Potency of IMP-10 metallo- β -lactamase in hydrolysing various antipseudomonal β -lactams

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Limited β -lactams show antipseudomonal activity. The rapid spread of IMP-type metallo- β -lactamases (MBLs), which have a broad spectrum of substrates and a poor susceptibility to clinically available inhibitors, further restricts β -lactam use. In the present study, we evaluated the potency of IMP-10 MBL in hydrolysing antipseudomonal β -lactams currently available in the clinic. Crude IMP-10 MBL was prepared from two clinical isolates of *Pseudomonas aeruginosa* harbouring the *bla*_{IMP-10} gene. The sensitivity of β -lactams to hydrolysis by IMP-10 MBL was determined by comparing the MICs of 14 antipseudomonal β -lactams against a susceptible strain of *P. aeruginosa* in the presence and absence of IMP-10 MBL. Carbapenems (imipenem, meropenem and panipenem) and extended-spectrum cepheims (ceftazidime, cefoperazone, cefsulodin and cefepime) were sensitive to the hydrolysing activity of IMP-10 MBL. By comparison, the fourth-generation cephem (ceftazidime), the extended-spectrum penicillins (carbenicillin, ticarcillin, piperacillin and mezlocillin) and monobactams (aztreonam and carumonam) were relatively resistant to IMP-10 MBL. The sensitivity profile of antipseudomonal β -lactams to IMP-10 MBL generated in the present study provides a valuable reference for antibiotic selection by medical professionals.

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INTRODUCTION

Pseudomonas aeruginosa is intrinsically resistant to a large range of antibiotics and is one of the most common pathogens in nosocomial infections and microbial substitutions. Limited β -lactam antibiotics have antipseudomonal activity; these include the extended-spectrum penicillins (carbenicillin, ticarcillin, piperacillin, mezlocillin and azlocillin), extended-spectrum cepheims (ceftazidime, cefoperazone, cefsulodin, cefepime and ceftazidime), carbapenems (imipenem, meropenem and panipenem) and monobactams (aztreonam and carumonam) (Gilbert *et al.*, 2005).

Carbapenems are highly resistant to most β -lactamases with the exception of the carbapenemases (Bush *et al.*, 1995; Rasmussen & Bush, 1997; Livermore & Woodford, 2000). Two types of carbapenemases have been described: serine β -lactamases and metallo- β -lactamases (MBLs) (Frere, 1995; Livermore, 1998). MBLs have a poor susceptibility to clinically available inhibitors, such as clavulanate and sulbactam (Livermore, 1998). EDTA and mercaptoacetate are only used as specific inhibitors of MBL

in vitro (Goto *et al.*, 1997). Several types of MBLs including IMP, VIM, SPM-1 and GIM-1 have been identified (Walsh *et al.*, 2005). Of these, the IMP-type MBLs are the most common and exhibit a worldwide distribution (Ito *et al.*, 1995; Bush, 1998; Nordmann & Poirel, 2002; Zhao *et al.*, 2007). IMP-1 MBL has been identified primarily in strains of *P. aeruginosa* and *Serratia marcescens* in Japan (Watanabe *et al.*, 1991; Osano *et al.*, 1994). Today, 18 types of *bla*_{IMP} have been identified worldwide from a variety of clinical isolates in the *Enterobacteriaceae* (Walsh *et al.*, 2005). In geographically diverse regions of Japan, approximately 1.9% of clinical isolates of *P. aeruginosa* have acquired MBL, and 90% of these are the IMP-1 type (Kimura *et al.*, 2005). *bla*_{IMP-10} is a point mutation derivative of *bla*_{IMP-1} with a single base replacement of G by T at nucleotide 145, which leads to an amino acid alteration of Val49 to Phe (Iyobe *et al.*, 2002). We identified 21 imipenem-resistant clinical isolates of *P. aeruginosa* from unrelated inpatients at our university hospital in 2007. Among the 21 isolates, seven strains (33%) harboured *bla*_{IMP-10}. We also identified two strains harbouring *bla*_{IMP-10} from seven IMP-type MBL-producing clinical isolates of *S. marcescens* collected at our university hospital between 1996 and 2002.

Here we present a profile of IMP-10 MBL potency in hydrolysing the antipseudomonal β -lactams currently available in the clinic.

Abbreviations: AZT, aztreonam; CAZ, ceftazidime; CBPC, carbenicillin; CFPM, cefepime; CFS, cefsulodin; CPR, ceftazidime; CPZ, cefoperazone; CRMN, carumonam; IPM, imipenem; MBL, metallo- β -lactamase; MEPM, meropenem; MZPC, mezlocillin; PAMP, panipenem; PCase, penicillinase; PIPC, piperacillin; TIPC, ticarcillin.

METHODS

Bacterial strains and media. Twenty-one imipenem-resistant *P. aeruginosa* isolates from unrelated inpatients at Showa University Hospital in 2007 were used. *P. aeruginosa* P11-2820, a clinical isolate susceptible to antipseudomonal β -lactams, and *Escherichia coli* ATCC 25922, a reference strain used in susceptibility tests recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 2000), were used for MIC determination. Mueller–Hinton (MH) broth (Becton Dickinson) supplemented with 25 mg Ca^{2+} l^{-1} and 12.5 mg Mg^{2+} l^{-1} was used for bacterial cell culture and antibiotic susceptibility testing.

Reagents. The following reagents were purchased from the indicated commercial sources: imipenem (IPM), mezlocillin (MZPC) and aztreonam (AZT) (US Pharmacopeia); piperacillin (PIPC), ceftazidime (CAZ) and meropenem (MEPM) (LKT Laboratories); panipenem (PAPM) (Sankyo Organic Chemicals); ticarcillin (TIPC), cefoperazone (CPZ) and penicillinase (PCase) from *Bacillus cereus* (Sigma); carbenicillin (CBPC), sodium mercaptoacetate and clavulanate (Wako Pure Chemical Industries); carumonam (CRMN) (Takeda Pharmaceutical); cefsulodin (CFS) (MP Biomedicals); cefepime (CFPM) (Bristol-Myers Squibb); ceftiofime (CPR) (Sanofi-Aventis); and nitrocefin (Oxoid).

Gene detection by PCR amplification and DNA sequencing. The presence of the *bla*_{IMP-1}-like gene was identified by PCR analysis using the specific primers 1241 F 5'-CTA CCG CAG CAG AGT CTT TG-3' and 1828 R 5'-AAC CAG TTT TGC CTT ACC AT-3' (Arakawa *et al.*, 1995), and a 588 bp fragment was amplified. The amplicons were purified using the QIAquick PCR purification kit and sequenced on an Applied Biosystems 3730xl DNA analyser (Applied Biosystems).

Preparation of IMP-10 MBL. *P. aeruginosa* P0717 and P0706, which are positive for the *bla*_{IMP-10} gene but negative for *bla*_{VIM-1,2}, *bla*_{TEM-1} and *bla*_{SHV-1}, were used as a source of IMP-10 MBL. The two strains were cultured in MH broth until an OD₆₀₀ of 0.5–0.7 units was reached. The bacterial cells were disrupted by sonication and insoluble cell debris was removed by centrifugation. The supernatants were collected, sterilized via filtration, and used as the crude IMP-10 MBL preparation. Enzyme activity was assayed using nitrocefin as a substrate, as described previously (O'Callaghan *et al.*, 1972; Zhao *et al.*, 2002).

Determination of IMP-10 MBL activity in hydrolysing β -lactams. The MIC of the antibiotic was determined by the broth microdilution method based on the guidelines of the NCCLS (2000). The β -lactam hydrolysing activity of β -lactamases was evaluated by adding a crude IMP-10 MBL extract to MH broth containing twofold serial dilutions of β -lactam. Bacterial cells (5×10^5 ml^{-1}) were then inoculated into the MH broth and cultured at 35 °C for 18 h. An increase in the MIC in the presence of IMP-10 MBL indicates β -lactamase hydrolysing activity. The hydrolysing activity of PCase was determined in parallel as a control.

Data presentation. All experiments were performed three times or more to confirm repeatability.

RESULTS AND DISCUSSION

Sensitivity of carbapenems to IMP-10 MBL

The carbapenem hydrolysing activity of IMP-10 MBL was evaluated by adding a crude enzyme extract from *P.*

aeruginosa P0717 to serial dilutions of the carbapenems IPM, MEPM and PAPM in MH broth. The MIC against *P. aeruginosa* P11-2820 was determined. The addition of IMP-10 MBL (5 U ml^{-1}) increased the MIC of IPM, MEPM and PAPM from 2, 0.25 and 16 $\mu\text{g ml}^{-1}$ to 32 (16-fold), 128 (512-fold) and 64 $\mu\text{g ml}^{-1}$ (4-fold), respectively (Fig. 1a). As a control, the addition of PCase (5 U ml^{-1}) increased the MIC of IPM, MEPM and PAPM 4-, 16- and 2-fold, respectively. The increase in MIC over controls indicates that IPM, MEPM and PAPM are hydrolysed by IMP-10 MBL.

Sensitivity of extended-spectrum cepheims to IMP-10 MBL

P. aeruginosa P11-2820 was susceptible to the third-generation (CAZ, CPZ and CFS) and fourth-generation (CFPM and CPR) cepheims with MICs of 2–8 $\mu\text{g ml}^{-1}$. The addition of IMP-10 MBL (5 U ml^{-1}) increased the MIC to 16 (8-fold), 128 (16-fold), 64 (32-fold) and 32 $\mu\text{g ml}^{-1}$ (4-fold) for CAZ, CPZ, CFS and CFPM, respectively, while the MIC of CPR remained unchanged (Fig. 1b). As a control, the addition of PCase (5 U ml^{-1}) increased the MIC of CPZ to 512 $\mu\text{g ml}^{-1}$ (64-fold) and that of CFS to 8 $\mu\text{g ml}^{-1}$ (4-fold), while the MIC of CAZ, CFPM and CPR remained unchanged. These data indicate that the third-generation cepheims (CAZ, CPZ and CFS) and a fourth-generation cephem, CFPM, are sensitive to IMP-10 MBL, while another fourth-generation cephem, CPR, is resistant to IMP-10 MBL.

Sensitivity of extended-spectrum penicillins to IMP-10 MBL

The MIC of the penicillins CBPC, TIPC, PIPC and MZPC against *P. aeruginosa* P11-2820 was 4–32 $\mu\text{g ml}^{-1}$. The addition of IMP-10 MBL (5 U ml^{-1}) failed to increase the MIC of these penicillins (Fig. 1c). By comparison, PCase at 0.05 U ml^{-1} significantly increased the MIC of these penicillins to 256–1024 $\mu\text{g ml}^{-1}$. These results suggest that the extended-spectrum penicillins CBPC, TIPC, PIPC and MZPC are resistant to IMP-10 MBL.

Sensitivity of monobactams to IMP-10 MBL

P. aeruginosa P11-2820 was also susceptible to the monobactams AZT and CRMN, with a MIC of 4 $\mu\text{g ml}^{-1}$. Both IMP-10 MBL and PCase at 5 U ml^{-1} failed to increase the MIC of these monobactams, indicating that AZT and CRMN are resistant to IMP-10 MBL and PCase (Fig. 1d).

The outer-membrane barrier and the MexAB–OprM efflux system also affect the susceptibility of *P. aeruginosa* to β -lactams (Li *et al.*, 2000). The effect of these factors on the MIC of β -lactams was assessed by testing *E. coli* ATCC 25922 in parallel (Table 1). The trends of the MIC changes in *E. coli* ATCC 25922 were consistent with those of *P.*

Fig. 1. Sensitivity of antipseudomonal β -lactams to IMP-10 MBL. (a) Carbapenems; (b) extended-spectrum cepheims; (c) extended-spectrum penicillins; (d) monobactams. Symbols in (d) apply also to (a), (b) and (c). IMP-10 MBL at 5 U ml^{-1} was added in all the experiments; $5 \text{ U PCase ml}^{-1}$ was added in the experiments of (a), (b) and (d), while $0.05 \text{ U PCase ml}^{-1}$ was added in the experiments of (c). IPM, imipenem; MEPM, meropenem; PAPM, panipenem; CAZ, ceftazidime; CPZ, cefoperazone; CFS, cefsulodin; CFPM, cefepime; CPR, ceftipime; CBPC, carbenicillin; TIPC, ticarcillin; PIPC, piperacillin; MZPC, mezlocillin; AZT, aztreonam; CRMN, carumonam.

aeruginosa P11-2820, indicating that IMP-10 MBL induces the observed changes in the MIC. Similar results were obtained using an additional IMP-10 MBL preparation, extracted from *P. aeruginosa* P0706.

In this study, we used a crude preparation of IMP-10 MBL to evaluate the potency of IMP-10 MBL. To exclude the possibility of serine-type carbapenemases mixed in the crude preparation, inhibition testing of β -lactamase activity was performed. More than 90 % of the activity in the crude β -lactamase preparation (2.5 U ml^{-1}) was inhibited by $3 \text{ }\mu\text{M}$ sodium mercaptoacetate, a specific MBL inhibitor, while less than 0.5 % of the activity was inhibited by clavulanate, a specific serine-type β -lactamase inhibitor, indicating that possible effects of non-MBL in the preparation can be ignored. It is known that *bla*_{AmpC} and *bla*_{OXA-50} are two naturally encoded β -lactamase genes in *P. aeruginosa* (Lodge *et al.*, 1990; Stover *et al.*, 2000). The expression of chromosomal *ampC* genes is mainly induced when the bacteria are exposed to some β -lactams as inducers (Hanson & Sanders, 1999). We did not add any inducers when the MBL was prepared. In addition, we used a strain of *bla*_{AmpC}⁺/*bla*_{IMP}⁻ *P. aeruginosa* as a control to exclude the possible effect of AmpC. The effect of OXA-50 can also be excluded because PIPC is one of the restricted substrates of OXA-50 (Girlich *et al.*, 2004), while the crude preparation in our experiment did not hydrolyse PIPC (Fig. 1c).

The efficiencies of IMP-10 MBL for hydrolysing some β -lactams have been reported by another group using purified IMP-10 MBL (Iyobe *et al.*, 2002). The $K_{\text{cat}}/K_{\text{m}}$ values indicate that IPM, MEPM and CAZ are sensitive to IMP-10 MBL, while CBPC and PIPC are relatively resistant. These results are consistent with our data, which were obtained using a simple microbiological test, in the presence and absence of crude IMP-10 MBL.

The hydrolysing activities of IMP-10 for penicillins, except for carbenicillin, were very low compared to those of IMP-1, indicating that a single amino acid alteration, resulting from a point mutation, caused a decrease in penicillin-hydrolysing activity (Iyobe *et al.*, 2002). Compared to the frequent reports on *bla*_{IMP-1}, there are very limited reports on *bla*_{IMP-10}. The possibility cannot be excluded that some *bla*_{IMP-10} strains might be misinterpreted as *bla*_{IMP-1} strains based on PCR analysis alone. Sequencing analyses of PCR products are essential to distinguish *bla*_{IMP-10} from *bla*_{IMP-1}.

In conclusion, the carbapenems (IPM, MEPM and PAPM), the third-generation cepheims (CAZ, CPZ and CFS) and a fourth-generation cephem, CFPM, are sensitive to IMP-10 MBL. By comparison, another fourth-generation cephem, CPR, the extended-spectrum penicillins (CBPC, TIPC, PIPC and MZPC) and the monobactams (AZT and CRMN) are relatively resistant to IMP-10 MBL. The present study has generated a comprehensive profile of antipseudomonal β -lactam sensitivity to IMP-10 MBL, thereby providing a valuable reference for antibiotic selection by medical professionals.

Table 1. Sensitivity of antipseudomonal β -lactams to IMP-10 MBL

IPM, imipenem; MEPM, meropenem; PAMP, panipenem; CAZ, ceftazidime; CPZ, cefoperazone; CFS, cefsulodin; CFPM, cefepime; CPR, ceftipime; CBPC, carbenicillin; TIPC, ticarcillin; PIPC, piperacillin; MZPC, mezlocillin; AZT, aztreonam; CRMN, carumonam.

β -Lactam	β -Lactamase	MIC ($\mu\text{g ml}^{-1}$)	
		<i>P. aeruginosa</i> P11-2820	<i>E. coli</i> ATCC 25922
Carbapenem			
IPM	None	2	0.125
	IMP-10 MBL (5 U ml ⁻¹)	32	8
	IMP-10 MBL (2.5 U ml ⁻¹)	4	2
	IMP-10 MBL (1.25 U ml ⁻¹)	2	1
	PCase (5 U ml ⁻¹)	8	2
MEPM	None	0.25	0.03
	IMP-10 MBL (5 U ml ⁻¹)	128	128
	IMP-10 MBL (2.5 U ml ⁻¹)	32	32
	IMP-10 MBL (1.25 U ml ⁻¹)	8	4
	PCase (5 U ml ⁻¹)	4	0.5
PAPM	None	16	0.125
	IMP-10 MBL (5 U ml ⁻¹)	64	8
	IMP-10 MBL (2.5 U ml ⁻¹)	32	2
	IMP-10 MBL (1.25 U ml ⁻¹)	16	0.5
	PCase (5 U ml ⁻¹)	32	0.5
Cephem			
CAZ	None	2	0.25
	IMP-10 MBL (5 U ml ⁻¹)	16	2
	IMP-10 MBL (2.5 U ml ⁻¹)	4	0.5
	IMP-10 MBL (1.25 U ml ⁻¹)	2	0.25
	PCase (5 U ml ⁻¹)	2	0.25
CPZ	None	8	0.5
	IMP-10 MBL (5 U ml ⁻¹)	128	256
	IMP-10 MBL (2.5 U ml ⁻¹)	64	32
	IMP-10 MBL (1.25 U ml ⁻¹)	32	8
	PCase (5 U ml ⁻¹)	512	512
CFS	None	2	64
	IMP-10 MBL (5 U ml ⁻¹)	64	256
	IMP-10 MBL (2.5 U ml ⁻¹)	16	64
	IMP-10 MBL (1.25 U ml ⁻¹)	4	64
	PCase (5 U ml ⁻¹)	8	128
CFPM	None	8	0.03
	IMP-10 MBL (5 U ml ⁻¹)	32	0.25
	IMP-10 MBL (2.5 U ml ⁻¹)	8	0.06
	IMP-10 MBL (1.25 U ml ⁻¹)	8	0.03
	PCase (5 U ml ⁻¹)	8	0.03
CPR	None	8	0.03
	IMP-10 MBL (5 U ml ⁻¹)	8/16	0.03/0.06
	IMP-10 MBL (2.5 U ml ⁻¹)	8	0.03
	IMP-10 MBL (1.25 U ml ⁻¹)	8	0.03
	PCase (5 U ml ⁻¹)	8/16	0.03/0.06
Penicillin			
CBPC	None	32	8
	IMP-10 MBL (5 U ml ⁻¹)	32	8
	PCase (0.1 U ml ⁻¹)	512	256
	PCase (0.05 U ml ⁻¹)	256	64
TIPC	None	32	8
	IMP-10 MBL (5 U ml ⁻¹)	32	8
	PCase (0.1 U ml ⁻¹)	512	512
	PCase (0.05 U ml ⁻¹)	256	128

Table 1. cont.

β -Lactam	β -Lactamase	MIC ($\mu\text{g ml}^{-1}$)	
		<i>P. aeruginosa</i> P11-2820	<i>E. coli</i> ATCC 25922
PIPC	None	4	2
	IMP-10 MBL (5 U ml^{-1})	4	2
	PCase (0.1 U ml^{-1})	2048	1024
	PCase (0.05 U ml^{-1})	512	512
MZPC	None	32	2
	IMP-10 MBL (5 U ml^{-1})	32	2
	PCase (0.1 U ml^{-1})	2048	2048
	PCase (0.05 U ml^{-1})	1024	1024
Monobactam			
AZT	None	4	0.125
	IMP-10 MBL (5 U ml^{-1})	4	0.125
	PCase (5 U ml^{-1})	4	0.125
CRMN	None	4	0.125
	IMP-10 MBL (5 U ml^{-1})	4/8	0.125/0.25
	PCase (5 U ml^{-1})	4	0.125

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