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Microdilution Technique for Antimicrobial Susceptibility Testing of Anaerobic Bacteria

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A microdilution technique using commercially available media and materials was developed and used to determine the minimal inhibitory concentrations (MICs) of clindamycin, chloramphenicol, tetracycline, minocycline, ampicillin, carbenicillin, cephalothin, and gentamicin for 101 anaerobic isolates. Representative strains of *Bacteroides*, *Clostridium*, *Fusobacterium*, *Peptococcus*, and *Peptostreptococcus* were tested. The use of Schaedler broth at pH 7.2, an inoculum of 10^5 to 10^7 colony-forming units per ml, and incubation at 35 C in an anaerobic glove box with an atmosphere of 80% nitrogen, 10% hydrogen, and 10% carbon dioxide resulted in good growth and easily interpretable results. After 48 h of incubation, 97% of strains tested were inhibited by 3.1 μg or less of clindamycin per ml and 98% were inhibited by 12.5 μg or less of chloramphenicol per ml. Tetracycline and minocycline inhibited 81 and 88% of strains tested in concentrations of 6.2 μg or less per ml and 1.6 μg or less per ml, respectively. Ampicillin inhibited all strains other than *B. fragilis* in concentrations of 3.1 μg or less per ml. Excluding certain strains of *Bacteroides* and *Clostridium*, carbenicillin in concentrations of 12.5 μg or less per ml and cephalothin in concentrations of 6.2 μg or less per ml inhibited all strains tested. Gentamicin was inactive although some strains of anaerobic cocci and *Bacteroides* were inhibited by 3.1 μg or less per ml. After 18 to 24 h of incubation, eight of the 101 strains had not grown sufficiently for MICs to be determined; for the 93 strains which had grown sufficiently, 93% of 744 MICs were the same or one concentration lower than the 48-h MICs.

Presently there is no standard method for antimicrobial susceptibility testing of anaerobes although both agar and broth dilution methods are available (18). The broth dilution method is generally considered to be cumbersome but recent developments in instrumentation have made small volume versions of this procedure more feasible for routine use. Because the efficacy of microdilution techniques for broth dilution antimicrobial susceptibility testing has been demonstrated for rapidly growing facultative and aerobic bacteria (2, 5), a microdilution method was developed and used to test the susceptibility of 101 strains of anaerobic bacteria to eight antibiotics. (This paper was presented in part at the 74th Annual Meeting of the American Society for Microbiology, Chicago, Ill., 1974.)

MATERIALS AND METHODS

Microorganisms. Ninety isolates from patients at University Hospitals, Columbus, Ohio and 11 clinical isolates supplied by the Ohio Department of Health Laboratories were studied. The isolates included 38 strains of *Bacteroides fragilis*, two strains of *B. melaninogenicus*, two strains of *B. clostridium*, one strain of *B. oralis*, seven strains of *Fusobacterium*, 19 strains of *Clostridium perfringens*, 13 strains of other clostridia including one strain each of *C. cochlearium*, *C. bifermentans*, *C. ramosum*, *C. pseudotetanicum*, *C. fallax*, *C. histolyticum*, *C. septicum*, *C. sordellii*, *C. glycolicum*, two strains each of *C. tertium* and *C. sporogenes*, and 19 strains of gram-positive anaerobic cocci. Isolates were identified by the University Hospitals Clinical Bacteriology Laboratory and the Ohio State Department of Health Laboratories by the criteria established by Dowell and Hawkins (3), Holdeman and Moore (7), and Sutter and Finegold (16). Stock cultures in Schaedler broth (BBL) with 10% glycerol were maintained frozen in liquid nitrogen and were subcultured onto anaerobically stored 5% sheep blood agar plates before use.

Antibiotics. Antibiotics were prepared by dissolving laboratory standard powders in sterile distilled water and diluting them to a concentration of 400 μg per ml. The drugs were stored for a maximum of 1

month at −20 C in sterile plastic Microdel bottles (Cooke Laboratory Products, Alexandria, Va.) with tips calibrated to deliver 0.05 ml per drop. Before use, a fresh bottle of each drug was thawed slowly in the refrigerator for 3 h. Antibiotics tested were clindamycin, chloramphenicol, tetracycline, minocycline, ampicillin, carbenicillin, cephalothin, and gentamicin.

Media. Schaedler broth was used for all tests. It was stored for 1 to 7 days before use in an anaerobic glove box (Coy Manufacturing, Ann Arbor, Mich.) which contained 80% nitrogen, 10% hydrogen, and 10% carbon dioxide at 60% relative humidity. Before autoclaving, the pH of the media was 8.4; after autoclaving and equilibration in the glove box the pH was 7.2. For anaerobic cocci, heat-inactivated (56 C for 0.5 h) horse serum was added to a final concentration of 1% before use.

Minimal inhibitory concentrations. Minimal inhibitory concentrations (MICs) for the eight antibiotics were determined by a microdilution method. Anaerobically stored standard U-bottom microdilution plates (Linbro Chemical Co., New Haven, Conn.) each with eight horizontal rows of 12 wells each were prepared in duplicate for each organism. Using a calibrated disposable pipette (Cooke Laboratory Products, Alexandria, Va.), one drop (0.05 ml) of Schaedler broth was added to each well. One drop (0.05 ml) of each of the eight antibiotics was then added to the wells in the first vertical row so that the first well in each horizontal row contained a different antibiotic. The antibiotics in the first wells were then simultaneously diluted in serial twofold decreasing concentrations through the 11 wells using a manual multimicrodiluter handle equipped with eight 0.05-ml microdiluters (Cooke Laboratory Products, Alexandria, Va.). The 12th vertical row of wells received no antibiotic. The microdilution plates were then transferred to the glove box and allowed to equilibrate for at least 0.5 h. The antibiotic-containing wells and every other well in the 12th vertical row of each plate were then inoculated with one drop (0.05 ml) of a diluted 18-h broth culture using a disposable pipette.

To prepare the inocula, two or three colonies of each test strain were subcultured to 5 ml of Schaedler broth and incubated in the glove box overnight. For suspensions which were faintly turbid, corresponding to an optical density of approximately 0.1 to 0.3 at 650 nm, a 1:10 dilution was used; for suspensions which were more turbid, a 1:100 dilution was used. Adjustment of the inocula to a specific turbidity standard was not appropriate because there was marked variation in the number of colony-forming units present in cultures of the various genera of anaerobes which were adjusted to given turbidity standards.

The final inocula ranged from 10^5 to 10^7 colony-forming units per ml. The final antibiotic concentrations ranged from 100 to 0.1 μg per ml. The 12th vertical rows consisted of alternating growth and sterility controls. Plates were sealed with cellophane tape (Cooke Laboratory Products, Alexandria, Va.) to prevent evaporation and incubated at 35 C in the glove box.

MICs were read as the lowest concentrations of antibiotics which inhibited visible growth after incubation for 18 to 24 and 48 h. Growth was indicated by diffuse turbidity, a single large dot, or a filamentous network of growth. All MICs were read independently by two observers. When duplicate plates gave results which varied by more than one concentration, the test was repeated. A strain of *C. perfringens* or a strain of *B. fragilis* with known MICs was tested each day and served as a system control.

RESULTS

The 48-h MICs of the eight antibiotics tested for the 101 anaerobic isolates are shown in Tables 1 through 8. Clindamycin (Table 1) inhibited 100% of *Bacteroides*, *Fusobacterium*, and anaerobic cocci, 95% of *C. perfringens*, and 77% of other clostridia in concentrations of 3.1 μg or less per ml. Two strains of *C. tertium* and one strain each of *C. perfringens* and *C. sporogenes* were more resistant. Chloramphenicol (Table 2) inhibited 98% of all strains in concentrations of 12.5 μg or less per ml. One strain each of *B. clostridium* and *C. perfringens* was more resistant. Tetracycline (Table 3) and minocycline (Table 4) inhibited 81 and 88% of strains tested in concentrations of 6.2 μg or less per ml and 1.6 μg or less per ml, respectively. Forty-three percent and 71% of the isolates were highly susceptible with MICs of 0.1 μg or less per ml to each of the tetracyclines, respectively. Ampicillin (Table 5) inhibited all anaerobes other than *B. fragilis* in concentrations of 3.1 μg or less per ml; only 32% of *B. fragilis* were susceptible to those concentrations. Carbenicillin (Table 6) inhibited all strains tested except

TABLE 1. *Susceptibility of anaerobic isolates to clindamycin*

Organism	Strains tested	Cumulative percent inhibited at various concn (μg/ml)										
		0.1	0.2	0.4	0.8	1.6	3.1	6.2	12.5	25	50	100
Bacteroides fragilis	38	84	92		95	100						
Bacteroides sp.	5	60			100							
Fusobacterium sp.	7	100										
Clostridium perfringens	19	42	53	68	89	95						
Clostridium sp.	13	31	46	54	69		77		84		92	100
Gram-positive cocci	19	90	95	100								

TABLE 2. *Susceptibility of anaerobic isolates to chloramphenicol*

Organism	Strains tested	Cumulative percent inhibited at various concn (μg/ml)										
		0.1	0.2	0.4	0.8	1.6	3.1	6.2	12.5	25	50	100
Bacteroides fragilis	38			13	18	55	92	95	100			
Bacteroides sp.	5	20					40		80	100		
Fusobacterium sp.	7		14	29	43	57	86		100			
Clostridium perfringens	19					11	37	68	95		100	
Clostridium sp.	13		8			23	62	85	100			
Gram-positive cocci	19	5			16	42	63	95	100			

TABLE 3. *Susceptibility of anaerobic isolates to tetracycline*

Organism	Strains tested	Cumulative percent inhibited at various concn (μg/ml)										
		0.1	0.2	0.4	0.8	1.6	3.1	6.2	12.5	25	50	100
Bacteroides fragilis	38	26	34			39	47	71	82	92	100	
Bacteroides sp.	5	40						80			100	
Fusobacterium sp.	7	57	86	100								
Clostridium perfringens	19	53			63	74		89	100			
Clostridium sp.	13	69						77	85		92	100
Gram-positive cocci	19	42	53	63	74		84			89	100	

TABLE 4. *Susceptibility of anaerobic isolates to minocycline*

Organism	Strains tested	Cumulative percent inhibited at various concn (μg/ml)										
		0.1	0.2	0.4	0.8	1.6	3.1	6.2	12.5	25	50	100
Bacteroides fragilis	38	61	76	78	84	92	95		97	100		
Bacteroides sp.	5	40						60		100		
Fusobacterium sp.	7	100										
Clostridium perfringens	19	84			89	95		100				
Clostridium sp.	13	92							100			
Gram-positive cocci	19	58		63	68	74	79	84	89	100		

TABLE 5. *Susceptibility of anaerobic isolates to ampicillin*

Organism	Strains tested	Cumulative percent inhibited at various concn (μg/ml)										
		0.1	0.2	0.4	0.8	1.6	3.1	6.2	12.5	25	50	100
Bacteroides fragilis	38	3	5	8		16	32	47	66	74	79	84
Bacteroides sp.	5	60		80	100							
Fusobacterium sp.	7	86	100									
Clostridium perfringens	19	100										
Clostridium sp.	13	54	69	85	92		100					
Gram-positive cocci	19	95	100									

TABLE 6. *Susceptibility of anaerobic isolates to carbenicillin*

Organism	Strains tested	Cumulative percent inhibited at various concn (μg/ml)										
		0.1	0.2	0.4	0.8	1.6	3.1	6.2	12.5	25	50	100
Bacteroides fragilis	38			5	8		26	45	63	76	82	
Bacteroides sp.	5	40					60	80	100			
Fusobacterium sp.	7	43	57	100								
Clostridium perfringens	19	53	74	95	100							
Clostridium sp.	13	8	38	54	62			69		77	85	92
Gram-positive cocci	19	21		53	79	95	100					

for some strains of *B. fragilis* and clostridia at concentrations of 12.5 μg or less per ml. Sixty-three percent of *B. fragilis* were inhibited by those concentrations and 82% were inhibited by 50 μg or less per ml. Sixty-nine percent of clostridia other than *C. perfringens* were inhibited by 12.5 μg or less per ml and 85% were inhibited by 50 μg or less per ml. Cephalothin (Table 7) inhibited many of the strains tested in concentrations of 6.2 μg or less per ml although 89% of *B. fragilis*, 40% of other bacteroides, and 16% of clostridia were more resistant. Gentamicin (Table 8), unlike the other seven antibiotics tested, showed little activity against most of the anaerobes, although concentrations of 3.1 μg or less per ml inhibited 35% of the anaerobic cocci and one strain of *Bacteroides*.

Table 9 shows the changes in MICs which occurred between the 18- to 24-h and 48-h readings. Eight of the 101 test strains did not grow sufficiently to read MICs at 18 to 24 h. For the remaining 93 strains, 79% of the 744 MICs did not change during day 2 of incubation. Fourteen percent increased one concentration, 5% increased two concentrations, and 2% increased three or more concentrations during day 2. Whereas the 18- to 24-h readings of 93% of the 744 interpretable MICs were the same or only one concentration lower than the corresponding 48-h readings, 24-h results for 99% of the more rapidly growing clostridia were within one concentration of those 48-h readings.

Ninety percent of both the 24-h and 48-h MICs for the control organisms were the same or within one concentration of the geometric mean MIC for each antibiotic; 8% varied by two concentrations and 2% varied more than two concentrations.

DISCUSSION

MICs determined by the method presented were comparable to those reported by others (1, 4, 8–13, 15, 17, 19–22). Clindamycin and chloramphenicol were the most consistently active antimicrobial agents against the anaerobes studied. Tetracycline was highly active against many strains in all genera but a significant number of strains were resistant. Minocycline, a tetracycline derivative, was active against a

TABLE 9. *MICs at 24 h compared to 48 h for 93 strains of anaerobic bacteria*[a]

Drug	% of strains			
	Same MIC	1 Concn lower	2 Concn lower	≥3 Concn lower
Clindamycin	91	7	1	1
Gentamicin	84	11	3	2
Ampicillin	86	10	2	2
Carbenicillin	80	18	1	1
Tetracycline	71	18	10	1
Minocycline	84	9	3	4
Cephalothin	79	14	5	2
Chloramphenicol	63	27	8	2

[a] With 8 additional strains there was insufficient growth at 24 h for reading.

TABLE 7. *Susceptibility of anaerobic isolates to cephalothin*

Organism	Strains tested	Cumulative percent inhibited at various concn (μg/ml)										
		0.1	0.2	0.4	0.8	1.6	3.1	6.2	12.5	25	50	100
Bacteroides fragilis	38					3	5	11	18	21	53	84
Bacteroides sp.	5	40					60					100
Fusobacterium sp.	7	71		86			100					
Clostridium perfringens	19		11		26	68	84	89	100			
Clostridium sp.	13	8	23	46	62	69	77		92	100		
Gram-positive cocci	19	42	58	74	79	100						

TABLE 8. *Susceptibility of anaerobic isolates to gentamicin*

Organism	Strains tested	Cumulative percent inhibited at various concn (μg/ml)										
		0.1	0.2	0.4	0.8	1.6	3.1	6.2	12.5	25	50	100
Bacteroides fragilis	38							3		11	21	58
Bacteroides sp	5	20							60	100		
Fusobacterium sp.	7									57	86	
Clostridium perfringens	19									5	26	53
Clostridium sp.	13							15		62	77	84
Gram-positive cocci	19	5		10	15	25	35	50	55	70	85	100

larger number of strains at comparable concentrations. This increased activity, also observed by others (13, 15), is of unknown clinical significance since obtainable serum concentrations with minocycline are lower than with tetracycline and there has been little clinical experience with minocycline in treating anaerobic infections. Ampicillin was active against all strains other than *B. fragilis*. Carbenicillin and cephalothin were highly active against fusobacteria, anaerobic cocci, and many clostridia, but some clostridia and bacteroides, especially *B. fragilis*, were resistant. Gentamicin was generally inactive. Resistance of the anaerobic isolates tested was usually apparent after 18 to 24 h of incubation, whereas susceptibility could only be determined with certainty after 48 h incubation.

The method reported utilized commercially available media and materials and was convenient to perform. Bacterial growth in the Schaedler broth was abundant, as has been found by others (14) and MIC end points were easily determined with virtually no reader variation. As a microdilution method, the test could be used to perform large numbers of MICs and is suitable for the determination of bactericidial activity (6).

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