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Prevalence of AmpC over-expression in bloodstream isolates of *Pseudomonas aeruginosa*

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ABSTRACT

This study examined the contribution of AmpC over-expression to β-lactam resistance in clinical isolates of *Pseudomonas aeruginosa* obtained from a hospital in Houston, TX, USA. Seventy-six non-repeat bloodstream isolates obtained during 2003 were screened for ceftazidime resistance in the presence and absence of clavulanic acid 4 mg/L. AmpC was identified by isoelectric focusing (with and without cloxacillin inhibition); stable derepression was ascertained phenotypically by a spectrophotometric assay (with and without preceding induction by imipenem) using nitrocefin as the substrate, and was confirmed subsequently by quantitative RT-PCR of the *ampC* gene. The clonal relatedness of the AmpC-over-expressing isolates was assessed by pulsed-field gel electrophoresis. In addition, the *ampC* and *ampR* gene sequences were determined by PCR and sequencing. For comparison, two standard wild-type strains (PAO1 and ATCC 27853) and three multidrug-susceptible isolates were used as controls. AmpC over-expression was confirmed in 14 ceftazidime-resistant isolates (overall prevalence rate, 18.4%), belonging to seven distinct clones. The most prevalent point mutations in *ampC* were G27D, V205L and G391A. Point mutations in *ampR* were also detected in eight ceftazidime-resistant isolates. AmpC over-expression appears to be a significant mechanism of β-lactam resistance in *P. aeruginosa*. Understanding the prevalence and mechanisms of β-lactam resistance in *P. aeruginosa* may guide the choice of empirical therapy for nosocomial infections in hospitals.

Keywords AmpC, β-lactamase, derepression, over-expression, *Pseudomonas aeruginosa*, resistance

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INTRODUCTION

Pseudomonas aeruginosa is an important pathogen associated with serious nosocomial infections such as pneumonia and sepsis. In 2003, *P. aeruginosa* was the most commonly isolated Gram-negative species causing nosocomial pneumonia, and the third most commonly isolated Gram-negative species causing nosocomial urinary tract infection in the USA [1]. Bloodstream infections caused by *P. aeruginosa* have been associated with mortality rates of 18–62% [2]. Furthermore, *P. aeruginosa* is also associated with multiple mechanisms of antibiotic resistance, including β-lactamase production, porin channel deletion, efflux pump over-expression, multi-functional group transferases, and target site mutations. [3].

Treatment of pseudomonal infections often represents a challenge for clinicians. Some of the mechanisms of resistance are highly specific for one specific antimicrobial agent, while others affect a broad spectrum of agents and confer different levels of resistance. There are very few new antimicrobial agents in an advanced stage of development that are designed to target Gram-negative bacteria, and none are expected to be available for clinical use in the next decade. Given that the drug development process takes many years, it is critical that the clinical efficacy of currently available agents is preserved and that new agents are developed against these bacteria.

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AmpC is normally a chromosomally-mediated β-lactamase that is expressed constitutively, with expression regulated transcriptionally by *ampR*. In contrast to a large number of plasmid-mediated β-lactamases, the hydrolytic activity of AmpC is not inhibited by β-lactamase inhibitors (e.g., clavulanic acid) [4]. Point mutations in *ampR* have been associated with stable derepression of *ampC*, resulting in β-lactam resistance in *Enterobacter cloacae* [5], but it is not known whether this is also true for *P. aeruginosa*.

The prevalence of ceftazidime resistance among clinical isolates of *P. aeruginosa* in France has been reported as 6%, primarily because of AmpC overexpression [6]. The objective of the present study was to determine the prevalence of AmpC overexpression among bloodstream isolates of *P. aeruginosa* in the USA. An improved understanding of the prevalence and mechanisms of β-lactam resistance in *P. aeruginosa* may help to guide formulary decisions and the choice of empirical therapy for nosocomial infections in hospitals.

MATERIALS AND METHODS

Bacteria and resistance screening

All non-repeat (>7 days apart) bloodstream isolates of *P. aeruginosa* from 2003, together with their susceptibility results, were obtained from the clinical microbiology laboratory of St Luke's Episcopal Hospital, a 664-bed university-affiliated teaching hospital in Houston, TX, USA. The isolates were screened for ceftazidime resistance (defined as an MIC >8 mg/L) in the presence and absence of clavulanic acid 4 mg/L, using Etests (AB Biodisk, Piscataway, NJ, USA), according to the manufacturer's instructions, to assess possible resistance caused by AmpC over-expression.

Spectrophotometric assay

Increased β-lactamase activity was confirmed by a spectrophotometric assay using nitrocefin as the substrate (with and without preceding induction by imipenem) [7]. In brief, 1 mL of an overnight culture was diluted 20-fold with pre-warmed, cation-adjusted Mueller–Hinton broth (Becton Dickinson, Sparks, MD, USA) and incubated in a shaking water bath at 37°C for 90 min. Imipenem was added to a concentration of 0.5 mg/L (0.25 × the MIC for the wild-type isolate) for enzyme induction. The cells were incubated for a further 2.5 h before being harvested by centrifugation (5000 *g* at 37°C for 15 min), washed once with 100 mM phosphate buffer (pH 7.0), and then resuspended in 1 mL of phosphate buffer. Lysis of the outer-membrane of the cells was achieved by two cycles of freezing (−20°C) and thawing. Membranes and residual cells were removed by centrifugation (20 000 *g* at 4°C for 30 min). The enzymic activity of the crude cell lysate was determined spectrophotometrically at 486 nm using 100 μM nitrocefin (Oxoid, Ogdensburg, NY, USA) in 100 mM phosphate buffer and 1 mM EDTA (pH 7.0) at 30°C. The protein content of the crude cell lysate was determined with a bicinchoninic acid protein assay reagent kit (Pierce Biotechnology, Rockford, IL, USA), following the manufacturer's instructions. The standard wild-type strain ATCC 27853 and three clinical multidrug-susceptible isolates were used as controls.

Analytical isoelectric focusing (IEF)

IEF was used to identify the β-lactamase(s) responsible for accelerated hydrolysis of nitrocefin. Bacterial lysates were prepared as described previously [8]. IEF standards were purchased from Bio-Rad (Hercules, CA, USA). Identification of β-lactamase bands was facilitated by overlaying gels with 1 mM nitrocefin in 100 mM phosphate buffer (pH 7.0). An analytical IEF inhibition assay was performed by overlaying the gel with 0.3 mM cloxacillin in 100 mM phosphate buffer (pH 7.0) before overlaying with nitrocefin [9].

Quantitative RT-PCR (RT-qPCR)

The relative level of *ampC* expression was determined by RT-qPCR. Total RNA was isolated from bacterial cultures (*c.* 2–4 × 10^8 cells in logarithmic growth phase) using an RNeasy mini kit and RNAprotect bacteria reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Reverse transcription to cDNA was performed using a GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA, USA) with random hexamers. The DNA samples were analysed in triplicate using an ABI PRISM 7000 (Applied Biosystems) in conjunction with the primers and dual-labelled fluorescent hybridisation probes listed in Table 1. The threshold cycle (C_t) of each sample, which identified the PCR cycle at which the fluorescence exceeded a threshold value, was calculated by the ABI PRISM software. The *rpoD* housekeeping gene was used as an internal standard [10]; *rpoD* was amplified in separate qPCR reactions to correct for percentage differences in RNA recovery. The relative expression of *ampC* was assessed using the $\Delta\Delta C_t$ method as described previously [11]. The wild-type strain ATCC 27853 was used as a control.

Pulsed-field gel electrophoresis (PFGE)

The clonal relatedness of the isolates that screened positive for AmpC over-expression was assessed using PFGE.

Table 1. Oligonucleotides used for amplification and/or sequencing

Primer/probe	Sequence	Position[a]
(A) RT-qPCR		
AMPC-F	5′-CGCCGTACAACCGGTGAT	673
AMPC-R	5′-GAAGTAATGCGGTTCTCCTTTCA	754
AMPC-probe	5′-AAGGCCAATGACATTCCGGGC	692
RPOD-F	5′-CAGCAATCTCGTCTGAAAGAGTTG	19
RPOD-R	5′-TTGATCCCCATGTCGTTGATC	167
RPOD-probe	5′-ACCACCTGCCGGAGGATATTTCCGAT	92
(B) PCR		
AMPC-F	5′-GGGGCGGTTTCTCATGCAGCCAACG	510
AMPC-R	5′-GAAGCGCTCATGGCACCATCATAGCC	1820
AMPR-F	5′-AGGATTGGCGTCCTTTGTC	13
AMPR-R	5′-CTTGAATCGCCTGCATAACC	1071

[a]Numbers correspond to the position of the first 5′ base of each oligonucleotide according to the numbering of the nucleotide sequences in GenBank, accession numbers X54719 (*ampC*), X67095 (*ampR*), and AE004494 (*rpoD*).

Chromosomal DNA was prepared from each strain as described previously [12], with the following modifications: overnight bacterial cultures were grown in cation-adjusted Mueller–Hinton broth; DNA was digested with *Xba*I (Invitrogen, Carlsbad, CA, USA) overnight at 35°C; and the samples were analysed using the CHEF MAPPER system (Bio-Rad Laboratories, Richmond, CA, USA) at 6.0 V/cm for 21 h, with linear pulse times ramped from 1 to 25 s.

Sequencing of *ampC* and *ampR*

The *ampC* and *ampR* genes of isolates that screened positive for AmpC stable derepression were amplified by PCR. Genomic DNA was extracted using a QIAamp DNA mini kit (Qiagen), following the manufacturer's instructions, and 50 ng of genomic DNA was used as a PCR template. Reaction mixtures (final volume 50 μL) contained 1 U of *Taq* DNA polymerase, 0.5 μM each primer (Table 1), 10 mM Tris-HCl, 50 mM KCl, 2.5 mM $MgCl_2$ and 250 μM each dNTP. The PCR comprised 94°C for 10 min, followed by 35 cycles of 94°C for 1 min, 58°C (*ampC*) or 55°C (*ampR*) for 1 min and 72°C for 2 min, with a final extension at 72°C for 10 min. Sequences of the PCR products were subsequently determined using an ABI 3730 XL DNA analyser (Applied Biosystems) and were compared with the wild-type PAO1 sequences in GenBank, accession numbers X54719 (*ampC*) and X67095 (*ampR*).

RESULTS

Bacteria and resistance screening

In total, 76 *P. aeruginosa* isolates (from 63 patients) were available, of which 15 (19.7%) were resistant to ceftazidime (MIC >8 mg/L). All patients had been hospitalised for ≥48 h, and no patient was diagnosed with cystic fibrosis. Susceptibilities of the isolates to other antimicrobial agents are shown in Fig. 1. Susceptibility to ceftazidime was not enhanced in the presence of clavulanic acid (ceftazidime MIC >4 mg/L for all 15 isolates), suggesting that an extended-spectrum β-lactamase (e.g., SHV- or TEM-type) was not the sole mechanism of ceftazidime resistance.

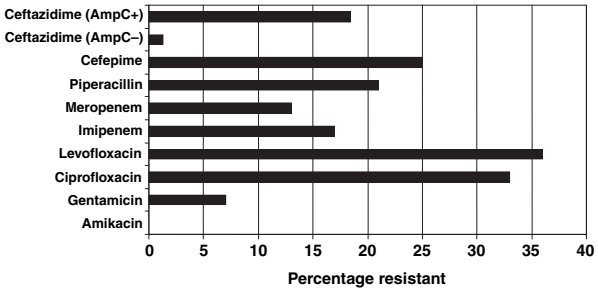

Fig. 1. Resistance profiles of *Pseudomonas aeruginosa* bloodstream isolates (n = 76) to antimicrobial agents (AmpC+, AmpC over-expressed; AmpC–, AmpC not over-expressed, i.e., similar to wild-type).

Spectrophotometric assay and IEF

The enzymic activities of the wild-type isolate were found to be 52.5 U/mg (non-induced) and 8760 U/mg (induced) (1 U of enzyme hydrolyses 1 nmol nitrocefin/min at 30°C with a nitrocefin concentration of 100 μM). Induction by imipenem resulted in a >150-fold increase in enzymic activity in the wild-type isolate. The enzymic activity of the multidrug-susceptible isolates (non-induced) was comparable with that of the wild-type isolate (all <four-fold different). In contrast, 14 of the 15 ceftazidime-resistant isolates (non-induced) had a >20-fold increase in enzymic activity compared with the wild-type (Table 2). Induction by imipenem resulted in a <ten-fold further increase in enzymic activity in these 14 resistant isolates (data not shown), consistent with (partially) stable derepression. The remaining ceftazidime-resistant isolate (PA 1616) had enzymic activity similar to that of the wild-type. This isolate was also resistant to piperacillin–tazobactam, meropenem, ciprofloxacin and levofloxacin, suggesting that over-expression of efflux pumps (e.g., MexAB–OprM) might be responsible for ceftazidime resistance, and was therefore not evaluated further.

IEF analysis of extracts from the remaining 14 ceftazidime-resistant isolates revealed a β-lactamase of pI 8.7 that was inhibited by cloxacillin in all 14 isolates, i.e., consistent with the chromosomal AmpC enzyme of *P. aeruginosa* [13]. Furthermore, only a single β-lactamase band was observed in 13 isolates; isolate PA 1872 produced an additional β-lactamase of pI 5.7, consistent with a β-lactamase belonging to the TEM family.

RT-qPCR

The relative expression of the *ampC* gene in the ceftazidime-resistant isolates is summarised in Table 2. All three multidrug-susceptible isolates showed similar expression of *ampC*. However, none of the ceftazidime-resistant isolates had a relative gene expression that was >five-fold that of the wild-type control. The enhanced enzymic activity observed could not be fully explained by the difference in transcription levels.

Table 2. Point mutations detected in *ampC*, relative expression (non-induced) and ceftazidime susceptibility of isolates

Isolate	Clone	Point mutations resulting in amino-acid changes at position					Relative expression (fold-difference)		Ceftazidime MIC (mg/L)
		21	27	97	205	391	Enzymic activity	Transcription	
PA 27853[a]	–						1.0 (reference)	1.0 (reference)	1
PA 2828[b]	–						3.9	0.9	1.5
PA 2599[b]	–						2.6	0.9	1
PA 2905[b]	–						1.9	0.8	1.5
PA 1843[c]	1						58.1	1.1	64
PA 1872[c]	2						386.4	1.0	48
PA 1962[c]	1						34.1	1.3	24
PA 1975[c]	1						38.6	2.1	48
PA 2026	3	T21A			V205L	G391A	2000.3	3.3	>256
PA 2040	3	T21A			V205L	G391A	1375.6	4.2	>256
PA 2041	3	T21A			V205L	G391A	2111.4	3.1	>256
PA 2205	4		G27D	A97V	V205L	G391A	107.4	3.5	48
PA 2300	4		G27D	A97V	V205L	G391A	22.0	1.0	32
PA 2431	5		G27D	A97V	V205L	G391A	280.3	1.5	96
PA 2484	4a		G27D	A97V	V205L	G391A	272.0	2.0	96
PA 2493	4		G27D	A97V	V205L	G391A	30.2	1.0	32
PA 2924	6		G27D		V205L	G391A	22.6	2.1	16
PA 2973	7		G27D	A97V	V205L	G391A	389.5	1.1	32

[a]ATCC standard wild-type strain.
[b]Clinical multidrug-susceptible isolate.
[c]Point mutation not detected compared with PA 27853.

PFGE typing

The patterns of the genomic DNAs digested with *Xba*I endonuclease revealed seven distinct clones (Table 2), as suggested by a difference of seven or more DNA bands [14]. Two sub-clones (differing by four bands) were identified for one of the clones.

Sequencing of *ampC* and *ampR*

Multiple point mutations in *ampC* were identified. Compared to PAO1, several point mutations (R79Q, T105A, G186S and A397R) were detected in the standard wild-type strain ATCC 27853 (susceptible to ceftazidime), suggesting that these point mutations were unlikely to be involved in ceftazidime resistance. A summary of the other point mutations resulting in amino-acid changes is provided in Table 2. Similarly, point mutation(s) in *ampR* were also detected in eight ceftazidime-resistant isolates. Point mutations (A12R and G237A) were detected in the standard wild-type strain ATCC 27853, suggesting that these point mutations were also unlikely to be involved in ceftazidime resistance. A summary of the other point mutations resulting in amino-acid changes is shown in Table 3.

Table 3. Point mutations detected in *ampR* among ceftazidime-resistant isolates of *Pseudomonas aeruginosa*

Isolate	Point mutations resulting in amino-acid changes at position			
	114	282	283	288
PA 1843[a]				
PA 1872[a]				
PA 1962[a]				
PA 1975[a]				
PA 2026[a]				
PA 2040			G283E	M288R
PA 2041[a]				
PA 2205			G283E	M288R
PA 2300			G283E	M288R
PA 2431			G283E	M288R
PA 2484		R282T	G283E	M288R
PA 2493			G283E	
PA 2924	E114A		G283E	M288R
PA 2973			G283E	M288R

[a]Point mutation not detected compared with the ATCC standard wild-type strain PA 27853.

DISCUSSION

P. aeruginosa is one of the most common pathogens implicated in serious nosocomial infections such as pneumonia and sepsis. Treatment of pseudomonal infections often represents a challenge to clinicians. Resistance to the β-lactams has been reported and is becoming more prevalent [15]. If the rapid spread of resistance is unchecked, there is a serious threat of a return to the pre-antibiotic ara, wiping out the great advances achieved a generation ago in the control of infectious diseases [16]. Clearly, understanding the prevalence and mechanisms of β-lactam resistance is important in optimising treatment of infections caused by these resistant pathogens.

Resistance to the β-lactams in *P. aeruginosa* may be mediated by several mechanisms, e.g., chromosomally-mediated β-lactamase (AmpC),

plasmid-mediated β-lactamases (SHV and TEM), integron-associated metallo-β-lactamases (VIM and IMP), outer-membrane porin (OprD) deletions, and the over-expression of efflux pumps (MexAB–OprM). With a good understanding of the mechanisms of β-lactam resistance prevalent in a hospital, effective empirical therapy for nosocomial infections can be formulated rationally. For example, β-lactam/β-lactamase inhibitor combinations may not be appropriate as empirical therapy in hospitals where AmpC over-expression is prevalent, as the hydrolytic activity of AmpC is not inhibited effectively by clinically-available β-lactamase inhibitors (as demonstrated with clavulanic acid in the present study). A carbapenem (e.g., meropenem or imipenem) or a non-β-lactam agent may be preferred as empirical therapy, since these agents are stable against hydrolysis mediated by AmpC.

The present study investigated the prevalence of AmpC over-expression as a mechanism of resistance responsible for β-lactam resistance in *P. aeruginosa* obtained from a hospital in Houston, TX, USA. In bloodstream isolates, the overall resistance rates ranged from <1% (amikacin) to 36% (levofloxacin). Among the β-lactams, resistance rates ranged from 13% (meropenem) to 25% (cefepime). Extended-spectrum β-lactamases were not prevalent among ceftazidime-resistant isolates, and ceftazidime resistance was associated primarily with AmpC over-expression, as identified by IEF and ascertained phenotypically in this study. While the increased β-lactamase activity (over-expression of AmpC) observed phenotypically correlated somewhat with ceftazidime susceptibility (MIC), it could not be explained simply by increased transcription of *ampC*. The increase in substrate hydrolysis among the ceftazidime-resistant isolates ranged from 22.6- to 2111.4-fold, compared with up to a 4.2-fold increase in *ampC* expression (transcription). The hydrolytic activity of AmpC could have been enhanced by translational differences. Alternatively, it is also possible that point mutation(s) in *ampC* may have increased the enzymic efficiency of AmpC. This latter suggestion is supported by the observation that the three isolates with the highest enzymic activity and MIC (i.e., PA 2026, PA 2040 and PA 2041) were clonally related and had identical point mutations in *ampC*. In particular, the T21A point mutation was not observed in any other isolate investigated, and could therefore be the focus of future studies. The results of the present study provide sufficient evidence to conclude that ceftazidime resistance was associated predominantly with enhanced activity of AmpC.

Point mutations in *ampR* have been shown to be associated with stable derepression of *ampC*, resulting in β-lactam resistance in *E. cloacae* [5] and *P. aeruginosa* [17]. However, the underlying molecular events leading to AmpC over-expression may be much more complex in *P. aeruginosa*. The *ampR* gene in *P. aeruginosa* is thought to be a global regulator for more than one gene; thus, it regulates the expression of *ampC*, *poxB*, *lasR*, etc., but does not auto-regulate its own promoter [18]. The *ampC–ampR* genetic region in isolates obtained from cystic fibrosis patients has been reported to be highly conserved [19]. However, in the present study, point mutation(s) in *ampR* were common in isolates derived from non-cystic fibrosis patients, despite their clonal diversity. The role of specific point mutations in *ampC* or *ampR* was not investigated using complementation studies, which would have been beyond the focus of the present study. However, there were four ceftazidime-resistant isolates without a point mutation in either *ampC* or *ampR*; therefore, ceftazidime resistance mediated by β-lactamase over-production could not be attributed solely to mutations in these genes. In this respect, it has been reported previously that AmpC hyper-production in clinical isolates of *P. aeruginosa* lacking mutations in *ampR* or the *ampC–ampR* intergenic region may be caused by AmpD/*ampD* inactivation [20,21]. An insertion sequence located in the *ampD* gene has been associated with high-level expression of AmpC [17], and transcription of *ampC* has been reported to be regulated in a stepwise manner by various AmpD homologues [22]. Furthermore, AmpE may also play an indirect role in ceftazidime resistance [20].

In conclusion, the molecular events leading to over-expression of AmpC are clearly important in understanding bacterial resistance. The present study found that the prevalence of AmpC over-expression was 18.4% among bloodstream isolates of *P. aeruginosa*. In contrast to the findings of a previous study [6], AmpC over-expression appears to be a significant mechanism of β-lactam resistance in *P. aeruginosa*. Understanding the prevalence and mechanisms of β-lactam resistance in *P. aeruginosa* may guide the choice of empirical therapy for nosocomial infections.

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