Genomic Characterization of Multi-Drug Resistant Pseudomonas aeruginosa Clinical Isolates: Evaluation and Determination of Ceftolozane/Tazobactam Activity and Resistance Mechanisms

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Resistance to ceftolozane/tazobactam (C/T) in Pseudomonas aeruginosa is a health concern. In this study, we conducted a whole-genome-based molecular characterization to correlate resistance patterns and β-lactamases with C/T resistance among multi-drug resistant P. aeruginosa clinical isolates. Resistance profiles for 25 P. aeruginosa clinical isolates were examined using disk diffusion assay. Minimal inhibitory concentrations (MIC) for C/T were determined by broth microdilution. Whole-genome sequencing was used to check for antimicrobial resistance determinants and reveal their genetic context. The clonal relatedness was evaluated using MLST, PFGE, and serotyping. All the isolates were resistant to C/T. At least two β-lactamases were detected in each with the blaOXA-4, blaOXA-10, blaOXA-50, and blaOXA-395 being the most common. blaIMP-15, blaNDM-1, or blaVIM-2, metallo-β-lactamases, were associated with C/T MIC >256 μg/mL. Eight AmpC variants were identified, and PDC-3 was the most common. We also determined the clonal relatedness of the isolates and showed that they grouped into 11 sequence types (STs) some corresponding to widespread clonal complexes (ST111, ST233, and ST357). C/T resistance was likely driven by the acquired OXA β-lactamases such as OXA-10, and OXA-50, ESBLs GES-1, GES-15, and VEB-1, and metallo- β-lactamases IMP-15, NDM-1, and VIM-2. Collectively, our results revealed C/T resistance determinants and patterns in multi-drug resistant P. aeruginosa clinical isolates. Surveillance programs should be implemented and maintained to better track and define resistance mechanisms and how they accumulate and interact.

Keywords: ceftolozane/tazobactam (C/T), Pseudomonas aeruginosa, AmpC, porins, beta lactamases
INTRODUCTION

Antimicrobial resistance has been a soaring global health care tolling problem (Tacconelli et al., 2018; Moghnieh et al., 2019). *Pseudomonas aeruginosa* is one of the leading multidrug resistant (MDR) nosocomial pathogens worldwide and defined by the World Health Organization as a critical health concern with limited effective treatment options and is associated with poor clinical outcomes (Weiner et al., 2016; Tacconelli et al., 2018). MDR *P. aeruginosa* isolates have a broad variety of mechanisms mediating antimicrobial resistance (Gellatly and Hancock, 2013; López-Causapé et al., 2018). These include the up-regulation of efflux pumps, loss of outer membrane porins, the production of AmpC, extended-spectrum β-lactamases (ESBLs) and carbapenemases, and modification of antimicrobial target sites (Lister et al., 2009; Zavascki et al., 2010; Bassetti et al., 2019). The overproduction of AmpC β-lactamase was also linked to cephalosporins resistance and which was not reversed by β-lactamase inhibitors such as tazobactam and clavulanic acid (Sligl et al., 2015).

Two drug combinations were developed to treat infections caused by resistant Gram-negative bacteria, namely ceftazidime/avibactam and ceftolozane/tazobactam (C/T) (Sold under the brand name Zerbaxa) (Wright et al., 2017). Ceftolozane is a cephalosporin derivative of ceftazidime with an intrinsic broad activity and is not hydrolyzed by most broad-spectrum β-lactamases such as ESBLs and AmpCs (van Duin and Bonomo, 2016). Ceftolozane is particularly active against *P. aeruginosa* exhibiting AmpC efflux pumps overexpression (Moyá et al., 2012; Giacobbe et al., 2018), and has a heavier pyrazole substituent at the 3-position side chain instead of the lighter pyridinium in ceftazidime, enhancing steric hindrance and exhibiting AmpC efherence and is not hydrolyzed by most broad-spectrum β-lactamase inhibitors such as tazobactam and clavulanic acid (Sligl et al., 2015).

The susceptibilities of the isolates, and conducting a genome-based molecular characterization.

**MATERIALS AND METHODS**

**Ethical Approval**

Ethical approval was not required. The isolates were collected as part of routine clinical care and patient data collection. No additional isolates were collected beyond those obtained from routine clinical care, and no diagnostic or treatment decisions were affected by the outcomes of this study.

**Bacterial Isolates and Identification**

A total of 25 *P. aeruginosa* isolates were collected and identified by the Matrix-Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF) system (Bruker Daltonik, GmbH, Bremen, Germany) at the clinical microbiology laboratory of the American University of Beirut Medical Center (AUBMC), Beirut, Lebanon between December 2017 and November 2018. AUBMC is around 350-bed tertiary care major hospital in the country. The isolates were designated as ZBX-P1 to ZBX-P25.

**Antimicrobial Susceptibility Tests**

Antimicrobial susceptibility was tested against 10 antibiotics including C/T, amikacin, aztreonam, ceftazidime, ciprofloxacin, gentamicin, imipenem, tazobactam and colistin. Data obtained were interpreted according to the CLSI guidelines (CLSI, 2022). For C/T breakpoints in *P. aeruginosa*, the established EUCAST (2022) (EUCAST, 2022) clinical zone diameter breakpoints were followed; S ≥ 24 mm and R < 24 mm and the MIC breakpoints of S ≤ 4 µg/L and R > 4 µg/mL. For disk diffusion, the disk content was set at CXA-TAZ 30-10 µg, by both EUCAST and CLSI (CLSI, 2022; EUCAST, 2022).

**Pulsed-Field Gel Electrophoresis (PFGE)**

PFGE fingerprinting was performed using the SpeI restriction enzyme (ThermoScientific, Waltham, MA, USA), 1% SeaKem agarose gel, and the universal laboratory standard *Salmonella enterica* subsp. enterica serovar Braenderup (ATCC® BAA664™) digested with XbaI restriction enzyme according to the standard PulseNet protocol (http://www.pulsenetinternational.org). Electrophoresis was performed using the Bio-Rad laboratories CHEF DR-III system (Bio-Rad Laboratories, Hercules, CA, USA) with a run time of 16 h and switch time of 5–40 s (https://www.cdc.gov/pulsenet/). Gels were stained with ethidium bromide. PFGE profiles were analyzed with the BioNumerics software version 7.6.1 (Applied Maths, Sint-Martens-Latem, Belgium), with banding patterns showing a difference in three or more bands being placed under distinct pulsotypes (Tenover et al., 1995). Pulsotypes were clustered using the BioNumerics software version 7.6.1 (Applied Maths, Sint-Martens-Latem, Belgium) with an optimization of 0.5% and tolerance of 0.5%.
Whole-Genome Sequencing
DNA extraction was performed using the NucleoSpin Microbial DNA kit (Macherey-Nagel, Germany) according to the manufacturer’s instructions followed by long-read sequencing of the isolates (ZBX-1 to ZBX-25). PacBio long-read sequencing on the Sequel I platform (Pacific Biosciences, CA, USA) was performed. Library preparation was according to the manufacturer’s instructions for microbial isolate multiplexing. G-tubes (Covaris, USA) were used for DNA shearing and no size selection was performed. Resulting contigs were polished with Pilon, v1.23 (Walker et al., 2014), and the overlapping ends of chromosomes were trimmed after manual inspection of reads mapped by BWA-MEM algorithm as implemented in BWA, with minimum seed coverage of 30x (Chin et al., 2013).

Genome Assembly
Genomes were annotated using the RAST server (http://rast.nmpdr.org) (Aziz et al., 2012). Resfinder (Zankari et al., 2012), the Comprehensive Antibiotic Resistance Database CARD v3.1.0 (Alcock et al., 2020), MLST v1.8 (Larsen et al., 2012), ISfinder database (Siguier et al., 2006), PlasmidFinder (Carattoli et al., 2014) and BLASTn (Wheeler et al., 2003) were used to identify resistance genes, sequence types (STs), ISs, and plasmid incompatibility group identification and manual curation of annotations, respectively. The serotypes of the isolates were determined using the PAst v1.0. (Thrane et al., 2016) PAst annotations, respectively. The serotypes of the isolates were incompatibility group identification and manual curation of annotations, respectively. The serotypes of the isolates were determined using the PAst v1.0. (Thrane et al., 2016) PAst types were split into 12 serogroups covering the 20 serotypes determined using the PAst v1.0. (Thrane et al., 2016) PAst annotations, respectively. The serotypes of the isolates were grouped into eleven STs respectively. The serotypes of the isolates were grouped into eleven STs (Thrane et al., 2016). Genome assembly was done using HGAP4 with minimum seed coverage of 30x (Chin et al., 2013).

Porin Analysis
Sequences of 27 genes encoding for porins were extracted from P. aeruginosa PAO1 reference genome (Accession no. GCF_000006765.1) available on the Pseudomonas Genome Database (https://pseudomonas.com/) (Winsor et al., 2016). These sequences were used to build a dataset in MyDbFinder 2.0 (https://cge.cbs.dtu.dk/services/MyDbFinder/). Genomes were blasted against the dataset using a 60% ID threshold and 40% minimum length for comparison.

Data Availability
The Whole Genome Shotgun project was deposited at DDBJ/ENA/GenBank under the accession numbers presented in S1 Table.

RESULTS
Patient Data
The mean patients’ age was 62.3 ± 18.9 years old. 64% (n=16) were males and 36% (n=9) were females and were collected from various sites of infection including: deep tracheal aspirate (DTA) (32%; n=8), urine (32%; n=8), wound (12%; n=3), sputum (12%; n=3), fluids, bone, and blood (4%; n=1).

Genome Statistics and Isolate Genotyping
The average genome size of the sequenced isolates was 6,972,377 ± 255,784 bp, and the average G+C content was 65.9 ± 0.2%. The clonal relatedness was evaluated using MLST, PFGE, and serotyping. The isolates were grouped into eleven STs (Figure 1) (one new: ST-3425). ST111 (n=4), ST654 (n=1), ST308 (n=1), and ST357 (n=4) are widespread “high-risk” clones (Woodford et al., 2011; Oliver et al., 2015; Papagiannitsis et al., 2017). The isolates were distributed within seven serotypes and 23 pulsotypes (PT1 to 23) showing ≥84% similarity based on PFGE (Figure 1).

Antibiotic Susceptibilities
All the isolates were resistant to C/T with MICs ranging between 2 to >256 μg/mL, while most also showed resistance to the other tested β-lactams including ceftazidime (88%; n=22) and imipenem (68%; n=17) (Figure 2). All the isolates were susceptible to colistin, followed by ciprofloxacin (68%; n=17), gentamicin (n=13; 52%) and amikacin (n=11; 44%) (Figure 2).

Resistance Genomics
Whole-genome sequencing was used to check for antimicrobial resistance determinants and reveal their genetic context (Figure 3 and Table 1). Between two to four β-lactamases were detected in the sequenced genomes; all were chromosomal except for blaGES-15 (Table 1). blaIMP-15, blaNDM-1, or blaVIM-2, metallo-β-lactamases, were associated with C/T MIC >256 μg/mL. Other β-lactamases were also detected with the most common being blaOXA-39s, blaOXA-50, blaOXA-4, and blaOXA-10, respectively (Figure 3). MIC distributions are shown in (Figure 2). All C/T resistant isolates with MIC >256 were either intermediate (n=1) or resistant to ceftazidime (n=15). Of these, nine had four β-lactamases with one being a metallo-β-lactamase.

All the isolates were intrinsic AmpC producers showing eight ampC-type variants (PDC) (Figure 3). We checked for polymorphisms in the AmpR (blaPDC, transcriptional regulator) aligning against PA4109 in P. aeruginosa PAO1 reference strain. Several substitutions were detected including D135N in isolates with C/T MIC < 256 μg/mL (ZBX-P9 MIC: 8 μg/mL, ZBX-P11 (MIC: 4 μg/mL, and ZBX-P24 MIC: 12 μg/mL), and another restricted to the ones typed as ST111 (E287G C/T MIC > 256 μg/mL). We also looked for OprD polymorphisms and detected the characteristic mutation (Q142X) in ZBX-P12, ZBX-P16, and ZBX-P23 (all with C/T MIC > 256 μg/mL).

Furthermore, blaNDM-1 along with floR and msrE were on a chromosomal 74.2 kb integrative and conjugative element (ICE) ICEPaP4371:6385. blaOXA-48s was also chromosomal, while blaVIM-2, blaOXA-4, and blaOXA-16 were all detected on chromosomal class I integrons, and blaGES-15 on IncP-6 plasmid (Table 1).

Finally, we compared 27 porin encoding genes with that of P. aeruginosa PAO1. The highest variability was observed in oprD, opdP (PA4501 and PA1025), and oprQ. Deletions and premature stop codons (truncations) were detected throughout
FIGURE 1 | β-lactamases and AmpC antibiotic resistance genes identified in the isolates. Isolate’s ST is also shown.

FIGURE 2 | PFGE dendrogram and Serotype in the C/T P. aeruginosa isolates. Dendrogram was generated by BioNumerics software version 7.6.1 showing the relationship of the isolates based on their banding patterns generated by SpeI restriction digestion. Isolates’ STs, and pulsortypes (PT) are shown.
OprD, and the characteristic OprD mutation (Q142X) associated with C/T resistance (Fraile-Ribot et al., 2018) was found in ZBX-P12 and ZBX-P16, and ZBX-P23 (C/T MIC > 256 µg/mL).

**DISCUSSION**

Choosing the appropriate antimicrobial agent to treat infections caused by resistant bacteria would significantly decrease infection-linked morbidity and mortality. C/T has increased activity against resistant *P. aeruginosa* isolates and is an important treatment option in institutions with high rates of pseudomonal infections (Humphries et al., 2017; Hirsch et al., 2020). The emergence of C/T resistance in MDR/XDR *P. aeruginosa* isolates, resistant to most β-lactams and having several resistance mechanisms, is very likely to happen. In this study, we used long-read whole-genome sequencing approach to study the resistance genomics and clonal relatedness of 25 C/T resistant *P. aeruginosa*.

The isolates belonged to 11 STs and 23 pulsotypes, exhibited C/T MIC of 1.5 to > 256 µg/mL, showed resistance to the other tested β-lactams including ceftazidime (88%; n=22), ciprofloxacin (68%; n=17), and imipenem (68%; n=17). Moreover, isolates had two to four β-lactamases and 10 were positive for *bla*<sub>IMP-15</sub>, *bla*<sub>NDM-1</sub>, or *bla*<sub>VIM-2</sub>, and were intrinsic AmpC producers.

PDC variants in this study were assigned according to substitutions in AmpC described by Rodriguez-Martinez et al. (2009) and accordingly we identified eight *ampC* variants, PDC-3 being the most common. T79A (T105A non-processed peptide) detected in PDC-3 (Rodríguez-Martínez et al., 2009), was previously found to be prevalent in carbapenem-resistant clinical isolates. Berrazeg et al. (2015), however, overexpressed PDC-3 (T79A) in porin OprD-negative strain to test if it would potentiate the AmpC action and revealed that T79A variation doesn’t broaden the substrate spectrum of AmpC (Berrazeg et al., 2015). They concluded that PDC common polymorphisms had negligible impact on AmpC activity while confirming that mutations occurring in specific regions of the substrate-binding pocket could enhance the catalytic efficiencies and as a result increase the hydrolytic activity of *P. aeruginosa* AmpC on cephalosporins including ceftolozane. On the other hand, Fernández-Esgueva et al. (2020) showed that C/T resistance was associated with AmpC mutations including a novel one (PDC-388; G183V) (Fernández-Esgueva et al., 2020) and in line with this Fraile-Ribot et al. (2018) also cloned AmpC variants (PDC-221, 222, and 223) in *ampC*-deficient derivative of PAO1 (Fraile-Ribot et al., 2018). The cloned AmpC variants showed increased ceftolozane/tazobactam and ceftazidime/avibactam MICs compared with wild type AmpC with the associated polymorphisms being located within the Ω loop and selected mutations. **FIGURE 3** | Detailed phenotypic and genotypic resistance information of the C/T resistant *P. aeruginosa* isolates. ST, Sequence type. Classes of antibiotic resistance genes are marked as follows: aminoglycoside, phenicol, macrolide, tetracycline, fosfomycin, quinolone, rifampicin, trimethoprim, and sulphonamide resistance genes. MIC, Minimum inhibitory concentration (µg/ml), DD, Diffusion diameter (mm). Black, present.
in vitro upon C/T exposure (Cabot et al., 2014; Haidar et al., 2017). We detected the characteristic OprD mutation (Q142X) associated with C/T resistance (Fraile-Ribot et al., 2018) in three of the study isolates, ZBX-P12, ZBX-P16, and ZBX-P23 (C/T MIC > 256 μg/mL).

Moreover, C/T resistance emergence was previously reported in isolates producing horizontally acquired β-lactamases such as OXA-10 and OXA-2 (Fraile-Ribot et al., 2018), and ESBLs (Ortiz de la Rosa et al., 2019). In general, C/T may be ineffective against isolates carrying carbapenemases including class A and class D (OXA) β-lactamases and it’s inactive against metallo-β-lactamases (Hirsch et al., 2020; Karlowsky et al., 2021). Extended-spectrum OXAs were also noted as infrequent cause of ceftolozane. Horizontally acquired β-lactamases IMP-15, NDM-1, or OXA-lactamases including OXA-10, OXA-50, ESBLs GES-1, GES-15 and VEB-1, and metallo-β-lactamases IMP-15, NDM-1, and VIM-2. Collectively, our results highlight the need to maintain active surveillance programs to better track and define resistance mechanisms and how they accumulate and interact.

Infections caused by MDR P. aeruginosa could be treated with C/T, clinical studies evaluating optimal dosing and using combined therapy are recommended (Fraile-Ribot et al., 2018). Mutations occurring within the substrate-binding pocket could increase the hydrolytic activity of P. aeruginosa AmpC on cephalosporins including ceftolozane. Horizontally acquired β-lactamases IMP-15, NDM-1, or OXA-lactamases such as OXA-10, OXA-50, ESBLs GES-1, GES-15 and VEB-1, and metallo-β-lactamases IMP-15, NDM-1, and VIM-2. Collectively, our results highlight the need to maintain active surveillance programs to better track and define resistance mechanisms and how they accumulate and interact.

### DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.
AUTHOR CONTRIBUTIONS

GA: Conceptualization, Validation, Writing – review and editing. ST: Conceptualization, Funding acquisition, Project administration, Supervision, Validation, Writing – review and editing. IB: Funding acquisition, Investigation, Methodology, Writing – review and editing. TS: Investigation, Methodology, Visualization, Writing – original draft, Writing – review and editing. GM: Investigation, Writing – review and editing. JH: Investigation, Methodology. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ficmb.2022.922976/full#supplementary-material

Supplementary Table 1 | List of the isolates’ accession numbers.

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