β-Lactam Resistance Mechanisms in *Pseudomonas aeruginosa* Isolates Analyzed Using Whole Genome Sequencing (WGS) and Transcriptome Analysis and Their Impact in Resistance to New β-Lactam/β-Lactamase Inhibitors

Mariana Castanheira[1], Timothy B. Doyle[1], Cory Hubler[1], Matthew Healy[1§], Caitlin J. Smith[1§], Rodrigo E. Mendes[1]

[1]JMI Laboratories, North Liberty, IA, USA; [§]Former affiliation

Introduction

- Analyzing 20 years of SENTRY Antimicrobial Surveillance Program data demonstrates that worldwide multidrug resistance (MDR; ≥3 antimicrobial classes) rates in *Pseudomonas aeruginosa* are approximately 25%, with the highest rates in Latin America and Europe
  - Additionally, almost 18% of *P. aeruginosa* isolates are extensively drug-resistant (XDR; only susceptible to ≤2 classes)
  - Treatment options for MDR and XDR in *P. aeruginosa* are limited
- New β-lactam/β-lactamase inhibitor combinations, such as ceftazidime-avibactam and ceftolozane-tazobactam, display excellent antipseudomonal activity and are active against many MDR and XDR isolates
- *P. aeruginosa* susceptibility against these agents can be affected by acquired resistance genes and mutations, but the understanding of these resistance mechanisms beyond metallo-β-lactamase production is limited
- We used whole genome and transcriptome analysis to evaluate resistance mechanisms against ceftazidime-avibactam and ceftolozane-tazobactam among 142 *P. aeruginosa* isolates collected in Asia-Pacific, Europe, and Latin America during 2017

Materials and Methods

- Among 1,909 *P. aeruginosa* isolates collected during 2017 as part of the SENTRY Antimicrobial Surveillance Program in 63 hospitals located in Asia-Pacific, Europe, and Latin America, 142 isolates were randomly selected for further analysis
- Isolates were susceptibility tested by reference broth microdilution against anti-pseudomonal agents according to Clinical Laboratories and Standards Institution (CLSI) guidelines
  - Quality control (QC) was performed according to CLSI procedures and MIC results were within acceptable ranges, as published by CLSI (M100, 2019)
  - Categorical interpretations were those found in European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoint tables (version 9.0, January 2019), the CLSI criteria in M100 (2019), or the US Food and Drug Administration (FDA) website
- High quality DNA from isolates was submitted to whole genome sequencing on a MiSeq (Illumina, San Diego, California, USA) instrument targeting a 30X coverage
  - Sequences were *de novo* assembled and searched for the presence of acquired β-lactamases using a curated library and applying criteria of >94% sequencing identity and 40% minimum length coverage
- Total RNA was extracted using the RNeasy® Mini Kit (Qiagen, Valencia, CA) and DNase treated
  - Up to 2 µg of RNA was subjected to rRNA depletion using Ribo-Zero® rRNA Removal Kit (Illumina)
  - rRNA depleted, mRNA generated cDNA library (TruSeq™ Stranded mRNA Library Prep, Illumina) sequencing was performed on a MiSeq
- Trimmed reads were aligned against the *P. aeruginosa* PAO1 reference genome (ASM676v1) using EDGE-pro
  - Counts were normalized across samples using trimmed mean of M-values (TMM) and evaluated for differential gene expression (DE) analysis using the edgeR5 package
  - Non-ribosomal genes showing the lowest quartile coefficient of variance (QCV) across all samples was estimated and used to generate a negative binomial (NB) distribution
  - Using the per-prep wild-type sample as our control, fold change expression was calculated according to an exact test based on a quantile-adjusted conditional maximum likelihood (qCML) method
  - Gene synonyms and gene ontology (GO) terms were collected from UniProt8 to help interpret generated heatmaps, PCA plots, and raw data
- Data was analyzed using custom software and logistic regression

Results

- Selected *P. aeruginosa* clinical isolates were collected in Europe (n=80), Asia-Pacific (n=35), and Latin America (n=27)
- Isolates carrying metallo-β-lactamases (n=24) were resistant to nearly all β-lactams, including ceftazidime-avibactam and ceftolozane-tazobactam (Figure 1)
  - The only comparator compound inhibiting >50% of the isolates was colistin
- Extended-spectrum β-lactamase genes (bla_{VEB-1} or bla_{VEB-9}), some oxacillinases, and PDC variants caused resistance to ceftazidime-avibactam and ceftolozane-tazobactam (Table 1)
  - The presence of bla_{PER-1}, bla_{GES-5}, and bla_{GES-6} led to resistance to ceftolozane-tazobactam, but not to ceftazidime-avibactam
- Analysis of sequences of various genes demonstrated that disruptions of *ampR* (PDC regulator) and *glnD* (nitrogen metabolism) were associated with resistance to ceftazidime-avibactam and ceftolozane-tazobactam (Table 1)
  - The disruption of *armZ* (anti-repressor of *mexZ*) was only related to ceftolozane-tazobactam resistance
  - Alterations in *dnaJ* (chaperone) and *oprM* were only related to ceftolozane-tazobactam resistance
- The combination of wild-type sequences of various genes was negatively related to resistance to ceftazidime-avibactam and ceftolozane-tazobactam
- Transcriptome analysis revealed AmpC overexpression (>10X) among 51/142 isolates (Table 2)
  - The addition of avibactam was able to lower the ceftazidime MIC values >8-fold in the absence of acquired genes
- Elevated expression of MexXY-OprM (>5X) was noted among 85 isolates for *mexY* and 76 isolates for *mexX*
- *MexAB-OprM* expression was noted among 31% of the isolates
- PmrAB overexpression was noted among 22-24 isolates (depending on the gene tested) and was only noted among MDR isolates
- Expression levels of PBPs, RND pumps MexCD-OprJ, MexJK, and MexGHI, or the intrinsic β-lactamase (OXA-50) or oxacillinase (PIB-1) did not correlate with specific resistance traits in this initial analysis (Table 2; Figure 1)

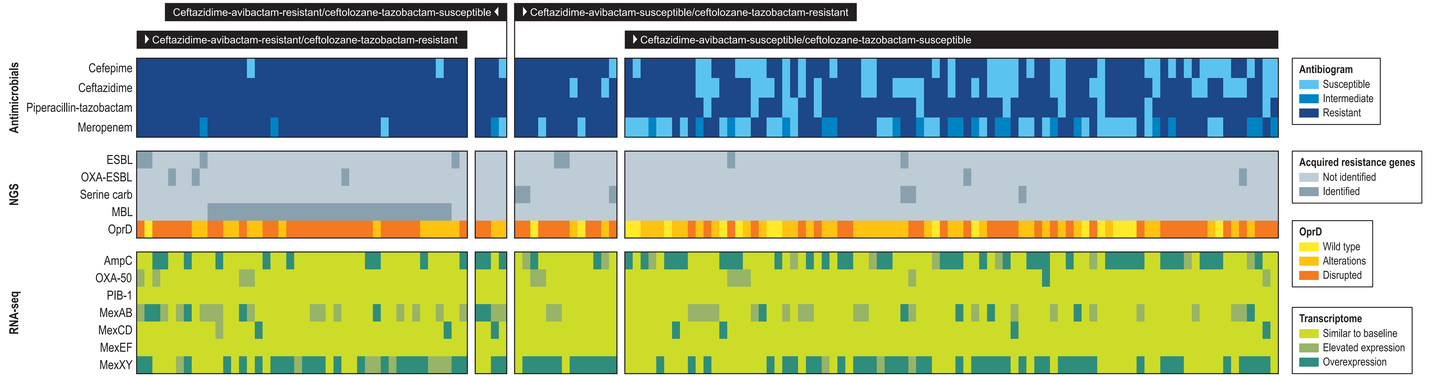
Table 1 Resistance mechanisms observed in the whole genome sequencing analysis of the ceftazidime-avibactam- and ceftolozane-tazobactam-resistant isolates

Resistance genes	Ceftazidime-avibactam-resistant isolates	Ceftolozane-tazobactam-resistant isolates
MBLs	X	X
PER-1		X
GES-5		X
GES-6		X
VEB-1	X	X
VEB-9	X	X
PME-1	X	X
PDC-19a	X	X
PDC-23	X	X
PDC-59	X	X
PDC-97		X
OXA-142	X	X
OXA-19	X	X
OXA-4	X	X
OXA-15	X	X
OXA-485	X	X
OXA-485 alterations	X	X
ARMZ disrupted		X
AMPR disrupted	X	X
GLND disrupted	X	X

Table 2 Transcriptome analysis of genes involved in β-lactam resistance

		No. of isolates at expression levels compared to PAO1 control			
Gene function	Gene	<3X	3-4X	5-9X	>10X
β-lactamases	AmpC	70	11	10	51
	OXA-50	95	37	9	1
Efflux components/ regulators	MexA	59	39	32	12
	MexB	60	36	36	10
	MexY	53	4	12	73
	MexX	60	6	20	56
	MexC	129	2	4	7
	MexD	132	2	2	6
	MexJ	116	14	11	1
	MexK	125	7	9	1
	MexG	141	0	0	1
	MexH	139	2	0	1
	MexI	140	0	1	1
LPS pathway	PmrA	113	7	6	16
	PmrB	113	8	8	13

Figure 1 Susceptibility results and resistance mechanisms for *P. aeruginosa* isolates



Conclusions

- Antimicrobial resistance in *P. aeruginosa* is an interplay of mechanisms that include limited permeability, efflux upregulation, target alterations, and enzymatic modification of the antimicrobial molecules
- Resistance mechanisms statistically associated with ceftazidime-avibactam resistance in *P. aeruginosa* were noted among ceftolozane-tazobactam-resistant isolates, but some mechanisms were only observed among ceftolozane-tazobactam-resistant isolates
- mRNA-sequencing data did not show strong correlations with ceftazidime-avibactam and ceftolozane-tazobactam resistance or with expression of genes involved in β-lactam resistance, but further analyses will expand the genes analyzed
- The richness of results employing these 2 methodologies requires further investigations that are being performed to evaluate sequences and expression alterations

Acknowledgements

This study was sponsored by Pfizer Inc. The authors would like to thank Timothy Collingsworth for the transcriptome analysis.

References

Clinical and Laboratory Standards Institute (2018). *M07Ed11. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard: eleventh edition*. Wayne, PA: CLSI.

Clinical and Laboratory Standards Institute (2019). *M100Ed29. Performance standards for antimicrobial susceptibility testing: 29th informational supplement*. Wayne, PA: CLSI.

Contact

Mariana Castanheira, PhD
JMI Laboratories
345 Beaver Kreek Centre, Suite A
North Liberty, Iowa 52317
Phone: (319) 665-3370
Fax: (319) 665-3371
Email: mariana-castanheira@jmilabs.com