

Review article

Analysis of multidrug-resistant *Pseudomonas aeruginosa* in a hospital with a dominant population of geriatric inpatients

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Abstract

Bacterial resistance and opportunistic human pathogens have become serious problems in hospitals, particularly those in which the dominant population consists of immunocompromised elderly inpatients. *Pseudomonas aeruginosa* is a prevalent opportunistic human pathogen that causes acute pneumonia, cystic fibrosis, and septicemia, and it is also known to possess various mechanisms of natural and acquired resistance to antibiotics. Carbapenems have been used to treat infections caused by β -lactamase-producing gram-negative bacteria including *P. aeruginosa*, but metallo- β -lactamase (MBL) can render carbapenems ineffective, and so action is required to prevent the development of strains that produce MBL. Between September 2010 and August 2011, 8 multidrug-resistant *P. aeruginosa* (MDRP) strains were isolated from pressure ulcer, urine, or expectoration or aspiration sputum samples of patients with different infections in a hospital in Osaka Prefecture, Japan. After confirming MBL production, we identified 5 strains that produced IMP-type MBL. The strain variations of isolated MDRP were determined by multiple-locus variable-number tandem repeat analysis. Because the 5 IMP-type MBL-producing strains were of the same genotype, it was suggested that the infection had spread within the wards. The remaining non-MBL-producing MDRP strains were also of the same genotype, but antimicrobial breakpoint tests revealed that they were not exactly the same, and the wards from which they had been isolated were not close to each other. Therefore, more accurate typing of the non-MBL-producing genotype group is required to determine whether diffusion between the wards had occurred.

Key words: Multidrug-resistant *Pseudomonas aeruginosa*, Metallo- β -lactamase, strain

INTRODUCTION

Bacterial resistance has become a serious worldwide medical issue. In Japan, with the rapid increase in the elderly population, an increased incidence of impaired resistance to infection is expected in elderly inpatients. Recently, large-scale infections within hospitals have been reported, and the data have been compiled into a database (<http://www.nih-janis.jp/section/index.html>).

Therefore, the expansion of preventive measures against multidrug-resistant opportunistic pathogens such as *Pseudomonas aeruginosa*, *Acinetobacter* spp., and *Klebsiella pneumoniae* has become especially important.

In particular, *P. aeruginosa* is a prevalent opportunistic human pathogen that causes acute pneumonia, cystic fibrosis, and septicemia in immunocompromised individuals, including elderly patients with chronic conditions (Govan and Deretic,

1996). *P. aeruginosa* is also known to possess various mechanisms of natural and acquired resistance to antibiotics (Aloush *et al.*, 2006), and the strains that are resistant to carbapenems, aminoglycosides, and fluoroquinolones are defined as multidrug-resistant *P. aeruginosa* (MDRP), according to the Infectious Diseases Control Law in Japan. Carbapenems have been used to treat infections caused by β -lactamase-producing gram-negative bacteria, but bacterial strains that produce metallo- β -lactamase (MBL), which render almost all broad-spectrum β -lactams and carbapenems ineffective, have been detected with the use of carbapenems (Senda *et al.*, 1996a). The MBL genes are found in integrons frequently located in plasmids or transposons, which contribute to their dissemination (Fluit and Schmitz, 1999).

P. aeruginosa thrives in soil and aqueous environments, including areas around nursing-station sinks and bathrooms of medical facilities, where it survives because of environmental adaptations that allow it to utilize a variety of nutrients (Nishijyo *et al.*, 2001). Because the spread of infection may occur through various routes, it is important to evaluate the spread of the same strain between patients. Methods frequently used to identify the variety of *P. aeruginosa* strains include multi-locus sequence typing (Curran *et al.*, 2004), pulsed-field gel electrophoresis (Romling *et al.*, 2000), and multiple-locus variable-number tandem repeat analysis (MLVA) (Onteniente *et al.*, 2003), which initially used 7 sets of polymorphic tandem repeat loci. More recently, modifications of the MLVA method using 15 sets of repeat loci for accurate typing (Vu-Thien *et al.*, 2007) and 4 sets of repeat loci (MLVA-4) for simple and easy detection (Hayakawa *et al.*, 2009) have been developed.

MATERIALS AND METHODS

Bacterial isolates and growth conditions

Between September 2010 and August 2011, *P. aeruginosa* strains were isolated from pressure ulcer, urine, or expectoration or aspiration sputum samples of patients with different infections in a hospital (approximately 1,000 beds); the dominant population in this hospital in Osaka Prefecture, Japan consists of immunocompromised elderly inpatients. Genus classification of the strains was performed using the MicroScan WalkAway Plus (Siemens, Munich, Germany) fully automated instrument. Isolated *P. aeruginosa* for which minimum inhibitory concentrations of imipenem

(IMP), amikacin, and ciprofloxacin are ≥ 16 , ≥ 32 , and ≥ 4 $\mu\text{g}/\text{mL}$, respectively, are defined as MDRP. PAO1 is a standard strain of *P. aeruginosa* for which the complete genomic sequence is available (Stover *et al.*, 2000).

All strains were cultured at 37°C in Mueller-Hinton medium (Oxoid, Sunnyvale, USA) supplemented with antibiotics when appropriate.

DNA techniques

DNA purification and other DNA manipulations were conducted as previously described (Nakada and Itoh, 2002; Sambrook *et al.*, 2001). A polymerase chain reaction (PCR) was performed with KOD-plus DNA polymerase (Toyobo Biochemicals, Osaka, Japan) in a GeneAmp PCR System 2700 thermal cycler (Applied Biosystems, Foster City, USA) under the reaction conditions recommended by the supplier.

Antibiotic susceptibility test

Antimicrobial breakpoint tests were conducted by FALCO Biosystems Ltd. using the MicroScan WalkAway Plus fully automated instrument. Isolates were evaluated for susceptibility to piperacillin, ceftazidime, ceftazidime/ceftiofur, imipenem, minocycline, amikacin, and ciprofloxacin and categorized as susceptible (S), intermediate (I), or resistant (R) according to the antibiotic breakpoint guidelines of the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2006) for *P. aeruginosa*.

Screening of MBL producers by SMA test

The screening of all MDRP isolates to detect MBL producers was carried out using a sodium mercaptoacetic acid (SMA) (Eiken, Tokyo, Japan) disk method, with Kirby-Bauer disks (Eiken) of ceftazidime and SMA.

Immunochromatographic detection of IMP-type MBLs

The IMP-type MBLs were detected by immunochromatography (Quick Chaser IMP; Mizuho Medy, Saga, Japan), in which colloidal gold-labeled anti-IMP rat monoclonal antibodies were applied to a test strip, in all SMA-positive isolates.

PCR detection of *bla*_{IMP}

The IMP-type MBL genes (*bla*_{IMP}) were detected by PCR in all isolates for which the immunochromatographic method yielded positive results. According to the PCR detection method by Shinoda *et al.* (2006), primers (forward 5' - CTACCGCAGCAGAGTCTTTG-3' and reverse

5'-GTGGCITGAACCTTACCGTC-3') were prepared and the amplification reaction was carried out under the following cycling conditions: 94°C for 2 min; 30 cycles of 94°C for 30 sec, 57°C for 1.5 min, and 68°C for 1.5 min; and a final extension at 68°C for 2 min. The PCR products were determined by 2.0% agarose gel electrophoresis. For the positive control of *bla_{IMP}*, we used a *bla_{IMP-1}*-possessing plasmid that our laboratory had detected previously.

Genotyping by MLVA-4

The strain variations of all isolated MDRP were determined by MLVA-4 (Hayakawa *et al.*, 2009). The PCR primers used to amplify 4 sets of polymorphic tandem repeat loci and the amplicon size and repeat copy number of PAO1 are listed in Table 1. PCR conditions for MLVA-4 were as follows: 94°C for 2 min; 30 cycles of 96°C for 20 sec, 64°C for 30 sec, and 68°C for 1.5 min; and a final extension at 68°C for 2 min. The fragment size of the PCR products was determined by 2.0-4.0% agarose gel electrophoresis.

RESULTS

Isolated strains of MDRP

Within 1 year, we identified 8 patients at the hospital who had developed MDRP infection, and we obtained clinical isolates from pressure ulcer,

urine, or expectoration or aspiration sputum samples (Table 2). The patients included those on geriatric chronic-stage wards (A and C), specific disease wards (B), and an acute-phase ward (D). The antimicrobial breakpoint test showed that all 8 isolates were amikacin-, imipenem-, and ciprofloxacin-resistant; moreover, AUH-55, AUH-56, AUH-59, AUH-63, AUH-66, and AUH-68 strains were resistant to all antibiotics tested. Of the remaining 2 strains, both isolated from ward C patients, AUH-57 was intermediate against ceftazidime and AUH-62 was intermediate and susceptible to ceftazidime and ceftazidime, respectively.

Detection of MBL

Of the 8 strains evaluated by the screening tests using disks containing SMA, 5 strains (AUH-55, AUH-56, AUH-59, AUH-66, and AUH-68) appeared to produce MBL (Table 2). The SMA-positive strains were also confirmed to produce IMP-type MBL by immunochromatography (Table 2). However, of the 5 IMP-type MBL-producing strains, *bla_{IMP}* was not amplified by the common specific PCR method (data not shown).

MLVA-4 of isolated MDRPs

MLVA-4 of the 8 clinically isolated MDRPs revealed the same electrophoretic pattern for AUH-55, AUH-56, AUH-59, AUH-66, and AUH-

Table 1 PCR primers for the detection of tandem repeat loci

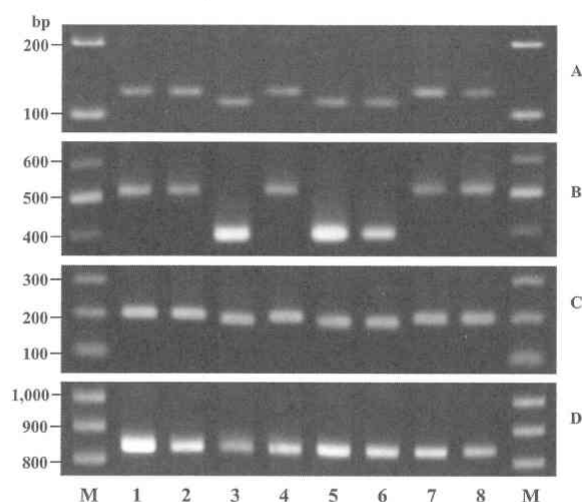
Locus name	Associated ORF	Primer	Oligonucleotide sequence (5'-3')	Amplicon size bp* (repeat copy no.)
ms-61	<i>pscP</i>	PscP-L	CTTGCCGTGCTACCGATCC	127 (12)
		PscP-R	CCCCATGCCAGTTGC	
ms-77	<i>pcoA</i>	PcoA-L	GCGTCATGGTCTGCATGTC	422 (4)
		PcoA-R	TATACCCTCTTCGCCAGTC	
ms-142	PA3463	PA3463-L	AGCAGTGCCAGTTGATGTTG	890 (7)
		PA3463-R	GTGGGGCGAAGGAGTGAG	
ms-172	PA4541	PA4541-L	GGATTCTCTCGCAGGAGGT	789 (12)
		PA4541-R	TACGTGACCTGACGTTGGTG	

*Corresponding to *P. aeruginosa* PAO1

Table 2 Specimens, antimicrobial breakpoints, and MBL detection of MDRP isolates

Strain	Hospital ward	Specimens	Breakpoint							SMA test	IC-IMP test	
			PIPC	CAZ	CZOP	IPM	MINO	AMK	CPFX			
AUH-55	A	pressure ulcer	R	R	R	R	R	R	R	R	+	+
AUH-56	B	urine	R	R	R	R	R	R	R	R	+	+
AUH-57	C	urine	R	R	I	R	R	R	R	R	-	-
AUH-59	B	urine	R	R	R	R	R	R	R	R	+	+
AUH-62	C	aspiration sputum	R	I	S	R	R	R	R	R	-	-
AUH-63	D	expectoration	R	R	R	R	R	R	R	R	-	-
AUH-66	B	urine	R	R	R	R	R	R	R	R	+	+
AUH-68	B	urine	R	R	R	R	R	R	R	R	+	+

A and C represent geriatric chronic-stage wards, B represents specific disease wards, and D represents an acute-phase ward. PIPC, piperacillin; CAZ, ceftazidime; CZOP, ceftazidime; IPM, imipenem; MINO, minocycline; AMK, amikacin; CPFX, ciprofloxacin; IC-IMP, IMP-type MBL immunochromatography.



A, ms-61; B, ms-77; C, ms-142; D: ms-172. Lane M, 100-bp DNA marker; 1, AUH-55; 2, AUH-56; 3, AUH-57; 4, AUH-59; 5, AUH-62; 6, AUH-63; 7, AUH-66; 8, AUH-68

Figure 1 Electrophoresis for the MLVA-4 of the isolated MDRP strains

Table 3 Genotypes of the reference strain PAO1 and isolated MDRP strains

Strain	Hospital ward	Allele at the following locus maker			
		ms-61	ms-77	ms-142	ms-172
PAO1	—	12	4	7	12
AUH-55	A	13	6	1	13
AUH-56	B	13	6	1	13
AUH-57	C	10	3	1	13
AUH-59	B	13	6	1	13
AUH-62	C	10	3	1	13
AUH-63	D	10	3	1	13
AUH-66	B	13	6	1	13
AUH-68	B	13	6	1	13

68 strains (Figure 1). From the repeat copy number of each locus deduced by the amplicon size, this group was identified as genotype 13-6-1-13 (Table 3). In the same way, AUH-57, AUH-62, and AUH-63 showed the same electrophoretic pattern and were identified as genotype 10-3-1-13. From these results, it appears that only 2 strain types were isolated from 8 different patients within 1 year.

DISCUSSION

Over the course of 1 year, 8 patients at the hospital were found to have an MDRP infection, and the MDRP detection rate (number of newly infected patients/total number of hospitalized patients) was 0.39%. Because of the high number of chronic-stage elderly patients who are hospitalized, stringent nosocomial infection measures are necessary.

After confirming the presence of MDRP based

on a drug sensitivity test and MBL production by a SMA test, we could divide the bacterial strains into 2 groups: SMA-positive (AUH-55, AUH-56, AUH-59, AUH-66, and AUH-68) and SMA-negative (AUH-57, AUH-62, and AUH-63). The SMA-positive group consisted of highly resistant strains that also produced IMP-type MBL, and all of these strains showed resistance to all antibiotics that we examined. However, we were unable to amplify the domain for *bla*_{IMP} by the general *bla*_{IMP}-detection PCR (Shinoda *et al.*, 2006; Senda *et al.*, 1996b). These results indicate that the SMA-positive strains maintain the production of IMP-type MBL that cannot be detected by the primers. With respect to the IMP-type MBL, 48 kinds have now been confirmed, and this number is increasing annually (<http://www.lahey.org/Studies/>). Among these, there are alleles that cannot be amplified by the general *bla*_{IMP}-detection PCR, such as *bla*_{IMP-7} (Koh *et al.*, 2004). Furthermore, an unidentified allele that cannot be amplified by the PCR may be present. Subsequently, we plan to use touch down PCR or multiplex PCR based on the *bla*_{IMP} sequence diversities, and to confirm the findings by complete sequencing of the *bla*_{IMP} detected this time.

Strain identification with the MLVA-4 method revealed that all 5 SMA-positive strains had the same genotype (13-6-1-13), and the antimicrobial breakpoint tests showed that the 5 strains were all similarly resistant (Table 2). These results indicate that the spread of infection on the B ward had occurred via the same genotype strain. The MLVA-4 method also revealed that the 3 SMA-negative strains obtained from the patients on the C and D wards were also all of the same genotype (13-6-1-13), but, according to the antimicrobial breakpoint tests, the 3 strains were not exactly the same (Table 2). It is noteworthy that, in the hospital, the C and D wards are not close to each other. Because MLVA-4 is a simplified detection method, detailed analysis using MLVA-15 or pulsed-field gel electrophoresis is necessary to conclude that diffusion spreads on the C and D wards.

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