Umezawa K, et al., J Emerg Med Trauma Surg Care 2016, 3: 017
DOI: 10.24966/ETS-8798/100017

HSOA Journal of
Emergency Medicine Trauma and Surgical Care

Research Article

Molecular Characteristics of a Series of Clinical Isolates of Drug-Resistant *Acinetobacter baumannii* ST219 Strain: The Implications of a Sequence Analysis of the *bla*$_{\text{OXA-51-like}}$

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Abstract

The drug-resistant *Acinetobacter baumannii* ST219 strain was sporadically isolated in the emergency intensive care unit of Tokai University Hospital in 2014 after an outbreak in 2013. The isolates were identical in their antimicrobial susceptibility pattern, the fingerprint pattern of rep-PCR, and the molecular properties including mutations in drug-resistant genes and decreased expression of the efflux pump and the outer membrane porin genes, but they were found to possess different sequence types based on the findings of multilocus sequence typing: ST208 and 219. The analysis of the *bla*$_{\text{OXA-51-like}}$ sequences showed that all were *bla*$_{\text{OXA-66}}$. Given these findings, all of the isolates were considered to be subclones derived from the same strain. A sequence analysis of the *bla*$_{\text{OXA-51-like}}$ of *A. baumannii* would therefore be useful for investigating the relationship of nosocomial infections.

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Citation: Umezawa K, Asai S, Iwashita H, Minakawa Y, Ohashi M, et al. (2016) Molecular Characteristics of a Series of Clinical Isolates of Drug-Resistant *Acinetobacter baumannii* ST219 Strain: The Implications of a Sequence Analysis of the *bla*$_{\text{OXA-51-like}}$. J Emerg Med Trauma Surg Care 3: 017.

Received: January 28, 2016; **Accepted:** June 17, 2016; **Published:** June 30, 2016

Keywords: *Acinetobacter baumannii*; Drug resistance; Outer membrane porin; OXA-type β lactamase; ST219

Introduction

Acinetobacter baumannii is a strictly aerobic, non-fermenting, non-fastidious, non-motile, catalase-positive, oxidase-negative, gram-negative bacteria that is known to be a major pathogen of nosocomial infection in immunocompromised patients [1]. Outbreaks of antimicrobial-resistant *A. baumannii* have become a major clinical problem [1-3]. All strains of *A. baumannii* possessed a chromosomally encoded *bla*$_{\text{OXA-51-like}}$, some of which provided resistance to carbapenems when the molecular milieu around the gene promoted its expression, such as IS*Aba1* [4]. The *bla*$_{\text{OXA-51-like}}$ has been reported to have sequence variations with over 40 variants [5,6].

We previously reported an outbreak of an amikacin- and ciprofloxacin-resistant *A. baumannii* sequence type (ST)219 strain that affected 15 patients in the emergency intensive care unit of Tokai University Hospital from September to October in 2013 [3]. Intensive control measures were implemented, including the replacement of the water supply system, which was considered to be a bacterial reservoir, and thus could successfully control the outbreak. However, sporadic cases of *A. baumannii,* with an identical pattern of antimicrobial susceptibility, were subsequently detected in 2014. The present study was undertaken to elucidate the molecular characteristics for antimicrobial resistance and typing for epidemiology in drug resistant (DR) *A. baumannii* in 2014.

Materials and Methods

After an outbreak of DR-*A. baumannii* that affected 15 patients from September to October 2013 [3], DR-*A. baumannii* was sporadically detected in sputum specimens from six patients from January to October 2014 (TS-*A. baumannii*-2014-1 to -6). These patients were treated in the emergency intensive care unit (57 beds, including 3 beds in the severe burn care unit) of Tokai University Hospital (total 804 beds) for serious burns, traffic injuries, or cerebral hemorrhaging. Routine microbial examinations were performed on a weekly basis in clinical specimens from the patients' sputum, urine (via catheter), venous blood and wounds, among other bodily fluids. One drug-susceptible *A. baumannii* (TS-*A. baumannii*-2014-7) was used as a control for quantitative RT-PCR (qRT-PCR).

Antimicrobial susceptibility testing, and screening of carbapenemase, MBL, ESBL and AmpC

Bacterial identification and antimicrobial sensitivity tests were performed using the MiroScan WalkAway 96 Plus kit (Beckman Coulter, Inc., CA, USA) in accordance with the CLSI 2010 guidelines [7] for Imipenem (IPM), Meropenem (MEPM), Piperacillin (PIPC), Ceftazidime (CAZ), Cefcapene (CFPM), Sulbactam/Cefoperazone (S/C), Aztreonam (AZT), Cefozopran (CZOP), Gentamicin (GM), Tobramycin (TOB), Amikacin (AMK), Levofloxacin (LVFX), Ciprofloxacin (CPFX), Minocycline (MINO), Fosfomycin (FOM) and Sulfamethoxazole-Trimethoprim (ST). The criteria for multiple drug-resistant *A. baumannii* was resistance to IPM (MIC > 16 μg/mL), AMK

(MIC > 32 μg/mL) and CPFX (MIC > 4 μg/mL), and DR-*A. baumannii* was defined as resistance to at least 2 of the drugs, according to the Japanese National Guideline concerning the prevention of infections and medical care for patients with infections.

A. baumannii isolates were screened for the production of metallo-β-lactamase (MBL) by Double-Disk Synergy Tests (DDST) (Eiken Chemical Co., Ltd. Tokyo, Japan) using extended-spectrum β lactamase (ESBL), in accordance with the CLSI 2010. The isolates were screened for the production of AmpC using 3-aminophenylboronic acid monohydrate (Kanto Chemical Co., Inc., Tokyo, Japan).

Molecular typing

The first detected isolate of *A. baumannii* in each patient was used for the following molecular characterizations. DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany). *A. baumannii* isolates were screened for gene homology by the repetitive-sequence-based-polymerase chain reaction (rep-PCR), as described previously [8]. The BOX-PCR primer was used (5'-CTA CGG CAA GGC GAC GCT GAC G-3'). The temperature profiles were as follows: initial denaturation at 95°C for 3 min, 30 cycles of denaturation at 90°C for 30 s, annealing at 32°C for 1 min, extension at 65°C for 8 min, and a final extension at 65°C for 16 min.

Multilocus sequence typing (MLST) was performed as described previously [9]. The MLST sequences were uploaded into the *A. baumannii* MLST sequence type database (http://pubmlst.org/abaumannii/) to determine the alleles and sequence types (ST). Clonal complexes (CCs) were assigned using the eBURST V3 software program (http://eburst.mlst.net/v3/) and defined as single locus variants (SLVs) and double locus variants (DLVs). The annealing temperature of the PCR amplification used in this study was 55°C for *gltA, gyrB, recA* and *cpn60,* and 50°C for *gdhB, gpi* and *rpoD*. The amplification products were purified with a QIAGEN DNA purification kit (QIAGEN GmbH). The DNA sequencing was performed using an ABI3500xL Genetic Analyzer (Applied Biosystems, Life Technologies Japan Ltd., Tokyo, Japan).

PCR assay for β-lactamase and *armA*

The resistance genes were examined via a multiplex PCR for *bla*$_{OXA-23-like}$, *bla*$_{OXA-24-like}$, *bla*$_{OXA-51-like}$, *bla*$_{OXA-58-like}$, and IS*Aba1* genes, as described previously [2,10-12]. The amplification of the *bla*$_{OXA-51-like}$ genes was performed as described previously [6,11]. The *armA* gene, which encodes 16S rRNA methylases and confers high resistance to aminoglycosides (AGs), was screened by PCR using primers as described previously [13].

Sequencing of OXA-type β-lactamase, and *gyrA*, and *parC*

Sequencing of OXA-type β-lactamase was performed as described previously [14]. The quinolone resistance-determining regions (QRDRs) of *gyrA* and *parC* were amplified and analyzed as described previously [14,15]. The DNA sequencing of the amplified DNA products was performed using an ABI3500xL Genetic Analyzer (Applied Biosystems).

Quantitative RT-PCR (qRT-PCR)

The RNA templates were extracted using the RNeasey® Mini Kit (QIAGEN GmbH). The expression of three different resistance-nodulation-division (RND) family pump-encoding genes (*adeB, adeG, adeJ*) and two outer membrane porin-encoding genes (*oprD* and *carO*) were analyzed by quantitative RT-PCR using the StepOnePlus™ Real-Time PCR System (Applied Biosystems) [2,16-18]. The three previously characterized genes *adeB*, *adeG* and *adeJ* encoded the RND pumps in the *adeABC*, *adeFG* and *adeIJK* operons, respectively. The housekeeping gene 16S rRNA was used as a control [19-21]. Reactions (20 μL) were set up using 400 nM primers and 2 μL of the cDNA template (diluted 1:10) with SYBR® Premix Ex Taq™ II (Tli RNase H Plus) and ROX plus (Takara Bio Inc., Shiga, Japan).

The data were analyzed using the StepOne™ software program. The expression of each target gene was normalized based on the level of the 16S rRNA mRNA gene and was expressed as a relative rate compared to that in the susceptible isolate of each pair. The expression of drug-susceptible *A. baumannii* (TS-*A. baumannii*-2014-7) was set as 1.0 [2]. The experiments were conducted at least three times independently, and all of the reactions were performed in triplicate.

Results

Antimicrobial susceptibility testing, and screening of carbapenemase, MBL, ESBL and AmpC

The antimicrobial susceptibility patterns of 6 isolates of *A. baumannii* in 2014 (TS-*A. baumannii*-2014-1 to -6) was shown in Table 1. They were resistant to PIPC, CAZ, CFPM, S/C, AZT, CZOP, GM, TOB, AMK, LVFX, CPFX, MINO, FOM and ST. They were negative for MBL, ESBL and AmpC on DDSTs.

Molecular typing

The molecular genotyping of isolates by a MLST analysis revealed a sequence type of ST219 for TS-*A. baumannii*-2014-1, -3, -4, -5 and -6 (*gltA, gyrB, gdhB, recA, cpn60, gpi, rpoD*: 1, 3, 3, 2, 2, 101, 3) and ST208 for TS-*A. baumannii*-2014-2 (*gltA, gyrB, gdhB, recA, cpn60, gpi, rpoD*: 1, 3, 3, 2, 2, 97 and 3) (Table 2). The difference in the MLST sequence between ST208 and ST219 was confirmed to be a single *gpi* base by sequencing the PCR product (data not shown). TS-*A. baumannii*-2014-7 showed an unknown sequence type (*gltA, gyrB, gdhB, recA, cpn60, gpi, rpoD*: 15, 48, 58, 42, 36, 54, 41). All of the isolates (TS-*A. baumannii*-2014-1 to -6,) except for TS-*A. baumannii*-2014-7 shared the same fingerprint pattern in the rep-PCR (Figure 1).

Characteristics of the regions involved in antimicrobial resistance

The results of the gene analysis are shown in Table 2. Among the OXA-β-lactamases, *bla*$_{OXA-51-like}$ was detected in all isolates, together with IS*Abe1* in TS-*A. baumannii*-2014-1 to -6. The investigation of the *bla*$_{OXA-51-like}$ sequences resulted that TS-*A. baumannii*-2014-1 to -6 carried *bla*$_{OXA-66}$. They showed the expression of *armA* for AGs resistance. PCR and sequencing of TS-*A. baumannii*-2014-1 to -6 revealed a point mutation of *gyrA* (83-Serine to Leucine) and *parC* (80-Serine to Leucine) for fluoroquinolones (FQs) resistance. The findings for the TS-*A. baumannii*-2014-1 and -2 as the representative strains from each group with the same susceptibility pattern were shown in Table 3. The results of qRT-PCR showed decreased expression of efflux pump (*adeB*, *adeG* and *adeJ*) and outer membrane porin-encoding genes (*oprD* and *carO*).

Discussion

Clinical isolates of an outbreak in 2013 and subsequent sporadic detection in 2014 of DR-*A. baumannii* in the emergency intensive care unit of Tokai University Hospital showed resistance to a broad spectrum of antimicrobials except for carbapenems, and were found to have *bla*$_{OXA-51-like}$ carrying *bla*$_{OXA-66}$ with IS*Aba1,* alterations

Strain	MIC (μg/mL)															
	β-Lactams								AGs			FQs		Other agents		
	IPM	MEPM	PIPC	CAZ	CFPM	S/C	AZT	CZOP	GM	TOB	AMK	LVFX	CPFX	MINO	FOM	ST
TS-*A. baumannii*-2014 -1,-2,-3,-4,-5,-6	≤1(S)	≤1(S)	>64(R)	>16(R)	16(R)	<16(S)	>16(R)	16(R)	>8(R)	>8(R)	>32(R)	>4(R)	>2(R)	4(S)	>16(R)	>2(R)
TS-*A. baumannii*-2014 -7	≤1(S)	≤1(S)	≤8(S)	4(S)	<4(S)	<16(S)	8(S)	8(S)	2(S)	2(S)	8(S)	≤0.5(S)	1(S)	≤2(S)	<16(S)	≤2(S)

Table 1: The antimicrobial susceptibility pattern of the clinical isolates of *Acinetobacter baumannii*.

MIC- minimum inhibitory concentration; AGs- aminoglycosides; FQs- fluoroquinolones; IPM- imipenem; MEPM- meropenem; PIPC- piperacillin; CAZ- ceftazidime; CFPM- cefepime; S/C- sulbactam/cefoperazone; AZT- aztreonam; CZOP- cefozopran; GM- gentamicin; TOB- tobramycin; AMK- amikacin; LVFX- levofloxacin; CPFX- ciprofloxacin; MINO- minocycline; FOM- fosfomycin; ST- sulfamethoxazole/ trimethoprim; R- resistant; S- susceptible.

	TS-*A. baumannii*-2014 -1, -3, -4, -5, -6	TS-*A. baumannii*-2014 -2	TS-*A. baumannii*-2014 -7
Sequence type	219	208	unknown*
bla$_{OXA-23-like}$	-	-	-
bla$_{OXA-24-like}$	-	-	-
bla$_{OXA-58-like}$	-	-	-
bla$_{OXA-51-like}$	+	+	+
bla$_{OXA-66}$	+	+	-
IS*Aba1*	+	+	-
armA	+	+	-
QRDRs			
gyrA	83 Ser→Leu	83 Ser→Leu	83 Ser
parC	80 Ser→Leu	80 Ser→Leu	80 Ser

Table 2: The characteristics of antimicrobial resistance in the clinical isolates of *Acinetobacter baumannii*.

QRDRs- quinolone resistance-determining regions.

* TS-*A. baumannii*-2014-7 showed an unknown sequence type (*gltA, gyrB, gdhB, recA, cpn60, gpi, rpoD*: 15, 48, 58, 42, 36, 54, 41).

	TS-*A. baumannii*-2014-1	TS-*A. baumannii*-2014-2	TS-*A. baumannii*-2014-7
Efflux pump expression			
abeB	0.34	0.49	1.00
abeG	0.17	0.57	1.00
abeJ	0.69	0.55	1.00
Outer membrane porin expression			
oprD	0.17	0.01	1.00
carO	0.02	0.33	1.00

Table 3: Relative expression of efflux pumps and outer membrane porin in clinical isolates *Acinetobacter baumannii* by quantitative RT-PCR.

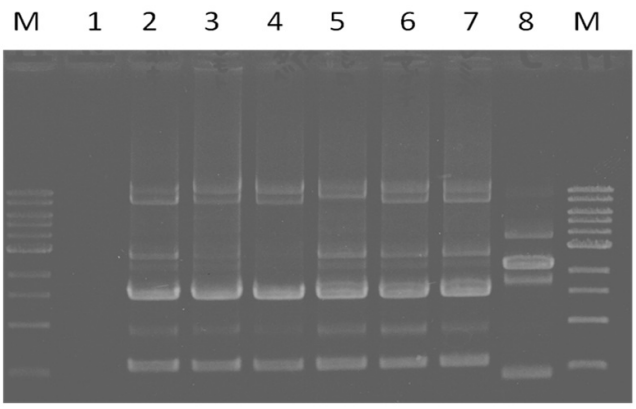

Figure 1: The results of the rep-PCR in clinical isolates of *A. baumannii*.

Lanes 1 was negative control of the PCR. Lanes 2 to 7 indicate drug resistant-*A. baumannii* (TS-*A. baumannii*-2014-1 to -6), and lane 8 is drug susceptible-*A. baumannii* (TS-*A. baumannii*-2014-7), respectively. Lane M is a marker. TS- *A. baumannii*-2014-1 to -6 showed an identical pattern with a homologous rate of identity > 97%. TS-*A. baumannii*-2014-7 had different fingerprint pattern (less than 70% similarity, respectively).

of genes responsible for AGs and FQs, and decreased expression of the efflux pump and outer membrane porin-encoding genes. These findings suggested that these isolates of *A. baumannii* shared a molecular basis for the same susceptibility pattern to antimicrobials. These findings are compatible with collateral susceptibility to carbapenem and consistent with a previous report indicating that the combination of OXA-type β lactamases with IS*Aba1* and deficiency of outer membrane porins deficiency alone does not confer carbapenem resistance, and that overexpression of the efflux pumps may be necessary [2,12,22].

Isolates of TS-*A. baumannii*-2014-1 to -6 showed identical molecular characteristics, such as the fingerprint pattern, OXA type, drug resistant genes and expression of efflux pumps. *A. baumannii* can survive for long-term periods of time in the hospital environment, causing sporadic and endemic infection [2,3,23]. We experienced an outbreak twice in the past in the emergency intensive care unit and burn unit of our University Hospital. DR-*A. baumannii* ST208 was involved in an outbreak in 2011, where the air fluidity bed was identified as a reservoir. An outbreak of DR-*A. baumannii* in 2013 was detected from the water systems including hands-free automatic tap and water

mixture side of the joint tube. Effective measures to minimize the risk in the wet environmental reservoir included strict sanitary management of the water systems in order to prevent future outbreaks. The MLST sequences between ST208 and ST219 differed by only a single *gpi* base, and these isolates closely resembled one another in the molecular characteristics for resistance against each drug. This finding suggested that the ST208 and ST219 isolates were closely related in terms of genetics and might have been derived as subclones from the same origin.

In the detection of subsequent isolates, a reservoir was not identified despite environmental sampling for bacterial culture at several times. A more intensive environmental surveillance will be needed to identify the reservoir should DR-*A. baumannii*-2014 continue to be detected. However, the reinforcement of environmental disinfection, including clinical surfaces, and ensuring hand hygiene with alcohol containing antiseptic will be crucial for reducing the risk of cross-transmission in healthcare facilities [24].

In conclusion, a detailed molecular analysis of DR-*A. baumannii* would provide important knowledge for controlling nosocomial infections. Even when a different ST strain is detected, the sequencing of the *bla*$_{OXA-51-like}$ would be useful for determining the clonality, which would thus make it possible to identify the relationship of *A. baumannii* infection.

Acknowledgments

This work was supported by JSPS KAKENHI Grant Numbers 25670768 and 26460657.

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