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Susceptibility Testing of Anaerobic Bacteria: Myth, Magic, or Method?

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GENERAL CONSIDERATIONS

Anaerobes are increasingly being recognized as clinically significant, and, concomitantly, their antibiograms are becoming less predictable. Physicians need to rely on published data or data generated in their own hospital laboratories to predict whether a given antibiotic will be effective against a particular organism. Also, the plethora of new antimicrobial agents with variable activity against anaerobes requires testing to determine antianaerobic activity.

A review of titles of recent articles reveals some of the confusion and disorder in susceptibility testing of anaerobes: "Susceptibility Testing of Anaerobes—Fact, Fancy and Wishful Thinking" (52); "Anaerobic Susceptibility Testing: Myth, Magic, or Method?" (115), "Revisiting Anaerobic Susceptibility Testing" (128); and "Son of Anaerobic Susceptibility Testing—Revisited" (6). The conclusion may be correctly drawn that consensus has not yet been achieved in this area.

Although there has been a National Committee for Clinical Laboratory Standards (NCCLS)-approved reference technique for some years, not all laboratories use this method. The Wadsworth Anaerobe Laboratory uses brucella–laked-blood agar rather than the Wilkins-Chalgren agar described in the earlier reference method (83); we found that Wilkins-Chalgren medium did not adequately support the growth of a number of important anaerobes. The newest NCCLS-approved standard does include the method used by the Wadsworth Anaerobe Laboratories as an approved alternative (85). However, there are still a number of well-respected large laboratories that use different media or different methods of inoculum preparation or both (29, 81, 105). Some of these centers are understandably reluctant to change because of the large data base they have built up over many years. Presumably, all laboratories use the quality control standards outlined by the NCCLS and ensure that their test results conform to those standards. However, because of the allowable error of the technique and the fact that MICs of many antimicrobial agents cluster near critical concentrations, a slight variance in test results may still be enough to change reported antibiograms radically. The reader must be very careful when reviewing data to ascertain exactly what kind of test was done.

NEED

Routine susceptibility testing of anaerobes is generally not required (6, 47). Exact identifications and susceptibility results generated days after therapy has been initiated are likely to be of less use to the clinician than presumptive identification of the likely pathogens. Also, determining the relative importance of the antimicrobial susceptibility of each strain isolated from a mixed infection is probably not possible. A recent study from our laboratory showed an average of 12 isolates from each patient with perforated or gangrenous appendicitis (11) and found that clinical cures occurred even when patients were treated with agents shown to be inactive against the *Bacteroides fragilis* group strains present (48). Some agents are effective against essentially all anaerobes (e.g., chloramphenicol or metronidazole [for all but nonsporeforming gram-positive rods]), but the toxicity of some of the most effective agents has prompted physicians to look toward some of the newer cephalosporins, which have widely differing efficacies against anaerobes. Consequently, susceptibility testing of the organisms as an indicator of developing patterns has become essential. Differences in antibiograms among species can generally be summarized, and reports published by reliable large research centers can often serve as a guide for the clinician in initiating appropriate therapy. Reliance on published data is frequently the primary mode by which smaller laboratories make predictions about the susceptibility of particular isolates. However, regional variation (even hospital to hospital) exists and is undoubtedly influenced by each institution's formulary and antibiotic usage patterns. Thus, when possible, each hospital should batch isolates and test them periodically to monitor any possible changes in relevant antibiograms, particularly if there is any reason to suspect developing resistance. Also, there are specific instances when testing of individual isolates should be performed.

When To Test

Isolates from patients with the following infections should undergo testing: brain abscess, other central nervous system infections, endocarditis, osteomyelitis, joint infection, prosthetic-device infection, and refractory or recurrent bacteremia. Anaerobes isolated in pure culture should also be tested. Susceptibility data on isolates from patients with mixed infections who are being treated medically, rather than surgically, will be of use to the clinician (6). Obviously, isolates from infections not responsive to empiric therapy should also be tested.

Organisms To Test

Isolates that may be considered for testing include the *Bacteroides fragilis* group isolates, *B. gracilis*, *Fusobacterium varium*, *F. mortiferum*, *Clostridium perfringens*, *C. ramosum*, *C. innocuum*, *C. clostridioforme*, and *Bilophila wadsworthia*. *B. fragilis* group strains are the most commonly found anaerobes in clinical infections and can be very resistant to a number of commonly used antimicrobial agents. *B. gracilis* (a member of the *B. ureolyticus* group) is found in serious, deep-seated infections and is quite resistant to many antimicrobial agents. The clinical significance of *Bilophila wadsworthia* is not yet fully established (2), but this organism is encountered frequently in patients with intra-abdominal infections and is very resistant to a number of antimicrobial agents that usually have good activity against anaerobes. Occasional strains of clostridia (including *C. perfringens*) may be resistant to penicillins; one-third of strains of clostridia other than *C. perfringens* are resistant to cefoxitin, and many are also resistant to clindamycin. *C. ramosum* is the most resistant *Clostridium* species.

Agents To Test

The hospital formulary should serve as a guide in considering the agents for testing. Imipenem, chloramphenicol, metronidazole (for all but nonsporeforming gram-positive rods), and the β-lactam/β-lactamase inhibitor combinations

are presently almost uniformly active against anaerobes in the United States and do not need to be tested except under unusual circumstances (e.g., a patient nonresponsive to empiric therapy or when there is evidence of resistance to one of these agents). Of course, these agents must be monitored periodically in large centers to detect any developing resistance. There are reports that 5% of strains in the *B. fragilis* group are resistant to metronidazole in France (19) and 10% are resistant to imipenem in Japan (112). Many cephalosporins, penicillins, and clindamycin are variably active against anaerobes, and one cannot predict the susceptibility of a particular strain on the basis of published patterns. The quinolones presently on the market show relatively poor activity against anaerobes; some of the newer ones being developed have an improved spectrum against anaerobes.

METHODS

The NCCLS has recently published a revised approved standard for susceptibility testing of anaerobic bacteria (85). The methods include agar dilution testing, broth microdilution, and broth macrodilution. The following sections briefly discuss these methods, as well as the broth disk elution method which is no longer approved by the NCCLS.

This section is not intended to be a "methods manual" but rather a brief description and evaluation of the techniques available for anaerobic susceptibility testing.

Preparation of Antimicrobial Stock Solutions and Dilutions

Stock solutions of antimicrobial agents generally may be prepared in advance and frozen in aliquots at −70°C. After thawing, they should not be refrozen. Some antibiotics (imipenem, for example) are not very stable upon freezing (even at −70°C) and should be made fresh. An appropriate dilution scheme, as well as the solvents and diluents used, may be found in the NCCLS protocol (85).

Agar Dilution

The agar dilution technique is most appropriate for laboratories doing large-scale testing of organisms for surveillance of susceptibility patterns. Thirty to 60 organisms can be tested with 5 to 10 antibiotics in a reasonable period; however, since the plated media are not stable and should not be stored for longer than 1 week, agar dilution is not a practical method for small laboratories doing only a few strains at a time. The NCCLS-recommended reference procedure uses Wilkins-Chalgren agar prepared the week of the test, and the antimicrobial dilutions are added to the agar the day of the test (83); the most recent Standard also includes the technique used by the Wadsworth Anaerobe Laboratories, which utilizes supplemented brucella–laked-blood agar (85). The inocula are prepared by diluting a culture growing in brucella broth (or other clear broth) to a certain density standard or by suspending colonies from an agar plate to the appropriate density (15, 80). The NCCLS protocol (85) also describes a modified agar dilution test in which one to four concentrations of each antimicrobial agent may be tested in divided plates. The highest concentration of antimicrobial agents is chosen to represent the resistant breakpoint.

Broth Macrodilution

Conventional broth dilution tests are useful for testing spreading organisms (such as some *Clostridium* spp.) or for determining MBCs. Values for broth tests (especially microdilution) will often be 1 dilution lower than those for agar dilution tests. The MIC is read as the lowest concentration showing no visible growth.

MBCs may be determined by streaking 0.1 ml of material from each tube onto a blood agar plate. Plates are incubated anaerobically for 48 h, and the MBC is read as the lowest concentration of drug resulting in a 99.9% killing rate on the basis of the original inoculum size. MBC determinations are generally requested for endocarditis and meningitis, infections in which anaerobes do not usually play a role. However, for infections such as osteomyelitis, or in patients with impaired host defenses, the cidal activity of the agent may be important. The clinical laboratory should be aware of the appropriate methods for performing the test (i.e., using broth macro- and not microdilution).

Broth Microdilution

For broth microdilution, antimicrobial solutions are prepared and diluted as described previously (85) and dispensed into each of the wells. This can be done manually (which is very cumbersome) or, more commonly, with a mechanized system (e.g., MIC-2000 [Dynatech Laboratories, Inc., Alexandria, Va.]). The trays are stored in sealed plastic bags at −70°C. Before use, the trays are thawed and the medium is allowed to prereduce in an anaerobic atmosphere. The organism may be inoculated with a hand-held inoculator or mechanically (e.g., with the MIC-2000). Details regarding the performance of the test may be found in the NCCLS protocol (85). The plates are read after 24 to 48 h, and the MIC is reported as the lowest concentration of antimicrobial agent that does not permit macroscopic growth (84).

Commercially Available Broth Microdilution Panels

Jones and colleagues (63) evaluated the Micro Media Systems anaerobe susceptibility panel (Micro Media Systems, Cleveland, Ohio) and reported inter- and intralaboratory agreements of 98.0 and 97.3%, respectively (using ±1 twofold dilution). Interpretive agreements (by category) with the NCCLS reference agar and broth dilution methods were 91.0 and 95%, respectively. Clindamycin showed a correlation of only 56.5% (strains within ±1 twofold dilution) when compared with the reference agar dilution results. Major interpretive errors were also noted with cefoxitin when it was tested against *B. thetaiotaomicron*. Jones et al. also caution that 20 to 40% of clinically isolated anaerobes may not grow in the medium used in these systems.

Baron and Bruckner (5) compared the commercial panel produced by MicroScan, Campbell, Calif., with the NCCLS reference agar procedure and found an average agreement (within 1 twofold dilution) of 83.2% between the two methods. Twenty-one percent of the strains failed to grow in the commercial panel. Less than 80% agreement was found between strains of *Clostridium* species, *Fusobacterium* species, and one strain of *Peptostreptococcus* sp. Seven of 110 strains also failed to grow on Wilkins-Chalgren agar. Only 4.2% of the results would have resulted in interpretive errors. Baron and Bruckner thought that the MicroScan system was an acceptable alternative for anaerobic susceptibility testing.

Hussain and colleagues (59) found good correlation on the basis of interpretive agreement between the Sceptor Anaerobe MIC panel (BBL Microbiology Systems, Cockeysville, Md.) and the reference agar dilution system for 92% of the

strains tested. However, 13.3% of the values (i.e., 212 strains) differed by more than 2 twofold dilutions from the reference value, and they concluded that further studies are needed before the Sceptor panel can be recommended for routine use. Using the categories "susceptible," "moderately susceptible," and "resistant," Heizmann and colleagues (54) found that the rate of "essential agreement" (complete agreement plus minor interpretative errors) with agar dilution was 93% for the Dynatech MIC system and 98% for the Sceptor Anaerobe MIC system.

One of the biggest problems with the use of these panels has been that the choice of drugs available did not reflect the appropriate range of drugs prescribed for anaerobic infections. Innovative Diagnostic Systems, Atlanta, Ga., has marketed a new frozen microdilution panel for anaerobes that includes many of the relevant antimicrobial agents. There have been no independent evaluations of that panel to date. PML Microbiologicals, Tualatin, Ore., will supply custom-poured MIC panels for institutions whose needs are not met by one of the existing panels.

Broth Disk Elution Method—No Longer Approved

The newly revised NCCLS protocol for susceptibility testing of anaerobic bacteria (85) does not include broth disk elution. Although this method was approved in the past, the committee members thought that the unreliability of the method and the poor correlation of the results with those of the reference method did not warrant its inclusion as an approved alternative. A number of studies found good correlation between this method and other reference techniques (56, 64, 100, 129), but in general, problems were found when *B. fragilis* group isolates for which MICs were near the breakpoint concentrations were tested. Since this is the most frequently tested group of organisms, the problem is not trivial. Tests with organisms for which MICs are near the breakpoint were often very difficult to read (i.e., the growth was somewhere between the sterility control but not nearly as turbid as the growth control). In some cases, the turbidity caused by the partial disintegration of the large number of disks needed caused an increased density that could be confused with growth of the test organism. The decision to exclude this method from the new document was made with the awareness that broth disk elution is probably the most commonly used technique in clinical laboratories in this country; however, the committee members deemed that the problems associated with this method warranted its exclusion. Offering clinicians inaccurate information about the susceptibility of an organism is certainly no better than providing no information. The committee also thought that the void created by eliminating the broth disk technique would enhance efforts to develop a reliable alternative.

β-Lactamase Tests

The β-lactamase test is simple and rapid, may be used as a supplement to conventional susceptibility tests, and may provide the microbiologist with some useful information. If a strain is β-lactamase positive, it will probably be resistant to the β-lactamase-labile agents (e.g., penicillins and early-generation cephalosporins) and may be susceptible to the agents when combined with a β-lactamase inhibitor such as sulbactam, tazobactam, or clavulanic acid or even advanced-generation cephalosporins. However, one cannot assume that a strain will be susceptible to agents simply because it does not produce β-lactamase. *B. gracilis* (61), *Bilophila wadsworthia* (7), and many strains of *B. distasonis* (70) do not produce β-lactamase and are quite resistant to β-lactam agents. Anaerobes that may produce β-lactamase are listed below. The most reliable method for detecting β-lactamases in anaerobes utilizes Cefinase disks (BBL) which are impregnated with the chromogenic cephalosporin nitrocefin.

Newer Methods

Spiral gradient endpoint (SGE) system. The spiral streaker (Spiral Systems Instruments, Bethesda, Md.) deposits a specific amount of antimicrobial stock solution in a spiral pattern on an agar plate, producing a concentration gradient that decreases radially from the center of the plate. After the antimicrobial agents are allowed to diffuse for 3 to 4 h, the isolates (prepared to a 0.5 McFarland density standard) are deposited on the plate with an automated inoculator or manually streaked from the center to the edge of the plate. The plates are incubated for 48 h in an anaerobic atmosphere, and then the endpoints of growth are marked and the distance is measured in millimeters from the center of the plate to the point where growth stops. The data are then entered into a computer software program provided by the manufacturer that determines the concentration of drug from the radius of growth and the molecular weight (i.e., diffusion characteristics) of the antimicrobial agent. Details of the procedure may be found in the manufacturer's guidelines. Comparisons of this procedure with standard agar dilution have been favorable (57, 123). In a study conducted in the Wadsworth Anaerobe Laboratory (123), spiral gradient MICs were determined by calculating antimicrobial concentrations at growth endpoints and rounding up to the next twofold incremental concentration. Overall agreement between the two techniques (within 1 doubling dilution) was 90.6%. In general, discrepancies between the two methods could be attributed to one of two causes: (i) endpoints were difficult to read on one or both tests, or (ii) MICs were close to breakpoint concentrations (and thus MICs determined by the two techniques could be within 1 twofold dilution of each other, yet one test would result in a "susceptible" and one would result in a "resistant" designation).

The increased sensitivity of the technique may yield information not available by using standard twofold dilutions of the agar dilution technique. For example, the inocula are prepared by diluting a culture growing in brucella broth (or other clear broth) to a certain density standard or by suspending colonies from an agar plate to the appropriate density (15, 80). These two methods of inoculum preparation apparently yield comparable results in agar dilution testing (15). However, using the SGE method, we have found that preparing the inoculum from a broth culture in logarithmic phase yields consistently lower MICs than preparing an inoculum by suspending colonies (presumably in stationary phase) to the proper density (116). The SGE technique retains the advantages of an agar dilution system (particularly good growth of fastidious organisms) as well as some of the labor-saving advantages of the simpler techniques. (However, the equipment required is expensive.)

ε test strip. The ε test strip produced by AB Biodisk (Sweden) uses a plastic strip coated with an antibiotic gradient on one side and an interpretive scale on the other. The strip is laid on the surface of an agar plate that has been inoculated with the organism, and the plate is anaerobically incubated for 24 h. The point at which the teardrop-shaped zone of inhibition intersects the interpretive scale is consid-

ered the endpoint. The use of these strips for anaerobic susceptibility testing has been evaluated. Good correlation with standard techniques was seen despite some discrepancies (26). We have not yet evaluated this method in our laboratory.

Agar disk diffusion tests. In 1973, Sutter and colleagues described the agar disk diffusion technique for use with rapidly growing anaerobes (104). Recently, modifications of the technique were evaluated, and good correlation with the reference method was seen for certain antimicrobial agents (8, 10, 58). For many agents, however, correlation is poor. At present, the test is not considered generally appropriate for anaerobic susceptibility testing.

Membrane transfer assay. It has long been recognized that NCCLS reference methods are not appropriate for evaluating antimicrobial agents applied topically for ocular or dental infections. Caulfield et al. (24) devised an assay that was designed to predict more closely the bactericidal effects of antimicrobial agents applied topically for short periods against dense aggregates of bacteria. They tested strains of periodontopathic organisms, including *B. gingivalis*, *B. intermedius*, and *F. nucleatum*. In this system, membranes that had been inoculated previously and that contained a lawn of bacteria were placed for 5 min on an agar plate on which disks containing various dilutions of antibiotic solution had been placed. The membranes were then removed and transferred onto fresh medium. After 12 h of incubation, MBCs for organisms were determined with the aid of the tetrazolium chloride indicator dye (TTC), which has been used to indicate the presence of viable cells (60, 78). The authors did not evaluate the use of the technique in predicting clinical outcome.

FACTORS CONTRIBUTING TO VARIABILITY IN MIC RESULTS

Technical Variability among Laboratories

Large laboratories use a variety of agar dilution and broth macro- and microdilution testing procedures. In a review of susceptibility testing with imipenem (117), the Wadsworth Anaerobe Laboratory found at least six different media being used, incubation periods ranging from 18 to 48 h, and inoculum sizes of 10^3 to 10^7. The various procedures may result in minor or major variations in the susceptibility results for single isolates and, in fact, do result in a wide variability of resistance rates reported that may often be a reflection of the particular procedure rather than of a shift in susceptibility or a geographical variation in the strains. Variations in inoculum (10^4 to 10^6) have been shown to have an effect on the MICs of certain antimicrobial agents. Several studies attest to the marked variation in MICs of ceftizoxime that are primarily related to the size of inoculum and type of medium (14, 25). We have observed significant differences in growth with certain anaerobes on various media (e.g., some *Fusobacterium* and *Peptostreptococcus* species grow poorly or not at all on Wilkins-Chalgren agar or on brain heart infusion agar), and some of the variability in MIC readings may be due to an unintended "inoculum effect." The choice of method (i.e., broth versus agar dilution) may also have significant effects on the results. Smith et al. (102) found that the MIC for 50% of the *B. fragilis* group isolates tested was approximately twice as high in Wilkins-Chalgren agar with lysed sheep blood as in the broth formulation of Wilkins-Chalgren agar. We have also found significant variation of MICs when we used different media and methods for certain antibiotics (3, 120).

Preparation of the inoculum and, more specifically, the growth phase of the organisms at the time of testing may affect results, although these differences may not be detectable within the allowable error range of standard dilution tests. Previous studies showed that preparing the inoculum by diluting a culture and allowing it to grow to a 0.5 McFarland density standard or, alternatively, by suspending colonies to the desired density gave equivalent results (15). However, with the allowable 1 twofold dilution error in agar dilution testing, differences of less than 1 or 2 dilutions will not be detected. As mentioned above, using the SGE method, we found that an inoculum prepared from a broth culture in logarithmic phase yields consistently lower MIC values than an inoculum prepared by suspending colonies (presumably in stationary phase) to the proper density (116). Brook (21) found that differences in growth occurred depending on the medium on which the organisms were inoculated: exponential growth occurred in agar media within 4 to 8 h, while such growth was delayed in broth media and occurred within 12 to 24 h after inoculation. Some antimicrobial agents exhibit strong inoculum effects (i.e., greatly reduced activity with increased inocula) (119). Brook postulates that this phenomenon may explain why resistance to antimicrobial agents that manifest an inoculum effect may be higher when the agent is tested in agar. However, the faster transition to log-phase growth may also have the opposite effect: there is a positive correlation with the killing rate of the agent (especially β-lactam agents) and the generation time of the organism. Organisms that enter log-phase growth more rapidly may also be killed or inhibited more effectively, as we found when testing two different inoculum preparations. Brown et al. recently reviewed the influence of growth rate on susceptibility to antimicrobial agents (22). They mention that changes in the limiting factors (e.g., carbon, phosphorus, magnesium, or nitrogen) correlate with changes in the cell envelope and may modify the action of antimicrobial agents in a number of ways.

Difficulties in Interpretation

The NCCLS agar dilution reference protocol (85) defines the endpoint as "the lowest concentration of drug yielding no growth, a haze, one discrete colony or multiple tiny colonies. And in the case of persistent (slight) growth, the MIC is read at that concentration where a **marked** change occurs in the appearance of growth as compared to the control plate." For certain organism-antibiotic combinations, applying different parts of the definition would allow different concentrations to be chosen as the MIC. Recently, in the Wadsworth Anaerobe Laboratory, three or four experienced microbiologists studied a series of agar dilution plates inoculated with *B. fragilis*; at least three different MIC readings were obtained. The problem generally occurs with gram-negative organisms and cell wall-active drugs (i.e., β-lactams). The difficulty is exacerbated with organisms that grow poorly. This problem is discussed further below (see subsection, Endpoint Definition).

Organisms Chosen for Testing

An additional source of variability in the test results is the proportion of the various *B. fragilis* group species used in the studies. The difference between the susceptibility of *B. fragilis* and those of other members of that group to most of

the commonly used agents is significant. *B. fragilis* tends to be much more susceptible than the other members of the group to many cephalosporins and to clindamycin. By increasing the proportion of *B. fragilis* in a given study sample, the percentage of strains susceptible at breakpoint would be increased. In studies in the Wadsworth Anaerobe Laboratory, we use the same proportions of species as are seen clinically at our institution, but this can vary among laboratories. Because the susceptibility pattern of *B. fragilis* is so different from those of the other *B. fragilis* group species, these values should be reported separately and not combined into a "group." The other members of the group (with the exception of *B. vulgatus*, which seems to be intermediate in resistance between *B. fragilis* and the rest of the group) tend to have similar antibiograms and may be combined. However, it is always useful to report the species separately so that any species differences may be detected.

Clustering of MICs

With certain antimicrobial agents (most β-lactam agents, clindamycin, and chloramphenicol), the MICs for a large percentage of strains cluster within 1 dilution of the breakpoint. In studies completed in the Wadsworth Anaerobe Laboratory, we found that 50 to 60% of all anaerobes were within 1 twofold dilution of the breakpoint for cefoperazone, cefotaxime, ceftizoxime, cefoxitin, and penicillin G and 38% were within 1 twofold dilution of the breakpoint for clindamycin (118). An even higher percentage (approximately 70%) of *B. fragilis* group isolates were within 1 twofold dilution of the breakpoint (46% for clindamycin) (118). Clustering about the breakpoint is a characteristic of the organism-drug interaction and is seen, to some degree, in all of the testing methods. When a MIC is near the breakpoint (e.g., in the case of the *B. fragilis* group and β-lactam antibiotics), an organism termed susceptible on one occasion may be retested and termed resistant. A system that provides a continuous concentration gradient, rather than the twofold dilution steps of the agar dilution system, minimizes the uncertainty caused by this clustering. The standard allowable error for agar dilution is considered to be ±1 two-fold dilution (±100%), and this has been confirmed in studies in our laboratory (122). In one study, agar dilution MICs were within 1 dilution of the 32-μg/ml breakpoint for 70% of the *B. fragilis* group strains tested; thus these strains might be variably interpreted as susceptible or resistant. However, in the same study, with the SGE test (assuming a ~20% experimental error [i.e., ±6.4 μg/ml]), MICs were within this breakpoint range for 19% of the strains (123). The greater precision available with the SGE test reduced the numbers of strains that may be variably labeled susceptible or resistant.

In light of these considerations, it is apparent that, when evaluating new techniques, using a technique with a ±100% error rate as the gold standard is problematic at best. It is unreasonable to discuss interpretive "very major," "major," and "minor" errors when the gold standard technique would undoubtedly show many "very major" errors when compared with itself. Evaluating alternative techniques may necessitate deviating from the twofold dilution scheme of the reference method, particularly when MICs cluster near the breakpoint.

Breakpoints

Until recently, there was little agreement as to breakpoints used for the various agents; some laboratories used NCCLS breakpoints, some used breakpoints on Food and Drug Administration-approved package inserts, and some used neither. The NCCLS has recently published a set of breakpoints (85) that are in close alignment with Food and Drug Administration breakpoints. It is extremely important that the breakpoint used for defining resistance be clearly stated; in light of the clustering effect described above, changing the breakpoint by 1 twofold dilution could (and often does) result in extreme differences in resistance rates. Interpretation of data from other countries is also complicated by disagreement about or total lack of approved breakpoints. Scavizzi and Bronner propose a global, data-processed model for the determination of breakpoints for each bacterial species, each antibiotic group, and each measurement technique (98). A working party of the British Society for Antimicrobial Chemotherapy has recently written a thoughtful review on general approaches to consideration and determination of breakpoints (92), mentioning that the clinical, pharmacological, and microbiological aspects must be taken into account.

Variability in Reporting

An additional source of confusion in interpreting results from different laboratories stems from the different formats adopted to report the results: the MIC for 50% of the strains (MIC_{50}), MIC_{90}, MIC_{100}, and "percent susceptible at breakpoint" are all used by various laboratories. It is impossible to project from one of these values what the others might be and hence to compare them with other investigators' results. Davies suggests that geometric mean MICs give more accurate information than MIC_{50}s, MIC_{75}s, or MIC_{90}s (36). The trend (and, at times, editorial pressure) for reporting only MIC_{50}s or MIC_{90}s is disturbing in light of these considerations. Thus, we recommend that workers report the range, the MIC_{90}, and the percentage of susceptible strains at a reasonable range of values about the breakpoint (e.g., a range of 3 twofold dilutions).

Problems in Taxonomic Definitions

Olsson-Liljequist and Nord (89) commented that, in their study, only 70% of the organisms classified as anaerobic cocci were susceptible to metronidazole, yet Watt and Jack (113) suggest that anaerobic cocci should be defined as cocci that grow only under anaerobiosis, do not grow in 10% carbon dioxide in air, and are susceptible to metronidazole. Anomalies in the taxonomy of anaerobic gram-positive cocci may account for the differences in susceptibility findings reported from various laboratories.

ADVANTAGES AND DISADVANTAGES IN EACH SYSTEM

There are benefits and drawbacks to each testing system. For centers monitoring trends in susceptibility patterns or evaluating new antimicrobial agents, the agar dilution or broth microdilution method is the most accurate and economical. The agar technique has the added advantage of supporting the growth of the more fastidious anaerobes (some pigmented *Bacteroides* spp., some *Fusobacterium* spp., and some anaerobic cocci) that will not grow in

microdilution trays. When individual strains must be tested and there is an urgent need for results, the microdilution technique is probably the best choice. It is hoped that continued validation of the newer methods (e.g., SGE test and ε test) will increase the options open to the clinical laboratory.

CONTROVERSIES IN SUSCEPTIBILITY TESTING

Discrepancies between Methods

With some antimicrobial agents, the discrepancies in results obtained with different methods may be extreme. The activity of ceftizoxime, in particular, has been controversial, and different resistance rates have been reported (2, 120). Aldridge and Sanders compared the agar dilution and broth microdilution methods and found that the MICs for 72% of the isolates in agar were at least 4 twofold dilutions higher than the MICs in broth (1). In another test, 50 strains of the *B. fragilis* group were exchanged between the Wadsworth Anaerobe Laboratories and Louisiana State University Medical Center. Again, the broth microdilution method produced MICs significantly lower than those determined by the agar dilution method (for ceftizoxime, 88% of the MICs were >2 twofold dilutions apart). The percentages of susceptible organisms (at a 32-μg/ml breakpoint) were 43% for the agar method and 94% for the microdilution method (3). In an effort to clarify the discrepant reports, the Wadsworth Anaerobe Laboratory recently tested 90 strains of *B. fragilis* group organisms from patients with perforated or gangrenous appendicitis. Eight different susceptibility testing procedures were used; as expected, the MICs were dependent on the technique used (120). However, the number of clinical failures was too low to allow us to make statistically valid judgments regarding the predictive values of the various techniques. All isolates cultured from superinfections in the two clinical failures (six *B. fragilis* group strains) were designated borderline resistant by agar dilution techniques. Only one strain was also designated resistant by the broth methods. However, most strains from patients who were treatment successes were also resistant by the agar technique and susceptible by the broth method. Clearly, however, the resistance rate as obtained by the agar dilution method was not matched by poor clinical activity. A similar study tested cefoxitin by several techniques and compared in vitro results with clinical outcomes (48, 121). Again, while three treatment failures occurred in infections with "resistant" organisms, many patients were successfully treated even though organisms with MICs of >64 μg/ml were isolated.

Reliability of Results and Quality Control Limits

Concern that the combination of the clustering effect with the inherent variability of the technique might result in significant nonmeaningful variability in reported results prompted the Wadsworth Anaerobe Laboratory to analyze statistically the significance of a given reported MIC (122). The study was designed to estimate the variance components in the determination of the cefoxitin MIC for isolates of the *B. fragilis* group. The analysis showed that a MIC determination is subject to a rounding bias as well as to significant sources of variation such as test (day) and reader effects. Specimen handling and test-to-test variations introduce significant errors. Replicate plating of the same strain did not necessarily yield identical MICs, although the MICs were always within ±1 twofold dilution of each other. However, in some cases the result was that the same strain was called resistant in one test and susceptible in another, even when only one person read the results. On the basis of these observations, we recommend that workers report the percent susceptible at a reasonable range of values about the breakpoint (e.g., a 3-twofold dilution range).

McDermott and Hartley (73) advise a more stringent control of the antimicrobial stock solutions and agar dilution plates than is required by the NCCLS. While the solutions and plates are undoubtedly sources of variability, we found that replicate strains varied even on the same plate (122).

Traditionally, MIC quality control limits include a range of 3 doubling dilutions. Barry et al. (9) suggest that broth or agar dilution susceptibility tests can be more precisely performed and that more stringent control limits can be applied. As they observe, major deviations would be necessary before technical problems would be detected if the allowable range is 3 twofold dilutions. They suggest that MIC control limits be the smallest range that includes >95% of the values from at least 100 MIC determinations. In their evaluation, half the drug-microorganism combinations would have control limits narrower than the usual 3-dilution range.

ISSUES THAT REQUIRE FURTHER INVESTIGATION

Endpoint Definition

The difficulty of endpoint determination with certain organism-antimicrobial agent combinations has plagued our laboratory, and others, for some time. The first extensive studies that our laboratory conducted with these "fuzzy endpoints" came about some years ago when we recorded results with *Fusobacterium* spp. and some β-lactam agents that were inconsistent with our previously published results. After retesting both the newer and the older strains, it became clear that the susceptibility of the organisms had not changed but the reading of endpoints had (even though the same technician had been reading them for 20 years!). For those strains, we found that the haze represented cell wall-deficient forms of the *Fusobacterium* strains and that these forms remained viable in cefoxitin concentrations of up to 16,000 μg/ml (62). The cell wall-deficient forms reverted to the parent morphology after two passages on drug-free medium. We recommended that, in these cases, the haze be ignored for purposes of susceptibility testing.

However, the problems of fuzzy endpoints are applicable to more than *Fusobacterium* spp. In some cases, different parts of the NCCLS criterion for reading endpoints could describe half or more of the plates in the dilution series. The problem exists to some extent with most gram-negative anaerobic bacteria. It may be particularly troublesome with those organisms that grow poorly, so that the growth control itself is fairly light. In some instances, there may be evidence of light growth (or multiple persistent tiny colonies) persisting in the presence of very high concentrations of drug, despite a sharp drop-off in growth at a much lower drug level. In the case of *Fusobacterium* spp., this light growth has been shown to be due to the persistence of cell wall-deficient forms of the bacteria (62). In these cases, the point at which the growth drops off sharply should be read as the MIC, and the persistence of haze should be noted. In the case of some organisms (e.g., *B. gracilis*, some pigmented *Bacteroides* spp., some anaerobic cocci, and *Bilophila wadsworthia*), there may be no sharp drop-off of growth, and the

endpoint may be very difficult to determine. Reading the plate against a background of transmitted light (rather than reflected light) may be helpful. Determination of the endpoint in some cases is necessarily arbitrary, and undue importance should not be ascribed to these results.

Viable dyes. In recent experiments, the Wadsworth Anaerobe Laboratory has investigated the use of the dye TTC as an aid in determining MICs. TTC is reduced by many bacteria to formazan, a red compound. For a selected antimicrobial agent-organism combination, we have found that TTC reduction endpoints correspond to counts of viable bacteria on spots removed from agar dilution plates (see below). The significant change in the color development was frequently easier to determine than was change in growth compared with that on the control plate. Preliminary results suggest that this method may be of use in determining MICs for the strains that are typically difficult to read.

Time-kill studies. Although the use of TTC allowed an easier determination of an endpoint, what that endpoint represented remained unclear. Therefore, experiments were designed to measure the viability of the organisms on either side of our determined endpoint (77). Agar plates containing concentrations of antibiotics bracketing the endpoint were poured and inoculated in accordance with the NCCLS protocol. At defined intervals after inoculation, the agar spots were cut out, vortexed, and plated to determine viable counts. Replicate spots were layered with TTC, and TTC endpoints were determined. Visual MICs were determined in the normal manner as well. We found that the TTC endpoints corresponded to antimicrobial concentrations that resulted in no net growth of the organisms. This type of study allows us to quantitatively describe the viability of the inoculum spots in the agar dilution tests.

One Method and Medium for All Anaerobes?

The *B. fragilis* group isolates generally grow in most of the test situations and media described above, although interpretation of the results may still be problematic. However, many of the more fastidious anaerobes will not grow in the microdilution panels available commercially. Also, some organisms may need supplements to produce sufficient growth for observable MICs. In preliminary experiments, we found that, for a strain of *B. gracilis* that has been characterized as resistant to ceftizoxime on at least four occasions, MICs of ceftizoxime were 0.25 μg/ml when the strain was grown with formate-fumarate as a supplement (77). We are looking at other strains of *B. gracilis* to see how widespread this phenomenon is. It seems clear that, to actually measure the MIC, good growth on the control medium must be achieved in order to compare this growth with that on the antibiotic-containing media. Also, for many of these agents, activity of the agent is dependent on cell growth. Since different groups of anaerobes need different supplements to achieve reasonable growth, it may be that, as in the case of some aerobes, different anaerobes need their own specific medium for testing.

MECHANISMS OF RESISTANCE IN ANAEROBES

β-Lactam Agents

Production of β-lactamases. Studies on the development of resistance to β-lactam antibiotics in anaerobic bacteria have focused on the production of β-lactamase enzymes (30, 33, 46, 126). β-Lactamases in *B. fragilis* are constitutive, are localized in the periplasmic space, and are primarily cephalosporinases (68). Transfer of resistance due to β-lactamase has been demonstrated (34, 96). Cephalosporins with a 7-alpha methoxy group (e.g., cefoxitin, cefotetan, and moxalactam) are considered resistant to hydrolysis by most β-lactamases. However, strains of *Bacteroides* sp. with β-lactamases capable of hydrolyzing "stable" agents, including imipenem, have been reported by a number of laboratories (46, 112, 126). Cuchural et al. (34) reported transfer of β-lactamase-associated cefoxitin resistance in *B. fragilis*. Some strains of the following species have been shown to produce β-lactamase enzymes: the *B. fragilis* group, *B. coagulans*, *B. splanchnicus*, pigmented *Prevotella* and *Porphyromonas* species, *Prevotella oris*, the *Prevotella oralis* group, *Prevotella buccae*, *Prevotella disiens*, *Prevotella bivia*, *F. nucleatum*, *F. mortiferum*, *F. varium*, *Megamonas hypermegas*, *Mitsuokella multiacida*, *C. ramosum*, *C. clostridioforme*, and *C. butyricum*, and *Bilophila wadsworthia*.

Changes in PBPs. Piddock and Wise described cefoxitin-resistant mutants of *B. fragilis* group organisms with altered mechanisms of resistance (93). In that study, decreased susceptibility to cefoxitin could be correlated with decreased affinity of either penicillin-binding protein 1 (PBP-1) or PBP-2, while three isolates also had outer membrane protein changes, with a 49,000- to 50,000-molecular-weight band apparently absent. A strain of *B. fragilis* in which reduced affinity for PBP-3 was apparently responsible for resistance to ceftezole, cefazolin, and cephalothin has been studied by Yotsuji and coworkers (127). Wexler and Halebian (114) isolated a cefoxitin-resistant laboratory mutant and the cefoxitin-susceptible revertant and showed that decreased susceptibility to cefoxitin could be correlated with changes in the PBP-1 complex. Resistance to benzylpenicillin in a strain of *C. perfringens* was shown to be mediated by a decreased affinity of PBP-1 for the antibiotic (124).

Changes in outer membrane permeability to β-lactams. The role of porins in the permeability of gram-negative aerobic bacteria and in antimicrobial resistance has been extensively studied (23, 53, 74, 91, 97, 110) and recently reviewed (86, 87). Harder et al. (53) found that mutants of *Escherichia coli* resistant to certain β-lactam compounds lack the OmpF porin molecule, while there was no difference between the drug-resistant mutant and the wild-type strain in terms of the level of β-lactamase or the pattern of PBPs. Sawai et al. (97) found diminished uptake of cefazolin in porin-deficient mutant strains of *E. coli*, *Proteus mirabilis*, and *Enterobacter cloacae*. Similarly, Godfrey and Bryan (51) found diminished permeation of β-lactam antibiotics in a mutant of *Pseudomonas aeruginosa* with a structurally modified porin. Recently, Trias et al. (110) found decreased permeability of imipenem in an imipenem-resistant strain of *Pseudomonas aeruginosa*. The outer membrane protein profile of members of the *B. fragilis* group are much more complex than those of *E. coli* (39, 66), and specific permeability functions have not been assigned to any molecular species in the genus *Bacteroides*. Therefore, the role of permeability barriers in antibiotic resistance in anaerobic bacteria could be approached only indirectly (88). Crosby and Gump (28) investigated the effect of EDTA on the efficacy of cefoperazone against *B. fragilis* and found that EDTA enhanced the activity of cefoperazone, particularly against resistant β-lactamase-negative isolates. They suggested that impermeability of the cell is a major mechanism of resistance to cefoperazone in β-lactamase-negative isolates. Recently, Cuchural et al. (30) investigated the permeability of the *B. fragilis*

outer membrane to various antibiotics and found that ionic charge, hydrophobicity, and molecular weight influenced β-lactam uptake. As in *E. coli*, increased negative charge and increased molecular weight were associated with decreased drug uptake in *B. fragilis*. However, unlike with *E. coli*, increased drug hydrophobicity was associated with increased uptake. Malouin and Lamothe (70) showed roles for both β-lactamase and the permeability barrier in the activity of cephalosporins against members of the *B. fragilis* group but found that the species-specific exception was *B. distasonis*, which showed only a permeability barrier to all antibiotics tested. *B. distasonis*, though one of the most resistant of the group, has one of the lowest proportions of β-lactamase-positive strains.

Other Agents

Clindamycin and erythromycin resistance in *B. fragilis* has been extensively studied (71, 72, 108, 109) and reviewed (101, 106, 107). The mechanism is probably similar to that of macrolide-lincosamide-streptogramin resistance seen in aerobic bacteria. Three transfer factors (pBFTM10, pBF4, and PBI136) that confer clindamycin resistance have been described. Constitutively resistant strains have been selected from *B. fragilis* group isolates that exhibit inducible resistance to clindamycin and erythromycin (94).

Chloramphenicol inactivation may occur by reduction of chloramphenicol by nitroreductase (69). A second mechanism, proposed by Britz and Wilkinson, was shown to be due to a constitutively produced acetyltransferase (20).

Metronidazole resistance in anaerobes is mediated by a decrease in nitroreduction of the compound to the active agent (82, 107, 109). Approximately one-half of the nonsporeforming gram-positive rods are resistant to metronidazole. Resistance among gram-negative anaerobes is quite rare. We have found several strains of metronidazole-resistant *B. gracilis*, and metronidazole-resistant strains with the resistance factor on a transferable plasmid have been reported (19).

A self-transferable plasmid conferring tetracycline and chloramphenicol resistance was described in *C. perfringens* (17). Mays and colleagues (72) reported non-plasmid-associated transfer of tetracycline and clindamycin resistance from a strain of *B. fragilis* to a strain of *B. uniformis*. Transfer of tetracycline resistance among strains of *C. perfringens* isolated from human feces was reported, and Miyoshi surmised that the resistance was transferred by a conjugationlike process (76).

CURRENT PATTERNS OF ANAEROBIC RESISTANCE

United States

Researchers at the Tufts-New England Medical Center have been conducting nationwide surveys on the susceptibilities of isolates of the *B. fragilis* group. The method used for this survey is not the NCCLS reference method but is considered comparable. Again, in light of the significant differences in reports due to subtle difference in technique, care must be taken when these studies are compared with those performed by other methods. However, these studies are extremely useful, since a single laboratory performed these studies with isolates obtained from various U.S. hospitals and since the antibiograms have been surveyed for several years (29, 31, 32, 35, 105). With the current NCCLS breakpoints, resistance rates of the strains tested ranged as follows: piperacillin, 8 to 12%; clindamycin: 3 to 7%; cefoxitin, 2 to 3%; cefotaxime, 23 to 35%; and cefoperazone, 27 to 33%. No obvious increase from 1981 to 1983 was seen. No metronidazole- or chloramphenicol-resistant isolates were found. Clustering of resistance by centers was found with cefoxitin, moxalactam, and clindamycin. It should be noted that the resistance rates published by this group for isolates from the Wadsworth Anaerobe Laboratories (27, 32, 35, 105) are considerably lower than our own values (see Table 1 and reference 118) and undoubtedly reflect the problems referred to above concerning the comparison of data between laboratories. The data from strains collected in 1986 and 1987 revealed 3% resistance to cefoxitin, 11% to piperacillin, 15% to cefotetan, 15% to ceftizoxime (using a 32-μg/ml breakpoint), 27% to cefoperazone, 33% to cefotaxime, 74% to ceftazidime, and 3 to 6% to clindamycin (27). No isolates resistant to chloramphenicol or metronidazole were found, and for the 3 years that imipenem was tested, only one isolate of *B. distasonis* was found for which the MIC was 16 μg/ml.

Data on strains from the Wadsworth Anaerobe Laboratories are summarized in Table 1. In an effort to simplify the presentation and interpretation, antibiotics with similar efficacies against groups of anaerobes have been grouped and the range of percent susceptible strains has been used to define the group. The ranking within a group does not reflect the degree of activity. This presentation also minimizes the significance of differences of <10%. At times an antibiotic was borderline between two groups, and the decision about its grouping was necessarily arbitrary.

Other Countries

A survey of the literature yielded susceptibility data from Australia (40, 79), Austria (75), Brazil (37), France (18, 38, 41–45, 95), Germany (49, 55), Italy (16, 90), Poland (65), Russia (13), Spain (12, 67), Turkey (111), Switzerland (125), and the United Kingdom (50). A perusal of the articles with English abstracts was undertaken both to obtain data regarding antibiograms from foreign hospitals and to determine the kinds of techniques used in anaerobic susceptibility testing in other countries. The search underscored the difficulty in comparing studies from various laboratories. Few, if any, of the reports indicated in the abstract the breakpoint used to determine percent susceptible strains, and it was often not very obvious or was absent in the text of the report as well. Further, there is no general consensus on appropriate breakpoints (4). When reasonable data could be obtained either from the English abstract or from tables in the article, the results were summarized (see below). If not mentioned below, the breakpoint used corresponded to the currently accepted NCCLS breakpoints. It would be very useful if authors in the United States and abroad included the method and the breakpoints used to generate the susceptibility data given in the abstract. Also, if the percent susceptible strains for a range of concentrations is included, the data can be compared even when the concentration chosen as the breakpoint differs. In this way, data from various laboratories could legitimately be compared, and trends of developing resistance could be spotted. If studies performed according to NCCLS-approved procedures were periodically solicited from large testing laboratories abroad, interlaboratory comparisons would be further facilitated.

Australia. Munro (79) investigated patterns of resistance in Sydney during 1987 and 1988 and reported very high levels of cefoxitin resistance (12 to 92% of strains resistant with a

TABLE 1. Susceptibility of anaerobes to antimicrobial agents

% of strains susceptible	Antimicrobial agent(s)									
	B. fragilis	Other *B. fragilis* group[a]	*B. gracilis*	Other *Bacteroides* spp.[b]	*Fusobacterium* spp.	*Bilophila wadsworthia*	*Peptostreptococcus* spp.	*C. perfringens*	Other *Clostridium* spp.	Other nonspore-forming gram-positive rods
>95	Ampicillin + sulbactam Cefoperazone + sulbactam Piperacillin + tazobactam Ticarcillin + clavulanate Chloramphenicol Imipenem Metronidazole	Ampicillin + sulbactam Cefoperazone + sulbactam Piperacillin + tazobactam Ticarcillin + clavulanate Chloramphenicol Imipenem Metronidazole	Chloramphenicol Imipenem	Ampicillin + sulbactam Cefoperazone + sulbactam Ticarcillin + clavulanate Cefoperazone Cefotaxime Cefoxitin Chloramphenicol Clindamycin Imipenem Piperacillin	Ampicillin + sulbactam Piperacillin + tazobactam Ceftizoxime Chloramphenicol Clindamycin Imipenem Metronidazole Penicillin G Piperacillin	Chloramphenicol Metronidazole	Ampicillin + sulbactam Cefoperazone + sulbactam Ticarcillin + clavulanate Cefoperazone Cefotetan Ceftazidime Ceftriaxone Chloramphenicol Imipenem Moxalactam Penicillin G Piperacillin	(All drugs active at >95%)	Ampicillin + sulbactam Amoxicillin Ampicillin Carbenicillin Chloramphenicol Imipenem Metronidazole Penicillin G Piperacillin Ticarcillin	Ampicillin + sulbactam Ticarcillin + clavulanate Cefotaxime Ceftizoxime Chloramphenicol Imipenem Penicillin G Piperacillin
85–95	Cefotetan Cefoxitin Clindamycin Piperacillin	Piperacillin	Metronidazole	Cefotetan Ceftazidime Ceftizoxime Ceftriaxone	Cefoperazone + sulbactam Ticarcillin + clavulanate Cefoperazone Cefotaxime Cefotetan Cefoxitin Ceftriaxone	Clindamycin	Metronidazole		Cefoxitin Clindamycin Moxalactam	Cefoperazone + sulbactam Cefotetan Cefoxitin Ceftazidime Ceftriaxone Clindamycin
70–84	Ceftizoxime Moxalactam	Cefoxitin Ceftizoxime Clindamycin	Ampicillin + sulbactam Cefoperazone + sulbactam Moxalactam Piperacillin	Moxalactam Penicillin G	Ceftazidime Moxalactam	Cefotaxime Cefotetan Imipenem	Clindamycin			Cefoperazone Moxalactam
50–69	Cefoperazone Cefotaxime Ceftazidime Ceftriaxone	Cefoperazone Cefotetan Moxalactam	Ticarcillin + clavulanate Cefoperazone Cefotaxime Cefotetan Cefoxitin Ceftizoxime Clindamycin Penicillin G						Cefoperazone Cefotaxime Ceftizoxime Ceftriaxone	Metronidazole
<50	Penicillin G	Cefotaxime Ceftazidime Ceftriaxone Penicillin G	Ceftazidime			Ticarcillin + clavulanate Cefoxitin Ceftizoxime Penicillin G Ticarcillin			Ceftazidime	

[a] Excluding *B. fragilis*.
[b] Includes *Porphyromonas* spp., *Prevotella* spp., and several other (non-*B. fragilis* group) *Bacteroides* spp.

breakpoint of 16 μg/ml and 42 to 83% resistant with a breakpoint of 32 μg/ml among the *B. fragilis* group isolates). Resistance rates to clindamycin with a breakpoint of 8 μg/ml were 10% for the *B. fragilis* group. Metronidazole resistance was seen only in the group of anaerobic gram-positive nonsporeforming rods. Resistance to ampicillin and sulbactam was noted in five strains of *B. distasonis* and two anaerobic gram-positive cocci (the breakpoint used was 8 μg/ml; the current accepted breakpoint is 16 μg/ml).

Austria. Four laboratories in Austria participated in a study of the susceptibilities of 197 strains of the *B. fragilis* group tested by an agar dilution method (75). This study illustrates how differing determinations of "susceptibility" may be obtained on the basis of different interpretations of the results. In the summary, the authors mention "favorable activity with penicillin G" at a MIC_{90} of 32 μg/ml, although only 9% of *B. fragilis* group organisms were susceptible to penicillin G at 4 μg/ml (the NCCLS-approved breakpoint). Susceptibilities to some of the other agents tested were 98% for cefoxitin (32 μg/ml), 96% for piperacillin (64 μg/ml), 95% for clindamycin (4 μg/ml), and 98% for metronidazole (16 μg/ml) (one strain of *B. vulgatus* and two strains of unidentified *B. fragilis* group species were resistant). Variations in susceptibility by species were noted as well: 11% of strains of *B. thetaiotaomicron* were resistant to clindamycin, 0 to 4% were resistant to cefoxitin, and 0 to 4% were resistant to piperacillin and mezlocillin (both at 64 μg/ml). No chloramphenicol resistance (at 16 μg/ml) was found, but one strain of *B. vulgatus* was metronidazole resistant.

Brazil. The susceptibility of 228 strains of the *B. fragilis* group isolated from fecal samples in Brazil was tested by an agar dilution method, using brain heart infusion broth and an inoculum of 3×10^4 CFU/ml (37). (The method is that used in the surveys at the Tufts University Medical Center mentioned above [29, 105].) In the *B. fragilis* group, no metronidazole-resistant strains were found and chloramphenicol MICs were 32 μg/ml for two strains. Of the strains tested, 21% were resistant to cefoxitin and a very high percentage (37%) were resistant to clindamycin.

France. In one study, Dubreuil et al. (43) reported from several French hospitals on susceptibility to metronidazole, clindamycin, and cefoxitin (using an early description of the NCCLS reference method [103]). In this study, no metronidazole-resistant *B. fragilis* group strains were found, and resistance to cefoxitin (i.e., MIC of >32 μg/ml) and clindamycin (i.e., MIC of >4 μg/ml) was 2 and 10%, respectively. In a later report, Dubreuil and co-workers (42) found that resistance rates to cefoxitin (MIC, >32 μg/ml) varied between 2 and 16% depending on the institution. The breakpoint for clindamycin in this study was 8 μg/ml (the current NCCLS-recommended breakpoint is 4 μg/ml), so it is difficult to compare resistance rates with those published elsewhere, but approximately 9 to 14% of strains were resistant. Clindamycin resistance increased in all three institutions included in this study between 1986 and 1987. Rates of resistance to piperacillin (MIC, >128 μg/ml) were found to increase from 0% before 1985 to 4 to 10% after 1986. Again, the currently accepted NCCLS breakpoint is 64 μg/ml for piperacillin, so care must be taken in comparing resistance rates. Metronidazole, imipenem, thiamphenicol, and amoxicillin-clavulanate were active against all strains tested in this study. At least one group in France reported results for anaerobes tested on Mueller-Hinton agar supplemented with horse blood (95). Another study (99) used the NCCLS method and found no resistance to amoxicillin with either clavulanic acid or sulbactam added and no resistance to metronidazole. Rates of resistance to piperacillin, cefoxitin, and cefotaxime were 4, 8, and 62%, respectively. Breuil and colleagues have reported (19) up to 5% transferable metronidazole resistance in *Bacteroides* spp. They also reported, using the NCCLS technique, a survey of 300 *B. fragilis* isolates (18) from 22 hospitals in France in 1987 and 1988. They found amoxicillin-clavulanate and imipenem resistance in two strains and metronidazole resistance in a few strains as well. No resistance to chloramphenicol was found. Two percent of the strains were resistant to cefoxitin, and between 12 and 25% were resistant to cefotetan, cefotaxime, ceftriaxone, and ceftizoxime. Nineteen percent of the strains were clindamycin resistant in this study.

Germany. Focht and coworkers reported on a large study with isolates from 24 hospitals in the Moers area of Germany (49). MICs were determined by a microdilution technique with the broth formulation of Wilkins-Chalgren agar. When the current NCCLS-recommended breakpoint of 8 μg/ml for amoxicillin (+4 μg/ml for clavulanic acid) was used, 25% of the *B. thetaiotaomicron* strains were resistant and 3% of the *B. fragilis* strains were resistant. All other anaerobes tested were susceptible.

Italy. Using an agar dilution technique, Braga et al. confirmed the "widespread" resistance in Italy to clindamycin and cefoxitin reported in the literature (16). The journal article was not available, and the percent resistant was not listed in the abstract, nor were the locations of the hospitals from which the strains were collected.

Poland. In Poland, Kedzia and Kaówski studied the effects of chlorhexidine gluconate on nonsporeforming anaerobes from the oral cavity (65). MBCs were determined after the organisms were exposed to various concentrations of the agent for 5, 10, and 15 min. Portions were removed and incubated for 7 days at 37°C, and the MBCs were determined.

Russia. Among nonsporeforming anaerobic bacteria in Russia, no resistance was seen to cefoxitin, cefotaxime, mezlocillin, or carbenicillin (all MICs were <31.2 μg/ml). The testing method described serial dilution with Schaedler anaerobe agar, an inoculum of 10^5 CFU per spot, and incubation for 48 h in GasPak jars (13).

Spain. Using the NCCLS reference method, Betriu et al. in Spain found no strains resistant to imipenem, metronidazole, amoxicillin-clavulanic acid, or chloramphenicol (12). Of the strains tested, 21% were resistant to clindamycin at 4 μg/ml, and 6% were resistant to cefoxitin, 9% to cefotetan, and 32% to cefmetazole, all at 32 μg/ml. Like others, Betriu et al. found that *B. fragilis* was more susceptible to many agents than were the other members of the group.

Turkey. Tunèkanat and Günalp reported resistance rates in Turkey to tetracycline, cephalothin, erythromycin, and penicillin G of 66, 29, 16, and 8%, respectively (111). They found no resistance to carbenicillin, clindamycin, or chloramphenicol. The method of testing and the breakpoints used were not outlined in the abstract.

United Kingdom. The antibiotic susceptibility of *B. fragilis* group isolates from 14 centers in the United Kingdom was surveyed (50). Researchers tested 862 strains by the microdilution method. No isolates resistant to chloramphenicol were found; one isolate of *B. vulgatus* was resistant to metronidazole. Fewer than 1% of the strains were resistant to clindamycin. The breakpoints did not correspond to those of the NCCLS, so exact comparisons were difficult. For example, 10% of the isolates were resistant to >1 μg of clindamycin per ml, but it is not clear how many were resistant to 4 μg/ml (the NCCLS breakpoint). Similarly, the

breakpoint used for cefoxitin and cefotetan was 8 μg/ml, and resistance rates for mezlocillin, cefoxitin, and cefotetan were 13, 16, and 7%, respectively. In general, very low resistance was found in this study, particularly in view of the low breakpoints used. MICs with broth microdilution systems are typically at least 1 dilution lower than those obtained with agar methods (85); this may account for part of the particularly low rate.

FUTURE CHALLENGES?

The challenge facing those who design and implement anaerobic susceptibility protocols is to find a testing method that will correlate laboratory results with clinical outcome. Unfortunately, this may be a near-impossible task. When anaerobes are found in pure culture in an infection (e.g., bacteremia), the task may be simpler. However, infections involving anaerobes are typically mixed; it may not be necessary to eradicate all of the organisms to effect a cure. Appropriate surgical manipulations and the age and health of the patient will have a significant impact on the outcome, regardless of whether a particular isolate is susceptible to the antimicrobial regimen. The microenvironment at the site of the infection (e.g., pH, available nutrients, and clearance of organisms by host defenses) will undoubtedly influence the interaction between the organism and the antimicrobial agent. Actually, the questions typically put to the clinical microbiologist, i.e., (i) Does the agent inhibit the organisms in vitro? and (ii) Will the agent work in a particular setting?, may not have the same answer. Obviously, the entire purpose of susceptibility testing in the clinical laboratory is to aid the clinician in predicting which agent will be effective in combatting the infection. However, the best possibilities for eventual correlation lie in the development of a susceptibility test that accurately defines the "bug/drug" interaction. Clinical correlations can then be derived from adjustments of interpretative breakpoints or other factors. Although clinical correlation studies are difficult to design and accumulation of data will take some time, such studies will allow the development of the most predictive test possible. The use of animal models of infection, though different in many respects from the infection in humans, may be of help in ultimately designing tests and ways of interpreting the results that will correlate with the clinical outcome. It may be that, for extensive studies of the drug-organism interaction, research laboratories will be better served by looking at more tightly defined kinetic studies of bacterial inhibition. In the meantime, clinicians need to evaluate the available data in light of the caveats outlined in this review.

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