Outbreak of PER-1 and diversity of \( \beta \)-lactamases among ceftazidime-resistant *Pseudomonas aeruginosa* clinical isolates

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A growing number of \( \beta \)-lactamases have been reported in *Pseudomonas aeruginosa* clinical isolates. The aim of this study was to investigate the diversity of \( \beta \)-lactamases in the collection of 51 ceftazidime-resistant *P. aeruginosa* clinical isolates in four hospitals of southern China. Among these isolates, variable degrees of resistance to other \( \beta \)-lactam and non-\( \beta \)-lactam agents were observed. Pulsed-field gel electrophoresis (PFGE) revealed a high degree of clonality with five main genotypes. Of the 51 isolates tested, 35 (68.6 %) were identified as extended-spectrum \( \beta \)-lactamase (ESBL) producers, with 35 producing PER-1, 1 CTX-M-3, 7 CTX-M-15 and 1 CTX-M-14. Most (82.9 %, 29/35) PER-1-producing isolates were collected from two hospitals between January and April in 2008 and belonged to the same PFGE pattern (pattern B) with similar antibiogram and \( \beta \)-lactamase profiles, which suggested an outbreak of this clone at the time. The prevalence of CTX-M-type ESBL (17.6 %, 9/51) was unexpectedly high. One isolate was identified as producing VIM-2. Furthermore, we also reported an occurrence of a novel OXA-10 variant, OXA-246, in 14 *P. aeruginosa* isolates. In addition, AmpC overproduction was found to be the \( \beta \)-lactamase-mediated mechanism responsible for ceftazidime resistance in 6 isolates (11.8 %). Our results revealed an overall diversity of \( \beta \)-lactamases and outbreak of a PER-1-producing clone among ceftazidime-resistant *P. aeruginosa* in southern China.

INTRODUCTION

*Pseudomonas aeruginosa* has the potential to become resistant to any of the antibiotics used to treat Gram-negative nosocomial infections. The development of \( \beta \)-lactam resistance in this opportunistic pathogen can be caused by several mechanisms: a stable overexpression of intrinsic AmpC cephalosporinase, upregulation of efflux systems, acquisition of transferable genes that code for various \( \beta \)-lactamases, reduced permeability, or a combination of these mechanisms (Livermore, 2002). Resistance to expanded-spectrum cephalosporins in *P. aeruginosa* is associated in most cases with the overproduction of AmpC (Lister et al., 2009). However, a growing number of Ambler class A extended-spectrum \( \beta \)-lactamases (ESBLs), class B metallo-\( \beta \)-lactamases (MBLs), and class D extended-spectrum oxacillinases (ES-OXAs) have been reported in *P. aeruginosa* clinical isolates (Weldhagen et al., 2003; Yatsuyanagi et al., 2004; Fournier et al., 2010).

Class A ESBLs reported in *P. aeruginosa* include PER, TEM, SHV, VEB, BEL and GES types and, more recently, CTX-M type (Picão et al., 2009a). PER-1 was first identified in a *P. aeruginosa* clinical isolate recovered from a Turkish patient in France, in 1991 (Nordmann et al., 1993). Later, PER-1 was found in European and Asian countries (Akinci & Vahaboglu, 2010). Although PER-1 was identified in *Acinetobacter* spp. in China, to our knowledge, data on the
Outbreak of PER-1 and diversity of β-lactamases in P. aeruginosa

prevalence of PER-1 in P. aeruginosa in China are limited (Hou et al., 2007). Five types of acquired MBLs of class B β-lactamases have been identified in P. aeruginosa, including IMP, VIM, SPM, GIM and AIM (Gupta, 2008). IMP-1, -4, and -9 and VIM-2 were found in China (Walsh et al., 2005; Dong et al., 2008). As to class C AmpC cephalosporinase, P. aeruginosa isolates with wild-type AmpC produce only low basal levels of the enzyme and are susceptible to the antipseudomonal penicillins, penicillin–inhibitor combinations, cephalosporins and carbapenems. Resistance to ceftazidime mostly results from overexpression of its naturally occurring AmpC-type cephalosporinase in P. aeruginosa (De Champs et al., 2002). In addition, class D OXA-type ESBLs have been found in P. aeruginosa. Several of the OXA-type ESBLs have been derived from OXA-10 (OXA-11, -14, -16, -17 and -28) in P. aeruginosa (De Champs et al., 2002). The relatedness of PFGE patterns was analysed using the Clinical and Laboratory Standards Institute (CLSI, 2012).

Phenotypic detection of β-lactamases. ESBL producers were identified by the combination disk method (Danel et al., 1995). Briefly, disks of ceftazidime, cefotaxime, cefotaxime–clavulanic acid and cefotaxime–clavulanic acid (Oxoid) were placed on MH agar plates at a distance of 30 mm (centre to centre) from each other. ESBL production was inferred if the zones produced by the disks with clavulanate were >5 mm larger than those without inhibitor and combined disk tests were performed on plates containing 250 μg ml⁻¹ cloxacillin (Sigma) for all ESBL screens. MBL production was identified by the imipenem–EDTA disk method. Briefly, two imipenem disks (Oxoid) were placed on an MH agar plate (Oxoid), and 930 μg EDTA (0.5 μl; Sigma) solution was added to one of them. The inhibition zones of the imipenem and imipenem–EDTA disks were compared after 18 h incubation at 37 °C (Pitout et al., 2005). Overproduction of the chromosomal AmpC was evaluated by a disk diffusion test with 30 μg ceftazidime disks on an MH agar plate with or without cloxacillin (Sigma) at 500 μg ml⁻¹ as described before (De Champs et al., 2002).

Genotypic detection of β-lactamase genes by PCR. PCR analyses were performed to identify various β-lactamase genes, including MBL genes (blaTEM, blaTIM, blaSPM, blaGIM, blaSIM) (Ellington et al., 2007) and potential ESBL genes (blaTEM, blaSHV, blaGES, blaCAZ, blaPER, blaCTX-M, 1, 4, 9 and VIM-2) were found in China (Walsh et al., 2005; Dong et al., 2008). As to class C AmpC cephalosporinase, P. aeruginosa isolates with wild-type AmpC produce only low basal levels of the enzyme and are susceptible to the antipseudomonal penicillins, penicillin–inhibitor combinations, cephalosporins and carbapenems. Resistance to ceftazidime mostly results from overexpression of its naturally occurring AmpC-type cephalosporinase in P. aeruginosa (De Champs et al., 2002). In addition, class D OXA-type ESBLs have been found in P. aeruginosa. Several of the OXA-type ESBLs have been derived from OXA-10 (OXA-11, -14, -16, -17 and -28) in P. aeruginosa (De Champs et al., 2002). The relatedness of PFGE patterns was analysed using the Clinical and Laboratory Standards Institute (CLSI, 2012).

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Quantification of the expression of ampC by real-time PCR. The expression of ampC was determined by using real-time reverse transcriptase PCR. An overnight culture was grown until OD600nm 0.5 and placed on ice immediately before extraction. RNA was isolated using Trizol (Invitrogen, Life Technologies) according to the manufacturer’s recommendations and also treated with DNase I (Tiangen). Purified RNA was used for two-step reverse transcription using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) with random primers. The diluted cDNA was then used as the template for real-time PCR using SYBR Green Master Mix (Applied Biosystems). The rpsL gene encoding the ribosomal protein was used as the reference for normalizing the transcriptional levels of target genes (Dumas et al., 2006). The experiment was repeated three times, including RNA extractions and duplicate PCRs. Overexpression of ampC relative to PAO1 (which was assigned a value of 1.0) was considered when transcriptional levels were >10-fold (Xavier et al., 2010).

Cloning experiments. To assess whether the amino acid substitutions observed in the novel OXA-10 variant OXA-246 affected the level of resistance to β-lactams, E. coli clones producing OXA-246 and its putative progenitor OXA-10 were constructed. The genes corresponding to the enzymes were amplified with primers OXA-10-F-BamHI (5’-GGCGGATCCATGAAAAACATTITGGCCGATATG-3’) and OXA-10-R-HindIII (5’-GGCAAGCTTTAGCCACCAATGATG-3’) (underlining indicates the recognition sites for restriction enzymes BamHI and HindIII, respectively). Purified PCR products were ligated with pSTV-28 (Takara Biotechnology) carrying a chloramphenicol resistance marker as the vector and transformed in E. coli DH10B as the recipient. Recombinant plasmids were selected on lysogenic agar plates containing 30 μg ml⁻¹ chloramphenicol. The cloned DNA fragments of recombinant plasmids were sequenced on both strands. Molecular strain typing. Molecular strain types of all P. aeruginosa isolates were evaluated by pulsed-field gel electrophoresis (PFGE) using restriction enzyme XbaI (MBI Fermentas). The DNA fragments were separated on 1.0% agarose gels in 0.5× Tris/borate–EDTA buffer with a CHEF-Mapper XA apparatus (Bio-Rad) for 20 h at 6 V cm⁻¹ and 14 °C. The relatedness of PFGE patterns was analysed using BioNumerics software, version 7.0 (Applied Maths). A dendrogram was generated from the cluster analysis of Dice similarity indices. Isolates were considered to belong to the same PFGE group if their similarity index was ≥80%.

RESULTS

Antimicrobial susceptibility

Two hundred and seventeen P. aeruginosa isolates were investigated in this study. Fifty-one out of the 217 P. aeruginosa isolates (23.5%) displayed ceftazidime resistance. Their antimicrobial susceptibility profiles are summarized in Table 1. Among the 51 isolates, resistance to third or fourth generation cephalosporins ranged from 92.2% to
100%. Of note, 92.2% (47/51) and 90.2% (46/51) of the isolates were resistant to imipenem and meropenem, respectively. In addition, variable degrees of resistance to non-β-lactam agents were observed, including 96.1% (49/51) to gentamicin, 78.4% (40/51) to amikacin and 86.3% (44/51) to ciprofloxacin.

**Table 1. Antimicrobial susceptibility profiles of 51 ceftazidime-resistant P. aeruginosa clinical isolates**

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>No. (%) of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Susceptible</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Cefepime</td>
<td>2 (3.9)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>2 (3.9)</td>
</tr>
<tr>
<td>Amikacin</td>
<td>9 (17.7)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>2 (3.9)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>4 (7.8)</td>
</tr>
<tr>
<td>Meropenem</td>
<td>4 (7.8)</td>
</tr>
</tbody>
</table>

**Table 2. Secondary β-lactamases detected in the 51 collected ceftazidime-resistant P. aeruginosa clinical isolates**

<table>
<thead>
<tr>
<th>Secondary β-lactamase(s)*</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2</td>
</tr>
<tr>
<td>PER-1</td>
<td>35</td>
</tr>
<tr>
<td>CARB</td>
<td>29</td>
</tr>
<tr>
<td>TEM</td>
<td>6</td>
</tr>
<tr>
<td>CTX-M-3</td>
<td>1</td>
</tr>
<tr>
<td>CTX-M-15</td>
<td>7</td>
</tr>
<tr>
<td>CTX-M-14</td>
<td>1</td>
</tr>
<tr>
<td>OXA-10</td>
<td>1</td>
</tr>
<tr>
<td>OXA-246</td>
<td>14</td>
</tr>
<tr>
<td>VIM-2</td>
<td>1</td>
</tr>
<tr>
<td>PER-1, CARB</td>
<td>12</td>
</tr>
<tr>
<td>PER-1, CARB, TEM</td>
<td>2</td>
</tr>
<tr>
<td>PER-1, OXA-246</td>
<td>1</td>
</tr>
<tr>
<td>PER-1, CARB, OXA-246</td>
<td>5</td>
</tr>
<tr>
<td>PER-1, CARB, TEM, OXA-246</td>
<td>2</td>
</tr>
<tr>
<td>PER-1, CARB, CTX-M-15</td>
<td>3</td>
</tr>
<tr>
<td>PER-1, CARB, TEM, CTX-M-15</td>
<td>1</td>
</tr>
<tr>
<td>PER-1, CARB, OXA-246, CTX-M-15</td>
<td>3</td>
</tr>
<tr>
<td>PER-1, CARB, TEM, OXA-246, CTX-M-14</td>
<td>1</td>
</tr>
<tr>
<td>VIM-2, OXA-246</td>
<td>1</td>
</tr>
</tbody>
</table>

*Secondary β-lactamases with an extended spectrum are shown in bold type.

Thirty-seven (72.5%) isolates displayed the ESBL phenotype, and a ESBL genes could be detected by PCR in 35 isolates, including 35 **bla**<sub>PER-1</sub>, 29 **bla**<sub>CARB</sub>, 7 **bla**<sub>CTX-M-15</sub>, 1 **bla**<sub>CTX-M-3</sub> and 1 **bla**<sub>CTX-M-14</sub>. The MBL-encoding gene **bla**<sub>VIM-2</sub> was identified in 1 isolate. Furthermore, 15 isolates possessed **bla**<sub>OXA-1</sub> group. Among them, a novel OXA-1 group gene was identified in 14 out of 15 isolates and designated **bla**<sub>OXA-1</sub> (accession number KF711993), which has 1 amino acid substitution at Lys141Asn compared with OXA-10. The remaining isolate was identified to possess **bla**<sub>OXA-10</sub>. No other β-lactamase genes were detected by PCR in this study. In addition, the phenotype consistent with AmpC overproduction was identified in 8 out of 51 isolates. Among them, overexpression in 6 isolates was verified with high expression of **ampC** by real-time PCR, with the fold changes ranging from 220.3 to 2574.3 compared with PAO1 (Fig. 1). In addition, 1 isolate (DY76) demonstrated overproduction of **ampC** as well as production of an ESBL (CTX-M-3).

**Molecular strain typing**

The PFGE patterns of 51 P. aeruginosa isolates are shown in Fig. 2. Fifty-one isolates clustered into five main PFGE patterns as follows: pattern A (n=2), pattern B (n=31), pattern C (n=3), pattern D (n=2), pattern E (n=4). The remaining 9 isolates showed unique patterns. Thirty-five PER-1-producing isolates mainly belonged to two clusters, including 29 isolates in pattern B and 3 isolates in pattern E. All PER-1-producing isolates were recovered from two hospitals and were clonally related. On the other hand, 15 OXA-producing (1 OXA-10 and 14 OXA-246) isolates mainly belonged to two patterns with 11 isolates in pattern B and 2 isolates in pattern E. Ten out of 14 OXA-246-producing isolates were clonally related and present only in one hospital. In addition, 9 CTX-M-producing isolates

**Fig. 1.** AmpC expression in P. aeruginosa clinical isolates with positive for phenotypic test of AmpC overexpression. Overexpression of **ampC** relative to P. aeruginosa PAO1 (which was assigned a value of 1.0) was defined by expression values >10-fold.
were mainly located in two different patterns, including 5 isolates in pattern B and 2 isolates in pattern E. Six AmpC overproducers belonged to five different genotypes. Cloning and sequencing of OXA10/246  
Several recombinant E. coli DH10B strains were obtained on chloramphenicol-containing lysogenic agar plates after cloning of an 801 bp PCR-amplified product for blaOXA\textsubscript{10} and blaOXA\textsubscript{246}. Two sequence-confirmed recombinants, one with blaOXA\textsubscript{10} and the other with blaOXA\textsubscript{246}, were retained for further experiments as below. Expression of recombinant blaOXA\textsubscript{10} in E. coli DH10B was confirmed by nitrocefin hydrolysis. The β-lactam MICs of E. coli DH10B (pOXA-246) did not differ significantly from those of E. coli DH10B (pOXA-10), with resistance to ampicillin.

**Fig. 2.** Dendrogram based on PFGE profiles of 51 ceftazidime-resistant P. aeruginosa clinical isolates.
DISCUSSION

The prevalence of ceftazidime resistance among *P. aeruginosa* was high (23.5%) in this study. Our results showed that this high rate is mainly due to production of ESBLs as opposed to AmpC overproduction. Interestingly, AmpC overproduction as a single enzymic mechanism for ceftazidime resistance was observed in *E. coli* DH10B (pOXA-246) (0.5 µg ml⁻¹) than in *E. coli* DH10B (pOXA-10) (0.25 µg ml⁻¹), which did not appear significant. These results suggested that the overall kinetic properties of OXA-246 were likely not altered by the substitution compared with its progenitor OXA-10.

In this study, the rate of MBL production in imipenem-resistant isolates, 2.1% (1/47), was lower than previous reports in the same area and other Asian countries (Kouday et al., 2009; Lee et al., 2009; Qu et al., 2009). However, a very high proportion of those isolates showed resistance to imipenem and meropenem. It suggested that the mechanism of MBL might not be the major reason for the resistance of carbapenems. Other mechanisms like loss of OprD or overexpression of efflux pumps may explain the resistance to carbapenems.

We also showed here an unexpectedly high proportion, 17.6% (9/51), producing CTX-M genes (CTX-M-3, CTX-M-15 and CTX-M-14). Although CTX-M-15 and CTX-M-14 were frequently detected in *E. coli* (Bou et al., 2002; Brasme et al., 2007), their identification in *P. aeruginosa* remains rare (al Naiemi et al., 2006; Celenza et al., 2006; Picão et al., 2009b). Previously, the difficulty of detecting CTX-M genes was noted, since many CTX-M variants confer a higher degree of resistance to cefotaxime but not ceftazidime (Rossolini et al., 2008). Since our selection criterion was based on ceftazidime resistance, the rate of CTX-M genes might be underestimated in this study.

This study investigated the prevalence of ES-OXA as well. Although, this survey did not detect any ES-OXA in the ceftazidime-resistant isolates, we found a new variant of OXA-10 β-lactamase, OXA-246, with one non-synonymous mutation at position A397C (Lys141Asn). Amino acid substitution at this position has not been found among the variants of OXA-10, and it is very close to the Y-G-N motif (positions 144 to 146). We cloned the new gene and compared the MIC of OXA-246 with OXA-10 in *E. coli* DH10B. The result for OXA-246 only showed a slight increase in ceftazidime resistance, without resistance for expanded-spectrum cephalosporins or carbapenems. Hence, we suggest that the novel OXA type, OXA-246, should be described as a non-ESBL β-lactamase. There are several derivatives of OXA-10 possessing increased activity to

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>MIC (mg l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> DH10B (pOXA-10)</td>
<td><em>E. coli</em> DH10B (pOXA-246)</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>64</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>256</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>64</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>128</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>0.125</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>0.25</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>0.5</td>
</tr>
<tr>
<td>Imipenem</td>
<td>0.125</td>
</tr>
</tbody>
</table>

This was largely due to the presence of *bla*"PER-1", which was identified in 68.6% (35/51) of the ceftazidime-resistant *P. aeruginosa* isolates and 94.6% (35/37) of the isolates with the ESBL phenotype. Most (82.9%, 29/35) of the PER-1-producing isolates belonged to PFGE pattern B with similar antibiogram and β-lactamase profiles, and were recovered from two hospitals between January and April in 2008. All these together indicated an outbreak of PER-1-producing isolates belonging to a specific clonal group. For years, PER-1 producers have been increasingly reported in Europe and Asian countries (Jeong et al., 2005; Naas et al., 2006, 2007; Yamano et al., 2006), reports on outbreaks are limited, with *P. aeruginosa* in Poland and *Acinetobacter* spp. in Korea (Jeong et al., 2005; Empel et al., 2007). We report here an outbreak of PER-1-producing *P. aeruginosa* in China.

**Table 3. Minimum inhibitory concentrations (MICs) of OXA-246/10-producing *P. aeruginosa* clinical isolates and isogenic clones**
hydrolise expanded-spectrum cephalosporins, but very few OXA-10-like enzymes (OXA-7, -13) possessing a narrow spectrum of hydrolysis have been described before (Scoulia et al., 1995; Mugnier et al., 1998).

In conclusion, we report an outbreak of PER-1 ESBL in southern China with the single clone spread in two hospitals. We also report a novel OXA-type β-lactamase, OXA-246, which possesses a narrow spectrum of hydrolysis of ampicillin, penicillin G, carbenicillin and ticarcillin. The cephalosidime resistance among P. aeruginosa in southern China appears to be mainly caused by the acquisition of ESBLs.

ACKNOWLEDGEMENTS

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