

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

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16

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

18

19 **Abstract**

20 **Serine cephalosporinases and carbapenemases are dominant causes of critically**
21 **important β -lactam resistance in *Klebsiella pneumoniae*. This has led to the recent**
22 **clinical deployment of new serine β -lactamase inhibitors used in combination with β -**
23 **lactams. Starting with clinical *K. pneumoniae* isolates and adding plasmids carrying**
24 **the OXA-48-like class D carbapenemase, OXA-232, the class A carbapenemase KPC-3,**
25 **the class A cephalosporinase CTX-M-14 and mutant derivatives of these enzymes, we**
26 **set out to identify the steps required to give resistance to the recently approved β -**
27 **lactam/ β -lactamase inhibitor pairs ceftazidime/avibactam and**
28 **meropenem/vaborbactam when both are used together. We show that four steps:**
29 ***ompK36* and *ramR* loss-of-function plus carriage of OXA-232 and KPC-3-D178Y, all of**
30 **which have been observed in clinical isolates, allow *K. pneumoniae* to resist the**
31 **combined use of both β -lactam/ β -lactamase inhibitor pairs. These findings have**
32 **implications for decision making about sequential and combinatorial use of β -**
33 **lactam/ β -lactamase inhibitor pairs to treat *K. pneumoniae* infections, and suggest**
34 **simple surveillance activities that might identify intermediate stages in resistance**
35 **acquisition and therefore guide therapy to reduce the emergence of dual resistant**
36 **strains.**

37 Introduction

38 Cephalosporin- and carbapenem-resistant *Klebsiella pneumoniae* are critically important
39 pathogens (1). *K. pneumoniae* can become resistant to a variety of antimicrobials by
40 increased production of two RND efflux pumps, AcrAB and OqxAB (2-5) or reduced
41 production of two porins, OmpK35 and OmpK36 (6). *K. pneumoniae* can also acquire a wide
42 range of serine β -lactamases (7) the most clinically significant of which are enzymes of the
43 CTX-M (class A cephalosporinase [8]), KPC (class A carbapenemase/cephalosporinase [9])
44 and the OXA-48-like (class D carbapenemase [10]) types.

45 In response to the rise of CTX-M and KPC, serine β -lactamase inhibitors have been
46 developed. Avibactam contains a diazabicyclo-octane cyclic core (11) and is used in
47 combination with the third-generation cephalosporin ceftazidime. In vitro, avibactam shows
48 good activity against many class A (TEM-1, SHV-1, CTX-M and KPC), class C (AmpC) and
49 even some class D (OXA-24 and OXA-48) β -lactamases (12,13). When used *in vivo*,
50 ceftazidime/avibactam resistance has been seen to develop in KPC producers, caused by a
51 series of point mutations in the *bla*_{KPC} gene, for example resulting in D179Y or V239G
52 substitutions in KPC (14,15). A similar mechanism of ceftazidime/avibactam resistance was
53 seen in CTX-M producers, with mutations in *bla*_{CTX-M}, for example leading to a P170S
54 change, alone or in addition to T264I (16).

55 Vaborbactam is a cyclic boronate and is used in combination with the carbapenem
56 meropenem (17). It has potent activity against class A carbapenemases including KPC. It
57 has also been shown to have inhibitory activity against other class A (TEM, SHV and CTX-
58 M) and class C (AmpC) β -lactamases, although to a lesser extent when compared to its
59 activity against KPC (18). Meropenem/vaborbactam resistance has been observed due to
60 loss of function mutations in *ompK36* and *ompK35* (18,19). Mutations in KPC that confer
61 reduced vaborbactam inhibition have not yet been observed.

62 Given the appearance of *K. pneumoniae* clinical isolates and laboratory selected mutants
63 that are resistant to meropenem/vaborbactam or ceftazidime/avibactam, there has been
64 some discussion in the literature about whether a combination of both β -lactam/ β -lactamase
65 inhibitor

66 pairs given together would overcome isolates resistant to either, and to both when used
67 separately (20,21). In the work reported here we identified the steps required to generate
68 resistance to each β -lactam/ β -lactamase inhibitor pair, both pairs when used separately, and
69 both pairs when used together. To do this we started with *K. pneumoniae* clinical isolates
70 susceptible to both meropenem and ceftazidime.

71

72 **Results and Discussion**

73 *Permeability mutations influencing Meropenem/Vaborbactam MICs in K. pneumoniae Ecl8*
74 *carrying OXA-232 or KPC-3*

75 There are four main loss-of-function mutations that affect accumulation of antibacterial drugs
76 in *K. pneumoniae* clinical isolates: loss of the transcriptional repressors OqxR (2,3) and
77 RamR (4,5), and loss of the porins OmpK35 (22,23) and OmpK36 (24,25). Starting with a
78 laboratory workhorse *K. pneumoniae* strain, Ecl8, we determined the influence of these
79 mutations on meropenem MIC in the presence or absence of $8 \mu\text{g.mL}^{-1}$ vaborbactam when
80 the strains carried one of two carbapenemases commonly encountered in the clinic: the
81 OXA-48-like enzyme OXA-232 (26) or the class A carbapenemase KPC-3 (15). OXA-232 is
82 a relatively weak carbapenemase, and in our hands does not give meropenem resistance in
83 this otherwise wild-type background (**Table 1**) as we saw in the same background when
84 using OXA-48 (27). Each loss-of-function mutation conferred a 4-fold increase in
85 meropenem MIC, but in no case was the mutant meropenem resistant. In the presence of
86 vaborbactam, meropenem MIC only marginally reduced, which was expected because
87 vaborbactam is a poor inhibitor of OXA-48-like enzymes (28). In contrast, KPC-3 is an

88 excellent carbapenemase and gives meropenem resistance even in this wild-type
89 background (**Table 1**). All four loss-of-function mutations increased meropenem MICs, but
90 only by one or two doubling dilutions. Vaborbactam is a strong inhibitor of KPC-3 (17), and
91 meropenem susceptibility was restored in Ecl8 and all mutants producing this enzyme. The
92 highest MIC was against the *ompK36* mutant, but it remained meropenem/vaborbactam
93 susceptible. This was expected, since it has been shown that meropenem/vaborbactam
94 resistance in derivatives of *K. pneumoniae* clinical isolates is primarily caused by *ompK36*
95 loss in a background where *ompK35* has already been lost (19) and we have previously
96 reported that Ecl8 actually produces more OmpK35 than clinical isolates (29).

97 *Generating derivatives of K. pneumoniae clinical isolates resistant to ceftazidime/avibactam*
98 *or meropenem/vaborbactam but not both.*

99 *K. pneumoniae* clinical isolate KP21 produces low levels of OmpK35 (29). Experimentally, it
100 has been shown that *ramR* loss of function downregulates OmpK35 production in *K.*
101 *pneumoniae* (27) and KP21 carries a frameshift mutation in *ramR* (29) whilst whole genome
102 sequencing confirmed that *ompK35* itself is wild type. We introduced a natural OXA-232
103 plasmid into this isolate, and the MIC of meropenem in the presence of vaborbactam against
104 KP21(pOXA-232) was 1 $\mu\text{g.mL}^{-1}$ (**Table 2**) which is below the resistance breakpoint and
105 equivalent to our findings for Ecl8 *ramR*(pOXA-232) (**Table 1**). We next selected a
106 spontaneous mutant of KP21(pOXA-232) which grew on 16 $\mu\text{g.mL}^{-1}$ meropenem in the
107 presence of vaborbactam, and so it is resistant. Proteomics confirmed that this mutant, KP21
108 M(pOXA-232), had undetectable OmpK36 porin levels, and whole genome sequencing
109 identified a single mutation resulting in a stop at codon 125 in *ompK36*, explaining loss of the
110 porin. The MICs of meropenem with or without vaborbactam against KP21
111 M[*ompK36*](pOXA-232) and against the constructed mutant KP21 *ompK36*(pOXA-232) were
112 256 $\mu\text{g.mL}^{-1}$ (**Table 2**). This shows that *ompK36* loss confers meropenem/vaborbactam
113 resistance in *ramR* mutant *K. pneumoniae* clinical isolates (i.e. with reduced OmpK35 levels)

114 producing OXA-48-like enzymes. Loss-of-function mutations in *ompK35* are not necessary
115 for this phenotype.

116 The meropenem/vaborbactam resistant KP21(pOXA-232) *ompK36* mutant derivatives
117 remained susceptible to ceftazidime (**Table 2**) as expected since OXA-232 is a weak
118 cephalosporinase (26). Also as expected, introduction of a low-copy-number vector carrying
119 *bla*_{CTX-M-14} alongside its native promoter into either KP21(pOXA-232), KP21 *ompK36*(pOXA-
120 232) or KP21 M[*ompK36*](pOXA-232) conferred ceftazidime resistance. The derivatives
121 remained ceftazidime susceptible in the presence of 4 µg.mL⁻¹ avibactam (**Table 2**) which is
122 a potent inhibitor of CTX-M (11). Replacement of *bla*_{CTX-M-14} with a gene encoding a variant
123 associated with reduced ceftazidime/avibactam susceptibility, CTX-M-14-P170S (16) drove
124 the ceftazidime MIC against KP21 *ompK36*(pOXA-232) and KP21 M[*ompK36*](pOXA-232) in
125 the presence of avibactam up to 8 µg.mL⁻¹, which is still susceptible, but only one doubling
126 dilution below the breakpoint for resistance. Disruption of *ompK35* in KP21
127 M[*ompK36*](pOXA-232)(pCTX-M-14-P170S) did not further increase the
128 ceftazime/avibactam MIC, confirming that *ramR* loss-of-function phenotypically mimics
129 *ompK35* loss-of-function in this regard (**Table 2**). Accordingly, it was not possible to generate
130 derivatives resistant to both meropenem/vaborbactam and ceftazidime/avibactam using a
131 CTX-M-14 variant associated with reduced susceptibility to ceftazidime/avibactam, even in a
132 *ramR* plus *ompK36* double mutant background co-producing OXA-232.

133 Introduction of KPC-3 and its derivatives associated with reduced ceftazidime/avibactam
134 susceptibility into KP21(pOXA-232) was also attempted. In this case, both KPC-3 derivatives
135 tested (D178Y or V239G) conferred ceftazidime/avibactam resistance. The D178Y derivative
136 did this at the expense of reducing meropenem MIC into the intermediate-resistant zone.
137 Essentially, there is a trade-off between meropenem hydrolytic activity and reduced
138 avibactam inhibition (or perhaps increased ceftazidime hydrolytic activity) as reported
139 previously (14). The V239G mutant did not suffer from such a large drop in meropenem MIC,
140 but neither S178Y or V239G significantly altered the ability of vaborbactam to inhibit KPC-3,

141 as confirmed because all KP21(pOXA-232) derivatives carrying these KPC-3 variants were
142 meropenem/vaborbactam susceptible (**Table 2**). Accordingly, it was not possible to generate
143 derivatives resistant to both meropenem/vaborbactam and ceftazidime/avibactam using
144 KPC-3 variants associated with ceftazidime/avibactam resistance in a background having
145 wild-type *ompK36*, even with co-production of OXA-232.

146

147 *Combining ramR loss, OXA-232, ompK36 loss and KPC-3-D178Y confers dual*
148 *meropenem/vaborbactam plus ceftazidime/avibactam resistance in K. pneumoniae.*

149 The above observations left us with the hypothesis that adding a KPC-3 derivative
150 associated with ceftazidime/avibactam resistance to a background where meropenem non-
151 susceptibility is conferred by mutation of *ramR* (i.e. causing OmpK35 downregulation)
152 *ompK36* loss and OXA-232 production would generate a derivative resistant to
153 ceftazidime/avibactam and meropenem/vaborbactam when used separately. This proved to
154 be correct. MICs of meropenem/vaborbactam and ceftazidime/avibactam were $\geq 64 \mu\text{g.mL}^{-1}$
155 against KP21 M[*ompK36*](pOXA-232) or KP21 *ompK36*(pOXA-232) carrying either the
156 D178Y or V239G derivatives of KPC-3. Additional disruption of *ompK35* only marginally
157 further increased MICs, again confirming that OmpK35 down-regulation caused by *ramR*
158 loss-of-function gives essentially the same phenotype as *ompK35* loss-of-function (**Table 2**).

159 Despite our use of OXA-232-producing strains for this analysis, production of OXA-232 was
160 not found to be essential for resistance to meropenem/vaborbactam and
161 ceftazidime/avibactam, when used separately. After constructing KP21 *ompK36*(pKPC-3-
162 D178Y) and KP21 *ompK36*(pKPC-3-V239G) – i.e. lacking OXA-232 – we found them to be
163 resistant to both β -lactam/ β -lactamase inhibitor pairs (**Table 2**). Another interesting finding
164 from our analysis was that ceftazidime/avibactam and meropenem/vaborbactam resistance
165 can be conferred in the presence of wild-type KPC-3 and OXA-232 in this *ramR* background
166 also lacking *ompK36* (**Table 2**). Clinical *K. pneumoniae* isolates with *ompK35* and *ompK36*

167 loss-of-function mutations and elevated KPC-3 production have recently been identified that
168 are resistant to ceftazidime/avibactam (30) which supports our conclusion that KPC
169 mutations are not essential for resistance.

170 Finally, we wanted to test whether the two β -lactam/inhibitor pairs would work synergistically,
171 to give hope of clinical efficacy for the double combination therapy that has been discussed
172 as a possibility for clinical use in the literature (20,21). Checkerboard assays (**Figure**)
173 confirmed that KP21 M[*ompK36*](pOXA-232) and KP21 *ompK36*(pOXA-232) carrying pCTX-
174 M14-P170S, pKPC-3 or pKPC-3-V239G are all susceptible to meropenem ($\text{MIC} \leq 8 \mu\text{g.mL}^{-1}$)
175 in the presence of vaborbactam plus ceftazidime/avibactam suggesting that combined
176 therapy would still work. However, KP21 M[*ompK36*](pOXA-232) carrying pKPC-3-D178Y
177 and KP21 *ompK36*(pOXA-232) carrying pKPC-3-D178Y were resistant to meropenem (MIC
178 $= 16 \mu\text{g.mL}^{-1}$) and ceftazidime ($\text{MIC} \geq 64 \mu\text{g.mL}^{-1}$) in the presence of vaborbactam plus
179 avibactam. Further disruption of *ompK35* did not alter this result, again confirming that *ramR*-
180 loss-mediated OmpK35 downregulation (as seen in all KP21 derivatives [29]) mimics the
181 phenotypic effect of *ompK35* loss.

182 We have, therefore, generated *K. pneumoniae* derivatives resistant to ceftazidime/avibactam
183 plus meropenem/vaborbactam when both pairs are used together. This was achieved in four
184 steps relative to wild-type (*ramR* loss-of-function, acquisition of OXA-232, *ompK36* loss-of-
185 function, acquisition of KPC-3-D178Y) each replicating an event previously identified in
186 clinical *K. pneumoniae* isolates. When we constructed a derivative of clinical isolate KP47
187 (wild type for *ramR*) carrying OXA-232 and KPC-3-D178Y and with *ompK36* inactivated, the
188 derivative was meropenem/vaborbactam and ceftazidime/avibactam resistant when the pairs
189 were used separately (**Table 2**) but not when both pairs were used at the same time
190 (**Figure**). This confirms that OmpK35 downregulation caused by *ramR* loss-of-function is
191 necessary to confer this dual-resistant phenotype. When we constructed the derivative KP21
192 *ompK36* carrying KPC-3-D178Y or KPC-3-V239G but without carriage of OXA-232 it was
193 also resistant to both pairs when used separately (**Table 2**) but not resistant when the pairs

194 were used together (**Figure**). Therefore, production of an OXA-48 like enzyme is also
195 necessary for dual resistance.

196 Overall, this work confirms the remarkable capacity of *K. pneumoniae* to acquire resistance
197 to the latest combination therapies available in the clinic by layering resistance mechanisms.
198 Essentially, one low frequency step (specific mutations in a gene) is needed for
199 ceftazidime/avibactam resistance; three steps (two high frequency loss-of-function mutations
200 and a gene acquisition event, whose frequency depends on many factors) are needed for
201 meropenem/vaborbactam resistance. If one of the three steps is acquisition of a KPC
202 associated with ceftazidime/avibactam resistance, then these three steps lead to resistance
203 to both ceftazidime/avibactam and meropenem/vaborbactam when used separately; finally
204 adding in an fourth step: acquisition of an OXA-48-like carbapenemase is all that is needed
205 to give resistance to both β -lactam/ β -lactamase inhibitor pairs when used together. Of
206 course, the order that these steps occur does not affect the result. So, it would seem prudent
207 to make every effort to identify, via molecular diagnostics, intermediate stages in this
208 acquisition process, e.g. *ramR* or *ompK35* mutants carrying KPC or an OXA-48-like enzyme.
209 It would also seem prudent not to rely on sequential use of β -lactam/ β -lactamase inhibitor
210 pairs, which might select for the dual-resistant phenotype observed here. In **table 3** we
211 attempt to address the issue of how different starting genotypes influence the chances of
212 obtaining derivatives with specific resistance phenotypes. Whilst the frequencies reported for
213 plasmid transmission events will vary widely dependent on local plasmid ecology, we hope
214 that this table stimulates discussion around clinical decision making to reduce the
215 emergence of resistance at the site of infection or in the patient's gut flora. And will increase
216 the desire to obtain relevant genotypic information about circulating bacterial populations,
217 and even the ecology of resistant organisms in the patient's gut microbiome prior to
218 choosing therapy.

219

220

221 **Experimental**

222 *Materials, bacterial isolates and plasmids*

223 Chemicals were from Sigma and growth media from Oxoid, unless otherwise stated.
224 Meropenem was from Sequoia Research Products, vaborbactam and avibactam were from
225 MedChemExpress. Strains used were *K. pneumoniae* Ecl8 (31) the TEM-1-producing *ramR*
226 mutant (Arg44FS) clinical isolate KP21, the wild-type clinical isolate KP47 and the in vitro-
227 selected Ecl8-derived *oqxR* (Tyr109STOP) or *ramR* (Thr124Pro) loss-of-function mutants
228 (29). A plasmid carrying *bla*_{OXA-232} (pOXA-232) was recovered from *K. pneumoniae* clinical
229 isolate KP11 (29) using a Qiagen plasmid purification kit, with the plasmid then used to
230 transform *E. coli* DH5 α to reduced piperacillin/tazobactam susceptibility (8 $\mu\text{g.mL}^{-1}$
231 piperacillin and 4 $\mu\text{g.mL}^{-1}$ tazobactam) using electroporation. Recombinants were confirmed
232 to be cefotaxime susceptible before the plasmid was re-purified and used to transform *K.*
233 *pneumoniae* isolates. The *bla*_{OXA-232}-encoding region was confirmed as being unchanged
234 from the original using PCR sequencing.

235

236 *Selection and generation of mutants*

237 To select meropenem/vaborbactam resistant mutants, 100 μL aliquots of overnight cultures
238 of KP21 grown in Nutrient Broth (NB) were spread onto Mueller Hinton agar containing 16
239 $\mu\text{g.mL}^{-1}$ meropenem in the presence of 8 $\mu\text{g.mL}^{-1}$ of vaborbactam, which were then
240 incubated for 24 h. Insertional inactivation of *ompK35* or *ompK36* was performed using the
241 pKNOCK suicide plasmid (32). The *ompK35* and *ompK36* DNA fragments were amplified
242 with Phusion High-Fidelity DNA Polymerase (NEB, UK) from *K. pneumoniae* Ecl8 genomic
243 DNA using primers *ompK35* KO FW (5'-TCCCAGACCACAAAAACCCG-3') and *ompK35* KO
244 RV (5'-CCAGACCGAAGAAGTCGGAG-3'); *ompK36* KO FW (5'-
245 CGTTCAGGCGAACAACACTG-3') and *ompK36* KO RV (5'-AAGTTCAGGCCGTCAACCAG-
246 3'). Each PCR product was ligated into the pKNOCK-GM at the SmaI site. The recombinant

247 plasmid was then transferred into wild-type *K. pneumoniae* isolates by conjugation. Mutants
248 were selected for gentamicin non-susceptibility ($5 \mu\text{g.mL}^{-1}$) and the mutation was confirmed
249 by PCR using primers *ompK35* full length FW (5'-CACTTCGATGTATTTAACCAG-3') and
250 *ompK35* full length RV (5'-ATGATGAAGCGCAATATTCTG-3') *ompK36* full length FW (5'-
251 GAGGCATCCGGTTGAAATAG-3') and *ompK36* full length RV (5'-
252 ATTAATCGAGGCTCCTCTTAC-3').

253

254 *Determining MICs of antimicrobials and checkerboard assays*

255 MICs were determined using CLSI broth microtiter assays (33) and interpreted using
256 published breakpoints (34). Checkerboard assays were performed using an adapted
257 microtiter MIC assay. Briefly, a PBS bacterial suspension was prepared to obtain a stock of
258 $\text{OD}_{600}=0.01$. The final volume in each well of a 96-well cell culture plate (Corning Costar)
259 was 200 μL and included 20 μL of the bacterial suspension. All wells contained Cation
260 Adjusted Muller Hinton broth (CA-MHB) with avibactam ($4 \mu\text{g.mL}^{-1}$) and vaborbactam (8
261 $\mu\text{g.mL}^{-1}$) with a serial dilution of meropenem from right to left and ceftazidime from the
262 bottom to the top of the plate. Bacterial growth was determined after 20 h of incubation by
263 measuring optical density at 600 nm (OD_{600}) using a POLARstar Omega spectrophotometer
264 (BMG Labtech).

265

266 *Proteomics*

267 Five hundred microlitres of an overnight CA-MHB culture were transferred to 50 mL CA-MHB
268 and cells were grown at 37°C to 0.6 OD_{600} . Cells were pelleted by centrifugation (10 min,
269 $4,000 \times g$, 4°C) and resuspended in 20 mL of 30 mM Tris-HCl, pH 8 and broken by
270 sonication using a cycle of 1 s on, 0.5 s off for 3 min at an amplitude of 63% using a Sonics
271 Vibracell VC-505TM (Sonics and Materials Inc., Newton, Connecticut, USA). The sonicated
272 samples were centrifuged at 8,000 rpm (Sorval RC5B PLUS using an SS-34 rotor) for 15

273 min at 4°C to pellet intact cells and large cell debris. For envelope preparations, the
274 supernatant was subjected to centrifugation at 20,000 rpm for 60 min at 4°C using the above
275 rotor to pellet total envelopes. To isolate total envelope proteins, this total envelope pellet
276 was solubilized using 200 µL of 30 mM Tris-HCl, pH 8 containing 0.5% (w/v) SDS.

277 Protein concentrations in all samples were quantified using Biorad Protein Assay Dye
278 Reagent Concentrate according to the manufacturer's instructions. Proteins (5 µg/lane for
279 envelope protein analysis) were separated by SDS-PAGE using 11% acrylamide, 0.5% bis-
280 acrylamide (Biorad) gels and a Biorad Min-Protein Tetracell chamber model 3000X1. Gels
281 were resolved at 200 V until the dye front moved approximately 1 cm into the separating gel.
282 Proteins in all gels were stained with Instant Blue (Expedeon) for 20 min and de-stained in
283 water.

284 The 1 cm of gel lane was subjected to in-gel tryptic digestion using a DigestPro automated
285 digestion unit (Intavis Ltd). The resulting peptides from each gel fragment were fractionated
286 separately using an Ultimate 3000 nanoHPLC system in line with an LTQ-Orbitrap Velos
287 mass spectrometer (Thermo Scientific). In brief, peptides in 1% (v/v) formic acid were
288 injected onto an Acclaim PepMap C18 nano-trap column (Thermo Scientific). After washing
289 with 0.5% (v/v) acetonitrile plus 0.1% (v/v) formic acid, peptides were resolved on a 250 mm
290 x 75 µm Acclaim PepMap C18 reverse phase analytical column (Thermo Scientific) over a
291 150 min organic gradient using 7 gradient segments (1-6% solvent B over 1 min, 6-15% B
292 over 58 min, 15-32% B over 58 min, 32-40% B over 5 min, 40-90% B over 1 min, held at
293 90% B for 6 min and then reduced to 1% B over 1 min) with a flow rate of 300 nL/min.
294 Solvent A was 0.1% formic acid and Solvent B was aqueous 80% acetonitrile in 0.1% formic
295 acid. Peptides were ionized by nano-electrospray ionization MS at 2.1 kV using a stainless-
296 steel emitter with an internal diameter of 30 µm (Thermo Scientific) and a capillary
297 temperature of 250°C. Tandem mass spectra were acquired using an LTQ-Orbitrap Velos
298 mass spectrometer controlled by Xcalibur 2.1 software (Thermo Scientific) and operated in
299 data-dependent acquisition mode. The Orbitrap was set to analyse the survey scans at

300 60,000 resolution (at m/z 400) in the mass range m/z 300 to 2000 and the top twenty
301 multiply charged ions in each duty cycle selected for MS/MS in the LTQ linear ion trap.
302 Charge state filtering, where unassigned precursor ions were not selected for fragmentation,
303 and dynamic exclusion (repeat count 1; repeat duration 30 s; exclusion list size 500) were
304 used. Fragmentation conditions in the LTQ were as follows: normalized collision energy of
305 40%; activation q 0.25; activation time 10 ms; and minimum ion selection intensity 500
306 counts.

307 The raw data files were processed and quantified using Proteome Discoverer software v1.4
308 (Thermo Scientific) and searched against the UniProt *K. pneumoniae* strain ATCC 700721 /
309 MGH 78578 database (5126 protein entries; UniProt accession 272620) using the
310 SEQUEST (Ver. 28 Rev. 13) algorithm. Peptide precursor mass tolerance was set to 10
311 ppm, and MS/MS tolerance was set to 0.8 Da. Search criteria included
312 carbamidomethylation of cysteine (+57.0214) as a fixed modification and oxidation of
313 methionine (+15.9949) as a variable modification. Searches were performed with full tryptic
314 digestion and a maximum of 1 missed cleavage was allowed. The reverse database search
315 option was enabled and all peptide data was filtered to satisfy false discovery rate (FDR) of 5
316 %. The Proteome Discoverer software generates a reverse “decoy” database from the same
317 protein database used for the analysis and any peptides passing the initial filtering
318 parameters that were derived from this decoy database are defined as false positive
319 identifications. The minimum cross-correlation factor filter was readjusted for each individual
320 charge state separately to optimally meet the predetermined target FDR of 5 % based on the
321 number of random false positive matches from the reverse decoy database. Thus, each data
322 set has its own passing parameters. Protein abundance measurements were calculated from
323 peptide peak areas using the Top 3 method (35) and proteins with fewer than three peptides
324 identified were excluded. The proteomic analysis was repeated three times for each parent
325 and mutant strain, each using a separate batch of cells.

326

327

328

329 *Whole genome sequencing to Identify mutations*

330 Whole genome resequencing was performed by MicrobesNG (Birmingham, UK) on a HiSeq
331 2500 instrument (Illumina, San Diego, CA, USA). Reads were trimmed using Trimmomatic
332 (36) and assembled into contigs using SPAdes 3.10.1 (<http://cab.spbu.ru/software/spades/>).
333 Assembled contigs were mapped to the *K. pneumoniae* Ecl8 reference genome (GenBank
334 accession number GCF_000315385.1) by using progressive Mauve alignment software (37).

335

336 *Cloning bla_{CTX-M-14} and bla_{KPC-3} and site-directed mutagenesis*

337 *bla_{CTX-M-14}* was amplified from a human urinary *E. coli* isolated from primary care (38) by PCR
338 with Phusion High-Fidelity DNA Polymerase (NEB, UK) using primers CTX-M-14 FW (5'-
339 CCGGAATTC AATACTACCTTGCTTTCTGA-3') and CTX-M-14 RV (5'-
340 CCGGAATTC CGTAGCGGAACGTTTCATCAG-3') and ligated into pUBYT (39) at the EcoRI
341 site. CTX-M-14 site-directed mutagenesis (*bla_{CTX-M-14-P170S}*) was performed using the
342 QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent, USA) with the primer CTX-M-
343 14-P170S-FW (5'-TCTGGATCGCACTGAATCTACGCTGAATACCGC-3').

344 *bla_{KPC-3}* was amplified from pKpQIL isolated from *K. pneumoniae* KP30 (29) as above using
345 primers KPC-3 FW (5'- CCGGAATTCGTAAAGTGGGTCAGTTTTTCAG-3') and KPC-3 RV
346 (5'- GGCTCTGAAAATCATCTATTGGAATTCGG-3') and ligated into pUBYT at the EcoRI
347 site. KPC-3 site-directed mutagenesis (*bla_{KPC-3-D178Y}* and *bla_{KPC-3-V239G}*) was performed using a
348 two-step, PCR-based site-directed mutagenesis strategy. *bla_{KPC-3-D178Y}* was constructed
349 using primers KPC-3-D178Y-FW (5'-AGGCGATGCGCGCTATACCTCATCGCC-3') and
350 KPC-3-D178Y-RV (5'- GGCGATGAGGTATAGCGCGCATCGCCT-3'); *bla_{KPC-3-V239G}* was
351 constructed using primers KPC-3-V239G-FW (5'-

352 GGAACCTGCGGAGGGTATGGCACGGCA-3') and KPC-3-V239G-RV (5'-
353 TGCCGTGCCATACCCTCCGCAGGTTCC-3'). Carriage of all pUBYT plasmids was selected
354 using kanamycin (30 µg.mL⁻¹)

355

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365

366 **We declare no conflicts of interest.**

367 **Figure Legend**

368

369 **Figure. Checkerboard assays for ceftazidime and meropenem in the presence of**
370 **avibactam and vaborbactam.**

371 Each image represents duplicate assays for an 8x8 array of wells in a 96-well plate. All wells
372 contained CA-MHB including avibactam ($4 \mu\text{g.mL}^{-1}$) and vaborbactam ($8 \mu\text{g.mL}^{-1}$). A serial
373 dilution of meropenem (MEM, x-axis) and ceftazidime (CAZ, y-axis) was created from 32
374 $\mu\text{g.mL}^{-1}$ in each plate as recorded. All wells were inoculated with a suspension of bacteria,
375 made as per CLSI microtiter MIC guidelines (33), and the plate was incubated at 37°C for 20
376 h. Growth was recorded by measuring OD₆₀₀ and growth above background (broth) is
377 recorded as a yellow block. Growth at $8 \mu\text{g.mL}^{-1}$ ceftazidime and $8 \mu\text{g.mL}^{-1}$ meropenem (this
378 position indicated in red) in the presence of vaborbactam and avibactam defines resistance
379 based on CLSI breakpoints (34). Bacterial suspensions used were: for images in the top
380 row, KP21[*ramR*] *ompK36*; second row, KP21[*ramR*] *ompK36*(pOXA-232); third row,
381 KP21[*ramR*] M[*ompK36*](pOXA-232); fourth row, KP21[*ramR*] M[*ompK36*] *ompK35*(pOXA-
382 232); fifth row, KP47 *ompK36*(pOXA-232). In each case, bacteria also carry the following
383 plasmids (where tested): images in first column, pCTX-M-14 P170S; second column, pKPC-
384 3; third column, pKPC-3-D178Y; fourth column, pKPC-3-V239G.

385

386 **Tables**

387 **Table 1. MICs ($\mu\text{g}\cdot\text{mL}^{-1}$) of meropenem with or without vaborbactam against *K.***
388 ***pneumoniae* Ecl8 derivatives.**

| | Meropenem | Meropenem/Vaborbactam |
|-------------------------------------|------------------|------------------------------|
| Ecl8(pOXA-232) | 0.125 | 0.125/8 |
| Ecl8 <i>ompK35</i>(pOXA-232) | 1 | 0.5/8 |
| Ecl8 <i>ompK36</i>(pOXA-232) | 1 | 1/8 |
| Ecl8 <i>ramR</i>(pOXA-232) | 1 | 0.5/8 |
| Ecl8 <i>oqxR</i>(pOXA-232) | 1 | 0.5/8 |
| Ecl8(pKPC-3) | 64 | 0.125/8 |
| Ecl8 <i>ompK35</i>(pKPC-3) | 128 | 0.125/8 |
| Ecl8 <i>ompK36</i>(pKPC-3) | 256 | 2/8 |
| Ecl8 <i>ramR</i>(pKPC-3) | 128 | 0.125/8 |
| Ecl8 <i>oqxR</i>(pKPC-3) | 128 | 0.125/8 |

389

390 Shading indicates resistance according to CLSI breakpoints (34).

391 **Table 2. MICs ($\mu\text{g}\cdot\text{mL}^{-1}$) of meropenem with or without vaborbactam and of ceftazidime**
 392 **with or without avibactam against derivatives of *K. pneumoniae* clinical isolates.**

| | Meropenem | Meropenem/ Vaborbactam | Ceftazidime | Ceftazidime/ Avibactam |
|---|-----------|---------------------------|-------------|---------------------------|
| KP21[<i>ramR</i>](pOXA-232)(pUBYT) | 1 | 1/8 | 2 | 0.5/4 |
| KP21[<i>ramR</i>] pOXA-232)(pCTX-M-14) | 1 | 1/8 | 32 | 1/4 |
| KP21[<i>ramR</i>] pOXA-232)(pCTX-M-14 P170S) | 1 | 1/8 | 256 | 4/4 |
| KP21[<i>ramR</i>](pOXA-232)(pKPC-3) | 128 | 2/8 | >256 | 8/4 |
| KP21[<i>ramR</i>](pOXA-232)(pKPC-3-D178Y) | 2 | 2/8 | >256 | 256/4 |
| KP21[<i>ramR</i>](pOXA-232)(pKPC-3-V239G) | 64 | 4/8 | >256 | 128/4 |
| KP21[<i>ramR</i>] <i>ompK35</i> (pOXA-232)(pUBYT) | 4 | 4/8 | 2 | 0.5/4 |
| KP21[<i>ramR</i>] <i>ompK35</i> (pOXA-232)(pCTX-M-14) | 4 | 4/8 | 32 | 1/4 |
| KP21[<i>ramR</i>] <i>ompK35</i> (pOXA-232)(pCTX-M-14 P170S) | 2 | 2/8 | >256 | 4/4 |
| KP21[<i>ramR</i>] <i>ompK35</i> (pOXA-232)(pKPC-3) | 128 | 1/8 | >256 | 0.125/4 |
| KP21[<i>ramR</i>] <i>ompK35</i> (pOXA-232)(pKPC-3-D178Y) | 4 | 4/8 | >256 | 128/4 |
| KP21[<i>ramR</i>] <i>ompK35</i> (pOXA-232)(pKPC-3-V239G) | 64 | 4/8 | >256 | 32/4 |
| KP21[<i>ramR</i>] <i>ompK36</i> (pOXA-232)(pUBYT) | 256 | 256/8 | 2 | 0.5/4 |
| KP21[<i>ramR</i>] <i>ompK36</i> (pOXA-232)(pCTX-M-14) | 256 | 256/8 | 32 | 1/4 |
| KP21[<i>ramR</i>] <i>ompK36</i> (pOXA-232)(pCTX-M-14 P170S) | 256 | 256/8 | >256 | 8/4 |
| KP21[<i>ramR</i>] <i>ompK36</i> (pOXA-232)(pCTX-M-14 P170S) | >256 | 256/8 | >256 | 16/4 |

| | | | | |
|--|------|-------|------|--------|
| 232)(pKPC-3) | | | | |
| KP21[<i>ramR</i>] <i>ompK36</i> (pOXA-232)(pKPC-3-D178Y) | 256 | 256/8 | >256 | >256/4 |
| KP21[<i>ramR</i>] <i>ompK36</i> (pOXA-232)(pKPC-3-V239G) | >256 | 256/8 | >256 | 128/4 |
| KP21[<i>ramR</i>] <i>ompK36</i> (pKPC-3) | >256 | 16/8 | >256 | 8/4 |
| KP21[<i>ramR</i>] <i>ompK36</i> (pKPC-3-D178Y) | 64 | 16/8 | >256 | 256/4 |
| KP21[<i>ramR</i>] <i>ompK36</i> (pKPC-3-V239G) | >256 | 32/8 | >256 | 256/4 |
| KP21 M[<i>ramR ompK36</i>](pOXA-232)(pUBYT) | 256 | 256/8 | 2 | 1/4 |
| KP21 M[<i>ramR ompK36</i>](pOXA-232)(pCTX-M-14) | 128 | 128/8 | 32 | 1/4 |
| KP21 M[<i>ramR ompK36</i>](pOXA-232)(pCTX-M-14 P170S) | 256 | 128/8 | >256 | 8/4 |
| KP21 M[<i>ramR ompK36</i>](pOXA-232)(pKPC-3) | >256 | 256/8 | >256 | 8/4 |
| KP21 M[<i>ramR ompK36</i>](pOXA-232)(pKPC-3-D178Y) | 128 | 128/8 | >256 | 256/4 |
| KP21 M[<i>ramR ompK36</i>](pOXA-232)(pKPC-3-V239G) | >256 | 256/8 | >256 | 64/4 |
| KP21 M[<i>ramR ompK36 ompK35</i>](pOXA-232)(pUBYT) | 256 | 256/8 | 2 | 1/4 |
| KP21 M[<i>ramR ompK36 ompK35</i>](pOXA-232)(pCTX-M-14) | 256 | 256/8 | 32 | 1/4 |
| KP21 M[<i>ramR ompK36 ompK35</i>](pOXA-232)(pCTX-M-14 P170S) | 256 | 256/8 | 256 | 8/4 |
| KP21 M[<i>ramR ompK36 ompK35</i>](pOXA-232)(pKPC-3) | >256 | 256/8 | >256 | 16/4 |
| KP21 M[<i>ramR ompK36</i>] | 256 | 256/8 | >256 | >256/4 |

| | | | | |
|---|------|-------|------|--------|
| <i>ompK35</i>(pOXA-232)(pKPC-3-D178Y) | | | | |
| KP21 M[<i>ramR ompK36</i>] <i>ompK35</i>(pOXA-232)(pKPC-3-V239G) | >256 | 256/8 | >256 | 128/4 |
| KP47 <i>ompK36</i>(pKPC-3) | >256 | 2/8 | >256 | 8/4 |
| KP47 <i>ompK36</i>(pKPC-3-D178Y) | 8 | 2/8 | >256 | 256/4 |
| KP47 <i>ompK36</i>(pOXA-232)(pKPC-3) | >256 | 16/8 | >256 | 16/4 |
| KP47 <i>ompK36</i>(pOXA-232)(pKPC-3-D178Y) | 16 | 16/8 | >256 | >256/4 |

393 Shading indicates non-susceptibility (resistance or intermediate resistance) according to

394 CLSI breakpoints (34).

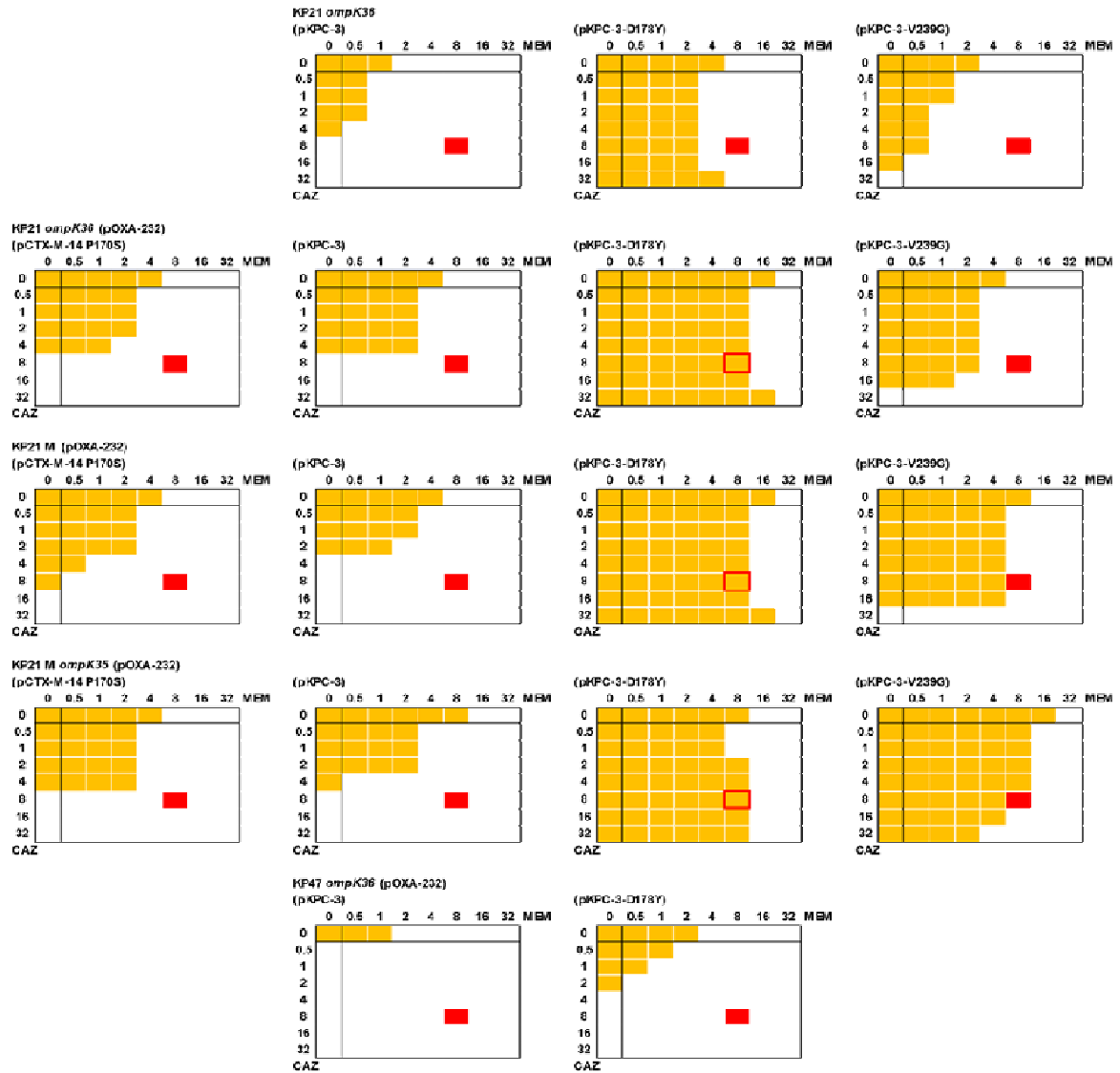
395 **Table 3 Rough relative estimates for risk of acquiring resistance to β -lactam/ β -lactamase inhibitor pairs, singularly or in combination**
 396 **based on starting genotype.**

| Phenotype > | MER/VAB | AVI/CAZ | MER/VAB and AVI/CAZ separately | MER/VAB and AVI/CAZ together |
|-------------------------------|--|--|---|--|
| Genotype Needed > | <i>ramR/ompK35+ompK36+KPC/OXA-232</i> | KPC-MUT or <i>ramR/ompK35+ompK36+KPC+OXA-232</i> | <i>ramR/ompK35+ompK36+KPC+OXA-232</i> or <i>ramR/ompK35+ompK36+KPC-MUT</i> | <i>ramR/ompK35+ompK36+KPC-MUT+OXA-232</i> |
| Actual Genotype | Needed (est. frequency) | Needed (est. frequency) | Needed (est. frequency) | Needed (est. frequency) |
| WT+KPC | <i>ramR/ompK35+OmpK36</i> (10^{-14}) | KPC-MUT (10^{-9}) | <i>ramR/ompK35+ompK36+KPC-MUT</i> (10^{-23}) | <i>ramR/ompK35+ompK36+KPC-MUT+OXA-232</i> (10^{-33})** |
| | | <i>ramR/ompK35+ompK36+OXA-232</i> (10^{-23})** | <i>ramR/ompK35+ompK36+OXA-232</i> (10^{-23})** | <i>ramR+ompK36+KPC-MUT+OXA-232</i> (10^{-33})** |
| <i>ramR/ompK35+KPC</i> | <i>ompK36</i> (10^{-7}) | KPC-MUT (10^{-9}) | <i>ompK36+KPC-MUT</i> (10^{-16}) | <i>ompK36+KPC-MUT+OXA-232</i> (10^{-26})** |
| | | <i>ompK36+OXA-232</i> (10^{-16})** | <i>ompK36+OXA-232</i> (10^{-16})** | <i>ompK36+KPC-MUT+OXA-232</i> (10^{-26})** |
| <i>ompK36+KPC</i> | <i>ramR/ompK35</i> (10^{-7}) | KPC-MUT (10^{-9}) | <i>ramR/ompK35+KPC-MUT</i> (10^{-16}) | <i>ramR/ompK35+KPC-MUT+OXA-232</i> (10^{-26})** |
| | | <i>ramR/ompK35+OXA-232</i> (10^{-16})** | <i>ramR/ompK35+OXA-232</i> (10^{-16})** | <i>ompK36+KPC-MUT+OXA-232</i> (10^{-26})** |
| WT+KPC-MUT | <i>ramR/ompK35+OmpK36</i> (10^{-14}) | -- | <i>ramR/ompK35+OmpK36</i> (10^{-14}) | <i>ramR/ompK35+OmpK36+OXA-232</i> (10^{-24})** |
| <i>ramR/ompK35+KPC-MUT</i> | <i>ompK36</i> (10^{-7}) | -- | <i>ompK36</i> (10^{-7}) | <i>ompK36+OXA-232</i> (10^{-17})** |
| <i>ompK36+KPC-MUT</i> | <i>ramR/ompK35</i> (10^{-7}) | -- | <i>ramR/ompK35</i> (10^{-7}) | <i>ramR/ompK35+OXA-232</i> (10^{-17})** |
| <i>ramR/ompK35+ompK36+KPC</i> | -- | KPC-MUT (10^{-9}) | KPC-MUT (10^{-9}) | KPC-MUT+OXA-232 (10^{-19})** |
| | | OXA-232 (10^{-10})** | OXA-232 (10^{-10})** | KPC-MUT+OXA-232 (10^{-20})** |
| WT+KPC+OXA-232 | <i>ramR/ompK35+OmpK36</i> (10^{-14}) | KPC-MUT (10^{-9}) | <i>ramR/ompK35+OmpK36+KPC-MUT</i> (10^{-23}) | <i>ramR/ompK35+OmpK36+KPC-MUT</i> (10^{-23}) |
| | | <i>ramR/ompK35+OmpK36</i> (10^{-14}) | <i>ramR/ompK35+OmpK36</i> (10^{-14}) | <i>ramR/ompK35+OmpK36+KPC-MUT</i> (10^{-23}) |
| <i>ramR/ompK35+</i> | <i>ompK36</i> (10^{-7}) | KPC-MUT (10^{-9}) | <i>ompK36+KPC-MUT</i> (10^{-16}) | <i>ompK36+KPC-MUT</i> (10^{-16}) |

| | | | | |
|-------------------------------------|--|----------------------------------|--|--|
| KPC+OXA-232 | | | | |
| | | <i>ompK36</i> (10^{-7}) | <i>ompK36</i> (10^{-7}) | <i>ompK36</i> +KPC-MUT (10^{-16}) |
| <i>ompK36</i> +KPC+OXA-232 | <i>ramR/ompK35</i> (10^{-7}) | KPC-MUT (10^{-9}) | <i>ramR/ompK35</i> +KPC-MUT (10^{-16}) | <i>ramR/ompK35</i> +KPC-MUT (10^{-16}) |
| | | <i>ramR/ompK35</i> (10^{-7}) | <i>ramR/ompK35</i> (10^{-7}) | <i>ramR/ompK35</i> +KPC-MUT (10^{-16}) |
| WT+KPC-MUT+OXA-232 | <i>ramR/ompK35+OmpK36</i> (10^{-14}) | -- | <i>ramR/ompK35+OmpK36</i> (10^{-14}) | <i>ramR/ompK35+OmpK36</i> (10^{-14}) |
| <i>ramR/ompK35</i> +KPC-MUT+OXA-232 | <i>ompK36</i> (10^{-7}) | -- | <i>ompK36</i> (10^{-7}) | <i>ompK36</i> (10^{-7}) |
| <i>ompK36</i> +KPC-MUT+OXA-232 | <i>ramR/ompK35</i> (10^{-7}) | -- | <i>ramR/ompK35</i> (10^{-7}) | <i>ramR/ompK35</i> (10^{-7}) |

397 **The risk of plasmid transfer is impossible to assess. It will depend, amongst many other factors, on the transmissibility and abundance of the
 398 plasmid that must be transferred. KPC plasmids of the pKpQIL type, in perfect laboratory conditions transfer at a frequency of 10^{-6} to 10^{-8}
 399 transconjugants per donor cell (40); IncL/M OXA-48 plasmids transfer is at a frequency of almost 10^{-6} (41). So, in mixed populations, such as
 400 might be seen in the gut microbiota, we have assessed the maximum possible transfer frequency as 10^{-10} for each. Of course, if a patient is
 401 clear of any plasmid-encoded KPC or OXA-48 producing bacteria, the observed transfer frequency for the genes encoding these enzymes will
 402 be close to zero. Frequency of spontaneous resistance in KPC was equated with the observed mutation frequency (10^{-9}) for streptomycin
 403 resistance in *Escherichia coli*, since this represents a similarly specific set of mutations (42). A mutator phenotype could increase this by two
 404 orders of magnitude (43). We set the frequency of loss-of-function mutation (*ramR*, *ompK35* or *ompK36*) as 100-fold higher than the
 405 spontaneous mutation frequency because of the number of possible mutations that could disrupt a gene, but this is likely to be a very
 406 conservative estimate as previously described (44). Abbreviations: MER, meropenem, VAB, vaborbactam, CAZ, ceftazidime, AVI, avibactam,
 407 KPC-MUT, KPC derivatives associated with AVI/CAZ resistance.

408 **Figure**



409
410

411 **References**

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