

Differences in Susceptibilities of Species of the *Bacteroides fragilis* Group to Several β -Lactam Antibiotics: Indole Production as an Indicator of Resistance

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Clinical isolates of the members of the *Bacteroides fragilis* group differ markedly in their susceptibilities to a variety of β -lactam antibiotics, including cefoperazone, moxalactam, cefotaxime, cefoxitin, cefamandole, cephalothin, ceftazolin, and carbenicillin, as determined by dilution techniques. The minimum concentrations required to inhibit at least 50% of the strains tested (MIC₅₀) for the entire *B. fragilis* group were lowest with moxalactam and cefoxitin, 4 and 8 μ g/ml, respectively, whereas the MIC₉₀s of cefoperazone, cefotaxime, moxalactam, cefoxitin, and carbenicillin were equivalent (64 μ g/ml); the MIC₉₀s of cefamandole, cephalothin, and ceftazolin were higher. Indole-positive members of the group (*B. ovatus*, *B. thetaiotaomicron*, and *B. uniformis*) were significantly more resistant to every antibiotic tested than were indole-negative members (*B. fragilis*, *B. distasonis*, and *B. vulgatus*). In a 6-month survey of clinical laboratory data, indole-positive strains comprised 40% of the *B. fragilis* group isolates and 22% of all *Bacteroides* isolates; *B. fragilis* was the most common species isolated (23%). The increased use of second-generation and the introduction of third-generation cephalosporins may dictate that clinical microbiology laboratories routinely identify members of the *B. fragilis* group as to species or, alternatively, test for indole production in addition to performing more extensive susceptibility testing.

Until recently, many microbiologists viewed all *Bacteroides fragilis* isolates as members of a single species. Considerable evidence has accumulated to justify separating this group into seven distinct species, four indole positive (*B. thetaiotaomicron*, *B. ovatus*, *B. uniformis*, and *B. eggerthii*) and three indole negative (*B. fragilis*, *B. distasonis*, and *B. vulgatus*). Inclusion of *B. uniformis* and *B. eggerthii* in this group is based largely on the DNA homology work of Johnson and Ault (11, 12). Further evidence that these two species should be included in the *B. fragilis* group stems from work evaluating direct fluorescent-antibody detection of *B. fragilis* group members. *B. eggerthii* and *B. uniformis* demonstrated significant fluorescence by this procedure (3). Due to the expense and time involved in the performance of required biochemical procedures, most clinical microbiology laboratories continue the practice of reporting all isolates within the group as *B. fragilis*, which would be an acceptable practice if the species had similar antibiotic susceptibilities.

Critical analysis of the many antibiotic studies with the *B. fragilis* group in the literature has proven difficult due to the variety of reporting practices utilized by microbiology laboratories. Such articles make reference to, e.g., antibiotic susceptibility patterns of the *B. fragilis* group (4), *B. fragilis* subsp. *fragilis* (17), *B. fragilis* (species or group unspecified) (1), *B. fragilis* and other members of the *B. fragilis* group (8), *B. fragilis* "strains" (7), the species *B. fragilis* (2), several distinct species within the *B. fragilis* group (6, 14, 15), and two subgroups, one consisting of *B. fragilis*, *B. vulgatus*, and *B. distasonis* and the other comprised of *B. thetaiotaomicron* and *B. ovatus* (13). This multitude of designations makes it difficult, if not impossible, to intelligently evaluate susceptibility data presented in the literature. This confusion clearly must extend to the clinician attempting to empirically choose an appropriate antimicrobial based on such information. Future articles should present results in a consistent and logical manner to eliminate any potential confusion regarding which organism or group of organisms is being reported.

This investigation was designed to determine whether clinical isolates of species within the *B.*

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fragilis group differ in their susceptibility patterns to a variety of β -lactam antibiotics and to determine the relative incidence of the individual species. This study was also designed to show the relative in vitro activity of some of the newer β -lactam antibiotics and to demonstrate the agreement of results that can be obtained between the reference agar (16) and microdilution methods.

MATERIALS AND METHODS

Antibiotics. Laboratory standard reference powders were furnished by the following firms: cefoperazone and carbenicillin by Pfizer Inc., New York, N.Y.; moxalactam, cefamandole, cefazolin, and cephalothin by Eli Lilly & Co., Indianapolis, Ind.; cefotaxime by Hoechst-Roussel Pharmaceuticals, Inc., Somerville, N.J.; and cefoxitin by Merck & Co., Inc., Rahway, N.J.

Bacterial strains. A total of 133 isolates, 112 strains from clinical material submitted to the microbiology laboratory at Mount Sinai Medical Center, Milwaukee, Wis., during an 18-month period, supplemented with 21 strains donated by other laboratories, was utilized in this study (Table 1). At the outset, 20 strains of each species were sought, but at the end of the 18-month collection period, the less common species were supplemented from frozen stocks or from other sources. In addition to the seven recognized species of the *B. fragilis* group, we also included four strains of an organism with a unique biotype, *B. "3452A"*, which can be recognized by the use of the API 20A anaerobe strip (Analytab Products, Plainview, N.Y.). This organism has characteristics similar to those of *B. distasonis*.

The majority of *B. fragilis* group isolates were maintained in frozen skim milk during the collection period. Isolates were identified by using Gram stain, conventional biochemical and gas-liquid chromatographic techniques, and the criteria described in the Virginia Polytechnic Institute *Anaerobe Manual* (10). In addition, xylan fermentation was determined on indole-positive members to separate conclusively *B. ovatus* from *B. thetaiotaomicron* (9). Atypical strains were also confirmed on API 20A anaerobe identification strips. The following National Committee for Clinical Laboratory Standards (NCCLS) control organisms were also included: *B. thetaiotaomicron* ATCC 29741, *B. fragilis* ATCC 25285, and *Clostridium perfringens* ATCC 13124.

MIC determinations. Minimal inhibitory concentrations (MICs) were determined by a microdilution technique and the proposed *Standard Reference Agar Dilution Procedure for Antimicrobial Susceptibility Testing of Anaerobic Bacteria* (16). Concentrations of antibiotics tested ranged from 0.125 to 256 μ g/ml. All 133 isolates were tested by the microdilution technique, but only 39 strains were tested by agar dilution for comparison purposes.

Microdilution plates with Wilkins-West broth (23) were prepared with a MIC 2000 dispenser (Dynatech Laboratories, Alexandria, Va.), and the plates were stored frozen at -70°C until needed. After thawing at room temperature in ambient atmosphere, the plates were "preconditioned" for 2 h before inoculation in a

holding jar being flushed with anaerobe-grade CO_2 . The final inoculum was approximately 10^6 colony-forming units per ml. The inoculum was prepared from a 24-h hemin- and vitamin K1-enriched thioglycollate broth culture, supplemented with NaHCO_3 after steaming and cooling. The plates were removed only long enough for inoculation (MIC 2000 inoculator) and were returned to the holding jars before anaerobic incubation (48 h at 35°C). In the agar dilution technique, inocula were applied to antibiotic-containing Wilkins-Chalgren agar (25) (Difco Laboratories, Detroit, Mich.) with a Steers replicator (19).

Microdilution and agar dilution plates were incubated anaerobically at 35°C in modified GasPak jars (BBL Microbiology Systems, Cockeysville, Md.) by using an evacuation-replacement technique. Two evacuations to 20 in. Hg and replacement with nitrogen and one evacuation-replacement with a gas mixture of 80% N_2 -10% CO_2 were utilized. After 48 h of incubation, the MIC for each strain was recorded as the lowest antibiotic concentration allowing no growth for the microdilution or, for the agar dilution technique, as no growth, one colony, or a fine, barely visible haze as detected with the unaided eye.

RESULTS

The comparative in vitro activities of eight β -lactam antibiotics against 133 isolates of the *B. fragilis* group are listed in Table 1. For reference purposes, the organisms are grouped by their ability to produce indole. When the concentrations at which 50% of the strains were inhibited ($\text{MIC}_{50\text{s}}$) for the entire *B. fragilis* group were examined, we found moxalactam and cefoxitin to have the most in vitro activity on a weight basis at 4 and 8 μ g/ml, respectively. The $\text{MIC}_{90\text{s}}$ of cefoxitin, cefoperazone, moxalactam, and carbenicillin were equivalent, in the range of 64 μ g/ml, but those of cefamandole, cefazolin, and cephalothin were higher. When the *B. fragilis* group was split into indole-positive and indole-negative subgroups, it became evident that the indole-positive members were more resistant than the indole-negative strains. An analysis of the data with the Wilcoxon-Mann-Whitney test for two independent samples (24) indicated that the indole-positive subgroup was significantly more resistant ($P \leq 0.01$) to every antibiotic tested than the indole-negative subgroup.

During a recent 6-month period, 714 clinical specimens at this institution which were inoculated onto media appropriate for the isolation of anaerobes yielded 121 strains of *Bacteroides*. Sixty-seven isolates were members of the *B. fragilis* group, 40 were indole negative, and 27 were indole positive. These were identified by conventional biochemicals. If the API 20A anaerobe strip were used for identification of the *Bacteroides* species, a limited number of strains with a unique biotype might be identified. This biotype has been given the numerical designation "3452A." Biochemically, it is similar to *B.*

TABLE 1. Comparative susceptibilities of the *B. fragilis* group to eight β -lactam antibiotics

Organism (no. of isolates)	Antibiotic	MIC range ($\mu\text{g/ml}$)	MIC ₅₀ ($\mu\text{g/ml}$)	MIC ₉₀ ($\mu\text{g/ml}$)	
Indole negative					
<i>B. fragilis</i> (24)	Cefoperazone	8->256	32	64	
	Cefotaxime	1-256	16	32	
	Moxalactam	0.5-4	0.5	4	
	Cefoxitin	2-16	8	8	
	Cefamandole	16-256	32	128	
	Cefazolin	8->256	32	128	
	Cephalothin	16->256	64	128	
	Carbenicillin	4->256	16	64	
	<i>B. vulgatus</i> (19)	Cefoperazone	0.5 ^a -64	16	32
		Cefotaxime	0.125-32	2	16
Moxalactam		\leq 0.125-4	0.5	2	
Cefoxitin		0.5-8	2	4	
Cefamandole		1 ^a -64	32	64	
Cefazolin		\leq 0.5-64	8	64	
Cephalothin		0.5 ^a -64	16	64	
Carbenicillin		\leq 0.125-128	2	16	
<i>B. distasonis</i> (16)		Cefoperazone	8-32	8	16
		Cefotaxime	0.25-64 ^a	1	8
	Moxalactam	4-128	16	64	
	Cefoxitin	4-32	8	16	
	Cefamandole	32->256	256	>256	
	Cefazolin	1->256	16	128	
	Cephalothin	4->256	32	128	
	Carbenicillin	2->256	32	128	
	<i>B. "3452A"</i> (4)	Cefoperazone	8-32	8	32
		Cefotaxime	1-8	2	8
Moxalactam		1-32	2	32	
Cefoxitin		4-16	8	16	
Cefamandole		16-128	16	128	
Cefazolin		8-64	16	32	
Cephalothin		16-64	32	64	
Carbenicillin		8-16	8	16	
Indole positive					
<i>B. thetaiotaomicron</i> (22)		Cefoperazone	16-128	32	64
	Cefotaxime	4-128	32	64	
	Moxalactam	2-64	8	32	
	Cefoxitin	8-32	16	32	
	Cefamandole	32-128	64	128	
	Cefazolin	4 ^a -256	32	128	
	Cephalothin	8 ^a -256	128	256	
	Carbenicillin	2 ^a -64	32	32	
	<i>B. ovatus</i> (27)	Cefoperazone	16-128	64	128
		Cefotaxime	8-64	32	64
Moxalactam		8-128	64	128	
Cefoxitin		16-32	16	32	
Cefamandole		64->256	128	256	
Cefazolin		16-256	128	256	
Cephalothin		64-256	256	256	
Carbenicillin		16-128	32	64	
<i>B. uniformis</i> (18)		Cefoperazone	4-256 ^a	16	64
		Cefotaxime	2-256	16	64
	Moxalactam	0.5-256	1	16	
	Cefoxitin	1-64	4	32	
	Cefamandole	16->256	32	256	
	Cefazolin	2-256	32	256	
	Cephalothin	16-256	64	256	
	Carbenicillin	4->256	32	128	
	<i>B. eggerthii</i> (3)	Cefoperazone	2-8	4	8
		Cefotaxime	1-8	1	8
Moxalactam		0.25-2	0.5	1	
Cefoxitin		0.5-1	1	1	
Cefamandole		16-32	32	32	
Cefazolin		16-32	16	32	
Cephalothin		16-64	16	64	
Carbenicillin		2-8	4	8	

^a Only one isolate at extreme (see text).

distasonis. Laboratories that elect to use abbreviated forms of the Virginia Polytechnic Institute (10), Wadsworth (21), or Centers for Disease Control methodology (5) for identifying *Bacteroides* species may not easily recognize this particular biotype. In addition, its antibiotic susceptibility pattern follows that of the other indole-negative species.

A wide range of susceptibilities was observed in every species-antibiotic comparison. Likewise, no one antibiotic displayed a narrow range of activity against isolates tested in this study. In several instances, the MIC of a given antibiotic was extreme (high or low) for a single strain of a species. For example, for one strain of *B. vulgatus*, the cefoperazone MIC was 0.5 µg/ml; the MIC of this antibiotic ranged from 8 to 64 µg/ml for all other isolates. For the same strain, the cephalothin MIC was 0.5 µg/ml, and the cefamandole MIC was 1.0 µg/ml; the MIC ranges for the other 21 isolates were 8 to 64 and 16 to 64 µg/ml, respectively. Similarly, for one isolate of *B. thetaiotaomicron*, the MIC of cefazolin was 4 µg/ml, the MIC of cephalothin was 8 µg/ml, and the MIC of carbenicillin was 2 µg/ml; the lowest MICs of these antibiotics for the other 21 strains were 16, 64, and 8 µg/ml, respectively. Conversely, for one *B. distasonis* strain, the MIC of cefotaxime was 64 µg/ml. All other strains were susceptible within the 0.25 to 8 µg/ml range. Finally, 256 µg of cefoperazone per ml was needed to inhibit one isolate of *B. uniformis*; the other 17 strains were inhibited by 64 µg/ml or less.

Figure 1 makes even more evident the difference in susceptibilities of the indole-positive and indole-negative subgroups of the *B. fragilis* group; the actual percentages of strains in the sensitive, intermediate, and resistant ranges can be seen. Note that with the third-generation β-lactams (cefoperazone, cefotaxime, and moxalactam), the majority of isolates, especially the indole-negative species, were inhibited by less than the accepted breakpoint of susceptibility, 16 µg/ml. A significant number of indole-positive strains were susceptible to intermediate levels of the newer agents. Of the indole-positive strains, *B. uniformis* and *B. eggerthii* were the most susceptible. Those strains susceptible to less than 8 µg/ml were all members of these species. In that only three isolates of *B. eggerthii* were tested, this finding may not be a true reflection of all members of the species.

In comparison, our data with the second-generation agents, cefamandole and cefoxitin, confirmed the earlier and recent reports of in vitro activity with these organisms, cefoxitin being more active than cefamandole (1, 2, 13, 14, 17). Note again that the indole-negative species are more susceptible than indole-positive spe-

cies, and in the case of cefoxitin, all but a very limited number of strains were inhibited by 16 µg/ml or less. The distributions of the MICs for indole-positive and indole-negative subgroups of *B. fragilis* of cefazolin, cephalothin, and carbenicillin are also different. It is important to note that in the case of carbenicillin, the breakpoint for susceptibility of which is considerably higher (64 µg/ml), the majority of all isolates are susceptible.

A comparison of MICs as determined by agar and broth dilution techniques revealed that microdilution (broth dilution) MICs tended to be one dilution lower than those obtained with agar dilution. For only 40% of the strains tested, the MICs were the same with the two techniques; for 34% of strains, the microdilution MICs were one dilution lower, and for only 12% the microdilution MICs were higher. Cefoxitin appeared to have a greater degree of activity by microdilution than by agar dilution, as illustrated by the fact that 64% of the microdilution results were one dilution lower than those of agar dilution. If we accept results ± one dilution of the reference agar dilution value, there was 86% overall agreement between microdilution and agar dilution results. Notably, only cefotaxime produced results that were more discrepant; a high percentage (27%) of the results exceeded the standard method result by greater than one dilution (16). Another observation was that moxalactam endpoints were difficult to read. This may have contributed to the different comparative results with this antibiotic. This "trailing" was also observed by Rolfe and Finegold (18). Factors known to affect the in vitro activity of β-lactam antibiotics against the *B. fragilis* group include choice of medium, incubation time of the inoculum, final readout time of the susceptibility test (24 or 48 h postinoculation), and inoculum density (22). These data suggest that the agreement of techniques is a function not only of the nature of the organism and the inoculum size, but also of the specific antimicrobial agent tested.

Some of the antibiotics that were evaluated in this study were not examined during the collaborative study of the proposed reference method for testing anaerobes (20). These included cefoperazone, cefotaxime, moxalactam, cefamandole, and cefazolin. Our agar dilution results with cefoxitin and carbenicillin for the three control strains agreed with the mode results of the published standard (16) or were within the acceptable range (Table 2). The results with the same two antibiotics and the control strains as determined by microdilution were all within the acceptable range, that is, within one dilution of the published mode, except for carbenicillin with *Clostridium perfringens*, which was two dilutions lower. With all the other antibiotics,

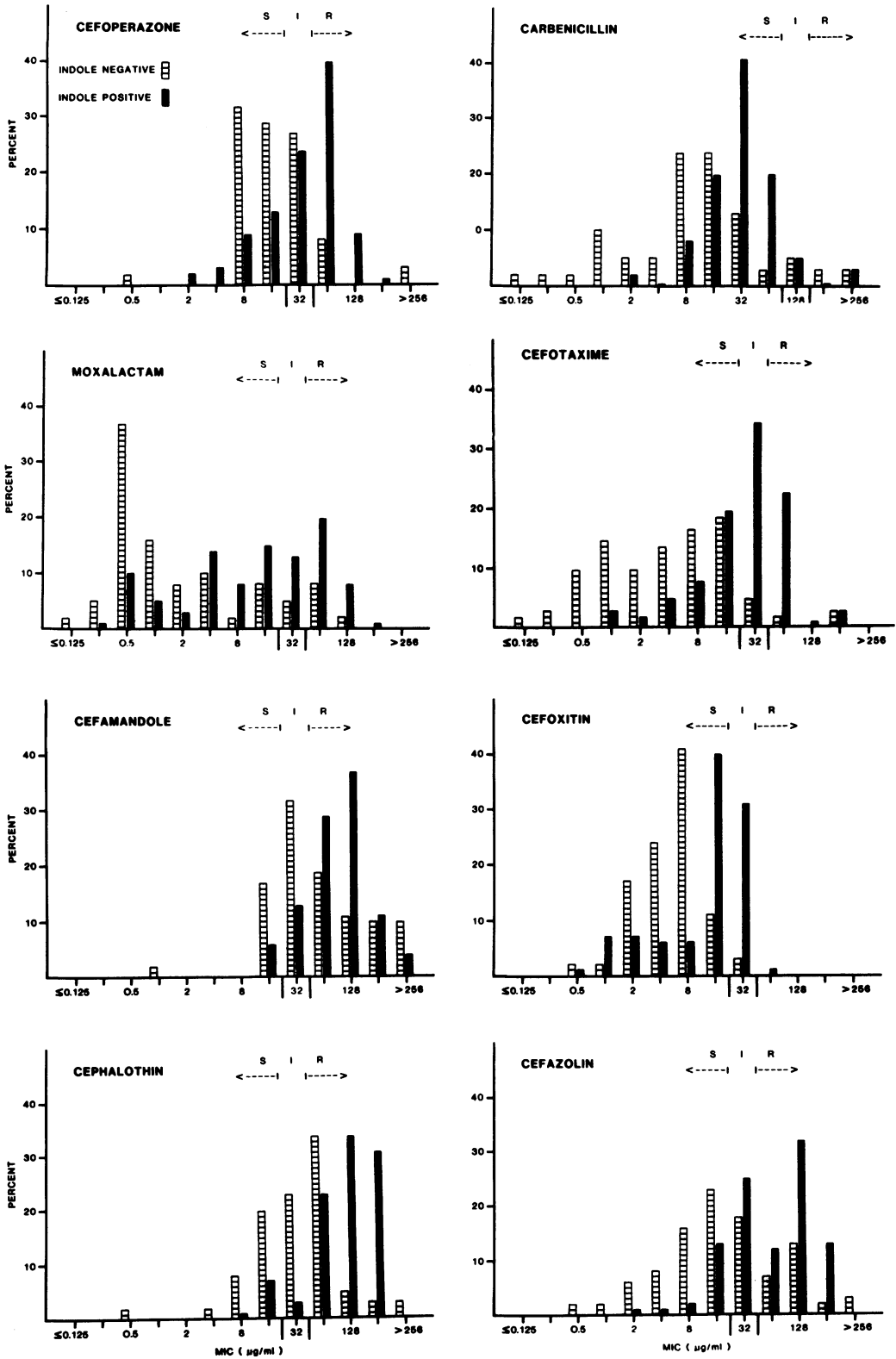


FIG. 1. Percent of indole-negative and indole-positive strains of the *B. fragilis* group susceptible to different concentrations of β -lactam antibiotics. Breakpoints for susceptible (S), intermediate (I), and resistant (R) are indicated.

TABLE 2. MICs for NCCLS control strains

Antibiotic	MIC ($\mu\text{g/ml}$) ^a					
	<i>B. fragilis</i> (ATCC 25285)		<i>B. thetaiotaomicron</i> (ATCC 29741)		<i>C. perfringens</i> (ATCC 13124)	
	Agar method	Microdilution method	Agar method	Microdilution method	Agar method	Microdilution method
Cefoperazone	64	64	64	32	≤ 0.125	≤ 0.125
Cefotaxime	16	32	32	16	≤ 0.125	≤ 0.125
Moxalactam	0.5	0.5	8	4	≤ 0.125	≤ 0.125
Cefoxitin	8	4	32	16	0.5	0.5
Cefamandole	64	64	64	64	≤ 0.125	≤ 0.125
Cefazolin	128	128	32	32	0.5	0.5
Cephalothin	128	64	128	64	0.25	≤ 0.125
Carbenicillin	32	32	32	16	0.25	≤ 0.125

^a Mode MIC for six determinations.

there was good agreement between agar and broth techniques for the control strains. *C. perfringens*, being the most susceptible strain, was at or below the level of susceptibility of our concentration range for most of the antibiotics we examined.

DISCUSSION

Species within the *B. fragilis* group remain the most prevalent of the intestinal anaerobes isolated from bacteremic patients and the most frequently isolated anaerobes from mixed or soft tissue infections. Most laboratories do not identify species of the *B. fragilis* group, which would be an acceptable practice if the species had similar antibiotic susceptibilities. Data from this study indicate that significant differences do exist in the susceptibility patterns of members of this group with a variety of β -lactam antibiotics, including representatives of the first-, second-, and third-generation cephalosporins and even carbenicillin. This phenomenon has been observed by others but has not been a consistent feature from laboratory to laboratory.

The observation that indole-positive members of this group are generally more resistant is of consequence, since in our experience these isolates represent a sizable fraction of all *Bacteroides* isolates. Of the indole-negative members of the *B. fragilis* group, *B. vulgatus* was more susceptible than *B. fragilis* or *B. distasonis*. Within the indole-positive group, *B. ovatus* was the most resistant. *B. uniformis*, although more susceptible than *B. thetaiotaomicron* or *B. ovatus*, had a wider range of susceptibility. Also, as indicated above, *B. eggerthii* was the most susceptible species within the *B. fragilis* group, but results for three isolates may not be representative of the general susceptibility pattern of the species.

A major determinant of the relative susceptibility of these agents is the breakpoint for the

resistance interpretation as established for the antibiotic in question. This information is supplied by the manufacturer and is projected from attainable serum levels and half-life values. Clearly, antibiotics with long half-lives or high serum levels will be relatively more active than antibiotics with lower values, as in the case of cefoperazone and cefotaxime.

MIC data from this investigation are not in total agreement with other recent findings in the literature. This may be a function of differences in reporting practices from institution to institution, i.e., use of the "generic" term *B. fragilis* group for all isolates that can now be given species status. Another factor potentially responsible for these discrepancies might be the different methodologies that have been described for anaerobic susceptibility testing utilizing different inoculum sizes and media. A common practice has been to utilize the same inoculum size for microdilution as for agar dilution. Even increasing the inoculum size for microdilution (10^6 versus 10^5) had only a modest effect in bringing the microdilution results into greater agreement with the proposed standard. It is also clear from this study that individual antibiotics can perform differently when tested by the two techniques, specifically, moxalactam and cefoxitin.

Based on the observation that indole-positive members of the *B. fragilis* group are generally more resistant to β -lactam antibiotics than are indole-negative strains, it may be advisable for clinical microbiology laboratories to routinely identify species of the *B. fragilis* group. As an alternative, some laboratories might choose to perform an indole test in addition to carrying out susceptibility testing with selected antibiotics. The NCCLS agar dilution reference method is an expensive, technically involved, and time-consuming procedure. It is not practical for routine use in many clinical laboratories. In

addition, results are not available to the clinician for 48 h after isolation of the organism or inoculation of the plates. Although also requiring 48 h for final results, microdilution susceptibility testing of anaerobes may represent a viable alternative. This technique is less time consuming than agar dilution. If the susceptibility trends described above hold true at other institutions, species identification or grouping based on indole production, in conjunction with published antibiograms of the individual species isolated at each institution, might provide clinically relevant information useful to physicians in initiating appropriate antimicrobial therapy. In laboratories in which antibiotic testing of anaerobes is routinely done for surveillance purposes, the procedures must be properly controlled. Therefore, the use of control strains which provide the proper reference values with all appropriate antibiotics is necessary. These control values for all new antibiotics must be made available for laboratories to utilize for quality control purposes as soon as the drugs are available to clinicians for therapeutic use.

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