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Comparison of Agar Dilution, Microdilution, and Disk Elution Methods for Measuring the Synergy of Cefotaxime and Its Metabolite against Anaerobes

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The activities of cefotaxime (CTX) and desacetyl cefotaxime (des-CTX) were tested both singly and in combination against 173 anaerobic clinical isolates. The MIC of CTX for 50% of 60 *Bacteroides fragilis* isolates was 22.4 μg/ml in broth, compared with 47.4 μg/ml in agar. This reduced efficacy in agar was seen with all species tested and is in apparent conflict with reported clinical efficacy of the drug. Synergy between CTX and des-CTX was observed with 70 to 100% of the isolates, including 60% of all *Bacteroides* spp. tested. The susceptibility results in a synergy system correlated well with those noted in a broth-disk elution method incorporating 32 μg of CTX and 8 μg of des-CTX per ml. The correlation was poorer when the broth-disk method contained 16 μg of CTX and 8 μg of des-CTX per ml.

Cefotaxime (CTX), a new beta-lactamase stable cephalosporin, differs from most other beta-lactam drugs in that its major metabolite, desacetyl cefotaxime (des-CTX), has significant antimicrobial activity and, moreover, that the metabolite acts synergistically with the parent compound against both strict and facultative anaerobes (1, 7, 8, 12). At present the clinical significance of this synergy is speculative, particularly with respect to facultative bacteria that are inhibited by low concentrations of CTX. However, many anaerobes are susceptible at concentrations that are close to maximum achievable levels in tissue (2, 13), and synergy with the desacetyl derivative may well add significantly to the efficacy of the drug. Thus, an anaerobic organism resistant to CTX in vitro may be susceptible in infected tissues when subjected to the combined action of CTX and des-CTX. Unfortunately, existing susceptibility-testing methods, with the exception of synergy-testing systems, do not allow the clinical laboratory to assess the susceptibility of anaerobic bacteria to the combination of CTX and its metabolite. Synergy methodologies are all cumbersome and expensive and beyond the scope of most diagnostic laboratories as routine procedures. Therefore we undertook to examine the feasibility of a disk elution system (16) for CTX susceptibility testing of anaerobes with both CTX and des-CTX and to compare the results of susceptibility tests with agar dilution (10), microdilution broth (11), and checkerboard (4) synergy tests.

MATERIALS AND METHODS

Bacteria. The isolates tested included 150 from the diagnostic laboratory of Vancouver General Hospital and 23 *Bacteroides fragilis*-group isolates kindly supplied by Ronald Jones, Kaiser Permanente Medical Care Program (Portland Region), Portland, Oregon. The species of these were determined by the methods described by the Virginia Polytechnic Institute and State University (6). The bacteria were *B. fragilis* (60 isolates), *Bacteroides thetaiotaomicron* (16 isolates), *Bacteroides distasonis* (9 isolates), *Bacteroides vulgatus* (10 isolates), *Bacteroides ovatus* (9 isolates), *Bacteroides ureolyticus* (4 isolates), *Clostridium difficile* (12 isolates), *Clostridium perfringens* (16 isolates), *Clostridium sordellii* (8 isolates), *Clostridium* spp. (18 isolates), and anaerobic cocci (19 isolates). The control isolates included in all test runs were *B. fragilis* ATCC 25285, *B. thetaiotaomicron* ATCC 29741, and *C. perfringens* ATCC 13124.

Bacterial suspensions for agar dilution and for microdilution broth methods were prepared with a Spectronic 21 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.) and by dilution were adjusted to give 5×10^5 cells per spot inoculum and 5×10^5 cells per ml of broth in the microdilution broth system.

Antimicrobial agents. Both CTX and des-CTX in dry powder form of known potency were generously donated by Roussel Canada Inc. Stock solutions were prepared and frozen at −70°C until required.

Microdilution broth testing. The microdilution tests of MIC and synergy were performed in sterile 96-well microtiter plates. The concentrations ranged from 128 to 0.12 μg of either antimicrobial agent per ml. An antibiotic-free well contained a positive growth control. The broth medium was Wilkins-Chalgren broth (Oxoid Canada Inc.). The MIC was the lowest concentration that completely inhibited growth of a test organism.

Agar dilution. Wilkins-Chalgren agar at 110% of the recommended strength was autoclaved, dispensed in 20-ml volumes, and supplemented at 48 to 56°C with 1 ml of lysed sheep blood and the appropriate concentration of antimicrobial agent. These volumes were poured into sterile 90-mm-diameter petri dishes and, when solidified, spot inoculated with bacterial suspensions by using a Steers (14) multiple inoculator.

Broth-disk elution. We used a modification of a method described by Wilkins and Theil (16) for broth-disk elution. Sterile 6-mm-diameter paper disks (Schleicher & Schuell, Inc., Keene, N.H.) containing des-CTX were prepared by us and stored at −70°C until required for testing. To each disk was added manually 20 μl of an aqueous solution of des-CTX containing 1.6 g/liter. Wilkins-Chalgren broth in 5.5-ml vol-

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TABLE 1. Reproducibility of MICs of CTX and des-CTX against three control strains by microdilution, agar dilution, and broth-disk elution systems

Organism and method	MIC range (μg/ml) of[a]:			Test for susceptibility to[b]:	
	CTX[c]	des-CTX	CTX + des-CTX[d]	CTX	CTX + des-CTX
B. fragilis ATCC 25285					
Microdilution	16–32	32–64	4–8/16–32		
Agar dilution	32–64	>128	32–64/8		
Broth-disk elution				S	S
B. thetaiotaomicron ATCC 29741					
Microdilution	32–64	32–64	6–16/16–32		
Agar dilution	32–64	64–128	32–64/8		
Broth-disk elution				R	R
C. perfringens ATCC 13124					
Microdilution	0.06–0.25	0.5–2.0	0.06/0.06		
Agar dilution	0.25–0.5	0.5–2.0	≤0.25/8		
Broth-disk elution				S	S

[a] Values are the range found in seven repeat test runs.
[b] For the broth-disk elution method, 16 μg of CTX per ml was used with or without 8 μg of des-CTX per ml to test susceptibility (S) and resistance (R).
[c] The expected MICs of CTX were as follows: *B. fragilis*, 8 to 32 μg/ml; *B. thetaiotaomicron*, 16 to 64 μg/ml; *C. perfringens*, 0.06 to 0.25 μg/ml.
[d] The first value is for CTX, and the second value is for des-CTX.

umes was supplemented with CTX by adding three or six 30-μg disks (BBL Microbiology Systems, Cockeysville, Md.) to achieve a concentration of 16 or 32 μg/ml, respectively. In addition, some 5.5-ml tubes of Wilkins-Chalgren broth were supplemented with 16 or 32 μg of CTX per ml and 8 μg of des-CTX per ml. An antimicrobial agent-free Wilkins-Chalgren broth was included as a positive growth control. For susceptibility testing, an inoculum of 55 μl of a 1/10 dilution of a McFarland 0.5 standardized broth was added to the tubes which were incubated anaerobically for 48 h. Absence of visible growth or turbidity less than one-half that in a growth control tube indicated a susceptible isolate. This was assessed by adding an equal volume of uninoculated Wilkins-Chalgren broth to the growth control and comparing turbidities in the growth control and test broths.

Synergy. Synergy was detected by calculating the fractional inhibitory concentration (FIC) as described by others (1, 3).

$$\text{FIC} = \left[\frac{\text{MIC}_{(\text{CTX + des-CTX})}}{\text{MIC}_{\text{CTX}}}\right] + \left[\frac{\text{MIC}_{(\text{des-CTX + CTX})}}{\text{MIC}_{(\text{des-CTX})}}\right]$$

The FIC scale was as follows: <0.5, full synergy; 0.50 to 0.75, partial synergy; >0.75, $\text{MIC}_{(\text{CTX + des-CTX})}$ and $\text{MIC}_{(\text{des-CTX + CTX})}$ each are the MIC of CTX and des-CTX used in combination, MIC_{CTX} is the MIC of CTX, and $\text{MIC}_{\text{des-CTX}}$ is the MIC of des-CTX.

In one set of observations, susceptibility was defined as a reduction of the MIC of CTX to ≤32 μg/ml in the presence of ≤8 μg of des-CTX per ml. Similarly in another set of calculations, susceptibility was defined as a CTX MIC of ≤16 μg/ml in the presence of ≤8 μg of des-CTX per ml.

The reproducibility of MICs in the synergy system was tested by running each of the three ATCC control strains against CTX and des-CTX singly and in combination in each test run.

MIC_{50} and MIC_{90}. The concentrations that inhibited 50% (MIC_{50}) and 90% (MIC_{90}) of the strains were calculated for each of the antimicrobial agents singly and at a fixed concentration of 8 μg of des-CTX per ml combined with various concentrations of CTX. The formula of geometric means was used as follows:

$$\text{MIC}_{50} = (M < 50) + \frac{(n - x) \times [(M > 50) - (M < 50)]}{y}$$

where M < 50 is the MIC of the highest cumulative percentage below 50%, M > 50 is the MIC of the lowest cumulative percentage above 50%; n is 50% of the number of organisms tested, x is the number of organisms in the group at M < 50, and y is the number of organisms in the group at M > 50.

For example, at a MIC of 1, 2, 4, 8, and 16, respectively, the number of organisms is 12, 6, 4, 5, and 2; the total number of organisms is 12, 18, 22, 27, and 29; and the cumulative percentage is 41, 62, 75, 93, and 100%.

$$\text{MIC}_{50} = 1 + \frac{(14.5 - 12) \times (2 - 1)}{6}$$

$$= 1 + \frac{(2.5 \times 1)}{6} = 1 + \frac{2.5}{6} = 1.41.$$

Therefore, the MIC_{50} is 1.41. Note that the MIC_{90} is calculated by substituting 90% for 50% in the MIC_{50} formula. Thus "n" will become 90% of 29, or 26.1, and the MIC_{90} will be 7.28.

RESULTS

The MICs of CTX and des-CTX singly and in combination against the three control strains were highly reproducible within each test system (Table 1).

The MICs of both CTX and des-CTX against 173 anaerobic isolates are shown in Table 2. It is noteworthy that the range of MICs in the agar system is higher than that in broth of all groups of anaerobes. *Clostridium difficile* on repeated testing by the microtiter broth method displayed numerous "skipped wells" of no growth as well as no growth in growth control wells, and the organism was always resistant at high concentrations by the agar dilution method. Among the remainder of the anaerobes, the *B. fragilis* group was the most resistant. However, there was a marked difference between the agar and the broth methods with respect to the MIC_{50} of CTX and des-CTX against *B. fragilis*. It was also quite pronounced when des-CTX at 8 μg/ml was combined with CTX (11.4 versus 43.6 μg/

TABLE 2. Susceptibility of 173 anaerobic isolates in broth and agar dilution to CTX and des-CTX singly and to various concentrations of CTX with a fixed concentration of des-CTX

Organism (*n*) and dilution medium	MIC of CTX (μg/ml)			des-CTX (μg/ml)			CTX + des-CTX (μg/ml)[a]		
	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}
B. fragilis (60)									
Broth	1.0–>64	22.4	60	2.0–>64	50.1	65.4	0.25–>128	11.4	57.6
Agar	1.0–>256	47.4	128	1.0–>256	166	249.6	8.0–>256	43.6	89.9
B. thetaiotaomicron (16)									
Broth	0.25–>64	22.8	60.1	0.5–>64	64	62.4	0.25–>128	6.4	23.5
Agar	8.0–>256	48	115.2	8.0–>256	142.2	233.2	0.25–>256	58.7	121.6
B. distasonis (9)									
Broth	0.25–>64	16.8	56.8	0.25–>64	28	65.1	0.25–>128	0.25	35.2
Agar	1.0–>256	35.2	58.2	8.0–>256	80	217.6	0.25–>256	37.3	134.4
B. vulgatus (10)									
Broth	0.5–>64	4	48	0.25–>64	16	57.6	0.25–>128	0.5	8
Agar	4.0–>256	12	64	32.0–>256	51.2	192	2.0–>256	16	64
B. ovatus (9)									
Broth	8.0–>64	18.7	49.6	32.0–>64	48	64.5	1.0–128	7	24.8
Agar	0.25–>256	18.7	70.4	0.5–>256	74.7	108.8	0.25–>256	24	99.2
B. ureolyticus (4)									
Broth	16.0–>64	10	39.5	32.0–>64	48	60.8	4.0–>128	5	7.4
Agar	1.0–>256	6	108.8	4.0–>256	6	13.6	0.25–>256	0.25	0.68
C. difficile (12)[b]									
Agar	64–>256	96	162	64.0–>256	192	232.5	64–>256	96	179
C. perfringens (16)									
Broth	0.25–>64	0.33	2.8	0.25–>64	0.94	8.8	0.25–>128	0.25	0.25
Agar	0.25–>256	0.5	1.8	2.0–>256	2.5	3.7	0.25–>256	0.25	0.25
C. sordellii (8)									
Broth	0.06–>64	0.06	0.2	0.06–>64	0.06	0.4	0.06–>28	0.06	0.06
Agar	0.12–>256	0.17	0.23	0.25–>256	0.42	1.2	0.06–>256	0.06	0.06
Clostridium spp. (10)									
Broth	0.25–>64	2	38.4	1.0–>64	20	38.4	0.06–>128	0.06	1.0
Agar	0.25–>256	4	8	1.0–>256	32	98	0.06–>256	0.5	25.6
Anaerobic cocci (19)									
Broth	0.06–>64	0.06	0.06	0.06–>64	0.06	0.44	0.25–>128	0.25	2.5
Agar	0.06–>256	0.56	3.85	0.12–>256	0.75	7.6	0.25–>256	0.25	1.7

[a] Values shown are for CTX. des-CTX was used at a concentration of 8μg/ml.
[b] Endpoints could not be determined in broth owing to skipped wells, which showed no growth.

ml). This pattern was repeated with most of the species tested.

Either partial or full synergy was present with 70 to 100% of the isolates tested, 43.3% of *B. fragilis* isolates showed full synergy, and 66% of all *Bacteroides* spp. showed full or partial synergy (Table 3). Table 4 extends these observations and shows that four isolates of *B. fragilis* that were resistant in agar dilution were susceptible at ≤32 μg of CTX per ml when 8 μg of des-CTX per ml was added to the CTX in agar. The same four isolates showed synergy in the microdilution broth synergy system, increasing the number of isolates susceptible in the microdilution broth from 45 (75%) to 49 (81.7%). In the broth-disk elution method, 51 (85%) were susceptible to CTX and 54 (90%) were susceptible when 8 μg of des-CTX per ml was added to the CTX. A similar pattern was seen with all other species tested, and there is a striking correlation between susceptibility results in the microdilution broth synergy studies and by the broth-disk elution method, particularly when the latter was supplemented with 8 μg of des-CTX per ml.

Table 5 displays the susceptibility patterns of the same isolates with inhibition at ≤16 μg of CTX per ml to denote susceptibility. This made little difference to the number of non-*Bacteroides* isolates susceptible to CTX. Moreover, there was little change in the number of isolates susceptible in the broth-disk elution tests both with and without CTX except for *B. thetaiotaomicron*. However, there was a marked reduction in the number of *B. fragilis* isolates that were susceptible by agar dilution synergy and microdilution broth tests. A point of note is that the number of category changes from resistant to susceptible shown in Tables 4 and

TABLE 3. Number of isolates showing full or partial synergy in the presence of both CTX and des-CTX by the microbroth dilution method

Organism (*n*)[a]	No. (%) of isolates showing synergy:		No. (%) of additive isolates[d]
	Full[b]	Partial[c]	
B. fragilis (60)	26 (43.3)	10 (16.7)	24 (40)
B. thetaiotaomicron (16)	9 (56.3)	2 (12.5)	5 (31.3)
B. distasonis (9)	2 (22.2)	1 (11.1)	6 (66.6)
B. vulgatus (10)	7 (70)	2 (20)	1 (10)
B. ovatus (9)	6 (66.7)	2 (22.2)	1 (11)
B. ureolyticus (4)	2 (50)	2 (50)	0
C. perfringens (16)	6 (37.5)	5 (31.25)	5 (31.25)
C. sordellii (8)	0	0	8 (100)
Clostridium sp. (8)	5 (62.5)	1 (12.5)	2 (25)

[a] Endpoints could not be determined for the 12 *C. difficile* isolates, owing to skipped wells.
[b] Full synergy was shown when isolates had an FIC of <0.5.
[c] Partial synergy was shown when isolates had an FIC betwene 0.51 and 0.75.
[d] Additive isolates had an FIC >0.75.

TABLE 4. Susceptibility of 173 anaerobic isolates to ≤32 μg of CTX per ml alone and in combination with des-CTX by agar dilution, microdilution broth, and broth-disk elution systems

Organism (*n*)	No. of isolates showing resistance or susceptibility by[a]:											
	Agar dilution				Microdilution broth				Broth-disk elution			
	CTX		CTX + des-CTX		CTX		CTX + des-CTX		CTX		CTX + des-CTX	
	S	R	S	R	S	R	S	R	S	R	S	R
B. fragilis (60)	18	42	22	38	45	15	49	11	51	9	54	6
B. thetaiotaomicron (16)	5	11	5	11	11	5	13	3	9	7	12	4
B. distasonis (9)	4	5	4	5	6	3	8	1	8	1	8	1
B. vulgatus (10)	8	2	8	2	8	2	9	1	9	1	9	1
B. ovatus (9)	5	4	4	5	7	2	7	2	6	3	7	2
B. ureolyticus (4)	3	1	3	1	4	0	4	0	4	0	4	0
C. perfringens (16)	16	0	16	0	16	0	16	0	16	0	16	0
C. difficile (12)	0	12	0	12	—[b]	—	—	—	0	12	0	12
C. sordelli (8)	8	0	8	0	8	0	8	0	8	0	8	0
Clostridium spp. (10)[c]	10	0	10	0	8	0	8	0	10	0	10	0
Anaerobic cocci (19)	19	0	19	0	NG[d]	NG	NG	NG	19	0	19	0

[a] CTX was used at 32 μg/ml with or without 8 μg of des-CTX per ml. S, Susceptible; R, resistant.
[b] —, These isolates could not be analyzed, owing to skipped wells.
[c] Two isolates of *Clostridium novyi* failed to grow in microdilution broth tests.
[d] NG, No growth.

5 are less impressive than the numbers of isolates that displayed synergy (Table 3). The highest percentage change was in microdilution broth, when 4 of 16 isolates of *B. thetaiotaomicron* changed from resistant to susceptible at ≤16 μg/ml.

DISCUSSION

We have used a variety of test methods and two susceptibility breakpoints in testing the action of CTX and des-CTX against a number of anaerobic clinical isolates. The results confirm the findings of others that significant synergy can exist between CTX and its desacetyl metabolite (1, 9). Furthermore, we confirm the substantial difference between agar dilution and broth microdilution susceptibility results of tests on *Bacteroides* species that others have reported (7). One could speculate that these differences may be attributable to differences in inoculum concentrations on the agar surface compared with the concentration when the inoculum is dispersed in broth. It is of interest that while the MICs of CTX against the control isolates were in accordance with established standards (10), the MICs of des-CTX were a little higher than those reported by Aldridge and his colleagues (1).

An important question that we sought to answer was whether a broth-disk elution method incorporating 8 μg of des-CTX per ml in the broth might correlate with microdilution checkerboard synergy results. This concentration of des-CTX was selected somewhat arbitrarily but also because it is achievable in tissues (2, 13). It is clear that at a susceptibility breakpoint of ≤32 μg/ml, there was a good correlation between the results of synergy tests and those of

TABLE 5. Susceptibility of 173 anaerobic isolates to ≤16 μg of CTX per ml alone and in combination with des-CTX by agar dilution, microdilution broth, and broth-disk elution methods

Organism (*n*)	No. of isolates showing resistance or susceptibility by[a]:											
	Agar dilution				Microdilution broth				Broth-disk elution			
	CTX		CTX + des-CTX		CTX		CTX + des-CTX		CTX		CTX + des-CTX	
	S	R	S	R	S	R	S	R	S	R	S	R
B. fragilis (60)	7	53	7	53	24	36	30	30	50	10	52	8
B. thetaiotaomicron (16)	1	15	1	15	6	10	10	6	5	11	5	11
B. distasonis (9)	2	7	3	6	6	3	6	3	8	1	8	1
B. vulgatus (10)	6	4	5	5	7	3	9	1	9	1	9	1
B. ovatus (9)	4	5	5	4	5	4	5	4	5	4	5	4
B. ureolyticus (4)	3	1	3	1	4	0	4	0	4	0	4	0
C. perfringens (16)	16	0	16	0	16	0	16	0	16	0	16	0
C. difficile (12)	0	12	0	12	—[b]	—	—	—	0	12	0	12
C. sordelli (8)	8	0	8	0	8	0	8	0	8	0	8	0
Clostridium spp. (10)[c]	10	0	10	0	8	0	8	0	10	0	10	0
Anaerobic cocci (19)	19	0	19	0	NG[d]	NG	NG	NG	0	19	0	19

[a] CTX was used at 16 μg/ml with or without 8 μg of des-CTX per ml. S, susceptible; R, resistant.
[b] —, These isolates could not be analyzed, owing to skipped wells.
[c] Two isolates of *Clostridium novyi* failed to grow in microdilution broth tests.
[d] NG, No growth.

broth-disk elution with 8 μg of des-CTX per ml. However, the correlation was poorer when a CTX breakpoint concentration of ≤16 μg/ml was used in the synergy and broth-disk elution methods. This difference in correlation is due to the greater number of isolates that were susceptible at ≤16 μg/ml in the broth-disk elution tests.

Two major conclusions can be drawn from this study and perhaps from others. Firstly, while agar dilution is regarded by many as the reference method, it is not clear that this can apply to the testing of CTX against anaerobes. There is a need, therefore, to redefine the appropriate reference method for this antimicrobial agent in tests of anaerobes, since agar dilution results do not appear to correlate well with clinical experience (5, 15), and synergy test results do. Secondly, if synergy is to be considered a clinically significant attribute of this drug, a method such as the broth-disk elution method that we describe here may have to be used since full checkerboard synergy tests are too cumbersome for routine diagnostic laboratories. In this study, the broth-disk elution results correlated well with the results of the microdilution synergy tests taking ≤32 μg/ml as the susceptibility breakpoint but less well at ≤16 μg/ml. Based on these observations, appropriate breakpoints in the broth-disk elution method may be ≤32 μg of CTX per ml combined with 8 μg of des-CTX per ml.

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