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Pseudomonas pseudomallei, a Common Pathogen in Thailand That Is Resistant to the Bactericidal Effects of Many Antibiotics

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The purpose of this investigation was to identify newer antimicrobial agents that may be useful in the therapy of melioidosis. The in vitro susceptibilities of 199 clinical isolates of *Pseudomonas pseudomallei* to 22 antibiotics were determined by standard disk diffusion, and those to 13 antibiotics were determined by agar dilution. Over 90% of the isolates were susceptible to imipenem, piperacillin-tazobactam, piperacillin, ceftazidime, ticarcillin-clavulanate, ampicillin-sulbactam, and carumonam by both methods. Standard disk diffusion yielded unacceptably high false-susceptibility results with aztreonam, ciprofloxacin, and temafloxacin. Piperacillin, ceftazidime, imipenem, and ciprofloxacin were not bactericidal for three selected *P. pseudomallei* strains as determined by time-kill curve methods. Furthermore, addition of ciprofloxacin to piperacillin, ceftazidime, or imipenem did not enhance bactericidal activity. One hundred ninety-four strains showed weak β-lactamase production that did not increase upon incubation with cefoxitin. These findings suggest that several newer antimicrobial agents may prove useful in the treatment of melioidosis. However, results of susceptibility studies involving *P. pseudomallei* and newer agents must be interpreted with caution.

Melioidosis, an infection caused by *Pseudomonas pseudomallei*, is endemic in Thailand, other parts of Southeast Asia, and northern Australia (3, 19, 21, 28). The diagnosis of melioidosis is being made with increasing frequency in Thailand, where over 700 cases were reported at the National Workshop on Melioidosis in 1986 (28). Septicemic melioidosis was seen in 57.4% of 686 patients evaluated, and 44.9% had pulmonary involvement. The overall mortality rate among infected patients exceeded 40% (28). Treatment with conventional antibiotics, such as chloramphenicol, tetracycline, kanamycin, or trimethoprim-sulfamethoxazole, is usually ineffective in patients with disseminated septicemic infections, whose mortality rates approach 90% (21, 28). New antimicrobial agents with enhanced activity against *P. pseudomallei* are needed to reduce the morbidity and mortality associated with melioidosis. To identify new, potentially more effective therapy, we determined the susceptibilities of 199 clinical isolates of *P. pseudomallei* to several new antimicrobial agents by using disk diffusion and agar dilution. β-Lactamase activity was measured by hydrolysis of nitrocephin before and after cefoxitin induction. Selected antibiotics were tested for bactericidal activity and synergy against representative strains by using the time-kill method.

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MATERIALS AND METHODS

Bacterial strains. With permission from the U.S. Public Health Service and Department of Agriculture, 200 clinical isolates of *P. pseudomallei* recovered from blood, sputum, urine, and exudate specimens of different patients were imported from Srinagarind Hospital, Khon Kaen, Thailand. These isolates were identified as *P. pseudomallei* by colony morphology, staining reaction, motility, biochemical, and growth characteristics (15). *P. pseudomallei* ATCC 11668, ATCC 15682, and ATCC 23343 were tested for comparison. *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as control strains in susceptibility tests. Cultures were prepared in Todd-Hewitt broth–10% glycerol, and multiple samples of each were stored at −80°C.

Antimicrobial agents. The following antibiotics were kindly provided by the manufacturers: ampicillin-sulbactam (Roerig Pfizer Pharmaceuticals, New York, N.Y.); amoxicillin-clavulanate and ticarcillin-clavulanate (Beecham Laboratories, Piscataway, N.J.); cefixime, piperacillin, and tazobactam (Lederle Laboratories, Pearl River, N.Y.); carumonam (Hoffmann-La Roche Inc., Nutley, N.J.); aztreonam (E.R. Squibb & Sons, Princeton, N.J.); imipenem (Merck Sharp & Dohme, West Point, Pa.); ceftazidime (Glaxo Inc., Durham, N.C.); cefpirome (Hoechst-Roussel Pharmaceuticals Inc., Somerville, N.J.); ofloxacin (Ortho Pharmaceutical Corp., Raritan, N.J.); ciprofloxacin (Miles Pharmaceuticals, West Haven, Conn.); and temafloxacin (Abbott Laboratories, North Chicago, Ill.). Amdinocillin, ampicillin, rifampin, chloramphenicol, tetracycline, kanamycin, trimethoprim-sulfamethoxazole, ticarcillin, and cefoperazone were purchased from Baxter Scientific Products, North Kansas City, Mo.

Antimicrobial susceptibility. Disk diffusion tests were performed by standard methods (25) with Mueller-Hinton agar. Commercially available disks of ampicillin-sulbactam, amoxicillin-clavulanate, and ticarcillin-clavulanate were used. Piperacillin (100 μg)-tazobactam (30 μg) disks were prepared in our laboratory. All antibiotics except cefixime were tested by disk diffusion. MICs of 13 selected antibiotics

TABLE 1. Antibiotic susceptibility patterns of 199 strains of *P. pseudomallei* determined by standard disk diffusion and agar dilution methods

Antibiotic	% of strains susceptible by:		MIC (μg/ml)[a]		
	Disk diffusion	Agar dilution	Range	50%	90%
Imipenem	100	100	0.06–4	0.5	0.5
Piperacillin-tazobactam	100	ND[b,c]	0.25–8	1	1
Piperacillin	100	100	0.25–16	1	2
Ceftazidime	100	99.5	0.125–16	1	2
Cefoperazone	99.5	ND	ND	ND	ND
Amoxicillin-clavulanate	99.5	ND	ND	ND	ND
Ticarcillin-clavulanate	99.5	99.5	1.0–>256	4	4
Carumonam	98	99.5	0.5–>256	2	4
Chloramphenicol	97	ND	ND	ND	ND
Tetracycline	97	ND	ND	ND	ND
Ampicillin-sulbactam	93.5	99.5	0.25–128	4	8
Temafloxacin	60.3	9.5	0.25–32	8	16
Ciprofloxacin	56.8	5.5	0.125–16	4	8
Cefpirome	40.7	ND	ND	ND	ND
Ofloxacin	39.7	ND	ND	ND	ND
Aztreonam	31.7	17	8–>256	16	32
Trimethoprim-sulfamethoxazole	18.6	ND	ND	ND	ND
Kanamycin	12.6	ND	ND	ND	ND
Ticarcillin	3.5	0.5	64–>256	>256	>256
Ampicillin	2	2	0.25–>512	32	32
Rifampin	1.5	ND	ND	ND	ND
Amdinocillin	1	ND	ND	ND	ND
Cefixime	ND	41.5	1–16	2	4

[a] MIC breakpoints (micrograms per milliliter): imipenem, ≤4 and ≥16; ceftazidime, carumonam, ampicillin, ampicillin-sulbactam, and aztreonam, ≤8 and ≥32; piperacillin, ticarcillin, and ticarcillin-clavulanate, ≤16 and ≥128; cefixime, ≤1 and ≥4; ciprofloxacin, ≤1 and ≥4; temafloxacin, ≤2 and ≥8. 50% and 90%, MICs for 50 and 90% of the strains, respectively.
[b] Piperacillin-tazobactam breakpoints have not been established.
[c] ND, Not done.

were determined. For MICs, an inoculum of 10^5 CFU per spot was applied to freshly prepared drug-containing Mueller-Hinton agar plates with a Steers replicator. Antibiotic powders were dissolved in appropriate buffer as directed by the manufacturer. When clavulanic acid, tazobactam, or sulbactam was used in combination with other drugs, a fixed inhibitor concentration of 2 μg/ml was used. The MIC was defined as the lowest concentration of antibiotic that inhibited bacterial growth after overnight incubation (26). Disk diffusion and agar dilution tests were not performed simultaneously. Freshly thawed samples of organisms were used for each test. Since interpretive criteria have not been established for *P. pseudomallei*, National Committee for Clinical Laboratory Standards criteria for members of the family *Enterobacteriaceae* and *P. aeruginosa* were used to determine susceptibility in both assays. Correlation coefficients for disk diffusion zone diameters versus agar dilution MICs were determined by regression line analysis (1).

Time-kill method. Time-kill curves were performed on three representative strains which were susceptible to piperacillin, ceftazidime, and imipenem. Two of these strains, 1 and 2, were susceptible to ciprofloxacin; the third, 3, was resistant to ciprofloxacin. A logarithmic-phase culture was prepared by inoculating 10 ml of Mueller-Hinton broth with 0.1 ml of thawed stock cultures. After incubation for 18 to 20 h at 37°C, cell numbers were determined by plating for the viable count. The culture was diluted with normal saline to give an initial inoculum of 10^5 to 10^6 CFU/ml. Tests were done in 100-ml bottles containing 10-ml samples of the above-described broth cultures with the required concentrations of antibiotics. Piperacillin, ceftazidime, and imipenem were tested either alone or in combination with ciprofloxacin at breakpoint concentrations (64, 8, 4, and 1 μg/ml). The cultures were incubated at 37°C on a shaker at 150 rpm. Samples (100 μl) were removed at 0, 4, and 24 h, and surviving bacteria were determined by plating 10-fold serial saline dilutions in duplicate on Mueller-Hinton agar. Bactericidal effect was determined as ≥2-order-of-magnitude killing at 24 h. Similar experiments were also performed with strains 2 and 3 at the MIC and at one-half of the MBC of the above-described antibiotics.

β-Lactamase assay. β-Lactamase activity was determined by a chromogenic microtiter assay with a nitrocefin substrate. Nitrocefin was eluted with 10 ml of 0.1 M phosphate buffer (pH 7.0) from 50 commercially prepared disks (Cefinase disks; BBL Microbiology Systems, Cockeysville, Md.). Overnight Mueller-Hinton broth cultures of *P. pseudomallei* were diluted 1:20 in Mueller-Hinton broth, and 150 μl was added to each of two wells on a microtiter plate. After 2 to 3 h at 37°C, cefoxitin (10 μg/ml in 0.1 M phosphate buffer) was added to one well to induce β-lactamase production. An equal volume of phosphate buffer was added to uninduced wells. After 2 h at 37°C, 25 μl of nitrocefin was added to each well. After overnight incubation at room temperature, wells were examined for pinkness indicating nitrocefin hydrolysis. A mutant of *P. aeruginosa* that produces high levels of chromosomal β-lactamase constitutively (provided by Christine C. Sanders, Creighton University) was used as the positive control.

RESULTS

Disk diffusion tests with 22 antibiotics were performed on 199 isolates of *P. pseudomallei*. These included 189 shiny, 6 mucoid, and 4 wrinkled yellow-to-white colony morphotypes of the organism. As shown in Table 1, less than 20% of the strains were susceptible to trimethoprim-sulfamethoxazole and kanamycin, two drugs that are often part of

TABLE 2. Association between results of agar dilution MIC and standard disk diffusion

Antibiotic	MIC by agar dilution (μg/ml)	No. of isolates	No. (%) with the following result by standard disk diffusion[a]:		
			S	I	R
Aztreonam	≥32	39	2 (5)[b]	25 (64)	12 (31)
	16	126	38 (30)	83 (66)	5 (4)
	≤8	34	24 (71)	10 (29)	0
Ciprofloxacin	≥4	158	77 (49)[b]	70 (44)	11 (7)
	2	30	26 (87)	4 (13)	0
	≤1	11	10 (91)	1 (9)	0
Temafloxacin	≥8	146	75 (52)[b]	53 (36)	18 (12)
	4	34	26 (76)	8 (24)	0
	≤2	19	19 (100)	0	0

[a] S, Susceptible; I, intermediate; R, resistant.
[b] False-susceptibility results.

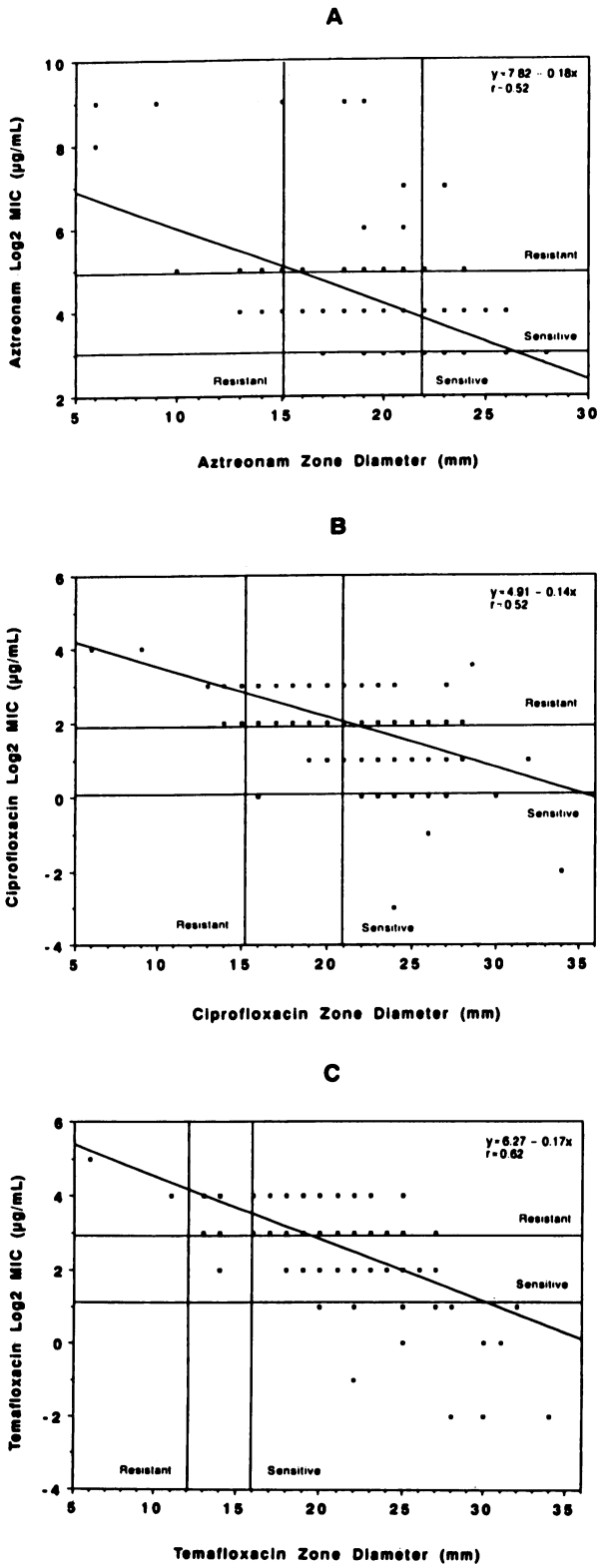

FIG. 1. Regression line analysis plots of disk diffusion zone diameters in millimeters versus agar dilution MICs for 199 *P. pseudomallei* strains tested against aztreonam (A), ciprofloxacin (B), and temafloxacin (C). On the basis of National Committee for Clinical Laboratory Standards criteria for members of the family *Enterobacteriaceae* and *P. aeruginosa*, the horizontal lines represent the recommended MIC interpretive breakpoints and the vertical lines represent the recommended disk diffusion breakpoints. The correlation coefficient (*r*) and the equation for the regression line are shown in the upper right-hand corner of each panel.

conventional therapy for melioidosis (21). The most active of the newer antibiotics tested were imipenem, piperacillin-tazobactam, piperacillin, ceftazidime, cefoperazone, amoxicillin-clavulanate, ticarcillin-clavulanate, carumonam, and ampicillin-sulbactam. Each of these compounds inhibited 90% or more of the isolates. Less than 70% of the isolates were susceptible to quinolones, cefpirome, and aztreonam. Most of the isolates were resistant to ticarcillin, ampicillin, rifampin, and amdinocillin.

By agar dilution testing using 13 antibiotics and all 199 isolates, imipenem, piperacillin-tazobactam, piperacillin, ceftazidime, carumonam, ticarcillin-clavulanate, and ampicillin-sulbactam were the most active agents against *P. pseudomallei*. Aztreonam, ciprofloxacin, and temafloxacin were less active (Table 1). The American Type Culture Collection strains of *P. pseudomallei* demonstrated similar susceptibility patterns.

Comparisons of results obtained by standard disk diffusion and agar dilution tests were made for 12 antibiotics. For most of the antibiotics, the results obtained with the two methods generally agreed (Table 1). However, for aztreonam, ciprofloxacin, and temafloxacin, the false-susceptibility rates by standard disk diffusion were 5, 49, and 52%, respectively (Table 2). Similarly, regression line analysis plots indicated poor correlation between disk diffusion and agar dilution cutoff points for these three drugs (Fig. 1).

In time-kill curve studies, none of the antibiotics examined produced progressive killing over 24 h (Fig. 2). At 4 h, each of the antibiotics examined had produced a weak bactericidal effect; however, this was not sustained over the 24 h of the test. Addition of ciprofloxacin to each of the β-lactam antibiotics did not enhance killing (Fig. 2). Similar results were obtained by using antibiotic concentrations equal to the breakpoint, the MIC, and one-half of the MBC (data not shown). Drug carryover was unlikely under our testing conditions, since decreasing numbers of surviving bacterial colonies were observed as the original culture samples were serially diluted.

One hundred ninety-four strains weakly produced β-lactamase. Of the five that did not produce β-lactamase, three were susceptible to ampicillin, ticarcillin, quinolones, and rifampin. Cefoxitin induction only minimally increased β-lactamase production. Virtually all isolates were resistant to ampicillin and ticarcillin but susceptible to ampicillin-sulbactam and ticarcillin-clavulanate (Table 1). Two β-lactamase-producing strains were highly resistant to β-lactam–β-lactamase inhibitors with a ticarcillin-clavulanate MIC of >256 μg/ml for one strain and an ampicillin-sulbactam MIC of 128 μg/ml for the other.

DISCUSSION

Recommendations for the use of tetracycline, chloramphenicol, kanamycin, and trimethoprim-sulfamethoxazole for treatment of melioidosis are based on previous in vitro studies (4, 12–14, 17, 18). We confirmed the previously reported high prevalence of susceptibility among *P. pseudomallei* to tetracycline and chloramphenicol, but most of our isolates were resistant to kanamycin and trimethoprim-sulfamethoxazole. Treatment failures with these agents

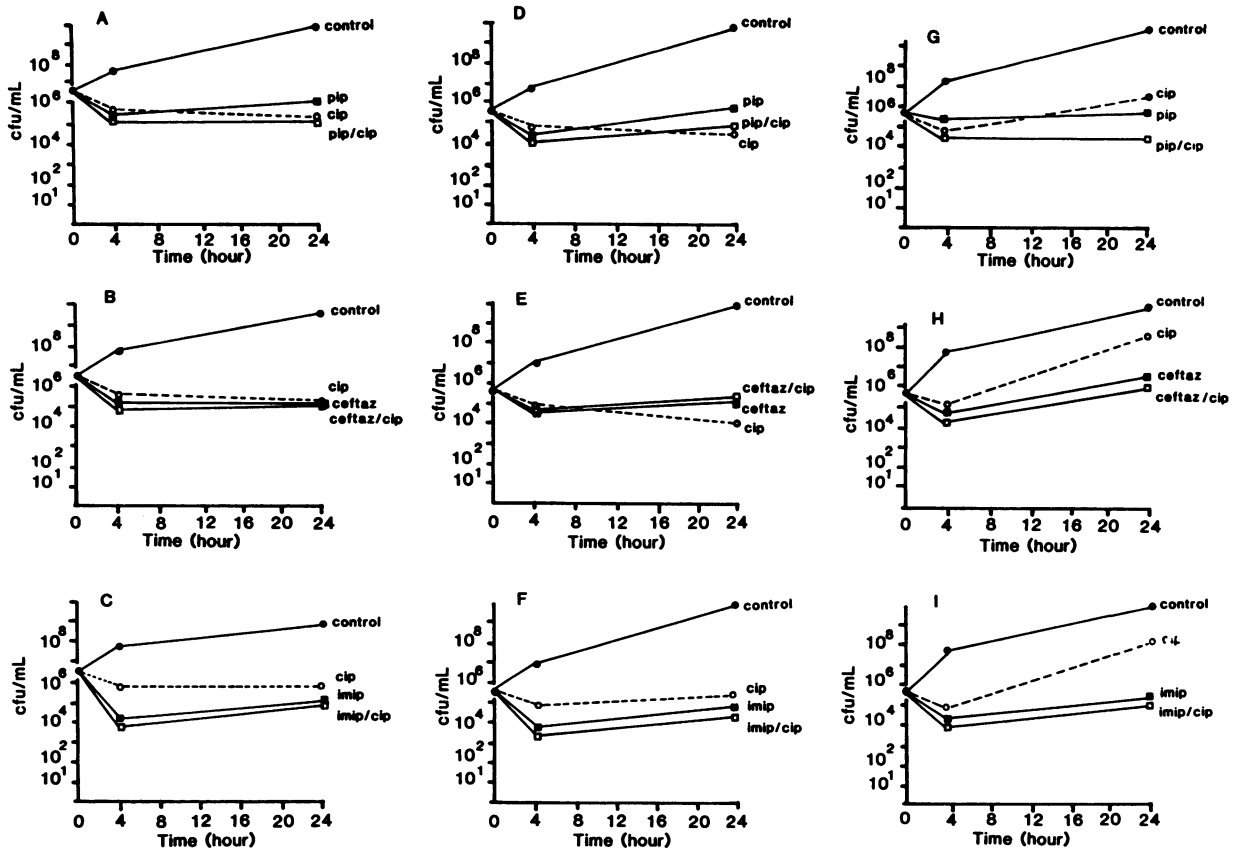

FIG. 2. Time-kill curves of *P. pseudomallei* 1 (A, B, and C), 2 (D, E, and F), and 3 (G, H, and I) against piperacillin (pip), ciprofloxacin (cip), and piperacillin plus ciprofloxacin (pip/cip) (upper row); ceftazidime (ceftaz), ciprofloxacin, and ceftazidine plus ciprofloxacin (ceftaz/cip) (middle row); and imipenem (imip), ciprofloxacin, and imipenem plus ciprofloxacin (imip/cip) (lower row).

may be explained in part by their bacteriostatic activities and the in vitro antagonism demonstrated by most combinations (9, 12).

A number of recent in vitro studies have tested *P. pseudomallei* strains for susceptibility to newer antibiotics (2, 3, 6–8, 16, 20, 23, 28, 29, 31, 33, 34). Potentially useful antibiotics with MICs for 90% of the strains tested well below therapeutic levels in serum included imipenem, piperacillin, ceftazidime, carumonam, azlocillin, mezlocillin, amoxicillin-clavulanate, and ticarcillin-clavulanate. Agents with MICs for 90% of the strains tested at or above peak therapeutic levels included cefoperazone, cefotaxime, and aztreonam. The MICs of moxalactam, quinolones (ciprofloxacin, ofloxacin, norfloxacin, pefloxacin, amifloxacin, and enoxacin), and ticarcillin for 90% of the strains tested uniformly exceeded therapeutic levels in blood. *P. pseudomallei* is intrinsically resistant to narrow- and expanded-spectrum cephalosporins and the aminoglycosides, with the exception of kanamycin (3, 12, 17).

Our isolates had susceptibility patterns similar to those shown in prior studies. Imipenem was the most active drug tested, followed by piperacillin-tazobactam, piperacillin, ceftazidime, carumonam, ticarcillin-clavulanate, and ampicillin-sulbactam. These drugs inhibited 90% of strains at concentrations of 1- to 32-fold lower than the achievable therapeutic levels. Moderate-to-poor activities were observed with cefixime, cefpirome, aztreonam, ampicillin, ticarcillin, amdinocillin, and rifampin.

P. pseudomallei is a facultative intracellular pathogen able to survive and multiply within phagocytes (10, 11, 27, 32). The new broad-spectrum quinolone antibiotics penetrate phagocytes well (32). In general, however, they have shown limited activity against *P. pseudomallei* in vitro (2, 6, 8, 20, 33, 34). Our results with ofloxacin, ciprofloxacin, and temafloxacin confirmed these findings. In addition, we found major discrepancies between disk diffusion and agar dilution susceptibility results for ciprofloxacin, temafloxacin, and the monobactam aztreonam. The false-susceptibility results seen with these three antibiotics exceeded the proposed acceptable rate of 1% (24). Our linear regression results suggest that disk diffusion is unsuitable for testing of *P. pseudomallei* susceptibility to ciprofloxacin, temafloxacin, and aztreonam.

Several β-lactams and quinolones have been considered bactericidal against *P. pseudomallei* on the basis of their MBC/MIC ratios of 2 to 4 (2, 8). These include azlocillin, piperacillin, cefotaxime, ceftriaxone, imipenem, carumonam, ciprofloxacin, and norfloxacin. Using time-kill curves, we studied bactericidal activities of three of the most active antibiotics, piperacillin, ceftazidime, and imipenem, against three susceptible strains of *P. pseudomallei*. Ciprofloxacin was chosen for synergy studies in combination with each of these antibiotics because of its availability in oral form, its unique site of action, and a recent report of bactericidal activity against *P. pseudomallei* (8). Despite their low MBC/MIC ratios, piperacillin, ceftazidime, imipenem, and cipro-

floxacin did not demonstrate bactericidal activity against these three strains. We were unable to demonstrate either an additive or synergistic effect when ciprofloxacin was added to these drugs in vitro.

Previous assays for β-lactamase have been positive for nearly all *P. pseudomallei* isolates tested by the chromogenic cephalosporin method (3, 6). Our isolates weakly produced β-lactamase and were not strongly inducible. The potential role of β-lactamase in *P. pseudomallei* antibiotic resistance was reflected by the increasing activity of β-lactam antibiotics against β-lactamase-producing isolates when β-lactamase inhibitors were added. Since clavulanic acid is an inhibitor of non-class 1 *P. pseudomallei* β-lactamase (5), it is likely that our strains produced this enzyme. It has been identified as a membrane-associated chromosomal cephalosporinase resembling the cefuroximases of *P. cepacia* and *Proteus vulgaris* (22). The β-lactamase-producing strains in our study which were highly resistant to ticarcillin-clavulanate also had relatively high imipenem MICs (4 μg/ml). This suggests the possibility of other mechanisms of antibiotic resistance, such as decreased outer membrane permeability, a known mechanism of resistance to imipenem in *P. aeruginosa* (30).

In summary, by both standard disk diffusion and agar dilution MIC, the most active antibiotics against 199 *P. pseudomallei* strains were imipenem, piperacillin-tazobactam, piperacillin, ceftazidime, carumonam, ticarcillin-clavulanate, and ampicillin-sulbactam. Piperacillin, ceftazidime, and imipenem were not bactericidal for *P. pseudomallei* in vitro. However, these agents and the newer β-lactam–β-lactamase inhibitor combinations warrant further study for treatment of melioidosis. The therapeutic value of ciprofloxacin in this setting is limited by the frequency of resistant strains of *P. pseudomallei*, the unacceptably high false-susceptibility rates seen with disk diffusion testing, and the lack of in vitro synergy when tested in combination with piperacillin, ceftazidime, and imipenem. The findings of β-lactamase production in 97% of the strains, a 2- to >64-fold reduction in MICs when β-lactamase inhibitors were added to β-lactam antibiotics, and susceptibilities to β-lactam antibiotics in β-lactamase-negative strains suggest a role for β-lactamase in the resistance of *P. pseudomallei* to β-lactam antibiotics. Resistance to β-lactam–β-lactamase inhibitor combinations, as well as decreased susceptibility of *P. pseudomallei* to new oral quinolones and some expanded-spectrum β-lactams, indicates that there are additional resistance mechanisms.

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