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Original Article

Molecular Epidemiology and Clinical Implications of Metallo-β-Lactamase-Producing *Pseudomonas aeruginosa* Isolated from Urine

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We conducted a study on molecular epidemiology and clinical implications of metallo-β-lactamase (MBL)-producing *Pseudomonas aeruginosa* isolated from urine. Over a 10-year period from 2001 through 2010, a total of 92 MBL-producing *P. aeruginosa* urine isolates were collected from patients (one isolate per patient) who were admitted to 5 hospitals in Okayama Prefecture, Japan. When cross-infection was suspected in the hospital, pulsed-field gel electrophoresis was performed. In the resulting dendrogram of 79 MBL-producing *P. aeruginosa* urine isolates, no identical isolates and 7 pairs of isolates with ≥80% similarity were found. The biofilm-forming capabilities of 92 MBL-producing *P. aeruginosa* urine isolates were significantly greater than those of 92 non-MBL-producing urine isolates in a medium of modified artificial urine. The imipenem resistance transferred in 16 of 18 isolates tested, and these frequencies were in the range of 10^{-3} to 10^{-9}. All of 18 isolates tested belonged to internationally spread sequence type 235 and had 3 gene cassettes of antimicrobial resistance genes in the class 1 integron. The strong biofilm-forming capabilities of MBL-producing *P. aeruginosa* urine isolates could be seriously implicated in nosocomial infections. To prevent spread of the organism and transferable genes, effective strategies to inhibit biofilm formation in medical settings are needed.

Key words: *Pseudomonas aeruginosa*, metallo-β-lactamase, molecular epidemiology, biofilm, urinary tract infection

Pseudomonas aeruginosa is one of the most common pathogens in complicated urinary tract infections (UTIs), and is mostly responsible for nosocomial or catheter-associated UTIs [1]. *P. aeruginosa* presents a serious therapeutic challenge. The most problematic characteristic of the pathogen is its ability to rapidly develop resistance during the course of treating an infection [2]. Moreover, the continuation of ineffective antimicrobial therapy promotes resistance of *P. aeruginosa* and biofilm formation. *P. aeruginosa* has a tendency to form biofilms on the surface of urinary catheters in particular [3]. Antimicrobial therapy for *P. aeruginosa*-biofilm infections in the urinary tract is limited [4].

Acquisition of a metallo-β-lactamase (MBL) gene will invariably mediate broad-spectrum β-lactam resis-

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tance in *P. aeruginosa* [5, 6]. The genes encoding MBLs are often procured by class 1 integrons, which are found in transposons, resulting in highly transmissible genetic elements [7]. Moreover, other gene cassettes within the integrons often confer resistance to aminoglycosides, precluding their use as an alternative treatment. In 1991, the first mobile MBL in a *P. aeruginosa* strain was reported in Japan [8]. The imipenem-resistance allele was found on a transferable conjugative plasmid that could be readily mobilized to other *P. aeruginosa* strains. The identical gene was reported in *Serratia marcescens* isolated from a hospital in Japan in 1991 and later designated $bla_{\mathrm{IMP\text{-}1}}$ [9]. Since then, a large variety of MBLs have been described in bacteria of many genera and species worldwide [5, 6, 10]. MBL-producing *P. aeruginosa* is one of the most recalcitrant hospital-associated pathogens and some outbreaks of infections with multidrug-resistant *P. aeruginosa* or MBL-producing *P. aeruginosa* in Japanese hospitals have been reported [11-13]. There is thus an urgent need for strategies to prevent the spread of this organism in a medical setting.

MBL-producing *P. aeruginosa* was isolated first at Okayama University Hospital in 2001. Since then, we have investigated cross-infections in 5 hospitals in Okayama prefecture, Japan by using pulsed-field gel electrophoresis (PFGE). In this study, we specifically focused on MBL-producing *P. aeruginosa* isolated from urine in order to develop more effective strategies for infection control in hospitals. We examined MBL-producing *P. aeruginosa* isolated from urine to determine its biofilm-forming capabilities and its antimicrobial resistance transfer, and performed additional studies on the molecular epidemiology of these isolates by using multilocus sequence typing (MLST) and analysis of integron cassettes.

Materials and Methods

Clinical isolates. Over a 10-year period from 2001 to 2010, a total of 92 MBL-producing *P. aeruginosa* urine isolates were collected from patients (one isolate per patient) who were admitted to 5 hospitals in Okayama Prefecture, Japan. For purpose of comparison, 92 non-MBL-producing *P. aeruginosa* urine isolates, which were collected from patients (one isolate per patient) attending the urology clinic of Okayama University Hospital over a 10-year period from 2001 to 2010, were used.

Polymerase chain reaction (PCR) analysis. The presumed MBL-producing *P. aeruginosa* urine isolates were tested by PCR analysis to confirm the presence of the $bla_{\mathrm{IMP\text{-}1}}$ gene by using the primers (IMP1-f and IMP1-r) described by Arakawa *et al.* [14] (Table 1). All isolates were cultured on Mueller-Hinton agar plates overnight. The resulting *P. aeruginosa* colonies were picked with sterilized toothpicks. They were shaken in microtubes (volume, 1.5 ml) with 50 μl of 7.5% Chelex-100 solution (Bio-Rad Laboratories K. K., Tokyo, Japan) in distilled water, and were heated at 100℃ for 10 min to prepare crude genomic DNA lysates. The mixture was vortexed for approximately 30 sec at room temperature and then centrifuged at 10,000 $\times g$ for 1 min. The supernatant (2.5 μl) was mixed with 22.5 μl of prepared reaction mixture to start the reaction. The 25-μl reaction volume contained 0.1 μM of each primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), and 0.625 U of *Taq* DNA polymerase (TaKaRa Bio Inc., Otsu, Japan). PCR amplification was carried out with the following thermal cycling profile: initial denaturation at 94℃ for 2 min, 30 cycles of amplification (denaturation at 94℃ for 1 min, annealing at 55℃ for 1 min, and extension at 72℃ for 1.5 min), and a final extension at 72℃ for 5 min in a GeneAmp 9700 PCR system (Life Technology Corp. (formerly Applied Biosystems Inc.), Carlsbad, CA, USA). PCR products were analyzed on a 1.2% SeaKem ME agarose gel (Lonza Walkersville Inc. (formerly FMC BioProducts Inc.), Walkersville, MD, USA) with 1 × Tris-borate-EDTA buffer. After electrophoresis, the gels were stained with ethidium bromide (1 mg/l) and photographed under a UV transilluminator. A 100-bp DNA ladder (New England Biolabs Japan Inc., Tokyo, Japan) was used as the molecular size marker and the fragment size of the PCR product was 587 bp. In this study, $bla_{\mathrm{IMP\text{-}1}}$-positive isolates of *P. aeruginosa* were used as MBL-producing isolates.

Pulsed-field gel electrophoresis (PFGE). PFGE of MBL-producing *P. aeruginosa* urine isolates was performed by using the standard procedures according to the manufacturer's protocol (Bio-Rad Laboratories). Briefly, isolates were grown in tryptic

soy broth at 37℃ overnight. Bacterial cultures were embedded in 0.7% agarose plugs, lysed, washed, and digested separately with SpeI restriction enzyme. Size separation of the resulting DNA fragments was performed for 18.5 h in 0.8% agarose gels by using a CHEF DR-III apparatus (Bio-Rad Laboratories); voltage was set at 6V/cm with switch times of 1 sec to 23 sec at 14℃. A lambda ladder was used as a reference size standard. Pattern images were captured with a GelDoc XR (Bio-Rad Laboratories). The relatedness of each PFGE fingerprint was determined using Fingerprinting II (Bio-Rad Laboratories) and interpreted by the criteria proposed by Tenover *et al.* [15]. In the dendrogram analysis, isolates with band patterns that were 100% similar were considered to be of identical type, and those with ≥ 80% similarity were considered to have clonal relationships.

Susceptibility testing. Antimicrobial susceptibilities of MBL-producing *P. aeruginosa* urine isolates were determined by the standard broth microdilution method using a dry plate DP-25 (Eiken Chemical Co., Ltd., Tokyo, Japan) in accordance with Clinical and Laboratory Standards Institute guidelines [16]. According to the Law Concerning the Prevention of Infections and Medical Care for Patients with Infections of the Japanese Ministry of Health, Labour and Welfare, multidrug-resistant *P. aeruginosa* (MDRP) was defined as an isolate for which the minimum inhibitory concentrations (MICs) of imipenem, ciprofloxacin and amikacin were ≥16, ≥4 and ≥32 μg/ml, respectively [17].

Biofilm formation assay. We used an *in vitro* microtiter plate assay to quantify biofilm formation [18]. The MBL-producing and non-MBL-producing *P. aeruginosa* urine isolates were grown overnight at 37℃ in medium of artificial urine supplemented with 0.4% nutrient broth (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) [19]. The culture was diluted 1 : 100 in medium, and 200 μl of this cell suspension was used to inoculate sterile flat-bottomed 96-well polystyrene microtiter plates (Nunc 167008; Thermo Fisher Scientific K. K., Yokohama, Japan). After 24 h at 37℃ without shaking, the wells were gently washed 3 times with 300 μl of distilled water, dried in an inverted position for 10 min, and stained with 200 μl of 0.3% crystal violet solution in water for 45 min. After staining, the plates were washed 3 times with distilled water. Quantitative analysis of biofilm production was performed by adding 300 μl of ethanol-acetic acid (95 : 5, vol/vol) to destain the wells. One hundred microliters from each well was transferred to a new microtiter plate, and the level (optical density; OD) of crystal violet present in the destaining solution was measured at 570 nm using a model 680 microplate reader (Bio-Rad Laboratories). Each assay was performed in triplicate. As a control, uninoculated medium was used to determine the background OD. The mean OD_{570} value from the control wells was subtracted from the mean OD_{570} value of tested wells. The biofilm-forming capabilities were arbitrarily classified into 3 groups: strong ($OD_{570} \geq 1$), medium ($OD_{570} \geq$ 0.5 to < 1) and weak ($OD_{570} \geq 0$ to < 0.5) biofilm formers [20]. Data are expressed as the mean values ± standard error (SE). The Mann-Whitney U test was used to compare the biofilm-forming capabilities between the 2 groups of MBL-producing and non-MBL-producing isolates. All results were considered statistically significant at the $p < 0.05$ level.

Conjugative transfer experiments. Filter mating was performed using each of the MBL-producing *P. aeruginosa* isolates as a donor and *P. aeruginosa* ML5017 as a recipient to determine the transferability of the carbapenem-resistance determinant. The *P. aeruginosa* ML5017 recipient strain was kindly provided by Shizuko Iyobe [21]. Overnight cultures of 50 μl of donor and 0.5 ml of recipient were added to 4.5 ml of fresh Mueller-Hinton broth, and the mixtures were immediately collected on a membrane (25 mm width 0.45 μm pore size filter, type HA; EMD Millipore Corp., Billerica, MA, USA), which was inverted onto the surface of a Mueller-Hinton agar plate. After incubation at 37℃ for 20 h, the cells were suspended in 1 ml of Mueller-Hinton broth and the portions (0.1 ml) of the mixed and diluted culture were then plated on Mueller-Hinton agar plates with appropriate selective antimicrobial agents. Transconjugants were selected on Mueller-Hinton agar plates supplemented with 8 μg/ml of imipenem and 100 μg/ml of rifampicin. Colonies were counted after 20 h of incubation at 37℃. Separate platings where donors alone were selected provided a basis for estimating the transfer frequency (per donor). Transfer frequencies were calculated as the number of transconjugants per donor cell.

Multilocus sequence typing (MLST). MLST was performed according to the protocols described on

the *P. aeruginosa* MLST Database website (http://pubmlst.org/paeruginosa/) (accessed August, 2013). PCR and sequencing were performed for 7 housekeeping genes (*acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA* and *trpE*) [22]. Briefly, the amplification product was purified using a QIAquick PCR Purification Kit (QIAGEN K. K., Tokyo, Japan). The DNA concentration of the purified product was measured with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). The purified product with 8 ng of DNA was used for direct sequencing on each DNA strand, and a BigDye Terminator version 1.1 Cycle Sequencing Kit (Life Technology) was used to perform fluorescent labeling. Unincorporated dye terminators were removed by precipitation with 70% ethanol. The resulting product was dehydrated and stored at 4℃. The dried PCR product was dissolved in 15 μl of Hi-Di™ formamide, and then sequencing was performed. An ABI 3130 xl Genetic Analyzer (Life Technology) at the Central Research Laboratory, Okayama University Medical School was used to decode the base sequences. The nucleotide sequences of these genes were compared with the sequences submitted to the MLST database to determine the allelic numbers and sequence types (STs).

PCR amplification of variable regions of the bla$_{IMP-1}$-containing integrons. To determine the content and order of genes in the variable regions of the *bla*$_{\text{IMP-1}}$-containing integrons, PCR amplifications were carried out with the primers listed in Table 1. The presence of the genes (*intI1*, *bla*$_{\text{IMP-1}}$, *aac6'-Iae*, *aadA1*, *qacEΔ1*, *sul1*, *orf5*, IS*1326*, IS*1353*, *tniBΔ1*, *tniAΔ*, IS*26*) and the position of integration of these genes were verified. The gene cassette array (Fig. 1) was composed as reported previously according to the sequence of entire variable regions encoding integrase (*intI1*), metallo-β-lactamase (*bla*$_{\text{IMP-1}}$), aminoglycoside acetyltransferase (*aac6'-Iae*); aminoglycoside adenyltransferase (*aadA1*); resistance to quaternary ammonium compound disinfectants (*qacEΔ1*), sulfonamide resistance (*sul1*); an open reading frame (*orf5*) of unknown function; IS, insertion sequence (IS*1326*, IS*1353*, IS*26*) and a partial integron transposition module (*tniBΔ1*, *tniAΔ*) [11, 23]. PCR procedures were performed as described above, and PCRs were amplified with TaKaRa *Taq*, Hot Start Version according to the manufacturer's protocol (TaKaRa Bio). The amplification conditions of the primer pairs shown in Table 1 (amplicon number: 1, 2, 4 to 11 and 16 in Fig. 1) were 95℃ for 2 min, followed by 30 cycles of 94℃ for 1 min, 50℃ for 1 min, and 72℃ for 3 min, and those of the primer pairs in Table 1 (amplicon number: 12 to 15, 17 and 18 in Fig. 1) were 95℃ for 2 min, followed by 25 cycles of 98℃ for 10 sec, 50℃ for 30 sec, and 72℃ for 1 min. PCR amplification using the primer pair shown in Table 1 (amplicon number 3 in Fig. 1) and the conditions described above was used to confirm the presence of the *bla*$_{\text{IMP-1}}$ gene.

Results

PFGE analysis. PFGE analysis was performed when cross-infection was suspected in the hospital. In the resulting PFGE dendrogram of 79 MBL-producing *P. aeruginosa* urine isolates, no identical isolates and 7 pairs of isolates with ≥80% similarity were found (Fig. 2). The 7 pairs of isolates having a clonal relationship were identified in 3 differ-

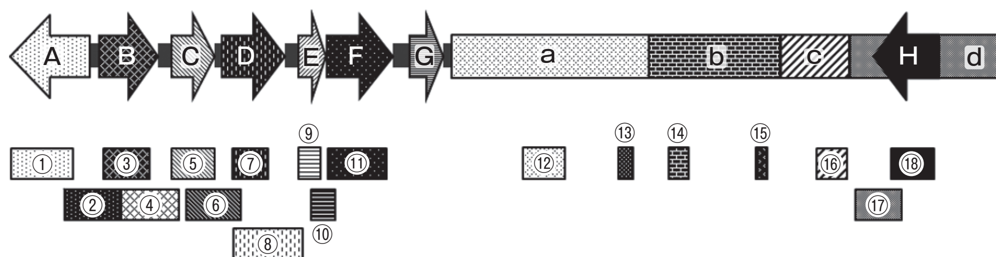

Fig. 1 PCR amplification of variable regions of the *bla*$_{\text{IMP-1}}$-containing integrons. The genes (A: *intI1*; B: *bla*$_{\text{IMP-1}}$; C: *aac6'-Iae*; D: *aadA1*; E: *qacEΔ1*; F: *sul1*; G: *orf5*; H: IS*26*; a: IS*1326*; b: IS*1353*; c: *tniBΔ1*; d: *tniAΔ*) in *bla*$_{\text{IMP-1}}$-containing integrons were amplified by PCR with primers as previously reported (Table 1). Open reading frames are shown as arrows indicating the orientation of the coding sequence. The rectangles (amplicon number: 1 to 18) indicate the position and size of PCR products.

Table 1 PCR primers used in this study

Name of primer	Sequence (5'→3')	Expected size of amplicon (bp)	Amplicon number in Fig. 1	Reference number
int1-F int1-R	TGCGTGTAAATCATCGTCGT CGAAGTCGAGGCATTTCTGT	766	1	11
int1imp1-F int1imp1-R	AGCACCTTGCCGTAGAAGAA TTTTATAGCCACGCTCCACA	695	2	11
IMP1-f IMP1-r	ACCGCAGCAGAGTCTTTGCC ACAACCAGTTTTGCCTTACC	587	3	14
imp1aacS1-F imp1aacS1-R	AAAGGCAGCATTTCCTCTCA GACGGCCAAGAATCGAAAT	737	4	11
aacS1-FC aacS1-RC	ATGAAATACAACATTGTTAATATTA TTACATTATATTTTTCCACATTAAT	552	5	11
aacS1aadA1-F aacS1aadA1-R	ATTGTGTGGTTGGGTTGGAT GGAGAATCTCGCTCTCTCCA	691	6	11
aadA1-F aadA1-R	TGATTTGCTGGTTACGGTGA TACTGCGCTGTACCAAATGC	451	7	11
aadA1qacEd-F aadA1qacEd-R	TGATTTGCTGGTTACGGTGA ATGCGGATGTTGCGATTACT	873	8	11
qacEdelta-F qacEdelta-R	TGAAAGGCTGGCTTTTTCTT GCAATTATGAGCCCCATACC	286	9	11
qacEdsul-F qacEdsul-R	TCGGTGTTGCTTATGCAGTC ACATCCACGACGTCTGATCC	306	10	11
sul-F sul-R	TCACCGAGGACTCCTTCTTC GGGTTTCCGAGAAGGTGATT	759	11	11
istA-955f istA-r	CGAGTCACTTCAACCTGCCT TAGCGGTTCACGTTGGCCTG	530	12	23
istA-105f istA-294r	GTATTTGCAAAGCAAGGTGG TCGGTCATAAGACCCGGTAA	190	13	23
AB104852-8121f AB104852-8380r	AGTAGATCACTATATCGAAC AGTTATACAACGTTGGTCGC	260	14	23
AB104852-9211f AB104852-9350r	GAAAATGCCCAATTAACGCG ATGAGGTCTTTCAGCGTCAC	140	15	23
tniB-F tniB-R	CAGAGCCAGTTGCTCCATTT ATCATCGACCTGTCCCACCT	399	16	11
IS26R-414f tniA-1622r	TAAAGCGCCCGCCTATGGTC TCGGCAATATCCGTATCCGG	595	17	23
IS26R-f IS26R-558r	ATGAACCCATTCAAAGGCCG CGTGGCGCCGATTATCCGTT	558	18	23

ent hospitals, and each of 2 pairs of isolates from 2 different hospitals was detected over a 4-day or 5-day interval. According to our records, the results confirmed that no spread occurred between 5 different hospitals and suggested that a cross-infection occurred within each of 2 hospitals. The other 5 pairs of isolates from 3 different hospitals were detected over approximately 1-month to 3-month intervals.

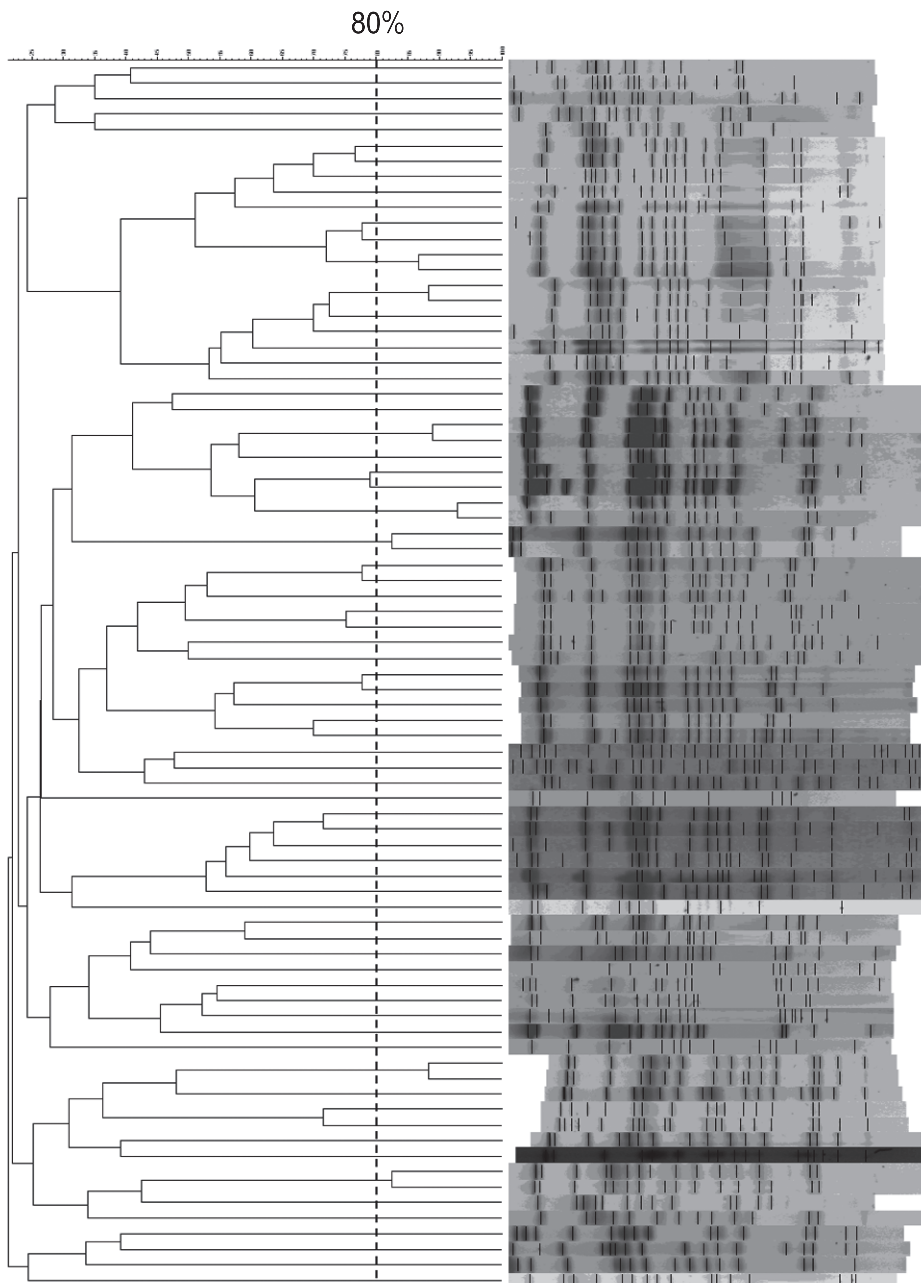

Fig. 2 PFGE dendrogram of 79 MBL-producing *P. aeruginosa* urine isolates. The corresponding banding pattern after SpeI digestion and subsequent PFGE is shown for each isolate. The scale bar at the top of the dendrogram indicates the similarity. The dotted line indicates the cutoff value of 80% similarity. This cutoff for clonal relationship was chosen if the PFGE patterns differed by changes consistent with a single genetic event, *i.e.*, a point mutation or an insertion or deletion of DNA [15], which in this PFGE setup corresponded to approximately 80% similarity.

Antimicrobial susceptibility and biofilm-forming capabilities. Of the 92 MBL-producing isolates, 83 (90.2%) were resistant to imipenem, ciprofloxacin and amikacin (MDRP), and 9 (9.8%) were resistant to imipenem and ciprofloxacin. Of the 92 isolates, 41 (44.6%), 37 (40.2%) and 14 (15.2%) isolates exhibited strong, medium and weak biofilm formation, respectively. In these 3 groups, 37 of 41 (90.2%), 35 of 37 (94.6%) and 11 of 14 (78.6%) isolates were MDRP, that is, more than 90% of MDRP exhibited strong or medium biofilm formation. As shown in Fig. 3, box-and-whisker plots were used to compare the biofilm-forming capabilities of MBL-producing and non-MBL-producing *P. aeruginosa* isolates in a medium of modified artificial urine. The average values of OD_{570} (mean ± SE) for each group (n = 92) were 1.17 ± 0.08 and 0.34 ± 0.05, respectively. The biofilm-forming capabilities of MBL-producing isolates were significantly greater than those of non-MBL-producing isolates ($p < 0.001$).

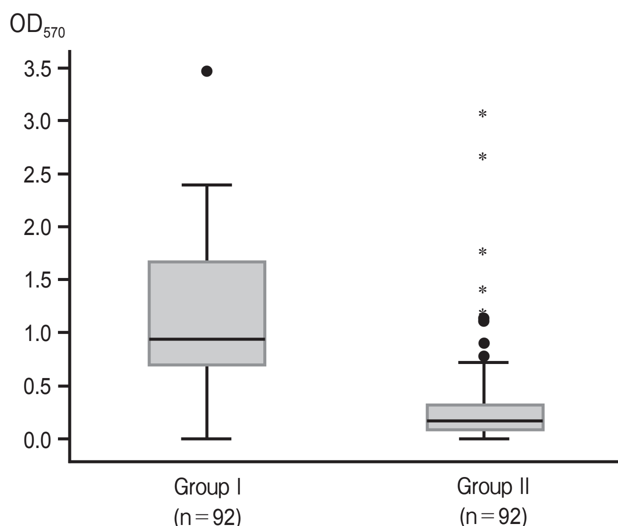

Fig. 3 Box-and-whisker plots of the biofilm-forming capabilities (OD_{570} values) of *P. aeruginosa* urine isolates in 2 groups (n = 92). A biofilm formation assay was performed in medium of artificial urine supplemented with 0.4% nutrient broth [19]. The heights of the boxes indicate the interquartile lengths. The central bar in each box is the median. Whiskers extend to the most extreme values at 1.5 box lengths from the edge of the box. Black dots and asterisks indicate outliers beyond 1.5 and 3.0 box lengths from the edge of the box, respectively. Group I is MBL-producing *P. aeruginosa* (mean ± SE: 1.17 ± 0.08). Group II consists of non-MBL-producing *P. aeruginosa* (mean ± SE: 0.34 ± 0.05). The *p*-value was < 0.001.

Carbapenem-resistance transfer. A total of 18 MBL-producing isolates (MDRP) classified into 3 groups according to their biofilm-forming capabilities (8, 6 and 4 isolates in the strong, medium and weak biofilm formation groups, respectively) were selected on the basis of their distinct PFGE patterns with less than 55% similarity. The imipenem resistance was transferred in 16 of 18 isolates tested, and these frequencies were in the range of 10^{-3} to 10^{-9} (Table 2). In the 3 groups according to biofilm-forming capability, 2 of 8, 1 of 6 and 1 of 4 isolates which exhibited strong, medium and weak biofilm formation, respectively, had relatively higher frequencies in the range of 10^{-3} to 10^{-5}. There was no relationship between the biofilm-forming capabilities and frequencies of carbapenem-resistance transfer.

Multilocus sequence typing (MLST). All of the 18 MBL-producing isolates (MDRP) tested above (Table 2) were genotyped as ST235 based on the presence of all 7 MLST alleles (*acsA* 38, *aroE* 11, *guaA* 3, *mutL* 13, *nuoD* 1, *ppsA* 2 and *trpE* 4).

Structure of the bla_{IMP-1}-containing integrons. As shown in Fig. 1, PCR amplification of variable regions of the bla_{IMP-1}-containing integron was performed in the 18 MBL-producing isolates (MDRP). All of the 18 amplicons were detected in 16 of 18 isolates tested. In the remaining 2 isolates, no amplification of the amplicon number 17 was detected. As summarized in Table 2, the bla_{IMP-1} gene was present in the class 1 integron (*intl1-qacEΔ1-sul1-orf5*) in agreement with the previous studies [7, 11, 23]. In the integrons, 3 gene cassettes (bla_{IMP-1}, *aac6'-Iae* and *aadA1*) encoding antimicrobial-resistance determinants and 3 insertion sequences (IS*1326*, IS*1353* and IS*26*) were detected in all of the 18 MBL-producing isolates tested. The insertion of IS*26* into the *tniA*-coding region was detected in 16 of 18 isolates.

Discussion

Over a 10-year period from 2001 to 2010, we have performed PFGE analysis on the MBL-producing *P. aeruginosa* isolates when cross-infection was suspected in the hospital. The resulting PFGE dendrogram suggested that the cross-infection occurred directly or indirectly on 7 pairs of isolates having a clonal relationship. These pairs of isolates were detected over a 4-day or 5-day interval or over approximately 1-month

Table 2 Summary of 18 MBL-producing isolates (MDRP) tested

Isolate number	Biofilm assay (OD_{570})	Conjugation frequencies	MLST	*bla*$_{IMP-1}$-containing integron			
				Class 1 integron[a]	Antimicrobial resistance genes[b]	Insertion sequences[c]	Truncated *tni* module[d]
13	2.070	7.0×10^{-5}	ST235	+	+	+	+
9	2.060	1.6×10^{-5}	ST235	+	+	+	+
14	2.005	1.1×10^{-7}	ST235	+	+	+	+
11	1.870	7.9×10^{-6}	ST235	+	+	+	+
26	1.849	1.0×10^{-7}	ST235	+	+	+	+
144	1.816	1.3×10^{-7}	ST235	+	+	+	+
5	1.226	1.5×10^{-6}	ST235	+	+	+	+
112	1.113	2.5×10^{-8}	ST235	+	+	+	+
2	0.828	7.1×10^{-7}	ST235	+	+	+	+
121	0.790	7.2×10^{-5}	ST235	+	+	+	−
105	0.789	Not detected	ST235	+	+	+	+
30	0.685	Not detected	ST235	+	+	+	+
118	0.650	2.5×10^{-7}	ST235	+	+	+	−
65	0.616	5.0×10^{-8}	ST235	+	+	+	+
21	0.486	1.7×10^{-3}	ST235	+	+	+	+
24	0.426	1.1×10^{-8}	ST235	+	+	+	+
32	0.425	4.5×10^{-8}	ST235	+	+	+	+
100	0.379	7.1×10^{-9}	ST235	+	+	+	+

[a]Class 1 integron (*intl1-qacEΔ1-sul1-orf5*) [7].
[b]Antimicrobial resistance genes (*bla*$_{IMP-1}$, *aac6'-lae*, *aadA1*) [11, 23].
[c]Insertion sequences (IS*1326*, IS*1353*, IS*26*) [11, 23].
[d]Truncated *tni* module (*tniBΔ1*, *tniAΔ*) with insertion of IS*26* into the *tniA*-coding region [11, 23].

to 3-month intervals. Such cross-infection can occur when an individual is in direct contact with a contaminated individual or through contaminated instruments or environments (equipment, work surface, clothes, *etc.*). PFGE is considered as the gold standard method for outbreak analysis [15]. However, MBL-producing *P. aeruginosa* isolates likely possess highly transmissible genetic elements [7]. The interpretation of PFGE patterns potentially related to cross-infection should be made with caution.

P. aeruginosa is a problematic organism in acute and chronic human infections, including UTIs. *P. aeruginosa* may produce biofilm, particularly in cases of chronic infection and device-related infection [3, 4, 24, 25]. Bacterial biofilms, which are microcolonies encased in extracellular polysaccharide material, result from the adherence of bacteria to all types of surfaces. The innate tolerance of biofilms to antimicrobial therapy has led to problems in their eradication and in the management of patients with device-related infections. Biofilms may also interfere with the immune clearance of infection. In addition, traditionally described resistance mechanisms in *P. aeruginosa*, such as loss of porins, efflux pumps, and enzymatic production, are responsible for the antimicrobial resistance [26]. The dissemination of distinct types of β-lactamase with hydrolytic activity against carbapenems has been increasing worldwide [5, 6]. The main carbapenemases produced by *P. aeruginosa* include the MBLs, which severely limit the treatment options for patients.

In this study, we used a microtiter plate assay to quantify biofilm formation in a medium of modified artificial urine. The biofilm-forming capabilities of MBL-producing *P. aeruginosa* urine isolates were significantly greater than those of non-MBL-producing urine isolates (Fig. 3). Perez *et al.* reported that *P. aeruginosa* harboring an MBL gene isolated from the sputa of patients with and without cystic fibrosis showed a high-level ability to produce biofilm [27].

They warned of an "overlapping of mechanisms" that can collectively heighten the challenge of treating pulmonary infections caused by *P. aeruginosa*. Biofilm formation by bacteria occurs not only with indwelling devices but also in response to any bacterial factor that mediates adherence to components of the extracellular matrix of the host [3, 4, 24, 25]. On the other hand, the ability of bacteria to survive in the hospital environment for long periods is linked to their antimicrobial resistance and their ability to form biofilms. The strong biofilm-forming capabilities of MBL-producing *P. aeruginosa* can cause serious problems both in patients with nosocomial infections, including UTIs, and in the hospital environment.

Zilberberg *et al.* reported on the secular trends in Gram-negative resistance among UTI hospitalizations in the United States, from 2000 to 2009 [28]. In this 10-year period, the frequency of UTI hospitalizations increased by approximately 50% and infections caused by multidrug-resistant *P. aeruginosa* increased by approximately 50%, whereas infections due to all Gram-negative bacteria followed a similar trajectory. Catheter-associated UTI (CAUTI) is responsible for 40% of nosocomial infections, making it the most common cause of nosocomial infection [1]. Catheterization of the urinary tract is one of the most common factors predisposing the host to complications [29]. Colli *et al.* reported on the national trends in hospitalization due to indwelling urinary catheter complications in the United States, from 2001 to 2010 [30]. In this 10-year period, the complications increased almost 4-fold and the majority of these patients had UTIs.

Biofilm formation is one of the prominent factors in bacterial diversification and is responsible for the horizontal spread of antimicrobial resistance and virulence genes [31, 32]. Our data showed that there was no relationship between the biofilm-forming capabilities and frequencies of carbapenem-resistance transfer of MBL-producing *P. aeruginosa* isolates (Table 2). Since some of the isolates at each level (strong, medium or weak) of biofilm-formation strength had high frequencies of gene transfer (10^{-3} to 10^{-5}), it was likely that the carbapenem-resistance determinant of these isolates was located on a plasmid. Antimicrobial-resistance genes on transferable conjugative plasmids are more problematic than those on chromosomes in medical settings, because the transmission and dissemination of antimicrobial-resistance determinants occurs more frequently among clinically relevant pathogenic bacteria [33]. The MBL-producing *P. aeruginosa* urine isolates must be taken into consideration in regard to the dissemination of antimicrobial resistance and virulence genes.

PFGE is still the gold standard method for investigating outbreaks and local epidemiology, and MLST may be more relevant to investigations exploring evolutionary and population biology relationships [34, 35]. Clinical isolates of *P. aeruginosa* ST235 have been reported worldwide and have often been shown to harbor clinically important acquired β-lactamases [36, 37]. In Japan, MBL-producing *P. aeruginosa* ST235 have undergone clonal expansion in medical settings [38]. In this study, all 18 of the MBL-producing *P. aeruginosa* urine isolates (MDRP) tested belonged to the internationally spread strain ST235. A limited number of *P. aeruginosa* genotypes, including ST235, that are known to be high-risk clones are responsible for epidemics of nosocomial infections by multidrug-resistant *P. aeruginosa* worldwide [39].

A large variety of MBLs have been described in bacteria of many genera and species worldwide [5, 6, 10]. MBLs belonging to the IMP, VIM, GIM, and SIM types have been detected primarily in *P. aeruginosa*. Among them, IMP and VIM are the most common types of MBL worldwide and the IMP type harboring $bla_{\text{IMP-1}}$ is predominant in Japan [11, 23]. Integrons are often found on the plasmid or as a part of the transposons or transmissible genetic elements (IS*CR* family) that are able to mobilize chromosomal elements, resulting in an increase in the number of antimicrobial-resistant Gram-negative bacteria [33, 40]. Kouda *et al.* reported 6 variants in the $bla_{\text{IMP-1}}$-containing integron (polymorphs A to F). Polymorph F, which was formed by insertion of IS*26* into the *intl1* gene of polymorph E, was first disseminated in 2005 in Hiroshima prefecture [23]. Our data confirmed that all 18 of the MBL-producing *P. aeruginosa* urine isolates (MDRP) tested belonged to polymorph E (Fig. 1), and no members of polymorph F were found. Kitao *et al.* reported the emergence of multidrug-resistant *P. aeruginosa* isolates that produce both IMP-type MBLs and an aminoglycoside-modifying enzyme [AAC(6')-Iae]; these isolates belong to polymorph E and have become a serious problem in medical settings in Japan [38].

In recent years, clones that are resistant to many antimicrobial agents and carry virulence factors have spread globally, and are considered highly successful or high-risk clones [33]. In *P. aeruginosa*, AmpR, which regulates the expression of the chromosomal β-lactamase AmpC, is a global transcriptional regulator involved in quorum sensing, alginate production, biofilm formation, and the expression of several other virulence factors [41]. Mulet *et al.* explored the potential biological parameters that may explain the success of widespread *P. aeruginosa* high-risk clones producing invasive infections. The bloodstream isolates belonged to high-risk clones showed significantly increased biofilm formation and mutant frequencies, but significantly reduced motility (twitching, swimming, and swarming), production of pyoverdine and pyocyanin, and fitness [42]. They suggested that the defined biological markers of high-risk clones, resembling those resulting from adaptation to chronic infections, could be useful for the design of specific treatment and infection control strategies. Although we have not yet studied virulence factors other than biofilm formation on MBL-producing *P. aeruginosa* urine isolates, such factors will be an important research subject for the development of effective strategies to inhibit biofilm formation [43].

In conclusion, the strong biofilm-forming capabilities of MBL-producing *P. aeruginosa* urine isolates could be seriously implicated in nosocomial infections. To prevent spread of the organism and transferable genes, effective strategies to inhibit biofilm formation in medical settings are needed.

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