SUSCEPTIBILITY TESTING

Susceptibility Testing of Anaerobic Bacteria—The State of the Art

Hannah M. Wexler

From the Research Service, Veterans Administration Medical Center, West Los Angeles, Wadsworth Division, and the Department of Medicine, UCLA School of Medicine, Los Angeles, California

Demand for susceptibility testing of anaerobes has increased, but no consensus on procedure and interpretation has been achieved. The need for reliable methods for testing anaerobic bacteria extends from small hospital laboratories to large research centers. Agar dilution testing is too costly and labor intensive for many clinical laboratories. Microbroth dilution is more convenient; however, some fastidious anaerobes do not grow well enough in this system, and the choice of antimicrobial agents may not reflect the hospital formulary. Disapproval of the broth disk elution system leaves fewer options open to clinical laboratories and emphasizes the need for more convenient and reliable techniques. Some newer methods are undergoing evaluation. Variables in susceptibility testing of anaerobes include the media and methods used, organisms chosen, breakpoints chosen, and endpoint determination. This latter variable is probably the most problematic since no endpoint based on interaction of organism and antimicrobial agent rather than on subjective observation has been defined. Also, clustering of MICs around the breakpoint may lead to significant variability in reported methods. A more accurate MIC measurement is needed. Adherence of laboratories to approved methods and careful reporting of methods and the interpretive breakpoints would facilitate interlaboratory comparisons and the identification of emerging resistance.

The plethora of recent articles about susceptibility testing of anaerobes reflects the dissatisfaction with the status quo. The titles "Anaerobic Susceptibility Testing—Fact, Fancy, and Wishful Thinking?" [1]; "Anaerobic Susceptibility Testing; Myth, Magic, or Method?" [2]; "Revisiting Anaerobic Susceptibility Testing" [3]; and "Son of Anaerobic Susceptibility Testing—Revisited"[4] reflect the lack of consensus in this area. Resistance of anaerobic bacteria to antimicrobial agents has increased, as has the need for information about the antimicrobial agents likely to be effective against a given organism. At this symposium, a consensus group concluded that susceptibility testing of anaerobes was warranted because (1) anaerobes are significant pathogens and (2) their susceptibility patterns cannot be reliably predicted [5].

The introduction to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS) suggests that while routine susceptibility testing of anaerobes is generally not considered necessary, some clinical settings require such testing. Realistically, both for medical and legal needs and for the confidence of the clinician prescribing therapy, testing often is performed more frequently than required by the NCCLS recommendations, and laboratories need a reliable, cost-effective testing method that can be used on a routine basis. A general list of types of isolates that should be tested include those from brain abscesses, endocarditis, osteomyelitis, joint infections, prosthetic device infections, and refractory or recurrent bacteremia and anaerobes isolated in pure culture; isolates from any infection not responsive to empirical therapy; and isolates from patients who will be treated medically rather than surgically. Isolates that should be considered for testing include *Bacteroides fragilis* group isolates, *Bacteroides gracilis*, *Clostridium perfringens*, and *Bilophila wadsworthia*.

For the research laboratory, anaerobic susceptibility testing is necessary on a large-scale basis because the patterns are not as predictable as once thought, and new agents must be evaluated. While much of the regional and hospital-to-hospital variation is related to technique, there certainly are some bona fide pockets of resistance, and any hospital that suspects that they are encountering such a phenomenon is encouraged to do large-scale testing. Also, large numbers of isolates in any hospital should be tested from time to time to monitor trends. Reports published by reliable large research centers can serve as a guide for the clinician in initiating appropriate therapy. In many cases, however, neither published results from other institutions nor even antibiograms derived from their own institution give entirely adequate guidance for therapy in many cases [5], although sometimes these are the only guides a clinician has when initiating therapy before laboratory results become available. Thus, the

Reprints or correspondence: Dr. Hannah M. Wexler, Microbial Diseases Research Laboratory, Building 304, Room E3-224, VA Wadsworth Medical Center 691/151J, Los Angeles, California 90073.

Clinical Infectious Diseases 1993;16(Suppl 4):S328–33

two settings in which susceptibility testing would occur, namely research centers and clinical laboratories, may have different criteria about what constitutes a reasonable, efficient mode of testing. Yet, the purpose of testing is ultimately the same: to determine whether a given antimicrobial agent is an effective inhibitor of a given organism.

The purpose of this brief review is twofold: to outline some of the challenges facing clinical and research laboratories performing anaerobic susceptibility tests and reiterate caveats to be aware of in interpreting susceptibility tests and to suggest the need for a more meaningful and reliable test and propose that a search for this improved test must begin with an unambiguous definition of an MIC.

Problems in Susceptibility Testing

Technical Variability Among Laboratories

Techniques used in different laboratories vary, and these variations may result in nontrivial differences in results. For example, it is well known that the efficacy of ceftizoxime in vitro is much greater in microbroth dilution tests than in agar dilution tests and that this difference is technique-dependent [6, 7]. The most popular technique in the past was the broth disk elution technique. However, in its latest reference protocol, the NCCLS, after fairly lengthy and agonized discussion, deleted the broth disk technique from its approved protocol [8]. The most economical technique is microbroth dilution, although some of the more fastidious organisms do not grow well enough in this system to allow reliable determination of MICs and may, in fact, produce β-lactamase (e.g., some strains of *Fusobacterium*, *Prevotella*, and *Porphyromonas*).

Organisms Chosen for Testing

When reports on the *B. fragilis* group are evaluated, the proportions of the various species used must be considered. *B. fragilis* itself is much more susceptible to certain agents than are other members of the group. Species other than *B. fragilis* comprise more than one-half of our clinical isolates at the Wadsworth Anaerobe Laboratory. By altering the proportions of the various species, one can radically alter the outcome of the tests. Also, strains tested should be fresh clinical isolates rather than stock laboratory cultures.

Clustering of MIC Values Around the Breakpoint

In our laboratory, the MICs of many β-lactam agents fall within one twofold dilution of the breakpoint for 50%–60% of all anaerobes and the MICs of clindamycin fall within one twofold dilution of the breakpoint for 38%. Even higher percentages of the MICs for *B. fragilis* group isolates fall within one twofold dilution of the breakpoint (~70% for many β-lactam agents and 46% for clindamycin) [9]. The accuracy of any of the currently accepted susceptibility tests is at best plus or minus one twofold dilution. Thus, a strain for which the MIC is 32 μg/mL on one day could have an MIC of 16 μg/mL or 64 μg/mL on another occasion, *within the accepted error rate for the test!* In practical terms, a large proportion of strains may be called susceptible on one day and resistant on the next. With such variation, coupled with any other slight shifts caused by technical variation (e.g., inoculum preparation, differences in media, or size of the inoculum), wide swings in reported susceptibilities may be meaningless. Systems that form a continuous concentration gradient, rather than the twofold dilution steps of the agar dilution system, minimize the uncertainty caused by this clustering.

The concept of introducing indeterminate or intermediate zones for anaerobes has been raised as a rejoinder to the problem of clustering of MICs. Many physicians and microbiologists feel that the relatively crude measures currently used to determine in vitro susceptibility can discriminate only between the "susceptible" and "resistant" groups of organisms and that attempts to "fine tune" such tests are not likely to be successful [5]. Although this question is entirely legitimate, we still need a test that yields more accurate, reliable results. With such a test, the number of isolates for which susceptibility results are indeterminate would be minimized.

The Breakpoint Used

Until a few years ago, there were even discrepancies between breakpoints recommended by the NCCLS [8] and those included on the package inserts approved by the Food and Drug Administration. These have since been brought into alignment. However, there are still articles in the literature that do not conform, particularly studies done outside the United States. Often, the regulatory bodies of different countries have established different breakpoints or have not agreed on breakpoints and leave the decision to the individual author.

Variability in Values Reported

The percentage of strains susceptible at breakpoint cannot be deduced from reports of MIC_{50} or MIC_{90} values. It is impossible to project from one of these values what the others might be and hence to compare values with those of other investigators. Geometric mean MICs may yield more accurate information than MIC_{50}, MIC_{75}, or MIC_{90} values [10]. The trend (and at times editorial pressure) for reporting only the MIC_{50} or the MIC_{90} is disturbing in light of these considerations. Many journals no longer will accept finlandiograms (which actually give the most information) but at least will accept reports of the percentage of isolates susceptible at a range of concentrations near the breakpoint. Thus, we recom-

mend that workers report the percentage of susceptible strains at a reasonable range of values about the breakpoint (e.g., a range of three twofold dilutions).

Changes in Taxonomic Definitions Not Consistent Among Laboratories

Olsson-Liljequist and Nord [11] commented that in their study only 70% of the organisms classified as anaerobic cocci were susceptible to metronidazole, yet Watt and Jack [12] suggested that anaerobic cocci should be defined as cocci that grow only under anaerobic conditions, 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 the susceptibility results reported by various laboratories. The newer taxonomic changes, especially for anaerobic gram-negative rods, will have an impact on whether a laboratory groups most of the non-*fragilis* group *Bacteroides* together or separates them into the newer genera (e.g., *Prevotella* and *Porphyromonas*).

Interpretation of Results

The issue is really twofold: that of interpreting what is seen on the susceptibility test; and that of the accuracy of the test itself. The first issue is perhaps the simpler one and will be addressed first.

Difficulties in determining an endpoint. One of the greatest sources of variability is in interpretation of the test, i.e., the determination of the MIC. The NCCLS agar dilution reference protocol defines the breakpoint as that dilution at which there is "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" [8].

For certain organism–antimicrobial agent combinations, application of different parts of the definition would result in different choices of MIC. The problem generally occurs with gram-negative organisms and cell wall–active drugs (i.e., β-lactam agents). With some organism–antimicrobial agent combinations, different parts of the definition might describe one-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, such as *B. gracilis*, where even the amount in the growth control is relatively light. Our laboratory first investigated these "fuzzy endpoints" some years ago when we noticed inconsistent results with *Fusobacterium* and some β-lactam agents. After retesting these strains, and then retesting some of our previously tested strains, we realized that the difference was not in the susceptibility of the organisms but in the interpretation of results. For those strains, we found that the haze seen on agar dilution tests represented cell wall–deficient (CWD) forms of the *Fusobacterium* strains and that these forms remained viable in concentrations of cefoxitin of up to 16,000 μg/mL. The CWD forms reverted back to the parental morphology after two passages in drug-free media. We recommended that in these cases the haze be ignored for purposes of susceptibility testing [13], primarily because these CWD forms are generally thought to be unimportant clinically, although they are so hard to grow under normal cultural conditions that detection would be very difficult. However, some investigators believe that these CWD forms may have clinical significance hitherto unrecognized [14].

Although we had decided to ignore these "hazes," we thought our decision was somewhat arbitrary even though our determination met the NCCLS criterion. This uncertainty prompted us to try to determine the significance of this growth (H. M. Wexler et al., unpublished data). We looked particularly at the growth of *B. fragilis* group organisms on agar plates containing increasing concentrations of ceftizoxime. As concentrations of ceftizoxime increased, there was a sharp transition from white, opaque growth to a region of clearer translucent growth. This region often extended over a broad range of ceftizoxime concentrations (equivalent to several twofold dilutions). This phenomenon is seen both on conventional twofold dilution agar plates and on antibiotic gradient plates made by a spiral streaker [16–18]. The purpose of that study was to ascertain whether viable cells were present in these "tail" regions and to see whether triphenyltetrazolium chloride (TTC) would be a useful aid in determining a ceftizoxime endpoint. TTC is reduced by many bacteria to formazan, a red insoluble compound. We used the formation of formazan (i.e., the development of a red color) within the bacterial colony as an indicator of cellular viability [19, 20]. We previously showed that MICs determined by the spiral gradient endpoint (SGE) system (Spiral Biotech, Bethesda, MD) correlated with those determined by agar dilution [18]. We exploited the fact that changes in both the amount of growth and the formation of the formazan dye on SGE plates were more evident along a continuous streak than on agar dilution plates. We also used the SGE plates to pinpoint an MIC because the MIC determination along a continuous concentration gradient is more precise than one from a twofold dilution series. At various times during incubation, we removed bacterial growth from areas on agar dilution plates and counted the number of cfu present at different ceftizoxime concentrations. We found that the TTC "endpoints" correlated with the concentration of antimicrobial agent that permitted no net growth of the organisms.

Another example of our "endpoint research" concerns an organism discovered a few years ago in studies of perforated and gangrenous appendicitis. *Bilophila wadsworthia*, isolated from patients with perforated and gangrenous appendicitis [21], appeared to be one of the most resistant anaerobes we had seen. Growth of *Bilophila* generally is weak and slow,

and the control growth in susceptibility tests is fairly transparent. Early evaluations comparing growth on antimicrobial agent–containing media suggested that the organism was growing even in the presence of high concentrations (>128 μg/mL) of some antimicrobial agents (e.g., imipenem, an agent with potent activity against anaerobes). However, our earlier experience led us to question hazy growth, and viability data obtained as described above indicated a far lower MIC than that recorded visually. Electron microscopic studies revealed that the haze was composed of cell wall–deficient spheroplasts that were strikingly large (as compared with the normal rod-shaped organism [P. Summanen et al., unpublished data]).

Another dilemma that must be resolved is whether the addition of growth-enhancing factors to the test medium is justified. Generally, it is considered permissible to add an enhancer as long as it does not interfere with the antimicrobial agent. In experiments in our laboratory, we were confronted with the opposite situation. We noticed that MICs obtained for *B. gracilis* grown on formate/fumarate (a growth enhancer for *B. gracilis*) were much lower than those obtained for the strains grown on conventional brucella blood agar [15]. Viability data indicated that without formate/fumarate, the initial inoculum essentially was maintained at concentrations of ceftizoxime near the determined MIC, whereas with the addition of formate/fumarate, the percentage of viable cells decreased more rapidly, in accordance with the principle that a faster generation time allows more rapid killing [23]. These findings raise these questions: Is it necessary that the organism be growing optimally to determine what the activity of the agent really is? Does the addition of a supplement make the test less relevant to the clinical setting, or does the in vitro setting itself make the test distinct from the clinical setting?

Accuracy of the determined MIC. Earlier, we identified the problem of endpoint definition as twofold: the decision about an endpoint and the accuracy of the determined MIC. Both the agar dilution and microbroth dilution techniques use a twofold dilution scheme in which the permissable rate of error is one twofold dilution. This variation was borne out in our own study of 20 organisms [24]. Five organisms were tested in triplicate, five in duplicate, and five singly. Tests were done on four separate days using four different inocula and read independently by two technicians. Even within triplicates (i.e., the same inoculum put into separate wells on the Steers replicator and stamped on the same plate), variation within one twofold dilution was seen—even with the same reader. More significantly, when the MICs were near the breakpoint (the case for a significant majority of the strains), the reading could alternately be one of resistance or one of susceptibility. Since this error occurred even within such a carefully controlled and defined study (in which the known purpose of the study was the assessment of variability), we must assume that the variability in the typical clinical laboratory may be greater. Also, in data presented to the NCCLS to determine quality-control values for anaerobic susceptibility testing, sometimes a fourfold dilution range was needed to encompass 95% of the values generated. Again, these tests are performed in highly qualified laboratories accustomed to anaerobic susceptibility testing. If the readings for quality-control organisms can easily vary within two or three twofold dilutions and if most of the MIC values fall within one dilution of the breakpoint, many organisms could be identified as resistant on one occasion and as sensitive on another within the testing parameters allowed.

Yet, this extraordinarily imprecise test is used as a gold standard for the evaluation of other procedures. It is increasingly clear that such a technique not only is too imprecise to be used as a "gold standard" for evaluating other techniques, but it is certainly not good enough to get a meaningful number on its own or to allow us to ascertain clinical correlations. These measurements also do not allow us to compare results of different laboratories or worldwide patterns of drug resistance.

The description of the interaction between antimicrobial agent and organism should not be dependent on technique but should be based on more fundamental criteria. One possible definition of an MIC has been offered by S. Schalkowsy (personal communication). This model may serve as a springboard for a reasonable, defensible definition of an MIC. In this model, we look at the following equation: $N = N_i \times (2p)^n$, where N is the viable cell density, N_i is the initial cell density, p is the probability of successful division, and n is the number of divisions. Then, if $p = 1$, which it would in the absence of an antimicrobial agent, the population doubles with each division. When $p = .5$, the likelihood of successful division and that of nonsuccessful division are equal, i.e., $N = N_i$. In other words, the initial population size remains unchanged. The probability that any particular division will be successful will be dependent only on the interaction between organism and antimicrobial agent. We can define the MIC as that concentration at which the initial density of viable cells remains unchanged, i.e., as that concentration at which $p = .5$. Schalkowsky et al. [25] have shown that when the log (N/N_i) is plotted against time of exposure to the antimicrobial agent at a series of concentrations, a series of lines with different slopes are obtained. As the concentration of antimicrobial agent increases, the rate of population growth decreases and the slope becomes less steep. At a particular concentration, the MIC, there is no net change in the population and the slope is 0. At higher concentrations, there is a net decrease in the population and a negative slope (i.e., the killing region). The concentration at which the slope is 0 (i.e., $N = N_i$) would be defined as the MIC. One could approximate this value from the series of slopes obtained. However, if these slopes are plotted as a function of drug concentration, a curve will be obtained, and the MIC is the point at which the curve crosses the axis (i.e.,

slope = 0). This discrete MIC value (as opposed to the values obtained by the twofold dilution techniques) can be generated by performing killing curves at various antimicrobial concentrations bracketing the endpoint, determining the slopes of the lines generated, and plotting those values against the antimicrobial concentration. This MIC should not be dependent on the generation time (which might well be technique-dependent). Studies by Tuomonen and coworkers showed that the rate of killing with an antimicrobial agent is dependent on generation time, with the slowest generation times corresponding to the slowest killing rates. On the other hand, when the rate of killing (plotted as the percentage of survivors) is plotted against the number of generations, the lines coalesce, indicating that as a function of the number of generations, the rate of killing is constant [23]. In other words, if we concentrate on a definition of MIC as that concentration at which the probability of successful division is 0.5 for any generation, the generation time will not be a factor.

The reader should note that the above discussion is not a description of a method but is rather a proposal of a definition of the MIC and a suggestion about how to obtain this discrete MIC value. The purpose of introducing this definition is to initiate a springboard for discussion among microbiologists that leads to a consensus definition of an MIC. This is of critical importance for both the research laboratory and the clinical microbiologist, since we cannot evaluate any "new and better" techniques until we have clearly defined what they are supposed to measure. We do not mean to imply that the clinical microbiologist would determine killing curves. However, the research laboratory can and should ascertain that the values obtained in accepted methods correspond to a real description of the interaction between the organism and the antimicrobial agent. Once we arrive at a definition, we can decide whether a given test performs this measurement adequately. Perhaps this work should be done for certain classes of organisms and antimicrobial agents for which results are hard to interpret. Viability studies can determine if the "hazes" seen are simply slower-growing organisms or represent the accumulation of dead cells. A series of killing curves for certain organisms and antibiotics can be generated to determine a "real" MIC. At that point, a test for which an easily determinable visible change correlates with the real MIC must be developed for use in the clinical laboratory.

A more accurate and reliable test will also enable us to evaluate some of the newer and more practical options such as the spiral gradient technique [17, 26], the Etest [27–29], and modifications of the disk diffusion technique [30]. The spiral streaker 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. Comparisons of this procedure with the standard agar dilution method have been favorable [17, 26]. In a study conducted in my laboratory [17], 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 one doubling dilution) was 90.6%. In general, discrepancies between the two methods could be attributed to one of two causes: endpoints were difficult to read on one or both tests or MICs were close to breakpoint concentrations (and thus MICs determined by the two techniques for one strain could be within one twofold dilution yet one resulted in a "sensitive" designation and the other, in a "resistant" designation). The Etest 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 placed on the surface of an agar plate that has been inoculated with the organism, and the plate is incubated anaerobically for 24 hours. The point at which the teardrop-shaped zone of inhibition intersects the interpretive scale is considered the endpoint. The use of these strips for anaerobic susceptibility testing has been evaluated. Good correlation with standard techniques was seen despite some discrepancies [27–29]. Sutter and colleagues described the use of the agar disk diffusion test with rapidly growing anaerobes in 1973 [31]. Recently, modifications of the technique were evaluated and for certain antimicrobial agents the correlation with the reference method was good [30, 32, 33]. For many agents, however, correlation was poor. At present, the test is not considered generally appropriate for anaerobic susceptibility testing.

The clinician can gain important information from anaerobic susceptibility tests, but an expectation of an exact correlation between in vitro results and clinical outcome is unrealistic. Most anaerobic infections are mixed infections. It may not be necessary to eradicate all of the organisms to effect a cure, and factors such as age and health of the patient and the use of appropriate surgical treatment are important variables in the equation. Eventually, we will need careful studies evaluating the correlation of in vitro data to clinical outcome. To this end, we will need laboratory-derived in vitro values that are meaningful and accurate. Given the complexity of a typical clinical anaerobic infection, the clinical microbiologist may never be able to answer the question of whether a particular antimicrobial agent will cure the patient. However, the clinical microbiologist should be able to ascertain the activity of this antimicrobial agent against this organism. Accurate information regarding the efficacy of a certain agent in inhibiting or killing the organism will certainly give the clinician useful information with which to choose a therapeutic agent, and a consensus group of infectious disease clinicians recently concluded that, for the most serious infections involving anaerobes, there is a correlation between the results of susceptibility tests and the clinical response [5].

References

1. Goldstein EJC, Citron DM. Susceptibility testing of anaerobes—fact, fancy, and wishful thinking. Clin Ther **1989**;11:710–23.
2. Wexler HM. Susceptibility testing of anaerobic bacteria: myth, magic, or method? Clin Microbiol Rev **1991**;4:470–84.
3. Zabransky RJ. Revisiting anaerobic susceptibility testing. Clinical Microbiology Newsletter **1990**;11:185–8.
4. Baron EJ, Citron DM, Wexler HM. Son of anaerobic susceptibility testing—revisited. Clinical Microbiology Newsletter **1990**;12:69–72.
5. Rosenblatt JE, Brook I. Clinical relevance of susceptibility testing of anaerobic bacteria. Clin Infect Dis **1993**;16(suppl 4):S446–8.
6. Aldridge KE, Sanders CV. Antibiotic- and method-dependent variation in susceptibility testing results of *Bacteroides fragilis* group isolates. J Clin Microbiol **1987**;25:2317–21.
7. Aldridge KE, Wexler HM, Sanders CV, Finegold SM. Comparison of in vitro antibiograms of *Bacteroides fragilis* group isolates: differences in resistance rates in two institutions because of differences in susceptibility testing methodology. Antimicrob Agents Chemother **1990**;34:179–81.
8. Methods for antimicrobial susceptibility testing of anaerobic bacteria: approved standard. 2nd ed. Publication M11-A2. Villanova, Pennsylvania: National Committee for Clinical Laboratory Standards, **1990**;10(15).
9. Wexler HM, Finegold SM. Antimicrobial resistance in *Bacteroides* [review]. J Antimicrob Chemother **1987**;19:143–6.
10. Davies BI. The importance of the geometric mean MIC [letter]. J Antimicrob Chemother **1990**;25:471–2.
11. Olsson-Liljequist B, Nord CE. Antibiotic resistance among anaerobic bacteria [in Swedish]. Lakartidningen **1981**;78:4032–4.
12. Watt B, Jack EP. What are anaerobic cocci? J Med Microbiol **1977**;10:461.
13. Johnson CC, Wexler HM, Becker S, Garcia M, Finegold SM. Cell-wall-defective variants of *Fusobacterium*. Antimicrob Agents Chemother **1989**;33:369–72.
14. Mattman LH. Cell wall deficient forms: stealth pathogens. Boca Raton, FL: CRC Press, **1992**.
15. This reference was deleted at the galley stage.
16. Paton JH, Holt HA, Bywater MJ. Measurement of MICs of antibacterial agents by spiral gradient endpoint compared with conventional dilution method. Int J Exp Clin Chemother **1990**;3:31–8.
17. Schalkowsky S, Schalkowsky E, Tawfig S. Reference data for determination of antimicrobial endpoints by the spiral gradient agar dilution method. Bethesda, Maryland: Spiral Systems Instruments, **1985**.
18. Wexler HM, Molitoris E, Jashnian F, Finegold SM. Comparison of spiral gradient with conventional agar dilution for susceptibility testing of anaerobic bacteria. Antimicrob Agents Chemother **1991**; 35:1196–202.
19. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods **1983**;65:55–63.
20. Isenberg HD, D'Amato RF. Rapid methods for antimicrobic susceptibility testing. In: Cunha BA, Ristuccia AM, eds. Antimicrobial therapy. New York: Raven Press, **1984**:157–70.
21. Baron EJ, Summanen P, Downes J, Roberts MC, Wexler H, Finegold SM. *Bilophila wadsworthia*, gen. nov. and sp. nov., a unique gram-negative anaerobic rod recovered from appendicitis specimens and human faeces. J Gen Microbiol **1989**;135:3405–411.
22. This reference was deleted at the galley stage.
23. Tuomanen E, Cozens R, Tosch W, Zak O, Tomasz A. The rate of killing of *Escherichia coli* by beta-lactam antibiotics is strictly proportional to the rate of bacterial growth. J Gen Microbiol **1986**;132:1297–304.
24. Wexler HM, Lavin PT, Molitoris E, Finegold SM. Statistical analysis of the effects of trial, reader, and replicates on MIC determination for cefoxitin. Antimicrob Agents Chemother **1990**;34:2246–9.
25. Schalkowsky S. Measures of susceptibility from a spiral gradient of drug concentrations [abstract]. Proceedings of the Symposium on Antimicrobial Susceptibility Testing: Critical Issues of the 90s, Philadelphia, **1993** (in press).
26. Hill GB, Schalkowsky S. Development and evaluation of the spiral gradient endpoint method for susceptibility testing of anaerobic gram-negative bacilli. Rev Infect Dis **1990**;12(suppl 2):S200–9.
27. Citron DM, Ostovari MI, Karlsson A, Goldstein EJC. Evaluation of the epsilometer for susceptibility testing of anaerobic bacteria [abstract no 876]. In: Program and abstracts of the 29th Interscience Conference on Antimicrobial Agents and Chemotherapy. Washington, DC: American Society for Microbiology, **1989**:248.
28. Citron DM, Ostavari MI, Karlsson A, Goldstein EJC. Evaluation of the epsilometer (E-test) for susceptibility testing of anaerobic bacteria. J Clin Microbiol **1991**;29:2197–203.
29. Nachnani S, Scuteri A, Newman MG, Avanessian AB, Lomeli SL. E-test: a new technique for antimicrobial susceptibility testing for periodontal microorganisms. J Periodontol **1992**;63:576–83.
30. Barry AL, Fuchs PC, Gerlach EH, et al. Multilaboratory evaluation of an agar diffusion disk susceptibility test for rapidly growing anaerobic bacteria. Rev Infect Dis **1990**;12(suppl 2):S210–7.
31. Sutter VL, Kwok YY, Finegold SM. Susceptibility of *Bacteroides fragilis* to six antibiotics determined by standardized antimicrobial disk susceptibility testing. Antimicrob Agents Chemother **1973**;3:188–93.
32. Barry AL. Anaerobic susceptibility tests: differences between disc diffusion, agar dilution and broth microdilution test results [letter]. J Antimicrob Chemother **1990**;25:875–7.
33. Horn R, Bourgault AM, Lamothe F. Disk diffusion susceptibility testing of the *Bacteroides fragilis* group. Antimicrob Agents Chemother **1987**;31:1596–9.