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Comparison of Three Different In Vitro Methods of Detecting Synergy: Time-Kill, Checkerboard, and E test

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An in vitro method of detecting synergy which is simple to perform, accurate, and reproducible and has the potential for clinical extrapolation is desirable. Time-kill and checkerboard methods are the most widely used techniques to assess synergy but are time-consuming and labor-intensive. The Epsilometer test (E test), a less technically demanding test, has not been well studied for synergy testing. We performed synergy testing of *Escherichia coli* ATCC 35218, *Enterobacter cloacae* ATCC 23355, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 29213 with various combinations of cefepime or ceftazidime with tobramycin or ciprofloxacin using time-kill, checkerboard, and E test techniques. Time-kill testing was performed against each organism alone and in combinations at one-fourth times the MIC (1/4× MIC) and 2× MIC. With checkerboard tests, the same combinations were studied at concentrations ranging from 1/32× to 4× MIC. Standard definitions for synergy, indifference, and antagonism were utilized. E test strips were crossed at a 90° angle so that the scales met at the MIC of each drug alone, and the fractional inhibitory concentration index was calculated on the basis of the resultant zone of inhibition. All antimicrobial combinations demonstrated some degree of synergy against the test organisms, and antagonism was infrequent. Agreement with time-kill testing ranged from 44 to 88% and 63 to 75% by the checkerboard and E test synergy methods, respectively. Despite each of these methods utilizing different conditions and endpoints, there was frequent agreement among the methods. Further comparisons of the E test synergy technique with the checkerboard and time-kill methods are warranted.

The accurate prediction of clinically relevant antibiotic synergy based upon the results of in vitro testing has been a goal of researchers for some time. There are, in fact, examples of such a correlation existing. Combinations of antimicrobial agents which have been shown to be synergistic in vitro have been associated with a more favorable clinical outcome in neutropenic patients with gram-negative sepsis (1, 9, 15, 17) and in the treatment of enterococcal endocarditis (29). Increased bactericidal activity in patient serum has also been documented with antibiotic combinations which are synergistic in vitro (16).

A number of methods used to detect in vitro synergy between antibiotics have been described; however, the checkerboard and time-kill curve methods are the most widely used techniques. The checkerboard method is a relatively easy test to perform; however, it is merely a gauge of inhibitory activity. The time-kill method of synergy testing assesses bactericidal activity but is time-consuming and labor-intensive. Several studies have compared results generated by the checkerboard and time-kill methods (2, 8, 21, 25, 27, 28, 30). While some studies have shown excellent agreement between these methods (30), most have not (2, 8, 21, 25, 27, 28), and controversy about the comparability of results generated by these techniques (3, 4, 12, 19, 20, 24) exists. Each method has its advocates.

The Epsilometer, or E test, is a relatively new agar diffusion method for performing antimicrobial susceptibility testing. The E test is a plastic strip coated with a continuous gradient of antibiotic concentrations on one side and an interpretation scale of the antimicrobial agent on the other side. The strip is placed onto the surface of an agar plate inoculated with bacteria and incubated overnight, and an elliptical zone of inhibited growth is formed around the strip. The MIC is read at the intersection of the zone with the strip. If the use of the E strip could be standardized for synergy testing and subsequent results could be demonstrated to be similar to those obtained by established methods, this new method of performing synergy tests would represent an attractive alternative to its labor-intensive predecessors. Further, this method could be performed on a routine basis in a clinical microbiology laboratory.

The main objective of the present study was to examine the degree of agreement of a possible new method utilizing the E test with the conventional methodologies used in in vitro synergy testing. The secondary objective was to define and compare the degree of synergistic activity of cefepime when combined with tobramycin or ciprofloxacin to that of similar combinations with ceftazidime.

Cefepime is a newer, broad-spectrum cephalosporin with excellent activity against gram-negative and gram-positive pathogens. The activity of cefepime against *Pseudomonas aeruginosa* and *Enterobacter cloacae* is similar to that of ceftazidime; however, cefepime is more active than ceftazidime against *Staphylococcus aureus* (11). Even though cefepime has activity against these microorganisms, these bacteria are more likely to develop resistance. Therefore, combination therapy which is additive or synergistic has often been used to treat life-threatening infections caused by these pathogens. To date, synergy data for cefepime are very limited.

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MATERIALS AND METHODS

Antimicrobial agents. Standard laboratory powders of cefepime (Bristol-Myers Squibb, Princeton, N.J.), ceftazidime (Glaxo, Research Triangle Park, N.C.), and ciprofloxacin (Miles Inc., New Haven, Conn.) were used in this study. A standard solution of tobramycin (10 mg/ml; Eli Lilly and Company, Indianapolis, Ind.) was used because of the hygroscopic nature of tobramycin powder. E strips of ceftazidime, cefepime, ciprofloxacin, and tobramycin (AB Biodisk, Solna, Sweden) were also used.

Microorganisms and media. *Escherichia coli* ATCC 35218, *E. cloacae* ATCC 23355, *P. aeruginosa* ATCC 27853, and *S. aureus* ATCC 29213 were used in this study. Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) was prepared immediately prior to use and adjusted to 25 and 12.5 mg/liter with $CaCl_2$ and $MgCl_2$, respectively, according to National Committee for Clinical Laboratory Standards guidelines. Antibiotic-free Mueller-Hinton agar plates (BBL, Cockeysville, Md.) were used for MIC and synergy testing using the E strips and colony count determination for the time-kill study.

MIC determination. The MICs of cefepime, ceftazidime, ciprofloxacin, and tobramycin for the test organisms were determined by broth microdilution and E test techniques. For broth microdilution, the MICs were determined in replicates of eight. The modal value was used in determining the concentrations of each antimicrobial agent for the synergy test. Stock solutions, corrected for potency, were prepared immediately prior to testing. The MICs were determined according to National Committee for Clinical Laboratory Standards guidelines for broth microdilution (23). The MIC was defined as the lowest concentration of antibiotic that completely inhibited the growth of the organism as detected by the unaided eye. The final inoculum of approximately 5×10^5 CFU/ml was verified with the Spiral Plater (Spiral System, Inc., Cincinnati, Ohio).

Determinations of the MICs by the E test were performed in triplicate according to the manufacturer's recommendations. The concentration range was 0.016 to 256 μg/ml for cefepime, ceftazidime, and tobramycin and 0.002 to 32 μg/ml for ciprofloxacin. The inoculum was matched to a McFarland standard of 0.5, and then with a cotton swab a lawn of bacteria was created on the Mueller-Hinton agar plates. Four E test strips were placed onto each Mueller-Hinton agar plate. The plates were incubated for 18 h at 35°C. The MIC was interpreted as the value at which the inhibition zone intersected the scale on the E strip. E strip results were rounded up to the nearest twofold dilution values for purposes of comparison with broth microdilution MIC results.

Synergy testing. Three different methods were compared for the determination of synergy: checkerboard, time-kill, and E test.

(i) Checkerboard. The combinations tested (in quadruplicate) against each microorganism were cefepime plus ciprofloxacin, cefepime plus tobramycin, ceftazidime plus ciprofloxacin, and ceftazidime plus tobramycin, and modal values were used in further calculations. The concentration range of each antimicrobial agent in combination ranged from 1/32 times the MIC (1/32 × MIC) to 4× MIC. Dilutions of drugs A and B were made with a twofold diluter (Dynatech Autodilutor III). The initial inoculum was prepared as described above.

To evaluate the effect of the combinations, the fractional inhibitory concentration (FIC) was calculated for each antibiotic in each combination (10). The following formulas were used to calculate the FIC index: FIC of drug A = MIC of drug A in combination/MIC of drug A alone, FIC of drug B = MIC of drug B in combination/MIC of drug B alone, and FIC index = FIC of drug A + FIC of drug B. Synergy was defined as an FIC index of ≤0.5. Indifference was defined as an FIC index of >0.5 but of ≤4. Antagonism was defined as an FIC index of >4.

(ii) Time-kill. The time-kill method of synergy testing was performed by the broth macrodilution technique and followed the guidelines set by the National Committee for Clinical Laboratory Standards (22). Each organism was tested against each antimicrobial agent alone and in combination. The combinations tested against each organism were the β-lactam (cefepime or ceftazidime) with ciprofloxacin and tobramycin (i.e., cefepime plus ciprofloxacin, cefepime plus tobramycin, ceftazidime plus ciprofloxacin, and ceftazidime plus tobramycin). The concentrations of each antimicrobial agent tested alone or in combination were 1/4× and 2× MIC. Therefore, the combinations tested against each organism were 2× MIC of both antimicrobial agents, 2× MIC of a β-lactam and 1/4× MIC of ciprofloxacin or tobramycin, 1/4× MIC of a β-lactam and 2× MIC of ciprofloxacin or tobramycin, and 1/4× MIC of both antimicrobial agents. A total of eight concentrations of the drugs alone and 16 combinations were tested against each microorganism.

The time-kill studies were performed with a final inoculum of approximately 5×10^5 CFU/ml in a final volume of 30 ml. The final inoculum was verified with the Spiral Plater. The tubes were continuously shaken on an orbital shaker and incubated at 35°C. Samples were obtained at 0, 4, 18, and 24 h. Duplicate samples were obtained at 0 and 24 h, as results from these points are used in the definition of synergy. At each sample time, 500 μl was withdrawn from each tube, and 10-fold dilutions were prepared when necessary. A 50-μl aliquot of the diluted and/or undiluted samples was plated onto each Mueller-Hinton agar plate with the Spiral Plater. The plates were incubated for 15 to 24 h at 35°C, and colony counts were determined.

Synergy was defined as a ≥100-fold or 2-$\log_{10}$ decrease in colony count at 24 h by the combination compared with that by the most active single agent and as a ≥100-fold decrease in colony count compared with the starting inoculum. Additivity or indifference was defined as a <10-fold change in colony count at 24 h by the combination compared with that by the most active single agent. Antagonism was defined as a ≥100-fold increase in colony count at 24 h by the combination compared with that by the most active drug alone.

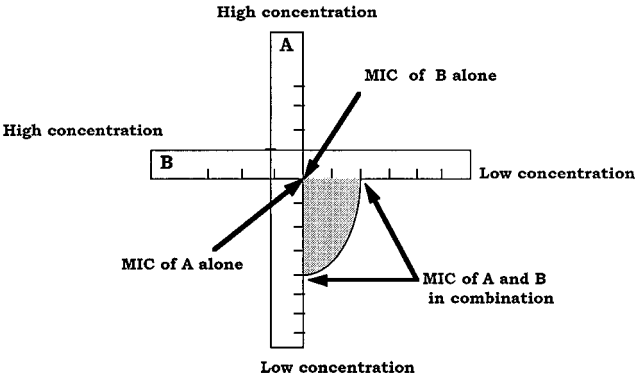

FIG. 1. Diagram of strip placement for E test synergy method.

(iii) E test. The same combinations of antimicrobial agents used for the checkerboard and time-kill methods were evaluated by the E test. The inoculum and streaked Mueller-Hinton agar plates were prepared as previously described. The E test strips were placed on the Mueller-Hinton agar in a cross formation, with a 90° angle at the intersection between the scales at their respective MICs for the organism (Fig. 1). The E test method was performed in duplicate for all combinations. The plates were then incubated for 18 h at 35°C. After incubation, the zones of inhibition were read as described above for the determination of the MIC by the E test. The nature of the drug interaction (synergy, additivity, indifference, or antagonism) was determined on the basis of the calculated FIC index as described above for the checkerboard method.

RESULTS

MICs. The modal MIC results are presented in Table 1. In general, there was good agreement between the E test and broth microdilution methods.

Synergy. For purposes of comparison, the time-kill test was used as the reference method. All antibiotic combinations demonstrated synergy against *P. aeruginosa* and *E. cloacae* at one or more concentration combinations. With *E. coli*, only the combination of cefepime and ciprofloxacin demonstrated synergy. *S. aureus* was affected synergistically only by the combination of cefepime and tobramycin. Overall, indifference occurred in 56, 94, 75, and 63% of instances with *E. cloacae*, *E. coli*, *S. aureus*, and *P. aeruginosa*, respectively. No combinations were antagonistic for *P. aeruginosa*, *E. cloacae*, or *E. coli*. Combinations of ceftazidime with either ciprofloxacin or tobramycin were antagonistic for *S. aureus.* An example of a synergistic combination with the E test is displayed in Fig. 2C. All results are presented in Table 2.

Agreement of method results. The checkerboard and E test

TABLE 1. Modal MIC results by broth microdilution and E test methods

Antimicrobial agent	MIC (μg/ml) determined by indicated test for:							
	P. aeruginosa ATCC 27853		*E. cloacae* ATCC 23355		*E. coli* ATCC 35218		*S. aureus* ATCC 29213	
	E test	Broth[a]	E test	Broth	E test	Broth	E test	Broth
Cefepime	1.00	2.00	0.03	0.03	0.05	0.06	2.00	2.00
Ceftazidime	1.50	1.00	4.00	4.00	0.13	0.25	12.00	16.00
Ciprofloxacin	0.19	0.50	0.02	0.06	0.02	0.03	0.25	0.25
Tobramycin	1.00	2.00	0.50	1.00	2.00	2.00	1.00	0.50

[a] Broth microdilution.

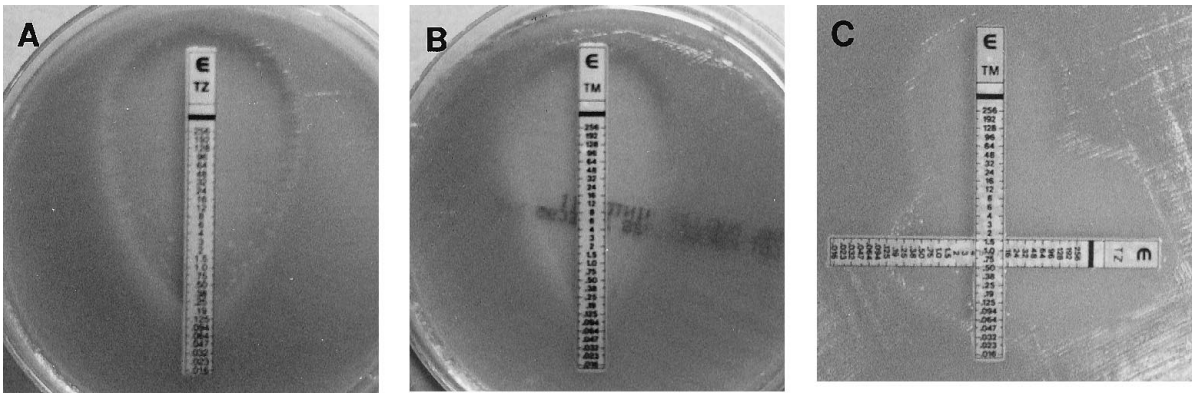

FIG. 2. Photographs of E test results for MIC of ceftazidime (A), MIC of tobramycin (B), and synergy test of ceftazidime plus tobramycin (C) with *E. cloacae* ATCC 23355.

results agreed in 12 of 16 instances (1 case of synergy; 11 cases of indifference). All four instances in which checkerboard and E test results disagreed occurred with *P. aeruginosa* and *E. cloacae*. Three of the four disagreements were with combinations that included tobramycin, and in each of these cases, the checkerboard result indicated synergy while the E test result indicated indifference. Agreement between the E test and the time-kill method ranged from 63 to 75%, with the best agreement occurring with time-kill combinations involving 1/4× MIC of each drug. Agreement between the checkerboard and time-kill methods ranged from 44 to 88%. The agreement data are presented in Table 3.

DISCUSSION

A reliable in vitro test that could accurately predict the in vivo synergy of antibiotic combinations has been sought for many years. The two most extensively used in vitro methods for detecting synergy, checkerboard and time-kill, have yielded mixed results in pertinent evaluations. Further, their respective results, when used in parallel to evaluate synergy in comparative studies, have often been at odds. This is not entirely surprising, as the two methods measure different phenomena. The checkerboard technique, based upon MICs, reflects the inhibition of bacterial growth, whereas the time-kill methodology measures the extent of killing. The checkerboard method has been questioned as an appropriate technique by some investigators (14) but is vigorously defended by others (5).

Although it appears that some investigators have found the time-kill method to be a reliable predictor of in vivo synergy (2, 7), it is not without problems. Shortcomings of the time-kill method include the effect of inoculum size, the difficulties in interpretation of results because relatively few antibiotic concentrations are examined, and the reliance on the reading at one time point (usually 24 h) as the sole determinant of the interaction. Another obvious disadvantage is that time-kill ex-

TABLE 2. Synergy method results[a]

Organism	Antibiotic combination	Checkerboard FIC index (interpretation)	Time-kill interpretation with combination:				E test FIC index (interpretation)
			2× MIC + 2× MIC	2× MIC + 1/4× MIC[b]	1/4× MIC + 2× MIC[c]	1/4× MIC + 1/4× MIC	
P. aeruginosa ATCC 27853	CTZ + CIP	4.0 (I)	S	I	I	I	0.501 (I)
	CTZ + TOB	0.5 (S)	I	S	I	S	0.503 (I)
	CFP + CIP	2.0 (I)	S	I	S	I	0.607 (I)
	CFP + TOB	0.5 (S)	I	S	I	I	0.695 (I)
E. cloacae ATCC 23355	CTZ + CIP	2.0 (I)	I	I	S	S	0.118 (S)
	CTZ + TOB	0.5 (S)	I	I	I	S	0.125 (S)
	CFP + CIP	4.0 (I)	S	S	S	I	0.617 (I)
	CFP + TOB	0.5 (S)	I	S	I	I	1.109 (I)
E. coli ATCC 35218	CTZ + CIP	1.0 (I)	I	I	I	I	1.278 (I)
	CTZ + TOB	1.0 (I)	I	I	I	I	1.502 (I)
	CFP + CIP	1.0 (I)	I	I	S	I	1.020 (I)
	CFP + TOB	1.0 (I)	I	I	I	I	1.341 (I)
S. aureus ATCC 29213	CTZ + CIP	1.0 (I)	I	I	I	A	0.730 (I)
	CTZ + TOB	1.0 (I)	I	I	A	A	0.713 (I)
	CFP + CIP	1.0 (I)	I	I	I	I	0.876 (I)
	CFP + TOB	2.0 (I)	I	I	I	S	0.753 (I)

[a] Abbreviations for antibiotics: CTZ, ceftazidime; CFP, cefepime; CIP, ciprofloxacin; TOB, tobramycin. Abbreviations for interpretations: S, synergy; A, antagonism; I, indifference.
[b] Combination of 2× MIC of cefepime or ceftazidime and 1/4× MIC of tobramycin or ciprofloxacin.
[c] Combination of 2× MIC of tobramycin or ciprofloxacin and 1/4× MIC of cefepime or ceftazidime.

TABLE 3. Agreement between synergy detection methods

Method of determining FIC index	% Agreement with result of method[a]:					
	E test FIC index	Checkerboard FIC index	Time-kill with combination:			
			2× MIC + 2× MIC	2× MIC + 1/4× MIC[b]	1/4× MIC + 2× MIC[c]	1/4× MIC + 1/4× MIC
E test		75	63	69	69	75
Checkerboard	75		56	88	44	63

[a] For each method, $n = 16$ (four organisms × four antibiotic combinations).
[b] Combination of 2× MIC of cefepime or ceftazidime and 1/4× MIC of tobramycin or ciprofloxacin.
[c] Combination of 2× MIC of tobramycin or ciprofloxacin and 1/4× MIC of cefepime or ceftazidime.

periments are labor-intensive and time-consuming. Moreover, a review of the relevant literature reveals that, in addition to using different definitions of synergy, investigators employ a variety of concentrations (clinically achievable concentrations and fractions of the MIC) and various bacterial inocula. The former is a serious limitation, as when one does not include a concentration of one antibiotic that does not affect the growth curve, it is difficult to distinguish between synergy and additivity (19). Finally, the timing of the colony count determination has varied from one investigation to the next and it has been shown, for example, that different synergy results are obtained at 24 and 48 h (2).

A variety of investigators have found discordance between checkerboard and time-kill results. In a study of clinical isolates of *Klebsiella pneumoniae*, frequent discordance regarding the presence of synergy as measured by checkerboard and time-kill methods was observed (25). In general, synergy was detected with a higher frequency by the time-kill method. While all strains affected synergistically according to the checkerboard method were also affected synergistically according to the time-kill method, the reverse was not true. This lack of consistent agreement has been reported by other investigators as well (2, 16, 18, 21, 28). Importantly, the checkerboard technique can predict antagonism when, in vivo, apparent synergy occurs (2, 21). Lastly, time-kill and checkerboard results can be diametrically opposed (synergy compared with antagonism), as illustrated in the present study. At the very least, it appears appropriate to conclude that these methods are not interchangeable.

The results of the E test method for detecting synergy evaluated in this study appear to agree fairly well with results from checkerboard and time-kill testing despite differences in endpoints (inhibition compared with killing) and media (broth compared with agar). Agreement between E test and time-kill results with 1/4× MIC of both drugs and with checkerboard results was 75%. If one considers agreement between E test and time-kill results for those combinations that contained 1/4× MIC of at least one drug, concordance was present in 67% of cases. However, the E test indicated synergy in only 25% of cases in which time-kill with 1/4× MIC of at least one drug detected synergy. Neither the checkerboard nor E test results suggest antagonism in the face of synergy results from the time-kill method. Because of the manner in which we placed the E strips on the agar (scales intersecting at the MICs), we would have been able to detect only dramatic instances of antagonism while mild cases would go undetected because the zone of inhibition ran under the crossed strips and was therefore unreadable and interpreted as indifference. Thus, it is possible that the E strip test results occasionally contradict time-kill results when antagonism is observed. Further work is needed to clarify this issue.

Use of the E test to evaluate antimicrobial combinations against mycobacteria (6, 13) and *Xanthomonas maltophilia* (26) has been described by other investigators. In those studies, E test results were compared with those of the checkerboard rather than the time-kill technique, however. We have used a method for performing the E test synergy evaluations not described by the other investigators. Previous methods have entailed either placing one antibiotic strip onto an agar plate for 1 h, removing it, and placing the second antibiotic strip on the same location (6, 26) or incorporating the second antibiotic into the medium itself (13). How our results would compare with those generated with either of the other techniques is unknown but is worthy of evaluation.

Our results suggest that rates of synergy for cefepime and ceftazidime for the test organisms in this study are comparable, although antagonism was observed more frequently with the latter. On the basis of our results, the method of synergy testing utilizing E strips appears to be a possible alternative to other in vitro methods. More extensive work with a variety of drugs and far more isolates is needed to confirm this impression, but we believe that such work is warranted on the basis of the results presented in this work and the relative simplicity of the E test.

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