Proposed Standardized Method for Testing and Interpreting Susceptibility of *Bacteroides fragilis* to Tetracycline

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One hundred twenty-four strains of *Bacteroides fragilis* were examined for susceptibility to tetracycline disks and by minimal inhibitory concentration (MIC) determinations. MIC values and zone sizes around 30-μg tetracycline disks were determined by using selected test conditions which included Mueller-Hinton agar supplemented with sheep blood, vitamin K, and hemin and an incubation temperature of 35 C in an atmosphere of 80% N_2, 10% H_2, and 10% CO_2. Strains were separated into two distinct populations by geometric mean MIC and disk tests. Of the 124 strains, 78 were resistant and 46 were susceptible. The resistant strains had geometric mean MIC's of 8 μg/ml or greater, whereas the geometric mean MIC's of sensitive strains were 5 μg/ml or less. The disk test proved to be more reproducible than the MIC test and completely separated the resistant and susceptible populations. An interpretive scheme for *B. fragilis* to tetracycline was statistically derived on the basis of the distribution of zone sizes of susceptible and resistant strains: resistant, 18 mm or less; indeterminant, 19 to 20 mm; and susceptible, 21 mm or greater. These zone sizes compared closely with the Kirby-Bauer criteria for aerobic bacteria.

Because of an increased awareness of the importance of nonsporulating anaerobes in infections, it is necessary that all clinical microbiology laboratories provide the physician with antibiotic susceptibility data by a convenient, standardized method. Even though certain species of *Bacteroides* have been known as potential pathogens since 1897 (20), their occurrence in clinical specimens has often been undetected. Now, with better methods for cultivating nonsporulating anaerobes, it has been shown that one of the most common pathogenic organisms is *Bacteroides fragilis* which is isolated frequently from infections and septicemias (11, 12, 15, 16, 22).

Bacteroides fragilis is resistant to many chemotherapeutic agents, and resistance to tetracycline has emerged in the last several years (19). Because of multiple resistance, the choice of antibiotic is most important for the welfare of a patient with a *B. fragilis* infection. As reported recently by Nobles (14), 60% of the patients with *B. fragilis* septicemia who did not receive effective antibiotic therapy died, whereas those who were treated with an appropriate chemotherapeutic agent showed only a 12% mortality rate.

Recently several disk methods were described for determining the antibiotic susceptibility of nonsporulating anaerobes to chemotherapeutic agents (18, 19, 21). These workers employed different testing methods and proposed different interpretive schemes. This study was undertaken to provide the clinical laboratory with a simple method of antibiotic susceptibility testing by disk diffusion as well as an accurate method of interpretation. Tetracycline against *B. fragilis* was chosen as a model system for using the suggested methods. Zone size measurement experiments using *Escherichia coli*, various types of media, and anaerobic atmospheres were conducted to select an optimal antibiotic test procedure for anaerobic bacteria. These experiments showed that Mueller-Hinton agar supplemented with sheep blood, mendione, and hemin and an anaerobic atmosphere of 80% N_2, 10% H_2, and 10% CO_2 produced the smallest changes in zone size. Because of this finding and in a desire to duplicate as closely possible the Bauer-Kirby antibiotic susceptibility test for aerobes, the above-specified medium and anaerobic atmosphere were selected for the disk test as well as for determination of the minimal inhibitory concentration (MIC) proce-

dure for anaerobic bacteria. This technique was a standardized modification of the Kirby-Bauer (1) procedure for rapidly growing aerobes, and utilized analysis of the population characteristics of the strains to derive interpretive schemes for the disk test.

MATERIALS AND METHODS

Bacterial strains. One hundred twenty-four strains of *B. fragilis* were used in this study. Eighteen of these strains were supplied from the Virginia Polytechnic Institute, courtesy of Tracy D. Wilkins. The rest of the organisms were clinical isolates collected over a 3-year period at Emory University Hospital and stored at −65 C. The number of subspecies used was as follows: 81 *fragilis*, 26 *thetaiotaomicron*, 5 *vulgatus*, 10 *distasonis*, 1 *ovatus*, and 1 NGF (Virginia Polytechnic Institute designation, no good fit). These strains were identified by the method of Holdeman and Moore (9).

Anaerobiosis. All work, unless otherwise noted, was performed in an anaerobe chamber (Coy Manufacturing Co., Ann Arbor, Mich.). The atmosphere in the chamber was 80% N_2, 10% H_2, and 10% CO_2 (Matheson Gas Company, Atlanta, Georgia).

Media. The organisms were maintained in brain heart infusion broth (BHIB) (Difco) supplemented with vitamin K (0.00005%) (Sigma) and hemin (0.0005%) (Sigma). Mueller-Hinton broth (MHB) (Difco) with vitamin K and hemin (VKH) added were used for diluting the inoculum. Mueller-Hinton agar (MHA) (Difco) with VKH was supplemented with 5% sheep blood and used for zone size determinations. Brain heart infusion agar (Difco) supplemented with VKH and 5% sheep blood was used for purity checks. All broth media were placed in the anaerobe chamber while hot. The agar plates were allowed to harden and placed in the anaerobe chamber.

Antibiotics. Tetracycline powder (Pfizer, Inc., New York, N.Y.) had an activity of 980 µg/ml and was dissolved in sterile distilled water. Standard disks (BBL) had a content of 30 µg of tetracycline.

Antibiotic disk method. Fresh MHA plates with blood and VKH less than 3 h old were used. The medium was adjusted to around pH 7.5 prior to autoclaving, and constant amounts of agar were placed in each dish. The inoculum consisted of a 24-h culture grown in BHIB-VKH at 35 C. The cultures were adjusted to one-half the turbidity of a no. 1 McFarland nephelometer standard using MHB-VKH. Sterile cotton swabs were used to remove the organisms, and each swab was pressed against the tube wall to remove the excess liquid. The plates were streaked evenly in three directions and allowed to dry for 3 to 5 min before a single disk was added to the surface of each plate. The plates were inverted and incubated at 35 C for 22 h. The zone sizes were measured with a vernier caliper and recorded to the nearest millimeter and included the diameter of the disk. The reproducibility of the test was determined by performing the technique on three different occasions.

MIC determinations. MIC values were deter-

mined by using twofold antibiotic dilutions in agar ranging from 128 to 0.062 µg/ml. One milliliter of each dilution was placed in each of two sterile petri dishes. Nineteen milliliters of MHA-VKH with blood were then pipetted into each dish. The plates were allowed to harden and then were placed in the chamber to incubate at 35 C for 22 h.

The inocula were grown in BHIB-VKH for 22 h at 35 C. By using MHB-VKH the cultures were adjusted to match the turbidity of one-half of a no. 1 McFarland nephelometer standard. A 1 ml amount of this solution was then added to 9 ml of MHB-VKH. An Oxford automatic pipettor was used to make the 1:10 dilutions. This dilution of each culture was then transferred to the surface of the plates by using a Steers replicating device (17).

After the inoculum dried, the plates were inverted and incubated at 35 C. The results were determined after 22 h of incubation. The MIC was recorded as the lowest concentration of antibiotic which completely inhibited the macroscopic growth of each organism.

RESULTS

The results of our experiments confirm the existence of two distinct populations of MICs of *B. fragilis* to the action of tetracycline (Fig. 1). This phenomenon has previously been described by other workers (10, 11). The bimodal distribution is clearly a nonrandom occurrence, and the interpretation of this disk test was therefore based on zone size characteristics of the two populations.
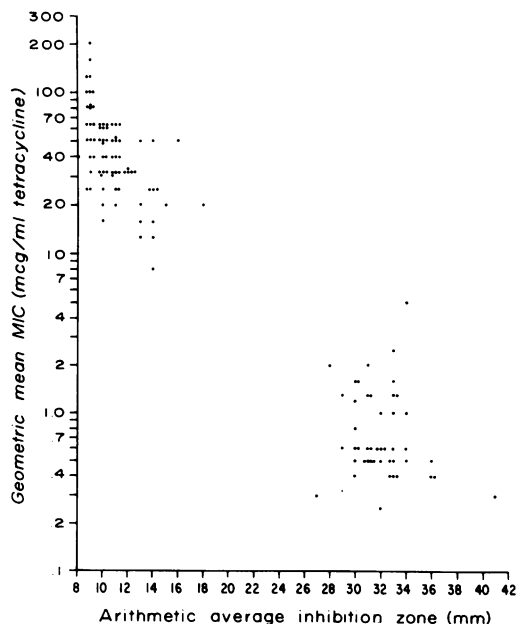

FIG. 1. *Correlation of MIC values and zone diameters around 30-µg tetracycline disks.*

The combined MIC and zone size data were utilized to classify the susceptibility of strains. Of the 124 strains tested, 78 belonged to a resistant population and 46 to a susceptible population. Resistant strains had geometric mean MICs of 8 μg/ml or greater, and susceptible strains had MICs of 5 μg/ml or less. Thus, the two populations are separated by less than one twofold dilution. Bimodal populations are clearly more apparent by the distribution of zone sizes than by the distribution of MIC values (Fig. 2 and 3). A separation of 8 mm between the two populations was observed with the disk test.

The reproducibility of the two testing methods was compared when determinations were made on three different days by both methods (Table 1). Separate assessments of reproducibility were made for strains in the susceptible and resistant populations. The coefficients of variation (standard deviations divided by the arithmetic average values) indicated an 8- to 20-fold greater variability in the MIC test as compared with the disk test both with susceptible and resistant populations. Therefore, not only does the disk test separate populations more clearly, but it is also more reproducible and thus less subject to misclassification of the susceptibility of strains.

To establish outcomes of a single disk test, only one of the three zone size values for each strain was selected by using a table of random numbers. The mean zone size of inhibition of the resulting resistant population was 10.86 mm, whereas the mean zone size of the susceptible population was 31.96 mm. To establish interpretive criteria, exact probabilities were calculated for outcomes in the critical area between the two populations of zone sizes
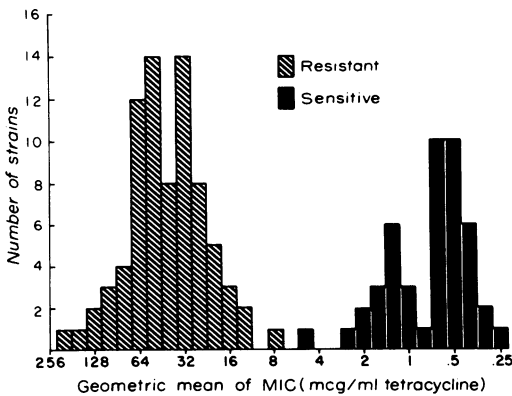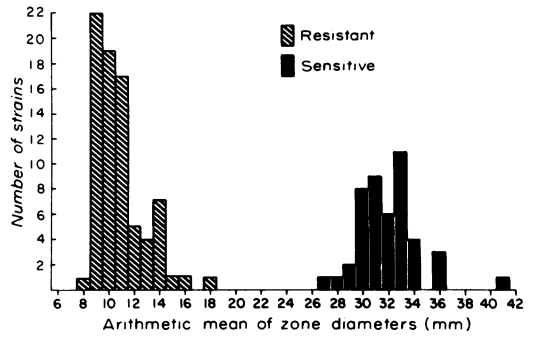

FIG. 3. Distribution of the arithmetic mean zone diameters of 124 strains of B. fragilis to tetracycline.

TABLE 1. Reproducibility of antibiotic susceptibility tests

Population	Standard deviation	Arithmetic avg	Coefficient of variation (%)
Susceptible			
Disk test	1.6 mm	31.9 mm	4.9
MIC	0.9 μg/ml	1.1 μg/ml	81.5
Resistant			
Disk test	0.7 mm	10.8 mm	6.9
MIC	28.6 μg/ml	55.1 μg/ml	51.9

(Table 2). Calculations were made to establish the theoretical frequency distribution of both populations. Based on this data, only four in one million susceptible strains would have zones of 18 mm, but 56 of 100,000 resistant strains would have this value. Thus, the relative chance that a strain with an 18-mm zone would be resistant was greater than 99%. In contrast, strains with a single zone determination of 21 mm had only a 3.5% chance of belonging to the resistant population and a 96.5% chance of belonging to the susceptible population. In order to make correct decisions, at least 95% of the time zones of 19 to 20 mm were called indeterminant, because they did not allow this degree of confidence. By using the specified test conditions, the following interpretive scheme is recommended for B. fragilis against tetracycline: resistant, 18 mm or less; indeterminant, 19 to 20 mm; and sensitive, 21 mm or greater.

Based on the sample of strains used, indeterminant readings are expected only once in 5,000 tests.

DISCUSSION

Because of the relative safety of tetracycline and because many strains of B. fragilis were reported susceptible, this chemotherapeutic

FIG. 2. Distribution of geometric mean MIC values of 124 strains of B. fragilis to tetracycline.

TABLE 2. *Probabilities of obtaining zone diameters from strains in the resistant and susceptible populations*

Recorded zone (mm)	Actual range of measurements (mm)	Probability that zone was produced by a strain of either population		Relative chance (%) that strain is	
		Susceptible	Resistant	Susceptible	Resistant
18	17.5 to 18.4	0.000004	0.000560	0.7	99.3
19	18.5 to 19.4	0.000017	0.000080	21.3	78.8
20	19.5 to 20.4	0.000080	0.000023	71.3	28.8
21	20.5 to 21.4	0.000200	0.000007	96.5	3.5

agent has been the drug of choice by many physicians (3, 4, 6, 15). In the 1960s most isolates of *B. fragilis* were susceptible to tetracycline; however, during the last decade a large percentage has become resistant (18). It is interesting to speculate that this resistance has probably emerged due to oral ingestion of this antibiotic. This finding has been noted with tetracycline-resistant strains of *Escherichia coli* from the intestinal tract of humans (8). Due to the multiple resistance pattern shown by this organism, chloramphenicol has frequently been used by clinicians, although its potential toxicity is certainly a risk.

Substantial differences in interpretive schemes exist among various workers. These differences are derived from the use of different test conditions including various media, anaerobic atmospheres, and inoculum size, and the way that results are analyzed to develop interpretations for the disk test. Within the past 2 years, several groups of workers have proposed interpretive criteria for *B. fragilis* to tetracycline based on regression line analysis. Regression line analysis to establish zone interpretive schemes is valid only when the variables are normally distributed along the regression line. Furthermore, the regression line approach places primary emphasis on the MIC test, which is less reproducible than the disk test. If a regression line approach is used it is still necessary for interpretive schemes to take into account the accuracy of predicting MIC values from zones.

Bennett et al. (2) successfully employed population analysis to derive a disk interpretive scheme for meningococci and sulfonamides. An identical statistical approach was used to develop interpretive zone sizes of *B. fragilis* to tetracycline in our study. It is suggested that this approach be used whenever clearly bimodal populations of strains are present and where the populations are separated by MIC values that bear a *reasonable similarity to clinically obtainable blood levels.* This proposal is based on the greater reproducibility of the disk test and the

lesser chance of misclassifying strains. Furthermore, it recognizes that the absolute value of a particular MIC test may be affected by different test conditions. Thus, the population distribution of MICs of strains may be relatively more helpful in classifying strains than the absolute MIC values. In fact, the distribution of zones may be very helpful in identifying MIC breakpoints between two populations, as illustrated in Fig. 1.

Similarity was observed in our zone interpretive schemes to the proposed criteria for aerobes and facultative anaerobes. This close correspondence was attributed in large part to deliberate selection of an anaerobic test method which closely corresponded to standardized testing for aerobes. Under aerobic conditions, organisms with zones of 19 mm or more are considered susceptible. Our data indicated 21 mm or more as a cutoff point for organisms incubated anaerobically with 10% CO_2 present.

The proposed disk test is a relatively simple test that can be performed in all clinical laboratories. The amount of carbon dioxide during incubation is critical (10). We used 10% CO_2 in an anaerobic chamber, but the GasPak (BBL) system also provides this concentration of CO_2 (5, 13). Therefore, we recommend that GasPaks be used by clinical laboratories when an anaerobe chamber is not available.

Therapeutic response of antibiotics in animals and humans is needed to validate the designation of an anaerobic strain as susceptible or resistant as derived from in vitro data.

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