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Occurrence of PER-1 Producing Clinical Isolates of *Pseudomonas aeruginosa* in Japan and their Susceptibility to Doripenem

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Abstract The acquisition of resistance by extended-spectrum β-lactamases (ESBL) has been reported primarily for *Enterobacteriaceae*, but there are few reports on the isolation of ESBL-producing *Pseudomonas aeruginosa*. PER-1-type ESBL producing *P. aeruginosa* has been found in various regions around the world but there are no reports of clinical isolates in Japan. During our susceptibility surveillance studies over a 10 year period, we found four clinical isolates resistant to ceftazidime due to production of PER-1. They were resistant to ceftazidime but susceptible in the presence of clavulanic acid, a class A β-lactamase inhibitor. The strains had the ability to hydrolyze ceftazidime. They also had the gene for PER-1-type ESBL. This is the first report of the isolation of PER-1 producing strains in Japan. These four strains were resistant to ceftazidime, cefepime and aztreonam with MICs of 64 μg/ml or more, but were more susceptible to carbapenem antibiotics. In particular, doripenem, which is a novel carbapenem antibiotic, showed good antibacterial activity with a MIC of 2 or 4 μg/ml, which was more potent than meropenem and imipenem. Doripenem also showed good therapeutic efficacy against a systemic infection of mice with a PER-1 producing strain, and was also more potent *in vivo* than imipenem or meropenem.

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Introduction

Pseudomonas aeruginosa is an opportunistic pathogen causing serious infection in immunocompromised hosts. *P. aeruginosa* has intrinsic resistance to many antibiotics and produces a variety of virulence factors. The intrinsic resistance of *P. aeruginosa* to various antibiotics is generally due to its low outer membrane permeability and the production of efflux pumps [1]. Although there are various antipseudomonal agents such as β-lactams, quinolones, and aminoglycosides, resistance to these antibiotics is acquired by *P. aeruginosa* at a relatively high rate by various resistant mechanisms.

P. aeruginosa, like other Gram-negative pathogens, is known to acquire resistance by producing various β-lactamases, which can be classified into chromosomal AmpC β-lactamase and plasmid-mediated β-lactamases. Among plasmid-mediated β-lactamases, extended-spectrum β-lactamases (ESBL) are generally known to hydrolyze cephamycins and/or carbapenems which can not be easily hydrolyzed by traditional β-lactamases [2]. Hence, ESBL production caused high resistance to various β-lactam antibiotics, which is a serious problem in clinic. Although the production of ESBL has been reported primarily in *Enterobacteriaceae*, there are few reports on the isolation of ESBL-producing *P. aeruginosa* [3]. PER-1-type β-lactamase was first detected as an ESBL in *P. aeruginosa* and was reported to hydrolyze various β-lactams except for

imipenem, oxacillin and cephamycins [4, 5]. Although there had been no reports on clinical isolates in Japan, PER-1 producing highly resistant strains have been found to be prevalent in *P. aeruginosa* and *Acinetobacter* spp. worldwide [6～12].

Among the antipseudomonal β-lactams, carbapenem antibiotics such as imipenem and meropenem display potent antipseudomonal activity and high stability to many of the serine-type β-lactamases including class C AmpC β-lactamase and ESBL. Doripenem, a novel carbapenem, has broad antibacterial spectrum and potent antipseudomonal activity [13～16]. In particular, the antibacterial activity of doripenem against clinical isolates of *P. aeruginosa* has been reported to be more potent than other carbapenem antibiotics [14～17]. In addition, doripenem and other carbapenem antibiotics have potent activity against ESBL-producing *Enterobactriaceae* [18].

In this study, we report the first isolation of PER-1 producing clinical isolates of *P. aeruginosa* in Japan, and the *in vitro* and *in vivo* antibacterial activities of doripenem against PER-1 producing *P. aeruginosa*.

Materials and Methods

Bacterial Strains and Susceptibility Testing

P. aeruginosa SR24836, SR24837, SR24838, SR24839 and SR24840 were isolated at a Japanese clinical facility in the year 2002. *P. aeruginosa* SR24823, SR24897, SR24858, and SR27001 were isolated at other Japanese clinical facilities in 2002. *P. aeruginosa* PAO1 and SR24 are generally susceptible to antipseudomonal antibiotics, and strain SR24-12, which is a resistant mutant derived from strain SR24, displays constitutive production of chromosomal AmpC β-lactamase.

Antibiotics

Doripenem was synthesized at Shionogi & Co., Ltd. Meropenem, imipenem, ceftazidime, cefepime and aztreonam were purchased from U.S. Pharmacopeia (Rockville, MD). Clavulanic acid, a β-lactamase inhibitor, and cilastatin, a dehydropeptidase-I inhibitor, were also purchased from U.S. Pharmacopeia.

Susceptibility Testing

MICs were determined using cation-adjusted Mueller-Hinton broth (Becton Dickinson, Sparks, MD) by the microbrothdilution method according to the Clinical and Laboratory Standards Institution guidelines (formerly National Committee for Clinical Laboratory Standards) [19]. For the detection of ESBL production, MICs were determined in the presence of clavulanic acid, an inhibitor for the class A β-lactamases.

Detection of ESBL Genes

The genes for five types of ESBL (PER, VEB, GES, SHV and TEM-types) were detected by polymerase chain reaction (PCR), which was performed using the primers described by Weldhagen *et al.* [3]. The complete PER-1 gene was amplified by using two primers, which correspond to *N*-terminal or *C*-terminal regions, followed by determination of the nucleotide sequence by the direct sequencing method.

Preparation of β-Lactamase

A crude cell extract of SR24840 was prepared by disruption of exponentially growing cells by repeated freeze-thawing and sonication at 4°C, followed by removal of the cell debris by centrifugation at 8,000×*g* for 15 minutes. The supernatant, as a crude cell extract, was prepared by centrifugation at 15,000×*g* for 30 minutes, followed by filtration through a filter with a pore size of 0.22 μm. The protein content of the crude cell extract was determined using a BCA protein assay kit (Pierce, Rockford, IL). The hydrolysis of ceftazidime and nitrocefin was examined by incubation of 100 μM ceftazidime and nitrocefin at 30°C in the presence of diluted crude cell extracts. The hydrolysis rates of ceftazidime and nitrocefin were determined by photometric methods at wavelengths of 260 and 490 nm, respectively [20, 21].

Analysis of Macrorestriction Patterns of Genomic DNA

The analysis was performed using genomic DNA of PER-1 producing clinical isolates by the pulsed-field gel electrophoresis (PFGE) reported by Pagani *et al.* [11]. Briefly, genomic DNA was extracted from the clinical isolates and was digested with *Spe*I using CHEF Genomic DNA Plug Kits (Bio-rad, Hercules, CA). The digested DNA fragments were separated on agarose gel by PFGE using the CHEF-DRII system (Bio-rad).

Therapeutic Efficacy against Systemic Infection Model

Five-week-old female mice were infected intraperitoneally with 0.5 ml of the bacterial suspension of strain SR24840 containing 6.4×10^5 cfu/ml. All test antibiotics were subcutaneously administered at 1, 3 and 5 hours after infection. For the carbapenem antibiotics doripenem, meropenem and imipenem, equal amounts of cilastatin was simultaneously administered to prevent the degradation of carbapenem antibiotics by renal dehydropeptidase-I [22, 23]. Seven mice were used at each dosage of the compound. The efficacy was determined from the survival

Table 1 Susceptibility of ceftazidime-resistant strains of *Pseudomonas aeruginosa* to various β-lactam antibiotics

	Ceftazidime	Cefepime	Doripenem	Meropenem	Imipenem	Aztreonam
SR24837	>64	>64	4	8	16	>64
SR24838	>64	64	2	4	16	>64
SR24839	>64	>64	4	8	16	>64
SR24840	>64	64	2	8	16	>64
SR24	1	0.5	0.125	0.25	1	4
SR24-12	64	64	0.25	0.25	1	>64

MICs (μg/ml) were determined by microdilution method using cation-adjusted Mueller-Hinton medium.

Table 2 Effect of clavulanic acid on susceptibility of the strains to ceftazidime

	MIC of ceftazidime in the presence of	
	None	10 μg/ml of clavulanic acid
SR24837	>64	2
SR24838	>64	2
SR24839	>64	2
SR24840	>64	2
SR24	1	1
SR24-12	64	64

MICs (μg/ml) were determined by microdilution method using cation-adjusted Mueller-Hinton medium.

rates of the mice at 7 days after infection, and ED_{50} and 95% confidence limits were calculated by the logit method.

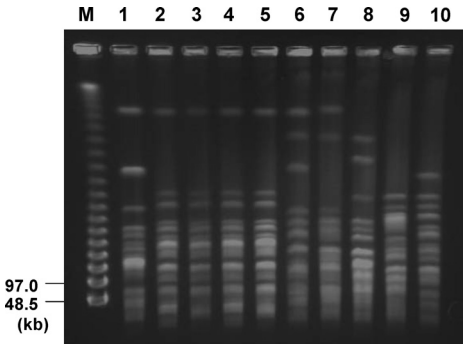

Fig. 1 Pulse field gel electrophoresis profiles of clinical isolates.

*Spe*I-digested DNA fragments of genomic DNA derived from PER-1 producing strains SR24837 (lane 2), SR24838 (lane 3), SR24839 (lane 4) and SR24840 (lane 5), and non PER-1 producing strains SR24836 (lane 1), SR24823 (lane 6), SR24897 (lane 7), SR24858 (lane 8) and SR27001 (lane 9) were separated on agarose gel by pulse field gel electrophoresis apparatus. Lambda ladder markers were shown on lane M.

Results

Occurrence of Ceftazidime-resistant Strains and Their Susceptibilities to Various Antibacterials

We have been performing susceptibility surveillance studies every two years since 1992 using clinical isolates collected from about 17 medical facilities in Japan [24~29]. For *P. aeruginosa*, about 100 strains have been collected each year. We found that four strains isolated at one medical facility in 2002 had unique characteristics. The strains were highly resistant to ceftazidime with MIC of >64 μg/ml (Table 1), but were susceptible to ceftazidime in the presence of 10 μg/ml of clavulanic acid, an inhibitor for class A β-lactamase (Table 2). Other ceftazidime-resistant strains, which were isolated at a frequency of about 10 to 20% in each surveillance study, were resistant even in the presence of clavulanic acid, suggesting that they did not produce class A β-lactamase including ESBL like PER-1. Generally, many of the ceftazidime-resistant strains as well as strain SR24-12 were reported to produce constitutively chromosomal AmpC β-lactamase, and were not susceptible to ceftazidime even in the presence of clavulanic acid. Some strains have been reported to acquire high resistance to ceftazidime even in the presence of clavulanic acid due to the production of class B metallo-β-lactamase.

Because these four resistant strains were isolated at one medical facility, the isogenicity between these four strains was observed by comparing the macro restriction patterns of genomic DNA of these strains by PFGE. Figure 1 showed that these four strains showed almost identical macro restriction patterns, although other strains showed significantly different macro restriction patterns. These results suggested that these four strains were isogenic, so strain SR24840 was used for further studies.

Table 3 Hydrolysis of β-lactams by PER-1 producing strains

	Hydrolysis rate (mM/min/mg-protein)	
	Nitrocefin	Ceftazidime
SR24840	280	2.1
SR24-12	1160	<0.01

The hydrolysis rate was determined by a photometric method at the concentration of 100 μM substrate.

Table 4 Therapeutic efficacy against murine systemic infection caused by *P. aeruginosa*

	ED_{50} (mg/kg)	95% confidence limit
Doripenem	2.83	1.37～6.42
Meropenem	7.72	3.55～15.7
Imipenem	5.52	2.72～12.3
Ceftazidime	>32	NC
Cefepime	>32	NC

Infection: Five-week-old female mice were infected intraperitoneally with 6.4×10^5 cfu/ml of *P. aeruginosa* SR24840. Treatment: Antibiotics were subcutaneously administered at 1, 3 and 5 hours after infection. Doripenem, meropenem and imipenem were administered in concentrations equal to cilastatin. NC: not calculated.

The hydrolysis of ceftazidime by the cell free extracts of strain SR24840 was detected, but not by the cell free extract of SR24-12, AmpC producer (Table 3). On the other hand, the hydrolysis of nitrocefin did not differ significantly between these two strains. These results strongly suggested that strain SR24840 had the ability to hydrolyze ceftazidime.

Detection of PER-1 Production

There have been few reports on the production of ESBL in *P. aeruginosa*, but some ESBLs are known to cause high resistance in *P. aeruginosa* [3]. The presence of genes for various ESBLs having the ability to hydrolyze ceftazidime was investigated using PCR. As a result, the PER-1 gene was detected in all four strains, and the nucleotide sequence of the amplified PER-1 gene of SR24840 was identical to that of the PER-1 gene which was previously reported [5].

These results showed that these four strains were resistant to ceftazidime because they were PER-1 producers. This is the first report of the isolation of PER-1 producing strains of *P. aeruginosa* in Japan.

Therapeutic Efficacy against Systemic Infection Models

As shown in Table 1, these strains were highly resistant to ceftazidime, cefepime and aztreonam with MIC of 64 μg/ml or more. On the other hand, they were more susceptible to carbapenem antibiotics, with doripenem showing the most potent activity with MIC of 2 to 4 μg/ml. The MIC of meropenem against these four strains was 2- to 4-fold higher than that of doripenem, and the MIC of imipenem was 4- to 8-fold higher than that of doripenem.

The efficacy of various β-lactams was tested in mice infected with PER-1 producing strain SR28480. For carbapenem antibiotics, cilastatin, an inhibitor of DHP-I, was administered simultaneously at a ratio of 1 : 1 in order to inhibit the effect of murine dehydropeptidase-I on pharmacokinetic profiles. Ceftazidime and cefepime were not active in this model, however, carbapenem antibiotics showed good therapeutic efficacy. The therapeutic efficacy of doripenem was 2- and 3-fold better than that of imipenem and meropenem (Table 4). These results showed that doripenem was also the most effective even against infection models caused by a PER-1 producing strain of *P. aeruginosa*.

Discussion

We report the first instance of isolation of PER-1 producing strains of *P. aeruginosa* in Japan. In 1991, PER-1 producing strains of *P. aeruginosa* were found in Turkey [4] and have been reported in European countries and USA [8～12]. However, there had been no reports on the emergence of PER-1 producing strains of *P. aeruginosa* in Asian countries, although some strains of *Acinetobacter* spp. were reported to carry PER-1 in various countries including Korea [30]. PER-1 was reported to be transferable to various Gram-negative bacteria [3], and strains carrying PER-1 and metallo-β-lactamase or OXA-type ESBL have also been isolated [12, 31].

In the case of the four strains discovered in this study, the PFGE and antibiotic susceptibility profiles were almost identical although they were different from those of other strains without PER-1 production (Fig. 1). These results strongly suggest the spread of an isogenic resistant strain in a hospital. Although no other PER-1 producing *P. aeruginosa* has yet been reported in Japan, carefully monitoring is needed.

Doripenem is a novel carbapenem which has a broad antibacterial spectrum and potent antipseudomonal activity. In particular, the activity of doripenem against clinical isolates of *P. aeruginosa* was reported to be superior to

other carbapenem antibiotics [14~17]. Doripenem was also reported to be active against PER-1 producing transconjugants susceptible to ceftazidime and cefepime [32]. This study showed that doripenem was active against PER-1 producing clinical isolates which were resistant to ceftazidime and cefepime. The differences of susceptibility between PER-1 producers might be due to the amount of production of PER-1.

Doripenem and meropenem can be used clinically without a dehydropeptidase-I inhibitor due to their high stability to human renal dehydropeptidase-I [22, 33]. Doripenem is also more stable against murine dehydropeptidase-I than meropenem and imipenem, and the therapeutic efficacy by single administration of doripenem against various infection models has been found to be comparable to or better than that of the combinational administration of meropenem : cilastatin and imipenem : cilastatin [14, 16]. However, Tsuji *et al.* also reported that the pharmacokinetic profile of doripenem was improved by cilastatin and suggested evaluation by the combination administration of doripenem and cilastatin [14]. Hence, the therapeutic efficacy of carbapenem antibiotics including doripenem was evaluated by combination use with cilastatin in this study. Under these conditions, doripenem was shown to have potent therapeutic efficacy against *P. aeruginosa* which produces PER-1 ESBL, being more potent than meropenem and imipenem.

These results showed that, among carbapenem antibiotics tested, doripenem displays the most potent therapeutic efficacy against *P. aeruginosa*, regardless of PER-1 production.

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