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New Datum Handling Methods for the Quality Control of Antibiotic Solutions and Plates Used in the Antimicrobial Susceptibility Test

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Quality control programs are described for monitoring both the antibiotic solutions and the plates used in the dilution antimicrobial susceptibility test. For the quality control of antibiotic solutions, a simple comparative disk diffusion assay is used in which the data are expressed as zone ratios calculated from the following formula: zone ratios = mean zones of inhibition for disks impregnated with antibiotic under test/mean zones of inhibition of previously prepared quality control disks impregnated with the same antibiotic. The rejection limits set for this method are twice as stringent as for MIC estimation, and its precision is four times greater. For quality control of the antibiotic agar plates, fluid was absorbed directly into filter paper disks placed on the surface of the medium. These disks were then transferred to a seeded plate to produce zones of inhibition. These zones of inhibition were then converted to zone ratios by dividing the zone of inhibition for the individual antibiotics by the mean of the total zones of inhibition for all the antibiotics tested on the same plate. We further normalized these data by expressing each entry as a standard normal deviate calculated from the mean and standard deviation of the cumulative data for each individual antibiotic. The use of zone ratios reduced batch-to-batch variation, and standard normal deviate values gave a uniform result for convenience. The method has a sensitivity equal to that of an MIC estimation if it were possible to estimate the MICs of antibiotics at these low levels.

Antibiotic solutions. Laboratories which perform the agar dilution antimicrobial susceptibility test require and must store all the standard antibiotic powders used in these tests. Most of these powders are unstable and have stated expiry dates based on a 10% loss of potency under ideal storage conditions. Because it is impractical for laboratories performing routine testing to accurately assay these powders, they are obliged to rely on the information on stated potency and expiry date provided by the manufacturers. Stock solutions are prepared from these powders and may be stored by the laboratory. We believe that it is essential to perform quality control (QC) testing of these solutions and have developed a method for monitoring the concentration of the antibiotic in these solutions to ensure that reliable and consistent results are obtained when pouring antibiotic-containing plates from these solutions or using them in MIC estimations.

In one of the methods currently available to perform QC testing of these solutions, the broth dilution method recommended by the National Committee for Clinical Laboratory Standards (9), stock solutions which may have an antibiotic content of between 50 and 200% of the desired target are regarded as acceptable. A further disadvantage is that the test does not provide quantitative data on antibiotic concentrations, since the results are expressed relative to an arbitrarily selected dilution series. Antibiotic assays in which diffusion methods are used are complex and time-consuming to perform, because fresh standards must be prepared to obtain calibration line data needed to calculate the concentration of the antibiotics. This method, however, has the advantage of compensating for interassay fluctuations.

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As an alternative, Cooper and Linton (2, 3) have shown that a single disk containing a known concentration of antibiotic can be used to calculate the critical concentration of the antibiotic required to inhibit the growth of an organism under controlled conditions. We have extended this concept and developed a method to perform QC testing of antibiotic solutions by using single-strength assay standard disks that we refer to as quality control disks (QCD). These disks contain a lower antibiotic concentration than commercially available disks, which produce too large a zone of inhibition (DZI) and consequently lack sensitivity. The results of tests are compared with those of the QCDs and expressed as a zone ratio (ZR) to compensate for interassay fluctuations.

TABLE 1. Data used to illustrate the ZR approach to the QC of antibiotic susceptibility test solutions

Plate no.	DZI for disk impregnated with test solution (mm)	DZI for QCD (mm)	ZR
1	14.6	12.6	1.16
2	14.2	12.0	1.18
3	14.2	12.1	1.17
4	14.2	13.9	1.02
5	14.8	12.7	1.17
6	15.0	13.0	1.15
7	14.1	12.0	1.18
8	14.8	12.8	1.16
9	14.8	12.7	1.17
10	16.2	14.4	1.13
11	15.3	13.4	1.14
12	14.6	12.6	1.16
13	15.1	13.1	1.15
14	16.0	14.1	1.13
Mean	14.9	12.9	1.148
SD	0.644	0.758	0.0402
CV%	4.3	5.9	3.5

TABLE 2. Summary of the data-processing procedure illustrated with reference to a typical data set

Day	DZI (mm) for following antibiotic:				Mean DZI (mm)	ZR for following antibiotic:				SND for following antibiotic:			
	A	B	C	D		A	B	C	D	A	B	C	D
Mon	18	23	31	35	26.8	0.67	0.86	1.16	1.31	0.77	−1.20	1.61	1.00
Tues	16	22	27	33	24.5	0.65	0.90	1.10	1.35	−0.77	0.40	−0.32	0.33
Wed	15	20	25	30	22.5	0.67	0.89	1.11	1.33	0.77	0	0	−0.33
Thurs	13	18	22	28	20.3	0.64	0.89	1.08	1.38	−1.54	0	−0.97	1.33
Fri	12	17	20	24	18.3	0.66	0.93	1.09	1.31	0	1.60	−0.65	−1.00
Mean						0.66	0.89	1.11	1.34				
SD						0.013	0.025	0.031	0.030				

Antibiotic plates. The second procedure in the agar dilution antimicrobial susceptibility test which requires QC testing is the preparation of the antibiotic plates containing a wide range of antibiotics at a variety of concentrations. Most laboratories rely on the use of QC organisms for which the antibiotic MICs are known (5, 9), but these will detect only gross errors of preparation. Franklin (6) proposed that QC testing of antibiotic plates be carried out by producing DZIs from cores of agar cut out of the plates; acceptance and rejection limits were set at ±1 and ±2 mm of the target DZI, respectively. Unfortunately this method mutilates the plates, and no data were given to support its ability to detect errors.

We have developed a method in which filter paper disks were used to absorb antibiotic solution from the plate surface, thus avoiding mutilation of the plate. The panel of antibiotic disks containing antibiotic solutions from all the bilayered plates used in the agar dilution antimicrobial susceptibility test were then tested on a single plate to produce DZIs. The individual DZIs were then divided by the mean DZI of all the DZIs on the bilayered plate to convert them to ZRs, which compensate for interassay fluctuations. The single-layered plates were treated in a similar manner, but the data were processed separately. From these data, 95% confidence limits were established for the test deviations, and any fluctuations greater than these were considered abnormal and were a warning to repeat the test. If a particular antibiotic plate failed the test twice, that batch of plates was discarded. To simplify the interpretation of the result from an individual plate, we converted all the results to standard normal deviate (SND) values. We have demonstrated that this method can clearly differentiate populations which have a one-dilution difference in concentration.

TABLE 3. Antibiotics used to establish the analytical sensitivity of the plate method

Antibiotic	Use of antibiotic at following concn (μg/ml)[a]:										
	0.5	1	2	4	8	16	32	64	128	256	512
Amoxicillin		×	×	×	×	×	×				
Ampicillin		×	×	×	×	×	×				
Imipenem		×	×	×	×	×	×				
Cephalothin		×	×	×	×	×	×				
Azlocillin					×	×	×	×	×		
Cefoxitin			(×)	(×)	×	×	×				
Gentamicin			×	×	×						
Tobramycin	(×)	(×)	×	×	×						
Tetracycline		×	×	×	×	×					
Nalidixic acid					×	×	×				
Norfloxacin					×	×	×				
Sulfadiazine									(×)	×	×
Trimethoprim			(×)	×	×						

[a] ×, The antibiotic was used at this concentration. (×), These plates produced no DZIs or inconsistent small DZIs.

MATERIALS AND METHODS

Theoretical considerations and experimental design. (i) Antibiotic stock solution testing. Our approach is based on the theory that when a test concentration equals the QCD concentration, the ZR must be 1.0. In practice, the ratio fluctuates in the vicinity of 1.0. We proposed that by expressing the results for our test solutions as a normalized ZR, we would reduce the effects of interassay fluctuations and make it possible to perform a quantitative assay by using single-strength assay standards. The process of the calculations is illustrated in Table 1 for a representative data set, and the reduction in the coefficient of variation (CV%) for the data expressed as the ZR can be appreciated.

(ii) Antibiotic plate testing. The design of the part of the study devoted to antibiotic plate testing was based on the hypothesis that interassay fluctuations in the DZIs are both random within a test and proportional to each other, despite being produced by different antibiotics. We proposed that by expressing individual DZIs as a ZR to the mean DZI for all the antibiotics tested on a single plate on the same day, we would reduce the obscuring effect of random intratest fluctuation on trends in the cumulative records of data for tests on different batches.

Representation of data was further simplified by expressing all the ZRs in the tables of cumulative results for the

TABLE 4. Concentrations of antibiotics used in routine tests

Type of plate	Antibiotic	Use of antibiotic at following concn (μg/ml)[a]:						
		1	2	4	16	32	64	256
Double-layered	Amoxicillin		×		×			
	Amoxicillin-clavulanic acid		×		×			
	Cephalothin	×			×			
	Imipenem			×	×			
	Norfloxacin				×			
	Gentamicin			×				
	Tobramycin			×				
	Azlocillin				×		×	
	Imipenem			×	×			
	Norfloxacin				×			
Single-layered	Tetracycline	×		×				
	Cefoxitin			×	×			
	Trimethoprim				×			
	Sulfadiazine							×
	Nalidixic acid					×		

[a] ×, The antibiotic was used at this concentration.

TABLE 5. Antibiotic concentrations in the stock solutions used to impregnate the QCD

Antibiotic	Stock solution	
	Concn (mg/ml)	μg/disk
Amoxicillin	1	2
Cephalothin[a]	1	0.2
Tetracycline	1	2
Gentamicin	1	2
Tobramycin	1	2
Nalidixic acid	3	6
Sulfadiazine	16	32
Trimethoprim	0.4	0.8
Cefoxitin	2	4
Norfloxacin	1	2
Azlocillin	8	16
Imipenem[a]	1	0.2

[a] These stock solutions were diluted 1/10 before 2 μl was pipetted onto each disk.

antibiotics as SND units (1). This process of data manipulation is illustrated in Table 2 for a hypothetical panel of four typical antibiotics, A to D, studied daily for 5 days.

The first data set in Table 2 shows the cumulative DZI data in millimeters. Visual inspection and assessment of this panel can only be subjective, and this assessment is complicated by the wide range in DZIs (12 to 35 mm) encountered in this selection of four antibiotics. The general observation that all DZIs appear to be decreasing in size is probably the only comment that can be made. The ZR data are probably easier for most observers to interpret, since the target ZRs (as shown by the column means) are 0.66, 0.89, 1.11, and 1.34. However, even the data in this panel cover a wide dynamic range (0.64 to 1.38), and this is the principal justification for calculating the SNDs. All the SND data, regardless of their column, can be subjected to the same assessment based upon the common statistical acceptance criterion of mean ± 1.97 standard deviations ($P < 0.05$). The target mean for an SND is zero by definition. From this set of SNDs, it is obvious that the readings for antibiotic C on Monday and antibiotic B on Friday were the most discrepant. In retrospect, it would have been difficult to support this conclusion from the DZI data alone. An additional advantage of the SND data is that they have a statistical meaning, and a table of "Z values" (1) can be consulted to assign actual statistical probabilities to all the results, e.g., SND = 1.60, $P = 0.890$. In practice, a method which rejects results outside the 95% confidence limits will reject 1 in 20 sets of

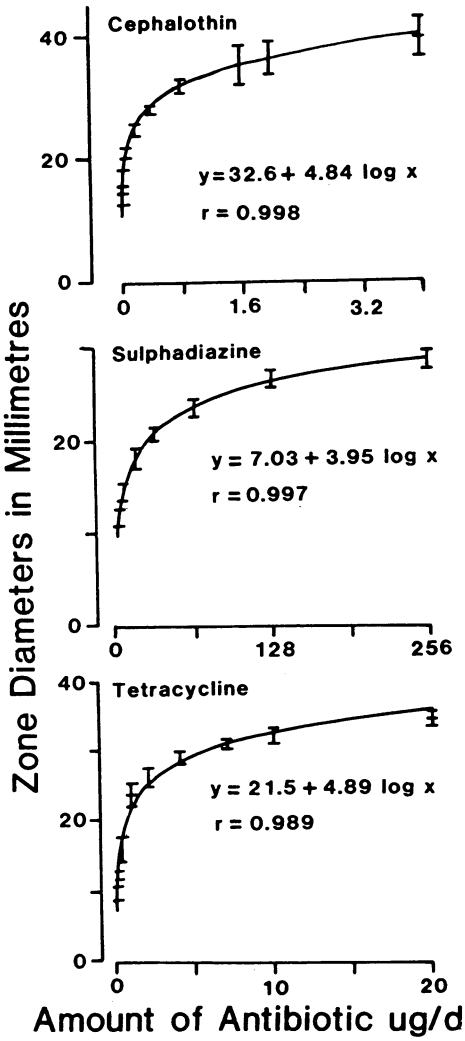

FIG. 1. Wide-range calibration curves for cephalothin, sulfadiazine, and tetracycline in the disk diffusion method.

plates even though the antibiotic concentration may be acceptable. To overcome this problem, we reject only batches of plates which are outside the 95% confidence limits when retested.

A weakness in this approach is that it requires a number of plate batches to be tested before the statistics of calculating

TABLE 6. Evaluation of the variability of the DZI measurement versus the ZR measurement for 11 antibiotic stock solutions

Antibiotic	No. of tests	QCD DZI		Stock solution DZI		ZR		CV% from calculation of ZR only
		Mean (mm)	CV%	Mean (mm)	CV%	Mean	CV%	
Amoxicillin	29	23.480	7.971	23.598	8.166	1.003	1.302	0.195
Cephalothin	27	25.434	7.238	25.248	7.077	0.993	2.043	0.161
Tetracycline	30	23.805	6.416	23.152	7.268	0.971	2.840	0.852
Gentamicin	45	20.392	6.919	20.569	6.675	1.007	1.939	0.244
Tobramycin	22	20.741	4.987	20.863	5.052	1.011	1.749	0.065
Nalidixic acid	20	17.709	8.787	18.108	7.562	1.024	2.423	1.225
Sulfadiazine	25	18.938	16.519	17.674	18.138	0.930	4.673	1.619
Trimethoprim	24	21.607	10.316	20.834	10.196	0.965	2.318	0.12
Cefoxitin	26	24.084	8.241	24.344	7.629	1.010	2.193	0.612
Norfloxacin	24	24.649	3.371	24.732	3.704	1.005	2.352	0.333
Azlocillin	20	20.083	8.796	20.012	9.478	0.998	1.573	0.682

TABLE 7. Calibration curves for the 11 antibiotic stock solutions expressed as diameters of the DZI and as ZRs versus concentrations

Antibiotic	QCD concn (μg/disk)	Data set	Slope	Intercept	r^a	ZR for following concn:			
						50%	75%	125%	150%
Amoxicillin	2	A	3.212	23.44	0.9781				
		B	2.833	21.73	0.9618				
		C	0.1219	0.9113	0.9696	0.911	0.961	1.023	1.045
Cephalothin	0.2	A	4.665	33.23	0.9789				
		B	4.097	31.52	0.9736				
		C	0.1735	1.282	0.9748	0.883	0.953	1.042	1.073
Tetracycline	2	A	3.826	21.49	0.9715				
		B	4.391	20.02	0.9897				
		C	0.1749	0.8824	0.9763	0.882	0.953	1.042	1.075
Gentamicin	2	A	2.825	19.83	0.9821				
		B	2.675	19.00	0.9577				
		C	0.1299	0.9148	0.9675	0.914	0.967	1.034	1.057
Tobramycin	2	A	3.019	16.31	0.9751				
		B	3.157	19.33	0.9764				
		C	0.1558	0.8951	0.9736	0.895	0.958	1.038	1.066
Nalidixic acid	6.4	A	3.984	12.18	0.9559				
		B	4.443	10.05	0.9795				
		C	0.2248	0.5895	0.9651	0.851	0.942	1.057	1.097
Sulfadiazine	32	A	7.533	−11.32	0.9741				
		B	4.289	6.877	0.9730				
		C	4.652	7.240	0.9819				
		D	3.693	11.18	0.9662				
		E	0.2614	0.0852	0.8508	0.810	0.916	1.049	1.097
Trimethoprim	0.8	A	4.487	21.04	0.9795				
		B	4.714	21.69	0.9456				
		C	0.2286	1.060	0.9598	0.851	0.944	1.060	1.102
Cefoxitin	4	A	5.124	18.34	0.9855				
		B	5.159	17.47	0.9873				
		C	0.2048	0.7151	0.9817	0.857	0.940	1.045	1.082
Norfloxacin	2	A	5.218	21.77	0.9967				
		B	5.279	21.46	0.9887				
		C	0.2070	0.8546	0.9891	0.854	0.938	1.044	1.082
Azlocillin	3.2	A	3.088	17.17	0.9604				
		B	3.029	17.19	0.9621				
		C	0.1487	0.8343	0.9381	0.904	0.964	1.040	1.067

[a] r, Correlation coefficient for the logarithmic equation $y = a + b\log_e x$. For the number of datum points, see the text.

the column mean and column standard deviations of the ZRs becomes reliable; 10 batches is the minimum number we were prepared to consider. Unfortunately, it is impractical in routine work to wait for many months to accumulate these base-line data, and we compromised by setting up 10 base-line plates over a 2-week period from the same production batch of plates. This compromise provided us with data sets which actually underestimate the true batch-to-batch variation. However, experience with this QC system has proved that this is an advantage, because it raises the sensitivity of the approach, particularly when there are gradual drifts in QC results. In this situation, no action is usually taken, and the ZRs and SNDs slowly adjust to new plateau values. If, however, the base-line data are always included, the QC procedure becomes less prone to this problem. We have incorporated the following rules into our computer program so that the base-line data are always included: (i) if the batch of 10 plates for base-line data plus the total number of plates, NP, assayed to date is less than 20, all the data i.e., 10 + NP, are used; (ii) if NP is greater than 20, the base-line data plus data from the last 10 plates assayed are used. In this way, we ensure that the latest data are always compared with the base-line data set. Having defined the statistical basis of the method, we proceeded to design an experiment to determine its analytical sensitivity. We needed to determine what the statistically significant deviations, if any, would mean in terms of antibiotic concentrations in those plates.

Our work on the antibiotic solutions reported in this paper demonstrated that large DZIs were usually associated with poor analytical sensitivity, and we assumed that the same restriction would apply to this part of the study. Single plates of each antibiotic and concentration were prepared (Table 3). The range of antibiotic concentrations was selected to enable us to compare results for one dilution above and below the routinely used concentrations. Since the antibiotic concentrations on numerous plates were tested, it was

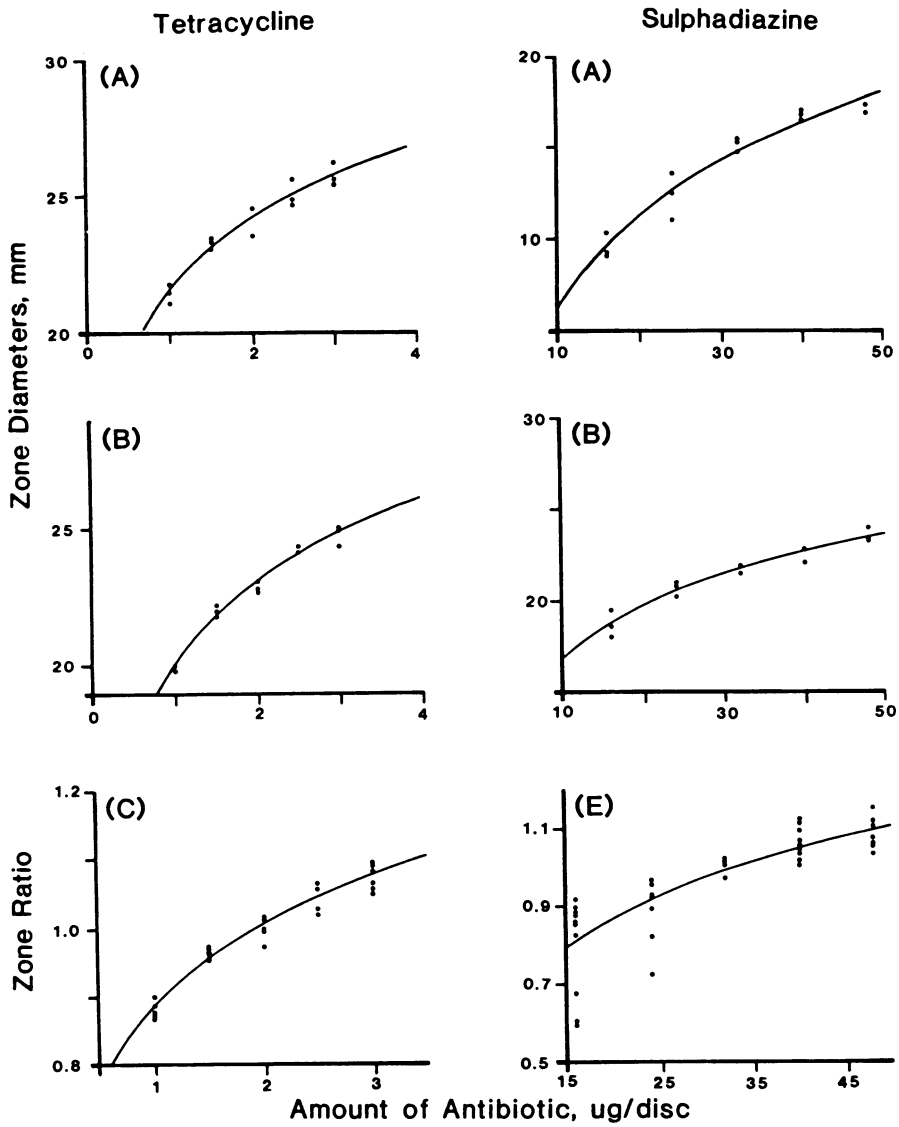

FIG. 2. Narrow-range calibration curves for tetracycline and sulfadiazine expressed as both diameters of the DZI of two separate test runs (A and B) and ZR (C and E). The equations for the lines of best fit are given in Table 6.

necessary to use three seeded test plates for the complete range of tests. The same plate containing each concentration of antibiotic was tested 10 times over a 2-week period. The DZIs of the routinely used antibiotic concentrations, together with the one dilution above and below were divided by the mean of the routinely used concentration DZIs to produce ZRs and standard deviations. A 2-standard-deviation bar chart was constructed from these results and examined for possible overlap of the mean ± 2 standard deviations for the routine concentration with those for one dilution above and below this concentration. Overlapping would have been interpreted as evidence of poor analytical sensitivity.

The base-line data for the routine prospective quality control of agar plates were established as follows. Bottles containing 200 ml of agar were prepared to include the complete range of antibiotics and concentrations shown in Table 4. Ten 9-cm agar plates were prepared from each of these bottles. The plates were stored at 4°C in plastic screw-top containers. Each of these plates was tested prior to being used for routine antibiotic susceptibility testing.

The QC of routinely used antibiotic plates was performed by testing one plate from each batch of 10 plates containing the antibiotics and concentrations shown in Table 4. The data obtained from both the routine QC and the base-line data testing were processed as outlined in Table 2.

Materials and organisms used. Antibiotic assay filter disks (diameter, 6 mm) AA grade filter paper; Whatman, Maidstone, England) were chosen for the QC method after studies showed these disks to have a high uptake of fluid when placed on the surface of agar plates. The mean weight of the fluid absorbed was 24.14 μg, with a 95% confidence limit of 1.5756 μg; subsequent QC testing of new batches of disks has given good reproducibility of these data. The balance used to obtain these results was an H10W (Mettler, Zurich, Switzerland), which gave 95% confidence limits of 0.00092 g with a QC weight of 0.5586 g.

Ultra-micro Samplers (Oxford Instruments, St. Louis,

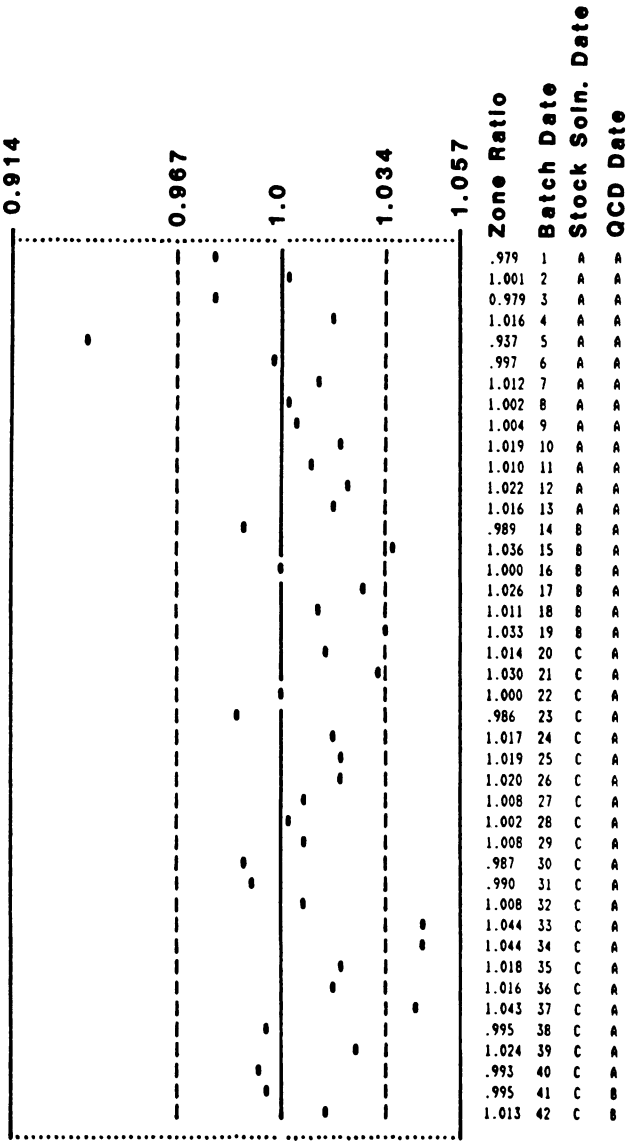

FIG. 3. QC chart for gentamicin QCD ratio measurements. Batch dates: batch 1, 3 February 1987; batch 42, 13 October 1987. Stock solution dates: solution A, 28 August 1986; solution B, 21 January 1987; solution C, 10 April 1987. QCD dates: QCD A, 20 January 1987; QCD B, 7 August 1987.

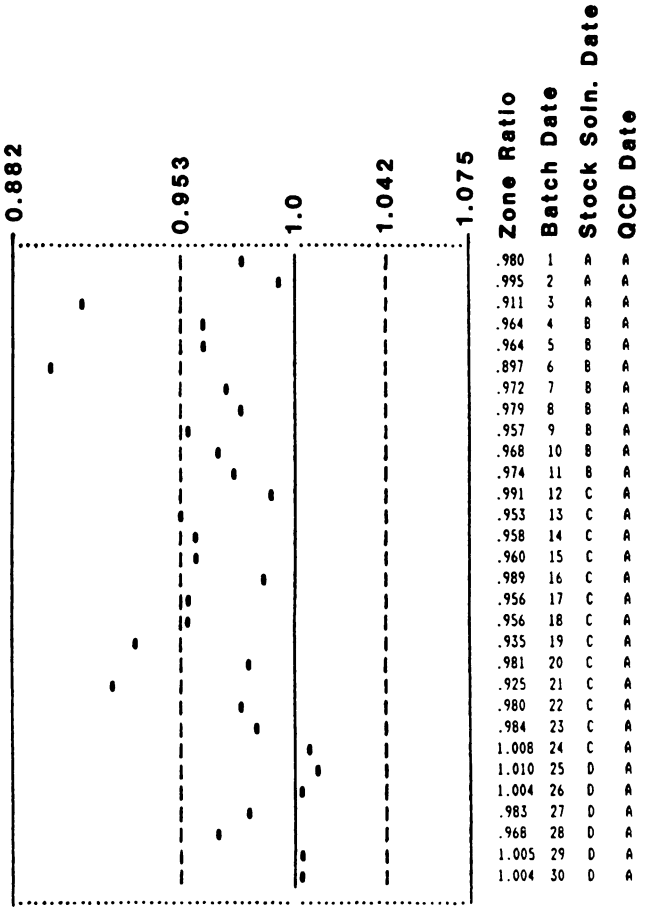

FIG. 4. QC chart for tetracycline QCD ratio measurements. Batch dates: batch 1, 9 December 1986; batch 30, 13 October 1987. Stock solution dates: solution A, 4 November 1986; solution B, 27 January 1987; solution C, 26 February 1987; solution D, 18 August 1987. QCD A date, 10 January 1985.

Mo.) delivering 2 μl with a specified accuracy of 3% and a reproducibility of 0.04 μl were chosen to load the QCDs with antibiotic solution. This volume was selected to avoid diluting stock solutions. Davis et al. (4) considered this the lowest practical volume usable for antibiotic disk assays.

The indicator organism chosen for the assays was a *Bacillus* species (IMVS FD0101) which has been used in antibiotic assays for many years at the Institute of Medical and Veterinary Science. This organism was used as a spore suspension in distilled water with a viable count adjusted to 10^8 CFU by the plate count method of Miles and Misra (8) and is stable for many years at 4°C. This organism does not form β-lactamase and gives DZIs which lie in the area of optimal sensitivity of the calibration curves used in this paper.

Large-diameter polystyrene petri dishes (15 cm) and Iso-Sensitest agar (CM 471; Oxoid, Basingstoke, England) stored in 20-ml aliquots was used throughout this trial, unless otherwise stipulated.

Preparation of bilayered assay plates. The 15-cm bilayered assay plates were used to assay both antibiotic solutions and antibiotic dilution plates. The bottoms of all the plates used were checked for flatness with a straight edge, and pouring was done on a preleveled surface. A base layer of 20 ml of Iso-Sensitest agar was poured and allowed to set before the seeded layer was poured onto this surface. The seeded layer contained 20 ml of Iso-Sensitest agar which had been cooled to 50°C before addition of 0.1 ml of the *Bacillus* spore suspension. After pouring, the plates were left at room temperature with their lids off so that the surface dried, and they were used within 1 h of being prepared to avoid premature growth of the indicator organism in relation to the diffusion of antibiotic from the disks tested.

Preparation of QCDs. Fifty filter disks were placed across the bottom of a sterile disposable petri dish (diameter, 9 cm), and 2 μl of the appropriate antibiotic was pipetted into the center of each disk. They were dried for 1 h at 37°C before being placed in plastic containers holding a layer of silica gel desiccant. The lids of the containers were sealed with imbedding wax before they were placed in long-term storage at −20°C. The concentrations of the stock solutions and the

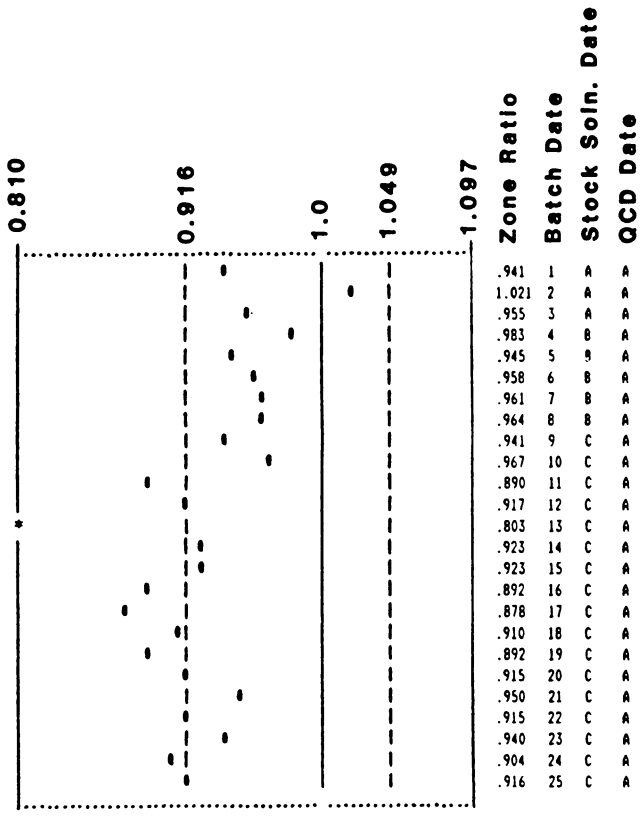

FIG. 5. QC chart for sulfadiazine QCD ratio measurements. Batch dates: batch 1, 10 December 1986; batch 25, 13 October 1987. Stock solution dates: solution A, 10 April 1986; solution B, 11 December 1986; solution C, 23 March 1987. QCD A date, 10 October 1985.

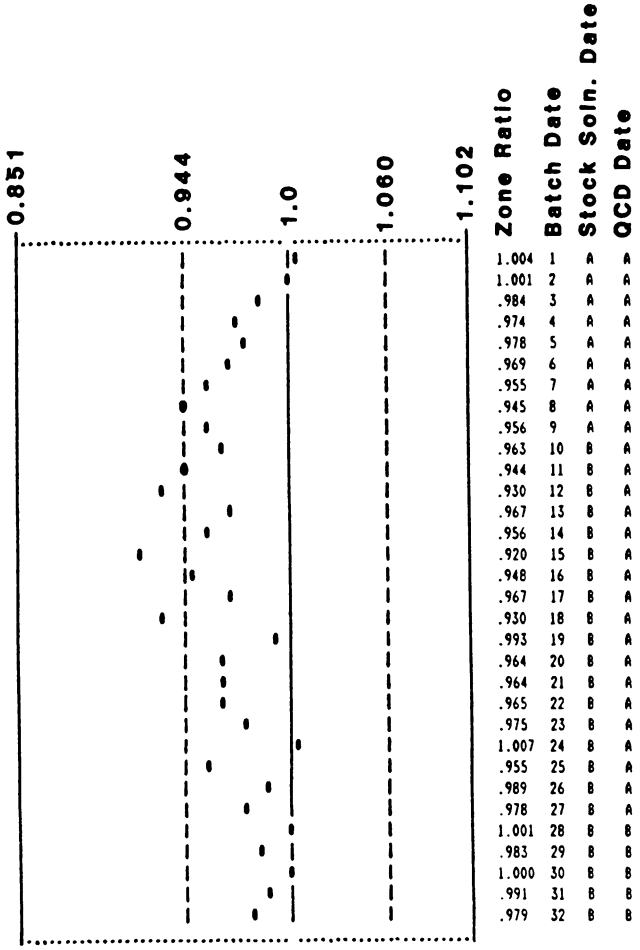

FIG. 6. QC chart for trimethoprim QCD ratio measurements. Batch dates: batch 1, 1 December 1986; batch 32, 9 November 1987. Stock solution dates: solution A, 25 November 1986; solution B, 23 March 1987.

amounts of antibiotic used on each QCD are shown in Table 5.

Routine testing method for antibiotic solutions. Two antibiotic solutions were tested on each 15-cm bilayered assay plate. Each antibiotic and its QCDs were tested in quadruplicate. Test disks were placed adjacent to the appropriate QCDs, with no attempt being made to achieve random distribution. Templates were used to facilitate the work so that the disks could be rapidly positioned no closer than 3 cm to each other and to reduce the prediffusion time between disks. Our standard procedure was to place the four QCDs for the first antibiotic on the plate, position the four test disks, and then pipette 2 μl of the appropriate antibiotic onto the center of each of the test disks. This procedure was then repeated for the second antibiotic solution. The petri dishes were incubated at 37°C for 18 h with a spacer between the dish and the lid to allow sufficient ventilation to encourage a luxuriant growth of the indicator organism. For reading, the petri dishes were placed bottom uppermost over an incident light source. DZIs were measured with vernier calipers and recorded to the nearest 0.1 mm. ZRs for antibiotic stock solution testing were calculated as follows: ZR = mean DZI for four test disks/mean DZI for four QCDs. Data are valid only when the tests and QCDs are performed on the same plate.

Routine testing of plates. A filter disk (diameter, 6 mm) was placed close to the edge of the agar on each plate to be tested and left to absorb fluid for a minimum of 30 min before being transferred aseptically to an appropriately seeded plate. These seeded plates were incubated and read as above. Disks from up to 20 different antibiotic plates can be subjected to QC testing on a single 15-cm plate. The range tested will be dictated by the normal range used and reported by the laboratory, but a minimum of five antibiotic plates should be used. The full range of antibiotics in use must be tested on each run for the results to be valid. If the laboratory changes a single antibiotic in its range, a new data base must be compiled. The ranges used in our laboratory are shown in Table 5. ZRs for antibiotic plate testing were calculated as follows: ZR = DZI of test disk/mean of all DZIs on the test plate. Data are valid only if all measurements are performed on a single plate containing a full range and all dilutions of antibiotics. SNDs for antibiotic plate testing were calculated as follows: SND = (ZR for an antibiotic − mean ZR for this antibiotic observed to date)/standard deviation of the ZRs for this antibiotic observed to date. The derivation of this mean ZR is discussed below.

Full concentration range calibration curves to establish the optimal working range of the disk method. Three antibiotics were chosen in the calibration curve study: cephalothin, sulfadiazine, and tetracycline. Disks were prepared to contain nine different concentrations of each antibiotic. The ranges were 0.02 to 4 μg per disk for cephalothin, 0.1 to 20 μg per disk for tetracycline, and 0.8 to 256 μg per disk for

TABLE 8. Typical DZI, ZR, and SND data set for five antibiotics[a]

Day	DZI Matrix							Means of Rows	Zone Ratio Matrix							Standard Normal Deviate Table						
	A	B	C	D	E	F	G		H	I	J	K	L	M	N	A	B	C	D	E	F	G
1	12.8	12.9	13.8	17.2	18.8	26.8	15.5	16.829	0.761	0.767	0.820	1.022	1.117	1.593	0.921	-0.09	0.13	-0.87	-0.75	0.39	0.58	0.28
2	14.7	15.3	16.7	22.0	23.1	31.5	17.0	20.043	0.733	0.763	0.833	1.098	1.153	1.572	0.848	-0.62	0.06	-0.67	0.19	0.72	0.40	-1.68
3	15.8	15.8	17.8	22.0	21.8	30.6	18.5	20.329	0.777	0.777	0.876	1.082	1.072	1.505	0.910	0.23	0.37	-0.04	-0.00	-0.04	-0.19	-0.01
4	15.1	14.8	17.1	21.7	19.1	27.3	17.7	18.971	0.796	0.780	0.901	1.144	1.007	1.439	0.933	0.59	0.43	0.34	0.76	-0.66	-0.77	0.61
5	15.5	15.7	17.1	21.2	19.2	27.5	17.7	19.129	0.810	0.821	0.894	1.108	1.004	1.438	0.925	0.87	1.33	0.23	0.32	-0.68	-0.78	0.40
6	15.5	15.3	17.3	21.2	17.6	26.7	17.1	18.671	0.830	0.819	0.927	1.135	0.943	1.430	0.916	1.25	1.30	0.72	0.65	-1.26	-0.85	0.14
7	15.4	15.2	17.5	21.5	19.0	27.2	17.3	19.014	0.810	0.799	0.920	1.131	0.999	1.431	0.910	0.86	0.86	0.63	0.60	-0.73	-0.84	-0.02
8	15.0	14.4	17.2	21.2	18.8	26.7	16.8	18.586	0.807	0.775	0.925	1.141	1.012	1.437	0.904	0.81	0.31	0.70	0.72	-0.61	-0.79	-0.18
9	15.0	14.8	16.4	20.9	16.2	24.4	16.3	17.714	0.847	0.835	0.926	1.180	0.915	1.377	0.920	1.58	1.65	0.71	1.20	-1.53	-1.31	0.26
10	15.0	15.0	16.8	20.7	17.2	25.1	17.7	18.214	0.824	0.824	0.922	1.136	0.944	1.378	0.972	1.13	1.39	0.65	0.67	-1.25	-1.30	1.65
A	13.1	13.4	13.3	16.5	24.2	31.9	16.0	18.343	0.714	0.731	0.725	0.900	1.319	1.739	0.872	-0.99	-0.66	-2.28	-2.26	2.30	1.87	-1.03
B	10.6	11.5	11.5	14.0	20.7	29.3	16.0	16.229	0.653	0.709	0.709	0.863	1.276	1.805	0.986	-2.17	-1.15	-2.53	-2.71	1.88	2.45	2.04
C	15.6	15.4	17.9	22.1	23.2	31.6	19.4	20.743	0.752	0.742	0.863	1.065	1.118	1.523	0.935	-0.26	-0.40	-0.23	-0.21	0.40	-0.03	0.67
D	13.6	13.1	17.2	20.4	19.7	27.7	17.3	18.429	0.738	0.711	0.933	1.107	1.069	1.503	0.939	-0.53	-1.10	0.82	0.30	-0.07	-0.21	0.76
E	14.0	13.2	16.7	19.7	22.8	31.6	16.1	19.157	0.731	0.689	0.872	1.028	1.190	1.650	0.840	-0.67	-1.58	-0.10	-0.67	1.08	1.08	-1.89
F	13.6	13.4	17.0	20.5	19.8	28.3	17.2	18.543	0.733	0.723	0.917	1.106	1.068	1.526	0.928	-0.62	-0.84	0.57	0.28	-0.08	-0.00	0.46
G	15.0	14.8	16.9	20.9	19.2	28.5	17.1	18.914	0.793	0.782	0.894	1.105	1.015	1.507	0.904	0.54	0.48	0.23	0.28	-0.58	-0.17	-0.17
H	15.0	14.1	17.2	21.2	20.0	28.2	16.3	18.857	0.795	0.748	0.912	1.124	1.061	1.495	0.864	-.58	-0.28	0.50	0.52	-0.15	-0.27	-1.24
I	13.3	13.6	15.5	19.2	21.5	30.6	17.0	18.671	0.712	0.728	0.830	1.028	1.151	1.639	0.910	-1.02	-0.71	-0.72	-0.67	0.71	0.99	-0.00
J	13.5	13.5	19.0	22.5	21.4	30.3	17.1	19.614	0.688	0.688	0.969	1.147	1.091	1.545	0.872	-1.49	-1.60	1.34	0.80	0.14	0.16	-1.04

[a] Means of columns A to G (DZI): 14.36, 14.26, 16.49, 20.33, 20.16, 28.59, and 17.06. SDs of columns A to G (DZI): 1.28, 1.14, 1.74, 2.14, 2.14, 2.24, and 0.91. CV% of columns A to G (DZI): 8.9, 8.0, 10.6, 10.5, 10.6, 7.8, and 5.3. Means of columns H to N: 0.765, 0.761, 0.878, 1.083, 1.076, 1.527, and 0.911. SDs of columns H to N: 0.052, 0.045, 0.067, 0.081, 0.106, 0.114, and 0.037. CV% of columns H to N: 6.8, 5.9, 7.6, 7.5, 9.9, 7.5, and 4.1. Column headings: A and H, gentamicin (4 μg/ml); B and I, tobramycin (4 μg/ml); C and J, azlocillin (16 μg/ml); D and K, azlocillin (64 μg/ml); E and L, imipenem (4 μg/ml); F and M, imipenem (16 μg/ml); G and N, norfloxacin (16 μg/ml). Underlined SND values exceed the ±1.97 rejection limits. See the text.

TABLE 9. DZI, ZR, and SND data for six antibiotics[a]

Day	DZI Matrix A	B	C	D	E	F	G	H	I	J	Means of Rows	Zone Ratio Matrix K	L	M	N	O	P	Q	R	S	T	Standard Normal Deviate Table A	B	C	D	E	F	G	H	I	J
1	14.0	21.0	14.1	21.1	15.7	19.4	30.2	21.5	30.5	17.3	20.480	0.684	1.025	0.688	1.030	0.767	0.947	1.475	1.050	1.489	0.845	0.03	0.23	0.03	-0.24	1.46	0.05	0.57	-0.55	-0.46	-0.38
2	12.4	18.9	12.7	19.4	31.0	18.3	27.7	20.0	29.2	16.2	18.780	0.660	1.006	0.676	1.033	0.692	0.974	1.475	1.065	1.555	0.863	-0.51	-0.31	-0.33	-0.15	-0.37	0.76	0.58	-0.33	0.25	0.17
3	11.5	17.8	11.7	18.5	13.0	18.2	26.4	18.8	27.5	15.7	17.910	0.642	0.994	0.653	1.033	0.726	1.016	1.474	1.050	1.535	0.877	-0.93	-0.66	-1.00	-0.15	0.46	1.86	0.56	-0.56	0.04	0.59
4	13.8	20.0	14.0	20.4	15.2	18.5	28.1	23.0	31.6	16.8	20.140	0.685	0.993	0.695	1.013	0.755	0.919	1.395	1.142	1.569	0.834	0.06	-0.68	0.23	-0.81	1.17	-0.71	-1.01	0.79	0.41	-0.70
5	13.4	20.3	13.2	20.2	14.4	17.9	28.4	22.4	31.7	16.6	19.850	0.675	1.023	0.665	1.018	0.725	0.902	1.431	1.128	1.597	0.836	-0.17	0.15	-0.6	-0.65	0.45	-1.15	-0.30	0.59	0.71	-0.63
6	11.5	18.1	11.9	18.0	12.6	16.9	27.7	19.8	28.2	16.3	18.100	0.635	1.000	0.657	0.994	0.696	0.934	1.530	1.094	1.558	0.901	-1.08	-0.49	-0.88	-1.41	-0.28	-0.31	1.68	0.09	0.29	1.32
7	13.2	20.7	13.3	20.9	14.4	18.4	28.6	19.0	26.5	17.1	19.210	0.687	1.078	0.692	1.088	0.750	0.958	1.489	0.989	1.379	0.890	0.11	1.70	0.15	1.63	1.04	0.33	0.85	-1.44	-1.66	1.00
8	10.5	16.9	11.1	17.2	12.4	17.7	26.0	19.2	27.5	15.5	17.400	0.603	0.971	0.638	0.989	0.713	1.017	1.494	1.103	1.580	0.891	-1.82	-1.30	-1.45	-1.60	0.13	1.89	0.96	0.23	0.53	1.02
9	12.0	17.9	12.1	18.5	13.1	17.8	27.4	18.2	32.3	16.9	18.620	0.644	0.961	0.650	0.994	0.704	0.956	1.472	0.977	1.735	0.908	-0.87	-1.58	-1.20	-1.44	-0.09	0.28	0.51	-1.61	2.22	1.53
10	13.4	21.7	14.3	21.0	13.9	17.8	30.0	23.5	32.3	16.3	20.420	0.656	1.063	0.700	1.028	0.681	0.872	1.469	1.151	1.582	0.798	-0.60	1.28	0.38	-0.30	-0.66	-1.95	0.46	0.92	0.55	-1.79
A	14.5	20.4	14.7	20.7	13.6	17.8	27.8	19.7	27.7	17.3	19.420	0.747	1.050	0.757	1.066	0.700	0.917	1.432	1.014	1.426	0.891	1.48	0.94	2.05	0.92	-0.17	-0.76	-0.29	-1.07	-1.55	1.02
B	15.3	20.8	14.9	21.7	14.4	19.0	27.7	22.4	28.6	16.8	20.160	0.759	1.032	1.739	1.076	0.714	0.942	1.374	1.111	1.419	0.833	1.76	0.41	1.53	1.26	0.17	-0.08	-1.43	0.34	-1.24	-0.72
C	13.5	20.7	13.7	21.3	15.2	19.0	28.7	19.7	27.5	16.8	19.610	0.688	1.056	0.699	1.086	0.775	0.969	1.464	1.005	1.402	0.857	0.14	1.08	0.33	1.58	1.67	0.62	0.35	-1.21	-1.41	-0.01
D	13.8	20.4	13.2	21.2	14.5	18.9	28.9	20.9	28.4	16.3	19.650	0.702	1.027	0.672	1.079	0.738	0.962	1.471	1.064	1.445	0.830	0.46	0.59	-0.46	1.34	0.75	0.43	0.49	-0.35	-0.94	-0.84
E	12.6	19.4	12.2	19.5	13.2	17.1	27.5	21.2	29.9	16.3	18.890	0.667	1.027	0.646	1.032	0.699	0.905	1.456	1.122	1.583	0.863	-0.35	0.28	-1.22	-0.18	-0.21	-1.06	0.20	0.50	0.56	0.17
F	15.1	23.0	16.6	22.8	15.5	20.7	30.1	25.4	32.3	17.8	21.930	0.689	1.049	0.757	1.040	0.707	0.944	1.373	1.158	1.473	0.812	0.14	0.89	2.05	0.06	-0.01	-0.04	-1.46	1.03	-0.64	-1.38
G	13.5	20.1	13.7	20.4	12.7	19.2	27.6	24.0	31.7	15.9	19.880	0.679	1.011	0.689	1.026	0.639	0.966	1.388	1.207	1.595	0.800	-0.08	-0.17	0.05	-0.38	-1.69	0.54	-1.15	1.74	0.69	-1.74
H	15.4	19.8	13.8	20.7	13.6	18.9	26.0	19.4	29.5	17.1	19.420	0.793	1.020	0.711	1.066	0.700	0.973	1.339	0.999	1.519	0.881	2.55	0.07	0.69	0.92	-0.17	0.73	2.13	-1.30	-0.14	0.71
I	12.6	19.0	12.7	19.0	12.2	17.8	28.1	21.2	28.3	16.5	18.740	0.672	1.014	0.678	1.014	0.651	0.950	1.499	1.131	1.510	0.880	-0.23	-0.09	-0.28	-0.77	-1.39	0.11	1.07	0.63	-0.24	0.71
J	13.0	17.9	13.1	20.1	11.8	17.0	27.2	22.9	32.2	16.4	19.160	0.678	0.934	0.684	1.049	0.616	0.887	1.420	1.195	1.681	0.856	-0.09	-2.34	-0.11	0.37	-2.25	-1.53	-0.52	1.57	1.63	-0.04

[a] Means of columns A to J (DZI): 13.25, 19.74, 13.35, 20.13, 13.72, 18.32, 28.01, 21.11, 29.67, and 16.59. SDs of columns A to J (DZI): 1.30, 1.51, 1.27, 1.37, 1.15, 0.92, 1.20, 1.98, 1.99, and 0.57. CV% of columns A to J (DZI): 9.8, 7.6, 9.5, 6.8, 8.4, 5.0, 4.3, 9.4, 6.7, and 3.4. Means of columns K to T: 0.682, 1.017, 0.687, 1.038, 0.707, 0.945, 1.446, 1.088, 1.532, and 0.857. SDs of columns K to T: 0.043, 0.035, 0.034, 0.031, 0.041, 0.038, 0.050, 0.069, 0.091, and 0.033. CV% of columns K to T: 6.3, 3.4, 4.9, 3.0, 5.8, 4.0, 3.5, 6.3, 5.9, and 3.9. Column headings: A and K, amoxicillin (2 μg/ml); B and L, amoxicillin (16 μg/ml); C and M, amoxicillin-clavulanic acid (2 μg/ml); D and N, amoxicillin-clavulanic acid (16 μg/ml); E and O, gentamicin (4 μg/ml); F and P, cephalothin (2 μg/ml); G and Q, cephalothin (16 μg/ml); H and R, imipenem (4 μg/ml); I and S, imipenem (16 μg/ml); J and T, norfloxacin (16 μg/ml). Underlined SND values exceed the ±1.97 rejection limits.

sulfadiazine. A full 15-cm plate was used to test each range, and each range was tested 10 times in a single run. Regression lines were constructed, and the standard deviation was calculated from the data. All concentrations of these antibiotics tested, with the exception of the 0.8- and 1.6-μg sulfadiazine disks, produced DZIs.

Narrow-range calibration curves used to establish the analytical sensitivity of the QCD method. New stock solutions were made up at a 50% higher concentration than the normal stock solution concentration used (Table 5). These stock solutions were diluted to prepare a series of antibiotic concentrations in solution. The following scheme of dilutions was used in all cases: for 150% concentration, 5 ml of solution and no distilled water; for 125% concentration, 5 ml of solution and 1 ml of distilled water; for 100% concentration, 2 ml of solution and 1 ml of distilled water; for 75% concentration, 1 ml of solution and 1 ml of distilled water; and for 50% concentration, 1 ml of solution and 2 ml of distilled water. Each concentration level of the antibiotic was tested in triplicate on a single 15-cm plate on two separate days. All testing was carried out under the same conditions, and all the DZIs were measured by the same operator. The calibration curves obtained from this study were used to calculate the acceptance and rejection limits of the test.

Testing procedure to establish the analytical sensitivity of the antibiotic plate method. Plates were prepared to contain the range of antibiotics shown in Table 4. One dilution above and below the target range was included for each antibiotic. Because the antibiotic concentrations of numerous plates were tested, it was necessary to use three seeded test plates for the complete range of tests. A plate containing the same set of concentrations of antibiotic was tested 10 times over a 2-week period. The DZIs of the routinely used antibiotic concentrations and of the concentrations above and below this were divided by the mean of the routinely used concentration DZIs to produce ZRs. The standard deviations of the ZRs were also calculated. A mean ± 2 standard deviations bar chart for the three concentrations was constructed from these results and examined for overlap of results (see Fig. 7).

Establishing base-line data for the plate testing method. Bottles containing 200 ml of agar were prepared to include the complete range of antibiotics and concentrations used in our laboratory. Ten agar plates (diameter, 9 cm) were poured from each of these bottles. The plates were stored at 4°C in plastic screw-top containers. Each of these plates was tested before being used for routine antibiotic susceptibility testing.

RESULTS AND DISCUSSION

Antibiotic solutions. In Fig. 1 we present calibration curve data for three antibiotics, cephalothin, sulfadiazine, and tetracycline, over very wide concentration ranges. It can be seen that the slopes of the curves became very flat at concentrations equal to and greater than 0.8 μg of cephalothin per disk, 64 μg of sulfadiazine per disk, and 4 μg of tetracycline per disk. The error bars shown on these graphs are equal to ±2 standard deviations, and each point represents the mean of 10 zone sizes. In our test system the commercial susceptibility disks produced by Oxoid (cephalothin, 30 μg per disk; sulfadiazine, 256 μg per disk; and tetracycline, 10 μg per disk) would theoretically give DZIs of 49, 29, and 33 mm, respectively, and a single experiment confirmed that these antibiotics gave DZIs of 52, 25, and 32 mm, respectively, for these antibiotics. Diameters of this size fall on the portion of the curve where the error bars

TABLE 10. DZI, ZR, and SND data for five antibiotics showing increase in CV% from DZI to ZR for cefoxitin[a]

Day	DZI Matrix					Means of Rows	Zone Ratio Matrix					Standard Normal Deviate Table				
	A	B	C	D	E		F	G	H	I	J	A	B	C	D	E
1	14.6	17.1	14.5	14.2	12.5	14.580	1.001	1.173	0.995	0.974	0.857	1.72	0.52	-1.31	-0.83	1.06
2	13.3	15.6	13.4	11.8	11.1	13.040	1.020	1.196	1.028	0.905	0.851	2.19	0.78	-0.56	-1.51	0.83
3	12.3	16.2	12.6	11.7	10.8	12.720	0.967	1.274	0.991	0.920	0.849	0.83	1.64	-1.40	-1.37	0.75
4	14.2	17.2	15.6	17.2	11.7	15.180	0.935	1.133	1.028	1.133	0.771	0.02	0.08	-0.56	0.73	-2.15
5	13.6	16.9	14.7	14.9	11.7	14.360	0.947	1.177	1.024	1.038	0.815	0.32	0.57	-0.65	-0.21	-0.52
6	13.1	16.6	13.6	12.9	11.5	13.540	0.968	1.226	1.004	0.953	0.849	0.85	1.11	-1.09	-1.04	0.76
7	13.3	16.9	14.2	14.7	11.8	14.180	0.938	1.192	1.001	1.037	0.832	0.09	0.73	-1.15	-0.22	0.13
8	12.1	16.4	15.1	14.7	11.6	13.980	0.866	1.173	1.080	1.052	0.830	-1.77	0.53	0.62	-0.07	0.04
9	13.7	18.0	15.7	13.4	12.4	14.640	0.936	1.230	1.072	0.915	0.847	0.03	1.15	0.45	-1.41	0.68
10	14.4	17.6	14.6	14.9	12.5	14.800	0.973	1.189	0.986	1.007	0.845	0.99	0.70	-1.49	-0.51	0.59
A	14.5	16.0	16.5	19.5	12.0	15.700	0.924	1.019	1.051	1.242	0.764	-0.28	-1.18	-0.04	1.81	-2.38
B	14.5	16.0	17.0	18.3	13.5	15.860	0.914	1.009	1.072	1.154	0.851	-0.52	-1.29	0.43	0.94	0.83
C	13.5	14.8	15.9	16.9	12.3	14.680	0.920	1.008	1.083	1.151	0.838	-0.38	-1.30	0.69	0.91	0.34
D	14.1	15.2	15.9	18.0	12.6	15.160	0.930	1.003	1.049	1.187	0.831	-0.11	-1.36	-0.09	1.27	0.09
E	14.3	15.5	16.1	17.5	12.4	15.160	0.943	1.022	1.062	1.154	0.818	0.22	-1.14	0.21	0.94	-0.40
F	13.5	15.0	16.8	17.6	12.0	14.980	0.901	1.001	1.121	1.175	0.801	-0.86	-1.37	1.55	1.15	-1.02
G	14.5	16.3	17.2	18.0	13.1	15.820	0.917	1.030	1.087	1.138	0.828	-0.46	-1.05	0.78	0.78	-0.03
H	12.8	15.9	14.9	13.7	12.0	13.860	0.924	1.147	1.075	0.988	0.866	-0.28	0.24	0.51	-0.69	1.37
I	13.5	17.5	16.5	15.0	12.0	14.900	0.906	1.174	1.107	1.007	0.805	-0.73	0.54	1.23	-0.51	-0.87
J	12.0	15.8	15.8	14.5	11.5	13.920	0.862	1.135	1.135	1.042	0.826	-1.86	0.10	1.86	-0.17	-0.10

[a] Means of columns A to E (DZI): 13.59, 16.32, 15.33, 15.47, and 12.05. SDs of columns A to E (DZI): 0.82, 0.89, 1.26, 2.26, and 0.64. CV% of columns A to E (DZI): 6.0, 5.4, 8.2, 14.6, and 5.3. Means of the columns F to J: 0.935, 1.126, 1.053, 1.059, and 0.829. SDs of columns F to J: 0.039, 0.090, 0.044, 0.101, and 0.027. CV% of columns F to J: 4.2, 8.0, 4.2, 9.5, and 3.3. Column headings: A and F, tetracycline (4 μg/ml); B and G, cefoxitin (16 μg/ml); C and H, trimethoprim (4 μg/ml); D and I, sulfadiazine (256 μg/ml); E and J, nalidixic acid (32 μg/ml). Underlined SND values exceed the ±1.97 rejection limits.

indicate considerable overlap. Hence, if these disks were used, it would be difficult to discriminate either the next lowest or the next highest concentration from the target concentration. We concluded that these commercial disks, although readily available, could not be used as a convenient source of QCDs.

In Table 6 we present data which illustrate that the CV% for DZIs (column 6) fell from a median of 7.6% (range, 3.7 to 18.1%) to a median of 2.2% (range, 1.3 to 4.7%) when the data were expressed as ZRs (column 8). This improvement was not due to the combination of statistical errors in the calculation of the ZRs. This calculation is shown in column 9 of Table 6. The calculation of the ZRs does not appear to be a statistical artifact (7). We concluded that the use of the ratio was a very effective means of minimizing those components of batch-to-batch variation which were not due to laboratory errors.

Having minimized the batch-to-batch variation by this technique, we evaluated the ZR measurement as a potential QC parameter. Our first task was to determine action limits for each antibiotic. We expected that these limits would be asymmetric, because the response curve of antibiotic concentrations (in micrograms per disk) on the x axis versus either the DZI or the zone ratio on the y axis is logarithmic and is of the form $y = a + b\log_e x$. Calibration curves were constructed from triplicate readings on two separate occasions at five concentration levels, namely, 50, 75, 100, 125, and 150% of the concentration normally used to impregnate the QCDs (Table 5) for each of the 11 antibiotics. For sulfadiazine these experiments were performed on four separate occasions, because this antibiotic gives highly variable DZIs. The data obtained for the 11 antibiotics are summarized in Table 7. Least-squares regression analyses of the data given for DZI versus $\log_e x$ were used to obtain the equations for the lines of best fit for the data expressed as concentration versus DZI and as the concentration of antibiotic in each QCD versus ZR.

TABLE 11. Improved CV% with problematic antibiotics using *B. subtilis* NCTC 8236 (ATCC 11774)

Antibiotic and concn (μg/ml)	DZI (CV%)	ZR (CV%)
Tetracycline		
1	9.7	9.3
4	11.4	9.5
Cefoxitin		
4	6.8	7.0
16	10.8	8.6
Sulfadiazine (25)	10.5	7.8
Trimethoprim (4)	9.6	9.1

Excellent fits were obtained, as indicated by the correlation coefficient, r, which at the $P = 0.001$ level of significance for 15 datum points is 0.7419. It was possible to pool the data from data sets A and B (Table 7) after they had been expressed as ZRs. This gave 30 datum points per calibration curve (60 datum points for sulfadiazine), and again the correlation coefficients confirmed an excellent agreement with the logarithmic model. For 30 datum points, $r = 0.5620$ is significant at the $P = 0.001$ level of significance. Figure 2 illustrates the graphs obtained for tetracycline (data sets A, B, and C) and sulfadiazine (data sets A, B, and E).

The equations for these calibration curves permitted us to calculate some practical QC action limits in ZR units, which could be used prospectively in our QC scheme. We arbitrarily elected to use warning limits of 75 and 125% and rejection limits of 50 and 150% of the target concentration of the antibiotic on the QCD. These limits are considerably more

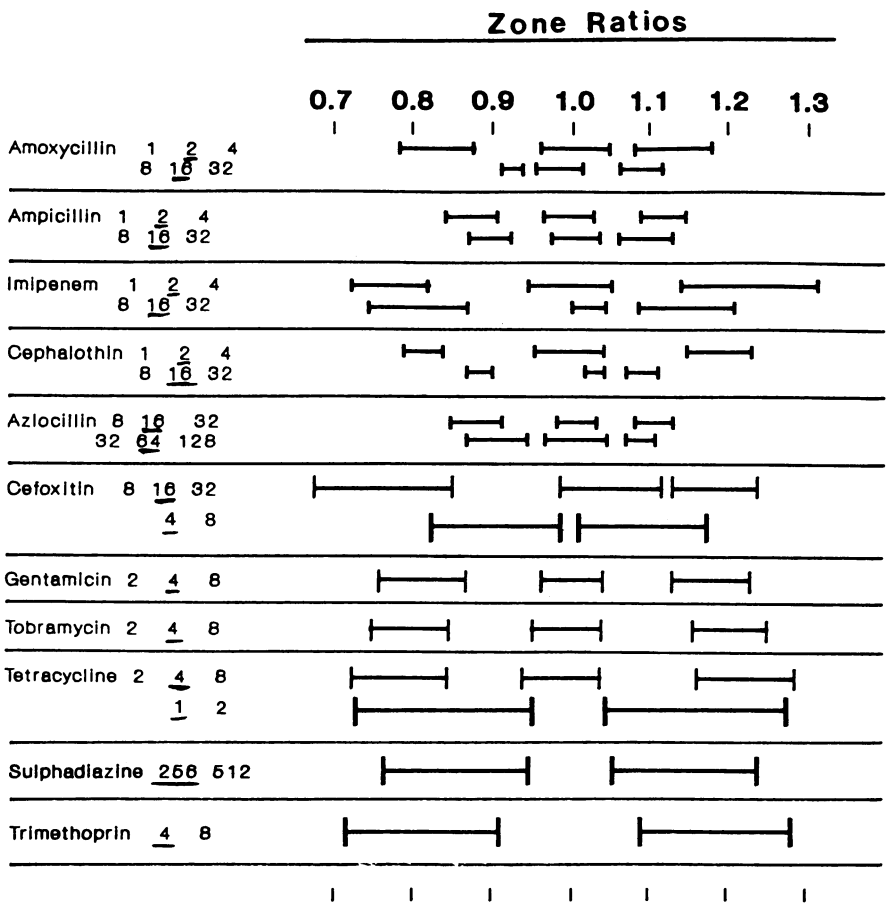

FIG. 7. Analytical sensitivity 2-standard-deviation bar chart for antibiotic plate testing. See text for calculation of ZR data. The numbers after the antibiotic names are the concentrations of the antibiotics (in micrograms per milliliter) incorporated in the plates. The underlined values are the concentrations incorporated into routinely produced plates.

stringent than those in the conventional MIC approach, which has acceptance limits of 50 and 200% and rejection limits of 25% below and 400% above the target MIC. The actual values of the ZR limits for each antibiotic were calculated by substituting values of 50, 75, 125, and 150% of the QCD concentrations (Table 7, column 2) into the regression equations. The results of these calculations are shown in Table 7, columns 7 to 10.

We have used these limits prospectively to evaluate the QC data accumulated for the 11 antibiotics over an 8-month period. Figure 3 illustrates the data we have collected for gentamicin; they are typical of the data we obtained for antibiotics giving the most highly reproducible results, i.e., amoxicillin, cephalothin, gentamicin, tobramycin, nalidixic acid, cefoxitin, norfloxacin, and azlocillin. The data for tetracycline, sulfadiazine, and trimethoprim are shown in Fig. 4, 5 and 6, respectively. The ZRs for these three antibiotics tended to be low, with means of 0.970, 0.928, and 0.966, respectively, probably because the solutions used to prepare the QCDs of batch A had 17 to 21% higher concentrations than anticipated when the stock solutions were produced. Some further support for this hypothesis was discernible in the data for trimethoprim (Fig. 6), which showed some improvement when QCDs of batch B were used. The four datum points for batch B had a mean of 0.994.

Comparison of ZRs and SNDs with the diameters of DZIs in antibiotic plates. Table 8 is a summary of a typical data set for gentamicin, tobramycin, azlocillin, imipenem, and norfloxacin at the various concentrations given in Table 3. The data for 10 base-line plates plus data from 10 routine assays are shown. Means, standard deviations, and CV% are given in the footnote. The CV% ranged from 5.3 to 10.6% for the DZI results. The corresponding ZR data are given in the second panel, and the individual statistics for ZR are also given in the footnote. A column-by-column comparison of the CV%s clearly demonstrated that the theoretical advantage of calculating ZRs was again realized in practice. These CV%s ranged from 4.1 to 9.9%. The panel of SND data could therefore be confidently scanned for items which exceeded the ±1.97 rejection limits. Eight items highlighted in this third panel exceeded these limits, indicating that the plates tested on days A and B were unacceptable. This conclusion was reached easily from the SND data but was harder to detect from the ZR data matrix and even less discernible from the DZI data matrix.

Table 9 presents the DZI, ZR, and SND data for amoxicillin, amoxicillin-clavulanic acid (Augmentin), gentamicin, cephalothin, imipenem, and norfloxacin at the concentrations given in Table 3. Again, a column-by-column comparison of the CV%s substantiated the theoretical advantage of calculating the ZRs. For this set of six antibiotics, 9 of the 10 columns of data showed improvements in the CV%s of between 0.3 and 4.2%. However, no apparent improvement was shown for norfloxacin, and this may be attributed to the

exceptionally good DZI CV% result obtained for this antibiotic. The SND data showed several instances when the value was equal to or greater than the ±1.97 warning limits. Three of these occurred singly on days 9, A, and F. Days H and J both had two values outside these warning limits. This appeared to be a relatively high warning rate and was one of the reasons we chose to retain seemingly unacceptable data, such as those for imipenem at 16 μg/liter on day 9, within the 10 base-line data sets. Their intentional inclusion was designed to reduce the sensitivity of this QC test to realistic and practical levels. When batches of plates which had exceeded the warning limit were retested, the results almost invariably fell within the acceptance limits, confirming this observation of oversensitivity. This is illustrated by the fact that the probability of a deviation of ±1.97 is $P = 0.025$ and the probability of two consecutive deviations of ±1.97 is $P = (0.025)^2 = 0.0006$. An example of a test failure is shown in Table 8, rows A and B, for azlocillin plates with concentrations of 16 and 64 mg/liter.

The data in Table 10 illustrate a further feature of this QC approach. In Tables 8 and 9 there was an improvement in the CV%s of the ZR compared with the DZI data, but the data in Table 10 demonstrate that this is not always the case. The CV%s for the cefoxitin data increased from 5.4% for the DZI data to 8.0% for the ZR data. This was unexpected, and a further examination revealed that this phenomenon was due to the highly variable data for trimethoprim and sulfadiazine (CV% = 8.2 and 14.6% respectively), which distorted the results for cefoxitin. If the columns of data for trimethoprim and sulfadiazine were omitted and the data for tetracycline, cefoxitin, and nalidixic acid were retained and processed again, the following satisfactory results for the CV% of the ZR data were obtained: tetracycline, 3.5%; trimethoprim, 4.4%; nalidixic acid, 3.6%. Hence, in all cases, the CV%s of the ZR data were reduced, as was previously anticipated.

Further work has revealed that the indicator organism used in this study may not be optimal for testing for trimethoprim and sulfadiazine. Use of *B. subtilis* NCTC 8236 (ATCC 11774) for a similar panel of antibiotics which included the problematic sulfadiazine and trimethoprim provided more satisfactory results (Table 11).

Analytical sensitivity. Figure 7 presents a graphical summary of the data collected for the 13 antibiotics in the plates examined in this final part of the study. When using the ZR method, it was quite clear that it was possible to distinguish between the target concentration of any of these antibiotic plates and one dilution above or below this target concentration. The statistical confidence with which we could make this distinction was better than $P = 0.05$ for the four antibiotics sulfadiazine, trimethoprim, cefoxitin, and tetracycline; i.e., we were 95% confident that we would be able to identify differences. It was possible to detect errors in one direction only, i.e., one dilution below their target concentrations.

Conclusions. QC should be easy to perform, inexpensive, and reproducible. These were our priorities in designing the methods; however, it would appear that in the case of the QCDs, we have achieved improvements in analytical precision. The methods are simple, do not involve complicated procedures, and can be easily incorporated into the routine work of the laboratory.

The criteria used for assessment of antibiotic solution QC are quantitative and are suitable for cumulative assessment in graphical form. We have developed a computer program to assist with the filing and display of the results to encourage the ready acceptance of this method in the busy routine of the laboratory. Should the method gain wide acceptance, a commercial source of QCDs would further simplify the adoption of this approach in smaller laboratories and facilitate both interlaboratory QC surveys and method development.

The criteria used for the assessment of antibiotic plates are qualitative and do not assay the antibiotic concentration contained in the agar; however, they do detect abnormal fluctuations within the normal deviations of the method. This method of QC is a further extension of the ZR technique applied to the QC of antibiotic susceptibility testing plates. It is simple to apply and provides a uniform criterion, the SND, on which to make QC decisions which have statistical respectability and confidence. It can be easily incorporated into a computerized QC data recording system and should be applicable to the particular antibiotic susceptibility testing panels in any laboratory, provided that care is taken to recognize that inclusion of antibiotics which are known to have highly variable performance characteristics may degrade the overall performance and potential of this approach.

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