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Comparison of Kill-Kinetic Studies with Agar and Broth Microdilution Methods for Determination of Antimicrobial Activity of Selected Agents against Members of the *Bacteroides fragilis* Group

CHARLES W. STRATTON,[1]* LYNDELL S. WEEKS,[1] AND KENNETH E. ALDRIDGE[2]

Department of Pathology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232,[1] and Department of Medicine, Louisiana State University School of Medicine, New Orleans, Louisiana 70112[2]

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Kill-kinetic studies often are used to determine the rate and degree of killing of aerobic bacteria by antimicrobial agents. Few studies, however, make use of this method for determining antimicrobial activity against anaerobic bacteria. To evaluate kill-kinetic studies for anaerobes, kill-kinetic studies were performed for selected antimicrobial agents against members of the *Bacteroides fragilis* group and compared with MICs obtained by using a reference agar dilution method and a broth microdilution method. Results of the kill-kinetic studies showed that the degree of killing over a 24-h test period was related to the MIC for the test organism. In general, the higher the MIC of an antimicrobial agent for a test organism, the less the killing observed. In addition, these studies demonstrate subtle differences in bactericidal activity at various concentrations of the antimicrobial agents, which cannot be determined by agar or broth dilution methods. Kill-kinetic studies are a useful addition to dilution methods for the evaluation of antimicrobial agents against anaerobes.

Kill-kinetic studies often are used to determine the rate and degree of killing of aerobic bacteria by an antimicrobial agent (or combination of agents) compared with other agents. Such studies are not as frequently used to study antimicrobial activity of agents against anaerobic bacteria (5, 14, 16). To evaluate this method for anaerobes, we did kill-kinetic studies for selected antimicrobial agents against members of the *Bacteroides fragilis* group and compared these with MICs obtained by using the National Committee for Clinical Laboratory Standards reference agar dilution method (13) and a broth microdilution method.

MATERIALS AND METHODS

Microorganisms. Twenty-one clinical isolates of the *B. fragilis* group were studied. Included in this group were *B. fragilis* (15 isolates), *B. ovatus* (3 isolates), *B. thetaiotaomicron* (1 isolate), *B. distasonis* (1 isolate), and *B. vulgatus* (1 isolate). Species identfication was performed by established methodology (7), which included biochemical profiles and gas-liquid chromatography patterns. In addition, two ATCC strains (*B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29741) were used in all susceptibility test procedures. Stock cultures were maintained frozen at −70°C and were cloned twice on prereduced anaerobically stored 5% sheep blood agar plates before use.

Antimicrobial agents. Four antimicrobial agents (clindamycin, metronidazole, chloramphenicol, and cefoxitin) most likely to be used in a clinical situation involving anaerobes were selected (4). Standard powders were obtained from the following sources: clindamycin, The Upjohn Co., Kalamazoo, Mich.; metronidazole, Searle Laboratories, Chicago, Ill.; chloramphenicol, Sigma Chemical Co., St. Louis, Mo.; and cefoxitin, Merck Sharp & Dohme, Rahway, N.J. Antimicrobial stock solutions were prepared according to the instructions of the manufacturers and stored at −70°C until use. Final concentrations were prepared on the day they were used.

Media. Wilkins-Chalgren (WC) agar and Anaerobe Broth Experimental (ABE; Difco Laboratories, Detroit, Mich.) were used for the agar and broth microdilution methods. ABE has the same formulation as WC agar except that the agar is omitted. All broth media were supplemented by the manufacturer with 0.1 μg of vitamin K per ml and 5 μg of hemin per ml. Before use, all media were reduced in an anaerobic jar for at least 4 h. Before the time-kill-kinetic studies, ABE and a commercially available broth-bottle system for anaerobic blood cultures (BACTEC 7D bottle; Johnston Laboratories, Inc., Towson, Md.) initially were compared by using six isolates (two *B. fragilis*, one *B. ovatus*, one *B. thetaiotaomicron*, one *B. distasonis*, and one *B. vulgatus*) to determine their ability to support growth of strains of the *B. fragilis* group. These isolates were tested with and without cefoxitin (32 μg/ml) in both ABE and in the 7D broth.

Inoculum. Portions of five or more colonies of a plate culture were inoculated into prereduced thioglycolate broth and incubated in an anerobic jar overnight. The turbidity of the logarithmic-phase culture was adjusted to a 0.5 McFarland standard with thioglycolate broth. Further dilutions were done as determined by the specific susceptibility test method by using the prereduced broth for that method. The inoculum used for each susceptibility test method was carefully controlled, and the final concentration was confirmed by a modified surface colony count method (11, 21). For this method, 10-fold dilutions were made in physiologic saline, and a calibrated micropipette was used to deliver five 0.05-ml samples from each dilution onto a prereduced anaerobic sheep blood agar plate. These plates were incubated anaerobically for 48 h, colonies were counted and averaged, and the final inoculum was calculated. If the final

* Corresponding author.

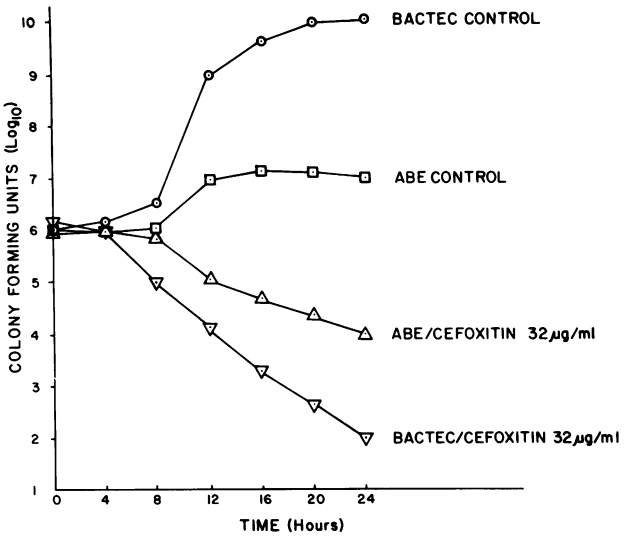

FIG. 1. Comparison of time-kill curves for BACTEC 7D anaerobic broth and ABE with or without cefoxitin (32 μg/ml) for *B. fragilis* ATCC 25285.

inoculum was not between 5×10^5 and 5×10^6 for broth methods, the test was repeated.

Agar dilution method. The agar dilution procedure was done on two occasions as described by the National Committee for Clinical Laboratory Standards with WC agar (Difco) as the test medium (27). Serial twofold dilutions (ranging from 0.25 to 128 μg/ml) were prepared in agar and used to test each isolate. The plates were inoculated with a multipoint replicator delivering approximately 3 μl. ATCC strains (*B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29741) were included for quality control. In addition, the size of the inoculum delivered to the plate was determined by the modified surface colony count method described above.

Broth microdilution method. A microdilution method modified from the National Committee for Clinical Laboratory Standards method for aerobic bacteria (12) was used. ABE was used as the test medium. Serial twofold dilutions (ranging from 0.25 to 128 μg/ml) were prepared in broth and used to test each isolate. The final inoculum was 5×10^5 CFU per well and was confirmed each time by the modified surface colony count method described above. A micropipette delivering 0.05 ml was used to inoculate each well of the microdilution plate. The final volume in each well was 0.1 ml. Microdilution plates (Dynatech Laboratories, Inc., Alexandria, Va.) with appropriate twofold dilutions were allowed to reduce for at least 4 h in an anaerobic jar before being inoculated. After 48 h of incubation in an anaerobic jar at 35°C, the plates were removed and read by using a microtiter mirror. The MIC was the lowest concentration of the antimicrobial agent that inhibited growth of the test organism. MICs were determined in duplicate on at least two occasions. ATCC strains (*B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29741) were used for quality assurance.

Kill-kinetic studies. All kill-kinetic studies were done with a final volume of 30 ml of broth in glass bottles. Borosilicate glass bottles without medium were kindly supplied by Johnston Laboratories. The desired medium was placed in each bottle, and the bottles were capped and sterilized. In addition, commercially available bottles for anaerobic blood cultures (BACTEC 7D bottle) were used for comparison. That the commercially available blood culture bottles contained 30 ml of medium was confirmed by actual determination of the volume in randomly selected bottles. Volumes of subsequent bottles were confirmed by comparing the broth levels with a known level. Both the antimicrobial agent to be tested and the inoculum were added to most bottles in a small volume (1 ml) so as to maintain a final volume of approximately 30 ml. If a larger volume (3 ml) was needed, the added volume was included in the calculations for the final concentration and inoculum size. The inoculum added was calculated to achieve a final concentration of 10^6 CFU/ml. Each isolate was tested in time-kill-kinetic studies to establish the effect of subinhibitory (1/2× MIC), inhibitory (1× MIC; 2× MIC), and suprainhibitory (4× MIC) concentrations of the antibiotics. Higher concentrations which would not be achievable in serum with clinical use of the agent usually were not tested for isolates with high MICs. Bottles were incubated anaerobically on a shaker (Johnston Laboratories) at 35°C for 24 h, which allowed growth in the control bottles to reach a level of 10^9 to 10^{10} CFU/ml. Bottles were usually subcultured for colony counts at 0, 4, 8, 12, 16, 20, and 24 h of incubation. Colony counts were performed by removing 0.5 ml of the broth with a syringe and by making 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} dilutions in sterile saline. A 0.02-ml sample of each dilution was plated onto prereduced blood agar plates and incubated for 48 h. The number of colonies was counted and used to determine the number of viable CFU at each sampling time. Kill-kinetic curves were then plotted, showing time versus CFU per milliliter of

TABLE 1. MICs (MIC for reference strains, range for clinical isolates) of selected antimicrobial agents for members of the *B. fragilis* group with agar dilution and broth dilution methods

Antimicrobial agent	MIC (μg/ml)[a] for:				Range of MICs (μg/ml)[b] for *B. fragilis* group (21 isolates)	
	B. fragilis ATCC 25285		*B. thetaiotaomicron* ATCC 29741			
	Agar dilution	Broth dilution	Agar dilution	Broth dilution	Agar dilution	Broth dilution
Chloramphenicol	4	4	8	8	4 (2–8)	4 (2–8)
Clindamycin	1	1	2	2	1 (0.25–>128)	1 (0.25–>128)
Metronidazole	1	1	2	2	0.5 (0.25–2)	0.5 (0.25–2)
Cefoxitin	4	4	8	8	8 (2–32)	8 (2–32)

[a] Mean values observed for eight determinations.
[b] Mean values and ranges observed for duplicate determinations.

TABLE 2. Inoculum killed with 1/2× MIC of each antibiotic at various times for 21 clinical isolates of *B. fragilis* group

Time (h)	Mean % killed by:			
	Chloramphenicol	Clindamycin	Metronidazole	Cefoxitin
4	0[a]	71.5	46.6	42.0
8	0	86.8	0	0
12	0	88.7	0	0
24	0	6.8	0	0

[a] A zero indicates that the CFU per milliliter were equal to or greater than the initial inoculum.

broth. Time-kill curves were done on at least two occasions for each isolate against each concentration of antibiotic to determine reproducibility.

RESULTS

All 21 *Bacteroides* organisms and the two ATCC strains grew in the broth control well for the broth microdilution method and on the growth control agar plate for the agar dilution method, demonstrating that the ABE and WC agar used were able to support the growth of these microorganisms. However, growth of the six isolates in ABE over a 24-h period as determined by colony counts was less than that observed for the BACTEC bottles. In ABE, all isolates had an increase of only 10^1 to 10^2 CFU over this time. Figure 1 is representative and illustrates the growth of *B. fragilis* ATCC 25285 in supplemented ABE and in the commercial 7D broth with or without 32 μg of cefoxitin per ml. Growth of the other isolates was similar to that of the ATCC strain.

Agar and broth dilution methods. The inoculum delivered to the surface of the agar plates for the reference method and to each well in the broth microdilution method was comparable (5×10^5 CFU). The MICs for the control strains (Table 1) were the same as those published previously (13). MICs determined by both methods (Table 1) were comparable and were consistent with those reported previously (5, 10, 19, 22, 23).

Kill-kinetic studies. Table 2 indicates the bactericidal effect of each antibiotic at subinhibitory concentrations (1/2× MIC) for susceptible isolates of the *B. fragilis* group studied. Chloramphenicol showed only a static effect over the 24-h period. Both metronidazole and cefoxitin showed moderate killing at 1/2× MIC up to 4 h and rapid regrowth thereafter (8 h). Clindamycin showed significant killing (82%) for up to 12 h, but regrowth then occurred. Table 3 indicates the effect of each antibiotic at inhibitory concentrations (1× MIC) on susceptible strains. Cefoxitin showed significant killing at 4 h, but regrowth of the organisms occurred by 8 h. Metronidazole had activity similar to that of cefoxitin at 4 h, with less killing at 8 h and regrowth by 12 h. At 1× MIC, both chloramphenicol and clindamycin showed the most bactericidal activity, killing greater than 95% of the inoculum at 24 h. Table 4 indicates the bactericidal effect of each antibiotic at suprainhibitory concentrations (4× MIC) on susceptible strains tested. All drugs showed excellent bactericidal activity at both 12 and 24 h. When time-kill curves for isolates with different MICs were compared, the activity seen was proportional to the MIC. Figure 2 illustrates this, comparing time-kill curves for three isolates of *B. fragilis* which have different MICs. The isolates with differences in MICs of the other antimicrobial agents tested also had activities proportional to the MIC (Table 5).

TABLE 3. Inoculum killed with 1× MIC of each antibiotic at various times for 21 clinical isolates of *B. fragilis* group

Time (h)	Mean % killed by:			
	Chloramphenicol	Clindamycin	Metronidazole	Cefoxitin
4	15.0	74.2	68.1	61.2
8	51.0	91.0	43.2	0
12	86.3	97.2	0[a]	0
24	90.1	94.9	0	0

[a] A zero indicates that the CFU per milliliter were equal to or greater than the initial inoculum.

TABLE 4. Inoculum killed with 4 × MIC of each antibiotic at various times for 21 clinical isolates of *B. fragilis* group

Time (h)	Mean % killed by:			
	Chloramphenicol	Clindamycin	Metronidazole	Cefoxitin
4	16.0	84.3	93.0	92.1
8	80.4	91.2	95.7	94.2
12	90.6	97.6	97.9	96.5
24	94.9	98.1	99.3	97.8

DISCUSSION

Kill-kinetic studies are time-consuming and technically difficult but allow the demonstration of the rate and degree of killing by one agent compared with those of another agent. We assessed the bactericidal activity of selected antimicrobial agents against members of the *B. fragilis* group by performing serial colony counts over a 24-h period from cultures that were incubated anaerobically in the presence of specific concentrations of antimicrobial agents. We used four agents (clindamycin, metronidazole, chloramphenicol, and cefoxitin) which have proven effective against anaerobic bacteria in clinical settings. We compared the results of the kill-kinetic studies with results of agar and broth dilution methods.

We found that the results of the agar and broth dilution methods were comparable. Previous studies (1, 9, 17) also have shown that results with broth dilution methods are very

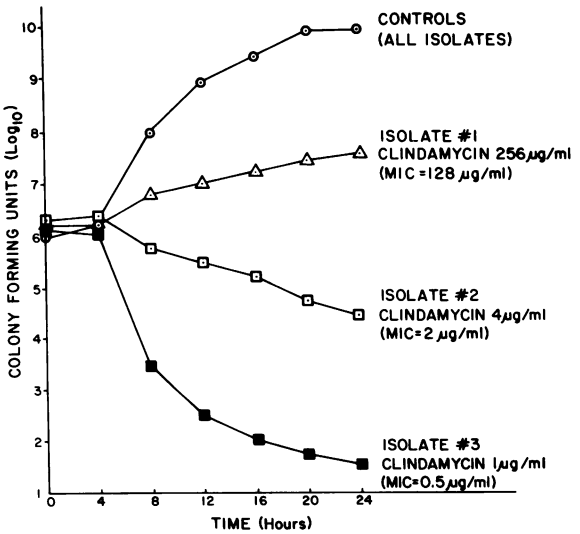

FIG. 2. Comparison of time-kill curves for three different isolates of *B. fragilis* with different clindamycin MICs. Time-kill curves were determined with a concentration twice the respective MIC.

TABLE 5. Bactericidal activity of cefoxitin, clindamycin, and chloramphenicol against strains of *B. fragilis* group with high, moderate, and low MICs[a]

Antimicrobial agent (no. of strains)[b]	Mean % killed at:			
	4 h	8 h	12 h	24 h
High MICs				
Cefoxitin (4)	90.1	91.2	92.8	93.7
Clindamycin (3)	79.4	90.0	93.0	96.2
Chloramphenicol (5)	8.2	68.5	86.9	90.7
Moderate MICs				
Cefoxitin (13)	92.3	95.1	97.8	99.0
Clindamycin (12)	84.2	91.5	96.3	98.1
Chloramphenicol (12)	13.6	81.4	92.2	95.9
Low MICs				
Cefoxitin (6)	95.0	98.6	99.0	99.9
Clindamycin (8)	90.4	93.2	97.0	98.0
Chloramphenicol (6)	25.7	86.6	95.1	99.0

[a] Data given for antibiotics tested at 4× MIC

[b] The following criteria were used to categorize MICs (micrograms per milliliter): cefoxitin, <8 = low, 16 to 32 = moderate, >64 = high; clindamycin, <0.5 = low, 1 to 4 = moderate, >8 = high; chloramphenicol, < 8 = low, 16 = moderate, >32 = high.

similar to those provided by the standard agar dilution method. A difference in the ability of ABE (WC broth) to support the growth of *B. fragilis* isolates compared with the 7D broth was demonstrated. Others have reported differences in the growth rate of *B. fragilis* strains caused by differences in media (6, 18, 25). The poor growth observed in ABE was also associated with diminished bactericidal activity of cefoxitin. This would be expected because beta-lactam antimicrobial agents are more active against rapidly growing microorganisms. The differences observed for the agents evaluated in this study were not due to differences in broths, because all comparisons were made with only the results of kill-kinetic studies performed in 7D broth. Kill-kinetic studies were comparable to agar and broth dilution methods in that the higher the MIC, the less bactericidal activity observed at various concentrations of antimicrobial agent. Kinetic studies, however, illustrate that the inhibitory activity of an antibiotic expressed as an MIC does not always relate to the bactericidal activity of that compound (15, 28). Our data suggest that even at subinhibitory concentrations, antibiotics can vary widely in their bactericidal activity. Such information obtained at 12 to 24 h with kill-kinetic studies may have more relevance than a MIC obtained after 48 h of incubation. As the concentration of the antibiotics increased, so did the bactericidal activity of each agent.

When the antibiotics tested were compared, both cefoxitin and metronidazole showed poor bactericidal activity at 1/2× and 1× MIC but had excellent activity at 4× MIC. Clindamycin had excellent activity at 1/2× and 1× MIC; this was not unexpected, because clindamycin has been shown to exhibit better bactericidal activity against *B. fragilis* than cefoxitin (20). Chloramphenicol, although bactericidal at 4× MIC, was the least active of the agents studied. This may explain, in part, the occasional clinical failures in both patients and experimental animals (2, 8, 24, 26). In addition, enzymatic inactivation of chloramphenicol has been described (3) and may be a factor in treatment failures.

In conclusion, time-kill-kinetic studies appear to accurately reflect the antimicrobial activity of those agents tested against members of the *B. fragilis* group. In addition, these studies demonstrate subtle differences in bactericidal activity at various concentrations of the antimicrobial agent. Kill-kinetic studies for anaerobes can be simplified by the use of the BACTEC 7D anaerobic broth. Kill-kinetic studies are useful additions to dilution methods for the evaluation of antimicrobial agents against anaerobes because they allow the rate and degree of killing for antimicrobial agents at various concentrations and times to be compared. We conclude that this method is a useful means of assessing and comparing the antimicrobial activity of agents against members of the *B. fragilis* group.

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