JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1984, p. 912–916
0095-1137/84/110912-05$02.00/0
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Vol. 20, No. 5

Determination of Susceptibility of Anaerobic Bacteria to Cefotetan and Cefoxitin by the Thioglycolate Disk Elution Method

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Received 30 March 1984/Accepted 13 August 1984

The in vitro activities of two cephamycins, cefotetan and cefoxitin, against 107 anaerobic bacteria were evaluated. The aerobically incubated thioglycolate disk elution technique of Kurzynski et al. (Antimicrob. Agents Chemother. 10:727–732, 1976) was also evaluated to establish the appropriate number of 30-μg disks to be added to each tube, assuming that strains with MICs of ≤16 μg/ml are susceptible. Optimal predictive values were obtained when two tubes were prepared for each antimicrobial agent, one with two 30-μg disks and the other with three 30-μg disks. After 48 h of incubation, resistant strains grew in both tubes and susceptible strains provided no growth or growth ≤50% of that in the control broth. Growth in one tube but not in the other was considered an equivocal test result indicating the need for additional tests; 5 to 7% of our strains gave equivocal results. Reproducibility studies confirmed that broth microdilution tests were more reproducible than disk elution susceptibility tests.

Cefotetan and cefoxitin are both cephamycins having a 7α-methoxy configuration which renders them both relatively resistant to hydrolysis by many β-lactamases (1). Both cephamycins have a broad spectrum of antibacterial activity (1, 2, 6, 10), including activity against anaerobic, aerobic, and facultatively anaerobic bacteria. Cefotetan may prove effective for treating some infections involving anaerobic bacteria (2, 6). Since some anaerobes are resistant to the cephamycins, it may be prudent for clinical laboratories to perform susceptibility tests with selected isolates.

The aerobically incubated thioglycolate disk elution method of Kurzynski et al. (5) is commonly used for testing clinical isolates. This technique is a disk elution test which delivers a drug to 5-ml volumes of thioglycolate by adding one or more disks manufactured for diffusion tests. The purpose of this study was to determine the optimal number of disks that will accurately predict susceptibility to cefotetan and cefoxitin.

MATERIALS AND METHODS

Antimicrobial agents. Cefotetan was kindly provided by Stuart Pharmaceuticals, Div. of ICI Americas, Inc., Wilmington, Del. Cefoxitin was provided by Merck Sharp & Dohme, West Point, Pa. Commercially manufactured disks (BBL Microbiology Systems, Cockeysville, Md.) were utilized for the elution tests. Bioassays of the 30-μg cefotetan and cefoxitin disks demonstrated average potencies of 98 to 103% of labeled content.

Microorganisms. Clinical isolates of anaerobic bacteria were collected from 10 clinical laboratories throughout the United States. Most of the strains were recovered from patients participating in ongoing clinical trials of cefotetan therapy. *Bacteroides thetaiotaomicron* ATCC 29741, *B. fragilis* ATCC 25285, *B. vulgatus* ATCC 29327, and *Clostridium perfringens* ATCC 13124 were also included for internal control. Cefoxitin MICs with the latter control strains were identical to those defined for the National Committee for Clinical Laboratory Standards agar dilution reference method (7).

Microdilution susceptibility tests. The broth microdilution technique of Jones et al. (4) was utilized as the reference procedure for this study. A broth version of Wilkens-Chalgren medium (9) was used to prepare doubling dilutions of either drug (1.0 to 64 μg/ml), with half-dilution interval steps of 6, 12, and 24 μg/ml. The trays were frozen at −40°C and, just before use, allowed to thaw at room temperature. An inoculum of approximately 10^6 CFU/ml was obtained by adjusting the turbidity of an overnight culture in Wilkens-Chalgren broth. MICs were recorded after 48 h of anaerobic incubation in GasPak jars (BBL). For purposes of this study, strains with MICs of ≤16 μg/ml were considered susceptible, those with MICs of 24 or 32 μg/ml were considered intermediate or moderately susceptible, and those with MICs of ≥64 μg/ml were considered resistant. This categorization differs from the current National Committee for Clinical Laboratory Standards breakpoints for aerobes (8), which define the susceptible category as an MIC of ≤8.0 μg/ml and the resistant category as an MIC of >16 μg/ml. The slightly greater breakpoints seem appropriate when the pharmacokinetic characteristics of cefotetan and cefoxitin (3, 11) are considered with the type of dosage schedules likely to be used when treating anaerobic infections, i.e., maximal doses.

Disk elution tests. The broth disk procedure of Kurzynski et al. (5) was performed with thioglycolate medium without indicator (no. 7160; Acumedia, Baltimore, Md.) enriched with vitamin K (0.1 μg/ml) and hemin (5.0 μg/ml) and prepared in 5-ml volumes in screw-capped tubes (13 by 100 mm). Just before use, the thioglycolate was heated in a boiling water bath for 10 min and then rapidly cooled. Cefotetan or cefoxitin disks were added to the boiled and cooled thioglycolate broth and allowed to elute for 2 h before inoculation. Two, three, and four 30-μg disks were eluted to provide theoretical concentrations of 12, 18, and 24 μg/ml, respectively. Antibiotic-containing broths and a growth control broth without antibiotics were each inoculated with 0.1 ml of an 18- to 24-h thioglycolate broth culture. This deviates only slightly from the inoculum used by Kurzynski et al. (5), who used 2 drops of an overnight chopped-meat–glucose broth culture for inoculation.

The thioglycolate cultures were incubated aerobically at 35°C with the screw-caps tightened. At 24 h and 48 h of incubation, the tubes were examined for the presence or absence of any more than a faint haze of growth and for the

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presence of growth greater than 50% of that seen in the growth control. The latter endpoint was used to determine whether the test was positive or negative (resistant or susceptible), but the possibility of using a growth-no growth endpoint was also evaluated.

RESULTS

The 107 anaerobes that were included in this study are described in Table 1. Cefoxitin was generally more active than cefotetan, but the percentage of strains susceptible to 16 μg/ml did not differ greatly (72% susceptible to cefoxitin versus 63% susceptible to cefotetan). Intermediate MICs (24 or 32 μg/ml) were seen with 7 of the 107 strains tested against cefotetan, but 19 of the 107 strains had intermediate susceptibility to cefoxitin. When evaluating a test procedure in which a single breakpoint is used for separating susceptible and resistant populations, it is important to recognize the proportion of challenge strains with MICs at or near that breakpoint. Over half (59%) of our strains were either susceptible to ≤6.0 μg or resistant to 32 μg of cefotetan per ml. With cefoxitin, 58% of the strains showed MICs of <8.0 or >32 μg/ml. With both cephamycins, MICs of 16 μg/ml ± 1 doubling dilution were obtained with 41 to 42% of the challenge strains.

The results of disk elution tests are summarized in Table 2. A larger proportion of false-susceptible test results was recorded when the tests were read after 24 h of incubation than after 48 h. This result is not surprising, since our reference MICs were recorded after 48 h of incubation. If the disk elution tests had been read as negative when there was no growth or only a faint haze of growth, the number of false-susceptible tests would have been decreased and false-resistant tests would have been increased, but the overall agreement with MIC categories would not have changed appreciably. Optimally, the thioglycolate disk tests should be read for turbidity >50% of that in the growth control after 48 h of incubation. After overnight incubation, strains showing definite turbidity may be reported to be resistant to the study drug, but susceptibility should not be reported until 48 h have elapsed.

Additional analysis was based on the assumption that the one-tube disk elution test should separate susceptible strains (MIC, ≤16 μg/ml) from those that are not susceptible (MIC, >16 μg/ml); i.e., intermediate or moderately susceptible strains are considered resistant, since a single concentration is being tested. The predictive values of positive and negative cefoxitin disk elution tests were calculated (Table 3). Inhibition in a single tube with 30-μg cefoxitin disks had a 93.6% predictive value (6.4% of the negative strains were resistant by the microdilution method), but growth in the same tube had only an 86.2% predictive value (13.8% of the positive strains were susceptible by the microdilution method). With more disks, the predictive value for a positive test improved but the predictive value of a negative result decreased.

To optimize the disk elution test, one can prepare two tubes, one with two disks and the other with three disks. Growth in both tubes had 95.2% predictive value, and inhibition in both tubes had a 93.6% predictive value. Inhibition in one tube but not in the other should be considered an equivocal result rather than indicating intermediate susceptibility. Such equivocal tests occurred with eight (7.5%) or our isolates. When other combinations of two and four or three and four disks were evaluated, the proportion of equivocal results was increased without significant improvement in the predictive values.

Similar data with cefotetan disk elution tests are presented in Table 4. Two tubes, one with two 30-μg disks and the other with three 30-μg disks, provided optimal results, and only five (4.7%) of our isolates gave equivocal results.

Reproducibility of thioglycolate disk tests and of the microdilution MICs was evaluated by testing 21 selected isolates each on three separate days. The isolates included 14 *B. fragilis*, 2 *B. thetaiotaomicron*, 2 *B. melaninogenicus*, 1 *B. distasonis*, 1 *B. vulgatus*, and 1 *B. ovatus* isolates.

The range of variability between triplicate MICs of cefoxi-

TABLE 1. Susceptibilities of anaerobic isolates used to evaluate the thioglycolate disk elution procedure

Species (no. tested)	Antimicrobial agent	No. of isolates with MIC (μg/ml) of:								
		≤4	6	8.0	12	16	24	32	64	>64
B. fragilis (39)										
	Cefotetan	0	2	3	16	10	2	1	2	3
	Cefoxitin	11	2	11	8	1	5	1	0	0
Other *B. fragilis* group (26)[a]										
	Cefotetan	1	0	0	0	1	0	1	5	18
	Cefoxitin	2	0	3	0	1	10	3	3	4
Other *Bacteroides* species (19)[b]										
	Cefotetan	7	4	1	2	1	1	2	0	1
	Cefoxitin	15	1	2	0	0	0	0	1	0
Gram-positive cocci (12)[c]										
	Cefotetan	8	1	0	2	0	0	0	1	0
	Cefoxitin	12	0	0	0	0	0	0	0	0
Clostridium species (11)[d]										
	Cefotetan	7	0	0	1	0	0	0	0	3[e]
	Cefoxitin	8	0	0	0	0	0	0	0	3[e]

[a] Includes 14 *B. thetaiotaomicron*, 5 *B. ovatus*, 4 *B. vulgatus*, and 3 *B. distasonis* isolates.
[b] Includes nine *B. bivius*, six *B. melaninogenicus*, and one *B. capillosus* isolates and three unidentified species.
[c] Includes five *Peptostreptococcus anaerobius*, six *Peptococcus asaccharolyticus*, and 1 *Peptococcus magnus* isolates.
[d] Includes five *C. perfringens*, three *C. difficile*, two *C. sporogenes*, and one *C. bifermentans* isolates.
[e] Represents three of three *C. difficile* isolates.

TABLE 2. Thioglycolate disk elution tests (30-μg disks) versus microdilution MICs

Antimicrobial agent	MIC (μg/ml)	No. of isolates resistant or susceptible to[a]:											
		2 Disks				3 Disks				4 Disks			
		24 h		48 h		24 h		48 h		24 h		48 h	
		R	S	R	S	R	S	R	S	R	S	R	S
Cefoxitin	≥64	10	1	10	1	10	1	10	1	5	6	6	5
	32	3	1	3	1	2	2	3	1	1	3	3	1
	24	4	11	12	3	1	14	7	8	1	14	2	13
	16	0	2	1	1	0	2	0	2	0	2	0	2
	12	0	8	1	7	0	8	0	8	0	8	0	8
	≤8	1	66	2	65	0	67	1	66	0	67	0	67
No. false-susceptible[b]			13		5		17		10		23		19
No. false-resistant[b]		1		4		0		1		0		0	
Cefotetan	≥64	32	1	33	0	31	2	31	2	26	7	27	6
	32	2	2	3	1	2	2	3	1	1	3	2	2
	24	2	1	2	1	1	2	2	1	2	1	2	1
	16	2	10	2	10	1	11	1	11	1	11	2	10
	12	1	20	1	20	0	21	0	21	0	21	0	21
	≤8	2	32	2	32	1	33	1	33	1	33	1	33
No. false-susceptible			4		2		6		4		11		9
No. false-resistant		5		5		2		2		2		3	

[a] Two, three, and four 30-μg disks were allowed to elute in 5 ml of thioglycolate to yield a theoretical concentration of 12, 18, and 24 μg/ml, respectively. Results are recorded as presence or absence of growth >50% of that in a growth control after 24 and 48 h of aerobic incubation. R, Resistant; S, susceptible.
[b] Assuming strains with MICs of ≤16 μg/ml are susceptible and all others are resistant.

tin was not greater than 0.5 $\log_2$ dilution intervals. With cefotetan, MICs varied no more than 1.5 $\log_2$ dilution intervals: 18 of 21 strains varied no more than 0.5 $\log_2$ dilution intervals. Interpretive agreement among triplicate cefotetan MICs was seen with 19 of the 21 strains (90.5% agreement) and with 20 of the 21 strains tested against cefoxitin (95.2% agreement). All of the discrepancies involved tests that varied from susceptible to intermediate and never from susceptible to resistant.

Triplicate disk elution tests were also performed. Cefoxitin disks provided seven, six, and four sets of discrepant results, with two, three, and four disks per tube, respective-

TABLE 3. Predictive values of cefoxitin disk elution tests with one or two tubes, with two, three, or four 30-μg disks, plus a growth control

No. of tubes tested[a]	Results[b] with (no. of 30-μg disks per tube):			MIC (μg/ml)[c]			Predictive value (%)[d]
	2	3	4	≤16 (S)	24–32 (I)	≥64 (R)	
1	Neg			73	4	1	93.6
	Pos			4	15	10	86.2
1		Neg		76	9	1	88.4
		Pos		1	10	10	95.2
1			Neg	77	14	5	80.2
			Pos	0	5	6	100
2	Neg	Neg		73	4	1	93.6
	Pos	Neg		3	5	0	—[e]
	Pos	Pos		1	10	10	95.2
2	Neg		Neg	73	4	1	93.6
	Pos		Neg	4	10	4	—
	Pos		Pos	0	5	6	100
2		Neg	Neg	76	9	1	88.4
		Pos	Neg	1	5	4	—
		Pos	Pos	0	5	6	100

[a] Five milliliters of thioglycolate with two, three, or four disks, providing theoretical concentrations of 12, 18, or 24 μg/ml, respectively.
[b] Negative (Neg) or positive (Pos) for growth >50% of the growth control after 48 h of aerobic incubation.
[c] No. of isolates in each MIC category. S, Susceptible; I, intermediate; R, resistant.
[d] Predictive value of a negative or positive result with MIC breakpoints of ≤16 μg/ml for susceptible and >16 μg/ml for resistant.
[e] With two tubes, different results are considered equivocal test results; therefore, no predictive values were calculated.

TABLE 4. Predictive values of cefotetan disk elution tests with using one or two tubes, with two, three, or four 30-μg disks, plus a growth control

No. of tubes tested[a]	Results[b] with (no. of 30-μg disks per tube):			MIC (μg/ml)[c]			Predictive value (%)[d]
	2	3	4	≤16 (S)	24–32 (I)	≥64 (R)	
1	Neg			62	2	0	96.9
	Pos			5	5	33	88.4
1		Neg		65	2	2	94.2
		Pos		2	5	31	94.7
1			Neg	64	3	6	87.7
			Pos	3	4	27	91.2
2	Neg	Neg		62	2	0	96.9
	Pos	Neg		3	0	2	—[e]
	Pos	Pos		2	5	31	94.7
2	Neg		Neg	62	2	0	96.9
	Pos		Neg	2	1	6	—
	Pos		Pos	3	4	27	91.2
2		Neg	Neg	64	2	2	94.1
		Pos	Neg	1	1	4	—
		Pos	Pos	2	4	27	93.9

[a] Five milliliters of thioglycolate with two, three, or four disks, providing theoretical concentrations of 12, 18, or 24 μg/ml, respectively.
[b] Negative (Neg) or positive (Pos) for growth >50% of the growth control after 48 h of aerobic incubation.
[c] No. of isolates in each MIC category. S, Susceptible; I, intermediate; R, resistant.
[d] Predictive value of a negative or positive result with MIC breakpoints of ≤16 μg/ml for susceptible and >16 μg/ml for resistant.
[e] With two tubes, different results are considered equivocal test results; therefore, no predictive values were calculated.

ly. Cefotetan broth disks provided three, five, and four discrepant results, with two, three, and four disks per tube, respectively. Consistency among triplicate tests was obtained with 73% of all of the cefoxitin disk elution tests and 81% of all of the cefotetan disk elution tests; this result may be compared with the 95.2 and 90.5% interpretive agreements obtained with microdilution tests.

With a two-tube system (two and three disks per tube), cefoxitin provided nine discrepant sets of triplicate test results, but five of these varied from resistant to equivocal, and only four (19%) varied from susceptible to resistant. With two and three 30-μg cefotetan disks, only five sets of triplicate disks were discrepant: three varied from susceptible to resistant and two varied from resistant to equivocal. With the two-tube system, essential agreement (disregarding equivocal results) among triplicate tests was obtained with 81% of the cefoxitin tests and 86% of the cefotetan tests. This result may be compared with the 100% essential interpretive agreement obtained with microdilution tests.

DISCUSSION

The reference microdilution tests cannot be expected to give results that are identical to those obtained with the thioglycolate disk test. The two test systems differ in a number of ways; i.e., the test medium, inoculum density, volume of broth, and incubation conditions all differ. To estimate the appropriate number of disks to be tested in the disk elution procedure, correlative studies must first be performed. Estimates of the theoretical concentration of eluted drug in each tube of thioglycolate is only the first step. Confirmatory studies are then needed. Our data suggests that, for both cephamycins, two 30-μg disks in 5 ml of thioglycolate best predict susceptibility (MIC, ≤16 μg/ml), even though the theoretical concentration is only 12 μg/ml.

For optimal results with single-breakpoint screening tests such as the thioglycolate disk technique, inevitable interpretive discrepancies should be in the direction of false-resistant rather than false-susceptible. With cefoxitin, three 30-μg disks (18 μg/ml) provided somewhat better overall agreement with MICs than did tests with two 30-μg disks. However, more false-susceptible results were obtained with three disks. For this reason, we recommend testing two 30-μg disks for both cephamycins.

To optimize predictive values for the disk elution test, two tubes may be tested for each drug. One tube contains the number of disks that provide the best predictive values for a susceptible (negative) result, and the other tube contains the number of disks that give the best predictive value for a resistant (positive) results. Strains that grow in one tube but not in the other tube cannot be categorized: such tests are truly equivocal. To be effective, the combination of two tubes should not provide more than 5 to 10% equivocal results with the type of isolates that are normally tested.

In our hands, the thioglycolate disk test was not nearly as reproducible as the microdilution technique. Part of the problem involves the rather subjective estimate of whether a tube contains growth >50% of that in a control tube. Our results indicate that a growth-no growth endpoint may not be as effective in terms of correlation with reference MICs. Use of two tubes for each drug rather than one tube should serve as an internal control that can be helpful in reading the disk elution tests.

Difficulties that we encountered with the cephamycins might not be seen with other types of drugs. Nearly half of our challenge strains had MICs at or near the breakpoint of 16 μg/ml, and thus, day-to-day variability in single-breakpoint testing might be anticipated. Other drugs that display a marked bimodal distribution of MICs are more likely to provide clear-cut results with the disk elution test. Additional efforts to better standardize the inoculation procedure, endpoint determination, and actual disk content might

reduce day-to-day variability and improve accuracy of the thioglycolate disk elution technique.

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