



# Emergence of Ceftazidime-Avibactam Resistance Due to Plasmid-Borne *bla*<sub>KPC-3</sub> Mutations during Treatment of Carbapenem-Resistant *Klebsiella pneumoniae* Infections

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**ABSTRACT** Ceftazidime-avibactam is a novel  $\beta$ -lactam/ $\beta$ -lactamase inhibitor with activity against carbapenem-resistant *Enterobacteriaceae* (CRE) that produce *Klebsiella pneumoniae* carbapenemase (KPC). We report the first cases of ceftazidime-avibactam resistance to develop during treatment of CRE infections and identify resistance mechanisms. Ceftazidime-avibactam-resistant *K. pneumoniae* emerged in three patients after ceftazidime-avibactam treatment for 10 to 19 days. Whole-genome sequencing (WGS) of longitudinal ceftazidime-avibactam-susceptible and -resistant *K. pneumoniae* isolates was used to identify potential resistance mechanisms. WGS identified mutations in plasmid-borne *bla*<sub>KPC-3</sub>, which were not present in baseline isolates. *bla*<sub>KPC-3</sub> mutations emerged independently in isolates of a novel sequence type 258 sublineage and resulted in variant KPC-3 enzymes. The mutations were validated as resistance determinants by measuring MICs of ceftazidime-avibactam and other agents following targeted gene disruption in *K. pneumoniae*, plasmid transfer, and *bla*<sub>KPC</sub> cloning into competent *Escherichia coli*. In rank order, the impact of KPC-3 variants on ceftazidime-avibactam MICs was as follows: D179Y/T243M double substitution > D179Y > V240G. Remarkably, mutations reduced meropenem MICs  $\geq 4$ -fold from baseline, restoring susceptibility in *K. pneumoniae* from two patients. Cefepime and ceftriaxone MICs were also reduced  $\geq 4$ -fold against D179Y/T243M and D179Y variant isolates, but susceptibility was not restored. Reverse transcription-PCR revealed that expression of *bla*<sub>KPC-3</sub> encoding D179Y/T243M and D179Y variants was diminished compared to *bla*<sub>KPC-3</sub> expression in baseline isolates. In conclusion, the development of resistance-conferring *bla*<sub>KPC-3</sub> mutations in *K. pneumoniae* within 10 to 19 days of ceftazidime-avibactam exposure is troubling, but clinical impact may be ameliorated if carbapenem susceptibility is restored in certain isolates.

**KEYWORDS** ceftazidime-avibactam, resistance, carbapenem-resistant *Enterobacteriaceae*, *Klebsiella pneumoniae*, *Klebsiella pneumoniae* carbapenemase, sequence type 258

Carbapenem-resistant *Enterobacteriaceae* (CRE) have emerged worldwide as causes of infections that are resistant to front-line antibiotics (1). Mortality rates among patients with serious CRE infections are as high as 70% (2–4). The most common determinants of carbapenem resistance among *Enterobacteriaceae* in the United States

Received 28 September 2016 Returned for modification 16 November 2016 Accepted 18 November 2016

Accepted manuscript posted online 28 December 2016

**Citation** Shields RK, Chen L, Cheng S, Chavda KD, Press EG, Snyder A, Pandey R, Doi Y, Kreiswirth BN, Nguyen MH, Clancy CJ. 2017. Emergence of ceftazidime-avibactam resistance due to plasmid-borne *bla*<sub>KPC-3</sub> mutations during treatment of carbapenem-resistant *Klebsiella pneumoniae* infections. *Antimicrob Agents Chemother* 61:e02097-16. <https://doi.org/10.1128/AAC.02097-16>.

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are *Klebsiella pneumoniae* carbapenemases (KPCs), especially KPC-2 and KPC-3. Treatment of CRE infections has relied heavily upon salvage antibiotics like colistin, aminoglycosides, and tigecycline, which are limited by suboptimal pharmacokinetics, toxicity, and/or emergence of resistance (5, 6).

Avibactam is a novel  $\beta$ -lactamase inhibitor that inactivates KPCs, OXA-48 carbapenemases, AmpC, and extended-spectrum  $\beta$ -lactamases (ESBLs), but not metallo- $\beta$ -lactamases such as NDM, VIM, or IMP (7). The agent, by itself, is not active against KPC-producing *Enterobacteriaceae* (KPC-*Enterobacteriaceae*). In combination, however, avibactam restores ceftazidime activity against KPC-*Enterobacteriaceae* *in vitro* (8). Ceftazidime-avibactam was approved in 2015 by the U.S. Food and Drug Administration for treatment of complicated intra-abdominal infections (in conjunction with metronidazole) and urinary tract infections (9–11). Data against KPC-*Enterobacteriaceae* infections are limited, but ceftazidime-avibactam is endorsed as front-line therapy due to its activity *in vitro*, its safety profile, and the lack of effective alternative regimens (7).

In a recent study, we described our experience at the University of Pittsburgh Medical Center (UPMC) with ceftazidime-avibactam in treating 37 patients with CRE infections (12). Clinical success and survival at 30 days were 59% and 76%, respectively, rates that were comparable to those given in previous reports of CRE-infected patients treated with  $\geq 2$  *in vitro*-active agents (4). Ceftazidime-avibactam was well tolerated, with a 10% rate of acute kidney injury after  $\geq 7$  days of treatment, which was considerably lower than the  $\sim 30\%$  rate that we observed with colistin- or aminoglycoside-containing regimens (13, 14). As a counterpoint to these promising findings, ceftazidime-avibactam resistance emerged in three patients during treatment of KPC-producing *K. pneumoniae* infections. To our knowledge, these were the first descriptions of resistance developing during ceftazidime-avibactam treatment. To date, resistance mechanisms have not been described in KPC-producing clinical isolates.

Our objectives in this study were to provide detailed case reports of the emergence of ceftazidime-avibactam resistance, identify and validate resistance mechanisms using genomic and molecular genetic approaches, and study the impact of ceftazidime-avibactam resistance mechanisms on susceptibility to meropenem and other  $\beta$ -lactam agents.

(These data were presented at ID Week 2016, October 2016, New Orleans, LA.)

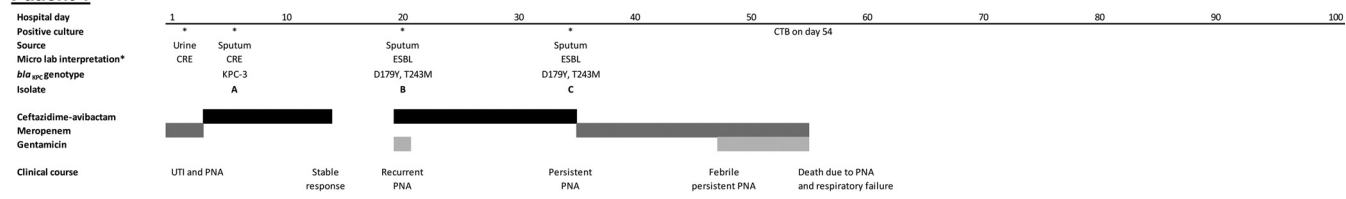
## RESULTS

**Case reports.** Clinical and microbiologic details, timelines, and antimicrobial therapy are summarized in Fig. 1 and Table 1. Baseline isolates from each patient (isolates 1-A, 2-A, and 3-A) were meropenem resistant and ceftazidime-avibactam susceptible. Ceftazidime-avibactam was dosed at 2.5 g intravenously every 8 h (adjusted based on renal function) (9).

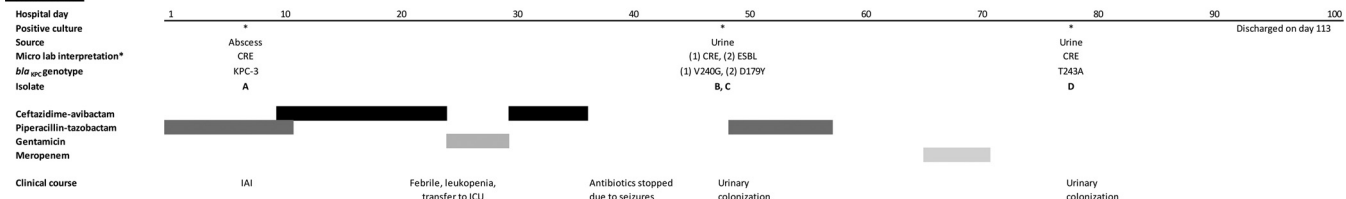
Patient 1, a double-lung transplant recipient in her 40s, was diagnosed with carbapenem-resistant *K. pneumoniae* pneumonia and urinary tract infection (isolate 1-A, recovered from sputum; ceftazidime-avibactam MIC, 2  $\mu\text{g/ml}$ ; meropenem MIC, 128  $\mu\text{g/ml}$ ) in November 2015. She was treated with 10 days of ceftazidime-avibactam. Four days after completing treatment, ceftazidime-avibactam and gentamicin were initiated for recurrent pneumonia. Sputum culture (isolate 1-B) grew meropenem-susceptible *K. pneumoniae* (MIC = 0.5  $\mu\text{g/ml}$ ), which was reported as ESBL producing. Ceftazidime-avibactam was discontinued after 14 days, and meropenem was administered because meropenem-susceptible (MIC = 0.25  $\mu\text{g/ml}$ ), ESBL *K. pneumoniae* was still identified in sputum culture (isolate 1-C). Both meropenem-susceptible isolates were ceftazidime-avibactam resistant (MICs = 256  $\mu\text{g/ml}$ ). Meropenem was given for 17 days (alone and then combined with gentamicin), but the patient died of respiratory failure. There were no epidemiological links between patient 1 and patient 2 or 3.

Patient 2, a woman in her 50s, was diagnosed with a subphrenic abscess due to carbapenem-resistant *K. pneumoniae* (isolate 2-A; ceftazidime-avibactam MIC, 4  $\mu\text{g/ml}$ ; meropenem MIC, 32  $\mu\text{g/ml}$ ) in January 2016. She was successfully treated with abscess drainage and 19 days of ceftazidime-avibactam. Subsequent hospitalization was com-

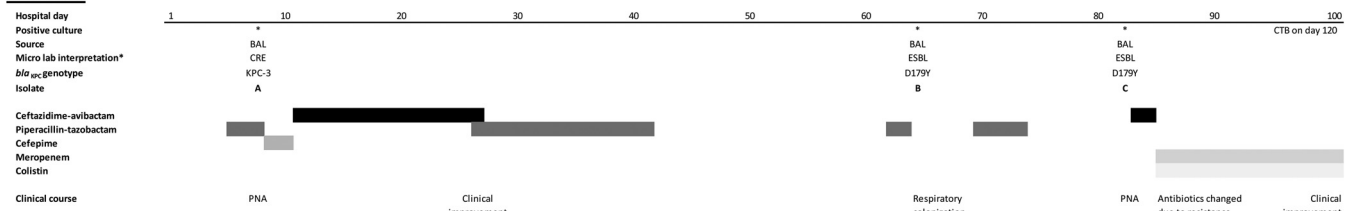
**Patient 1**



**Patient 2**



**Patient 3**



**FIG 1** Time courses of infection and treatment among patients in whom ceftazidime-avibactam-resistant *K. pneumoniae* emerged. Black and shaded bars represent duration of treatment with respective antibiotics. \*, interpretation of *K. pneumoniae* phenotype, as reported by the clinical microbiology laboratory. Abbreviations: CRE, carbapenem-resistant *Enterobacteriaceae*; CTB, ceased to breathe; ESBL, extended-spectrum  $\beta$ -lactamase; IAI, intra-abdominal infection; PNA, pneumonia; UTI, urinary tract infection.

plicated by recurrent fevers and worsening of comorbid conditions. Work-up during these febrile episodes revealed urinary tract colonization with three different carbapenem-resistant *K. pneumoniae* isolates, which was not treated (isolates 2-B, -C, and -D; meropenem MICs, 4 to 8  $\mu$ g/ml). Two colonizing isolates (isolates 2-B and -C) were ceftazidime-avibactam resistant (MICs, 32 and >256  $\mu$ g/ml). The patient was ultimately discharged.

Patient 3, a man in his 70s who had undergone esophagectomy for cancer, was diagnosed with pneumonia due to carbapenem-resistant *K. pneumoniae* (isolate 3-A; ceftazidime-avibactam MIC, 2  $\mu$ g/ml; meropenem MIC, 32  $\mu$ g/ml) in January 2016. He was treated with ceftazidime-avibactam for 15 days. Thereafter, meropenem-susceptible, ESBL-producing *K. pneumoniae* isolates were identified as a respiratory tract colonizer or cause of recurrent pneumonia (isolates 3-B, 3-C; MICs, 0.25 and 0.125  $\mu$ g/ml, respectively). Both isolates were ceftazidime-avibactam resistant (MICs, 64 and 128  $\mu$ g/ml). Recurrent pneumonia was treated successfully with meropenem plus colistin for 14 days.

**Characterization of *K. pneumoniae* isolates.** All 10 *K. pneumoniae* isolates were sequence type (ST) 258, clade II (*cps-2*, *wzi* allele 154) clones (Table 2) (15). Whole-genome sequences of these isolates were compared to those of 23 ST258, clade II isolates from our center (2010 to 2011) and others (Fig. 2). A total of 892 core single nucleotide polymorphisms (SNPs) were identified across the 33 isolates; 152 SNPs that were located at repetitive or prophage regions were excluded from phylogenetic analyses. An average of 91 SNPs (range, 1 to 214) were identified in pairwise comparisons of isolates, which is in agreement with our previous data that ST258, clade II isolates differed by ~82 SNPs (range, 2 to 231) (15). The 10 isolates from this study clustered tightly with, but were divergent from, the other ST258, clade II *K. pneumoniae* isolates (Fig. 2 and 3; see also Table S1 in the supplemental material).

All isolates had genes encoding TEM-1, SHV-11, and OXA-9  $\beta$ -lactamases; none had genes encoding NDM, other metallo- $\beta$ -lactamases, or OXA-48-like carbapenemases.

**TABLE 1** *K. pneumoniae* isolates recovered from patients treated with ceftazidime-avibactam<sup>a</sup>

Patient- isolate ID	Days from admission to culture (source)	Ceftazidime-avibactam exposure at time of culture (days)	MIC ( $\mu\text{g}/\text{ml}$ ) <sup>b</sup>											Relative fold change in $bla_{\text{KPC}}$ expression by qRT-PCR <sup>c</sup>
			Ceftazidime-avibactam	Ceftazidime	Meropenem	Ertapenem	Piperacillin-tazobactam	Cefepime	Ceftriaxone	Aztreonam	KPC-3 variant <sup>c</sup>	Aztreonam	Ceftazidime	
<b>1-A</b>	<b>6</b> (sputum)	<b>0</b>	<b>2</b> (S)	<b>512</b>	<b>128</b>	<b>256</b>	<b>256</b>	>512	>128	>128	>128	>256	<b>Wild-type</b>	<b>Reference</b>
1-B	20 (sputum)	10	256	>512	0.5 (S)	1	1	>512	16	32	32	8	D179Y, T243 M	↓ 2.64-fold
1-C	42 (sputum)	24	256	>512	0.25 (S)	2	2	>512	16	32	32	16	D179Y, T243 M	NP <sup>e</sup>
<b>2-A</b>	<b>7</b> (abscess)	<b>0</b>	<b>4</b> (S)	<b>256</b>	<b>32</b>	<b>8</b>	<b>8</b>	>512	>128	>128	>128	>256	<b>Wild-type</b>	<b>Reference</b>
2-B	48 (urine)	19	32	>512	8	8	8	>512	>128	>128	>128	>256	V240G	<2-fold
2-C	48 (urine)	19	>256	>512	4	16	16	>512	16	4	4	8	D179Y	↓ 10.45-fold
2-D	78 (urine)	19	4 (S)	256	4	8	8	>512	>128	>128	>128	>256	T243A	<2-fold
<b>3-A</b>	<b>6</b> (BALF)	<b>0</b>	<b>2</b> (S)	<b>256</b>	<b>32</b>	<b>8</b>	<b>8</b>	>512	>128	>128	>128	>256	<b>Wild-type</b>	<b>Reference</b>
3-B	64 (BALF)	15	128	512	0.25 (S)	1	1	256	4	16	16	4 (S)	D179Y	↓ 2.05-fold
3-C	82 (BALF)	15	64	512	0.125 (S)	0.5	0.5	256	8	8	16	4 (S)	D179Y	NP

<sup>a</sup>BALF, bronchoalveolar lavage fluid; S, susceptible, based on CLSI interpretive criteria (susceptibility breakpoints: ceftazidime-avibactam,  $\leq 8$   $\mu\text{g}/\text{ml}$ ; ceftazidime,  $\leq 4$   $\mu\text{g}/\text{ml}$ ; meropenem,  $\leq 1$   $\mu\text{g}/\text{ml}$ ; piperacillin-tazobactam,  $\leq 16$   $\mu\text{g}/\text{ml}$ ; cefepime,  $\leq 2$   $\mu\text{g}/\text{ml}$ ; ceftriaxone,  $\leq 1$   $\mu\text{g}/\text{ml}$ ; aztreonam,  $\leq 4$   $\mu\text{g}/\text{ml}$ ). Boldface rows represent baseline isolate from each patient.

<sup>b</sup>MICs within the susceptible range are indicated by "(S)"; all other MICs indicated resistance.

<sup>c</sup>When relevant, amino acid substitutions within KPC-3 are listed.

<sup>d</sup>RT-PCR was performed for a representative isolate from each patient that expressed a given KPC-3 variant. Data show differences in gene expression relative to the corresponding baseline isolate (reference).

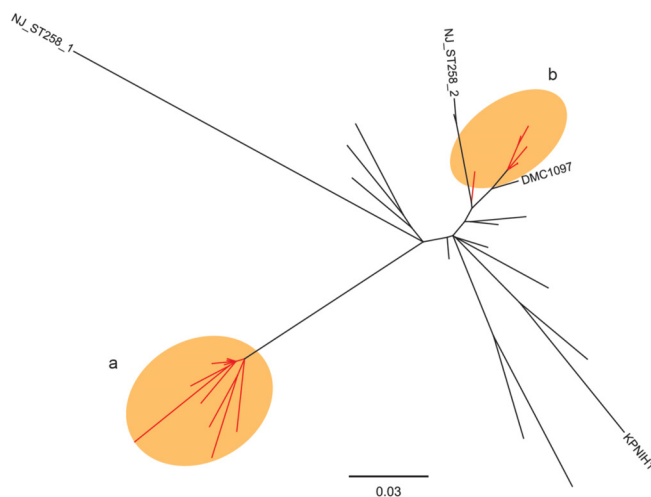
<sup>e</sup>NP, RT-PCR not performed.

**TABLE 2** Molecular characteristics of *K. pneumoniae* isolates from the present study<sup>a</sup>

Patient no.	Strain ID	OmpK36	KPC	<i>bla</i> <sub>KPC-3</sub>	Other resistance genes	Plasmid InC
1	1-A	IS5 promoter ins	KPC-3	<i>bla</i> <sub>KPC-3</sub>	<i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>SHV-11</sub> , <i>bla</i> <sub>OXA-9</sub> , <i>aadA1</i> , <i>aac(6')-lb</i> , <i>strAB</i> , <i>sul2</i> , <i>dfrA14</i>	ColpVC,Col(MG828), pBK30683 repA, repB, pNJST258N3
1	1-B	WT	AA179D→Y, 243T→M	532G→T, 725C→T	<i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>SHV-11</sub> , <i>bla</i> <sub>OXA-9</sub> , <i>aadA1</i> , <i>aac(6')-lb</i> , <i>strAB</i> , <i>sul2</i> , <i>dfrA14</i>	ColpVC,Col(MG828), pBK30683 repA, repB, pNJST258N3, L/M
1	1-C	AA288stop	AA179D→Y, 243T→M	532G→T, 725C→T	<i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>SHV-11</sub> , <i>bla</i> <sub>OXA-9</sub> , <i>aadA1</i> , <i>aac(6')-lb</i> , <i>strAB</i> , <i>sul2</i> , <i>dfrA14</i>	ColpVC,Col(MG828), pBK30683 repA, repB, pNJST258N3, L/M
2	2-A	WT	KPC-3	<i>bla</i> <sub>KPC-3</sub>	<i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>SHV-11</sub> , <i>bla</i> <sub>OXA-9</sub> , <i>aadA1</i> , <i>aac(6')-lb</i> , <i>strAB</i> , <i>sul2</i> , <i>dfrA14</i>	pBK30683 repB, pNJST258N3
2	2-B	WT	AA240V→G (KPC-8)	716T→G	<i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>SHV-11</sub> , <i>bla</i> <sub>OXA-9</sub> , <i>aadA1</i> , <i>aac(6')-lb</i> , <i>strAB</i> , <i>sul2</i> , <i>dfrA14</i>	pBK30683 repB, pNJST258N3
2	2-C	IS5 ins 827 bp	AA179D→Y	532G→T	<i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>SHV-11</sub> , <i>bla</i> <sub>OXA-9</sub> , <i>aadA1</i> , <i>aac(6')-lb</i> , <i>strAB</i> , <i>sul2</i> , <i>dfrA14</i>	pBK30683 repB, pNJST258N3
2	2-D	WT	AA243T→A	724G→A	<i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>SHV-11</sub> , <i>bla</i> <sub>OXA-9</sub> , <i>aadA1</i> , <i>aac(6')-lb</i> , <i>strAB</i> , <i>sul2</i> , <i>dfrA14</i>	pBK30683 repB, pNJST258N3
3	3-A	WT	KPC-3	<i>bla</i> <sub>KPC-3</sub>	<i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>SHV-11</sub> , <i>bla</i> <sub>OXA-9</sub> , <i>aadA1</i> , <i>aac(6')-lb</i> , <i>strAB</i> , <i>sul2</i> , <i>dfrA14</i>	pBK30683 repB, pNJST258N3
3	3-B	WT	AA179D→Y	532G→T	<i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>SHV-11</sub> , <i>bla</i> <sub>OXA-9</sub> , <i>aadA1</i> , <i>aac(6')-lb</i> , <i>strAB</i> , <i>sul2</i> , <i>dfrA14</i>	pBK30683 repB, pNJST258N3
3	3-C	WT	AA179D→Y	532G→T	<i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>SHV-11</sub> , <i>bla</i> <sub>OXA-9</sub> , <i>aadA1</i> , <i>aac(6')-lb</i> , <i>strAB</i> , <i>sul2</i> , <i>dfrA14</i>	pBK30683 repB, pNJST258N3

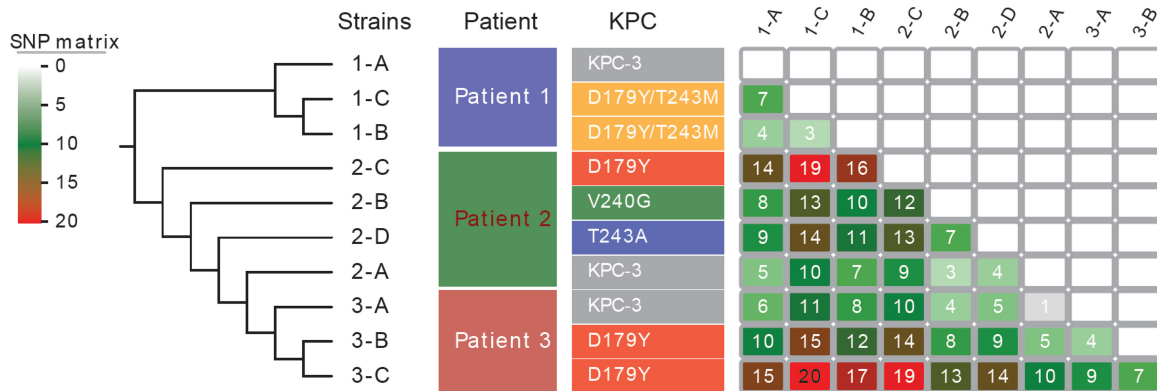
<sup>a</sup>Abbreviations: InC, incompatibility group; KPC, *Klebsiella pneumoniae* carbapenemase; WT, wild type. All isolates had the following features: sequence type (ST) 258; *wzi* allele 154; clade *cps-2*; OmpK35, AA89stop.

Baseline isolates carried *bla*<sub>KPC-3</sub>. Subsequent isolates carried mutant *bla*<sub>KPC-3</sub> encoding variant KPC-3 enzymes (Table 2). The most common KPC-3 variant featured a tyrosine for aspartic acid substitution at Ambler amino acid position 179 (D179Y), either alone (isolates 2-C, 3-B, and 3-C) or in combination with a methionine-for-threonine substitution at position 243 (D179Y/T243M; isolates 1-B and 1-C). Alanine-for-threonine and glycine-for-valine substitutions were observed at positions 243 (T243A) and 240 (V240G), respectively, in other isolates from patient 2; the V240G variant corresponds to KPC-8 (16). Each wild-type and mutant *bla*<sub>KPC-3</sub> gene was in a ΔTn1331-Tn4401d nested



NJ\_ST258\_1/2: New York/New Jersey ST258, clade II reference strains. DMC1097: Michigan ST258, clade II reference strain. KPNIH1: Maryland ST258, clade II reference strain (NIH outbreak).

**FIG 2** Phylogenetic comparison of ST258, clade II *K. pneumoniae* isolates from our center and others in the United States. The phylogenetic tree, based on whole-genome SNP analysis, was generated with the use of the maximum-likelihood optimality criterion. Branch lengths are proportional to the number of evolutionary changes, and all nodes were supported by 100% bootstrap. Ten *K. pneumoniae* isolates from the present study and 22 isolates collected from hospitals in New York, New Jersey, Pennsylvania, Maryland, and Michigan in previous studies were included (15, 37, 39). Isolates from our medical center are presented in yellow ovals. Isolates in cluster a are from the present study, and isolates in cluster b were collected from our center in 2010 to 2011.



**FIG 3** SNP matrix of the 10 *K. pneumoniae* isolates from the present study. Numbers of SNPs for each pairwise comparison of isolates are shown.

transposon element, which was found within a common ~40-kb contig. This contig is virtually identical to the corresponding region on IncFIA plasmid pBK30683, a predominant *bla*<sub>KPC-3</sub>-bearing plasmid in New York and New Jersey hospitals (17).

*K. pneumoniae* isolates with wild-type *bla*<sub>KPC-3</sub> were ceftazidime-avibactam susceptible and meropenem resistant. Ceftazidime-avibactam MICs were increased  $\geq 4$ -fold against isolates with mutant *bla*<sub>KPC-3</sub> encoding D179Y/T243M, D179Y, and V240G variants compared to corresponding baseline isolates (Table 1); increased MICs conferred clinical drug resistance. Meropenem MICs against ceftazidime-avibactam-resistant isolates were reduced  $\geq 4$ -fold, which restored meropenem susceptibility to isolates from patients 1 and 3. Cefepime, ceftriaxone, and aztreonam MICs were reduced  $\geq 4$ -fold against D179Y/T243M and D179Y variant isolates. All isolates remained resistant to cefepime and ceftriaxone. The expression of *bla*<sub>KPC-3</sub> encoding D179Y/T243M and D179Y variants was reduced compared to *bla*<sub>KPC-3</sub> expression in corresponding baseline isolates. No mutations were evident within promoter sequences of mutant *bla*<sub>KPC-3</sub> compared to wild-type *bla*<sub>KPC-3</sub>.

**Validation of *bla*<sub>KPC-3</sub> mutations.** Genes encoding D179Y/T243M, D179Y, and V240G KPC-3 variants were validated as causes of ceftazidime-avibactam resistance through three lines of evidence (Table 3). First, deletion of mutant *bla*<sub>KPC-3</sub> in ceftazidime-avibactam-resistant *K. pneumoniae* restored drug susceptibility. Second, laboratory transfer of mutant *bla*<sub>KPC-3</sub>-containing plasmids from *K. pneumoniae* to *Escherichia coli* DH10B resulted in  $\geq 4$ -fold increases in ceftazidime-avibactam MICs, compared to control, wild-type *bla*<sub>KPC-3</sub> transformants. Third, ceftazidime-avibactam MICs also increased against *E. coli* DH5 $\alpha$  harboring plasmid constructs with cloned mutant *bla*<sub>KPC-3</sub>, compared to *E. coli* DH5 $\alpha$  with wild-type *bla*<sub>KPC-3</sub> constructs. For mutant *bla*<sub>KPC-3</sub> encoding D179Y/T243M, D179Y, and V240G variants, ceftazidime-avibactam MICs were increased  $\geq 128$ -fold,  $\geq 16$ -fold, and  $\geq 4$ -fold, respectively. Mutant *bla*<sub>KPC-3</sub> encoding D179Y/T243M and D179Y variants conferred  $\geq 4$ -fold reductions in meropenem, cefepime, ceftriaxone, and aztreonam MICs against *E. coli* DH5 $\alpha$  compared to wild-type *bla*<sub>KPC-3</sub>.

## DISCUSSION

Ceftazidime-avibactam resistance due to plasmid-borne *bla*<sub>KPC-3</sub> mutations appeared within 10 to 19 days of treatment in three patients with carbapenem-resistant *K. pneumoniae* infections. These findings are particularly concerning because *K. pneumoniae* isolates were all ST258, the most successful CRE worldwide (18), and ceftazidime-avibactam resistance arose through a variety of *bla*<sub>KPC-3</sub> mutations. For some ceftazidime-avibactam-resistant isolates, *bla*<sub>KPC-3</sub> mutations restored meropenem susceptibility, suggesting that their clinical impact may be ameliorated. However, carbapenem susceptibility was not restored against all ceftazidime-avibactam-resistant isolates, and the stability of the phenotype is unknown. MICs of other  $\beta$ -lactams were

**TABLE 3** MICs against *bla*<sub>KPC-3</sub> deletion mutants and *E. coli* transformants<sup>a</sup>

KPC-3 variant	Description	MIC range (μg/ml)						
		Ceftazidime-avibactam	Ceftazidime	Meropenem	Piperacillin-tazobactam	Cefepime	Ceftriaxone	Aztreonam
<b>Wild-type</b>	<b>Clinical isolates 1-A, 2-A, 3-A</b>	<b>2–4</b>	<b>256–512</b>	<b>32–128</b>	<b>&gt;512</b>	<b>&gt;128</b>	<b>&gt;128</b>	<b>&gt;256</b>
	Clinical isolate with <i>bla</i> <sub>KPC</sub> deletion <sup>b</sup>	0.5	256	≤0.125	16–32	8	32	>256
	<i>E. coli</i> with <i>bla</i> <sub>KPC</sub> -containing plasmid from clinical isolate <sup>c</sup>	1	128	2	256	32	>128	>256
	<i>E. coli</i> with pET30a plasmid into which <i>bla</i> <sub>KPC</sub> was cloned <sup>d</sup>	≤0.25–0.5	64–128	2–8	>512	>128	>128	>256
<b>D179Y, T243 M</b>	<b>Clinical isolates 1-B, 1-C</b>	<b>256</b>	<b>&gt;512</b>	<b>0.25–0.5</b>	<b>&gt;512</b>	<b>16</b>	<b>32</b>	<b>8</b>
	Clinical isolate with <i>bla</i> <sub>KPC</sub> deletion <sup>b</sup>	≤0.25	128	≤0.125	128	8	4	2
	<i>E. coli</i> with <i>bla</i> <sub>KPC</sub> -containing plasmid from clinical isolate <sup>c</sup>	64	256	≤0.125	64	8	32	64
	<i>E. coli</i> with pET30a plasmid into which <i>bla</i> <sub>KPC</sub> was cloned <sup>d</sup>	64	≥512	≤0.125	2–4	2–8	4–8	2–4
<b>D179Y</b>	<b>Clinical isolates 2-C, 3-B, 3-C</b>	<b>64 to &gt;256</b>	<b>≥512</b>	<b>0.125–4</b>	<b>256 to &gt;512</b>	<b>4–16</b>	<b>4–16</b>	<b>4–8</b>
	Clinical isolate with <i>bla</i> <sub>KPC</sub> deletion <sup>b</sup>	≤0.25	256	≤0.125	32–128	16	32	>256
	<i>E. coli</i> with <i>bla</i> <sub>KPC</sub> -containing plasmid from clinical isolate <sup>c</sup>	16	128	≤0.125	16	4	4	2
	<i>E. coli</i> with pET30a plasmid into which <i>bla</i> <sub>KPC</sub> was cloned <sup>d</sup>	8	64	≤0.125	1	1	4	2
<b>V240G</b>	<b>Clinical isolate 2-B</b>	<b>32</b>	<b>&gt;512</b>	<b>8</b>	<b>&gt;512</b>	<b>&gt;128</b>	<b>&gt;128</b>	<b>&gt;256</b>
	Clinical isolate with <i>bla</i> <sub>KPC</sub> deletion <sup>b</sup>	0.5	128	≤0.125	32	8	32	>256
	<i>E. coli</i> with <i>bla</i> <sub>KPC</sub> -containing plasmid from clinical isolate <sup>c</sup>	4	512	1	256	32	>128	>256
	<i>E. coli</i> with pET30a plasmid into which <i>bla</i> <sub>KPC</sub> was cloned <sup>d</sup>	2	256	1	128	128	>128	>256

<sup>a</sup>Boldface rows represent baseline carbapenem-resistant *K. pneumoniae* isolates.

<sup>b</sup>*bla*<sub>KPC</sub> deletions were performed for a representative clinical isolate with each KPC-3 variant.

<sup>c</sup>IncFIA pBK30683-like plasmids were extracted from representative clinical isolates and transferred into *E. coli* DH10B.

<sup>d</sup>*bla*<sub>KPC</sub> genes from representative clinical isolates were cloned into a pET30a expression vector plasmid, which was transferred into *E. coli* DH5α. Ceftazidime-avibactam, ceftazidime, and meropenem MICs against *E. coli* DH5α carrying pET30a without *bla*<sub>KPC</sub> genes were ≤0.25, ≤0.5, and ≤0.125 μg/ml, respectively.

also reduced against *bla*<sub>KPC-3</sub> mutant *K. pneumoniae*. Nevertheless, isolates generally remained resistant to these drugs, highlighting that other plasmid-mediated β-lactamases contribute to overall resistance phenotypes. Taken together, the data raise questions of whether *bla*<sub>KPC</sub> mutations that confer ceftazidime-avibactam resistance will emerge in CRE at other centers, mutant *bla*<sub>KPC</sub> will disseminate through horizontal gene transfer, and carbapenems will serve as effective salvage agents in treating at least some infections by such pathogens.

In rank order, the impact of KPC-3 variants on ceftazidime-avibactam MICs was as follows: D179Y/T243M > D179 > V240G. D179Y/T243M and D179Y variants caused comparable reductions in meropenem MICs, which were greater than with V240G. The results are in keeping with published laboratory data. Ceftazidime-avibactam resistance and KPC-3 amino acid substitutions arose at low frequency (~10<sup>-9</sup>) in *K. pneumoniae* and *Enterobacter cloacae* during *in vitro* selection (19). D179Y emerged most frequently; a proline-for-threonine substitution at position 243 (T243P) was less common (19). D179Y and T243P mutants reverted to carbapenem susceptibility. In another study, introduction of substitutions at KPC-2 amino acid positions 179 and 243 by site-directed mutagenesis conferred ceftazidime-avibactam resistance in *E. coli* (20). *E. coli* recombinants carrying KPC-8 also exhibited significant increases in ceftazidime-avibactam MICs compared to control *E. coli* (16).

The mechanisms by which *bla*<sub>KPC-3</sub> mutations mediate ceftazidime-avibactam resistance and reversion to carbapenem susceptibility are not defined. The KPC Ω-loop encompasses amino acid positions 165 through 179. KPC variants with Ω-loop substitutions demonstrate enhanced ceftazidime affinity, which is postulated to prevent binding of avibactam (20). Therefore, Ω-loop substitutions exert ceftazidime-related effects, but they are not thought to confer avibactam resistance *per se* (19, 20). KPC-3 has 9- to 30-fold-greater catalytic efficiency than KPC-2 against ceftazidime (21, 22), and

we previously demonstrated that ceftazidime-avibactam MICs were significantly higher against *bla*<sub>KPC-3</sub>-producing than *bla*<sub>KPC-2</sub>-producing *K. pneumoniae* (8). These factors may predispose KPC-3-*K. pneumoniae* to ceftazidime-avibactam resistance (8). Stepwise mutations are well recognized to result in KPC variants with reduced hydrolytic activity against carbapenems (18). Kinetic studies are needed to determine if the KPC-3 substitutions described here attenuate carbapenemase activity. Finally, expression of mutant *bla*<sub>KPC-3</sub> encoding D179Y/T243M or D179Y variants was significantly diminished; these preliminary data suggest that transcriptional regulation may modulate meropenem and ceftazidime-avibactam susceptibility patterns.

These are the first reports of ceftazidime-avibactam resistance emerging during treatment of CRE infections. Preexisting resistance is reported in up to 2 to 3% of *Enterobacteriaceae* in clinical trials and large biorepositories (8, 10, 11, 23, 24). In a recent report from a large U.S. cancer center, ceftazidime-avibactam resistance was detected in 6 CRE isolates that were shown subsequently to carry NDM-1 (25). Since NDM-1 is not inhibited by avibactam, the study described baseline ceftazidime-avibactam resistance rather than the emergence of resistance as documented in the present study. Two case reports, including one from the same cancer center, described ceftazidime-avibactam resistance in non-NDM-carrying *K. pneumoniae* isolates recovered from untreated patients (25, 26). One isolate harbored KPC-3, and the other did not carry a carbapenemase, but mechanisms of resistance were not identified. The three cases of drug resistance presented here accounted for 30% of microbiologic failures in our early experience with ceftazidime-avibactam against CRE infections (12).

Our WGS data indicate that ceftazidime-avibactam resistance arose within a novel *K. pneumoniae* ST258 sublineage. It is unclear at present if this sublineage is uniquely predisposed to the development of *bla*<sub>KPC-3</sub> mutations or if mutations will be encountered at similar frequency in other ST258 populations, different STs, and diverse *Enterobacteriaceae*. Clearly, the 10 isolates here share a recent common ancestry. Nevertheless, resistance-conferring *bla*<sub>KPC-3</sub> mutations were more likely to have evolved independently in different isolates (and in different patients) than to have emerged in a single parent isolate that subsequently disseminated. In support of this conclusion, patient 1 did not have any epidemiological links with patients 2 and 3. In addition, KPC variants had mutations at three distinct amino acid positions; even isolates from patients 2 and 3, who resided in the same unit, exhibited distinct amino acid substitutions. Longitudinal isolates from each of the patients were also heterogeneous in their core genomes, carrying noncumulative SNPs that varied from isolate to isolate; isolates from patients 1 and 2 demonstrated a variety of *ompK36* porin genotypes. Therefore, patients appeared to harbor subpopulations of *K. pneumoniae* that evolved through different pathways from a common ancestor rather than in a direct lineage. Taken together, the findings highlight the well-recognized genetic plasticity and speed of evolutionary changes manifested by carbapenem-resistant *K. pneumoniae* (27).

Due to the potential for rapid KPC spread and evolution, it is imperative that centers detect CRE isolates and infected patients in a timely fashion. Our cases illustrate the difficulties faced by clinical laboratories in this regard. Indeed, several ceftazidime-avibactam-resistant isolates were reported as meropenem-susceptible, ESBL-producing *K. pneumoniae* and were not identified as KPC producing. Such misidentification was inevitable since carbapenemase screening by our laboratory, as at many centers, is triggered by elevated carbapenem MICs. Routine molecular screening for *bla*<sub>KPC</sub> has been advocated for rapid detection of carbapenem resistance (28). However, such assays will need to detect specific genes, since KPC variants have different antibiotic affinities and hydrolytic properties. As shown here, sequencing the *bla*<sub>KPC</sub> gene directly or by next-generation genomics technologies may be necessary to identify and interpret the loss or gain of carbapenem and ceftazidime-avibactam susceptibility.

In conclusion, ceftazidime-avibactam is one of the few new antibiotics at an advanced stage of development that have anti-CRE activity. In order to preserve ceftazidime-avibactam, the medical community must define optimal dosing strategies (including the role of antibiotic combination regimens), adhere to rigorous stewardship



and infection control practices, develop assays for detecting resistance and resistance determinants expeditiously, and understand resistance mechanisms. Together with reports of the recent emergence of MCR-1, the first plasmid-mediated colistin resistance determinant in *Enterobacteriaceae* (29), our findings attest to the urgent need for new drugs and treatment strategies against Gram-negative bacterial infections.

## MATERIALS AND METHODS

**Susceptibility testing.** MICs were determined by broth microdilution according to Clinical Laboratory Standard Institutes (CLSI) guidelines (30). Antimicrobial agents were purchased from the UPMC pharmacy. Avibactam (kindly provided by AztraZeneca) was tested at a fixed concentration of 4  $\mu$ g/ml in combination with ceftazidime (8). Susceptibility was defined according to CLSI interpretive criteria (31). Screening for ESBL production by the UPMC clinical microbiology laboratory used standard disc diffusion methods (31). In brief, isolates that were nonsusceptible to aztreonam and/or ceftriaxone were tested against ceftazidime and cefotaxime with and without clavulanic acid. Isolates demonstrating a  $\geq$ 5-mm increase in zone diameter for either agent in combination with clavulanic acid were classified as ESBL.

**Whole-genome sequencing (WGS) and analysis.** Genomic DNA was prepared from overnight cultures using a Wizard Genomic DNA purification kit (Promega, Madison, WI). Genome libraries were prepared using the Nextera DNA Library Preparation kit and sequenced using the Illumina NextSeq platform (paired end reads of 150 bp). Sequences were trimmed with sickle (GitHub), followed by *de novo* genome assembly using SPAdes 3.8 (32). *In silico* multilocus sequence typing and capsular *wzi* typing were performed using the BIGSdb online server (<http://bigsdb.pasteur.fr/klebsiella/klebsiella.html>). Antimicrobial resistance genes and plasmid replicons were identified using SRST2 (33). Novel plasmid replicons from our previous studies also were included in the analysis (15, 17).

Single nucleotide polymorphisms (SNPs) and insertion-deletions (indels) were called using BWA (34) and Samtools (35). SNP and indel annotation was conducted with SnpEff (36). *K. pneumoniae* isolate DMC1097 (37), an ST258 clade II (*cps-2*) strain from Detroit, MI, was used as reference. For core SNP analysis, SNPs located at repetitive (identified by nucmer from MUMmer) or prophage (identified by PHAST) regions of the genome were excluded. SNP phylogenetic analysis was performed using RAxML 8.0.0 (38). In addition, 22 ST258 clade II isolates from New Jersey (NJST258\_1, NJST258\_2, 1748, 1750, 1756, 1757) (15), New York (1765, 1790, 1789, 1814, 1801, 1780, 1764, 1824) (15), Maryland (KPNIH1, accession number CP008827) (39), Michigan (DMC1097, CP011976) (37), and UPMC (1818, 1808, 1809, 1816, 1811, 1817) (15) were included in the core SNPs analysis.

**qRT-PCR.** DNase-treated RNA was obtained from late-exponential-phase cultures (Mueller-Hinton broth, 37°C; RiboPure-Bacteria kit, ThermoFisher Scientific, MA). cDNA was made using qScript cDNAMix (Quanta Biosciences, MD). Quantitative reverse transcription (qRT)-PCR was performed using the Applied Biosystems 7900 system, with established *bla*<sub>KPC</sub> primers and the SYBR green kit (Quanta Biosciences, MD) (40). *bla*<sub>KPC</sub> expression was normalized to housekeeping gene *rpoB*. In each case, relative expression was calibrated against corresponding baseline isolates that were susceptible to ceftazidime-avibactam.

***bla*<sub>KPC</sub> gene deletion.** *bla*<sub>KPC</sub> genes in *K. pneumoniae* isolates were deleted using the Red/ET system (Gene Bridges, Heidelberg, Germany), according to the manufacturer's protocol with slight modifications. In brief, *K. pneumoniae* isolates were transformed with the pRED/ET plasmid, which contains all genes necessary for homologous recombination. A linear DNA fragment, comprising the FRT-PGK-gb2-arr3-FRT cassette (in which a kanamycin resistance gene was replaced by an *arr3* rifampin resistance gene) with 50-bp homology arms targeting the upstream and downstream sequence of *bla*<sub>KPC</sub>, was PCR amplified. The amplicon was electroporated into *K. pneumoniae* cells, resulting in replacement of *bla*<sub>KPC</sub> through recombination. Transformants, selected on lysogeny broth (LB) agar containing 100  $\mu$ g/ml of rifampin, were confirmed by PCR and sequencing.

**Plasmid transfer.** Plasmid DNA from *K. pneumoniae* isolates, extracted using a Qiagen Plasmid Midi kit (Qiagen, Valencia, CA), was electroporated into *Escherichia coli* DH10B (Invitrogen) using a Gene Pulser II instrument (Bio-Rad Laboratories). *E. coli* DH10B transformants were selected on LB agar plates with 20  $\mu$ g/ml ceftazidime. Transformants were screened by multiplex real-time PCR for the presence of *bla*<sub>KPC</sub> genes (41) and checked by S1-PFGE (S1 nuclease digestion of plasmid DNA followed by pulsed-field gel electrophoresis) for the presence of a single *bla*<sub>KPC</sub>-harboring plasmid. Mutations within *bla*<sub>KPC</sub> genes were confirmed using standard PCR and sequencing.

***bla*<sub>KPC-3</sub> cloning.** *bla*<sub>KPC</sub> and flanking regions (600 bp upstream, 240 bp downstream) were amplified from plasmid DNA by PCR using primers KPC3\_For\_KpnI (CGGGGTACCCTGCTGGTGATCGATGAGAT) and KPC3\_Rev\_EcoRI (CCGGAAATCTTAAATGGGGCGGCTCAAAT), which contained introduced KpnI and EcoRI restriction sites, respectively (underlined). Amplicons were digested and ligated in the antisense direction into pET30a (Novagen, Madison, WI); inserts were sequenced on both strands. Plasmids were transformed into *E. coli* DH5 $\alpha$  by electroporation. Successful transfer of *bla*<sub>KPC</sub> genes was confirmed by PCR and sequencing.

**Accession number(s).** Sequence data have been deposited in NCBI under BioProject number PRJNA326665.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.02097-16>.

**TEXT S1**, PDF file, 0.06 MB.

## ACKNOWLEDGMENTS

This work was funded, in part, by grants from the National Institutes of Health (K08AI114883 to R.K.S., R21AI117338 to L.C., R01AI090155 to B.N.K., UM1AI104681 to M.H.N., and R21AI111037 to C.J.C.).

We declare that we have no conflicts of interest.

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