In Vitro Susceptibility of Multidrug-resistant *Pseudomonas Aeruginosa* Following Treatment-Emergent Resistance to Ceftolozane-Tazobactam

by

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In Vitro Susceptibility of Multidrug-resistant *Pseudomonas Aeruginosa* Following Treatment-Emergent Resistance to Ceftolozane-Tazobactam

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Abstract

Background: Multidrug-resistant (MDR) *Pseudomonas aeruginosa* is a major public health threat. Treatment with ceftolozane-tazobactam improves patient outcomes compared to salvage therapy; however, resistance has emerged in ~15% of patients following courses ranging from 7 to 53 days. Understanding the development and mechanisms of resistance in these difficult to treat MDR *P. aeruginosa* has public health importance. Our objective was to study the *in vitro* activity of alternative β-lactams in the setting of ceftolozane-tazobactam resistance.

Methods: Isolates from 23 patients in whom ceftolozane-tazobactam resistance emerged were selected for analysis. Minimum inhibitory concentrations (MICs) were determined by standard broth microdilution in triplicate and interpreted by CLSI breakpoints. Mechanisms of resistance and relatedness of isolates were explored through whole-genome sequence (WGS) analysis in 15 patients from whom baseline and post-treatment isolates were available.

Results: 23 baseline and 32 post-treatment isolates were included. The median baseline ceftolozane-tazobactam MIC was 2 µg/mL (range: 0.5 – 8 µg/mL). 75%, 25%, 82.6%, and 83.3% of baseline isolates were non-susceptible to ceftazidime, ceftazidime-avibactam, imipenem, and piperacillin-tazobactam respectively. Following a median 16 (range: 3- 60) days of therapy, the median post-exposure ceftolozane-tazobactam MIC was 64 µg/mL (range: 8 – >256 µg/mL). 100%, 72.7%, 69.6%, and 79.2% of post-treatment isolates were resistant to ceftazidime, ceftazidime-avibactam, imipenem, and piperacillin-tazobactam. The corresponding MIC

foldchanges were 4, 8, -2, and 0, respectively. Median imipenem-relebactam MICs did not change before or after treatment with ceftolozane-tazobactam (median= 2 µg/mL for both) and 16.7% were classified as resistant. WGS data revealed several mutations in *ampC* and *ampR* sequences.

Discussion: Our findings show that resistance to ceftolozane-tazobactam impacts the susceptibility of alternative β-lactams. Cross resistance occurs with ceftazidime and ceftazidimeavibactam (median 4 and 8 fold MIC increase, respectively). Imipenem MICs are decreased 2fold potentially demonstrating collateral sensitivity. Piperacillin-tazobactam MICs were unchanged and isolates remained resistant. Importantly, imipenem-relebactam MICs were unchanged suggesting the mechanism of ceftolozane-tazobactam resistance may be due to structural changes in *ampC*. WGS data showed a number of different mutations in both *ampC* and *ampR*. Certain mutations, such as F147L and mutations found in positions 234-244, were found to promote resistance to ceftolozane-tazobactam.

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Preface

Acknowledgments: Kreiswirth Lab for whole genome sequencing and analysis of isolates, Imipenem-relebactam and ceftolozane-tazobactam were supplied from Merck. Other collaborators or co-investigators include Ellen Kline, Chelsea Jones, Cornelius Clancy, and Hong Nguyen.

1.0 Background

Pseudomonas aeruginosa is a common pathogen causing nosocomial infection. Antibiotic resistance in this bacterium results in multidrug-resistant (MDR) *P. aeruginosa,* which presents a major public health threat. 2.8 million antibiotic resistant infections occur in the United States every year, and 35,000 people will die as a result (Centers, 2019). Additionally, it is important to note everyone is at risk of contracting antibiotic resistant infections. However, certain populations, such as the elderly or immunocompromised persons, have a higher risk of contracting an antibiotic infection. The CDC released an antibiotic resistant threat report in 2019 which covers 18 different antibiotic resistant infections. Each infection was given a classification as urgent threat, serious threat, concerning threat, or watch list. Classifications of urgent and serious threat require the most attention and immediate action. MDR *P. aeruginosa* was listed as a serious threat in this report. This was listed as a serious threat, because in 2019 there were 32,600 cases, and 2,700 deaths due to MDR *P. aeruginosa,* as well as $767 million in attributable health care costs (Centers, 2019).

Patients with MDR *P. aeruginosa* infections represent a significant therapeutic challenge to clinicians. A new antibiotic combination, ceftolozane-tazobactam has provided new hope in treating MDR *P. aeruginosa.* Treatment with ceftolozane-tazobactam improves patient outcomes compared to salvage therapy (Haidar, 2017; Shortridge, 2017; van Duin, 2016). At UPMC Presbyterian, treatment of MDR *P. aeruginosa* with ceftolozane-tazobactam resulted in 30-day clinical cure and survival rates of 55% and 77% respectively. However, on balance with these encouraging results, resistance to ceftolozane-tazobactam emerged in ~15% of patients following treatment courses ranging from 7 to 53 days. This novel resistance seen in isolates was found to

have mutations *ampC* and *ampR* sequences (Haidar, 2017). In MDR *P. aeruginosa ampC* is a chromosomally encoded and inducible protein. *AmpR* is also chromosomally encoded in MDR *P. aeruginosa* and can induce *ampC* (Livermore, 1982; Torrens, 2019). These are proteins of interest because *ampC* is capable of drug hydrolysis. Ceftolozane-tazobactam was selected as a therapeutic measure for MDR *P. aeruginosa* because it has a bulky R2 side chain (Barnes, 2018). This side chain makes ceftolozane-tazobactam too large to fit into the binding site and be hydrolyzed by *ampC*. Understanding the development and mechanisms of resistance in these difficult to treat MDR *P. aeruginosa* has public health importance as it could increase overall clinical cure and survival rates by identifying optimal treatment regimens and new therapeutic targets.

Additionally, it is important to understand cross resistance or collateral sensitivity that arises in light of resistance. Cross resistance occurs when isolates develop resistance to the treatment antibiotic and therefore causes resistance to other antibiotics to develop that were not used for treatment. Whereas, collateral sensitivity is when resistance develops to the treatment antibiotic and causes increased susceptibility to other antibiotics. Ceftolozane-tazobactam has the same backbone as ceftazidime but also has the bulky R2 side chain (Barnes, 2018). Therefore, testing ceftazidime for cross resistance is imperative to guide therapeutic measures for clinicians and promote public health. Collateral sensitivity can present a unique opportunity to clinicians. While ceftolozane-tazobactam resistance develops mutations occurring in *ampC* optimize ceftolozane-tazobactam as the substrate. Therefore, carbapenems with a chemically dissimilar structure might become less optimal substrates for *ampC* hydrolysis. As a result, collateral sensitivity could occur in carbapenems imipenem and piperacillin-tazobactam.

Imipenem-relebactam is a novel carbapenem-b-lactamase inhibitor combination that was FDA approved in July 2019. Relebactam has been shown to have activity agains *ampC* and

potentially against mutated *ampC* (Karaiskos, 2019). Therefore, imipenem-relebactam might provide a novel therapeutic option for clinicians treating ceftolozane-tazobactam resistant MDR *P. aeruginosa*.

1.1 Hypotheses

1.1.1 Cephalosporins

I hypothesize the development of ceftolozane-tazobactam resistance in MDR *P. aeruginosa* isolates causes at least 4-fold increases in ceftazidime and ceftazidime-avibactam MICs due to structural similarities between ceftolozane and ceftazidime.

1.1.2 Carbapenems

I hypothesize the development of ceftolozane-tazobactam resistance in MDR *P. aeruginosa* isolates causes at least 4-fold decreases in imipenem and piperacillin-tazobactam MICs due to mutations in *ampC.*

1.1.3 Imipenem-relebactam

I hypothesize imipenem-relebactam will retain potent *in vitro* activity against isolates before and after treatment with ceftolozane-tazobactam due to relebactam's activity against *ampC.*

1.1.4 *AmpC*

I hypothesize that mutations in *ampC* are responsible for ceftolozane-tazobactam resistance in MDR *P. aeruginosa*.

2.0 Methods

2.1 MIC Study

2.1.1 Screening

This was a retrospective cohort study of an initial 28 patients with MDR *P. aeruginosa* infections treated with >72 hours of ceftolozane-tazobactam from August 2015 to May 2019. Patients were screened for development of ceftolozane-tazobactam resistance. Development of resistance was identified by measuring ceftolozane-tazobactam MICs using broth microdilution (BMD) reference methods as recommended by the Clinical Laboratory Standard Institute (CLSI) methods. Briefly, 96-well plates were used to create doubling dilutions of ceftolozane-tazobactam from 0.25-256 µg/mL with tazobactam at a fixed concentration of 4 µg/mL. Ceftolozanetazobactam MICs were measured in triplicate and susceptible, intermediate, and resistant MICs were defined according to CLSI breakpoints. Quality control (QC) strains *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used throughout. All QCs were in acceptable ranges. Nonsusceptible was defined as an isolate with an MIC $^{3}8$ µg/mL. From the initial 28 patients, 23 patients were selected for this study based on the following inclusion criteria: the patient must have been infected with *P. aeruginosa*, treated with ceftolozane-tazobactam, had a baseline isolate that was collected prior to treatment with ceftolozane-tazobactam, and had a nonsusceptible isolate collected post-exposure. Each patient had one baseline and at least one postexposure isolate.

Relatedness of baseline and post-exposure isolates were compared by whole genome sequence (WGS) analysis. Post-exposure isolates were defined as those collected during or post treatment. Additionally, individual isolates were removed from analysis if WGS did not identify them as *P. aeruginosa*, or if they were susceptible to ceftolozane-tazobactam post-exposure. The resulting study cohort was comprised of 23 patients with 23 baseline isolates and 32 post-exposure isolates.

2.1.2 Susceptibility Testing

A susceptibility profile was developed for the 55 isolates. MICs were measured as described previously. Ranges tested for all isolates included Ceftazidime 0.5-512 µg/mL, ceftazidime-avibactam 0.25-256 µg/mL, ceftolozane-tazobactam 0.25-256 µg/mL, imipenem 0.03-32 µg/mL, Imipenem-relebactam 0.03-32 µg/mL and piperacillin-tazobactam 0.5-512 µg/mL (avibactam, relebactam, and tazobactam tested at a fixed concentration of 4 µg/mL).

2.1.3 Analysis

Consensus MICs for each isolate were used for analysis. Consensus was identified for each drug, using the modal value when possible, and when not, the median of the triplicate tests was used. MIC fold-changes were calculated for each patient from baseline to post-exposure for each drug. Fold-changes were used to assess changes in susceptibility that develop in response to development of ceftolozane-tazobactam resistance. Median fold-changes were identified for each drug in order to determine collateral sensitivity and cross resistance.

2.2 WGS Study

2.2.1 Isolate Selection

A total of 39 isolates, 16 baseline and 23 post-exposure, from 15 patients were sent to the Kreiswirth Lab for WGS. Patients selected for WGS had large ceftolozane-tazobactam foldchanges, reliable ceftolozane-tazobactam treatment dates, and post-exposure isolates collected in close proximity to the treatment dates.

2.2.2 Analysis

Raw WGS data were received and examined for mutations in *ampC* and *ampR* protein sequences. PAO1 was used as the reference wild-type strain. Any amino acid variation from the wild-type sequence was recorded as a mutation. Mutations of interest were defined as mutations that arose in post-exposure isolates but were not present in baseline isolates for that patient. Relatedness of isolates was assessed using data received on sequence type (ST) and single nucleotide polymorphisms (SNP) variation. Isolates with the same ST and £300 SNP variations were considered to be related. If only ST or SNP data were available, one was considered sufficient to assess relatedness.

3.0 Results

3.1 MIC Study

MIC screening revealed that patients readily developed resistance to ceftolozanetazobactam. Baseline isolates had a median MIC of 2 µg/mL (range: 0.5-8 µg/mL) and postexposure isolates had a median MIC of 64 µg/mL (range: 8-512 µg/mL). Figure 1 shows the MIC distributions between baseline and post-exposure isolates. Ceftolozane-tazobactam MICs increased a median of 32-fold from baseline to post-exposure. Figure 2 shows MIC fold-changes—approximately a normal distribution.

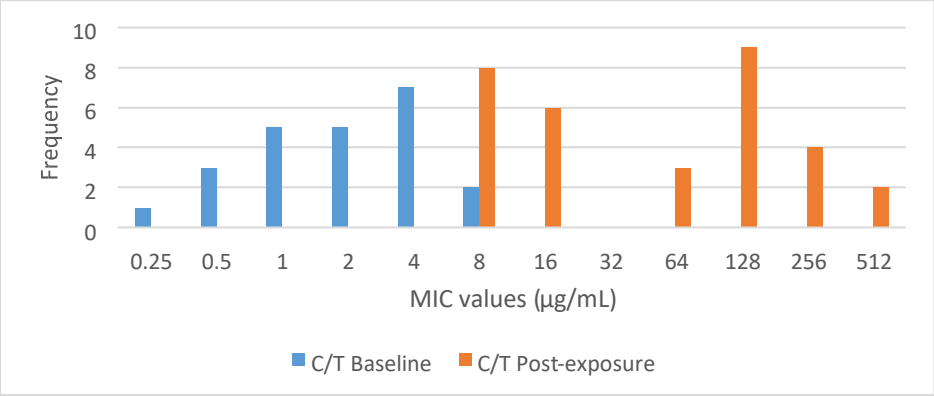

Figure 1: Ceftolozane-tazobactam (C/T) Baseline and Postexposure MIC frequencies

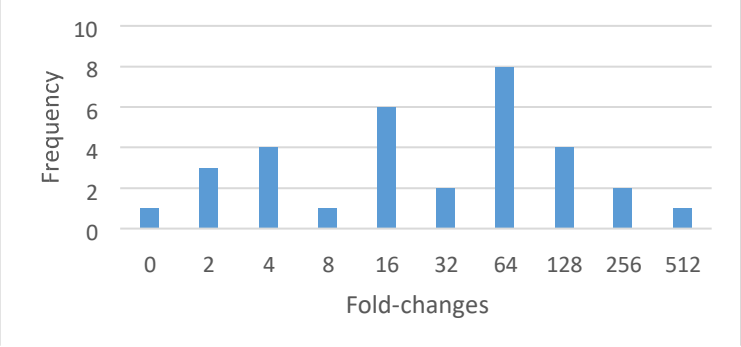

Figure 2: C/T MIC Fold-Changes

Isolates were tested for cross resistance to cephalosporins, ceftazidime and ceftazidimeavibactam. Median MICs for baseline isolates against ceftazidime and ceftazidimeavibactam were 32 and 4 µg/mL respectively (ranges: 1-256 µg/mL; 1-32 µg/mL). Post-exposure isolates had a median ceftazidime and ceftazidime-avibactam MICs of 128 and 64 µg/mL respectively (ranges: 32-1024 µg/mL; 4-512 µg/mL). Resulting median MIC fold-changes for ceftazidime and ceftazidime-avibactam were 4 and 8 respectively. All MIC fold-changes are shown in Figures 3 and 4; both have approximately normal distributions. Outliers were seen at a fold-change of 512 for ceftazidime and -4 for ceftazidime-avibactam. Additionally, 73.9% and 26.1% of baseline isolates were non-susceptible to ceftazidime and ceftazidime-avibactam, respectively, compared to 100% and 71.9% of post-exposure isolates, respectively. Figure 5 shows the MIC fold-changes of cephalosporins compared to ceftolozane-tazobactam.

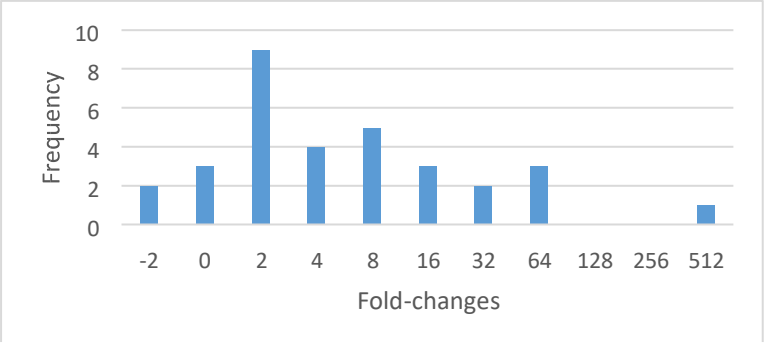

Figure 3: Ceftazidime (CAZ) MIC Fold-Changes

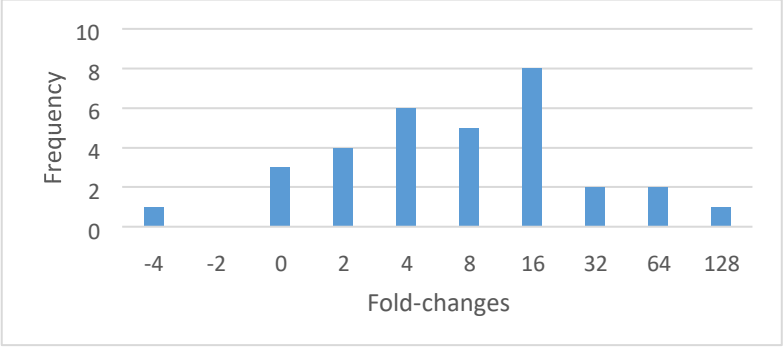

Figure 4: Ceftazidime-avibactam (CZA) MIC Fold-Changes

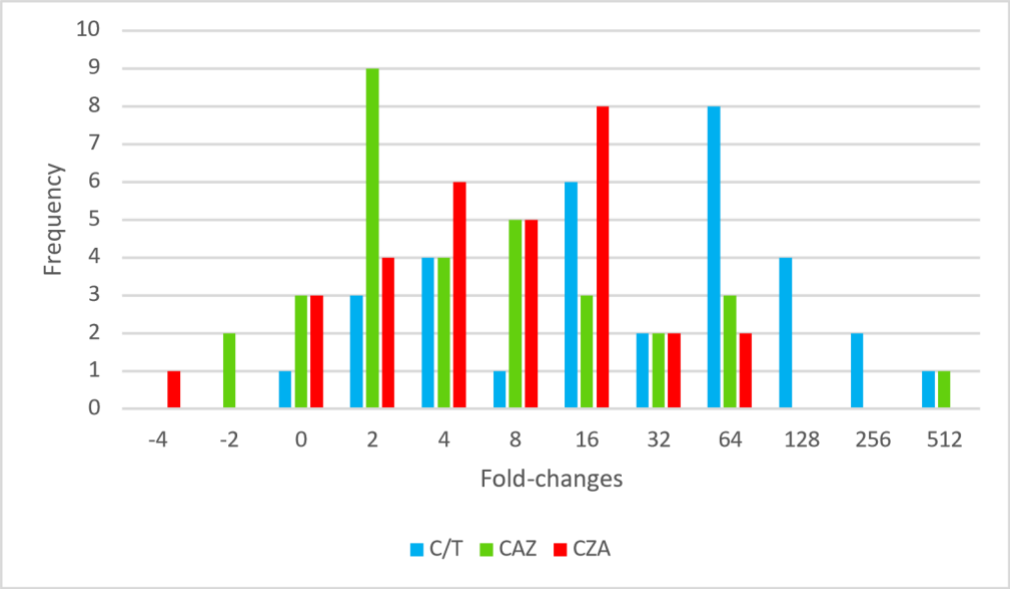

Figure 5: MIC Fold-Changes for Ceftolozane-tazobactam vs. Cephalosporins

Piperacillin-tazobactam and imipenem were tested for collateral sensitivity. Baseline isolates had median MICs of 128 and 16 µg/mL for piperacillin-tazobactam and imipenem respectively (ranges: 1-512 µg/mL; 0.12-32 µg/mL). Comparatively, post-exposure isolates had median MICs of 32 and 2 µg/mL (ranges: 4-1024 µg/mL; 0.5-64 µg/mL). Median MIC foldchanges were 0 and -2 for piperacillin-tazobactam and imipenem, respectively. Figures 6 and 7 show MIC fold-changes for piperacillin-tazobactam and imipenem. Both piperacillintazobactam and imipenem had a nearly bimodal distribution. Baseline isolates were found to be 82.6% and 81.8% non-susceptible to piperacillin-tazobactam and imipenem respectively; whereas postexposure isolates were 84.4% and 50% non-susceptible. Figure 8 displays MIC fold-changes of carbapenems compared to ceftolozane-tazobactam.

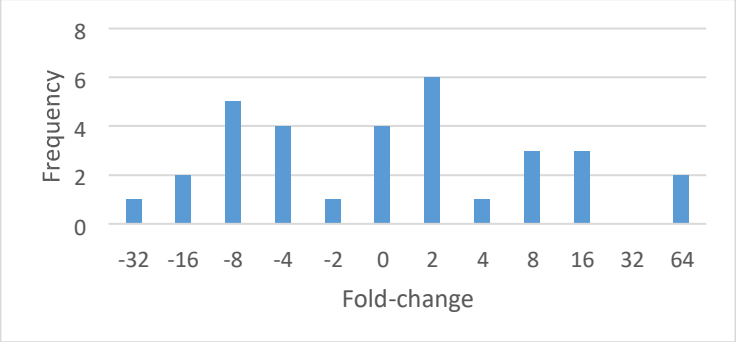

Figure 6: Piperacillin-tazobactam (P/T) MIC Fold-Changes

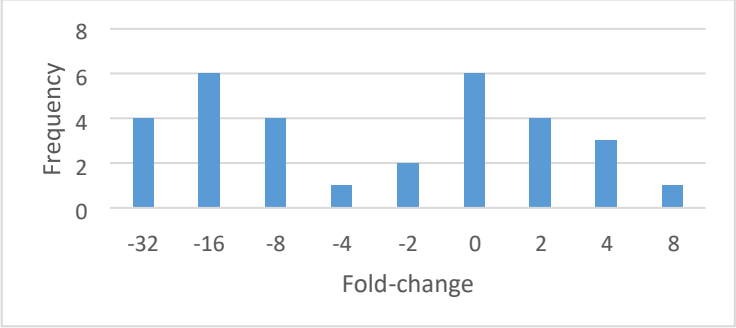

Figure 7: Imipenem (IMI) MIC Fold-Changes

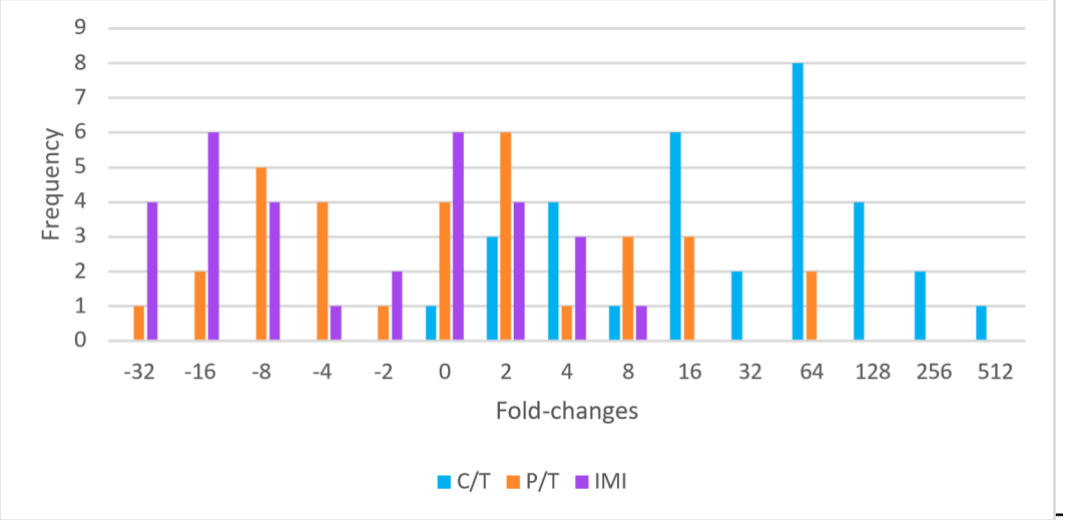

Figure 8: MIC Fold-Changes for Ceftolozane-tazobactam vs. Carbapenems

Lastly, imipenem-relebactam was tested to see if it maintained potent *in vitro* activity against isolates before and after developing resistance to ceftolozane-tazobactam. Median MICs

were found to be 2 µg/mL for both baseline and post-exposure isolates (ranges: 0.06-16 µg/mL; 0.25-16 µg/mL). The resulting fold-change was 0. 21.7% of baseline isolates were resistant to imipenem-relebactam compared to 12.5% of post-exposure isolates. Figure 9 shows all foldchanges and shows approximately a normal distribution.

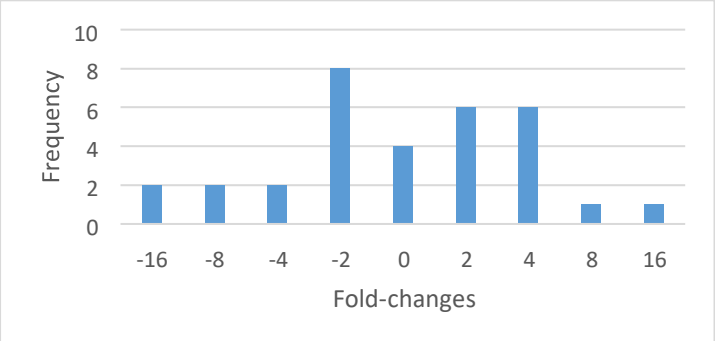

Figure 9: Imipenem-relebactam MIC Fold-Changes

Figures 10 and 11 show of baseline and post-exposure MICs for each drug compared to ceftolozane-tazobactam. The red bar denotes the median MIC for each respective category.

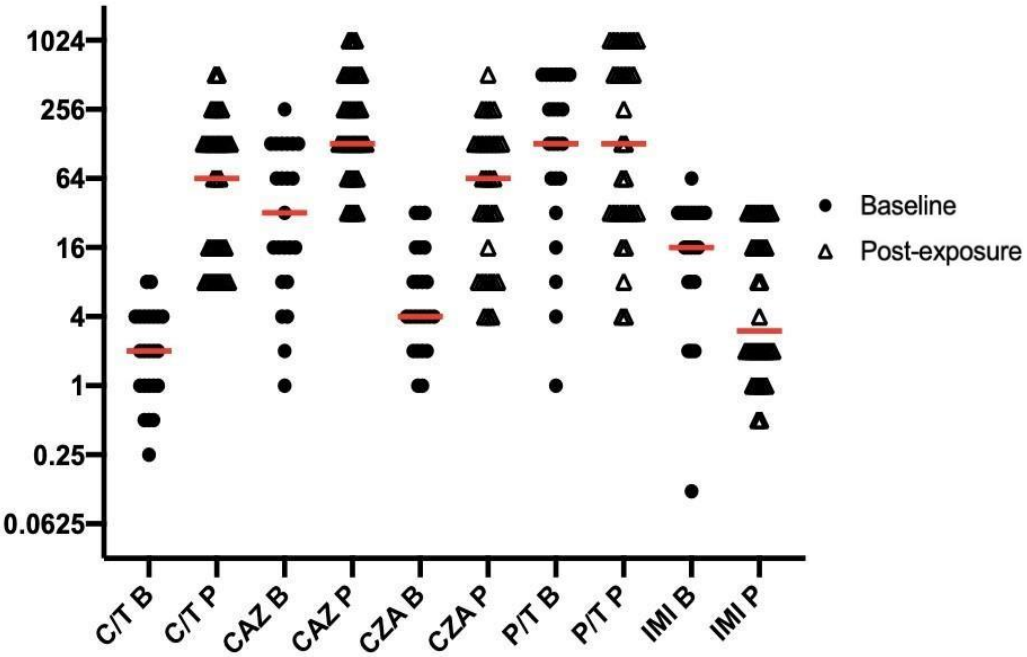

Figure 10: MICs Baseline (B) to Post-exposure (P) and their medians denoted by the red lines

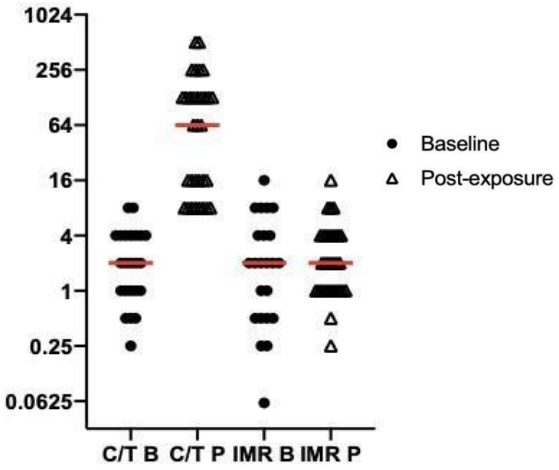

Figure 11: MICs Baseline (B) to Post-exposure (P) and their medians denoted by the red lines

3.2 WGS Study

Isolates from 14 of 15 patients had the same ST and were within 300 SNP variations and were therefore considered to be related. Median SNP variation was 7 between baseline and postexposure isolates (range 0-291). Patient 2 had SNP variations of 22,953 and 22,956 between baseline and post-exposure isolates and was excluded from further analysis due to lack of a related baseline isolate. Patient 9 had two baseline isolates, however only one baseline isolate was considered to be related to all of the post-exposure isolates. One baseline isolate from patient 9 was >300 SNP variations from a post-exposure isolate.

Four patients had mutations in *ampR*, which were found in both the baseline and postexposure isolates. All 14 patients had mutations in *ampC* protein sequence. The most frequent mutation was T105A which was found in 87.1% of isolates. Other common mutations included R79Q, G391A, and F147L. Table 1 shows the full list of mutations and MIC values for each drug.

Mutations of interest have been bolded. Additionally, other beta-lactamases (BL'ases) were explored in WGS analysis. All isolates had some other BL'ases the most common was OXA-50. No isolate had metallo-beta-lactamases.

Table 1: WGS Data

Clinical Designation	Patient	ampC	ampR	BL'ases	C/T MIC	CAZ MIC	CZA MIC	IMI MIC	P/T MIC	IMR MIC
Baseline	1	T21A ; G391A ; T105A	--	OXA-494	2	64	2	32	256	4
Post-exposure	1	T21A; G391A ; T105A	--	OXA-494	8	128	32	32	>512	16
Baseline	3	R79Q ; T105A	R244W ; G283E ; Stop_lost + 9aa at 3'(recomb at 3")	OXA-396	4	128	16	16	256	2
Post-exposure	3	R79Q **; 234-241 del** ; T105A	R244W ; G283E ; Stop_lost + 9aa at 3'(recomb at 3")	OXA-396	256	512	256	1	32	1
Baseline	4	L176H ; E335K ; T105A	--	OXA-488	8	256	8	16	512	4
Post-exposure	4	**F147L;**L176H;**G183D;** E335K ; T105A	--	OXA-488	64	128	64	0.5	16	1
Baseline	7	T21A ; G391A ; T105A	--	OXA-486	1	16	4	32	128	8
Post-exposure	7	T21A ; G391A ; **D245 del** ; T105A	--	OXA-486	16	32	8	1	32	1
Baseline	8	T105A	G283E ; Stop_lost + 9aa at 3'(recomb at 3")	CARB-2; OXA-395; OXA-9	1	8	4	32	512	2
Post-exposure	8	T105A	G283E ; Stop_lost + 9aa at 3'(recomb at 3")	CARB-2; OXA-395; OXA-9	1	8	4	32	512	2
Post-exposure	8	**G183R** ; T105A	G283E ; Stop_lost + 9aa at 3'(recomb at 3")	CARB-2; OXA-395; OXA-9	64	128	64	2	512	1
Baseline	9	R79Q ; T105A	--	OXA-396	1	8	1	16	64	2
Baseline	9	R79Q ; T105A	--	OXA-396	1	32	4	16	128	2
Post-exposure	9	R79Q ; **P243S ;** T105A	--	OXA-396	16	32	4	2	16	1
Post-exposure	9	R79Q **; D234G ; P243S** ; T105A	--	OXA-396	128	256	128	2	32	1
Post-exposure	9	R79Q ; **G183D ; 243G ins** ; T105A	--	OXA-396	128	128	128	1	32	2
Baseline	10	--	--	OXA-50	2	128	32	32	256	8
Post-exposure	10	**Δ237-243**	--	OXA-50	128	512	256	2	32	2
Post-exposure	10	**Δ237-243**	--	OXA-50	>256	>512	>256	2	128	1
Baseline	12	T105A	--	OXA-50	2	64	4	16	128	0.5
Post-exposure	12	**T96I,** T105A	--	OXA-50	256	32	8	2	8	2
Post-exposure	12	**T96I,** T105A	--	OXA-50	128	128	64	2	32	2
Baseline	13	R79Q;T105A	R244W ; G283E ; Stop_lost + 9aa at 3'(recomb at 3")	OXA-488	1	16	8	2	128	0.5
Post-exposure	13	R79Q ; **F147L** ; T105A	R244W ; G283E ; Stop_lost + 9aa at 3'(recomb at 3")	OXA-488	256	512	128	16	256	8
Baseline	16	T105A	D135G	OXA-50	4	64	4	2	512	0.5
Post-exposure	16	T105A	D135G	OXA-50	16	512	64	8	>512	2
Baseline	18		--	OXA-50	<0.25	4	4	0.12	16	0.06
Post-exposure	18	**F147L**	--	OXA-50	16	64	4	0.5	128	0.25
Baseline	19	T105A		OXA-395	2	16	2	32	64	4
Post-exposure	19	**241R ins** ; T105A	--	OXA-395	128	>512	128	32	512	4
Post-exposure	19	**241R ins** ; T105A	--	OXA-395	128	>512	128	32	>512	4
Baseline	20	R79Q ; T105A	--	OXA-50	4	128	8	16	512	1
Post-exposure	20	R79Q ; T105A	--	OXA-50	8	128	16	32	>512	2
Baseline	25	R79Q ; T105A	--	OXA-396	4	64	16	32	256	2
Post-exposure	25	R79Q ; **G183D** ; T105A	--	OXA-396	>64	128	128	1	32	1
Post-exposure	25	R79Q ; T105A	-	OXA-396	4	128	16	32	256	2

Only patient 2 had a mutation of interest in the *ampR* sequence, however this could be due to the non-relatedness of the baseline isolate and therefore was not considered further for analysis. In 11 patients at least one mutation of interest was identified in the *ampC* sequence. Patient 1, 16, and 20 had no change in mutations from baseline to post-exposure isolates. However, these patients had a small ceftolozane-tazobactam fold-change of 4, 4, and 2-fold increase. The post-exposure MIC was 8 µg/mL which is 8-fold below the median ceftolozanetazobactam MIC (64 µg/mL). Mutation F147L and G183D were both found in 3 patients each and were the most frequent mutations of interest. In patients 18 and 13 F147 was the only mutation of interest identified and resulted in 64 and 256-fold increase. In patient 4 F147 and G183D were the mutations of interest and resulted in an 8-fold increase. Patient 25 had G183D as the only mutation of interest and experienced a 32-fold increase. Patient 9 had G183D and 243Gins (insertion) as the mutations of interest and resulted in a 128-fold increase. Additionally, patient 8 had a mutation at position 183, mutation G183R, and they experienced a 64-fold increase. Other notable mutations of interest occurred between positions 234-244. Patients 3, 7, 9, 10, 19 all had mutations of interest within these positions. MIC changes were also observable in Table 1. The changes discussed in previous hypothesis can be seen in a molecular context. Patient 9 and 25 are important examples. Patient 9 has observable stepwise MIC increases in ceftolozane-tazobactam, ceftazidime, and ceftazidime-avibactam, and decreases in piperacillintazobactam and imipenem that occur with mutation acquisition. Patient 25 is notable because the final isolate loses the mutation of interest and has a similar MIC susceptibility profile as the baseline isolate.

4.0 Discussion

4.1 Cephalosporins

P. aeruginosa readily developed ceftolozane-tazobactam resistance in infected patients. This resistance development led to cross resistance in cephalosporins. Cross resistance is extremely problematic when considering therapeutic options. When clinicians consider alternative treatments to accommodate resistance, they must also be aware of potential cross reactivity in order to prescribe effective treatment. Ceftazidime exhibited a 4-fold increase from baseline to postexposure isolates and ceftazidime-avibactam exhibited an 8-fold increase. Results shown in this study support the hypothesis that when ceftolozane-tazobactam resistance develops in MDR *P. aeruginosa* isolates, ceftazidime and ceftazidime-avibactam MICs will increase at least 4-fold. The 4-fold change was selected as the breakpoint change to confirm cross resistance because changes 2-fold or less could be due to random error. In addition, baseline isolates were 75% and 25% non-susceptible to ceftazidime and ceftazidime-avibactam which increased to 100% and 72.7% in post-exposure isolates. These data confirm cross resistance was occurring in both ceftazidime and ceftazidime-avibactam and therefore these antibiotics would not be good therapeutic options for clinicians. Figure 5 visually represents both ceftazidime and ceftazidimeavibactam trend together with ceftolozane-tazobactam. This also suggests avibactam is not an effective inhibitor drug combination. Comparatively, Figure 10 which has the MIC values and medians, shows trends are similar between baseline and post-exposure isolates for ceftazidime and ceftazidime-avibactam. There were outliers in the data: patient 5 had a 512-fold increase in ceftazidime and patient 23 had a 4-fold decrease in ceftazidime-avibactam. Patient 5 also had the

largest fold-change in ceftolozane-tazobactam (512-fold increase), which could be responsible for the outlier value in ceftazidime. Patient 23 was resistant to ceftazidime-avibactam at baseline but decreased to susceptible in the post-exposure isolate.

4.2 Carbapenems

Piperacillin-tazobactam had a median of 0-fold change from baseline to post-exposure isolates while imipenem had a median 2-fold decrease. However, both drugs had nearly bimodal distribution. Considering this, roughly 50% of patients supported my hypothesis when ceftolozanetazobactam resistance develops in MDR *P. aeruginosa* isolates, piperacillintazobactam and imipenem MICs will decrease at least 4-fold, while roughly 50% did not. There was not a uniform response to ceftolozane-tazobactam resistance development. These carbapenems were selected because as *ampC* mutates to hydrolyze ceftolozane-tazobactam, piperacillin-tazobactam and imipenem become less optimal substrates for *ampC*. As less optimal substrates for *ampC* piperacillin-tazobactam and imipenem, might demonstrated collateral sensitivity. Again, roughly 50% of patients demonstrated collateral sensitivity. Most patients that fell outside of the hypothesized fold-change were the same for piperacillin-tazobactam and imipenem. Additionally, all patients with *ampR* mutations fell outside the hypothesis fold-change range. These data suggest there is some molecular similarity between patients outside the hypothesis range. However, there was not a single commonality observed in all of the patients that fell outside the hypothesis range. As a result, further testing is needed to uncover the genetic reasons for some patients having a positive fold-change compared to those that had a negative fold-change.

4.3 Imipenem-relebactam

Imipenem-relebactam maintained potent *in vitro* activity between baseline and postexposure isolates. This was supported by both fold-change data and median MICs: no foldchange was observed, and the median MIC remained 2 µg/mL for baseline and post-exposure isolates. Additionally, only 21.7% of baseline isolates were found to be resistant to imipenemrelebactam, and only 12.5% of post-exposure isolates were resistant. Figure 11 visually represents how MICs remain unchanged despite development of ceftolozane-tazobactam resistance. These data support that relebactam has activity against *ampC* and a mutated *ampC,* and that imipenemrelebactam may be a viable therapeutic option for patients with ceftolozane-tazobactam resistant MDR *P. aeruginosa*.

4.4 *AmpC*

4.4.1 Relatedness

14 of 15 patients were considered to be related and the resulting post-exposure isolates likely evolved from the baseline isolates. Patient 2 was removed from analysis, because they had no baseline that was related to the two post-exposure isolates. No assumptions can be made about mutations in *ampC* or *ampR* in patient 2 that yield ceftolozane-tazobactam resistance. Patient 9 had two baselines however the post-exposure isolates likely evolved from one baseline isolate due to SNP variations. Therefore, assumptions about *ampC* and *ampR* mutations will be based on the more related baseline to the post-exposure isolates.

4.4.2 Mutations

Several different mutations were identified in *ampC* and *ampR* protein sequences. *AmpR* acts as a promotor for *ampC* and therefore was essential to examine mutation in the *ampR* sequence as it ultimately would affect the expression of the *ampC* protein (Livermore, 1982; Torrens, 2019). Other BL'ases were explored in order to identify if any isolates had metallo-beta-lactamases. Metallo-beta-lactamases have been shown to confer ceftolozane-tazobactam resistance (Karaiskos, 2019; Livermore, 2017). Therefore, it was necessary to rule out metallo-betalactamases as the agent conferring ceftolozane-tazobactam resistance. Since no isolates had metallo-betalactamases it can be assumed a different agent is responsible for this resistance development.

In 11 patients, at least one mutation of interest was identified in the *ampC* sequence. Notable mutations of interest were observed at F147L, position 183, and positions 234-244. Position 183 had two different mutations G183D and G183R. Therefore, it can be assumed position 183 is important in development of ceftolozane-tazobactam resistance. A previous study had found that the mutation G183D was responsible for ceftolozane-tazobactam resistance in their isolates (MacVane, 2017). Similarly, positions 234-244 had different mutations. These positions might fall in the omega loop of the *ampC* protein (Berrazeg, 2015). The omega loop is a known hotspot for mutations. Any mutations that occur here often effect the active site of *ampC*, therefore altering the overall function of *ampC*. However, further research is still needed into the importance of positions 234-244 to understand how it promotes ceftolozane-tazobactam resistance. Lastly, the largest fold-changes that occurred (256-fold increase) were F147L and deletion 237-243. This suggests that these two mutations are of particular interest in future studies. These data support the hypothesis that mutations in *ampC* are responsible for ceftolozane-tazobactam resistance in MDR *P. aeruginosa*.

4.5 Conclusions

These data supported all four of my hypotheses. Cross resistance was evident between ceftazidime and ceftazidime-avibactam and ceftolozane-tazobactam. Therefore, clinicians should not use ceftazidime or ceftazidime-avibactam to treat these MDR *P. aeruginosa* isolates. Collateral sensitivity was also evident in roughly half of patients for piperacillin-tazobactam and imipenem. There were bimodal distributions in piperacillin-tazobactam and imipenem which showed a non-uniform response to ceftolozane-tazobactam resistance development. More molecular research is necessary to identify commonalities to the isolates that responded as hypothesized compared to those isolates that responded differently than hypothesized. Additionally, imipenem-relebactam had potent *in vitro* activity against isolates before and after treatment with ceftolozane-tazobactam. Imipenem-relebactam is an effective treatment method for MDR P. aeruginosa isolates. Lastly, in 11 of 14 patients a mutation of interest was found in *ampC*, suggesting a mutated *ampC* is responsible for ceftolozane-tazobactam resistance.

However, these data also leave more questions. Moving forward I wish to recreate mutations seen in these isolates into the PAO1 strain. This method will allow me to use Koch postulates to further support my hypotheses. After recreating these mutations, I will observe if similar resistance and fold-changes occur. I hypothesize creating mutant *ampC* in isolates will yield ceftolozane-tazobactam resistant MICs due to these mutations being found in resistant ceftolozane-tazobactam isolates.

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