

In Vitro Double and Triple Synergistic Activities of Polymyxin B, Imipenem, and Rifampin against Multidrug-Resistant *Acinetobacter baumannii*

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Eight unrelated clinical *Acinetobacter baumannii* isolates resistant to all commonly used antibiotics were subjected to three-dimensional checkerboard microtiter plate dilution and time-kill studies at one-fourth of their MICs of polymyxin B, imipenem, and rifampin. Synergy was demonstrated with combinations of polymyxin B and imipenem, polymyxin B and rifampin, and polymyxin B, imipenem, and rifampin. Double combinations of polymyxin B and imipenem and of polymyxin B and rifampin were bactericidal for seven of eight isolates, and triple combinations were bactericidal for all isolates within 24 h. Future clinical studies using double and triple therapy with these antibacterials may provide an effective option against potentially lethal infection due to multiresistant *Acinetobacter baumannii*.

In recent years, *Acinetobacter baumannii* has emerged as one of the more ubiquitous antibiotic-resistant gram-negative nosocomial pathogens among critically ill patients (4, 23). Although the carbapenems, ampicillin-sulbactam, and amikacin have retained excellent in vitro and clinical activities against susceptible strains of *A. baumannii*, a growing number of reports have documented resistance to these antibacterials (1, 2, 10, 12–14, 16). As a result nontraditional agents, including polymyxin B and colistin, have been used to treat patients infected with multiresistant *A. baumannii* (6, 9, 15). However, pulmonary infections have not responded well to such monotherapy, and resistance has occurred among strains that have persisted during treatment (6, 15, 24).

In this communication, we describe the in vitro double and triple interactions of polymyxin B, imipenem, and rifampin against eight unique strains of multidrug-resistant *A. baumannii* using checkerboard microdilution and time-kill methods.

MATERIALS AND METHODS

Eight multidrug-resistant clinical strains of *A. baumannii* were obtained from the Clinical Microbiology Laboratory at New York Hospital Queens. All isolates had different pulsed-field gel electrophoresis patterns and were considered unrelated according to the criteria established by Tenover et al. (21). MICs of selected individual antibiotics were then determined using microdilution and E-test methods (AB Biodisk North America Inc., Piscataway, N.J.) by the Infectious Disease Research Laboratory. E-test metallo-beta-lactamase (E-test MBL) strips were used to detect the presence of molecular class B beta-lactamases (25). Rifampin and polymyxin B were obtained from Sigma (St. Louis, Mo.), and imipenem powder was supplied by Merck and Co. (Rahway, N.J.). MIC determinations were performed using checkerboard microdilution methods with Mueller-Hinton broth and a final inoculum of approximately 5×10^5 CFU/ml. Microdilution plates for evaluation of triple drug combinations were performed in the following manner. The first microtiter plate contained no imipenem and increasing concentrations of rifampin ranging from 0 to 32 $\mu\text{g/ml}$ on the *x* axis and increasing concentrations of polymyxin B ranging from 0 to 32 $\mu\text{g/ml}$ on the *y* axis. Each of the subsequent six plates contained a fixed concentration of

imipenem ranging from 1 to 32 $\mu\text{g/ml}$ with increasing concentrations of rifampin ranging from 0 to 32 $\mu\text{g/ml}$ on the *x* axis and increasing concentrations of polymyxin B ranging from 0 to 32 $\mu\text{g/ml}$ on the *y* axis. MICs and fractional inhibitory concentrations (FICs) were determined after 24 h of growth. The MIC was defined as the well in the microtiter plate with the lowest drug combination at which no visible growth was observed. A bactericidal effect was defined as $\geq 99.9\%$ killing of the starting inoculum. FICs of < 1.0 , 1.0, and > 1.0 were used to define synergy, addition, and antagonism, respectively, according to previously published methods (3, 27). Time-kill studies were also performed according to earlier described techniques (7, 17). *Escherichia coli* strain ATCC 25922 was used as a control for all experiments. The limit of detection in these studies was $2 \log_{10}$ CFU/ml. Three-dimensional isobolograms were generated using Surfer 8 (Golden Software, Inc., Golden, Colo.).

RESULTS

The MICs of the three antibiotics studied for all eight isolates are shown in Table 1. All eight isolates with imipenem MICs of 32.0 $\mu\text{g/ml}$ or $> 32.0 \mu\text{g/ml}$ did not demonstrate an eightfold MIC reduction in the presence of EDTA using the E-test MBL procedure and were presumed not to possess metallo-beta-lactamases, according to the manufacturer's specifications. The results of time-kill experiments using 0.25 μg of polymyxin B/ml, 0.5 μg of rifampin/ml, and 8.0 μg of imipenem/ml for the eight unique clinical isolates of *A. baumannii* tested in this study are also summarized in Table 1. All isolates were killed within 24 h using polymyxin B-imipenem-rifampin, one isolate showed regrowth with imipenem plus polymyxin B (isolate E), and one isolate showed regrowth with polymyxin B plus rifampin (isolate A). Using three-dimensional checkerboard microdilution with polymyxin B, rifampin, and imipenem, the combined concentrations of each antibiotic showing synergy and their sum FICs (ΣFICs) of less than 1.0 against isolates A and B are presented in Table 2. Double combinations of polymyxin B (0.5 $\mu\text{g/ml}$) and imipenem (2.0 to 8 $\mu\text{g/ml}$) were synergistic against isolate A. Double combinations of polymyxin B (0.25 to 0.5 $\mu\text{g/ml}$) and imipenem (0.25 to 8.0 $\mu\text{g/ml}$) were synergistic against isolate B. Synergy was observed with a combination of polymyxin B and rifampin at concentrations of 0.5 $\mu\text{g/ml}$ of each drug against isolate A.

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TABLE 1. MICs of each antibiotic alone and time-kill results for the eight clinical *A. baumannii* isolates

<i>A. baumannii</i> isolate	MIC ($\mu\text{g/ml}$)			Time (h) and antibacterial effect ^a		
	IPM	PB	RIF	IPM+PB+RIF	IPM+PB	PB+RIF
A	32	1	2	2, bactericidal	4, bactericidal	8, regrowth
B	32	1	2	4, bactericidal	2, bactericidal	2, bactericidal
C	>32	2	8	2, bactericidal	8, bactericidal	8, bactericidal
D	>32	2	12	4, bactericidal	24, bactericidal	8, bactericidal
E	>32	1	5	8, bactericidal	2, regrowth	24, bactericidal
F	>32	1	5	8, bactericidal	8, bactericidal	24, bactericidal
G	>32	8	1	8, bactericidal	8, bactericidal	24, bactericidal
H	>32	1.5	>32	24, bactericidal	8, bactericidal	24, bactericidal

^a Time-kill results are for experiments using double and triple combinations of imipenem (IPM), polymyxin B (PB), and rifampin (RIF) at 8, 0.25, and 0.5 $\mu\text{g/ml}$, respectively.

Double combinations of polymyxin B (0.25 to 0.5 $\mu\text{g/ml}$) and rifampin (0.03 to 0.5 $\mu\text{g/ml}$) were synergistic. Triple combinations of polymyxin B (0.12 to 0.5 $\mu\text{g/ml}$), rifampin (0.03 to 1.0 $\mu\text{g/ml}$), and imipenem (1.0 to 16.0 $\mu\text{g/ml}$) were also synergistic against isolates A and B. These results are also shown as three-dimensional isobolograms for isolates A and B (Fig. 1). The areas of concavity correspond to the aforementioned con-

centration ranges of double and triple combinations. The graphs of time-kill experiments using one-fourth MICs of each agent (0.25 μg of polymyxin B/ml, 0.5 μg of rifampin/ml, 8.0 μg of imipenem/ml) alone and in combination are shown for four isolates (A, B, E, and H) in Fig. 2.

DISCUSSION

Several in vitro and animal model studies have demonstrated synergy with polymyxin B plus rifampin or imipenem and with polymyxin B plus meropenem against multidrug-resistant *A. baumannii* (23, 26; N. X. Chin, B. Scully, and P. Della-Latta, Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemother., abstr. E-56, 1998). Another study has shown synergy between polymyxin B and rifampin against *Serratia marcescens* isolates from infected patients, even though the isolates were resistant to each antibiotic alone (19). In vitro triple synergistic studies with polymyxin B, trimethoprim, and sulfonamide have shown efficacy against multidrug-resistant *Burkholderia cepacia*, as has the combination of polymyxin E (methane sulfonate, colistimethate), trimethoprim, and sulfamethoxazole against *S. marcescens* (20, 22).

In the present study, double combinations of polymyxin B plus imipenem and polymyxin B plus rifampin were bactericidal against seven out of eight isolates tested. The triple combination of polymyxin B-rifampin-imipenem was synergistic against all isolates by checkerboard and time-kill studies. In these studies FICs (concentrations or MICs of the antibiotics in the combination divided by the MIC of the antibiotic alone) were calculated for two- and three-drug combinations (Table 2). All MICs have been graphically represented in a three-dimensional isobologram (Fig. 1). Synergy for three antibiotics has been defined by several investigators as a fractional sum of <1.0 (ΣFIC of <1.0) (3, 27). Plotted values showing concave surface areas on the isobolograms correspond to synergy, and convex areas represent antagonism (3, 27). The probable role of polymyxin B in such synergy is its rapid permeabilization of the outer membrane, allowing enhanced penetration and activity of both imipenem and rifampin. Synergy between polymyxin B or other peptide antibacterials and the carbapenems would be expected when carbapenem resistance is due to porin protein defects. Synergy may not be evident if the organism possesses significant carbapenemase activity due to class B beta-lactamases. Metallo-carbapenemases were most likely not contributing to imipenem resistance in the eight isolates, since

TABLE 2. Individual and sum total FICs of the three antibiotics

FIC ($\mu\text{g/ml}$) for isolate A			ΣFIC ($\mu\text{g/ml}$) of PB+RIF+IPM	FIC ($\mu\text{g/ml}$) for isolate B			ΣFIC ($\mu\text{g/ml}$) of PB+RIF+IPM
PB	RIF	IPM		PB	RIF	IPM	
0.5	0.25	0	0.750	0.25	0.015	0	0.265
0.5	0.25	0	0.750	0.5	0.03	0	0.530
0.5	0.015	0.0313	0.546	0.5	0.06	0	0.560
0.5	0.03	0.0313	0.561	0.5	0.125	0	0.625
0.5	0.06	0.0313	0.591	0.5	0.25	0	0.750
0.5	0.125	0.0313	0.656	0.5	0.015	0.0313	0.546
0.25	0.25	0.0313	0.531	0.5	0.03	0.0313	0.561
0.12	0.5	0.0313	0.651	0.25	0.06	0.0313	0.341
0.5	0.015	0.0625	0.578	0.25	0.125	0.0313	0.406
0.5	0.03	0.0625	0.593	0.25	0.25	0.0313	0.531
0.5	0.06	0.0625	0.623	0.12	0.5	0.0313	0.651
0.5	0.125	0.0625	0.688	0.5	0.015	0.0625	0.578
0.5	0.25	0.0625	0.813	0.5	0.03	0.0625	0.593
0.25	0.5	0.0625	0.813	0.5	0.06	0.0625	0.623
0.5	0.015	0.1250	0.640	0.5	0.125	0.0625	0.688
0.5	0.03	0.1250	0.655	0.25	0.25	0.0625	0.563
0.5	0.06	0.1250	0.685	0.25	0.5	0.0625	0.813
0.5	0.125	0.1250	0.750	0.5	0.015	0.1250	0.640
0.25	0.25	0.1250	0.625	0.5	0.03	0.1250	0.655
0.25	0.5	0.1250	0.875	0.5	0.06	0.1250	0.685
0.25	0.015	0.2500	0.515	0.5	0.125	0.1250	0.750
0.5	0.03	0.2500	0.780	0.25	0.25	0.1250	0.625
0.5	0.06	0.2500	0.810	0.25	0.5	0.1250	0.875
0.25	0.125	0.2500	0.625	0.25	0.015	0.2500	0.515
0.12	0.25	0.2500	0.620	0.25	0.03	0.2500	0.530
0.06	0.5	0.2500	0.810	0.25	0.06	0.2500	0.560
0.25	0.015	0.5000	0.765	0.5	0.125	0.2500	0.875
0.25	0.03	0.5000	0.780	0.25	0.25	0.2500	0.750
0.25	0.06	0.5000	0.810	0.12	0.5	0.2500	0.870
0.12	0.125	0.5000	0.745	0.12	0.015	0.5000	0.635
0.06	0.25	0.5000	0.810	0.12	0.03	0.5000	0.650
0.03	0.25	0.5000	0.780	0.25	0.06	0.5000	0.810
0.5	0	0.0625	0.563	0.12	0.125	0.5000	0.745
0.5	0	0.1250	0.625	0.06	0.25	0.5000	0.810
0.5	0	0.2500	0.750	0	0.25	0.5000	0.750
				0.5	0	0.0078	0.508
				0.5	0	0.0625	0.563
				0.25	0	0.1250	0.375
				0.25	0	0.2500	0.500
				0.25	0	0.5000	0.750

^a Abbreviations: PB, polymyxin B; RIF, rifampin; IPM, imipenem.

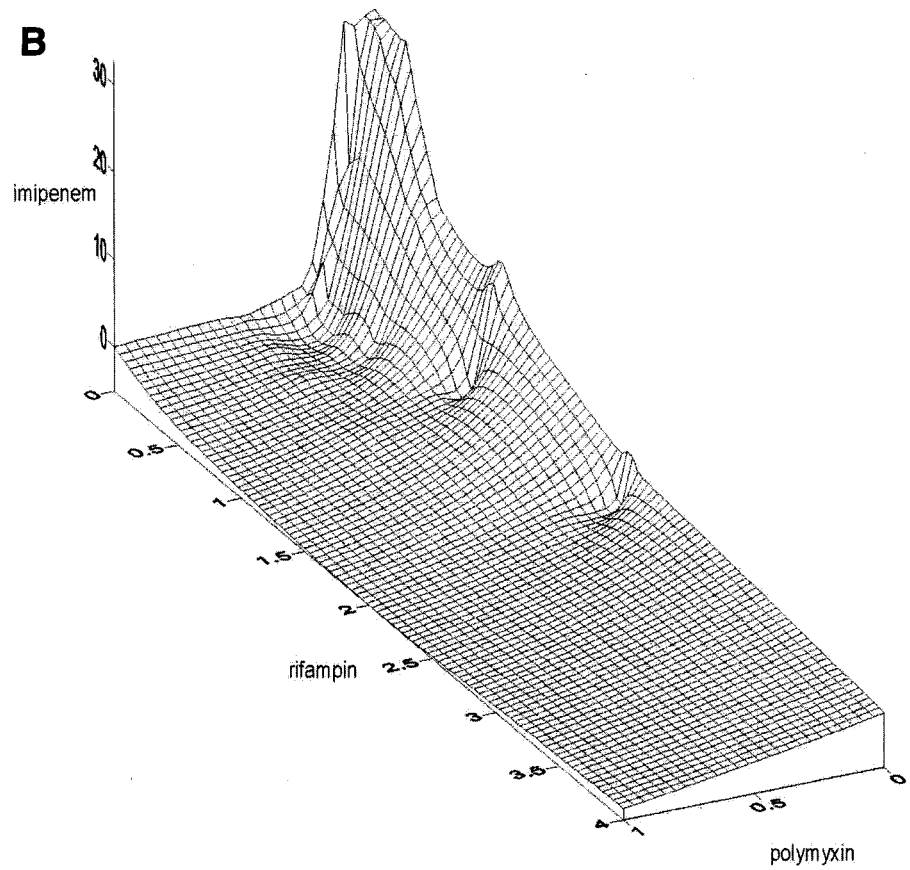
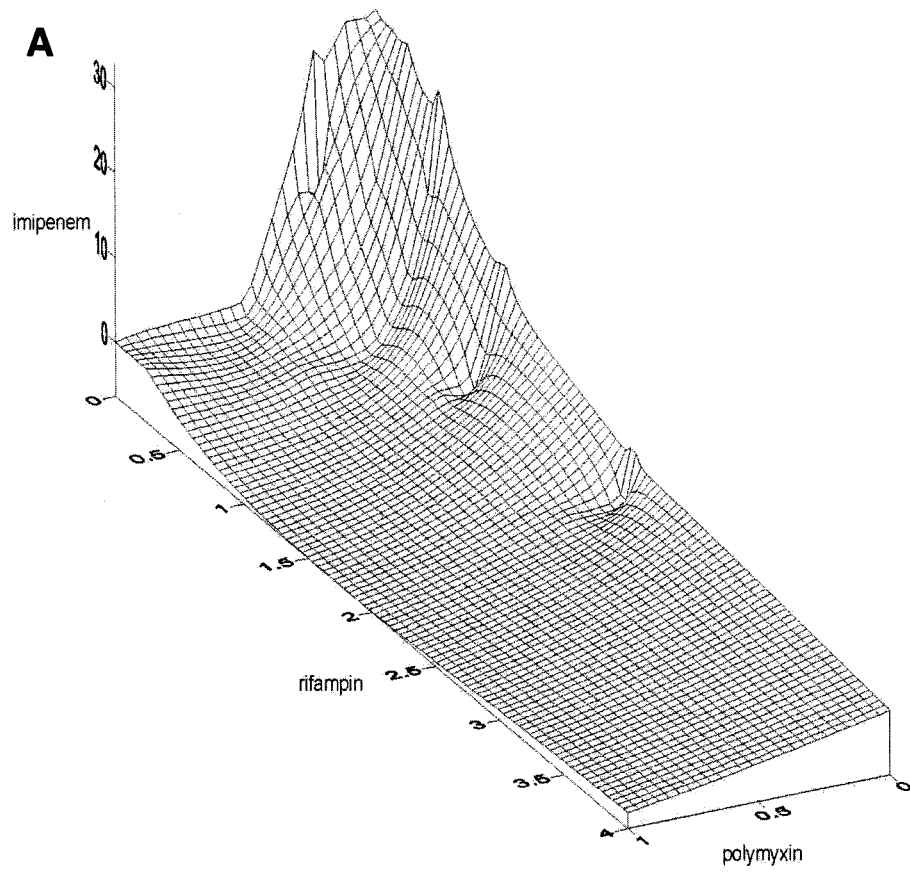


FIG. 1. (A) Three-dimensional isobologram for *A. baumannii* isolate A. The areas on the isobologram showing depressions (concave sinks) that approach zero represent the greatest synergy ($\Sigma\text{FIC} \leq 1.0$). (B) Results for isolate B.

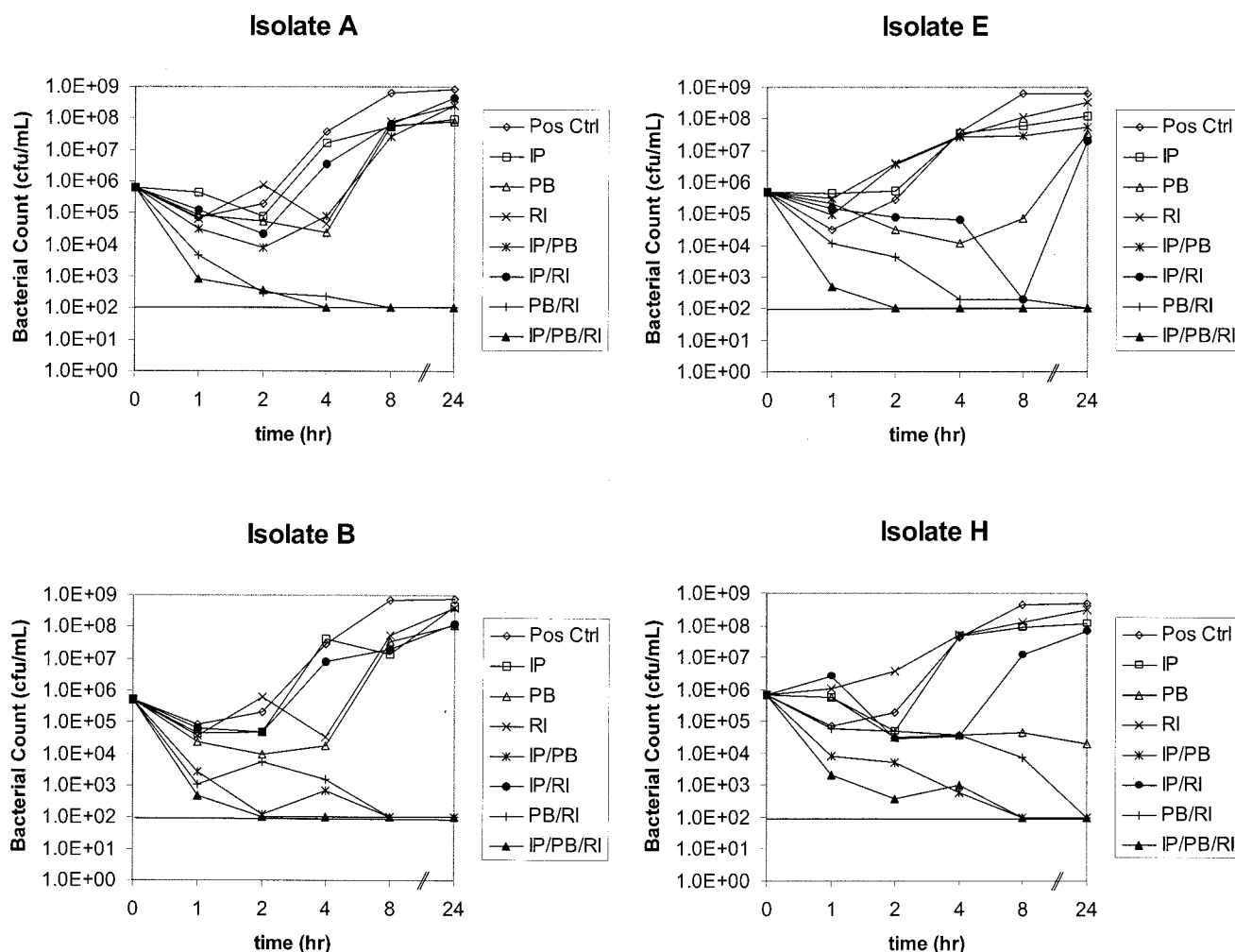


FIG. 2. Time-kill experiments using one-fourth the MIC of each agent (0.25 μg of polymyxin B/ml, 0.5 μg of rifampin/ml, and 8.0 μg of imipenem/ml) alone and in combination for isolates A, B, E, and H. The dotted lines indicate the lower limit of detection of $2 \log_{10}$ CFU/ml.

EDTA using MBL strips did not reverse resistance. Numerous mechanisms other than class B enzymes have been implicated in carbapenem resistance in *Acinetobacter*, including other beta-lactamase classes, porin protein losses or their reduced expression, and penicillin-binding protein changes (8, 23).

Although the use of two or more agents is accepted as appropriate treatment of patients with tuberculosis or human immunodeficiency virus infection, this approach has not been tested in controlled clinical trials against multidrug-resistant *Acinetobacter*. However, it is known that the selection and isolation of mutants from existing populations of cells depend on a number of variables, including the bacterial inoculum at the site of infection, antibacterial agent(s) used, and mechanisms of resistance associated with targeted bacteria. It therefore seems reasonable to use at least two agents for treatment of selected patients infected with multidrug-resistant pathogens that have demonstrated mutation-related progressive resistance during single-drug therapy. Others have also suggested combination therapy as a tool to prevent the emergence of bacterial resistance and for improved outcomes in severely

ill patients infected with gram-negative bacteria known to develop resistance on single therapy (5, 18).

Our studies showed that the combination of polymyxin B plus imipenem was as effective as polymyxin B plus rifampin and may provide an alternative for treatment even when *Acinetobacter* isolates are resistant to the carbapenems due to mechanisms other than metallo-carbapenemases. The addition of rifampin as a third component remains controversial. Synergy between colistin and rifampin has been reported previously in vitro (11). However, all except one of our eight isolates were killed within 24 h by imipenem and polymyxin B. Clinical trials will be necessary to determine whether combination therapy with two or three drugs is more effective than use of a single agent.

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