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Ceftolozane/Tazobactam Resistance and Mechanisms in Carbapenem-Nonsusceptible *Pseudomonas aeruginosa*

Jocelyn Qi-Min Teo,[a,b] Jie Chong Lim,[c] Cheng Yee Tang,[b] Shannon Jing-Yi Lee,[a*] Si Hui Tan,[a] James Heng-Chiak Sim,[d] Rick Twee-Hee Ong,[b] Andrea Lay-Hoon Kwa[a,e,f]

[a]Department of Pharmacy, Singapore General Hospital, Singapore, Singapore
[b]Saw Swee Hock School of Public Health, National University of Singapore and National University Health System, Singapore, Singapore
[c]Department of Pharmacy, National University of Singapore, Singapore, Singapore
[d]Department of Microbiology, Singapore General Hospital, Singapore, Singapore
[e]Singhealth Duke-NUS Medicine Academic Clinical Programme, Singapore, Singapore
[f]Emerging Infectious Diseases, Duke-National University of Singapore Medical School, Singapore, Singapore

Jocelyn Qi-Min Teo and Jie Chong Lim contributed equally to this work. Author order was determined by random coin toss. Rick Twee-Hee Ong and Andrea Lay-Hoon Kwa also contributed equally to this work.

ABSTRACT This study established the *in vitro* activity of ceftolozane/tazobactam (C/T) and its genotypic resistance mechanisms by whole-genome sequencing (WGS) in 195 carbapenem-nonsusceptible *Pseudomonas aeruginosa* (CNSPA) clinical isolates recovered from Singapore between 2009 and 2020. C/T susceptibility rates were low, at 37.9%. Cross-resistance to ceftazidime/avibactam was observed, although susceptibility to the agent was slightly higher, at 41.0%. Whole-genome sequencing revealed that C/T resistance was largely mediated by the presence of horizontally acquired β-lactamases, especially metallo-β-lactamases. These were primarily disseminated in well-recognized high-risk clones belonging to sequence types (ST) 235, 308, and 179. C/T resistance was also observed in several non-carbapenemase-producing isolates, in which resistance was likely mediated by β-lactamases and, to a smaller extent, mutations in AmpC-related genes. There was no obvious mechanism of resistance observed in five isolates. The high C/T resistance highlights the limited utility of the agent as an empirical agent in our setting. Knowledge of local molecular epidemiology is crucial in determining the potential of therapy with novel agents.

IMPORTANCE *Pseudomonas aeruginosa* infection is one of the most difficult health care-associated infections to treat due to the ability of the organism to acquire a multitude of resistance mechanisms and express the multidrug resistance phenotype. Ceftolozane/tazobactam (C/T), a novel β-lactam/β-lactamase inhibitor combination, addresses an unmet medical need in patients with these multidrug-resistant *P. aeruginosa* infections. Our findings demonstrate geographical variation in C/T susceptibility owing to the distinct local molecular epidemiology. This study adds on to the growing knowledge of C/T resistance, particularly mutational resistance, and will aid in the design of future β-lactams and β-lactamase inhibitors. WGS proved to be a useful tool to understand the *P. aeruginosa* resistome and its contribution to emerging resistance in novel antimicrobial agents.

KEYWORDS *Pseudomonas aeruginosa*, ceftolozane/tazobactam, molecular characterization

Address correspondence to Rick Twee-Hee Ong, ephoth@nus.edu.sg, or Andrea Lay-Hoon Kwa, andrea.kwa.l.h@sgh.com.sg.

* Present address: Shannon Jing-Yi Lee, NovogeneAIT Genomics Singapore Pte Ltd., Singapore, Singapore.

*P*seudomonas aeruginosa is one of the most common pathogens implicated in hospital-acquired infections (1). Aside from its intrinsic resistance to several antibiotics, its propensity to acquire resistance is responsible for its multidrug resistance profile,

TABLE 1 Activities of antimicrobial agents against 195 clinical isolates of carbapenem-nonsusceptible *Pseudomonas aeruginosa*

Antimicrobial agent	MIC (mg/liter)			Susceptibility[a]		
	50%	90%	Range	% S	% I	% R
Ceftolozane/tazobactam[b]	≥128/4	≥128/4	≤0.5/4 to ≥128/4	37.9	3.1	59.0
Other β-lactam agents						
Imipenem	32	≥64	1 to ≥64	3.6	8.7	87.7
Meropenem	32	≥64	≤0.25 to ≥64	13.3	8.2	78.5
Doripenem	32	≥64	≤0.25 to ≥64	16.4	8.7	74.9
Aztreonam	32	≥128	4 to ≥128	13.9	22.0	64.1
Cefepime	≥128	≥128	≤1 to ≥128	17.4	10.8	71.8
Piperacillin/tazobactam	128/4	≥256/4	4 to ≥256/4	13.9	8.7	77.4
Ceftazidime/avibactam[b]	32/4	≥128/4	1/4 to ≥128/4	41.0		59.0
Other classes						
Amikacin	8	≥128	≤1 to ≥128	58.0	6.1	35.9
Gentamicin[c]				35.4	2.0	62.6
Levofloxacin	32	≥64	≤0.25 to ≥64	18.0	8.7	73.3
Polymyxin B	1	2	≤0.25 to ≥32		96.9	3.1

[a]S, susceptible; I, intermediate; R, resistant.
[b]Susceptibility was determined using gradient MIC test strips.
[c]Susceptibility was determined using disk diffusion or Vitek routinely at the microbiology laboratory.

rendering the pathogen a therapeutic challenge (2). Carbapenems are the drugs of choice in the management of severe *P. aeruginosa* infections. Unfortunately, resistance to this class of agents has developed, resulting in carbapenem-nonsusceptible *P. aeruginosa* (CNSPA). Carbapenem nonsusceptibility rates in clinical *P. aeruginosa* isolates at Singapore General Hospital have hovered at approximately 8 to 10% since 2011 (3). This is congruent to the nation's overall carbapenem resistance rate in *P. aeruginosa* clinical isolates derived from public hospitals (https://www.moh.gov.sg/resources-statistics/reports/one-health-report-on-antimicrobial-utilisation-and-resistance-2017). Additionally, carbapenem nonsusceptibility was detected in 24% of *P. aeruginosa* hospital-acquired infections in Singapore (1).

Ceftolozane/tazobactam (C/T) is a novel broad-spectrum new-generation cephalosporin/β-lactamase inhibitor combination that is highly active against *P. aeruginosa*. This novel agent has been designed to "escape" many of *P. aeruginosa*'s common resistance mechanisms, including AmpC hydrolysis, drug efflux, and OprD porin inactivation (2, 4, 5). Several studies have also demonstrated high C/T susceptibilities against CNSPA, supporting its empirical use in such infections, in which most other antibiotics are rendered ineffective (6–8). However, antibiotic susceptibilities are subject to geographical and institutional variations. The lack of local surveillance data has limited our understanding of the clinical utility of C/T in the local context. The objectives of this study were to establish the *in vitro* activity of C/T in a collection of CNSPA isolates recovered from Singapore and to characterize the genotypic profiles of C/T-nonsusceptible CNSPA.

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RESULTS AND DISCUSSION

Antimicrobial susceptibility profiles. A total of 195 CNSPA isolates were included in the study. Only 74 (37.9%) isolates were susceptible (inhibited at <8 mg/liter). Table 1 shows the susceptibility patterns for various antibiotics against CNSPA. C/T demonstrated better activity than the other β-lactams, with the exception of ceftazidime/avibactam (CZA), which had a slightly higher susceptibility rate (41.0%). Only 66 (33.8%) isolates were susceptible to both C/T and CZA. Considerably higher

TABLE 2 Genotypic characteristics of 195 CNSPA isolates

Parameter	C/T-nonsusceptible isolates (n = 121)		C/T-susceptible isolates (n = 74)
	Carbapenemase producers (n = 86)	Non-carbapenemase producers (n = 35)	
No. of STs	12	14 (13 + 1 new)	58 (55 + 3 new)
Known STs[a]	**233**, 235, 244, **308**, 316, 357, **621**, 773, **823**, **964**, **3440**, **3444**	155, **179**, 235, 244, **252**, 274, **313**, **316**, 357, **664**, 815, 1076, **1666**	11, 17, 27, 111, 155, 207, 235, 244, 245, 253, 266, 274, 292, 298, 314, 357, 389, 408, 446, 463, 471, 485, 508, 553, 560, 564, 569, 606, 620, 645, 697, 708, 773, 792, 815, 840, 882, 1076, 1247, 1649, 1930, 2013, 2021, 2033, 2069, 2326, 2476, 2651, 3078, 3311, 3439, 3442, 3443, 3445, 3446
Harbors acquired β-lactamase	10	24	5
AmpC and regulator alteration[b]	10	6	
PBP3 alteration[b]	3	1	
Ceftazidime/avibactam susceptible	4	10	66

[a]STs in bold were observed only in the C/T-nonsusceptible population in our study.
[b]Only alterations unique to the C/T-nonsusceptible population are reported. Refer to Fig. 1 and 2 for the specific observed alterations for each isolate.

susceptibility rates were observed for the non β-lactam antibiotics, such as amikacin (58.0%). Resistance remained rare for polymyxin B (3.1%).

Whole-genome sequencing (WGS) revealed carbapenemase production among 86 of the 195 isolates (44.1%); all 86 were nonsusceptible to C/T, as expected (C/T MIC$_{50}$, ≥128/4 mg/liter; MIC$_{90}$, ≥128/4 mg/liter). Hence, all 74 C/T-susceptible CNSPA isolates were observed in the 109 remaining non-carbapenemase-producing isolates, resulting in a susceptibility rate of 67.9% (C/T MIC$_{50}$, 2/4 mg/liter; MIC$_{90}$, ≥128/4 mg/liter) in this cohort.

The low C/T susceptibility rate (37.9%) is in contrast to several other studies conducted elsewhere, in which moderate to high susceptibility rates (ranging from 67 to 88%) were observed in multidrug-resistant or carbapenem-resistant *P. aeruginosa* (9–11). Even among non-carbapenemase-producing CNSPA isolates, moderate C/T susceptibility (67.9%) was observed. This suggests that C/T has limited utility as an empirical agent for suspected *P. aeruginosa* hospital-acquired infections in our setting, and susceptibility testing for the agent or knowledge of carbapenemase status is imperative prior to its use. Notably, most of the C/T-nonsusceptible isolates were recovered prior to the introduction of C/T into our institution, even among non-carbapenemase-producing CNSPA, substantiating that drivers of C/T resistance are likely not limited to C/T usage.

Genomic profiles of 121 C/T-nonsusceptible *P. aeruginosa* isolates. A brief summary of the genomic characteristics of all 195 CNSPA isolates is presented in Table 2. C/T nonsusceptibility was detected in 22 sequence types (STs) (21 known STs and 1 novel ST) in the 121 C/T-nonsusceptible CNSPA isolates. C/T resistance in the high-risk clones of ST235 (46/121 [38.0%]) and ST308 (33/121 [27.3%]) were the most prevalent. ST175 *P. aeruginosa*, the international high-risk clone with AmpC hyperproduction plus OprD inactivation which has been associated with C/T resistance, was not found in our study (12). In contrast, the ST types in C/T-susceptible isolates were even more widely distributed (58 different STs).

We analyzed the resistance mechanisms for the 121 C/T-nonsusceptible isolates. Figures 1 and 2 depict the isolates' characteristics and the potential mechanisms responsible for C/T nonsusceptibility in 86 carbapenemase-producing and 35 non-carbapenemase-producing CNSPA isolates, respectively. C/T nonsusceptibility can be explained primarily by the presence of horizontally acquired carbapenemases in a large proportion of the C/T-nonsusceptible isolates (86/121 [71.1%]). The predominant types of genes detected were metallo-β-lactamases: *bla*$_{NDM}$ (35 isolates), *bla*$_{IMP}$ (31 isolates), and *bla*$_{VIM}$ (11 isolates). Carbapenem-hydrolyzing *bla*$_{GES-5}$ accounted for the remaining isolates, with the exception of two isolates which harbored *bla*$_{KPC-2}$ and *bla*$_{OXA-232}$.

Isolate	C/T MIC, mg/L	CZA MIC, mg/L[a]	ST	Acquired resistance mechanisms		Chromosomal mutations[b]			
				Carbapenemase	Other β-lactamases	ampC PDC subtype	ampD	dacB	ftsI (PBP3)
PA0742	8	**4**	235	GES-5		PDC-35			
PA0747	16	**8**	235	GES-5		PDC-35			
PA1357	16	**4**	235	GES-5		PDC-35			
PA0433	32	16	235	GES-5		PDC-35			
PA1273	64	16	235	GES-5		PDC-35			
PA1060	≥128	64	235	GES-5		PDC-35			
PA1049	≥128	≥128	235	IMP-7		PDC-35			
PA0147	≥128	≥128	235	IMP-39		PDC-35			
PA0407	≥128	≥128	235	IMP-39		PDC-35			
PA0413	≥128	≥128	235	IMP-39		PDC-35			
PA0443	≥128	≥128	235	IMP-39		PDC-35			
PA0446	≥128	≥128	235	IMP-39		PDC-35			
PA0455	≥128	≥128	235	IMP-39		PDC-35			
PA0483	≥128	≥128	235	IMP-39		PDC-35			
PA0542	≥128	≥128	235	IMP-39		PDC-35			
PA0557	≥128	≥128	235	IMP-39		PDC-35			
PA0581	≥128	≥128	235	IMP-39		PDC-35			
PA0592	≥128	≥128	235	IMP-39		PDC-35			
PA0740	≥128	≥128	235	IMP-39		PDC-35			
PA0741	≥128	≥128	235	IMP-39		PDC-35			
PA0881	≥128	≥128	235	IMP-39		PDC-35			
PA0896	≥128	≥128	235	IMP-39		PDC-35			
PA0963	≥128	≥128	235	IMP-39		PDC-35			
PA1068	≥128	≥128	235	IMP-39		PDC-35			
PA1169	≥128	≥128	235	IMP-39		PDC-35			
PA0151	≥128	≥128	235	IMP-39		PDC-35	S75F		
PA0325	≥128	≥128	235	IMP-39		PDC-35	Start variant		
PA0336	≥128	≥128	235	IMP-43		PDC-35	S31T, L58R		
PA1002	≥128	≥128	235	IMP-43	OXA-10	PDC-35	S31T, L58R		
PA0595	≥128	≥128	235	IMP-39		PDC-35		G287D	
PA1227	≥128	≥128	235	IMP-39		PDC-35		T428P	
PA0507	≥128	≥128	235	IMP-39		PDC-35			P215L
PA0628	≥128	≥128	235	IMP-39		PDC-35			PG215LD
PA0601	≥128	64	235	VIM-2		PDC-35			
PA0736	≥128	≥128	235	VIM-2		PDC-35			
PA0745	≥128	64	235	VIM-2		PDC-35			
PA0749	≥128	32	235	VIM-2		PDC-35			
PA0767	≥128	64	235	VIM-2		PDC-35			
PA0197	≥128	≥128	308	NDM-1		PDC-19a			
PA0393	≥128	≥128	308	NDM-1		PDC-19a			
PA0395	≥128	≥128	308	NDM-1		PDC-19a			
PA0408	≥128	≥128	308	NDM-1		PDC-19a			
PA0424	≥128	≥128	308	NDM-1		PDC-19a			
PA0436	≥128	≥128	308	NDM-1		PDC-19a			
PA0453	≥128	≥128	308	NDM-1		PDC-19a			
PA0472	≥128	≥128	308	NDM-1		PDC-19a			
PA0473	≥128	≥128	308	NDM-1		PDC-19a			
PA0486	≥128	≥128	308	NDM-1		PDC-19a			F507L
PA0556	≥128	≥128	308	NDM-1		PDC-19a			
PA0834	≥128	≥128	308	NDM-1		PDC-19a			
PA0874	≥128	≥128	308	NDM-1		PDC-19a			
PA0887	≥128	≥128	308	NDM-1		PDC-19a			
PA0895	≥128	≥128	308	NDM-1		PDC-19a			
PA0900	≥128	≥128	308	NDM-1		PDC-19a	S18L		
PA0939	≥128	≥128	308	NDM-1		PDC-19a			
PA0954	≥128	≥128	308	NDM-1		PDC-19a			
PA0971	≥128	≥128	308	NDM-1		PDC-19a			
PA0972	≥128	≥128	308	NDM-1		PDC-19a			
PA1030	≥128	64	308	NDM-1		PDC-19a			
PA1041	≥128	≥128	308	NDM-1		PDC-19a			
PA1079	≥128	≥128	308	NDM-1		PDC-19a			
PA1111	≥128	≥128	308	NDM-1		PDC-19a			
PA1155	≥128	≥128	308	NDM-1		PDC-19a			
PA1212	≥128	≥128	308	NDM-1		PDC-19a			
PA1216	≥128	≥128	308	NDM-1		PDC-19a			
PA1250	≥128	≥128	308	NDM-1		PDC-19a	P41S		
PA1284	≥128	≥128	308	NDM-1		PDC-19a			
PA1318	≥128	≥128	308	NDM-1		PDC-19a			
PA1347	≥128	≥128	308	NDM-1		PDC-19a			
PA1352	≥128	≥128	308	NDM-1		PDC-19a			
PA1353	≥128	≥128	308	NDM-1		PDC-19a			
PA0474	16	**2**	244	GES-5		PDC-1			
PA1231	≥128	≥128	244	VIM-6	OXA-10	PDC-1			
PA0399	≥128	≥128	357	IMP-7	OXA-2	PDC-11	P42L		
PA1367	≥128	≥128	357	NDM-1	OXA-10, VEB-like	PDC-11			
PA1065	≥128	≥128	357	VIM-2	OXA-10, LCR-1	PDC-11	T166A		
PA0376	≥128	≥128	773	VIM-2	OXA-10	PDC-16			
PA0996	≥128	≥128	773	VIM-2	OXA-10	PDC-16			
PA0287	≥128	16	823	VIM-2		PDC-38			
PA1021	≥128	16	823	VIM-2		PDC-38			
PA0382	≥128	≥128	233	IMP-39	OXA-4, OXA-10	PDC-3			
PA1010	≥128	≥128	316	NDM-1	OXA-10, CTX-M-15	PDC-36			
PA0545	≥128	≥128	621	IMP-13		PDC-5			
PA0004	≥128	≥128	964	IMP-39		PDC-3			
PA1147	16	16	3444	OXA-232		PDC-3			
PA0419	≥128	≥128	3440	KPC-2	OXA-10, VEB-like, CARB-2-like	PDC-3			

FIG 1 Mechanisms of ceftolozane/tazobactam (C/T) resistance in 86 carbapenemase-producing CNSPA isolates. a, Bold values indicate ceftazidime/avibactam (CZA) susceptibility. b, The main chromosomal mutations (*ampC*, *ampR*, *dacB*, and *ftsI*) leading to amino acid substitutions compared to the reference wild-type comparator amino acid sequences from *Pseudomonas aeruginosa* PAO1 are shown. The list of nonsynonymous variations were refined to include only those more likely to be involved in the C/T-resistant phenotype, i.e., (i) mutations with known effect on resistance according to published evidence and (ii) mutations with predicted functional impact (i.e., deleterious) and not identified in wild-type/susceptible isolates. There were no mutations found in *ampR* in this set of isolates. PDC, *Pseudomonas*-derived cephalosporinase; ST, sequence type.

Isolate	C/T MIC, mg/L	CZA MIC, mg/L[a]	ST	Acquired resistance mechanisms	Chromosomal mutations[b]				
				β-lactamases	*ampC* PDC subtype	*ampD*	*ampR*	*dacB*	*ftsI* (PBP3)
PA0292	8	**2**	179	OXA-10	PDC-8				
PA0735	16	32	179	OXA-14	PDC-8				
PA1271	16	64	179	OXA-14	PDC-8				
PA0739	32	64	179	OXA-14	PDC-8				
PA0484	≥128	≥128	179	OXA-14	PDC-8				
PA0751	≥128	≥128	179	OXA-14	PDC-8				
PA0756	≥128	≥128	179	OXA-14	PDC-8				
PA0901	≥128	≥128	179	OXA-14	PDC-8				
PA1304	≥128	≥128	179	OXA-14	PDC-8				
PA0345	16	**4**	235	OXA-10	PDC-35				
PA0190	≥128	≥128	235	OXA-10	PDC-35				
PA0178	≥128	**8**	235	GES-1	PDC-35				
PA0183	≥128	**8**	235	GES-1	PDC-35				
PA0251	≥128	**8**	235	GES-1	PDC-35				
PA0748	≥128	32	235	GES-1	PDC-35				
PA0023	≥128	≥128	235	GES-1	PDC-35				
PA1236	≥128	≥128	235		PDC-35				
PA0006	≥128	32	357	OXA-10, VEB-like	PDC-11				
PA0279	≥128	64	357	OXA-10, VEB-like	PDC-11				
PA0374	≥128	≥128	664	OXA-796, OXA-10	PDC-98				
PA0421	≥128	≥128	664	OXA-796, OXA-10	PDC-98				
PA0427	≥128	≥128	664	OXA-796, OXA-10-like*	PDC-98				
PA1238	8	16	244		PDC-5		D135N		
PA0637	32	**8**	244	OXA-10	PDC-1-like^			E275K	
PA1277	≥128	≥128	244		PDC-1-like+				
PA0409	8	**2**	815	OXA-10	PDC-100				
PA0578	8	**8**	252		PDC-3	FS#			
PA0411	8	64	155		PDC-5		D135N		
PA1156	≥128	64	Novel		PDC-16		D135N		
PA0659	≥128	32	313		PDC-393				P215L
PA1073	≥128	≥128	316	OXA-10	PDC-36				
PA1225	8	**2**	316		PDC-36				
PA1215	8	≥128	1666		PDC-3				
PA0753	32	≥128	274		PDC-24				
PA0358	8	**4**	1076		PDC-16				

FIG 2 Mechanisms of C/T resistance in 35 non-carbapenemase-producing CNSPA isolates. a, Bold values indicate CZA susceptibility. b, The main chromosomal mutations (*ampC*, *ampR*, *dacB*, and *ftsI*) leading to amino acid substitutions compared to the reference wild-type comparator amino acid sequences from *Pseudomonas aeruginosa* PAO1 are shown. The list of nonsynonymous variations was refined to include only those more likely to be involved in the C/T-resistant phenotype, i.e., (i) mutations with known effect on resistance according to published evidence and (ii) mutations with predicted functional impact (i.e., deleterious) and not identified in wild-type/susceptible isolates. *, G439C amino acid substitution in OXA-10; ^, A163T amino acid substitution in AmpC; +, ΔK74-E75 in AmpC; #, frameshift (FS) at position 149.

In the 35 non-carbapenemase-producing C/T-nonsusceptible CNSPA isolates, horizontally acquired extended-spectrum β-lactamases (ESBLs) were frequently observed (24/35 [68.6%]). Notably, bla_{OXA-14}, the extended-spectrum variant of bla_{OXA-10} which has been associated with C/T resistance (13), was detected in eight isolates, all of which were ST179. bla_{VEB}, bla_{GES-1}, bla_{OXA-10}, and other bla_{OXA} variants were also detected. C/T appeared to have variable activity in *P. aeruginosa* with secondary ESBLs. Various ESBLs such as those encoded by bla_{GES} and bla_{VEB} have been shown to inactivate C/T (14).

We noted that the distribution of these exogenous β-lactam resistance elements was limited primarily to three main clones, ST235 (*n* = 46), ST308 (*n* = 33), and ST179 (*n* = 9), which accounted for 72.7% of the C/T-nonsusceptible isolates. Within each clone, there was little or no intraclonal variation. This suggests that multidrug resistance, including C/T resistance, is contributed primarily by a limited number of clones which have gained a strong foothold in our setting, although *P. aeruginosa* organisms of other diverse STs could also acquire these ESBLs/carbapenemases over time, resulting in broad-spectrum resistance.

As ceftolozane is neither affected by efflux pumps nor transported via OprD, resistance is driven primarily by acquisition of ESBLs, AmpC hyperproduction, AmpC structural modifications, or mutations in PBP3 (15, 16). Since we observed a number of C/T-nonsusceptible isolates without any carbapenemases/ESBLs or harboring only narrow-spectrum beta-lactamases like bla_{OXA-10}, we analyzed the chromosomal genes related to AmpC and its expression (*ampC* and the regulator genes *ampD*, *ampR*, and *dacB*), as well as the *ftsI* gene (encoding PBP3), which is the target binding site of C/T. Our analysis revealed that most of the 121 C/T-nonsusceptible CNSPA isolates

harbored single nucleotide polymorphisms (SNPs) resulting in nonsynonymous AmpC amino acid substitutions. The number of amino acid substitutions ranged from 0 to 5 (median, 5). This is congruent to the high sequence polymorphism of AmpC reported for *P. aeruginosa* (16). Classification of the isolates based on *Pseudomonas*-derived cephalosporinase (PDC) subtypes showed a total of 17 different subtypes. The PDC-35 subtype was the most prevalent; it was detected solely in the 46 ST235 isolates. This was followed by PDC-19a, which was found exclusively in the 33 ST308 isolates. The majority of these AmpC amino acid substitutions were unlikely to be associated with C/T nonsusceptibility, as they were either similarly found in the susceptible strains in our study or have been described for wild-type strains elsewhere. We did not detect any SNPs implicated in C/T nonsusceptibility which had been described in literature previously (4, 16, 17). However, we did observe potentially deleterious variants (A163T in PA0637 and a 2-amino-acid deletion, K74-E75, in PA1277) in two isolates (Fig. 2). Deleterious SNPs in the other *ampD*, *ampR*, and *dacB* regulator genes and PBP3 variants were infrequently observed, occurring in only approximately 16% of the isolates. There were five nonsusceptible isolates (highlighted in dark purple in Fig. 2) which did not appear to have any ESBLs/carbapenemases or alterations in AmpC or PBP3. The identified deleterious SNPs in this study have not been reported in the literature, and thus, their role in mediating C/T resistance requires further validation.

Although the aim of this study did not include a detailed investigation of the mechanisms of CZA resistance, we noted that there were differential susceptibilities in the two agents. Cross-resistance was high due to the high prevalence of metallo-β-lactamases, which both agents were inactive against. In contrast to tazobactam, avibactam was designed to have potent activity against class C β-lactamases and have a slightly broader anti β-lactamases activity (inclusive of KPC and OXA-48) (14, 17). This could explain the observation of the 14 (11.6%) isolates among the 121 C/T-nonsusceptible CNSPA isolates which remained susceptible to CZA (Table 2). These isolates primarily harbored GES and OXA β-lactamases (Fig. 1 and 2), which can be inhibited by avibactam. Additionally, 8 (10.8%) of the 74 C/T-susceptible CNSPA were resistant to CZA, which had moderate MICs near the breakpoint (16 mg/liter). We postulate that resistance in these isolates may be attributed to drug efflux and/or decreased cell permeability in the presence of low levels of AmpC overexpression which may still be overcome by C/T (18–20). Nevertheless, due to the slight difference in the activities of the two agents, there may be a role for each agent, depending on the molecular epidemiology of the setting.

Concluding remarks. CNSPA is a major treatment challenge due to a lack of available effective agents. Novel agents such as the AmpC-stable C/T are introduced in a bid to expand the armamentarium against these difficult-to-treat organisms. In this study, we assessed the rates of *in vitro* susceptibility to C/T and the molecular mechanisms mediating C/T resistance in CNSPA recovered from a large tertiary hospital in Singapore where C/T has only recently (January 2019) been introduced into its formulary.

The observed high C/T nonsusceptibility rates in our CNSPA, together with cross-resistance to CZA, the other novel β-lactamase inhibitor combination, signify a severe therapeutic challenge in CNSPA infections. Our results also affirm the limited use of C/T as an empirical agent in our setting, reserving the agent for culture-directed indications. Aside from polymyxin B and amikacin, which are often associated with toxicities, there are no safer and tolerable options for our multidrug-resistant CNSPA, prompting the urgent need to explore the use of other novel agents, such as cefiderocol or combination therapy, to fill the gaps in the armamentarium against CNSPA in our setting (21).

The high nonsusceptibility rates may be corroborated by our molecular findings. There is a high prevalence of well-established multidrug-resistant carbapenemase-producing *P. aeruginosa* high-risk clones (ST235, ST308, and ST179) among the C/T-

nonsusceptible isolates. Additionally, diverse STs can also acquire ESBLs/carbapenemases, leading to reduced β-lactam susceptibilities. The role of constitutive AmpC variants leading to structural modifications and/or hyperproduction in mediating C/T resistance appeared to be minimal in our population. The *ampC* gene in *P. aeruginosa* is highly polymorphic, and mutations did not necessarily translate to changes in C/T phenotype.

A limitation of this study is that we did not measure the change in expression levels in AmpC. AmpC hyperproduction mediated by mutations in other unstudied genes may have been responsible for C/T resistance. Though they appear to be rare, we are not aware of the true proportion of AmpC hyperproducers in our population. However, more importantly, C/T resistance in strains not producing carbapenemases/acquired ESBLs predates the introduction of C/T into our clinical practice. This highlights that C/T resistance could result independently of C/T use, which could be due to the rampant use of other β-lactam antibiotics that are able to induce or derepress AmpC production (22). There is also a possibility that C/T resistance is independent of AmpC or β-lactamase activity. Mechanisms driving C/T resistance still need to be further explored.

Although C/T has been reported to be highly active against *P. aeruginosa* and retained susceptibility in CNSPA elsewhere in the world, susceptibility is not universal. The prevalence of C/T resistance is related to the molecular epidemiology of *P. aeruginosa*, which can vary temporally and geographically. In our setting, where prevalence of acquired β-lactamases is high, the utility of C/T is limited. Knowledge of the molecular epidemiology and genotypes is important in evaluating the place of therapy with novel agents.

MATERIALS AND METHODS

Collection of bacterial isolates. Nonduplicate clinical CNSPA isolates, which exhibited nonsusceptibility to at least one carbapenem (doripenem, meropenem, imipenem), collected at the Singapore General Hospital (SGH) Pharmacy Research Laboratory between 2009 and 2020 from various culture sites (blood [53.5%], lower respiratory specimens [16.1%], skin and soft tissue [11.6%], urine [5.8%], and other sites, including bone, gastrointestinal tract, etc. [13.0%]), were studied. Isolates were randomly selected for testing from the laboratory's repository, which comprised CNSPA isolates collected from an informal carbapenem-nonsusceptible Gram-negative pathogen surveillance study of hospital inpatients initiated in 2015. Isolates from prior to 2015 were collected via convenience sampling or were submitted to the laboratory for antibiotic combination testing.

These isolates were subjected to genus identification and confirmation as per the institution's microbiology laboratory routine procedures, i.e., using Vitek GNI+ cards with the Vitek 2 instrument (bioMérieux, Hazelwood, MO) and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) system (Bruker Daltonik, Germany), if necessary. All isolates were preserved in Microbank cryovials (Pro-Lab Diagnostics, Richmond Hill, ON, Canada) at −80°C and subcultured twice on Trypticase soy agar-5% sheep blood plates (BD, Sparks, MD) before experimental testing.

Antibiotic susceptibilities. Susceptibilities to meropenem, imipenem, doripenem, cefepime, piperacillin/tazobactam, levofloxacin, amikacin, and polymyxin B were determined using customized 96-well broth microdilution plates (TREK Diagnostics, East Grinstead, UK) in accordance with the manufacturer's recommendations. Gradient MIC test strips were used to determine ceftazidime/avibactam (bioMérieux, Marcy l'Etoile, France) and ceftolozane/tazobactam (Liofilchem, Roseto degli Abruzzi, Italy) susceptibilities. Ceftazidime susceptibility was not routinely tested in this institution, as the agent was reserved primarily for the treatment of melioidosis. All MICs were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (23). *P. aeruginosa* ATCC 27853 was used as the quality control strain.

DNA preparation and whole-genome sequencing. Genomic DNAs were extracted and purified from overnight bacterial cultures with the DNeasy blood and tissue kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. Paired-end whole-genome sequencing (WGS) was performed on the genomic DNAs using the MiSeq/HiSeq systems (Illumina Inc., CA), with a resultant coverage of at least 100-fold. Raw sequences were assessed for quality using FastQC (v0.11.3, Babraham Institute), followed by removal of adaptors and poor-quality bases using Trimmomatic (24, 25). Trimmed sequences were then assembled *de novo* using SPAdes software (26).

Genotypic profiling. Acquired resistance genes were identified using the SRST2 package (v0.2.0), which mapped raw short reads to the ARG-ANNOT database (27, 28). Selected chromosomal gene targets related to C/T susceptibility were analyzed by aligning assembled sequences to the PAO1 reference genome (GenBank accession no. AE004091.2), and variants were called with the pipeline Snippy (v4.6.0) (available at https://github.com/tseemann/snippy). The Protein Variation Effect Analyzer (PROVEAN) software tool was used to predict the impact of identified amino acid substitutions on protein biological

function, i.e., whether the amino acid substitution was neutral or deleterious (http://provean.jcvi.org/index.php) (29). Sequence types (STs) were identified using the Basic Local Alignment Search Tool (BLAST) against the PubMLST database (https://pubmlst.org/paeruginosa/).

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