Characterization of Intrinsic β-lactam Resistance Mechanisms Among Contemporary Ceftazidime Nonsusceptible *Pseudomonas aeruginosa* From USA Hospitals

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INTRODUCTION

Pseudomonas aeruginosa is an opportunistic nosocomial pathogen that causes significant mortality rates and represents a challenge for antimicrobial chemotherapy due to its intrinsic low permeability to various antimicrobial agents and potential to acquire resistance mechanisms. Among valuable therapeutic options for *P. aeruginosa* infections, cephalosporins with antipseudomonal activity and carbapenems are important due to their low toxicity profile. Resistance against these β-lactam agents in *P. aeruginosa* includes AmpC de-repression, up-regulation of resistance-nodulation-division efflux systems, and loss of the outer membrane channel OprD.

Until recently, ceftazidime was considered the most active cephalosporin against *P. aeruginosa* strains; however, this antimicrobial agent is affected by up-regulation of efflux systems, mainly MexAB-OprM, de-repression of AmpC, and less commonly by the acquisition of β-lactamases. Ceftolozane/tazobactam is an antibacterial that combines an antipseudomonal cephalosporin that has improved stability against the chromosomal AmpC produced by *P. aeruginosa*, and tazobactam, a penicillanic acid-sulfone β-lactamase inhibitor that has modest activity against constitutive Ambler class C and various class A enzymes. Ceftolozane/tazobactam was at least 4-fold more potent than ceftazidime against a large collection of global *P. aeruginosa* isolates, including multidrug-resistant (MDR) and extremely drug-resistant (XDR) isolates.

In this study, we evaluated the expression of intrinsic resistance mechanisms to β-lactams among ceftazidime nonsusceptible *P. aeruginosa* collected in USA hospitals from the 2012 surveillance study for ceftolozane/tazobactam. Isolates were selected according to ceftazidime minimum inhibitory concentration (MIC) values to represent mostly ceftazidime-nonsusceptible isolates, including strains with modestly elevated ceftazidime susceptibility results (MIC results of 4 and 8 mg/L).

MATERIALS AND METHODS

Bacterial Isolates and Susceptibility Testing

A total of 998 *P. aeruginosa* clinical isolates were collected during 2012 in 27 USA hospitals and tested against ceftolozane/tazobactam and comparator antimicrobial agents as part of a surveillance initiative. These isolates were collected from nosocomial respiratory tract infections (n = 500), skin/soft-tissue infections (n = 201), urinary tract infections (n = 103), bloodstream infections (n = 92), intra-abdominal infections (n = 70), or other sources (n = 32).

One isolate per patient per episode that was deemed by the participant investigator to be the cause of infection was included in the surveillance study. Isolates were susceptibility tested using the broth microdilution method as described by the Clinical and Laboratory Standards Institute (CLSI) and categorical interpretations applied were those found in M100-S24 and on the the European Committee on Antimicrobial Susceptibility Testing (EUCAST) website. Quality control (QC) was performed using *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853. All QC results were within specified ranges as published in CLSI documents. Antimicrobial agents used for this analysis were ceftazidime, cefepime, ceftolozane/tazobactam, piperacillin/tazobactam, imipenem, and meropenem.

One hundred (10.0%) isolates were selected for further evaluation and these strains had ceftazidime MIC values at ≥4 mg/L as follows: 15 isolates were selected with MIC values at 4 (10.3% of the all isolates displaying MIC result), 8 (19.7%), and 16 (45.5%) mg/L, respectively; 20 (45.5%) isolates at 32 mg/L; and 35 (46.6%) at >32 mg/L. All isolates further investigated were identified using the MALDI Biotyper (Bruker Daltonics, Billerica, Massachusetts, USA) according to the manufacturer instructions.

Expression Analysis of the Chromosomally Encoded AmpC and Efflux Pumps

The expression of *ampC*, *mexA* (MexAB-OprM), *mexC* (MexCD-OprJ), *mexE* (MexEF-OprN), and *mexX* (MexXY-OprM) was determined by quantitative real-time polymerase chain reaction (PCR) (qRT-PCR) using DNA-free RNA preparations. Total RNA was extracted from mid-log-phase bacterial cultures (cell density at OD_{606} of 0.3-0.5) using RNA Protect Reagent and RNeasy Mini Kit (Qiagen, Hilden, Germany) in the Qiacube workstation (Qiagen) and residual DNA was eliminated with RNase-free DNase (Promega, Wisconsin, USA).

Quantification of mRNA and sample quality was assessed using the RNA 6000 Pico kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA) according to manufacturer instructions. Only preparations with an RNA integrity number (RIN) >8 that showed no visual degradation were used for experiments. Relative quantification of target genes was performed in triplicate by normalization to an endogenous reference gene (*rpsL*) on the StepOne Plus instrument (Life Technologies, Carlsbad, California, USA) using Power SYBR Green RNA-to-CT kit (Life Technologies) and custom-designed primers showing efficiency >98.0%. Transcription levels were considered significantly different if at least a 5- or 10-fold difference was noted compared with *P. aeruginosa* PAO1 for efflux pumps and AmpC, respectively.

Porin Detection

Outer membrane proteins were purified in the FastPrep-24 instrument (MP Biomedicals, Solon, Ohio, USA), according to the manufacturer's instructions. Normalized concentrations of purified outer membrane proteins were electrophoretically separated and transferred onto polyvinylidene difluoride (PVDF) membranes. Western blots were probed with an affinity-purified polyclonal antibody raised in rabbits using the synthetic OprD peptide N'-SDKTGTGNLPVMNDGKPPD-C' (Thermo Fisher Scientific, Rockford, Illinois, USA) and revealed with the WesternBreeze chromogenic kit (Life Technologies). *P. aeruginosa* PAO1 and 2 OprD down-regulated laboratory constructs were used as positive and negative controls for comparative analysis.

RESULTS

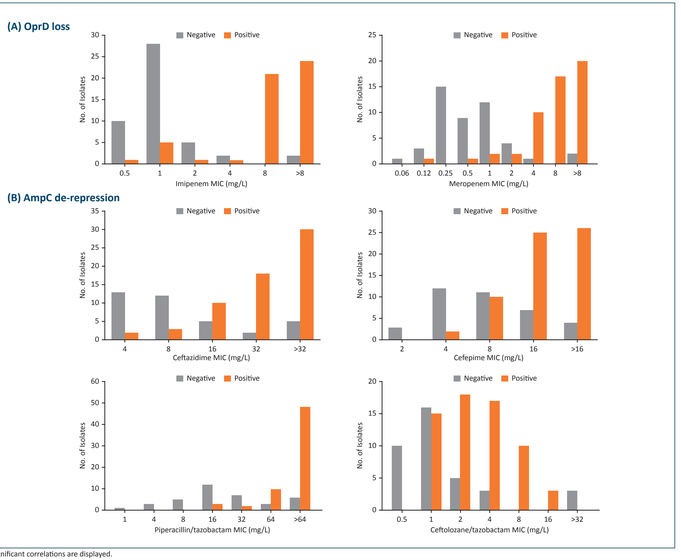
- De-repressed AmpC was noted among 63 (63.0%) isolates with levels 5-fold greater than the baseline isolate *P. aeruginosa* PAO1 and 39 (39.0%) had 10-fold greater levels of this chromosomal enzyme (**Table 1**). Additionally, 47 (47.0%; **Table 1**) strains tested showed no OprD band.
- Overall, 39.0% of the tested strains had elevated expression of the MexAB-OprM system. This resistance mechanism was expressed in greater levels (≥10X) in 23 (23.0%) isolates (**Table 1**). Elevated expression of MexCD-OprJ and MexXY-OprM was noted among 10 (10.0%) and 32 (32.0%) isolates tested, respectively (**Table 1**).
- When compared with PAO1 as a baseline, none of the isolates tested displayed elevated expression of MexEF-OprN; however, when the results were analysed using a different baseline strain, 34 isolates had modestly and highly elevated expression of this efflux system (**Table 1**).
- Resistance mechanisms described above were observed alone (21 isolates) or in combinations. Overall, 21 phenotypes were noted and 10 (10.0%) isolates carried none of the resistance mechanisms analysed (**Table 2**).
- The most prevalent resistance mechanism was AmpC de-repression alone (11 strains); followed by a combination of AmpC de-repression and OprD loss with or without elevated expression of efflux systems (29 and 10 isolates, respectively; **Table 2**).
- Among 10 isolates carrying none of the resistance mechanisms evaluated, 8 had ceftazidime MIC values of 4 to 8 mg/L (**Table 2**) and all of them displayed ceftolozane/tazobactam at ≤4 mg/L (**Table 2**). Cefepime, imipenem, and meropenem MIC results were also low (all isolates at ≤4, ≤4, and ≤1 mg/L, respectively; data not shown).
- OprD loss displayed a correlation with greater carbapenem MIC values and almost all isolates displaying imipenem and/or meropenem MIC values ≥4 mg/L had OprD loss (**Figure 1A**).
- De-repression of chromosomal AmpC was noted among *P. aeruginosa* isolates displaying ceftazidime and/or cefepime MIC values ≥16 and ≥8 mg/L, respectively (**Figure 1B**). Furthermore, the vast majority of isolates displaying piperacillin/tazobactam MIC results at ≥64 mg/L had elevated AmpC expression.
- In general, the expression of efflux pumps did not display strong correlations with MIC values of the antimicrobial agents selected (data not shown).

Table 1. Overall Results for Expression of Chromosomal Cephalosporinase (AmpC), Efflux Pumps, and Phenotypic Presence/Absence of Outer Membrane Protein for *P. aeruginosa* Isolates Collected From USA Hospitals

Resistance Determinant/Difference From the Control[a]	No. of Isolates (% of Isolates Tested)	Resistance Determinant/Difference From the Control[a]	No. of Isolates (% of Isolates Tested)
AmpC		**MexEF-OprN[a]**	
<5-fold	37 (37.0)	<5-fold	100 (100.0; 66 [66.0%])
5.0-9.9-fold	24 (24.0)	5.0-9.9-fold	0 (0.0; 20 [20.0%])
10.0-99.9-fold	33 (33.0)	10.0-99.9-fold	0 (0.0; 14 [14.0%])
≥100-fold	6 (6.0)	≥100-fold	0 (0.0)
MexAB-OprM		**MexXY-OprM**	
<5-fold	61 (61.0)	<5-fold	68 (68.0)
5.0-9.9-fold	16 (16.0)	5.0-9.9-fold	27 (27.0)
10.0-99.9-fold	23 (23.0)	10.0-99.9-fold	5 (5.0)
≥100-fold	0 (0.0)	≥100-fold	0 (0.0)
MexCD-OprJ		**OprD Western blot**	
<5-fold	90 (90.0)	No band	47 (47.0)
5.0-9.9-fold	6 (6.0)	Similar to control	53 (53.0)
10.0-99.9-fold	4 (4.0)		
≥100-fold	0 (0.0)		

[a]*P. aeruginosa* PAO1 was used as baseline for the analysis of the results. Due to PAO1 high expression levels of MexEF-OprN, the expression of this efflux system was also analyzed using another *P. aeruginosa* wild-type laboratory strain.

Figure 1. Distribution of Isolates Displaying (A) OprD Loss and (B) AmpC De-repression ([Positive] in Orange) and With Basal Levels ([Negative] in Gray) at MIC Values for β-lactam Agents[a]



[a]Only significant correlations are displayed.

Table 2. Distribution of Resistance Phenotypes Detected Among 100 *P. aeruginosa* Isolates at Ceftazidime and Ceftolozane/Tazobactam MIC Values

Phenotype	No. of Isolates	No. of Isolates at MIC (mg/L): Ceftazidime 4	8	16	32	>32	Ceftolozane/Tazobactam 0.5	1	2	4	8	16	>32
AmpC overexpression	11		2	1	7	1		3	7	1			
OprD loss, AmpC + MexAB-OprM overexpression	11	1	1	2	3	4		4	2	3	2		
OprD loss, AmpC overexpression	10			2	1	7		1	2	3	4		
OprD loss, AmpC + MexXY-OprM overexpression	8	1		2	1	4		3	1	4			
AmpC + MexXY-OprM overexpression	6			2	2	2		1	3			2	
AmpC + MexAB-OprM overexpression	6			2		4		2	1		3		
OprD loss, AmpC + MexAB-OprM + MexXY-OprM overexpression	6			1	1	4		1	1	2	1	1	
OprD loss, MexAB-OprM overexpression	5	4	1				3	2					
OprD loss, MexXY-OprM overexpression	5		2			3		1	1	1			2
MexAB-OprM overexpression	4	1	1		1	1		2	1	1			
OprD loss	4	3		1			1	2	1				
AmpC + MexCD-OprJ overexpression	3					3				3			

Phenotype	No. of Isolates	No. of Isolates at MIC (mg/L): Ceftazidime 4	8	16	32	>32	Ceftolozane/Tazobactam 0.5	1	2	4	8	16	>32
MexAB-OprM + MexXY-OprM overexpression	3		2	1			1	1	1				
AmpC + MexAB-OprM + MexCD-OprJ + MexXY-OprM overexpression	1					1				1			
MexCD-OprJ overexpression	1		1					1					
MexCD-OprJ + MexXY-OprM overexpression	1	1					1						
MexXY-OprM overexpression	1					1							1
OprD loss, AmpC + MexAB-OprM + MexCD-OprJ overexpression	1				1			1					
OprD loss, AmpC + MexAB-OprM + MexCD-OprJ + MexXY-OprM overexpression	1				1			1					
OprD loss, AmpC + MexCD-OprJ overexpression	1				1				1				
OprD loss, MexAB-OprM + MexCD-OprJ overexpression	1		1					1					
None	10	4	4	1	1		4	4	1	1			
Total No. of Isolates Tested	**100**	**15**	**15**	**15**	**20**	**35**	**10**	**31**	**23**	**20**	**10**	**3**	**3**

CONCLUSIONS

- AmpC de-repression and OprD loss were the most common intrinsic resistance mechanisms detected in selected *P. aeruginosa* strains collected in USA hospitals. Hyperexpression of efflux pumps was also detected, but the majority of these isolates also harboured AmpC de-repression and/or OprD loss.
- In general, ceftazidime-resistant isolates carried one or more of the resistance mechanisms evaluated and most (8/10) isolates displaying ceftazidime MIC results at 4 or 8 mg/L showed negative results for these intrinsic mechanisms.
- The presence of multiple intrinsic resistance mechanisms could explain elevated MIC values for the β-lactams and β-lactam/β-lactamase inhibitor combinations analyzed against the majority of the *P. aeruginosa* isolates tested. However, the presence of extended-spectrum β-lactamases not evaluated in this study could have contributed to the elevated MICs for the cephalosporins and β-lactam/β-lactamase inhibitor combinations.

SELECTED REFERENCES

Cabot G, Bruchmann S, Mulet X, Zamorano L, Moya B, Juan C, Haussler S, Oliver A. *Pseudomonas aeruginosa* ceftolozane-tazobactam resistance development requires multiple mutations leading to overexpression and structural modification of AmpC. *Antimicrob Agents Chemother*. 2014 [in press].

Castanheira M, Deshpande LM, Costello A, Davies TA, Jones RN. Epidemiology and carbapenem resistance mechanisms of carbapenem-non-susceptible *Pseudomonas aeruginosa* collected during 2009-2011 in 14 European and Mediterranean countries. *J Antimicrob Chemother*. 2014 [in press].

Clinical and Laboratory Standards Institute (2012). *M07-A9. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard: ninth edition*. Wayne, PA: CLSI.

Clinical and Laboratory Standards Institute (2014). *M100-S24. Performance standards for antimicrobial susceptibility testing: 24th informational supplement*. Wayne, PA: CLSI.

European Committee on Antimicrobial Susceptibility Testing (2014). Breakpoint tables for interpretation of MICs and zone diameters. Version 4.0, January 2014. Available at: http://www.eucast.org/clinical_breakpoints/. Accessed January 1, 2014.

Farrell DJ, Flamm RK, Sader HS, Jones RN. Antimicrobial activity of ceftolozane/tazobactam tested against Enterobacteriaceae and *Pseudomonas aeruginosa* with various resistance patterns isolated in U.S. hospitals (2011-2012). *Antimicrob Agents Chemother*. 2013;57:6305-6310.

Livermore DM, Mushtaq S, Ge Y, Warner M. Activity of cephalosporin CXA-101 (FR264205) against *Pseudomonas aeruginosa* and *Burkholderia cepacia* group strains and isolates. *Int J Antimicrob Agents*. 2009;34:402-406.

Queenan AM, Shang W, Bush K, Flamm RK. Differential selection of single-step AmpC or efflux mutants of *Pseudomonas aeruginosa* by using cefepime, ceftazidime, or ceftobiprole. *Antimicrob Agents Chemother*. 2010;54:4092-4097.

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