Evaluation of Agar Dilution Method for Determination of Sensitivity of Bacteria to Antibiotics.* (20183)

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There has developed recently renewed interest in methods of determination of sensitivity of bacteria to antibiotics. This interest has paralleled use of the newer antibiotics and has stimulated the quest for a method for determining quickly the most effective antibiotic in the treatment of an infection. Several methods having the principle of agar diffusion have been used and there has been much discussion concerning each of those methods (1,2). Because of the great practical importance of this problem it was decided to compare an agar dilution and an agar diffusion method of sensitivity determination with the serial broth dilution method, the latter being used as a standard for comparison. An agar dilution method was chosen for evaluation because it offers 4 inherent advantages: 1) it obviates the need for diffusion of the antibiotic to be tested, 2) a direct estimate of the concentration of antibiotic necessary for inhibition of an organism can be obtained, 3) the plates may be prepared in quantity for later use, and 4) a single set of antibiotic dilutions may be used to measure the sensitivity of a number of bacterial cultures.

Materials and methods. Source and identity of bacteria. Ninety-eight strains of bacteria, isolated in the routine diagnostic laboratory serving the University Hospitals of Cleveland, were tested for sensitivity to 6 antibiotics, each organism being tested with all 6 antibiotics by the agar dilution, agar diffusion, and tube dilution methods. The following bacteria were tested: 29 strains of *Escherichia coli,* 20 strains of *Staphylococcus,* 15 strains of *Aerobacter aerogenes,* 9 strains of *Streptococcus,* 9 strains of *Pseudomonas aeruginosa,* 4 strains of *Proteus,* 3 strains of *Pneumococcus,* 3 strains of *Klebsiella pneumoniae,* 2 strains of *Alkaligenes fecalis,* and one strain each of *Salmonella typhosa, Salmonella* sp. (antigenic group C_2), *Shigella paradysenteriae,* and *Shigella sonnei. Culture media.* Tryptose blood agar base medium (Difco) with citrated human blood incorporated in a concentration of 5% was used in the preparation of antibiotic-containing plates. Tryptose agar (Difco) was used in the preparation of plates employed in agar diffusion testing. Tryptose broth was used in the preparation of dilutions used in tube dilution testing. *Antibiotics.* Standard commercial preparations of terramycin,† aureomycin,‡ penicillin, bacitracin, streptomycin, and chloramphenicol were employed. Unbuffered crystalline aureomycin hydrochloride was used for aureomycin determinations. For tests involving the agar diffusion method, commercially prepared antibiotic discs were used ("Dia-Discs," Commercial Solvents Corporation); the disc having the greater quantity of antibiotic was used routinely. *Agar dilution method.* The agar to be used was melted and then cooled to 45°C. An antibiotic was mixed with the agar and plates were poured. The various antibiotics were used in concentrations of 20, 5, 1.25, .312, and .08 units (or mcg) per ml. All plates were stored in a refrigerator at 4°C until they were used. In this test, a sector of the surface of each of a series of plates was streaked with a 10^{-3} dilution of an 18-hour broth culture of the organism to be tested; 6 to 8 different cultures were placed on each plate. After overnight incubation at 37°C the plates were examined. Complete inhibition of growth was chosen as the end-point.

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‡ Aureomycin was kindly supplied by Dept. of Clinical Research, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

Agar diffusion method. In this test the surface of a tryptose agar plate (blood agar if a *Streptococcus* was being tested) was heavily inoculated with the organism to be tested, antibiotic-containing discs were placed on the surface, and the plate was incubated at 37°C. After overnight incubation the plate was examined and, if growth of the organism had been inhibited, the diameter of the zone of inhibition was recorded. *Tube dilution method.* Five concentrations of an antibiotic were prepared by dilution in tryptose broth. To 0.5 ml quantities of the antibiotic-containing broth were added 0.5 ml quantities of a 10^{-3} dilution of an 18-hour broth culture of the organism to be tested. The final concentrations of the antibiotic were 20, 5, 1.25, .312 and .08 units (or mcg) per ml. Citrated human blood was incorporated in broth used in the dilution of cultures of streptococci. The tubes were examined after overnight incubation. Complete inhibition of growth, indicated by absence of visible turbidity, was selected as the end-point.

Experimental results. A total of 98 bacterial strains, of 13 genera, were tested for sensitivity to penicillin, bacitracin, streptomycin, chloramphenicol, aureomycin, and terramycin by 3 methods currently in use, *viz.* agar dilution, agar diffusion, and tube dilution. The results of simultaneous tests of sensitivity to 3 of the 6 antibiotics by the agar dilution and tube dilution methods are shown in Tables I-III. It is seen that agreement between the two methods is good, less than 10% of the tests differing by as much as a single 4-fold dilution; in all the determinations, which amount to a cumulative total of 1176, there are only 5 instances in which there is a discrepancy greater than a 4-fold dilution.

TABLE I. Comparison of Tube Dilution and Agar Dilution Methods of Testing Sensitivity to Penicillin. Bacteria tested: *Staphylococcus*, 20 strains; *Streptococcus*, 9 strains; *Pneumococcus*, 3 strains.

Inhibiting concentration, tube dilution test (units/ml)	Inhibiting concentration, agar dilution test (units/ml) .08	.312	1.25	5	20	N.I.*
N.I.*						9
20					2	1
5				4		
1.25			1			1
.312						
.08	12†	1	1			

* N.I. = Not inhibited.

† No. of strains, having indicated sensitivity in tube dilution test which were inhibited by given concentration of antibiotic in agar dilution test.

TABLE II. Comparison of Tube Dilution and Agar Dilution Methods of Testing Sensitivity to Streptomycin. Bacteria tested: *Escherichia coli*, 29 strains; *Staphylococcus*, 20 strains; *Aerobacter aerogenes*, 15 strains; *Streptococcus*, 9 strains; *Pseudomonas aeruginosa*, 9 strains; *Proteus*, 4 strains; *Pneumococcus*, 3 strains; *Klebsiella pneumoniae*, 3 strains; *Alkaligenes fecalis*, 2 strains; 1 strain each of *Salmonella typhosa*, *Salmonella* sp. (antigenic group C_2), *Shigella paradysenteriae*, and *Shigella sonnei*.

Inhibiting concentration, tube dilution test (μg/ml)	Inhibiting concentration, agar dilution test (μg/ml) .08	.312	1.25	5	20	N.I.*
N.I.*					2	40
20					16	
5			2	28	2	1
1.25			5	1		
.312		1†				
.08						

* N.I. = Not inhibited. † See Table I.

TABLE III. Comparison of Tube Dilution and Agar Dilution Methods of Testing Sensitivity to Aureomycin. Bacteria tested: See Table II.

Inhibiting concentration, tube dilution test (μg/ml)	Inhibiting concentration, agar dilution test (μg/ml) .08	.312	1.25	5	20	N.I.*
N.I.*						23
20					13	
5			2	33	1	
1.25		1	10	1		
.312		12†	1			
.08			1			

* N.I. = Not inhibited. † See Table I.

In Tables IV-VI a comparison of the agar diffusion and tube dilution test results is presented. For the purpose of tabulation, the results of the tests were used to classify each organism as "resistant," "sensitive," or "intermediate." The concentrations of antibiotic chosen to limit each of these groups was based on the plasma levels of the antibiotic that can be obtained by administration of a reasonable dose of the drug(3-8). Since the blood level that can be obtained varies with each antibiotic, the "yardstick" used to define each of the 3 groups also varies with each antibiotic. The zone diameters chosen to limit each of the groups were based on the over-all range of the diameters of the zones of inhibition produced by an antibiotic-containing disc in the

TABLE IV. Comparison of Tube Dilution and Agar Diffusion (Disc) Methods of Testing Sensitivity to Penicillin. Bacteria tested: See Table I.

Classified by tube dilution test	Classified by Agar Diffusion Test: Sensitive (inhibition zone 20-45 mm)	Intermediate (inhibition zone 8-19 mm)	Resistant (inhibition zone ≤8 mm)
Sensitive: 14 (inhibited by .08 unit/ml)	12*	1	1
Intermediate: 6 (inhibited by .312-20 units/ml)		4	2
Resistant: 12 (inhibited by 20 units/ml)	2	4	6

* No. of strains, having indicated sensitivity in tube dilution test which were classified as "sensitive," "intermediate," or "resistant" in agar diffusion test (see text).

TABLE V. Comparison of Tube Dilution and Agar Diffusion (Disc) Methods of Testing Sensitivity to Streptomycin. Bacteria tested: See Table II.

Classified by tube dilution test	Classified by Agar Diffusion Test: Sensitive (inhibition zone 22-35 mm)	Intermediate (inhibition zone 12-21 mm)	Resistant (inhibition zone ≤12 mm)
Sensitive: 7 (inhibited by <5 μg/ml)	4*	3	
Intermediate: 49 (inhibited by 5 & 20 μg/ml)	11	37	1
Resistant: 42 (inhibited by >20 μg/ml)		10	32

* See Table IV.

testing of the 98 strains of bacteria used. There was wide variation in the zones of inhibition produced by the different discs; therefore, the zone diameters that define each of the 3 groups also vary with each antibiotic. The diameters of the zones of inhibition and the antibiotic concentrations of the broth dilutions chosen for division of the organisms into the groups are shown in Tables IV-VI. It can be seen from examination of these tables that in the case of penicillin fair agreement exists between the results of the disc and tube dilution tests. The agreement between results of the 2 tests with bacitracin is also fair. There is poor agreement between the results of the 2 tests with regard to the other 4 antibiotics, however. With aureomycin, while there is agreement between the tests in selecting resistant strains, many were classified as resistant by the agar diffusion method that were classified as sensitive by the tube dilution method. The reverse was true in the case of streptomycin, in that many organisms that were classified as resistant by the dilution method were intermediate as tested by the diffusion method; also, many classified as intermediate by the dilution method were sensitive according to the diffusion method. There was similar rather poor correlation of the results obtained in the testing of chloramphenicol and terramycin.

The effect of the size of inoculum used in agar dilution tests was evaluated by repeating sensitivity determinations using different dilutions of the same broth culture; the dilutions used were 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}. In such tests with various strains it was found that the size of the inoculum used did not alter the results.

In order to test the stability of the antibiotic-containing plates the following experi-

TABLE VI. Comparison of Tube Dilution and Agar Diffusion (Disc) Methods of Testing Sensitivity to Aureomycin. Bacteria tested: See Table II.

Classified by tube dilution test	Classified by Agar Diffusion Test: Sensitive (inhibition zone 13-26 mm)	Intermediate (inhibition zone 8-12 mm)	Resistant (inhibition zone ≤8 mm)
Sensitive: 14 (inhibited by <1.25 μg/ml)	7*	6	1
Intermediate: 48 (inhibited by 1.25 & 5 μg/ml)	8	7	33
Resistant: 36 (inhibited by 20 μg/ml)			36

* See Table IV.

ment was performed. Several series of plates were prepared with each antibiotic and the plates were then stored at 4°C. The plates were streaked with the same strains of bacteria at weekly intervals over a period of 4 weeks and the apparent sensitivity recorded. The aureomycin-containing plates showed no loss in potency during the first 3 weeks; after that period the potency decreased by one-half. The other 5 antibiotic-containing plates that were tested maintained their potency over the full 4-week period.

Discussion. There is evident need in the clinical laboratory for a method of determining quickly, the antibiotic sensitivity of bacteria isolated from infectious processes. The simplicity of the disc method of testing recommends it over the cumbersome tube dilution method. However, question has been raised as to the accuracy of the former method of testing. The results of this study demonstrate that there is poor agreement between the values obtained by the disc and tube dilution methods with certain antibiotics. It is therefore felt that there is a sacrifice of accuracy for simplicity of testing when the disc method of sensitivity determination is used.

There was good agreement between the results of the agar dilution and tube dilution methods of testing. The fact that the antibiotic-containing plates can be kept in the refrigerator over a period of 4 weeks (3 weeks in the case of aureomycin) without loss of potency means that series of plates can be prepared once every 2 to 3 weeks and stored until needed for testing. There are several points that are important in the preparation of antibiotic-containing plates. It is important, especially with aureomycin, to avoid letting solutions of antibiotics stand before incorporating them in plates because some decrease in potency occurs(7,9). Of prime importance in the preparation of such plates is the cooling of the agar to 45°C before adding an antibiotic; this must be observed in order to prevent heat destruction of the antibiotic. Plates to be stored must be kept in a refrigerator at a temperature of 4°C.

In these experiments the size of the inoculum used did not alter the results of the agar dilution method. These findings are in agreement with those of others(10-12).

The agar dilution method of testing is simple in its performance and its accuracy agrees with that of the tube dilution method within limits of clinical usefulness. Like the latter method, the agar dilution method offers a direct estimate of the concentration of antibiotic necessary for inhibition of an organism.

Conclusions. 1. The agar dilution and tube dilution methods of determining bacterial sensitivity are equally reliable. 2. Aureomycin-containing plates can be stored for 3 weeks without loss of potency; plates containing penicillin, bacitracin, streptomycin, chloramphenicol, and terramycin can be stored for 4 weeks without loss of potency. 3. The agar diffusion method of sensitivity determination is not fully reliable, as shown by poor correlation of the results obtained by that method and the tube dilution method of testing with some antibiotics.

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1. Editorial, *New England J. Med.,* 1951, v245, 868.

2. Erlanson, P., *Acta Pathol. et Microbiol. Scandinavica. Supp.,* 1951, v85, 1.

3. Boxer, G. E., Jelinek, V. C., Tompsett, R., Dubois, R., and Edison, A., *J. Pharmacol. Exp. Therap.,* 1948, v92, 226.

4. Werner, L. A., Knight, V., and McDermott, W., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 261.

5. Glazko, A. J., Wolf, L. M., Dill, W. A., and Bratton, C., *J. Pharmacol. Exp. Therap.,* 1949, v96, 445.

6. Sanders, M., Rumball, J. M., Solomon, C., Soret, M. G., and Ricci, N. I., *J. Clin. Invest.,* 1949, v28, 1006.

7. Herrell, W. E., Heilman, F. R., Wellman, W. E., *Mayo Clinic Proc. Staff Meeting,* 1950, v25, 183.

8. Scudi, J. V., Clift, M. E., and Krueger, R. A, PROC. SOC. EXP. BIOL. AND MED., 1947, v65, 9.

9. Price, C. W., Randall, W. A., and Welch, M., *Ann. N. Y. Acad. Sci.,* 1948, v51, 211.

10. Parker, R. F., PROC. SOC. EXP. BIOL. AND MED., 1946, v63, 443.

11. May, J. R., and Morley, C. W., *Lancet,* 1952, v1, 636.

12. Paine, T. F., Collins, H. S., and Finland, M., *Ann. N. Y. Acad. Sci.,* 1948, v51, 228.

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