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Methods of Measuring Zones of Inhibition with the Bauer-Kirby Disk Susceptibility Test

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Standard Bauer-Kirby disk tests were performed with 85 selected isolates, each tested in triplicate by four different investigators. Each disk test was observed, and zone diameters were measured, under two lighting conditions (transmitted light and reflected light). The two lighting systems produced similar zone measurements (±2 mm) with 96% of the tests. When there were greater differences, zones appeared to be larger when observed with reflected light. Interlaboratory reproducibility was much greater when using reflected light rather than transmitted light. We concluded that zone diameters should be measured from the back of the plate while it is resting on, or held 2 to 3 inches [ca. 5.1 to 7.6 cm] above, a black, nonreflecting, flat surface, illuminated by a reflected light source.

In 1966, Bauer et al. (1) published a detailed description of a standardized single-disk method for performing the antimicrobic susceptibility test. This procedure has been widely accepted as the preferred reference method. In the original manuscript (1), zones of inhibition were to be measured by holding a ruler on the underside of the petri dish or with calipers held next to the agar surface; the angle and source of light were not specified.

In 1975, the National Committee for Clinical Laboratory Standards defined the method of Bauer et al. in somewhat greater detail (2). In that document, zone diameters were to be measured to the nearest whole millimeter by holding a ruler or sliding calipers on the back of the petri dish, illuminated with reflected light. The Committee's document goes on to state that systems using transmitted light may be used if the expected zone sizes are obtained with quality control microorganisms.

A recent collaborative study provided the opportunity to determine whether the method of illiminating the test plates could affect the zone measurements. Four investigators each tested 85 selected isolates in triplicate. All zones were measured with two lighting conditions (reflected light and transmitted light). Differences in zone measurements obtained with the two lighting systems were documented, and inter- and intralaboratory variability was evaluated and compared.

MATERIALS AND METHODS

Bacterial strains. The 85 study strains included in this report were selected and distributed by C. Thornsberry and C. N. Baker (Center for Disease Control, Atlanta, Ga.). The isolates included 25 *Escherichia coli*, 15 *Klebsiella pneumoniae*, 12 *Proteus mirabilis*, 1 *Proteus vulgaris*, 2 *Proteus rettgeri*, 2 *Proteus morganii* (*Morganella morganii*), 3 *Providencia stuartii*, 1 *Providencia alcalifaciens*, 3 *Serratia marcescens*, 1 *Serratia rubidea*, 1 *Enterobacter cloacae*, 1 *Enterobacter hafniae*, 1 *Citrobacter diversus*, 1 *Salmonella enteritidis*, 1 *Acinetobacter calcoaceticus* var. *anitratus*, 5 *Pseudomonas aeruginosa*, 8 *Staphylococcus aureus*, and 2 *S. epidermidis*. In addition, subcultures of *E. coli* (ATCC 25923), *S. aureus* (ATCC 25922), and *P. aeruginosa* (ATCC 27853) were distributed for quality control purposes.

Disk test procedures. Each investigator tested each isolate on three separate days, using the procedure outlined by the National Committee for Clinical Laboratory Standards (2). All antimicrobial disks and Mueller-Hinton agar plates were provided from a common source. Each investigator was instructed to measure all zones of inhibition by two different methods (illuminated by reflected light and by transmitted light). The lighting source was defined as follows.

(i) Reflected light. The test plates were placed, medium side up, on a black, nonreflecting surface and were illuminated with reflected light from a desk lamp. One investigator held the test plates 2 to 3 inches (ca. 5.1 to 7.6 cm) above the black surface to expedite zone measurement. The zones were then measured by holding a metric ruler against the back of the petri plate. The present study excludes strains which require the addition of blood to the agar medium; in that situation,

the zones would have to be measured from the surface, illuminated with reflected light and with the cover removed.

(ii) Transmitted light. The test plates were held in front of a desk lamp, and the zones were measured with a ruler held against the back of the petri plate.

In either situation, the diameters of the zones of inhibited growth were measured to the nearest whole millimeter, including the diameter of the 0.25-inch (6.35-mm) disk. If there was no inhibition, a zone of 6 mm was recorded. The endpoint was to be taken as the area showing no obvious growth that could be detected with the unaided eye, not including a faint haze of growth or tiny colonies which can be detected only with difficulty at the edge of the zone of inhibited growth. If a few large colonies appeared within an otherwise clear zone of inhibition, contamination was suspected, and the test was repeated after checking for purity. When testing swarming *Proteus* spp., the investigators were instructed to ignore a thin veil of swarming growth inside an otherwise clearly defined zone of inhibition.

Statistical analysis. Standard deviation estimates for intra- and interlaboratory variability were derived by using variance component analysis (3). This allows the overall experimental error to be partitioned into various sources of variability. The sources of variability that affect observations in this study include (i) the variability of responses for different tests with different strains within each group of related microorganisms, (ii) variability due to repeated observations in the same clinical laboratory, and (iii) the variability observed between clinical laboratories evaluating the same isolate.

RESULTS

Differences in zone diameters. A total of 12,011 pairs of zone measurements were available for analysis. Forty-seven tests were omitted because of incomplete report forms submitted by the investigators. In addition, 182 tests with ampicillin disks were not performed because of an early supply problem. Zone measurements obtained with the two lighting systems were compared directly (Tables 1 and 2). Mean differences between the two types of measurements were all less than 1.0 mm. Correlation coefficients calculated for each antimicrobial agent varied from 0.88 to 0.99. No differences were seen with 52% of the tests, and 96% of the tests displayed differences of ≤2 mm. When there was a discrepancy, the zones observed with reflected light tended to be larger than those recorded with transmitted light. Chloramphenicol accounted for one-third of the major discrepancies (differences, ≥4 mm), and another third involved tests with the aminoglycosides or trimethoprim/sulfamethoxazole. The type of microorganism being tested did not appear to influence the comparison of zone measurements (Table 2). The number of tests with no differences would have been reduced somewhat if we had eliminated all tests with no zone of inhibition. The number of tests with discrepancies would not be

TABLE 1. *Direct comparison of zone diameters observed with reflected light versus transmitted light; 17 antimicrobial agents*

Drug	No. of tests	No. of tests with differences in zone diameters[a]: ≤−4 mm	−3 mm	−2 mm	−1 mm	0 mm	+1 mm	+2 mm	+3 mm	≥+4 mm	Mean difference (±SD)[b]
Amakacin[c]	895	0	1	5	93	449	267	60	15	5	0.4 ± 0.91
Ampicillin	837	0	0	10	72	572	151	23	3	6	0.2 ± 0.78
Carbenicillin[c]	897	2	0	19	135	443	206	67	14	11	0.3 ± 1.17
Cephalothin	1,015	2	2	26	120	614	209	32	6	4	0.1 ± 0.95
Chloramphenicol	1,016	1	2	28	125	405	233	122	53	47	0.7 ± 1.47
Clindamycin[d]	119	0	0	5	7	63	30	10	1	3	0.4 ± 1.07
Erythromycin[d]	119	0	0	2	8	71	22	10	4	2	0.4 ± 1.03
Gentamicin	1,016	0	2	18	115	426	296	107	34	18	0.5 ± 1.12
Kanamycin	1,016	0	0	19	92	520	241	90	40	14	0.5 ± 1.09
Nalidixic acid[c]	897	1	0	21	119	493	221	36	4	2	0.2 ± 0.92
Nitrofurantoin[c]	898	2	1	14	93	501	236	38	9	4	0.2 ± 0.88
Oxacillin[d]	119	0	0	0	11	77	24	2	5	0	0.3 ± 0.82
Penicillin[d]	119	0	0	1	9	80	26	2	1	0	0.2 ± 0.66
Tetracycline	1,014	0	0	8	83	613	196	78	28	8	0.4 ± 0.95
Trimethoprim/sulfamethoxazole	1,017	2	1	21	135	479	269	71	25	14	0.4 ± 1.13
Tobramycin[c]	898	1	1	13	126	378	253	87	29	10	0.4 ± 1.14
Vancomycin[d]	119	0	0	3	21	64	31	0	0	0	0.0 ± 0.74

[a] Zone diameter observed by reflected light minus zone diameter observed with transmitted light; + values = reflected light larger; − values = reflected light smaller. Expressed as number of tests in each category.

[b] SD, Standard deviation.

[c] Tested against 75 gram-negative bacilli only; each isolate tested 12 times.

[d] Tested against 10 *Staphylococcus* spp. only; each isolate tested 12 times.

TABLE 2. *Direct comparison of zone diameters observed with reflected light versus transmitted light; different microorganism groups*

Genus	No. of tests	Percent of tests with differences[a] in zone diameters:									Mean difference (±SD)[b]
		≤−4 mm	−3 mm	−2 mm	−1 mm	0 mm	+1 mm	+2 mm	+3 mm	≥+4 mm	
Escherichia	3,438			0.7	12.0	49.4	25.6	7.3	3.3	1.6	0.4 ± 1.07
Klebsiella	2,116			0.8	7.0	47.2	29.4	11.0	3.3	1.3	0.6 ± 1.03
Enterobacter	288			2.4	11.1	44.8	24.7	12.5	4.5		0.5 ± 1.07
Serratia	575		0.5	3.5	12.3	48.0	25.0	7.5	1.7	1.4	0.3 ± 1.11
Proteus											
P. mirabilis	1,724	0.5	0.3	3.2	14.8	50.9	23.0	5.5	0.6	1.0	0.2 ± 1.21
Other species	720	0.1		0.7	8.8	55.4	24.9	6.0	1.9	2.2	0.4 ± 1.06
Providencia	574			4.2	15.2	54.9	19.9	4.2	0.5	1.2	0.1 ± 1.16
Pseudomonas	720			0.7	6.9	80.0	10.7	1.0	0.6	0.1	0.1 ± 0.56
Other gram-negative bacilli	429			2.6	16.9	52.2	21.0	5.4	2.1		0.2 ± 0.93
Staphylococcus											
S. aureus	1,139			2.3	12.3	55.0	23.1	5.6	1.4	0.3	0.2 ± 0.89
S. epidermidis	288			6.6	10.8	44.1	25.7	5.9	2.8	4.2	0.4 ± 1.35
Total no. of tests	12,011	11	10	213	1,364	6,248	2,911	835	271	148	
Total %		0.1	0.1	1.8	11.4	52.0	24.2	7.0	2.3	1.2	

[a] Zone diameters observed by reflected light minus zone diameters observed with transmitted light; + values = reflected light larger; − values = reflected light smaller. Expressed as percentage of tests within each microorganism group.

[b] SD, Standard deviation.

affected significantly by eliminating such "no zone" responses.

Precision of zone measurements. Each isolate was tested on 3 separate days by each of four investigators. Consequently, we could compare intra- and interlaboratory variability of the two measuring systems.

Intralaboratory variability was calculated by comparing triplicate tests reported by each investigator. Table 3 expresses the results as the average standard deviation for each group of related microorganisms. The reproducibility of the two measuring methods was essentially the same, i.e., each laboratory could reproduce its own results with either method of illumination.

Interlaboratory variability was calculated by comparing the mean zone diameter recorded by each of the four participants. Since these data compare the means of triplicate determinations, somewhat greater precision might be anticipated. Table 3 summarizes the results, expressed as the standard deviation for each group of related microorganisms. The greatest variability between laboratories was observed with *P. mirabilis*. With other microorganisms, zones observed with reflected light were much more reproducible than those measured with transmitted light.

Quality control tests. The results of replicate tests with three control strains are summarized in Table 4. The mean zone diameters were 0 to 1 mm larger when measured with reflected light versus transmitted light. Standard deviations calculated for results obtained with the two lighting systems were essentially the same. At least with the three control strains, precision of the two measuring techniques did not differ greatly.

DISCUSSION

For the sake of standardization, every aspect of the disk diffusion susceptibility test must be defined and carefully controlled. The present report documents the fact that zone measurements can be influenced by the method of illuminating the test plates. Although most microorganism-drug combinations were not greatly affected, some tests demonstrated rather profound differences. Tests with the three standard control microorganisms were not markedly affected by the method of measuring zone diameters.

At this time, we cannot determine which measuring procedure produces the most accurate results; we can only document the fact that there may be some rather large differences. Precision of zone measurements was improved when the test plates were observed with reflected light, and for that reason reflected light is recommended. We found no differences between zones measured with the test plate lying flat on the work bench and those measured with the test

TABLE 3. *Intra- and interlaboratory variability in zone diameters observed with reflected light versus zones seen with transmitted light*

Genus	Intralaboratory variability[a]		Interlaboratory variability[a]	
	Reflected	Transmitted	Reflected	Transmitted
Escherichia	±1.91	±1.99	±1.56	±2.40
Klebsiella	±1.97	±1.91	±1.90	±2.55
Enterobacter	±1.99	±1.90	±2.75	±3.53
Serratia	±2.35	±2.22	±1.68	±2.25
Proteus				
P. mirabilis	±2.55	±2.63	±3.54	±3.37
Other species	±2.34	±2.30	±1.76	±2.15
Providencia	±2.08	±2.08	±1.82	±2.49
Pseudomonas	±1.81	±1.80	±1.13	±1.55
Other gram-negative bacilli	±2.09	±2.04	±1.43	±2.38
Staphylococcus				
S. aureus	±2.01	±1.97	±1.49	±1.80
S. epidermidis	±2.34	±2.27	±1.46	±2.37

[a] Expressed as ±2 standard deviations (millimeters) to represent the approximate 95% confidence limit for a single observed value.

TABLE 4. *Comparison of zone diameters observed by either a reflected light or a transmitted light source for quality control organisms*

Drug	Zone diameters[a] (mm) (reflected light/transmitted light)					
	E. coli (ATCC 25922)		*P. aeruginosa* (ATCC 27853)		*S. aureus* (ATCC 25923)	
	Mean	SD	Mean	SD	Mean	SD
Amikacin	19.1/18.5	0.72/0.77	17.4/17.1	0.84/1.06	NT[b]	
Ampicillin	18.5/17.9	1.07/1.16	NZ/NZ[c]		31.5/29.8	1.34/1.15
Carbenicillin	24.7/24.2	0.97/1.00	19.7/19.5	0.97/1.08	NT	
Cephalothin	19.5/19.4	1.27/1.37	NZ/NZ		33.6/31.8	1.52/1.03
Chloramphenicol	23.5/22.7	0.97/1.18	NZ/NZ		24.3/23.4	1.23/1.14
Clindamycin	NT		NT		26.5/25.5	1.13/0.83
Erythromycin	NT		NT		27.1/26.0	1.16/1.36
Gentamicin	22.2/21.2	0.58/0.88	18.0/18.0	0.77/0.81	23.2/22.3	0.98/0.92
Kanamycin	21.8/20.7	0.92/1.08	NZ/NZ		22.4/21.9	0.84/0.87
Nalidixic acid	23.6/23.4	1.04/1.00	NZ/NZ		NT	
Nitrofurantoin	21.6/21.5	0.83/0.99	NZ/NZ		NT	
Oxacillin	NT		NT		21.4/20.8	0.91/0.96
Penicillin	NT		NT		31.8/31.1	1.51/1.06
Tetracycline	22.9/22.1	1.28/1.37	11.8/11.7	1.13/1.11	27.9/26.9	1.18/1.12
Tobramycin	20.9/19.9	0.84/0.92	21.6/21.4	0.93/0.91	NT	
Trimethoprim/sulfamethoxazole	26.4/26.3	0.99/0.93	NZ/NZ		29.3/28.9	1.14/1.36
Vancomycin	NT		NT		17.7/17.4	0.53/0.47

[a] Based on 59 tests with *E. coli*, 60 tests with *P. aeruginosa*, and 24 tests with *S. aureus*.
[b] NT, This antimicrobial agent was not tested with this organism.
[c] NZ/NZ, No zone of inhibition present for either light source.

plate held 2 to 3 inches (ca. 5.1 to 7.6 cm) above the black surface.

The reasons for the differences in zone measurements are not always obvious. In most cases, there is a faint inner ring of growth just inside an otherwise well-defined zone of inhibition. This inner haze of growth may be seen when the plate is held in front of a desk lamp but is not seen when the plate is observed with reflected light and a black background. Consequently, zones observed with reflected light might appear to be 1 mm to 4 mm larger than those observed with transmitted light. This type of response is particularly common with chloramphenicol and other bacteriostatic drugs. Because reflected light tends to identify the somewhat better-de-

fined zone edges, the measurements tend to be more reproducible. Within a given laboratory, precise zone measurements can be performed with transmitted light, but between different individuals, zones measured with reflected light are more reproducible, i.e., there is a significant subjective element in selecting the appropriate endpoint.

Tests with *Proteus* spp. present a unique problem since swarming growth frequently obliterates the zone edge, and consequently precise zone measurements are very difficult to determine. With either method of illumination, zones of inhibition with *Proteus* spp. are difficult to measure reliably.

As a result of this study, we recommend that disk susceptibility test plates should be examined while resting on, or held less than 3 inches (ca. 7.6 cm) above, a black, nonreflecting, flat surface, and zones should be measured with a reflected light source.

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LITERATURE CITED

1. **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.
2. **National Committee for Clinical Laboratory Standards.** 1975. Performance standards for antimicrobial disc susceptilibity tests. National Committee for Clinical Laboratory Standards, Villanova, Pa.
3. **Searle, S. R.** 1971. Linear models, p. 376–420. John Wiley and Sons, Inc., New York.