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Disk Diffusion Susceptibility Testing of the *Bacteroides fragilis* Group

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The susceptibilities of 225 isolates of the *Bacteroides fragilis* group to six antibiotics were determined by a new disk diffusion test in Wilkins-Chalgren agar and by the standard agar dilution method. For disk diffusion, the bacteria were directly suspended in saline and immediately swabbed onto 15-cm agar plates. Disks of cefoxitin (30 μg), chloramphenicol (30 μg), clindamycin (2 μg), moxalactam (30 μg), imipenem (10 μg), and ticarcillin (75 μg) were applied, and the plates were incubated at 37°C in an anaerobic atmosphere. Zone sizes were measured at 24 h. The results of disk diffusion and agar dilution were compared by regression analysis by the method of least squares and by the error rate-bounded method. Zones were easily measured for 216 strains (96%). The correlation between the MICs and diameters of inhibition for cefoxitin, clindamycin, moxalactam, and ticarcillin was generally good. A correlation could not be established for chloramphenicol and imipenem, as there were too few resistant strains. With the recommended resistance breakpoints, the following susceptible and resistant zone diameter breakpoints could be established: cefoxitin, ≤19 and ≥21 mm; clindamycin, ≤14 and ≥18 mm; moxalactam, ≤21 and ≥25 mm; and ticarcillin, ≤15 and ≥16 mm. By applying these zone criteria, the percentage of false-susceptible strains was <1% and of false-resistant strains was <4% for the drugs tested.

The need to perform susceptibility testing on anaerobic bacteria has been, until recently, controversial (W. J. Martin, Clin. Microbiol. Newsl. 3:111–112, 1981). In the last few years, the introduction of new antimicrobial agents and the increased resistance to some widely used antibiotics have created the need for wider susceptibility testing of clinically significant anaerobes (2, 4, 5, 15). Most of the currently available techniques for antimicrobial susceptibility testing of these organisms, although allowing testing of the majority of clinical isolates, are long, cumbersome, and costly for routine use in clinical laboratories (9, 10, 18). Disk diffusion susceptibility testing of anaerobes has been evaluated in the past but has not gained general acceptance (7, 13, 14, 17). Because of the need for a rapid, simple method for susceptibility testing of anaerobes, we have devised a new disk diffusion test which can be done on the day the organism is isolated and interpreted in 24 h. The following study describes this technique for the *Bacteroides fragilis* group and compares the results with those of the standard reference agar dilution method (9).

MATERIALS AND METHODS

Bacterial strains. A total of 225 clinical isolates of the *Bacteroides fragilis* group were tested: *Bacteroides fragilis* (112 strains), *Bacteroides thetaiotaomicron* (33 strains), *Bacteroides ovatus* (23 strains), *Bacteroides vulgatus* (21 strains), *Bacteroides distasonis* (17 strains), *Bacteroides uniformis* (5 strains), and *Bacteroides* spp. (14 strains). The organisms were identified by standard methods (6, 12) and maintained frozen in 15% glycerol at −70°C. Before testing, bacterial suspensions were thawed and plated onto tryptic soy agar supplemented with yeast extract, hemin (5 μg/ml), vitamin K (0.1 μg/ml), and 5% sheep blood and subcultured on the same medium. The following control strains were also included: *Clostridium perfringens* ATCC 13124, *Bacteroides fragilis* ATCC 25285, and *Bacteroides thetaiotaomicron* ATCC 29741.

Antimicrobial agents. The following laboratory-standard antibiotic powders were tested: cefoxitin and imipenem (Merck Frosst Canada Inc., Pointe-Claire, Quebec); moxalactam (Eli Lilly & Co., Indianapolis, Ind.); clindamycin (The Upjohn Co., Kalamazoo, Mich.); ticarcillin (Beecham Laboratories, Pointe-Claire, Quebec); and chloramphenicol (Parke-Davis Canada Inc., Brockville, Ontario). Standard disks of cefoxitin (30 μg), chloramphenicol (30 μg), clindamycin (2 μg), moxalactam (30 μg), and ticarcillin (75 μg) were used (Difco Laboratories, Detroit, Mich.). Disks of imipenem (10 μg) were kindly provided by Merck Frosst Canada.

Susceptibility testing. The MICs were determined by the standard reference agar dilution procedure for antimicrobial susceptibility testing of anaerobic bacteria (9) with Wilkins-Chalgren agar (16). The antibiotic concentrations tested ranged from 0.06 to 128 μg/ml.

The disk diffusion susceptibility tests were performed in the following manner. The inocula were prepared by directly suspending colonies from the agar plates into 2 ml of sterile saline to match the turbidity equivalent to that of one-half of a number one McFarland standard. The inoculum was then immediately swabbed in three directions onto 15-cm plates containing 60 ml of Wilkins-Chalgren agar (depth, approximately 4 mm). The plates were allowed to dry for 5 min, and the antibiotic disks were applied to the surface of each plate so as to prevent overlapping of zones. The plates were then inverted and incubated at 37°C in an anaerobic chamber (Coy Laboratories Inc., Ann Arbor, Mich.). The zone sizes were measured with calipers at 24 h. The three control organisms were tested each time the test was performed.

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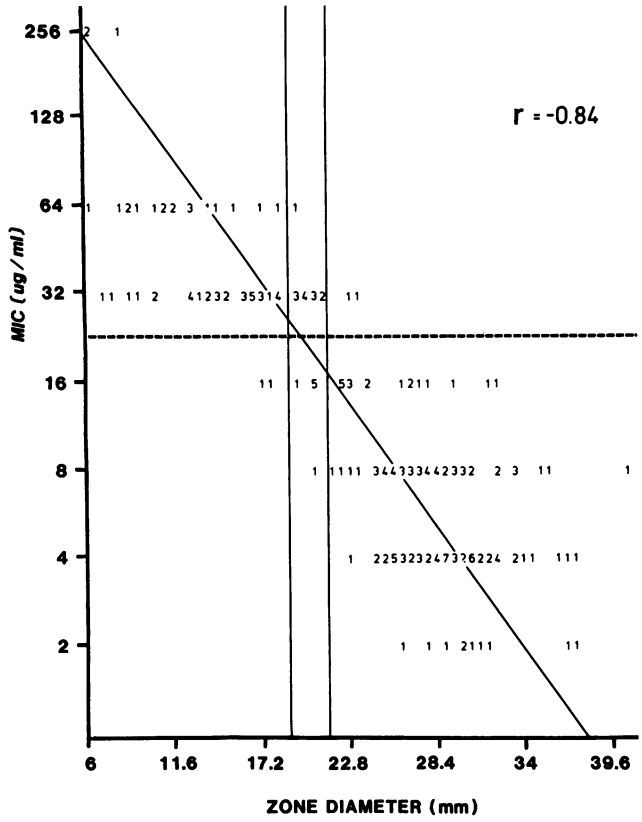

FIG. 1. Correlation of MICs and zone diameters around cefoxitin (30 μg) disks.

Statistical analysis. The susceptibility results obtained by both methods were plotted as scattergrams; the MICs were correlated with the growth inhibition zone diameters, and regression lines were calculated by least-squares analysis (3). With the recommended (9) resistance MIC breakpoints for the antimicrobial agents tested, corresponding susceptible and resistant zone diameter breakpoints were established by the error rate-bounded method.

RESULTS

Of the 225 strains of the *B. fragilis* group tested, 216 grew well within 24 h, and inhibition zone diameters were easily measured. The nine strains that did not grow at 24 h (*B. distasonis*, 2; *B. fragilis*, 2; *B. ovatus*, 2; *B. uniformis*, 2; and *Bacteroides* spp., 1) did not grow at 48 h either.

Cefoxitin. The scatter plot and regression lines for cefoxitin are shown in Fig. 1. MICs for 70 of 216 strains were ≥32 μg/ml, and these 70 were considered resistant. With resistant and susceptible zone diameter breakpoints of ≤19 and ≥21 mm, respectively, two strains each (0.9%) fell within the false-resistant and false-susceptible regions. Twenty (9.2%) strains fell within the intermediate zone. The correlation coefficient was −0.84.

Clindamycin. The scatter plot and regression line for clindamycin are shown in Fig. 2. MICs for 12 strains were ≥8 μg/ml (MICs were ≥128 μg/ml for six), and these strains were considered resistant. With resistant and susceptible zone diameter breakpionts of ≤14 and ≥18 mm, respectively, eight strains (3.7%) were classified as falsely resistant, but there were no falsely susceptible strains. The strains for which MICs were ≥128 μg/ml did not produce any zone around the disk. There were 19 strains (8.7%) that fell within the intermediate zone. The correlation coefficient was −0.73.

Moxalactam. The MICs of moxalactam for 30 strains were ≥32 μg/ml, and these strains were considered resistant (Fig. 3). With resistant and susceptible zone diameter breakpoints of ≤21 and ≥25 mm, respectively, two (0.9%) isolates were classified as falsely susceptible and four (1.8%) as falsely resistant. However, 41 (18.9%) strains fell within the intermediate zone. The correlation coefficient was −0.88.

Ticarcillin. The MICs of ticarcillin for 36 strains were ≥128 μg/ml, and these strains were considered resistant (Fig. 4). With resistant and susceptible zone diameter breakpoints of ≤15 and ≥16 mm, respectively, only one strain (0.5%) was classified as falsely susceptible, and none were classified as falsely resistant. There was no intermediate zone for ticarcillin. The correlation coefficient was −0.89.

Chloramphenicol. All 216 isolates were susceptible to 8 μg or less of chloramphenicol per ml (Fig. 5). Zones of inhibition ranged from 26 to 49 mm. A regression line was not calculated because most strains were susceptible over a very narrow range of antibiotic concentrations.

Imipenem. A total of 215 strains were susceptible to ≤4 μg of imipenem per ml (Fig. 6). The MIC for one multiresistant strain was 64 μg/ml. This strain showed no zone on disk diffusion. The other zone sizes ranged from 28 to 61 mm. A regression line was not calculated because most strains were

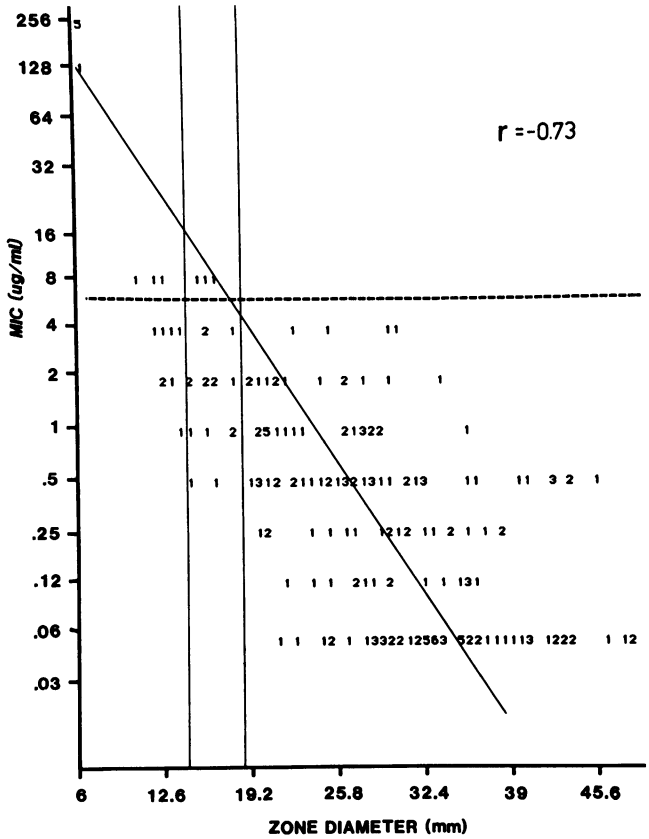

FIG. 2. Correlation of MICs and zone diameters around clindamycin (2 μg) disks. See Fig. 1 legend for details.

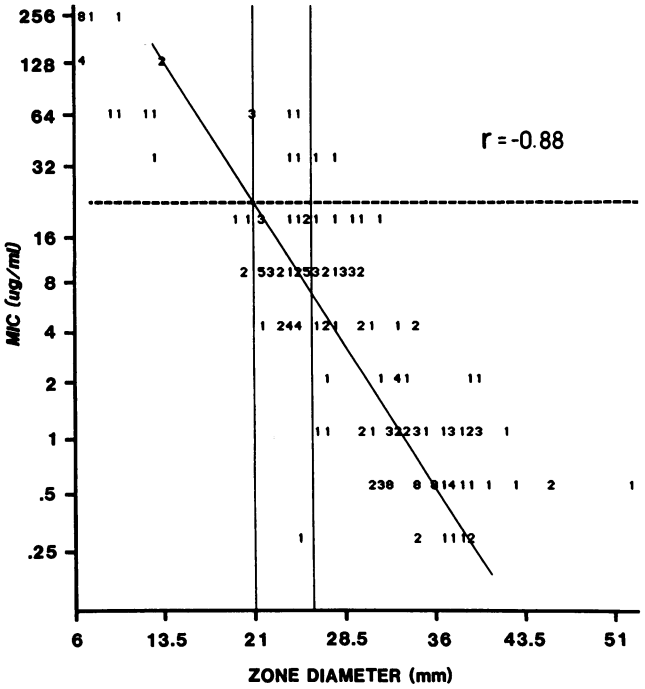

FIG. 3. Correlation of MICs and zone diameters around moxalactam (30 μg) disks. See Fig. 1 legend for details.

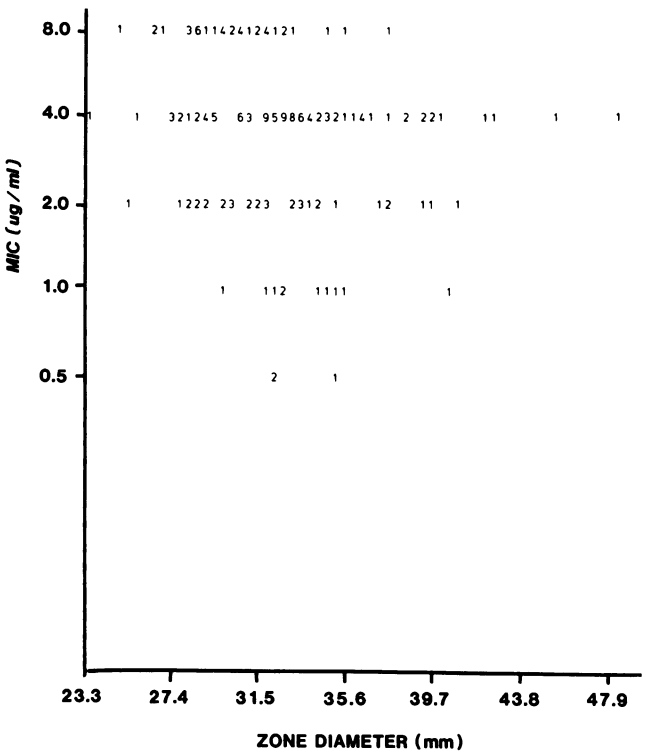

FIG. 5. Correlation of MICs and zone diameters around chloramphenicol (30 μg) disks. See Fig. 1 legend for details.

susceptible over a very narrow range of antibiotic concentrations.

DISCUSSION

Disk diffusion methods for susceptibility testing of anaerobes were described in the early 1970s but have not been recommended for general use due to the complexities of standardization introduced by the variability of growth of different anaerobes, the need for a standard complex enriched medium, and the effect of the anaerobic atmosphere on the activity of some drugs (1, 7, 11, 13, 14, 17). In addition, reports of tests with anaerobes performed by several groups have shown that variation about the regression line was much greater for anaerobes than for aerobes. Despite these pitfalls, most authors have reported satisfactory results when testing *B. fragilis* group strains (14, 17). Some of these earlier difficulties can now be overcome. A new, standardized, well-defined susceptibility testing medium has been developed by Wilkins and Chalgren (16) and accepted as the reference medium for agar dilution tests. The standard agar dilution method (9) can serve as a reference comparison for the accuracy of other practical methods. Finally, Metzler and De Haan have proposed a bivariate "error rate-bounded" classification scheme for relating the MIC and zone size for bacteria readily applicable to antimicrobial susceptibility testing of anaerobic bacteria (8). This latter method requires the determination of the relative importance of two types of errors, false-resistant classification and false-sensitive classification, and determination of the acceptable rate of error of false classification.

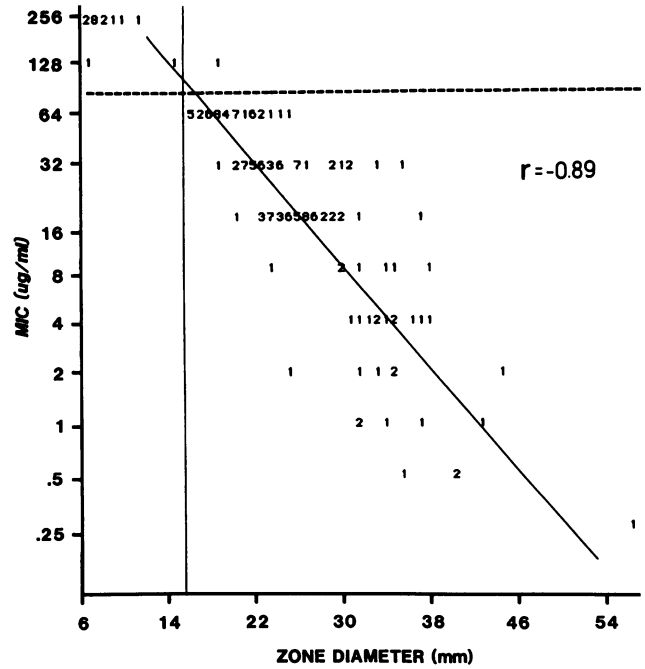

FIG. 4. Correlation of MICs and zone diameters around ticarcillin (75 μg) disks. See Fig. 1 legend for details.

The disk diffusion test described herein was simple to perform and could be easily adapted by any laboratory that uses the Kirby-Bauer method for aerobes. The Wilkins-Chalgren medium is commercially available. Only 9 of our 225 strains failed to grow: these strains were scattered among the different species. It is worth noting that these strains did not grow well on primary subculture either. The inoculum was easily prepared, and the test gave rather sharply clearcut zones of inhibition which were easy to read at 24 h. With the error rate-bounded method, zone criteria could be established so that the percentage of falsely sus-

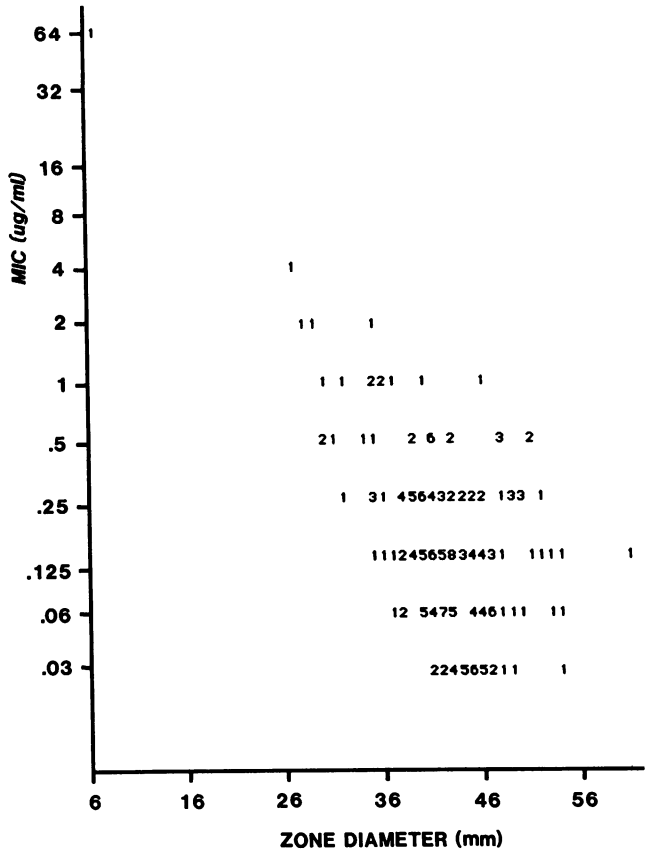

FIG. 6. Correlation of MICs and zone diameters around imipenem (10 μg) disks. See Fig. 1 legend for details.

ceptible strains was <1% and that of falsely resistant strains was ≤3.7% for the drugs tested. Although the percentage of strains that fell within the intermediate zone was high for moxalactam, it was 10% for the other agents. It should be noted that a weakness in this analysis was the relatively few resistant strains represented in the bacterial population studied. This paucity of resistant strains requires us to be only tentative in our recommendations for zone size interpretative criteria. Chloramphenicol and imipenem zone size standards were not recommended because there were no resistant strains. However, tentative zone size standards of ≥26 mm for imipenem and ≥23 mm for chloramphenicol can be proposed for the susceptible categories. Strains with smaller zones should be suspected and retested by other methods.

In summary, the proposed disk test is a simple and rapid procedure for testing the *B. fragilis* group, members of which are the most common anaerobic organisms found in important clinical infections. It gives results in 24 h and can be performed in most clinical laboratories. The fact that results are obtained shortly after isolation of the organisms is highly desirable and may increase the use of this information by clinicians. The test can give relatively accurate, valuable, preliminary information which could later be confirmed by dilution techniques. It should be stressed, however, that the method described and the zone diameter interpretative criteria are limited to the *B. fragilis* group and should not be construed to apply to other anaerobic bacteria. Because of the simplicity of this method, further evaluation and modification of the interpretative zone standards would seem to be in order.

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