ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 1975, p. 15-21

Vol. 7, No. 1
Printed in U.S.A.

Broth-Dilution Method for Determining the Antibiotic Susceptibility of Anaerobic Bacteria

DONALD R. STALONS AND CLYDE THORNSBERRY*

*Department of Microbiology, Health Sciences Center, Temple University, Philadelphia, Pennsylvania 19140; and Antimicrobics Investigation Section, Center for Disease Control, Atlanta, Georgia 30333**

Received for publication 6 September 1974

A broth-dilution method for performing antimicrobial susceptibility tests on anaerobic bacteria has been proposed. The medium used in the test was Schaedler broth, with incubation in a glove box with an atmosphere of 5% CO_2, 10% H_2, and 85% N_2, or in the GasPak system. Minimal inhibitory concentrations for selected antibiotics were determined, under these conditions, by using a conventional twofold dilution scheme for the antibiotics and a "categorization three-tube method" in which two or three clinically significant concentrations of each antibiotic were used. Minimal inhibitory concentrations obtained by both methods were very similar. The categorization method could be used routinely to test the antimicrobial susceptibility of anaerobic bacteria.

Because anaerobic bacteria are being isolated from clinical material with increasing frequency, the diagnostic microbiology laboratory is more often called upon to test the susceptibility of these organisms to a variety of antimicrobial agents. The determination of the drug susceptibility of anaerobic organisms is generally more complicated than for aerobic organisms, because of the difficulty in isolating and identifying them, the obligation to test them in the anaerobic environment, and the lack of a standard method for testing their susceptibility.

The approach of many investigators has been to use conventional techniques for aerobic and facultative organisms to test the drug susceptibility of anaerobes in an oxygenless environment. Therefore, antimicrobial susceptibilities have been determined by disk-diffusion, agar-dilution, and broth-dilution techniques. Although there are several common sources of variation for these techniques, e.g., inoculum concentrations, pH of the medium, and components of the medium, one major disadvantage of the disk-diffusion and agar-dilution techniques for testing anaerobic bacteria in comparison with the broth-dilution technique is that the organisms are much more likely to be subjected to lethal doses of oxygen when they are spread onto the surface of agar. Furthermore, the addition of agar to a broth medium adds to the complexity of the medium and to the possibility of some effects on the susceptibility test. For these reasons we chose to use a broth-dilution technique as our reference method for determining minimal inhibitory concentrations (MICs) of several antimicrobial agents for various anaerobic bacteria.

The initial consideration in attempts to standardize a method for susceptibility testing should be the achievement of optimal growth conditions for the organisms being tested. We have previously shown that Schaedler broth (BBL) in an atmosphere of 5% CO_2, 10% H_2, and 85% N_2 was superior to eight other media and other combinations of gas mixtures for growing anaerobic bacteria likely to be isolated from clinical material (10). A broth-dilution method, with this medium and atmosphere, were used in this study. Susceptibility tests were also performed with a modified broth-dilution technique in which only two or three concentrations of each antimicrobial agent are used. We think that this test can be readily adapted to routine use in a diagnostic laboratory.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study were obtained from the stock-culture collection of the Anaerobe Unit, Center for Disease Control, and from the American Type Culture Collection. Each strain was characterized by a battery of microscopic, cultural, and biochemical tests, as described by Dowell and Hawkins (1) and Holdeman and Moore (5). These strains of anaerobes included many that are commonly encountered in human infections (1).

Media. Schaedler broth was used in this study because it supported the best growth of the majority of anaerobic organisms (10). It was prepared according to the manufacturer's specifications and prere-

duced in the anaerobic atmosphere that we have found to best support the growth of most anaerobic strains isolated from clinical material, i.e., 85% N_2, 10% H_2, and 5% CO_2 (10). Mueller-Hinton broth (BBL) was used in certain comparative studies, since it is the medium in which standard MICs of antibiotics are well documented. Stock cultures were maintained on Schaedler agar (BBL) that had been enriched with 5% defibrinated rabbit blood and 0.5% yeast extract (Difco); hemin, which stimulates the growth of various *Bacteroides* species, and menadione, a growth requirement of some *Bacteroides melaninogenicus* strains (4), were added to all media in final concentrations of 5 μg/ml and 0.1 μg/ml, respectively.

Antibiotics. The antibiotics used in this study were supplied by the following organizations: cephalothin, erythromycin, and vancomycin, Eli Lilly Co. (Indianapolis); clindamycin and lincomycin, Upjohn Co. (Kalamzaoo, Mich.); chloramphenicol, Parke-Davis Co. (Detroit); gentamicin, Schering Laboratories (Bloomfield, N. J.): penicillin, Wyeth Laboratories (Philadelphia, Pa.); and tetracycline, Chas. Pfizer and Co., Inc. (New York). The antibiotics were diluted according to the protocol described by Gavan et al. (3), so that the final concentration of each stock solution was 2,560 μg of active antibiotic per ml. In the tests on the effect of medium and atmosphere on MICs, the effect of inoculum size on MICs, and in the determination of base line MICs, the stock solutions were diluted so that the final concentration in the first tube would be 128 μg/ml and in succeeding tubes, serial twofold dilutions of this concentration. For the categorization test, the concentrations of antibiotics shown in Table 5 were achieved by diluting the stock antibiotic solution in Schaedler broth.

Anaerobic systems. The anaerobic systems used were the GasPak (BBL) and an anaerobic glove box (Coy Manufacturing, Ann Arbor, Mich.). A palladium-coated aluminum catalyst (Englehard Industries, Newark) was used with each of the anaerobic systems.

Determination of effect of medium and atmosphere on antibiotic activity. MICs were determined on two standard strains of facultative organisms by the microtiter broth-dilution technique described by Gavan et al. (3), with Mueller-Hinton and Schaedler broths and aerobic and anaerobic incubation, to determine whether the medium and atmosphere selected for optimal growth in earlier studies (10) had an effect on antibiotic activity. Two standard reference strains, *Staphylococcus aureus* (ATCC no. 25923) and *Escherichia coli* (ATCC no. 25922) that have been recommended as reference strains for the standardized agar diffusion test (7) but have been studied extensively by dilution tests, were used in this phase of the study. The antibiotics tested are listed in Table 1. The cultures were incubated for 24 h at 35 C in the appropriate atmosphere.

Effect of inoculum size on MICs. The effect of variation in inoculum on the MIC of antibiotics was determined. Three-tenths milliliter of each antibiotic suspension listed in Table 1 was transferred to screw-cap tubes (16 by 125 mm) containing 2.7 ml of Schaedler broth; this gave an additional 1:10 dilution of the antibiotic (the broth medium was concentrated 10% to compensate for this dilution factor). The media containing the antibiotics were then prereduced in the anaerobic glove box with a gaseous environment of 5% CO_2, 10% H_2, and 85% N_2 for 24 h. After prereduction, sterile, precalibrated droppers (Cooke Laboratory Products no. 220-65) were used to inoculate each tube and a series of controls (3.0 ml of medium without antibiotic) with 0.025 ml of the appropriate concentration of organism. Standard inocula of four representative anaerobic organisms (*Bacteroides fragilis* ssp. *fragilis*, *Peptostreptococcus* Center for Disease Control group 2, *Eubacterium alactolyticum*, and *Clostridium perfringens*) were diluted serially to yield cultures containing 5×10^8, 5×10^7, 5×10^6, and 5×10^5 organisms per ml. Therefore, the final concentrations of organisms used in this comparative study were 4×10^6, 4×10^5, 4×10^4, and 4×10^3 per ml. After incubation at 35 C for an appropriate time, depending upon the growth re-

TABLE 1. *MICs of selected antibiotics for two reference strains of bacteria obtained with a microtiter broth-dilution method, with Mueller-Hinton and Schaedler broths, and aerobic and anaerobic atmospheres*

Antibiotics	*Staphylococcus aureus*				*Escherichia coli*			
	Mueller-Hinton		Schaedler		Mueller-Hinton		Schaedler	
	$\underline{O}_2$[a]	$\overline{O}_2$[b]	$\underline{O}_2$	$\overline{O}_2$	$\underline{O}_2$	$\overline{O}_2$	$\underline{O}_2$	$\overline{O}_2$
Chloramphenicol	4–8[c]	8	8	8	4	4	8	8
Clindamycin	0.5	0.25	0.25	0.25	64	32	128	128
Erythromycin	0.25	0.5	0.5	0.5	32	64	32	32
Gentamicin	≤0.015	≤0.015	≤0.015	0.12	1	2	2	8
Lincomycin	2	2	2	1	>128	>128	>128	>128
Penicillin	0.06	0.03	0.12	0.03	32	32	64	64
Tetracycline	0.06	0.12	1	0.25	1	0.5	2	2
Vancomycin	2	1	2	1	>128	>128	>128	>128

[a] $\underline{O}_2$, aerobic incubation.
[b] $\overline{O}_2$, anaerobic incubation.
[c] MICs (μg/ml). Concentration of antimicrobial agents tested were serial twofold dilutions ranging from 128 to 0.015 μg/ml.

sponse of the organism, the end points and corresponding MIC values were determined through visual interpretation of growth.

Reference MIC determinations. Base line MICs were determined on some commonly encountered anaerobic bacteria using a broth dilution method. The antibiotics were diluted in sterile distilled water to contain 10 times the desired final concentration. Three-tenths milliliter of each antibiotic concentration was added to 2.7 ml of supplemented Schaedler broth, thus achieving another 10-fold dilution of antibiotic. The Schaedler broth had been concentrated 10% to compensate for the dilution of the broth. The tests were performed in screw-capped tubes (16 by 125 mm). This media was prereduced by placing it in the anaerobic glove box containing an atmosphere of 5% CO_2, 10% H_2, and 85% N_2 for 24 h. The inocula were prepared by growing the organisms in Schaedler broth until the desired turbidity (corresponding to 10^8 colony-forming units/ml) was achieved or surpassed (generally overnight). The turbidity was determined spectrophotometrically at 540 nm. Adjustment of the turbidity of the culture was made with Schaedler broth. The adjusted inoculum was added to each of the tubes with antibiotic-containing broth to achieve a final concentration of approximately 10^6 colony-forming units/ml (0.025 ml into 3 ml of broth). The tubes were incubated at 35 C for 18 to 24 h (or longer if growth not adequate) in the anaerobic glove box containing 5% CO_2, 10% H_2, and 85% N_2. The MIC was the least concentration of antibiotic that prevented visible growth. To measure the reproducibility of the test, triplicate determinations were performed at each of three testing episodes, using various lots of antibiotics, media, and anaerobic gas.

Categorization method for determining antibiotic susceptibility of anaerobic bacteria. An abbreviated version of the reference broth-dilution method was also evaluated. The antibiotics were tested in two or three concentrations that permitted clinically useful categorization of susceptibility, similar to the characterization obtained by the method described by Ericsson and Sherris (2). The concentrations of antibiotics used in these tests are shown in Table 5. The antibiotics were diluted in sterile distilled water to 10 times the desired final concentrations. Three-tenths milliliter of the antibiotic solution was added to 2.7 ml of Schaedler broth (contained in screw-capped in tubes either 16 by 125 mm or 13 by 100 mm), and prereduced as in the reference method. The inoculum was also prepared as in the reference method, that is, grown in Schaedler broth and adjusted spectrophotometrically to contain 10^8 colony-forming units/ml. Therefore, for each antibiotic, there were two or three antibiotic concentrations (depending upon the antibiotic) and one control tube of antibiotic-free medium. The tubes were incubated in the glove box containing 5% CO_2, 10% H_2, and 85% N_2, and/or the GasPak jar, at 35 C for 18 to 24 h (or longer to achieve visible turbidity). The end points were the lowest concentrations of antibiotics that permitted visible growth. The MICs obtained by this method could then be used to classify the organisms into one of four categories of susceptibility. We prefer the categories suggested by Ericsson and Sherris (2).

This test was evaluated by comparing results obtained with the category method to those obtained with the reference broth-dilution method, using strains of *B. fragilis* ssp. *fragilis*, *Peptostreptococcus* CDC group 2, *E. alactolyticum*, and *C. perfringens*. These evaluations were performed in the anaerobic glove box. In subsequent studies, the GasPak environment was also evaluated for use with the category test by comparing results obtained in the glove box with those obtained in the GasPak jar. In these studies, the inoculum preparation and subsequent inoculation of broth were done in the glove box in duplicate and one set was removed to the GasPak jar. Therefore, the only differences were the two atmospheres. Additional studies were also performed where the preparation of the inoculum, as well as the inoculation itself, were conducted under aerobic conditions to determine the effect on MIC values in the categorization method.

RESULTS

Determination of effect of medium and atmosphere on antibiotic activities. The effect of Schaedler broth and an anaerobic atmosphere of 5% CO_2, 10% H_2, and 85% N_2 on antimicrobical activity was determined by comparing the minimal concentrations of certain antibiotics required to inhibit the growth of reference facultative bacteria in this medium and atmosphere to those obtained in standard testing conditions. MICs of selected antibiotics for the reference cultures, *S. aureus* (ATCC no. 25923) and *E. coli* (ATCC no. 25922), obtained in Schaedler broth and in Mueller-Hinton broth in both aerobic and anaerobic conditions are shown in Table 1. In general, the MICs obtained in Schaedler broth, under both aerobic and anaerobic conditions, compared favorably with those obtained with the standard medium indicating that the medium and the atmosphere proposed for this procedure had little adverse effect on the action of the antibiotics. Therefore, any change in MIC from aerobic to anaerobic conditions would most likely be due to a change in growth response of the facultative organisms.

Effect of inoculum size on MICs. When the number of organisms in the test inoculum was varied, different MIC values were obtained. Tenfold dilutions of a culture in the mid-log-linear growth phase produced progessively smaller MIC values. These results are shown in Tables 2 and 3. The most marked variations in MIC patterns produced by changes in inoculum concentration were with the slower growing organisms, e.g., *E. alactolyticum* (Table 3), as compared to a rapid grower such as *C. perfringens* (Table 2).

Determination of baseline MICs of selected antibiotics. Baseline MICs of antibiotics that have been reported to be efficacious in the

TABLE 2. *MICs of selected antibiotics for different concentrations of Bacteroides fragilis ssp. fragilis and Clostridium perfringens*

Antibiotics	*B. fragilis*				*C. perfringens*			
	10^{6} [a]	10^{5}	10^{4}	10^{3}	10^{6}	10^{5}	10^{4}	10^{3}
Cephalothin	64[b]	32	16	8	0.25	0.25	≤0.12	≤0.12
Chloramphenicol	2	1	0.5	0.5	2	2	1	1
Clindamycin	≤0.03	≤0.03	≤0.03	≤0.03	0.25	0.12	0.12	0.06
Erythromycin	0.25	0.12	0.06	≤0.03	0.5	0.25	0.25	0.25
Gentamicin	>128	>128	128	128	32	32	32	16
Lincomycin	4	2	1	0.5	2	1	0.5	0.5
Penicillin	16	8	4	4	≤0.03	≤0.03	≤0.03	≤0.03
Tetracycline	0.25	0.12	0.06	0.06	0.06	0.06	0.06	0.06
Vancomycin	8	8	4	4	0.12	0.12	0.06	0.06

[a] Numbers represent 4 × 10^{6}, 4 × 10^{5}, etc., organisms per ml.

[b] MICs (μg/ml). Concentrations of antimicrobial agents tested were serial twofold dilutions ranging from 128 to 0.12 or 0.03 μg/ml.

TABLE 3. *MICs of selected antibiotics for different concentrations of Peptostreptococcus Center for Disease Control group 2 and Eubacterium alactolyticum*

Antibiotics	*Peptostreptococcus*				*E. alactolyticum*			
	10^{6} [a]	10^{5}	10^{4}	10^{3}	10^{6}	10^{5}	10^{4}	10^{3}
Cephalothin	0.03[b]	≤0.015	≤0.015	≤0.015	0.06	≤0.015	≤0.015	≤0.015
Chloramphenicol	2	1	0.03	≤0.015	1	0.25	≤0.015	≤0.015
Clindamycin	≤0.015	≤0.015	≤0.015	≤0.015	≤0.015	≤0.015	≤0.015	≤0.015
Erythromycin	≤0.015	≤0.015	≤0.015	≤0.015	0.06	≤0.015	≤0.015	≤0.015
Gentamicin	8	4	≤0.015	≤0.015	8	2	≤0.015	≤0.015
Lincomycin	0.5	0.12	0.03	≤0.015	≤0.015	≤0.015	≤0.015	≤0.015
Penicillin	0.03	0.03	≤0.015	≤0.015	0.03	≤0.015	≤0.015	≤0.015
Tetracycline	0.25	0.12	0.06	0.06	0.12	0.03	≤0.015	≤0.015
Vancomycin	8	8	4	4	0.5	0.5	≤0.015	≤0.015

[a] Numbers represent 4 × 10^{6}, 4 × 10^{5}, etc., organisms per ml.

[b] MICs (μg/ml). Concentrations of antimicrobial agents tested were serial twofold dilutions ranging from 128 to 0.015 μg/ml.

treatment of patients with anaerobic bacterial infections were determined by a modified broth-dilution method in which Schaedler broth and an anaerobic glove box atmosphere of 5% CO_2, 10% H_2, and 85% N_2 were used.

Each organism was tested in triplicate on different days to measure the reproducibility of the test. The mean MICs of selected antibiotics for several commonly encountered anaerobic organisms are shown in Table 4. Results obtained by this test were very reproducible; there was no variation in the MICs obtained at any one testing episode and no greater than one dilution variation between episodes.

Three-tube categorization method for determining antibiotic susceptibility of anaerobes. The susceptibility patterns of the four representative anaerobes used in this study were also determined by a three-tube broth-dilution categorization method. The results of six replicate tests (Tables 5 and 6) are consistent with the base line MIC values shown in Table 4. Moreover, the data shows that when the GasPak system was used as the mode of anaerobic incubation, identical, or very similar, MIC values were obtained. When the inoculum preparation and inoculation were done under aerobic conditions, the MIC values were also identical, or very similar, to those obtained when these manipulations were performed under anaerobic conditions.

DISCUSSION

Much effort has been exerted in recent years to standardize the techniques for antibiotic susceptibility testing. For the most part, the success of these efforts has been limited to the aerobic and facultative bacteria. The development of standard procedures for the anaerobes has been more difficult. Consequently, one of the main purposes of this study and of an earlier

TABLE 4. *MICs (μg/ml) of nine selected antibiotics against anaerobic organisms commonly encountered in clinical infections by using a broth-dilution technique with Schaedler broth and an anaerobic glove box atmosphere of 5% CO_2, 10% H_2, and 85% N_2*

Organisms	Antibiotics[a]								
	Ceph	Chlo	Clin	Ery	Gen	Lin	Pen	Tet	Van
Bacteroides fragilis ssp. *fragilis* no. 9053	32	4	0.12	0.5	>32	4	8	0.25	16
B. fragilis ssp. *fragilis* no. 11239	>32	4	0.12	0.5	>32	2	8	0.25	16
B. fragilis ssp. *fragilis* no. 11732	32	4	0.06	0.5	>32	2	8	0.25	16
B. fragilis ssp. *fragilis* no. 11736	32	4	0.25	0.5	>32	4	16	2	16
B. fragilis ssp. *fragilis* no. 11737	32	2	0.06	0.5	>32	4	8	0.25	16
B. fragilis ssp. *fragilis* no. 23745	>32	4	≤0.015	0.12	>32	0.5	16	0.25	8
B. fragilis ssp. *thetaiotaomicron*	0.12	8	0.06	0.12	1	0.5	0.03	2	2
Fusobacterium nucleatum no. 9052	0.5	4	1	0.03	32	8	0.06	0.12	2
F. nucleatum no. 14371	0.03	2	0.06	4	16	0.25	0.06	0.12	>32
Eubacterium alactolyticum	0.12	2	≤0.015	0.03	8	0.03	0.06	0.06	0.5
Propionibacterium granulosum	0.5	0.5	≤0.015	≤0.015	0.25	0.06	0.06	0.5	0.25
Clostridium perfringens	0.12	1	0.06	0.5	32	0.5	0.06	0.06	0.12
C. ramosum	0.5	4	2	0.25	8	4	1	0.5	2
Peptostreptococcus CDC group 2	0.03	1	0.25	≤0.015	8	0.25	≤0.015	0.25	0.25

[a] Antibiotics tested were cephalothin, chloramphenicol, clindamycin, erythromycin, gentamicin, lincomycin, penicillin, tetracycline, and vancomycin, respectively. They were tested in concentrations that were serial twofold dilutions ranging from 32 to 0.015 μg/ml.

TABLE 5. *MICs of nine antibiotics against Bacteroides fragilis and Peptostreptococcus Center for Disease Control group 2*[a]

Antibiotics	Concn tested (μg/ml)	*Bacteroides fragilis* ssp. *fragilis* 9053		*Peptostreptococcus* CDC group 2	
		Glove box	GasPak	Glove box	GasPak
Cephalothin	(2, 16, 128)	>16, ≤128	>16, ≤128	≤2	≤2
Chloramphenicol	(1, 8)	>1, ≤8	>1, ≤8	≤1	≤1
Clindamycin	(1, 4, 64)	≤1	≤1	≤1	≤1
Erythromycin	(1, 4, 64)	≤1	≤1	≤1	≤1
Gentamicin	(0.5, 4, 64)	>64	>64	>4, ≤64	>4, ≤64
Lincomycin	(1, 4, 64)	>1, ≤4	>1, ≤4	≤1	≤1
Penicillin	(0.25, 16, 128)	>0.25, ≤16	>0.25, ≤16	≤0.25	≤0.25
Tetracycline	(1, 8, 32)	≤1	≤1	≤1	≤1
Vancomycin	(4, 16)	>4, ≤16	>4, ≤16	≤4	≤4

[a] The MICs were determined by a categorization method using Schaedler broth and two atmospheres (anaerobic glove box with 5% CO_2, 10%H_2, and 85% N_2; GasPak).

study (10) was to consider the prerequisites that might lead to the recommendation of a reference method and routine method that would be accepted for use in a diagnostic laboratory.

Of the three testing techniques commonly used for determining antibiotic susceptibility, the broth-dilution method has fewer sources for variation in testing anaerobic bacteria. Although contamination and the restriction to test one organism per series of tubes are drawbacks of the broth-dilution method, the use of broth media minimizes the amount of oxygen exposure and thus may increase the growth response or reduce the time required to achieve eugonic

TABLE 6. *MICs on nine antibiotics against Eubacterium alactolyticum and Clostridium perfringens*[a]

Antibiotics	Concn tested (μg/ml)	MICs			
		Eubacterium alactolyticum		*Clostridium perfringens*	
		Glove box	GasPak	Glove box	GasPak
Cephalothin	(2, 16, 128)	≤2	≤2	≤2	≤2
Chloramphenicol	(1, 8)	>1, ≤8	>1, ≤8	>1, ≤8	>1, ≤8
Clindamycin	(1, 4, 64)	≤1	≤1	≤1,	≤1
Erythromycin	(1, 4, 64)	≤1	≤1	≤1	≤1
Gentamicin	(0.5, 4, 64)	>4, ≤64	>4, ≤64	>4, ≤64	>4, ≤64
Lincomycin	(1, 4, 64)	≤1	≤1	≤1	≤1
Penicillin	(0.25, 16, 128)	≤0.25	≤0.25	≤0.25	≤0.25
Tetracycline	(1, 8, 32)	≤1	≤1	≤1	≤1
Vancomycin	(4, 16)	≤4	≤4	≤4	≤4

[a] The MICs were determined by a categorization method using Schaedler broth and two atmospheres (anaerobic glove box with 5% Co_2, 10% H_2, and 85% N_2; GasPak).

growth in culture. Other advantages of the broth-dilution technique are that it can be used for simultaneous determination of minimal inhibitory and bactericidal concentrations; it can be used to test several antibiotics in synergism studies; and it is more easily adapted to automated procedures that have been used for susceptibility testing. (3, 6; C. Thornsberry, J. C. Sherris, L. D. Thrupp, J. M. Matsen, J. Washington, T. Gavan, L. D Sabbath, A. Balows, and F. Schoenknecht. Prog. Abstr. Intersci. Conf. Antimicrob. Ag. Chemother., 13th, Washington, D.C., Abstr. 204, 1973).

Although the agar dilution method is well suited for the simultaneous testing of large numbers of organisms, the broth-dilution method is a more efficient method for routine use in most laboratories where only a few anaerobic isolates would be tested at any one time.

The medium and atmosphere used in these broth-dilution tests had little deleterious effect on MIC values as compared to values achieved through standard methods with reference facultative organisms. A higher cation concentration in the Schaedler medium may contribute to variations in the MICs obtained with gentamicin and tetracycline, since effectiveness of animoglycosides and tetracyclines may be associated with ion concentration (2), but anaerobiosis and increased CO_2 could have played a role (8).

Even though the data obtained with this reference method are reliable, it is not an optimal method for the routine diagnostic laboratory because of the antibiotic dilution protocol and the physical requirements for the handling and processing of a large number of tubes. Consequently, our approach to overcoming this cumbersome problem was to abbreviate the number of antibiotic dilutions used in the test. Similar systems have been used by Schneirson and Amsterdam in a broth-dilution test (9) and by Washington in an agar-dilution test (11). Ericsson and Sherris and the World Health Organization—International Collaborative Study Group (2) also advocate such an approach to in vitro susceptibility testing. They recommend an extended dilution scheme in which two or three concentrations of each antibiotic are used so that susceptibility can be categorized according to levels attainable in blood, urine, or other body fluids or tissues, and according to efficacy in clinical experience. By using this method, there would generally be four categories of susceptibilities. Ericsson and Sherris (2) suggested that these be called groups 1, 2, 3 and 4. Group 1 would include the very susceptible organisms that would probably respond to the usual dosage of the antimicrobial agent being tested. Group 2 would include those organisms that cause systemic infections that would be likely to respond to the antimicrobial agent if it is given in high doses. Group 3 would include organisms causing localized infections that could probably be treated successfully because the antimicrobial agent is concentrated by physiological processes, as in the urine, or by direct application. Group 4 organisms would be those with degrees of resistance that make clinical response unlikely and, therefore, would be considered resistant without qualifaction. If these group definitions were to be used in the clinical laboratory, the report should contain a definition of each group. Examples of the application of this system are discussed in the International Collaborative Study report of Ericsson and Sherris (2).

The test concentrations of each antibiotic that we have used in this study were not adopted just for testing anaerobic bacteria, but for testing all bacteria. General agreement on what the most clinically useful test concentrations should be may not occur, but with this method each organization could set its own test levels.

Although we must test more strains, our results lead us to believe that the methods proposed here can be used routinely in the clinical bacteriology laboratory to test the antimicrobial susceptiblility of anaerobic bacteria. The three-tube categorization method, performed with the GasPak system, can be used as the routine method. If more definitive MICs are desired, the more conventional, twofold dilution scheme in the same broth and atmosphere can be used.

The categorization would be even more practical for the clinical laboratory, if paper disks were used to deliver the antibiotic to the broth. This method has been used by Isenberg et al. (6) and Thornsberry et al. (C. Thornsberry et al. Prog. Abstr. Intersci. Conf. Antimicrob. Ag. Chemother., 13th, Washington, D.C., Abstr. 204, 1973) for automated or mechanized susceptibility tests on aerobic and facultative organisms, and by Wilkins and Thiel (12) for anaerobic bacteria. This modification of the categorization method is being studied.

LITERATURE CITED

1. Dowell, V. R., Jr., and T. M. Hawkins. 1973. Laboratory methods in anaerobic bacteriology. U.S. Department of Health, Education, and Welfare Publication no. (HSM) 73-8222.
2. Ericsson, H. M., and J. C. Sherris. 1971. Antibiotic sensitivity testing: Report of an international collaborative study. Acta Pathol. Microbiol. Scand. Sect B. Supplement no. 217.
3. Gavan, T. L., H. W. McFadden, Jr., and E. L. Cheatle. 1971. Antimicrobial susceptibility testing. American Society of Clinical Pathologists, Chicago, Ill.
4. Gibbons, R. J., and J. B. MacDonald. 1959. Hemin and vitamin K compounds as required growth factors for the cultivation of certain strains of *Bacteroides melaninogenicus*. J. Bacteriol. **80**:164-170.
5. Holdeman, L. V., and W. E. C. Moore. 1972. Anaerobe laboratory manual. Virginia Polytechnic Institute and State University, Blacksburg, Va.
6. Isenberg, H. D., A. Reichler, and D. Wiseman. 1971. Prototype of a fully automated device for determination of bacterial antibiotic susceptibility in the clinical laboratory. Appl. Microbiol. **22**:980-986.
7. National Committee for Clinical Laboratory Standards Subcommittee on Antimicrobial Susceptibility Testing. 1974. Performance standards for antimicrobial susceptibility tests as used in clinical laboratories, p. 138-155. *In* A. Balows (ed.), Current techniques for antibiotic susceptibility testing. Charles C Thomas, Springfield, Ill.
8. Rosenblatt, J. E., and F. Schoenknecht. 1972. Effect of several components of anaerobic incubation on antibiotic susceptibility test results. Antimicrob. Ag. Chemother. **1**:433-440.
9. Schneirson, S. S., and D. Amsterdam. 1959. A simplified tube procedure for the routine determination of bacterial sensitivity to antibiotics. Amer. J. Clin. Pathol. **31**:81-86.
10. Stalons, D. R., C. Thornsberry, and V. R. Dowell. 1974. The effect of culture medium and carbon dioxide concentrations on the growth of anaerobic bacteria from clinical specimens. Appl. Microbiol. **27**:1098-1104.
11. Washington, J. A. The agar dilution method, p. 127-141. *In* T. L. Gavan, H. W. McFadden, and E. L. Cheatle (ed.), Antimicrobial susceptibility testing. American Society of Clinical Pathology, Chicago, Ill.
12. Wilkins, T. D., and T. Thiel. 1973. Modified broth-disk method for testing the antibiotic susceptibility of anaerobic bacteria. Antimicrob. Ag. Chemother. **3**:350-356.