore suitable for use in individual microbiology laboratories.

Thus, the importance of species-specific gentamicin breakpoints in disk diffusion antibiotic

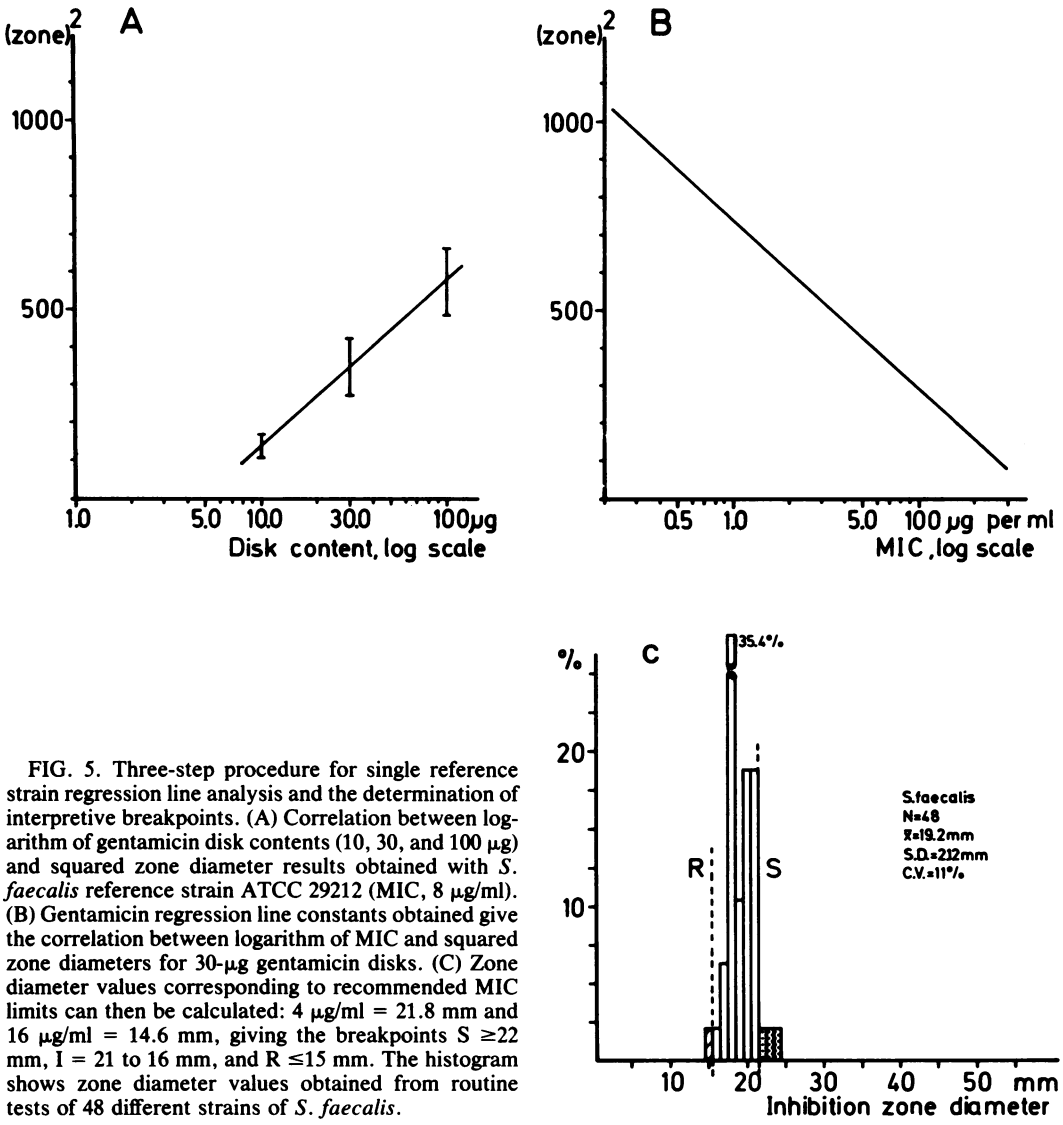

FIG. 5. Three-step procedure for single reference strain regression line analysis and the determination of interpretive breakpoints. (A) Correlation between logarithm of gentamicin disk contents (10, 30, and 100 µg) and squared zone diameter results obtained with *S. faecalis* reference strain ATCC 29212 (MIC, 8 µg/ml). (B) Gentamicin regression line constants obtained give the correlation between logarithm of MIC and squared zone diameters for 30-µg gentamicin disks. (C) Zone diameter values corresponding to recommended MIC limits can then be calculated: 4 µg/ml = 21.8 mm and 16 µg/ml = 14.6 mm, giving the breakpoints S ≥22 mm, I = 21 to 16 mm, and R ≤15 mm. The histogram shows zone diameter values obtained from routine tests of 48 different strains of *S. faecalis*.

susceptibility testing is apparent from the fact that different bacterial species show different regression lines. The definition of such alternative breakpoints is possible by using single reference strain analysis. Another equally important aspect of species-specific breakpoints must also be emphasized. From Fig. 5C, it is clear that *S. faecalis* strains with a gentamicin MIC of 8 µg/ml should be assigned to the I category according to Scandinavian limits (S ≤4 µg/ml; R >16 µg/ml) (2). The methodological variation in the disk test for one single strain tested repeatedly gives a range of 8 to 12 mm (14, 25, 27). To accurately assign *S. faecalis* strains with a mean zone value of 19.2 mm to the I category, the breakpoints for this bacterial species should be adjusted to account for the methodological variation. Since individual bacterial species have only a limited number of resistance mechanisms against each antibiotic, the number of zone diameter populations for each bacterial species is also limited (17, 25, 28, 29). Thus, species-related alternative breakpoints should also be defined on a statistical basis to improve the correct designation of susceptibility categories (24).

In calculations with the traditional correlation between 2 log MICs and inhibition zone sizes, a linear relationship is usually assumed (8, 15). The present study indicates that such a correlation is not valid when several different bacterial species are included. In a collaborative study by Ericsson and Sherris, linearity was significant in only 26% of the cases, suggesting some basic

error in the mathematical expressions or in the materials used (15). Other objections to the existing type of regression analysis in describing the correlation between dilution tests and disk diffusion results have also been raised (24, 26). These authors suggest the application of polynomic functions to account for the fact that both variables might be considered dependent. However, the use of MICs as the independent variable in regression analysis, according to equations in current use and to the novel equation described here, provides the only basis for considerations of therapeutic activity and must be adhered to (3, 7, 8, 11, 14, 15, 20, 26). It should also be pointed out that zone size squared gives better linearity in experimental studies as compared with both the zone diameter value and the theoretically more appropriate value of zone radius minus disk radius squared (11). Equation (3), which includes both disk potency and MICs for their correlation with growth inhibition zone size, therefore might be superior to other equations used at present.

Disk diffusion tests with gentamicin as well as other aminoglycoside antibiotics are subject to problems regarding their standardization (22). Despite such inherent difficulties, the application of the present single strain analysis to gentamicin susceptibility testing permitted the correct setting of inhibition zone diameter breakpoints. For antibiotics other than aminoglycosides, the number of reference strains required in routine quality control tests due to species differences will probably be comparatively small. In combination with currently accepted methods for the control of precision (6, 27), the single reference strain analysis described might therefore provide individual microbiological laboratories with a procedure for improving the accuracy of susceptibility tests. Extension of the present studies to other antibiotics, as well as to bacterial strains with resistance based on the production of inactivating enzymes known to give rise to atypical regression lines, is presently under way.

From the present study, some conclusions regarding quality control in antibiotic susceptibility testing can be drawn. First, there is a need for a set of internationally available reference strains representing different bacterial species commonly encountered in clinical practice. These strains should be well characterized in regard to MICs of antibiotics, preferably with dilution steps closer than twofold (7). Second, antibiotic disks with different potencies must be made available for control purposes from the major disk manufacturers. Third, when new antibiotics are introduced, MICs for the international reference strains should be given to permit laboratories to calculate zone diameter breakpoints which correspond to recommended MIC limits. With these basic provisions, it might be possible for individual microbiology laboratories to set up quality control schemes to determine accuracy and precision and to calculate regular breakpoints as well as some critical species-specific breakpoints by using single reference strain analysis.

ACKNOWLEDGMENTS

Figures were drawn and photographed by Åke Christensson. The preparation of growth media was performed by Vasile Soltesz. Their cooperation as well as the assistance of laboratory technicians at the Clinical Microbiology Laboratory, University Hospitals, Lund, is gratefully acknowledged.

LITERATURE CITED

1. **Acar, J. F.** 1980. The disc susceptibility test, p. 24–54. *In* V. Lorian (ed.), Antibiotics in laboratory medicine. Williams & Wilkins, London.
2. **Alestig, K., K. Dornbusch, C. Ericsson, L. O. Kallings, C. Kamme, F. Nordbring, R. Norrby, and G. Wallmark.** 1981. A revised system for antibiotic sensitivity testing. The Swedish reference group for antibiotics. Scand. J. Infect. Dis. **13**:148–152.
3. **Arvidson, S., K. Dornbusch, and H. Ericsson.** 1981. Interpretation of the agar diffusion method for bacterial susceptibility testing. J. Antimicrob. Chemother. **7**:5–14.
4. **Balows, A., and T. L. Gavan.** 1980. Quality control methods for in vitro antibiotic susceptibility testing, p. 409–417. *In* V. Lorian (ed.), Antibiotics in laboratory medicine. Williams & Wilkins, London.
5. **Barry, A. L.** 1980. Procedure for testing antibiotics in agar media: theoretical considerations, p. 1–23. *In* V. Lorian (ed.), Antibiotics in laboratory medicine. Williams & Wilkins, London.
6. **Barry A. L., and C. Thornsberry.** 1980. Susceptibility testing: diffusion test procedures, p. 463–474. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and J. P. Truant (ed.), Manual of clinical microbiology, 3rd ed. American Society for Microbiology, Washington, D.C.
7. **Barry, A. L., C. Thornsberry, and R. N. Jones.** 1981. Gentamicin and amikacin disk susceptibility tests with *Pseudomonas aeruginosa*: definition of minimal inhibitory concentration correlates for susceptible and resistant categories. J. Clin. Microb. **13**:1001–1003.
8. **Bauer, A. W., W. M. M. Kirby, J. C. Sherris, and M. Turck.** 1966. Antibiotic susceptibility testing by a standardized single disc method. Am. J. Clin. Pathol. **45**:493–496.
9. **Brimley, R. C.** 1949. Quantitative paper chromatography. Nature (London) **163**:215–216.
10. **Cooper, K. E.** 1955. Theory of antibiotic inhibition zones in agar media. Nature (London) **176**:510–511.
11. **Cooper, K. E.** 1964. The theory of antibiotic inhibition zones, p. 1–86. *In* F. Kavanagh (ed.), Analytical microbiology. Academic Press, Inc., New York.
12. **Cooper, K. E., A. H. Linton, and S. N. Sehgal.** 1958. The effect of inoculum size on inhibition zones in agar media using staphylococci and streptomycin. J. Gen. Microbiol. **18**:670–687.
13. **Cooper, K. E., and D. Woodman.** 1946. The diffusion of antiseptics through agar gels, with special reference to the agar cup assay method of estimating the activity of penicillin. J. Pathol. Bacteriol. **58**:75–84.
14. **Ericsson, H. M., and J. C. Sherris.** 1971. Antibiotic sensitivity testing. Report of an international collaborative study. Acta. Pathol. Microbiol. Scand. Sect. B **217**(Suppl.):1–90.
15. **Ericsson, H., G. Tunevall, and K. Wickman.** 1960. The paper disc method for determination of bacterial sensitivity to antibiotics. Relationship between the diameter of the zone of inhibition and the minimum inhibitory concen-

tration. Scand. J. Clin. Lab. Invest. **12**:414–422.
16. **Erlanson, P.** 1951. Determination of the sensitivity in vitro of bacteria to chemotherapeutic agents. Acta. Pathol. Microbiol. Scand. Sect. B **85**(Suppl):1–162.
17. **Furtado, G. L., and A. A. Medeiros.** 1980. Single-disk diffusion testing (Kirby-Bauer) of susceptibility of *Proteus mirabilis* to chloramphenicol: significance of the intermediate category. J. Clin. Microbiol. **12**:550–553.
18. **Gavan, T. L., and A. L. Barry.** 1980. Microdilution test procedures, p. 459–462. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and J. P. Truant (ed.), Manual of clinical microbiology, 3rd ed. American Society for Microbiology, Washington, D.C.
19. **Gavan T. L., R. N. Jones, A. L. Barry, P. C. Fuchs, E. H. Gerlach, J. M. Matsen, L. B. Reller, C. Thornsberry, and L. D. Thrupp.** 1981. Quality control limits for ampicillin, carbenicillin, mezlocillin, and piperacillin disk diffusion susceptibility tests: a collaborative study. J. Clin. Microbiol. **14**:67–72.
20. **Greenwood, D.** 1981. In vitro veritas? Antimicrobial susceptibility tests and their clinical relevance. J. Infect. Dis. **144**:380–385.
21. **Humphrey, J. H., and J. W. Lightbown.** 1952. A general theory for plate assay of antibiotics with some practical applications. J. Gen. Microbiol. **7**:129–143.
22. **Kahlmeter, G.** 1980. Some causes for variation in aminoglycoside plate assays. J. Antimicrob. Chemother. **6**:43–52.
23. **Knowles, R. C., and T. D. Moore.** 1979. Quality control of agar diffusion susceptibility tests. Data from the quality assurance service microbiology program of the College of American Pathologists. Am. J. Clin. Pathol. **72**:365–371.
24. **Krasemann, C., and G. Hildenbrand.** 1980. Interpretation of agar diffusion tests. J. Antimicrob. Chemother. **6**:181–187.
25. **Kronvall, G., and A. Runehagen.** 1981. Improved antibiotic susceptibility testing of bacteria using species-related SIR interpretive breakpoints (in Swedish). Läkartidningen **78**:3483–3485.
26. **Metzler, C. M., and R. M. DeHaan.** 1974. Susceptibility tests of anaerobic bacteria: statistical and clinical considerations. J. Infect. Dis. **130**:588–594.
27. **National Committee for Clinical Laboratory Standards.** 1975. Performance standards for antimicrobial disc susceptibility tests. ASM-2. National Committee for Clinical Laboratory Standards, Villanova, Pa.
28. **O'Brien, T. F., R. L. Kent, and A. A. Medeiros.** 1969. Computer-generated plots of results of antimicrobial susceptibility tests. J. Am. Med. Assoc. **210**:84–92.
29. **O'Brien, T. F., and the International Antibiotic Resistance Survey Group.** 1978. Multicenter sensitivity studies. International collaborative antibiotic resistance survey, p. 534–536. *In* W. Siegenthaler and R. Lüthy (ed.), Current chemotherapy. Proceedings of the Xth International Congress of Chemotherapy. American Society for Microbiology, Washington, D.C.
30. **Reimer, L. G., C. W. Stratton, and L. B. Reller.** 1981. Minimum inhibitory and bactericidal concentrations of 4 antimicrobial agents against three standard control strains in broth with and without human serum. Antimicrob. Agents. Chemother. **19**:1050–1055.
31. **Shannon, R., A. J. Hedges, and R. J. Edwards.** 1975. Distribution of levels of penicillin resistance among freshly isolated strains of *N. gonorrhoeae*. Application of a novel sensitivity assay. Br. J. Vener. Dis. **51**:246–250.
32. **Thornsberry, C., T. L. Gavan, and E. H. Gerlach.** 1977. Cumitech 6, New developments in antimicrobial agent susceptibility testing. Coordinating ed., J. C. Sherris. American Society for Microbiology, Washington, D.C.
33. **Vincent, J. G., and H. W. Vincent.** 1944. Filter paper disc modification of the Oxford cup penicillin determination. Proc. Soc. Exp. Biol. Med. **55**:162–164.
34. **Washington, J. A., II, and V. L. Sutter.** 1980. Dilution susceptibility test: agar and macro-broth dilution procedures, p. 453–458. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and J. P. Truant (ed.), Manual of clinical microbiology, 3rd ed. American Society for Microbiology, Washington, D.C.