AAC Accepted Manuscript Posted Online 25 September 2017
Antimicrob. Agents Chemother. doi:10.1128/AAC.01183-17

Version Date: 09/20/2017

For Submission to *Antimicrobial Agents and Chemotherapy*

Emergence of Ceftolozane-Tazobactam Resistant *Pseudomonas aeruginosa* During Treatment is Mediated by a Single AmpC Structural Mutation

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Running title: Emergence of Ceftolozane-tazobactam resistant *Pseudomonas*

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ABSTRACT (75/75 words)

Ceftolozane-tazobactam is a cephalosporin β-lactamase-inhibitor combination that exhibits potent *in vitro* activity against *Pseudomonas aeruginosa*, including strains resistant to other β-lactams. Emergence of ceftolozane-tazobactam resistance has rarely been described among clinical isolates of *P. aeruginosa*. Here we characterized ceftolozane-tazobactam resistant *P. aeruginosa* strains that were recovered from a patient treated with this agent for 6 weeks for a recurrent wound infection. The result showed that the resistance is mediated by a single AmpC structural mutation.

40 **BODY TEXT**

41 *Pseudomonas aeruginosa* is a nosocomial pathogen associated with significant morbidity and
42 mortality (1). Ceftolozane-tazobactam is a cephalosporin β-lactamase-inhibitor combination antibiotic
43 that has better outer membrane permeability and improved stability against chromosomal AmpC β-
44 lactamase than other β-lactam antibiotics, resulting in potent *in vitro* activity against *P. aeruginosa*,
45 including multidrug-resistant strains (2, 3). *In vitro* selection for ceftolozane-tazobactam resistance in *P.*
46 *aeruginosa* requires multiple mutations leading to overexpression and structural modification in AmpC
47 (4). Emergence of *P. aeruginosa* ceftolozane-tazobactam resistance due to AmpC overexpression and
48 structural modifications was recently reported in two patients during prolonged courses of ceftolozane-
49 tazobactam, albeit at different amino acid positions than the *in vitro* selection study (5). Here we
50 investigated the mechanism(s) leading to *in vivo* ceftolozane-tazobactam resistance development in
51 sequential clinical *P. aeruginosa* isolates following six weeks of ceftolozane-tazobactam treatment.

52 A 75-year-old man presented to a tertiary-care hospital in South Carolina in April 2015 with left
53 neck wound dehiscence suspicious for infection. The patient had experienced recurrent wound
54 infections following resection and X-ray therapy with mixed gram-positive and gram-negative organisms
55 including *Pseudomonas aeruginosa* (pan-susceptible) dating back to December 2014. The patient
56 continuously received antimicrobial therapy since December 2014 due to inadequate closure of the
57 fistula including courses of vancomycin plus piperacillin-tazobactam (and subsequently meropenem),
58 followed by trimethoprim-sulfamethoxazole and ciprofloxacin.

59 An operating room (OR) wound culture in April 2015 revealed heavy growth of *P. aeruginosa*
60 (two morphotypes; PA-105A [spready] and PA-105B [round]) and scant growth of mixed gram-positive
61 organisms. The patient was started on vancomycin and cefepime. Antimicrobial susceptibility testing
62 (AST) results (**Table 1**) revealed non-susceptibility of PA-105B to all routinely tested antipseudomonal β-

lactams (6), but PA-105A and PA-105B were both susceptible to ceftolozane-tazobactam according to the FDA breakpoint (minimum inhibitory concentration [MIC] ≤ 4/4 µg/mL)(7). All MICs were performed using broth microdilution (**Table 1**) by ARUP Laboratories (Salt Lake City, UT). Cefepime was then transitioned to ceftolozane-tazobactam 1.5g intravenous (IV) every 8 hours (q8h) and vancomycin IV was continued. Therapy was planned to continue until surgery. Approximately 6 weeks later, patient was taken to OR for debridement and pectoral flap closure of wound. OR cultures grew *P. aeruginosa* of 2 morphotypes (PA-147A [spready] and PA-147B [round]), methicillin-resistant *Staphylococcus aureus* (MRSA), and *Candida tropicalis*. PA-147A and PA-147B were resistant (≥ 32/4 µg/mL) to ceftolozane-tazobactam. Ceftolozane-tazobactam therapy was discontinued, and imipenem-cilastatin 1g IV q8h (extended infusion of 3 hours) and tobramycin 7 mg/kg IV were started. Tobramycin was stopped after 8 weeks of therapy, and the patient remained on vancomycin, imipenem, and micafungin indefinitely. Subsequent *P. aeruginosa* isolates recovered in March 2016 were imipenem resistant and ceftolozane-tazobactam susceptible, but were not available for further testing. The patient ultimately was transitioned to palliative care and passed away 8 months later, after several additional courses of antibiotics.

Multilocus sequence typing showed that all four *P. aeruginosa* isolates were the same sequence type (ST), ST-316 (https://pubmlst.org/paeruginosa/) (8, 9). PCR and Sanger sequencing of the full-length β-lactamase genes (AmpC gene bla_{PDC} and bla_{OXA-50}, including the promoter region) were then performed. The results showed that there is an aspartic acid to glycine substitution at Ambler amino acid position 183 (G183D) in the AmpC gene (bla_{PDC}) of PA-147A and PA-147B, recovered after 42 days of ceftolozane-tazobactam treatment. In comparison to PA-105A and PA-105B, PA-147A and PA-147B were resistant to ceftolozane-tazobactam with ≥ 3-fold increase in MIC (**Table 1**)(10). No mutations were found in the promoter region of the mutant bla_{PDC} (PA-147A and PA-147B) compared with baseline bla_{PDC} (PA-105A and PA-105B), or on bla_{OXA-50}.

In order to examine if the ceftolozane-tazobactam and ceftazidime-avibactam resistance in PA-147A and PA-147B were due to the G183D substitution in the AmpC gene, we cloned the full-length bla_{PDC} gene from PA-105A and PA-147A, along with its native promoter region (using primerPDC-F and PDC-R, **Table 2**), into pGlow vector (Invitrogen) in *E. coli* TOP10. The resultant pGlow vectors carrying wild-type and G183D mutation were subsequently electroporated into PA-105A (named as PA105A-WT and PA105A-MT). Further susceptibility testing of the PA-105A transconjugates showed that the mutant bla_{PDC} encoding the G183D variant increased the ceftolozane-tazobactam MIC ≥ 6-fold compared to the corresponding wild type bla_{PDC} isogenic strain (**Table 1**), providing good evidence that the gene encoding the mutant bla_{PDC} is responsible for the ceftolozane-tazobactam resistance observed in the clinical isolates.

We also investigated whether the ceftolozane-tazobactam resistance is associated with the over-expression of the two β-lactamase genes by quantitative reverse transcription PCR (RT-qPCR). Gene expression of bla_{PDC} and bla_{OXA-50} in the four *P. aeruginosa* strains (PA-105A, PA-105B, PA-147A, and PA-147B) were tested using primers listed in **Table 2**. RT-qPCR revealed no significantly changes of bla_{PDC} expressions between ceftolozane-tazobactam susceptible and resistant isolates from the same morphotypes (between PA-105A and PA-147A, or between PA-105B and PA-147B), however, the expression levels of bla_{PDC} in round isolates (PA-105B and PA-147B, ~150 fold in comparison to *P. aeruginosa* PAO1) were significantly higher than those in spready isolates (PA-105A and PA-147A, ~1.2 fold in comparison to PAO1). We suspect isolates of different morphotypes may have different genetic signatures involving expression regulation of bla_{PDC}. However, the G183D substitution in bla_{PDC}, instead of the bla_{PDC} high expression, primarily contributes to the ceftolozane-tazobactam resistance. No differences of bla_{OXA-50} expression were observed among these four isolates (PA-105A, PA-105B, PA-147A, and PA-147B).

Interestingly, compared with PA105A-WT, PA105A-MT had 4-8 fold MIC increase for aztreonam, cefepime, piperacillin-tazobactam, and ceftazidime-avibactam, but MIC decrease for imipenem (≥ 4 fold doubling dilution) (**Table 1**), suggesting the bla_{PDC} G183D substitution also contribute to the resistance changes to these β-lactams antibiotics in *P. aeruginosa*. Of note, similar increased ceftazidime-avibactam resistance but restoring carbapenem susceptibility was described in *Klebsiella pneumoniae*, due to point mutation in bla_{KPC-3} gene(11). However, the molecular mechanisms underlying the multidrug resistance in PA-105B, which doesn't harbor the G183D substitution, remain unclear. Multiple mechanisms, including the high bla_{PDC} expression as well as other mechanisms (efflux, porin, etc.) may be involved. A further whole genome sequencing and transcriptome analysis may help to decipher the molecular mechanisms of diverse resistance profile between the parent and subsequent strains, and between isolates of different morphotypes (**Table 1**)

Previous studies by *in vitro* selection and characterization of ceftolozane-tazobactam resistant mutants in *P. aeruginosa* strains associated the development of high-level resistance with structural modifications in the conserved residues of AmpC (F147L, Q157R, G183D, E247K, or V356I)(4). Similar *in vitro* studies with ceftazidime-avibactam resistant mutants in *P. aeruginosa* also found the G183D mutation, which is less effectively inhibited by avibactam (12). However, *in vivo* development of ceftolozane-tazobactam resistance among clinical patients has only recently been observed following eight days of treatment and was mediated by AmpC overexpression and associated with mutations within the AmpC Ω-loop (5). To our knowledge, the current case is the first report of clinical emergence of *P. aeruginosa* ceftolozane-tazobactam resistance mediated by the G183D mutation in AmpC. More importantly, we have proved this mutation is the cause of the ceftolozane-tazobactam resistance. Development of resistance to ceftolozane-tazobactam occurred after several weeks of therapy. Notably, our patient received ceftolozane-tazobactam 1.5 g IV every 8 hours, the dosage regimen approved for complicated urinary tract and intra-abdominal infections (7). A higher

pharmacokinetic/pharmacodynamic-derived dose of 3g IV every 8 hours is currently being investigated in nosocomial pneumonia clinical trials (13). Our finding of emergence of co-resistance to ceftazidime-avibactam is concerning. Further studies on the implications of this mutation on the susceptibility of other β-lactam antibiotics are warranted.

ACKNOWLEDGMENTS

Financial support. This work was in part supported by the following grants from the National Institute of Allergy and Infectious Diseases (R01AI090155 to B.N.K and R21AI117338 to L.C).

Potential conflicts of interest. All authors report no conflicts of interest relevant to this article.

142 **Table 1. Antimicrobial Susceptibility Testing Results of Clinical and Transconjugate Isolates**

Organism	PA-105A	PA-105B	PA-147A	PA-147B	PA-105A-WT (vector with wild type bla_{PDC})	PA-105A-MT (vector with G183D variant)
Antimicrobial Agent	Interpretative Criteria or MIC value (µg/mL)					
Amikacin	4 (S)	8 (S)	16 (S)	16 (S)	2 (S)	16 (S)
Aztreonam	16 (I)	≥ 64 (R)	≥ 64 (R)	32 (R)	16 (I)	≥ 64 (R)
Cefepime	4 (S)	32 (R)	32 (R)	32 (R)	4 (S)	32 (R)
Ceftazidime	4 (S)	≥ 32 (R)	≥ 32 (R)	≥ 32 (R)	4 (S)	≥ 32 (R)
Ciprofloxacin	≥ 8 (R)	≥ 8 (R)	≥ 8 (R)	4 (R)	≥ 8 (R)	4 (R)
Colistin	1 (S)	1 (S)	1 (S)	1 (S)	1 (S)	1 (S)
Gentamicin	2 (S)	4 (S)	8 (I)	8 (I)	2 (S)	8 (I)
Imipenem	≥ 32 (R)	≥ 32 (R)	1 (S)	1 (S)	≥ 32 (R)	2 (S)
Meropenem	8 (R)	≥ 16 (R)	8 (R)	1 (S)	8 (R)	8 (R)
Piperacillin-tazobactam	16/4 (S)	≥ 128/4 (R)	64/4 (I)	64/4 (I)	16/4 (S)	64/4 (I)
Tobramycin	0.5 (S)	1 (S)	2 (S)	1 (S)	1 (S)	1 (S)
Ceftazidime-avibactam	4/4 (S)	≥ 32/4 (R)	≥ 32/4 (R)	≥32/4 (R)	4/4 (S)	≥ 32/4 (R)
Ceftolozane-tazobactam	1/4 (S)	8/4 (I)	≥ 64/4 (R)	≥ 64/4 (R)	1/4 (S)	≥ 64/4 (R)

143 Note: Antimicrobial susceptibility testing performed by broth microdilution. Interpretative criteria based on CLSI M100-S25 or package insert, as
144 appropriate.

145 Abbreviations: I, Intermediate; ND, not done; PA, *Pseudomonas aeruginosa*; R, Resistant; S, Susceptible

146 **Table 2. Primers used for cloning and RT-PCR in this study**

Primer	Sequences (5'-3')	Purpose	Size (bp)
PDC-F	CGAACCAATCTCTGCTCCAA	Clone the full-length	1,350
PDC-R	TCAGCGCTTCAGCGGCACCTTGGC	bla_{PDC} gene	
PDC-F(RT)	ACTCGGTGCAGAAGGACCAG	RT-qPCR	102
PDC-R(RT)	CGATGCTCGGGTTGGAATAG		
OXA50-F(RT)	GGCACCTTCGTCCTCTACGA	RT-qPCR	139
OXA50-R(RT)	ATTTAACCGCCCCTGTGGAT		
rpsL-F(RT)	TATACACCACCACGCCGAAA	Internal control for	103
rpsL-R(RT)	CCTTCACCACCGATGTACGA	RT-qPCR	

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