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Standardized Single-Disc Method for Antibiotic Susceptibility Testing of Anaerobic Bacteria

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A method was developed for determination of the antibiotic susceptibility of anaerobic bacteria by use of a single-disc diffusion technique and incorporation of the inoculum in pour plates. The method was standardized by correlation of zone diameters with minimal inhibitory concentrations determined in broth. Zone diameters could be used to approximate the minimal inhibitory concentrations of the seven antibiotics tested: ampicillin, bacitracin, carbenicillin, cephalothin, clindamycin, penicillin, and tetracycline.

Anaerobic bacteria are being isolated from tissue infections with increasing frequency as clinical anaerobic methods have improved. At present, no standardized method is available for determination of the antibiotic susceptibility of these anaerobic isolates. The standardized Bauer-Kirby procedure (2), used by increasing numbers of clinical laboratories for rapidly growing aerobic pathogens, was not designed for use with anaerobic organisms. Any standard method proposed for use with anaerobic bacteria should be based on data obtained by using the anaerobic bacteria commonly isolated in clinical laboratories. The present study describes the standardization of a technique for susceptibility testing of anaerobic bacteria which has been used successfully in this laboratory for several years.

MATERIALS AND METHODS

Bacterial strains. The strains of anaerobes used in this study were from our culture collection. The majority were clinical isolates sent to this laboratory for identification. Organisms were identified according to methods previously described (1). All strain numbers refer to our collection numbers.

Media. Prereduced media were prepared according to methods and formulas previously published (1) and contained 0.05% cysteine. The medium used for antibiotic susceptibility testing was prereduced BBL brain heart infusion broth supplemented with 0.0005% heme, 0.0002% menadione, and 0.5% yeast extract (BHI-S). For agar plates, 2.5% agar (Difco) was added. Inocula were grown in prereduced chopped meat (CM) broth, prereduced chopped meat glucose (CMG) broth, or prereduced chopped meat carbohydrate (CMC) broth. The CMC medium was CM with 0.1% cellobiose, 0.1% soluble starch, and 0.4% glucose added. CMG medium was CM medium with 0.5% glucose added.

Antibiotics. Sources of antibiotics were as follows: penicillin G, potassium, 1,595 units/mg (E. R. Squibb & Sons Inc., New Brunswick, N.J.); ampicillin, sodium for injection (Beecham Pharmaceuticals, Clifton, N.J.); carbenicillin, disodium for injection (Pfizer Inc., New York, N.Y.); bacitracin, 50,000 units per vial for injection (The Upjohn Co., Kalamazoo, Mich.); cephalothin, sodium, 945 μg/mg (Eli Lilly & Co., Indianapolis, Ind.); tetracycline, HCl for injection (Lederle Laboratories, Pearl River, N.Y.); clindamycin, HCl sensitivity powder (The Upjohn Co.). Antibiotics were diluted in sterile distilled water. All antibiotic discs were purchased from Difco and were the standard high-concentration discs.

Determination of MIC. All minimal inhibitory concentration (MIC) values were determined by use of a modification of the broth-dilution method. Rubber-stoppered tubes each containing 5 ml of BHI-S medium prepared under O_2-free N_2 were opened under O_2-free CO_2, and 0.01 ml of an appropriate concentration of antibiotic was added by means of an automatic pipetting device (Eppendorf, Curtin Scientific Co., Houston, Tex.). The tubes were then inoculated with one drop (from a Pasteur pipette) of an 18- to 24-hr culture of the test organism in CM, CMG, or CMC broth. The initial concentration of bacteria was between 10^6 and 10^7 per ml, and incubation was at 37 C for 16 to 20 hr. The MIC was the lowest concentration of antibiotic in which there was no visible growth, or, in the case of bacteriostatic antibiotics, the lowest concentration in which there was no turbidity greater than the faint turbidity present in all tubes.

Disc susceptibility determinations. Approximately 1.5 ml of an 18- to 24-hr culture at maximal turbidity in CMG or CMC broth was added to 10 ml of melted and cooled (50 C) BHI-S agar medium in rubber-stoppered tubes. The contents of each tube were mixed

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twice by inversion and then poured into a plastic petri dish (90 by 15 mm) to solidify at room temperature. A commercial dispenser was used to apply antibiotic discs immediately before the plates were placed into an anaerobic jar. The plates were not inverted for incubation. No precautions were taken to exclude air until the plates were in the anaerobic jar. Anaerobic conditions were then achieved either by use of a GasPak (BBL) or by replacement of the air by 10% CO_2 and 90% H_2 after each of three evacuations of the jar to 60 cm of Hg. All anaerobic jars contained fresh catalyst (GasPak, BBL). The anaerobic jars were incubated for 18 to 24 hr at 37 C. Zone diameters were measured with a ruler. Zones were observed against a black background illuminated with a high-intensity lamp to provide transmitted light. Inhibition of approximately 80% or more of the growth around a disc was considered to be a zone of inhibition. Some strains exhibited an inner area of light growth immediately around the antibiotic disc with an obvious area of inhibition outside this hazy growth. In these instances, the outer zone of inhibition was measured. The most obvious zone of inhibition was measured in those instances when several concentric zones were observed around an antibiotic disc.

Total cell counts. Total numbers of bacterial cells were determined by use of a Petroff-Hausser bacterial counting chamber. Cells in chains were counted individually. Optical densities were determined by use of a Bausch & Lomb Spectronic-20 colorimeter with a 1-cm light path at 600 nm.

Regression plots. Least square lines were determined by computerized methods. Points corresponding to values of "greater than" or "less than" were not used for the least square determinations. Zone diameters of 7 mm corresponded to no measurable zone of inhibition since the antibiotic discs were between 6 and 7 mm in diameter.

RESULTS AND DISCUSSION

Influence of inoculum size on zone diameters. Figure 1 shows the effect of varying the amount of CMC culture used as inoculum on the resulting zone diameters produced by discs containing either penicillin or tetracycline. Since there was little difference between zone diameters resulting from inocula of 2.0 to 0.75 ml, an inoculum of intermediate size, 1.5 ml, was chosen as a standard inoculum. This allowed considerable variation in pipetting accuracy without affecting the zone diameters. A Pasteur pipette and rubber bulb can be used to approximate the 1.5-ml inoculum with no demonstrable loss of accuracy.

We chose to use a large inoculum for anaerobic susceptibility plates for several reasons: (i) growth was reliable and zone diameters were reproducible, (ii) zone diameters could be read in less than 24 hr, (iii) dilution in anaerobic fluid was not required and deleterious dilution in aerobic fluid was avoided, and (iv) zone diameters were relatively small and distinct so that several discs could be placed on one 90- to 100-mm petri plate. Petri plates larger than 100 mm could not be used because they do not fit the majority of anaerobic jars. When small inocula were used, we did not always obtain good growth, zone diameters were often very large, and the results were not

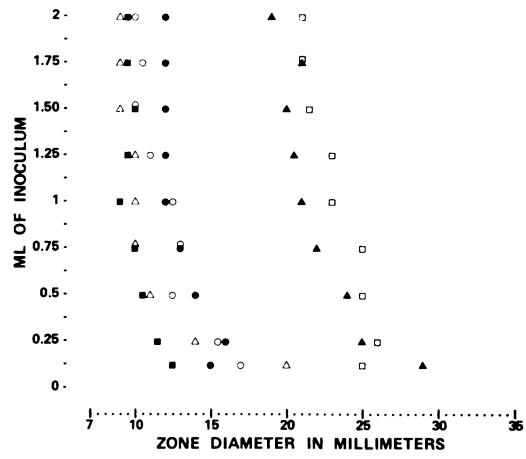

FIG. 1. *Effect of amount of inoculum on zone diameters. Inocula were from cultures at maximal turbidity in CMC broth. Open characters represent penicillin 10-unit discs; closed characters represent tetracycline 30-μg discs; circles, Bacteroides fragilis 6461; triangles, B. fragilis 6957B; squares, Clostridium innocuum 5511.*

TABLE 1. *Comparison of total cell numbers at maximal turbidity and at a standard optical density (OD)*

Organism	Culture at maximal turbidity		Diluted to 0.1 OD at 600 nm	
	Cells/ml	Ratio[a]	Cells/ml	Ratio[a]
Clostridium perfringens 7091	1.5×10^9	1.0	3×10^7	1.0
C. perfringens 6682	1.5×10^9	1.0	3×10^7	1.0
Peptostreptococcus anaerobius 6535A	2×10^9	1.3	2×10^8	6.6
P. anaerobius 6557	3×10^9	2.0	1.5×10^8	5.3
Bacteroides fragilis 6539B	4×10^9	2.6	1×10^8	3.3
B. fragilis 6831	4×10^9	2.6	1×10^8	3.3

[a] Ratio to *C. perfringens*.

TABLE 2. *Effect of method of preparing BHI-S medium on resulting zone diameters with penicillin discs*

Organism	Method of preparing media	
	Fresh	Prereduced
Bacteroides fragilis 0444C	7[a]	7[a]
Clostridium haemolyticum 2167	—[b]	40[c]
C. perfringens 6779	18	17
C. perfringens 5384	13	14
Eubacterium lentum 6818A	22	22
Peptostreptococcus anaerobius 6395B	15	14
P. anaerobius 6601B	20	16

[a] No measurable zone of inhibition; antibiotic discs were 7 mm in diameter.
[b] No growth on plate.
[c] Zone diameter in millimeters.

as reproducible with the same strain tested on different days.

In CMG or CMC broth, the rapidly growing anaerobes most commonly isolated from clinical material grew to maximal turbidity within 24 hr. Such cultures had similar numbers of cells at the end of the log phase of growth (Table 1). Indeed, inherent differences in cell size, and thus in refractility of light, among anaerobic organisms often resulted in more variability among inocula standardized by dilution to a specific optical density than among cultures at maximal turbidity (Table 1).

Effect of method of media preparation. Freshly made BHI-S agar medium without added cysteine, prepared without special precautions to exclude oxygen, was compared with prereduced BHI-S agar medium. Table 2 shows that the zone diameters obtained with both media were comparable for all organisms tested except *Clostridium haemolyticum*. This organism is a strict anaerobe which would grow only in the prereduced agar medium. Although zone diameters were comparable in both types of media, the zones were more clearly defined on the prereduced medium. Prereduced media, either prepared in the laboratory or purchased (Robbin Laboratories, Chapel Hill, N.C.), have an added advantage of immediate availability because they have a relatively long shelf life.

Influence of time and method of achieving anaerobic conditions. The effect of varying the time between pouring the inoculated agar medium into plates and achieving an anaerobic environment in anaerobic jars is shown in Table 3. Exposure to air for 30 min after the plates were poured had no effect on the zone diameters with the organisms tested. A pour plate rather than a swabbed surface plate was used to avoid, as much as possible, the deleterious effects of oxygen. Organisms streaked onto the surface of agar are directly exposed to atmospheric oxygen while the inoculum is drying and until anaerobic conditions have been established in the jar. Organisms in the medium

TABLE 3. *Effect of length of preincubation exposure of plates to atmospheric oxygen on zone diameters with penicillin discs*

Organism	Exposure time (min)	Zone diam (mm)
Clostridium ramosum 4496A	5	11
	10	11
	20	11
	30	10
Eubacterium limosum 6489	5	16
	10	15
	20	17
	30	15
Peptostreptococcus anaerobius 6283	5	17
	10	17
	20	18
	30	17
Peptostreptococcus intermedius 6270	5	23
	10	23
	20	25
	30	24

TABLE 4. *Effect of method of achieving anaerobic atmosphere on resulting zone diameters to penicillin discs*

Organism	Method of achieving anaerobic atmosphere	
	Replacement	GasPak
Bacteroides fragilis 0444C	9[a]	9
B. fragilis 6053A	10	10
Clostridium perfringens 6401C	18	16
C. perfringens 5190	24	23
Eubacterium limosum 6489	16	16
E. limosum 5244	22	21
Peptostreptococcus anaerobius 6311	29	30
P. anaerobius 6601B	13	15

[a] Zone diameter in millimeters. Antibiotic discs were 7 mm in diameter.

TABLE 5. *Effect of incubation time on zone diameters*

Organism	Incubation (hr)	Zone diam (mm)		
		Penicillin	Tetracycline	Clindamycin
Bacteroides fragilis 6461	4	12	15	22
	8	12	13	21
	12	12	12	22
	24	12	13	20
B. fragilis 6957-B	4	11	22	24
	8	7[a]	21	23
	12	7[a]	21	23
	24	8	20	22
Clostridium innocuum 5511	4	18	14	21
	8	26	13	22
	12	23	9	19
	24	25	10	19
C. perfringens 5922	4	12	10	12
	8	14	10	13
	12	14	11	13
	24	14	10	13
Peptostreptococcus anaerobius 6601B	4	12	12	21
	8	13	11	20
	12	12	12	19
	24	13	10	19

[a] No measurable zone of inhibition.

are somewhat protected. However, pour plates should be placed in anaerobic jars as soon as possible after pouring since fresh isolates or other strains, or species, of anaerobic bacteria might be more sensitive to the effects of oxygen than those organisms listed in Table 3.

We found that overlaying the pour plates with another layer of agar for further protection from atmospheric oxygen was not necessary, and we thought that it might be deleterious. The time required to pour the overlay and to allow it to harden would delay putting the plates into an anaerobic environment.

The method used to achieve anaerobic conditions in the anaerobic jars did not affect the results. Table 4 shows that the use of either the GasPak anaerobic system (BBL) or replacement jar technique with 10% CO_2–90% H_2 gave essentially the same results.

Influence of incubation time of susceptibility plates on zone diameters. Incubation times of 4, 8, 12, and 24 hr were tested to determine the earliest time at which zone diameters could be accurately measured (Table 5). With the organisms tested, zones could be measured after 4 hr of incubation. The zone diameters changed only a few millimeters with continued incubation and did not change further after 12 hr of incubation.

Correlation of zone diameter with MIC. To predict the susceptibility of organisms to antibi-

SYMBOL KEY FOR REGRESSION PLOTS

- ● *Bacteroides fragilis* ss. *fragilis*
- ★ *Bacteroides fragilis* ss. *ovatus*
- ■ *Bacteroides melaninogenicus*
- θ *Bacteroides ruminicola* ss. *brevis*
- ρ *Clostridium difficile*
- α *Clostridium indolis*
- γ *Clostridium paraputrificum*
- O *Clostridium perfringens* type A
- φ *Clostridium ramosum*
- ☆ *Clostridium sordellii*
- δ *Clostridium sporogenes*
- η *Clostridium subterminale*
- X *Clostridium tertium*
- ⊕ *Eubacterium lentum*
- □ *Eubacterium limosum*
- π *Fusobacterium varium*
- ◆ *Peptococcus asaccharolyticus*
- ▲ *Peptostreptococcus anaerobius* ss. *anaerobius*
- △ *Peptostreptococcus anaerobius* ss. *foetidus*
- ◆ *Peptostreptococcus anaerobius* ss. *putridus*
- ◇ *Peptostreptococcus intermedius*
- κ *Peptostreptococcus productus*

Overlapping symbols represent points at the bottom position.

FIG. 2. *Key for symbols used in Fig. 3–9.*

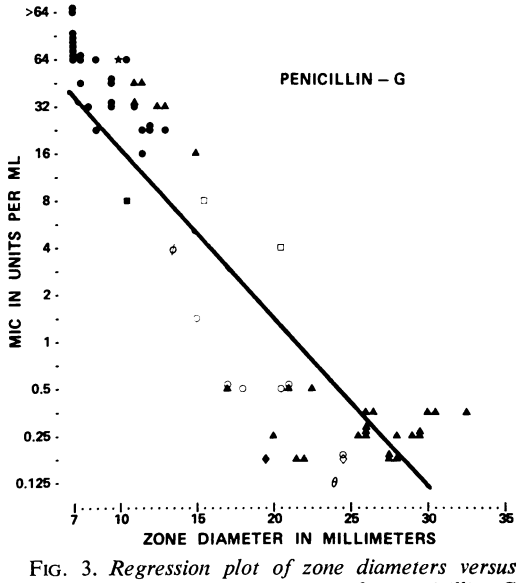

FIG. 3. *Regression plot of zone diameters versus minimal inhibitory concentrations for penicillin G (10-unit discs). Zone diameters of 7 mm are equivalent to no zone of inhibition. A symbol key is provided separately for all regression plots (Fig. 2).*

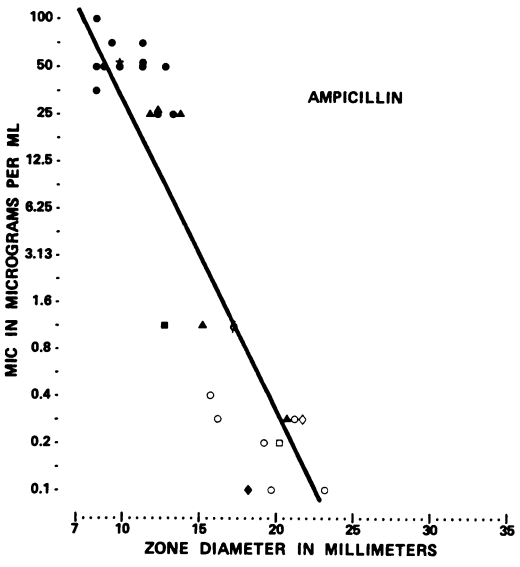

FIG. 4. *Regression plot of zone diameters versus minimal inhibitory concentrations for ampicillin (10-μg discs). See Fig. 2 for symbol key.*

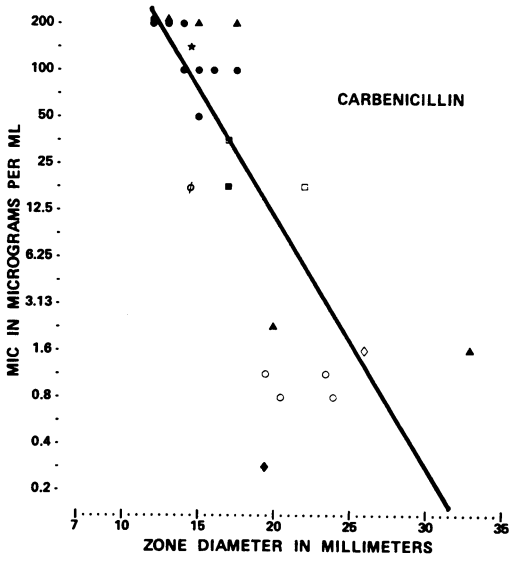

FIG. 5. *Regression plot of zone diameters versus minimal inhibitory concentrations for carbenicillin (50-μg discs). See Fig. 2 for symbol key.*

otics by use of zone diameters, we established regression plots of MIC values and zone diameters for seven antibiotics (Fig. 3–9). A symbol key is provided separately (Fig. 2). Averages of two determinations of zone diameters and two MIC values were used for each organism tested. To obtain a better definition of regression lines, we selected strains to represent a continuum of zone diameters. These were chosen from our culture collection on the basis of previous tests. Therefore, the strains used in this portion of the study do not represent the normal distribution of susceptibilities for the species. *Bacteroides fragilis*

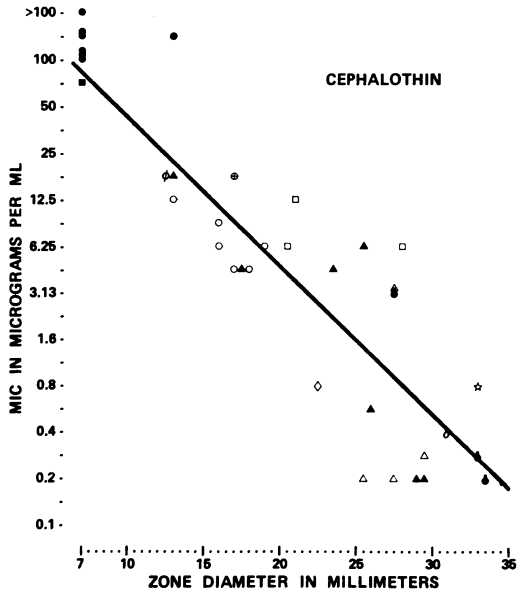

FIG. 6. *Regression plot of zone diameters versus minimal inhibitory concentrations for cephalothin (30-μg discs). See Fig. 2 for symbol key.*

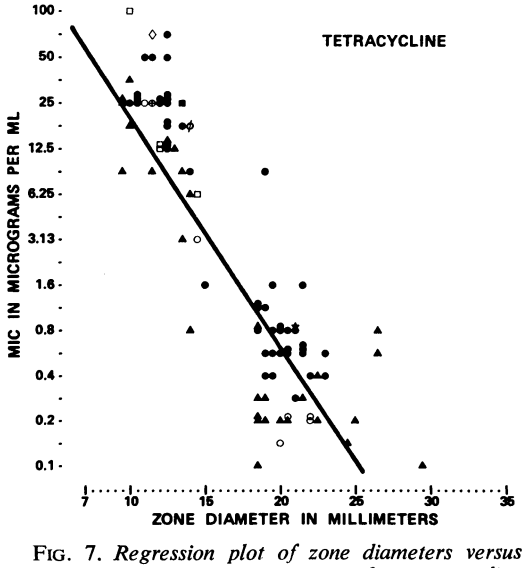

FIG. 7. *Regression plot of zone diameters versus minimal inhibitory concentrations for tetracycline (30-μg discs). See Fig. 2 for symbol key.*

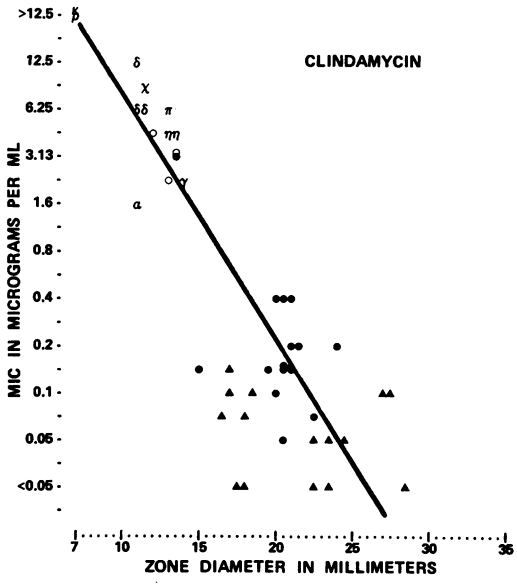

FIG. 8. *Regression plot of zone diameters versus minimal inhibitory concentrations for clindamycin (2-μg discs). See Fig. 2 for symbol key.*

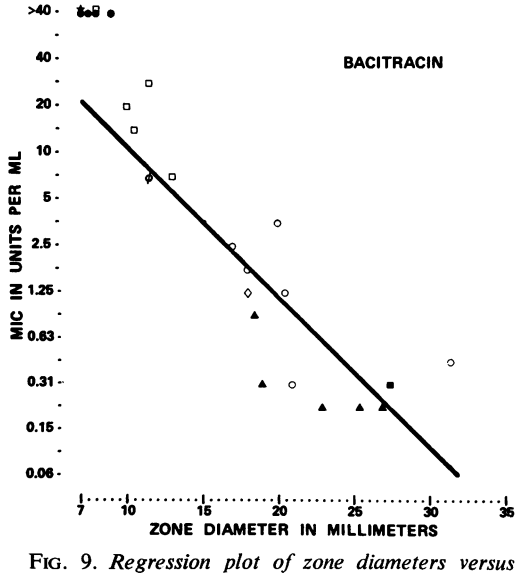

FIG. 9. *Regression plot of zone diameters versus minimal inhibitory concentrations for bacitracin (10-unit discs). See Fig. 2 for symbol key.*

subsp. *fragilis* and *Peptostreptococcus anaerobius* strains, however, were selected randomly. Satisfactory correlation was obtained between MIC values and zone diameters. The zone diameter therefore could be used as an approximation of the MIC.

Only a small percentage of strains had intermediate MIC values or produced intermediate zone diameters with some antibiotics. There was a division of organisms into resistant and susceptible groups with only a few intermediates. This division was especially pronounced with the unselected strains of *B. fragilis* and *P. anaerobius*.

Frequency distributions of zone diameters of unselected strains. To determine how unselected strains would be distributed according to zone diameters, we determined zone diameters for several antibiotics using 50 or more strains of *B. fragilis* subsp. *fragilis* and 50 strains of *P. anaerobius* chosen at random from our culture collection. Figures 10 to 15 show the distribution of strains according to the number of strains having each zone diameter. Odd-numbered zone diameters were rounded to the next higher even number.

When tested with penicillin discs, no strains of *B. fragilis* subsp. *fragilis* tested had zone diameters greater than 16 mm (Fig. 10), and against all of these strains the MIC was greater than 8 units per ml. *P. anaerobius* strains, however, were divided into two groups. The eight strains with zone diameters of 16 mm or less had MIC values of above 8 units per ml, and all but one strain with a zone diameter greater than 16 mm had an MIC of less than 2 units per ml. Since 2 units of

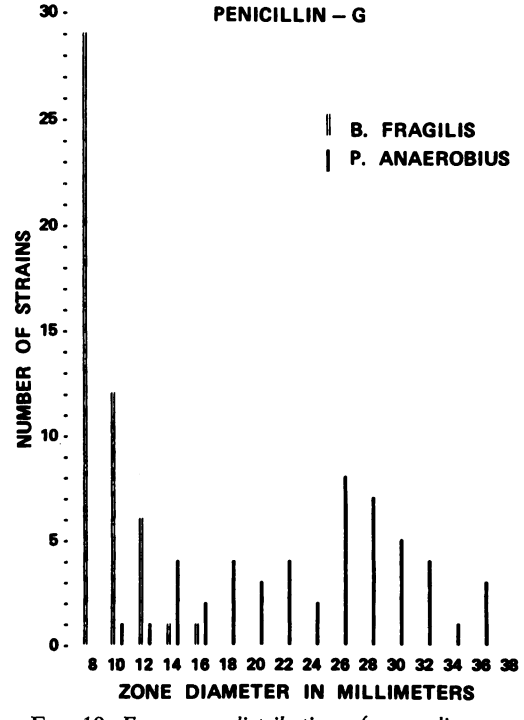

FIG. 10. *Frequency distribution of zone diameters of unselected strains to penicillin 10-unit discs.*

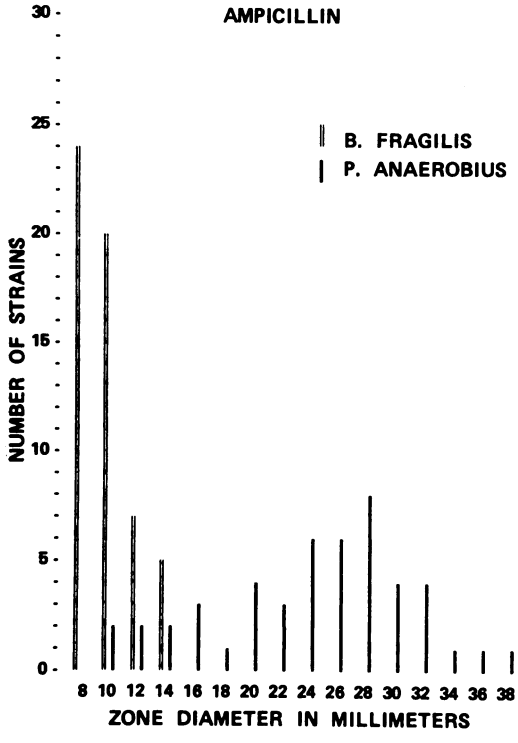

FIG. 11. *Frequency distribution of zone diameters of unselected strains to ampicillin 10-μg discs.*

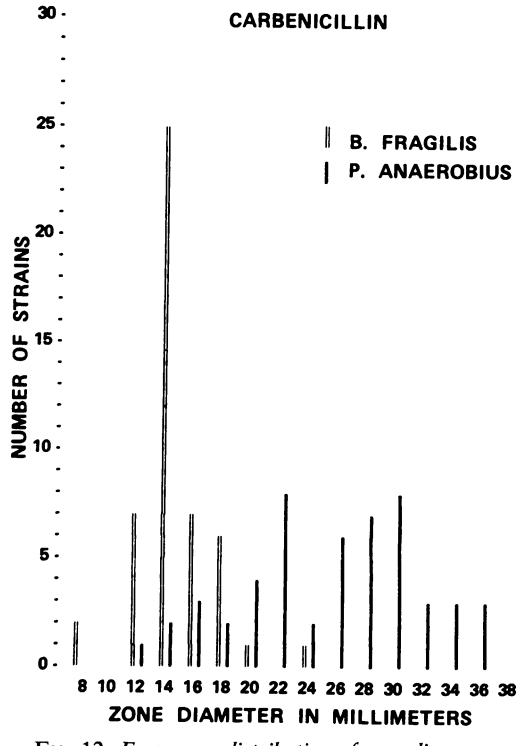

FIG. 12. *Frequency distribution of zone diameters of unselected strains to carbenicillin 50-μg discs.*

penicillin represents a peak blood concentration obtainable with 100,000 units of intramuscular penicillin G (3), those strains of *P. anaerobius* with zone diameters larger than 16 mm could be considered susceptible to penicillin by this criterion. The regression plot shows that 2 units corresponds to a zone diameter of approximately 18 mm (Fig. 3).

Figures 11, 12, and 13 show that approximately the same distribution of strains obtained with penicillin was obtained with ampicillin, carbenicillin, and cephalothin as well. In each case, the *B. fragilis* strains were separated from the majority of the *P. anaerobius* strains. Matsen et al. (4) suggested for cephalothin an MIC break-point of 6.3 μg per ml below which organisms should be considered susceptible to this antibiotic. From the regression plot given in Fig. 6, this value corresponds to a zone diameter of 16 to 18 mm, which is in the area of the break-point between the *B. fragilis* strains and the *P. anaerobius* strains. In the case of ampicillin, an MIC break-point of 3.1 μg per ml was calculated from the data of Matsen et al. On the regression plot given in Fig. 4, this corresponds to a zone diameter of 15 mm. Again, this is in the region of the zone diameter break-point between *B. fragilis* and *P. anaerobius* strains

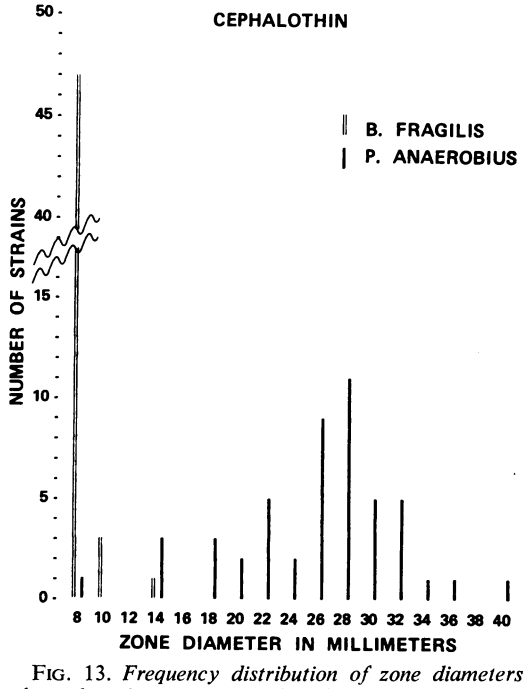

FIG. 13. *Frequency distribution of zone diameters of unselected strains to cephalothin 30-μg discs.*

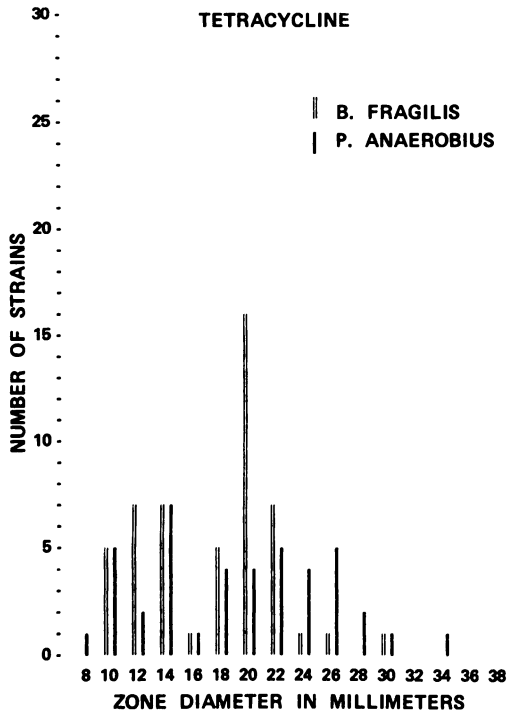

FIG. 14. *Frequency distribution of zone diameters of unselected strains to tetracycline 30-μg discs.*

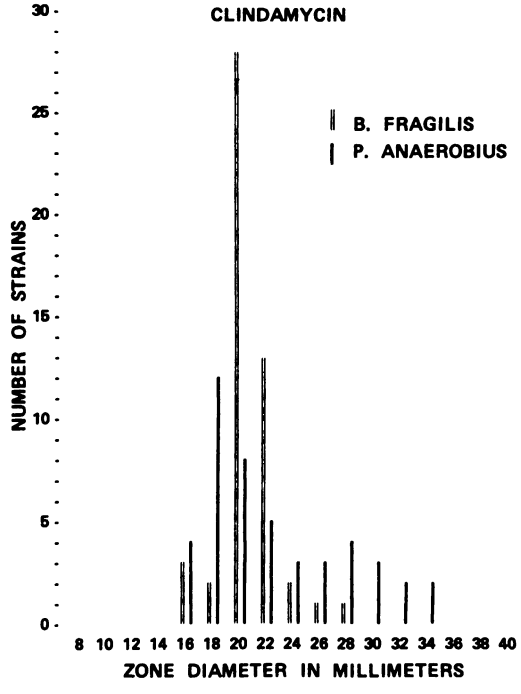

FIG. 15. *Frequency distribution of zone diameters of unselected strains to clindamycin 2-μg discs.*

(Fig. 10). With carbenicillin, a break-point of 25 μg per ml was suggested by Silverblatt and Turck (5). This corresponds to a zone diameter of approximately 18 mm (Fig. 5). The major break-point between *B. fragilis* and *P. anaerobius* strains was between 18 and 20 mm (Fig. 12). Concentrations of carbenicillin in the blood can be raised considerably higher than 25 μg per ml, however, and many of the *B. fragilis* strains would then be considered susceptible.

With tetracycline (Fig. 14), there was no separation of *B. fragilis* strains from *P. anaerobius* strains in regard to zone diameter. A biphasic distribution was evident; only 2 of 100 cultures had zone diameters of 15, 16, or 17 mm. According to the data of Matsen et al. (4), an MIC of 6.3 μg of tetracycline per ml is indicated as a susceptibility break-point. As calculated from our regression plot (Fig. 7), this corresponds to a zone diameter of approximately 14 to 15 mm. Determination of the MIC for the 50 *B. fragilis* and 50 *P. anaerobius* strains showed that all but one strain with a zone diameter of less than 15 mm had an MIC of 6.3 μg per ml or greater. All but one culture with a zone diameter of greater than 15 mm had an MIC of less than 3.1 μg per ml. The two methods thus correlated to give the approximate zone diameters corresponding to susceptibility to this antibiotic. Of the strains tested, 40% of the *B. fragilis* strains and 32% of the *P. anaerobius* strains were resistant to tetracycline.

TABLE 6. *Interpretative zone diameter break-points currently used in this laboratory*

Antibiotic	Resistant	Intermediate	Susceptible
Ampicillin.....	<15	15–17	>17
Carbenicillin....	<15	15–18	>18
Cephalothin.....	<15	15–18	>18
Clindamycin....	<15	15–17	>17
Penicillin.....	<15	15–17	>17
Tetracycline.....	<15	15–16	>16

With clindamycin (7-chloro, 7-deoxy lincomycin) there also was no separation between the *B. fragilis* and *P. anaerobius* strains (Fig. 15). MIC determinations on those strains with the smallest zone diameters showed that the MIC was consistently 0.4 μg or less per ml. During preparation of the regression plot for this antibiotic (Fig. 8), very few organisms could be found with average zone diameters of 14, 15, or 16 mm, and all strains tested with zone diameters of less than 15 mm had an MIC of 1.6 μg or more per ml. The anaerobes tested appeared to divide into resistant and susceptible groups at an MIC of approxi-

mately 1.6 μg of clindamycin per ml with a corresponding zone diameter of 15 mm.

A regression plot was determined for bacitracin (Fig. 9), but no attempt was made to determine MIC break-points. This antibiotic normally is only used topically. However, approximate MIC values could be calculated from the regression plot.

Use in clinical laboratories. The techniques described can be followed by most clinical laboratories without large expenditures for equipment. The only requirements are anaerobic jars or disposable GasPak systems (BBL) and oxygen-free CO_2 for inoculation of CMG or CMC medium. Colonies can be picked directly into tubes of CMC or CMG medium (6 ml) which are incubated until maximal growth occurs (normally within 18 hr) and then used as inocula for both susceptibility plates and biochemical tests. After overnight incubation, susceptibility results can be reported. In emergencies, preliminary results can often be reported after only 4 hr of incubation and then confirmed with a duplicate set of plates incubated for 18 hr.

As in all standard methods for determination of antibiotic sensitivity, it is very important that the exact procedures used to develop the standard zone diameter break-points be routinely followed in the clinical laboratory. Differences in media or inocula size can noticeably influence zone diameters. It is also essential that new or freshly regenerated catalysts be used with anaerobic jars.

The majority of clinical isolates grow rapidly to a high turbidity in prereduced CMC broth and can be assayed for antibiotic susceptibility by this procedure. The method should not be used for those organisms: (i) which grow very slowly, (ii) that only grow to low turbidity in CMC broth, (iii) that do not produce confluent growth in pour plates, or (iv) with which zones of inhibition are very indistinct or otherwise difficult to interpret.

Currently in this laboratory, we are using the interpretative zone diameters given in Table 6 to determine whether organisms are resistant, intermediate, or susceptible to the antibiotics for which regression lines have been determined. These criteria are subject to change as more data become available.

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