



Increased *bla*_{KPC} Copy Number and OmpK35 and OmpK36 Porins Disruption Mediated Resistance to Imipenem/Relebactam and Meropenem/Vaborbactam in a KPC-Producing *Klebsiella pneumoniae* Clinical Isolate

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ABSTRACT Herein, we report the *in vivo* evolution of imipenem/relebactam-resistance in KPC-producing *K. pneumoniae* (KPC-Kp) isolated from a critically-ill patient treated with multiple combination therapies based on ceftazidime-avibactam or meropenem-vaborbactam. Imipenem/relebactam-resistance was associated to meropenem-vaborbactam cross-resistance and was due to truncated OmpK35 and OmpK36 porins and increased copy of *bla*_{KPC} copy number. Genome analysis demonstrated that imipenem/relebactam-resistant KPC-Kp harbored a second copy of *bla*_{KPC}-carrying Tn4401 in a ColRNAI plasmid as a consequence of a transposition event.

KEYWORDS cross-resistance, Tn4401, copy number, public health, transposition mechanisms

Development of novel antimicrobial molecules represents a key to overcome clinical and microbiological challenge of the difficult-to-treat resistance (DTR) infections (1). The introduction of novel β -lactam/ β -lactamase inhibitor combinations (BLICs) partially solved the pressing need to treat DTR (2). Imipenem-cilastatin-relebactam (IMI-REL) is a novel combination recently approved by the FDA in 2019 for patients with limited or no alternative therapeutic options (3). Although IMI-REL represents a valuable option for the treatment of infections due to DTR, emerging IMI-REL-resistant isolates have been recently reported (4). Herein, we characterized the evolution of the resistance to IMI-REL in a series of KPC-producing *Klebsiella pneumoniae* (KPC-Kp) following ceftazidime-avibactam (CAZ-AVI) antimicrobial exposure.

A 35-year-old woman suffering from systemic lupus erythematosus with end-stage renal disease and autoimmune liver cirrhosis was admitted for simultaneous liver-kidney transplantation. Surveillance rectal swabs on admission revealed that the patient was colonized by KPC-Kp (isolate KPC-Kp_TO1). After 25 days of hospitalization, patient presented with pneumonia, and subculture from broncho-alveolar lavage fluid yielded a CAZ-AVI-susceptible KPC-Kp strain (isolate KPC-Kp_TO2). According to the antimicrobial susceptibility pattern, ceftazidime-avibactam with fosfomycin was started for an overall therapy of 10 days with patient clinical improvement. On day 69, blood cultures collected yielded a KPC-Kp strain (isolate KPC-Kp_TO3) susceptible to imipenem, meropenem, and CAZ-AVI; thus, a treatment with meropenem in association with CAZ-AVI and tigecycline was started for an overall therapy of 21 days. On hospital day 92, patient underwent simultaneous liver-kidney transplantation, and meropenem-vaborbactam (MER-VAB) plus tigecycline were administered for 7 days as empirical therapy. After 16 days from transplantation, patient presented with fever and abdominal pain,

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TABLE 1 Antimicrobial susceptibility profiles of KPC-producing *Klebsiella pneumoniae* strains included in this study^a

Isolate	Sample	MIC (ug/mL)								
		FOS	GEN	TGC	IPM	MEM	CAZ/AVI	MEM/VAB	IPM/REL	CFD
KPC-KP_TO1	Rectal swab	64	<2	≤0.5	> 32	> 32	0.5	1	0.25	0.5
KPC-KP_TO2	BAL	≤16	<2	≤0.5	> 32	> 32	2	2	0.5	0.5
KPC-KP_TO3	Blood	≤16	<2	≤0.5	0.125	1	4	1	0.125	0.5
KPC-KP_TO4	ADF	> 64	<2	≤0.5	> 32	> 32	1	2	2	0.125
KPC-KP_TO5	Blood	> 64	<2	≤0.5	> 32	> 32	8	≥ 256	8	0.25

^aBAL, broncho-alveolar lavage fluid; ADF, abscess drainage fluid; FOS, fosfomycin; GEN, gentamicin; TGC, tigecycline; IPM, imipenem; MEM, meropenem; CAZ/AVI, ceftazidime-avibactam; MER/VAB, meropenem-vaborbactam; IPM/REL, imipenem/relebactam; CFD, cefiderocol. Resistance is shown in bold.

and abdominal-pelvic computer tomography disclosed a peri-hepatic abscess that was promptly drained. Empirical therapy based on 3-h infusion of MER-VAB was started. Subculture from abscess drainage fluid yielded a CAZ-AVI-susceptible KPC-Kp strain (isolate TO4). According to antimicrobial susceptibility results, fosfomycin was added to MER-VAB for an overall therapy of 14 days with progressive clinical improvement. On day 134, patient presented with septic shock requiring fluid resuscitation and vasopressor support. Empirical therapy based on 3-h infusion of MER-VAB plus 4-h infusion of tigecycline and high-dose extended interval gentamicin was started. Blood cultures collected yielded a KPC-Kp strain (isolate TO5) resistant to MER-VAB and carbapenem and susceptible to CAZ-AVI. Based on clinical conditions and limited antimicrobial options, MER-VAB in association with gentamicin was continued, and despite initial clinical and analytic improvement, patient died 16 days later.

Antimicrobial susceptibility testing was performed using automated system MicroScan Walkaway-96 (Beckman Coulter, USA). The MICs for IMI-REL, MER-VAB, and CAZ-AVI were determined by using Microtiter Sensititre Plate MDRGN2F (ThermoFisher, USA) and confirmed with MIC test strips (Liofilchem, Italy). MIC values were interpreted following European Committee for Antimicrobial Susceptibility Testing (EUCAST) breakpoints v11.0. Antimicrobial susceptibility patterns of KPC-Kp strains showed that all strains were susceptible to CAZ-AVI (MICs range 0.5–8 μg/L) and cefiderocol (MICs range 0.125–0.5 mg/L) (Table 1). At the same time, four out of five KPC-Kp strains were susceptible to MER-VAB (MIC range 1–2 mg/L), while KPC producer isolated from the last fosfomycin episode (named KPC-Kp_TO5) exhibited an increased MIC of 2–6-fold for IMI-REL, 7–8 -fold for MER-VAB, and 2–4 -fold for CAZ-AVI compared to parental strains.

WGS was performed using Illumina iSeq100 (Illumina, USA) and Oxford Nanopore MinION (ONT, UK). Hybrid assemblies were performed using Unicycler v.0.4.7 and polished with Pilon v.1.23. Antimicrobial resistance genes, plasmid content, antimicrobial resistance genes, and MLST analysis were performed as previously described (5). Genomic analysis demonstrated that all KPC-Kp belonged to the Sequence Type ST512, harbored the same plasmid replicon types [i.e., ColRNAI, IncFIB (pQIL), IncFII(K)], and shared similar antimicrobial encoding genes (Table 2). In detail, analysis of β-lactamase genes showed that all strains harbored *bla*_{TEM1A'}, *bla*_{OXA-9'} and *bla*_{SHV-11'}, while carbapenemase gene was present with *bla*_{KPC-3} in KPC-Kp_TO1 and KPC-Kp_TO5 strains, and *bla*_{KPC-66} in KPC-Kp_TO3. Analysis of porin genes showed that all strains harbored truncated ompK35 porin, while OmpK36 porin was present with GD134 insertion within IMI-REL-susceptible strains and resulted truncated in the IMI-REL-resistant KPC-Kp_TO5 strain. Sequence analysis of circular plasmids demonstrated that *bla*_{KPC}-harboring Tn4401 transposon was present in single copy in IMI-REL-susceptible KPC-Kp, while it was present in double copies within IMI-REL-resistant KPC-Kp_TO5 (Fig. 1). In particular, Tn4401a transposon was located within IncFIB- IncFII(K) 115Kb plasmid in all sequentially-isolated KPC-Kp strain, while the second copy in the IMI-REL-resistant KPC-Kp_TO5 was located in a 25 Kb plasmid. Deep sequence analysis of 25 Kb plasmid demonstrated that the Tn4401 has been inserted into a ColRNAI plasmid common to parental KPC-Kp strains susceptible to IMI-REL and flanked by 5-bp direct repeats (DR) (Fig. 1).

The *bla*_{KPC} copy number was evaluated using the C_T value of target gene (*bla*_{KPC})

TABLE 2 Genomic characteristics of KPC-producing *Klebsiella pneumoniae* strains^a

Isolate	ST	Antimicrobial resistance determinants			Porins		Plasmid_replicons (InC)	<i>bla</i> _{KPC} copy no.
		Beta-lactam	Aminoglycosides		<i>ompK35</i>	<i>ompK36</i>		
KPC-KP_TO1	512	<i>bla</i> _{KPC-3} <i>bla</i> _{TEM1A'} <i>bla</i> _{OXA-9}	<i>aadA2b</i> , <i>aac(6')-Ib-cr</i>		Truncated at aa 41	GD insertion at aa 134-135	ColRNAI, IncFIB (pQIL), IncFII(K)	1 ^b
KPC-KP_TO3	512	<i>bla</i> _{KPC-66} <i>bla</i> _{TEM1A'} <i>bla</i> _{OXA-9}	<i>aadA2b</i> , <i>aac(6')-Ib-cr</i>		Truncated at aa 41	GD insertion at aa 134-135	ColRNAI, IncFIB (pQIL), IncFII(K)	0,85
KPC-KP_TO5	512	<i>bla</i> _{KPC-3} <i>bla</i> _{TEM1A'} <i>bla</i> _{OXA-9}	<i>aadA2b</i> , <i>aac(6')-Ib-cr</i>		Truncated at aa 41	Truncated at aa 310	ColRNAI, IncFIB (pQIL), IncFII(K)	4,59

^aST, sequence type; ND, not determined; GD, glycine and aspartic acid.

^bUsed as reference strain.

normalized to endogenous control gene (16S rDNA), as previously described (6). Relative quantification (RQ) analysis of *bla*_{KPC} gene demonstrated that IMI-REL-resistant KPC-Kp_TO5 had a *bla*_{KPC} copy number higher 4.59-fold than IMI-REL-susceptible KPC-Kp isolates (Table 1).

Comparative analysis based on Breseq software (7) demonstrated that Illumina reads of IMI-REL-susceptible strains aligned against IMI-REL-resistant KPC-Kp annotated genome identified respectively a total of 79 and 100 predicted mutations, most of them annotated as hypothetical proteins. Also, phylogenetic analysis generated based on the core genome SNPs analysis showed that KPC-Kp_TO3 and KPC-Kp_TO5 genomes differed by 175 and 167 SNPs with KPC-Kp_TO1, used as reference, respectively, thus demonstrating close genetic relationship.

In this study, we identified the mechanism at the basis of IMI-REL resistance in KPC-Kp isolated from a critically-ill patient treated with multiple antimicrobial combination therapies based on ceftazidime-avibactam or meropenem-vaborbactam. Our results are consistent with previous studies showing that increased level of expression of

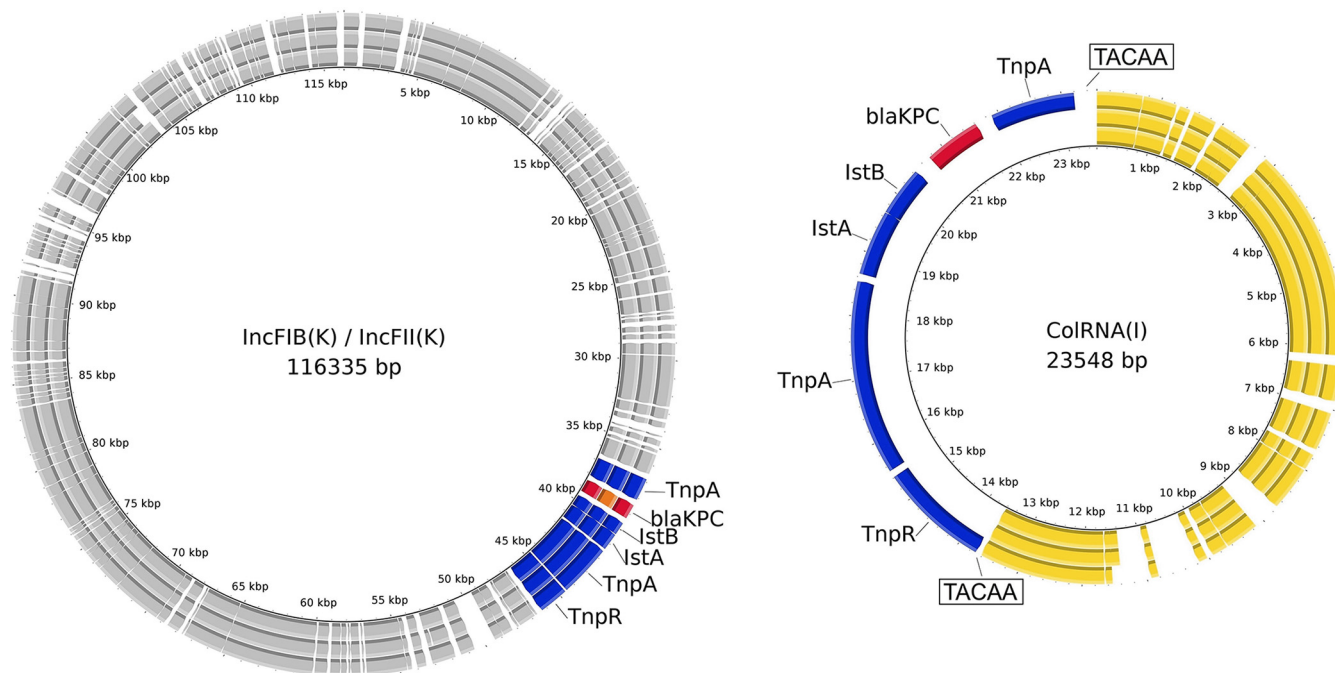


FIG 1 Circular representation of the IncFIB/IncFII and ColRNAI plasmids in the KPC-producing *Klebsiella pneumoniae* included in this study. From outside to inside: KPC-Kp_TO5 strains, KPC-Kp_TO3, and KPC-Kp_TO1. The CDS are color-coded with the direction of transcription indicated by arrowheads. The Tn4401 transposons are displayed in blue, KPC-3 in red, KPC-66 in orange, ColRNAI backbone in yellow, and other CDS in gray. Boxes indicate direct repeats (DRs).

*bla*_{KPC} gene in combination with reduced porins expression impacted the activity of IMI-REL, thus reducing its *in vitro* efficacy (4, 8, 9).

Clinical history showed that cross-resistance to meropenem-vaborbactam and IMI-REL emerged following multiple antimicrobial exposure to novel BLICs, thus suggesting the *in vivo* genomic adaption of *K. pneumoniae*. In line with this hypothesis, the first bacteremic episode was due to a KPC-Kp closely related to other KPC-Kp strains isolated from the same patient and that harbored a *bla*_{KPC-66} gene, a derivative of *bla*_{KPC-3} with an amino acid duplication at positions 167 and 168 (EL167-168). Although this variant did not confer resistance to IMI-REL, it should be noted that KPC-Kp carrying *bla*_{KPC-66} variant exhibited and increased MIC 3-fold for CAZ-AVI, reverted susceptibility to carbapenem in comparison to parental strain (i.e., KPC-Kp_TO1), and emerged after ceftazidime-avibactam-based therapy following pneumonia episode, thus suggesting a strength antimicrobial selection of mutated population after BLICs exposure in this anatomical site (10). These data are in accordance with previous studies showing that SNPs mutations within Ω-loop of the *bla*_{KPC} gene conferring CAZ-AVI-resistance was associated to increased susceptibility to carbapenems (11–12).

Lastly, IMI-REL-resistance was associated with an increased MIC for meropenem-vaborbactam and ceftazidime-avibactam, thus suggesting that the mechanisms conferring this type of resistance could be related to a cross-resistance for novel BLICs. Further studies are necessary to elucidate the microbiological impact of different therapies based on novel BLICs to selected mutant-resistant strains and to clarify the impact of cross-resistance on the clinical outcome.

Data availability. The sequences of KPC-Kp genomes included in this study were deposited at EMBL/EBI under the project study (EBI project [PRJNA794363](https://www.ebi.ac.uk/PRJNA794363)) with the following accession numbers: KPC-KP_TO1 ([SAMN24648676](https://www.ebi.ac.uk/PRJNA794363/SAMN24648676)), KPC-KP_TO3 ([SAMN24648677](https://www.ebi.ac.uk/PRJNA794363/SAMN24648677)), KPC-KP_TO5 ([SAMN24648678](https://www.ebi.ac.uk/PRJNA794363/SAMN24648678)).

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