

1 **Selection of mutants with resistance or diminished**
2 **susceptibility to ceftazidime/avibactam from ESBL- and**
3 **AmpC- producing Enterobacteriaceae**

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27
28 **Running head:** CAZ-AVI-resistant mutants

29

30 **Abstract**

31 **Introduction.** Difficult Gram-negative infections are increasingly treated with new β -
32 lactamase inhibitor combinations e.g. ceftazidime/avibactam. Disturbingly, mutations in KPC
33 carbapenemases can confer ceftazidime/avibactam resistance, sometimes selected in
34 therapy. We explored whether this risk extended to AmpC- and ESBL- enzymes. **Materials**
35 **and Methods.** Mutants were selected by plating AmpC-derepressed strains, ESBL
36 producers and ceftazidime-susceptible controls on agar containing ceftazidime + avibactam,
37 1 or 4 mg/L. MICs were determined by CLSI agar dilution; WGS was by Illumina
38 methodology. **Results** Using 2x MIC of ceftazidime + 1 mg/L avibactam, mutants were
39 selected from all strain types at frequencies of 10^{-7} - 10^{-9} . Rates diminished to $<10^{-9}$ with 4
40 mg/L avibactam or higher MIC multiples, except with AmpC-derepressed
41 Enterobacteriaceae. Characterised mutants (n=10, MICs 4-64 mg/L) of AmpC-derepressed
42 strains had modifications in *ampC*, giving Arg168Pro/His, Gly176Arg/Asp, Asn366Tyr or
43 small deletions around positions 309-314. Mutants of ESBL producers (n=20; MICs 0.5-16
44 mg/L) mostly had changes affecting permeability, efflux or β -lactamase quantity; only one
45 had an altered β -lactamase, with an Asp182Tyr substitution in CTX-M-15, raising the
46 ceftazidime/avibactam MIC but abrogating other cephalosporin resistance. Mutants of
47 ceftazidime-susceptible strains were not sequenced, but phenotypes suggested altered drug
48 accumulation or, for *Enterobacter cloacae* only, AmpC derepression. In further experiments,
49 avibactam reduced, but did not abolish, selection of AmpC-derepressed Enterobacteriaceae
50 by ceftazidime. **Conclusions.** Most mutants of AmpC-derepressed Enterobacteriaceae had
51 structural mutations in *ampC*; those of ESBL producers mostly had genetic modifications
52 outside β -lactamase genes, commonly affecting uptake efflux or β -lactamase quantity. The
53 clinical significance of these observations remains to be determined.

55 Introduction

56 Avibactam is the first diazabicyclooctane β -lactamase inhibitor to enter clinical use,
57 formulated with ceftazidime and now licensed in both the US and the EU. An
58 aztreonam/avibactam combination is in advanced development; development of a ceftaroline
59 combination was pursued into Phase II but is now in abeyance.¹ Avibactam inhibits Class A
60 β -lactamases, including ESBLs and KPC types, as well as Class C (AmpC) types.² Inhibitory
61 activity against Class D β -lactamases is variable but, few of these are potent ceftazidimases.
62 Metallo- (Class B) enzymes evade inhibition. As with all β -lactamase inhibitor combinations,
63 activity also depends on the amount of β -lactamase, the underlying spectrum of the
64 partner β -lactam and the permeability and efflux traits of the target strain.^{3,4}

65 Single amino acid substitutions can reduce binding of clavulanate and penicillanic
66 acid sulphones by TEM and SHV penicillinases,⁵ but in-therapy selection of sequence
67 variants of these enzymes is very rare.^{6,7} Less is yet known on the potential of avibactam
68 combinations to select resistance. In-vitro studies with ceftaroline/avibactam⁸ yielded: (i) a
69 single mutant of CTX-M-15 enzyme with a Lys237Gln substitution, and (ii)
70 ceftaroline/avibactam-resistant mutants of AmpC-derepressed *Enterobacter* with deletions in
71 the Ω -loop of AmpC, with Asn366His/Ile substitutions in AmpC or with porin modifications.
72 The CTX-M-15 mutant conferred resistance to ceftaroline/avibactam but lost the ability of
73 classical CTX-M-15 to cause resistance to other oxyimino-cephalosporins; the AmpC
74 mutants were associated with broad resistance. We failed to select stable resistance to
75 ceftaroline/avibactam in Enterobacteriaceae with other ESBLs besides CTX-M-15 or in those
76 with KPC β -lactamase.⁸ More recently, in-vitro and clinical selections of
77 ceftazidime/avibactam-resistant mutants of Enterobacteriaceae with KPC carbapenemases
78 have been described. Several mutations were seen in the laboratory mutants, mostly re-
79 configuring the Ω -loop.⁹ These alterations included Asp179Tyr, which has since been
80 selected, during ceftazidime/avibactam therapy, in clinical mutants.¹⁰ Its effect is to increase
81 binding of ceftazidime,¹¹ protecting the KPC enzyme from inactivation by avibactam. Such

82 mutants show only small MIC rises for avibactam combinations other than
83 ceftazidime/avibactam and often have reduced resistance to meropenem and aztreonam.^{9,10}
84 Clinical selection of reduced susceptibility, with the ceftazidime/avibactam MIC rising from 1
85 to 8 mg/L, was also described in a *Klebsiella pneumoniae* with OXA-48 and CTX-M-14. This
86 was associated with Pro170Ser and Thr264Ileu mutations in the CTX-M-14 enzyme;¹² OXA-48,
87 which lacks ceftazidimase activity, remained unchanged.

88 These observations led us to undertake selection studies with ceftazidime/avibactam,
89 investigating a wide range of ESBLs and AmpC enzymes

90

91 **Materials and Methods**

92 *Test strains*

93 The test strains are detailed in Table 1 and were either reference organisms, isolates from
94 survey collections.^{13,14} Work centred on *Escherichia coli*, *K. pneumoniae*, *Enterobacter*
95 *cloacae* and *Citrobacter freundii*, as the major opportunistic Enterobacteriaceae and on
96 TEM, SHV, CTX-M-15 and AmpC as the prevalent β -lactamases of concern.

97 β -Lactamase types were initially identified from phenotypes and PCR, but later
98 confirmed by WGS. Controls for MIC testing comprised *E. coli* ATCC 25922, *E. coli* ATCC
99 35218 and *K. pneumoniae* ATCC 700603.

100

101 *Antibiotics*

102 Avibactam was provided by AstraZeneca (Wilmington, DE, USA), as were ceftazidime and
103 ceftaroline; other antimicrobials were obtained from Sigma (Poole, UK), except ertapenem
104 (Merck Sharp & Dohme, Hoddesdon, UK) and meropenem (AstraZeneca, Alderley Park,
105 UK).

106

107 *Single step mutant selection*

108 Selection was undertaken as previously described for ceftaroline/avibactam.⁸ Briefly *c.* 10⁹
109 cfu from an overnight broth culture were spread on Mueller-Hinton agar
110 (Thermofisher/Oxoid, Basingstoke, UK) containing ceftazidime/avibactam (fixed 1 or 4 mg/L
111 concentration) at 2-16 x the MIC found previously by CLSI agar dilution. Colonies were
112 counted after overnight incubation, and representatives retained for MIC determination and
113 sequencing. Dilutions of the same overnight nutrient broth cultures were serially diluted and
114 spread on to antibiotic-free Mueller-Hinton agar to provide a viable count, as a denominator
115 for calculation of mutation frequencies.

116

117 *Multi-step selection using β -lactamase producers*

118 Inocula of 10⁸ cfu were added to 10-mL amounts of nutrient broth containing
119 ceftazidime/avibactam (with avibactam at 1 or 4 mg/L) at the ceftazidime/avibactam MICs
120 found previously on agar with the same avibactam concentration, but otherwise by standard
121 CLSI methodology,, and incubated up to 48 h.⁸ This was repeated sequentially, each time
122 doubling the ceftazidime concentration but keeping the avibactam concentration unchanged.

123

124 *Selectivity for AmpC-derepressed mutants*

125 Like other oxyimino-cephalosporins, ceftazidime can select AmpC-derepressed mutants
126 from AmpC-inducible populations.¹⁵ To test how avibactam might affect this phenomenon we
127 plated *c.* 10⁹ cfu of ceftazidime-susceptible (i.e., wild-type, AmpC-inducible, MIC \leq 2 mg/L)
128 cells of *E. cloacae* or *C. freundii* on to Mueller-Hinton agar with ceftazidime at 8 x MIC with
129 or without 1 or 4 mg/L avibactam. After overnight incubation the colonies were counted and
130 mutant frequencies calculated relative to the viable counts contained in the inocula.

131

132 *MIC determinations*

133 MICs were measured by CLSI agar dilution¹⁶ for ceftazidime and for β -lactams in
134 combination with β -lactamase inhibitors at the specified concentrations:
135 ceftazidime/avibactam 1 and 4 mg/L, ceftazidime/cloxacillin 100 mg/L,
136 ceftazidime/clavulanate 4 mg/L, ceftazidime/ tazobactam 4 mg/L, cefotaxime, cefepime,
137 piperacillin/tazobactam 4 mg/L, ertapenem, meropenem, gentamicin and ciprofloxacin.

138

139 *Genomic sequencing and bioinformatics analysis*

140 Parent and mutant DNA were fragmented and tagged for multiplexing using the NexteraXT
141 library preparation Kits (Illumina, Cambridge, UK) and sequenced on an Illumina HiSeq
142 platform to produce 2x100 bp reads. Reads were assembled *de novo* using VelvetOptimiser
143 software (<http://www.vicbioinformatics.com/software.velvetoptimiser.shtml>) with *k-mer* values
144 from 55 to 75. The presence of β -lactamase variants was confirmed by BLAST searches,
145 using the newly assembled genomes as query sequences against a reference database
146 downloaded from the NCBI β -lactamase data ([https://www.ncbi.nlm.nih.gov/pathogens/beta-](https://www.ncbi.nlm.nih.gov/pathogens/beta-lactamase-data-resources)
147 [lactamase-data-resources](https://www.ncbi.nlm.nih.gov/pathogens/beta-lactamase-data-resources)) resources.

148 Genomic alterations in mutants were identified as previously described.¹⁷ sequencing
149 reads for mutants were mapped to the de-novo assembled genome of the corresponding
150 parent using Bowtie2 (<http://bowtie-bio.sourceforge.net/bowtie2>), and variants were called
151 with Samtools with default paramters.¹⁸ The variant calling files thereby generated were then
152 parsed line by line to determine apparent alterations, with the accuracy of these predictions
153 assessed based on read depth and mapping quality as described previously.¹⁷ Sequences
154 flanking confirmed alterations for 2-5 Kb on either side were extracted from the parent
155 assemblies and manually inspected for gene structure and functional annotation with Blast
156 searches so as to determine whether the detected changes were located in an open reading
157 frame, promoter or intergenic region. Changes within structural genes were confirmed by
158 aligning the protein-encoding sequences extracted from the parent and mutant assemblies.
159 In high throughput sequencing, reads are randomly sampled, meaning that the number of

160 reads for any gene or gene fragment reflects its copy number: We therefore counted the
161 number of reads that mapped to the β -lactamase genes relative to those for the single-copy
162 chromosomal *gyrA* and *parC* genes, thereby assessing whether the β -lactamase genes had
163 been amplified in the mutants. Parent and mutant sequencing data are deposited in the
164 European Nucleotide Archive under study number PRJEB27344 (www.ebi.ac.uk/ena).

165

166 **Results**

167 *Mutant selection frequencies*

168 Mutants were obtained from most strains, including β -lactamase-negative controls, when
169 using ceftazidime/avibactam 1 mg/L at 2 x MIC, with frequencies of 10^{-7} to 10^{-9} (Table 1).
170 Mutant frequencies were much reduced at higher MIC multiples or with 4 mg/L avibactam in
171 the selective media. With 8- or 16-fold MIC multiples, mutants were obtained only from
172 strains with stably derepressed AmpC; none was detected from ESBL producers or controls,
173 even when the avibactam concentration was only 1 mg/L. Attempts to 'train' highly resistant
174 mutants by multi-step procedures in broth were unsuccessful, with few mutants obtained;
175 again mostly from strains with stably derepressed AmpC β -lactamases.

176

177 *Mutants of AmpC derepressed strains*

178 MICs were determined for 53 mutants selected from AmpC-derepressed *E. cloacae* or *C.*
179 *freundii*. The MICs of ceftazidime/avibactam 4 mg/L rose from 0.5-2 mg/L for the parent
180 strains to 4-64 (mostly 8-16) mg/L for the mutants; in 23/53 cases the values for the mutants
181 were >8 mg/L, exceeding the CLSI/EUCAST breakpoint. MICs of ceftazidime combined
182 with cloxacillin 100 mg/L (which inhibits AmpC) also were widely, though not universally,
183 raised: MICs of ceftaroline/avibactam rose little, generally only from 0.25-2 mg/L to 1-2 mg/L.
184 Shifts in the MICs of other β -lactams were erratic: some mutants showed rises in cefotaxime

185 and cefepime MICs whereas others showed falls. Likewise, a few mutants showed increases
186 in ertapenem MIC, but falls were commoner.

187 This diversity is illustrated in Table 2 for the 10 mutants of AmpC-derepressed *E.*
188 *cloacae* and *C. freundii* selected for WGS. All proved to have modifications in *ampC*,
189 resulting in amino acid substitutions, including Arg168Pro (three representatives), Arg168His
190 (two representatives), Gly176Arg/Asp (one representative each) Asn366Tyr (one
191 representative) or two- to four- amino acid deletions around positions 309-314 (two
192 representatives). The Arg168Pro substitution was associated with reduced resistance to all
193 cephalosporins, in both *E. cloacae* and *C. freundii* along with the complete loss of synergy
194 between avibactam and both ceftazidime and ceftaroline, whereas Arg168His and
195 Gly176Arg/Asp were associated with retention of broad cephalosporin resistance and
196 ceftaroline/avibactam synergy coupled with markedly reduced ceftazidime/avibactam
197 synergy.

198

199 *Characterisation of selected mutants: ESBL producers*

200 Among 63 mutants of nine ESBL producers (the tenth failed to yield any mutants), only 12
201 achieved resistance to ceftazidime/avibactam as defined by CLSI, with MICs of 16+4 or
202 32+4 mg/L. All these 12 were selected from the same parent, *E. coli* J53-1 with TEM-10
203 ceftazidimase. The ceftazidime/avibactam MICs for other mutants were raised, but with
204 values $\leq 8+4$ mg/L. MICs for 20 mutants selected to represent phenotype diversity are
205 shown in Table 3, along with details of the genetic modifications revealed by WGS.

206 Most mutants of ESBL producers had sequence changes in genes related to
207 permeability, efflux or β -lactamase expression, not in β -lactamase coding genes. Thus, 7/20
208 had modifications in *ompR/envZ*, which regulates expression of porins OmpC and OmpF;²⁰
209 2/20 had identical alterations in *acrAB* efflux gene components; and 9/20 either yielded
210 increased reads of β -lactamase genes relative to *gyrA* and *parC* during WGS, implying gene

211 amplification, or had sequence changes upstream of β -lactamase genes that putatively
212 might increase their expression, though this was not investigated by experiment. Almost all
213 these mutants of ESBL producers showed broad upward rises for β -lactam MICs, including
214 other inhibitor combinations besides ceftazidime/avibactam. A representative ('Mutant 5') of
215 the group of *E. coli* J53-1 TEM-10 mutants with ceftazidime/avibactam MICs of 16+4 mg/L
216 (i.e. the most-resistant mutants selected from ESBL producers, see above) had changes in
217 both *envZ* and upstream of *bla*_{TEM}; MICs of 4+4 mg/L were recorded for Mutants 2, 3 and 4
218 of the same parent, and these only had the lesion upstream of *bla*_{TEM}, not that in *envZ*.

219 Just one of the 20 mutants sequenced – *E. coli* EO 553 Mutant 3 – had a lesion in its
220 ESBL-encoding gene, leading to an Asp182Tyr substitution in CTX-M-15. Compared with its
221 parent, this mutant lost resistance to ceftaroline, cefotaxime and cefepime, and the
222 ceftazidime MIC was reduced two-fold from 32 to 16 mg/L. Synergy was completely lost
223 between ceftazidime and clavulanate or tazobactam whilst the ceftazidime/avibactam MIC
224 rose 8-fold, from 0.25 mg/L to 2 mg/L.

225 One further mutant – Mutant 8 of *K. pneumoniae* Mei 838 – had a lesion in *mdrA*,
226 which encodes penicillin-binding protein 2. This may act as a secondary target for
227 diazabicyclooctanes, though this effect is much weaker for avibactam than for the
228 developmental analogues nacubactam and zidebactam.^{21,22} The significance of this lesion is
229 difficult to judge.

230 Finally, several mutants had changes in proteins with no obvious link to β -lactam or
231 diazabicyclooctane action, including (i) aspartate semialdehyde dehydrogenase (Mutant 7 of
232 *K. pneumoniae* Mei 838), (ii) 4-cytidine 5-diphospho-2-C-methyl D erythritol kinase and
233 putative sulphate transporter (both in Mutant 8 of *K. pneumoniae* Mei 838) and (iii) the DNA-
234 binding protein HLP-II pleiotropic regulator (Mutant 14 of *K. pneumoniae* Mei 254).

235

236 *Mutants from control Enterobacteriaceae lacking ceftazidime resistance*

237 Mutants of ceftazidime-susceptible *E. coli* and *K. pneumoniae* were obtained under selection
238 with ceftazidime/avibactam 1 mg/L, though not ceftazidime/avibactam 4 mg/L (Table 1).
239 MICs of ceftazidime/avibactam 1 mg/L rose from 0.06 to 0.25 mg/L for the parent strains to
240 0.5-4 mg/L for the mutants, whilst those of ceftazidime/avibactam 4 mg/L rose from 0.015-
241 0.25 mg/L to 0.12-2 mg/L. These shifts were accompanied by small, generalised, rises in
242 the MICs of other β -lactams and inhibitor combinations and – often – ciprofloxacin. Given
243 this spectrum, permeability or efflux mechanisms are likely, and these were not pursued
244 further.

245 Similar small but broad MICs shifts were seen for 2/12 characterised mutants of the
246 AmpC-inducible *Enterobacter* strain LN07013 selected with low MIC multiples of
247 ceftazidime/avibactam 1 mg/L. However, 10/12 mutants had antibiograms suggesting
248 AmpC-derepression, with high-level resistance to ceftazidime, cefotaxime, ceftaroline and
249 piperacillin/tazobactam (MICs rising from ≤ 1 mg/L to ≥ 128 mg/L) but not to cefepime or
250 carbapenems. Despite selection with ceftazidime/avibactam, ceftazidime resistance
251 continued to be largely reversed by avibactam, with the MIC falling from ≥ 128 mg/L for the
252 unprotected cephalosporin to 2 mg/L with 1 mg/L avibactam and to 0.5-2 mg/L with 4 mg/L
253 avibactam or to 1-8 mg/L with 100 mg/L cloxacillin.

254 These observations led us to investigate the effect of avibactam on the selectivity of
255 ceftazidime for AmpC-derepressed mutants from AmpC-inducible population of *E. cloacae*
256 and *C. freundii*. We plated five AmpC-inducible, ceftazidime-susceptible strains of each of
257 these species on to agar with ceftazidime alone or with avibactam at 1 or 4 mg/L, always at
258 8 x MIC. Large numbers of colonies were recovered on plates containing ceftazidime alone,
259 indicating mutation frequencies of 10^{-6} to 10^{-7} , as is typical for these species.²³ With
260 avibactam at 1 mg/L, the numbers of colonies that grew were reduced by >75% in all cases
261 and, for 8/10 strains, fell below the detection limit of 10^{-9} ; with avibactam at 4 mg/L, the
262 mutant frequency fell below 10^{-9} for 9/10 strains.

263

264 **Discussion**

265 Mutational resistance to ceftazidime/avibactam has become a concern in respect of isolates
266 with KPC carbapenemases, with reports of resistance emerging during therapy as well as *in*
267 *vitro*.^{9,10} In the light of this concern we explored whether ceftazidime/avibactam could also
268 select resistant mutants from AmpC-derepressed Enterobacteriaceae and ESBL producers;
269 the work followed a similar previous study for ceftaroline/avibactam.⁸ We included common
270 and representative ESBLs, specifically CTX-M-15, as the most prevalent type; CTX-M-1, as
271 common from animal isolates; SHV-2 and -5 as major ESBL mutants of SHV-1 and TEM-10
272 as a ceftazidimase-type ESBL. *E. cloacae* and *C. freundii* were prioritised as the major
273 species where high-level expression AmpC is a resistance issue. Clearly there are further
274 enzymes that merit investigation in the future, notably including Group 9 CTX-M- types
275 (CTX-M-9 or -14), which are globally frequent, and broad-spectrum TEM ESBLs (e.g. CTX-
276 M-3).

277 Unlike for KPC carbapenemases, where we found much higher mutation frequencies
278 to ceftazidime/avibactam than to ceftaroline/avibactam,^{8,9} the present data substantially
279 mirror those obtained with ceftaroline/avibactam, with (i) low mutation frequencies ($<10^{-8}$ at
280 above 2 x MIC), particularly when selection was done with 4 mg/L avibactam, (ii) with mostly
281 small rises in ceftazidime/avibactam MICs, rather than frank resistance and (iii) with most
282 emerging resistance seen among AmpC derepressed strains rather than those with ESBLs.

283 Critically, and also in keeping with the previous ceftaroline/avibactam work, we found
284 that the mutants of AmpC derepressed strains typically had changes within *ampC* whereas
285 mutants derived from ESBL producers largely had mutations affecting efflux, permeability or,
286 putatively, β -lactamase quantity. Nevertheless, there were differences: hot spots for
287 mutations associated here with ceftazidime/avibactam non-susceptibility in AmpC
288 hyperproducers were amino acids 168, 176, 309-314 and 366; those conferring
289 ceftaroline/avibactam resistance were around the Ω loop (amino acids 213-226) or, again,
290 residue 366. Mutations at Arg168 – the site most often affected in this study – had differing

291 effects: Arg168Pro reduced resistance to all cephalosporins and abrogated synergy between
292 avibactam and both ceftazidime and ceftaroline. This behaviour would be compatible with
293 loss of affinity for avibactam, though this was not investigated biochemically. On the other
294 hand, Arg168His (and Gly176Arg/Asp) raised ceftazidime/avibactam MICs but had little
295 effect on cephalosporin resistance overall or on synergy between ceftaroline and avibactam
296 – behaviours that are more compatible with the mutation conferring increased affinity for
297 ceftazidime. The substitutions were all at conserved positions, with a caveat that the
298 background variation among the primary sequences of AmpC β -lactamases from *E. cloacae*
299 was about 40% in one study.²⁴ Asn366 (designated as Asn346 after discounting the 20-
300 amino acid signal peptide) has previously been described as a key residue for avibactam
301 binding.²⁴ The deletions around positions 309–314 observed here are in helix H10, close to
302 the enzyme active site, and at the location where a 6-amino-acid deletion in clinical isolate *E.*
303 *cloacae* CHE was associated with expansion of the enzyme's activity and diminished
304 susceptibility to avibactam combinations.¹⁹

305 The sole mutant of an ESBL enzyme selected here was an Asp185Tyr variant of
306 CTX-M-15. This change was associated with the reduction or loss of resistance to other
307 cephalosporins besides ceftazidime. It seems unlikely that a mutant with such a narrowed
308 resistance spectrum would be successful in evolutionary terms, implying little public health
309 risk. Similar points were made previously in respect of a Lys237Gln mutant selected with
310 ceftaroline/avibactam:⁸ compared with its parent organism this gained ceftaroline/avibactam
311 resistance but lost resistance to other oxyimino-cephalosporins, including ceftazidime. The
312 other mutants selected in the present study from ESBL producers largely had efflux or
313 permeability modifications, or had mutations and amplifications suggesting increased
314 β -lactamase expression - a known general correlate with reduced susceptibility to β -
315 lactam/ β -lactamase inhibitor combinations.⁴

316 Mutants selected from cephalosporin-susceptible AmpC and ESBL-negative *E. coli*
317 and *K. pneumoniae* only showed small increases in ceftazidime/avibactam MICs and were

318 not characterised in detail. Again, however, their antibiograms suggested permeation- or
319 efflux-related changes. By contrast, most of the *E. cloacae* mutants had antibiograms
320 suggesting AmpC derepression. Although avibactam inhibits this enzyme, MICs of
321 ceftazidime/avibactam for AmpC derepressed organisms are not quite so low as for
322 inducible ones and there appears to be a small concentration window (as e.g. with
323 cefepime)²⁵ in which derepressed mutants may be selected, though these remain
324 susceptible to ceftazidime/avibactam at breakpoint in the absence further changes to *ampC*
325 itself

326 Only clinical experience will show whether the present observations have clinical
327 significance. Overall, they suggest that the potential for emerging resistance
328 ceftazidime/avibactam is greater with AmpC producers than ESBL producers. Thus far we
329 are unaware of any reports of emerging resistance during clinical use against AmpC
330 producers. There is a single report¹² of emerging resistance in a pneumonia patient, with a
331 *K. pneumoniae* producing OXA-48 carbapenemase together with CTX-M-14, an ESBL not
332 studied here. The patient was treated first with ceftazidime plus colistin and later, after the
333 ceftazidime MIC had risen from 4 to >256 mg/L, with ceftazidime/avibactam plus
334 meropenem. During this latter phase of therapy the ceftazidime/avibactam MIC rose from 1
335 to 8 mg/L and the CTX-M-14 enzyme acquired Pro170Ser and Thr264Ile substitutions, whilst
336 the OXA-48 carbapenemase remained unaltered. This one case must, however, be set
337 against the clinical trials, where ESBL producers were well represented, without emerging
338 resistance;²⁶⁻²⁸ these support our view that the selection risk with ESBL producers is low
339 whereas that with AmpC-derepressed organisms will only be clarified by clinical experience.

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342

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344 including ceftazidime/avibactam, to Pfizer.

345 **Transparency declaration**

346 **DML:** Advisory Boards or ad-hoc consultancy for Accelerate, Achaogen, Adenium, Allecra,
347 AstraZeneca, Auspherix, Basilea, BioVersys, Centauri, Discuva, Meiji, Nordic, Pfizer,
348 Roche, Shionogi, T.A.Z., Tetrphase, The Medicines Company, VenatoRx, Wockhardt,
349 Zambon, Zealand. Paid lectures – Astellas, AstraZeneca, bioMérieux, Beckmann Coulter,
350 Cardiome, Cepheid, Merck, Pfizer and Nordic. Relevant shareholdings in– Dechra, GSK,
351 Merck, Perkin Elmer, Pfizer amounting to <10% of portfolio value. **WWN:** At the time of the
352 study, WWN was an employee of AstraZeneca and owns shares in that company. He has
353 also been a paid consultant for Pfizer. **All other authors:** none to declare. However,
354 PHE's AMRHAI Reference Unit has received financial support for conference attendance,
355 lectures, research projects or contracted evaluations from numerous sources, including:
356 Accelerate Diagnostics, Achaogen Inc, Allecra Therapeutics, Amplex, AstraZeneca UK Ltd,
357 AusDiagnostics, Basilea Pharmaceutica, Becton Dickinson Diagnostics, bioMérieux, Bio-Rad
358 Laboratories, The BSAC, Cepheid, Check-Points B.V., Cubist Pharmaceuticals, Department
359 of Health, Enigma Diagnostics, Food Standards Agency, GlaxoSmithKline Services Ltd,
360 Helperby Therapeutics, Henry Stewart Talks, IHMA Ltd, Innovate UK, Kalidex
361 Pharmaceuticals, Melinta Therapeutics, Merck Sharpe & Dohme Corp, Meiji Seika Pharma
362 Co., Ltd, Mobidiag, Momentum Biosciences Ltd, Neem Biotech, Nordic Pharma Ltd, Norgine
363 Pharmaceuticals, Rempex Pharmaceuticals Ltd, Roche, Rokitan Ltd, Smith & Nephew UK
364 Ltd, Shionogi & Co. Ltd, Trius Therapeutics, VenatoRx Pharmaceuticals and Wockhardt Ltd.

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452 **Table 1.** Frequencies of single step mutants selected on agar containing MIC multiples of ceftazidime/avibactam

Isolate No.	Species	Mechanism	Parent MIC (mg/L)			Initial count X 10 ⁹	Selection frequency x 10 ⁻⁹ using							
			CAZ	CAZ-AVI 1	CAZ-AVI 4		CAZ-AVI 1 at MIC multiple				CAZ-AVI 4 at MIC multiple			
							2 x	4 X	8 X	16 X	2 x	4 X	8 X	16 X
Mei 633	<i>K. pneumoniae</i>	SHV-2	>256	4	1	1.11	<	<	<	<	1.8	<	<	<
Mei 838	<i>K. pneumoniae</i>	SHV-2	64	4	0.5	1.23	8.9	<	<	<	3.3	<	<	<
Mei 254	<i>K. pneumoniae</i>	SHV-5	256	4	0.5	1.10	57.4	<	<	<	1020.0	16.4	<	<
Mei 679	<i>K. pneumoniae</i>	SHV-5	>256	4	1	1.63	<	<	<	<	<	<	<	<
LN01001	<i>K. pneumoniae</i>	CTX-M-1	64	0.5	0.5	1.46	2.7	<	<	<	<	<	<	<
LN01028	<i>K. pneumoniae</i>	CTX-M-1	256	2	0.5	1.41	2.9	<	<	<	<	<	<	<
EO 553	<i>E. coli</i>	CTX-M-15	16	0.5	0.25	1.27	3.2	<	<	<	0.8	<	<	<
EO 499	<i>E. coli</i>	CTX-M-15	32	0.25	0.25	0.92	1.1	<	<	<	<	<	<	<
NCTC13352	<i>E. coli</i>	TEM-10	>256	2	0.5	1.04	183.9	<	<	<	28.7	<	<	<
J53 TEM-10	<i>E. coli</i>	TEM-10	>256	2	1	1.22	26.3	1.7	<	<	18.9	6.6	<	<
LN03019	<i>E. cloacae</i>	AmpC SDR	64	1	0.5	1.51	26.6	<	<	<	<	<	<	<
LN07047	<i>E. cloacae</i>	AmpC SDR	64	1	0.5	1.04	835.6	7.7	1.0	1.9	21.2	4.8	1.9	<
LN10061	<i>C. freundii</i>	AmpC SDR	256	2	1	1.13	<	<	<	<	<	<	<	<
SE01073	<i>C. freundii</i>	AmpC SDR	128	1	0.5	0.91	25.3	7.7	3.3	<	8.8	2.2	2.2	<
LN01QC09	<i>E. coli</i>	CAZ S	0.125	0.125	0.125	1.10	Cont.	<	<	<	<	<	<	<
LN04QC03	<i>E. coli</i>	CAZ S	0.125	0.125	0.125	1.01	Cont.	<	<	<	13.9	<	<	<
LN07013	<i>E. cloacae</i>	CAZ S	0.5	0.25	0.25	1.60	267.8	14.4	<	<	1.3	<	<	<

LN09063A	<i>E. cloacae</i>	CAZ S	0.25	0.25	0.25	1.63	0.6	<	<	<	<	<	<	<
Mei 60	<i>K. pneumoniae</i>	CAZ S	0.12	0.12	0.12	1.14	3.5	<	<	<	<	<	<	<
Mei 888	<i>K. pneumoniae</i>	CAZ S	0.12	0.12	0.25	1.40	982.8	22.9	<	<	0.7	<	<	<

453

454 <: Below detection limit of c. 0.5 x10⁻⁹

455 Abbreviations: CAZ, ceftazidime, CAZ-AVI1 ceftazidime with 1 mg/L avibactam; CAZ-AVI4 ceftazidime with 4 mg/L avibactam; Cont: contaminated; CAZ-S

456 ceftazidime-susceptible; SDR, stably derepressed

457

458 **Table 2.** Characterisation of mutants selected from AmpC derepressed *E. cloacae* and *C. freundii*

Strain/mutant and selection conditions	AmpC mutation ^a	Porin status	Other	MIC (mg/L)														
				Ceftazidime					Ceftaroline		Others							
				Alone	+Avi, 1 mg/L	+Avi, 4 mg/L	+Clox 100 mg/L	+Clav 4 mg/L	+Taz 4 mg/L	Alone	+Avi, 4 mg/L	CTX	CPM	PTZ	MEM	ERP	GEN	CIP
<i>E. cloacae</i> LN07047		OmpC/F functional		128	1	0.5	2	128	64	>32	0.5	256	0.5	64	0.06	0.5	0.5	0.015
Mutant 4 (CAZ2-AVI1)	Gly176Arg	Unchanged		>256	256	64	>256	>256	>256	>32	2	>256	2	16	0.03	0.25	0.5	0.015
Mutant 7 (CAZ4-AVI1)	Gly176Asp	Unchanged		128	32	16	128	256	128	>32	2	64	0.125	8	0.03	0.06	0.5	0.015
Mutant 15 (CAZ1-AVI4)	Deletion Leu313, Ala314	Unchanged	OppB oligopeptide/nickel transporter Tyr272Asp	256	16	8	8	256	128	16	1	16	16	32	0.03	0.125	0.5	0.015
Mutant 19 (CAZ2-AVI4)	Arg168Pro	Unchanged		16	16	16	16	64	32	4	2	2	2	8	0.03	0.03	0.5	0.015
Mutant 24 (CAZ4-AVI4)	Arg168Pro	Unchanged		16	16	16	16	32	32	2	2	0.5	2	8	0.03	0.03	0.5	0.03
<i>C. freundii</i> SE01073		OmpF inactivated (IS)		128	1	0.5	2	128	64	32	0.125	32	1	64	0.06	0.25	0.5	0.015
Mutant 5 (CAZ2-AVI1)	Arg168Pro	Unchanged		16	32	8	32	32	32	0.5	0.25	2	4	16	0.015	0.015	1	0.008
Mutant 8 (CAZ4-AVI)	Deletion309-312 Ser-Lys-Val-Ala; Leu313Met	Unchanged		256	32	8	128	256	256	8	0.25	8	4	64	0.03	0.03	1	0.008
Mutant 9 (CAZ4-AVI1)	Arg168His	Unchanged		256	32	4	128	256	256	32	0.25	32	16	64	0.03	0.06	1	0.008

Mutant 12 (CAZ8-AVI1)	Asn366Tyr	Unchanged	DnaK Molecular chaperone Leu273Gln	128	64	16	64	128	128	16	2	16	4	64	0.03	0.03	1	0.03
Mutant 22 (CAZ4-AVI4)	Arg168His	Unchanged	Aldehyde dehydrogenase Arg407His	128	64	32	64	256	128	16	2	16	4	64	0.03	0.03	1	0.015

459

460 Parent strains are shown in bold font

461 ^a Numbering here is from the first amino acid of the coding sequence as in figure 3 of ref¹⁹. The first 20 amino acids comprise a signal peptide, cleaved from the mature

462 protein and are discounted in some numberings

Table 3. Characterisation of mutants selected from ESBL producers

Strain and selective conditions	β -lactamase(s)	Porin status ^a	Efflux ^a	Other ^a	MICs (mg/L)														
					Ceftazidime					Ceftaroline		others							
					Alone	+Avi, 1 mg/L	+Avi, 4 mg/L	+Clox 100 mg/L	+Clav 4 mg/L	+Taz 4 mg/L	Alone	+Avi, 4 mg/L	CTX	CPM	PTZ	MEM	ERP	GEN	CIP
<i>E. coli</i> EO 499	CTX-M-15, OXA-1, TEM-1	OmpC OmpF both active			32	0.25	0.125	32	0.25	0.5	>32	0.03	256	16	16	0.03	0.06	1	>16
Mutant 1 CAZ1-AVI1 ^b	<i>bla</i> _{CTX-M-15} up from c. 5 to 40 copies; <i>bla</i> _{TEM} / <i>bla</i> _{OXA} unchanged				>256	2	0.5	>256	2	32	>32	0.06	>256	>64	>256	0.125	0.5	1	>16
<i>E. coli</i> EO 553		OmpC OmpF both active			32	0.5	0.25	32	1	1	>32	0.06	256	32	4	0.03	0.06	0.5	>16
Mutant 2 CAZ1-AVI1			AcrB Phe615Ser		64	2	2	64	2	4	>32	0.125	>256	64	0.5	0.03	0.06	1	>16
Mutant 3 CAZ1-AVI1	CTX-M-15 Asp182Tyr				16	8	2	16	8	16	0.5	0.125	0.5	0.25	4	0.03	0.03	1	>16
Mutant 5 CAZ0.5-AVI4		EnvZ : Val132Gly	AcrB Phe615Ser		64	4	4	64	4	4	>32	0.5	>256	64	0.5	0.06	0.06	0.5	>16
<i>K. pneumoniae</i> Mei 838	SHV-2	OmpK35, OmpK36 both active			64	2	0.25	64	1	32	>32	0.06	64	16	>256	0.03	0.125	0.5	0.03
Mutant 6 CAZ1-AVI4	<i>bla</i> _{SHV-2} up from c. 15 to 70 copies	OmpR: Arg15His			>256	32	8	>256	8	>256	>32	0.5	>256	>64	>256	0.06	1	1	0.06
Mutant 7 CAZ1-AVI4	<i>bla</i> _{SHV-2} up from c. 15 to 45 copies			Aspartate-semialdehyde dehydrogenase	>256	32	2	>256	8	>256	>32	0.5	>256	>64	>256	0.06	1	1	0.03

				Gln247Leu															
Mutant 8 CAZ1-AVI4				4-Cytidine 5'- diphospho- 2-C-methyl- D-erythritol kinase Ala270Gly	>256	32	2	>256	8	>256	>32	0.5	256	>64	>256	0.06	0.5	1	0.06
				Putative sulphate transporter (<i>ychM</i>) Ala99Gly															
Mutant 9 CAZ1-AVI4				Penicillin binding protein 2 <i>mdrA</i> : Asp354Ala	>256	16	8	>256	8	256	>32	0.5	>256	>64	>256	0.06	0.5	1	0.06
<i>K. pneumoniae</i> LNO1028	CTX-M-15	OmpK35, OmpK36 both active			256	1	0.5	256	16	8	>32	0.125	256	64	32	0.125	2	>32	2
Mutant 1 CAZ4-AVI1		EnvZ : Arg397Cys			>256	16	4	>256	4	>256	>32	0.125	>256	64	>256	0.06	0.5	>32	4
Mutant 2 CAZ4-AVI1				Peptido- glycan- associated outer membrane lipo-protein Met1Ileu	>256	16	4	>256	8	>256	>32	0.25	>256	>64	>256	0.06	0.5	>32	4
Mutant 4 CAZ4-AVI1		EnvZ : Ileu412Leu			>256	16	4	>256	4	>256	>32	0.125	>256	>64	>256	0.06	0.5	>32	4
<i>K. pneumoniae</i> Mei 254	SHV-5	OmpK35, OmpK36 both active			256	1	0.25	256	0.5	2	8	0.125	16	2	8	0.03	0.06	0.5	1

Mutant 7 CAZ1-AVI4					>256	32	8	>256	8	>256	>32	0.25	256	>64	>256	0.125	1	0.5	1
Mutant 14 CAZ2-AVI4				DNA-binding protein HLP- II pleiotropic regulator Ser2Arg	>256	16	4	>256	4	>256	>32	0.25	128	32	>256	0.125	1	0.5	1
<i>E. coli</i> NCTC 13352	TEM-10	OmpC, OmpF both active			>256	1	0.5	256	0.5	2	>32	0.06	1	1	4	0.03	0.03	0.5	0.015
Mutant 9 CAZ1-AVI4	<i>bla</i> _{TEM} copy number up from 80 to 200	EnvZ Gln115Arg			>256	16	8	>256	8	>256	>32	0.25	32	>64	>256	0.125	1	1	0.03
Mutant 10 CAZ1-AVI4	<i>bla</i> _{TEM} copy number up from 80 to 160	EnvZ Gln115Arg			>256	16	4	>256	4	128	>32	0.125	16	32	>256	0.06	0.25	1	0.015
<i>E. coli</i> J53 TEM-10	TEM-10	OmpC, OmpF both active			>256	1	0.5	256	0.5	2	32	0.06	1	1	4	0.03	0.03	0.5	0.015
Mutant 2 CAZ4-AVI1	Mutation upstream of <i>bla</i> _{TEM} (-548) regulatory region possibly Affecting expression?				>256	16	4	>256	4	256	>32	0.125	32	>64	>256	0.06	0.25	1	0.015
Mutant 3 CAZ4-AVI1	Mutation upstream of <i>bla</i> _{TEM} (-548) regulatory region possibly Affecting expression?				>256	16	4	>256	4	>256	>32	0.25	32	>64	>256	0.06	0.25	0.5	0.015

Mutant 4 CAZ4-AVI1	Mutation upstream of <i>bla</i> _{TEM} (-548) regulatory region possibly Affecting expression?				>256	16	4	>256	4	>256	>32	0.125	32	>64	>256	0.06	0.25	0.5	0.015
Mutant 5 CAZ4-AVI1	Mutation upstream of <i>bla</i> _{TEM} (-548) regulatory region possibly Affecting expression?	EnvZ : Leu35Gln			>256	32	16	>256	16	>256	>32	0.5	32	>64	>256	0.25	2	1	0.03

464

465 **Abbreviations:** Avi, avibactam; Clav, clavulanate; CP, ciprofloxacin; CTX, cefotaxime, Ert, ertapenem, GENT, gentamicin; MEM, meropenem; PTZ, piperacillin/tazobactam
466 and Taz, tazobactam

467 ^a Numbering is from the first amino acid of the coding sequence, irrespective of whether this is cleaved as a signal peptide

468

469 ^b Selective conditions, CAZ 1 AVI1 means ceftazidime 1 mg/L plus avibactam 1 mg/L

470 Parent strains are shown in bold font