AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

MECHANISMS OF RESISTANCE

Resistance to Ceftazidime/Avibactam plus Meropenem/Vaborbactam When Both Are Used Together Is Achieved in Four Steps in Metallo-β-Lactamase-Negative *Klebsiella pneumoniae*

Punyawee Dulyayangkul,[a] Edward J. A. Douglas,[a] Filip Lastovka,[a] Matthew B. Avison[a]

[a]School of Cellular & Molecular Medicine, University of Bristol, Bristol, United Kingdom

Punyawee Dulyayangkul and Edward J. A. Douglas contributed equally to this work. Punyawee Dulyayangkul finished the work for publication and so is first author.

ABSTRACT Serine β-lactamases are dominant causes of β-lactam resistance in *Klebsiella pneumoniae* isolates. Recently, this has driven clinical deployment of the β-lactam–β-lactamase inhibitor pairs ceftazidime/avibactam and meropenem/vaborbactam. We show that four steps, i.e., *ompK36* and *ramR* mutation plus carriage of OXA-232 and KPC-3-D178Y variant β-lactamases, confer ceftazidime/avibactam and meropenem/vaborbactam resistance when both pairs are used together. These findings have implications for decision making about sequential and combinatorial use of these β-lactam–β-lactamase inhibitor pairs to treat *K. pneumoniae* infections.

KEYWORDS KPC, OXA-48, β-lactamase, porin

Klebsiella pneumoniae isolates can become resistant to third-generation cephalosporins (3GC) and/or carbapenems through acquisition of a wide range of serine β-lactamases (1). Most clinically significant are the CTX-M (2), KPC (3), and OXA-48-like (4) types. In response to the rise in KPC, which can confer 3GC and carbapenem resistance, serine β-lactamase inhibitors, including avibactam and vaborbactam, have recently been developed. Avibactam is used in combination with the 3GC ceftazidime (5). It inhibits CTX-M, KPC, and OXA-48-like β-lactamases (6, 7). Ceftazidime-avibactam (CAZ/AVI) nonsusceptibility can be caused by KPC changes, e.g., D179Y or V239G (8, 9), or by a P170S change in CTX-M (10). Vaborbactam is used in combination with the carbapenem meropenem (11). It has potent activity against KPC and, to a lesser extent, CTX-M (12). Meropenem-vaborbactam (MER/VAB) resistance in *K. pneumoniae* isolates is caused by loss of OmpK36 and OmpK35 porins in backgrounds carrying KPC or OXA-48-like enzymes (12, 13).

Given the appearance of *K. pneumoniae* clinical isolates resistant to MER/VAB or CAZ/AVI, it has been suggested that a combination of both given together would overcome isolates resistant to one or the other when used separately (14, 15). The aim of the work reported here was to identify steps that can generate resistance to MER/VAB and CAZ/AVI when used together.

First, a plasmid (pOXA-232) encoding the OXA-48-like carbapenemase OXA-232 was purified from *K. pneumoniae* clinical isolate KP11 and used to transform the *ramR* frameshift (Arg44FS) mutant clinical *K. pneumoniae* isolate KP21 using 8 $\mu g \cdot ml^{-1}$ piperacillin and 4 $\mu g \cdot ml^{-1}$ tazobactam as selection (16). This isolate was chosen because *ramR* mutation causes overproduction of the AcrAB-TolC efflux pump and reduces production of the OmpK35 porin, and this enhances the spectrum of resistance conferred by OXA-48 and other β-lactamases in *K. pneumoniae* isolates (17). However, despite its *ramR* mutation, the MIC of meropenem (CLSI microtiter assay [18]) in the

Citation Dulyayangkul P, Douglas EJA, Lastovka F, Avison MB. 2020. Resistance to ceftazidime/avibactam plus meropenem/vaborbactam when both are used together is achieved in four steps in metallo-β-lactamase-negative *Klebsiella pneumoniae*. Antimicrob Agents Chemother 64:e00409-20. https://doi.org/10.1128/AAC.00409-20.

Address correspondence to Matthew B. Avison, bimba@bris.ac.uk.

Received 3 March 2020
Returned for modification 23 April 2020
Accepted 7 July 2020
Accepted manuscript posted online 13 July 2020
Published 21 September 2020

TABLE 1 MICs of meropenem with or without vaborbactam and of ceftazidime with or without avibactam against derivatives of *K. pneumoniae* clinical isolates

K. pneumoniae derivative	MIC (μg · ml^{-1}) of[a]:			
	Meropenem	MER/VAB CAZ/AVI	Ceftazidime	CAZ/AVI
KP21[*ramR*] (pOXA-232)(pUBYT)	1	1/8	2	0.5/4
KP21[*ramR*] pOXA-232)(pCTX-M-14)	1	1/8	32	1/4
KP21[*ramR*] pOXA-232)(pCTX-M-14 P170S)	1	1/8	256	4/4
KP21[*ramR*] (pOXA-232)(pKPC-3)	128	2/8	>256	8/4
KP21[*ramR*] (pOXA-232)(pKPC-3-D178Y)	2	2/8	>256	256/4
KP21[*ramR*] (pOXA-232)(pKPC-3-V239G)	64	4/8	>256	128/4
KP21[*ramR*] *ompK35*(pOXA-232)(pUBYT)	4	4/8	2	0.5/4
KP21[*ramR*] *ompK35*(pOXA-232)(pCTX-M-14)	4	4/8	32	1/4
KP21[*ramR*] *ompK35*(pOXA-232)(pCTX-M-14 P170S)	2	2/8	>256	4/4
KP21[*ramR*] *ompK35*(pOXA-232)(pKPC-3)	128	1/8	>256	0.125/4
KP21[*ramR*] *ompK35*(pOXA-232)(pKPC-3-D178Y)	4	4/8	>256	128/4
KP21[*ramR*] *ompK35*(pOXA-232)(pKPC-3-V239G)	64	4/8	>256	32/4
KP21[*ramR*] *ompK36*(pOXA-232)(pUBYT)	256	256/8	2	0.5/4
KP21[*ramR*] *ompK36*(pOXA-232)(pCTX-M-14)	256	256/8	32	1/4
KP21[*ramR*] *ompK36*(pOXA-232)(pCTX-M-14 P170S)	256	256/8	>256	8/4
KP21[*ramR*] *ompK36*(pOXA-232)(pKPC-3)	>256	256/8	>256	16/4
KP21[*ramR*] *ompK36*(pOXA-232)(pKPC-3-D178Y)	256	256/8	>256	>256/4
KP21[*ramR*] *ompK36*(pOXA-232)(pKPC-3-V239G)	>256	256/8	>256	128/4
KP21[*ramR*] *ompK36*(pKPC-3)	>256	16/8	>256	8/4
KP21[*ramR*] *ompK36*(pKPC-3-D178Y)	64	16/8	>256	256/4
KP21[*ramR*] *ompK36*(pKPC-3-V239G)	>256	32/8	>256	256/4
KP21 M[*ramR ompK36*] (pOXA-232)(pUBYT)	256	256/8	2	1/4
KP21 M[*ramR ompK36*] (pOXA-232)(pCTX-M-14)	128	128/8	32	1/4
KP21 M[*ramR ompK36*] (pOXA-232)(pCTX-M-14 P170S)	256	128/8	>256	8/4
KP21 M[*ramR ompK36*] (pOXA-232)(pKPC-3)	>256	256/8	>256	8/4
KP21 M[*ramR ompK36*] (pOXA-232)(pKPC-3-D178Y)	128	128/8	>256	256/4
KP21 M[*ramR ompK36*] (pOXA-232)(pKPC-3-V239G)	>256	256/8	>256	64/4
KP21 M[*ramR ompK36*] *ompK35*(pOXA-232)(pUBYT)	256	256/8	2	1/4
KP21 M[*ramR ompK36*] *ompK35*(pOXA-232)(pCTX-M-14)	256	256/8	32	1/4
KP21 M[*ramR ompK36*] *ompK35*(pOXA-232)(pCTX-M-14 P170S)	256	256/8	256	8/4
KP21 M[*ramR ompK36*] *ompK35*(pOXA-232)(pKPC-3)	>256	256/8	>256	16/4
KP21 M[*ramR ompK36*] *ompK35*(pOXA-232)(pKPC-3-D178Y)	256	256/8	>256	>256/4
KP21 M[*ramR ompK36*] *ompK35*(pOXA-232)(pKPC-3-V239G)	>256	256/8	>256	128/4
KP47 *ompK36*(pKPC-3)	>256	2/8	>256	8/4
KP47 *ompK36*(pKPC-3-D178Y)	8	2/8	>256	256/4
KP47 *ompK36*(pOXA-232)(pKPC-3)	>256	16/8	>256	16/4
KP47 *ompK36*(pOXA-232)(pKPC-3-D178Y)	16	16/8	>256	>256/4

[a]Shading indicates nonsusceptibility (resistance or intermediate resistance) according to CLSI breakpoints (19).

presence of 8 μg · ml^{-1} vaborbactam (MedChemExpress) against KP21(pOXA-232) was 1 μg · ml^{-1} (Table 1), making it MER/VAB susceptible according to CLSI breakpoints (19). We therefore selected a spontaneous MER/VAB-resistant derivative. To do this, 100-μl aliquots of overnight cultures of KP21(pOXA-232) grown in nutrient broth were spread onto Mueller-Hinton agar containing 16 μg · ml^{-1} meropenem and 8 μg · ml^{-1} vaborbactam, which was then incubated for 24 h at 37°C.

Liquid chromatography-tandem mass spectrometry envelope proteomics, performed as described previously (17), confirmed that this putative MER/VAB-resistant mutant, KP21 M(pOXA-232), had undetectable OmpK36 porin levels. Whole-genome sequencing, performed as described previously (20), identified a single mutation resulting in a stop at codon 125 in *ompK36* in KP21 M(pOXA-232). To confirm the effect of this *ompK36* point mutation, we insertionally inactivated *ompK36* in the parent strain KP21(pOXA-232) using the pKNOCK suicide plasmid (21). The *ompK36* DNA fragment was amplified from *K. pneumoniae* strain Ecl8 genomic DNA using *ompK36* KO FW (5′-CGTTCAGGCGAACAACACTG-3′) and RV (5′-AAGTTCAGGCCGTCAACCAG-3′) primers (22). The PCR product was ligated into the pKNOCK-Gm at the SmaI site. The recombinant plasmid was then transferred into KP21(pOXA-232) by conjugation. Mutants were selected using gentamicin (5 μg · ml^{-1}) and confirmed by PCR using *ompK36* full-length FW (5′-GAGGCATCCGGTTGAAATAG-3′) and RV (5′-ATTAATCGAGGCTCCTCT

TAC-3′) primers. The MER/VAB MIC against the *ompK36* point mutant KP21 M(pOXA-232) and against the insertionally inactivated mutant KP21 *ompK36*(pOXA-232) was 256/8 μg · ml^{-1}, confirming resistance (Table 1). This finding reveals that OmpK35 downregulation seen in *ramR* mutants mimics the effect of OmpK35 loss previously shown to be essential alongside OmpK36 loss for MER/VAB resistance in *K. pneumoniae* isolates producing an OXA-48-like enzyme (12, 13).

Both KP21(pOXA-232) *ompK36* mutants remained susceptible to ceftazidime as expected, since OXA-232 only weakly hydrolyses ceftazidime (23) (Table 1). Aiming to increase ceftazidime MICs, we introduced the low-copy-number recombinant plasmid pCTX-M-14. To make pCTX-M-14, $bla_{\text{CTX-M-14}}$ was amplified along with its native promoter from human urinary *Escherichia coli* isolated from a primary care setting (24) using CTX-M-14 FW (5′-CCGGAATTCAATACTACCTTGCTTTCTGA-3′) and RV (5′-CCGGA ATTCCGTAGCGGAACGTTCATCAG-3′) primers and ligated into pUBYT at the EcoRI site (25). Carriage of pUBYT recombinants was selected using kanamycin (30 μg · ml^{-1}).

Even though CTX-M-14 only weakly hydrolyses ceftazidime (26), in the *ramR* mutant KP21, carriage of pCTX-M-14 conferred ceftazidime resistance, as seen in KP21(pOXA-232)(pCTX-M-14), KP21 M(pOXA-232)(pCTX-M-14), and KP21 *ompK36*(pOXA-232)(pCTX-M-14) (Table 1). However, these derivatives remained ceftazidime susceptible in the presence of 4 μg · ml^{-1} avibactam (MedChemExpress), which is a potent inhibitor of CTX-M (5) (Table 1). Replacement of $bla_{\text{CTX-M-14}}$ with $bla_{\text{CTX-M-14-P170S}}$, encoding a variant associated with reduced CAZ/AVI susceptibility (10), representing the naturally occurring variant CTX-M-19 (27), drove the CAZ/AVI MIC against KP21 *ompK36*(pOXA-232) and KP21 M(pOXA-232) up to 8/4 μg · ml^{-1}, which is one doubling dilution below the breakpoint for resistance. To enable this, CTX-M-14 site-directed mutagenesis was performed directly on the recombinant plasmid pCTX-M-14 using the QuikChange Lightning site-directed mutagenesis kit (Agilent, USA) with the CTX-M-14-P170S-FW primer (5′-TCTGGATCGCACTGAATCTACGCTGAATACCGC-3′).

Next, we insertionally inactivated *ompK35* in KP21 M[*ompK36*](pOXA-232)(pCTX-M-14-P170S) using the method described above but with the *ompK35* KO FW (5′-TCCC AGACCACAAAAACCCG-3′) and RV (5′-CCAGACCGAAGAAGTCGGAG-3′) primers and checked with *ompK35* full-length FW (5′-CACTTCGATGTATTTAACCAG-3′) and RV (5′-A TGATGAAGCGCAATATTCTG-3′) primers. However, this did not further increase the CAZ/AVI MIC, confirming that *ramR* mutation phenotypically mimics OmpK35 loss (Table 1). Accordingly, it was not possible for us to generate derivatives resistant to both MER/VAB and CAZ/AVI using CTX-M-14-P170S, even in a *ramR ompK36 ompK35* triple mutant background coproducing OXA-232.

KPC-3 is a carbapenemase that can confer ceftazidime resistance (9). To create the recombinant plasmid pKPC-3, $bla_{\text{KPC-3}}$ was amplified from pKpQIL isolated from *K. pneumoniae* strain KP30 by PCR using KPC-3 FW (5′-CCGGAATTCGTAAAGTGGGTCAGT TTTCAG-3′) and RV (5′-GGCTCTGAAAATCATCTATTGGAATTCCGG-3′) primers and ligated into pUBYT at the EcoRI site (16). We also generated KPC-3 variants associated with CAZ/AVI resistance (8). KPC-3 site-directed mutagenesis was performed using a two-step PCR-based strategy: $bla_{\text{KPC-3-D178Y}}$ was constructed using KPC-3-D178Y-FW (5′-AGGCGATGCGCGCTATACCTCATCGCC-3′) and -RV (5′-GGCGATGAGGTATAGCGCGC ATCGCCT-3′) primers, and $bla_{\text{KPC-3-V239G}}$ was constructed using KPC-3-V239G-FW (5′-G GAACCTGCGGAGGGTATGGCACGGCA-3′) and -RV (5′-TGCCGTGCCATACCCTCCGCAGGT TCC-3′) primers. The common flanking primers were KPC-3 FW and RV, as above.

When introduced into KP21(pOXA-232) (i.e., with wild-type *ompK36*), both pKPC-3-D178Y and pKPC-3-V239G conferred CAZ/AVI resistance, but pKPC-3 did not. The D178Y variant did this at the expense of reducing meropenem MIC into the intermediate-resistant zone, as reported previously (8) (Table 1). The V239G variant did not suffer from such a large drop in meropenem MIC, but MER/VAB MICs were the same (and in the susceptible range) against KP21(pOXA-232) derivatives producing KPC-3 or its D178Y or V239G variants (Table 1). Accordingly, it was not possible to generate derivatives resistant to both MER/VAB and CAZ/AVI using KPC-3 variants in a background having wild-type *ompK36*.

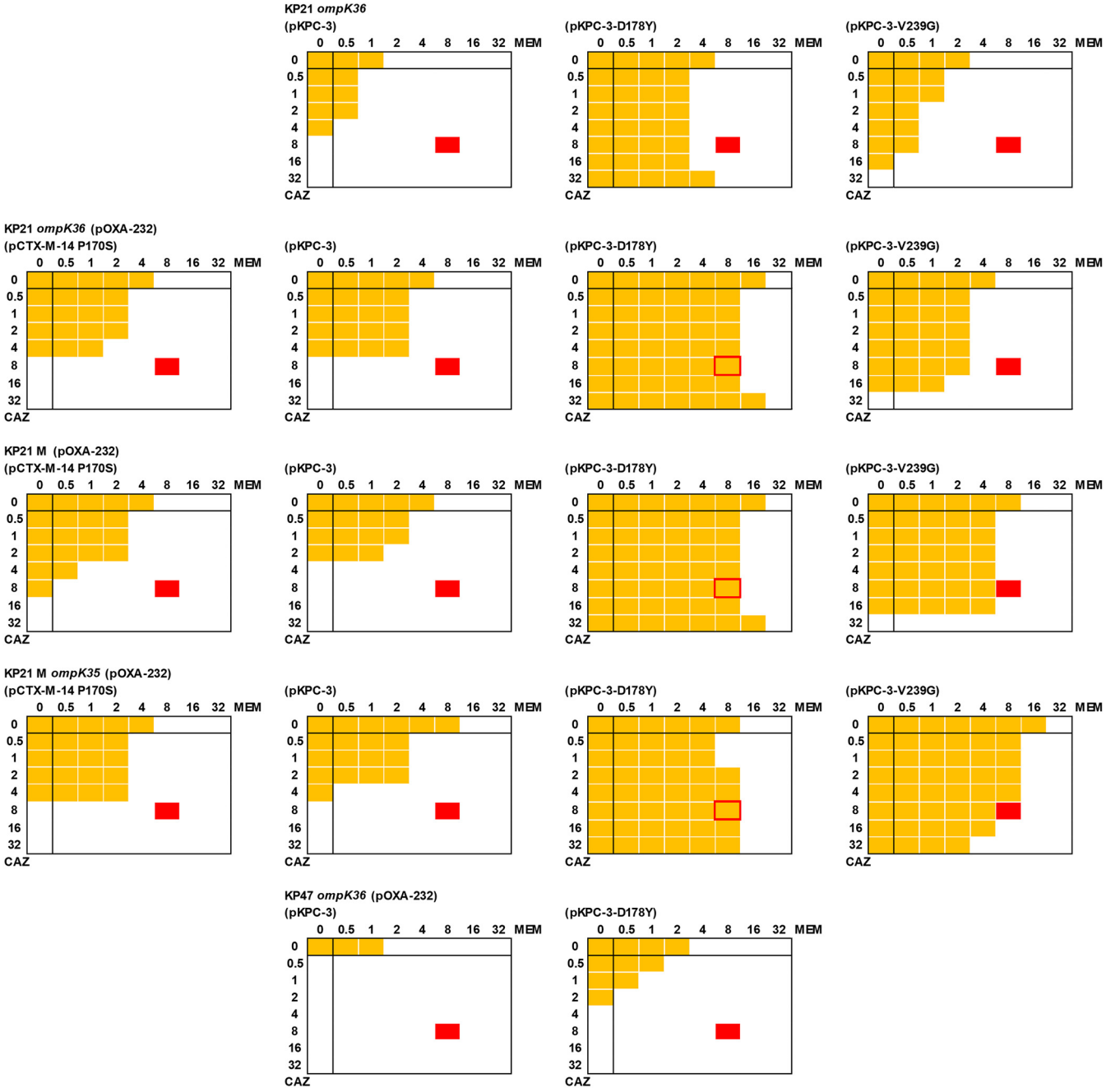

FIG 1 Checkerboard assays for ceftazidime and meropenem in the presence of avibactam and vaborbactam. Each image represents duplicate assays for an 8-by-8 array of wells in a 96-well plate. All wells contained CA-MHB, including avibactam (4 μg · ml^{-1}) and vaborbactam (8 μg · ml^{-1}). A serial dilution of meropenem (MEM; *x* axis) and ceftazidime (CAZ, *y* axis) was created from 32 μg · ml^{-1} in each plate as recorded. All wells were inoculated with a suspension of bacteria, made according to CLSI microtiter MIC guidelines (18), and the plate was incubated at 37°C for 20 h. Growth was recorded by measuring OD_{600}, and growth above background (broth) is shown as a yellow block. Growth at 8 μg · ml^{-1} ceftazidime and 8 μg · ml^{-1} meropenem (indicated in red) in the presence of vaborbactam and avibactam defined resistance based on CLSI breakpoints (19). Bacterial suspensions used were as follows: top row, KP21[*ramR*] *ompK36*; second row, KP21[*ramR*] *ompK36*(pOXA-232); third row, KP21[*ramR*] M[*ompK36*](pOXA-232); fourth row, KP21[*ramR*] M[*ompK36*] *ompK35*(pOXA-232); and fifth row, KP47 *ompK36*(pOXA-232). In each case, bacteria also carried the following plasmids (where tested): first column, pCTX-M-14 P170S; second column, pKPC-3; third column, pKPC-3-D178Y; and fourth column, pKPC-3-V239G.

Next, we introduced plasmids encoding the KPC-3 variants associated with CAZ/AVI resistance into the *ompK36* mutant MER/VAB-resistant derivatives KP21 M(pOXA-232) and KP21 *ompK36*(pOXA-232). As expected, the result was resistance to CAZ/AVI and MER/VAB when used separately (Table 1). OXA-232 production is not essential for this phenotype because KP21 *ompK36*(pKPC-3-D178Y) and KP21 *ompK36*(pKPC-3-V239G),

lacking OXA-232, were also resistant to CAZ/AVI and MER/VAB when used separately (Table 1). Furthermore, we found that CAZ/AVI plus MER/VAB resistance can be conferred by production of wild-type KPC-3 in a *ramR ompK36* double mutant carrying OXA-232 (Table 1). Clinical *K. pneumoniae* isolates with *ompK35* and *ompK36* mutation and elevated KPC-3 production that are CAZ/AVI resistant have recently been identified (28), which supports our finding that KPC-3 mutation is not essential.

Finally, checkerboard assays were performed using an adapted microtiter MIC assay to test whether CAZ/AVI and MER/VAB would work synergistically against derivatives resistant to both pairs when used separately (Fig. 1). Briefly, a phosphate-buffered saline bacterial suspension was prepared to obtain a stock optical density at 600 nm (OD_{600}) of 0.01. The final volume in each well of a 96-well cell culture plate (Corning Costar) was 200 μl and included 20 μl of the bacterial suspension. All wells contained cation-adjusted Muller-Hinton broth (CA-MHB) with avibactam (4 $\mu g \cdot ml^{-1}$) and vaborbactam (8 $\mu g \cdot ml^{-1}$) with serial dilutions of meropenem and ceftazidime. Bacterial growth (OD_{600}) was determined after 20 h using a POLARstar Omega spectrophotometer (BMG Labtech). These assays confirmed that KP21 M(pOXA-232) and KP21 *ompK36*(pOXA-232) carrying pCTX-M14-P170S, pKPC-3, or pKPC-3-V239G were susceptible to meropenem in the presence of vaborbactam plus CAZ/AVI, suggesting that combined therapy would still work (Fig. 1). Further disruption of *ompK35* did not alter this result. However, KP21 M(pOXA-232)(pKPC-3-D178Y) and KP21 *ompK36*(pOXA-232)(pKPC-3-D178Y) were resistant to meropenem (MIC, 16 $\mu g \cdot ml^{-1}$) and ceftazidime (MIC, $\geq$64 $\mu g \cdot ml^{-1}$) in the presence of vaborbactam plus avibactam.

We have, therefore, generated *K. pneumoniae* derivatives resistant to CAZ/AVI and MER/VAB, when both pairs are used together. This was achieved in four steps relative to wild-type isolates: *ramR* frameshift mutation, acquisition of OXA-232, *ompK36* frameshift mutation (or insertional inactivation), and acquisition of KPC-3-D178Y. When we constructed a derivative of clinical isolate KP47 (wild type for *ramR* [16]) carrying pOXA-232 and pKPC-3-D178Y and with *ompK36* insertionally inactivated as described above, the derivative was resistant to CAZ/AVI and MER/VAB when used separately (Table 1) but not when used together (Fig. 1). When we constructed a derivative of KP21 *ompK36* carrying pKPC-3-D178Y but not pOXA-232, it was also resistant to CAZ/AVI and MER/VAB when used separately (Table 1) but not when used together (Fig. 1). This confirms that *ramR* mutation and production of OXA-232 are both necessary for the dual-resistant phenotype. Because OXA-232 has a relatively low level of carbapenemase activity compared with the more commonly encountered OXA-48 (23), we are confident that this finding is representative of the wider family of OXA-48-like enzymes.

In conclusion, this work confirms the remarkable capacity of *K. pneumoniae* isolates to acquire resistance to the latest combination therapies available in the clinic by layering mechanisms. Of course, the order in which the four identified steps occur does not affect the result. So, it would be prudent to make every effort to identify, via molecular diagnostics, intermediate stages in this acquisition process. It would also be prudent to not rely on sequential use of CAZ/AVI and MER/VAB, which might select for the dual-resistant phenotype observed here.

ACKNOWLEDGMENTS

This work was funded by grant MR/S004769/1 to M.B.A. from the Antimicrobial Resistance Cross Council Initiative supported by the seven United Kingdom research councils and the National Institute for Health Research. Additional funding was provided by a bequest from the estate of the late Graham Ayliffe.

Genome sequencing was provided by MicrobesNG (http://www.microbesng.uk/), which is supported by the BBSRC (grant number BB/L024209/1).

We are grateful to Kate Heesom, School of Biochemistry, University of Bristol, for performing the proteomics analysis and to Jacqueline Findlay, School of Cellular & Molecular Medicine, University of Bristol, for purifying the plasmid pOXA-232.

We have no conflicts of interest to declare.

REFERENCES

1. Holt KE, Wertheim H, Zadoks RN, Baker S, Whitehouse CA, Dance D, Jenney A, Connor TR, Hsu LY, Severin J, Brisse S, Cao H, Wilksch J, Gorrie C, Schultz MB, Edwards DJ, Nguyen KV, Nguyen TV, Dao TT, Mensink M, Minh VL, Nhu NT, Schultsz C, Kuntaman K, Newton PN, Moore CE, Strugnell RA, Thomson NR. 2015. Genomic analysis of diversity, population structure, virulence, and antimicrobial resistance in *Klebsiella pneumoniae*, an urgent threat to public health. Proc Natl Acad Sci U S A 112:E3574–E3581. https://doi.org/10.1073/pnas.1501049112.
2. Cantón R, González-Alba JM, Galán JC. 2012. CTX-M enzymes: origin and diffusion. Front Microbiol 3:110. https://doi.org/10.3389/fmicb.2012.00110.
3. Kitchel B, Rasheed JK, Patel JB, Srinivasan A, Navon-Venezia S, Carmeli Y, Brolund A, Giske CG. 2009. Molecular epidemiology of KPC-producing *Klebsiella pneumoniae* isolates in the United States: clonal expansion of multilocus sequence type 258. Antimicrob Agents Chemother 53:3365–3370. https://doi.org/10.1128/AAC.00126-09.
4. Poirel L, Potron A, Nordmann P. 2012. OXA-48-like carbapenemases: the phantom menace. J Antimicrob Chemother 67:1597–1606. https://doi.org/10.1093/jac/dks121.
5. Ehmann DE, Jahić H, Ross PL, Gu RF, Hu J, Kern G, Walkup GK, Fisher SL. 2012. Avibactam is a covalent, reversible, non-β-lactam β-lactamase inhibitor. Proc Natl Acad Sci U S A 109:11663–11668. https://doi.org/10.1073/pnas.1205073109.
6. Castón JJ, Lacort-Peralta I, Martín-Dávila P, Loeches B, Tabares S, Temkin L, Torre-Cisneros J, Paño-Pardo JR. 2017. Clinical efficacy of ceftazidime/avibactam versus other active agents for the treatment of bacteremia due to carbapenemase-producing Enterobacteriaceae in hematologic patients. Int J Infect Dis 59:118–123. https://doi.org/10.1016/j.ijid.2017.03.021.
7. Wang DY, Abboud MI, Markoulides MS, Brem J, Schofield CJ. 2016. The road to avibactam: the first clinically useful non-β-lactam working somewhat like a β-lactam. Future Med Chem 8:1063–1084. https://doi.org/10.4155/fmc-2016-0078.
8. 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_{KPC-3} mutations during treatment of carbapenem-resistant *Klebsiella pneumoniae* infections. Antimicrob Agents Chemother 61:e02097-16. https://doi.org/10.1128/AAC.02097-16.
9. Woodford N, Tierno PM, Jr, Young K, Tysall L, Palepou MF, Ward E, Painter RE, Suber DF, Shungu D, Silver LL, Inglima K, Kornblum J, Livermore DM. 2004. Outbreak of *Klebsiella pneumoniae* producing a new carbapenem-hydrolyzing class A β-lactamase, KPC-3, in a New York medical center. Antimicrob Agents Chemother 48:4793–4799. https://doi.org/10.1128/AAC.48.12.4793-4799.2004.
10. Both A, Büttner H, Huang J, Perbandt M, Belmar Campos C, Christner M, Maurer FP, Kluge S, König C, Aepfelbacher M, Wichmann D, Rohde H. 2017. Emergence of ceftazidime/avibactam non-susceptibility in an MDR *Klebsiella pneumoniae* isolate. J Antimicrob Chemother 72:2483–2488. https://doi.org/10.1093/jac/dkx179.
11. Hecker SJ, Reddy KR, Totrov M, Hirst GC, Lomovskaya O, Griffith DC, King P, Tsivkovski R, Sun D, Sabet M, Tarazi Z, Clifton MC, Atkins K, Raymond A, Potts KT, Abendroth J, Boyer SH, Loutit JS, Morgan EE, Durso S, Dudley MN. 2015. Discovery of a cyclic boronic acid β-lactamase inhibitor (RPX7009) with utility vs class A serine carbapenemases. J Med Chem 58:3682–3692. https://doi.org/10.1021/acs.jmedchem.5b00127.
12. Lomovskaya O, Sun D, Rubio-Aparicio D, Nelson K, Tsivkovski R, Griffith DC, Dudley MN. 2017. Vaborbactam: spectrum of β-lactamase inhibition and impact of resistance mechanisms on activity in *Enterobacteriaceae*. Antimicrob Agents Chemother 61:e01443-17. https://doi.org/10.1128/AAC.01443-17.
13. Sun D, Rubio-Aparicio D, Nelson K, Dudley MN, Lomovskaya O. 2017. Meropenem-vaborbactam resistance selection, resistance prevention, and molecular mechanisms in mutants of KPC-producing *Klebsiella pneumoniae*. Antimicrob Agents Chemother 61:e01694-17. https://doi.org/10.1128/AAC.01694-17.
14. Pogue JM, Bonomo RA, Kaye KS. 2019. Ceftazidime/avibactam, meropenem/vaborbactam, or both? Clinical and formulary considerations. Clin Infect Dis 68:519–524. https://doi.org/10.1093/cid/ciy576.
15. Athans V, Neuner EA, Hassouna H, Richter SS, Keller G, Castanheira M, Brizendine KD, Mathers AJ. 2018. Meropenem-vaborbactam as salvage therapy for ceftazidime-avibactam-resistant *Klebsiella pneumoniae* bacteremia and abscess in a liver transplant recipient. Antimicrob Agents Chemother 63:e01551-18. https://doi.org/10.1128/AAC.01551-18.
16. Wan Nur Ismah WAK, Takebayashi Y, Findlay J, Heesom KJ, Jiménez-Castellanos JC, Zhang J, Graham L, Bowker K, Williams OM, MacGowan AP, Avison MB. 2017. Prediction of fluoroquinolone susceptibility directly from whole-genome sequence data by using liquid chromatography-tandem mass spectrometry to identify mutant genotypes. Antimicrob Agents Chemother 62:e01814-17. https://doi.org/10.1128/AAC.01814-17.
17. Jiménez-Castellanos JC, Wan Nur Ismah WAK, Takebayashi Y, Findlay J, Schneiders T, Heesom KJ, Avison MB. 2018. Envelope proteome changes driven by RamA overproduction in *Klebsiella pneumoniae* that enhance acquired β-lactam resistance. J Antimicrob Chemother 73:88–94. https://doi.org/10.1093/jac/dkx345.
18. Clinical and Laboratory Standards Institute. 2015. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard—10th ed. CLSI document M07-A10. Clinical and Laboratory Standards Institute, Wayne, PA.
19. Clinical and Laboratory Standards Institute. 2019. Performance standards for antimicrobial susceptibility testing; 29th informational supplement. CLSI document M100-S29. Clinical and Laboratory Standards Institute, Wayne, PA.
20. Dulyayangkul P, Wan Nur Ismah WAK, Douglas EJA, Avison MB. 2020. Mutation of *kvrA* causes OmpK35 and OmpK36 porin downregulation and reduced meropenem-vaborbactam susceptibility in KPC-producing *Klebsiella pneumoniae*. Antimicrob Agents Chemother 64:e02208-19. https://doi.org/10.1128/AAC.02208-19.
21. Alexeyev MF. 1999. The pKNOCK series of broad-host-range mobilizable suicide vectors for gene knockout and targeted DNA insertion into the chromosome of Gram-negative bacteria. Biotechniques 26:824–828. https://doi.org/10.2144/99265bm05.
22. George AM, Hall RM, Stokes HW. 1995. Multidrug resistance in *Klebsiella pneumoniae*: a novel gene, *ramA*, confers a multidrug resistance phenotype in *Escherichia coli*. Microbiology 141:1909–1920. https://doi.org/10.1099/13500872-141-8-1909.
23. Potron A, Rondinaud E, Poirel L, Belmonte O, Boyer S, Camiade S, Nordmann P. 2013. Genetic and biochemical characterisation of OXA-232, a carbapenem-hydrolysing class D β-lactamase from Enterobacteriaceae. Int J Antimicrob Agents 41:325–329. https://doi.org/10.1016/j.ijantimicag.2012.11.007.
24. Findlay J, Gould VC, North P, Bowker KE, Williams MO, MacGowan AP, Avison MB. 2020. Characterization of cefotaxime-resistant urinary *Escherichia coli* from primary care in south-west England 2017–18. J Antimicrob Chemother 75:65–71. https://doi.org/10.1093/jac/dkz397.
25. Takebayashi Y, Wan Nur Ismah WHK, Findlay J, Heesom KJ, Zhang J, Williams OM, MacGowan AP, Avison MB. 2017. Prediction of cephalosporin and carbapenem susceptibility in multi-drug resistant Gram-negative bacteria using liquid chromatography-tandem mass spectrometry. BioRxiv:138594. https://doi.org/10.1101/138594.
26. Ma L, Ishii Y, Chang FY, Yamaguchi K, Ho M, Siu LK. 2002. CTX-M-14, a plasmid-mediated CTX-M type extended-spectrum β-lactamase isolated from *Escherichia coli*. Antimicrob Agents Chemother 46:1985–1988. https://doi.org/10.1128/AAC.46.6.1985-1988.2002.
27. Poirel L, Naas T, Le Thomas I, Karim A, Bingen E, Nordmann P. 2001. CTX-M-type extended-spectrum β-lactamase that hydrolyzes ceftazidime through a single amino acid substitution in the omega loop. Antimicrob Agents Chemother 45:3355–3361. https://doi.org/10.1128/AAC.45.12.3355-3361.2001.
28. Coppi M, Di Pilato V, Monaco F, Giani T, Conaldi PG, Rossolini GM. 2020. Ceftazidime-avibactam resistance associated with increased bla_{KPC-3} gene copy number mediated by pKpQIL plasmid derivatives in ST258 *Klebsiella pneumoniae*. Antimicrob Agents Chemother 64:e01816-19. https://doi.org/10.1128/AAC.01816-19.