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Note

Acquisition of antimicrobial-resistant variants in repeated infections caused by *Pseudomonas aeruginosa* revealed by whole genome sequencing[☆]

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ABSTRACT

Pseudomonas aeruginosa, responsible for serious nosocomial-acquired infections, possesses intrinsic antibiotic resistance mechanisms and commonly exhibits multidrug resistance. Here, we report the evolving resistance profiles of strains isolated from the sputum of a patient being treated for repeated *P. aeruginosa* infections following cancer resection. Whole genome sequencing of six isolates obtained over a 2-month period revealed two key single nucleotide polymorphisms in the *mexR* and *gyrB* genes that affected efflux pump expression and antimicrobial resistance.

Pseudomonas aeruginosa is a major opportunistic pathogen causing community or nosocomial-acquired pneumonia [1]. Infections caused by *P. aeruginosa* are often hard to treat due to the organism's intrinsic resistance and high frequency of acquisition of resistance mechanisms to antibiological agents during treatment [2]. Antimicrobial resistance mechanisms include the acquisition of extended spectrum β-lactamases, carbapenemases, aminoglycoside-modifying enzymes, and 16S ribosomal ribonucleic acid methylases [3,4]. In the absence of such acquired resistance determinants, chromosomally-encoded genetic mechanisms, which include lower outer membrane permeability, the upregulation of multidrug efflux pumps, and mutations within antimicrobial targets, mediate high-level multidrug resistance [5]. In particular, hyperexpression of MexAB-OprM effux pump, which is member of resistance-nodulation-cell division (RND) family, has known to be able to pump out various antibiotics, owing to lead to multidrug resistant phenotype [6]. Specific mutations within antimicrobial resistance genes in *P. aeruginosa* have been reported by analysis using next generation sequencing [7].

Here, we report investigation of bacterial isolates from a 60-year-old male patient who was admitted to hospital for surgical resection of multiple liver metastases and sigmoidectomy to treat sigmoid cancer in April 2016. Our study was approved by the Ethics Committee of Kobe University Graduate School of Health Sciences (approval no. 472-3). We observed six repeated infections and the gradual acquisition of antimicrobial resistance caused by *P. aeruginosa* in the patient over a 2-month period (Table 1). Strains isolated from the patient were identified using a MALDI Biotyper (Bruker Daltonics K.K., Yokohama, Japan) and the minimal inhibitory

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Table 1
Genetic characterization and the antibiotic susceptibility of *Pseudomonas aeruginosa* isolates derived from repeat infections.

No. of isolate	Day of isolation after surgery	Specimen	ST[a]	MICs (μg/ml)[b]						*mexA* expression level	DDBJ accession no. of read data
				PIPC	CAZ	IPM	MEPM	AMK	CPFX		
KPA16005	day 3	Aspirate sputum	1239	3	0.75	1.5	0.064	4	0.064	1[c]	DRR101530
KPA16006	day 6	Aspirate sputum	1239	3	1	1.5	0.094	4	0.094	1.14 ± 0.06	DRR101531
KPA16007	day 20	Aspirate sputum	1239	2	1	3	0.125	6	0.75	1.22 ± 0.10	DRR101532
KPA16008	day 23	Aspirate sputum	1239	16	3	>32	6	4	2	10.5 ± 0.98[d]	DRR101533
KPA16009	day 27	Aspirate sputum	1239	16	3	>32	12	4	2	7.14 ± 0.56[d]	DRR101534
KPA16010	day 67	Sputum	1239	>256	128	>32	>32	32	8	11.3 ± 0.52[d]	DRR101535

[a] ST, sequence type by multilocus sequence typing.

[b] piperacillin (PIPC), ceftazidime (CAZ), imipenem (IPM), meropenem (MEPM), amikacin (AMK), ciprofloxacin (CPFX).

[c] mRNA expression of the *mexA* gene (mean of triplicate values ± standard error) relative to the expression of KPA16005, which is assigned a value of 1.

[d] $p < 0.05$. The p values are based on ANOVA.

concentrations (MICs) of piperacillin (PIPC), ceftazidime (CAZ), imipenem (IPM), meropenem (MEPM), amikacin (AMK) and ciprofloxacin (CPFX) were determined using an E-test (bioMérieux Japan Ltd., Tokyo, Japan) and assessed according to the manufacturer's guidelines. The therapeutic history and clinical evolution after the surgery in the patient were shown in Fig. 1. On day 3 after the surgery, dyspnea and consciousness disturbance appeared in the patient. The empiric antimicrobial agents, MEPM and vancomycin, were used following the diagnosis of pneumonia. On days 3 and 6 post-surgery, *P. aeruginosa* with susceptibility to all antimicrobial drugs tested was isolated from a sputum culture, as shown in Table 1 (KPA16005 and KPA16006). MEPM and levofloxacin were administered as the target therapy for pneumonia caused by *P. aeruginosa*. On day 20, *P. aeruginosa* that was not resistant to all antimicrobial drugs was detected from a sputum culture (KPA16007). On days 23 and 27, *P. aeruginosa* with resistance to IPM, MEPM, and CPFX was detected from a sputum culture (KPA16008 and KPA16009). The administration of MEPM was replaced by CAZ and inhalation of tobramycin in accordance with the results of antimicrobial susceptibility tests. CAZ and inhalation of tobramycin were administrated for 30 days. On day 67, the *P. aeruginosa* with not susceptible to PIPC, CAZ, IPM, MEPM, AMK and CPFX was isolated from a sputum culture (KPA16010). Although the *P. aeruginosa* isolate (KPA16010) did not have the acquired resistance determinants mentioned above, we analyzed single nucleotide variations by whole genome sequencing to reveal the genetic relationship between the isolate and antimicrobial resistance mechanisms. Whole genome sequencing DNA libraries were constructed using a QIAseq FX DNA Library kit (QIAGEN, Hilden, Germany), and paired-end sequences (2 × 300 bp) were generated using the MiSeq system (Illumina, San Diego, CA, USA) with a MiSeq reagent kit v.3 (Illumina). All of the generated reads were assembled into contigs using A5-miseq [8]. Gene predictions and annotations were performed with PROKKA [9], and the sequence types were determined by PubMLST (https://pubmlst.org/). Drug resistant genes were searched using Resfinder 2.0 [10], but not specifically detected in resistant isolates. For comparative analysis, a core single nucleotide polymorphism (SNP) matrix was generated using kSNP (v. 3.0) with a k-mer size of 23 [11]. The whole genome sequencing reads are available from the DDBJ/EMBL/GenBank Sequence Read Archive under accession number DRA006160, and are listed in Table 1. Analysis by kSNP was used to elucidate the mechanism by which the isolates changed from a susceptible to a multidrug-resistant phenotype. A total of 41 core SNPs were detected among the six isolates analyzed in this study. Among them, five SNPs detected were common mutations in drug-resistant isolates (Table 2). Of five mutations, two mutations (SNP nos. 4 and 5) found in three isolates (KPA16008 to KPA16010) showed higher MICs for IPM, MEPM and CPFX than the MICs of isolates KPA16005

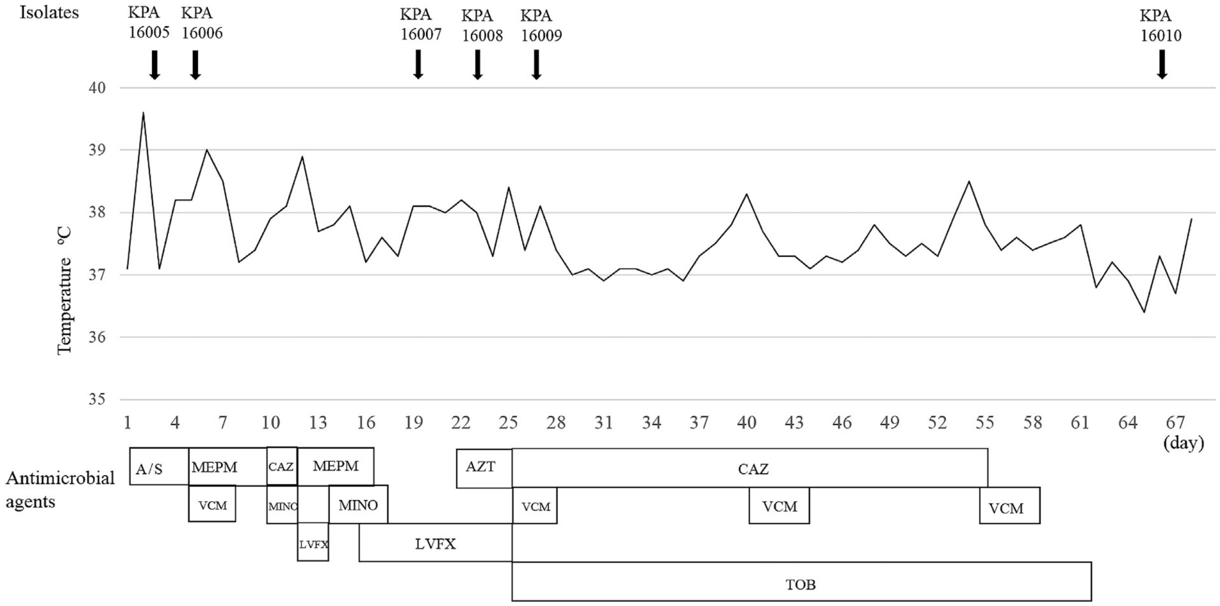

Fig. 1. Therapeutic history and clinical evolution during the 68 days-hospitalization after surgery in a patient *Pseudomonas aeruginosa* isolated. Antibiotics are follows: ampicillin/sulbactum (A/S), aztreonam (AZT), ceftazidime (CAZ), meropenem (MEPM), minocycline (MINO), levofloxacin (LVFX), tobramycin (TOB) and vancomycin (VCM).

Table 2
SNPs and common mutations in *Pseudomonas aeruginosa* isolates.

SNP no.	Annotation of gene[a]	SNP position (Base from strat of ORF)	Sequence of isolates [c]						Codon change resulting SNPs
			KPA16005	KPA16006	KPA16007	KPA16008	KPA16009	KPA16010	
1	Type 4 fimbrial biogenesis protein; PilZ	238	C	C	C	C	C	**T**	Gln→stop codon
2	Group 4 glycosyl transferase[b]	553	C	C	C	C	C	**T**	Gln→stop codon
3	Two-component sensor; ParS	1154	G	G	G	G	G	**A**	Arg→His
4	Multidrug resistance operon repressor; MexR	52	C	C	C	**T**	**T**	**T**	Gln→stop codon
5	DNA gyrase subunit B; GyrB	1397	C	C	C	**T**	**T**	**T**	Ser→Phe

[a] Refer to annotation of *P. aeruginosa* PAO1 except for SNP no. 2.
[b] Refer to annotation of *P. aeruginosa* ATCC 14886.
[c] Boldface data indicate the different sequence from those of the reference strains.

to KPA16007. SNP no. 4 was found at position 52 in the *mexR* gene, encoding a transcriptional repressor of the *mexAB–oprM* operon [12], leading to the generation of a stop codon. Another related mutation, SNP no. 5 (a C to T mutation), was found at position 1397 in the *gyrB* gene and that resulted in a substitution in the 466th amino acid (from Ser to Phe), leading to higher MICs for fluoroquinolone [13]. These results indicated that the *mexR* and *gyrB* genes were involved in the resistance of *P. aeruginosa* to IPM, MEPM, and CPFX according to use of several antimicrobial agents in this study. In addition, to support the finding that this novel mutation in *mexR*, we examined the expression of *mexA* genes in all isolates by qRT-PCR, as described previously [14]. The expression levels of the *mexA* gene in the three isolates, KPA16008 to KPA16010, harboring a mutated *mexR* gene and showing antimicrobial resistance, were found to be approximately 10-fold higher than that of isolate KPA16005 showing antimicrobial susceptibility ($p < 0.05$; Table 1). Moreover, *mexEF*, *mexXY* operons and *oprD* porin regulated by ParS/ParR, which were two component signal transduction system, were not present or pseudogene in the genome of 6 strains used in this study. Therefore, it was unlikely that SNP of *parS* was involved in multidrug resistance of KPA 16010.

In conclusion, we identified two key SNPs in *mexR* and *gyrB* that play a role in altering antimicrobial resistance by analyzing the genomic sequence and efflux pump expression of six *P. aeruginosa* isolates from a single patient. Our findings may give insights into understanding the role of single nucleotide variants in antimicrobial resistance and use of antimicrobial agents.

Conflict of interest

All authors declare that they have no competing interests.

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