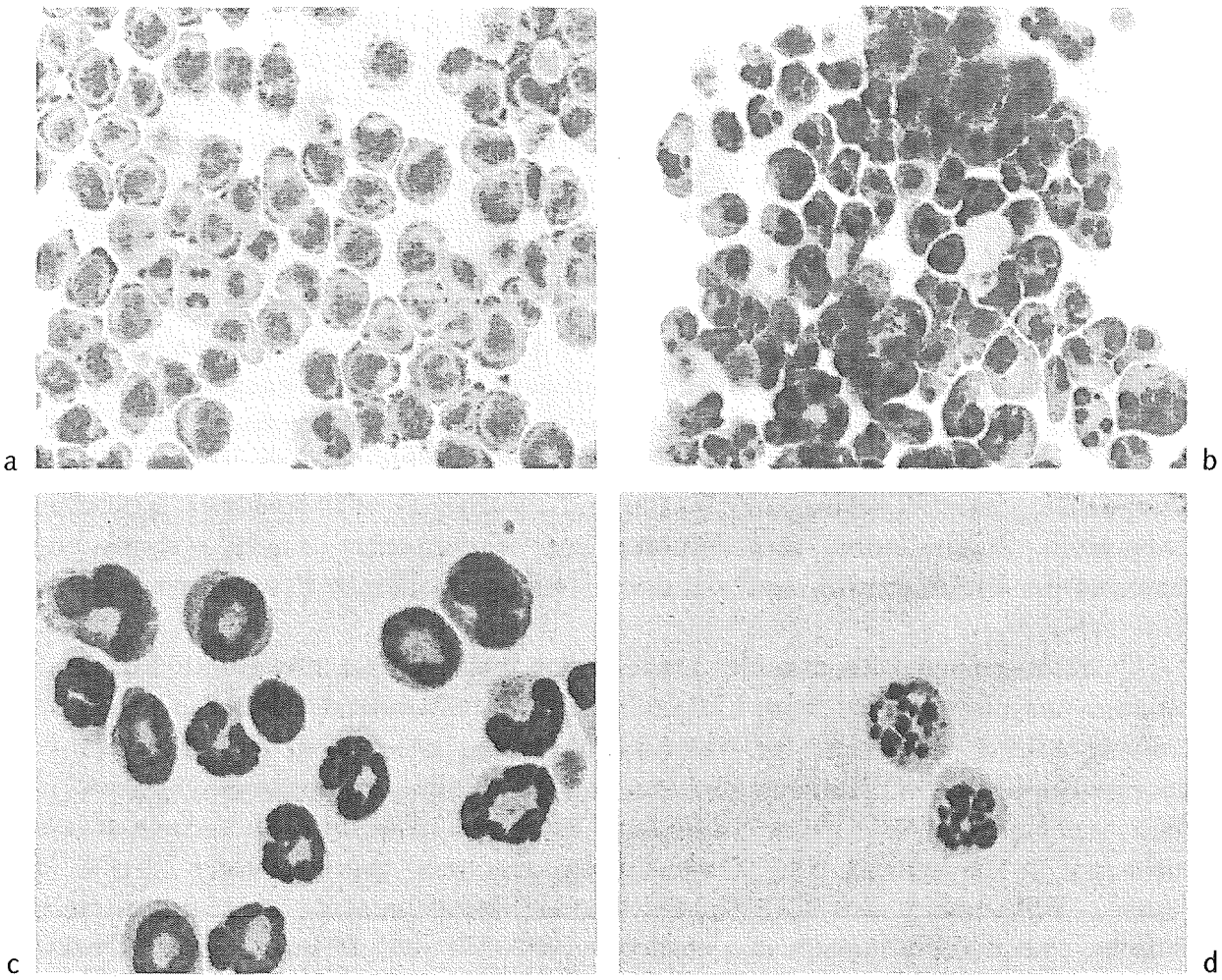

Figure 7
Induction of apoptosis by Pseudomonas *3-oxo-C_{12}-HSL in macrophage and neutrophil*
Macrophage cell line U-937 and mouse neutrophil were incubated with or without 3-oxo-C_{12}-HSL, and then morphology of cells was examined at 4 h after incubation.
a: U-937 cell, control. b: U-937 cell, 3-oxo-C_{12}-HSL. c: neutrophil, control. d: neutrophil, 3-oxo-C_{12}-HSL [59].

Potential of macrolides as quorum-sensing inhibitors

The discovery that gram-negative bacteria employ HSL autoinducer molecules to globally regulate the production of virulence determinants has identified a novel target for therapeutic intervention. The ability to interfere with bacterial virulence by jamming signal generation or signal transduction is intellectually seductive and pharmaceutically appealing, and may also be of considerable clinical importance. Strategies to inhibit quorum-sensing systems include chemical antagonists and specific antibody to inhibit the autoinducers, HSL-destroying enzyme lactonase, and

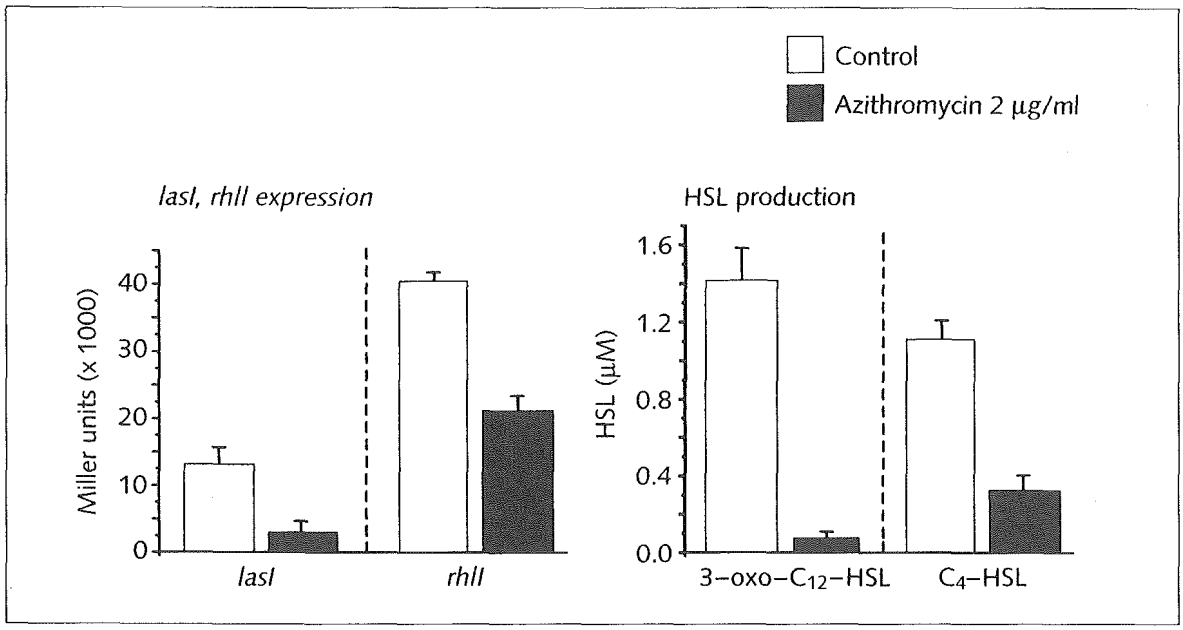

Figure 8
Effects of azithromycin on quorum-sensing systems of P. aeruginosa
P. aeruginosa *was incubated with or without azithromycin 2 μg/ml for 10 hours, and then autoinducer synthase expression* (lasI, rhlI) *and HSL production were examined [67].*

suppression of quorum-sensing by interfering with associated genes and gene products. Several investigators have reported the feasibility of HSL-analogues [64, 65] and synthetic derivatives of natural furanone as means to inhibit bacterial quorum-sensing systems [66].

Clinical and experimental data described above provided a hint that certain macrolides and their analogues may function as *Pseudomonas* quorum-sensing inhibitors. As shown in Figure 8, 2 μg/ml of azithromycin significantly suppressed transcription of *lasI* by 80% and *rhlI* by 50% in *P. aeruginosa* PAO1 [67]. Additionally, the production of 3-oxo-C_{12}-HSL and C_4-HSL was inhibited to approximately 6% and 28% of the control, respectively. In contrast, azithromycin treatment did not alter the expression of the *xcpR* gene, which codes for a structural protein belonging to the type II secretion pathway. These data suggested that azithromycin suppressed quorum-sensing systems in *P. aeruginosa*, and azithromycin's effects on these bacteria are somewhat selective in nature. Importantly, we have observed suppression of *lasI* gene expression by erythromycin, clarithromycin and roxithromycin, but not by oleandomycin and josamycin. These results suggested that the clinically effective macrolides are also the macrolides that are active in suppressing quorum-sensing system, and are consistent with the notion that macrolides might reduce the production of *Pseudomonas* virulence factors by inhibiting the synthesis of the autoinducer molecules.

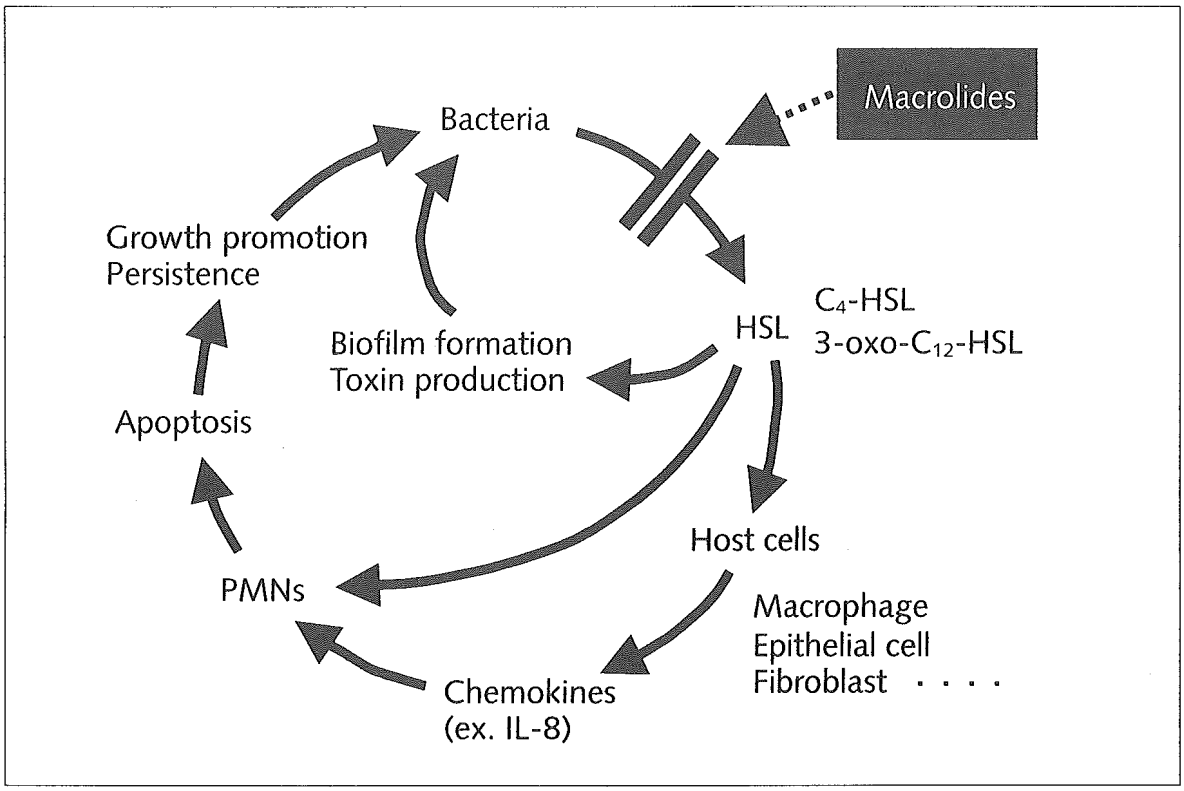

Figure 9
Inhibition of HSL production by macrolides and its impact on pathogenesis of chronic P. aeruginosa *pulmonary infection [59].*

Figure 9 demonstrates several potential mechanisms by which macrolide antibiotics may suppress quorum-sensing systems and highlight their contribution to clinical efficacy in chronic *P. aeruginosa* pulmonary infections. Activation of the quorum-sensing cascade promotes biofilm formation at the site of infection, which make conditions more favorable for bacterial persistence in the lung. Bacterial autoinducers, especially 3-oxo-C_{12}-HSL, stimulates several types of cells, such as epithelial cells, fibroblasts, and macrophages, to induce production of neutrophil chemotactic factors (IL-8 in humans and MIP-2 in mice). Migrated neutrophils are triggered to produce several toxic substances for killing of bacteria, but these molecules, in conjunction with bacterial virulence factors, promote tissue destruction that is a hallmark of the lungs of CF patients. In sites where bacteria are actively producing autoinducers and autoinducer-regulated virulence factors, host cells come in contact with these bacterial factors. In these sites, neutrophils begin to undergo apoptosis, and this process may be accelerated by the presence of bacterial factors, such as 3-oxo-C_{12}-HSL. Apoptotic neutrophils, in addition to secreted mucus and other cell debris, may serve as nutrients for the growth of bacteria and a niche for

their survival. Macrolide antibiotics strongly suppress *Pseudomonas* quorum-sensing systems, particularly autoinducer production, which may contribute to suppression of virulence factor expression and biofilm formation. Additionally, macrolides may alter pathogen-driven host responses, such as IL-8 production and apoptosis in neutrophil. Taken together, this evidence supports a potential role of certain macrolides as *Pseudomonas* quorum-sensing inhibitors, which may explain at least in part clinical efficacy of this class of antibiotics in chronic *P. aeruginosa* pulmonary infections. Further research regarding the mechanisms of action and putative target molecules of bacterial quorum-sensing systems, is warranted.

Conclusions

Clinical and basic science data summarized in this review suggests the potential of macrolides as a prototypic inhibitor of bacterial quorum-sensing systems. Given that clinical efficacy of macrolides is associated with suppression of bacterial virulence, including quorum-sensing activity, further investigation aimed at characterizing molecular mechanisms involved may prove fruitful in identifying novel strategies of antimicrobial chemotherapy against antibiotic resistant organisms and biofilm disease.

Acknowledgement
We thank Y. Ishii, S. Kimura and E. Tuzuki (Toho University) for their helpful assistance and discussion. We also express our appreciation to H. Hashimoto, S. Miyairi, M. Horikawa, N. Gotoh, M. Ishiguro (Quorum-sensing group member) and J.C. Pechere, C. Van Delden (University of Geneva) for their helpful suggestion and critical discussion.

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ORIGINAL ARTICLE

'Break-point Checkerboard Plate' for screening of appropriate antibiotic combinations against multidrug-resistant Pseudomonas aeruginosa

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Abstract

Increase of multiple drug resistant Pseudomonas aeruginosa (MDRP) is becoming a serious problem in the clinical setting. Although the checkerboard method to determine FIC index and synergistic effects of antibiotic combinations is useful, it is not well adapted to a routine test, mainly because of its time-consuming and labor-intensive nature. Here we report 'Break-point Checkerboard Plate', in which breakpoint concentrations, such as 'S' (sensitive) and 'I' (intermediate), were combined in a microtiter plate with 8 antibiotics, including carbapenem, aminoglycoside and fluoroquinolone. The results obtained from 12 strains of MDRP demonstrated a strong synergistic effect of some antibiotic combinations at clinically relevant concentrations. Our data suggest a usefulness of 'Break-point Checkerboard Plate' to screen appropriate antibiotic combinations against drug resistant organisms, including MDRP.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that causes a wide range of acute and chronic infections, including sepsis, wound and pulmonary infections. Multiple drug-resistant P. aeruginosa (MDRP) is becoming a serious problem in the clinical setting worldwide [1]. Since MDRP demonstrates resistance to almost all antibiotics available, including carbapenems, amynoglycosides and fluoroquinolones, combination therapy is usually required empirically in the absence of any in vitro synergy data, which can result in a less than optimal treatment outcome for these patients.

A number of methods including the 2-dimensional microtiter checkerboard method and the time-kill method have been widely employed to investigate combinations of antibiotics [2]. However, the time-consuming and labor-intensive nature of these tests is a disincentive to their routine use. Moreover, it may be difficult to expect therapeutic responses if synergistic effects were observed only in clinically non-relevant concentrations of the antibiotics. An in vitro method of determining the combination activity of antibiotics at clinically achievable levels, which is simple and convenient to perform and which could be used routinely in clinical microbiology laboratories, is desirable.

Method

For these situations we have designated 'Break-point Checkerboard Plate', in which combinations of 8 antibiotics were arranged in a plate (ceftazidime,-CAZ: piperacillin, PIPC: imipenem, IPM: aztreonam, AZT: gentamicin, GM: ciprofloxacin, CPFX: polymyxin-B, PB: rifampin, RFP), and examined its usefulness to screen appropriate antibiotic combinations against clinical isolates of P. aeruginosa (n = 12, unrelated strains). The NCCLS methodology of antibiotic susceptibility testing, such as preparation of the plate, inoculation of the organism and incubation time, was applied in this study [3]. We selected 2 concentrations from each antibiotic by considering breakpoints of these antibiotics, in addition to tissue and serum concentrations [3–5],

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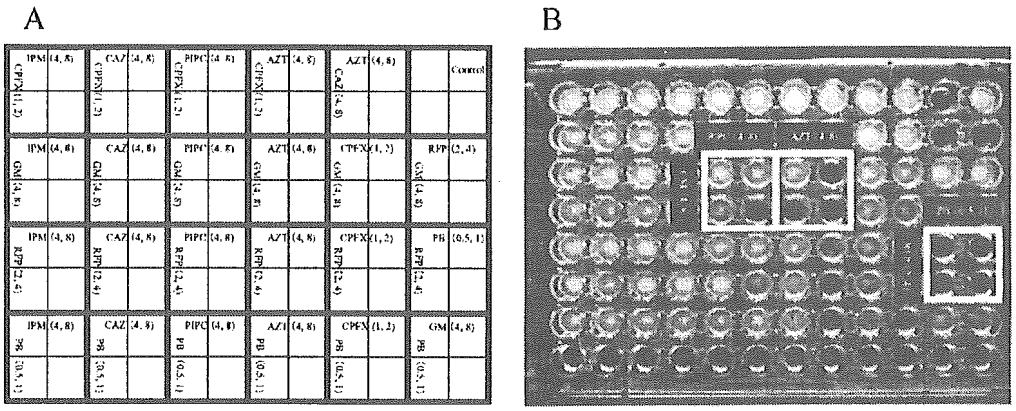

Figure 1. 'Break-point Checkerboard Plate' examined in this study.
Antibiotic combinations and concentrations were designed in 96-well microplate, as shown in Figure 1A. Figure 1B shows an example of the results, in which synergistic effects were demonstrated in several combinations of antibiotics.

as shown in Figure 1A. Figure 2 demonstrates MICs of these antibiotics to P. aeruginosa, which were examined in a broth micro-dilution method as described previously [6]. All these strains were judged to be resistant to IPM, GM and CPFX. Additionally, the majority of these strains were resistant to CAZ, PIPC and AZT. The ranges of MICs of RFP and PB were 16–32 mg/l and 1–4 mg/l, respectively.

Figure 1B demonstrates an example of the results in 'Break-point Checkerboard Plate', in which several synergistic effects were observed. As noted, we observed suppression of growth of this strain in some antibiotic combinations, such as GM+PIPC, GM+AZT and RFP+PB. In particular, the combination effect of RFP+PB was strong, in which growth of bacteria was inhibited at 2 mg/l of RFP plus 0.5 mg/l of PB.

Figure 3 demonstrates the results of 12 strains of MDRP in β-lactam+GM (A) and β-lactam+CPFX (B). The shaded area indicates concentrations of antibiotics used in 'Break-point Checkerboard Plate'. Closed circles in this area demonstrate suppression of growth in these combinations, and the circle was connected to another closed circle by-line, which indicates MICs of each antibiotic (non-combination). For example, a closed circle with asterisk in Figure 3A demonstrates that MICs of AZT and GM to this strain are >64 and 64 mg/l, respectively, and a combination of AZT (8 mg/l) plus GM (8 mg/l) inhibited growth of this strain. In contrast, open circles exhibit MICs of each antibiotic,

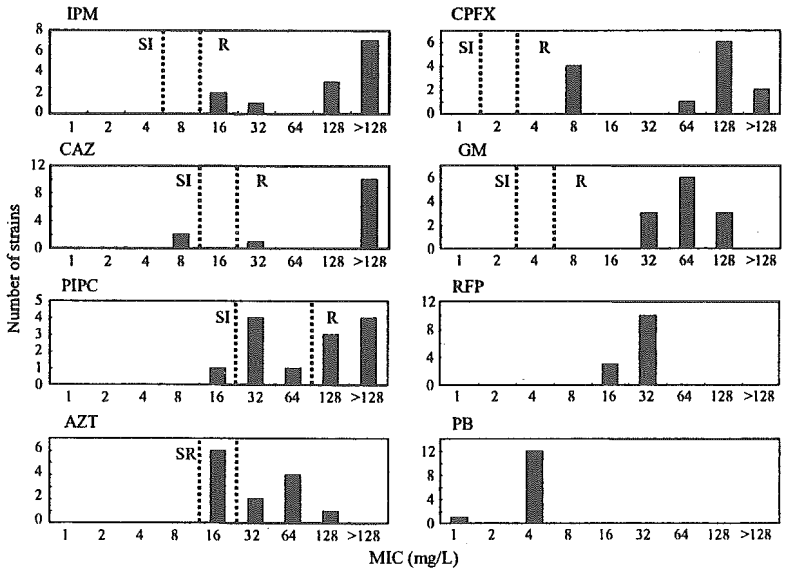

Figure 2. MIC distribution of several antibiotics against MDRP.
MICs of several antibiotics against 12 strains of MDRP were examined in a micro-broth method, as described in the text. Dotted lines indicate break points of 'S', 'I', 'R' in each antibiotic.

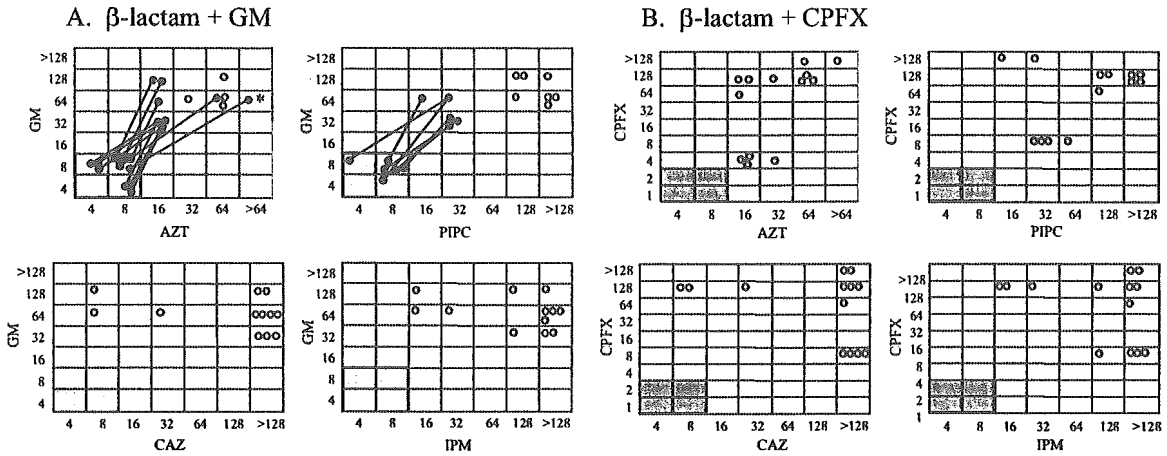

Figure 3. The results of 'Break-point Checkerboard Plate' in β-lactams plus GM, CPFX.
Figure 3A and 3B show the results of combinations of β-lactams+GM or β-lactams+CPFX, respectively.

in which inhibition of growth was not observed in any combination of this plate. In AZT+GM and PIPC+GM, suppression of growth was observed in 8 and 5 of 12 strains, respectively, whereas no combination effect was observed in CAZ+GM and IPM+GM (Figure 3A). Figure 3B demonstrates the results of combination of β-lactams+CPFX, in which there was no synergy in P. aeruginosa examined.

Figure 4 demonstrates the results of PB+β-lactam (A) and PB+other class antibiotics (B). Interestingly, we observed strong synergy in all antibiotics examined in the presence of PB. A concentration of 1 mg/l of PB, which is one quarter of MIC of all strains except 1, decreased MICs of counterpart antibiotics to lower levels in a majority of the strains. In particular, drastic combination effects were observed in PB+RFP, in which growth inhibition was observed at 0.5 mg/l of PB plus 2 mg/l of RFP in 9 of 12 strains examined.

These data suggest that the 'Break-point Checkerboard Plate' may be useful to screen appropriate antibiotic combination against MDRP. Since antibiotic concentrations used were clinically relevant and achievable at the site of infections, the synergy observed strongly suggests effectiveness of this combination in the clinical setting. On the other hand, we could not evaluate antagonism, post-antibiotic effects and antibiotic sequencing effects in combinations, which may be weak points of this plate. Other points of consideration for this method may include how to prepare the plate (freeze or freeze-dry), shelf-life, and quality control, in addition to comparison with and complementation by a regular microbroth dilution method.

Owing to the problems of increasing antibiotic resistance in P. aeruginosa, it is now standard clinical practice to use 2 or more antibiotics to treat these patients with MDRP infections. As a result, a less time-consuming and convenient test for seeking

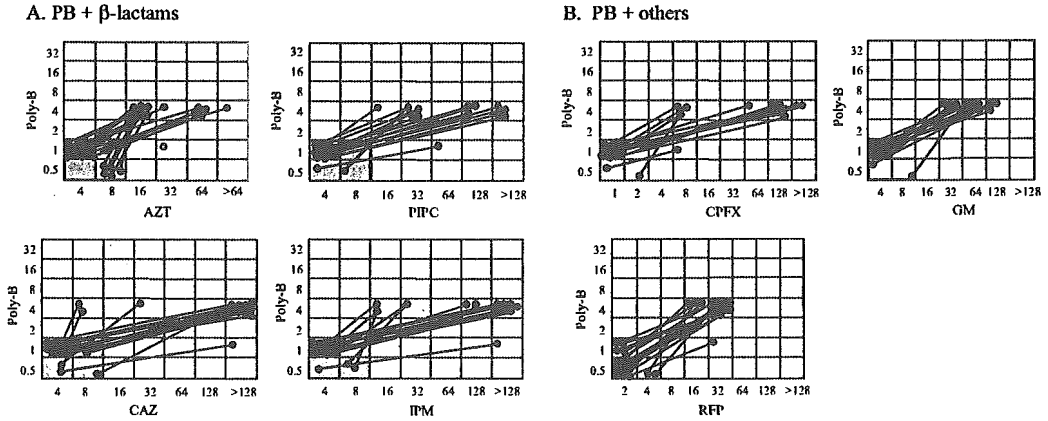

Figure 4. The results of "Break-point Checkerboard Plate" in PB plus β-lactams, others.
Figure 4A and 4B show the results of combinations of PB+β-lactams or PB+others, respectively.

an appropriate antibiotic combination is desired. Bonapace et al. [7] have reported a new test, in which 2 antibiotics of E-test strips were combined in a vertical position. More recently, Tunney and Scott have reported a broth macrodilution sensitivity method based on breakpoint MICs in CAZ and tobramycin [8]. The 'Break-point Checkerboard Plate' presented in this study is principally in line with these methods, and further extended to screening of multiple antibiotic combinations at clinically achievable levels. Once the plates were prepared, the test was easy to perform, less labor-intensive and more feasible to daily routine test, which suggests a potential of this plate for wide application in hospital microbiology laboratories.

Several investigators have reported efficacy of synergy in certain antibiotic combinations, such as β-lactams plus aminoglycosides, against antibiotic resistant organisms. Saiman et al. have reported combination effects with macrolides against antibiotic resistant organisms, including Burkholderia cepacia, Stenotrophomonas maltophilia and P. aeruginosa [9]. Okazaki et al. have investigated effectiveness of phosphomycin combination against MDRP by the efficacy time index [10]. Oie et al. have reported that the combination of AZT and amikacin was the most effective, inhibiting proliferation in MDRP [11]. Our data also suggested synergistic effects of GM, especially in combination with AZT and PIPC. Although a panel of study was done in combination with fluoroquinolones against MDRP, a majority of those studies demonstrated only a minor role for fluoroquinolones in combinations [12–16]. In our research, no combination effects were observed even at half MIC of CPFX plus half MIC of AZT in MDRP examined, as shown in Figure 3B. To confirm these results, we need further investigation by applying more clinical isolates of MDRP and different fluoroquinolone antibiotics.

The polypeptide antibiotics, such as PB and colistin, were first made available for clinical use in the late 1950s and early 1960s. However, as a result of concerns about adverse reactions, such as nephro- and neuro-toxicity, the parenteral use of these agents has been rather limited. Worldwide increases in antibiotic resistant bacteria, in addition to no introduction of new effective antibiotics to combat these organisms, have changed the strategy of antimicrobial chemotherapy. These pressures forced us to look for new antibiotic combinations and to rediscover older agents, such as the polypeptide antibiotics. Recent accumulating evidence from basic science and clinical data has shown a resurgence of these agents as an important salvage therapeutic option for patients with otherwise untreatable serious infections [17,18]. Several in vitro data have demonstrated strong synergy of the polypeptide antibiotics with other antibiotics against multidrug-resistant organisms, such as A. baumannii and MDRP [19–21]. Although the number of patients is still limited, Levin [22], Linden [23] and Sobieszczyk [24] have reported the use of parenteral polypeptide antibiotics for the treatment of serious infection due to antibiotic resistant organisms in 60, 23 and 25 patients, respectively.

The present data demonstrate that 1 mg/l of PB, one quarter of MICs for most strains, drastically decreased MICs of counterpart antibiotics to clinically susceptible levels. The probable role of PB in such synergy is its rapid permeabilization of the outer membrane, allowing enhanced penetration and activity of other antibiotics [4]. Clinical trial data of intravenous (2.5–3 mg/kg on d 1) and/or aerosolized PB (−2.5 mg/kg/d) to critically ill patients ($n = 25$) with antibiotic resistant organisms demonstrated efficacy of this compound, although nephrotoxicity was observed in 10% and did not result in discontinuation of therapy [24]. The pharmacokinetic data of colistin in patients with cystic fibrosis showed serum peak concentrations of 21.4 and 23 mg/l in first dose ($n = 30$) and steady state ($n = 27$), respectively, when the patients were started on colistin 5–7 mg/kg/d administered intravenously in 3 equally divided doses [25]. Although clinical and pharmacokinetic data of PB are limited, it is critical to seek for appropriate counterpart agents to maximize combination effects, which may reduce exposure and toxicity of the polypeptide antibiotics. Our data further stress potential of the polypeptide antibiotics as an enhancement of antibacterial activities of counterpart antibiotics in combination.

In the present study, we have observed the strongest combination effects in PB plus RFP against MDRP examined. Previously, several researchers have reported the potential of combination of polypeptide antibiotics and RFP as a therapeutic regimen for infections with multiple-resistant organisms [21,26–28]. Our data are substantially consistent with the previous results and further confirmed synergistic effects of combination of PB and RFP at clinically relevant concentrations against MDRP. Given the large number of patients with MDRP infections who are treated empirically, the results of this study are of great clinical importance. In this respect, Tascini et al. have reported clinical efficacy of a colistin and RFP combination in 4 patients with MDRP infections [29]. Further studies for evaluation of 'Break-point Checkerboard Plate' in multiple strains, in addition to modification of antibiotic

concentrations and its association with clinical responses in patients with MDRP infections, are warranted.

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Kinetics Study of KPC-3, a Plasmid-Encoded Class A Carbapenem-Hydrolyzing β-Lactamase

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The kinetic activity of KPC-3, a plasmid-encoded class A carbapenemase, was studied. It hydrolyzed penicillins, cephalosporins, carbapenems, and even sulbactam. The best substrate was cephalothin (k_{cat}/K_m = 3.48 μM^{-1} s^{-1}). The efficiency of the enzyme was similar for imipenem and meropenem (k_{cat}/K_m, 1.4 and 1.94 μM^{-1} s^{-1}, respectively).

Carbapenem use has increased during the past 2 decades. This is due, in part, to their broad-spectrum of antibacterial activity and their resistance to hydrolysis by extended spectrum β-lactamases (1, 10, 14, 17). However, the appearance of carbapenemases and other carbapenem resistance mechanisms is threatening the effectiveness of this antibiotic class. In gram-negative bacteria, carbapenem resistance has been attributed to three main mechanisms: the combination of high-level production of an AmpC β-lactamase and the loss of outer membrane proteins (5, 13), changes in the affinity of penicillin binding proteins for carbapenems (7, 8), and the production of a carbapenem-hydrolyzing β-lactamase (11, 16). Although clinically significant, carbapenem-hydrolyzing β-lactamases remain rare, but their frequency has been increasing. The β-lactamases involved belong to Ambler molecular classes A, B, and D (16, 19). A small number of class A enzymes have been found to be able to hydrolyze carbapenems (6). They belong to group 2f, as defined by Bush and colleagues (4, 23). They hydrolyze ampicillin and early cephalosporins more efficiently than carbapenems and can be inhibited by clavulanic acid. Class A carbapenemases can be chromosomally encoded (NMC-A, Sme-1 to -3, IMI-1) (9, 15, 21, 22, 24, 27) or plasmid encoded (KPC-1, KPC-2, GES-2) (18, 20, 25, 26, 28, 29, 30).

KPC-type β-lactamases have become one of the most frequently encountered carbapenem-hydrolyzing enzymes on the East Coast of the United States (2). KPC-3 is the most recently reported enzyme in that group (T. Hong, E. S. Moland, B. Abdalhamid, et al., Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. C1–665, 2003; K. Young, P. Tierno, Jr., L. Tysall, et al., Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. C2–50, 2003). KPC-3 is closely related to its predecessors, differing by only 1 amino acid from KPC-2 (H272Y) and by 2 amino acids with KPC-1 (S174G, H272Y). It has been recovered from isolates of *Klebsiella pneumoniae* (Young et al., 43rd ICAAC), *Escherichia coli* (Hong et al., 43rd ICAAC), and *Enterobacter cloacae* (3).

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In this study we purified KPC-3 and subjected the enzyme to kinetic characterization.

An isolate of *E. coli* (isolate 233) showing reduced susceptibility to carbapenems was referred to Creighton University from Hackensack University Medical Center. It was subsequently found to produce KPC-3 (report in press).

The isolate was grown in 4 liters of Luria-Bertani broth at 37°C (250 rpm) for 8 h, harvested, and suspended in 30 mM Tris-HCl buffer (pH 8.0) containing 30% sucrose. The periplasmic content was extracted as described previously (12). Purification was achieved using a HiPrep 16/10 SP XL column (Amersham Biosciences AB, Uppsala, Sweden) equilibrated in 10 mM acetate buffer (pH 5.0). Fractions displaying β-lactamase activity, observed as the initial rate of hydrolysis of nitrocefin (100 μM) ($\Delta\varepsilon_{482}$ = +10,000 $M^{-1}cm^{-1}$) (Oxoid Ltd., Hampshire, United Kingdom) were obtained after elution with a linear gradient of NaCl (0 to 400 mM). After concentration using Amicon ultrafiltration membranes (Millipore Corporation, Bedford, MA) and overnight dialysis in 10 mM morpholineethanesulfonic acid (MES) buffer (pH 5.5) at 4°C, the sample was reloaded onto a Mono S HR 5/5 column (Amersham Pharmacia Biotech, Uppsala, Sweden), equilibrated in MES buffer (pH 5.5), and eluted with a linear gradient of NaCl (0 to 300 mM). The entire purification process was done with an AKTA purifier (Amersham Pharmacia Biotech). The purity of the β-lactamase preparation was controlled using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue. The purity achieved was above 90%. The purified enzyme was then dialyzed overnight at 4°C in phosphate buffer (pH 7.0) and concentrated. Four liters of culture yielded a total of 0.27 mg of pure enzyme. After determination of the protein concentration using a Bio-Rad (Richmond, Calif.) protein assay, 20 μg/ml bovine serum albumin was added. The N-terminal sequence was determined using a Procise 492cLC-1 protein sequencer (Applied Biosystems, Foster City, Calif.), and the kinetic parameters were determined with the pure enzyme.

All kinetics studies were done by measuring hydrolysis rates with a Shimadzu (Kyoto, Japan) UV-2550 spectrophotometer connected to a personal computer. To determine the kinetic parameters, 6 to 10 different concentrations of each β-lactam were used. Each reported parameter is an average of three

TABLE 1. Comparison of kinetic parameters for KPC-1, KPC-2, and KPC-3[a]

Substrate	K_m or K_i (μM)			k_{cat} (s^{-1})			k_{cat}/K_m (μM^{-1} s^{-1})		
	KPC-1	KPC-2	KPC-3	KPC-1	KPC-2	KPC-3	KPC-1	KPC-2	KPC-3
Ampicillin	130	239	65 (±5)	110	210	77 (±4)	0.9	0.9	1.2
Nitrocefin	NA[b]	NA	42 (±5)	NA	NA	107 (±5)	NA	NA	2.6
Cephaloridine	560	500	261 (±1)	340	530	364 (±4)	0.6	1.1	1.4
Cephalotin	53	82	44 (±3)	75	69	153 (±5)	1.4	0.8	3.5
Cefotaxime	160	220	95 (±8)	14	22	52 (±4)	0.1	0.1	0.5
Ceftazidime	94	NA	88 (±1)	0.1	0.1	3 (±0.06)	0.001	NA	0.03
Cefoxitin[c]	120	180	970 (±65)	0.3	0.3	0.05[d] (±0.001)	0.002	0.002	0.5[d]
Moxalactam[c]	NA	NA	14 (±1.6)	NA	NA	0.008 (±0.0002)	NA	NA	0.05
Meropenem	12	15	4 (±1)	3	4	6 (±0.09)	0.3	0.3	1.4
Imipenem	81	51	23 (±6)	12	15	45 (±0.04)	0.2	0.3	1.9
Sulbactam	NA	NA	30 (±0.9)	NA	NA	4 (±0.1)	NA	NA	0.1

[a] Kinetic values used for KPC-1 and KPC-2 were reported by H. Yigit et al. (29, 30).
[b] NA, not available.
[c] K_i values were obtained using 100 μM nitrocefin as a reporter substrate.
[d] k_{cat} values were obtained by hydrolyzing a low concentration of substrate with a high concentration of enzyme.

separate measurements. All kinetic parameters were acquired by measuring the initial hydrolysis rate of the β-lactam under study at a constant temperature of 30°C, using 50 mM phosphate buffer (pH 7.0). Analysis of the data was done using the Hanes-Woolf linearization of the Michaelis-Menten equation. For all poor substrates, the competitive inhibition constant (K_i) was determined by competition experiments between the tested β-lactam and 100 μM nitrocefin. Hydrolysis of the substrate at a concentration 10 times the K_m (K_i) value or higher yielded the catalytic constants (k_{cat}) for poor substrates. The k_{cat} value for cefoxitin was obtained using 100 μM substrate with 1.1×10^{-7} M enzyme.

The N-terminal sequence of KPC-3 was determined as LT NLVAEPFAKLE. Table 1 shows a comparison between previously reported k_{cat}, K_m (K_i), and k_{cat}/K_m values for KPC-1 and KPC-2 (29, 30) and the parameters obtained for KPC-3 in this study. KPC-3 hydrolyzed penicillins, cephalosporins, and carbapenems. Among the substrates tested, the highest hydrolytic efficiency was seen with nitrocefin and cephalothin (k_{cat}/K_m, 2.55 μM^{-1} s^{-1} and 3.48 μM^{-1} s^{-1}, respectively). The k_{cat} and K_m values for cephaloridine were very high (k_{cat}, 364 s^{-1}; K_m, 261 μM). Imipenem and meropenem were hydrolyzed by KPC-3 with good efficiencies (k_{cat}/K_m, 1.94 μM^{-1} s^{-1} and 1.40 μM^{-1} s^{-1}, respectively), which were similar overall to those exhibited for ampicillin and cephaloridine. The substrate profiles for the three enzymes were similar overall, although the catalytic efficiency of KPC-3 appeared to be somewhat higher with some substrates, including oxyiminocephalosporins and carbapenems. One of the notable differences is the behavior of this enzyme with ceftazidime. The catalytic activity (k_{cat}) with ceftazidime (3.0 s^{-1} ± 0.01) was approximately 30 times higher than those of KPC-1 and KPC-2 (0.1 s^{-1} for both). Due to this, KPC-3 was 30 times more efficient than KPC-1 toward this substrate. Moreover, KPC-3 showed a lower affinity for cefoxitin than the other enzymes. The present findings, therefore, suggest that the amino acid substitution that differentiates KPC-3 from KPC-2 (H272Y) could have a functional significance. A molecular modeling analysis based on the structure of the TOHO-1 enzyme (25), now called CTX-M-44, suggested that the H272Y mutation (which would be at a position similar to that of R274 in TOHO-1) could influence the positions of R209, which interacts with the substrate carboxylate. Further investigation will be necessary to clarify these matters.

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Note

Evaluation of dipicolinic acid for detection of IMP- or VIM- type metallo-β-lactamase–producing *Pseudomonas aeruginosa* clinical isolates

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Abstract

We evaluated dipicolinic acid (DPA) as a chelating agent for detection of IMP- or VIM-type metallo-β-lactamase (MBL)–producing *Pseudomonas aeruginosa* clinical isolates. Using the broth microdilution testing in the presence or absence of DPA, MBL producers exhibited 100%, 92%, or 100% of ≥8 times (media, 32 times) reduction of MICs in presence of DPA for ceftazidime, imipenem, or meropenem, respectively. In disk diffusion testing, expansion of growth inhibitory zone of these clinical isolates was clearly observed. Thus, DPA could be useful in the detection for MBL-producing *P. aeruginosa* clinical isolates.

Keywords: Metallo-β-lactamase; Dipicolinic acid; IMP; VIM

Carbapenems are the most potent β-lactam agents with a broad-spectrum activity against Gram-negative and Gram-positive bacteria. They are stable in the presence of penicillinases and cephalosporinases. However, some class A, B, and D β-lactamase as defined by Ambler et al. can hydrolyze carbapenems (Nordmann and Poirel, 2002). In particular, class B β-lactamases, termed *metallo-β-lactamases* (MBLs), are an increasingly serious clinical problem because they have a very broad substrate profile that includes penicillins, expanded-spectrum cephalosporins and carbapenems, and exclude only aztreonam as a monobactam. It has been reported that IMP-1 MBL-producing *Serratia marcescens* was first isolated in 1991 (Osano et al., 1994). During the past decade, various kinds of MBLs, including IMP and VIM types, have increased in prevalence in *Pseudomonas aeruginosa* around the world (Nordmann and Poirel, 2002). Therefore, we need a method for rapid, specific, and sensitive detection of MBL-producing *P. aeruginosa*. Recently, some researchers have proposed screening methods to detect MBL producers by using thiol compounds, 2-mercaptopropionic acid (2-MPA), EDTA or EDTA plus 1,0-phenanthroline (Arakawa et al., 2000; Lee et al., 2001, 2003; Migliavacca et al., 2002). However, a number of these inhibitors possess action of bacterial growth inhibition or hydrolyze some antibiotics. On the other hand, it has been reported that dipicolinic acid (DPA) will be an adequate chelating agent against some MBLs chelators in biochemical studies (Murphy et al., 2003; Franceschini et al., 2000; Laraki et al., 1999). The aim of this study is to evaluate and develop a screening method for MBL-producing *P. aeruginosa* by using DPA.

The strains used in this study included 13 MBL-producing clinical isolates of *P. aeruginosa*. Known VIM-1– and VIM-2–producing clinical isolate (*P. aeruginosa* VR-143/97 and SAP-1, respectively) were kindly provided by Rossolini et al. (Lauretti et al., 1999; Rossolini et al., 2000). Ten nonduplicated MBL-producing clinical isolates (Kimura et al., 2005) carried the gene encoding IMP-1–type MBL, and 1 isolate carried a VIM-2–type MBL. Seven MBL-nonproducing *P. aeruginosa* clinical isolates were also used in this study (Kimura et al., 2005). *P. aeruginosa* strain ATCC 27853 was used as the reference strain. Bacterial strains were evaluated by antimicrobial susceptibility testing using the Clinical Laboratory Standards Institute (CLSI) broth microdilution method with cation-adjusted Mueller-Hinton (MH) broth (Difco, Detroit, MI) against several agents in the presence (200 μg/mL) or absence of DPA (Sigma-Aldrich, St. Louis, MO). Reference antibiotic

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Table 1
Measurement of hydrolysis of antibiotics β-lactams by DPA and 2-MPA

Antibiotics	Hydrolyzing rate ($\times 10^{-8}$ mol/L per min)	
	2-MPA	DPA
Ampicillin	nd	nd
Ceftazidime	nd	nd
Aztreonam	nd	nd
Imipenem	5.12	nd
Meropenem	5.09	nd

nd = not detected.

powders were obtained from their manufacturers: ceftazidime (Sanwa Kagaku, Tokyo, Japan), imipenem (Banyu Pharmaceutical, Tokyo, Japan), meropenem (Sumitomo Pharmaceutical, Tokyo, Japan), and aztreonam (Eizai, Tokyo, Japan). These antibiotics were used at concentrations of 0.06–128 μg/mL. In disk diffusion testing, we prepared the MH agar medium with DPA and then autoclaved it for 20 min at 121 °C. A colony of each bacterial strain was suspended and diluted with saline to 10^6 CFU/mL and then spread on simple MH agar plate and on one containing DPA (200 μg/mL) with a cotton swab. The Kirby–Bauer disks (Eiken, Tokyo, Japan) containing ceftazidime (30 μg/disk), imipenem (10 μg/disk), or aztreonam (30 μg/disk) were placed on the plate and incubated at 37 °C. Hydrolysis of β-lactam antibiotics was detected by monitoring the variation in the absorbance of the β-lactam solution in 50 mmol/L phosphate buffer (pH 7.0). Hydrolysis rates were measured with Shimadzu UV-2550 spectrophotometer (Shimadzu, Kyoto, Japan), connected to a personal computer. Measurement of hydrolysis of β-lactam antibiotics was reported by Kimura et al. (2004).

Firstly, to address whether the compound DPA possesses growth inhibitory effect against *P. aeruginosa*, we demonstrated that *P. aeruginosa* strain ATCC 27853 was grown in the MH medium containing various concentrations (from 3.9 to 1000 μg/mL) of DPA. The growth of the strain was not significantly inhibited by less than 400 μg/mL DPA (data not shown). Therefore, 200 μg/mL of DPA was used for the following experiments. Next, we demonstrated whether the β-lactam antibiotics were stable against chelators. The hydrolysis of β-lactam antibiotics by DPA or 2-MPA (Wako Pure Chemical Industries, Osaka, Japan) was monitored by a spectrophotometer (Table 1). 2-MPA hydrolyzed imipenem and meropenem, but did not hydrolyze ampicillin, ceftazidime, and aztreonam. However, DPA did not hydrolyze all of the selected antibiotics. Thus, β-lactam antibiotics are not affected by DPA, suggesting DPA may be a suitable inhibitor to detect MBL-producing *P. aeruginosa*. We next demonstrated that MBL-producing clinical isolates of *P. aeruginosa* were detected by using microdilution method with DPA. MBL-producing strains showed a decrease of ≥8-fold dilution of β-lactam MICs for ceftazidime (100%), imipenem (92%), and meropenem (100%) in the presence of DPA (200 μg/mL) when compared with that of the β-lactam alone, whereas no reduction or 2 times reduction of aztreonam MIC was observed in the presence or absence of DPA (Table 2). In MBL-nonproducing *P. aeruginosa* clinical isolate, no reduction or 2-fold reduction of these antibiotic MICs were observed except for 1 case which showed 4-fold reduction of meropenem MIC in the presence of DPA (Table 3). These results clearly show that DPA is a suitable compound for detection of the MBL-producing *P. aeruginosa* clinical isolate by using microdilution method. Next, we developed a simple screening test for detection of MBL producers in clinical microbiology laboratory: a disk susceptibility test carried out using a disk containing ceftazidime, imipenem, or aztreonam on agar plate containing DPA (200 μg/mL). Fig. 1 shows that expansion of growth inhibitory zone of *P. aeruginosa* carrying bla_{IMP-1} was clearly observed by using ceftazidime or imipenem disk, whereas no influence was observed with aztreonam disk. Among a total of 20 clinical isolates screened by DPA, all MBL-producing *P. aeruginosa* were clearly detected by this method (data not shown). Interestingly, false-positive or false-negative strains by the broth microdilution method previously mentioned were also significantly detected by this method. This result indicates that this could be a suitable simple method to detect MBL-producing *P. aeruginosa* clinical isolates.

In some biochemical studies, it has been reported that purified MBL such as IMP-1, VIM-1, and SPM-1 appeared to be more susceptible to DPA than EDTA or other chelators (Murphy et al., 2003; Franceschini et al., 2000; Laraki et al., 1999). In particular, the detailed mechanism of inactivation of IMP-1 by each chelator has been well studied by Siemann et al. (2002). They reported that 6 types of chelators

Table 2
Evaluation of DPA against MBL-producing clinical isolates by broth microdilution testing in the presence or absence of DPA

Antimicrobial	MIC (μg/mL) for												
	1630	1631	1672	1673	1682	1683	1708	1709	1710	1721	1732	1733	1757
Imipenem	128	128	>128	8	64	64	64	64	>128	64	64	>128	64
Imipenem + DPA[a]	8	8	1	0.13	1	4	2	2	4	16	4	8	8
Meropenem	128	64	>128	64	>128	>128	>128	>128	>128	>128	>128	>128	>128
Meropenem + DPA	4	4	4	0.5	4	16	4	4	16	16	8	4	4
Ceftazidime	>128	32	64	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128
Ceftazidime + DPA	4	4	2	1	4	8	4	4	8	8	4	4	4
Aztreonam	16	16	32	8	32	64	32	32	64	32	128	32	16
Aztreonam + DPA	8	8	32	8	16	64	32	32	64	16	128	16	16

[a] Evaluated by CLSI broth microdilution method against several agents in the presence of DPA (200 μg/mL).