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

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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 MS, a partial sequence analysis of *rpoB* and a PCR-based ORF typing (POT) method. The POT method can not only identify the species of ACB complexes but also simultaneously determine the international epidemic clones and the genetic identities of *Acinetobacter baumannii* in several hours. Carbapenem resistance gene detection by PCR, molecular epidemiological analysis by PFGE and Pasteur Institute multilocus sequence typing (MLST) analysis were performed. All three methods identified 18 isolates as *A. baumannii* (*n* = 10), *Acinetobacter pittii* (*n* = 4) and *Acinetobacter nosocomialis* (*n* = 4). A metallo-β-lactamase gene in all strains of *A. pittii* and *A. nosocomialis* and an IS*Ab1* gene in the upstream of the *bla*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 sequence type 2. These results were consistent with those obtained using the 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.

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 *Acinetobacter 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 epidemiological clones of *A. baumannii* in hospital-acquired infection control. The prevalence of carbapenem-resistant *Acinetobacter* spp. amongst *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 (Kuo 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 chromo-
some-encoded carbapenemases as a result of insertion of
\( IS_{Abai} \) into the upstream region of a \( bla_{OXA-51} \)-like gene, thereby providing a strong promoter. Aside from carbape-
menemases, acquired mechanisms such as decreased expres-
sion of outer membrane proteins (OMP s), overexpression of multidrug efflux pumps and alteration in the affinity of
penicillin-binding proteins have been reported (Dijkshoorn
\textit{et al.}, 2007). The loss of a 29 kDa OMP known as \textit{CarO}
is also reportedly associated with carbapenem resistance
(Peleg \textit{et al.}, 2008). An AdeABC gene, which was chromo-
somally encoded as a resistance-nodulation-division
family type efflux pump, was also reportedly involved in
multidrug resistance (Vila \textit{et al.}, 2007).

Genotyping methods, such as multilocus sequence typing
(MLST), in order to identify international epidemic clones
of the \textit{A. baumannii} are not performed easily in common
clinical laboratories. However, the PCR-based ORF typing
(POT) method is a molecular typing method using multi-
plex PCR without performing nucleotide sequencing anal-
yses of multiple genes as with MLST. The Cica Genes(TM)
Acineto POT KIT (Kanto Chemical) can identify the species
of \textit{Acinetobacter calcocalicus-baumannii} (ACB) complex.
Moreover, it can determine the international epidemic
clones and the genetic identities of the species identified as
\textit{A. baumannii}. This method is applicable to common clinical
laboratories because all the results can be obtained easily
and rapidly (Suzuki \textit{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 conven-
tional biochemical method. Then, we compared them with
the results obtained using a Cica Genes(TM) Acineto POT KIT.

\section*{METHODS}

\subsection*{Bacterial isolates}
This study examined 18 isolates of a carbapenem-
resistant ACB complex from the clinical isolates in our hospital during
2004–2013. Biochemical identification of the isolates was accomplished
using a panel (Neg Com 6.12J; Beckman Coulter) and a MicroScan
WalkAway-96 SI system (Beckman Coulter). Species identification was
confirmed from a partial sequence analysis of \textit{rpoB} gene (La Scola
\textit{et al.}, 2006). Species identification was confirmed using matrix-assisted laser
desorption ionization–time-of-flight MS (MALDI-TOF MS). The sample
was applied directly on a stainless steel target plate. Then
\( \alpha \)-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 5627 entries; Bruker Daltonics). The reliability score
value recommended by the manufacturer was used for species identifica-
tion with scores of >2.000.

\subsection*{Antimicrobial susceptibility testing}
The MICs were determined using a microdilution broth method based on the recommendation by the
Clinical and Laboratory Standards Institute (CLSI, 2012). Commer-
cially available dry plates for antibiotic susceptibility tests were pur-
chased from Eiken Chemical. Susceptibilities to the antibiotics were
based on the CLSI criteria (CLSI, 2012). Antimicrobial agents tested in
this study were the following: imipenem (IPM), meropenem (MEPM),
sulbactam/ampicillin (SBT/ABPC), amikacin and ciprofloxacin (CPFX).

\subsection*{DNA amplification analysis}
The carbapenem resistance genes and their associated upstream insertion sequence (IS) elements, including
\( bla_{IMP-1} \), \( bla_{VIM-2} \), \( bla_{OXA-51} \)-like, \( bla_{OXA-23} \)-like, \( bla_{OXA-24} \)-like and
\( bla_{OXA-58} \)-like \( IS_{Abai} \) and \( IS_{Aba3} \)-like, were detected under the same
PCR conditions with the same primers described previously (Poirel
& Nordmann, 2006; Shibata \textit{et al.}, 2003; Turton \textit{et al.}, 2006). The insertion
of \( IS_{Abai} \) into the \textit{carO} gene was confirmed as previously

\subsection*{POT method}
The POT method was performed using a commercially available Cica Genes(TM) Acineto POT KIT (Kanto Chemical) according to
the manufacturer’s instructions. The several ORFs specific to the cer-
tain strains of \textit{Acinetobacter} spp were detected using multiplex PCR.
The distribution patterns of the ORFs were calculated from three catego-
ries of the POT codes. The first category of the POT code indicates the
species identification, such as <1000 for \textit{A. baumannii}, 1000–1999 for
\textit{Acinetobacter pittii}, 2000–2999 for \textit{Acinetobacter nosocomialis}, 3000–3999
for \textit{Acinetobacter} spp close to \textit{A. nosocomialis} and 4000 and more for the other
\textit{Acinetobacter} spp. Furthermore, the first category of the POT code
denotes the types of international clones, for example, 69 for inter-
national clone I and no. 122 for international clone II. If the identified
species is \textit{A. baumannii} and also if they are obtained from an outbreak,
all three categories of POT code will be the same (Suzuki \textit{et al.}, 2014).

\subsection*{PFGE analysis}
Chromosomal DNAs were digested with Apal restriction
enzyme (Takara Bio). DNA fragments were electrophoresed in 1 %
agarose gel in a 0.5× Tris/borate/EDTA buffer with GenePath™ systems
(Bio-Rad Laboratories) at 14 C and 6 V cm⁻¹ for 18.5 h 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 \textit{et al.} (1995).

\subsection*{MLST analysis}
MLST analysis was performed according to the proto-
ocol of the Pasteur Institute’s MLST schemes (http://www.pasteur.fr/
recherche/genre/pol/MLST/Abaumannii.html). Amongst the isolates
showing the different PFGE pattern, nos 9, 10 and 15 were chosen as
representatives for MLST analysis.

\section*{RESULT}

\subsection*{Species identification}
Eighteen isolates of ACB complex were identified as \textit{A. baum-
nannii} \((n=10)\), \textit{A. pittii} \((n=4)\) and \textit{A. nosocomialis} \((n=4)\). All three methods (MALDI-TOF MS, POT method and
\textit{rpoB} analysis) yielded the same results. MALDI-TOF MS
yielded score values of >2,000 for all isolates (data not shown).

\subsection*{DNA amplification analysis}
Three strains of \textit{A. pittii} and four strains of \textit{A. nosocomialis}
were positive for a \( bla_{IMP-1} \) gene. These seven isolates were positive for a \( bla_{OXA-58} \)-like gene and also for an \( IS_{Abai} \)-like gene in its upstream region. One isolate of \textit{A. pittii} was posi-
tive for a \( bla_{VIM-2} \) gene. Ten isolates of \textit{A. baumannii} were
positive only for a \( IS_{Abai} \) gene. Eight of those strains were positive for an \( IS_{Abai} \) gene in their upstream region. The \textit{carO} gene encoding OMPs was positive in all
\textit{A. baumannii}. However, a PCR amplicon for the \textit{carO} gene
that had an approximate size of 1700 bp, which was larger
Ten isolates of Acinetobacter baumannii were classified into three types (types A, B1 and B2) using PFGE analysis. Nine isolates of A. baumannii by two major types (A and B) and two subtypes (B1 and B2) demonstrated that all these isolates belonged to sequence type 2 (ST2). The first POT codes of 10 isolates of A. baumannii were all no. 122 (Table 2, Fig. 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 mg l⁻¹. 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 and 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 into three types. This result agreed with those of PFGE analysis. The MLST analysis for each representative isolate was reported by Kishii et al. (2014) with 86.0% (246/286 strains) and by Hsueh 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-TOF MS is able to identify the species rapidly and precisely. Moreover, the results provide useful data for choosing antimicrobial therapies and infection control policies.

### 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 differed in the resistance genes carried 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, Acinetobacter 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 MALDI-TOF MS and rpoB analysis were reported by Kishii et al. (2014) with 72.4% (89/123 strains) and by Hsueh 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-TOF MS is able to identify the species rapidly and precisely. Moreover, the results provide useful data for choosing antimicrobial therapies and infection control policies.

Table 1. Characteristics of carbapenem-resistant Acinetobacter spp.

<table>
<thead>
<tr>
<th>No.</th>
<th>MALDI-TOF MS/rpoB</th>
<th>POT code</th>
<th>Carbapenemase gene</th>
<th>carO</th>
<th>MIC (mg l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>MBL</td>
<td>OXA</td>
<td>ISAb1-bla₁ₓₓₓ₁,₅₁</td>
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<tr>
<td>1</td>
<td>A. pittii</td>
<td>1078-0-0</td>
<td>IMP-1</td>
<td>OXA-58</td>
<td>NT</td>
</tr>
<tr>
<td>2</td>
<td>A. nosocomialis</td>
<td>2105-0-0</td>
<td>IMP-1</td>
<td>OXA-58</td>
<td>NT</td>
</tr>
<tr>
<td>3</td>
<td>A. nosocomialis</td>
<td>2105-0-0</td>
<td>IMP-1</td>
<td>OXA-58</td>
<td>NT</td>
</tr>
<tr>
<td>4</td>
<td>A. pittii</td>
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<td>IMP-1</td>
<td>OXA-58</td>
<td>NT</td>
</tr>
<tr>
<td>5</td>
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<td>VIM-2</td>
<td>–</td>
<td>NT</td>
</tr>
<tr>
<td>6</td>
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<td>IMP-1</td>
<td>OXA-58</td>
<td>NT</td>
</tr>
<tr>
<td>7</td>
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<td>OXA-58</td>
<td>NT</td>
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<td>8</td>
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<td>NT</td>
</tr>
<tr>
<td>9</td>
<td>A. baumannii</td>
<td>122-26-54</td>
<td>–</td>
<td>OXA-51</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>A. baumannii</td>
<td>122-26-55</td>
<td>–</td>
<td>OXA-51</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>A. baumannii</td>
<td>122-26-55</td>
<td>–</td>
<td>OXA-51</td>
<td>–</td>
</tr>
<tr>
<td>12</td>
<td>A. baumannii</td>
<td>122-26-55</td>
<td>–</td>
<td>OXA-51</td>
<td>–</td>
</tr>
<tr>
<td>13</td>
<td>A. baumannii</td>
<td>122-26-55</td>
<td>–</td>
<td>OXA-51</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>A. baumannii</td>
<td>122-26-55</td>
<td>–</td>
<td>OXA-51</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>A. baumannii</td>
<td>122-26-53</td>
<td>–</td>
<td>OXA-51</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td>A. baumannii</td>
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<td>–</td>
<td>OXA-51</td>
<td>+</td>
</tr>
<tr>
<td>17</td>
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<td>–</td>
<td>OXA-51</td>
<td>+</td>
</tr>
<tr>
<td>18</td>
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<td>122-26-55</td>
<td>–</td>
<td>OXA-51</td>
<td>+</td>
</tr>
</tbody>
</table>

MALDI-TOF MS, matrix-assisted laser desorption ionization–time-of-flight MS; POT, PCR-based ORF 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.
No plasmid-related OXA \( \beta \)-lactamase was detected in 10 isolates of \( A. \) baumannii. In addition, IS\(Abal\) in the upstream of the OXA-51-like gene was identified in eight strains of \( A. \) baumannii, but not in two isolates (nos 11 and 12). We previously reported that resistance to carbapenem in strain no. 11 resulted from increased expression of a \(bla_{ADC}\) gene and an efflux pump system (\(adeB\)) gene (Yamada & Suwabe, 2013) and we also reported that resistance to carbapenem in isolate no. 12 was caused by a loss of \(carO\) OMPs. However, metallo-\( \beta \)-lactamase genes were negative in 10 isolates of \( A. \) baumannii and were positive in eight strains of non-\( A. \) baumannii. These results agreed with the report showing that metallo-\( \beta \)-lactamase-producing \( A. \) baumannii was rare (Zarrilli et al., 2013) and also with the report that \( A. \) baumannii and non-\( A. \) baumannii differed in the resistance genes carried (Kouyama et al., 2012; Matsui et al., 2014). These reports and our results underscore the extreme importance of clarifying resistance mechanisms when planning therapeutic strategies and infection control. Antimicrobial susceptibility test results showed that isolates of \( A. \) baumannii were multidrug resistant (resistance to carbapenems, fluoroquinolones and aminoglycosides), and these strains showed lower MIC levels to IPM (4–8 mg l\(^{-1}\)). Eight strains of non-\( A. \) baumannii showed higher MIC levels to IPM and MEP, probably caused by metallo-\( \beta \)-lactamases, which had a 100–1000-fold higher carbapenem-hydrolysing activity than OXA-type carbapenemases (Zarrilli et al., 2013), although they showed lower MIC levels to SBT/ABPC and CPFX. These results were apparently consistent with those reported by Lee et al. (2007).

The PFGE analysis classified \( A. \) baumannii into three types by two major types (types A and B) and two subtypes (types B1 and B2). Nine strains of \( A. \) baumannii (nos 10–18) belonging to subtypes B1 and B2 were interpreted as ‘closely related clones’ based on criteria presented by Tenover et al. (1995). On the other hand, these strains were also classified into two types (122-26-55 and 122-26-53) using the POT method. Based on the manufacturer’s instructions, the different POT codes 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, and 15) of \( A. \) baumannii as ST2, belonging to an international clone II. A POT method using all 10 strains of \( A. \) baumannii also demonstrated results suggesting an international clone II. Because rapid detection of the appearance and international clones of \( A. \) baumannii of their kind is important in hospital infection control, 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., several limitations need to be considered in interpreting our results. First, the number of samples was quite small (\( n=18\)) because we studied the samples from a single institution. This is indeed a limitation of this study. Second, only three types of the ACB complex isolates were studied, and the other types of \( Acinetobacter\) spp. were not investigated. Third, 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 multicentre study is expected to be necessary to evaluate the usefulness of the POT method.

In conclusion, the *Acinetobacter* spp. determined as ACB complex in most clinical laboratories are identifiable precisely using MS, *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 regarded as a useful tool for infection control measures.

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