

Susceptibility of Anaerobic Bacteria to Carbenicillin

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One hundred and seventy-one strains of anaerobes were tested for susceptibility to carbenicillin by using agar dilution, broth dilution, and two disk diffusion methods. The minimal inhibitory concentration (MIC) for 67% of 51 strains of *Bacteroides fragilis*, 7 of 9 strains of *Bacteroides melaninogenicus*, and all of 8 strains of *Eubacterium* was 100 μg or less per ml. The MICs of the remaining anaerobes were 50 μg or less per ml. The broth dilution results were felt to be the most accurate of the four methods utilized.

The clinical record of carbenicillin has established its value as an antibiotic for use in certain infections due to bacteria routinely resistant to other penicillins and to the cephalosporins. Susceptible organisms include *Pseudomonas aeruginosa*, *Enterobacter* sp., *Proteus vulgaris*, *P. morgani*, *P. rettgeri*, *Providencia*, and *Escherichia coli*. Anaerobic organisms are being recognized with increasing frequency as etiologic agents in clinical infections. Little has been reported with respect to the action of carbenicillin against this group of bacterial pathogens, whereas it is known, for example, that most *Bacteroides fragilis* are resistant to other penicillins and the cephalosporins.

This study was carried out, therefore, to determine the susceptibility of anaerobic organisms to carbenicillin.

MATERIALS AND METHODS

Test organisms. One hundred and seventy-one strains of anaerobic bacteria, comprising nine genera, were tested. All organisms were isolated from clinical specimens in the University of Minnesota Hospital's Diagnostic Microbiology Laboratory and were identified according to the methods of Holdeman and Moore (2). Prereduced media (Scott Laboratories) and a VPI anaerobic apparatus (Bellco) were used for culturing.

Methods of susceptibility testing. Two different dilution methods and two different disk diffusion methods were used because no standard method of susceptibility testing for anaerobes has been defined.

For the agar dilution method (4), plates were prepared with brain-heart infusion (BHI) agar, 5% sheep blood, and twofold dilutions of carbenicillin at concentrations ranging from 400 to 0.2 $\mu\text{g}/\text{ml}$. The plates were kept at room temperature and used the day after preparation. For the inoculum an overnight culture in prereduced chopped meat glucose (CMG) was diluted 1:100 in prereduced BHI-S broth (BHI

supplemented with hemin and vitamin K). The diluted cultures were inoculated on the plates with the replicating device of Steers et al. (6). A plate without antibiotic was included as a growth control. Plates were incubated at 35 C in a vented GasPak jar (BBL), evacuated, and filled with 90% CO_2 :10% H_2 three times. The plates were read at 24 and 48 h. The minimal inhibitory concentration (MIC) was determined as the lowest concentration of carbenicillin showing no growth on the plate. Single-colony growth or very hazy growth was ignored in reading these plates as per the international collaborative study (1) recommendations for reading agar dilution susceptibility tests.

For the broth dilution test (8) twofold dilutions of carbenicillin (400 to 0.2 $\mu\text{g}/\text{ml}$) were made by adding 0.01 ml of the appropriate carbenicillin concentration to 5 ml of prereduced BHI-S broth by using an Eppendorf pipette and a VPI anaerobic apparatus. Tubes were stored at room temperature and used the day after addition of the antibiotic. One drop of an undiluted overnight culture in CMG was added to each tube and to a BHI-S without antibiotic as growth control. Tubes were incubated for 18 to 24 h at 35 C. The MIC was recorded as the lowest concentration of carbenicillin showing no turbidity.

One method of disk diffusion testing was that of Wilkins et al. (8). Ten milliliters of prereduced BHI agar supplemented with hemin and vitamin K (BHIA-S) were melted and cooled to 50 C. A 1.5-ml amount of an undiluted overnight culture in CMG was added to the cooled agar; the agar was mixed and poured into a sterile Petri dish (100 by 15 mm). Two pour plates were prepared for each organism. After hardening, two 50- μg carbenicillin disks were placed on one plate and two 100- μg carbenicillin disks were placed on the other plate. The plates were incubated in a vented GasPak jar at 35 C for 18 to 24 h; after incubation, inhibition zones were measured with a caliper.

The other disk diffusion method was a modification of that of Sutter et al. (7). Brucella agar (Pfizer) was prepared according to manufacturer's directions.

After the medium was autoclaved and cooled, 5 ml of sheep blood and 1 ml of hemin-vitamin K (Scott Laboratories) were added for each 100 ml of agar. Twenty-five milliliters of agar was poured into sterile petri dishes (100 by 15 mm). Plates were held at room temperature and used the day after preparation. For inoculation an overnight culture in CMG was diluted 1:10 in prereduced BHI-S. Two plates were swabbed with the dilution of each organism. Two 50- μ g carbenicillin disks were placed on one plate and two 100- μ g carbenicillin disks were placed on the other plate. Plates were incubated as above for 18 to 24 h; inhibition zones were measured with a caliper.

Tests by all four methods were performed on the same day by using the same CMG culture of each organism for the inoculum.

E. coli (ATCC 25922) was included in each day's tests as a control for all four methods.

RESULTS

The results of the broth dilution studies are shown in Table 1. Sixty-seven percent of 51 *Bacteroides fragilis* strains required 100 μ g or less of carbenicillin per ml for inhibition of growth. Strains of *Bacteroides melaninogenicus* were variable in their susceptibility, whereas the MIC for *B. oralis* and *B. ruminicola* was 25 μ g or less per ml. The MICs for the remaining anaerobes were 50 μ g or less per ml with the exception of one *Eubacterium* which had an MIC of 100 μ g/ml.

In most cases the results of the agar dilution test agreed with the broth dilution test or

showed only a one- or two-dilution difference; when there was a difference, the agar dilution method yielded higher MICs. Results with the agar dilution method were the same at both 24 and 48 h of incubation in almost every instance. In a few cases, with *Lactobacillus*, *Bifidobacterium*, and *B. oralis*, no growth appeared on the agar dilution control plate, so MICs could not be determined by that method.

Zone sizes versus MICs were plotted for both disk methods and both disk contents (Fig. 1-4). The correlation between MICs and zone sizes obtained by the surface disk diffusion method appeared to be better than that with the pour plate disk diffusion method. However, some strains of *B. melaninogenicus*, *B. oralis*, and *Fusobacterium* did not grow on the surface disk plates.

Overall, the best growth of organisms occurred in the broth dilution tests.

The MIC of *E. coli* (incubated anaerobically) was reproducible and ranged from 25 to 12.5 μ g/ml.

DISCUSSION

With the exception of the 17 strains of *B. fragilis* and 2 strains of *B. melaninogenicus*, all of the anaerobes tested were susceptible to concentrations of carbenicillin easily achieved in the blood. Because anaerobes commonly occur with facultative organisms in clinical specimens, if patients were being treated with

TABLE 1. Results of broth dilution susceptibility tests with carbenicillin

| Organism | No. of strains tested | MIC (μ g/ml) | | | | | | | | | | | |
|---------------------------------------|-----------------------|-------------------|-----|-----|----|----|------|-----|-----|-----|-----|-----|------------|
| | | ≥ 400 | 200 | 100 | 50 | 25 | 12.5 | 6.3 | 3.1 | 1.5 | 0.8 | 0.4 | ≤ 0.2 |
| <i>Bacteroides fragilis</i> | 51 | 10 | 7 | 20 | 9 | 4 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>Bacteroides melaninogenicus</i> | 9 | 0 | 2 | 1 | 0 | 0 | 2 | 1 | 0 | 0 | 1 | 2 | 0 |
| <i>Bacteroides oralis</i> | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 |
| <i>Bacteroides ruminicola</i> ... | 6 | 0 | 0 | 0 | 0 | 1 | 1 | 2 | 0 | 0 | 0 | 2 | 0 |
| <i>Fusobacterium</i> sp. | 4 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 |
| <i>Lactobacillus</i> sp. | 5 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 1 | 1 | 0 | 0 | 1 |
| <i>Eubacterium</i> sp. | 8 | 0 | 0 | 1 | 0 | 3 | 2 | 1 | 1 | 0 | 0 | 0 | 0 |
| <i>Bifidobacterium</i> sp. | 20 | 0 | 0 | 0 | 0 | 3 | 3 | 4 | 5 | 2 | 2 | 1 | 0 |
| <i>Peptococcus magnus</i> | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 4 | 4 | 1 |
| <i>Peptococcus prevotii</i> | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 | 1 | 1 | 3 |
| <i>Peptococcus asaccharolyticus</i> | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 7 |
| <i>Peptostreptococcus anaerobius</i> | 11 | 0 | 0 | 0 | 3 | 1 | 0 | 0 | 1 | 0 | 2 | 3 | 1 |
| <i>Peptostreptococcus intermedius</i> | 5 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 |
| <i>Veillonella</i> sp. | 9 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 1 | 4 | 1 | 0 | 0 |
| <i>Clostridium perfringens</i> ... | 10 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 6 | 2 | 0 |
| Total | 171 | 10 | 9 | 22 | 13 | 15 | 14 | 9 | 11 | 16 | 21 | 18 | 13 |

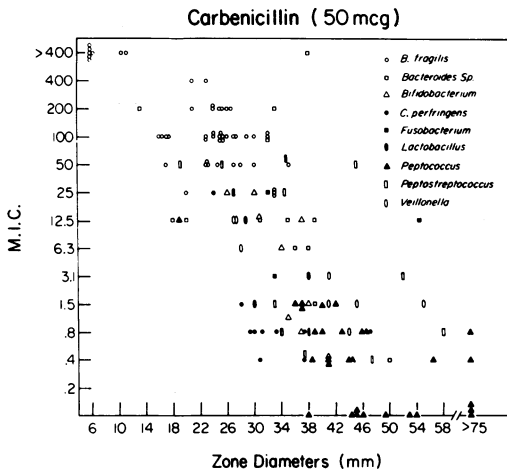


FIG. 1. Comparison of surface-swab disk diffusion and broth dilution susceptibility test results obtained with 50- μ g carbenicillin disks and eight genera of anaerobic bacteria.

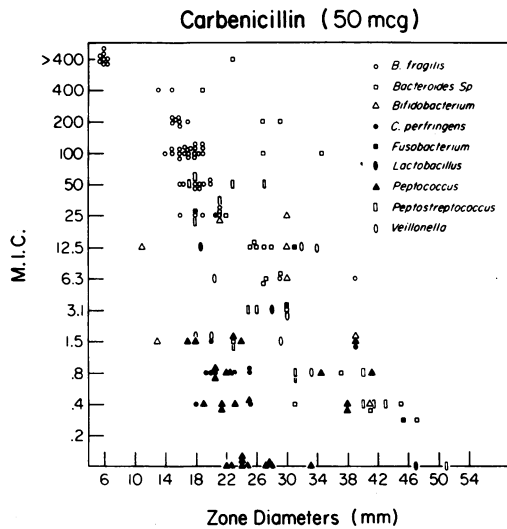


FIG. 2. Comparison of pour plate disk diffusion and broth dilution susceptibility test results obtained with 50- μ g carbenicillin disks and eight genera of anaerobic bacteria.

carbenicillin for the facultative organisms this antibiotic might be effective against those anaerobes other than some strains of *B. fragilis* and *B. melaninogenicus*. However, because serum levels of 100 to 200 μ g of carbenicillin per ml may be achieved by increasing the dosage, it is possible that carbenicillin might also be effective against the more resistant *B. fragilis* and *B. melaninogenicus*.

Our results disagree somewhat with those of

Kislak (3), who reported 90% of 40 strains of *B. fragilis* to be inhibited by 100 μ g or less of carbenicillin per ml. His agar dilution method was different from the one used in this study and used an inoculum of approximately 2×10^6 organisms; we did not do viable counts, but according to Wilkins et al. (8) an overnight culture in CMG broth usually yields $1.5^{-4} \times 10^9$ organisms/ml for an appreciable number of anaerobes. Thus, our inoculum was probably in the range of $1.5^{-4} \times 10^4$ organisms. With this smaller number of organisms, one might have expected the MICs to be lower than was the

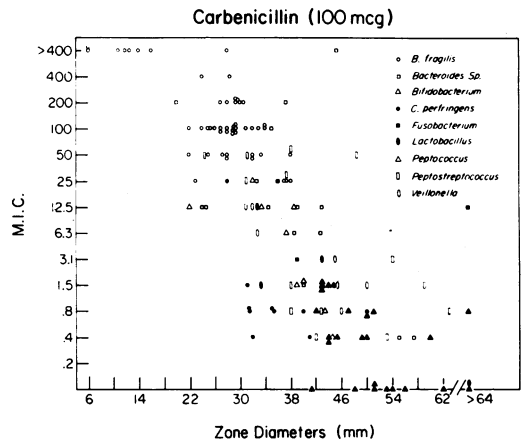


FIG. 3. Comparison of surface-swab disk diffusion and broth dilution susceptibility test results obtained with 100- μ g carbenicillin disks and eight genera of anaerobic bacteria.

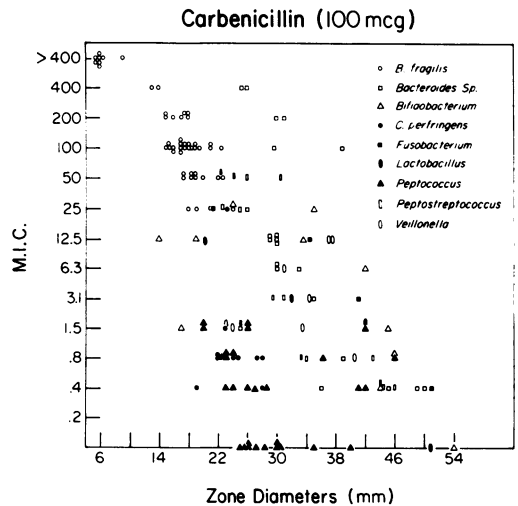


FIG. 4. Comparison of pour plate disk diffusion and broth dilution susceptibility test results obtained with 100- μ g carbenicillin disk and eight genera of anaerobic bacteria.

case. Apparently, our strains of *B. fragilis* are more resistant organisms than those tested by Kislak.

Because carbenicillin is less active at an acid pH, one might think that the atmosphere of 90% CO₂ and 10% H₂ used in our study might cause some acid inactivation of the antibiotic. However, the pH of the media after incubation was found to be 7.0.

Zone sizes were difficult to measure with both disk diffusion methods. There was often an inner zone of growth within the zone of inhibition in the pour plate method. In the surface disk diffusion method the zones were seldom clearly demarcated, and it was not easy to determine the actual zone size.

The best regression curve was obtained with the 50- μ g carbenicillin disk and the surface disk diffusion method, although even this curve would not be satisfactory for use in a disk diffusion method. Most strains of *Clostridium perfringens* were aberrant in their position on the curve, which was undoubtedly due to the rapid growth of this species, resulting in smaller zone sizes in relation to its MIC. Separate curves would have to be used for organisms with differing growth rates in order for the disk diffusion method to be accurate. This is in agreement with the work of Sutter et al. (7), who have shown that different zone sizes must be used to indicate susceptibility of different anaerobes to the various antibiotics.

These in vitro results would tend to indicate that carbenicillin, at the serum levels achieved by dosages usually employed in systemic infections, would be effective in inhibiting those anaerobes other than *B. fragilis* and *B.*

melaninogenicus. Whether in vitro results correlate with in vivo efficacy with respect to anaerobic infections can be determined only by clinical therapeutic trials. The known predisposition of some organisms to develop resistance to carbenicillin (5) would also have to be considered with respect to its possible effectiveness against anaerobes.

ACKNOWLEDGMENTS

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