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Susceptibility Testing of Clinically Isolated Anaerobic Bacteria by an Agar Dilution Technique

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Agar dilution minimal inhibitory concentrations (MICs) of penicillin, tetracycline, chloramphenicol, and clindamycin were determined using Wilkens-Chalgren agar for 1,266 clinical isolates of anaerobic bacteria. In addition, a reference strain of *Bacteroides fragilis* was repeatedly tested and demonstrated the precision of the technique. Fifty-six percent of our *Bacteroides melaninogenicus* strains were resistant (MIC ≥4.0 μg/ml) to penicillin. Resistance to this antibiotic was also seen among other anaerobes, but the results are more in accord with previous reports. Resistance to tetracycline (MIC ≥4.0 μg/ml) was found in 60% of our isolates. Chloramphenicol proved to be the most effective agent in vitro with only 2.0% of strains resistant (MIC ≥16 μg/ml). Only 5% of strains were resistant to clindamycin (MIC ≥8.0 μg/ml), and this included 10 isolates of *B. fragilis* and 4 of *B. melaninogenicus*. The incidence of resistance of anaerobic bacteria to these frequently used antibiotics is greater than previous reports and indicates the need for reliable susceptibility testing of anaerobic bacteria.

Numerous techniques have been proposed during the last 10 years for susceptibility testing of anaerobic bacteria. These include disk diffusion (11, 24), broth-disk methods (10, 16, 26), and minimal inhibitory concentration (MIC) methods using broth dilution (14, 16) and agar dilution (23, 27; V. L. Sutter, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 16th, Chicago, Ill., abstr. no. 453, 1976). These procedures have been reviewed and evaluated (3, 22). The agar dilution procedure has the greatest degree of precision and is recommended as a reference method for anaerobic susceptibility testing (19). A recent report compared various practical methods with the agar dilution reference method (13) and found similar results with all the methods. The relatively easy-to-perform and reliable broth-disk procedures are the techniques that most clinical microbiology laboratories should have available. The choice of when to perform susceptibility tests depends on the experience of the medical staff and the type of patients and procedures found in the institution.

The qualitative results observed with the broth-disk technique contribute a limited amount of data to our overall information on the susceptibility of anaerobic bacteria and on the emergence of resistance among anaerobic bacteria, which is similar to what has occurred among the facultative anaerobic and aerobic bacteria. Therefore, larger institutions with the volume, facilities, and expertise in infectious and laboratory medicine should be doing quantitative testing on anaerobic bacteria. We report here our MIC information on a significant number of recently isolated anaerobic bacteria from clinical infections during a 2-year period.

MATERIALS AND METHODS

Organisms. Most of the bacteria tested were recent clinical isolates from anaerobic specimens submitted to the diagnostic microbiology laboratory of Hutzel Hospital. A few of the isolates were obtained from other hospitals in the 2,300-bed Detroit Medical Center. Strains were tested from September 1977 through August 1979. *Bacteroides fragilis* ATCC 25285 was obtained from the American Type Culture Collection. Organisms were identified in the Hutzel Hospital diagnostic microbiology laboratory according to procedures recommended by Dowell and Hawkins (7) and Holdeman and Moore (8).

Media and susceptibility tests. Isolated organisms were inoculated into preduced chopped-meat-glucose medium (GIBCO Diagnostics, Madison, Wis.) and incubated at 35°C for 24 h or until the medium was visibly turbid. These cultures were diluted in Schaedler broth (BBL Microbiology Systems, Cockeysville, Md.) to a concentration equal to the turbidity of one-half the no. 1 MacFarland standard. Susceptibility tests were carried out in air and therefore were performed as soon as possible after making dilutions, to maintain the viability and number of bacteria at 1.5×10^8 cells per ml. An agar dilution susceptibility test was performed using Wilkens-Chalgren agar made according to the originators' procedure (23). A Steers replicator was used to inoculate the adjusted suspensions to the agar surfaces. Two control plates without antimicrobial agents were inoculated; one was incubated at 35°C in 10% CO_2 in air, and the other plate was incubated anaerobically with the antibiotic dilu-

tion plates in a Coy Anaerobic Chamber (Ann Arbor, Mich.). These plates served as contamination and growth controls. The plates were incubated for 48 h at 35°C in an anaerobic chamber with a gas mixture of 85% nitrogen, 5% carbon dioxide, and 10% hydrogen. The MIC was read as the lowest concentration of antimicrobial agent that allowed three or fewer discrete colonies, or a barely visible haze of growth which was definitely less than the growth on the growth control agar plate.

Antimicrobial agents. The four antibiotics included in our routine testing were: penicillin G (Wyeth Laboratories, Philadelphia, Pa.), tetracycline (Pfizer Inc., New York, N.Y.), chloramphenicol (Parke-Davis, Detroit, Mich.), and clindamycin (Upjohn Co., Kalamazoo, Mich.). Laboratory standard powders were weighed to make stock solutions of 4,800 μg of penicillin, tetracycline, and clindamycin per ml and 19,200 μg of chloramphenicol per ml. Aliquot solutions of each were frozen at −70°C. Each week a vial of each agent was thawed and diluted, and three sets of antibiotic agar dilution plates were prepared. These plates were stored at 2 to 5°C and were used within 1 week of preparation. Previous studies have demonstrated the stability of antibiotics under these conditions (27). The MIC breakpoint for each antimicrobial agent, above which an organism was considered to be resistant, was established as: penicillin G, 2 μg/ml; tetracycline, 2 μg/ml; chloramphenicol, 8 μg/ml; clindamycin, 4 μg/ml.

RESULTS

The MICs of penicillin, tetracycline, chloramphenicol, and clindamycin for 1,266 recent clinical isolates of anaerobic bacteria are shown in Tables 1 to 4. We used the criterion that any organism with an MIC of ≥4.0 μg/ml is resistant to penicillin, and 97% of the *B. fragilis* strains were resistant. This same breakpoint demonstrated 56% of the *B. melaninogenicus* group to be resistant to penicillin. Numerous resistant *Clostridium* strains were isolated; variation among species was considerable (Table 1). The incidence of resistance to penicillin was less frequent among other species.

TABLE 1. *Agar dilution MICs of penicillin for anaerobic bacteria*

Strain	No. of isolates	Cumulative % susceptible to concn:					
		≤0.25 μg/ml	0.5 μg/ml	1.0 μg/ml	2.0 μg/ml	4.0 μg/ml	8.0 μg/ml
Actinomyces sp.	4	100					
Bacteroides corrodens	6	100					
B. fragilis[a]	231	1	2		3	7	19
B. melaninogenicus[a]	124	33	36	39	40	46	61
Bacteroides sp.	39	39	41	49	59	64	70
Bifidobacterium sp.	8	75	100				
Clostridium bifermentans	4	100					
C. butyricum	1		100				
C. clostridiiforme	3			33			
C. difficile	4		25			75	100
C. innocuum	7	14	57		72		86
C. paraputrificum	5	20					
C. perfringens	44	68	80	86		89	
C. ramosum	14	29	79	86			
C. sordellii	1	100					
C. sporogenes	3	67	100				
C. symbiosum	2			100			
C. tertium	2				100		
Eubacterium contortum	6	83	100				
E. lentum	7	86	100				
E. sp.	6	83		100			
Fusobacterium mortiferum	2	50					
F. nucleatum	22	86	91		95		
Fusobacterium sp.	81	82	87	88	89	93	94
Gaffkya anaerobia	73	86	89	90		95	97
Peptococcus asaccharolyticus	178	80	85	86	89	91	94
P. magnus	63	95	100				
P. prevotii	64	75	81		86		88
P. sp.	31	90	94			97	100
Peptostreptococcus anaerobius	181	65	68	72	74	83	93
P. micros	4	100					
Peptostreptococcus sp.	14	100					
Propionibacterium sp.	11	100					
Veillonella sp.	21	52	67	86	91	95	

[a] Includes all former subspecies.

Organisms with MICs of ≥4.0 μg/ml were considered to be resistant to tetracycline. The endpoints for the isolates can be seen in Table 2. Variation was seen among the species, but a majority of the anaerobic clinical isolates were resistant to this agent.

Organisms with MICs of ≥16 μg/ml were considered to be resistant to chloramphenicol. Only a few of our isolates were resistant to this amount of chloramphenicol (Table 3).

Isolates with clindamycin MICs of ≥8.0 μg/ml were considered resistant (Table 4). Particularly interesting are the 4.4% of the *B. fragilis* group and the 3.2% of the *B. melaninogenicus* group that were resistant to this agent.

Quality assurance of our agar dilution procedure was checked by repeated testing of the reference strain *B. fragilis* ATCC 25285, as recommended by A. L. Barry (personal communication). It was tested 40 times with each antibiotic concentration, which resulted in mean MICs as follows: penicillin, >8.0 μg/ml; tetracycline, ≤0.25 μg/ml; chloramphenicol, 4.0 μg/ml; clindamycin, 1.0 μg/ml. The chloramphenicol and clindamycin MICs were never more than ±1 dilution from the mean.

DISCUSSION

There have been several reports of susceptibility testing of anaerobic bacteria, but most deal with techniques and only include a relatively small number of clinical isolates. There have been only a few reports presenting data on several hundred isolates, such as the extensive study by Sutter and Finegold (20), but there is no report with information on a large number of recent clinical isolates using Wilkens-Chalgren agar and a technique similar to what has recently been proposed as the reference method of MIC testing of anaerobic bacteria (19).

Our procedure differs from the proposed reference method in several respects. We used

TABLE 2. *Agar dilution MICs of tetracycline for anaerobic bacteria*

Strain	No. of isolates	Cumulative % susceptible to indicated concn:					
		≤0.25 μg/ml	0.5 μg/ml	1.0 μg/ml	2.0 μg/ml	4.0 μg/ml	8.0 μg/ml
Actinomyces sp.	4	50			75	100	
Bacteroides corrodens	6	83		100			
B. fragilis[a]	231	6	15	26	29	32	36
B. melaninogenicus[a]	124	23	30	34	36	41	49
Bacteroides sp.	39	26	31	38	41	56	64
Bifidobacterium sp.	8		13		25	50	75
Clostridium bifermentans	4	75	100				
C. butyricum	1	100					
C. clostridiiforme	3		33				
C. difficile	4	25		75	100		
C. innocuum	7	43		72			86
C. paraputrificum	5	80	100				
C. perfringens	44	23			25	34	45
C. ramosum	14	7	29	50			57
C. sordellii	1						100
C. sporogenes	3	67	100				
C. symbiosum	2						
C. tertium	2				100		
Eubacterium contortum	6	50		83			100
E. lentum	7	29	43			57	
E. sp.	6	33		50		68	100
Fusobacterium mortiferum	2	50	100				
F. nucleatum	22	41	68	77	86		96
Fusobacterium sp.	81	53	64	75	85	88	93
Gaffkya anaerobia	73	30	42	45	53	56	63
Peptococcus asaccharolyticus	178	12	16	17	18	20	25
P. magnus	63	10	16	35	52	56	60
P. prevotii	64	16	23	34	39	41	50
P. sp.	31	16	26	36	45		52
Peptostreptococcus anaerobius	181	11	13	15	19	29	51
P. micros	4	50	75				
Peptostreptococcus sp.	14	21	29	43	50	57	64
Propionibacterium sp.	11	18	73	82	91		100
Veillonella sp.	21	10	38	81	91	95	

[a] Includes all former subspecies.

TABLE 3. *Agar dilution MICs of chloramphenicol for anaerobic bacteria*

Strain	No. of isolates	Cumulative % susceptible to concn:					
		1.0 μg/ml	2.0 μg/ml	4.0 μg/ml	8.0 μg/ml	16.0 μg/ml	32.0 μg/ml
Actinomyces sp.	4	75	100				
Bacteroides corrodens	6	100					
B. fragilis[a]	231	1	7	58	95	99	100
B. melaninogenicus[a]	124	34	72	99	100		
Bacteroides sp.	39	44	74	92	100		
Bifidobacterium sp.	8	13	38	63	100		
Clostridium bifermentans	4			75	100		
C. butyricum	1	100					
C. clostridiiforme	3	33		100			
C. difficile	4			75		100	
C. innocuum	7		14	29		100	
C. paraputrificum	5	40	100				
C. perfringens	44		25	93	98		
C. ramosum	14	14	36	79	86	100	
C. sordellii	1		100				
C. sporogenes	3			100			
C. symbiosum	2		100				
C. tertium	2			50			100
Eubacterium contortum	6	33	83	100			
E. lentum	7	57	71		100		
E. sp.	6	33	83	100			
Fusobacterium mortiferum	2	100					
F. nucleatum	22	50	86	100			
Fusobacterium sp.	81	72	91	96	100		
Gaffkya anaerobia	73	37	85	100			
Peptococcus asaccharolyticus	178	34	79	96	99	100	100
P. magnus	63	8	38	89	98	100	
P. prevotii	64	36	72	86	98		
P. sp.	31	32	84	97	100		
Peptostreptococcus anaerobius	181	60	90	98	100		
P. micros	4	50	100				
Peptostreptococcus sp.	14	43	79	100			
Propionibacterium sp.	11	64	100				
Veillonella sp.	21	53	92	96	100		

[a] Includes all former subspecies.

chopped-meat broth instead of thioglycolate medium to grow the organisms; we adjusted the inocula with Schaedler broth instead of Brucella broth; and we used two or fewer discrete colonies as our criterion of no growth when determining MICs, whereas the reference technique used no growth or one discrete colony. We did not have difficulty with growth of our isolates, whereas the collaborative study reported difficulty with certain species (19). Two factors that may have contributed to our results were that we prepared our Wilkens-Chalgren agar fresh each week from the individual ingredients (23) and that we incubated our tests in an anaerobic chamber, in contrast to having the medium premixed by the manufacturers and incubating the tests in GasPak jars.

The breakpoint at which one decides that a bacterium is resistant has not been unanimously agreed upon. For penicillin, some investigators have chosen a value of 20 μg (32 U)/ml (20), and others prefer 2 μg (3.2 U)/ml (13). We agree with the latter value for in vitro testing, although wide variation in serum levels of penicillin is achievable, and the nature of the infection and the clinical condition of the patient in conjunction with the microbial susceptibility results must be considered in deciding whether an isolate is treatable with a particular antimicrobial agent. Using the >2.0 μg/ml breakpoint for penicillin, we found that 56% of our *B. melaninogenicus* group of bacteria were resistant to penicillin. This value is in disagreement with the general view that this group of organisms is susceptible to penicillin, but it is in agreement with the findings of Murray and Rosenblatt (12), who found that 56% of their *B. melaninogenicus* strains produced beta-lactamase, and Appelbaum and Chatterton (1), who reported that 32% of their strains had MICs of >2.0 μg/ml. We did

TABLE 4. *Agar dilution MICs of clindamycin for anaerobic bacteria*

Strain	No. of isolates	Cumulative % susceptible to concn:					
		≤0.25 μg/ml	0.5 μg/ml	1.0 μg/ml	2.0 μg/ml	4.0 μg/ml	8.0 μg/ml
Actinomyces sp.	4	100					
Bacteroides corrodens	6	83		100			
B. fragilis[a]	231	42	60	79	88	96	97
B. melaninogenicus[a]	124	88	95		97		99
Bacteroides sp.	39	87	90	95		97	100
Bifidobacterium sp.	8	100					
Clostridium bifermentans	4	100					
C. butyricum	1			100			
C. clostridiiforme	3	33	67	100			
C. difficile	4					75	
C. innocuum	7	43		86	100		
C. paraputrificum	5					60	80
C. perfringens	44	55	70	89	95	98	
C. ramosum	14	21	36	50	71	100	
C. sordellii	1		100				
C. sporogenes	3						
C. symbiosum	2	100					
C. tertium	2					50	
Eubacterium contortum	6	67					
E. lentum	7	100					
E. sp.	6	67	83				
Fusobacterium mortiferum	2	100					
F. nucleatum	22	86	96			100	
Fusobacterium sp.	81	85	90	95	96		
Gaffkya anaerobia	73	86	95	96	97		
Peptococcus asaccharolyticus	178	65	78	89	92	93	94
P. magnus	63	48	70	83	90		92
P. prevotii	64	64	75	78	84	89	91
P. sp.	31	58	65	78	87	94	
Peptostreptococcus anaerobius	181	87	95	96	98	99	
P. micros	4	75	100				
Peptostreptococcus sp.	14	93					
Propionibacterium sp.	11	100					
Veillonella sp.	21	100					

[a] Includes all former subspecies.

not test our isolates for beta-lactamase activity. Other significantly resistant common clinical isolates included five *Clostridium perfringens* and 13 *Peptostreptococcus anaerobius* isolates, which had MICs greater than 8.0 μg/ml. Resistance of *C. perfringens* to penicillin has been reported, but our results are different from those of Appelbaum and Chatterton (1), who found that all of their peptostreptococci were inhibited by 1.0 μg/ml. This could be due to chance, since they were reporting on only nine isolates. Sutter and Finegold (20) found 90% of their peptostreptococci to be susceptible to less than 2.0 μg of penicillin per ml. Both groups only reported their isolates to the genus level. We found that all the resistant strains in this genus were *P. anaerobius*, but all other species were inhibited by ≤0.25 μg of penicillin per ml. For 26% of our *P. anaerobius* strains, the MICs were ≥4.0 μg/ml.

We observed a high incidence of resistance to tetracycline, which is in agreement with results previously reported for this agent (1, 6, 20). This confirms that tetracycline is not a drug of choice for anaerobic infections unless laboratory tests have demonstrated susceptibility of the isolate.

Twelve of our 231 *B. fragilis* strains were resistant to chloramphenicol, with MICs of ≥16 μg/ml. Other studies have found *B. fragilis* to be susceptible to this antibiotic (1, 14), but enzymatic resistance to chloramphenicol has been reported in *B. fragilis* (5), and Sutter and Finegold (20) showed that the MIC for 10 of 76 isolates was ≥16 μg/ml. Overall, our isolates were susceptible to this agent; only 25 strains among 1,266 anaerobic bacteria required MICs of ≥16 μg/ml.

One of our most interesting observations is the incidence of isolates resistant to clindamycin (i.e., with MICs of ≥8.0 μg/ml). *Clostridium* and

Peptococcus strains resistant to this agent have been reported (17, 18, 25). However, our incidence of resistance of *B. fragilis* and *B. melaninogenicus* was higher than previously reported (14, 17, 20). Blazevic (4) did have one isolate from this group with an MIC of 12.5 μg/ml, and Salaki et al. (15) reported two isolates with MICs of 25 and >100 μg of clindamycin per ml. Noteworthy in our findings is that 8 strains of the 231 *B. fragilis* group tested had MICs greater than 8.0 μg of clindamycin per ml. One of our resistant isolates was studied in another Detroit Medical Center laboratory and found to respond to an MIC of 512 μg of clindamycin per ml (2). These investigators, using a microbroth technique, found that the MICs for 12 out of the 92 *B. fragilis* they studied were ≥8.0 μg of clindamycin per ml. Our selection of 8.0 μg/ml as a breakpoint for determining resistance in this report has been influenced by the report of Jones et al. (9), who found slightly higher clindamycin MIC endpoints when they used Wilkens-Chalgren agar. We also found two isolates of the *B. melaninogenicus* group with MICs of ≥8.0 μg of clindamycin per ml. We are not aware of any other reports of this species being resistant to clindamycin, but it is not surprising that it has occurred, and we will most likely observe more resistance to clindamycin among anaerobic bacteria with its continued use.

Our recovery of a higher incidence of resistance among anaerobic bacteria may be due to our large sample of isolates compared to the previous reports, in which they may have missed the resistant strains. However, more likely our results reflect the situation of a large urban medical center with extensive use of antibiotics and many compromised patients. This situation produces conditions favorable for the emergence of resistance among anaerobic bacteria. These results emphasize that diagnostic laboratories should have available, either in their own institution or at reference laboratories, the capability of doing susceptibility tests on anaerobic bacteria.

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