Journal of Global Antimicrobial Resistance 32 (2023) 66–71

Contents lists available at ScienceDirect

Journal of Global Antimicrobial Resistance

journal homepage: www.elsevier.com/locate/jgar

In vitro-obtained meropenem-vaborbactam resistance mechanisms among clinical *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae* isolates

Jacqueline Findlay[a,*], Laurent Poirel[a,b], Patrice Nordmann[a,b,c]

[a] Medical and Molecular Microbiology, Faculty of Science and Medicine, University of Fribourg, Fribourg, Switzerland
[b] Swiss National Reference Center for Emerging Antibiotic Resistance, University of Fribourg, Fribourg, Switzerland
[c] Institute for Microbiology, University of Lausanne and University Hospital Centre, Lausanne, Switzerland

ARTICLE INFO

Article history:
Received 18 November 2022
Revised 22 December 2022
Accepted 26 December 2022
Available online 14 January 2023

Editor: Prof Holger Rohde

Keywords:
Klebsiella pneumoniae
Meropenem-vaborbactam
KPC
Carbapenemase
Antibiotic resistance

ABSTRACT

Objectives: A novel ß-lactam-β-lactamase inhibitor (BLBI), meropenem (MEM), combined with the boronate-based inhibitor vaborbactam (VAB), has recently been introduced for the treatment of infections caused by *Klebsiella pneumoniae* carbapenemase (KPC)-producing *Enterobacterales*. The purpose of this study was to select for MEM-VAB resistance using a collection of eight KPC-producing *K. pneumoniae* clinical isolates, including three that produce KPC variants conferring ceftazidime-avibactam (CAZ-AVI) resistance, and subsequently decipher the corresponding resistance mechanisms.

Methods: Mutants were selected in a stepwise process on agar plates containing different MEM-VAB concentrations. Susceptibility testing was performed by broth microdilution, and complementation assays were performed with wildtype *ompK36*. Whole genome sequencing was performed on mutants, and KPC copy number was assessed by quantitative polymerase chain reaction .

Results: Mutants were obtained from 6/8 tested isolates and reduced susceptibility to all tested β-lactams, and BLBIs, including CAZ-AVI, imipenem-relebactam, and aztreonam-AVI, were observed. No mutations were identified in the bla_{KPC}. However, mutations in *ompK36* were observed in four mutant lineages, and complementation with a wild-type *ompK36* resulted in a reduction of minimal inhibitory concentrations to both MEM-VAB and other ß-lactams/BLBIs. bla_{KPC} gene copy numbers were significantly increased in four mutant lineages. Whole genome sequencing identified genomic rearrangements in two lineages comprising mutations in the plasmid replicon encoding gene and duplication of the Tn*4401* transposon bearing the bla_{KPC} gene into a ColE-like, high copy number plasmid.

Conclusions: In contrast to what is observed with KPC-producing mutants exhibiting resistance to CAZ-AVI, mainly corresponding to mutated KPC enzymes, here the MEM-VAB-resistant mutants showed permeability defects combined with increased KPC production, resulting from genomic rearrangement.

© 2023 The Author(s). Published by Elsevier Ltd on behalf of International Society for Antimicrobial Chemotherapy.
This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/)

1. Introduction

Since their initial identification over two decades ago in the USA [1], *Klebsiella pneumoniae* carbapenemases (KPCs) have successfully disseminated globally and have become an increasingly significant threat to public health [2.3]. KPCs are predominantly found in Enterobacterales (particularly *K. pneumoniae*), usually located on plasmids, but they have also been identified in non-Enterobacterale species including *Pseudomonas* Spp. [2] and *Acinetobacter* Spp. [4]. Most KPC producers are multidrug resistant [2,3], harbouring resistance genes encoding resistance mechanisms that also compromise the efficacy of non-β-lactam antibiotics, subsequently limiting the treatment options for infections caused by those KPC-producing bacteria.

Vaborbactam (VAB) is a non-ß-lactam ß-lactamase inhibitor with activity against class A and C serine β-lactamases [5], and it is the first boronate-based inhibitor approved for clinical use. VAB has been developed for use alongside the carbapenem antibiotic, meropenem (MEM), to treat serious multidrug-resistant Gram-negative infections (e.g. bacteraemia, hospital acquired pneumonia,

* Corresponding Author: Medical and Molecular Microbiology Unit, Department of Medicine, Faculty of Science, University of Fribourg, Chemin du Musée 18, CH-1700 Fribourg, Switzerland
E-mail address: jacqueline.findlay@unifr.ch (J. Findlay).

https://doi.org/10.1016/j.jgar.2022.12.009
2213-7165/© 2023 The Author(s). Published by Elsevier Ltd on behalf of International Society for Antimicrobial Chemotherapy. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/)

Table 1
Characteristics of the eight parent KPC-producing *K. pneumoniae* strains.

			Porins		MIC (mg/L)	
Strain	KPC Variant	ST	OmpK35	OmpK36	MEM	MEM-VAB
N931	KPC-2	34	IS Prom	WT	32	≤ 0.06
N1118	KPC-2	11	TR	WT	64	≤ 0.06
N52	KPC-3	730	WT	WT	16	≤ 0.06
N119	KPC-3	405	WT	WT	32	≤ 0.06
N1762	KPC-3	512	TR	GD ins L3	> 256	4
N435	KPC-41	395	TR	WT	1	0.125
N2280	KPC-46	101	TR	TD ins L3	2	1
N859	KPC-50	258	TR	WT	4	0.125

GD ins L3, GD insertion in the L3 loop; MEM, meropenem; ST, sequence type; TD ins L3, TD insertion in the L3 loop; TR, truncated; VAB, vaborbactam; WT, wildtype

complicated urinary tract infections, complicated intraabdominal infections), particularly those producing KPC enzymes [5,6].

MEM-VAB was approved for use by the US Food and Drug Administration in 2017 [7] and by the European Medicines Agency in 2018 [8], and since then there have been only sparse reports of KPC-producing *K. pneumoniae* clinical isolates being resistant to this drug combination [9,10]. With the recent increasing incidence of ceftazidime-avibactam (CAZ-AVI) resistance emerging during treatment among KPC-producing *K. pneumoniae* isolates, which is predominantly attributed to mutations within the bla_{KPC} gene [11–14], MEM-VAB might be favoured over CAZ-AVI for the treatment of serious infections caused by KPC producers. It is therefore necessary to understand the mechanisms by which such resistance may arise.

Previously, in-vitro mutation studies have shown that MEM-VAB resistance can arise in KPC-producing *K. pneumoniae* via mechanisms including permeability changes and bla_{KPC} gene overexpression. However, such studies have mostly been performed on strains harbouring the "classical" KPC variants, KPC-2 and KPC-3 [10,15,16]. KPC variants that confer CAZ-AVI resistance usually do so at a cost of their ability to hydrolyse carbapenems [11–14], resulting in strains that become susceptible to both MEM and remain susceptible to MEM-VAB.

Additionally, a recent study has shown that MEM-VAB exhibits excellent activity against KPC-producing *K. pneumoniae*, regardless of whether they produce the "classic variants" or those that confer resistance to CAZ-AVI [16]. Nevertheless, resistance development to MEM-VAB in *K. pneumoniae* producing those KPC variants conferring resistance to CAZ-AVI has not been widely studied.

The purpose of this study was to select for MEM-VAB resistance in a collection of clinical KPC-producing *K. pneumoniae* isolates, including three isolates producing KPC variants that confer CAZ-AVI resistance, and subsequently ascertain the mechanisms responsible.

2. Methods and Materials

2.1. Bacterial isolates

Eight clinical KPC-producing *K. pneumoniae* isolates, all obtained from hospitals in Switzerland, were selected for this study: two KPC-2 producers (N931 and N1118), three KPC-3 producers (N52, N119 and N1762), and single isolates producing KPC variants resistant to CAZ-AVI. The latter either produced KPC-41 (N435) [12], KPC-46 (N2280), or KPC-50 (N859) [13]. Isolates had previously been subject to whole-genome sequencing (WGS) and were selected to represent diverse but clinically relevant sequence types (STs) and KPC variants. Characteristics of the isolates including STs, OmpK35 and OmpK36 porin genotypes, and minimal inhibitory concentrations (MICs) of MEM-VAB are summarized in Table 1.

2.2. Susceptibility testing

MICs of antimicrobial agents were determined by broth microdilution according to Clinical and Laboratory Standards Institute methodology [17] and interpreted according to the European Committee on Antimicrobial Susceptibility Guidelines [18]. The preparation of the ß-lactam / ß-lactamase inhibitors (BLBIs) were performed according to the Clinical and Laboratory Standards Institute guidelines [17], with a fixed concentration of the inhibitor at 4 mg/L for avibactam (AVI) and relebactam (REL), and 8 mg/L for VAB.

2.3. MEM-VAB mutant selection

K. pneumoniae mutant strains were selected by either single-step or multi-step mutation selection. Briefly, strains were grown overnight in a liquid culture (Luria-Bertani [LB]) and one hundred microliters of overnight culture were spread on LB agar plates containing varying concentrations of MEM and with VAB at a fixed concentration of 8 mg/L. Selection was initially attempted at the MEM-VAB breakpoint concentration (MEM at 8 mg/L) for all isolates. However, where mutants were not initially obtained, the MEM selection concentrations were reduced to 0.125–4 mg/L, respectively. Hence, mutants were obtained at sub-breakpoint concentrations were then selected on increasing MEM-VAB concentrations, in a stepwise process. Following overnight incubation at 37 °C, individual mutant colonies were selected and plated onto antibiotic-free agar for further analysis.

2.4. Whole genome sequencing

Total genomic DNA (gDNA) of isolates was extracted from a bacterial culture grown overnight using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA). WGS was performed using either a MiSeq (Illumina, San Diego, CA) or MinION Mk1C (Oxford Nanopore Technologies, Oxford, UK) platforms. For short-read sequencing, DNA libraries were constructed using the Nextera (Illumina) sample preparation method with 2 × 300 bp paired end reads and a coverage of ≥ 50X. For MinION sequencing, sequencing libraries were prepared using a native barcoding kit (EXP-NBD104; Oxford Nanopore Technologies) and 1D chemistry Ligation Sequencing Kit (SQK-LSK109; Oxford Nanopore Technologies). Sequencing was performed on a MinION Mk1C sequencer (Oxford Nanopore Technologies) using a R9.4.1 Flow Cell (FLO-MIN106; Oxford Nanopore Technologies).

Short-read sequencing reads were trimmed using Trimmomatic [19]. Assembles were performed using the Shovill pipeline (https://github.com/tseemann/shovill), and contigs were annotated using Prokka [20]. STs, the presence of resistance genes, plasmid replicon types, and the confirmation of speciation were determined using MLST 2.0, ResFinder 4.1 [21], PlasmidFinder 2.1 [22], and KmerFinder 3.2 [23] on the Center for Genomic Epidemiology platform (https://www.genomicepidemiology.org/). Long-read sequencing reads were trimmed and corrected using Canu [24]. Hybrid assembles, using both short- and long-read data, were performed using UniCycler [25].

Sequence data from this study was submitted to the National Center for Biotechnology Information's Sequence Read Archive (BioProject no. PRJNA897716).

2.5. ompK36 sequencing

ompK36 alleles were amplified by polymerase chain reaction (PCR) from all mutant isolates and clones using primers ompK 36 QQ1 (5'- GACAAGCTTTAAAAGGCATATAACAAACAG-3') and ompK36

QQ2 (5'- CTGGGATCCAGCGAGGTTAAACCGG-3') [26], and subjected to Sanger sequencing.

2.6. ompK36 cloning and complementation

A wild-type *ompK36* gene was amplified from *K. pneumoniae* MGH78578 (GenBank Accession No. NC_009648) and cloned into pACYC184 using either primers ompK36 QQ1 and ompK36 QQ2, or primers ompK36 QQ3 (5'- GACCCATGGTAAAAGGCATATAACAAACAG-3') and ompK36 QQ4 (5'- CTGCCATGGAGCGAGGTTAAACCGG-3'), to generate plasmid pACompK36 that was further selected by supplementing selective plates either with tetracycline (25 mg/L) or chloramphenicol (20 mg/L). Both the original plasmid pACYC184 and recombinant plasmid pACompK36 were transformed into strains and mutants of N931, N435 and N859.

2.7. bla_{KPC} gene copy number determination

Determination of the bla_{KPC} copy number was determined using cell lysates being prepared as previously described [15]. Quantitative PCR (qPCR) was performed on a Qiagen RotorGene using Promega Sybr Green PCR mix, using primers KPC-qF (5'-CGCCAATTTGTTGCTGAAGG-3') and KPC-qR (5'-GCCAATCAACAAACTGCTGC-3') for the bla_{KPC} gene, and primers 16S-qF (5'-TCATGGAGTCGAGTTGCAGA-3') and 16S-qR (5'- GCGCAACCCTTATCCTTTGT-3') for 16S rRNA. All assays were performed on three biological replicates and the delta-delta Ct method was used to calculate relative copy numbers of the KPC gene for mutants when compared with the parental strain set to 1.

3. Results and discussion

3.1. Phenotypic and genotypic profiling of MEM-VAB-resistant mutants

Direct selection of mutants on agar plates containing breakpoint concentrations of MEM-VAB (8/8 mg/L) did not result in selection of mutant colonies for any of the isolates, with the exception of N1762 (KPC-3 producer), for which there was confluent growth. Subsequently, a range of mutation selection concentrations were attempted using sub-breakpoint (0.125–4 mg/L) levels of MEM, whilst VAB remained fixed at 8 mg/L, in a stepwise process for the remaining seven isolates, and at greater than breakpoint level for strain N1762 (32 mg/L) (Table 2). A second step of mutant selection was performed on increasing MEM-VAB concentrations for all isolates that produced mutants after a single selection step, except N1762, which already exhibited high-level MEM-VAB resistance. Overall mutants were obtained from six parental isolates in either one or two steps of selection, respectively (Table 2).

Mutants and parents were subject to susceptibility testing to a range of antibiotics (Table 3). As expected, susceptibility levels to β-lactam antibiotics were reduced in line with increasing MICs of MEM-VAB. Of note, the MICs of three other BLBI combinations, namely CAZ-AVI, imipenem-REL, and aztreonam-AVI, were also significantly increased, highlighting a mechanistic cross-resistance. MICs of amikacin, chloramphenicol, tigecycline, and ciprofloxacin (typical substrates of efflux pump) [27] remained relatively unchanged between the parent and mutant strains, indicating that efflux was unlikely playing a role in the observed acquired resistance to MEM-VAB of these isolates (Table S1).

Amongst the parent isolates, different combinations of porin gene alterations (*ompK35* and *ompK36*) were represented (Table 1). Most isolates harboured substitutions resulting in truncation or disruption of the *ompK35* promoter region, likely resulting in no

Table 2
Susceptibility testing, identified mutations in OmpK36, and relative KPC copy number in parent strains and mutants.

Strain	MEM-VAB[1] Selection Concentration	Mutation Frequency	MICs (mg/L)										KPC relative copy number
			ETP	IPM	IPM-REL	MEM	MEM-VAB	CAZ	CAZ-AVI	ATM	ATM-AVI	FEP	OmpK36	
N931	NA	NA	32	8	0.25	16	≤ 0.06	64	0.5	256	0.25	8	WT	1.0
N931 M1	1	2.04×10^{-9}	> 256	256	4	>256	2	128	2	> 256	0.5	> 256	TR	1.0
N931 M2	4	3.51×10^{-9}	> 256	> 256	32	> 256	64	256	8	> 256	8	> 256	TR	5.1
N1118	NA	NA	128	16	0.5	64	≤ 0.06	64	1	> 256	0.125	128	WT	1.0
N1118 M1	0.5	5.48×10^{-9}	> 256	> 256	8	> 256	4	128	2	> 256	0.25	> 256	V319D	1.0
N1118 M2	16	1.35×10^{-7}	> 256	> 256	128	> 256	256	256	32	> 256	32	> 256	V319D	5.9
N1762	NA	NA	> 256	> 256	2	> 256	4	> 256	4	> 256	2	256	GD ins	1.0
N1762 M1	32	1.21×10^{-7}	> 256	> 256	32	> 256	256	> 256	32	> 256	16	> 256	GD ins	6.1
N435	NA	NA	8	4	0.25	1	0.125	256	256	64	1	16	WT	1.0
N435 M1	4	5.57×10^{-10}	256	128	16	64	16	> 256	> 256	128	2	128	Y326D	1.0
N435 M2	64	4.48×10^{-8}	> 256	> 256	128	> 256	128	> 256	> 256	> 256	8	> 256	Y326D	3.5
N859	NA	NA	16	8	1	4	0.125	> 256	> 256	128	4	> 256	WT	1.0
N859 M1	1	1.21×10^{-7}	> 256	128	2	128	8	> 256	> 256	128	32	> 256	TR	1.1
N859 M2	16	5.56×10^{-7}	> 256	256	8	256	64	> 256	> 256	128	64	256	TR	1.1
N2280	NA	NA	8	2	0.5	2	1	> 256	64	32	2	64	TD ins	1.0
N2280 M1	2	9.54×10^{-8}	64	8	8	16	16	> 256	128	32	4	128	TD ins	1.1
N2280 M2	8	2.62×10^{-8}	128	16	8	16	16	> 256	128	32	4	128	TD ins	1.2

[1] VAB concentration remained fixed at 8 mg/LATM, aztreonam; AVI, avibactam; CAZ, ceftazidime; ETP, ertapenem; FEP, cefepime; GD ins; GD amino acid insertion in L3, IS Prom; IS5 insertion sequence disrupting the promoter region. IPM, imipenem; REL, relebactam; TR, truncated; WT, wildtype

Table 3
MICs of parental and mutant strains with vector only, pACYC184 (P), or Ompk36 complemented, pACompK36 (PK36).

		MIC (mg/L)									
Strain	OmpK36	ETP	IPM	IPM-REL	MEM	MEM-VAB	CAZ	CAZ-AVI	ATM	ATM-AVI	FEP
N931/P	WT	32	8	0.25	16	≤ 0.06	64	0.5	256	0.25	8
N931/PK36		32	8	0.25	16	≤ 0.06	64	0.5	256	0.25	8
N931 M1/P	TR	> 256	256	2	> 256	2	128	2	> 256	0.5	> 256
N931 M1/PK36		64	16	0.25	16	≤ 0.06	64	0.5	> 256	0.25	32
N931 M2/P	TR	> 256	> 256	32	> 256	64	256	8	> 256	8	> 256
N931 M2/PK36		256	32	0.5	64	0.25	128	1	> 256	0.25	64
N435/P	WT	8	4	0.25	1	0.125	> 256	256	64	1	16
N435/PK36		8	4	0.25	1	0.125	> 256	256	64	1	16
N435 M1/P	Y326D	256	128	16	64	16	> 256	> 256	256	2	128
N435 M1/PK36		16	16	0.5	4	1	> 256	> 256	64	1	64
N435 M2/P	Y326D	> 256	> 256	128	> 256	128	> 256	> 256	256	8	256
N435 M2/PK36		64	32	2	16	8	> 256	> 256	128	1	64
N859/P	WT	16	8	1	4	0.125	> 256	> 256	128	4	128
N859/PK36		16	8	1	4	0.125	> 256	> 256	128	4	128
N859 M1/P	TR	> 256	128	2	128	8	> 256	> 256	128	32	> 256
N859 M1/PK36		32	64	1	8	1	> 256	> 256	128	8	256
N859 M2/P	TR	> 256	256	8	256	16	> 256	> 256	128	64	> 256
N859/PK36		32	64	2	16	1	> 256	> 256	128	16	256

ATM, aztreonam; AVI, avibactam; CAZ, ceftazidime; ETP, ertapenem; FEP, cefepime; GD ins; GD amino acid insertion in L3, IS Prom. IPM, imipenem; REL, relebactam; TR, truncated; WT, wildtype.

production of the porin. Interestingly, the two strains for which no mutants could be obtained (N52 and N119) both harboured a wild-type *ompK35*, in contrast to the other isolates in the study, suggesting that OmpK35 loss or weak production may be a prerequisite or favourable for further selection of MEM-VAB mutants. For four out of the six isolates used, mutants also exhibited mutations in the *ompK36* gene, corresponding to truncations of the gene in two strains (N931 and N859) and single amino acid changes in the corresponding protein for the remaining two (N435 and N1118). To our knowledge, the latter two mutations have not been reported previously. The two mutant lineages (lineage being defined as the mutants derived from a single parent) for which no changes in *ompK36* were observed (N1762 and N2280) already harboured insertions in the OmpK36 L3 loop (GD and TD, respectively), which are known to result in constriction of the pore and subsequently reduced permeability for both MEM and VAB [26,28]. In line with this observation, both parent isolates exhibited reduced susceptibility to MEM-VAB (MICs of 1 and 4 mg/L respectively) relative to those isolates producing wild-type OmpK36 (MICs ranging from ≤ 0.06–0.125 mg/L).

3.2. ompK36 *complementation*

Complementation assays were performed for three strain lineages where mutations in the *ompK36* gene sequence were found, namely N435, N859, and N931. Complementation assays were performed with a wild-type *ompK36* gene cloned in plasmid pACYC184 (pACompK36), resulting in a significant reduction in both MEM and MEM-VAB MICs across all mutants with *ompK36* mutations (Table 3). The mutation identified in the N435 M2 mutant, namely the Y326D amino acid change, was also shown to play a role in antibiotic permeation into the cell, although the exact structural mechanism of this remains unknown. This confirms that OmpK36 is the primary route by which VAB enters the cells in *K. pneumoniae* and that permeability defects are necessary for MEM-VAB resistance, in line with observations made in other studies [16,26,28].

3.3. *Determination of the* bla_{KPC} *copy numbers and genomic rearrangements*

The copy number of the bla_{KPC} gene in mutant relative to the parental strains was assessed by qPCR, showing that in four mutant lineages (N931, N1118, N1762, and N435) the bla_{KPC} gene copy number had significantly increased, likely resulting in increased bla_{KPC} expression. Two of these mutants were subject to long-read sequencing to determine the responsible mechanism(s). Parent strains and selected mutants derived from N931 (N931 M2) and N1762 (N1762 M1) were subject to both long- and short-read sequencing, which revealed that genomic rearrangements had occurred in both mutants. In the N931 parent strain, the transposon encompassing bla_{KPC-2} was encoded on a ~113 kb IncFIIK/FIB pKpQIL-like plasmid. In the mutant N931 M2, an IS*Kpn14* insertion sequence was identified, encoded within the *repA2* gene of the plasmid alongside a reduction in the plasmid size to ~32kb (Fig. 1), resulting in plasmid pKPC-M2 (Genbank Accession No. OP776642) (Fig. 1). The region of the pKpQIL-like plasmid that was lost was ~81 kb, including a ~35 kb region encoding the *tra* genes, likely rendering the plasmid non-conjugative. The *repA2* gene is responsible for the control of the plasmid copy number, and the presence of the insertion sequence likely results in increased copy

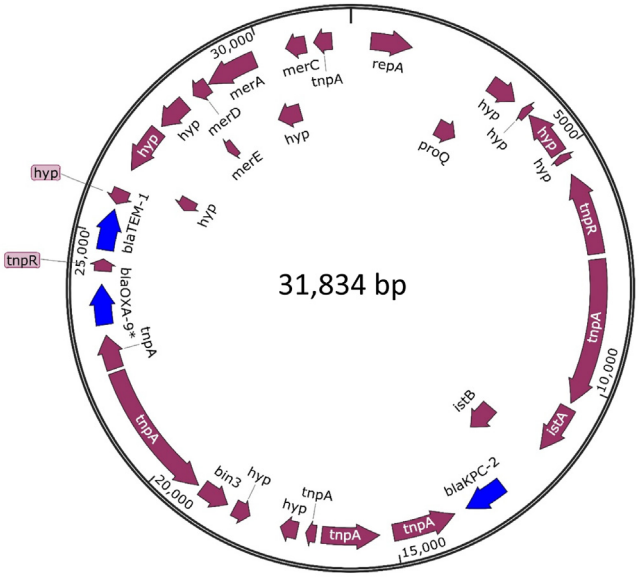

Fig. 1. The plasmid sequenced from strain N931_M2, pKPC-M2 (OP776642), which lost ~81 kb, including the *tra* region, compared with its parent, N931.

number of the plasmid and therefore increased bla_{KPC} expression, as has been suggested in a previous study where a similar insertion was found [15].

In the parent N1762, WGS identified two copies of the transposon bearing bla_{KPC-3} on an ~138 kb IncFIIK/FIB plasmid. In the N1762 M1 mutant a further duplication of the bla_{KPC-3}-encoding transposon was detected in which it had inserted onto a 16.8 kb ColE-like plasmid, pKPC_ColE (GenBank Accession No. OP745647), which are typically high copy number plasmids. Multiple copies of the bla_{KPC-3} gene in the parent isolate would explain the reduced susceptibility to MEM-VAB (4 mg/L) and subsequently why this was the only isolate for which MEM-VAB resistant mutants could be selected for directly [15].

4. Conclusion

MEM-VAB-resistant mutants were difficult to select in vitro for all strains in this study, and they were required to be selected in a stepwise process, with the exception of one strain that possessed two copies of bla_{KPC-3}. Mutants resistant to MEM-VAB did not result from structural modifications of the KPC protein as opposed to most of the previously reported CAZ-AVI-resistant mutants. Duplications of the bla_{KPC} gene in clinical *K. pneumoniae* isolates have been reported in relation to increased MEM-VAB resistance previously [15], albeit not very frequently. However, this may be due to the difficulty in detecting these duplications using the most common laboratory techniques such as PCR and short-read sequencing. Here, we showed that acquired resistance to MEM-VAB had occurred in some strains as a result of both permeability changes and bla_{KPC} gene amplification via duplication of transposon Tn*4401* and plasmid copy number changes. The mechanisms of the remaining strains have not yet been fully established, and further work is underway, but we have shown that isolates producing KPC variants resistant to CAZ-AVI can still become resistant to MEM-VAB. Cross resistance was observed between MEM-VAB and other BLBI combinations such as CAZ-AVI, IPM-REL, and to a much lesser extent ATM-AVI. Such cross resistance highlights the shared mechanisms by which resistance can emerge to both β-lactams and BLBIs and emphasises the need for awareness that the selection of some antimicrobial therapies can potentially compromise alternative treatments.

In contrast, no potential effect of efflux systems was evidenced. Overall MEM-VAB resistance in this study appeared to be related to a combination of two main mechanisms, i.e. permeability changes and more specifically, alteration of *ompK36*, and increased bla_{KPC} expression, with the former mechanism appearing to be most critical.

Funding

This work was financed by the Swiss National Science Foundation (grant FNS 310030_1888801).

Ethical approval

Not required.

Competing interests

None to declare.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2022.12.009.

References

[1] Yigit H, Queenan AM, Anderson GJ, Domenech-Sanchez A, Biddle JW, Steward CD, et al. Novel carbapenem-hydrolyzing β-lactamase, KPC-1, from a carbapenem-resistant strain of *Klebsiella pneumoniae*. Antimicrob Agents Chemother 2001;45:1151–61. doi:10.1128/AAC.45.4.1151-1161.2001.
[2] Munoz-Price LS, Poirel L, Bonomo RA, Schwaber MJ, Daikos GL, Cormican M, et al. Clinical epidemiology of the global expansion of *Klebsiella pneumoniae* carbapenemases. Lancet Infect Dis 2013;13:785–96. doi:10.1016/S1473-3099(13)70190-7.
[3] Bush K, Bradford BA. Epidemiology of β-Lactamase-Producing Pathogens. Clin Microbiol Rev 2020;33 e00047–19. doi:10.1128/CMR.00047-19.
[4] Robledo IE, Aquino EE, Santé MI, Santana JL, Otero DM, León CF, et al. Detection of KPC in *Acinetobacter* spp. in Puerto Rico. Antimicrob Agents Chemother 2010;54:1354–7. doi:10.1128/AAC.00899-09.
[5] Hecker SJ, Reddy KR, Totrov M, Hirst GC, Lomovskaya O, Griffith DC, et al. Discovery of a cyclic boronic acid beta-lactamase inhibitor (RPX7009) with utility versus class A serine carbapenemases. J Med Chem 2015;58:3682–92. doi:10.1021/acs.jmedchem.5b00127.
[6] Lomovskaya O, Sun D, Rubio-Aparicio D, Nelson K, Tsivkovski R, Griffith DC, et al. Vaborbactam: spectrum of beta-lactamase inhibition and impact of resistance mechanisms on activity in Enterobacteriaceae. Antimicrob Agents Chemother 2017;61 e01443–17. doi:10.1128/AAC.01443-17.
[7] US Food and Drug Administration VABOMERE™ (meropenem and vaborbactam) for injection, for intravenous use; 2017 https://www.accessdata.fda.gov/drugsatfda_docs/label/2017/209776lbl.pdf [accessed 01.11.22].
[8] European Medicines Agency Vabomere (meropenem/vaborbactam): an overview of Vabomere and why it is authorised in the EU; 2017 https://www.ema.europa.eu/en/documents/overview/vabomere-epar-medicine-overview_en.pdf [accessed 01.11.22].
[9] Gaibani P, Lombardo D, Bussini L, Bovo F, Munari B, Giannella M, et al. Epidemiology of meropenem/vaborbactam resistance in KPC-producing *Klebsiella pneumoniae* causing bloodstream infections in Northern Italy, 2018. Antibiotics (Basel) 2021;10:536. doi:10.3390/antibiotics10050536.
[10] Pfaller MA, Huband MD, Mendes RE, Flamm RK, Castanheira M. In vitro activity of meropenem/vaborbactam and characterisation of carbapenem resistance mechanisms among carbapenem-resistant Enterobacteriaceae from the 2015 meropenem/vaborbactam surveillance programme. Int J Antimicrob Agents 2018;52:144–50. doi:10.1016/j.ijantimicag.2018.02.021.
[11] Humphries RM, Yang S, Hemarajata P, Ward KW, Hindler JA, Miller SA, et al. First report of ceftazidime-avibactam resistance in a KPC-3-expressing *Klebsiella pneumoniae* isolate. Antimicrob Agents Chemother 2015;59:6605–7. doi:10.1128/AAC.01165-15.
[12] Mueller L, Masseron A, Prod'Hom G, Galperine T, Greub G, Poirel L, et al. Phenotypic, biochemical and genetic analysis of KPC-41, a KPC-3 variant conferring resistance to ceftazidime-avibactam and exhibiting reduced carbapenemase activity. Antimicrob Agents Chemother 2019;63 e01111-9. doi:10.1128/AAC.01111-19.
[13] Poirel L, Vuillemin X, Juhas M, Masseron A, Bechtel-Grosch U, Tiziani S, et al. KPC-50 confers resistance to ceftazidime-avibactam associated with reduced carbapenemase activity. Antimicrob Agents Chemother 2020;64 e00321–20. doi:10.1128/AAC.00321-20.
[14] Shields RK, Chen L, Cheng S, Chavda KD, Press EG, Snyder A, et al. Emergence of ceftazidime-avibactam resistance due to plasmid-borne blaKPC-3 mutations during treatment of carbapenem-resistant *Klebsiella pneumoniae* infections. Antimicrob Agents Chemother 2017;61 e02097–16. doi:10.1128/AAC.02097-16.
[15] Sun D, Rubio-Aparicio D, Nelson K, Dudley MN, Lomovskaya O. Meropenem-vaborbactam resistance selection, resistance prevention, and molecular mechanisms in mutants of KPC-producing *Klebsiella pneumoniae*. Antimicrob Agents Chemother 2017;61 e01694–17. doi:10.1128/AAC.01694-17.
[16] Wilson WR, Kline EG, Jones CE, Morder KT, Mettus RT, Doi Y, et al. Effects of KPC variant and porin genotype on the in vitro activity of meropenem-vaborbactam against carbapenem-resistant Enterobacteriaceae. Antimicrob Agents Chemother 2019;63 e02048–18. doi:10.1128/AAC.02048-18.
[17] Clinical and Laboratory Standards Institute Document M100-S30. In: Performance standards for antimicrobial susceptibility testing: twenty-seventh informational supplement. Wayne, PA: CLSI; 2020.
[18] EUCAST Clinical breakpoint table v.12.0; 2022 https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_12.0_Breakpoint_Tables.pdf [accessed 01.11.22].
[19] Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 2014;30:2114–20. doi:10.1093/bioinformatics/btu170.
[20] Seeman T. Prokka: rapid prokaryotic genome annotation. Bioinf 2014;30:2068–9. doi:10.1093/bioinformatics/btu153.
[21] Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, et al. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother 2012;67:2640–4. doi:10.1093/jac/dks261.
[22] Carattoli A, Zankari E, García-Fernández A, Voldby Larsen M, Lund O, Villa L, et al. In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrob Agents Chemother 2014;58:3895–903. doi:10.1128/AAC.02412-14.
[23] Larsen MV, Cosentino S, Lukjancenko O, Saputra D, Rasmussen S, Hasman H, et al. Benchmarking of methods for genomic taxonomy. J Clin Microbiol 2014;52:1529–39. doi:10.1128/JCM.02981-13.

[24] Koren S, Walenz BP, Berlin K, Miller JR, Bergman NH, Phillippy AM. Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. Genome Res 2017;27:722–36. doi:10.1101/gr.215087.116.
[25] Wick RR, Judd LM, Gorrie CL, Holt KE. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. PLoS Comput Biol 2017;13:e1005595. doi:10.1371/journal.pcbi.1005595.
[26] Fajardo-Lubian A, Ben Zakour NL, Agyekum A, Qi Q, Iredell JR. Host adaptation and convergent evolution increases antibiotic resistance without loss of virulence in a major human pathogen. PLoS Pathog 2019;15(3):e1007218. doi:10.1371/journal.ppat.1007218.
[27] Piddock LJ. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. Clin Microbiol Rev 2006;19:382–402. doi:10.1128/CMR.19.2.382-402.2006.
[28] David S, Wong JLC, Sanchez-Garrido J, Kwong HS, Low WW, Morecchiato F, et al. Widespread emergence of OmpK36 loop 3 insertions among multidrug-resistant clones of *Klebsiella pneumoniae*. PLoS Pathog 2022;18:e1010334. doi:10.1371/journal.ppat.1010334.