Frequencies and mechanisms of mutational resistance to ceftibuten/avibactam in Enterobacterales

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Running head: Ceftibuten/avibactam mutants

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Abstract

Background. Antibiotic resistance complicates treatment of urinary infections, particularly when these ascend above the bladder, with few oral options remaining. New oral β–lactamase inhibitor combinations present a potential answer, with ceftibuten/avibactam – now undergoing clinical trials – widely active against strains with ESBLs and serine carbapenemases. To inform its development we undertook mutant selection studies. **Methods and Materials**. Single-step mutants were sought from Enterobacterales (n=24) with AmpC, ESBL, OXA-48 and KPC β–lactamases. MICs were determined by CLSI agar dilution. Illumina WGS of selected mutants (n=50) was performed. **Results.** Even at low MIC multiples, mutant frequencies were mostly only *c.* 10^{-8}. β–Lactamase structural mutants were obtained only from KPC and AmpC enzymes. The KPC mutants had Trp105Arg or Ser130Thr substitutions, causing only small MIC shifts; the AmpC mutant had an Asn346Trp replacement, as previously selected with other avibactam combinations. No ESBL mutants were obtained. Rather, from *E. coli,* we predominantly selected mutants with modifications to *ftsI*, encoding penicillin-binding protein (PBP)3. From *Klebsiella pneumoniae* and *Enterobacter cloacae* we predominantly obtained variants with modification of uptake and efflux components or their regulators. *ftsI* mutants lacked cross-resistance to other avibactam combinations; uptake mutants had broader MIC rises. A few putative mutants had other lesion(s) of uncertain significance, or grew as small, stressed, colonies lacking detectable lesions. **Conclusions.** There seems little risk of ESBLs mutating to confer ceftibuten/avibactam resistance, though some risk may apply for KPC and AmpC enzymes. The propensity to select *E. coli ftsI*/PBP3 mutants is notable and was not seen with other avibactam combinations.

Introduction

Urinary tract infections (UTIs) are frequent and mostly caused by *Escherichia* coli. Treatment is complicated by increasing resistance;[1] fluoroquinolones and co-trimoxazole resistances are commonplace whilst cephalosporins have been undermined by ESBLs(1) and co-amoxiclav by OXA-1 penicillinase.[2] Multiresistant strains, notably *E. coli* ST131 have disseminated globally and present an especial challenge.[3] Nitrofurantoin, fosfomycin and mecillinam do remain widely active[4] but are effective only in uncomplicated lower UTIs; patients with ascending infection may require hospitalisation for parenteral antibiotics, or remain hospitalised owing to a lack of oral follow-on. It follows that there is an urgent need for new out-patient therapies.

Possible answers include oral (carba)penems or cephalosporin/β-lactamase inhibitor combinations.[5] Several such combinations are marketed in India, but clinical trials are remarkable by their absence and drug ratios are pharmacodynamically questionable.[6] Nevertheless, the principle is sound and novel oral cephalosporin/inhibitor combinations are being evaluated internationally. Ceftibuten is a favoured partner because it is well tolerated and near-stable to some ESBLs, facilitating protection.[7] One developmental combination is ceftibuten/avibactam-tomilopil. This protects ceftibuten with an orally-absorbed pro-drug of avibactam, diazabicyclooctane already combined with ceftazidime and aztreonam in parenteral formulations. Ceftibuten/avibactam 1+4 mg/L inhibited 97.6% of ESBL producers and the great majority of those with KPC and OXA-48 carbapenemases.[8]

We previously undertook in-vitro mutant selection studies for avibactam combined with the parenteral β-lactams ceftaroline,[9] ceftazidime[10,11] and aztreonam,[12] finding it easy to select AmpC sequence mutants, but difficult to select ESBL or OXA-48 mutants. The mutant risk with KPC carbapenemases varied with the combination, being greatest with ceftazidime/avibactam, where we readily selected Asp179Tyr mutants[10] identical to those later recovered during clinical use.[13] Besides β–lactamase mutants, our studies yielded many variants with changes to porin and efflux components or their regulators. Here, we describe similar selection studies with ceftibuten/avibactam.

Materials and methods

Bacteria

The test strains (Table 1) were clinical *Escherichia coli, Klebsiella pneumoniae* and *Enterobacter cloacae* group submitted to the UK Health Security Agency's Antimicrobial Resistance and Healthcare Associated Infections (AMRHAI) Reference Unit for investigation of resistance. β-Lactamase genes were identified by WGS prior to the present study, or as part of it. Within the three species – which are the most clinically-prevalent Enterobacterales – we represented the most prevalent serine carbapenemases (KPC and OXA-48-like types) and ESBLs (TEM-10, SHV-2 and -12, CTX-M-14 and -15) along with both acquired (CMY and DHA) and chromosomal (*E. cloacae*) AmpC enzymes. Metallo-carbapenemase producers were excluded, being resistant to ceftibuten/avibactam.[8]

Mutant selection

Selection followed the single-step procedure used previously.[9] Briefly, 100 μl volumes of overnight nutrient broth cultures were spread, giving an inoculum of *c.* 10^8 *to* 10^9 cfu, onto Mueller-Hinton agar containing ceftibuten plus avibactam, 1 or 4 mg/L at 2- to 16 x MIC. Colonies were counted after overnight incubation and referenced against those that grew when serial ten-fold dilutions of the original broth cultures were spread on antibiotic-free agar.

MIC determination

For each parent and each avibactam concentration (i.e. 1 or 4 mg/L) we retained up to six presumptive mutants, favouring those obtained at higher ceftibuten/avibactam concentrations. Species identification was confirmed by MALDI-ToF mass spectroscopy (Bruker Biotyper, Bruker Daltonics, Bremen, Germany). MICs were determined by CLSI agar dilution,[14] using antibiotics from suppliers as follows: ceftibuten, avibactam and ceftaroline (Pfizer, Netherlands); aztreonam (Alfa Aesar, Heysham, UK); ceftazidime, cefotaxime, cefepime, meropenem, piperacillin, ciprofloxacin and amikacin (Merck, Gillingham, UK). The non-β-lactam agents served solely as controls.

WGS of selected mutants

Mutants for sequencing were chosen: (i) to represent all parents that yielded variants with significantly (≥4-fold) raised ceftibuten/avibactam MICs and (ii) to represent the greatest diversity of MIC changes as compared with their parent strains. WGS and bioinformatic methods were as described previously[13] except that, additionally, the assembled genomes of parent strains were annotated using Prokka.[15] Sequences of predicted genes, along with potential regulatory regions (500bp upstream of the start codon), were used to perform BlastN searches in the assemblies of the corresponding mutants to confirm gene presence and to identify any alterations, including split alignments resulting from large insertions. Amino-acid numbering for Class A β–lactamases follows Ambler[16] that for Class C enzymes follows Mack *et al.*[17] all other numbering is against entire proteins.

Results

Mutation frequencies

Mutation frequences were low, mostly $<10^{-8}$ (Table 1). Moreover, except with *K. pneumoniae* PF22_03, with a KPC carbapenemase, and three strains with AmpC β-lactamases (*E. coli* PF22_37, *K. pneumoniae* PF_39 and *E. cloacae* PF_44) we only obtained mutants at detectable frequencies (typically $>1x10^{-8}$) when selection was at 2-4 x MIC, not at 8-16 x MIC, even with avibactam at 1 mg/L (Table 1). For AmpC hyperproducers we saw some overgrowth at 2 x MIC with 1 mg/L avibactam; at higher MIC multiple, or with 4 mg/L avibactam, we recovered few or no survivors, indicating that this overgrowth reflected the inhibitor being overwhelmed, not frequent mutation. It should be added that the use of avibactam at 1 as well as 4 mg/L is because, *in vivo*, bacteria are exposed to dynamically changing drug concentrations; a fixed 4 mg/ is the accepted standard for susceptibility testing but has no especial clinical relevance. Past experience, recapitulated here, suggests that mutants are often most readily selected with the lower inhibitor concentration.[9-12]

Mutant characterisation

Fifty putative mutants were sequenced: 13 from organisms with KPC carbapenemases, 10 from those with OXA-48 carbapenemases, eight from those with ESBLs and 19 from those with plasmid (DHA-1 or CMY-2) or chromosomal AmpC; 19 were *E. coli*, 14 *K. pneumoniae* and 17 *E. cloacae.* Only 5/50, four from KPC producers and one from an AmpC-derepressed *E. cloacae* had substitutions in β–lactamase structural genes; two more had mutations in regulatory components for AmpC (below). Far more (n=18), predominantly *E. coli*, had lesions in *ftsI*, encoding penicillin-binding protein (PBP)3, or in porin and efflux components or regulators. A few had other changes, of less certain significance, or no lesions found

Because non-β-lactamase mutants dominated, this Results section is organised by mutation type, not β–lactamase profile. Line listings of mutant versus parent MICs by β–lactamase group are, presented in Supplementary Tables S1-4; these also detail the conditions under which each mutant was selected.

β-Lactamase structural mutants

Four mutants, from three parents, had lesions in bla_{KPC} (Table 2): three of these had Trp105Arg (Ambler numbering: position 104 in the primary sequence) substitutions in KPC-2 or -3; one had a Ser130Thr (position 129 in the primary sequence) replacement in KPC-2.

Trp105Arg substitutions were associated with marked rises in the MICs of ceftibuten/avibactam 1 mg/L, from 0.12-0.5 mg/L to 4-32 mg/L. MICs of ceftibuten/avibactam 4 mg/L rose more modestly, from 0.06 to 0.5 mg/L. MICs of aztreonam/avibactam 4 mg/L rose too, from 0.12-0.25 to 1-2 mg/L whereas those of avibactam combinations with ceftazidime, cefepime and ceftaroline were essentially unaltered. MICs of unprotected ceftibuten rose whereas those cefepime, ceftaroline and meropenem fell. The conservative Ser130Thr change in *K. pneumoniae* PF22_02k was associated with small MIC rises for almost all avibactam combinations including, ceftibuten/avibactam 4 mg/L, where the MIC increased from 0.06 to 0.5 mg/L. Ceftaroline/avibactam 4 mg/L was an outlier,

with the MIC rising substantially, from 0.5 to 64 mg/L. MICs of unprotected cephalosporins, except ceftaroline, were reduced, as were those of aztreonam and meropenem.

The *E. cloacae* AmpC sequence mutant had an Asn346Tyr substitution (Asn366Tyr in the raw sequence, including the signal peptide). Like similar mutants selected previously with other avibactam combinations[9-12] this showed broad reductions in susceptibility, with the ceftibuten/avibactam MIC rising to 32+4 mg/L.

PBP3 (ftsI) mutants

Eighteen mutants had lesions in *ftsI*, encoding PBP3 (Table 3): 14 were *E. coli*, from 11 parent strains. These variously had: (i) deletion of one of more member(s) of the glycine tetrad at residues 480-483 (n=5): (ii) amino-acid substitutions, including Gly363Ser (n=3). Leu350Phe (n=2), Ser355Phe (n=1), Gly363Val (n=1), Ser365Tyr (n=1) or, (iii) insertion of arginine at position 336 (n=1) (Table 3). Among three *K. pneumoniae ftsI* mutants, from two parents, two had a Gly479Arg substitution and one had Val355Gly. The latter change was also present in the sole *E. cloacae ftsI* mutant.

Among the *E. coli ftsI* mutants, the highest ceftibuten/avibactam MICs were from 4+4 to 16+4 mg/L and were associated with Gly363Ser and Leu350Phe. The *E. cloacae* mutant (PF22_44b) with Val355Gly was only inhibited by ceftibuten/avibactam at 32+4 mg/L – the highest MIC for any single-step mutant obtained; the *K. pneumoniae* mutant (PF22_03a) with the same substitution had a much smaller MIC increase, from 0.25 to 1 mg/L.

A few *ftsI* mutants had secondary lesions of possible significance. *E. coli* PF22_51a, with the highest ceftibuten/avibactam MIC (16+4 mg/L) among the *E. coli* mutants, also had a lesion in *baeS*. Changes in this site, of themselves, can increase the MICs of avibactam combinations;[11-12] accordingly, this organism is also included in the total of permeability and efflux mutants below (Table 4 and fig 1b). Both mutants of *K. pneumoniae*, PF22_59 (f and o), with Gly479Arg in PBP3, also shared a Gln97Leu substitution in KdsC, a 3-deoxy-manno-octulosonate-8-phosphatase involved in LPS synthesis.[18] This may affect outer membrane structure and function. Less likely to be of significance:

(i) one *E. coli* mutant (PF22_51a) had a Arg5Cys substitution in the signal peptide of the ActS peptidoglycan amidase activator; (ii) two mutants of *K. pneumoniae* PF22_59 (f and o), had Glu87Gly substitutions in a thermonuclease; (iii) one *E. coli* mutant (PF22_13m) had a Leu177Ile substitution in the AsnA asparaginase and (iv) one *E. coli* mutant (PF22_52ai) had a Ile253Leu change in the KdpD regulator, which modulates virulence and motility.[19]

PBP3 modifications affected ceftibuten/avibactam more than to other avibactam combinations, or unprotected β–lactams (fig 1a). Specifically, geometric mean MIC rises for ceftibuten/avibactam 1 mg/L and 4 mg/L were 8.3- and 12.2- fold, respectively, versus ≤2-fold for other avibactam (4 mg/L) combinations.

Mutants with lesions to porin genes or efflux components

Twelve mutants had lesions in porins or efflux pumps and six had lesions in regulators known or likely to affect drug accumulation (Table 4); 14 were from *K. pneumoniae* or *E. cloacae* parents and only four from *E. coli*. One (PF22_51a; above) also with a substitution is *ftsI*.

Porin mutants included (i) *K. pneumoniae* PF22_26m, with early termination of OmpK36; (ii) *K. pneumoniae* PF22_02fk, with an Ala21Val substitution at the C terminus of the OmpK36 signal peptide, potentially affecting cleavage and, (iii) *E. coli* PF22_13c, with a Tyr337Cys substitution in OmpC. Variants with changes putatively affecting efflux included: (i) two mutants of *E. cloacae* PF22_07 (b and d) with Glu672Lys substitutions in AcrB, one also with a Cys159Phe substitution in PBP4 (DacB); (ii) one *E. coli* (PF22_47a) with a Gly141Asp substitution in AcrB; four mutants of *E. cloacae* PF22_43 (a, b, m, and n) with insertions in a Major Facilitator Superfamily (MFS) transporter and (iv) one *K. pneumoniae* (PF22_59m) with a lesion in a 60-amino-acid peptide encoded adjacent to *acrD*, possibly affecting its expression.

Two *E. cloacae* PF22_43 mutants had further changes, maybe relevant: PF22_43a had an IS3 transposon disrupting the *dacB* gene (PBP4) and PF22_43b had a *prs* lesion, leading to a Val204Met substitution in its ribose-phosphate pyrophosphokinase product. Ceftibuten/avibactam 4 mg/L MICs

for these mutants exceeded those for PF22_43m and PF22_43n, which had the same MFS protein inactivation but lacked these secondary mutations (Table 4). Besides its *acrD*-adjacent lesion, *K. pneumoniae* PF22_59m additionally had a Gln97Leu substitution in KdsC, an enzyme involved in LPS synthesis.(18) This change also was seen in two *ftsI* mutants (f and o) from the same parent (Table 3).

Regulatory mutations modulating or potentially modulating uptake and efflux included: (i) those leading to Gln163Leu or Arg416His substitutions in BaeS (part of a two-component sensor kinase system involved in stress responses) as in *E. coli* PF22_29o and PF22_51a; (ii) those causing Gly130Ala or Pro131Leu substitutions in the CpxA, kinase, as in *E. cloacae* PF22_42i and PF22_42j; and (iii) those causing early termination of the RseA anti-sigma factor regulator protein, as in *K. pneumoniae* mutants PF22_27n and PF22_39a. The significance of these mutations is taken up in the Discussion. The sole mutant in the 'regulatory group' with further relevant changes was PF22_29o, with both *ftsI* and *baeS* lesions (above).

As a group – albeit a heterogenous one – the 18 organisms with changes to uptake and efflux components showed broader MIC increases than *ftsI* mutants (fig 1a and b). Not only were the geometric mean MICs of ceftibuten/avibactam 1 and 4 mg/L raised by 10.6- and 15.4- fold, respectively but additionally, MICs of other avibactam combinations mostly were raised by around four-fold, as were those of meropenem.

Variants with other lesions, or none

Besides the mutants categorised above we sequenced another 10 organisms that grew on the selective plates (Table 5). Two, from a KPC-positive *E. cloacae* parent (PF22_07), had mutations in AmpC regulatory genes: one had an Asp135Ala substitution in AmpR and the other had a deletion at the start of *ampD*. Two further *E. cloacae* variants (PF22_45b and PF22_45l) had lesions in the Prs ribose-phosphate pyrophosphokinase, a protein also modified in the efflux mutant *E. coli* PF22_43b (Table 4). Of more doubtful significance, *K. pneumoniae* PF22_04f had a putative oxidoreductase truncated at amino-acid 152 and *E. cloacae* PF22_44k had a frameshift mutation affecting the Pgm

phosphoglucomutase, with early truncation. No lesions were found in four organisms; all gave small colonies on selective agar, suggesting stressed growth rather than genetic change.

Discussion

This paper follows earlier studies with ceftaroline/avibactam, ceftazidime/avibactam and aztreonam/avibactam.[9-12] The parent strains were chosen to represent prevalent β–lactamases, reflecting concern that altered β–lactamase structure can reduce inhibitor binding or increase affinity for a partner β–lactam, protecting the enzyme from inhibition.[20] In reality, just 5/50 studied variants had β–lactamase sequence changes: such changes were seen only in KPC and AmpC enzymes, not in ESBLs nor OXA-48. Far more mutants had changes affecting PBP3 or uptake components.

The KPC mutants variously had Trp105Arg (n=3) or Ser130Thr (n=1) substitutions. A Trp105Arg mutant was previously selected also with aztreonam/avibactam[12] and this lesion reduced susceptibility to both ceftibuten/avibactam and aztreonam/avibactam, with little effect on ceftazidime/avibactam and ceftaroline/avibactam. The Ser130Thr modification is novel. Whilst conservative, it affects the first residue in a Ser-Asp-Asn triad that is hugely conserved in Class A β-lactamases and has a direct role in catalysis.[21,22] We did not see KPC mutants with substitutions around the omega loop (residues Arg164 to Asp179) whereas these – particularly with Asp179Tyr – predominate among clinical mutants selected with ceftazidime /avibactam. [13,20] In the case of AmpC, the sole sequence change selected was Asn346Tyr, a substitution previously selected with aztreonam/avibactam[12] and ceftazidime/avibactam, causing broad rises in the MICS of avibactam combinations[11] Similar Asn346His/Ile substitutions were selected with ceftaroline/avibactam.[9] A full understanding of the effects of these mutations must await biochemical studies on the enzymes: in principle the MICs of an inhibitor combination can increase if either or both (a) enzyme affinity for the inhibitor reduces or (ii) affinity for the substrate increases (lower K_m) enabling it to protect the enzyme from the inhibitor. The latter explanation is likely to be dominant in cases – e.g. Trp105Arg substitutions in KPC – where the MIC for unprotected ceftibuten also rose.

The lack of ESBL mutants reinforces experience for other avibactam combinations, where few such mutants were obtained and where these substantially lost activity against other cephalosporins besides the selective agent.[9,11] Likewise, no selection of OXA-48 mutants was seen. This is unsurprising because, besides being inhibited by avibactam (as demonstrated by meropenem/ avibactam synergy[23]) OXA-48-like enzymes lack cephalosporinase activity.[24,25] A ceftibuten/avibactam-compromising mutation therefore would need both to create cephalosporinase activity *and* abrogate inhibition by avibactam. This combination seems unlikely to emerge in one step.

What was surprising were the numerous *ftsI* (PBP3) mutants selected, particularly from *E. coli*. In previous studies we only encountered one such mutant, an *E. cloacae* variant with Ala498Thr, selected with aztreonam/avibactam.[12] In the present study, 14/19 *E. coli* mutants had PBP3 modifications, comprising: (i) deletion of one or more members of the glycine tetrad at positions 480-483, or (ii) substitutions around positions 350 to 365, including Gly363Ser/Val, Leu350Phe, Ser355Phe or Ser365Tyr or, (iii) insertion of arginine at position 336. All these residues are close to the transpeptidase active serine (Ser307)[26] or to other active-site residues (Lys310, Ser359, Asn361, Lys494, Thr495, Gly496 and Thr497) or, in the case of arginine 336, are close to a site (333) where four-amino-acid insertions (Tyr-Arg-Ile-Asn/Lys) confer broad reductions in susceptibility to PBP3-targeted agents.[27] There is no modelling available for PBP3 from *K. pneumoniae* and *E. cloacae*, but their enzymes resemble the *E. coli* protein in primary sequence, with Ser307 Lys310, Ser359, Asn361, Lys494, Thr495, Gly496 and Thr497 all conserved. The substitutions found here, at positions 355 and 479, lie close to these critical residues.

The PBP3 modifications had little effect on other avibactam combinations besides ceftibuten/avibactam, likely explaining why we did not select them previously. The present study was not designed to compare the relative frequency of different mutant types among different species; nevertheless, the preponderance of *E. coli* mutants is striking and is in keeping with *E. coli* being the sole species where naturally-occurring PBP3 insertions have been seen as a source of resistance.

The other major groups of mutations seen were those affecting efflux or permeability. These were present in 14/31 *K. pneumoniae* and *E. cloacae* variants studied, compared with 4/19 *E. coli*. Mutations affecting porins included a Tyr337Cys substitution in *E. coli* OmpC, an early stop affecting *K. pneumoniae* OmpK36 and, also, in *K. pneumoniae*, a substitution at the end of the OmpK36 signal peptide, possibly impeding cleavage. Other mutations altered efflux components, notably including: (i) substitutions in AcrB and (ii) insertions or other changes leading to truncation of TolC and an MFS family protein. Although the latter changes would be expected to impede efflux, not to promote it, feedback may increase expression of other pumps. Similar mutations, affecting porins and efflux, were recorded in our studies on other avibactam combinations,[11,12] and porin loss is occasionally selected during therapy with newer β–lactamase inhibitor combinations.[28]

Mutations affecting regulators included: (i) those in *cpxA*, which modulates the *mar* multidrug resistance system, increasing AcrAB-TolC-mediated efflux and supressing OmpF expression;[29] (ii) those in *baeS*, which regulates efflux by MdtABC and MdtEF,[30] and (iii) those in the RseA anti-sigma E factor, which is associated with down-regulation of porins.[31] Whilst such mutations, particularly in *baeRS*, have regularly been encountered in our selection studies with avibactam combinations, they have not become a clinical issue and may be counter-selected *in vivo*. Curiously, we did not encounter mutants with changes in the EnvZ regulator, though we saw these extensively with ceftazidime/avibactam and aztreonam/avibactam.[11,12]

A few other mutations of possible significance were seen. First, *E. cloacae* PF22_07o, derived from a parent with a KPC carbapenemase, had a deletion in *ampD*. This would be predicted to derepress AmpC, and the combined activity of copious AmpC plus KPC enzymes may account for the small MIC shifts seen, with the ceftibuten/avibactam MIC rising from 0.5 to 4 mg/L. Another mutant (PF22_07l) from the same parent had a lesion in AmpR, which also regulates AmpC . Although this is intuitively a 'relevant mutation type,' the MIC rise for ceftibuten/avibactam 4 mg/L rose from 0.5 to 16 mg/L, and this seems an anomalously large shift. Secondly, two *E. cloacae* mutants, PF22_07c and PF22-43a, both also with efflux lesions, had lesions in DacB/PBP4 leading, in the case of PF22-43a, to

early termination. Although PBP4 is a non-essential peptidoglycan carboxypeptidase, modifications has previously been associated with raised β–lactam MICs.[32] Thirdly, several *E. cloacae* mutants had lesions in *prs*, encoding a ribose-phosphate pyro-phosphokinase (Tables 4 and 5). Although no link to β–lactam resistance is obvious, Prs is involved in core metabolism,[33] and disruption may cause metabolic stresses and contingent resistance shifts. Lastly, two isolates – both with PBP3 modifications – additionally had Gln97Leu substitutions in KdsC, involved in synthesis of an LPS-specific sugar.(18) The altered LPS may affect drug uptake.

The study has several limitations. These notably include: (i) resource constraints meant that only a limited number of organisms could be studied, and we cannot exclude the possibility that emerging resistance will involve some presently uncommon β-lactamase; (ii) selection studies were not done with multiple replicates, limiting the precision of the mutation frequencies shown in Table 1; (ii) we used parent strains likely to count as susceptible to ceftibuten/avibactam and did not include e.g. those already resistant to ertapenem through combinations of ESBL or AmpC and porin loss, although these may be 'primed' to develop resistance and (iv) in-vitro studies of this type cannot predict the fitness of the mutant selected: this is particularly pertinent to the novel *E. coli ftsI* mutants, which may or may not become a clinical issue.

Overall, these findings are encouraging. Over three-quarters of UTIs involve *E. coli* and the major constraint on cephalosporin utility is the spread of ESBLs.[1,4] Ceftibuten/avibactam is consistently active against ESBL-producing *E. coli*[8] and, as the present study shows, these do not readily mutate to evade the combination's activity. AmpC and KPC enzymes do have more potential to mutate so as to confer resistance but are less prevalent in *E. coli*. The potential for resistance via modification of *E. coli* PBP3 is noteworthy and seems to be a greater hazard with ceftibuten/avibactam than with other avibactam combinations. Nonetheless, mutation frequencies were low in absolute terms (mostly $<10^{-8}$) and the level of 'resistance' was low, with MICs of ceftibuten/avibactam 4 mg/L remaining $\leq$1 mg/L in 10/14 cases and exceeding 8 mg/L for only one.

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Table 1. Mutation frequencies observed for the test strains, with different ceftibuten/avibactam concentrations

			Baseline ceftibuten MICs (mg/L)			Observed mutation frequency with:							
			with avibactam at:			With ceftibuten + avibactam, 1 mg/L				With ceftibuten + avibactam, 4 mg/L			
	Primary β–lactamase	Other β–lactamases	0	1 mg/L	4 mg/L	2xMIC	4xMIC	8xMIC	16xMIC	2xMIC	4xMIC	8xMIC	16xMIC
K. pneumoniae PF22_02	KPC-2	OXA-9[a]; SHV-2; TEM-1	8	0.12	0.06	$1.0x10^{6}$	$<4.7x10^{-9}$	$<4.7x10^{-9}$	$<4.8x10^{-9}$	$4.8x10^{-8}$	$<4.7x10^{-9}$	$9.4x10^{-9}$	$<4.7x10^{-9}$
K. pneumoniae PF22_03	KPC-3	SHV-11; OXA-9[a], TEM-1	16	0.5	0.12	$<6.5x10^{9}$	$<6.5x10^{-9}$	$1.3x10^{-8}$	$<6.5x10^{-9}$	$1.6x10^{-7}$	$<6.5x10^{-9}$	$6.5x10^{-9}$	$<6.5x10^{-9}$
K. pneumoniae PF22_04	KPC-3	SHV11;	>128	4	2	$5.2x10^{-7}$	$<7.7x10^{-9}$	$<7.7x10^{-9}$	$<7.7x10^{-9}$	$<7.7x10^{-9}$	$<7.7x10^{-9}$	$<7.7x10^{-9}$	$<7.7x10^{-9}$
E. cloacae PF22_07	KPC-4	AmpC; OXA-129	>128	8	0.5	$4.7x10^{-8}$	<2.6E+09	<2.6E+09	<2.6E+09	$3.5x10^{-8}$	$2.0x10^{-8}$	<2.6E+09	<2.6E+09
E. coli PF22_09	KPC-2	TEM-1	32	0.12	0.06	$6.5x10^{-8}$	$<3.4x10^{-9}$	$<3.4x10^{-9}$	$<3.4x10^{-9}$	$1.7x10^{-8}$	$<3.4x10^{-9}$	$<3.4x10^{-9}$	$<3.4x10^{-9}$
E. coli PF22_10	KPC-2	OXA-1; OXA-9[a]; TEM-1	8	0.12	0.06	$1.7x10^{-8}$	$6.7x10^{-9}$	$<3.4x10^{-9}$	$<3.4x10^{-9}$	$<3.4x10^{-9}$	$3.4x10^{-9}$	$<3.4x10^{-9}$	$<3.4x10^{-9}$
E. coli PF22_12	CTX-M-15		16	0.25	0.03	$<1.2x10^{-9}$	$<1.2x10^{-9}$	$<1.2x10^{-9}$	$<1.2x10^{-9}$	$1.6x10^{-8}$	$<1.2x10^{-9}$	$<1.2x10^{-9}$	$<1.2x10^{-9}$
E. coli PF22_13	CTX-M-15	OXA-1	16	0.25	0.06	$9.3x10^{-9}$	$<2.7x10^{-9}$	$<2.7x10^{-9}$	$<2.7x10^{-9}$	$4.9x10^{-6}$	$<2.7x10^{-9}$	$<2.7x10^{-9}$	$<2.7x10^{-9}$
E. coli PF22_14	CTX-M-15	OXA-1; TEM-1	16	0.12	0.12	$3.5x10^{-8}$	$<8.7x10^{-9}$	$<8.7x10^{-9}$	$<8.7x10^{-9}$	$<8.7x10^{-9}$	$<8.7x10^{-9}$	$<8.7x10^{-9}$	$<8.7x10^{-9}$
E. coli PF22_20	CTX-M-14	TEM-1	4	0.5	0.25	$<5.3x10^{-9}$	$<5.3x10^{-9}$	$<5.3x10^{-9}$	$<5.3x10^{-9}$	$5.3x10^{-9}$	$<5.3x10^{-9}$	$<5.3x10^{-9}$	$<5.3x10^{-9}$
K. pneumoniae PF22_26	SHV-2	-	8	0.12	0.06	$2.1x10^{-8}$	$<3.5x10^{-9}$	$<3.5x10^{-9}$	$<3.5x10^{-9}$	$4.5x10^{-8}$	$<3.5x10^{-9}$	$<3.5x10^{-9}$	$<3.5x10^{-9}$
K. pneumoniae PF22_27	SHV-12	-	4	0.03	0.03	$8.0x10^{-8}$	$<4.2x10^{-9}$	$<4.2x10^{-9}$	$<4.2x10^{-9}$	$<4.2x10^{-9}$	$<4.2x10^{-9}$	$<4.2x10^{-9}$	$<4.2x10^{-9}$
E. coli PF22_29	TEM-10	-	1	0.12	0.06	$1.6x10^{-8}$	$5.3x10^{-9}$	$<5.3x10^{-9}$	$<5.3x10^{-9}$	$5.8x10^{-8}$	$<5.3x10^{-9}$	$<5.3x10^{-9}$	$<5.3x10^{-9}$
E. coli PF22_32	CMY-2	TEM-1	>128	1	0.25	Over-grown	Over-grown	$<5.6x10^{-9}$	$<5.6x10^{-9}$	$3.2x10^{-8}$	$1.8x10^{-8}$	$<5.6x10^{-9}$	$<5.6x10^{-9}$

E. cloacae PF22_42	AmpC	-	>128	8	2	Over-grown	$2.1x10^{-8}$	$<4.7x10^{-9}$	$<4.7x10^{-9}$	Over-grown	$2.1x10^{-8}$	$4.3x10^{-9}$	$<4.3x10^{-9}$
E. cloacae PF22_43	AmpC	-	>128	8	1	$2.0x10^{-6}$	$3.0x10^{-7}$	$<5.0\ x10^{-9}$	$<5.0\ x10^{-9}$	$3.0x10^{-6}$	$5.6x10^{-8}$	<5.0E-9	<5.0E-9
E. cloacae PF22_44	AmpC	-	>128	4	2	Over-grown	Over-grown	Over-grown	Over-grown	Over-grown	$1.1x10^{-8}$	$<2.7x10^{-9}$	$<2.7x10^{-9}$
E. cloacae PF22_45	AmpC	-	>128	8	1	Over-grown	Over-grown	$1.2x10^{-9}$	$<1.1x10^{-9}$	$8.8x10^{-8}$	$1.8x10^{-8}$	$<1.1x10^{-9}$	$<1.1x10^{-9}$
E. coli PF22_37	DHA-1	-	>128	2	0.03	Over-grown	Over-grown	$<6.5x10^{-9}$	$8.2x10^{-8}$	$1.8x10^{-6}$	Over-grown	$4.6x10^{-7}$	$6.5x10^{-9}$
K. pneumoniae PF22_39	DHA-1	SHV-1	>128	4	0.12	Over-grown	Over-grown	Over-grown	Over-grown	$3.7x10^{-7}$	$9.2x10^{-7}$	$5.5x10^{-7}$	$5.9x10^{-8}$
E. coli PF22_47	OXA-48	-	4	0.5	$\leq$0.015	$1.9x10^{-7}$	$3.1x10^{-9}$	$<3.2x10^{-9}$	$<3.2x10^{-9}$	$3.0x10^{-7}$	$6.7x10^{-9}$	$<6.7x10^{-9}$	$<6.7x10^{-9}$
E. coli PF22_51	OXA-48	TEM-1	4	0.5	0.5	$1.5x10^{-8}$	$<4.0x10^{-9}$	$<4.0x10^{-9}$	$<4.0x10^{-9}$	$<4.0x10^{-9}$	$<4.0x10^{-9}$	$<4.0x10^{-9}$	$<4.0x10^{-9}$
E. coli PF22_52	OXA-48	CTX-M-15; TEM-190	64	1	0.5	$<6.8x10^{-9}$	$<6.8x10^{-9}$	$<6.8x10^{-9}$	$<6.8x10^{-9}$	$1.5x10^{-7}$	$<6.8x10^{-9}$	$<6.8x10^{-9}$	$<6.8x10^{-9}$
E. coli PF22_53	OXA-48	CTX-M-24; TEM-1	16	0.5	0.25	$6.1x10^{-9}$	$<6.1x10^{-9}$	$<6.1x10^{-9}$	$<6.1x10^{-9}$	$<6.1x10^{-9}$	$<6.1x10^{-9}$	$<6.1x10^{-9}$	$<6.1x10^{-9}$
K. pneumoniae PF22_59	OXA-48	CTX-M-15; TEM-1; OXA-1; SHV-11;	64	0.12	0.12	$7.1x10^{-8}$	$1.3x10^{-8}$	$<6.5x10^{-9}$	$<6.5x10^{-9}$	$3.9x10^{-8}$	$<6.5x10^{-9}$	$<6.5x10^{-9}$	$<6.5x10^{-9}$
K. pneumoniae PF22_60	OXA-48	CTX-M-15; OXA-1; SHV-1	>128	0.5	0.25	$6.8x10^{-9}$	$<6.8x10^{-9}$	$<6.8x10^{-9}$	$<6.8x10^{-9}$	$<6.8x10^{-9}$	$<6.8x10^{-9}$	$<6.8x10^{-9}$	$<6.8x10^{-9}$

[a] Truncated at amino-acid 112 and almost certainly non-functional.

Table 2. Mutants with structural lesions in β–lactamase proteins

		MIC (mg/L)															
Organism and β–lactamase type (parent strains in bold font)	Mutations, based on Ambler[16] and Mack[17] numbering	CTB	CTB + AVI1	CTB + AVI4	AZT	AZT + AVI4	CAZ	CAZ + AVI4	FEP	FEP + AVI4	CPT	CPT + AVI4	CTX	CTX + AVI4	PTZ	MEM	CIP	AMK
***K. pneumoniae* PF22_02 (KPC-2)**		**8**	**0.12**	**0.06**	**256**	**0.25**	**64**	**1**	**64**	**0.06**	**128**	**0.5**	**128**	**0.12**	**256**	**64**	**0.06**	**2**
Mutant PF22_02k	KPC-2 Ser130Thr	2	1	0.5	2	0.25	16	4	2	0.5	256	64	4	0.5	64	4	0.06	1
***K. pneumoniae* PF22_03 (KPC-3)**		**32**	**0.5**	**0.25**	**256**	**0.25**	**256**	**1**	**256**	**0.25**	**256**	**0.5**	**256**	**0.5**	**256**	**256**	**128**	**64**
Mutant PF22_03l	KPC-3 Trp105Arg	256	32	0.5	256	2	256	0.5	16	0.12	32	0.12	256	0.5	256	16	128	64
Mutant PF22_03m	KPC-3 Trp105Arg	256	16	0.5	256	2	256	1	16	0.25	64	0.5	256	0.5	256	16	128	64
***E. coli* PF22_10 (KPC-2)**		**8**	**0.12**	**0.06**	**256**	**0.12**	**32**	**0.25**	**64**	**0.06**	**64**	**0.25**	**256**	**0.06**	**256**	**16**	**256**	**4**
Mutant PF22_10b	KPC-2 Trp105Arg	256	4	0.5	256	1	32	0.25	8	0.03	32	0.12	128	0.06	256	4	256	4
***E. cloacae* PF22_44**		**256**	**4**	**0.5**	**32**	**0.5**	**64**	**0.5**	**1**	**0.06**	**32**	**0.25**	**128**	**0.5**	**64**	**0.06**	**0.03**	**1**
Mutant PF22_44i	AmpC Asn346Tyr	64	64	32	16	8	128	64	1	0.5	64	16	32	16	32	0.03	0.03	2

Abbreviations: AMK, amikacin; AVI1, avibactam at 1 mg/L; AVI4, avibactam at 4 mg/L; AZT, aztreonam; CAZ, ceftazidime; CTX, cefotaxime; CPT, ceftaroline; CIP, ciprofloxacin; FEP, cefepime; MEM, meropenem and PTZ, piperacillin/tazobactam, 4 mg/L.

Table 3. MIC changes for ceftibuten and ceftibuten/avibactam among 18 *ftsI* mutants

			MIC (mg/L)					
			Parent			Mutant		
Mutant	**FtsI change**	**Other changes**	**Ceftibuten**	**Ceftibuten/ AVI, 1**	**Ceftibuten/ AVI, 4**	**Ceftibuten**	**Ceftibuten/ AVI, 1**	**Ceftibuten/ AVI, 4**
E. coli								
PF22_14a (CTX-M-15)	Deletion of Gly 480 and 481		8	0.12	0.06	32	0.5	0.5
PF22_32b (CMY-2)	Deletion of two glycines from the Gly tetrad at 480-483		128	1	0.12	256	4	1
PF22_09a (KPC-2)	Deletion of one glycine from the Gly tetrad at 480-483		256	0.5	0.06	128	1	0.5
PF22_32c (CMY-2)	Deletion of one glycine from the Gly tetrad at 480-483		128	1	0.12	256	4	0.5
PF22_32m (CMY-2)	Deletion of one glycine from the Gly tetrad at 480-483		128	1	0.12	256	4	0.5
PF22_20b (CTX-M-14)	Gly363Ser		4	0.25	0.12	16	2	1
PF22_51a (OXA-48)	Gly363Ser	ActS, Arg5Cys (in signal peptide) BaeS Arg416His	4	1	0.5	16	16	16
PF22_53a (OXA-48)	Gly363Ser		32	0.5	0.25	256	8	4
PF22_47o (OXA-48)	Gly363Val		2	0.5	0.3	16	8	4
PF22_52ai (OXA-48)	Leu350Phe	KdpD, Ile253Leu	64	1	0.5	256	8	8
PF22_12d (CTX-M-15)	Leu350 Phe		8	0.12	0.03	128	1	0.5
PF22_13m (CTX-M-15)	Ser355Phe	AnsA, Leu177Ile	64	0.25	0.06	128	2	0.5
PF22_47p (OXA-48)	Ser365Tyr		2	0.5	0.3	16	8	1
PF22_10a (KPC-2)	Arg inserted at 336		8	0.125	0.06	128	2	1
K. pneumoniae								
PF22_59f (OXA-48)	Gly479Arg	Thermonuclease family protein, Glu87Gly KdsC, Gln97Leu	64	0.125	0.125	256	2	1
PF22_59o (OXA-48)	Gly479Arg	Thermonuclease family protein Glu87Gly;	64	0.125	0.125	256	1	0.5

		KdsC Gln97Leu						
PF22_03a (KPC-3)	Val355Gly		32	0.5	0.25	256	4	1
E. cloacae								
PF22_44b (AmpC)	Val355Gly		256	4	0.5	128	64	32

Abbreviations: AMK, amikacin; AVI1, avibactam at 1 mg/L; AVI4, avibactam at 4 mg/L; AZT, aztreonam; CAZ, ceftazidime; CTX, cefotaxime; CPT, ceftaroline; CIP, ciprofloxacin; FEP, cefepime; MEM, meropenem and PZ, piperacillin/tazobactam, 4 mg/L

FtsI: PBP3
ActS: peptidoglycan amidase activator
BaeS: part of two-component sensor kinase system
KdpD: regulator of virulence and motility
KdsC: 3-deoxy-manno-octulosonate-8-phosphatase
AnsA: asparaginase

Table 4. MIC changes for ceftibuten and ceftibuten/avibactam among 14 mutants with changes in porins, efflux components or their proven or putative regulators

				MIC (mg/L)					
				Parent			Mutant		
Mutant	**Putative porin/efflux/ regulatory change**	**Category**	**Other changes**	**Ceftibuten**	**Ceftibuten/ AVI, 1**	**Ceftibuten /AVI, 4**	**Ceftibuten**	**Ceftibuten/ AVI, 1**	**Ceftibuten/ AVI, 4**
E. coli									
PF22_ 13c (CTX-M-15)	OmpC Tyr337Cys	Porin change	AnsA Leu177Ile	64	0.25	0.06	64	1	0.5
PF22_47a (OXA-48)	AcrB Gly141Asp	Efflux		2	0.5	0.03	4	1	0.5
PF22_29o (TEM-10)	BaeS Gln163Leu	Regulatory		2	0.12	0.06	4	0.5	1
PF22_51a (OXA-48)	BaeS Arg416His	Regulatory	PBP3 Gly363Ser ActS, Arg5Cys (in signal peptide)	4	1	0.5	16	16	16
K. pneumoniae									
PF22_02f (KPC-2)	OmpK36 Ala21Val (in signal peptide)	Porin change		8	0.12	0.06	32	2	0.5
PF22_26m (SHV-2)	OmpC Gln170 early stop	Porin change		8	0.06	0.06	128	1	0.5
PF22_39f (DHA-1)	TolC 181 early Stop	Efflux		256	4	0.03	256	8	2
PF22_59m (OXA-48)	60-amino-acid hypothetical protein adjacent to multidrug efflux permease AcrD: Cys53Tyr	Efflux or wall architecture	KdsC Gln97Leu Thermonuclease family protein Glu87Gly	64	0.12	0.12	64	1	0.5
PF22_27n (SHV-12)	RseA 95C nucleotide deletion, causing frame shift and codon 59 early stop.	Regulatory		4	0.06	0.06	32	0.5	0.12
PF22_39a (DHA-1)	RseA 33 early stop	Regulatory		256	4	0.03	256	256	1

E. cloacae									
PF22_07b (KPC-4)	AcrB Glu672Lys	Efflux	DacB Cys159Phe	256	2	0.5	256	16	8
PF22_07d (KPC-4)	AcrB Glu672Lys	Efflux		256	2	0.5	256	64	16
PF22_42i (AmpC)	CpxA Pro131Leu	Regulatory		256	4	1	256	256	16
PF22_42j (AmpC)	CpxA Gly130Ala	Regulatory		256	4	1	256	64	16
PF22_43a (AmpC)	Insertion at G429 of MFS transporter		IS3 transposon insertion in DacB	256	2	0.5	256	32	8
PF22_43b (AmpC)	Insertion at G429 of MFS transporter		Prs Val204Met	256	2	0.5	256	32	16
PF22_43m (AmpC)	Insertion at G429 of MFS transporter			256	2	0.5	256	64	0.5
PF22_43n (AmpC)	Insertion at G428 of MFS transporter			256	2	0.5	256	64	0.5

Abbreviations: AMK, amikacin; AVI1, avibactam at 1 mg/L; AVI4, avibactam at 4 mg/L; AZT, aztreonam; CAZ, ceftazidime; CTX, cefotaxime; CPT, ceftaroline; CIP, ciprofloxacin; FEP, cefepime; MEM, meropenem and PZ, piperacillin/tazobactam, 4 mg/L

ActS: peptidoglycan amidase activator
AnsA: asparaginase
BaeS: sensor kinase
CpxA: histidine kinase regulating OmpF and AcrD
DacB: PBP4
KdsC: 3-deoxy-manno-octulosonate-8-phosphatase
MFS: Major Facilitator Superfamily
Prs: ribose-phosphate pyrophosphokinase
RseA: anti-sigma-E factor

Table 5. MIC changes for ceftibuten and ceftibuten/avibactam among nine mutants with other changes of uncertain relevance, or none found

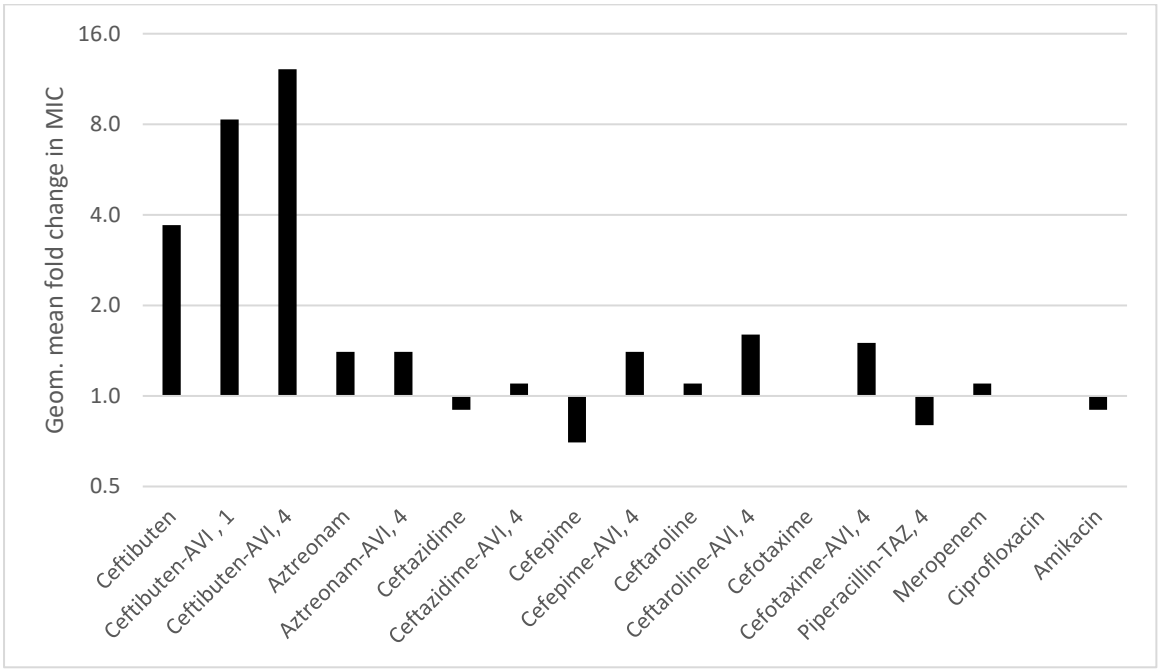
		MIC (mg/L)					
		Parent			Mutant		
Mutant	**Changes found**	**Ceftibuten**	**Ceftibuten/ AVI, 1**	**Ceftibuten/ AVI, 4**	**Ceftibuten**	**Ceftibuten/ AVI, 1**	**Ceftibuten/ AVI, 4**
E. coli							
PF22_37n	None found[a]	256	2	0.03	256	2	0.25
K. pneumoniae							
PF22_04f	Putative oxidoreductase truncated at amino-acid 152	256	4	1	256	64	4
PF22_60g	None found[a]	128	0.5	0.25	256	2	1
E. cloacae							
PF22_07l	AmpR D135A	256	2	0.5	256	64	16
PF22_07o	AmpD 29 base-deletion at start	256	2	0.5	256	64	4
PF22_42a	None found[a]	256	4	1	256	32	8
PF22_42b	None found[a]	256	4	1	256	64	16
PF22_44k	Phosphoglucomutase gene deletion leading to frame shift from G379 and truncation	256	4	0.5	256	256	8
PF22_45b	Prs Val113Phe	256	8	0.5	256	32	8
PF22_45l	Prs Stop316C	256	8	0.5	256	128	8

Abbreviations: AMK, amikacin; AVI1, avibactam at 1 mg/L; AVI4, avibactam at 4 mg/L; AZT, aztreonam; CAZ, ceftazidime; CTX, cefotaxime; CPT, ceftaroline; CIP, ciprofloxacin; FEP, cefepime; MEM, meropenem and PZ, piperacillin/tazobactam, 4 mg/L

[a]Observed as small colonies in the presence of antibiotics, suggesting stressed growth

Figure 1. Fold MIC shifts for comparator antibiotics among (panel a), 18 mutants with lesions in FtsI/PBP3, as included in Table 3 and (panel b), 14 mutants with lesions potentially affecting antibiotic uptake or efflux, as included in Table 4. (NB '1-fold change' means 'no change'; 2-fold is doubling; 0.5-fold is halving).

a)



b)