Evolving landscape of carbapenem-resistant *Pseudomonas aeruginosa* at a single centre in the USA

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Objectives: The increased identification of carbapenem-resistant *Pseudomonas aeruginosa* (CR-PA) is an ongoing concern. However, information on the evolving antimicrobial resistance profile and molecular epidemiology of CR-PA over time is scarce. Thus, we conducted a cross-sectional analysis to investigate the phenotypic and genotypic characteristics of CR-PA recovered over different time periods, focusing on the isolates exhibiting a ceftolozane/tazobactam resistance phenotype.

Methods: A total of 169 CR-PA isolated from clinical specimens at a single centre in Houston, TX, USA were studied. Among them, 61 isolates collected between 1999 and 2005 were defined as historical strains, and 108 collected between 2017 and 2018 were defined as contemporary strains. Antimicrobial susceptibilities against selected β-lactams was determined. WGS data were used for the identification of antimicrobial resistance determinants and phylogenetic analysis.

Results: Non-susceptibility to ceftolozane/tazobactam and ceftazidime/avibactam increased from 2% (1/59) to 17% (18/108) and from 7% (4/59) to 17% (18/108) from the historical to the contemporary collection, respectively. Carbapenemase genes, which were not identified in the historical collection, were harboured by 4.6% (5/108) of the contemporary strains, and the prevalence of ESBL genes also increased from 3.3% (2/61) to 16% (17/108). Genes encoding acquired β-lactamases were largely confined to the high-risk clones. Among ceftolozane/tazobactam-resistant isolates, non-susceptibility to ceftazidime/avibactam, imipenem/relebactam and cefiderocol was observed in 94% (15/16), 56% (9/16) and 12.5% (2/16), respectively. Resistance to ceftolozane/tazobactam and imipenem/relebactam was primarily associated with the presence of exogenous β-lactamases.

Conclusions: Acquisition of exogenous carbapenemases and ESBLs may be a worrisome trend in *P. aeruginosa*.

Introduction

Antimicrobial resistance is a serious threat to global public health, estimated to cause more than 2.8 million antibiotic-resistant infections and 35 000 deaths annually in the USA alone.[1] *Pseudomonas aeruginosa* is one of the most important healthcare-associated opportunistic pathogens, frequently implicated in infections in critically ill or immunocompromised patients, causing pneumonia, urinary tract infections and surgical site infections, among others. Infections due to *P. aeruginosa* often pose a great therapeutic challenge, since these organisms possess intrinsic resistance to a variety of antimicrobial agents.[2] Furthermore, *P. aeruginosa* can acquire resistance via mutation of core genes or acquisition of determinants through horizontal gene transfer of mobile genetic elements.[3]

Carbapenems have been a major therapeutic option for serious infections due to *P. aeruginosa*; however, carbapenem resistance is a growing problem, with 13.3% of *P. aeruginosa* isolates

related to healthcare-associated infections reported to the National Healthcare Safety Network (NHSN) showing carbapenem resistance in 2019.[4] In the USA, resistance to carbapenems in *P. aeruginosa* arises mainly through mutational processes that alter the expression and/or function of chromosomal genes encoding mainly the outer membrane porin OprD, and multidrug efflux pumps.[2] In contrast, resistance through the acquisition of carbapenemases is more prevalent outside of the USA.[3] In addition to carbapenem resistance, isolates displaying MDR or XDR phenotypes have been increasingly documented across the world, especially linked to global dissemination of high-risk clonal lineages, such as ST235, ST111 and ST175. These high-risk clones tend to harbour transmissible genetic determinants containing multiple resistance elements, particularly those encoding class B carbapenemases (MBLs; e.g. VIM, IMP) and ESBLs (e.g. VEB, PER, GES), as well as aminoglycoside-modifying enzymes (AMEs).[5,6] The production of carbapenemases and selected ESBLs substantially affects the susceptibility to ceftolozane/tazobactam, a last-resort antimicrobial agent for the treatment of drug-resistant *P. aeruginosa*.[7-9] In fact, we previously reported two strains of XDR *P. aeruginosa* belonging to ST309, harbouring the ESBLs bla_{GES-19} and bla_{GES-26} genetically clustered in tandem on a chromosomal class 1 integron, exhibiting high-level resistance to all β-lactam agents available at the time, including ceftolozane/tazobactam.[10]

Surveillance studies have been conducted at various locations to understand the local epidemiology of carbapenem-resistant *P. aeruginosa* (CR-PA).[11-13] However, information is scarce on how the molecular epidemiology and antimicrobial resistance profile of CR-PA changes over time, which would provide essential insights into the evolutionary dynamics of this 'difficult-to-treat' microorganism as new treatments are introduced. Hence, using high-throughput sequencing technology, we conducted a cross-sectional study to investigate phenotypic and genotypic characteristics of CR-PA strains collected between 1999 and 2015 (i.e. historical collection) and those between 2017 and 2018 (i.e. contemporary collection) at a large urban hospital network in Houston, TX, USA, especially focusing on the isolates exhibiting a phenotype of resistance to ceftolozane/tazobactam.

Methods

Study setting and bacterial isolates

A total of 169 non-duplicate CR-PA isolates, which were recovered from various clinical sites between 1999 and 2018 at a large urban hospital network in Houston, consisting of 15 distinct hospitals, and initially reported as non-susceptible to at least one anti-pseudomonal carbapenem (meropenem or imipenem), were used in this study. These isolates had been collected for surveillance purposes without a predefined criterion until 2017 when CR-PA isolates were starting to be systemically collected, following the emergence of XDR-PA in the hospital network.[10] The isolates were identified as *P. aeruginosa* by the standard microbiological procedure in the clinical microbiological laboratory. Among them, 61 isolates collected between 1999 and 2015 were defined as historical strains, of which one isolate was collected in 1999, 54 between 2004 and 2005, and 6 between 2010 and 2015. A total of 108 isolates collected between 2017 and 2018, when ceftolozane/tazobactam became widely available in clinical practice, were defined as contemporary strains, of which 4 strains were isolated in 2017 and 104 in 2018.

Antibiotic susceptibility testing

Frozen stocks of the isolates were steaked onto cetrimide agar plates containing meropenem (1 μL/mL) and grown overnight at 37°C to maintain the carbapenem-resistant phenotype. MICs of selected β-lactam agents (meropenem, aztreonam, ceftolozane/tazobactam and ceftazidime/avibactam) were determined by Etest strips (bioMérieux, Marcy l'Étoile, France) in accordance with the manufacturer's instructions, and the results were interpreted according to the CLSI criteria.[14] For strains that were resistant to ceftolozane/tazobactam, additional testing for susceptibility to imipenem/relebactam (via Etest) and cefiderocol (broth microdilution using iron-depleted Mueller–Hinton broth) was performed. *P. aeruginosa* PAO1 (ATCC 15692) was used as a quality control strain.

DNA preparation and WGS

Strains were incubated in lysogenic broth (LB) medium containing meropenem (1 μL/mL) at 30°C for 3–6 h, using a shaking incubator. Genomic DNA was extracted using the DNeasy blood and tissue kit (QIAGEN, Crawley, West Sussex, UK) as per the manufacturer's instructions. WGS was performed on all isolates with the MiSeq platform with 2×300 paired-end reads (Illumina, Inc., San Diego, CA, USA), with selected isolates also sequenced on GridION X5 with an R9.4.1 flow cell (Oxford Nanopore Technologies, Oxford Science Park, UK) for long reads to close the genome and plasmids. *De novo* genome assembly was performed with SPAdes (version v3.13.1) and annotations done with RAST.[15] Hybrid assembly was executed with a custom pipeline, utilizing Flye for initial long-read draft assembly with subsequent short-read polishing.[16] The assembly was then annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP), and additional antimicrobial resistance gene identification was carried out with NCBI Antimicrobial Resistance Gene Finder Plus (AMRFinderPlus).[17,18] Minimap2 was used for alignment of contigs within each assembly, and the resultant genomic maps were constructed using the circlize and gggenomes packages in R (v4.4.1).[19-21]

Identification of antimicrobial resistance genes and phylogenetic analysis

Acquired resistance genes were identified using ResFinder AMRFinder,[22] selecting hits with an identity percentage higher than 90% and a coverage higher than 80%. Selected chromosomal genes associated with antimicrobial resistance were identified via BLAST,[23] then underwent *in silico* translation with pairwise alignment of both nucleotide and predicted amino acid sequences using PAO1 as a reference. These chromosomal genes included those mediating β-lactam resistance via the *ampC* pathway (*ampC*, *ampD*, *ampDh2*, *ampDh3*, *ampG*, *ampR*, *dacB*, *dacC*, *pbpA*, *mpl*), genes involved in efflux systems (*mexA*, *mexB*, *oprM*, *mexR*, *mexT*, *parS*, *parR*, *mexS*, *mex E*, *mexF*, *oprN*, *nalD*, *nalC*, *mexC*, *mexD*, *oprJ*, *nfxB*, *mexX*, *mexY*, *mexZ*, *armZ*), *oprD* porin gene and those in the QRDRs of *gyrA*, *gyrB*, *parC* and *parE*. STs were identified by the MLST tool (https://github.com/tseemann/mlst).[24] Core genomes were determined using Roary16,[25] aligned with Muscle[26] and then concatenated. A maximum-likelihood phylogenetic tree was constructed with RAxML with 100 bootstrap resampling and plotted with iTOl.[27,28]

Statistical methods

Binary variables were analysed with the chi-squared test or Fisher's exact test. All *P* values were two-sided and *P* values <0.05 were considered statistically significant. The statistical analysis was performed using STATA 15.1.

Results

Antimicrobial susceptibility

The susceptibility to the selected β-lactams of 167 CR-PA, composed of 59 historical strains and 108 contemporary strains, are

shown in Figure 1; individual strain MICs are listed in Table S1 (available as Supplementary data at *JAC-AMR* Online). The susceptibility testing results were not available for two isolates from the historical collection due to poor growth. While all strains were reported as carbapenem resistant in the clinical laboratory, 20% of the historical collection and 6% of the contemporary collection were carbapenem susceptible on repeat testing in the research laboratory. A higher rate of resistance to antipseudomonal β-lactam agents, including the newer β-lactam/β-lactamase inhibitor combinations ceftolozane/tazobactam and ceftazidime/avibactam, was observed in the contemporary collection. The non-susceptibility rate to ceftolozane/tazobactam and ceftazidime/avibactam increased from 2% to 17% ($P < 0.01$) and from 7% to 17% ($P = 0.07$) from the historical to the contemporary collection, respectively, with only the former being statistically significant.

Antimicrobial resistance profiling: acquired resistance genes

At least one acquired β-lactamase gene was detected in 26% of the isolates in both the contemporary (28/108) and historical collections (16/61) (Table 1, Tables S1 and S2). While the most prevalent acquired β-lactamase was OXA-9 (21%; 13/61), a narrow-spectrum oxacillinase, in the historical collection, OXA-15 (9.3%; 10/108) was most frequently identified in the contemporary collection, which is an extended-spectrum variant of OXA-2. Presence of OXA-9 and CARB-3 was associated with the ST111 strains. Acquired carbapenemase genes were identified only in the contemporary collection, and all of them were MBLs: bla_{VIM-2} (four isolates), bla_{VIM-5} (one isolate) and bla_{NDM-1} (one isolate). Additionally, genes encoding acquired ESBLs were more common in the contemporary collection compared with the historical collection [17% (18/108) versus 3.3% (2/61), $P = 0.01$]. ESBL genes identified in the contemporary collection were bla_{OXA-15} (10 isolates), bla_{GES-19} (3 isolates), bla_{GES-26} (3 isolates), bla_{OXA-10} (2 isolates), bla_{OXA-21} (1 isolate), bla_{VEB-1a} (2 isolates) and $bla_{OXA-226}$ (2 isolates). Meanwhile, the prevalence rates of acquired aminoglycoside resistance genes [not including *aph(3')-IIb*], such as *aadA6*, *aadA2* and *rmtB*], were higher in the historical collection (43%; 26/61) compared with the contemporary collection (25%; 27/108), and these aminoglycoside resistance determinants were more likely to be associated with the presence of genes encoding exogeneous β-lactamases ($P < 0.001$). The acquired colistin resistance gene, *mcr-5*, and quinolone resistance gene, *qnrVC1*, remained rare in both historical and contemporary collections, with prevalence rates of 0.6% and 1.2% in the whole studied population, respectively.

Antimicrobial resistance profiling: intrinsic resistance genes

All isolates harboured the chromosomally encoded bla_{OXA-50}, *fosA* and *aph(3')-IIb* except for eight isolates. Twenty-three *Pseudomonas*-derived cephalosporinases (PDC) variants, which are the chromosomally encoded class C cephalosporinases (AmpC β-lactamase), were present in the studied population. Among them, bla_{PDC-3} and bla_{PDC-35} were the predominant variants in both contemporary and historical collections, comprising 39% and 64% of the AmpC variants in each collection, respectively. While bla_{PDC-35} was exclusively detected in ST235, bla_{PDC-3} was

found mainly in ST111. Deletion/insertion of sequences in genes encoding AmpR, AmpD and PBP4 (*dacB*), which have been described to regulate PDC expression, were observed in 15% (25/169), 13% (21/169) and 6.0% (10/169) of the studied population, respectively (Table S1). The occurrence of these mutations in the AmpC regulator genes did not differ significantly between the contemporary and historical collections [28% (30/108) versus 36% (22/61), $P = 0.26$]. Additionally, 80% of the isolates harboured frameshift mutations, premature stop codons, insertions, or deletions in the *oprD* gene, whose functional loss is a major contributor in mediating carbapenem resistance. No difference was observed in the prevalence of *oprD* mutations between the contemporary and historical collections [81% (87/108) versus 79% (48/61), $P = 0.77$]. Deletion or insertion mutations in genes encoding MexR and MexZ, which regulate expression of MexAB-OprM and MexXY-OprM multidrug efflux pump, respectively, was detected in 7.7% (13/169) and 23% (39/169) of the contemporary versus historical collections, respectively.

In silico MLST and phylogenetic analysis

A total of 169 CR-PA were grouped into 39 STs, of which, 32 isolates belonged to novel STs (Figure 2). ST235 was the most dominant clonal lineage (22%; 37/169), followed by ST111 (13%; 22/169), and they were the two most prevalent STs in both contemporary and historical collections (Table S3). While well-recognized high-risk clones, such as ST235, ST111, ST244, ST274, ST298, ST308, ST357, ST654 and ST773,[3,29] composed the majority of the historical isolates (69%; 42/61), the contemporary collection comprised a wider variety of STs, with lower representation of high-risk clonal lineages (39%; 42/108). Genes encoding acquired β-lactamases were largely confined to the high-risk clones compared with other clones [46% (39/84) versus 5.9% (5/85), $P < 0.01$] (Table S3). More specifically, carbapenemase (i.e. MBL) producers were primarily observed in ST111, while ESBL-encoding genes were detected in high-risk clones: ST235 (10 isolates), ST298 (3 isolates) and ST357 (2 isolates), as well as ST309 (3 isolates). Acquired aminoglycoside resistance determinants (i.e. AMEs, RmtB) were also more frequently observed in high-risk clones than non-high-risk clones [56% (47/84) versus 7.1% (6/85), $P < 0.01$].

Ceftolozane/tazobactam-resistant isolates

A summary of the phenotypic and genomic characteristics of the 16 ceftolozane/tazobactam-resistant isolates is presented in Figure 3, all of which were from the contemporary collection except for one isolate. Non-susceptibility to ceftazidime/avibactam, imipenem/relebactam and cefiderocol was observed in 94% (15/16), 56% (9/16, 1-I, 8-R) and 12.5% (2/16, 1-I, 1-R) of the ceftolozane/tazobactam-resistant strains, respectively. The predominant STs were ST111 (31%; 5/16) followed by ST309 (19%; 3/16), ST235 (13%; 2/16) and ST 357 (13%; 2/16). Acquired carbapenemase (MBL) genes were detected in 31% (5/16) of the isolates, which likely accounted for the ceftolozane/tazobactam resistance phenotype. Long-read sequencing was used to resolve the context of the MBLs in the isolates PA_HTX70, PA_HTX119 and PA_HTX147 (Figure S1). For PA_HTX70, a copy of the bla_{VIM-2} gene was carried in a class 1 integron on the chromosome associated with a Tn*3*-like element, as well as on a closed 159 kb circular
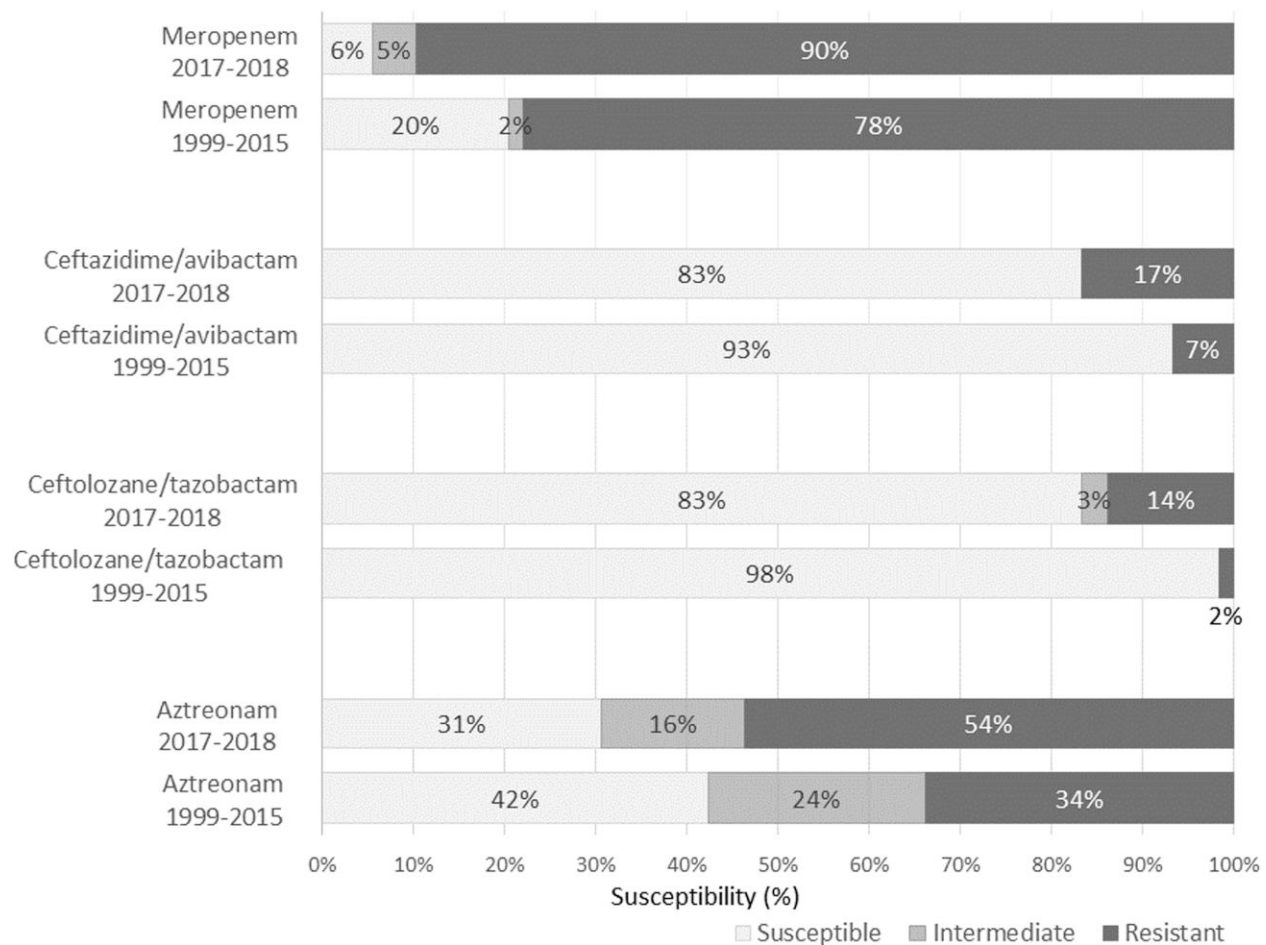
Figure 1. Susceptibility test results of CR-PA isolates collected during 1999–2015 (historical collection) and 2017–18 (contemporary collection). Antimicrobial susceptibility testing was performed by Etest, and the results were interpreted according to CLSI criteria (M-100, 31st edition). Percentages of susceptible (light-grey bars), intermediate (grey bars) and resistant (black bars) strains to the indicated antimicrobial agents are displayed.

contig likely representing a plasmid. PA_HTX119 carried the bla_{VIM-2} gene in a class 1 integron on the chromosome in a Tn*3*-like transposable element similar to that from PA_HTX70. PA_HTX147 carried a chromosomal copy of bla_{NDM-1} and the *rmtB4* aminoglycoside resistance determinant, as well as bla_{VIM-5} and bla_{OXA-21} on a plasmid. Additionally, 44% (7/16) of the isolates harboured genes encoding ESBLs, such as bla_{GES-19}, bla_{GES-26}, bla_{VEB-1a}, bla_{OXA-10}, bla_{OXA-15} and $bla_{OXA-226}$. Among them, bla_{GES} or bla_{VEB}, which are known to confer resistance to ceftolozane/tazobactam, were observed in five isolates.[30] The impact of OXA-15 and OXA-226, extended-spectrum oxacillinases derived from OXA-2, on the activity of ceftolozane/tazobactam is yet to be characterized. Resistance to imipenem/relebactam was associated with the presence of MBLs and the GES enzymes. Overall, cefiderocol maintained *in vitro* activity against most ceftolozane/tazobactam-resistant strains. The one resistant isolate carried three exogenous β-lactamases (NDM-1, VIM-5 and OXA-21), while the isolate with intermediate susceptibility possessed multiple changes in AmpC (T79A, L150R, H189Y, N321S). Mutations of bla_{PDC} and genes related to its

expression (*ampD*, *ampR* and *dacB*) as well as the *ftsI* gene, which encodes PBP3, were common. One strain without carbapenemase or ESBL genes carried a PDC variant containing E221K, a substitution previously reported to affect ceftolozane/tazobactam susceptibility.[9]

Discussion

In this study, phenotypic and genotypic profiles of 169 CR-PA, which were collected over different time periods at a large urban hospital network, were analysed using WGS. In the majority of the isolates, carbapenem resistance was associated with mutations in the genes related to expression of the porin OprD, efflux pumps and AmpC production. However, in the contemporary collection (i.e. CR-PA strains collected after 2017), 4.6% of the isolates harboured carbapenemase genes, which were not detected in any of the historical collection (i.e. CR-PA strains collected before 2015) and all of these carbapenemases were MBLs (VIM-2, VIM-5, NDM-1), as previous studies reported.[3,11] The prevalence of carbapenemase producers among

Table 1. Comparison of acquired resistance determinants and STs between historical and contemporary collections

|	Historical collection ($N=61$)	Contemporary collection ($N=108$)	P value
Isolate with at least one β-lactamase gene	26 (16)	26 (28)	0.97
Narrow-spectrum β-lactamase	43 (26)	10 (11)	<0.001
bla_{CARB-3}	20 (12)	3.7 (4)	
bla_{OXA-1}	1.6 (1)	0 (0)	
bla_{OXA-2}	0 (0)	2.8 (3)	
bla_{OXA-9}	21 (13)	3.7 (4)	
ESBL	3.3 (2)	17 (18)	0.014
bla_{GES-26}	0 (0)	2.8 (3)	
bla_{GES-19}	0 (0)	2.8 (3)	
bla_{OXA-10}	0 (0)	1.9 (2)	
bla_{OXA-15}	3.3 (2)	9.3 (10)	
bla_{OXA-21}	0 (0)	0.9 (1)	
$bla_{OXA-226}$	0 (0)	1.9 (2)	
bla_{VEB-1a}	0 (0)	1.9 (2)	
Carbapenemase	0 (0)	4.6 (5)	0.088
bla_{NDM-1}	0 (0)	0.9 (1)	
bla_{VIM-2}	0 (0)	3.7 (4)	
bla_{VIM-5}	0 (0)	0.9 (1)	
Isolate with at least one aminoglycoside resistance determinant	43 (26)	25 (27)	0.018
aadA6	18 (11)	9.3 (10)	
aadA2	20 (12)	3.7 (4)	
aadA	0 (0)	7.4 (8)	
aac(6′)-Ib7	3.3 (2)	1.9 (2)	
aph(3′)-IIa	8.2 (5)	0 (0)	
aph(6′)-Ic	8.2 (5)	0 (0)	
aac(6′)-29a	0 (0)	2.8 (3)	
aac(6′)-29b	0 (0)	2.8 (3)	
aac(6′)-33	0 (0)	2.8 (3)	
aac(6′)-IIa	1.6 (1)	1.9 (2)	
ant(2″)-Ia	0 (0)	0.9 (1)	
aph(3′)-Ib	0 (0)	0.9 (1)	
aph(3′)-VIa	0 (0)	0.9 (1)	
aph(6′)-Id	0 (0)	0.9 (1)	
rmtB	0 (0)	0.9 (1)	
mcr-5	1.6 (1)	0 (0)	0.18
qnrVC1	0 (0)	1.9 (2)	0.28
High-risk clonal lineage	69 (42)	39 (42)	<0.001
ST235	34 (21)	15 (16)	
ST111	21 (13)	8.3 (9)	
ST244	3.3 (2)	3.7 (4)	
ST274	3.3 (2)	1.9 (2)	
ST298	3.3 (2)	3.7 (4)	
ST308	3.3 (2)	2.8 (3)	
ST357	0 (0)	1.9 (2)	
ST654	0 (0)	0.9 (1)	
ST773	0 (0)	0.9 (1)	

Data are % (n).

the contemporary CR-PA isolates was slightly higher than that observed in another epidemiological study conducted in the USA (1.9%–2.3%),[11] although it was much lower than that reported in Europe and Asia (28%–51%).[12,31] Furthermore, the rate of ESBL producers increased dramatically in the contemporary collection compared with the historical collection. Genes encoding ESBLs from the families OXA-2 (OXA-15, OXA −226), OXA-10, GES (GES-19, GES-26), VEB (VEB-1a) were present in the studied population, which was comparable to a previous study.[32] These β-lactamase genes were frequently associated with the carriage of aminoglycoside resistance elements in well-recognized high-risk clones, such as ST111, ST235, ST298 and ST357, which have been disseminated across the world.[3,29] Likewise, exogenous β-lactamase genes were also found in ST309, a clonal lineage that circulates mainly in South America (i.e. Mexico and Brazil) and were reported to be linked to an XDR phenotype.[33,34]

It is well described that *P. aeruginosa* belonging to high-risk clones possess multiple transferable resistance elements, with some of them accompanied by acquired virulence determinants.[3,35] These clones are well adapted to survive in the hospital environment and are often associated with hospital outbreaks of nosocomial infections.[36] Given the serious impact of the clonal dissemination of drug-resistant *P. aeruginosa* in hospitals and local communities, molecular surveillance studies integrated with phenotypic testing provides essential data to inform public health and infection control practice, and guide empirical use of antimicrobials.

Ceftolozane/tazobactam, first approved by the FDA in 2014,[37] has been widely used as a therapeutic agent for infections due to MDR *P. aeruginosa*, as ceftolozane is stable against hydrolysis by AmpC enzymes, and is neither affected by common active efflux pumps nor loss of the OprD porin.[9,38] Nevertheless, resistance to ceftolozane/tazobactam has been reported in various studies, primarily driven by acquisition of carbapenemases or selected types of ESBLs, hyperproduction or structural modification of PDC enzymes, or certain substitutions in PBP3.[39] Emergence of ceftolozane/tazobactam resistance has also been reported to occur during treatment through mutations in bla_{PDC}.[40,41] In our study, the genomic profiles of 16 ceftolozane/tazobactam-resistant isolates were analysed, of which 11 isolates (11/16) had clearly defined mechanisms that could explain ceftolozane/tazobactam resistance. The majority of these isolates (10/11) carried acquired carbapenemases or ESBLs, except one isolate (PA_HTX110) harbouring a substitution mutation (E221K) in PDC, known to affect ceftolozane/tazobactam susceptibility.[9] This finding was consistent with a previous study conducted in Singapore.[42] In terms of the remaining strains, ceftolozane/tazobactam resistance might have been driven by OXA-type β-lactamases (PA_HTX7, 115, 165) or a combination of multiple bla_{PDC} mutations (PA_HTX95);[43–45] however, their impact on ceftolozane/tazobactam susceptibility is yet to be understood. There was one strain (PA_HTX164) that did not possess any obvious genotypic mechanism related to ceftolozane/tazobactam resistance.

In line with the growing prevalence of acquired resistance genes in CR-PA, non-susceptibility rates to ceftolozane/tazobactam also dramatically increased across time in our cohort, from 2% for historical isolates to 17% of the contemporary isolates. All except one of the ceftolozane/tazobactam-resistant isolates
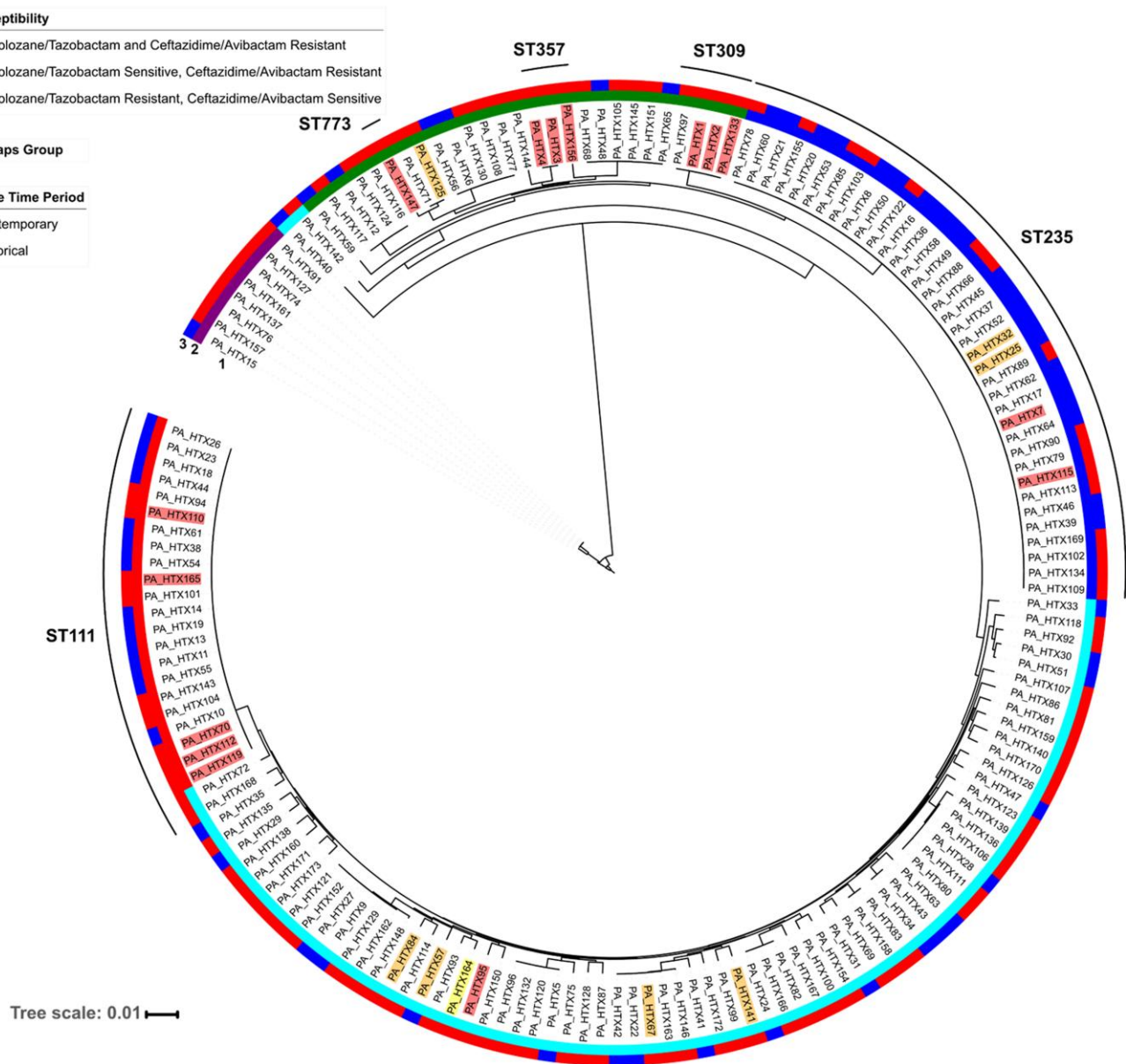
Figure 2. Core-genome phylogenetic tree of 169 CR-PA isolates. The maximum-likelihood phylogenetic tree was built using concatenated core-genome sequences with 100 bootstrap iterations. From inside to outside: (1) susceptibility to ceftolozane/tazobactam and ceftazidime/avibactam; (2) hierBAPS groups; (3) isolated time periods and STs.

also showed resistance to ceftazidime/avibactam, and susceptibility to imipenem/relebactam was less than 50% in these strains. The siderophore-conjugated cephalosporin cefiderocol largely retained activity against ceftolozane/tazobactam-resistant strains, although the presence of some ESBLs (VEB, GES) was associated with increases in MIC that still fell within the susceptible range (2–4 mg/L). The findings from our study suggest acquisition of exogenous carbapenemases and ESBLs may be a worrisome trend for *P. aeruginosa* isolates from our region, compromising the activity of novel antipseudomonal agents.

There are several limitations in this study. First, the historical isolates were not systematically collected, so the prevalence of STs and resistance determinants in this collection may not accurately represent the epidemiology of CR-PA at the time. Second, CR-PA were analysed based on the initial laboratory-reported phenotype, irrespective of *in vitro* reproducibility of the phenotype, to mirror carbapenem-resistant strains recovered in actual practice. Indeed, there were a few CR-PA isolates whose phenotypic test results (i.e. resistance to carbapenem) were not reproduced when being retested in the research lab, and was noted to be higher among historical isolates. This might reflect the heterogeneity among bacterial subpopulations, despite efforts to recover the carbapenem-resistant phenotype on selective media. This phenomenon has been reported in other large studies of MDR organisms.[46] Third, this was a single-centre study, and may not reflect trends from other geographical regions.

Strain	Year	ST	PDC Variant	AmpC Changes[a]	β-lac present	AmpR	AmpD	OprD	PBP3 (*ftsI*)	PBP4 (*dacB*)	MexR	MexZ	MICs (mg/L)						
													ATM	C/T	CZA	MEM	IPM/REL	FDC	CST
PA_HTX1	2017	309	PDC-37	G1D;T79A;V179L;V330I;G365A	GES-19, GES-26, OXA-2								>256	>256	96	>32	6	1	2
PA_HTX2	2017	309	PDC-37	G1D;T79A;V179L;V330I;G365A	GES-19, GES-26, OXA-2								>256	>256	>256	>32	8	2	1
PA_HTX3	2017	357	PDC-11	T79A;V179L;G365A	VEB-1a, OXA-10								>256	>256	>256	>32	2	4	0.5
PA_HTX4	2017	357	PDC-11	T79A;V179L;G365A	VEB-1a, OXA-10								>256	>256	96	>32	2	4	0.5
PA_HTX7	2004	235	PDC-35	G1D;A71V;T79A;V179L;G365A	OXA-15								24	12	32	>32	1	1	1
PA_HTX70	2018	111	PDC-3	T79A	VIM-2								32	>256	128	>32	>32	<0.5	0.75
PA_HTX95	2018	novel	PDC-205	T79A;L150R;H189Y;N321S	-								>256	24	>256	>32	4	8	0.25
PA_HTX110	2018	111	novel	A46D;T79A;E221K	CARB-2								96	128	48	>32	1.5	<0.5	2
PA_HTX112	2018	111	PDC-3	T79A	VIM-2								32	>256	64	>32	>32	<0.5	1
PA_HTX115	2018	235	PDC-35	G1D;A71V;T79A;V179L;G365A	OXA-226								48	192	96	>32	1	<0.5	0.5
PA_HTX119	2018	111	PDC-3	T79A	VIM-2								16	>256	24	>32	>32	<0.5	0.75
PA_HTX133	2018	309	PDC-37	G1D;T79A;V179L;V330I;G365A	GES-19, GES-26, OXA-2								>256	>256	96	>32	8	2	3
PA_HTX147	2018	773	PDC-16	G1D;T79A;V179L;G365A	NDM-1, VIM-5, OXA-21								4	>256	>256	>32	>32	16	0.38
PA_HTX156	2018	novel	PDC-16	G1D;T79A;V179L;G365A	VIM-2								16	96	12	>32	>32	1	1.5
PA_HTX164	2018	2450	PDC-3	T79A	-								>256	128	1.5	>32	1.5	<0.5	1
PA_HTX165	2018	111	PDC-3	T79A	OXA-9								32	>256	>256	>32	0.75	2	0.25

Figure 3. Phenotypic and genotypic characteristics of ceftolozane/tazobactam-resistant *P. aeruginosa*. Antimicrobial susceptibilities to selected antimicrobial agents and genotypic features of 16 ceftolozane/tazobactam-resistant *P. aeruginosa* are listed. White boxes represent gene sequence without changes as compared with *P. aeruginosa* PAO1; black boxes, gene sequence with missense mutation; red boxes, gene sequence with insertion, deletion, frameshift mutation or premature stop codon. ATM, aztreonam; β-lac, β-lactamase; C/T, ceftolozane/tazobactam; CST, colistin; CZA, ceftazidime/avibactam; FDC, cefiderocol; IPM/REL, imipenem/relebactam; MEM, meropenem; -, indicates no exogenous β-lactamase present. [a]Numbering of the amino acids in the AmpC peptide after removal of the 26 N-terminal amino acids of the signal peptide.

Fourth, the prevalence of PDC mutations or ESBLs conferring ceftolozane/tazobactam resistance and carbapenem hypersusceptibility, which have been reported to develop under ceftolozane/tazobactam treatment,[44] might be underestimated in this study due to our strain selection criteria. Finally, this surveillance set did not have any associated clinical data, so the impact on treatment outcomes, or whether the isolate was considered an active infection or colonization, cannot be determined.

In summary, an increase in the resistance rates of CR-PA to newer β-lactam agents (ceftolozane/tazobactam, ceftazidime/avibactam and imipenem/relebactam) was observed concurrently with a growing prevalence of acquired carbapenemases and ESBLs in our cross-sectional study. These findings underscore the importance that transmissible resistance determinants such as β-lactamases, frequently harboured by high-risk clonal lineages, play in the emergence of ceftolozane/tazobactam resistance. Susceptibility testing for CR-PA remains important to guide therapy, and further surveillance is needed to assess the frequency of acquired β-lactamases in *P. aeruginosa* isolates from the USA.

Acknowledgements
We thank Dr Yohei Doi for his thoughtful comments on the manuscript.

Transparency declarations
C.A.A. has received grants from Merck, MeMed Diagnostics and Entasis Therapeutics. W.R.M. has received grants and/or honoraria from Merck, Entasis Therapeutics and Shionogi. All other authors: none to declare.

Supplementary data
Figure S1 and Tables S1–S3 are available as Supplementary data at *JAC-AMR* Online.

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