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Disk Susceptibility Testing of Slow-Growing Anaerobic Bacteria

YUNG-YUAN KWOK,* FRANCIS P. TALLY, VERA L. SUTTER AND SYDNEY M. FINEGOLD

Veterans Administration, Wadsworth Hospital Center, Los Angeles, California 90073 and the Department of Medicine, University of California School of Medicine, Los Angeles, California 90024*

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The susceptibility of 55 strains of slow-growing anaerobes to eight clinically useful or potentially useful antibiotics was determined by agar dilution and disk diffusion tests. Strains of the genera *Peptococcus*, *Peptostreptococcus*, *Megasphaera*, *Veillonella*, *Eubacterium*, *Bifidobacterium*, *Clostridium*, and *Fusobacterium* were included. All strains were susceptible to chloramphenicol, but varied in their susceptibility to penicillin, lincomycin, clindamycin, tetracyclines, and vancomycin. Correlation between minimal inhibitory concentration and inhibition zone diameters was generally good. Prediction of susceptibility based on zone diameter measurements appeared satisfactory. Although routine susceptibility testing of anaerobic bacteria is not recommended, there are circumstances where such testing is relevant to the clinical situation. For those laboratories ill-equipped to do dilution tests, a disk diffusion test would give relatively accurate preliminary information. Quantitative susceptibility tests could then be done by a reference laboratory.

Our standardized methods for susceptibility testing of rapidly growing anaerobes have been described previously (4, 6, 7). However, many clinically significant anaerobes do not grow rapidly enough to be tested in this manner. This study was carried out to modify previously described methods to compensate for the slower growth rate of these organisms.

MATERIALS AND METHODS

Fifty-five assorted slow-growing anaerobes were included in the study (Table 1). Slow-growing strains were accepted as those which required an 18- to 24-h incubation to reach a turbidity greater than that of a no. 1 McFarland standard in the inoculum broth. The identification of strains tested against eight clinically useful or potentially useful antibiotics is depicted in Fig. 1 through 8. Although most of them were identified to the species level, they are grouped as genera in the figures since there were few representatives of each species and their susceptibility within the groups was essentially the same. Most of them were from clinical sources, seven were from human feces. Thirteen of the 55 were obtained from the Center for Disease Control, Atlanta, Ga., and the remainder were our own isolates.

Paper disk agar diffusion tests were performed in parallel with agar dilution tests. The inoculum for both tests consisted of an overnight culture of each strain in thioglycolate medium without indicator (BBL-135C) and supplemented with hemin (5 μg/ml) before autoclaving and $NaHCO_3$ (1 mg/ml) and vitamin K_1 (0.1 μg/ml) after autoclaving. This was adjusted to the turbidity of the no. 1 McFarland standard, giving 10^7 to 10^8 viable cells per ml. It was applied by swabbing to Brucella agar (Pfizer) containing 5% defibrinated sheep blood and vitamin K_1 (10 μg/ml); the plates had been dried in a 35 C incubator for 1 h prior to use. Antibiotic-containing disks were applied. Zones of inhibition were measured after 42 to 48 h of incubation at 35 C in GasPak jars.

One strain of *Peptococcus magnus* was included as a control. This strain was tested 50 times on different days with each antibiotic disk to determine reproducibility of results.

Agar dilution tests were done by incorporating serial twofold dilutions of the antibiotics (0.05 to 100 μg or U/ml) in Brucella agar with 5% laked sheep blood and vitamin K_1 (10 μg/ml). Plates were dried as above before use. The inoculum was applied by means of a Steers' replicator (3). Minimal inhibitory concentration (MIC) was determined after 48 h of incubation in GasPak jars. MIC was defined as the lowest concentration permitting no growth.

Diameters of zones of inhibition were correlated with the MIC values and regression lines were calculated by least squares analysis whenever results permitted this.

RESULTS

Penicillin G. All but nine strains were susceptible to 0.8 U or less of penicillin per ml (Fig. 1) and had zones of 27 mm or larger around 10-U disks. The nine strains with MICs of 1.6 U/ml and 3.1 U/ml had inhibition zone diameters of 20 to 28 mm. Regression line calculation showed

no significant deviation from linearity (Table 2).

Lincomycin and clindamycin. Thirty-nine of the strains were susceptible to lincomycin (3.1 μg or less per ml), eight were intermediate with an MIC of 6.2 to 12.5 μg/ml, and four were resistant with MICs of 25 μg or more per ml. Resistant strains were *Peptococcus* sp., *Eubacterium limosum*, and *Clostridium ramosum*.

With 2-μg lincomycin disks, some susceptible strains (3.1 μg or less per ml) and some with intermediate susceptibility (6.2 to 12.5 μg/ml) had no zones of inhibition or very small zones, whereas all of the resistant strains had no zones of inhibition. Disks containing 10 μg of lincomycin separated susceptible, intermediate, and resistant strains on the basis of inhibition zone diameters as shown in Fig. 2. All but one strain with MICs of 3.1 μg or less per ml had zones of 19 mm or more, intermediate strains had zones of 13 to 17 mm, and resistant strains had no zones of inhibition around the disk. The calculated regression line (Table 2) showed no significant deviation from linearity.

TABLE 1. *Strains of anaerobes tested*

Strain	Number
Peptococcus species	15
Peptostreptococcus species	13
Gram-negative cocci (*Megasphaera* and *Veillonella*)	3
Eubacterium species	18
Bifidobacterium species	2
Clostridium species	3
Fusobacterium species	1

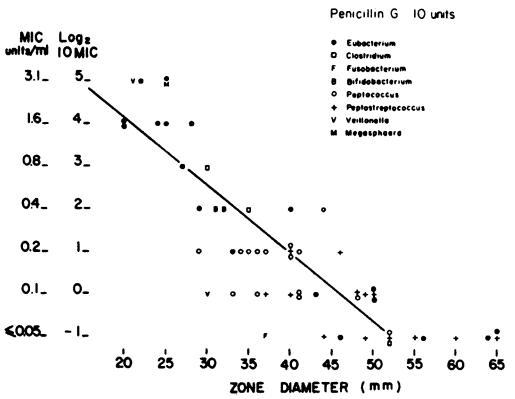

FIG. 1. *Relationship of zone diameters around 10-U penicillin disks and MIC values obtained with slow-growing anaerobes.*

All but seven strains were susceptible to clindamycin, 1.6 μg or less per ml. Resistant strains were *Peptococcus* sp., *E. limosum*, and *C. ramosum*. With 2-μg disks, the susceptible strains had zones of 12 mm or larger, intermediate strains (MIC 3.1 to 6.2 μg/ml) had zones of 6 to 12 mm, and resistant strains (MIC 12.5 μg or more per ml) had no zones of inhibition (Fig. 3). With 10-μg clindamycin disks, susceptible strains had zones of 23 mm or greater, intermediate strains had zones of 14 to 23 mm, and resistant strains had zones of 6 to 13 mm (Fig. 4). Although regression line calculations (Table 2) indicated no significant deviation from linearity when correlating MICs and inhibition zone diameters with either the 2- or 10-μg disks, the prediction of susceptibility by zone diameter measurements appeared better with the 10-μg disk.

Tetracyclines. The susceptibility of the several anaerobes to tetracycline and two of its analogues, doxycycline and minocycline, is shown in Fig. 5–7. Although partial results with tetracycline and doxycycline were previously reported (8), they are included here with more definitive identification of the organisms and for comparison of results with minocycline.

Strains susceptible to 1.6 μg of tetracycline or less per ml had zones of 31 mm or larger, except for one which had a zone diameter of 25 (Fig. 5). Two strains in the intermediate susceptibility category (3.1 μg/ml) had zones of 24 to 31 mm.

TABLE 2. *Relationship between MIC and inhibition zone diameters*[a]

Antibiotic	Disk potency (μg)[b]	Least squares line (y = a + bx)		Correlation coefficient	SE of estimate	F[c]	P
		a	b				
Penicillin	10	7.269	−0.158	−0.827	2.340	2.135	>0.05
Lincomycin	10	8.716	−0.199	−0.858	2.954	0.508	>0.9
Clindamycin	2	6.463	−0.208	−0.848	2.486	0.647	>0.75
Clindamycin	10	9.843	−0.253	−0.808	2.851	0.655	>0.75
Tetracycline	30	10.691	−0.214	−0.909	4.199	2.255	>0.05
Doxycycline	30	9.180	−0.211	−0.917	3.591	12.885	<0.005
Minocycline	30	9.856	−0.227	−0.863	3.759	1.429	>0.25
Vancomycin	30	8.889	−0.163	−0.678	2.144	0.469	>0.95

[a] The probability of deviation from a straight line is as follows: $P > 0.05$, not significant; $0.05 \geq P > 0.01$, almost significant; $0.01 \geq P > 0.001$, significant. SE, Standard error.

[b] Penicillin expressed in units.

[c] Test for linearity.

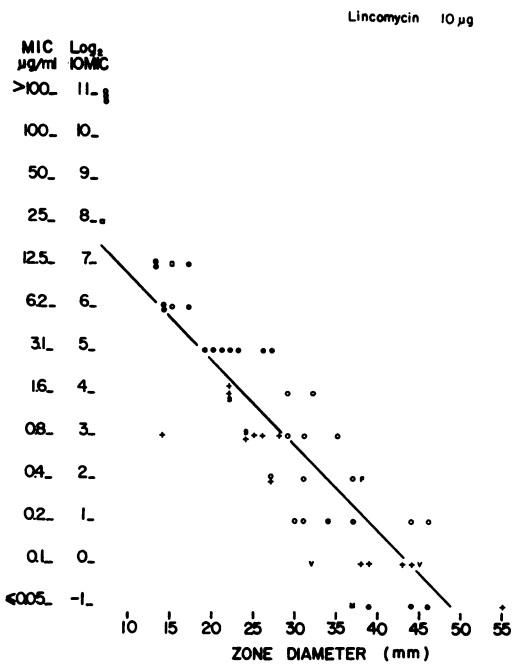

FIG. 2. *Relationship of zone diameters around 10-µg lincomycin disks and MIC values obtained with slow-growing anaerobes. Symbols for bacteria are shown in Fig. 1.*

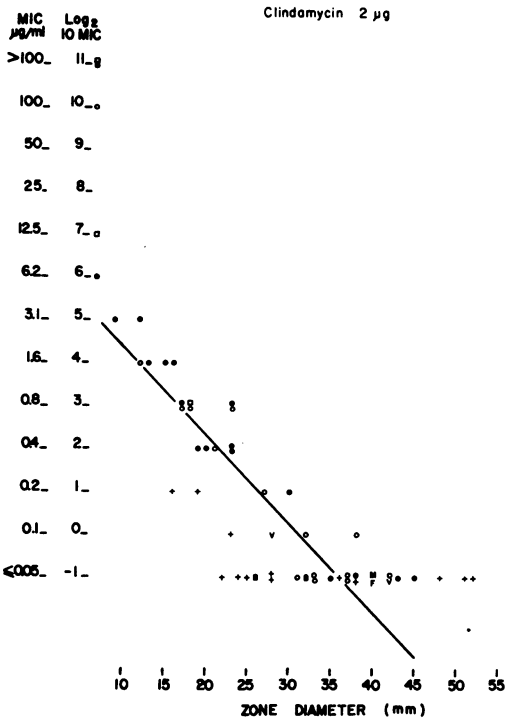

FIG. 3. *Relationship of zone diameters around 2-µg clindamycin disks and MIC values obtained with slow-growing anaerobes. Symbols for bacteria are shown in Fig. 1.*

Resistant strains (12.5 µg or more per ml) had zones of 17 mm or less.

With doxycycline, 40 strains were susceptible to 1.6 µg or less per ml and had zone diameters of 25 mm or larger. Strains with intermediate susceptibility (3.1 µg/ml) had zones of 19 and 20, and resistant strains (6.2 µg or more per ml) had zones of 10 to 18 mm in diameter (Fig. 6). Although there were no susceptible strains with zone diameters in the intermediate or resistant range and no resistant strains with zones in the intermediate and susceptible range, the regression line showed significant deviation from linearity.

Results obtained with minocycline are shown in Fig. 7. The 40 strains susceptible to 1.6 µg or less per ml had zones of 26 mm or larger. Those with MICs of 3.1 and 6.2 µg/ml had zones 19 to 27 mm; whereas those with MICs of 12.5 or more per ml had zones of 15 to 21 mm. There was no significant deviation of the regression line from linearity.

Chloramphenicol. All 55 strains tested were susceptible to chloramphenicol, 6.2 µg or less per ml. Inhibition zones around a 30-µg disk were all 15 mm or larger. No regression line was calculated.

Vancomycin. Forty-nine strains were found susceptible to vancomycin (6.2 µg or less per ml)

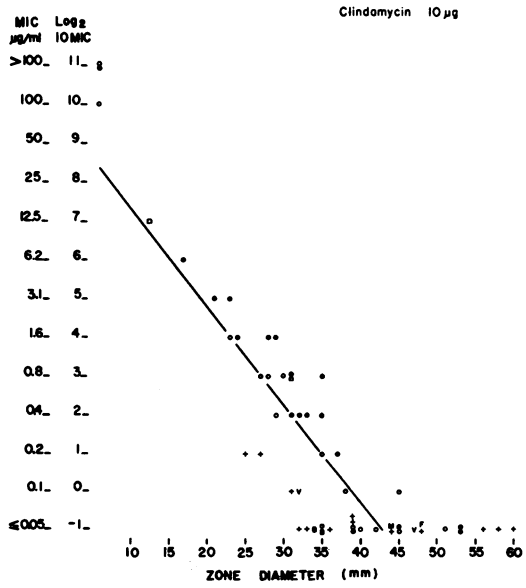

FIG. 4. *Relationship of zone diameters around 10-µg clindamycin disks and MIC values obtained with slow-growing anaerobes. Symbols for bacteria are shown in Fig. 1.*

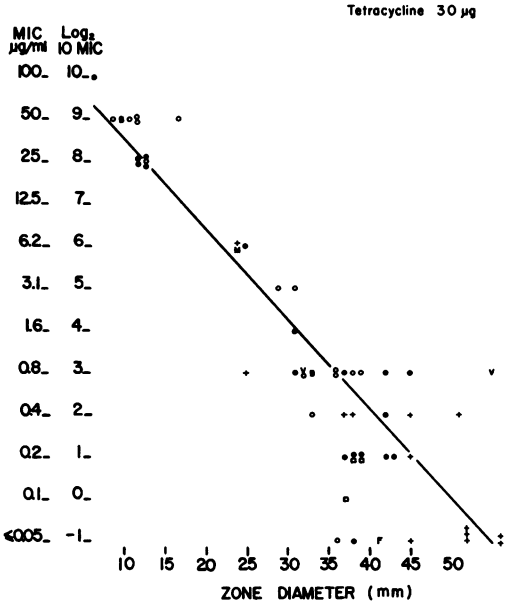

FIG. 5. *Relationship of zone diameters around 30-µg tetracycline disks and MIC values obtained with slow-growing anaerobes. Symbols for bacteria are shown in Fig. 1.*

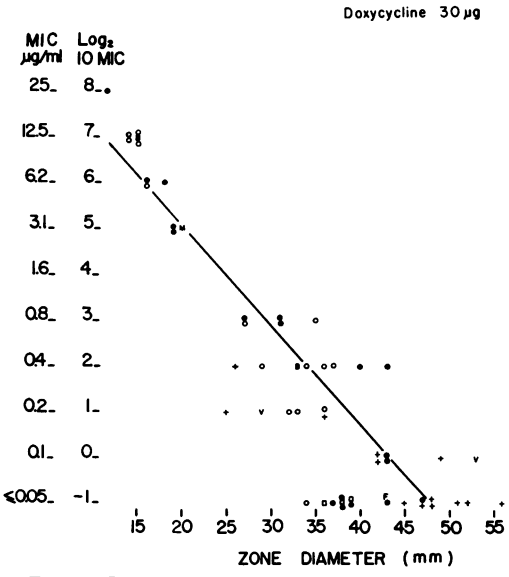

FIG. 6. *Relationship of zone diameters around 30-µg doxycycline disks and MIC values obtained with slow-growing anaerobes. Symbols for bacteria are shown in Fig. 1.*

and had zones of 21 mm or more. These were all gram-positive bacteria. Strains which had MICs of 25 µg or more per ml had zones of 6 to 20 mm. One *Peptostreptococcus productus* and one *Clostridium innocuum* were found to be resistant; the remainder of the resistant strains were gram-negative bacteria. The calculated regression line did not show significant deviation from linearity (Fig. 8, Table 2).

Variation in zone diameters. The results of tests to determine variation in inhibition zone diameters with each kind of antibiotic disk against the control strain are shown in Table 3. Variation in zone diameters was minimal with most of the antibiotics. Maximal variation of 12 mm was observed with doxycycline disks and minimal variation of 5 mm with lincomycin 10-µg disks.

Estimation of susceptibility by zone diameter measurements. Based on the data presented, conservative estimates of susceptibility of slow-growing anaerobic organisms can be made by inhibition zone diameter measurements as shown in Table 4. Using these zone diameter criteria, no resistant strains would be predicted susceptible and no susceptible strains would be predicted resistant. Whenever overlapping of zone diameters occurred among strains with susceptible MICs and intermediate MICs, or among strains with intermediate MICs and resistant MICs, these zone diameters were considered equivocal. A few resistant and susceptible strains would fall into the equivocal range.

A striking similarity between the regression lines and zone diameter criteria obtained with the slow-growing anaerobes and those previ-

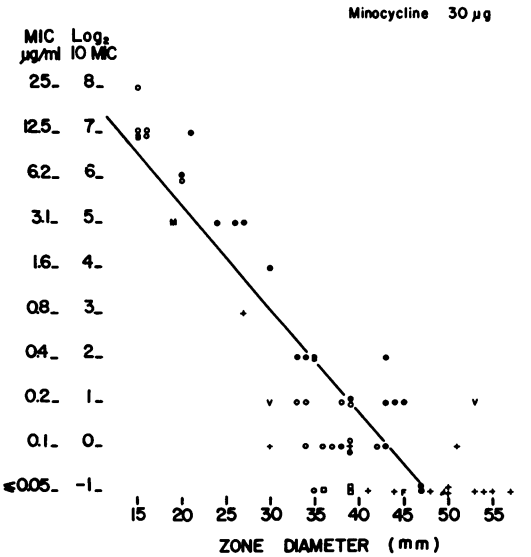

FIG. 7. *Relationship of zone diameters around 30-µg minocycline disks and MIC values obtained with slow-growing anaerobes. Symbols for bacteria are shown in Fig. 1.*

ously obtained with *Bacteroides fragilis* was observed. Theoretically, interpretation of zone diameters should therefore be very much the same. A comparison of predictions by criteria developed in this study (Table 4) and those developed for *B. fragilis* (6, 7) indicated that only minor differences in prediction would occur if the *B. fragilis* zone diameter criteria were used for the slow-growing anaerobes (Table 5).

There were no strains among those presently studied which were resistant to penicillin by the agar dilution test. Among the eight strains which were intermediate in susceptibility, none were predicted susceptible or resistant by criteria in Table 4, but one with an MIC of 1.6 U/ml would have been considered susceptible if *B. fragilis* criteria were used. The susceptible strain considered equivocal by Table 4 criteria would have been predicted susceptible by *B. fragilis* criteria. This strain had an MIC of 0.8 U/ml. Similar differences were observed with the other antibiotics.

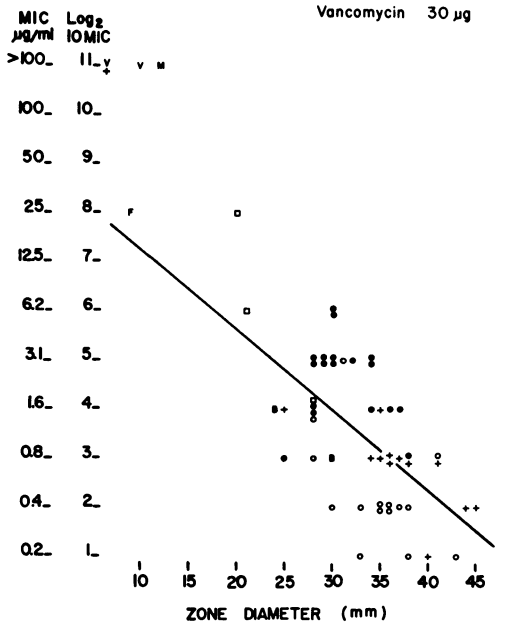

FIG. 8. *Relationship of zone diameters around 30-µg vancomycin disks and MIC values obtained with slow-growing anaerobes. Symbols for bacteria are shown in Fig. 1.*

DISCUSSION

The main objective of this study was to standardize a disk diffusion method for slow-growing anaerobes. The numbers of strains within groups such as gram-negative cocci, *Bifidobacterium*, *Clostridium*, and *Fusobacterium* were small, therefore conclusions regarding susceptibility patterns should not be drawn until more data for each group have been obtained.

Since there is evidence to indicate that strains which have been isolated from clinical specimens are quite tolerant to exposure to air during subculture (1,3; F. P. Tally, P. R. Stewart, J. E. Rosenblatt, and V. L. Sutter, Abstr. Annu. Meet. Amer. Soc. Microbiol. 1973, M60, p. 83), all manipulations of the tests were carried out in air to standardize the method under the simplest conditions possible.

Slow-growing anaerobes such as *Peptococcus*, *Peptostreptococcus*, many *Eubacterium*, and representatives of a number of other genera, account for approximately half of the isolates recovered from clinical specimens (2). Generally, it is thought that there is a high level of predictability of antimicrobial susceptibility of anaerobic organisms; this is the empirical basis for the initial therapy of anaerobic infections.

TABLE 3. *Variation of zone diameters with Peptococcus magnus (2508)*[a]

Antibiotic	Disk potency (µg)[b]	MIC (µg/ml)[b]	Zone diameter (mm)		
			Minimum-maximum	Mean	SD[c]
Penicillin G	10	0.1	40–47	44	1.58
Lincomycin	10	0.8	29–34	32	1.70
Clindamycin	2	0.8	14–20	17	1.46
Clindamycin	10	0.8	28–36	31	1.53
Tetracycline	30	0.8	33–43	38	2.21
Doxycycline	30	0.8	33–45	38	2.61
Minocycline	30	0.1	36–45	41	2.59
Chloramphenicol	30	3.1	29–37	34	1.81
Vancomycin	30	0.8	32–38	35	1.39

[a] Wadsworth Anaerobic Bacteriology Research Laboratory Culture Collection Number.
[b] Penicillin G expressed in units; MIC of penicillin G in units per milliliter.
[c] SD, Standard deviation.

TABLE 4. *Estimates of susceptibility by inhibition zone diameter measurements*

Antibiotic	Disk potency (μg)[a]	Zone diameter (mm)		
		Resistant	Intermediate or equivocal	Susceptible
Penicillin G	10		20–28	≧29
Lincomycin	10	≦12	13–18	≧19
Clindamycin	2		6–12	≧13
Clindamycin	10	6–13	14–23	≧24
Tetracycline	30	≦17	18–31	≧32
Doxycyline	30	≦18	19–24	≧25
Minocycline	30	≦16	17–27	≧28
Chloramphenicol	30			≧15
Vancomycin	30	≦12	13–20	≧21

[a] Penicillin G is expressed in units.

TABLE 5. *Equivocal predictions of susceptibility*[a]

Antibiotic disk	Zone diameter criteria	MIC (R)		MIC (I)		MIC (S)	
		E	S	R	S	E	R
Penicillin G (10 U)	Table 4			0	0	1	0
	B. fragilis[b]			0	1	0	0
Lincomycin (10 μg)	Table 4	4	0	0	0	1	0
	B. fragilis	2	0	0	1	1	0
Clindamycin (10 μg)	Table 4	0	0	0	0	1	0
	B. fragilis	0	0	0	1	0	0
Tetracycline (30 μg)	Table 4	0	0	0	0	3	0
	B. fragilis	0	0	0	1	1	0
Vancomycin (30 μg)	Table 4	1	0	0	0	0	0
	B. fragilis	1	0	0	0	1	0

[a] R, Resistant; I, intermediate susceptibility; S, susceptible; E, equivocal. Results show the number of strains predicted in each category.

[b] See references 6 and 7.

Recent reports indicate that these patterns of susceptibility are changing. A number of anaerobes, including anaerobic cocci and eubacteria, are resistant to tetracycline (2, 10); the data presented here substantiate this. A few strains of peptococci and *Bacteroides melaninogenicus* appear to be somewhat resistant to penicillin G, having MICs of 12.5 U or more per ml (2; V. L. Sutter et al., unpublished data). Resistance to clindamycin among some of the clostridia and fusobacteria has also been reported (2, 9, 11).

Although routine susceptibility testing of anaerobic or other fastidious organisms is not recommended, there are circumstances when such testing is relevant to proper management of the clinical situation. In serious infections such as endocarditis and brain abscess, the antimicrobial susceptibility of the individual isolate is of importance. For those laboratories ill-equipped to do dilution studies, a disk diffusion test would give relatively accurate preliminary information with the most commonly isolated anaerobes (*B. fragilis* and anaerobic cocci). Quantitative susceptibility studies could then be done by a reference laboratory.

ACKNOWLEDGMENTS

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