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Resistance to Ceftazidime/Avibactam Plus Meropenem/Vaborbactam When Both are Used Together Achieved in Four Steps from Metallo-β-Lactamase Negative *Klebsiella pneumoniae*.

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Running Title: Dual MER/VAB CAZ/AVI Resistance in *K. pneumoniae*

Abstract

Serine β-lactamases are dominant causes of β-lactam resistance in *Klebsiella pneumoniae*. Recently, this has driven clinical deployment of the β-lactam/β-lactamase inhibitor pairs ceftazidime/avibactam and meropenem/vaborbactam. We show that four steps: *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.

Text

K. pneumoniae can become third-generation cephalosporin (3GC) and/or carbapenem resistant through acquisition of a wide range of serine β-lactamases (1). Most clinically significant are the CTX-M (2), KPC (3) and the OXA-48-like (4) types. In response to the rise of KPC, which can confer 3GC and carbapenem resistance, serine β-lactamase inhibitors have recently been developed, including avibactam and vaborbactam. Avibactam (5) is used in combination with the 3GC ceftazidime. It inhibits CTX-M, KPC and OXA-48-like β-lactamases (6,7). Ceftazidime/avibactam (CAZ/AVI) non-susceptibility 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* 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 both when used separately (14,15). The aim of the work reported here was to identify steps that can generate resistance to MER/VAB or CAZ/AVI when used together.

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

LC-MS/MS 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* Ecl8 (22) genomic DNA using primers *ompK36* KO FW (5′-CGTTCAGGCGAACAACACTG-3′) and *ompK36* KO RV (5′-AAGTTCAGGCCGTCAACCAG-3′). 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 primers *ompK36* full length FW (5′-GAGGCATCCGGTTGAAATAG-3′) and *ompK36* full length RV (5′-ATTAATCGAGGCTCCTCTTAC-3′). 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} (**Table 1**) confirming resistance. 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* producing an OXA-48-like enzyme (12,13).

Both KP21(pOXA-232) *ompK36* mutants remained susceptible to ceftazidime (**Table 1**) as expected since OXA-232 only weakly hydrolyses ceftazidime (23). 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 alongside its native promoter from a human urinary *E. coli* isolated from primary care (24) using primers CTX-M-14 FW (5′-CCGGAATTCAATACTACCTTGCTTTCTGA-3′) and CTX-M-14 RV (5′-

CCGGAATTCCGTAGCGGAACGTTCATCAG-3′) and ligated into pUBYT (25) at the EcoRI site. 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**). These derivatives remained ceftazidime susceptible in the presence of 4 μg.mL^{-1} avibactam (MedChemExpress), however (**Table 1**), which is a potent inhibitor of CTX-M (5). 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 primer CTX-M-14-P170S-FW (5′-TCTGGATCGCACTGAATCTACGCTGAATACCGC-3′).

We next insertionally inactivated *ompK35* in KP21 M[*ompK36*](pOXA-232)(pCTX-M-14-P170S) using the method described above but with the primers *ompK35* KO FW (5′-TCCCAGACCACAAAAACCCG-3′) and *ompK35* KO RV (5′-CCAGACCGAAGAAGTCGGAG-3′) and checked with primers *ompK35* full length FW (5′-CACTTCGATGTATTTAACCAG-3′) and *ompK35* full length RV (5′-ATGATGAAGCGCAATATTCTG-3′). 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 co-producing OXA-232.

KPC-3 is a carbapenemase that can also confer ceftazidime resistance (28). To create the recombinant plasmid pKPC-3, *bla*$_{\text{KPC-3}}$ was amplified from pKpQIL isolated from *K. pneumoniae* KP30 (16) by PCR using primers KPC-3 FW (5′-CCGGAATTCGTAAAGTGGGTCAGTTTTCAG-3′) and KPC-3 RV (5′-

GGCTCTGAAAATCATCTATTGGAATTCCGG-3′) and ligated into pUBYT at the EcoRI site. 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*$_{KPC-3-D178Y}$ was constructed using primers KPC-3-D178Y-FW (5′-AGGCGATGCGCGCTATACCTCATCGCC-3′) and KPC-3-D178Y-RV (5′- GGCGATGAGGTATAGCGCGCATCGCCT-3′); *bla*$_{KPC-3-V239G}$ was constructed using primers KPC-3-V239G-FW (5′-GGAACCTGCGGAGGGTATGGCACGGCA-3′) and KPC-3-V239G-RV (5′-TGCCGTGCCATACCCTCCGCAGGTTCC-3′). The common flanking primers were KPC-3 FW and KPC-3 RV, as above.

When introduced into KP21(pOXA-232) (i.e. with wild-type *ompK36*), both pKPC-3-D178Y or 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 (**Table 1**) as reported previously (8). 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*.

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 since KP21 *ompK36*(pKPC-3-D178Y) and KP21 *ompK36*(pKPC-3-V239G) – i.e. 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 have recently

been identified that are CAZ/AVI resistant (29) which supports our finding here that KPC-3 mutation is not essential.

Finally, checkerboard assays (**Figure 1**) 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. Briefly, a PBS bacterial suspension was prepared to obtain a stock of OD_{600}=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 $\mu g.mL^{-1}$) and vaborbactam (8 $\mu g.mL^{-1}$) 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 (**Figure 1**) confirmed that KP21 M(pOXA-232) and KP21 *ompK36*(pOXA-232) carrying pCTX-M14-P170S, pKPC-3 or pKPC-3-V239G were all susceptible to meropenem in the presence of vaborbactam plus CAZ/AVI suggesting that combined therapy would still work. Further disruption of *ompK35* did not alter this result. However, KP21 M(pOXA-232)(pKPC-3-D178Y) or KP21 *ompK36*(pOXA-232)(pKPC-3-D178Y) were resistant to meropenem (MIC = 16 $\mu g.mL^{-1}$) and ceftazidime (MIC ≥ 64 $\mu g.mL^{-1}$) in the presence of vaborbactam plus avibactam.

We have, therefore, generated *K. pneumoniae* derivatives resistant to CAZ/AVI and MER/VAB, when used separately when both pairs are used together. This was achieved in four steps relative to wild-type: *ramR* frameshift mutation, acquisition of OXA-232, *ompK36* frameshift mutation (or insertional inactivation), 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 (**Figure 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 (**Figure 1**). This confirms that *ramR* mutation

and production of OXA-232 are both necessary for the dual-resistant phenotype. Since 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* to acquire resistance to the latest combination therapies available in the clinic by layering mechanisms. Of course, the order that the four identified steps occur does not affect the result. So, it would seem prudent to make every effort to identify, via molecular diagnostics, intermediate stages in this acquisition process. It would also seem prudent not to rely on sequential use of CAZ/AVI and MER/VAB, which might select for the dual-resistant phenotype observed here.

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We declare no conflicts of interest.

Figure Legend

Figure 1. Checkerboard assays for ceftazidime and meropenem in the presence of avibactam and vaborbactam.

Each image represents duplicate assays for an 8×8 array of wells in a 96-well plate. All wells contained CA-MHB including avibactam (4 $\mu g.mL^{-1}$) and vaborbactam (8 $\mu g.mL^{-1}$). A serial dilution of meropenem (MEM, x-axis) and ceftazidime (CAZ, y-axis) was created from 32 $\mu g.mL^{-1}$ in each plate as recorded. All wells were inoculated with a suspension of bacteria, made as per 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 recorded as a yellow block. Growth at 8 $\mu g.mL^{-1}$ ceftazidime and 8 $\mu g.mL^{-1}$ meropenem (this position indicated in red) in the presence of vaborbactam and avibactam defines resistance based on CLSI breakpoints (19). Bacterial suspensions used were: for images in the 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); fifth row, KP47 *ompK36*(pOXA-232). In each case, bacteria also carry the following plasmids (where tested): images in first column, pCTX-M-14 P170S; second column, pKPC-3; third column, pKPC-3-D178Y; fourth column, pKPC-3-V239G.

Tables

Table 1. MICs (µg.mL^{-1}) of meropenem with or without vaborbactam and of ceftazidime with or without avibactam against derivatives of *K. pneumoniae* clinical isolates.

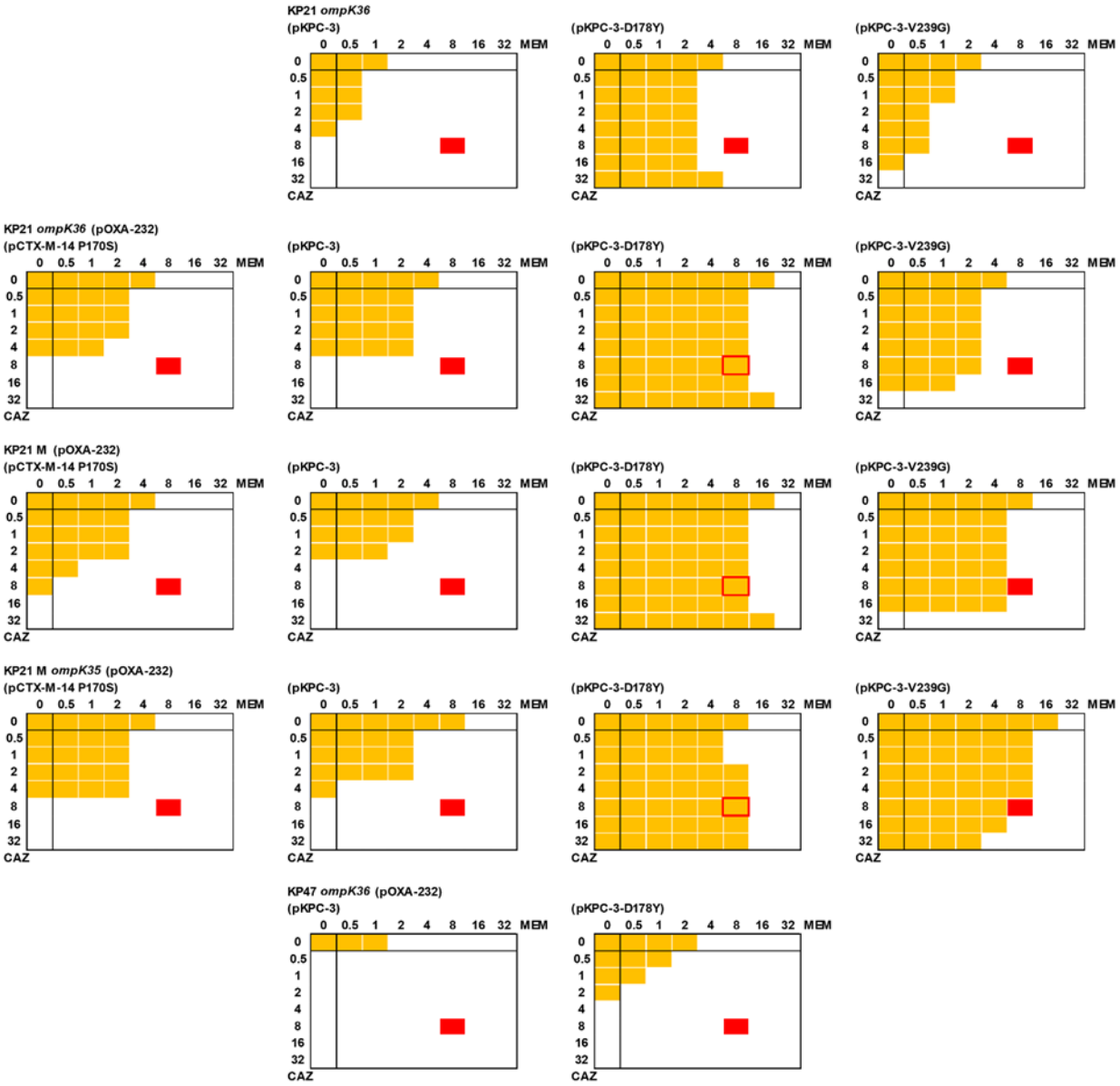
	Meropenem	Meropenem/ Vaborbactam	Ceftazidime	Ceftazidime/ Avibactam
KP21[*ramR*](pOXA-232)(pUBYT)	1	1/8	2	0.5/4
KP21[*ramR*] pOXA-232)(pCTX-M-14)	1	1/8	32	1/4
KP21[*ramR*] pOXA-232)(pCTX-M-14 P170S)	1	1/8	256	4/4
KP21[*ramR*](pOXA-232)(pKPC-3)	128	2/8	>256	8/4
KP21[*ramR*](pOXA-232)(pKPC-3-D178Y)	2	2/8	>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

Shading indicates non-susceptibility (resistance or intermediate resistance) according to CLSI breakpoints (19).

Figure 1


KP21 ompK36
(pKPC-3)
(pKPC-3-D178Y)
(pKPC-3-V239G)
KP21 ompK36 (pOXA-232)
(pCTX-M-14 P170S)
KP21 M (pOXA-232)
(pCTX-M-14 P170S)
KP21 M ompK35 (pOXA-232)
(pCTX-M-14 P170S)
KP47 ompK36 (pOXA-232)
0 0.5 1 2 4 8 16 32 MEM
CAZ

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