OXA-type beta-lactamases among extended-spectrum cephalosporin-resistant *Pseudomonas aeruginosa* isolates in a university hospital in southern Taiwan

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**Background and Purpose:** Data on the epidemiology of OXA-type extended-spectrum beta (β)-lactamases (ESBLs) are limited due to difficulty of identification by routine microbiology laboratories. We determined the prevalence rate of OXA-type β-lactamases among extended-spectrum cephalosporin (ESC)-non-susceptible *Pseudomonas aeruginosa* isolates at a university hospital in southern Taiwan.

**Methods:** A total of 1294 ESC-non-susceptible *P. aeruginosa* isolates collected between 1989 and 1996 (n = 42) and between December 1999 and December 2002 (n = 1252) were analyzed by polymerase chain reaction assays with primers specific for *bla*OXA genes and isoelectric focusing.

**Results:** Forty five isolates (3.5%) were found to produce an OXA-type β-lactamase. Overall, 2 OXA-type ESBLs, OXA-14 (n = 2) and OXA-17 (n = 35), were detected in 37 (2.9%) isolates, and the OXA-10-type narrow-spectrum β-lactamase was found in 8 (0.6%) isolates. OXA-10 and the 2 OXA-type ESBLs were detected in 6 (14.3%) and 4 (9.5%) of 42 ESC-non-susceptible isolates collected between 1989 and 1996. OXA-10 and OXA-17 were detected in 2 (0.2%) and 33 (2.6%) of 1252 ESC-non-susceptible isolates collected between December 1999 and December 2002.

**Conclusions:** These data indicate that OXA-17 was the most common OXA-type ESBL and that OXA-type β-lactamases have decreased in ESC-non-susceptible *P. aeruginosa* at this hospital in recent years. Pulsed-field gel electrophoresis revealed clonal diversity among the OXA-producing isolates.

**Key words:** beta-Lactamases, cephalosporin resistance, microbial sensitivity tests, polymerase chain reaction, *Pseudomonas aeruginosa*

**Introduction**

*Pseudomonas aeruginosa* is responsible for nosocomial infections and is intrinsically resistant to many antibiotics. *P. aeruginosa* is naturally susceptible to ureido- and carboxypenicillins and some extended-spectrum cephalosporins (ESCs) [1]. ESC resistance in *P. aeruginosa* is often associated with the overproduction of a naturally produced cephalosporinase [2-4]. Extended-spectrum beta (β)-lactamases (ESBLs) that can confer resistance to ESCs are common in *Enterobacteriaceae* and have spread worldwide [5]. Various Ambler’s class A ESBLs, such as TEM-, SHV-, VEB-, and PER-type ESBLs, and class D ESBLs, such as OXA-type ESBLs, have been identified in *P. aeruginosa* [2,5-8]. Among these enzymes in *P. aeruginosa*, OXA-type ESBLs have been encountered most commonly, and class A ESBLs are uncommon [2,4,6-8]. Most OXA-type ESBLs are OXA-2 or OXA-10 derivatives [6]. Unlike class A ESBLs, most OXA-type ESBLs are only weakly inhibited by clavulanic acid and cannot be identified at routine microbiology laboratories due to the lack of standard phenotypic detection methods [5]. Thus, the epidemiology of OXA-type ESBLs is not well known.
In Taiwan, a high proportion of \textit{P. aeruginosa} isolates from patients in intensive care units have been found to be resistant to ceftazidime \cite{9,10}. Moreover, although various \(\beta\)-lactamases of classes A, B, and C that confer resistance to ESCs in Gram-negative bacilli have been identified in Taiwan \cite{9,11}, the prevalence of class D ESBLs has not been reported. Thus, the aim of the present study was to determine the prevalence of OXA-type \(\beta\)-lactamases among ESC-non-susceptible \textit{P. aeruginosa} isolates at a university hospital in Taiwan. This is the first report of the prevalence of OXA-type \(\beta\)-lactamases in \textit{P. aeruginosa} in Taiwan.

\textbf{Methods}

\textbf{Bacterial isolates}

A total of 366 \textit{P. aeruginosa} isolates from blood samples were collected between 1989 and 1996, and a total of 8681 \textit{P. aeruginosa} isolates were isolated from various specimens between December 1999 and December 2002 at the Department of Pathology of National Cheng Kung University Hospital, a 900-bed teaching hospital in southern Taiwan. The disk diffusion susceptibility results of these isolates were analyzed according to the guidelines of the National Committee for Clinical Laboratory Standards \cite{12}. Isolates that demonstrated intermediate susceptibilities or resistance to ceftazidime, cefotaxime, cefoperazone, cefepime, or aztreonam were selected for further investigation.

\textbf{Detection of ESBL genes}

All ESC-non-susceptible isolates were subjected to polymerase chain reaction (PCR) assays to detect \textit{bla}_{OXA} genes. A fresh bacterial colony was suspended in 100 \(\mu\)L of sterile distilled water and boiled at 100°C for 10 min. After centrifugation, the supernatant was removed for PCR. Amplifications were performed with primers OPR1 (5’-GTCTTTTCGAGTGACG CATTAT-3’) and OPR2 (5’-ATTTCCTTAGCGA AC TTAC-3’) for \textit{bla}_{OXA,10}’-related genes and primers OXA-2,3 (5’-GCCAAGGGCACTATAGTTTGT-3’) and OXB-2,3 (5’-GCTTCGAGTGGACTGCGG-3’) for \textit{bla}_{OXA,2}’-related genes under PCR conditions as described previously \cite{8,13}. PCR products were purified with a commercial kit (Roche Molecular Biochemicals, Mannheim, Germany) and both strands of the amplicons were sequenced on an ABI PRISM 310 automated sequencer (PE Applied Biosystems, Foster City, CA, USA). \textit{bla}_{VEB}, \textit{bla}_{TEM}, and previously obtained PCR products of \textit{bla}_{TEM,1}, \textit{bla}_{SHV,1}, \textit{bla}_{CTX,M,9} and \textit{bla}_{CTX,M,14} \cite{11} were used as the templates to synthesize the digoxigenin-labeled probes. Colony hybridization was performed with these probes and was detected with Detection Starter Kit II (Roche Applied Science, Mannheim, Germany) according to the manufacturer’s instructions.

\textbf{Isoelectric focusing analysis}

Crude \(\beta\)-lactamase extracts were prepared using sonication as described previously \cite{15}. Isoelectric focusing was performed by the method of Matthew et al \cite{16} with an LKB Multiphor apparatus on prepared PAGplate gels (pH 3.5 to 9.5; Amersham Pharmacia Biotech, Hong Kong, China) as previously described \cite{11,16}.

\textbf{Susceptibility testing}

Minimal inhibitory concentrations (MICs) of various antimicrobial agents were determined by using E-test strips (AB BIODISK, Solna, Sweden).

\textbf{Pulsed-fielded gel electrophoresis analysis}

Pulsed-fielded gel electrophoresis (PFGE) was performed with a CHEF Mapper apparatus (Bio-Rad Laboratories, Hercules, CA, USA) according to the instruction manual. Chromosomal DNA was digested with \textit{XbaI} (New England Biolabs, Beverly, MA, USA) \cite{17}, and was separated on 1% agarose gels. PFGE patterns were interpreted according to the Tenover’s criteria \cite{17}.

\textbf{Results and Discussion}

Forty two (11.4\%) of the 366 \textit{P. aeruginosa} isolates collected between 1989 and 1996 and 1647 (19.0\%) of the 8681 \textit{P. aeruginosa} isolates isolated between December 1999 and December 2002 demonstrated intermediate susceptibilities or resistance to at least 1 of the 5 ESCs based on the results of disk diffusion tests. Among the 1647 ESC-non-susceptible isolates, 1252 isolates were collected. A total of 1294 ESC-non-susceptible isolates collected during the 2 periods were subjected to PCR and colony hybridization.

All 1294 isolates had negative results on colony hybridization assays with the \textit{bla}_{VEB}, \textit{bla}_{CTX,M}, \textit{bla}_{SHV}, and \textit{bla}_{TEM} probes. In the PCR assays, all 1294 isolates had negative results with the primers for \textit{bla}_{OXA,2}’-related genes, and only 45 (3.5\%) isolates from 21
patients yielded a 720-bp fragment with the primers for blaOXA-10-related genes. The results of colony hybridization were consistent with those of PCR assays for blaOXA genes. Nucleotide sequencing revealed that 8 isolates from 7 patients, 2 isolates from 2 patients, and 35 isolates from 12 patients carried blaOXA-10, blaOXA-14, and blaOXA-17, respectively. OXA-10 is a narrow-spectrum class D β-lactamase [18], and OXA-14 and OXA-17 are two OXA-10-derived class D ESBLs [19,20]. These data suggest that ESC resistance in P. aeruginosa at this university hospital was mostly due to mechanisms other than ESBL production, such as hyperproduction of intrinsic class C cephalosporinas and reduced accumulation of antibiotics [2-4]. In isoelectric focusing analysis, all blaOXA-10-positive and blaOXA-17-positive isolates demonstrated 2 bands at pH values for the isoelectric point (pIs) of 6.1 and 7.9, and both blaOXA-14-positive isolates demonstrated 2 bands at pIs of 6.2 and 7.9. The bands at pI of 7.9 were consistent with the expression of intrinsic class C β-lactamases [18], the bands at pI of 6.1 were consistent with the expression of OXA-10 or OXA-17 [18,19], and the bands at pI of 6.2 were consistent with the expression of OXA-14 [20]. Our results indicate that among the ESC-non-susceptible P. aeruginosa isolates at this university hospital, OXA-17 was the most common OXA-type ESBL, accounting for 35 (94.6%) of the 37 isolates producing an OXA-type ESBL.

Among the 42 ESC-non-susceptible isolates collected between 1989 and 1996, OXA-10 and OXA-type ESBLs were detected in 6 (14.3%) and 4 (9.5%) isolates (2 OXA-14-producing and 2 OXA-17-producing isolates), respectively. Among the 1252 isolates collected between December 1999 and December 2002, OXA-10 and OXA-17 were detected in 2 (0.2%) and 33 (2.6%) isolates, respectively. Thus, 23.8% of the ESC-non-susceptible isolates collected between 1989 and 1996 and 2.8% of the ESC-non-susceptible isolates collected between December 1999 and December 2002 produced an OXA-type β-lactamase. These results suggest that the prevalence rate of OXA-type ESBLs as well as OXA-type narrow-spectrum β-lactamases decreased among ESC-non-susceptible P. aeruginosa isolates at this hospital in recent years. The exact prevalence rate of OXA-type β-lactamases in P. aeruginosa could not be determined because ESC-susceptible isolates, which may not be available for analysis, were not included in our study.

Table 1. Antimicrobial susceptibilities and PFGE patterns of 21 non-repetitive OXA-producing Pseudomonas aeruginosa isolates

<table>
<thead>
<tr>
<th>Isolate/year of collection</th>
<th>Specimen type</th>
<th>pIs of β-lactamases</th>
<th>blaOXA gene</th>
<th>MIC (µg/mL)</th>
<th>PFGE profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-16/1989 Blood</td>
<td>7.9, 6.1</td>
<td>&gt;256 &gt;256 32 2 24 8 1 1.5 &gt;256 &gt;256 0.25</td>
<td>IIIa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>32-52/1991 Blood</td>
<td>7.9, 6.1</td>
<td>&gt;256 &gt;256 24 1.5 24 1 &gt;32 32 &gt;256 &gt;256 0.125</td>
<td>IV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>38-59/1991 Blood</td>
<td>7.9, 6.1</td>
<td>128 192 12 1 12 1 1 1 256 &gt;256 &gt;256 0.125</td>
<td>II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>39-69/1991 Blood</td>
<td>7.9, 6.1</td>
<td>&gt;256 &gt;256 48 2 16 12 1 1 1 &gt;256 &gt;256 0.094</td>
<td>II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>44-67/1992 Blood</td>
<td>7.9, 6.1</td>
<td>&gt;256 128 12 1 12 1 1 0.75 &gt;256 &gt;256 0.19</td>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>195/1996 Blood</td>
<td>7.9, 6.1</td>
<td>&gt;256 &gt;256 12 1 12 2 &gt;32 8 &gt;16 &gt;256 0.032</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>149/2002 Wound</td>
<td>7.9, 6.1</td>
<td>&gt;256 &gt;256 48 2 24 6 2 &gt;32 &gt;32 &gt;256 &gt;256 2</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>176/1996 Blood</td>
<td>7.9, 6.2</td>
<td>128 64 &gt;256 &gt;256 24 6 2 0.5 &gt;256 &gt;256 8</td>
<td>Va</td>
<td></td>
<td></td>
</tr>
<tr>
<td>178/1996 Blood</td>
<td>7.9, 6.2</td>
<td>&gt;256 &gt;256 &gt;256 &gt;256 16 24 8 &gt;2 &gt;0.75 &gt;125 &gt;256 12</td>
<td>Vb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>29-1/1991 Blood</td>
<td>7.9, 6.1</td>
<td>&gt;256 &gt;256 64 4 48 96 2 12 &gt;256 &gt;256 3 0.5</td>
<td>VI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>181/1996 Blood</td>
<td>7.9, 6.1</td>
<td>128 64 128 4 12 16 0.75 4 64 3 4</td>
<td>XII</td>
<td></td>
<td></td>
</tr>
<tr>
<td>611/2000 Wound</td>
<td>7.9, 6.1</td>
<td>&gt;256 &gt;256 64 2 128 12 3 &gt;00 &gt;256 &gt;256 12 0.125</td>
<td>VII</td>
<td></td>
<td></td>
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<tr>
<td>956/2000 Wound</td>
<td>7.9, 6.1</td>
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<td>VIII</td>
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<td></td>
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<tr>
<td>1110/2000 Sputum</td>
<td>7.9, 6.1</td>
<td>&gt;256 &gt;256 &gt;256 &gt;256 &gt;256 16 &gt;32 &gt;32 &gt;256 &gt;256 12 0.5</td>
<td>VIII</td>
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<td></td>
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<tr>
<td>1390/2000 Urine</td>
<td>7.9, 6.1</td>
<td>&gt;256 &gt;256 &gt;256 &gt;256 32 32 32 1.5 6 &gt;256 &gt;256 2</td>
<td>IXa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>103/2002 Urine</td>
<td>7.9, 6.1</td>
<td>&gt;256 &gt;256 &gt;256 &gt;256 128 &gt;256 32 1 1.5 &gt;256 &gt;256 16 &gt;32</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>106/2001 Sputum</td>
<td>7.9, 6.1</td>
<td>&gt;256 &gt;256 &gt;256 &gt;256 48 192 16 &gt;32 &gt;16 &gt;256 &gt;256 0.5</td>
<td>VIII</td>
<td></td>
<td></td>
</tr>
<tr>
<td>195/2001 Sputum</td>
<td>7.9, 6.1</td>
<td>&gt;256 &gt;256 &gt;256 &gt;256 8 64 6 1 1.5 &gt;256 &gt;256 12 &gt;32</td>
<td>XII</td>
<td></td>
<td></td>
</tr>
<tr>
<td>87/2002 Sputum</td>
<td>7.9, 6.1</td>
<td>&gt;256 &gt;256 &gt;256 &gt;256 &gt;256 32 256 15 &gt;32 &gt;32 &gt;256 &gt;256 12 &gt;32</td>
<td>IXb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>312/2002 Urine</td>
<td>7.9, 6.1</td>
<td>&gt;256 192 &gt;256 4 32 16 1 1.5 &gt;256 &gt;256 12 1</td>
<td>XI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>444/2002 CSF</td>
<td>7.9, 6.1</td>
<td>&gt;256 &gt;256 &gt;256 &gt;256 &gt;256 &gt;256 &gt;256 &gt;256 &gt;256 &gt;256 &gt;256 &gt;256</td>
<td>IXc</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: PFGE = pulsed-fielded gel electrophoresis; pIs = pH values for the isoelectric point; MIC = minimal inhibitory concentration; PIP = piperacillin; TZP = piperacillin/tazobactam; CTX = cefotaxime; CAZ = ceftazidime; FEP = cefepime; ATM = aztreonam; IPM = imipenem; MEM = meropenem; GEN = gentamicin; AMK = amikacin; CIP = ciprofloxacin; CSF = cerebrospinal fluid; NS = not successful.
produce narrow-spectrum OXA-type \( \beta \)-lactamases, were not analysed.

One OXA-producing isolate from each patient was selected for further analyses. The results of susceptibility testing are shown in Table 1. All 21 isolates showed high-level resistance to piperacillin and piperacillin/tazobactam and were non-susceptible to cefotaxime (MICs >8 µg/mL) and cefepime (MICs >8 µg/mL). Twelve and 5 of the 14 isolates that produced an OXA-type ESBL showed high-level resistance to cefotaxime (MICs ≥128 µg/mL) and cefepime (MICs ≥128 µg/mL), respectively. The MICs of ceftazidime for the 14 isolates producing an OXA-type ESBL ranged widely, from 2 to >256 µg/mL. All OXA-10-producing and both OXA-14-producing isolates showed high-level resistance to amikacin (>256 µg/mL), while all OXA-17-producing isolates were susceptible to this drug (≤16 µg/mL).

OXA-type \( \beta \)-lactamases have strong activity against oxacillin and cloxacillin and are predominantly penicillinases [18]. The OXA-type ESBLs provide weak resistance to oxyiminocephalosporins when cloned into Escherichia coli [5,19]. The OXA-17 \( \beta \)-lactamase confers resistance to cefotaxime and ceftriaxone but provides only marginal protection against ceftazidime [5,19]. In this study, 5 of the 12 \( \text{bla}^{\text{OXA-17}} \)-positive isolates were susceptible to ceftazidime (MICs ≤8 µg/mL). This result indicates that all ESC-resistant isolates rather than ceftazidime-resistant isolates only should be analyzed to determine the prevalence rate of OXA-type ESBLs in \( \text{P. aeruginosa} \).

Of the 21 non-repetitive OXA-producing isolates, 20 isolates were successfully typed by PFGE, and 13 major patterns were identified. Six typeable OXA-10-producing isolates revealed 4 major patterns (patterns I to IV), among which pattern III had 2 subtypes (Fig. 1A). Two OXA-14-producing isolates differed by two bands and were thus considered to be closely related. Eight major patterns (patterns VI to XIII) were identified among the 12 typeable OXA-17-producing isolates, and 6 of them were represented by a single isolate (Fig. 1B). Three pattern IX isolates were subgrouped into 3 subclones, and pattern VIII was shared by 3 isolates. The data indicate the spread of the OXA-gene among different \( \text{P. aeruginosa} \) clones.

In conclusion, this study indicates that the prevalence rate of OXA-type ESBLs among ESC-non-susceptible \( \text{P. aeruginosa} \) isolates has decreased in recent years at a Taiwanese university hospital. OXA-17 was the most common OXA-type ESBL and its genetic determinant has spread among different clones in \( \text{P. aeruginosa} \).

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