Susceptibility Testing of Anaerobic Bacteria with 100-μg Carbenicillin Disks

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A total of 245 strains of anaerobic bacteria were examined for their susceptibility to carbenicillin by the disk test method and by minimum inhibitory concentration (MIC) determinations. Standard-curve studies with a strain of Bacteroides fragilis subsp. fragilis that was minimally susceptible to carbenicillin and Escherichia coli (ATCC 25922) demonstrated that a disk containing 100 μg of carbenicillin was suitable for testing susceptibility of anaerobes to carbenicillin. Thus, the diameter of zones around the 100-μg carbenicillin disks and MIC values were determined under the following test conditions: Mueller-Hinton agar supplemented with sheep blood, vitamin K₁, and hemin; an incubation temperature of 35 C; and an atmosphere of 80% N₂, 10% H₂, and 10% CO₂. The strains were separated into two populations by correlating zone diameters and geometric mean MICs. The disk test more clearly separated the resistant and susceptible populations and was more reproducible than the MIC test. Thus, a statistical analysis based on the distribution of zone diameters of susceptible and resistant strains was used to derive an interpretive scheme for anaerobic bacteria tested with 100-μg carbenicillin disks. The following interpretive scheme is recommended for testing anaerobes with 100-μg disks of carbenicillin: resistant, 8 mm or less; indeterminate, 9 to 12 mm; and susceptible, 13 mm or greater.

Until the last few years, anaerobic organisms were rarely implicated as causative agents of human infection, although Bacteroides species had been accepted as a potential cause of infection since 1897 (39). Recent advances in culturing and identifying nonsporulating anaerobes have led to increased awareness of the role of these organisms in the disease process, and it is now well recognized that anaerobic bacteria are involved in a significant number of human infections (9, 10, 13, 19, 27, 28, 42).

This recognition of the role of anaerobes in the disease process has stimulated interest in the susceptibility of these organisms to antimicrobial agents. Recently, Nobles (25) reported a high mortality rate among patients with Bacteroides fragilis septicemia who received no effective antibiotic therapy, yet Lambe et al. (18) found a high survival rate among patients infected with B. fragilis subsp. fragilis if the patients received proper antibiotic therapy. B. fragilis has become increasingly resistant to certain antibiotics such as tetracycline (20, 38). Only chloramphenicol has been consistently effective in treating infections, including septicemias, caused by anaerobic bacteria (4).

Clindamycin has also been recommended for treatment of B. fragilis infections (11), but both chloramphenicol and clindamycin produce adverse effects that limit their usefulness (6, 8, 12, 30, 31, 33, 40). Carbenicillin is relatively nontoxic, and in vitro data (3, 5, 16, 25, 34, 41) indicate that most anaerobes are susceptible to this agent. Furthermore, one in vivo study has demonstrated a high rate of success in treating anaerobic infections with carbenicillin (23).

The purpose of the present study was to derive an interpretive scheme by the disk test method for evaluating the susceptibility of rapidly growing anaerobic bacteria to carbenicillin. The standard method that we proposed previously for antibiotic susceptibility testing of anaerobes (28) was used in this study. We developed this method initially because it most closely duplicated the Bauer-Kirby (1) antibiotic susceptibility test for rapidly growing aerobes. Population characteristics were analyzed to determine the interpretive scheme for the 100-μg carbenicillin disk test.

MATERIALS AND METHODS

Bacterial strains. There were 245 strains of anaerobic bacteria used in this study. Of these, 141 strains were B. fragilis, and the subspecies of B.
fragilis represented included 67 B. fragilis fragilis, 35 B. fragilis thetaiotaomicron, 27 B. fragilis distasonis, and 12 B. fragilis vulgatus. Of the 245 strains tested, 86 were anaerobic cocci, which included Peptococcus magnus (seven strains), Peptostreptococcus anaerobius (27 strains), Peptococcus prevoti (17 strains), Peptococcus asaccharolyticus (23 strains), Streptococcus constellatus (three strains), Streptococcus intermedius (eight strains), and Streptococcus morbillorum (one strain). Other anaerobes included Bacteroides group FS (proteolytic-saccharolytic) (one strain), Bacteroides oralis (one strain), Bacteroides ruminicola (one strain), Fusobacterium necrophorum (two strains), Fusobacterium nucleatum (one strain), Fusobacterium russii (one strain), Fusobacterium varium (two strains), Actinomycetales israelii (one strain), Actinomyces naeslundii (one strain), Bifidobacterium adolescentis (two strains), Eubacterium lentum (two strains), Eubacterium limosum (one strain), and Lactobacillus acidophilus (one strain).

An aerobe. All work was performed in an anaerobe chamber (Coy Manufacturing Co., Ann Arbor, Mich.) with an atmosphere of 80% N₂, 10% H₂, and 10% CO₂ (Matheson Gas Products, Atlanta, Ga.).

Media. Stock cultures of the test organisms were maintained in brain heart infusion broth (Difco) supplemented with vitamin K₃ (0.0005%) (Sigma) and hemin (0.0005%) (K & K Laboratories, Plainview, N.Y.). The inoculum was diluted with Mueller-Hinton broth (MHB) (Difco) containing vitamin K₃ and hemin (VKH). Zone size determinations and minimum inhibitory concentration (MIC) determinations were made on Mueller-Hinton agar (MHA) (Difco) supplemented with VKH and 5% defibrinated sheep blood (Nolan Biological Laboratories, Inc., Tucker, Ga.). Purity plates consisted of brain heart infusion agar supplemented with VKH and 5% defibrinated sheep blood. All broth media were prepared outside the anaerobe chamber and placed in the anaerobe chamber while warm. The agar plates were prepared outside the anaerobe chamber, allowed to solidify, and then placed inside the anaerobe chamber; the plates were used within a few hours after preparation.

Antibiotics. Carbenicillin powder (Pfizer, Inc., New York, N.Y.) was dissolved in sterile distilled water. Antibiotic disks (Pfizer, Inc., New York, N.Y.) had a content of 100 μg of carbenicillin.

Antibiotic disk method. The disk method described by Overman et al. (38) was used in this study. MHA plates supplemented with blood and VKH were prepared on the day of use. The medium was adjusted to pH 7.4 before autoclaving; the pH was rechecked after autoclaving and adjusted to pH 7.4 if necessary. After autoclaving, 30 ml of the medium was poured into sterile petri dishes (100 by 15 mm). An inoculum was prepared by growing a culture for 24 h in brain heart infusion broth-VKH at 35 C. The culture was adjusted to one-half the turbidity of a no. 1 McFarland nephelometer standard using MHB-VKH as the diluent. Sterile cotton swabs were inserted into the diluted culture; the swabs were pressed against the tube wall to remove excess inoculum, and the MHA plates were then swabbed evenly in three directions. After the surface of the medium was allowed to dry for 3 to 5 min, disks were placed on the surface of the medium. The plates were inverted and incubated anaerobically at 35 C for 24 h. A vernier caliper was used to measure the zone diameter, which included the diameter of the disk. The zone diameter was recorded to the nearest millimeter. For some cultures, an inner zone of diminished growth was observed; the zone diameter was measured to the inner edge of this zone. Only those strains which gave clearly readable zones in 24 h were included in this study. The reproducibility of the test was determined by performing the test on three different days.

MIC determinations. One milliliter of each twofold antibiotic dilution ranging from 4,096 to 0.062 μg/ml was placed in each of two sterile petri dishes. MHA supplemented with VKH and blood (19 ml) was pipetted into each dish. After the medium had solidified, the plates were placed in an anaerobe chamber.

The inocula were prepared by growing the cultures in brain heart infusion broth supplemented with VKH for 22 h at 35 C. The cultures were adjusted to one-half the turbidity of a no. 1 McFarland nephelometer standard by using MHB-VKH as the diluent. A 1:10 dilution was prepared by using an Oxford automatic pipette to deliver 1 ml of the diluted culture to 9 ml of MHB-VKH. A Steers replicating device (32) was used to transfer the diluted cultures to the surface of the MHA plates.

After the inocula had dried, the plates were inverted and incubated at 35 C for 22 h. The MIC was recorded as the lowest concentration of antibiotic that completely inhibited the growth of each organism as viewed macroscopically.

Standard curve. Antibiotic disks were prepared in our laboratory according to the following protocol. Gelman filter sheets (Scientific Products, Atlanta, Ga.) were punched with a two-hole punch (Punchodes) to yield disks with a diameter of 6 mm. The disks were placed in glass petri plates (15 by 150 mm) and autoclaved for 15 min. The disks were allowed to dry overnight at room temperature. Sterile disks were then placed on sterile nylon screens in petri dishes (15 by 90 mm). Various concentrations (in 20-μl amounts) of the antibiotic were applied to each disk, resulting in 11 different disk contents ranging from 1.5 to 800 μg. All disks were lyophilized (Virtila) overnight. All disks were used within 1 week of preparation.

Each disk content was tested seven times against a strain of Escherichia coli (ATCC 25922) and a carbenicillin-susceptible strain of B. fragilis subsp. fragilis; this B. fragilis strain produced a zone diameter close to the range ultimately defined as indeterminate. The antibiotic disk method described above was modified so that a large glass plate (38 by 26.7 cm) containing the medium was flooded with the appropriately diluted culture. The inoculum was
evenly distributed over the surface of the medium by gently rocking the plate. Excess fluid was removed with a Pasteur pipette. After the surface of the medium had dried, seven disks of each content (77 total disks) were placed on the surface of the medium. One plate containing E. coli and one plate containing B. fragilis were incubated anaerobically at 35°C for 24 h. Zone diameters around each disk were measured with a vernier caliper, and the readings from the replicated studies were averaged for each organism to give a value that could be used in plotting a standard curve.

RESULTS

The standard curves of carbenicillin for B. fragilis subsp. fragilis and E. coli are shown in Fig. 1. The results were plotted with the zone diameters on the abscissa and disk content on a log scale on the ordinate. This standard curve was useful in determining the appropriate disk content to be used for antibiotic susceptibility testing (7, 21, 22). With this standard curve, it was possible to assess two criteria required for the selection of the proper disk content: (i) the effect of antibiotic deterioration on the size of the zone diameter and (ii) the disk content that produced a zone size that was not too large for use in a single petri plate in conjunction with other antibiotic disks. There was a linear relationship from 25 to 400 μg for both organisms tested. The portion of the line between 25 and 400 μg was examined for linearity, because a disk content of 50 μg or less was insufficient to separate resistant and susceptible populations; thus, 25 μg was chosen as the lower limit of the range. Since the carbenicillin disk will be used in conjunction with other antimicrobial disks, the disk content ultimately chosen should not produce zone diameters that are exceedingly large. Thus, disks with a content greater than 400 μg would not be practical. Thus, within that range, for B. fragilis, a twofold reduction in disk content resulted in about a 4-mm reduction in zone size. One hundred-microgram disks, which fell on this linear segment and were commercially available, were then selected for the disk test method.

The correlation between MIC values and zone diameters around 100-μg carbenicillin disks for 245 anaerobic bacteria is shown in Fig. 2. The results suggested the existence of two distinct populations of organisms with respect to the coordinates and zone diameters for MICs. The separation of the two populations for carbenicillin was not as great as for tetracycline against the five subspecies of B. fragilis (26) or for B. fragilis (35). When the zone diameters and MICs for 141 strains of four subspecies of B. fragilis were correlated separately, the phenomenon of bimodality was also observed. Excluding B. fragilis, all of the other anaerobic bacteria tested appeared to be members of the susceptible population, except for two strains of P. anaerobius. The two strains of P. anaerobius were susceptible by zone size determination but had MICs greater than the achievable serum level for carbenicillin. Because of the nonrandom occurrence of the bimodal distribution, the interpretation of the disk test was based on

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**Fig. 1.** Standard curve of carbenicillin for E. coli (ATCC 25922) and B. fragilis subsp. fragilis (Emory University Hospital no. N-1216-72A) (the coefficient of correlation \( r \) for the B. fragilis strain was 0.9187; for the E. coli strain, \( r \) was 0.8786).

**Fig. 2.** Correlation of MIC values and zone diameters of anaerobic bacteria around 100-μg carbenicillin disks.
zone size characteristics of the two populations rather than by regression line analysis.

Both MIC and zone size data were used to classify the susceptibility of strains. The distribution of zone diameters for the 245 anaerobes tested is shown in Fig. 3. The bimodality of the population distribution was more apparent in the distribution of the zone diameters (Fig. 3) than in the distribution of the MICs (Fig. 4): 4 mm separated the two populations by the disk test, whereas there was no clear separation between the two populations by the MIC test alone.

An interpretive scheme was established by calculating the probability of outcomes in the area between the two zone sizes (Table 1). Calculations were then made to determine the probability that a given zone was produced by a strain of either the susceptible or resistant population. Thus, only 61 out of 10,000 susceptible strains would produce a zone of 8 mm, whereas 17 out of 100 resistant strains would produce a zone this size. Based on these data, there was a relative chance of 96% that a strain producing a zone diameter of 8 mm or less would be resistant. In contrast, there was a relative chance of 95% that a strain producing a zone diameter of 13 mm or greater would belong to the susceptible population and only a 5% chance that such a strain would belong to the resistant population. Since we wished to make correct decisions at least 95% of the time, those strains producing zones of 9 to 12 mm were termed "indeterminate." This type of statistical analysis was also calculated separately on the 141 strains of B. fragilis and the 86 strains of the anaerobic cocci. It was found that the susceptibility of none of the strains of B. fragilis and only one coccus would have been misclassified by using the interpretive scheme for all the anaerobes tested, rather than the interpretive scheme de-

![Fig. 3. Distribution of the arithmetic mean of zone diameters with 245 strains of anaerobic bacteria tested with carbenicillin.](image)

![Fig. 4. Distribution of MIC values of carbenicillin for 245 strains of anaerobic bacteria.](image)

### Table 1. Probabilities of obtaining zone diameters from strains in resistant and susceptible populations

<table>
<thead>
<tr>
<th>Recorded zone size (mm)</th>
<th>Actual range of measurements (mm)</th>
<th>Probability that zone was produced by a strain of either population</th>
<th>Relative chance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Susceptible</td>
<td>Resistant</td>
</tr>
<tr>
<td>8</td>
<td>7.5–8.4</td>
<td>0.00610</td>
<td>0.16828</td>
</tr>
<tr>
<td>9</td>
<td>8.5–9.4</td>
<td>0.00715</td>
<td>0.10243</td>
</tr>
<tr>
<td>10</td>
<td>9.5–10.4</td>
<td>0.00832</td>
<td>0.04659</td>
</tr>
<tr>
<td>11</td>
<td>10.5–11.4</td>
<td>0.00946</td>
<td>0.01462</td>
</tr>
<tr>
<td>12</td>
<td>11.5–12.4</td>
<td>0.01084</td>
<td>0.00343</td>
</tr>
<tr>
<td>13</td>
<td>12.5–13.4</td>
<td>0.01236</td>
<td>0.00060</td>
</tr>
</tbody>
</table>
developed for each of the two groups. Thus, by using the test conditions outlined, the following interpretive scheme is recommended for carbenicillin against all anaerobes that grow sufficiently to produce a zone of inhibition in 24 h: resistant, 8 mm or less; indeterminate, 9 to 12 mm; and susceptible, 13 mm or greater.

For the MIC and zone size methods, a comparison of the reproducibility of tests performed at different times is shown in Table 2. Zone size and MIC determinations were made on 3 separate days. A table of random numbers was used to select 60 strains in the susceptible population for calculation of the coefficient of variation. The reproducibility of the two testing methods was studied by examining the difference in the arithmetic mean of the zone diameters and MICs of the strain and each of the three separate results contributing to these averages. It was not possible to determine the reproducibility of the strains in the resistant population because most strains in this population had a mean zone diameter of 6 mm.

For those strains in the susceptible population, the MIC test was approximately 10-fold more variable than the disk test. Thus, as noted by others (2, 26), the disk test separates populations much more clearly and is more reproducible than determination of MICs. Therefore, use of the disk test reduces the chance of misclassifying the susceptibility of the strains.

**DISCUSSION**

Because of the potential toxicity of chloramphenicol and clindamycin, an effective, alternative antibiotic is needed for treating infections caused by anaerobic bacteria. A member of the relatively safe penicillin group of antibiotics is carbenicillin. A number of workers have demonstrated the in vitro susceptibility of anaerobes to carbenicillin (3, 5, 16, 25, 34, 41). Furthermore, Meny et al. (23) demonstrated the efficacy of carbenicillin in the treatment of infections caused by anaerobic bacteria.

Although some workers do not recommend routine susceptibility testing of anaerobic bacteria, the changing susceptibility pattern of these organisms now dictates that clinical laboratories should routinely perform antibiotic susceptibility testing of anaerobes (26, 35). To accomplish this objective, guidelines for a standard method for susceptibility testing of anaerobes were proposed by Overman et al. (26). Differences in interpretive schemes exist among various workers (17, 26, 29, 36, 41), but these differences result from the use of different inoculum sizes, media, atmospheric conditions and statistical methods for analyzing data.

Many workers have derived interpretive schemes through regression line analysis. In reports of antimicrobial susceptibility testing of anaerobes by Kwok et al. (17), Sapico et al. (29), Sutter et al. (34, 35, 36) and Wilkins et al. (41), considerable variation about the regression line was shown. As noted previously (26), regression line analysis is valid only if the variables are normally distributed along the regression line. Furthermore, in linear regression, emphasis is placed on the MIC test; as we and others (2, 26) have shown, the MIC test is not only less reproducible than the disk test but has been successful in separating resistant and susceptible populations even when geometric mean values have been used. Statistical difficulties inherent in the use of linear regression for relating MICs to zone diameters have been noted by others (24). We believe that the use of the regression line to develop interpretive schemes in certain studies (17, 29, 34, 35, 36, 41) can be questioned for the reasons cited.

Previously, Bennett et al. (2) and others (26) recommended the use of population analysis to determine interpretive schemes for an antibiotic if two criteria are met: bimodality must occur, and the two populations of strains must be separated by an MIC value that is comparable to a clinically obtainable blood level of the antibiotic in question. Our strains met these two criteria: bimodality occurred, and the two populations were separated by an MIC of 125 \( \mu \)g/ml, which is accepted as a clinically obtainable blood level of carbenicillin. Therefore, we used the population analysis to derive the interpretive scheme for the anaerobic strains and carbenicillin.

In clinical laboratories, 50-\( \mu \)g disks were originally employed in performing the Bauer-Kirby susceptibility testing of aerobes with carbenicillin. However, it was noted by some workers (22) that strains which were susceptible by MIC criteria were resistant by zone diameter measurements around the 50-\( \mu \)g disks. This discrepancy was probably due to the use of disks with too low a content of antibiotic. Matsen et al. (22) performed standard-curve studies with aerobic bacteria and demonstrated that there was no theoretical reason for limiting the

**Table 2. Reproducibility of antibiotic susceptibility tests**

<table>
<thead>
<tr>
<th>Population susceptible by:</th>
<th>Standard deviation</th>
<th>Arithmetic avg</th>
<th>Coefficient of variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disk test</td>
<td>2.0</td>
<td>27.3</td>
<td>7.3</td>
</tr>
<tr>
<td>MIC</td>
<td>33.3</td>
<td>43.5</td>
<td>76.6</td>
</tr>
</tbody>
</table>
content of carbenicillin disks to 50 μg. One hundred-microgram disks were then made available by the manufacturer.

Earlier work with 50-μg carbenicillin disks and anaerobes in our laboratory (unpublished data) and the work of others (41) demonstrated the inability of disks of this content to adequately separate resistant and susceptible populations. As was demonstrated by Matsen et al. (22) with aerobes and carbenicillin, a linear relationship between zone diameters and disk content was noted in the range of 10 to 150 μg; our work with anaerobes and carbenicillin in standard-curve studies showed a linear relationship in the range of 25 to 400 μg. A disk content that falls on or above the linear portion of the standard curve must be used in an attempt to minimize the effect of deterioration of the antibiotic in the disks on zone size reduction and interpretive errors.

There are several criteria that a disk content must satisfy before it is selected as the optimal disk mass for use in antibiotic susceptibility testing. First, standard-curve studies should be performed to determine the effect of antibiotic deterioration on zone diameters as well as to determine the absolute zone size produced by minimally susceptible strains. Once a disk content is considered it should be studied for its ability to separate resistant and susceptible populations. This is followed by the development of interpretive schemes. Since manufacturers are permitted tolerances of 60 to 140% of stated contents, it is thus important to again review the standard curve to ensure that a 40% decrease in content (100 to 60%) would not cause minimally susceptible strains to be erroneously labeled resistant. One hundred-microgram disks satisfied this requirement. Furthermore, the selected disk mass must give reproducible results. Only a variation of 7.3% in zones was observed with the 100-μg carbenicillin disks in our studies. For these and other reasons, we recommend the use of 100-μg carbenicillin disks for antibiotic susceptibility testing of anaerobes.

Under test conditions different from ours and with the use of 50-μg carbenicillin disks and regression line analyses, Wilkins et al. (41) recommended that anaerobic organisms with zone sizes greater than 18 mm be considered susceptible. With our test conditions and 100-μg carbenicillin disks, those strains producing zone diameters of 13 mm or greater were considered susceptible.

Between 1 January 1975 and 31 December 1975, the clinical laboratory of Emory University Hospital isolated and tested 411 anaerobic strains with 100-μg carbenicillin disks. Of these 411 strains, 91% (375 of 411 strains) were susceptible to carbenicillin using the test method that we proposed. A total of 257 of the 411 strains isolated were susceptible to carbenicillin; these strains included anaerobic cocci, Bifidobacterium species, Eubacterium species, Fusobacterium species, Lactobacillus species, and Bacteroides species, excluding B. fragilis. A total of 154 strains of four subspecies of B. fragilis were isolated during this time period. Of these strains, 77% (118 strains) were susceptible, 10% (15 strains) were indeterminate, and 13% (21 strains) were resistant. By MIC criteria, Tally et al. (37) reported that 60% of 33 strains of B. fragilis were susceptible to carbenicillin at achievable blood levels. A total of 129 anaerobes other than B. fragilis were tested by Tally et al. (37); 99% were susceptible to carbenicillin at 128 μg/ml.

The CO₂ content in the atmosphere used for antibiotic disk susceptibility testing is important since CO₂ content can alter zone diameters around antimicrobial disks (15). Earlier work in our laboratory (26) demonstrated less variation in zone size with use of 10% CO₂ in the atmosphere than with higher concentrations of CO₂. Therefore, with changing antimicrobial susceptibility patterns of anaerobes and with the availability of interpretive schemes that have been derived for various antimicrobial agents, we propose that clinical laboratories should routinely perform anaerobic antimicrobial susceptibility testing for clinically significant anaerobic isolates with appropriate, clinically useful antibiotics.

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LITERATURE CITED