Susceptibility of *Clostridium ramosum* to Antimicrobial Agents

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Received for publication 25 January 1974

The susceptibility of 49 strains of *Clostridium ramosum* to 10 antibiotics was determined by agar dilution and disk diffusion tests. Results showed that, among the anaerobes, *C. ramosum* is second only to *Bacteroides fragilis* in its resistance to antimicrobial agents. All strains were susceptible to penicillin, carbenicillin, chloramphenicol, vancomycin, and metronidazole at readily achievable blood levels. Most strains (83%) were susceptible to erythromycin. There was a high level of resistance to clindamycin in 16% of the strains. All isolates were resistant to rifampin and gentamicin, and most were resistant to lincomycin. Assessment of susceptibility by measurement of inhibition zone diameters with disk diffusion tests was not satisfactory.

Clostridium ramosum was first isolated from the pus of a patient with pulmonary gangrene and appendicitis and named *Bacillus ramosum* by Veillon and Zuber in 1898 (18). It was renamed *Ramibacterium ramosum* and was so designated until its ability to form spores was demonstrated in 1971 (6). It is present in high numbers in the normal fecal flora of man (1). The pathogenic potential of *C. ramosum* was reviewed by Prevot in 1949 (10). Recently, Thadepalli et al. found *C. ramosum* to be the clostridial species most frequently isolated from patients with wound infections after intraabdominal trauma (17). Inadequate identification techniques account for many failures to recognize this organism. We are currently preparing a manuscript on clinical significance of our isolates and the phenotypic characteristics utilized for the identification of *C. ramosum*.

With widespread use of antimicrobial agents there has been an emergence of resistance in anaerobic bacteria (8, 13). The resistance of *C. ramosum* to rifampin was noted in 1971 (12). There is significant resistance of this organism to tetracycline and doxycycline with good correlation between the agar diffusion and disk diffusion tests (15). Recent studies of anaerobic gram-positive bacilli in the Wadsworth Anaerobic Laboratory suggested that *C. ramosum* is resistant to other antimicrobial agents. Therefore, in cooperation with the Anaerobic Laboratory of the Center for Disease Control (CDC) in Atlanta, we undertook studies of the in vitro

susceptibility of recently isolated strains of *C. ramosum* to 10 antimicrobial compounds. The relationship of the diameter of the inhibition zone around antibiotic-containing disks to the minimal inhibitory concentration (MIC) was also assessed.

MATERIALS AND METHODS

Bacterial strains. Forty-nine strains of *C. ramosum* were included in the study: 32 strains from the Anaerobic Laboratory Collection of the CDC, Atlanta, Ga.; 16 strains from the Wadsworth Anaerobic Laboratory (WAL) Collection, and the neotype strain ATCC-25582. Thirty-three strains were isolated from clinical specimens, thirteen from normal fecal flora specimens, and the source of three strains, including the neotype strain, is unknown. Four strains from normal feces were isolated on rifampin selective medium, and the remaining strains were isolated on non-antibiotic-containing media.

Antimicrobial drugs. The drugs tested include those currently being used for treatment of anaerobic infections, analogues of older antibiotics, and one antimicrobial agent currently being used experimentally for anaerobic infections.

The following drugs were used: penicillin G, carbenicillin, chloramphenicol, erythromycin, vancomycin, metronidazole, rifampin, gentamicin, lincomycin, and clindamycin.

Procedures. The procedures used for both agar dilution and disk diffusion susceptibility studies were the same as the standardized methods previously described (13), except that vitamin K₁ was substituted for menadione. Briefly, the strains of *C. ramosum* studied, and one strain of *Bacteroides fragilis*

WAL #1887 (used as a control), were grown for 4 to 6 h in thioglycolate broth without indicator (BBL 135C) to which was added 5 μ g of filter-sterilized vitamin K₁ per ml after autoclaving. For the agar dilution studies, twofold dilutions of the drugs were freshly made in Brucella agar (Pfizer Diagnostics) containing 10 μ g of vitamin K₁ per ml and 5% laked sheep blood. The inocula were adjusted to the No. 1 McFarland nephelometer standard and applied to the surface of the plates by means of the Steers replicating device (10). The MIC was the lowest concentration of antibiotic showing no macroscopic growth.

For disk diffusion tests, the 4- to 6-h cultures were diluted to the density of the Bauer et al. standard (10⁶ viable U/ml) (3) and applied with cotton swabs to freshly prepared Brucella agar (Pfizer Diagnostics) plates containing 5% defibrinated sheep blood and 10 μ g of vitamin K₁ per ml. Petri dishes (90 by 15 mm) were used; the agar depth was 5 to 6 mm, and not more than four antibiotic disks were applied on each plate.

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

RESULTS

The strains were classified as susceptible, intermediate, or resistant to the drugs tested according to the criteria shown in Table 1. A summary of the agar dilution susceptibility results with the 10 antimicrobial agents, together with previously published tetracycline and doxycycline data (included for comparison), is presented in Table 2. The MIC values were correlated with inhibition zone diameters, and regression lines were calculated by the method

of analysis of least squares; results are shown in Table 3. Figure 1 exhibits the lack of correlation between agar and disk diffusion in the case of carbenicillin. The data plots with the other drugs were similar.

Penicillin G. The MIC of penicillin G against all strains tested was 6.2 units or less per ml. Twenty-four strains (48%) were classified as susceptible, and 25 strains were classified as intermediate. The zone diameters of the susceptible and intermediate strains overlapped, so definitive break points based on zone diameter could not be established.

Carbenicillin. All the strains tested were susceptible to 50 μ g of carbenicillin or less per ml. Prediction of susceptibility by zone diameter measurements with the 50- μ g disk was unsatisfactory.

Chloramphenicol. All the 49 strains tested had MICs of 6.2 μ g or less per ml and are classed as susceptible. The MICs of the isolates fit into a narrow range with most isolates susceptible to 3.1 or 6.2 μ g/ml. Most zone diameters were between 23 and 35 mm, with one zone of 19 mm and one zone of 39 mm.

Lincomycin. With 48 of the 49 tested, the MIC of lincomycin was 12.5 μ g or greater per ml; these were classified as resistant. One strain had intermediate susceptibility. With the 2- μ g lincomycin disk, all but one strain failed to show any inhibition of growth. With the 10- μ g disk, zone sizes for the resistant strains were from 6 to 20 mm, and the one intermediate strain had a zone diameter of 21 mm.

Clindamycin. Against 6 (12%) of the 49 strains tested, the MIC of clindamycin was 1.6 μ g or less per ml. Thirty-five strains (72%) were

TABLE 1. Classification of susceptibility to antimicrobials based on MIC^a

Antibiotic	MIC (μ g/ml) ^b		
	Susceptible (or less)	Intermediate	Resistant
Penicillin G	0.8	1.6 to 12.5	25 or more
Carbenicillin	50	100 to 200	More than 200
Chloramphenicol	12.5		25 or more
Lincomycin	3.1	6.2 to 12.5	25 or more
Clindamycin	1.6	3.1 to 6.2	12.5 or more
Vancomycin	6.2	12.5	25 or more
Erythromycin	1.6	3.1	6.2 or more
Metronidazole	6.2	12.5 to 50	100 or more
Rifampin	3.1	6.2	12.5 or more
Gentamicin	1.6	3.1 to 6.2	12.5 or more

^a This arbitrary classification is based on the assumption that strains with MIC equivalent to: blood levels readily achieved with conventional dosage are susceptible; those achieved with higher dosage are intermediate in their degree of susceptibility; and those which are not readily achieved without the hazard of toxicity are resistant.

^b Penicillin G is expressed in units per milliliter.

TABLE 2. Susceptibility of *C. ramosum* to antimicrobial agents

Antibiotic	No. of strains tested	Cumulative percent susceptible to indicated concn ($\mu\text{g/ml}^a$)												
		≤ 0.05	0.1	0.2	0.4	0.8	1.6	3.1	6.2	12.5	25	50	100	> 100
Penicillin G	49		4	6	22	49	82	94	100					
Carbenicillin	49				2	10	20	40	62	83	96	100		
Chloramphenicol	49						6	60	100					
Lincomycin	49								2	34	65	81	85	100
Clindamycin	49				4	6	12	30	84	88				100
Erythromycin	48					50	69	84						100
Vancomycin	49							49	98	100				
Metronidazole	48				2	4	20	50	76	94	100			
Rifampin	49													100
Gentamicin	49												72	100
Tetracycline ^b	47		2	24	26	40	44	49	53		55	76	93	100
Doxycycline ^b	47	17	34	38	50	55	58		64	92	97	100		

^a Penicillin G expressed in units per milliliter.^b Results from previously published data included for comparison (15).TABLE 3. Relationship between MIC and inhibition zone diameters^a

Antibiotics	Disk potency	Least square line ($y = a + b x$)	Correlation coefficient	SE of Estimate	F	P
Penicillin	10 units	8.537 -0.188	0.615	1.715	0.378	$P > 0.05$
Carbenicillin	50 μg	11.245 -0.176	0.441	1.988	1.147	$0.5 > P > 0.25$
Lincomycin	10 μg	9.837 -0.156	0.581	1.205	0.603	$0.1 > P > 0.75$
Clindamycin	2 μg	8.829 -0.233	0.675	1.419	2.980	$0.01 > P > 0.005$
	10 μg	9.754 -0.207	0.706	1.432	2.412	$0.025 > P > 0.01$

^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.

classified as intermediate, with MICs of 3.1 to 6.2 $\mu\text{g/ml}$. The remaining eight strains (16%) were resistant to clindamycin, requiring from 12.5 $\mu\text{g/ml}$ to greater than 100 $\mu\text{g/ml}$. With the 1- μg disk, the zone sizes were all less than 25 mm, with appreciable overlap between susceptible and intermediate strains. With the 10- μg disk, the zone diameters were larger (from 15 to 32 mm), but overlap between the susceptible and intermediate strains also occurred.

Erythromycin. Of the 48 strains, 69% were susceptible, and an additional 15% showed an intermediate degree of susceptibility. Seven strains were resistant; the MICs were greater than 100 $\mu\text{g/ml}$. With the 15- μg disk, zone diameters for the susceptible isolates were from 21 to 31 mm; there was no zone of inhibition with resistant isolates.

Vancomycin. For 48 (98%) of the 49 strains tested, the MIC of vancomycin was 6.2 μg or less per ml; these were classified as susceptible. One strain was intermediate. With all isolates, the 30- μg disk produced zone sizes ranging from 16 to 25 mm. With the one intermediate strain, the zone size was 20 mm.

Metronidazole. For 37 (76%) of the 48 strains

tested, the MIC of metronidazole was 6.2 μg or less per ml; these were classified as susceptible. The remaining strains were intermediate. There were no disks available for comparison of MIC and zone size.

Rifampin. All 49 strains tested were resistant to greater than 100 μg of rifampin per ml, and there was no inhibition of growth around the 15- μg disks.

Gentamicin. All 49 strains tested were resistant to 100 μg or more of gentamicin. With the 10- μg disk, there were occasional small inhibition zones (8 mm or less).

Control. The susceptibility of the *B. fragilis* control strain was within the limits of variability previously published (14).

DISCUSSION

There is a paucity of data in the literature on the susceptibility of *C. ramosum* to antimicrobial agents. Martin et al. (8) tested three strains of *Catenabacterium filamentosum* (now classified as *C. ramosum*) and found them susceptible to most antibiotics. Recently, Wilkins found four of 10 strains of *C. ramosum* resistant to clindamycin (19). Our in vitro

FIG. 1. Relationship of zone diameters around 50- μ g carbenicillin disks and MIC values. Carbenicillin, 50 μ g; *C. ramosum*, 47 strains.

susceptibility studies, a portion of which has been previously published, indicate that, of the anaerobic bacteria, *C. ramosum* is second only to *B. fragilis* in its resistance to various antimicrobial agents.

Based on our study, effective treatment of patients with infections involving *C. ramosum* would require high dosages of penicillin. Our data also show that carbenicillin has good activity against *C. ramosum*, but there is essentially no clinical data on treatment of patients with anaerobic infections with carbenicillin.

Our previous studies with tetracycline, referred to above, show that *C. ramosum* is often resistant to this drug, as are other anaerobes (4, 8, 11, 13). Doxycycline in our experience is somewhat more active.

Clindamycin shows good in vitro activity against most anaerobic bacteria and has been used successfully in the treatment of anaerobic infections (2, 15, 17). Recent evidence indi-

cates that, after intravenous administration of clindamycin, blood levels of 6.2 μ g/ml are readily and consistently achieved (5). With this blood level as the upper limit of the susceptibility range, 16% of our isolates of *C. ramosum* would be considered resistant.

The in vitro susceptibility of *B. fragilis* to lincomycin and erythromycin has varied widely depending upon the atmosphere in which tests are incubated (7, 14, 19). In our studies, we used an atmosphere containing 10% CO₂ in which, according to Ingham et al., the activity of these two antibiotics is decreased (7). Despite this, erythromycin showed good activity against *C. ramosum*, whereas lincomycin showed very poor activity.

All strains tested were sensitive to chloramphenicol and vancomycin. Chloramphenicol has remained the most consistently active antibiotic in vitro against anaerobic bacteria, but its use must be restricted because of its serious side

effects. Vancomycin, which is not very active against the gram-negative anaerobic bacteria (8), has excellent in vitro activity against *C. ramosum*; however, there has been no clinical evaluation of this drug in anaerobic infections.

Metronidazole, which has excellent in vitro activity against anaerobic bacteria and has given good results in early clinical trials (16), may also prove useful in infections involving *C. ramosum*. All strains were inhibited by 25 µg or less per ml, a level easily achievable with high dose oral therapy. Previous studies have also indicated that this antimicrobial agent is bactericidal against *B. fragilis* (9).

All isolates tested were resistant to greater than 100 µg of rifampin per ml and to 100 µg or more of gentamicin per ml. The incorporation of rifampin into agar plates for use as a selective medium, as described by Sutter et al. (12), facilitates the isolation of *C. ramosum* from mixed clinical specimens and in normal flora studies.

The disk diffusion susceptibility test, as described by Bauer et al. (3) for the aerobic and facultative organisms, is well accepted as a rapid, reliable method. Recent studies in this laboratory directed toward the standardization of a disk diffusion test for certain individual anaerobes, including *B. fragilis* and *C. perfringens*, have been encouraging (11, 13-15). The present studies indicate that the prediction of susceptibility of *C. ramosum* by the disk diffusion test is not satisfactory except in the cases of tetracycline and doxycycline (15).

The reason for the unsatisfactory prediction of susceptibility may have been subtle differences in the growth rates of our strains, which was not apparent during the study. All strains grew rapidly, but neither growth curves nor generation times were determined. Several other growth conditions which affect the measurement of susceptibility of anaerobes by disk diffusion have been reviewed by Sutter et al. and include anaerobiosis, effect of CO₂ on pH of the media, and difficulty in reading indistinct zones on an opaque media (14).

ACKNOWLEDGMENTS

This research was supported by Public Health Service research grant CC 00520 from the Center for Disease Control, Atlanta, Ga., and by grants from The Upjohn Co., The Roerich Co., and G. P. Searle & Co.

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