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Rapid, Modified Kirby-Bauer Susceptibility Test with Single, High-Concentration Antimicrobial Disks

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A rapid (6–7 hr), modified Kirby-Bauer disk-susceptibility method, by which derivatives of tetrazolium dyes are used to enhance delineation between areas of growth and zones of inhibition, has been developed. Inoculated petri plates, prepared by the Kirby-Bauer method, were sprayed, after 6 to 7 hr of incubation (37 C), with aqueous solutions of MTT-tetrazolium or INT-tetrazolium resulting in readily detectable zones of inhibition. Excellent correlation was obtained between the modified test and the standard Kirby-Bauer test when challenged with a variety of gram-negative bacteria and *Staphylococcus aureus* strains. Additionally, the modified test has demonstrated reproducibility comparable to the standard Kirby-Bauer test. It is demonstrated that the modified test is applicable to susceptibility determinations with representative, commercially available antimicrobial disks. This applicability indicates that the modified method could provide rapid in vitro guidelines for in vivo therapy.

The standard Kirby-Bauer in vitro disk-susceptibility method, when performed and evaluated correctly, has been extremely useful as a guide in choosing the antimicrobial agent best suited for in vivo therapy of infections due to gram-negative bacteria and staphylococci (1, 2). Recently, the Food and Drug Administration has recommended the Kirby-Bauer technique as a standardized procedure for the determination of antimicrobial disk susceptibilities (3). Recommended specifications have included standardization of the size and method of inoculation, standardization of the type and depth of agar, and utilization of specific types of petri plates.

General acceptance of the in vitro disk-susceptibility method has been aided by its simplicity and rapidity (1, 2, 4). However, the prolonged incubation interval required (18–20 hr) between the determination of susceptibility in vitro and the utilization of the antimicrobial agent in vivo has remained a distinct disadvantage. Recently, our laboratory has been examining the ability of various tetrazolium dyes to enhance the distinction between areas of bacterial growth and zones of inhibition produced by antimicrobial agents. In the course of these studies, we considered the potential application of this technique to in vitro disk susceptibility determinations. With this in mind, we examined the susceptibilities of a number of gram-negative bacteria and staphylococci by a rapid, modified Kirby-Bauer disk-susceptibility method. This report demonstrates that the bacterial susceptibilities determined by the modified method correlate extremely well with those obtained by the standard Kirby-Bauer method. The potential utilization of the modified method as a guideline for rapid in vivo therapy is discussed.

MATERIALS AND METHODS

Organisms. The organisms employed in the present study were clinical isolates obtained from the Albany Medical Center, Albany, N.Y., and the Veterans' Administration Hospital, Albany, N.Y. Some of the *Escherichia coli* and *Serratia marcescens* strains were obtained from the Sterling-Winthrop Research Institute Culture Collection.

Disk-susceptibility tests. Disk susceptibilities were determined by the Kirby-Bauer technique as recommended by the Food and Drug Administration (1, 2). Upon repeated testing, we have found that dilution of the overnight culture under test to an optical density of 0.10 (650 nm), with a Spectronic 20 spectrophotometer (Bausch & Lomb) is equivalent to the barium sulfate solution recommended for standardization of the inoculum. Hence, in most of our studies we used cultures adjusted to an optical density of 0.10 (650 nm) as the standard inoculum.

Commercial antibiotic disks were obtained from

Difco Laboratories, Detroit, Mich. Nalidixic acid disks were purchased from BBL Laboratories, Cockeysville, Md. Ampicillin, chloramphenicol, colimycin, and gentamicin disk potencies were 10, 30, 10, and 10 μg/disk, respectively. Disks (30 μg) of tetracycline, neomycin, nalidixic acid, and novobiocin were used. Where indicated, 15-μg erythromycin disks were used. All commercial disks were stored as recommended by the manufacturer and were used within 6 months of the purchase date.

Modified disk susceptibility test. Basically, the only modification of the standard Kirby-Bauer technique was in the period of incubation. Triplicate sets of inoculated plates, containing the antibiotic (or drug) disks under study, were prepared by the Kirby-Bauer technique. All plates were incubated at 37 C and, after 6.5 hr, two of the plates in each set were removed from the incubator and treated as follows. One set of plates was sprayed with an aqueous solution of 0.50% MTT-tetrazolium. The second set of plates was sprayed with an aqueous solution of 0.20% INT-tetrazolium. Utilization of an aerosol spray can (Sprayon Jet Pak, Sprayon Products, Inc., Cleveland, Ohio) resulted in a fine mist which prevented flotation of the disks and "puddle" formation on the plates. After 5 to 10 min, dye reduction resulted in a blue (MTT) or red (INT) color where growth occurred on the plate. Color development provided delineation of distinct zones of inhibition even when growth was light due to the shortened period of incubation. Within 15 to 20 min after removal from the incubator, the zones of inhibition could be measured with a caliper. This method is referred to as the "modified" Kirby-Bauer test. The third set of plates was maintained at 37 C overnight, and the zones of inhibition were measured with a caliper. This latter procedure is that recommended in the Kirby-Bauer technique and is referred to as such in the present study for comparison with the modified Kirby-Bauer test.

Statistical analysis. The degree of association, and significance thereof, between the modified and standard Kirby-Bauer methods was determined by utilizing a single antimicrobial agent, gentamicin. We used the Spearman rank-correlation coefficient (6) and treated the data as responses (zone diameters in millimeters) obtained by two independent test methods. This nonparametric measure of association was used in lieu of a statistically more powerful, though often inappropriate, parametric measure for a specific reason. An underlying assumption made when calculating a parametric correlation coefficient is that the zonal responses for both the modified and standard Kirby-Bauer methods are normally distributed. Because the distribution of zonal responses is highly dependent upon the selection of chemotherapeutic agents and their effect on the test organism, this important criterion could not always be met. Consequently, nonparametric tests were employed.

Chemicals. MTT-tetrazolium [3(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] and INT-tetrazolium [2-(*p*-iodophenyl)3-*p*-nitrophenyl-5-phenyl tetrazolium chloride] were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Disk susceptibility of E. coli ATCC 25922. The susceptibility of *E. coli* ATCC 25922 (the bacterium recommended to validate control of the sensitivity test) to nine antimicrobial agents was readily detectable when tested by the modified technique (Fig. 1). After 6.5 hr of incubation at 37 C, zones of inhibition were as definitive when sprayed with 0.20% INT-tetrazolium (Fig. 1A) or 0.50% MTT-tetrazolium (Fig. 1B) as when incubation was carried out overnight at 37 C, as in the standard Kirby-Bauer method (Fig. 1C). Incubation of inoculated plates for periods of less than 6 to 7 hr resulted in greater variation in zone diameters and less definitive demarcation between zones of inhibition and areas of growth.

The disk susceptibility of *E. coli* ATCC 25922 determined by the modified test was comparable to that determined by the Kirby-Bauer test (Fig. 2). The zones of inhibition produced by eight antimicrobial agents were in excellent agreement when measured by the modified test and the Kirby-Bauer test. The responses to seven antibiotics, determined by the modified test, fell within the range of zone sizes (vertical bars) anticipated for the control organisms used with the standard Kirby-Bauer test (1). Since no range of zone sizes for nalidixic acid is recommended by the Food and Drug Administration, the vertical bar illustrated simply represents the lower limit for categorizing the organism as susceptible (19 mm or more) according to the interpretation data of Bauer et al. (2).

Similar results were obtained when *Staphylococcus aureus* ATCC 25923 was tested by the modified test and the Kirby-Bauer method (Fig. 3). As observed for *E. coli*, the disk susceptibilities of *S. aureus* determined by the modified test and the Kirby-Bauer test were in excellent agreement. Again, the responses measured by the modified test were well within the range of zone sizes expected for these antibiotics against the control reference organism (1).

The reproducibility of the modified Kirby-Bauer test was comparable to that of the standard Kirby-Bauer test when daily susceptibilities were determined with *E. coli* ATCC 25922 (Table 1). The high degree of reproducibility of the modified method, where evaluated, indicates that this technique demonstrates a degree of control equivalent to that of the standard Kirby-Bauer method. When the modified method was used, the

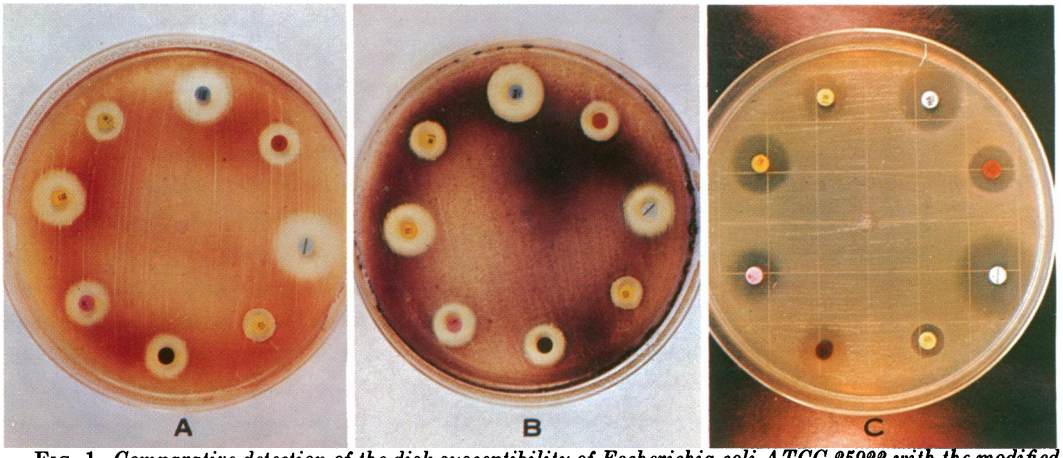

FIG. 1. *Comparative detection of the disk susceptibility of Escherichia coli ATCC 25922 with the modified Kirby-Bauer method and the standard Kirby-Bauer method. A, Modified Kirby-Bauer with 0.2% INT-tetrazolium; B, modified Kirby-Bauer with 0.5% MTT-tetrazolium; C, standard Kirby-Bauer method.*

maximum deviation from the mean zone diameter occurred with kanamycin (7% standard deviation, range 18.6 to 20.3 mm).

Susceptibility of E. coli strains. All of the strains categorized as susceptible to neomycin by the Kirby-Bauer test fell within the sensitive range when tested by the modified method (Fig. 4). Conversely, those organisms resistant to neomycin by the Kirby-Bauer method were resistant in the modified test. Similar results were obtained when colimycin and tetracycline susceptibility disks were used.

By the modified method, 93.3% of the *E. coli* strains challenged with neomycin disks gave zones of inhibition within 2 mm of those obtained when the Kirby-Bauer method was used (Fig. 4). Among these, 26.3% gave no deviation, whereas 47 and 20% deviated by 1 and 2 mm, respectively, from the zones obtained by the standard Kirby-Bauer method. Three strains (6.7%) demonstrated a deviation in zones of inhibition of 3 mm when tested by the two methods.

Similar results were obtained when colimycin disks were employed. The maximum deviation in zone response from the standard Kirby-Bauer method (2 mm) occurred with a single strain. Among the 44 remaining strains tested for susceptibility, 61% of the responses obtained were of equal zone diameter and 39% deviated by 1 mm.

With tetracycline disks, the maximum deviation from the standard Kirby-Bauer test was a 2-mm zone of inhibition. Of the organisms inhibited in the modified test, 50% had zone diameters equivalent to those obtained in the standard Kirby-Bauer test; 37 and 13% of the responses obtained in the modified test deviated by 1 and 2 mm, respectively, from those obtained in the Kirby-Bauer test.

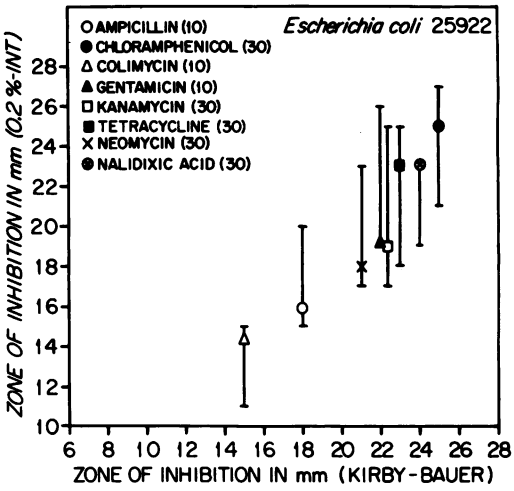

FIG. 2. *Comparative disk susceptibility of E. coli ATCC 25922 to eight antimicrobial agents with the standard Kirby-Bauer method and the modified Kirby-Bauer method (0.2% INT).*

Colimycin, neomycin, and tetracycline were tested here because organisms susceptible to these agents provide a definitive range of susceptibility responses from low zone sizes to high zone sizes. Susceptibility to colimycin is indicated by zone diameters of 11 mm or more, whereas neomycin and tetracycline susceptibility results in zone diameters of 17 mm or more and 19 mm or more, respectively.

Bacterial susceptibility to gentamicin. The susceptibilities determined by the modified Kirby-Bauer test and those obtained by the standard Kirby-Bauer test were significantly correlated ($r = 0.90$, $P < 0.001$) when a variety of gram-negative bacteria and *S. aureus* were challenged with gentamicin disks (Fig. 5). All of

the organisms tested by the Kirby-Bauer method fell into the susceptible category (13 mm or more). Similarly, all of the organisms were inhibited to zone diameters of 13 mm or more when tested by the modified Kirby-Bauer test. Among individual responses to gentamicin, the maximum deviation between the two methods was 4 mm. However, this is of minor concern since the variation in zone size within a given category should not influence the interpretation.

Comparative distributions of gram-negative bacteria and S. aureus. Excellent agreement was obtained between the modified Kirby-Bauer test (0.20% INT) and the standard Kirby-Bauer test when the susceptibilities of gram-negative bacteria and *S. aureus* to 10 antimicrobial agents were determined (Table 2). Where shifts in distribution did occur, the differences observed were based on 2- to 3-mm variations in zone diameters. In most cases, the changes in susceptibilities involved a redistribution from resistant to intermediate susceptibility or from sensitive to intermediate susceptibility.

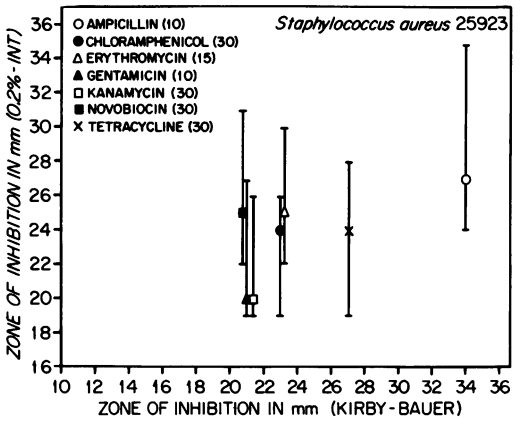

FIG. 3. *Comparative disk susceptibility of S. aureus ATCC 25923 to seven antimicrobial agents with the standard Kirby-Bauer method and the modified Kirby-Bauer method (0.2% INT).*

Less agreement was obtained between the modified test and the Kirby-Bauer test when 0.50% MTT-tetrazolium was employed in the modified test (Table 3). When tested by the modified method, 17% of the bacteria were resistant to chloramphenicol, whereas 29% were resistant when tested by the Kirby-Bauer method. Similarly, the distribution of organisms susceptible to kanamycin decreased 12% when tested by the modified technique, and intermediate susceptibility to neomycin increased to 65%.

DISCUSSION

The present results demonstrate the feasibility of utilizing the modified (0.20% INT-tetrazolium) Kirby-Bauer test for the determination of susceptibilities of bacteria to antimicrobial disks. The major advantage gained by this minor modification is the ability to determine susceptibilities within 6.5 hr after initiation of the test. This rapid determination of bacterial susceptibility would decrease significantly the time required to choose that antimicrobial agent best suited for therapy of infected patients. This would be especially suited in cases of severe septicemia where "follow-up" therapy may be required after initial, immediate therapy without susceptibility testing.

The modification employed should not alter the standards recommended by the Food and Drug Administration, since there is no inclusive change in the specifications designated for the Kirby-Bauer method. That is, no modification of inoculum standardization, medium, agar depth, etc., is employed. The dyes employed are not included in the agar but are simply sprayed onto

TABLE 1. *Comparative reproducibility of the standard Kirby-Bauer and modified Kirby-Bauer methods when tested against E. coli ATCC 25922*[a]

Agent	Amt (μg/disk)	0.20%-INT		Kirby-Bauer	
		Mean[b]	% SD	Mean	% SD
Chloramphenicol	30	24.8	5.0	24.7	5.0
Colimycin	10	14.1	2.8	13.9	4.3
Kanamycin	30	19.0	7.0	21.1	5.2
Tetracycline	30	22.3	6.0	23.0	3.0
Neomycin	30	16.1	2.5	17.0	3.8
Gentamicin	10	18.7	3.7	20.0	5.4
Nalidixic acid	30	23.2	4.7	23.9	2.1

[a] Disk susceptibility tests were carried out as described in Materials and Methods. The means and percent standard deviations (SD) represent the results obtained from 12 daily single assays for each antibiotic or drug.

[b] Mean zone diameter in millimeters.

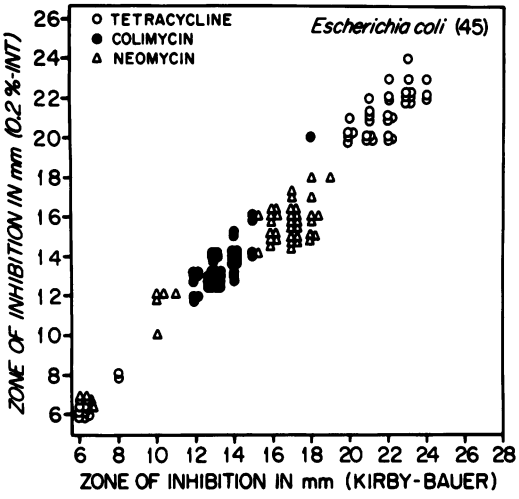

FIG. 4. *Comparative disk susceptibilities of E. coli strains determined by the modified Kirby-Bauer method and the standard Kirby-Bauer method. The potencies of tetracycline, colimycin, and neomycin were 30, 10, and 30 μg/disk, respectively.*

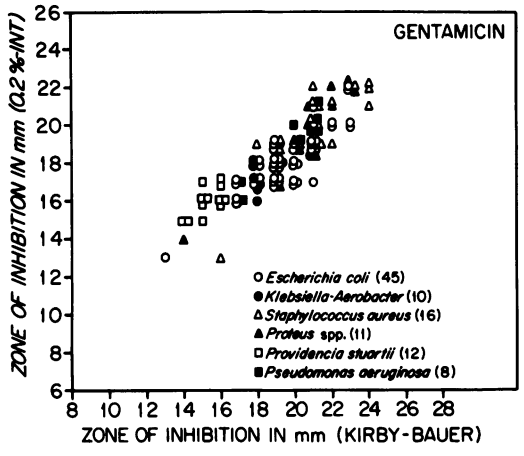

FIG. 5. *Comparative susceptibilities of gram-negative bacteria and S. aureus strains to gentamicin determined by the modified Kirby-Bauer method and the standard Kirby-Bauer method. Gentamicin concentration was 10 μg/disk.*

the plate at the end of the test, i.e., after removal of plates from the incubator and just prior to recording zone diameters. This eliminates any possible interference by the dye with growth of the organism or with the antibacterial activity of the agent impregnated into the disk. Undoubtedly, the only major modification, a decreased period of incubation, is distinctly advantageous.

As recommended for the Kirby-Bauer test, the modified test would appear to be most reliable with rapidly growing organisms such as the gram-negative bacteria and *S. aureus* strains employed in the present study. Further evaluation of the test system is required to determine susceptibilities of slower-growing organisms. However, the decreased incubation period employed would suggest that the modified method would not be applicable to these latter types of bacteria. Indeed, some preliminary results indicated that neither *Pseudomonas maltophilia* nor *P. cepacia* grew sufficiently rapidly to give readable zones of inhibition after 6 to 7 hr of incubation at 37 C.

The present studies have been carried out only with those organisms indicated. All of these bacteria were able to reduce the dyes employed, obviously a contingency upon which rests the potential application of the test method. Dye reduction by other gram-negative bacteria (except *S. marcescens* and *Salmonella* species, which do reduce the dyes but have not been evaluated) and gram-positive bacteria has not been examined.

Although the present modified method has been directed toward the Kirby-Bauer technique, there is no reason why this method could not be applied to other disk susceptibility testing methods. Certain reports have presented data on "all or none" disk susceptibility testing (5). Here, there is either a "zone" or "no zone" indicating susceptibility or resistance, respectively. Preferential utilization of this type of testing could easily apply the dye-reduction modification. Indeed, in such instances, it might be most efficacious, since interpretation of result eliminates ranking into resistant, intermediate, or susceptible categories based upon zone sizes.

Both the reproducibility of, and the susceptibility distributions determined by, the modified method closely paralleled the results obtained by the standard Kirby-Bauer method. Use of the modified test to evaluate the susceptibility of *E. coli* ATCC 25922 to chloramphenicol, colimycin, kanamycin, tetracycline, neomycin, gentamicin, and nalidixic acid demonstrated a high degree of control with variation comparable to that obtained with the standard Kirby-Bauer method. The excellent agreement between the results of the two methods suggests that, during generalized application, the interpretation of results of the modified test will closely parallel the interpretation of results obtained by the standard Kirby-Bauer method.

TABLE 2. *Comparative susceptibilities of gram-negative bacteria and S. aureus strains determined by the Kirby-Bauer and modified Kirby-Bauer methods*

Agent[c]	Distribution of susceptibilities (%)[a]					
	0.20% INT-6.5 hr[b]			Kirby-Bauer[b]		
	Res	Int	Sus	Res	Int	Sus
Ampicillin (10)[c]	43	10	47	44	8	48
Chloramphenicol (30)	20	2	78	23	4	73
Colimycin (10)	25	0	75	25	0	75
Gentamicin (10)	2	0	98	1	0	99
Kanamycin (30)	21	10	69	21	5	75
Tetracycline (30)	39	2	59	40	1	59
Neomycin (30)	22	66	11	24	40	36
Nalidixic Acid (30)	11	9	80	11	8	81
Novobiocin (30)	56	3	42	56	3	42
Erythromycin (15)	7	0	93	7	0	93

[a] A total of 100 bacterial strains was examined. *S. aureus* strains were tested against ampicillin, gentamicin, and tetracycline only.

[b] Resistant (Res), intermediate (Int), and susceptible (Sus) categories were classified according to the Kirby-Bauer Interpretative Charts for the respective agents.

[c] Numbers in parentheses represent the disk potencies in micrograms.

TABLE 3. *Comparative susceptibilities of gram-negative bacteria and S. aureus strains determined by the Kirby-Bauer and modified Kirby-Bauer methods*

Agent[c]	Distribution of susceptibilities (%)[a]					
	0.50% MTT-6.5 hr[b]			Kirby-Bauer[b]		
	Res	Int	Sus	Res	Int	Sus
Ampicillin (10)	45	7	48	46	5	49
Chloramphenicol (30)	17	4	79	29	2	69
Colimycin (10)	28	0	72	27	0	73
Gentamicin (10)	2	0	98	1	0	99
Kanamycin (30)	23	17	60	23	5	72
Tetracycline (30)	38	3	59	41	1	58
Neomycin (30)	20	65	15	25	34	41
Nalidixic Acid (30)	14	10	76	15	8	77
Novobiocin (30)	55	0	45	53	2	45
Erythromycin (15)	6	0	94	6	0	94

[a] A total of 100 bacterial strains was examined. *S. aureus* strains were tested against ampicillin, gentamicin, tetracycline, and erythromycin only.

[b] Resistant (Res), Intermediate (Int), or susceptible (Sus) according to the Kirby-Bauer Interpretative Charts for the respective agents.

[c] Numbers in parentheses represent the disk potencies in micrograms.

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