Collaborative Evaluation of a Proposed Reference Dilution Method of Susceptibility Testing of Anaerobic Bacteria

VERA L. SUTTER,¹* ARTHUR L. BARRY,² TRACY D. WILKINS,³ AND RONALD J. ZABRANSKY⁴

Wadsworth Veterans Administration Hospital Center and University of California (Los Angeles) School of Medicine, Los Angeles, California 90073¹; Clinical Microbiology Laboratories, University of California (Davis) Medical Center, Sacramento, California 95817²; Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061³; Department of Pathology and Laboratory Medicine, Mount Sinai Medical Center, Milwaukee, Wisconsin 53201⁴

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An agar dilution method for susceptibility testing of anaerobic bacteria was evaluated to determine whether results obtained would be consistent enough to recommend it as a reference method. The study was conducted in 10 laboratories where the minimum inhibitory concentrations of six antibiotics (carbenicillin, cefoxitin, chloramphenicol, clindamycin, penicillin G, and tetracycline) were determined against 10 bacterial strains on Wilkins-Chalgren agar prepared by three manufacturers. Minimum inhibitory concentrations falling on the modes varied from 57 to 80% of all determinations and on the mode or within $\pm 1 \log_2$ dilution of the mode from 87 to 100% within each laboratory. When data from all laboratories were pooled, minimum inhibitory concentrations from each laboratory agreed with the overall mode 48 to 71% of the time, with an overall agreement at $\pm 1 \log_2$ dilution of 96%. This degree of reproducibility allows for recommendation of the procedure as a reference method. Results with three of the test strains were very consistent, and these strains are recommended as control strains: *Clostridium perfringens* ATCC 13124, *Bacteroides fragilis* ATCC 25285 and *Bacteroides thetaiotaomicron* ATCC 29741. The minimum inhibitory concentrations for these strains were on the mode or within $\pm 1 \log_2$ dilution of the mode 98, 99, and 99% of the time, respectively. The remaining anaerobic bacteria are recommended as reference strains.

Procedures that have been recommended and used for antimicrobial susceptibility testing of anaerobic bacteria are principally the same as those for aerobic and facultatively anaerobic bacteria. These methods include broth dilution (both macro- and micro-), agar dilution, and agar diffusion methods. There have been substantial differences in the individual techniques that have been described within each class of methods.

As a result of discussions at the International Conference on Anaerobic Bacteria, Atlanta, Ga., 1972, several investigators formed a Working Group for Development of Standard Methods for Susceptibility Testing of Anaerobic Bacteria. This group was later appointed as an ad hoc working group for the National Committee for Clinical Laboratory Standards Subcommittee on Antimicrobial Susceptibility Testing. The primary goal of the working group was to reach agreement on the numerous variables inherent in susceptibility tests such as inoculum preparation and density, test medium, control and reference strains, and other precise technical

details. Secondary goals were to define simplified procedures which could be standardized for routine use.

The group agreed that the agar dilution technique was easier to read and interpret than was the broth dilution technique and therefore might be more reproducible. Individuals within the group then began investigations on various aspects of susceptibility testing of anaerobic bacteria. Studies concerning the development of the medium, inoculum requirements, and culture conditions have been published elsewhere (2-5, 7). Details of an agar dilution method were agreed upon and preliminary trials of the method were reported in 1976 (V. L. Sutter, A. L. Barry, W. J. Martin, J. E. Rosenblatt, V. R. Dowell, Jr., C. Thornsberry, T. D. Wilkins, and R. J. Zabransky, *Progr. Abstr. Intersci. Conf. Antimicrob. Agents Chemother.* 16th, Chicago, Ill., Abstr. no. 453, 1976). Results indicated that the method was reproducible and warranted further trial.

The present study was organized under the auspices of the National Committee for Clinical

Laboratory Standards under a contract negotiated with the Division of Anti-Infective Drugs of the Food and Drug Administration. An ad hoc committee of knowledgeable representatives from government, the professions, and industry reviewed the proposed reference methodology and the validity testing protocol, selected 10 laboratories in which to run the protocol, and reviewed the resulting test data.

The objectives of the present study were to determine: (i) whether the variation of minimum inhibitory concentrations (MICs) for anaerobic bacteria, as measured within individual participating laboratories and between those laboratories, would be within limits that would permit the method being considered as a reference procedure; (ii) the variability of MICs due to differences in media from three sources; and (iii) the variability of MICs for each bacterium-drug combination to identify control and reference strains for use in future work.

MATERIALS AND METHODS

The study consisted of the determinations of MICs of six antibiotics for 10 selected strains of bacteria by using Wilkins-Chalgren agar (7) compounded by three manufacturers. Ten laboratories each tested each bacterium-drug-medium combination on 3 separate days.

Participants were selected so that there would be representation of government-, industry-, and university-affiliated laboratories. The participants also had varying degrees of expertise in susceptibility testing of anaerobic bacteria. They were as follows: Arthur L. Barry and Robert E. Badal, University of California (Davis) Medical Center, Sacramento; Marie Coyle, Harborview Medical Center, Seattle, Wash.; Kenneth P. Cundy, Temple University School of Medicine, Philadelphia, Pa.; Paul Ensminger, Lilly Research Laboratories, Indianapolis, Ind.; Jimmy Evans, Food and Drug Administration, Washington, D.C.; Sharon Hansen, Veterans Administration Hospital, Baltimore, Md.; William J. Martin and Charles W. Hanson, University of California, Los Angeles; Jon E. Rosenblatt, Mayo Clinic and Foundation, Rochester, Minn.; Clyde Thornsberry, Center for Disease Control, Atlanta, Ga.; and Ronald J. Zabransky, Mount Sinai Medical Center, Milwaukee, Wis.

Test strains. Eight strains of anaerobic bacteria and two facultative anaerobic bacteria were used. These were strains which had been used as antibiotic susceptibility test control or reference strains in the laboratories of the Center for Disease Control, Virginia Polytechnic Institute and State University, and Wadsworth Veterans Administration Hospital. The strains were collected, deposited with the American Type Culture Collection (if not previously in the Collection), and distributed to the participating laboratories by T. D. Wilkins. They were as follows: *Clostridium perfringens* ATCC 13124; *Peptococcus variabilis* ATCC 14956; *Bacteroides fragilis* ATCC 25285; *Bacteroides vulgatus* ATCC 29327; *Peptococcus magnus* ATCC 29328; *Bacteroides thetaiotaomicron* ATCC 29741; *B.*

thetaitaomicron ATCC 29742, and *Peptococcus asaccharolyticus* ATCC 29743. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 completed the set of 10 strains used.

Antibiotics. Antibiotics selected for the study were those most commonly recommended for therapy of anaerobic infections. The β -lactamase-resistant cephamycin cefoxitin was also included since it appeared to be promising for therapy of anaerobic infections, particularly those involving the *B. fragilis* group.

Antibiotics for the study were kindly supplied as follows: carbenicillin, Roerig, New York, N.Y.; cefoxitin, Merck Sharp and Dohme, Inc., West Point, Pa.; chloramphenicol, Warner-Lambert/Parke-Davis, Detroit, Mich.; clindamycin, The Upjohn Co., Kalamazoo, Mich.; penicillin G, Eli Lilly and Co., Indianapolis, Ind.; and tetracycline, Pfizer, Inc., Brooklyn, N.Y. Each of the antibiotics was from a single lot number and was distributed to the participating laboratories by V. L. Sutter.

Culture media and other materials. Media for propagation of cultures and preparation of inocula for the tests as well as GasPak generator envelopes and indicators were kindly supplied by BBL Microbiology Systems, Cockeysville, Md. Wilkins-Chalgren agar was compounded and distributed to the laboratories by Difco Laboratories, Detroit, Mich., GIBCO Laboratories, Madison, Wis., and BBL Microbiology Systems. Each of the items supplied was from a single batch or lot.

Procedures. Each laboratory received 1 lyophilized culture of each of the 10 test strains. The lyophilized cultures were reconstituted and cultivated, and several vials of each culture were frozen in sterile skim milk and stored at -20°C or less in freezers that were not frost-free. Each week when cultures were required, a frozen vial of each culture was thawed, streaked on brucella blood agar supplemented with hemin (5 $\mu\text{g}/\text{ml}$) and vitamin K_1 (10 $\mu\text{g}/\text{ml}$) and incubated for 48 to 72 h. Portions of five or more colonies to provide a 3-mm loopful of growth were inoculated into thioglycollate medium without indicator which was supplemented with hemin (5 $\mu\text{g}/\text{ml}$) before autoclaving and with vitamin K_1 (0.1 $\mu\text{g}/\text{ml}$) and sodium bicarbonate (1 mg/ml) just before use. After overnight incubation, turbidity was adjusted with clear brucella broth to the turbidity of one-half the no. 1 McFarland standard.

Each week, agar from each of the three manufacturers was rehydrated and sterilized according to their directions. One agar preparation required the addition of vitamin K_1 and hemin, but these additives were incorporated into the manufactured formulation of the other two media. Antibiotic dilutions were prepared and incorporated into agar as suggested by Ericsson and Sherris (1). All antibiotics were dissolved and diluted in water except chloramphenicol which was first dissolved in phosphate buffer at pH 6.0 to give the stock solution of 2,560 $\mu\text{g}/\text{ml}$ and then further diluted in water. The antibiotic-containing agar plates were prepared on the same day that the tests were performed and were dried in a 35°C incubator for 30 to 45 min before use.

Inocula were applied to the antibiotic-containing plates with Steers replicator (6) and incubated anaer-

obically at 35°C in GasPak jars. At the beginning and end of each series of tests with each drug-medium combination, two plates of the test medium without antibiotics were inoculated. One was incubated anaerobically to serve as a growth control; the other was incubated aerobically to detect possible aerobic contamination. No longer than 5 h elapsed between the melting of the agar and the placement of inoculated plates in anaerobic jars. To avoid bias due to the order in which media were inoculated, the laboratories rotated the order of the source of the media for each of the three sets of inoculation in the three consecutive weeks.

After 48 h of incubation, the MIC of each strain was recorded as the lowest concentration of the antibiotic yielding no growth, one discrete colony, or a fine, barely visible haze as determined with the unaided eye.

Data from each laboratory were examined for each bacterium-drug-medium combination to determine the mode MIC and variation for each. Data were excluded if no growth or very poor growth occurred in the controls or if contamination was evident. Data from all the laboratories were pooled and examined for each bacterium-drug-medium combination to determine the mode MIC and variation for each. They were also examined to determine the mode MIC and variation for each. They were also examined to determine variation related to source of medium and the specific bacterium.

To preserve the anonymity of the participating laboratories with regard to specific results, the laboratories will be identified in the tables by number without respect to the order in which they were listed in Materials and Methods. Since the media manufacturers were provided with a formula for compounding the Wilkins-Chalgren agar but were given no guidelines for quality control, they will be identified by the letters X, Y, Z, also without respect to the order in which they were listed above.

RESULTS

The analysis of data from laboratory 1 for carbenicillin and cefoxitin is given in Table 1 to

illustrate the way in which data were analyzed from each laboratory. Mode values obtained with medium X were sometimes one dilution lower than mode values obtained with the media from the other sources. Similar differences between media were observed in all laboratories and thus should not affect the overall assessment of within-laboratory variation. The data from laboratory 1 for 1 day of determinations with carbenicillin and cefoxitin against *S. aureus* ATCC 25923 were deleted because of contamination. With cefoxitin and clindamycin against *P. variabilis* ATCC 14956, equal numbers of determinations occurred at two MICs, so both values were accepted as modes.

The lowest concentration tested was 0.015 µg/ml, a concentration low enough to record MICs with most organisms. However, *B. vulgatus* ATCC 29327 tested with clindamycin gave only one value on scale (0.03 µg/ml); the others were ≤0.015 µg/ml. The off-scale values were excluded. With clindamycin against *E. coli* ATCC 25922, one value was >256; that MIC was also excluded.

Data from all of the laboratories were examined and analyzed in the same way as those from laboratory 1. They are summarized in Table 2. A total of 540 determinations was expected from each laboratory. However, because of problems such as contamination or poor or no growth of a strain on the control medium, some data were not included in the tabulations and analyses. Reasons for eliminated or missing data were as follows. For laboratory 2, there was poor growth on the controls of *P. variabilis* ATCC 14956 on medium X during the tests with carbenicillin and cefoxitin, and one off-scale value with penicillin occurred with this strain. Cross-contamination occurred between *B. vulgatus* ATCC 29327 and *S. aureus* ATCC 25923 in one test so that the clindamycin MICs for these two bacte-

TABLE 1. Intralaboratory variation—laboratory 1

Culture (ATCC no.)	Carbenicillin				Cefoxitin			
	No. of tests	Mode MIC (µg/ml)	No. on mode ^a	Range	No. of tests	Mode MIC (µg/ml)	No. on mode ^a	Range
13124	9	0.25	6	0.25–0.5	9	0.5	7	0.25–1
14956	9	0.5	5	0.125–0.5	9	0.125/0.25	4/4	0.062–0.025
25285	9	32	4	16–64	9	4.0	6	4–8
29327	9	0.25	6	0.062–0.25	9	1.0	7	0.5–1
29328	9	0.5	8	0.125–0.5	9	0.125	6	0.062–0.125
29741	9	32	8	16–64	9	16	5	16–32
29742	9	64	9	0	9	32	8	32–64
29743	9	0.5	7	0.25–0.5	9	0.25	4	0.062–0.25
25922	9	16	7	8–32	9	8.0	6	4–8
25923	6	0.5	6	0	6	1.0	5	1–2

^a Number on mode or ±1 log₂ dilution from mode: for carbenicillin, 81; for cefoxitin, 86. Percent on mode or ±1 log₂ dilution from mode: for carbenicillin, 93; for cefoxitin, 99.

TABLE 2. Intra- and interlaboratory variation

Laboratory no.	Total no. of tests	% MICs on mode within laboratory	% MICs on mode or $\pm 1 \log_2$ dilution from mode within laboratory	% MICs on mode for all laboratories	% MICs on mode or $\pm 1 \log_2$ dilution from mode for all laboratories
1	525	75	98	69	97
2	529	80	99	57	94
3	533	79	99	71	99
4	539	81	100	69	99
5	444	73	98	54	93
6	516	76	98	50	95
7	540	70	96	58	95
8	526	57	87	48	86
9	523	80	98	71	99
10	497	77	100	60	100
Total	5,213			60	96

ria could not be determined, and one off-scale value of $\leq 0.015 \mu\text{g/ml}$ was omitted for *B. vulgatus* ATCC 29327 and clindamycin. One additional off-scale value occurred with clindamycin against *E. coli* ATCC 25922. For laboratory 3, off-scale values for six determinations of clindamycin MICs with *B. vulgatus* ATCC 29327 and one determination with cefoxitin against *P. magnus* ATCC 29328 were deleted. For laboratory 4, one off-scale value with clindamycin against *B. vulgatus* ATCC 29327 was deleted. For laboratory 5, there was no growth or poor growth of *P. variabilis* ATCC 14956 on medium X in all determinations except one with chloramphenicol and clindamycin and also in all determinations with medium Z. All nine values with clindamycin against *B. vulgatus* ATCC 29327 were off scale. There was poor or no growth of *P. magnus* ATCC 29328 on medium X on two occasions each with chloramphenicol, clindamycin, penicillin, and tetracycline and poor or no growth of this strain on medium Z in all determinations. There was one low off-scale endpoint with carbenicillin, two with cefoxitin, and one with penicillin. There was no growth of *P. asaccharolyticus* ATCC 29743 on medium X on one occasion during tests with penicillin and tetracycline and poor or no growth of this strain on medium Z on all occasions. One value with *E. coli* ATCC 25922 was off scale. For laboratory 6, seven low off-scale values were deleted for *P. variabilis* ATCC 14956; one was deleted with carbenicillin, three were deleted with cefoxitin, and three were deleted with penicillin. Nine MICs with clindamycin against *B. vulgatus* ATCC 29327 were also off scale. Seven omissions occurred with *P. magnus* ATCC 29328. Three MICs were off scale with cefoxitin and two were off scale with penicillin. There was no growth of this strain on medium X at the time of one determination with penicillin and tetracycline.

One MIC with penicillin against *S. aureus* ATCC 25923 was $\leq 0.015 \mu\text{g/ml}$. For laboratory 8, during 1 day of tests with clindamycin there was no growth of *B. vulgatus* ATCC 29327 on all three media controls. *P. asaccharolyticus* ATCC 29743 gave no growth on controls with medium Z and carbenicillin on one occasion and no growth on controls with medium X and medium Z and tetracycline on one occasion each. Two values with clindamycin against *E. coli* ATCC 25922 were deleted because they were >256 . On 1 day of tests with carbenicillin and cefoxitin, *S. aureus* ATCC 25922 was contaminated, eliminating three MICs for each drug. For laboratory 9, off-scale values were eliminated as follows: one for penicillin against *P. variabilis* ATCC 14956, eight for clindamycin against *B. vulgatus* ATCC 29327, and two for clindamycin against *E. coli* ATCC 25922. Contamination of *P. magnus* ATCC 29328 occurred during 1 day of tests with carbenicillin and cefoxitin, eliminating three values for each drug. For laboratory 10, all determinations with *P. variabilis* ATCC 14956 and *P. magnus* ATCC 29328 on medium X were omitted because of poor or no growth on the control plates. Seven determinations with clindamycin against *B. vulgatus* ATCC 29327 were off scale.

MICs falling on the modes within each laboratory varied from 57 to 80%, with nine of the laboratories obtaining 70% or more values on their own modes. When the data were examined to determine how frequently MICs were on the modes or within $1 \log_2$ dilution of the modes, the results varied between 87 and 100%, with nine of the laboratories obtaining 96% or more values within this range. When data from all laboratories were pooled and examined for interlaboratory variation, it was determined that MICs fell on the overall modes from 48 to 71% of the time, with an overall agreement of 60%. MICs were on

TABLE 3. Variation related to source of medium with carbenicillin

Culture (ATCC no.)	Medium X				Medium Y				Medium Z			
	No. of tests	Mode	No. on mode"	Range	No. of tests	Mode	No. on mode"	Range	No. of tests	Mode	No. on mode"	Range
13124	30	0.25	16	0.125-1	30	0.5	26	0.25-0.5	30	0.5	24	0.25-1
14956	21	0.25	10	0.031-16	30	0.5	23	0.25-16	27	0.5	21	0.25-1
25285	30	16	16	16-64	30	32	16	16-64	30	32	17	16-64
29327	30	0.062	10	0.031-0.5	30	0.125	11	0.031-8	30	0.25	9	0.031-0.5
29328	25	0.5	9	0.031-2	29	0.5	13	0.25-2	26	0.5	17	0.5-2
29741	30	32	20	16-64	30	32	24	32-64	30	32	20	16-64
29742	30	64	15	16-128	30	64	20	32-128	30	64	22	32-128
29743	30	0.25	19	0.031-0.5	30	0.5	27	0.25-0.5	26	0.5	26	0
25922	30	16	15	4-32	30	16	19	8-32	30	16	14	8-32
25923	28	0.5	15	0.25-1	28	0.5	15	0.5-1	28	0.5	13	0.25-1
Total	284		145		297		194		287		183	

"No. on mode or $\pm 1 \log_2$ dilution from mode: 255 for medium X, 290 for medium Y, and 278 for medium Z; percent on mode or $\pm \log_2$ dilution from mode: 90 for medium X, 98 for medium Y, and 97 for medium Z.

TABLE 4. Variation related to source of medium (all antibiotics)

Medium source	Total no. of tests	% MICs for each medium		% MICs for all media	
		On mode	On mode or $\pm 1 \log_2$ dilution from mode	On mode	On mode or $\pm 1 \log_2$ dilution from mode
X	1,686	63	95	51	92
Y	1,770	65	97	63	97
Z	1,716	67	97	65	97

the modes or within 1 $\log_2$ dilution of the modes 86 to 100% of the time, with an overall agreement of 96%.

Data were then examined to determine variation related to source of media. Table 3 illustrates results obtained with carbenicillin against the test strains on the three media. A summary of results obtained on the three media with all antibiotics is shown in Table 4. Data that were missing or deleted from the intra- and interlaboratory analysis of variation were also missing or deleted from the analysis of variation related to source of medium. Medium X had the fewest remaining results and the lowest rate of agreement for both intra- and inter-media analyses. Medium Z had fewer remaining results than did medium Y but had similar or slightly better intra- and intermedia agreement than that obtained with medium Y.

Data were examined to determine variation related to the individual test strains. Results are shown in Table 5. Strains exhibiting the least variation were *C. perfringens* ATCC 13124, *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29741. The most variation was exhibited by *P. variabilis* ATCC 14956 and *P. magnus* ATCC 29328. These latter two strains were also those which had the fewest results for analysis due to their inability to grow well on medium X

and medium Z. The mode MIC given for clindamycin against *B. vulgatus* ATCC 29327 may not represent the true value since a total of 49 off-scale values were deleted. If these had been included, 86 bits of data would have been considered yielding a mode of ≤ 0.015 , with 57% of the values on the mode and 81% of the values on the mode or within one dilution from the mode. This would indicate slightly greater variability with this organism than is shown in Table 5.

DISCUSSION

Results of this study indicate that a high degree of reproducibility was achieved, although there were differences in the performance of the individual laboratories. These differences did not appear to be related to the expertise of the laboratory. There were two laboratories which had no difficulties related to bacteria. All the data from these two laboratories were highly consistent. There were three laboratories that had more difficulty with the growth of some strains, particularly *P. variabilis* ATCC 14956 and *P. magnus* ATCC 29328, and many of the data with these bacteria were omitted. These difficulties appear to be in the growth-promoting factors in the individual media formulations as they relate to these organisms. One of the three

TABLE 5. Variation related to bacterial strains

Organism	Antibiotic	Tests	Mode MIC ^a	Range	% MICs	
					On mode	On mode or $\pm 1 \log_2$ dilution from mode
<i>C. perfringens</i> ATCC						
13124	Carbenicillin	90	0.5	0.125-1	68	98
	Cefoxitin	90	0.5	0.25-2	60	99
	Chloramphenicol	90	4	2-8	66	100
	Clindamycin	90	0.062	0.031-0.125	80	100
	Penicillin G	90	0.125	0.031-0.5	46	97
	Tetracycline	90	0.062	0.031-0.25	60	97
	Total	540			63	98
<i>P. variabilis</i> ATCC						
14956	Carbenicillin	78	0.5	0.031-16	60	88
	Cefoxitin	75	0.25	0.031-32	44	87
	Chloramphenicol	82	4	0.031-4	57	91
	Clindamycin	82	1	0.062-2	49	91
	Penicillin G	76	0.062	0.031-8	63	95
	Tetracycline	81	1	0.125-2	58	77
	Total	474			55	88
<i>B. thetaiotaomicron</i>						
ATCC 29742	Carbenicillin	90	64	16-128	63	99
	Cefoxitin	90	32	8-64	69	99
	Chloramphenicol	90	8	0.5-8	83	97
	Clindamycin	90	4	1-16	70	98
	Penicillin G	90	64	8-128	60	98
	Tetracycline	90	0.5	0.25-2	39	88
	Total	540			64	97
<i>P. asaccharolyticus</i>						
ATCC 29743	Carbenicillin	86	0.5	0.031-0.5	70	95
	Cefoxitin	84	0.25	0.031-0.5	50	86
	Chloramphenicol	87	2	0.5-4	74	97
	Clindamycin	87	128	0.25-256	52	99
	Penicillin G	86	0.25	0.031-0.5	63	91
	Tetracycline	84	32	8-128	49	98
	Total	514			60	94
<i>B. fragilis</i> ATCC						
25285	Carbenicillin	90	32	16-64	47	100
	Cefoxitin	90	8	4-8	56	100
	Chloramphenicol	90	4	2-8	87	100
	Clindamycin	90	1	0.25-2	62	97
	Penicillin G	90	32	8-64	89	99
	Tetracycline	90	0.25	0.125-2	50	96
	Total	540			65	99
<i>B. vulgatus</i> ATCC						
29327	Carbenicillin	90	0.125	0.031-8	30	83
	Cefoxitin	90	1	0.125-2	47	91
	Chloramphenicol	90	4	1-4	50	99
	Clindamycin	37	0.031	0.031-1.0	65	89
	Penicillin G	90	0.5	0.125-2	56	96
	Tetracycline	90	8	2-16	71	98
	Total	487			52	93
<i>P. magnus</i> ATCC						
29328	Carbenicillin	80	0.5	0.031-2	49	88
	Cefoxitin	75	0.125	0.031-0.25	55	95
	Chloramphenicol	82	4	0.5-16	48	91
	Clindamycin	82	1	0.125-2	48	94
	Penicillin	78	0.062	0.031-8	40	95
	Tetracycline	81	1	0.125-16	42	83
	Total	478			47	91

^a Concentration in micrograms per milliliter except for penicillin G, which is in units per milliliter.

TABLE 5—continued

Organism	Antibiotic	Test	Mode MIC ^a	Range	% MICs	
					On mode	On mode or $\pm 1 \log_2$ dilution from mode
<i>B. thetaiotaomicron</i> 29741	Carbenicillin	90	32	16-64	71	100
	Cefoxitin	90	16	8-32	60	100
	Chloramphenicol	90	8	0.5-8	79	98
	Clindamycin	90	4	0.5-8	78	99
	Penicillin G	90	32	8-64	78	99
	Tetracycline	90	16	4-32	62	98
	Total	540			71	99
<i>E. coli</i> ATCC 25922	Carbenicillin	90	16	4-32	53	99
	Cefoxitin	90	8	4-16	56	100
	Chloramphenicol	90	8	4-16	56	100
	Clindamycin	83	256	64-256	75	99
	Penicillin G	90	64	32-128	60	100
	Tetracycline	90	1	0.5-8	57	93
	Total	533			59	98
<i>S. aureus</i> ATCC 25923	Carbenicillin	84	0.5	0.25-1	51	100
	Cefoxitin	84	1	1-2	58	100
	Chloramphenicol	90	16	8-32	58	100
	Clindamycin	89	0.125	0.031-2	71	90
	Penicillin G	89	0.062	0.031-0.125	84	100
	Tetracycline	90	0.5	0.25-2	58	94
	Total	526			63	97

laboratories demonstrated highly consistent results with narrow ranges of values around the mode. The remaining laboratories submitted data within 93% or greater agreement with others.

Although there were differences in the results received with each of the media from different sources, the overall performance of the media was satisfactory. In this study the performance of these formulations was not compared with the original formulation as described by Wilkins and Chalgren (7), but Zabransky and Hauser (8) demonstrated that the original formulation would support the growth of some of the organisms evaluated during this study: *C. perfringens* ATCC 13124, *B. fragilis* ATCC 25285, *B. vulgatus* ATCC 29327, and *P. magnus* ATCC 29328. The fact that one manufacturer's formulation did not contain the vitamin K₁ and the hemin (medium Z) did not appear to be a contributing factor to the differences in results obtained or in the ability or disability of that medium to support the growth of the test strains. The difficulties as seen with the media in this study, particularly medium X, appear to be related to their abilities to support the growth of organisms selected for the evaluation.

The greatest difficulty in obtaining reproducible results involved tests with the anaerobic, gram-positive cocci. They tended to produce fairly poor growth in the thioglycollate medium

after overnight incubation. Somewhat better growth might have been achieved if the frozen stock cultures had been grown first on blood agar plates for two or three consecutive passes rather than just one pass. The anaerobic, gram-positive cocci also produced relatively poor growth on the Wilkins-Chalgren agar media. Differences in the nutritive capacity of the three lots of media became quite apparent with the *P. variabilis* and *P. magnus* strains. These microorganisms should be included in any battery of test strains that will be used to check the performance of different lots of Wilkins-Chalgren agars. The three manufacturers who provided experimental batches of Wilkins-Chalgren agar for this study obviously had difficulty in producing a single medium which performs uniformly. This suggests that there may be some difficulty in achieving an entirely satisfactory commercial product. A series of performance tests will be needed for the manufacturers to check the quality of each new lot of medium before it is released for sale. The reference strains included in this study should be useful for such purposes. Additional strains, especially *B. melaninogenicus*, *B. asaccharolyticus*, and other frequent isolates from anaerobic infections, should be examined for their ability to grow in commercial preparations.

It is unrealistic to expect any susceptibility test system to be capable of testing all anaerobic

microorganisms. However, a useful reference method should be capable of testing at least the various types of anaerobes selected for this study. Unusually slow-growing or nutritionally fastidious strains were intentionally omitted from the collection because they would not be useful for testing the reference method. The procedure will probably have to be modified to test the more fastidious anaerobes in much the same way that standard aerobic methods are modified to permit testing of fastidious aerobes and facultative anaerobes. Additional studies will be needed to determine how such procedural changes affect the end results with various antibiotics.

Some microorganism-drug combinations characteristically yielded rather poorly defined endpoints. By comparing the growth on each antibiotic-containing plate to that on the drug-free control plate, the endpoints can be selected consistently. A faint haze of growth was arbitrarily considered negative, whereas slightly heavier growth was positive. Considering the nature of the endpoints that were observed, it is reassuring to see the high degree of reproducibility that was documented in this report.

For quality control purposes, three of the eight anaerobes included in this series can be recommended. Those strains demonstrating the most consistent results were *C. perfringens* ATCC 13124, *B. fragilis* ATCC 25285, and *B. thetaio-taomicron* ATCC 29741. The five remaining anaerobes and two facultative organisms are recommended as reference strains for use in future studies. The susceptibility test results recorded in this report define the range of MICs that should be expected for each of the 10 strains tested against six antibiotics. It is our hope that future studies of other antibiotics will include replicate tests with these reference strains to further expand the data base.

The degree of overall agreement of data from the 10 laboratories allows the recommendation of this procedure for susceptibility testing of anaerobic bacteria. It will be proposed to the National Committee for Clinical Laboratory Standards as a reference method. Although this

method is sufficiently practical for use in many clinical laboratories, others may find it too cumbersome and seek simpler tests. With this as a reference method, any other susceptibility testing procedure for anaerobes that has been or would be developed should be based upon obtaining the same MICs with the same organism-antibiotic combinations as achieved in this study.

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