Virulence of Metallo- β -Lactamase-Producing *Pseudomonas aeruginosa* In Vitro and In Vivo

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We evaluated the virulence of *Pseudomonas aeruginosa* carrying *bla*_{IMP}, a metallo- β -lactamase gene, and the efficacy of ceftazidime, imipenem-cilastatin, and ciprofloxacin in the endogenous bacteremia model. The presence of *bla*_{IMP} did not practically change the virulence of the parent strain, and ciprofloxacin was effective against infection with *P. aeruginosa* carrying *bla*_{IMP}.

Pseudomonas aeruginosa is a major opportunistic pathogen in immunosuppressed patients. Carbapenems have been the predominant antibiotic class for treatment of *P. aeruginosa* infection because of their stability against most β -lactamases. However, metallo- β -lactamases able to hydrolyze carbapenem have been reported, and *P. aeruginosa* strains carrying metallo- β -lactamase genes are a recent clinical threat (1, 3, 12). The purpose of this study was to evaluate the virulence of *P. aeruginosa* carrying the *bla*_{IMP} gene by using the epithelial cell monolayer system and mouse models of endogenous bacteremia. Moreover, we examined the efficacy of ceftazidime (CAZ), imipenem-cilastatin (IPM-CS), and ciprofloxacin (CIP) in the endogenous bacteremia model using *P. aeruginosa* carrying *bla*_{IMP}.

P. aeruginosa PAO1 wild type (WT), used as the parent strain, is invasive and penetrates MDCK cell monolayers within 3 h after infection (2). The vector control strain pMS360 was PAO1 transformed with the vector plasmid *pMS360* with a broad host range and streptomycin resistance (7). The strain pMS363 (7) was PAO1 transformed with the vector *pMS363*, which includes *bla*_{IMP} and an integrase gene (8), in vector plasmid *pMS360*. The PAO1 WT-derived MexAB-OprM deletion mutant (Δ *mexAB-oprM*) (5), which does not penetrate MDCK cell monolayers until 6 h, was used as a negative control. All strains exhibited the same growth kinetics in Luria-Bertani broth. Noninvasive rabbit enterotoxigenic *Escherichia coli* RDEC-1 was used as another negative control and as an internal control of monolayer integrity (2).

MICs of all β -lactams for the pMS363 strain, except that of aztreonam (ATM), rose by up to 4- to 64-fold compared to those for the parent WT, whereas those for the pMS360 strain were similar to those for the WT. MICs of fluoroquinolones for pMS360 and pMS363 strains were identical to those for the WT (Table 1).

We evaluated the capacity of *P. aeruginosa* to cause invasive infection by using the MDCK monolayer assay as described previously (2, 5). Although pMS363 could penetrate MDCK

cell monolayers by 3 h (Fig. 1), the numbers of pMS363 CFU detected in basolateral medium were less than those of the WT strain. Clinically isolated strains, which have the capacity to cause invasive infections, penetrate MDCK cell monolayers by 3 h (2). Therefore, the differences in capacities to cause invasive infections between pMS363 and the WT did not seem to be practical in spite of the statistical difference in the detected bacterial numbers in the basolateral medium.

Our observations in mouse models demonstrated this conclusion. The experimental protocols were approved by the Animal Care and Use Committee of Nagasaki University. Male 5-week-old BALB/c specific-pathogen-free mice (Japan SLC Inc., Shizuoka, Japan) were used. Endogenous *P. aeruginosa* bacteremia was induced as previously described (4), with modifications. All endogenously bacteremic mice died by day 10 (Fig. 2), and similar lethality rates were found for the three *P. aeruginosa* strains. These results suggested that the *bla*_{IMP} gene raises MICs of β -lactam antibiotics, except that of ATM, for *bla*_{IMP}-carrying strains but does not decrease the virulence of these strains.

In a subsequent study, mice were treated with several doses of CAZ (Tanabe Seiyaku Co., Ltd., Osaka, Japan), IPM-CS (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan), and CIP (Bayer AG, Leverkusen, Germany) by intraperitoneal injection twice daily after day 5. These trials were repeated with several concentrations of antibiotics, as indicated below. Mice were observed up to day 12. In the pMS360 mouse bacteremia model, all control mice died by day 9; however, treatment with some concentrations of CAZ increased survival rates (400

TABLE 1. Antimicrobial susceptibilities of *P. aeruginosa* WT, pMS360, and pMS363 strains^a

Strain	MIC (μ g/ml)									
	PIP	CAZ	FEP	SUL-CFP	ATM	CPR	IPM	MEM	LVX	CIP
WT	2	1	1	8	4	2	1	≤ 0.5	≤ 0.5	≤ 0.5
pMS360	2	1	1	4	4	8	1	≤ 0.5	≤ 0.5	≤ 0.5
pMS363	16	64	≥ 32	≥ 32	4	≥ 32	64	≥ 32	≤ 0.5	≤ 0.5

^a PIP, piperacillin; FEP, cefepime; SUL-CFP, sulbactam-cefoperazone; CPR, ceftipime; IPM, imipenem; MEM, meropenem; LVX, levofloxacin. MICs were determined using a microbroth dilution method according to the recommendations of the National Committee for Clinical Laboratory Standards (10).

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FIG. 1. Extent of penetration of *P. aeruginosa* WT, $\Delta mexAB-oprM$ mutant, pMS360, pMS363, and *E. coli* RDEC-1 through MDCK cell monolayers at 3 and 6 h after inoculation. Bacteria were inoculated at 3.5×10^7 CFU/well onto apical surfaces of MDCK cell monolayers. The assay was done in triplicate, and results are expressed as averages $\pm$ standard deviations. All *P. aeruginosa* strains except the $\Delta mexAB-oprM$ mutant were recovered from basolateral medium by 3 h after inoculation, whereas *E. coli* RDEC-1 could not be recovered at either time point. Analysis of variance was used for the comparisons among the groups. *, $P < 0.001$; **, $P = 0.068$; ***, $P = 0.001$.

mg/kg of body weight/day, 100% survival rate; 200 mg/kg/day, 60%; 6.25 mg/kg/day, 20%). No mice injected with 3.125 mg of IPM-CS/kg/day survived, but higher concentrations of IPM-CS correlated with increased survival rates (25 and 75 mg/kg/day, 80%; 50 mg/kg/day, 60%). In mice treated with CIP, the survival rate rose dose dependently, with a 100% survival rate for

the group treated with 40 mg of CIP/kg/day. On the other hand, all saline-treated control mice died by day 9 in the pMS363 mouse bacteremia model. All mice treated with CAZ died by day 11, and not even treatment with 1,000 mg/kg/day rescued the pMS363 bacteremic mice. All mice treated with 3.125 and 25 mg of IPM-CS/kg/day died by days 10 and 11, but

FIG. 2. Survival kinetics of leukopenic mice given *P. aeruginosa* strains pMS360, pMS363, and WT in the endogenous bacteremia model. Mice were given 200 mg of cyclophosphamide (C) and 200 mg of ampicillin (A) per kg on the indicated days. Bacteria were given orally in drinking water in 0.45% saline at 10^7 CFU/ml between days 2 and 5. The survival rate in the pMS363 bacteremic mouse model was not significantly different from those in the WT ($P, 0.0671$ by log rank test) and pMS360 ($P, 0.4165$ by log rank test) models. Also, there was no significant difference between pMS360 and the WT ($P, 0.1462$ by log rank test).

TABLE 2. ED₅₀s of three antibiotics in the mouse endogenous bacteremia model

Strain	Mean ED ₅₀ ± SD (mg/kg/day) (95% confidence interval)		
	CAZ	IPM-CS	CIP
pMS360	186.9 ± 28.2 (137.2–266.5)	35.1 ± 7.1 (19.8–53.5)	10.0 ± 1.7 (6.9–14.8)
pMS363	>1,000	91.5 ± 24.7 (62.6–456.3)	7.1 ± 1.8 (4.3–14.41)

other doses of IPM-CS increased survival rates (12.5 and 50 mg/kg/day, 20%; 75 mg/kg/day, 40%). In CIP-treated mice, the survival rates increased dose dependently from 0 (0.15625 mg of CIP/kg/day) to 100% (40 mg of CIP/kg/day). The 50% effective doses (ED₅₀s) of the three antibiotics were thus calculated (Table 2). Although MICs of CAZ and IPM-CS for pMS363 and pMS360 were equivalent, ED₅₀s of CAZ were clearly higher than those of IPM-CS in both mouse models. This may be due to different initial bactericidal activities of CAZ and IPM-CS (9) or different levels of endotoxin release from *P. aeruginosa* in infections treated with CAZ and IPM-CS (11). The ED₅₀s of CAZ were higher than those of IPM-CS in both models, but those of CIP were almost equal, with CIP being the most effective antibiotic.

Although examination of several strains allows generalization, our results showed minor differences, which had insignificant practical influence on virulence, between the *P. aeruginosa* PAO1 strain carrying *bla*_{IMP} and the parent strain in vitro and in animal models. CIP was the most potent antibiotic against *P. aeruginosa* carrying *bla*_{IMP} in the endogenous bacteremia model. However, CIP resistance is more common than *bla*_{IMP} in *P. aeruginosa*, and multidrug-resistant *bla*_{IMP}-carrying *P. aeruginosa* isolates with resistance to aminoglycosides, fluoroquinolones, and β-lactam antibiotics are emerging (6). Therefore, it is necessary to develop new antibiotics with potent activity against multidrug-resistant *bla*_{IMP}-carrying *P. aeruginosa*. Currently, it is important to detect metallo-β-lactamase-producing bacteria rapidly and to judge whether or not the pathogens are sensitive to fluoroquinolones.

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