Rapid species identification and epidemiological analysis of carbapenem-resistant *Acinetobacter* spp. by a PCR-based open reading frame typing method

Running title: Rapid epidemiological analysis for *Acinetobacter*

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Summary

The spread of carbapenem-resistant *Acinetobacter* spp. has become a global problem. In this study, 18 carbapenem-resistant *Acinetobacter calcoaceticus-baumannii* (ACB) complexes, identified using a conventional biochemical method at our hospital during 2004 – 2013, were studied for species identification and epidemiological analyses. Species identification was performed using matrix-assisted laser desorption ionization – time of flight mass spectrometry (MALDI-TOF MS), a partial sequence analysis of *rpoB* and a PCR-based open reading frame typing (POT) method. The POT method can not only identify the species of ACB complexes but can also simultaneously determine the international epidemic clones and their genetic identities of *A. baumannii* in several hours. Carbapenem resistance gene detection by PCR, molecular epidemiological analysis by pulsed-field gel electrophoresis (PFGE), and Pasteur Institute multi-locus sequence typing (MLST) analysis were performed. All three methods identified 18 isolates as *A. baumannii* (*n*=10), *A. pittii* (*n*=4), and *A. nosocomialis* (*n*=4). A metallo β-lactamase gene in all strains of *A. pittii* and *A. nosocomialis* and an IS*Aba1* gene in the upstream of the $bla_{\text{OXA-51-like}}$ gene in eight strains of *A. baumannii* were detected respectively as carbapenemase-related genes. Results from PFGE demonstrated that nine strains of *A. baumannii* were closely related genetically. Results of MLST analysis showed that *A. baumannii* are classifiable to a sequence type 2. These results were consistent with those obtained using POT method. This POT method can easily and rapidly identify

the international epidemic clones and the identities of *A. baumannii*. It can be a useful tool for infection control.

Key words: carbapenem-resistant *Acinetobacter* spp., matrix-assisted laser desorption ionization – time of flight mass spectrometry, PCR-based open reading frame typing

Introduction

Acinetobacter spp. are important pathogens causing ventilator-associated pneumonias, bloodstream infections, and wound infections in patients with critical illness. Recently, hospital-acquired infection caused by multidrug-resistant *A. baumannii* showing resistance to antibiotic agents such as carbapenems, fluoroquinolones, and aminoglycosides has become a global problem (Peleg *et al*., 2008; Perez *et al*., 2007). Particularly the epidemic-type lineages of *A. baumannii* international clones I and II reportedly show multidrug resistance and cause hospital outbreaks (Diancourt *et al*., 2010). Therefore, it is apparently important to identify the types of the epidemiologic clones of *A. baumannii* in hospital-acquired infection control. The prevalence of carbapenem-resistant *Acinetobacter* spp. among *Acinetobacter* spp. has been reported as lower in Japan (3.6%) (Japan Nosocomial Infections Surveillance, http://www.nih-janis.jp/report/kensa.html), but increasing worldwide, by 34.4% in the USA (Master *et al*., 2013), 90.8% in China (Xu *et al*., 2013), 58.7% in Taiwan (Kou *et al*., 2012), and 41.4% in Korea (Park *et al*., 2012).

The mechanisms of carbapenem resistance of *A. baumannii* have been explained by production of plasmid-encoded carbapenemases such as OXA-carbapenemases and metallo β-lactamases and also by increased production of chromosome-encoded carbapenemases as a result of insertion of IS*Aba1* into the upstream region of a $bla_{\text{OXA-51-like}}$ gene, thereby providing a strong promoter. Aside from carbapenemases, acquired mechanisms such as decreased expression of outer membrane proteins

(OMPs), overexpression of multidrug efflux pumps, and alteration in the affinity of penicillin-binding proteins have been reported (Dijkshoorn *et al*., 2007). The loss of a 29-kDa OMP known as *CarO* is also reportedly associated with carbapenem resistance (Peleg *et al*., 2008). An AdeABC gene, which was chromosomally encoded as a resistance-nodulation-division family type efflux pump, was also reportedly involved in multidrug resistance (Vila *et al*., 2007).

Genotyping methods, such as multi-locus sequence typing (MLST), in order to identify international epidemic clones of the *A. baumannii* are not performed easily in common clinical laboratories. However, the PCR-based open-reading-frame (ORF) typing (POT) method is a molecular typing method using multiplex PCR without performing nucleotide sequencing analyses of multiple genes as with MLST. The Cica Geneus™ Acineto POT KIT (Kanto Chemical Co. Inc., Tokyo, Japan) can identify the species of *Acinetobacter calcoaceticus-baumannii* (ACB) complex. Moreover, it can determine the international epidemic clones and the genetic identities of the species identified as *A. baumannii*. This method is applicable to common clinical laboratories because all the results can be obtained easily and rapidly (Suzuki *et al*., 2014). In the present study, we investigated the strains, antimicrobial susceptibilities, and molecular biological characteristics of a carbapenem-resistant ACB complex, which is identified in a conventional biochemical method. Then we compared them with the results obtained using a Cica Geneus™ Acineto POT KIT.

Methods

Bacterial isolates

This study examined 18 isolates of a carbapenem-resistant ACB complex from the clinical isolates in the period during 2004–2013. Biochemical identification of the isolates was accomplished using a panel (Neg Com 6.12J; Beckman Coulter Inc., CA, USA) and a MicroScan WalkAway-96 *SI* system (Beckman Coulter Inc.). Species identification was confirmed from a partial sequence analysis of *rpoB* gene (La Scola *et al*., 2006). Species identification was confirmed using matrix-assisted laser desorption ionization – time of flight mass spectrometry (MALDI-TOF MS). The sample was applied directly on a target plate of the stainless steel. Then α-cyano-4-hydroxycinnamic acid matrix solution was added. After drying, it was measured using software with a library (MALDI Biotyper 3.1 ver.4.0.0.1 with 5,627 entries; Bruker Daltonics GmbH, Bremen, Germany). The reliability score value recommended by a manufacturer was used for species identification with scores of >2.000.

Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) were determined using a microdilution broth method based on the recommendation by the Clinical and Laboratory Standards Institute (CLSI). Commercially available dry plates for antibiotic susceptibility tests were purchased from Eiken

Chemical Co. Ltd. (Tokyo, Japan). Susceptibilities to the antibiotics were based on the CLSI criteria (CLSI M100-S22). Antimicrobial agents tested in this study were the following: imipenem (IPM), meropenem (MEPM), sulbactam/ampicillin (SBT/ABPC), amikacin (AMK), and ciprofloxacin (CPFX).

DNA amplification analysis

The carbapenem resistance genes and their associated upstream Insertion sequence (IS) elements, including $bla_{\text{IMP-1}}$, $bla_{\text{IPM-2}}$, $bla_{\text{VIM-2}}$, $bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-24-like}}$ and $bla_{\text{OXA-58-like}}$, IS*Aba1* and IS*Aba3*-like, were detected under the same PCR conditions with the same primers described previously (Poire *et al.*, 2006, Shibata *et al.*, 2003, Turton *et al.*, 2006). The insertion of *ISAba1* into the *carO* gene was confirmed as previously described (Lee *et al.*, 2011; Lu *et al.*, 2009).

PCR-based ORF Typing (POT) method

The POT method was performed using a commercially available Cica Geneus™ Acineto POT KIT (Kanto Chemical Co. Inc.) according to the manufacturer's instructions. The several ORFs specific to the certain strains of *Acinetobacter* spp. were detected using multiplex PCR. The distribution patterns of the ORFs were calculated from three categories of the POT codes. The first category of the POT code indicates the species identification such as < 1000 for *A. baumannii*, 1000–1999 for *A. pittii*, 2000–2999 for *A. nosocomialis*, 3000–3999 for *Acinetobacter* species close to *A. nosocomialis*,

and 4000 and more for the other *Acinetobacter* spp. Furthermore, the first category of the POT code denotes the types of international clones, for example, No. 69 for international clone I, No. 122 for international clone II. If the identified species are *A. baumannii* and also if they are obtained from an outbreak, all three categories of POT code will be the same (Suzuki *et al*., 2014).

Pulsed-field gel electrophoresis (PFGE) analysis

Chromosomal DNAs were digested with *Apa*I restriction enzyme (Takara Bio Inc., Tokyo, Japan). DNA fragments were electrophoresed in 1% agarose gel in a 0.5 × Tris-borate-EDTA buffer with GenePath® systems (Bio-Rad Laboratories Inc., CA, USA) at 14 °C and 6 V/cm for 18.5 hr with the initial and final pulse times of 1.0–17.0 s. The banding pattern was interpreted according to the methods described by Tenover *et al*. (1995).

Multi-locus sequence typing (MLST) analysis

MLST analysis was performed according to the protocol of the Institute Pasteur's MLST schemes (http://www.pasteur.fr /recherche/genopole/PF8/mlst/Abaumannii.html). Among the isolates showing the different PFGE pattern, Nos. 9, 10, and 15 were chosen as representatives for MLST analysis.

Result

Species identification

Eighteen isolates of ACB complex were identified as *A. baumannii* (*n*=10), *A. pittii* (*n*=4), and *A. nosocomialis* (*n*=4). All three methods (MALDI-TOF MS, POT method, *rpo*B analysis) yielded the same results. MALDI-TOF MS yielded score values of > 2.000 for all isolates (data not shown).

DNA amplification analysis

Three strains of *A. pittii* and four strains of *A. nosocomialis* were positive for a $bla_{\text{IMP-1}}$ gene. These seven isolates were positive for a $bla_{\text{OXA-58-like}}$ gene and also for an IS*Aba3*-like gene in its upstream region. One isolate of *A. pittii* was positive for a $bla_{\text{VIP-2}}$ gene. Ten isolates of *A. baumannii* were positive only for a $bla_{\text{OXA-51-like}}$ gene. Eight of those strains were positive for an IS*Aba1* gene in their upstream region. The *carO* gene encoding OMPs was positive in all *A. baumannii*. However, a PCR amplicon for the *carO* gene that had approximate size of 1,700 bp, which was larger than the expected size of 750 bp, was obtained from four isolates (Nos. 10, 12, 14, 16) (Table 1).

Antimicrobial susceptibility testing

All isolates were resistant to MEPM. Eight isolates of *A. pittii* and *A. nosocomialis* were resistant to IPM, but eight isolates of *A. baumannii* showed MIC range at 4–8 μg/mL. Six isolates of *A. pittii*

and *A. nosocomialis* were susceptible to SBT/ABPC. Ten isolates of *A. baumannii* were multidrug-resistant strains exhibiting resistance to carbapenems, aminoglycosides, and fluoroquinolones (Table 1).

Molecular epidemiology analysis

A. baumannii were classified into three types (types A, B1, B2) by two major types (A and B) and two subtypes (B1 and B2) using PFGE analysis. Nine isolates of *A. baumannii* (Nos. 10–18) were demonstrated to be close mutually related clones based on the Tenover criteria. The POT method classified *A. baumannii* in three types. This result agreed with those of PFGE analysis.The MLST analysis for each representative isolate (Nos. 9, 10, 15) from three different types (types A, B1, B2) demonstrated that all these isolates belonged to sequence type 2 (ST 2). The POT codes 1 of ten isolates of *A. baumannii* were all No. 122 (Table 2, Fig. 1).

Discussion

In this study, carbapenem-resistant ACB complexes were identified to *A. baumannii*, *A. pittii*, and *A. nosocomialis*. The identification results obtained using three methods (MALDI-TOF MS, POT method, and *rpoB* analysis) were in agreement with each other. Furthermore, *A. baumannii* and non-*A. baumannii* were different in the carrying resistance genes and also in the antimicrobial resistance

profiles. In most hospital laboratories, the *Acinetobacter* spp. identified using conventional biochemical methods are reported as ACB complex. Precise identification such as *A. baumannii*, *A. calcoaceticus*, *A. pittii*, and *A. nosocomialis* is difficult to perform. However, this identification of ACB complex is required because the *Acinetobacter* spp. display different antimicrobial resistance profiles depending on their species. Therefore, molecular biological methods such as *rpoB* sequence analysis are necessary for accurate identification. The consistent ratios of species identification between an MALDI-TOF MS and an *rpoB* analysis were reported by Kishii *et al*. (2014) with 72.4% (89/123 strains) and by Hsueh PR *et al*. (2014) with 86.0% (246/286 strains). In this study, 18 isolates identified using MALDI-TOF MS were completely consistent with those identified using *rpoB* analysis, although the samples were quite few. Nevertheless, it is noteworthy that the MALDI-TOFMS is able to identify the species rapidly and precisely. Moreover, the results provide useful data for choosing antimicrobial therapies and infection control policies.

No plasmid-related OXA β-lactamase was detected in 10 isolates of *A. baumannii*. In addition, IS*Aba1* in the upstream of the OXA-51-like gene was identified in eight strains of *A. baumannii*, but not in two isolates (Nos. 11, 12). We previously reported that resistance to carbapenem in No. 11 strain resulted from increased expressions of a bla_{ADC} gene and an efflux pump system (*adeB*) gene (Yamada *et al.*, 2013) and also reported that resistance to carbapenem in No. 12 was caused by a loss of *carO* OMPs. However, metallo-β-lactamases genes were negative in 10 isolates of *A. baumannii*

188 and were positive in eight strains of non-*A. baumannii*. These results agreed with the report showing
189 that metallo-β-lactamase producing *A. baumannii* was rare (Zarrilli *et al*., 2013) and also with the
190 report that *A. baumannii* and non- *A. baumannii* differed in the carrying resistance genes (Kouyama
191 *et al*., 2012; Matsui *et al*., 2014). These reports and our results underscore the extreme importance of
192 clarifying resistance mechanisms when planning therapeutic strategies and infection control.
193 Antimicrobial susceptibility test results showed that isolates of *A. baumannii* were
194 multidrug-resistant (resistance to carbapenems, fluoroquinolones, and aminoglycosides), and these
195 strains showed lower MIC levels to IPM (4-8 μg/mL). Eight strains of non-*A. baumanni*i showed
196 higher MIC levels to IPM and MEPM, probably caused by MBLs, which had a 100–1000-fold
197 higher carbapenem-hydrolyzing activity than OXA type carbapenemases (Zarrilli *et al*., 2013),
198 although they showed lower MIC levels to SBT/ABPC and CPFX. These results were apparently
199 consistent with those reported by Lee *et al.* (2007).
200 The PFGE analysis classified *A. baumannii* into three types by two major types (types A and B) and
201 two subtypes (types B1 and B2). Nine strains of *A. baumannii* (Nos. 10–18) belonging to subtype B1
202 and B2 were interpreted as "closely related clones" based on criteria presented by Tenover *et al*.
203 (1995). On the other hand, these strains were also classified into two types (122-26-55 and
204 122-26-53) using POT method. Based on the manufacturer's instructions, the different POT codes
205 are interpreted as different clones. However, it was suggested that they might be related clones in

consideration of the result of PFGE analysis and clinical course: they were obtained from the same outbreak. Pasteur's MLST analysis classified three representative strains (Nos. 9, 10, 15) of *A. baumannii* as ST2, belonging to an international clone II. A POT method using all ten strains of *A. baumannii* also demonstrated the result suggesting an international clone II. Because rapid detection of the appearance and international clones of *A. baumannii* of their kind are important in hospital infection controls, an easier POT method was substituted for a more complicated MLST method.

Although we demonstrated the usefulness of POT analysis in epidemiological analysis of carbapenem-resistant *Acinetobacter* spp., the several limitations need to be considered in interpreting our results. First, the number of the samples was quite small (n=18), because we studied the samples from a single institution. These are indeed limitations of this study. Secondly, only three types of the ACB complex isolates were subjected and the other types of *Acinetobacter* spp. were not investigated. Thirdly, all *A. baumannii* strains belonged to the same MLST type ST2. Since it is difficult to increase the number and the kinds of the samples in a single institution, a multicenter study is expected to be necessary to evaluate the usefulness of the POT method.

In conclusion, the species of *Acinetobacter* spp. determined as ACB complex in most clinical laboratories are identifiable precisely using mass spectrometry, *rpoB* analysis, and POT analysis. Results obtained from POT analysis were presented to demonstrate that it can identify an international epidemic clone of *A. baumannii* and their identities easily and rapidly. This method is

224 regarded as a useful tool for infection control measures.

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Table 1. Characterizations of carbapenem-resistant *Acinetobacter* species

No.	MALDI-TOF MS / *rpoB*	POT code	Carbapenemase gene				*carO*	MIC (mg/L)				
			MBL	OXA	IS*Aba1-bla*$_{\text{OXA-51}}$	IS*Aba3-bla*$_{\text{OXA-58}}$		IPM	MEPM	SBT/ABPC	AMK	CPFX
1	*A. pittii*	1078-0-0	IMP-1	OXA-58	NT	+	NT	> 8 (R)	> 8 (R)	8/16 (I)	> 32 (R)	0.06 (S)
2	*A. nosocomialis*	2105-0-0	IMP-1	OXA-58	NT	+	NT	> 8 (R)	> 8 (R)	≤ 2/4 (S)	> 32 (R)	0.5 (S)
3	*A. nosocomialis*	2105-0-0	IMP-1	OXA-58	NT	+	NT	> 8 (R)	> 8 (R)	≤ 2/4 (S)	> 32 (R)	0.25 (S)
4	*A. pittii*	1066-0-0	IMP-1	OXA-58	NT	+	NT	> 8 (R)	> 8 (R)	≤ 2/4 (S)	16 (S)	0.12 (S)
5	*A. pittii*	1066-0-0	VIM-2	-	NT	NT	NT	> 8 (R)	> 8 (R)	4/8 (S)	16 (S)	0.12 (S)
6	*A. nosocomialis*	2105-0-0	IMP-1	OXA-58	NT	+	NT	> 8 (R)	> 8 (R)	4/8 (S)	> 32 (R)	0.12 (S)
7	*A. nosocomialis*	2105-0-0	IMP-1	OXA-58	NT	+	NT	> 8 (R)	> 8 (R)	>8/16 (R)	> 32 (R)	0.12 (S)
8	*A. pittii*	1078-0-0	IMP-1	OXA-58	NT	+	NT	> 8 (R)	> 8 (R)	4/8 (S)	> 32 (R)	0.25 (S)
9	*A. baumannii*	122-26-54	-	OXA-51	+	NT	Intact	8 (I)	> 8 (R)	≤ 2/4 (S)	> 32 (R)	> 2 (R)
10	*A. baumannii*	122-26-55	-	OXA-51	+	NT	Lost	8 (I)	> 8 (R)	>8/16 (R)	> 32 (R)	> 2 (R)
11	*A. baumannii*	122-26-55	-	OXA-51	-	NT	Intact	8 (I)	> 8 (R)	>8/16 (R)	> 32 (R)	> 2 (R)
12	*A. baumannii*	122-26-55	-	OXA-51	-	NT	Lost	8 (I)	> 8 (R)	8/16 (I)	> 32 (R)	> 2 (R)
13	*A. baumannii*	122-26-55	-	OXA-51	+	NT	Intact	8 (I)	> 8 (R)	>8/16 (R)	> 32 (R)	> 2 (R)
14	*A. baumannii*	122-26-55	-	OXA-51	+	NT	Lost	8 (I)	> 8 (R)	8/16 (I)	> 32 (R)	> 2 (R)
15	*A. baumannii*	122-26-53	-	OXA-51	+	NT	Intact	4 (S)	> 8 (R)	>8/16 (R)	> 32 (R)	> 2 (R)
16	*A. baumannii*	122-26-55	-	OXA-51	+	NT	Lost	8 (I)	> 8 (R)	>8/16 (R)	> 32 (R)	> 2 (R)
17	*A. baumannii*	122-26-55	-	OXA-51	+	NT	Intact	> 8 (R)	> 8 (R)	>8/16 (R)	> 32 (R)	> 2 (R)
18	*A. baumannii*	122-26-55	-	OXA-51	+	NT	Intact	8 (I)	> 8 (R)	8/16 (I)	> 32 (R)	> 2 (R)

MALDI-TOF MS, matrix-assisted laser desorption ionization – time of flight mass spectrometry; POT, PCR-based open-reading-frame typing; MBL, metallo-β-lactamase; IPM, imipenem; MEPM, meropenem; SBT/ABPC, sulbactam/ampicillin; AMK, amikacin; CPFX, ciprofloxacin; OXA-51, OXA-51-like; OXA58, OXA-58-like; NT, not tested.

Table 2. Epidemiology analysis of PFGE, POT and MLST in *A. baumannii*

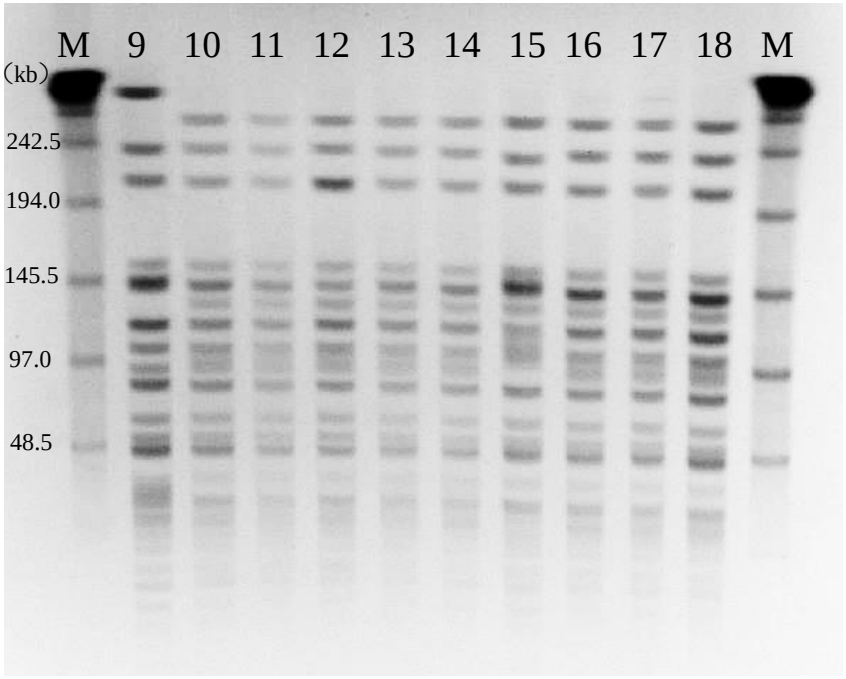
No.	Date of isolation	PFGE	POT code	MLST Pasteur's
9	2010/9	A	122-26-54	ST-2
10	2011/1	B1	122-26-55	ST-2
11	2011/2	B1	122-26-55	NT
12	2012/6	B1	122-26-55	NT
13	2012/10	B1	122-26-55	NT
14	2012/10	B1	122-26-55	NT
15	2012/11	B2	122-26-53	ST2
16	2013/2	B1	122-26-55	NT
17	2013/2	B1	122-26-55	NT
18	2013/3	B1	122-26-55	NT

PFGE, pulsed-field gel electrophoresis; POT, PCR-based open-reading-frame typing; MLST, multi-locus sequence typing.

Figure legend

Fig 1. Pulsed-field gel electrophoresis profile of *Apa* I- digested genomic DNA in *A. baumannii*

M, molecular-weight standard


M
9
10
11
12
13
14
15
16
17
18
M
(kb)
242.5
194.0
145.5
97.0
48.5