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Antibiotic- and Method-Dependent Variation in Susceptibility Testing Results of *Bacteroides fragilis* Group Isolates

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The susceptibilities of 36 isolates of the *Bacteroides fragilis* group to ceftizoxime, cefoxitin, clindamycin, and metronidazole were determined by using the National Committee for Clinical Laboratory Standards agar dilution reference method and a broth microdilution method using anaerobe, brucella, Schaedler, and brain heart infusion broths. MICs that were ≥fourfold higher or lower than those of the reference method were considered significant. Major and minor discrepancies in susceptibility interpretation (SI) were also noted. Ceftizoxime showed the greatest number of variations and SI discrepancies. In 72% of the cases, MICs in broth were ≥fourfold lower than those obtained by the reference method, resulting in 33% of the major and 22% of the minor discrepancies in SI. A total of 53% of the isolates were resistant to ceftizoxime by the reference method, but only 11 to 17% were resistant in the various broths. Significant variations in MICs of cefoxitin occurred in 19 to 22% of the isolates; 17 to 19% of the isolates showed major discrepancies and 31 to 58% showed minor discrepancies in SI. A total of 58% of the isolates were resistant to cefoxitin by the reference method, but only 19 to 28% were resistant in the various broths. Significant variations with clindamycin in broths ranged from 32 to 53% and resulted in 3 to 8% of the isolates showing major discrepancies and 33 to 44% showing minor discrepancies in SI. Metronidazole yielded significant variations in MICs in 6 to 28% of the isolates, but no major or minor SI discrepancies were noted. This study indicates that significant differences in susceptibility results, which appear to be method related, can result when isolates of the *B. fragilis* group are tested. Therefore, studies correlating in vitro results, determined by various methods, to clinical outcome are essential.

Susceptibility testing of aerobic and facultatively anaerobic clinical isolates of bacteria is for most clinical laboratories a well-accepted method which provides information to physicians that is helpful in choosing appropriate antimicrobial therapy. Collaborative studies have shown a good correlation between agar and broth dilution methods for a variety of bug-drug combinations (2). Thus, a technique can be chosen from a variety of methods to suit the needs of the laboratory.

Within the last 10 to 15 years, the clinical relevance of anaerobic bacteria has been better defined, and we now appreciate the frequency and significance of anaerobic bacteria in many types of infections (3). In the last several years, nationwide surveys noted increases in resistance levels of anaerobes, particularly *Bacteroides fragilis* group isolates, to several antibiotics (13). Susceptibility patterns have also been found to vary geographically (13). As a result of these susceptibility differences, it is important for more clinical laboratories to consider instituting antimicrobial susceptibility testing of anaerobic bacteria.

The current reference method sanctioned by the National Committee for Clinical Laboratory Standards (8) is an agar dilution method described by Sutter et al. (12). This method was shown to be reproducible and also established quality control guidelines. For most clinical laboratories, however, this method is laborious, time-consuming, and costly. Some laboratories have instituted the use of various broth methods in an effort to eliminate some of the disadvantages of the reference method. We compared susceptibility results for clinical isolates of the *B. fragilis* group in broth media by a broth microdilution method to the results obtained with the reference agar dilution method.

MATERIALS AND METHODS

Organisms. A total of 36 isolates of the *B. fragilis* group (16 *B. fragilis*, 13 *B. thetaiotaomicron*, 5 *B. distasonis*, and 2 *B. ovatus*) from clinical specimens were tested. Each organism was identified to the species level by selective media, biochemical profiles, and gas-liquid chromatography patterns (4, 6).

Antibiotics. Each of the following antibiotics was tested: cefoxitin (Merck Sharp & Dohme, Rahway, N.J.), ceftizoxime (Smith Kline & French Laboratories, Philadelphia, Pa.), clindamycin (The Upjohn Co., Kalamazoo, Mich.), and metronidazole (G. D. Searle, Chicago, Ill.). Each antibiotic was stored desiccated at −20°C until used.

Antimicrobial assays. (i) Reference method. The agar dilution method described by Sutter et al. (12) was used as the reference method, as recommended by the National Committee for Clinical Laboratory Standards (8). Serial twofold dilutions of each antibiotic were prepared in Wilkins-Chalgren (W-C) agar (Difco Laboratories, Detroit, Mich.), which is supplemented with vitamin K (0.5 μg/ml) and hemin (5 μg/ml) by the manufacturer. Agar dilutions ranged from 0.016 to 64 μg/ml. Agar plates containing no antibiotics were used as sterility and organism growth controls. Before being tested, each isolate was cloned twice on prereduced 5% sheep blood agar supplemented with vitamin K (0.5 μg/ml) and hemin (5 μg/ml) and incubated at 35°C in an anaerobic atmosphere (10% CO_2–10% H_2–80% N_2). The inoculum was prepared by picking three to five colonies of the test organism and inoculating them into 5 ml of prereduced thioglycolate broth supplemented with vitamin K (0.5 μg/ml) and

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hemin (5 μg/ml). The broth cultures were incubated overnight at 35°C and used to prepare an organism suspension in prereduced brucella broth (Difco) equivalent in density to a 0.5 McFarland standard. Each plate was then inoculated with a multipoint inoculating device (11) which delivered a final inoculum of approximately 10^5 CFU per spot. The inoculum size was verified by plating serial dilutions of the inoculum and performing colony counts. The plates were incubated at 35°C in an anaerobic chamber for 48 h. The MIC was defined as the lowest dilution of each antibiotic that inhibited the visible growth of the test organism. A single colony or a haze was interpreted as no growth.

(ii) Broth method. For comparison, the MIC endpoints were determined in four broth media by a broth microdilution method. Serial twofold dilutions of each antibiotic were prepared in each of the following broth media: anaerobe, brucella, Schaedler, and brain heart infusion (BHI) broths, all from Difco. All broth media were supplemented with vitamin K (0.5 μg/ml) and hemin (5 μg/ml). Antibiotic concentrations ranged from 0.016 to 64 μg/ml. Plates were prepared with a semiautomated dispenser (Dynatech Laboratories, Inc., Alexandria, Va.) with a final test volume of 100 μl per well. Wells containing antibiotic-free medium served as broth sterility and inoculum growth controls. The inoculum was prepared from the same thioglycolate culture used in the reference method. Appropriate dilutions of the overnight culture were prepared, and the anaerobically prereduced plates were inoculated with a multipoint inoculator (Dynatech) that delivered a final inoculum of approximately 10^5 CFU per well. The inoculum size was verified by performing colony counts on the inoculum. All plates were incubated in an anaerobic chamber for 48 h. The MIC was defined as the lowest concentration of each antibiotic that inhibited the visible growth of the test organism.

(iii) Quality assurance. Quality assurance of each susceptibility run was controlled by testing *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29741.

Comparison of results. (i) Agreement of broth MICs with the reference method. The MIC endpoints for each type of broth were compared with those obtained by the agar dilution method. Broth MICs equal to or within 1 twofold dilution of the MIC obtained by the reference method were considered acceptable, whereas results equal to or greater than 2 twofold dilutions of the reference method value were considered significantly different.

(ii) Comparison of the major and minor discrepancies. Discrepancies between the results of the broth microdilution and agar dilution reference methods were defined as follows: a major discrepancy existed when one method produced an MIC interpreted as susceptible and the other method produced an MIC interpreted as resistant; a minor discrepancy existed when the MICs by one method were interpreted as susceptible and the MICs by the other were interpreted as moderately susceptible, or as moderately susceptible by one method and resistant by the other. The following antibiotic concentrations (in micrograms per milliliter) were used for interpretation of MICs: for ceftizoxime, ≤8 showed susceptibility and ≥32 showed resistance; for cefoxitin, ≤8 showed susceptibility and ≥32 showed resistance; for clindamycin, ≤0.5 showed susceptibility and ≥8 showed resistance; and for metronidazole, ≤16 showed susceptibility and ≥32 showed resistance. MIC interpretation categories are based on recommendations from the National Committee for Clinical Laboratory Standards and the recommendations of the drug manufacturers.

RESULTS

Comparison of broth and agar MICs. Tables 1 and 2 show the agreement of the MICs determined by broth microdilution with those obtained by the agar dilution reference method. The MICs of ceftizoxime determined in broth were ≥fourfold lower than those determined by the reference method for 56 to 72% of the isolates. As a result, the mean

TABLE 1. Comparison of the activities of ceftizoxime, cefoxitin, clindamycin, and metronidazole in various media against 36 isolates of *B. fragilis* group

Antimicrobial agent[a]	Test medium	MIC (μg/ml)				% Resistant
		Range	Mean	50%	90%	
Ceftizoxime	W-C agar	0.5–≥128	16	32	128	53
	Anaerobe broth	0.063–≥128	4	4	64	17
	Brucella broth	0.063–64	4	8	64	11
	Schaedler broth	0.063–64	4	4	32	14
	BHI broth	0.063–≥128	4	4	64	11
Cefoxitin	W-C agar	8–64	32	32	32	58
	Anaerobe broth	2–64	16	16	32	28
	Brucella broth	0.5–64	16	16	32	25
	Schaedler broth	4–64	16	16	32	22
	BHI broth	4–64	16	16	32	19
Clindamycin	W-C agar	≤0.125–≥128	2	2	8	28
	Anaerobe broth	≤0.125–≥128	1	1	8	11
	Brucella broth	≤0.125–≥128	0.5	0.5	4	8
	Schaedler broth	≤0.125–≥128	1	0.5	2	8
	BHI broth	≤0.125–≥128	0.5	0.25	4	8
Metronidazole	W-C agar	0.25–2	1	0.5	2	0
	Anaerobe broth	0.5–4	1	1	2	0
	Brucella broth	0.25–2	1	1	2	0
	Schaedler broth	0.125–2	1	1	2	0
	BHI broth	0.125–4	1	1	1	0

[a] All isolates were tested with each antimicrobial agent.

TABLE 2. Agreement of MIC results of broth microdilution method with those of agar dilution reference method

Antimicrobial agent[a]	Test medium (broth)	No. (%) of isolates agreeing with the agar method at dilutions of:	
		≤1	≥2
Ceftizoxime	Anaerobe	10 (28)	26 (72)
	Brucella	16 (44)	20 (56)
	Schaedler	13 (36)	23 (64)
	BHI	13 (36)	23 (64)
Cefoxitin	Anaerobe	29 (81)	7 (19)
	Brucella	29 (81)	7 (19)
	Schaedler	28 (78)	8 (22)
	BHI	29 (81)	7 (19)
Clindamycin	Anaerobe	25 (69)	11 (31)
	Brucella	18 (50)	18 (50)
	Schaedler	20 (56)	16 (44)
	BHI	17 (47)	19 (53)
Metronidazole	Anaerobe	27 (75)	9 (25)
	Brucella	32 (89)	4 (11)
	Schaedler	27 (75)	9 (25)
	BHI	34 (94)	2 (6)

[a] Each of 36 isolates was tested with each antimicrobial agent.

MIC in the broths was 4 μg/ml, whereas the mean MIC in agar was 16 μg/ml. The MICs required to inhibit growth of 50 or 90% of the strains (MIC_{50} or MIC_{90}, respectively) in each of the broths were lower than those obtained by the reference method. Thus, less resistance was found when ceftizoxime was tested in broth than when it was tested in agar. MICs determined in anaerobe broth showed the highest percentage of variation from the MICs determined by the reference method; those in brucella broth showed the least variation. Regardless of the broth used, the MICs varied significantly for over half of the isolates tested.

By comparison, the MICs of cefoxitin in broth microdilution tests showed 78% or greater acceptable MICs compared with those determined by the reference method. As a result, the mean MIC and MIC_{50} dropped only 1 twofold dilution, whereas the MIC_{90} for all media was 32 μg/ml. The resistance levels, however, were lower in the broth dilution assays than in the reference method. Of isolates showing significant variation from the reference method, more than 95% of the broth microdilution MICs were fourfold lower than those of the reference method. The same isolates were responsible for the significant variations regardless of the broth medium.

MIC comparisons for clindamycin in the various media showed different degrees of significant variation for each broth medium (31 to 53%; Table 2) compared with those of the reference method. Resistance MICs were found 17 to 20% less often in broth media than in the reference method. MICs in anaerobe broth showed the best correlations with the reference method, whereas BHI broth MICs showed the least acceptable correlations. For a larger number of isolates, clindamycin MICs were ≥eightfold lower in BHI broth than in the reference method, compared with values determined in the other broths.

For metronidazole, the majority of the MICs produced in the various broth media were equal to or 1 twofold dilution higher than the MICs in the reference method. BHI and brucella broths gave the highest level of correlation of results with the reference method. No significant changes in the mean MIC, MIC_{50}s, or MIC_{90}s were observed when the

TABLE 3. Distribution into categories by MICs for 36 isolates of *B. fragilis* group

Antimicrobial agent	Growth medium	No. of isolates		
		Susceptible[a]	Intermediate[b]	Resistant[c]
Ceftizoxime	W-C agar	12	5	19
	Anaerobe broth	27	3	6
	Brucella broth	27	5	4
	Schaedler broth	30	1	5
	BHI broth	28	6	2
Cefoxitin	W-C agar	4	11	21
	Anaerobe broth	15	11	10
	Brucella broth	17	10	9
	Schaedler broth	13	15	8
	BHI broth	15	14	7
Clindamycin	W-C agar	7	19	10
	Anaerobe broth	15	17	4
	Brucella broth	21	12	3
	Schaedler broth	21	12	3
	BHI broth	22	11	3
Metronidazole	W-C agar	36		
	Anaerobe broth	36		
	Brucella broth	36		
	Schaedler broth	36		
	BHI broth	36		

[a] Antibiotic concentrations (in micrograms per milliliter) used for interpretation of MICs were ≤8 for ceftizoxime and cefoxitin, ≤0.5 for clindamycin, and ≤16 for metronidazole.

[b] Antibiotic concentrations (in micrograms per milliliter) used for interpretation of MICs were 16 for ceftizoxime and cefoxitin and 1 to 4 for clindamycin.

[c] Antibiotic concentrations (in micrograms per milliliter) used for interpretation of MICs were ≥32 for ceftizoxime, cefoxitin, and metronidazole and ≥8 for clindamycin.

TABLE 4. Comparison of major and minor discrepancies as a result of MIC variations between the broth microdilution and reference methods

Antimicrobial agent[a]	Test medium (broth)	No. (%) of isolates showing discrepancies	
		Major	Minor
Ceftizoxime	Anaerobe	10 (28)	8 (22)
	Brucella	12 (33)	6 (17)
	Schaedler	12 (33)	7 (19)
	BHI	12 (33)	6 (17)
Cefoxitin	Anaerobe	6 (17)	13 (36)
	Brucella	7 (19)	16 (44)
	Schaedler	6 (17)	21 (58)
	BHI	6 (17)	15 (42)
Clindamycin	Anaerobe	1 (3)	12 (33)
	Brucella	3 (8)	15 (42)
	Schaedler	3 (8)	15 (42)
	BHI	3 (8)	16 (44)
Metronidazole	Anaerobe	0	0
	Brucella	0	0
	Schaedler	0	0
	BHI	0	0

[a] Each of 36 isolates was tested with each antimicrobial agent.

broth medium results were compared with those of the reference method.

Comparison of MIC distributions and interpretation discrepancies. Tables 3 and 4 show the distributions of MICs into interpretative categories for each antibiotic and the degree of interpretive discrepancies produced. For ceftizoxime, the distribution of MICs showed most organisms to be susceptible by broth methods but resistant by the reference method. The change in MICs for individual organisms was predominantly (56 to 67%) from resistant by agar dilution to susceptible by broth dilution. For minor discrepancies, MICs determined by the broth microdilution method resulted in interpretive shifts from moderately susceptible to susceptible and from resistant to moderately susceptible compared with reference method results.

For cefoxitin, the distribution of MICs in the reference method fell largely in the moderately susceptible and resistant categories, whereas for MICs in broth dilution assays, the distribution was largely in the susceptible and moderately susceptible categories. The same organisms accounted for the major discrepancies when broth and agar results were compared. Most of the discrepancies (68 to 78%) were minor by definition and resulted in shifts in interpretation from resistant to moderately susceptible and from moderately susceptible to susceptible. Cefoxitin had an overall greater number of discrepancies but fewer major discrepancies than did ceftizoxime.

With clindamycin, the distribution of agar dilution MICs fell predominantly in the moderately susceptible and resistant categories, whereas results in broth dilution assays were predominantly in the susceptible and moderately susceptible categories. Few major interpretive discrepancies occurred in the clindamycin results in the various media tested; however, a significant number of minor discrepancies was noted. As with the cephalosporins, the minor discrepancies with clindamycin were shifts from resistant to moderately susceptible and from moderately susceptible to susceptible.

Since all metronidazole MICs, regardless of method or media, were in the susceptible category, no major or minor interpretive discrepancies were noted.

DISCUSSION

The agar dilution reference method described by Sutter et al. (12) and sanctioned by the National Committee for Clinical Laboratory Standards (8) is more suited for research laboratories than for most clinical laboratories. Although this method has been shown to be highly reproducible, it is laborious and expensive to perform. Various alternative broth methods are available (9) that are often used when limited susceptibility testing of anaerobes is desired. In this study, we compared the results of MIC determinations in a broth microdilution method with results in an agar dilution reference method. Four antibiotics (ceftizoxime, cefoxitin, clindamycin, and metronidazole) were tested. In addition, results with four broth media were compared with results of the agar dilution method.

For ceftizoxime, cefoxitin, and clindamycin, the broth microdilution MICs were, in general, lower than those from the reference method. The broth microdilution MICs of ceftizoxime and clindamycin were fourfold lower in 31 to 72% of tests with the various broths compared with MICs in the reference method. Variations in results for cefoxitin in broth ≥ 2 dilutions lower than in the agar dilution method have been reported to be from 8 to 24.1% (1, 5, 10). The same authors have reported that clindamycin discrepancies vary in 4 to 43.5% of tests when broth microdilution results were compared with results of the reference agar dilution method (1, 5, 10). Metronidazole has shown MIC discrepancies of ≥ 2 dilutions between the two methods 10.7 to 15.7% of the time (1, 5, 10). These differences in lower MICs produced both major and minor discrepancies in susceptibility determinations for these three antimicrobial agents. The broth microdilution MICs of ceftizoxime had the highest percentage of major discrepancies compared with the results of the reference method, whereas cefoxitin and clindamycin produced more frequent minor discrepancies than did ceftizoxime. When metronidazole was tested, MIC differences between the two methods were found with some isolates but were not significant enough to produce any major or minor interpretive discrepancies. Jones et al. (5) reported a significant interpretive discrepancy of 6.3% with cefoxitin when the broth microdilution and agar dilution methods were compared. For clindamycin interpretive discrepancies, rates of 2 to 4% have been reported, whereas no interpretive discrepancies were noted for metronidazole (5, 10).

From this study, it is evident that different methods used in antimicrobial susceptibility testing of anaerobes can produce significantly different results. These results can cause major changes in the interpretation of susceptibility patterns. The differences observed between these methods appear also to be antibiotic dependent and medium dependent, although no single medium tested appeared to be superior for testing the four antibiotics in the present study. The formulation of anaerobe broth is identical to that of W-C agar with the agar omitted; however, significantly different results were observed with the two media. Thus, the question still exists as to which results are most predictive of the therapeutic outcome of these antimicrobial agents in patients. This question can be answered only by comparing the various in vitro susceptibility results with the clinical outcome of patients with established infections caused by the organisms in question.

In conclusion, it is important that additional studies be done to examine other variables, such as medium osmolarity, inoculum size, and the effect of anaerobiosis on certain antibiotics. As data on these variables accumulate, an attempt to establish a standardized broth microdilution method will be easier.

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