Standardized Antimicrobial Disc Susceptibility Testing of Anaerobic Bacteria: In Vitro Susceptibility of *Clostridium perfringens* to Nine Antibiotics¹

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The in vitro susceptibility of 43 strains of *Clostridium perfringens* to nine antibiotics was determined by a standardized test for rapid-growing anaerobes. Good correlation was established between the agar dilution susceptibility and the disc diffusion susceptibility results. The inhibition zone diameters around the antibiotic discs, however, were generally much smaller than those of gram-negative anaerobes previously studied. All of the strains tested were susceptible in vitro to chloramphenicol, clindamycin, doxycycline, minocycline, penicillin, and vancomycin. Erythromycin showed poor in vitro activity against this organism, with only 7% of the strains susceptible, 72% intermediate in susceptibility, and 21% resistant. In tests of the 43 strains against lincomycin, 58% were susceptible, 32.5% were intermediate in susceptibility, and 9.5% were resistant. Against tetracycline, 37% of the strains were intermediate in susceptibility and the rest were susceptible.

Clostridium perfringens is said to be more widespread in nature than any other pathogenic bacterium (11), and human infections caused by this organism have been well described. Previous studies have shown that this organism is susceptible in vitro to a variety of commonly used antimicrobial agents (2, 5, 6, 8, 10, 15, 16). However, the constant, widespread use of antibiotics in the practice of both human and veterinary medicine necessarily entails the risk of the development of antibiotic resistance among some strains of *C. perfringens*. Indeed, we may be witnessing the beginning of such a phenomenon, particularly concerning the antibiotic tetracycline (4, 5; A. C. Sonnenwirth, St. Louis, Mo., personal communication).

We have studied the in vitro susceptibility of recently isolated strains of *C. perfringens* to nine antibiotics, including some commonly used drugs as well as newer ones and analogues of older antibiotics. The relationship of the diameter of the inhibition zones around antibiotic-containing discs to the minimal inhibitory concentration (MIC) was also assessed.

MATERIALS AND METHODS

Bacterial strains. Forty-three strains of *C. perfringens* isolated from 1968 through 1971 were used in the study. Seven of these were clinical isolates and the rest were from normal fecal flora. All of the strains were originally isolated from media that did not contain antibiotics.

Antibiotics. The antibiotics (standard powders and discs) used were as follows: penicillin G sodium (standard powder from Eli Lilly & Co., Indianapolis, Ind.; 2- and 10-unit discs from BBL), tetracycline hydrochloride (standard powder from Pfizer Inc., New York, N.Y.; 30- μ g discs from BBL), minocycline hydrochloride (standard powder and 30- μ g discs from Lederle Laboratories, Pearl River, N.Y.), doxycycline hyclate (standard powder from Pfizer Inc.; 30- μ g discs from BBL), erythromycin estolate (standard powder from Eli Lilly & Co.; 15- μ g discs from BBL), chloramphenicol (standard powder from Parke, Davis & Co., Detroit, Mich.; 30- μ g discs from BBL), lincomycin hydrochloride (standard powder from The Upjohn Co., Kalamazoo, Mich.; 2- and 10- μ g discs from BBL), clindamycin hydrochloride hydrate (standard powder and 2- and 10- μ g discs from The Upjohn Co.), and vancomycin hydrochloride (standard powder from Eli Lilly & Co.; 5- and 30- μ g discs from BBL).

Procedures. The procedures used for both the

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agar dilution susceptibility and disc diffusion studies were the same as the standardized methods described by Sutter, Kwok, and Finegold (14). Briefly, the strains of *C. perfringens* studied and two strains of *Bacteroides fragilis* used as controls were allowed to grow for 4 to 5 hr in Thioglycollate medium without indicator -135C (BBL) to which was added 5 μ g of hemin per ml prior to autoclaving and 1 mg of NaHCO_3 per ml plus 0.5 μ g of filter-sterilized menadione per ml after autoclaving. For the agar dilution studies, twofold dilutions of the antibiotics were freshly made in *Brucella* agar containing 0.5 μ g of menadione per ml and 5% laked sheep blood. The inocula were adjusted to the no. 1 McFarland nephelometer standard and then applied to the surface of the plates by means of the Steers, Foltz, and Graves replicating device (12).

For the disc diffusion tests, the 4- to 6-hr cultures were diluted to the density of one-half the turbidity of the no. 1 McFarland standard and applied with cotton swabs to freshly prepared *Brucella* agar plates containing 5% defibrinated sheep blood and 0.5 μ g of menadione per ml. Petri dishes 100 by 15 mm were used; the agar depth was 5 to 6 mm, and not more than four antibiotic discs were applied on each plate.

The plates were incubated at 37 C in GasPak jars containing palladium-coated alumina catalysts. The agar-dilution tests were read after about 48 hr of incubation, and the inhibition zone diameters around the antibiotic discs were measured by use of a Vernier caliper after about 24 hr of incubation.

RESULTS

The strains were classified as susceptible, intermediate, or equivocal in susceptibility, or as resistant to the antibiotic tested according to their MIC by the agar-dilution method and the standards shown in Table 1. A summary of the susceptibilities of the strains tested is shown in Table 2. Regression lines were calculated by the method of analysis of least squares (1), and interpretations of the diameters of zones of inhibition as related to the MIC are indicated in Table 3.

Penicillin G. The MIC of penicillin G against all of the 43 strains tested was 0.8 units or less/ml (range, 0.1 to 0.8 units/ml), and all of the strains were classified as susceptible. No regression line could be drawn, as all the strains tested were susceptible over a very narrow range of antibiotic concentrations. With a 2-unit disc, all strains tested had inhibition zone diameters of more than 20 mm, with a range of 21 to 29.5 mm. With a 10-unit disc, all of the strains had inhibition zone diameters of more than 24 mm, with a range of 24 to 34.5 mm.

Tetracycline. The MIC of tetracycline against 27 of the 43 strains tested (63%) was 1.6 μ g or less/ml; these strains were classified as susceptible (Fig. 1). The remaining strains (37%) were

TABLE 1. *Classification of organisms according to the degree of susceptibility to antibiotics based on the minimal inhibitory concentrations (MIC)*

Antibiotic	MIC (μ g/ml) ^a		
	Susceptible ^b	Intermediate or equivocal ^c	Resistant
Penicillin G.....†	0.8 or less	1.6-6.2	12.5 or more
Tetracycline.....	1.6 or less	3.1-6.2	12.5 or more
Doxycycline.....	1.6 or less	3.1-6.2	12.5 or more
Minocycline.....	1.6 or less	3.1-6.2	12.5 or more
Erythromycin...	1.6 or less	3.1	6.2 or more
Chlor- amphenicol...	12.5 or less		
Lincomycin.....	3.1 or less	6.2	12.5 or more
Clindamycin.....	3.1 or less	6.2	12.5 or more
Vancomycin.....	6.2 or less	12.5	25 or more

^a Penicillin G expressed in units per milliliter.

^b Blood concentrations achieved with ordinary dosage schedules of the drug.

^c Blood contractions achieved with parenteral or high dosage schedules of the drug, without risk of major toxicity.

intermediate or equivocal in susceptibility (MIC, 3.1 to 6.2 μ g/ml). Except for two strains whose inhibition zone diameters around the 30- μ g disc overlapped with those of the intermediate group, all of the susceptible strains had zone diameters greater than 21 mm, with an actual range of 23 to 31 mm. Those of intermediate susceptibility had zone diameters less than 21 mm, with a range of 16.5 to 20.5 mm.

Doxycycline. All of the 43 strains tested were susceptible to 0.4 μ g or less of doxycycline/ml, with 23 of the 43 strains (or 53.5%) susceptible to 0.05 μ g or less/ml. The inhibition zone diameters around the 30- μ g disc all exceeded 21 mm, with a range of 22 to 37.5 mm. Since all of the strains were susceptible over a very narrow range of antibiotic concentration, no regression line could be constructed.

Minocycline. All of the 43 strains tested were susceptible to 0.2 μ g or less of minocycline/ml, and 30 of the 43 (or 69.8%) were susceptible to 0.05 μ g or less/ml. The inhibition zone diameters around the 30- μ g disc ranged from 23.0 to 37.5 mm. Again, as with doxycycline, no regression line could be drawn.

Erythromycin. Only 3 of the 43 strains tested (7%) were susceptible to erythromycin, with an MIC of 1.6 μ g/ml. One of these three strains had an inhibition zone diameter of 18.5 mm and two had zone diameters of 23 mm. Of the 43 strains, 31 (75%) were intermediate in susceptibility, with an MIC of 3.1 μ g/ml and an inhibition zone diameter range of 17 to 21.5 mm. These zone diameters overlapped with those of the resistant strains and one of the susceptible

TABLE 2. Susceptibility of *Clostridium perfringens* to antimicrobial agents^a

Antibiotic	Cumulative percent susceptible at various concentrations ($\mu\text{g/ml}^b$)									
	≤ 0.05	0.1	0.2	0.4	0.8	1.6	3.1	6.2	12.5	25
Penicillin G		5	28	81	100					
Tetracycline	2	51	58			63	84	100		
Doxycycline	53	56	84	100						
Minocycline	70	98	100							
Erythromycin						7	79	98	100	
Chloramphenicol							98	100		
Lincomycin				16	47	49	58	91	98	100
Clindamycin	21	44	47	53	58	67	100			
Vancomycin				7	98	100				

^a Forty-three strains tested.^b Penicillin G expressed in units per milliliter.TABLE 3. Estimation of susceptibility of *Clostridium perfringens* to antibiotics based on diameters of zones of inhibition

Antibiotic	Disc potency	Diam of zone of inhibition (mm)		
		Resistant	Intermediate or equivocal	Susceptible
Penicillin G	2 units			21 or more
	10 units			24 or more
Tetracycline	30 μg		17-21	23 or more
Doxycycline	30 μg			22 or more
Minocycline	30 μg			23 or more
Erythromycin	15 μg		17-22	23 or more
Chloramphenicol	30 μg			21 or more
Lincomycin	2 μg		7-13	14 or more
	10 μg	11 or less	12-17	22 or more
Clindamycin	2 μg			9 or more
	10 μg			15 or more
Vancomycin	5 μg			16 or more
	30 μg			19 or more

strains. Nine of 43 (21%), with an MIC of 6.2 to 12.5 $\mu\text{g/ml}$, were classified as resistant. Their inhibition zone diameters ranged from 16.5 to 18.5 mm. The MIC values were distributed over a narrow range of antibiotic concentrations, and no regression lines could be drawn.

Chloramphenicol. All of the 43 strains tested were susceptible to chloramphenicol. There was remarkable uniformity of results, as all but one strain had an MIC of 3.1 $\mu\text{g/ml}$. The exception had an MIC of 6.2 $\mu\text{g/ml}$. All strains had inhibition zone diameters of more than 20 mm, with a range of 21 to 30.5 mm.

Lincomycin. Against 25 of the 43 strains studied (58%), the MIC of lincomycin was 3.1

FIG. 1. Relationship of inhibition zone diameters around 30- μg tetracycline discs and MIC values with 43 strains of *Clostridium perfringens*. Least squares line for $y = a + bx$; $b = -0.55$; $a = 15.3$.

FIG. 2. Relationship of inhibition zone diameters around 2- μ g lincomycin discs and MIC values with 43 strains of *Clostridium perfringens*. Least squares line for $y = a + bx$; $b = -0.3$; $a = 8.8$.

μ g or less/ml; these strains were classified as susceptible to lincomycin. With a 2- μ g disc (Fig. 2), all of these susceptible strains except for the four with an MIC of 3.1 μ g/ml had inhibition zone diameters measuring greater than 13 mm, with an actual range of 13.5 to 24 mm. Fourteen of the strains (32.5%) had an MIC of 6.2 μ g/ml and were classified as intermediate or equivocal in susceptibility. The inhibition zone diameters of the intermediate strains overlapped with those of the resistant strains (as well as with those of four susceptible ones). Four of the 43 (9.5%) were resistant, with an MIC of 12.5 to 25 μ g/ml and inhibition zone diameters ranging from 7 to 9 mm.

With the 10- μ g disc (Fig. 3), all of the susceptible strains except two with an MIC of 3.1 μ g/ml had inhibition zone diameters of greater than 17.5 mm, with an actual range of 22 to 30.5 mm. The intermediate group had zone diameters that also overlapped with those of the resistant group but with only two of those of the susceptible strains. The resistant strains had inhibition zone diameters ranging from 10.5 to 14.5 mm.

Clindamycin. All of the 43 strains tested were susceptible to 3.1 μ g or less of clindamycin/ml.

The MIC for 14 of the 43 (32.5%) was 3.1 μ g/ml. With a 2- μ g disc (Fig. 4), all of the strains studied had inhibition zone diameters of 9 mm or more, with a broad range of 9 to 30 mm. Thus, a few of the susceptible strains had inhibition zone diameters only a few millimeters larger than the disc diameter itself.

With a 10- μ g disc (Fig. 5), all of the strains tested had inhibition zone diameters of more than 14 mm, with a range of 15 to 34 mm.

Vancomycin. All of the 43 strains tested were susceptible to 1.6 μ g or less of vancomycin/ml. The MIC for 39 of the strains (90.5%) was 0.8 μ g/ml. With a 5- μ g disc, the inhibition zone diameters ranged from 16 to 20.5 mm. With a 30- μ g disc, they ranged from 19 to 24.5 mm.

DISCUSSION

Recent studies in this laboratory have been directed toward standardization of a disc diffusion method for susceptibility testing of anaerobes (14). Our results indicate that the standardized antimicrobial disc susceptibility test for rapidly growing anaerobes can be applied to *C. perfringens*. In general, the inhibition zone diameters around the antibiotic discs were smaller than

FIG. 3. Relationship of inhibition zone diameters around 10- μ g lincomycin discs and MIC values with 43 strains of *Clostridium perfringens*. Least squares line for $y = a + bx$; $b = -0.3$; $a = 10.3$.

FIG. 4. Relationship of inhibition zone diameters around 2- μ g clindamycin discs and MIC values with 43 strains of *Clostridium perfringens*. Least squares line for $y = a + bx$; $b = -0.39$; $a = 9.2$.

those observed with gram-negative anaerobes (V. L. Sutter, Y. Y. Kwok, and S. M. Finegold, Abstr. Annu. Meeting-1972, p. 95; Y. Y. Kwok, V. L. Sutter, and S. M. Finegold, Abstr. Annu. Meeting-1972, p. 95). This observation may be a function of the faster growth rate exhibited by this organism.

In the studies in which there were comparatively wide ranges of antibiotic susceptibility, there was generally good separation between the inhibition zone diameters of the susceptible strains on one hand and those of the intermediate and resistant strains on the other. Separation between the zone diameters of the latter two groups, however, was not clear-cut.

In the clindamycin study, although all of the strains tested were found to be uniformly susceptible, there were a few strains that produced inhibition zone diameters around the 2- μ g disc that were only slightly larger than the disc diameter itself. The problems that may arise with the 2- μ g disc test in the event of the occurrence of resistant strains are therefore obvious. This problem may be avoided by the use of the 10- μ g disc.

There was slightly less overlap between the inhibition zone diameters of the susceptible and intermediate strains and between the intermediate and resistant strains when the 10- μ g

FIG. 5. Relationship of inhibition zone diameters around 10- μ g clindamycin discs and MIC values with 43 strains of *Clostridium perfringens*. Least squares line for $y = a + bx$; $b = -0.45$; $a = 13$.

lincomycin disc was used than when the 2- μ g disc was used. The significance of these slight differences, however, is uncertain.

Although Table 3 indicates an interpretation of the diameters of zones of inhibition based on the results of this study, it is too early to accept these as standards for all anaerobes. A vast amount of additional data with other *Clostridium* species as well as other rapidly growing anaerobes must be accumulated before interpretive standards can be developed.

Penicillin G, chloramphenicol, and vancomycin are still uniformly active in vitro against *C. perfringens*. The previous observations of the activity of clindamycin against *C. perfringens* (5, 7) are confirmed by our studies. The organism, however, is developing in vitro resistance to tetracycline. Accordingly, this antibiotic should not remain as one of the foremost alternates to penicillin G in clinical situations. However, all of the strains tested (including those of intermediate susceptibility to tetracycline) were exquisitely sensitive to the newer tetracycline analogues doxycycline and minocycline. These findings support the previous reports of differ-

ences in the in vitro activity of tetracycline and its analogues (13). Further studies along this line are indicated.

Our results with erythromycin, in particular, but with lincomycin as well, were quite disappointing. There is a possibility, however, that the pH changes brought about by the presence of CO₂ in our anaerobic jars may have affected the activity of these antibiotics and thus have influenced the results (3). Results with these antibiotics differ from those of Martin and co-workers (5) in that their strains appeared more susceptible to both erythromycin and lincomycin. The problem of pH change in the presence of CO₂ cannot account for this difference, because both studies utilized a GasPak atmosphere with an incubation period of 48 hr. Major differences in methodology, such as age of inoculum and medium for testing, may account for these differences as well as others between the two studies. Other factors, such as sources of strains tested, patient population, and locality of institutions, probably also play a role, because with clindamycin our strains appeared to have about the same susceptibility as did theirs, and with the other drugs our strains appeared more resistant.

There are numerous problems associated with reliable susceptibility testing of anaerobes. In addition to factors which influence results of tests with aerobic bacteria, such as age and density of inoculum and incubation period, conditions inherent in or commonly used for growth of anaerobic bacteria may affect results of susceptibility tests. Components used in media for anaerobes may also influence them. Anaerobiosis itself, the presence and amount of carbon dioxide in the atmosphere, humidity, pH of media (both initial and subsequent), and blood and other materials added to media all undoubtedly influence the results (9). The degree of influence appears to vary with different drugs and organisms (3, 9). The exact definition and significance of such effects remain to be determined.

Because of the necessity to use special conditions to obtain satisfactory growth of many anaerobes, our approach has been to standardize the techniques and conditions of the test as carefully as possible and to determine the variability of results in such situations. Once the variability is known for each antimicrobial agent, then the effect that each factor (such as presence

of CO₂, blood, and others) has on the activity of antimicrobial agents can be determined.

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