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Decimal Assay for Additivity of Drugs Permits Delineation of Synergy and Antagonism

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Although there are many in vitro tests for drug interactions, few possess a linear, predictable dose-dependent end point or have a precise definition for additivity. Therefore, a new test with both of these features, the decimal assay for additivity, was developed. This test is based on a disk diffusion assay and the strict linear relationship between drug mass and size of the inhibition zone. When the decimal assay for additivity was applied to combinations known on a mechanistic basis to be additive, synergistic, or antagonistic, results of the new test always reflected the expected drug interaction. For example, synergy between trimethoprim and sulfamethoxazole was detected in tests with *Escherichia coli* and *Haemophilus influenzae*, as was antagonism between cefoxitin and cefotaxime in tests with *Enterobacter cloacae*. Quinolones plus chloramphenicol appeared to be antagonistic. In addition to correctly identifying the drug interaction, the decimal assay for additivity identified the drug ratio producing the maximal drug interaction. These results suggest that the decimal assay for additivity should prove very useful in future studies of drug interactions.

Numerous previous studies have focused on in vitro measurement of antibiotic interactions, and many others have been concerned with the appropriate interpretation of data generated in such tests. In fact, there has been quite a bit of controversy surrounding the terminology used to define antibiotic interactions (4). Nevertheless, there is general consensus that synergy is an effect greater than the sum of the activities of the individual agents (i.e., the additive effect), while antagonism is an effect less than additivity (1, 4, 5). Given these generally accepted definitions that are based upon additivity, it is very surprising to note that most in vitro tests for drug interactions do not utilize methodology that will generate predictable, dose-dependent data, and few have a precisely defined point for additivity. The two most widely used methods for assessing drug interactions are the time-kill technique and the checkerboard titration (1, 3). Both have important methodologic limitations.

In the time-kill technique, synergy and antagonism are not defined relative to the additive effect but are defined on the basis of the extent of killing over a specified time by the single most active agent (3, 11). Furthermore, the parameters of the test do not require that there be a predictable relationship between the extent of killing and the dose (concentration) utilized. Therefore, not only is the additive effect undefined, but it cannot be predicted on a dose-response basis because of the end point (killing) utilized in the test.

The checkerboard titration also has important limitations. In this test, all possible combinations of two drugs are prepared with serial twofold dilutions, and the end point measured is usually analogous to an MIC (3). To assess the extent of drug interaction, a fractional inhibitory concentration (FIC) is calculated for each drug by the formula FIC_A = concentration of drug A in an inhibitory combination/MIC of drug A alone. An FIC index is then calculated by the formula FIC index = $FIC_A + FIC_B$. Synergy is defined as an FIC index of ≤ 0.5, and antagonism is defined as an FIC index of >4 (1). These definitions take into account the twofold error of the method for both components in the combination. Thus, synergy requires that the concentration of both drugs in an inhibitory combination be no more than one-fourth of the MIC, while antagonism requires that the concentration of at least one drug be increased fourfold or more. Few have problems with this definition of antagonism, since most antagonisms observed are one-way, not mutual. However, problems arise in interpretation of FIC indices between 0.125 and 4 (1, 4) (Table 1). FIC indices between 0.75 and 4 are best described as indifference because the inhibitory effect of the combination could have resulted from the activity of just one of the two drugs in the combination because of the twofold error of the MIC (i.e., the true MIC may lie anywhere between 1/2× the MIC and 2× the MIC). Although FIC indices of ≤ 0.5 are usually defined as synergy, this allows no place for additivity if indices of 0.75 to 4 are truly indifference. An FIC index of 0.5 is most likely true additivity, since the inhibitory effect of the combination cannot be attributed to either component alone (Table 1). However, since an FIC index of 0.5 is only twofold below 1 (and thus not different from 1), such an index most likely represents the anticipated summation of the effects of the two components. If an FIC index of 0.5 is the true additive point, then synergy can be invoked only for indices at least fourfold below this to keep interpretation of data outside the inherent twofold error of the test (Table 1). All of these problems in interpretation of data generated in checkerboard titrations are complicated by the fact that the end point used, complete inhibition of growth, is not a quantitative parameter that can be predicted on a dose-response basis. It is a qualitative measure with no gradations between growth and no growth.

From the recognition of the various limitations of methods most often used to assess drug interactions, a study was designed to develop and evaluate a new test that would avoid these problems. The test, the decimal assay for additivity, was designed to have a quantitative end point which varied in a predictable dose-response fashion. The test also had a

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TABLE 1. Interpretation of FIC indices from serial twofold checkerboard titrations based on the twofold error of the method for each component antibiotic

FIC_A	FIC_B	FIC index	Interpretation
4	1	5	Antagonism
2[a]	2	4	Indifference
1	1	2	Indifference
1/2	1/2	1	Indifference
1/2	1/4	0.75	Indifference
1/4	1/2	0.75	Indifference
1/4	1/4	0.5	Additivity
1/8	1/4	0.375	Additivity
1/8	1/8	0.25	Additivity
1/16	1/8	0.1875	Additivity
1/16	1/16	0.125	Synergy

[a] Underlined values represent situations in which inhibition of growth by the combination could be the result of the single agent because of the inherent twofold error of the test.

precisely defined point for additivity so that interactions greater or less than additivity could be accurately identified. The validity of the decimal assay for additivity was assessed with combinations known on mechanistic bases to be additive, synergistic, or antagonistic. This approach was chosen over direct comparisons with other methods for drug interactions because it has been well established that there is poor agreement between these tests (1, 8, 10) and, as noted above, these other methods have inherent limitations of their own.

MATERIALS AND METHODS

Strains and antibiotics. Tests were performed with *Escherichia coli* ATCC 25922 and a single clinical isolate each of *Enterobacter cloacae*, *Haemophilus influenzae*, and *Staphylococcus aureus*. Diagnostic powders of antibiotics were either purchased from Sigma Chemical Company, St. Louis, Mo. (amoxicillin, chloramphenicol, and sulfamethoxazole) or obtained from their respective manufacturers. The latter group of antibiotics included ampicillin (Roerig Division, Pfizer Inc., New York, N.Y.), cefotaxime (Hoechst-Roussel Pharmaceuticals Inc., Somerville, N.J.), cefoxitin (Merck, Sharp & Dohme, West Point, Pa.), ciprofloxacin (Miles Inc., West Haven, Conn.), ofloxacin (Ortho Pharmaceutical Corp., Raritan, N.J.), and trimethoprim (Hoffmann-La Roche Inc., Nutley, N.J.). All antibiotic solutions were prepared on the day of use.

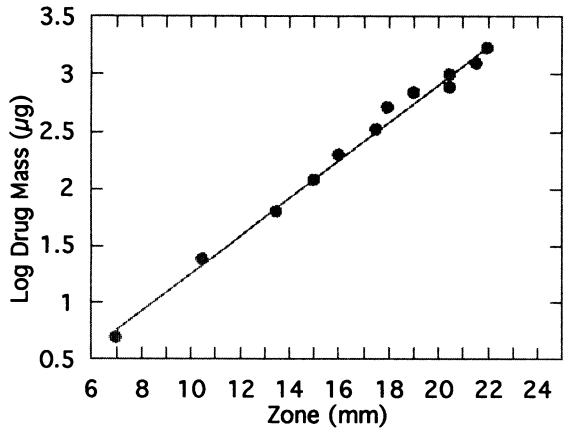

FIG. 1. Example of a standard dose-response curve generated in disk diffusion assays with *E. coli* ATCC 25922 and ampicillin.

TABLE 2. Mixtures used in the decimal assay for additivity

Decimal mixture	Decimal portion of BEF		Ratio (A:B)
	Drug A	Drug B	
0	1.0	0.0	10:0
1	0.9	0.1	9:1
2	0.8	0.2	8:2
3	0.7	0.3	7:3
4	0.6	0.4	6:4
5	0.5	0.5	5:5
6	0.4	0.6	4:6
7	0.3	0.7	3:7
8	0.2	0.8	2:8
9	0.1	0.9	1:9
10	0.0	1.0	0:10

Disk diffusion assays. All assays involving disk diffusion were performed by the method described by the National Committee for Clinical Laboratory Standards (9). Antibiotic disks were prepared the day of use by applying 20 μl of drug solution to 6-mm sterile paper disks (Difco Laboratories, Detroit, Mich.) before placing them on the agar medium. For tests with *H. influenzae*, Haemophilus test medium (Baltimore Biological Co., Cockeysville, Md.) was used, while for all other organisms, Mueller-Hinton agar (Unipath Ltd., Basingstoke, Hampshire, England) was used.

Decimal assay for additivity. Disk diffusion assays were performed initially with each antibiotic alone over a range of drug masses in order to derive a standard dose-response curve by linear regression analysis (Fig. 1). For each antibiotic, there is a linear relationship between the $\log_{10}$ of the drug mass (in micrograms) on the disk and the diameter of the zone of inhibition (in millimeters) produced. Once a standard curve for each drug alone had been derived, a target zone for each combination to be tested was selected.

TABLE 3. Parameters used to set up the decimal assay for additivity

Test organism	Antibiotic[a] combination	Target zone diam (mm)	BEF (μg)
E. coli ATCC 25922	AMP	15	8.90
	AMX	15	11.00
E. coli ATCC 25922	TRIM	16	0.73
	SULFA	16	52.40
E. cloacae 55	CX	15	154.00
	CTX	15	0.19
E. cloacae 55	OFX	15	0.16
	CHL	15	12.00
E. cloacae 55	CIP	15	0.024
	CHL	15	12.00
S. aureus 4	OFX	15	0.32
	CHL	15	5.70
S. aureus 4	CIP	15	0.13
	CHL	15	5.70
H. influenzae 4	AMP	20	1.41
	AMX	20	2.47
H. influenzae 4	TRIM	16	0.26
	SULFA	16	64.00

[a] Drug abbreviations: AMP, ampicillin; AMX, amoxicillin; TRIM, trimethoprim; SULFA, sulfamethoxazole; CX, cefoxitin; CTX, cefotaxime; OFX, ofloxacin; CHL, chloramphenicol; CIP, ciprofloxacin.

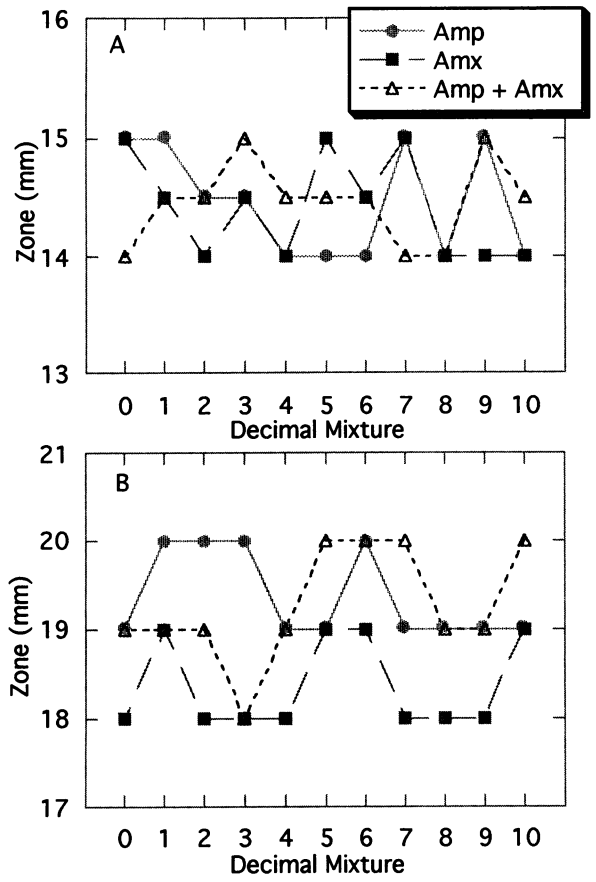

FIG. 2. Examples of additivity between ampicillin (Amp; 100% in combination decimal mixture 0) and amoxicillin (Amx; 100% in combination decimal mixture 10) in tests with *E. coli* ATCC 25922 (A) and *H. influenzae* 4 (B).

This target was selected to represent a zone in the midrange of the standard curve for each drug so that increases or decreases in the zone size resulting from drug interactions could be reliably detected. Once a target zone size was selected for a particular combination, the mass of each drug alone (in micrograms) required in order to produce this zone was calculated from the formula for the standard dose-response curve. This mass was defined as the biologic equivalence factor (BEF), since it represented the mass of each of the two drugs that would produce the same zone size.

Once the BEF had been calculated, a series of 11 decimal mixtures of the two drugs to be examined in combination was prepared (Table 2). This series represented all possible mixtures of the two drugs that, when combined in 10 parts, added up to one BEF. Thus, if no positive or negative interaction between the drugs occurred, each mixture should have generated the target zone size.

Since there are inherent errors in any standard curve and in preparation of the decimal mixtures, controls for these potential errors consisted of each drug added to itself in a similar series of decimal mixtures. The results generated by these 11 single-drug decimal mixtures represented the true additive response. Therefore, results obtained with combination decimal mixtures (mixtures 1 through 9 [Table 2]) had to lie outside the range of those obtained with the single-drug

TABLE 4. Statistical analysis of data obtained in the decimal assay for additivity

Organism	Antibiotic(s)[a]	Mean target zone diam (mm)	95% CI[b] (mm)
E. coli ATCC 25922	AMP	14.4	14.1–14.8
	AMX	14.4	14.1–14.7
	AMP + AMX	14.5	14.2–14.8
E. coli ATCC 25922	TRIM	15.3	15.0–15.5
	SULFA	16.2	15.9–16.5
	TRIM + SULFA	22.3	21.7–22.9
E. cloacae 55	CX	14.6	14.4–14.9
	CTX	14.1	13.9–14.4
	CX + CTX	10.3	8.4–12.3
E. cloacae 55	OFX	13.9	13.7–14.1
	CHL	15.7	15.4–16.0
	OFX + CHL	11.7	9.8–13.5
E. cloacae 55	CIP	14.5	14.0–14.9
	CHL	15.7	15.4–16.0
	CIP + CHL	12.2	10.5–13.9
S. aureus 4	OFX	13.3	13.0–13.7
	CHL	13.9	13.7–14.1
	OFX + CHL	9.3	7.0–11.6
S. aureus 4	CIP	12.0	12.0–12.0
	CHL	13.9	13.0–13.7
	CIP + CHL	9.3	7.2–11.5
H. influenzae 4	AMP	19.4	19.0–19.7
	AMX	18.4	18.0–18.7
	AMP + AMX	19.2	18.7–19.7
H. influenzae 4	TRIM	15.7	15.4–16.0
	SULFA	16.8	16.1–17.5
	TRIM + SULFA	24.7	24.0–25.3

[a] For explanation of drug abbreviations, see Table 3, footnote *a*.
[b] CI, confidence interval.

decimal mixtures in order to be considered indicative of a positive or negative interaction. Data were also analyzed statistically (Stat View II, Abacus Concepts, Berkeley, Calif.). The mean zone size ($\bar{x}_{drug}$) was calculated from the data obtained with the 11 single-drug decimal mixtures, and 95% confidence intervals (*t* distribution) were determined. For the combination decimal mixtures, the mean zone size ($\bar{x}_{comb}$) was calculated by using data obtained with mixtures 1 through 9 only, since mixtures 0 and 10 represented each drug alone (Table 2). Ninety-five percent confidence intervals (*t* distribution) were calculated for this mean as well. Results obtained with the combination were considered indicative of synergism if $\bar{x}_{comb}$ was larger than $\bar{x}_{drug\ A}$ and $\bar{x}_{drug\ B}$ and the 95% confidence intervals for $\bar{x}_{comb}$ did not overlap those for $\bar{x}_{drug\ A}$ or $\bar{x}_{drug\ B}$. Results were considered indicative of antagonism if $\bar{x}_{comb}$ was smaller than $\bar{x}_{drug\ A}$ and $\bar{x}_{drug\ B}$ and the 95% confidence intervals did not overlap. All other results were considered additive.

RESULTS

The target zone sizes selected from the standard dose-response curves for each drug evaluated in this study are shown in Table 3, as are the BEFs calculated from the formulae for the standard curves. Tests with ampicillin plus amoxicillin were performed with *E. coli* ATCC 25922 and *H. influenzae* 4. These drugs are so closely related chemically and mechanistically that they were expected to behave in an additive fashion. As shown in Fig. 2, all datum points for the combination decimal mixtures lay within the range of datum points for the single-drug decimal mixtures. Statistical analyses revealed the combinations to be additive as well (Table

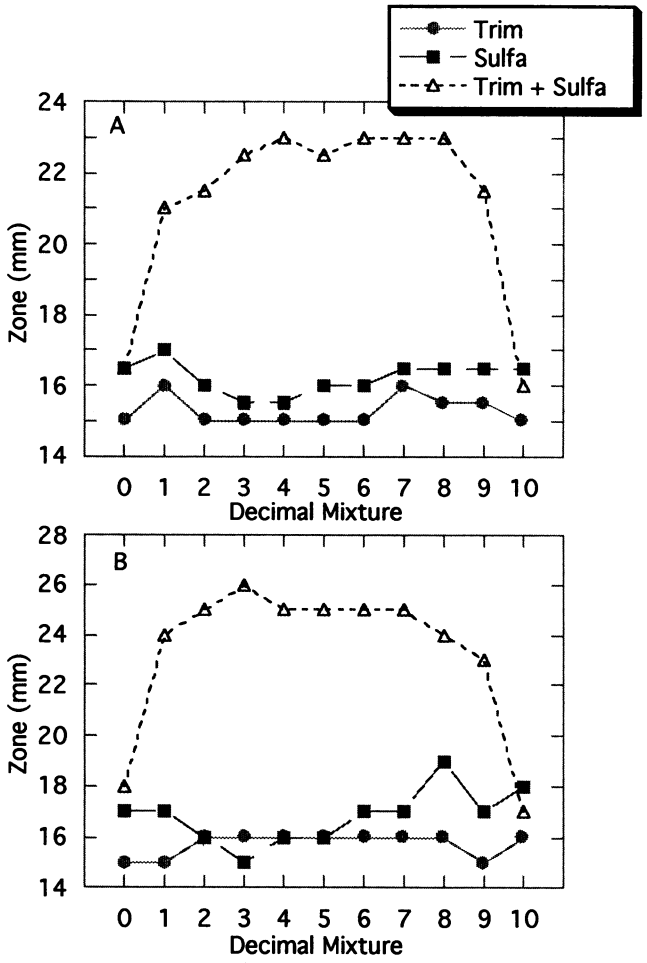

FIG. 3. Examples of synergy between trimethoprim (Trim; 100% in combination decimal mixture 10) and sulfamethoxazole (Sulfa; 100% in combination decimal mixture 0) in tests with *E. coli* ATCC 25922 (A) and *H. influenzae* 4 (B).

4). Tests with sulfamethoxazole plus trimethoprim were performed with *E. coli* ATCC 25922 and *H. influenzae* 4, since these drugs have been shown to interact synergistically in virtually every in vitro test examined and clinically as well (1, 2). As shown in Fig. 3, all datum points for the combination (decimal mixtures 1 to 9) lay outside the range of those for the single-drug decimal mixtures and indicated a fairly uniform synergy regardless of the ratio of the two components. Statistical analysis of the data confirmed the synergy (Table 4). Tests with cefotaxime plus cefoxitin were performed with *E. cloacae* 55 because this combination has been shown to be antagonistic with this organism and others that characteristically produce an inducible group 1 β-lactamase (1, 12). As shown in Fig. 4, antagonism was clearly evident in all combination decimal mixtures, especially when the ratio of cefotaxime to cefoxitin was high. Statistical analysis confirmed the antagonism (Table 4).

Numerous reports have described the antagonism of quinolones by bacteriostatic inhibitors of protein biosynthesis (7, 13, 14). Therefore, tests were performed with ciprofloxacin or ofloxacin plus chloramphenicol against *E. cloacae* 55 and *S. aureus* 4. As shown in Fig. 5, antagonism was evident, especially when the ratio of the quinolone to chloramphenicol was high. Statistical analyses confirmed the antagonism (Table 4).

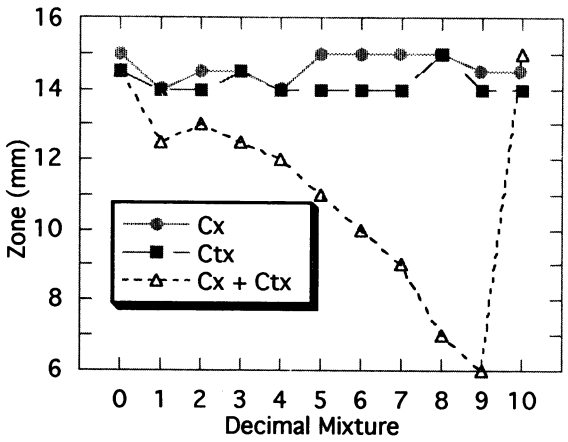

FIG. 4. Antagonism of cefotaxime (Ctx; 100% in combination decimal mixture 10) by cefoxitin (Cx; 100% in combination decimal mixture 0) in tests with *E. cloacae* 55.

DISCUSSION

The validity of the decimal assay for additivity has been demonstrated by using combinations known on mechanistic bases to be additive, synergistic, or antagonistic. In addition to correctly identifying the drug interaction, the decimal assay for additivity identified the component ratio at which drug interaction was maximal. Also, for one-way drug interactions, the skew of the graph toward one component made it possible to identify which component was affecting the other. Finally, from a comparison of the amounts of each drug required to produce the interaction, a projection of whether the interaction may occur at clinically attainable concentrations is possible. No other in vitro assay for drug interactions allows such a complete description of the interaction.

In tests with certain combinations, a significant interaction was detected in some but not all decimal mixtures. This was the case for the antagonism of quinolones by chloramphenicol. Datum points for combination decimal mixtures containing a high chloramphenicol-to-quinolone ratio were often within the range of datum points for the single-drug decimal mixtures (Fig. 5). Such skewed data could mask true interactions if $\bar{x}_{comb}$ is always calculated from all nine combination decimal mixtures. Thus, with certain combinations it is important to graph the datum points to determine whether only certain subsets of the combination data should be used to calculate $\bar{x}_{comb}$. In other combinations, drug interactions were found to occur uniformly regardless of the component ratio. Such was the case for synergy between trimethoprim and sulfamethoxazole. Perhaps this explains why this synergy is so easy to detect regardless of the in vitro assay utilized and why the combination has been so successful clinically (1, 2).

Other tests for drug interactions have utilized the basic principle of drug diffusion in agar (1, 6, 8). These include disk approximation tests and assays involving drug-impregnated paper strips. Although each has its own merits and utility, the decimal assay for additivity is the first agar diffusion test that is designed to target a single, predictable additive end point and includes a range of component ratios that allows

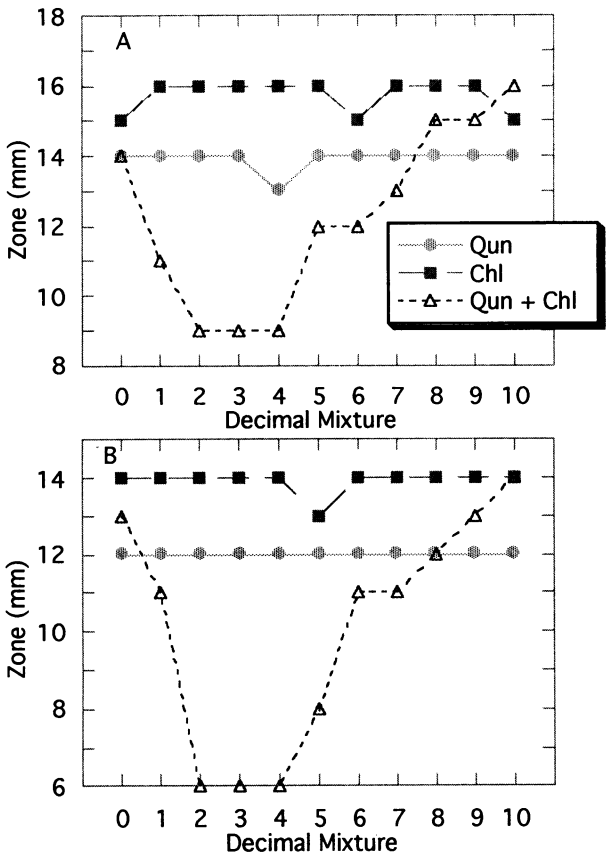

FIG. 5. Antagonism of ofloxacin (quinolone [Qun]; 100% in combination decimal mixture 0) (A) or ciprofloxacin (Qun; 100% in combination decimal mixture 0) (B) by chloramphenicol (Chl; 100% in combination decimal mixture 10) in tests with *E. cloacae* 55 (A) and *S. aureus* 4 (B).

more detailed description of any interaction detected. The results of this initial study are encouraging and indicate that other well-documented drug interactions should be studied by this test. However, it should be noted that, like other agar-based tests, the decimal assay for additivity does not detect the synergistic interactions between penicillins and the aminoglycosides against enterococci. Results in such tests indicate an additive rather than a synergistic interaction (data not shown). This is probably due to the fact that this particular synergistic interaction is essentially a conversion of two bacteriostatic effects into a bactericidal one—a conversion that may not be detectable as a change in zone size. Nevertheless, bactericidal-to-static antagonism, as is the case between quinolones and chloramphenicol (7, 13, 14), was detectable with the decimal assay for additivity. Therefore, certain changes in the qualitative action as well as quantitative effects of drug combinations may be detectable with this assay. It is clear, however, that it cannot replace the time-kill method for detection of all static-to-bactericidal interactions. If the validity of this test can be proven with other well-documented drug interactions, it may become a very useful method for assessing interactions between new combinations of antibiotics. Although less labor-intensive than time-kill techniques, the decimal assay for additivity may not be practical for use in routine clinical laboratories because of the preliminary tests necessary to define parameters for use in the final assay, i.e., target zone size and BEF. However, it should prove useful in research laboratories if its accuracy can be extended to a broad range of organisms and drug combinations. We propose that this new procedure be referred to as the DAA.

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