

Practical Anaerobic Broth-Disk Elution Susceptibility Test

JAMES H. JORGENSEN,* GARY A. ALEXANDER, AND JAMES E. JOHNSON

Departments of Pathology and Microbiology, The University of Texas Health Science Center, The Bexar County Hospitals, and Audie Murphy Veterans Administration Hospital, San Antonio, Texas 78284*

A modified broth-disk elution method for routine susceptibility testing of anaerobic bacteria was compared with the proposed reference agar dilution method under study by the National Committee on Clinical Laboratory Standards. The broth-disk elution method used a 24-h test incubation period, Schaedler broth medium supplemented with menadione, and GasPak (BBL Microbiology Systems) incubation. Results obtained by this method compared favorably with carbenicillin, chloramphenicol, clindamycin, and penicillin minimal inhibitory concentrations. The 24-h incubation period led to major discrepancies with tetracycline disk elution tests. This method represents a practical routine procedure for testing of anaerobic bacterial isolates in hospital clinical microbiology laboratories.

The recent trend toward relevance and cost-effectiveness in clinical microbiology has encouraged development of practical methods for anaerobic bacteriology. The combined use of techniques such as the GasPak (BBL Microbiology Systems) jar and gas-liquid chromatography and the availability of relatively simple biochemical test procedures have allowed many hospital laboratories to process cultures for anaerobic bacteria with relative ease and proficiency.

Although various techniques of susceptibility testing of anaerobes have been performed for a number of years, a universally standardized method for testing this group of microorganisms has not existed. Certain laboratories have employed either broth or agar dilution techniques (2, 4, 6), and others have attempted to utilize some type of disk diffusion methodology (1, 8). Variables in testing among laboratories have included the type of media, age and size of the inoculum, and the duration of anaerobic incubation.

A collaborative group recently proposed an anaerobic agar dilution reference susceptibility test method (7) for endorsement by the National Committee on Clinical Laboratory Standards. Although this medium and its methodology have not been formally adopted, it appears to be the method with which all future techniques will be compared for accuracy. It is recognized that this agar dilution technique will not be practical for use in most hospital laboratories, particularly those in small hospitals where fewer isolates are tested simultaneously. The current study describes a simple broth-disk elution susceptibility test method based upon use of Schaedler broth, GasPak jar incubation, and Bauer-Kirby suscep-

tibility disks. Results by this method have been compared with those obtained by the tentative reference technique of the National Committee on Clinical Laboratory Standards.

Bacterial isolates. One hundred isolates of anaerobic bacteria were obtained for use in this study from the Microbial Pathology Laboratories of the Bexar County Hospitals and the Audie Murphy Memorial Veterans Administration Hospital. The following kinds and numbers (within parentheses) of isolates were identified by the Minitek System (BBL Microbiology Systems) in conjunction with gas-liquid chromatography: *Actinomyces israelii* (2), *Actinomyces naeslundii* (1), *Bacteroides fragilis* (27), *Bacteroides distasonis* (5), *Bacteroides ovatus* (1), *Bacteroides thetaiotaomicron* (13), *Bacteroides vulgatus* (1), *Bacteroides melaninogenicus* (2), *Bacteroides corrodens* (1), *Bacteroides* CDC group F1 (1), *Bacteroides* CDC group F2 (1), *Clostridium cadaveris* (1), *Clostridium chauvoei* (1), *Clostridium paraputrificum* (2), *Clostridium perfringens* (18), *Clostridium sordellii* (3), *Clostridium* sp. (2), *Fusobacterium nucleatum* (1), *Peptococcus* sp. (3), *Peptostreptococcus* sp. (5), *Propionibacterium* sp. (8), and *Veillonella alcalescens* (1).

Agar dilution susceptibility test. Agar dilution susceptibility tests were performed with the medium of Wilkins and Chalgren (7) which contained various concentrations of carbenicillin, chloramphenicol, clindamycin, penicillin, and tetracycline. Inocula were prepared by overnight growth of isolates in Schaedler broth supplemented with 0.5 μ g of menadione per ml, adjustment to the 0.5 MacFarland opacity standard, and application of the suspension to surfaces of antibiotic-containing plates with a

Steers replicator. Plates were then incubated for 48 h at 35°C in an anaerobic environment (GasPak jars). The minimal inhibitory concentration (MIC) was interpreted as the lowest concentration of antibiotic which either entirely prevented growth on the agar surface or inhibited growth of more than three distinct colonies.

Broth-disk elution susceptibility test.

The Wilkins-Thiel disk elution method (9) was modified by the use of tubes containing 4 ml of Schaedler broth with the addition of 0.5 µg of menadione per ml in screw-cap tubes (16 by 125 mm). The appropriate number of antibiotic-containing disks (BBL) were added aseptically to each tube as indicated in Table 1. The drugs included in this study were carbenicillin, chloramphenicol, clindamycin, penicillin, and tetracycline. Each tube plus a control tube without antibiotic was inoculated with 1 drop (from a Pasteur pipette) of an undiluted, overnight Schaedler broth culture of each test organism. Caps were left loose, and the tubes were incubated at 35°C for 24 h in a GasPak jar. Susceptibility to an antibiotic was indicated by the absence of turbidity or by only a very slight haze after incubation. Resistance to an antibiotic could be seen by the production of turbidity resembling that of the positive control tube.

A summary of the results comparing agar dilution and broth-disk elution susceptibility testing is shown in Table 2. Results between the two techniques corresponded best with chloramphenicol, followed closely by penicillin, carbenicillin, and clindamycin. An overall agreement of 97.5% between the two techniques was observed with these four antibiotics. Conversely, many disagreements could be seen with tetracycline testing, which showed only a 68% agreement. Overall results in every instance indicated susceptibility with the broth-disk elution method and resistance with the agar dilution method. Specifically, with carbenicillin, discrepancies were noted with one isolate of *B. fragilis* and three isolates of *B. thetaiotaomicron*; with clindamycin, discrepancies were encountered with one isolate of *Peptococcus* sp., one isolate

TABLE 2. Summary of results comparing agar dilution and broth-disk elution methods

Antibiotic	No. of tests	No. that agree	No. that disagree
Carbenicillin	100	96	4
Chloramphenicol	100	100	0
Clindamycin	100	96	4
Penicillin	100	98	2
Tetracycline	100	68	32

of *C. perfringens*, one isolate of *B. thetaiotaomicron*, and one isolate of *B. distasonis*. Two discrepancies involving false disk susceptibility to penicillin were encountered with *B. fragilis*. In 7 of these 10 instances, the MIC for the isolate was very close to the disk elution concentration. False susceptibility with tetracycline disk elution tests occurred with 32 isolates, including 8 of *B. fragilis*, 10 of other *Bacteroides* spp., 6 of *C. perfringens*, 1 of *Clostridium* sp., 2 of *Peptococcus* sp., and 5 of *Peptostreptococcus* sp. In all cases with tetracycline, MICs were at least twice the disk elution test concentration.

In an effort to resolve the numerous disagreements seen with tetracycline, a subset of 20 isolates was retested by the same methodology but with incubation of all tests for 48 h instead of for 24 h. This extended incubation period resulted in agreement between tetracycline MICs and disk elution results for all isolates; however, two instances of false resistance by the disk elution technique occurred with penicillin and chloramphenicol.

The declining antibiotic susceptibilities of anaerobes (3, 5) emphasize the need for development of methods which allow convenient testing of small numbers of clinical isolates in hospital laboratories. The method described here allows reliable testing to be performed on one or more isolates simultaneously with commercially available media and reagents. We and others (4) have found that use of Schaedler broth yields much sharper endpoints than other comparable media for broth dilution testing. Moreover, Schaedler broth supplemented with menadione provides a satisfactory growth medium for all but the most fastidious anaerobic isolates (4).

The results of this study with an overnight broth-disk elution method compared favorably with the reference agar dilution method proposed to the National Committee on Clinical Laboratory Standards with four of five antibiotics tested; the exception was with tetracycline. An extended 48-h incubation period seemed essential for correct interpretation of tetracycline disk elution results. Similar discordance with tetracycline susceptibility testing was observed by Rosenblatt et al. (4). However, a 48-h incu-

TABLE 1. Antibiotic concentrations for broth-disk elution method

Antibiotic	Disk content	No. of disks per tube	Final concn (per ml)
Carbenicillin	100 µg	2	50 µg
Chloramphenicol	30 µg	1	7.5 µg
Clindamycin	2 µg	3	1.5 µg
Penicillin	10 U	1	2.5 U
Tetracycline	30 µg	1	7.5 µg

bation did not eliminate tetracycline discrepancies in their study. In view of these results and current trends in antimicrobial therapy for anaerobic infections, it may be superfluous to test for susceptibility to tetracycline initially.

Of the 10 discrepancies noted with antibiotics other than tetracycline, 7 can be readily explained. In all four discrepancies with carbenicillin, isolates appeared susceptible at 50 $\mu\text{g}/\text{ml}$ in the disk elution method, whereas they were shown to have an MIC of 64 $\mu\text{g}/\text{ml}$ by the agar dilution technique. In three of four instances, the MIC for clindamycin was 2 $\mu\text{g}/\text{ml}$, whereas the disk elution method yielded a susceptible result at a concentration of 1.5 $\mu\text{g}/\text{ml}$. Therefore, agreement between these disk elution and agar dilution results fell easily within the acceptable twofold dilutional error of the MIC values. In only three instances (two with penicillin and one with clindamycin) were the MICs sufficiently high to justify calling the results between agar dilution and broth elution methods a true disagreement. Thus, only 3 of 400 tests indicated disagreement, yielding an overall correlation of >99% between the two methods.

We have found the broth-disk elution method described here to be a practical, simple procedure during routine use by our two hospital clinical laboratories for more than 2 years. The advantages of this method are a 24-h inoculum preparation, a 24-h test incubation, commercially prepared inoculum and test media, and use of GasPak jars for provision of an anaerobic environment. This method provides flexibility for hospital laboratories to test various numbers of clinically significant anaerobic isolates. The ability to easily choose test concentrations by

altering the volume of test medium or numbers of disks added provides additional flexibility and allows more than one clinically relevant concentration of an antimicrobial agent to be tested simultaneously.

LITERATURE CITED

1. Dornbusch, K., C. E. Nord, and B. Olsson. 1975. Antibiotic susceptibility testing of anaerobic bacteria by the standardized disc diffusion method with special reference to *Bacteroides fragilis*. *Scand. J. Infect. Dis.* 7: 59-66.
2. Martin, W. J., M. Gardner, and J. A. Washington II. 1972. In vitro antimicrobial susceptibility of anaerobic bacteria isolated from clinical specimens. *Antimicrob. Agents Chemother.* 1:148-158.
3. Murray, P. R., and J. E. Rosenblatt. 1977. Penicillin resistance and penicillinase production in clinical isolates of *Bacteroides melaninogenicus*. *Antimicrob. Agents Chemother.* 11:605-608.
4. Rosenblatt, J. E., P. R. Murray, A. C. Sonnenwirth, and J. L. Joyce. 1979. Comparison of anaerobic susceptibility results obtained by different methods. *Antimicrob. Agents Chemother.* 15:351-355.
5. Salaki, J. S., R. Black, F. P. Tally, and J. W. Kislak. 1976. *Bacteroides fragilis* resistant to the administration of clindamycin. *Am. J. Med.* 60:83-88.
6. Stalons, D. R., and C. Thornsberry. 1975. Broth-dilution method for determining the antibiotic susceptibility of anaerobic bacteria. *Antimicrob. Agents Chemother.* 7:15-21.
7. Sutter, V. L., A. L. Barry, T. D. Wilkins, and R. J. Zabransky. 1979. Collaborative evaluation of a proposed reference dilution method of susceptibility testing of anaerobic bacteria. *Antimicrob. Agents Chemother.* 16:495-502.
8. Sutter, V. L., Y. Y. Kwok, and S. M. Finegold. 1972. Standardized antimicrobial disc susceptibility testing of anaerobic bacteria. I. Susceptibility of *Bacteroides fragilis* to tetracycline. *Appl. Microbiol.* 23:268-275.
9. Wilkins, T. D., and T. Thiel. 1973. Modified broth-disk method for testing the antibiotic susceptibility of anaerobic bacteria. *Antimicrob. Agents Chemother.* 3: 350-356.