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Economical Agar Dilution Technique for Susceptibility Testing of Anaerobes

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A reliable agar dilution technique has been developed enabling the clinical laboratory to perform antibiotic susceptibility tests on significant anaerobes with ease and economy.

The lack of a standardized disk diffusion test for anaerobic bacteria creates problems for the clinical laboratory wanting to determine the antibiotic susceptibility of these organisms. The technique recommended by the Food and Drug Administration–National Committee for Clinical Laboratory Standards for aerobes has not been found to be completely satisfactory for anaerobes (1). At the present time, disk diffusion tests, with possibly the exception of chloramphenicol and tetracycline, are not acceptable for routine anaerobic analyses. Dilution tests, however, have been shown to produce reliable and reproducible results (5, 6, 8). Based on our cost studies and those of others (2, 4) we feel that the agar dilution test for aerobes is more easily adapted to routine use than the awkward and expensive broth dilution procedure. It seemed appropriate, therefore, to devise a similar scheme for anaerobic organisms, which would be isolated in lower frequency than aerobic organisms.

By the use of an expanded agar dilution scheme (3) and quadrant plates, we developed a system which allowed reproducibility, ease, and economy in the clinical laboratory. The procedure uses four antibiotics, each tested at different concentrations: penicillin (5, 1, 0.1, 0.01 μg/ml); clindamycin (10, 1, 0.1, 0.01 μg/ml); chloramphenicol (20, 10, 5, 1 μg/ml); and tetracycline (10, 5, 1 μg/ml). These dilutions of antibiotics were chosen to be consistent with the aerobic agar dilution scheme in our laboratory and previously published procedures (3). By the use of a Steer replicator, as many as eight isolates could be tested simultaneously.

Antibiotic stock concentrations (1,000 μg/ml) were stored at −20 C or below and appropriate dilutions in water were made to reach the final concentrations in agar as shown above. Additional antibiotics may be tested by adding plates and by using the appropriate dilutions (3).

The agar dilution plates were prepared by combining 4.0 ml of molten and cooled Mueller-Hinton agar enriched with 0.5% yeast extract, 0.5 ml of lysed sheep erythrocytes (vol/vol sterile water and defibrinated sheep erythrocytes), and 0.5 ml of the appropriate antibiotic dilution. The fourth quadrant of the tetracycline plate contained no antibiotic and thus served as a control. Holding the tubes in a dry heat bath was the most convenient manner to prevent gelling of the agar. After mixing, the agar was poured into the appropriate quadrant of each plate; and when the agar had solidified, the plates were either dried by inverting them open in a 37 C incubator for about 30 min or stored in a refrigerator for up to 1 week. Anaerobic or aerobic storage at room temperature or in the refrigerator for up to 1 week did not alter the activity of the antibiotics, as determined by the repeated testing of standard strains.

Twenty-four-hour cultures of the organisms in the thioglycolate broth containing vitamin K_1, hemin, and horse serum were diluted in thioglycolate broth to a turbidity of McFarland no. 1 barium sulfate standard and used as the inocula. If sufficient growth was not reached in 24 h, which was seldom, the cultures were either reincubated for another 24 h or transferred to some other acceptable anaerobic broth media, such as peptone-yeast extract-glucose medium or Schaedler. The standardized cultures were dispensed into the wells of a Steer replicator so

that each quadrant received the same organism on the same position of the plate (Fig. 1). The number eight position was used for the control organism (*Staphylococcus aureus* ATCC 25923). This strain had the same minimal inhibitory concentration both aerobically and anaerobically: penicillin, 0.1 μg/ml; clindamycin, 0.1 μg/ml; chloramphenicol, 5 μg/ml; and tetracycline, 5 μg/ml. A blood agar plate, incubated aerobically, was used to check for contamination. The organisms may also be dispensed with a calibrated milk dilution loop (0.001 ml). The spots were allowed to dry and the plates were incubated in a GasPak jar for 24 h at 37 C.

Reading of the plates required careful examination of the anaerobic control sector for the degree of growth and then examination of each quadrant of the respective antibiotic dilution as it was rotated to the same position. The total inhibition of growth of the organisms was considered to be the end point (Fig. 2). Insufficient growth on the plates required reincubation for

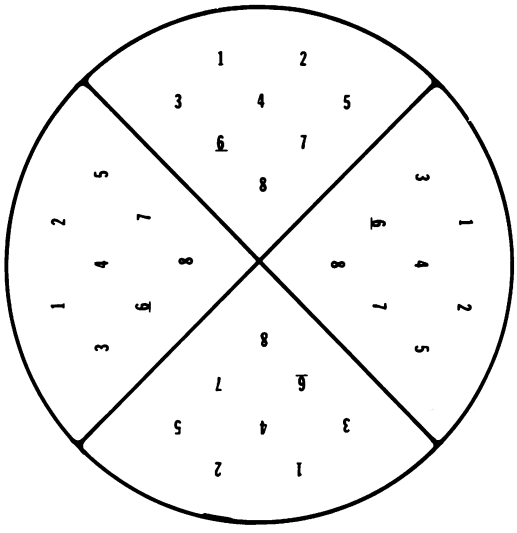

FIG. 1. *Inocula positions. Numbers indicate position of inocula; all quadrants are identical.*

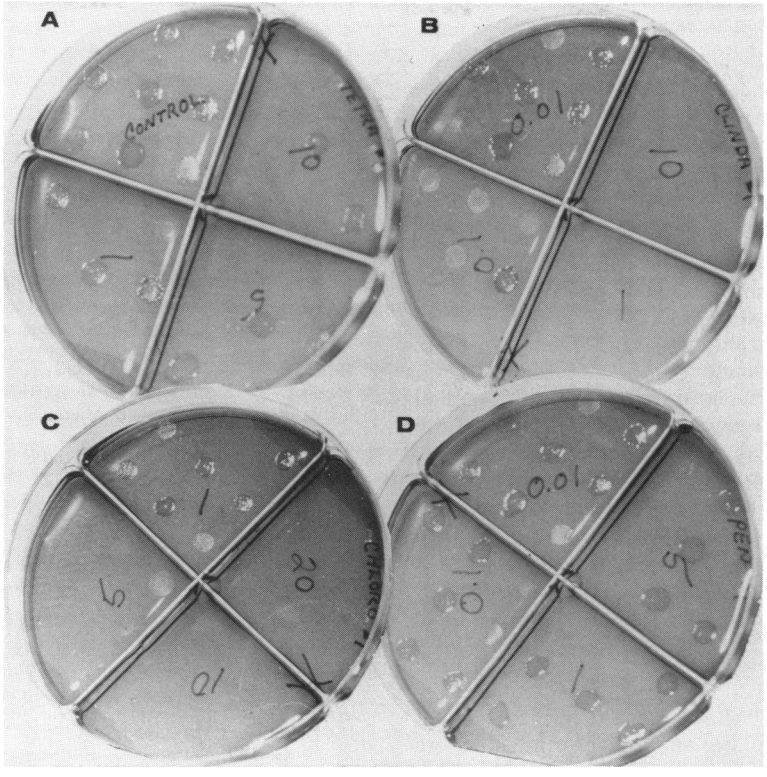

FIG. 2. *Quadrant plates: (A) tetracycline and control, (B) clindamycin, (C) chloramphenicol, and (D) penicillin. Organism positions: 1, 3, 4, 5, 7—Bacteroides fragilis; 2—Fusobacterium necrophorum; 6—Clostridium perfringens; 8—Staphylococcus aureus ATCC 25923.*

TABLE 1. *Number of strains inhibited at various concentrations of antibiotics tested*

Antibiotic	No. of strains inhibited						
	0.01[a]	0.1	1	5	10	20	>HLT[b]
Clindamycin							
B. fragilis	0	29	36	—[c]	3	—	1
Bacteroides sp.	14	8	6	—	1	—	0
Fusobacterium sp.	5	13	1	—	0	—	0
Clostridium sp.	3	0	2	—	1	—	1
Anaerobic cocci	2	7	3	—	0	—	0
Tetracycline							
B. fragilis	—	—	17	7	18	—	27
Bacteroides sp.	—	—	13	12	4	—	0
Fusobacterium sp.	—	—	11	4	0	—	4
Clostridium sp.	—	—	5	1	0	—	1
Anaerobic cocci	—	—	4	2	1	—	5
Penicillin							
B. fragilis	0	0	3	9	—	—	57
Bacteroides sp.	3	4	12	10	—	—	0
Fusobacterium sp.	3	3	12	1	—	—	0
Clostridium sp.	1	3	3	0	—	—	0
Anaerobic cocci	1	9	1	0	—	—	1
Chloramphenicol							
B. fragilis	—	—	0	35	29	5	0
Bacteroides sp.	—	—	5	19	4	1	0
Fusobacterium sp.	—	—	12	2	5	0	0
Clostridium sp.	—	—	0	6	0	0	1
Anaerobic cocci	—	—	5	6	1	0	0

[a] Micrograms of antibiotic per milliliter of medium.
[b] >HLT, Greater than highest level tested.
[c] —, Not tested.

another 24 h or performance of the test in a rich broth medium, such as peptone-yeast extract-glucose medium or Schaedler broth.

The results were reported as minimal inhibitory concentration; however, if an interpretation of susceptible (S) or resistant (R) was required, we used the following guide: penicillin, S was <1 μg/ml and R >5 μg/ml; clindamycin, S was <10 μg/ml and R was >10 μg/ml; chloramphenicol, S was <10 μg/ml and R was >20 μg/ml; and tetracycline, S was <10 μg/ml and R was >10 μg/ml. Results with this procedure, as indicated in the accompanying table, were in agreement with previously reported data (5, 6, 8; Table 1).

We have described a reliable and economical agar dilution technique for the susceptibility testing of clinically significant anaerobic bacteria. The use of the expanded dilution scheme and quadrant plates enables the clinical laboratory to determine the susceptibilities of eight organisms to as many as 12 antibiotics with the use of only one GasPak jar and a minimal amount of media, supplies, and time. Additional organisms may be tested by inoculation of additional sets of antibiotic plates.

LITERATURE CITED

1. Barry, A. L. 1973. Revised tentative standard. Performance standards for antimicrobial disc susceptibility tests as used in clinical laboratories. National Committee for Clinical Laboratory Standards, Los Angeles.
2. Ericsson, H. M., and J. C. Sherris. 1971. Antibiotic sensitivity testing-report of an international collaborative study. Acta Pathol. Microbiol. Scand. Sect. B **217**(Suppl.):64-73.
3. Gavan, T. L., H. W. McFadden, and E. L. Cheatle, (ed.). 1971. Agar dilution method, p. 127-141. *In* Antimicrobial susceptibility testing. Am. Soc. Clin. Pathol. Comm. Cont. Ed., Chicago.
4. Haltalin, K. C., A. H. Markley, and E. Wordman. 1973. Agar plate dilution method for routine antibiotic susceptibility testing in a hospital laboratory. Am. J. Clin. Pathol. **60**:384-394.
5. Kislak, J. W. 1972. The susceptibility of *Bacteroides fragilis* to 24 antibiotics. J. Infect. Dis. **125**:295-299.
6. Martin, W. J., M. Gardner, and J. A. Washington II. 1972. In vitro antimicrobial susceptibility of anaerobic bacteria isolated from clinical specimens. Antimicrob. Agents Chemother. **1**:148-158.
7. Steers, E., E. L. Foltz, and B. S. Graves. 1959. An inocula replicating apparatus for routine testing of bacterial susceptibility to antibiotics. Antibiot. Chemother. (Washington, D.C.) **9**:307-311.
8. Zabransky, R. J., J. A. Johnston, and K. J. Hauser. 1973. Bacteriostatic and bactericidal activities of various antibiotics against *Bacteroides fragilis*. Antimicrob. Agents Chemother. **3**:152-156.