Diversity of carbapenem resistance mechanisms in Acinetobacter baumannii from a Taiwan hospital: spread of plasmid-borne OXA-72 carbapenemase

Po-Liang Lu1,2, Michel Doumith3, David M. Livermore3, Tyen-Po Chen1,2 and Neil Woodford3*

1Department of Internal Medicine, Kaohsiung Medical University Hospital, Kaohsiung City, Taiwan; 2Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung City, Taiwan; 3Antibiotic Resistance Monitoring and Reference Laboratory, Centre for Infections, Health Protection Agency, London, UK

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Objectives: We investigated the molecular epidemiology of carbapenem-resistant Acinetobacter baumannii from a Taiwanese hospital and determined the mechanisms responsible for resistance.

Methods: Ninety-two consecutive meropenem-resistant A. baumannii isolates collected between January 2005 and June 2007 were screened for genes encoding OXA carbapenemases, metallo-β-lactamases and for the carO gene encoding an outer membrane protein. PFGE was used to define clonal relatedness. PCR mapping was used to examine the linkage of insertion sequences and blaOXA genes. Southern hybridization of plasmid extracts and I-CeuI-restricted chromosomal DNA was used to locate blaOXA-24-like genes. Sequences of selected blaOXA-24-like and carO genes were determined and loss of CarO expression was confirmed by SDS–PAGE.

Results: Most (70/92, 76%) isolates belonged to one of three PFGE pulsotypes, indicating clonal spread. Fifty-nine isolates, including the majority of those of pulsotypes I and III, produced OXA-72 carbapenemase. The blaOXA-72 gene was located on a 54 kb plasmid in selected isolates. Thirty-three (36%) isolates, including all 16 of pulsotype II, had ISAb1 preceding the blaOXA-51-like gene, promoting its expression. In addition to OXA-72 carbapenemase, two pulsotype I and three pulsotype III isolates did not produce CarO protein as the carO gene was disrupted by insertion of an ISAb1 element. Two isolates of a minor pulsotype had a blaOXA-58-like gene and a single PFGE-unique isolate had a blaOXA-23-like gene.

Conclusions: Although diverse mechanisms were identified, production of OXA-72 carbapenemase was the most common mechanism of carbapenem resistance in A. baumannii from this Taiwanese hospital. The plasmidic location of the gene had facilitated its spread to multiple strains.

Keywords: OXA, outer membrane proteins, ISAb1

Introduction

Carbapenem-resistant Acinetobacter spp. have been increasingly reported worldwide,1 raising serious concerns about the limited antimicrobial treatment options remaining.2 The most prevalent mechanism of carbapenem resistance is the production of class D (OXA) carbapenemases,2 including both acquired types (OXA-23-, OXA-24- and OXA-58-like) and ISAb1-enhanced expression of the intrinsic chromosomal blaOXA-51-like gene of Acinetobacter baumannii.3 Less common is the production of class B metallo-β-lactamases (MBLs) while, on a few occasions, resistance has been associated with altered penicillin-binding proteins,4 loss of outer membrane proteins (OMPs)5,6 and other β-lactamases.7 In particular, loss of CarO OMP, which forms non-specific monomeric porin channels,8 is reported to cause carbapenem resistance in A. baumannii;9 though the prevalence of this mechanism has not been reported; combinations of resistance mechanisms have been observed too,10–13 though, again, the role of CarO loss is unclear in this context.

There are marked geographic differences in the prevalence of these mechanisms of carbapenem resistance, with wide variability also reported in the transmissibility of acquired OXA and
metallo-carbapenemases, and in the clonality of resistant isolates.\textsuperscript{14–19} Better understanding of these aspects is required to optimize both infection control and treatment. Here we studied the molecular epidemiology and mechanisms of carbapenem resistance in \textit{A. baumannii} in a Taiwanese hospital, with emphasis on the role of carbapenemases and the loss of CarO OMP.

\textbf{Materials and methods}

\textbf{Isolates and susceptibility testing}

From January 2005 to June 2007, all \textit{Acinetobacter} spp. isolates with meropenem resistance by the CLSI disc method\textsuperscript{20} were collected from a 500 bed hospital in Taiwan. Identification of \textit{A. baumannii} was confirmed by \textit{recA} amplification and sequencing.\textsuperscript{21} Multiple isolates from a single patient with the same antibiogram and molecular characteristics were counted as one isolate. MICs were determined subsequently by agar dilution methodology following BSAC guidelines.\textsuperscript{22}

\textbf{PCR amplification and sequencing}

Multiplex PCRs were used to detect genes encoding OXA and metallo-carbapenemases.\textsuperscript{23,24} ISAba\textsubscript{1}, which can act as a promoter for \textit{bla\textsubscript{OXA-51-like}}, \textit{bla\textsubscript{OXA-23-like}} and \textit{ampC} genes, was sought with primers ISAba\textsubscript{1}F and ISAba\textsubscript{1}R.\textsuperscript{25} Its linkage upstream of the \textit{bla\textsubscript{OXA-51-like}} gene was sought with primers ISAba\textsubscript{1}F and OXA-51-likeR.\textsuperscript{3} PCR and sequencing of the \textit{carO} gene used primers 5\textsuperscript{'-CAT ATG AAA GTA TTA GTG TTA GTG-3'} and 5\textsuperscript{'-GTT ACC TTA CCA GTA GAA GTT TAC ACC-3'};\textsuperscript{3,9} the \textit{carO} promoter region was amplified with primers 5\textsuperscript{'-AAC GTG TTA CGT TTA ATT GAG GTA TAA GAA GTT TAC ACC-3'} and 5\textsuperscript{'-TTT TCT CCT TCT TAA GAA AAG GCT CTG-3'}. The PCR conditions for \textit{carO} amplification were: initial denaturation at 94°C for 1 min; 10 cycles of denaturation at 94°C for 20 s, primer annealing at 52°C for 10 s and primer extension at 68°C for 150 s; and 25 cycles of denaturation at 94°C for 20 s, primer annealing at 58°C for 10 s and primer extension at 68°C for 150 s. \textit{ampC} was sought with primers AC15 and AC16,\textsuperscript{15} while primers preABprom+ and preAB26\textsuperscript{26} were used to seek any upstream copy of ISAba\textsubscript{1}. Whether known \textit{Acinetobacter} IS elements immediately preceded \textit{bla\textsubscript{OXA-24-like}} and \textit{bla\textsubscript{OXA-23-like}} genes was sought using an IS-located forward primer—one of ISAba1B, ISAba2A, ISAba3C (\textit{mpA} of \textit{ ISAba3-like} and \textit{ ISAba3}), ISAba\textsubscript{1B} or ISAba\textsubscript{1A}—and a reverse primer located in the \textit{bla\textsubscript{OXA}} gene.\textsuperscript{25} Further characterization of the genetic structure surrounding \textit{bla\textsubscript{OXA-58-like}} genes was undertaken using previously described primers.\textsuperscript{25} Primers P1 and P2 were used to ascertain whether \textit{bla\textsubscript{OXA-24-related}} genes had flanking regions similar to those reported previously.\textsuperscript{10}

Sequencing of selected PCR products was performed with the primers used for amplification. In all cases, products were first purified with the GeneClean\textsuperscript{6} Turbo for PCR Kit (Q-BIOgene, Cambridge, UK). In general, sequencing was performed using dye terminator chemistry on a CEQ8000 Genetic Analyzer (Beckman Coulter, High Wycombe, UK), with sequencing of \textit{bla\textsubscript{OXA-24-like}} PCR products according to previously reported methods.\textsuperscript{29} We also sequenced the \textit{carO} gene when the PCR product was not of the expected size (741 bp).

\textbf{Hybridization of plasmid and genomic DNA}

Plasmid DNA was extracted from clinical isolates as described by Kado and Liu\textsuperscript{30} or with a High Purity Plasmid Miniprep Kit (Marligen Biosciences, Inc., Ijamsville, MD, USA). After electrophoresis, the DNA was transferred to Hybond N+ nylon membranes (Amersham, Little Chalfont, UK).

Genomic DNA was prepared with bacterial cells embedded in low-melting-point gel and treated with lysozyme and protease K\textsuperscript{31,32} and then restricted with I-\textit{CeuI} enzyme, which is a double-strand endonuclease yielding seven fragments that were separated on a CHEF-DR II apparatus (Bio-Rad, Hemel Hempstead, UK)\textsuperscript{31,3} and transferred to nylon membranes.

Digoxigenin-11-dUTP (Roche, Lewes, UK) was used to produce labelled probes by PCR with OXA-24-like primers\textsuperscript{24} and 16S rRNA primers.\textsuperscript{34} Labelling of probes and hybridization to blots of plasmid or I-\textit{CeuI}-digested genomic DNA were performed as previously reported.\textsuperscript{35}

Plasmids were sized by comparison with those of \textit{Escherichia coli} strain V517 (NCTC 50193).

\textbf{OMP analysis}

The OMPs of selected clinical isolates were analysed by SDS-PAGE using 12.5% gels.\textsuperscript{36} \textit{A. baumannii} ATCC 19606 was used as a positive control for CarO expression.

\textbf{PFGE}

DNA fingerprinting was done by PFGE of ApaI-digested genomic DNA\textsuperscript{37} with banding patterns analysed using BioNumerics software (BioNumerics, Sint-Martens-Latem, Belgium). Isolates were considered to be of the same pulstype if there was >85% similarity.

\textbf{Statistical analysis}

Categorical variables were evaluated using the Pearson $\chi^2$ test or Fisher’s exact test where more appropriate. The threshold for a significant difference was designated at $P<0.05$.

\textbf{Results}

\textbf{Characterization of \textit{A. baumannii} isolates}

According to local disc susceptibility testing results, 111 of 396 (28\%) \textit{Acinetobacter} spp. isolated in the hospital during the 30 month study period were resistant to meropenem. After exclusion of those isolates that were duplicates from particular patients, had meropenem MICs <16 mg/L or were \textit{Acinetobacter} genomic species 3, 92 \textit{A. baumannii} isolates were included: 47 from ventilated patients in the respiratory care unit, 29 from intensive care unit patients and 16 from patients on general wards. Fifty-three (57.6\%) isolates caused infection and 39 were considered to be colonization isolates.

Three major PFGE types accounted for 70 (76\%) of the 92 isolates (Figure 1): pulsotype I (23 isolates), II (16 isolates) and III (31 isolates). The remaining 22 isolates belonged to eight pulstypes. The temporal distribution of the three major pulstypes is shown in Figure 2: in the third quarter of 2005 and second quarter of 2006, only pulsotype III was present, whereas in the first quarter of 2006, only pulsotype I was isolated. No particular ward and unit was associated with any specific pulstype. No pulstype correlated significantly with a higher percentage of infections.

The MICs for the 92 \textit{A. baumannii} isolates are shown in Table 1. All were resistant to meropenem and ciprofloxacin.
Eleven (12.0%) retained intermediate susceptibility to imipenem. Otherwise, multiresistance was almost universal: only one (1.1%) isolate was susceptible to piperacillin/tazobactam, and five (5.4%) to gentamicin. Forty-five (48.9%) were susceptible to tigecycline at the BSAC breakpoint (MIC ≤ 1 mg/L), 14.1% were intermediately susceptible and 37% were resistant (Table 1). The percentage non-susceptible to tigecycline differed among the three major pulsotypes (43.5% of pulsotype I, 12.5% of pulsotype II and 74.2% of pulsotype III). Based upon a breakpoint of 4 mg/L, all 92 isolates were susceptible to colistin.

![PFGE typing of A. baumannii isolates. Isolates producing OXA-72 carbapenemase are shown (p) as is the presence (t) or absence (n) of an amplicon with primer pair P1/P2.](image-url)

Figure 1. PFGE typing of A. baumannii isolates. Isolates producing OXA-72 carbapenemase are shown (p) as is the presence (t) or absence (n) of an amplicon with primer pair P1/P2.
Carbapenemase production and influence on carbapenem MICs

All 92 isolates had blaoXA-51-like genes. Eighty-nine (96.7%) had ISAbal, and 33 (35.9%) had ISAbal immediately upstream of blaoXA-51-like. Fifty-nine (64.1%) had an acquired blaoXA-24-like gene, which was confirmed by sequencing from seven isolates (six from three major pulsotypes and one from a minor pulsotype) to encode OXA-72 carbapenemase (GenBank accession no. AY739646); only 29 of these yielded products with primer pair P1/P2, indicating that the flanking sequences differed significantly in the remaining 30 isolates from those published previously.10

The 59 OXA-72 enzyme producers included 17 of 23 pulsotype I isolates, 28 of 31 pulsotype III isolates and 14 of 22 isolates of minor pulsotypes (Table 2); those not giving a P1/P2 product were distributed among multiple pulsotypes (Figure 1). The blaoXA-72 gene was not detected in pulsotype II isolates, all of which had ISAbal before the blaoXA-51-like gene. Only four (6.8%) OXA-72 producers also had ISAbal before blaoXA-51-like genes (Table 2).

Genes encoding two other acquired carbapenemases (blaoXA-58-like and blaoXA-23-like) were detected. Two isolates of the same minor pulsotype (i.e. not belonging to pulsotypes I, II or III) had a blaoXA-58-like gene. These gave positive PCR results using: (i) forward primers ISAbal3C and ISAbal2A27 with a blaoXA-58-like reverse primer and (ii) the blaoXA-58-like forward primer with ISAbal3B, indicating the same genetic structure as found in A. baumannii MAD.27 with ISAbal3-like and ISAbal2 elements upstream of blaoXA-58-like and an ISAbal3 element downstream of it. One further isolate had blaoXA-23-like. None of these three isolates had ISAbal preceding the blaoXA-51-like gene, but ISAbal was located immediately upstream of the blaoXA-23-like gene.

None of the 92 isolates had any of the five MBL genes sought; all had an ampC gene and only one isolate lacked ISAbal before ampC.

The levels of imipenem and meropenem resistance associated with the two predominant resistance mechanisms differed. Production of OXA-72 carbapenemase was more often associated (56 of 59 isolates) with high-level imipenem resistance (MICs ≥ 32 mg/L) than was ISAbal-enhanced expression of a blaoXA-51-like gene (3 of 29 isolates; P < 0.001).

Plasmid location of blaoXA-72

Plasmid DNA from two isolates, skb94 and skb109, was hybridized with a blaoXA-72 probe and revealed blaoXA-72 to be located on a plasmid of ~54 kb. The 54 kb plasmid was found in one pulsotype I isolate, one pulsotype III isolate and in two isolates of minor pulsotypes that were randomly selected for plasmid profile experiments. Attempts to transfer this plasmid by conjugation or

Table 1. In vitro susceptibilities of 92 meropenem-resistant A. baumannii isolates

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC range (mg/L)</th>
<th>Number (%) of isolates*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>16 to &gt;256</td>
<td>0</td>
</tr>
<tr>
<td>Piperacillin/tazobactam</td>
<td>≤1 to &gt;64</td>
<td>1 (1.1)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>4 to &gt;128</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Meropenem</td>
<td>16 to &gt;32</td>
<td>2 (2.1)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>&gt;8</td>
<td>0</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>2 to &gt;32</td>
<td>0</td>
</tr>
<tr>
<td>Amikacin</td>
<td>1 to &gt;64</td>
<td>—</td>
</tr>
<tr>
<td>Sulbactam</td>
<td>≤1 to &gt;32</td>
<td>—</td>
</tr>
<tr>
<td>Colistin</td>
<td>&lt;0.5 to 1</td>
<td>—</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>0.5 to 32</td>
<td>45 (48.9)</td>
</tr>
</tbody>
</table>

Table 2. Carbapenem resistance mechanisms identified in 92 meropenem-resistant A. baumannii

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Total isolates</th>
<th>Pulsotype (number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXA-72 only</td>
<td>50</td>
<td>I 15 II 0 III 22 others 13</td>
</tr>
<tr>
<td>ISAbal preceding blaoXA-51-like only</td>
<td>29</td>
<td>I 5 II 16 III 3 others 5</td>
</tr>
<tr>
<td>OXA-72 + insertion inactivation of carO</td>
<td>5</td>
<td>I 2 II 0 III 3 others 0</td>
</tr>
<tr>
<td>OXA-72 + ISAbal preceding blaoXA-51-like</td>
<td>4</td>
<td>I 0 II 0 III 3 others 1</td>
</tr>
<tr>
<td>OXA-58-like only</td>
<td>2</td>
<td>I 0 II 0 III 2 others 0</td>
</tr>
<tr>
<td>OXA-23-like only</td>
<td>1</td>
<td>I 0 II 0 III 1 others 0</td>
</tr>
<tr>
<td>None of the above</td>
<td>1</td>
<td>I 1 II 0 III 0 others 0</td>
</tr>
</tbody>
</table>
Carbapenem-resistant *A. baumannii* in Taiwan

transformation were unsuccessful. The lack of hybridization of these plasmid preparations with a 16S rRNA probe excluded the possibility that hybridization with the bla*OXA*-72 probe was contingent on contaminating chromosomal DNA in the plasmid extract; moreover, the OXA-24-like probe did not hybridize with any I-CeuI-restricted chromosomal fragment.

**Loss of CarO OMP**

All isolates yielded a carO PCR product, but five gave an ampli-con of 1900 bp rather than the expected size of 750 bp; two of these were of pulstype I and three were of pulstype III; all were OXA-72 carbapenemase producers. In each of these five cases, the carO sequence was identical to GenBank accession no. EF646275, but was disrupted by the insertion of a diver-gently transcribed IS*Abal* element, which was flanked by direct repeats of CAACGTTTTA (positions 282–290 of carO; GenBank accession no. EF646275). This insertion of IS*Abal* formed a premature stop codon at position 315. The outer membrane profile of isolate skb60, taken as a representative, confirmed the absence of a 29 kb band (CarO) when compared with *A. baumannii* ATCC 19606. No mutations were found in the carO promoters for these 5 isolates, or in 10 selected isolates without IS*Abal* in their carO gene. Five OXA-72 producers with disrupted CarO had imipenem MICs of 64–128 mg/L. Ten randomly selected OXA-72 producers with normal CarO had MICs ranging from 32 to >128 mg/L; the only isolate with imipenem MIC >128 mg/L had both OXA-72 and IS*Abal* before the bla*OXA*-51-like gene.

**Discussion**

The OXA-24-like carbapenemases comprise OXA-40 (which is synonymous with OXA-24), OXA-25, OXA-26 and OXA-72 enzymes.38 OXA-72 was first reported from an *Acinetobacter* strain from Thailand in 2004 (GenBank accession no. AY739646), and has since been reported in a single *A. baumannii* isolate found during a surveillance study in mainland China.39 We have identified OXA-72 enzyme as the major mechanism of carbapenem resistance in *A. baumannii* in a hospital in Taiwan. OXA-72 enzyme-producing isolates were more resistant to carbapenems than those with IS*Abal*-enhanced expression of the intrinsic OXA-51-like enzyme. There were three concomitant outbreaks in the same hospital, involving three different *Acinetobacter* clones. The prevalence of OXA-72 enzyme in this hospital is in marked contrast to previous studies in Taiwan, which identified plasmid-borne OXA-58-like carbapenemase,40 IS*Abal*-enhanced expression of intrinsic OXA-51-like carbapenemase,13,41 endemic spread of IMP-1 metallo-carbapenemase,42 and spread of VIM-2, VIM-3, VIM-11 and IMP-8 metallo-carbapenemases43 as carbapenem resistance mechanisms in *Acinetobacter* spp. It also differs from the situation in mainland China, where OXA-23 carbapenemase is prevalent, being detected in 97.7% of 221 imipenem-resistant *Acinetobacter* isolates from one study.39

The bla*OXA*-24-like genes of *A. baumannii* have previously been reported on the chromosome17,33 on plasmids18 or, in some isolates, in both locations.16 In our isolates, bla*OXA*-72 was located on a 54 kb plasmid. In 29 of 59, it was flanked by sequences similar to those previously described10,18 while in other isolates, the negative PCR result with P1/P2 primers suggested different genetic organization(s). Despite this variation, the presence of the bla*OXA*-72 gene in isolates of diverse pulstypes, including major types I and III, indicates that both horizontal transfer and clonal spread played roles in the spread of this resistance mechanism in the hospital.

Multiple copies of IS*Abal* are present in most isolates of *Acinetobacter* spp., but this element has not been found in Enterobacteriaceae or *Pseudomonas aeruginosa*.25 It serves an important role as a ‘mobile promoter’.44 As evidence of this role, we found IS*Abal* immediately upstream of bla*ampC* and bla*OXA*-51-like genes in many of our isolates and, in the single OXA-23 carbapenemase producer, also before the bla*OXA*-23-like gene. It underlines further the role of IS*Abal* as a key genetic element regarding the overall genetic plasticity of *A. baumannii* and its consequences on expression of resistance. As with other IS elements, IS*Abal* can also cause insertional inactivation. Here we showed it to disrupt expression of CarO, an OMP that forms a non-specific monomeric channel that allows influx of carbapenems and is essential for L-ornithine uptake.45 Similar disruption of this pore’s expression, mediated by IS*Abu*125 and IS*Abu*825 elements, has been reported previously as the sole mechanism of carbapenem resistance in *Acinetobacter* isolates.9,17 IS*Abal* insertion in the carO gene was detected in 5.4% of our *A. baumannii* isolates. Moreover, the prevalence of this resistance mechanism was probably underestimated since our PCR-based strategy was only able to identify *carO* genes of abnormal length, not those with promoter mutations or those encoding proteins with structural mutations. Combinations of resistance mechanisms, such as carbapenemases together with decreased porin expression, may act additively to confer high-level carbapenem resistance in *A. baumannii*.11–13,38,46,47 Isolates without CarO protein were reported to have MICs of imipenem of 16 mg/L.9 However, most OXA-72-producing isolates had MICs of imipenem of more than 32 mg/L. The loss of CarO porin in isolates also producing OXA-72 carbapenemase does not produce a higher MIC value.

All of our isolates were susceptible to colistin, whereas non-susceptibility to tigecycline was seen in 51%. In view of this non-susceptibility rate and the low serum concentration attained,48 it is essential to check susceptibility when considering tigecycline for treatment of infections caused by carbapenem-resistant *A. baumannii*.

From the literature, it is clear that different hospitals experience different epidemiologies of carbapenem-resistant *A. baumannii*. The situation described here is unique in being the first where the OXA-72 variant of OXA-40 carbapenemase was the enzyme in outbreak strains and one of the few where plasmid transfer among strains was so important.

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Transparency declarations

N. W. and D. M. L. have received research grants and accepted speaking engagements/conference invitations from various companies. D. M. L. has a diversified share portfolio, including holdings in pharmaceutical companies. Other authors: none to declare.

References