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Microdilution Transfer Plate Technique for Determining In Vitro Synergy of Antimicrobial Agents

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A microdilution transfer plate technique for determining in vitro synergy of antimicrobial agents is described. Combinations of gentamicin-nalidixic acid against *Proteus mirabilis* and rifampin-amphotericin B against *Candida albicans* are used as examples to demonstrate the technique. Results correlate with published data obtained by conventional methods. The technique is effective for evaluating the in vitro effects of antimicrobial agent combinations against both bacteria and fungi. The technique enables one to produce a checkerboard gradient in a fast, convenient, and reproducible way; results are easily visualized.

In vitro synergy of antimicrobial agents is traditionally accomplished using a tube broth dilution "checkerboard" in which several concentrations of one drug are each combined with concentrations of a second drug (4, 14). Agar dilution can be applied to this technique (11). Several agar diffusion methods are also described (7, 9, 12).

Microdilution methods have been described for determining synergy (3, 5, 6, 15, 16). Two methods (15, 16) involve dilution of both drugs before pipetting each into the microdilution plate. The method of Kluge et al. (6) requires diluting one drug in the microdilution plate, then adding the second drug (already diluted) to the wells containing the first drug. In two other methods described (3, 5) both drugs are diluted in the same microdilution plate.

This study describes a microdilution technique using the transfer plate (Cooke Engineering Co., Alexandria, Va.). The technique permits one drug dilution sequence to be superimposed upon another. This results in a rapid, reproducible, quantitative determination with simple dilution calculations.

Two known synergistic combinations, gentamicin-nalidixic acid against *Proteus mirabilis* and rifampin-amphotericin B against *Candida albicans* were selected as examples to demonstrate the technique.

MATERIALS AND METHODS

Antimicrobial agents. Rifampin (lot 557303, assay 990 μg/mg) was purchased from Calbiochem. Gentamicin (lot GMC-4M-6231, assay 578 μg/mg) was a gift from Schering Corp. Amphotericin B (lot 3956-001, assay 876 μg/mg) was a gift from E. R. Squibb and Sons, Inc. Nalidixic acid (lot J9463) was a gift from Sterling-Winthrop. All drugs were solubilized in 50% Me_2SO-50% ethanol and diluted in distilled water. Final solvent concentration in the microdilution plate was ≤1.0%.

Cultures and growth media. *P. mirabilis* (ICN 40) was used for the nalidixic acid-gentamicin combination; brain heart infusion (Difco) was the medium used for this example. *C. albicans* (ICN 80) was used for the amphotericin B-rifampin combination; a medium containing a yeast-nitrogen base (Difco), L-asparagine, and dextrose was used (13).

All incubations were for 18 to 24 h at 35°C.

Synergy assay. The test for synergy was performed using a 96-well, flat-bottom microdilution plate (Microtest II, Falcon, Div. of Becton, Dickinson & Co., Oxnard, Calif.) and 50-μl microdiluters (Cooke) to dilute drug I. Drug II was diluted in a transfer plate (Cooke) using 25-μl microdiluters (Cooke). Wells in the transfer plate have a calibrated orifice designed to retain the material in the well by surface tension until contact is made with the fluid in the flat-bottom plate. This contact breaks the surface tension and allows all wells of the transfer plate to drain. The system includes a transfer plate holder and carrier, which were autoclaved before use. Pipetting into plates was accomplished using precision pipettes with sterile, disposable tips (Medical Laboratory Automation Inc., Mt. Vernon, N. Y.).

The flat-bottom plate was prepared by first pipetting 50 μl of growth media into each well. Next, 50 μl of drug I was pipetted into all wells of row B (12 wells across). Twofold serial dilutions were then made from row B through row G using 50-μl microdiluters.

The transfer plate was prepared by first pipetting 25 μl of growth media into each well. Next, 25 μl of drug II was pipetted into all wells of column 2 (8 wells across). Twofold serial dilutions were made from column 2 through column 11 using 25-μl microdiluters.

Before draining drug II from the transfer plate

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into the flat-bottom plate, the flat-bottom plate was oriented with the highest concentration of drug I at the top. The transfer plate was oriented above the flat-bottom plate with the highest concentration of drug II to the left. Contents of the transfer plate were added to the flat-bottom plate following the manufacturer's instructions. This transfer achieved a concentration gradient (highest in the upper left to lowest in the lower right) of both drugs in the flat-bottom plate (Fig. 1). Each well in the flat-bottom plate was inoculated with 200 μl of growth media containing ~8 10^5 cells/ml. Final well volume was 275 μl.

Plate layout of the assay is given in Fig. 1. The four-corner wells of the plate contained no drug and were used as positive growth controls. The minimum inhibitory concentration (MIC) of drug I alone was determined in columns 1 and 12. The MIC of drug II alone was determined in rows A and H.

After incubation, a lack of growth in wells below the MIC levels of both drugs was an indication of a possible synergistic effect; drug concentrations of these wells were plotted on an isobologram. Definition of synergy was that of Sabath (12).

Minimum lethal concentrations were determined by pipetting 50 μl of the well contents into a tube containing 5.0 ml of broth. No growth after incubation indicated a lethal effect.

The fractional inhibitory concentration (FIC) index and the fractional lethal concentration (FLC) index were calculated as follows (2, 10):

$$\text{FIC index} = \frac{\text{MIC of SMX in combination}}{\text{MIC of SMX alone}} + \frac{\text{MIC of TMP in combination}}{\text{MIC of TMP alone}}$$

(Abbreviations: SMX, sulfamethoxazole; TMP, trimethoprim.) Since rifampin was not inhibitory or lethal by itself, the highest concentration of the drug tested was used to calculate the FIC or FLC index.

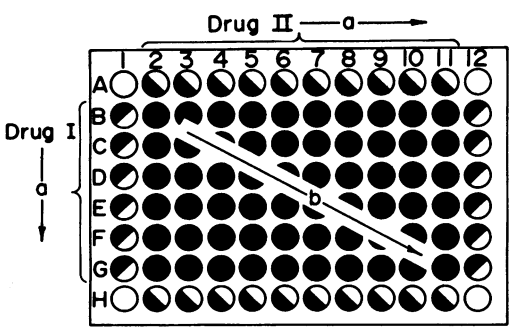

FIG. 1. *Plate layout of microdilution transfer plate synergy assay. a, direction of dilution; b, direction of concentration gradient; ◐, drug I; ◑, drug II; ●, I + II; ○, growth control.*

RESULTS

Nalidixic acid and gentamicin. The results of 10 consecutive tests of the nalidixic acid-gentamicin combination against *P. mirabilis* are given in Tables 1 and 2. Bacteriostatic and bactericidal synergy was produced by this combination. The FIC and FLC indexes were 0.654 ± 0.057 and 0.825 ± 0.038, respectively (tests by P. Dougherty).

Rifampin and amphotericin B. Results of the rifampin-amphotericin B combination against *C. albicans* are given in Tables 3 and 4. Both fungistatic and fungicidal synergies

TABLE 1. *Replicate testing of minimal inhibitory concentrations of gentamicin and nalidixic acid, alone and in combination, against Proteus mirabilis using the microdilution transfer plate technique*

Test	MIC (μg/ml)[a]				FIC index[b]
	Alone Gent	Alone Nal A	Combination		
			Gent	Nal A	
1	1.0	4.0	0.062	2.0	0.56
2	1.0	4.0	0.062	2.0	0.56
3	1.0	4.0	0.062	2.0	0.56
4	1.0	4.0	0.125	2.0	0.62
5	0.5	4.0	0.250	2.0	1.00
6	0.5	4.0	0.250	2.0	1.00
7	1.0	4.0	0.062	2.0	0.56
8	1.0	4.0	0.062	2.0	0.56
9	1.0	4.0	0.062	2.0	0.56
10	1.0	4.0	0.062	2.0	0.56

[a] Gent; Gentamicin; Nal A, nalidixic acid.
[b] Mean of FIC indexes ± standard error: 0.654 ± 0.057.

TABLE 2. *Replicate testing of minimal lethal concentrations of gentamicin and nalidixic acid, alone and in combination, against Proteus mirabilis using the microdilution transfer plate technique*

Test	MLC (μg/ml)[a]				FLC index[b]
	Alone Gent	Alone Nal A	Combination		
			Gent	Nal A	
1	1.0	8.0	0.25	4.0	0.75
2	1.0	8.0	0.50	4.0	1.00
3	1.0	8.0	0.25	4.0	0.75
4	1.0	8.0	0.25	4.0	0.75
5	1.0	8.0	0.50	2.0	0.75
6	0.5	8.0	0.25	4.0	1.00
7	1.0	8.0	0.25	4.0	0.75
8	1.0	8.0	0.50	4.0	1.00
9	1.0	8.0	0.25	4.0	0.75
10	1.0	8.0	0.25	4.0	0.75

[a] Gent, Gentamicin; Nal A, nalidixic acid.
[b] Mean of FLC indexes ± standard error: 0.825 ± 0.038.

TABLE 3. *Replicate testing of minimal inhibitory concentrations of amphotericin B and rifampin, alone and in combination, against Candida albicans using the microdilution transfer plate technique*

Test	MIC (μg/ml)[a]				FIC index[b]
	Alone Am B	Alone Rif	Combination		
			Am B	Rif	
1	1.0	>250	0.06	31.3	0.19
2	1.0	>250	0.06	15.6	0.12
3	1.0	>250	0.06	15.6	0.12
4	1.0	>250	0.06	7.8	0.09
5	1.0	>250	0.03	31.3	0.19
6	1.0	>250	0.06	7.8	0.09

[a] Am B, Amphotericin B; Rif, rifampin.
[b] Mean of FIC indexes ± standard error: 0.133 ± 0.018.

TABLE 4. *Replicate testing of minimal lethal concentrations of amphotericin B and rifampin, alone and in combination, against Candida albicans using the microdilution transfer plate technique*

Test	MCL (μg/ml)[a]				FLC index[b]
	Alone Am B	Alone Rif	Combination		
			Am B	Rif	
1	2.0	>250	0.5	7.8	0.28
2	4.0	>250	0.5	7.8	0.15
3	2.0	>250	0.5	7.8	0.28
4	4.0	>250	0.5	7.8	0.15
5	2.0	>250	0.25	15.6	0.19
6	2.0	>250	0.5	7.8	0.28

[a] Am B, Amphotericin; Rif, rifampin.
[b] Mean of FLC indexes ± standard error: 0.221 ± 0.026.

were observed. The FIC and FLC indexes were 0.133 ± 0.018 and 0.221 ± 0.026, respectively, for six consecutive tests (tests by D. Yotter).

DISCUSSION

The results of this quantitative study confirm the qualitative synergy data reported previously for these combinations. Bactericidal synergy of nalidixic acid and gentamicin against *Enterobacteriaceae* was reported by Michel et al. (9). The potentiation of rifampin by amphotericin B was reported by Beggs et al. (1) and Medoff et al. (8).

We have found the microdilution transfer plate technique useful for evaluating the in vitro effects of antimicrobial agent combinations against both bacteria and fungi. Quantitative inhibitory and lethal synergistic values are reproducible. The FIC and FLC indexes for the nalidixic acid-gentamicin combination, determined by using a single-plate method (3), were 0.625 and 0.75, respectively. These values compare favorably with the FIC and FLC indexes calculated from the microdilution transfer plate data.

The technique provides an efficient and convenient way to produce the desired checkerboard gradient. After the stock drug solutions and inocula are prepared, an experienced worker can set-up, dilute, and inoculate a plate in less than 40 min. Individual well concentrations are easily calculated. Additionally, the layout of the plate permits easy visualization of a possible synergistic effect. Each drug is diluted only once in its respective plate. This reduces the potential variation of diluting both drugs in the same plate.

Occasional problems have been encountered due to inadequate drainage of the transfer plate wells. These have been minimized by following the manufacturer's suggestions for the elimination of static charge.

The transfer plate technique represents a small investment and can be easily applied in any laboratory that uses the microdilution method for susceptibility testing. When preparation time, ease of interpretation, and cost of materials using this technique are compared with many conventional methods, the advantages of this technique are readily apparent.

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