Spread of OXA-23-producing Acinetobacter baumannii ST2 and ST246 in a hospital in Japan

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A total of 1085 strains of Acinetobacter sp. obtained from 280 medical institutions in the Chugoku and Shikoku areas of Japan were investigated between 2011 and 2013. Among these strains, 20 (1.84 %) showing meropenem or imipenem resistance with a MIC of >4 µg ml⁻¹ were detected. Of these 20 strains, the blaOXA-23 gene was detected in 17 strains isolated from the same institution. The PFGE patterns of the 17 strains were separated into two clusters, and multi-locus sequence typing showed the sequence types (STs) to be ST2 and ST246. This investigation demonstrated that A. baumannii ST2 of international clone II, which has rarely been isolated in Japan, has not yet spread nationwide.

INTRODUCTION

Recently, cases of multidrug-resistant isolates of Acinetobacter baumannii have been reported (Abbo et al., 2005). Acinetobacter spp., which are a frequent cause of nosocomial infections, have acquired various resistance mechanisms (Bergogne-Bérzin & Towner, 1996). The main resistance mechanism is associated with the production of metallo-β-lactamases belonging to Ambler class B and oxacillinases belonging to Ambler class D (Nordmann & Poirel, 2002; Brown & Amyes, 2006). It has been found that the blaOXA-51-like gene is intrinsic to A. baumannii, and acquired OXA genes are divided into three subgroups, blaOXA-23, blaOXA-40, and blaOXA-58-like genes (Evans & Amyes, 2014). The SENTRY Antimicrobial Surveillance Program, which investigated the situation of OXA-type carbapenemases in the Asia-Pacific region (excluding Japan) between 2006 and 2007, revealed that the blaOXA-23 carbapenemase gene was widely distributed in the Asia-Pacific region. The results of the investigation confirmed that, among 230 strains of carbapenem-non-susceptible Acinetobacter spp. isolated in six countries – China, Hong Kong, India, Korea, Singapore and Thailand – class D carbapenemases were detected in 160.

Of these 160 strains, 156 were A. baumannii, of which 134 were confirmed to have blaOXA-23 carbapenemase genes (Mendes et al., 2009). In contrast, outbreaks of multidrug-resistant A. baumannii in institutions (Yuji et al., 2011; Yoshida et al., 2014) and detection of metallo-β-lactamase-producing A. baumannii (Yokozawa et al., 2012) have been reported in Japan, but strains carrying the blaOXA-23 carbapenemase gene have been distributed only sporadically (Endo et al., 2012; Yasuhara et al., 2013).

In the present study, 1085 strains of Acinetobacter sp. isolated from clinical specimens were collected from 280 medical institutions in Japan, and molecular biological analyses were performed as a large-scale investigation of carbapenem-resistant A. baumannii.

METHODS

Bacterial isolates. We isolated one to 67 strains (median=2, interquartile range=3) of Acinetobacter sp. from the clinical specimens provided by 280 medical institutions within the Chugoku/Shikoku area in Japan between 2011 and 2013 (total of 1085 strains), and strains showing a meropenem or imipenem MIC of >4 µg ml⁻¹ were included for further analysis.

Identification and susceptibility testing. Identification and drug susceptibility testing of the 1085 strains were performed using the

Abbreviations: ADC, Acinetobacter-derived cephalosporinase; IEF, isoelectric focusing; MLST, multi-locus sequence typing
Microscan Neg Comb 6.23\textsuperscript{a} panel (Siemens Healthcare Diagnostics Japan) and they were analysed with a Microscan WalkAway 96\textsuperscript{b} system (Siemens Healthcare Diagnostics Japan). The Microscan WalkAway 96\textsuperscript{b} system was used for measurement, and Clinical and Laboratory Standards Institute criteria (CLSI, 2009\textsuperscript{a}) were used for determining susceptibility. In the carbapenem-resistant strains, the 16S–23S rRNA gene intergenic spacer was sequenced to identify the genomic species (Chang et al., 2005), and MICS of various antimicrobial agents were determined by DP35 panel (Eiken Co.) using the CLSI standard broth microdilution method (CLSI, 2009\textsuperscript{b}).

**Phenotypical analysis of \(\beta\)-lactamase.** A sodium mercuratoacetic acid inhibition test using disks of ceftazidime and meropenem (Arakawa et al., 2000) and a three-dimensional test using disks of meropenem and imipenem (Coudron et al., 2000) were performed to confirm carbapenemase production. A double-disk synergy test was also performed (Yagi et al., 2005) to confirm the production of AmpC \(\beta\)-lactamase, in which 20\textsuperscript{b} \(\mu\)l 3-aminocephalosphoronic acid at 30\textsuperscript{mg} ml\textsuperscript{\textminus}1 was added to a blank disk and set in the centre of Mueller–Hinton agar; the ceftazidime and meropenem disks were then placed close to the disk containing 3-aminocephalosphoronic acid.

**Genotypic analysis of \(\beta\)-lactamase genes and sequencing of the \(\textit{bla}_{\text{OXA-23}}\) and \(\textit{bla}_{\text{OXA-51}}\) genes.** The phylogenetic group of \(\textit{bla}_{\text{OXA-51}}, \textit{bla}_{\text{OXA-58}}, \textit{bla}_{\text{OXA-23}}\) and \(\textit{bla}_{\text{OXA-40}}\) was detected by multiplex PCR according to the method of Queenan and Bush (2007). Determination of the presence of the ISA\textit{ba1} insertion sequence upstream of the \(\textit{bla}_{\text{OXA-23}}\) and \(\textit{bla}_{\text{OXA-51}}\) genes was performed by PCR in combination with an amplifier primer as described in the method of Turton et al. (2006). Direct reaction sequencing of the PCR product was done using a BigDye Terminator v.3.1 Cycle Sequencing kit (Thermo Fisher Scientific) and it was analysed with an Applied Biosystems 3730xl DNA Analyser (Thermo Fisher Scientific).

**PFGE analysis.** PFGE was performed based on the method of Seifert et al. (2005). The restriction enzyme was \(ApaI\), and the device used was a CHEF-DR II system (Bio-Rad Laboratories). The reaction conditions were as follows: running parameters of 6.0\textsuperscript{V} cm\textsuperscript{\textminus}1, 5.3\textsuperscript{s} initial switching time, 34.9\textsuperscript{s} final switching time and 19.5\textsuperscript{h} total running time at 14\textdegree C. Dendrograms were calculated using Fingerprinting Plus v.1.12 (Bio-Rad Laboratories) with Dice coefficients and the unweighted pair group method using arithmetic averages. Multilocus sequence typing (MLST) analysis. The sequences of seven housekeeping genes (\(\textit{cpxD}, \textit{fusA}, \textit{gltA}, \textit{pyrG}, \textit{recA}, \textit{rplB}\) and \(\textit{rpoB}\)) were determined by MLST, which was performed according to the method developed at the Pasteur Institute (http://www.pasteur.fr/recherche/genopole/PF8/mlst/).

**Isoelectric focusing (IEF).** IEF was conducted according to the method of Komatsu et al. (2001). A polyacrylamide gel containing ampholyte (Bio-Lyte 3/10; Bio-Rad Laboratories) was prepared. Crude enzyme was extracted by freezing and thawing the culture medium, and the adjusted total protein concentration of 1\textsuperscript{mg} ml\textsuperscript{\textminus}1 was analysed by IEF using a model 111 Mini IEF Cell (Bio-Rad Laboratories). \(\beta\)-Lactamase activity was detected by overlaying the gel with a filter paper containing nitrocefin at 0.5\textsuperscript{mg} ml\textsuperscript{\textminus}1.

**RESULTS**

**Isolation frequency of carbapenem-resistant \textit{A. baumannii} and MICs**

A total of 1085 strains of Acinetobacter sp. were isolated from various clinical specimens obtained from 280 medical institutions between 2011 and 2013. Among these strains, 20 (1.84\%) that showed meropenem or imipenem resistance, as indicated by a MIC of >4\textsuperscript{mg} ml\textsuperscript{\textminus}1, was detected (Table 1). Of these 20 strains, 18 were isolated in the same institution, and the MICS of the 20 isolates showed that seven were susceptible to and 13 were resistant to ceftazidime. All 20 of these carbapenem-resistant strains were identified as \textit{A. baumannii}.

**Phenotypic analysis of \(\beta\)-lactamase**

The sodium mercuratoacetic acid inhibition test was negative in all 20 strains, and the three-dimensional test was positive in 19 strains. Only 13 strains showing resistance to ceftazidime were positive in the 3-aminocephalosphoronic acid inhibition test, and strains showing the 3-aminocephalosphoronic acid inhibitory reaction did not grow on the meropenem disk (Table 1).

**Genotyping of OXA-type \(\beta\)-lactamase and sequencing of the \(\textit{bla}_{\text{OXA-23}}\) and \(\textit{bla}_{\text{OXA-51}}\) genes**

When classified by OXA-type carbapenemase-producing genetic type, all 20 strains were confirmed to carry the \(\textit{bla}_{\text{OXA-51}}\)-like gene, and 17 strains carrying the \(\textit{bla}_{\text{OXA-23}}\) gene were also detected (Table 1). The sequence of the \(\textit{bla}_{\text{OXA-23}}\)-like gene completely matched that of GenBank accession no. AJ132105.1. In addition, in all strains, ISA\textit{ba1} was detected upstream of the \(\textit{bla}_{\text{OXA-23}}\) gene. The remaining three strains that did not carry the \(\textit{bla}_{\text{OXA-23}}\) gene were confirmed to have ISA\textit{ba1} upstream of the \(\textit{bla}_{\text{OXA-51}}\)-like gene. However, the 17 strains that carried the \(\textit{bla}_{\text{OXA-23}}\) were confirmed not to have ISA\textit{ba1} upstream of the \(\textit{bla}_{\text{OXA-51}}\)-like gene.

**PFGE analysis**

PFGE patterns and dendrograms of the 20 strains are shown in Fig. 1. Based on the dendrogram analysis (Fig. 1), the 20 strains were divided into two clusters. The 17 \(\textit{bla}_{\text{OXA-23}}\) gene-positive strains were divided into two clusters (PFGE cluster A and cluster B). Cluster A was divided into a group of 10 strains carrying the \(\textit{bla}_{\text{OXA-23}}\) gene and a group of three strains carrying the \(\textit{bla}_{\text{OXA-51}}\)-like gene and not the \(\textit{bla}_{\text{OXA-23}}\) gene. Members of cluster B carried the \(\textit{bla}_{\text{OXA-23}}\) gene and were susceptible to ceftazidime.

**MLST analysis**

All strains in the group classified as cluster A by PFGE were sequence type 2 (ST2) (allelic profile 2/2/2/2/2/2/2), and all strains in the group of cluster B were ST246 (allelic profile 1/49/3/4/5/2/36).

**IEF**

A band at isoelectric point (pl) >9.0, suggesting \textit{Acinetobacter}-derived cephalosporinase (ADC) \(\beta\)-lactamase, with
Table 1. PFGE patterns and analysis of the various β-lactamases and MICs

<table>
<thead>
<tr>
<th>Hospital no.</th>
<th>Isolate no.</th>
<th>β-lactamase gene</th>
<th>APB-DDST*</th>
<th>3D with IPM, MEPM disks†</th>
<th>MIC (µg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>PFGE type</td>
<td>CAZ</td>
<td>MEPM</td>
</tr>
<tr>
<td>I</td>
<td>2</td>
<td><em>blaOXA-23</em> and <em>blaOXA-51-like</em></td>
<td>A</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>I</td>
<td>26</td>
<td><em>blaOXA-23</em> and <em>blaOXA-51-like</em></td>
<td>A</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>I</td>
<td>43</td>
<td><em>blaOXA-23</em> and <em>blaOXA-51-like</em></td>
<td>A</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>I</td>
<td>56</td>
<td><em>blaOXA-23</em> and <em>blaOXA-51-like</em></td>
<td>A</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>I</td>
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<td>A</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
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<td>A</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
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<td>107</td>
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<td>A</td>
<td>+</td>
<td>–</td>
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<td>A</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>I</td>
<td>151</td>
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<td>+</td>
<td>–</td>
</tr>
<tr>
<td>I</td>
<td>202</td>
<td><em>blaOXA-23</em> and <em>blaOXA-51-like</em></td>
<td>A</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>I</td>
<td>86</td>
<td><em>blaOXA-23</em> and <em>blaOXA-51-like</em></td>
<td>B</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>I</td>
<td>87</td>
<td><em>blaOXA-23</em> and <em>blaOXA-51-like</em></td>
<td>B</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>I</td>
<td>153</td>
<td><em>blaOXA-23</em> and <em>blaOXA-51-like</em></td>
<td>B</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>I</td>
<td>155</td>
<td><em>blaOXA-23</em> and <em>blaOXA-51-like</em></td>
<td>B</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>I</td>
<td>171</td>
<td><em>blaOXA-23</em> and <em>blaOXA-51-like</em></td>
<td>B</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>I</td>
<td>183</td>
<td><em>blaOXA-23</em> and <em>blaOXA-51-like</em></td>
<td>B</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>I</td>
<td>201</td>
<td><em>blaOXA-23</em> and <em>blaOXA-51-like</em></td>
<td>B</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>II</td>
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<td><em>blaOXA-51-like</em></td>
<td>A</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>I</td>
<td>96</td>
<td><em>blaOXA-51-like</em></td>
<td>A</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>III</td>
<td>194</td>
<td><em>blaOXA-51-like</em></td>
<td>A</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

CAZ, ceftazidime; MEPM, meropenem; TAZ/P/IP, tazobactam/piperacillin; S/C, sulbactam/cefoperazone; CFPM, cefepime; IPM, imipenem; AZT, aztreonam; GM, gentamicin; AMK, amikacin; MINO, minocycline; LVFX, levofloxacin; CL, colistin; ST, trimethoprim/sulphamethoxazole.

*Double-disk synergy test (DDST) using a disk supplemented with 3-aminophenylboronic acid (APB).
†Sodium mercaptoacetic acid (SMA) inhibition test using a disk of SMA (Eiken Chemical Co.).
‡Three-dimensional (3D) test.
strong enzyme activity was observed in PFGE cluster A, and a band at pI > 9.0 with weak enzyme activity was observed in ceftazidime-susceptible cluster B. A band at pI 5.4 (TEM-1) was observed in cluster A (isolates 26 and 96), and cluster A was positive for the \textit{bla}_{TEM-1} gene.

**DISCUSSION**

Outbreaks of A. \textit{baumannii} carrying the \textit{bla}_{OXA-23} gene have been reported worldwide (Dalla-Costa \textit{et al.}, 2003; Naas \textit{et al.}, 2005; Kohlenberg \textit{et al.}, 2009). However, the epidemiological background of OXA-type carbapenemase-producing A. \textit{baumannii} has not yet been clarified in Japan. In the present study, 1085 clinical strains of \textit{Acinetobacter} sp. obtained from 280 medical institutions in Japan were investigated, from which the \textit{bla}_{OXA-23} carbapenemase gene was isolated in only one institution. Based on MLST, there were two STs, ST2 and ST246.

Jeon \textit{et al.} (2005) investigated the status of \textit{bla}_{OXA-23} gene detection in Asia and reported the detection of a strain carrying the \textit{bla}_{OXA-23} gene in Korea for the first time in Asia in 2005. They detected 52 (26.9%) imipenem-resistant strains from 193 \textit{A. baumannii} clinical isolates. Among these 52 strains, the \textit{bla}_{OXA-23} gene was detected in 36 (69.2%), and these strains were all similar in PFGE pattern. The SENTRY Program (Gur \textit{et al.}, 2008) conducted in Turkey in 2006 demonstrated that, among 75 \textit{A. baumannii} strains isolated in that country, 44 (58.6%) exhibited resistance to imipenem, meropenem and ceftazidime. Of the 44 strains, the \textit{bla}_{OXA-23}-like gene was detected in 26 (59.1%), and their PFGEs had identical or similar patterns. Compared with the reports described above, in this study, carbapenem-resistant strains were detected in only three of the 280 institutions. In addition, this study revealed that most of the carbapenem-resistant genes were the \textit{bla}_{OXA-23} gene, whose prevalence was extremely low, at 1.57%. In the present survey, the frequency of appearance of carbapenem-resistant \textit{Acinetobacter} sp. was estimated using clinical isolates obtained in a hospital without a bacterial laboratory, and the frequency could be underestimated in comparison with that in reports from large academic institutions.

Strains not carrying the \textit{bla}_{OXA-23} gene had IS\textit{Aba1} upstream of the \textit{bla}_{OXA-51} gene, whereas the strains that did carry the \textit{bla}_{OXA-23} gene did not have IS\textit{Aba1} upstream of the \textit{bla}_{OXA-51} gene. In addition, regarding carbapenems, the imipenem MICs of the three strains not carrying the \textit{bla}_{OXA-23} gene were one to two dilutions lower than those of the strains carrying the \textit{bla}_{OXA-23} gene. ST246 was reported as a new ST of fluoroquinolone-resistant \textit{A. baumannii} by Higuchi \textit{et al.} (2014). The ST246 isolated in this study was susceptible to levofloxacin (MIC of 2 or \textless; 1 \mu g ml\textsuperscript{-1}). ST246 is reportedly still rare, but attention should be paid to its future trend. The prevalence of different
bacterial clones carrying identical genes in a single institution indicates the possibility that, in *A. baumannii* carrying the blaOXA-23 gene, for which MLST has indicated outbreaks of ST2 in many countries, the plasmid was transmitted to ST246, which was concurrently detected.

On the basis of the IEF analysis of β-lactamase activity, Jeon *et al.* (2005) reported that all 36 *A. baumannii* strains carrying the blaOXA-23 gene had an acknowledged band at pl 6.65, and Zhou *et al.* (2007) confirmed a single band at pl 6.64. Naas *et al.* (2005) also confirmed the expression of the blaOXA-23 gene (pl 6.9), the chromosomal class C β-lactamase (pl > 9.0) and blaTEM-1 (pl 5.4). The results of our IEF analysis revealed that the strains carrying the blaOXA-23 gene had bands exhibiting strong enzyme activity at pl > 9.0, suggesting ADC β-lactamase, but no band equivalent to that of the blaOXA-23 gene was detected. Cefazidime resistance was presumed to be due to the enzyme activity of ADC β-lactamase, strains of which were positive in the 3-aminophenylboronic acid inhibition test but negative in the same test using the meropenem disk.

Meropenem and imipenem resistance mechanisms were suspected to originate from carbapenemase production because the three-dimensional test using the carbapenem disk was positive. However, the results were inconsistent with those of the IEF analysis, and thus it remains unclear whether the carbapenem-resistance mechanism originated from the blaOXA-23 gene. Yigit *et al.* (2002) reported that the carbapenem-resistance mechanism of *Enterobacter aerogenes* originated from chromosomal AmpC production and decreased expression of the bacterial strain due to the lack of outer-membrane protein. Lee *et al.* (2010) reported that excessive expression of the blaOXA-23 gene and AdeABC efflux pump, located on the chromosomes, were related to the acquisition of carbapenem resistance in *A. baumannii*. We considered the possibility that similar factors are involved in blaOXA-23 production and the carbapenem resistance of the *A. baumannii* carrying the blaOXA-23 gene in this analysis.

Consequently, our study demonstrated that, in comparison with investigations of carbapenem-resistant strains in other Asian countries, the isolation frequency of these strains in Japan is still very low. This report indicates that *A. baumannii* carrying the blaOXA-23 gene undoubtedly represents a new threat to healthcare-associated infection control, and clinical microbiological laboratories should have schemes in place to identify *A. baumannii* carrying the blaOXA-23 gene and pay attention to its future trend.

REFERENCES


