Selection and characterisation of mutational resistance to aztreonam/avibactam in β-lactamase-producing Enterobacterales

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Running head: Aztreonam/avibactam mutants

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Synopsis

Introduction. Aztreonam/avibactam is being developed for its broad activity against carbapenemase-producing Enterobacterales, including those with metallo-β-lactamases (MBLs). Its potential to select resistance in target pathogens was explored. Findings are compared with previous data for ceftazidime/avibactam and ceftaroline/avibactam. **Methods.** Single-step mutants were sought from 52 Enterobacterales with AmpC, ESBL, KPC, MBL and OXA-48-like enzymes. Mutation frequencies were calculated. MICs were determined by CLSI agar dilution. Genomes were sequenced using Illumina methodology. **Results**. Irrespective of β-lactamase type and of whether avibactam was used at 1 or 4 mg/L, mutants could rarely be obtained at >4x the starting MIC, and most MIC rises were correspondingly small. Putative resistance (MIC >8+4 mg/L) associated with changes to β-lactamases was seen only for mutants of AmpC, where it was associated with Asn346Tyr and Tyr150Cys substitutions. Asn346Tyr led to broad resistance to avibactam combinations; Tyr150Cys significantly affected only aztreonam/avibactam. MIC rises up to 4+4 mg/L were seen for producers of mutant KPC-2 or -3 enzymes, and were associated with Trp105Arg, Ser106Pro and Ser109Pro substitutions, which all reduced the MICs of other β-lactams. For producers of other β-lactamase types, we largely found mutants with lesions in *baeRS* or *envZ*, putatively affecting drug accumulation. Single mutants had lesions in *ampD*, affecting AmpC expression or *ftsI*, encoding PBP3. **Conclusion**. The risk of mutational resistance to aztreonam/avibactam appears smaller than for ceftazidime/avibactam where Asp179Tyr arises readily in KPC enzymes, conferring frank resistance. Asn346 substitutions in AmpC enzymes may remain a risk, having been repeatedly selected with multiple avibactam combinations *in vitro*.

Introduction

Combinations of β-lactams with diazabicyclooctane (DBO) β-lactamase inhibitors provide one answer to the challenge of multi-resistant Gram-negative bacteria.[1] Ceftazidime/avibactam, the first such combination, has been used successfully against Enterobacterales with KPC [2,3] and OXA-48[4,5] carbapenemases; Metallo-β-lactamase (MBL) producers however remain resistant. This limitation might be overcome by combining avibactam with aztreonam, which is stable to MBLs, but which requires protection from co-produced ESBLs and AmpC enzymes.[6] Aztreonam/avibactam 1.5+0.5g q6h is being progressed on this rationale, with Phase III trials underway[7] and with comprehensive activity demonstrated against carbapenemase-producing Enterobacterales *in vitro*.[8]

The combination's development, including assessment of resistance risks, is informed by experience with ceftazidime/avibactam and ceftaroline/avibactam, which was investigated but not progressed.[9-11] In vitro, we could select single-step resistance-conferring mutations - most often Asp179Tyr - in KPC carbapenemases with ceftazidime/avibactam,[9] but not ceftaroline/avibactam.[10] Both ceftazidime/avibactam and ceftaroline/avibactam readily selected resistance-conferring mutations in AmpC enzymes, but very rarely did so in ESBLs.[10,11] Since ceftazidime/avibactam entered use, concern has centred on mutations in KPC carbapenemases, where Asp179Tyr substitutions been associated with clinical failure.[12] This change increases ceftazidime binding by KPC enzymes, rendering them harder to inhibit.[13] Activity against other β-lactams is less affected or, as with meropenem, is decreased.[14] There is a single report of clinical selection of a CTX-M-14 β-lactamase mutant with reduced ceftazidime/avibactam susceptibility, but the case history is complicated by prior use of unprotected ceftazidime.[15]

In the present study we investigated the resistance selection risk with aztreonam/avibactam, using similar approaches to those employed previously with ceftazidime/avibactam and ceftaroline/avibactam.

Materials and methods

Bacteria

Test strains (Table 1) were clinical *Escherichia coli, Klebsiella pneumoniae* and *Enterobacter cloacae* submitted to PHE's Antimicrobial Resistance and Healthcare Associated Infections (AMRHAI) Reference Unit for investigation of resistance. β-Lactamase genes were identified by prior WGS. Within the species, we represented prevalent carbapenemases (KPC, IMP, NDM and VIM and OXA-48-like types), ESBLs (TEM-10, SHV-2 and -5, CTX-M-14 and -15 types) and both acquired (CMY-2 and -44) and chromosomal (*Enterobacter*) AmpC enzymes.

Selection methodology

Selection was with the single-step procedure used previously.[9-11] Briefly, overnight nutrient broth cultures were spread on to Mueller-Hinton agar containing aztreonam combined with avibactam 1 mg/L or 4 mg/L at 2, 4, 8 and 16 x MIC. Colonies were counted after overnight incubation. Serial dilutions of the broth cultures were also spread onto drug-free agar and these counts were used as denominators for calculation of the mutation frequency for each MIC multiple.

Determination of MICs for mutants

For each parent organism and selective condition we retained up to 6 presumptive mutants, confirming species identity by MALDI-ToF mass spectroscopy (Bruker Biotyper, Bruker Daltonics, Bremen, Germany) then determined MICs by CLSI agar dilution[16] for aztreonam alone (0.03-128 mg/L), aztreonam/avibactam 1 mg/L and 4 mg/L (0.03-128 mg/L), ceftazidime (0.03-128 mg/L), ceftazidime-avibactam 4 mg/L (0.03-128 mg/L), cefepime (0.03-128 mg/L), cefepime/avibactam 4 mg/L (0.03-128 mg/L), ceftaroline (0.03-128 mg/L) ceftaroline/avibactam 4 mg/L (0.03-128 mg/L), cefotaxime (0.03-128 mg/L), meropenem (0.03-128 mg/L), piperacillin/tazobactam 4 mg/L (0.03-128 mg/L), ciprofloxacin (0.03-128 mg/L) and amikacin (0.03-128 mg/L). In general, we favoured retaining and testing mutants obtained at the highest selective concentrations where they were obtained. Antibiotics were from suppliers

as follows: avibactam, and ceftaroline (Pfizer), aztreonam (Alfa Aesar, Heysham, UK), ceftazidime, cefotaxime, cefepime, meropenem, piperacillin, ciprofloxacin and amikacin (Merck, Gillingham, UK). MICs are based on single determinations; accordingly, single tube shifts should be viewed having low significance.

WGS of selected mutants

DNA was extracted as previously,[11] then fragmented and tagged for multiplexing using NexteraXT library preparation Kits (Illumina, Cambridge, UK). Sequencing was on an Illumina HiSeq platform to produce 2x100 bp reads. Parent strain genome assembly was performed using SPAdes 3.5.0 genome assembler software with *k-mer* values 55,77,91.[17] The presence of β-lactamase variants was confirmed by BLAST searches, using the newly assembled genomes as query sequences against a reference database downloaded from the NCBI β-lactamase data.[18] Sequencing reads for mutants were mapped to the corresponding parent using the PHEnix algorithm,[19] with variants called and filtered using Genome Analysis Toolkit v2.[20] Sequences flanking confirmed alterations were manually inspected for gene structure and functional annotation, with Blast searches determine whether any changes lay in an open reading frame, promoter or intergenic region. Changes within structural genes were confirmed by aligning the protein-encoding sequences for the parent and mutant assemblies. To detect gene amplifications we compared the number of reads that mapped to β-lactamase genes relative to those for the single copy chromosomal genes *gyrA* and *parC.*

Amino acid numbering for Class A β-lactamases follows Ambler's scheme;[21] that for Class C enzymes follows Mack *et al*.[22]

Results.

Mutation frequency

Susceptibility testing for avibactam combinations, including aztreonam/avibactam, is routinely performed with a fixed inhibitor concentration of 4 mg/L. Here, however, we undertook selection and corresponding MIC determinations with both 1 and 4 mg/L avibactam on the

rationale that bacteria in infections and the gut flora are exposed to dynamically changing concentrations of both aztreonam and avibactam. Unsurprisingly, mutants were more readily selected at with low MIC multiples, and when avibactam was used at 1 rather than 4 mg/L (Table 1). Nonetheless, even with avibactam at 1 mg/L we rarely obtained mutants at 8x MIC and never did so at 16 x MIC; with 4 mg/L avibactam we obtained mutants as 8 x MIC for only two strains. High mutation frequencies (i.e. $>10^{-7}$ range) were largely confined to tests with 2 to 4 x MIC, particularly with avibactam at 1 mg/L, and showed no clear association with particular β-lactamase types.

Specifically, for 11/12 parent isolates with KPC carbapenemases we only obtained mutants at two or four times the starting MIC; the exception was *E. cloacae* PF19_8 where, with 1 mg/L avibactam, we obtained mutants up to 16 x MIC. Among 18 isolates with MBLs we obtained mutants at 8 x MIC for one organism – *E. coli* PF19_51; otherwise mutants were only selected, if at all, at two of four times the parent MIC. Among four isolates with OXA-48-like enzymes, we only obtained mutants up to four times MIC. We tested 12 isolates chosen as ESBL producers, representing CTX-M, TEM and SHV enzymes. In addition, 14/34 isolates included primarily as carbapenemase producers also produced ESBLs. Among the former 12, we could obtain mutants at only two- to four- times the aztreonam/avibactam MIC, even with avibactam at 1 mg/L. Finally, among six isolates with plasmid-mediated or derepressed chromosomal AmpC (two *E. coli* with CMY-2 or CMY-44 enzymes and 4 *E. cloacae* respectively) we obtained mutants at eight times MIC in one case, but otherwise only at two to four times MIC.

MIC distributions for selected mutants

MICs of aztreonam/avibactam 1 or 4 mg/L and comparators were determined for a total of 314 mutants, derived from parents with principal β-lactamases as follows: KPC (n=69), MBLs (n=112), OXA-48 (n=32), ESBLs (n=61) and AmpC (n=40). Fold increases in MIC aztreonam/avibactam 4 mg/L compared with parent strains are shown in fig 1A, whilst fig 1B

illustrates the actual MICs. MIC increases were mostly two- to eight- fold, with rises ≥64-fold seen only for AmpC producers.

All the mutants selected from parents with KPC carbapenemases were inhibited by aztreonam/avibactam 4+4 mg/L, as were all those selected from producers of ESBLs or OXA-48-like enzymes. MICs exceeding the provisional breakpoint of 8+4 mg/L were seen only for mutants of strains with AmpC enzymes, where values of ≥64+4 were observed in four instances, and for those derived from *E. coli* with NDM-5 or -7 enzymes, where the 'starting' parent MICs were relatively high at 1-4 mg/L. Aztreonam/avibactam MICs >4+4 mg/L were not seen for mutants of other MBL producers, where the starting MICs were lower, at 0.06 to 0.25 mg/L. This nuance is apparent in figure 1, showing that the *fold* increases in MICs for the mutants of MBL producers were normally distributed, with a peak at four-fold, whereas the actual MICs were bimodal, with peaks at 0.25 and 16 mg/L.

Mutant characterisation

For WGS we preferred those mutants with the greatest rises in aztreonam/avibactam MICs and those with the greatest diversity of MIC changes to comparator antibiotics as tested alone or combined with avibactam. Accordingly, it should be noted that the MICs for most mutants were below those for the mutant studied in detail.

Strains with KPC enzymes. We sequenced 15 mutants from 10 parents with KPC carbapenemases; four parents also had ESBLs (Table 2). Seven of the 15 mutants had nucleotide changes leading to substitutions in their KPC enzymes. Four, variously from parents with KPC-2 and -3 enzymes, had Trp105Arg; two, from parents with KPC-2 and -3 enzymes, had Ser106Pro, and one had Ser109Pro (numbering based on Ambler's scheme; positions in the mature KPC protein itself are 104, 105 and 108). MICs of aztreonam/avibactam 4 mg/L for these variants ranged from 0.5 to 4 mg/L compared with 0.06 to 0.25 mg/L for their parent strains. The MICs of meropenem were reduced for all seven mutants, variously by four- to 32- fold. MICs of cefepime, ceftazidime and cefotaxime also

were widely reduced, although precise interpretation was confounded by (i) by off-scale cephalosporin MICs for some parent strains, precluding assessment of change, and (ii) by co-production of unaltered ESBLs, except in strains *K. pneumoniae* PF_19-1 and *E. coli* PF_19-9 and their mutants. In general, ceftaroline MICs were less reduced than those of other oxyimino cephalosporins, whilst MICs of all cephalosporin/avibactam combinations remained within one doubling dilution of those for the parent strains. Mutant *E. cloacae* PF_19-7e, with a Ser106Pro substitution in KPC-2 additionally had a mutation in its LamB maltoporin. Whilst this porin is not generally considered to be a major entry channel for β-lactams in Enterobacterales, one group associated decreased expression with broad, small, reductions in antibiotic susceptibility.[23]

The remaining eight sequenced mutants lacked changes in bla_{KPC}. They all showed small, generalised MIC rises for cephalosporins, cephalosporin/avibactam combinations and meropenem. One - *E. cloacae* PF_19-8h) - was an *ampD* mutant, with this lesion predicting derepression of its AmpC β-lactamase. Another – *E. cloacae* PF_19-6d – had a mutation in *baeS*, leading to an Asp111Val substitution in the corresponding histidine kinase, BaeS; this is part of the BaeSR sensor system and is linked to efflux pump and porin expression.[24,25] A third– *E. cloacae* PF_19-7h) – had a mutation affecting its LamB maltoporin (with no change to bla_{KPC}), though, as above, the significance of this is uncertain. Last, mutant *E. coli* PF_19-12c had a premature stop codon in *rfaG.* The product of this gene is involved in LPS biogenesis and its inactivation may affect outer membrane architecture. We failed to find any significant changes in the remaining four mutants sequenced and the basis of raised MICs for these organisms must, perforce, remain uncertain.

Strains with MBLs. We sequenced 16 mutants derived from 13 parents with MBLs, including VIM, IMP and NDM types (Table 3). Thirteen were selected at only 2x the starting the MIC, two at 4x and one at 8x, reflecting the paucity of mutants at high MIC multiples. Despite these 'mild' conditions, MIC rises for aztreonam/avibactam were raised up to 16- or 32-fold for *E. coli*

mutants PF_19-39b, PF_19-43c and PF_19-51e, PF_19-51n, though most rises for mutants of MBL producers were smaller, with a mode of 4-fold (fig. 1a).

The parent MBL strains were broadly resistant to comparator agents, reducing the scope to assess the wider effect of the mutations seen; nevertheless, generalised MICs rises clearly were frequent, and extended up to four- and eight-fold in the case of meropenem and *E. coli* mutants PF_19-43c and PF_19-44f. Significant MIC reductions were not seen for any antibiotic.

Eight of the 16 mutants had alterations in *baeS*, and five (one with a *baeS* lesion) had mutations in *envZ*, encoding an expression regulator of porins OmpC and OmpF and their equivalents. Mutations in genes not obviously linked to the activity of β-lactams were variously found in *asnS* (asparagine synthase), *rpoB* (mRNA polymerase), *smtA* (S-adenosylmethionine-dependent methyltransferase), and *araA* (L-arabinose isomerase). One *envZ* mutant *E. coli* (PF_19-44f) had an additional stop codon in an already inactivated *lamB* maltoporin gene. Four mutants had no mutations detected and, again, mechanisms here remain unresolved.

Strains with OXA-48-like enzymes. Only three mutants of isolates (two *E. coli* and one *K. pneumoniae*) with OXA-48-like carbapenemases were sequenced; two additionally had ESBLs (Table 4). These small numbers reflect the fact that few mutants were selected and that these showed little MIC diversity. These mutants exhibited two- to eight- fold MIC rises for aztreonam/avibactam and cephalosporin/avibactam, with no marked increases for the unprotected cephalosporins or meropenem. All three had lesions in *baeS*.

Strains with ESBLs. Five mutants of ESBL producers were sequenced – two *E. coli* and three *K. pneumoniae* (Table 5). These showed small MICs increases for some or all cephalosporin/avibactam combinations as well as up to 8 to 16-fold for aztreonam/avibactam and, also, in the case of *E. coli* PF_19-23d, for meropenem and piperacillin/tazobactam. MICs of aztreonam/avibactam remained in the likely clinical range. Two mutants had alterations

affecting the porin regulator EnvZ and one had a lesion in the histidine kinase sensor BaeS; no changes of relevance were found in the remaining two mutants.

Strains with AmpC enzymes. Eleven mutants were sequenced from six parents; three were from *E. coli* with acquired CMY enzymes and eight from *E. cloacae* strains with derepressed chromosomal AmpC (Table 6). The sequenced mutants included three with MICs exceeding the provisional aztreonam/avibactam breakpoint of 8+4 mg/L. These all had changes within *ampC*. Two – *E. coli* PF_19-26a (aztreonam/avibactam MIC 16+4 mg/L) and *E. cloacae* PF_19-30a (MIC 128+4 mg/L) – had Tyr150Cys substitutions, in acquired CMY-44 and in chromosomal AmpC respectively (numbering based on Mack *et al.*[22] and corresponding to the actual sequence of the mature proteins). Asides from resistance to aztreonam/avibactam, Tyr150Cys was associated with small rises in ceftaroline/avibactam MICs, unchanged low MICs for other cephalosporin/avibactam combinations and by frequent, though not universal, reductions in piperacillin/tazobactam and oxyimino-cephalosporins MICs. The third mutant with frank aztreonam/avibactam resistance– *E. cloacae* PF_19-30h (aztreonam/avibactam MIC 16+4 mg/L) – had an Asn346Tyr substitution. This was associated with raised MICs for all avibactam combinations.

The remaining eight mutants, all with aztreonam/avibactam MICs $\leq$8+4 mg/L, lacked mutations in *ampC* and largely showed unchanged MICs for other β-lactams besides aztreonam/avibactam, or showed only small changes. Only one – PF-28b – had a mutation with potential relevance, leading to an Ala498Thr substitution in PBP3. This mutant showed large MIC rises for ceftazidime/avibactam, from 0.5+4 to the breakpoint of 8+4 mg/L, and for cefepime, from 2+4 to 32+4 mg/L. The other mutations found were of doubtful significance, variously affecting the lipopolysaccharide transporter LptD, the replication initiation protein RepZ, the RNA polymerase RpoC, the glycerol-3-phosphotase UgpE and the UTP glucose-1-phosphate uridylphosphotransferase GalU.

Discussion

Aztreonam/avibactam is the third avibactam combination where we have undertaken selection studies, following ceftaroline/avibactam and ceftazidime/avibactam.[9-11]

With the 'standard' concentration of 4 mg/l avibactam we only obtained mutants at frequencies in the 'concerning' $10^{-6}/10^{-7}$ range at 2xMIC or, for two strains (*E. cloacae* PF19_28 with derepressed AmpC and *E. coli* PF19_39 with NDM-7) at 4xMIC; frequencies at 8xMIC were $<10^{-8}$ in all cases and, except for *E. cloacae* PF19_30 with derepressed AmpC and *E. coli* PF19_39 with NDM-7, were below detection limits (Table 1). We additionally undertook selection with 1 mg/L avibactam, on the rationale that bacteria in infections and the gut flora are exposed to dynamically changing concentrations of both aztreonam and avibactam and that selection may occur in milieux and body compartments where the avibactam exposure is reduced. Unsurprisingly, mutants were more readily selected with this lower avibactam concentration but, even so, frequencies in the $10^{-6}/10^{-7}$ range were only seen at >4xMIC with two parent strains, one (*E. cloacae* PF19_8) with KPC-2 and the other (*E. coli* PF19-51) with NDM-5 enzyme. These low mutation frequencies recapitulate experience with ceftaroline/avibactam.[10] For ceftazidime/avibactam, by contrast, it was notably easy to select single-step mutants of strains with KPC carbapenemases at 8 to16 x MIC even with 4 mg/L avibactam.[9]

In terms of the mutant types selected several patterns become apparent across the three studies. First, we have consistently selected substantially resistant AmpC mutants (MIC >8+4 mg/L) with each avibactam combinations, with Asn346 mutants invariably among those seen (these appear as Asn366 mutants in earlier publications, with numbering based on the entire protein, including the signal peptide). Here, and with ceftazidime/avibactam, we saw Asn346Tyr substitutions; previously, with ceftaroline/avibactam, we obtained Asn346His and Asn346Ile variants. Other AmpC mutants found in the various studies had proximate substitutions. Thus, here we twice selected mutants with Tyr150Cys, whereas with ceftazidime/avibactam we selected mutants with Arg148His and Gly156Arg/Asp and deletions around positions 289-294.[11] These observations are consistent with the known structure/function relationships of AmpC enzymes. Asn346 is involved in avibactam binding,

which is dramatically reduced if it is replaced by tyrosine, whereas this does not compromise cephalosporin hydrolysing activity.[26] Similarly, Tyr150 forms part of the active site[22,27] and is strongly conserved; consequently, it is unsurprising its replacement, or replacement of nearby residues (e.g., Arg148) have major effects. Notably, the present Tyr150Cys mutants gained resistance (MICs >8+4 mg/L) to aztreonam/avibactam but substantially lost resistance to oxyimino-cephalosporins. By contrast, as found repeatedly by ourselves and others, Asn346 mutants gain broad resistance to avibactam combinations without obvious compromise.[10,11,26] We accordingly anticipate Asn346 mutants may be seen in clinical practice, but would be surprised to see evolutionary success by the broadly compromised Tyr150 mutants.

Second, whereas it has consistently been possible to select AmpC mutants, it has repeatedly proved extremely difficult to select such mutations in ESBLs, of the CTX-M, SHV and TEM families.[10,11] No ESBL mutants were selected here and, although we did select mutants of CTX-M-15 with ceftaroline/avibactam and ceftazidime/avibactam, these were greatly compromised in respect to being able to confer resistance to other β-lactams. It is hard to believe that they could achieve evolutionary success in a 'real world' of diverse β-lactam usage. Clinically, emerging resistance with to ceftazidime/avibactam has been associated, once each, with mutations in CTX-M-14, -15 and VEB-1 enzymes;[15,28] it is unknown whether these changes also compromised aztreonam/avibactam.

Third, also consistent, is the fact that we have never been able to select resistance-conferring mutations in any β-lactamase that ordinarily lacks activity against the partner β-lactam. In context, aztreonam is stable to MBLs and OXA-48, whereas ceftazidime is stable to OXA-48 carbapenemases. To our knowledge, no monobactam-hydrolysing MBL has yet been described, so failure to select such activity in IMP, NDM and VIM enzymes is unsurprising. Failure to select β-lactamase-related resistance in organisms with OXA-48-like is more notable, insofar as some OXA-48-related enzymes (e.g. OXA-163) do hydrolyse oxyimino cephalosporins and aztreonam.[29] Others have selected OXA-48 laboratory mutants with raised ceftazidime and ceftazidime/avibactam MICs, though without frank resistance.[30] These had both Pro68Ala and Tyr211Ser mutations and substantially lost resistance to

piperacillin/tazobactam and carbapenems, as also applies with wild type OXA-163 enzyme.

Fourth, KPC carbapenemases present the least consistent case across the different avibactam combinations. With ceftaroline/avibactam we selected no resistant mutants.[10] With ceftazidime/avibactam we readily selected mutants with high-level resistance (MICs up to 128+4 mg/L) mostly with mutations around the Ω loop, principally Asp179Tyr. Here, with aztreonam/avibactam, we selected mutants with Trp105Arg or Ser106Pro substitutions or, in one case only, Ser109Pro. Asp179Tyr increases ceftazidimase activity and the stability of the ceftazidime-KPC acyl enzyme,[13] thus protecting the β-lactamase from inhibition by avibactam. The substitution *reduces* activity against many other β-lactams, including meropenem and aztreonam; resistance is not conferred to aztreonam/avibactam,[31] explaining the lack of selection here. The present mutations around positions 105-109, reduced the ability of KPC-2 and -3 enzymes to confer resistance to other β-lactams besides aztreonam; moreover, aztreonam/avibactam MICs never reached the clinical breakpoint, and ceftazidime/avibactam was not compromised. We therefore doubt that they present a significant potential threat. Trp105 ordinarily is a highly conserved residue in KPC and other Class A β-lactamases, forming part of the active site.[32] It is unsurprising that replacement with arginine, a dissimilar amino acid, has a substantial effect. Introduction of proline at position 106 or 109 is also likely to consequential, as the rigidity of this amino acid distorts protein secondary structure and, potentially, the alignment of Trp105.

Besides mutations affecting β-lactamase structure, we encountered multiple lesions affecting *baeRS*, which encode a histidine kinase sensor system co-regulating multiple efflux systems[24] and porins;[25] also in *envZ*, which co-regulates expression of major porins.[33] Mutations affecting *envZ* were widely encountered in our previous selection studies with ceftazidime/avibactam[11] whilst those affecting both *envZ* and *baeRS* were selected in studies with monobactam BOS-228.[34] Lesions in these genes were associated with broad, albeit small, rises in the MICs of β-lactams, compatible with general increases

in efflux or reductions in permeability. Another mutation of potential relevance led to an Ala498Thr substitution in PBP3 of *E. cloacae* PF_19-28b, an AmpC-derepressed strain. This mutant showed raised MICs for the all tested β-lactams, which universally target PBP3,[35-37] and for their inhibitor combinations; MICs remained within likely clinical ranges, though that for ceftazidime/avibactam reached the breakpoint of 8+4 mg/L. Lastly, mutant *E. cloacae* PF_19-8h, with a KPC carbapenemase, acquired an *ampD* lesion putatively causing derepression of its chromosomal AmpC β-lactamase. We previously showed that ceftazidime/avibactam could select AmpC-derepressed Enterobacter mutants, albeit less frequently than ceftazidime alone and that, as here, these caused small rises in the MIC of avibactam combinations.[11] Sequestration of avibactam by copious AmpC enzyme – up to 3-4% of total cell protein in a derepressed strain – likely reduces the amount available to inhibit the KPC carbapenemase.[38]

Overall, these results are favourable for aztreonam/avibactam. Single-step mutations in KPC carbapenemases did not confer resistance at the provisional breakpoint and reduced the enzyme's ability to confer resistance to other β-lactams, suggesting that they should be counter-selected in clinical settings. This contrasts to the single step Asp179Tyr mutants most often selected with ceftazidime/avibactam, which confers unequivocal resistance to that combination. There does appear to be a potential risk for selection of AmpC mutants, with Asn346 substitutions compromising all avibactam combination without degrading the enzymes' ability to cause resistance. Nonetheless we are not aware of this mutation having been selected clinically. The hazard cannot, however, be dismissed: AmpC sequence mutants of *P. aeruginosa* have been selected during ceftolozane/tazobactam therapy and show cross resistance to ceftazidime/avibactam.[39,40] There is no evidence that *baeRS* and *envZ* mutants will present a real hazard: these have been selected *in vitro* with other antibiotics, but have not been recorded as emerging during therapy. Mutants with lesions in porin structural genes were not seen in any of our avibactam studies but may be a risk, given that they have been selected e.g. with

meropenem/vaborbactam and occasionally during carbapenem usage.[41-43] Last, there remains the issue that four of the present *E. coli* isolates 'began' with aztreonam/avibactam MICs of 1-4 mg/L, rather than the more typical 0.06-0.25 mg/L, meaning that relatively small MIC rises raised the MICs above the breakpoint. Raised starting MICs for aztreonam/avibactam have been associated with Tyr-Arg-Ile-Asn/Pro inserts at position 334 in PBP3,[44,45] but these were not found. The parent has diverse OmpC sequences (not shown), and this may be pertinent. Although these isolates had NDM-5 and -7 MBLs rather than NDM-1 we do not count this as directly relevant, since all these NDM variants are similarly unable to raise aztreonam MICs when transferred into a new host strain.[46] In short, the resistance risk to aztreonam/avibactam seems relatively small, is not strongly associated with particular carbapenemases; in the case of KPC enzymes the hazard seems less than for ceftazidime/avibactam, which itself has proved a successful agent.

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Transparency declarations.

DML has undertaken Advisory Boards or ad-hoc consultancy for Accelerate, Antabio, Centauri, Discuva/Summit, Entasis, GlaxoSmithKline, J&J, Meiji, Menarini, Mutabilis, Nordic, Paion, ParaPharm, Pfizer, Pharmacytics, QPEX, Shionogi, T.A.Z., Sumitovant, Summit, Tetraphase, VenatoRx, Wockhardt and Zambon. He has received lectures fees from bioMérieux, Beckman Coulter, Cardiome, Cepheid, GSK, Hikma, Merck/MSD, Menarini, Nordic, Pfizer and Shionogi. He has direct relevant shareholdings or options in Dechra, GSK, Merck, Perkin Elmer, Pfizer, and T.A.Z, amounting to <10% of portfolio value. He also has nominated holdings in Angle, Arecor Therapeutics, Avacta, C4X Drug Discovery, Creo Medical,

DeepVerge, Deltex Medical, Directa Plus, Diaceutics, Diurnal, Evgen, Faron, Genedrive, Ixico, MaxCyte, Renalytics AI, Rua Life Sciences, Synairgen and Trellus Health (all with research/products pertinent to medical and diagnostic innovation) through Enterprise Investment Schemes but has no authority to trade these shares directly. **SM, AV, NE** and **NW** are members of PHE's Antimicrobial Resistance and Healthcare Associated Infections Reference Unit, which has received financial support for conference attendance, lectures, research projects, or contracted evaluations from numerous sources, including Accelerate Diagnostics, Achaogen Inc., Allecra Therapeutics, Amplex, AstraZeneca UK Ltd, AusDiagnostics, Basilea Pharmaceutica, Becton Dickinson Diagnostics, bioMérieux, Bio-Rad Laboratories, BSAC, Cepheid, Check-Points B.V., Cubist Pharmaceuticals, Department of Health, Enigma Diagnostics, Food Standards Agency, GlaxoSmithKline Services Ltd, Helperby Therapeutics, Henry Stewart Talks, IHMA Ltd, Innovate UK, Kalidex Pharmaceuticals, Melinta Therapeutics, Merck Sharpe & Dohme Corp, Meiji Seika Pharma Co. Ltd, Mobidiag, Momentum Biosciences Ltd, Neem Biotech, Nordic Pharma Ltd, Norgine Pharmaceuticals, Rempex Pharmaceuticals Ltd, Roche, Rokitan Ltd, Smith & Nephew UK Ltd, Shionogi & Co. Ltd, Trius Therapeutics, T.A.Z., VenatoRx Pharmaceuticals and Wockhardt Ltd.

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559 **Table 1.** Frequency of mutants in relation of strain and selective conditions

Species	Enzyme	Secondary β-lactamases	Frequency at MIC multiple, avibactam at 1 mg/L				Frequency at MIC multiple, avibactam at 4 mg/L			
			2x	4x	8x	16x	2x	4x	8x	16x
K. pneumoniae PF19_1	KPC-2	OXA-1, SHV-1	**$2.57x10^{-7}$**	**$1.71x10^{-7}$**	$<10^{-8}$	$<10^{-8}$	**$2.14x10^{-7}$**	$<10^{-8}$	$<10^{-8}$	$<10^{-8}$
K. pneumoniae PF19_2	KPC-2	SHV-1	$<10^{-8}$	$<10^{-8}$	$<10^{-8}$	$<10^{-8}$	$<10^{-8}$	$<10^{-8}$	$<10^{-8}$	$<10^{-8}$
K. pneumoniae PF19_3	KPC-2	OXA-9v, SHV-2, TEM-1	**$1.09x10^{-6}$**	$<3.77x10^{-9}$	$<3.77x10^{-9}$	$<3.77x10^{-9}$	$<3.77x10^{-9}$	$<3.77x10^{-9}$	$<3.77x10^{-9}$	$<3.77x10^{-9}$
K. pneumoniae PF19_4	KPC-3	SHV-11, OXA-9, TEM-1	$<5.97x10^{-9}$	$<5.97x10^{-9}$	$<5.97x10^{-9}$	$<5.97x10^{-9}$	$<5.97x10^{-9}$	$<5.97x10^{-9}$	$<5.97x10^{-9}$	$<5.97x10^{-9}$
E. cloacae PF19_5	KPC-2	AmpC, TEM-1	**$3.51x10^{-7}$**	$<2.60x10^{-9}$	$<2.60x10^{-9}$	$<2.60x10^{-9}$	**$3.38x10^{-7}$**	$<2.60x10^{-9}$	$<2.60x10^{-9}$	$<2.60x10^{-9}$
E. cloacae PF19_6	KPC-2	AmpC, TEM-1	**$1.15x10^{-7}$**	**$5.94x10^{-9}$**	$<1.98x10^{-9}$	$<1.98x10^{-9}$	**$5.54x10^{-7}$**	**$7.92x10^{-9}$**	$<1.98x10^{-9}$	$<1.98x10^{-9}$
E. cloacae PF19_7	KPC-2	AmpC, CTX-M-9, OXA-9, TEM-191	**$1.34x10^{-6}$**	**$1.82x10^{-7}$**	$<6.06x10^{-9}$	$<6.06x10^{-9}$	**$1.15x10^{-7}$**	$<6.06x10^{-9}$	$<6.06x10^{-9}$	$<6.06x10^{-9}$
E. cloacae PF19_8	KPC-2	AmpC	**$2.78x10^{-6}$**	**$1.05x10^{-6}$**	**$3.91x10^{-7}$**	**$3.35x10^{-7}$**	**$1.91x10^{-7}$**	$<3.23x10^{-9}$	$<3.23x10^{-9}$	$<3.23x10^{-9}$
E. coli PF19_9	KPC-2	TEM-1	**$2.33x10^{-8}$**	$<2.00x10^{-9}$	$<2.00x10^{-9}$	$<2.00x10^{-9}$	$<2.00x10^{-9}$	$<2.00x10^{-9}$	$<2.00x10^{-9}$	$<2.00x10^{-9}$
E. coli PF19_10	KPC-2		$<1.20x10^{-9}$	$<1.20x10^{-9}$	$<1.20x10^{-9}$	$<1.20x10^{-9}$	$<1.20x10^{-9}$	$<1.20x10^{-9}$	$<1.20x10^{-9}$	$<1.20x10^{-9}$
E. coli PF19_11	KPC-3	CTX-M-15, OXA-1	**$2.31x10^{-8}$**	**$5.76x10^{-9}$**	$<5.76x10^{-9}$	$<5.76x10^{-9}$	$<5.76x10^{-9}$	$<5.76x10^{-9}$	$<5.76x10^{-9}$	$<5.76x10^{-9}$
E. coli PF19_12	KPC-3		$<2.22x10^{-9}$	$<2.22x10^{-9}$	$<2.22x10^{-9}$	$<2.22x10^{-9}$	$4.24x10^{-7}$	$<2.22x10^{-9}$	$<2.22x10^{-9}$	$<2.22x10^{-9}$

E. coli PF19_35	NDM-1	CTX-M-15, OXA-1	**2.06×10^{-6}**	**3.01×10^{-9}**	$< 3.01 \times 10^{-9}$	$< 3.01 \times 10^{-9}$	**2.37×10^{-7}**	$< 3.01 \times 10^{-9}$	$< 3.01 \times 10^{-9}$	$< 3.01 \times 10^{-9}$
E. coli PF19_36	NDM-1		**2.12×10^{-7}**	$< 7.3 \times 10^{-9}$	$< 7.3 \times 10^{-9}$	$< 7.3 \times 10^{-9}$	**1.92×10^{-7}**	$< 7.3 \times 10^{-9}$	$< 7.3 \times 10^{-9}$	$< 7.3 \times 10^{-9}$
E. coli PF19_37	NDM-5	TEM-1	**1.79×10^{-9}**	$< 3.88 \times 10^{-9}$	$< 3.88 \times 10^{-9}$	$< 3.88 \times 10^{-9}$	$< 3.88 \times 10^{-9}$	$< 3.88 \times 10^{-9}$	$< 3.88 \times 10^{-9}$	$< 3.88 \times 10^{-9}$
E. coli PF19_38	NDM-5		$< 1.83 \times 10^{-9}$	$< 1.83 \times 10^{-9}$	$< 1.83 \times 10^{-9}$	$< 1.83 \times 10^{-9}$	$< 1.83 \times 10^{-9}$	$< 1.83 \times 10^{-9}$	$< 1.83 \times 10^{-9}$	$< 1.83 \times 10^{-9}$
E. coli PF19_39	NDM-7	OXA-33p, TEM-1	**2.47×10^{-6}**	**1.33×10^{-7}**	$< 3.41 \times 10^{-9}$	$< 3.41 \times 10^{-9}$	**2.15×10^{-6}**	**3.58×10^{-7}**	**3.41×10^{-9}**	$< 3.41 \times 10^{-9}$
E. coli PF19_40	NDM-7	OXA-1, TEM-1	**9.76×10^{-7}**	**1.58×10^{-8}**	$< 4.76 \times 10^{-9}$	$< 4.76 \times 10^{-9}$	**2.71×10^{-7}**	$< 4.76 \times 10^{-9}$	$< 4.76 \times 10^{-9}$	$< 4.76 \times 10^{-9}$
K. pneumoniae PF_41	NDM-1	CTX-M-15	$< 5.35 \times 10^{-9}$	$< 5.35 \times 10^{-9}$	$< 5.35 \times 10^{-9}$	$< 5.35 \times 10^{-9}$	$< 5.35 \times 10^{-9}$	$< 5.35 \times 10^{-9}$	$< 5.35 \times 10^{-9}$	$< 5.35 \times 10^{-9}$
K. pneumoniae PF_42	NDM-1	OXA-1	**3.57×10^{-6}**	**6.37×10^{-9}**	$< 6.37 \times 10^{-9}$	$< 6.37 \times 10^{-9}$	$< 6.37 \times 10^{-9}$	$< 6.37 \times 10^{-9}$	$< 6.37 \times 10^{-9}$	$< 6.37 \times 10^{-9}$
E. coli PF19_43	VIM-4	CTX-M-14, TEM-1	$< 8.26 \times 10^{-9}$	$< 8.26 \times 10^{-9}$	$< 8.26 \times 10^{-9}$	$< 8.26 \times 10^{-9}$	**7.60×10^{-7}**	$< 8.26 \times 10^{-9}$	$< 8.26 \times 10^{-9}$	$< 8.26 \times 10^{-9}$
E. coli PF19_44	VIM-1		1.64×10^{-9}	$< 4.1 \times 10^{-10}$	$< 4.1 \times 10^{-10}$	$< 4.1 \times 10^{-10}$	1.64×10^{-9}	$< 4.1 \times 10^{-10}$	$< 4.1 \times 10^{-10}$	$< 4.1 \times 10^{-10}$
K. pneumoniae PF19_45	VIM-4	CTX-M-15, SHV-100	$< 3.85 \times 10^{-9}$	$< 3.85 \times 10^{-9}$	$< 3.85 \times 10^{-9}$	$< 3.85 \times 10^{-9}$	$< 3.85 \times 10^{-9}$	$< 3.85 \times 10^{-9}$	$< 3.85 \times 10^{-9}$	$< 3.85 \times 10^{-9}$
K. pneumoniae PF19_46	VIM-4	SHV-11, TEM-1, LEN-11p	$< 2.34 \times 10^{-9}$	$< 2.34 \times 10^{-9}$	$< 2.34 \times 10^{-9}$	$< 2.34 \times 10^{-9}$	$< 2.34 \times 10^{-9}$	$< 2.34 \times 10^{-9}$	$< 2.34 \times 10^{-9}$	$< 2.34 \times 10^{-9}$
K. pneumoniae PF19_47	VIM-1	SHV-12	**7.63×10^{-7}**	$< 2.77 \times 10^{-9}$	$< 2.77 \times 10^{-9}$	$< 2.77 \times 10^{-9}$	$< 2.77 \times 10^{-9}$	$< 2.77 \times 10^{-9}$	$< 2.77 \times 10^{-9}$	$< 2.77 \times 10^{-9}$
K. pneumoniae PF19_48	VIM-1	SHV-100	**2.40×10^{-7}**	$< 1.04 \times 10^{-8}$	$< 1.04 \times 10^{-8}$	$< 1.04 \times 10^{-8}$	7.77×10^{-8}	$< 1.04 \times 10^{-8}$	$< 1.04 \times 10^{-8}$	$< 1.04 \times 10^{-8}$

K. pneumoniae PF19_49	IMP-1	CTX-M-15, OXA-1, SHV-36, TEM-1	**2.67×10^{-8}**	$< 5.35 \times 10^{-9}$	$< 5.35 \times 10^{-9}$	$< 5.35 \times 10^{-9}$	**2.56×10^{-7}**	$< 5.35 \times 10^{-9}$	$< 5.35 \times 10^{-9}$	$< 5.35 \times 10^{-9}$
K. pneumoniae PF19_50	IMP-1	SHV-53p	**1.43×10^{-6}**	**1.78×10^{-7}**	$< 5.78 \times 10^{-9}$	$< 5.78 \times 10^{-9}$	**9.83×10^{-7}**	**5.78×10^{-9}**	$< 5.78 \times 10^{-9}$	$< 5.78 \times 10^{-9}$
E. coli PF19_51	NDM-5	CTX-M-15, OXA-1, TEM-1	**1.94×10^{-6}**	**8.00×10^{-8}**	**1.20×10^{-7}**	$< 2.00 \times 10^{-8}$	**3.60×10^{-7}**	**2.00×10^{-8}**	$< 2.00 \times 10^{-8}$	$< 2.00 \times 10^{-8}$
E. coli PF19_52	NDM-7	CTX-M-15, OXA-1, TEM-1	**5.01×10^{-7}**	$< 2.43 \times 10^{-9}$	$< 2.43 \times 10^{-9}$	$< 2.43 \times 10^{-9}$	**2.92×10^{-8}**	$< 2.43 \times 10^{-9}$	$< 2.43 \times 10^{-9}$	$< 2.43 \times 10^{-9}$
E. coli PF19_31	OXA-48		**1.69×10^{-8}**	$< 8.44 \times 10^{-9}$	$< 8.44 \times 10^{-9}$	$< 8.44 \times 10^{-9}$	**2.53×10^{-8}**	$< 8.44 \times 10^{-9}$	$< 8.44 \times 10^{-9}$	$< 8.44 \times 10^{-9}$
E. coli PF19_32	OXA-48		**1.67×10^{-8}**	**$< 3.34 \times 10^{-9}$**	$< 3.34 \times 10^{-9}$	$< 3.34 \times 10^{-9}$	**2.87×10^{-6}**	**4.30×10^{-6}**	$< 3.34 \times 10^{-9}$	$< 3.34 \times 10^{-9}$
K. pneumoniae PF19_33	OXA-48	CTX-M-15, SHV-1	$< 4.23 \times 10^{-9}$	$< 4.23 \times 10^{-9}$	$< 4.23 \times 10^{-9}$	$< 4.23 \times 10^{-9}$	**7.61×10^{-8}**	$< 4.23 \times 10^{-9}$	$< 4.23 \times 10^{-9}$	$< 4.23 \times 10^{-9}$
K. pneumoniae PF19_34	OXA-48	SHV-1, LEN-11	**1.41×10^{-7}**	$< 6.12 \times 10^{-9}$	$< 6.12 \times 10^{-9}$	$< 6.12 \times 10^{-9}$	$< 6.12 \times 10^{-9}$	$< 6.12 \times 10^{-9}$	$< 6.12 \times 10^{-9}$	$< 6.12 \times 10^{-9}$
E. coli PF19_13	CTX-M-15	OXA-1, TEM-1	$< 5.99 \times 10^{-9}$	$< 5.99 \times 10^{-9}$	$< 5.99 \times 10^{-9}$	$< 5.99 \times 10^{-9}$	$< 5.99 \times 10^{-9}$	$< 5.99 \times 10^{-9}$	$< 5.99 \times 10^{-9}$	$< 5.99 \times 10^{-9}$
E. coli PF19_14		TEM-1	**1.39×10^{-7}**	$< 5.33 \times 10^{-9}$	$< 5.33 \times 10^{-9}$	$< 5.33 \times 10^{-9}$	$< 5.33 \times 10^{-9}$	$< 5.33 \times 10^{-9}$	$< 5.33 \times 10^{-9}$	$< 5.33 \times 10^{-9}$
K. pneumoniae PF19_15	CTX-M-15	OXA-9, SHV-39p, TEM-191p	**2.31×10^{-7}**	**5.01×10^{-9}**	$< 5.01 \times 10^{-9}$	$< 5.01 \times 10^{-9}$	**4.01×10^{-8}**	$< 5.01 \times 10^{-9}$	$< 5.01 \times 10^{-9}$	$< 5.01 \times 10^{-9}$
K. pneumoniae PF19_16	CTX-M-15	Not sequenced	$< 4.47 \times 10^{-9}$	$< 4.47 \times 10^{-9}$	$< 4.47 \times 10^{-9}$	$< 4.47 \times 10^{-9}$	$< 4.47 \times 10^{-9}$	$< 4.47 \times 10^{-9}$	$< 4.47 \times 10^{-9}$	$< 4.47 \times 10^{-9}$
E. coli PF19_17	CTX-M-14		**1.67×10^{-7}**	$< 8.37 \times 10^{-9}$	$< 8.37 \times 10^{-9}$	$< 8.37 \times 10^{-9}$	$< 8.37 \times 10^{-9}$	$< 8.37 \times 10^{-9}$	$< 8.37 \times 10^{-9}$	$< 8.37 \times 10^{-9}$

E. coli PF19_18	CTX-M-14		**$4.04x10^{-8}$**	$<4.49x10^{-9}$	$<4.49x10^{-9}$	$<4.49x10^{-9}$	$<4.49x10^{-9}$	$<4.49x10^{-9}$	$<4.49x10^{-9}$	$<4.49x10^{-9}$
K. pneumoniae PF19_19	CTX-M-14		**$8.70x10^{-8}$**	$<7.25x10^{-9}$	$<7.25x10^{-9}$	$<7.25x10^{-9}$	$<7.25x10^{-9}$	$<7.25x10^{-9}$	$<7.25x10^{-9}$	$<7.25x10^{-9}$
K. pneumoniae PF19_20	CTX-M-14		$<6.67x10^{-9}$	$<6.67x10^{-9}$	$<6.67x10^{-9}$	$<6.67x10^{-9}$	$<6.67x10^{-9}$	$<6.67x10^{-9}$	$<6.67x10^{-9}$	$<6.67x10^{-9}$
K. pneumoniae PF19_21	SHV-5	SHV-1	**$5.33x10^{-8}$**	$<5.33x10^{-9}$	$<5.33x10^{-9}$	$<5.33x10^{-9}$	**$1.13x10^{-8}$**	$<5.33x10^{-9}$	$<5.33x10^{-9}$	$<5.33x10^{-9}$
K. pneumoniae PF19_22	SHV-2	None	**$2.03x10^{-7}$**	$<6.15x10^{-9}$	$<6.15x10^{-9}$	$<6.15x10^{-9}$	$<6.15x10^{-9}$	$<6.15x10^{-9}$	$<6.15x10^{-9}$	$<6.15x10^{-9}$
E. coli PF19_23	TEM-10	None	**$2.36x10^{-5}$**	**$2.27x10^{-8}$**	$<2.27x10^{-8}$	$<2.27x10^{-8}$	**$2.11x10^{-6}$**	$<2.27x10^{-8}$	$<2.27x10^{-8}$	$<2.27x10^{-8}$
E. coli PF19_24	TEM-10	None	$<1.18x10^{-8}$	$<1.18x10^{-8}$	$<1.18x10^{-8}$	$<1.18x10^{-8}$	$<1.18x10^{-8}$	$<1.18x10^{-8}$	$<1.18x10^{-8}$	$<1.18x10^{-8}$
E. coli PF19_25	CMY-2	None	**$4.18x10^{-9}$**	$<5.36x10^{-9}$	$<5.36x10^{-9}$	$<5.36x10^{-9}$	$<5.36x10^{-9}$	$<5.36x10^{-9}$	$<5.36x10^{-9}$	$<5.36x10^{-9}$
E. coli PF19_26	CMY-44	None	**$7.71x10^{-8}$**	**$1.29x10^{-8}$**	$<6.43x10^{-9}$	$<6.43x10^{-9}$	$<6.43x10^{-9}$	**$6.43x10^{-9}$**	$<6.43x10^{-9}$	$<6.43x10^{-9}$
E. cloacae PF19_27	AmpC	-	**$1.94x10^{-7}$**	**$1.01x10^{-8}$**	$<3.37x10^{-9}$	$<3.37x10^{-9}$	**$1.34x10^{-7}$**	$<3.37x10^{-9}$	$<3.37x10^{-9}$	$<3.37x10^{-9}$
E. cloacae PF19_28	AmpC		**$1.04x10^{-6}$**	**$1.11x10^{-8}$**	$<3.71x10^{-9}$	$<3.71x10^{-9}$	**$1.01x10^{-6}$**	**$1.36x10^{-7}$**	$<3.71x10^{-9}$	$<3.71x10^{-9}$
E. cloacae PF19_29	AmpC		**$6.00x10^{-6}$**	$<2.21x10^{-9}$	$<2.21x10^{-9}$	$<2.21x10^{-9}$	**$1.00x10^{-6}$**	$<2.21x10^{-9}$	$<2.21x10^{-9}$	$<2.21x10^{-9}$
E. cloacae PF19_30	AmpC		**$8.82x10^{-7}$**	**$2.11x10^{-8}$**	**$4.67x10^{-9}$**	$<1.87x10^{-9}$	**$9.34x10^{-9}$**	**$<1.87x10^{-9}$**	**$3.78x10^{-9}$**	$<1.87x10^{-9}$

Bold font: measurable frequencies; plain font: below detection limits indicated

563 **Table 2.** Mutations selected with aztreonam/avibactam in isolates with KPC enzymes
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			MIC (mg/L)													
Strain	β-Lactamase(s)	Mutations found	ATM	ATM AVI1	ATM AVI4	CAZ	CAZ AVI4	FEP	FEP AVI4	CPT	CPT AVI4	AVI	CTX	MEM	TZP	CIP	AMK
***K. pneumoniae* PF_19-1**	**KPC-2; OXA-1; SHV-1**		**>128**	**0.25**	**0.12**	**64**	**1**	**>128**	**0.12**	**16**	**0.5**	**>4.0**	**>128**	**32**	**>128**	**4**	**16**
PF_19-1b (2 x MIC@4)[a]		KPC2 S109P	64	4	1	4	1	1	0.06	16	1	>4.0	8	2	>128	4	8
PF_19-1f (4 x MIC@1)		KPC2 W105R; TilS R17S	>128	16	2	16	0.5	2	0.06	16	1	>4.0	>128	1	128	4	32
***K. pneumoniae* PF_19-3**	**KPC-2; OXA-9v, SHV-2, TEM-1**		**>128**	**1**	**0.25**	**128**	**2**	**32**	**0.12**	**32**	**1**	**>4.0**	**128**	**64**	**>128**	**0.06**	**2**
PF_19-3h (2 x MIC@1)		DUF445 silent	>128	>128	2	>128	8	>128	2	>128	8	>4.0	>128	>128	>128	0.12	2
PF_19-3k (2 x MIC@1)		LdcC silent	>128	4	0.5	>128	4	128	0.12	32	2	>4.0	>128	64	>128	0.06	2
***K. pneumoniae* PF_19-4**	**KPC-3; SHV-11; OXA-9, TEM-1**		**>128**	**2**	**0.25**	**>128**	**2**	**>128**	**0.25**	**64**	**1**	**>4.0**	**>128**	**>128**	**>128**	**128**	**64**
PF_19-4b (2 x MIC@1)		KPC3 W105R	>128	128	4	>128	2	16	0.25	32	1	>4.0	>128	16	>128	128	64
PF_19-4c (2 x MIC@1)		KPC3 S106P	64	16	2	16	2	4	0.25	32	1	>4.0	8	8	>128	128	64
***E. cloacae* PF_19-5**	**KPC-2; AmpC; TEM-1**		**>128**	**0.25**	**0.06**	**16**	**0.5**	**16**	**0.12**	**8**	**0.5**	**>4.0**	**>128**	**8**	**>128**	**≤0.03**	**2**
PF_19-5c (2 x MIC@4)			>128	2	0.5	>128	1	>128	0.25	64	1	>4.0	>128	64	>128	≤0.03	2
***E. cloacae* PF_19-6**	**KPC-2; AmpC; TEM-1**		**>128**	**0.5**	**0.12**	**64**	**1**	**32**	**0.12**	**32**	**1**	**>4.0**	**>128**	**16**	**>128**	**≤0.03**	**2**
PF_19-6d (4 x MIC@4)		BaeS D111V	>128	4	2	>128	4	>128	0.5	32	2	>4.0	>128	64	>128	≤0.03	2
***E. cloacae* PF_19-7**	**KPC2; AmpC; CTX-M-9; OXA-9v; TEM191-P**		**>128**	**0.5**	**0.06**	**32**	**1**	**>128**	**0.25**	**32**	**1**	**>4.0**	**>128**	**16**	**>128**	**≤0.03**	**2**

PF_19-7e (2 x MIC@4)		KPC2 S106P; Maltoporin N347D	128	8	2	8	2	8	0.25	32	2	>4.0	64	1	128	≤0.03	2
PF_19-7h (4 x MIC@1)		Maltoporin N347D	>128	4	0.5	>128	2	>128	0.12	64	1	>4.0	>128	16	>128	0.06	2
***E. cloacae* PF_19-8**	**KPC-2, AmpC**		**>128**	**0.5**	**0.25**	**64**	**2**	**>128**	**0.25**	**16**	**1**	**>4.0**	**>128**	**16**	**>128**	**0.5**	**4**
PF_19-8a (2 x MIC@4)			>128	16	4	>128	4	>128	0.5	64	4	>4.0	>128	64	>128	2	2
PF_19-8h (16 x MIC@1)		AmpD I113S	>128	8	1	>128	2	>128	0.25	32	2	>4.0	>128	16	>128	0.5	2
***E. coli* PF_19-9**	**KPC-2; TEM-1**		**>128**	**0.25**	**0.12**	**32**	**0.5**	**128**	**0.06**	**32**	**1**	**>4.0**	**>128**	**8**	**>128**	**0.25**	**2**
PF_19-9a (2 x MIC@1)		KPC2 W105R	>128	8	1	8	0.5	1	≤0.03	8	0.5	>4.0	>128	2	32	≤0.03	2
***E. coli* PF_19-11**	**KPC-3; CTX-M-15; OXA-1**		**>128**	**0.12**	**0.12**	**128**	**0.25**	**128**	**≤0.03**	**8**	**0.25**	**>4.0**	**>128**	**4**	**>128**	**0.06**	**8**
PF_19-11b (2 x MIC@4)		KPC3 W105R	>128	4	0.5	64	0.5	64	0.06	8	0.5	>4.0	>128	0.12	32	0.06	8
***E. coli* PF_19-12**	**KPC-3**		**>128**	**0.25**	**0.06**	**>128**	**0.5**	**128**	**0.06**	**16**	**0.25**	**>4.0**	**>128**	**8**	**>128**	**≤0.03**	**64**
PF_19-12c (2 x MIC@4)		*rfaG* premature stop Q196*; pepP T421I	>128	2	1	>128	4	>128	0.12	32	1	>4.0	>128	32	>128	0.25	64

[a] 2 x MIC@4 means 'selected at 2 x MIC of aztreonam avibactam for the parent strain, as found using 4 mg/L avibactam etc.

Parent strains are shown in bold font

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

573 **Table 3.** Mutations selected with aztreonam/avibactam in Isolates with metallo- β-lactamases

			MIC (mg/L)													
Strain	β-Lactamase(s)	Mutations found	ATM	ATM AVI1	ATM AVI4	CAZ	CAZ AVI4	FEP	FEP AVI4	CPT	CPT AVI4	AVI	CTX	MEM	TZP	CIP	AMK
***E. coli* PF_19-35**	**NDM-1, CTX-M-15; OXA-1**		**>128**	**2**	**0.5**	**>128**	**>128**	**>128**	**128**	**>128**	**>128**	**>4.0**	**>128**	**64**	**>128**	**128**	**16**
PF_19-35b (2x MIC@4)[a]		BaeS R418L. AsnS H149Y	>128	8	4	>128	>128	>128	>128	>128	>128	>4.0	>128	64	>128	128	16
***E. coli* PF_19-36**	**NDM-1**		**0.12**	**0.12**	**0.12**	**>128**	**>128**	**64**	**64**	**>128**	**>128**	**>4.0**	**>128**	**64**	**>128**	**≤0.03**	**>128**
PF_19-36g (2x MIC@4)		BaeS G421D	0.5	0.5	0.5	>128	>128	64	128	>128	>128	>4.0	>128	64	>128	≤0.03	>128
***E. coli* PF_19-39**	**NDM-7, OXA-33-p*; TEM-1**		**2**	**1**	**2**	**>128**	**>128**	**>128**	**>128**	**>128**	**>128**	**>4.0**	**>128**	**>128**	**>128**	**>128**	**8**
PF_19-39b (4x MIC@4)		None found	16	16	16	>128	>128	>128	>128	>128	>128	>4.0	>128	>128	>128	>128	16
***E. coli* PF_19-40**	**NDM-7, OXA-1; TEM-1**		**2**	**1**	**1**	**>128**	**>128**	**>128**	**>128**	**>128**	**>128**	**>4.0**	**>128**	**>128**	**>128**	**>128**	**16**
PF_19-40b (2x MIC@4)		*glnA* silent; RpoB R451H	32	16	16	>128	>128	>128	>128	>128	>128	>4.0	>128	>128	>128	>128	32
PF_19-40f (2x MIC@4)		glnA silent; BaeS Q163L	16	16	16	>128	>128	>128	>128	>128	>128	>4.0	>128	>128	>128	>128	8
***K. pneumoniae* PF_19-42**	**NDM-1, OXA-1**		**0.12**	**0.12**	**0.06**	**>128**	**>128**	**128**	**64**	**>128**	**>128**	**>4.0**	**>128**	**64**	**>128**	**64**	**>128**
PF_19-42k (2x MIC@1)		Class I SAM-dependent methyltransferase P22T; EnvZ G145G	0.5	0.5	0.25	>128	>128	>128	>128	>128	>128	>4.0	>128	128	>128	128	>128
***E. coli* PF_19-43**	**VIM-4, CTX-M-14;TEM-1**		**64**	**0.25**	**0.06**	**32**	**16**	**>128**	**2**	**>128**	**128**	**>4.0**	**>128**	**4**	**>128**	**≤0.03**	**8**
PF_19-43c (2x MIC@4)		EnvZ V241G	>128	1	0.5	128	64	>128	8	>128	>128	>4.0	>128	16	>128	≤0.03	8
***E. coli* PF_19-44**	**VIM-1**		**0.12**	**0.12**	**0.06**	**>128**	**128**	**8**	**8**	**128**	**128**	**>4.0**	**64**	**2**	**>128**	**0.5**	**4**

PF_19-44f (2x MIC@4)		EnvZ A51E; LamB parent premature stop at 499, mutant has addition premature stop at 216.	2	1	1	>128	>128	32	64	>128	>128	>4.0	>128	16	>128	1	4
***K. pneumoniae* PF_19-47**	**VIM-1, SHV-12**		**>128**	**1**	**0.25**	**>128**	**>128**	**8**	**16**	**64**	**128**	**>4.0**	**64**	**2**	**128**	**8**	**2**
PF_19-47l (2x MIC@1)		None found	>128	4	0.5	>128	>128	32	>128	>128	>128	>4.0	>128	32	>128	2	2
***K. pneumoniae* PF_19-48**	**VIM-1, SHV-100[u]**		**0.06**	**0.25**	**0.12**	**>128**	**>128**	**32**	**32**	**>128**	**>128**	**>4.0**	**128**	**8**	**>128**	**1**	**2**
PF_19-48v (2x MIC@1)		None found	1	1	0.5	>128	>128	32	64	>128	>128	>4.0	>128	64	>128	2	4
***K. pneumoniae* PF_19-49**	**IMP-1; CTX-M-15; OXA-1; SHV-36; TEM-1**		**128**	**0.12**	**0.12**	**>128**	**>128**	**32**	**64**	**>128**	**>128**	**>4.0**	**>128**	**16**	**128**	**4**	**8**
PF_19-49h (2x MIC@4)		BaeS V295G; EnvZ I152N; *adhE* silent	128	2	1	>128	>128	>128	>128	>128	>128	>4.0	>128	32	>128	2	16
***K. pneumoniae* PF_19-50**	**IMP-1; SHV-53-p***		**0.12**	**0.06**	**0.06**	**>128**	**>128**	**32**	**64**	**>128**	**>128**	**>4.0**	**>128**	**32**	**>128**	**1**	**4**
PF_19-50k (4x MIC@1)		EnvZ I152S	1	0.5	0.25	>128	>128	128	128	>128	>128	>4.0	>128	32	>128	1	4
***E. coli* PF_19-51**	**NDM-5; CTX-M-15; OXA-1; T EM-1**		**>128**	**2**	**1**	**>128**	**>128**	**>128**	**>128**	**>128**	**>128**	**>4.0**	**>128**	**128**	**>128**	**>128**	**>128**
PF_19-51e (2x MIC@4)		BaeS Y42H	>128	16	16	>128	>128	>128	>128	>128	>128	>4.0	>128	64	>128	128	>128
PF_19-51n (8x MIC@1)		BaeS G241D; AraAQ26stop	>128	32	32	>128	>128	>128	>128	>128	>128	>4.0	>128	64	>128	>128	>128
***E. coli* PF_19-52**	**NDM-7; CTX-M-15; OXA-1; TEM-1**		**>128**	**8**	**4**	**>128**	**>128**	**>128**	**>128**	**>128**	**>128**	**>4.0**	**>128**	**>128**	**>128**	**128**	**>128**

PF_19-52d (2x MIC@4)		BaeR G23E	>128	32	16	>128	>128	>128	>128	>128	>128	>4.0	>128	>128	>128	128	>128
PF_19-52j (2x MIC@4)		BaeS F159L	>128	32	16	>128	>128	>128	>128	>128	>128	>4.0	>128	>128	>128	128	>128

[a] For mutant notation and abbreviations see Table 2

Parent strains are shown in bold font

Table 4. Mutations selected with aztreonam/avibactam in Isolates with OXA-48 β-lactamases

			MIC (mg/L)													
Strain	β-Lactamase(s)	Mutations found	ATM	ATM AVI1	ATM AVI4	CAZ	CAZ AVI4	FEP	FEP AVI4	CPT	CPT AVI4	AVI	CTX	MEM	TZP	CIP	AMK
***E. coli* PF_19-31**	**OXA-48, CTX-M-15, TEM-1**		**32**	**0.25**	**0.12**	**16**	**0.25**	**32**	**0.06**	**4**	**0.12**	**>4.0**	**>128**	**1**	**128**	**0.5**	**8**
PF_19-31m (2x MIC@1)[a]		BaeS P255A; RpoA L48A	64	1	0.25	16	0.5	16	0.06	4	0.06	>4.0	>128	2	128	1	8
***E. coli* PF_19-32**	**OXA-48**		**2**	**0.25**	**0.12**	**1**	**0.12**	**2**	**0.12**	**8**	**0.06**	**>4.0**	**8**	**64**	**>128**	**128**	**4**
PF_19-32n (2x MIC@1)		BaeS R416H	2	2	1	2	1	2	0.25	8	0.25	>4.0	8	64	>128	128	4
***K. pneumoniae* PF_19-33**	**OXA-48, CTX-M-15, SHV-1**		**128**	**0.12**	**0.06**	**64**	**0.5**	**>128**	**≤0.03**	**8**	**0.12**	**>4.0**	**>128**	**2**	**>128**	**0.06**	**4**
PF_19-33h (2x MIC@4)		BaeS T279I	128	1	0.25	64	1	>128	≤0.03	8	0.25	>4.0	>128	2	>128	≤0.03	2

[a] For mutant notation and abbreviations see Table 2

Parent strains are shown in bold font

Table 5. Mutations selected with aztreonam/avibactam in Isolates with ESBLs

			MIC (mg/L)													
Strain	β-Lactamase(s)	Mutations found	ATM	ATM AVI1	ATM AVI4	CAZ	CAZ AVI4	FEP	FEP AVI4	CPT	CPT AVI4	AVI	CTX	MEM	TZP	CIP	AMK
***K. pneumoniae* PF_19-15**	**CTX-M-15; OXA-9; SHV-39; TEM-191-p**		**>128**	**0.5**	**0.25**	**>128**	**1**	**64**	**0.06**	**8**	**0.5**	**>4.0**	**>128**	**0.06**	**8**	**4**	**16**
PF_19-15f (2x MIC@4)[a]	CTX-M-15	None found	>128	4	1	>128	2	64	0.06	8	0.5	>4.0	>128	0.06	>128	4	32
***E. coli* PF_19-17**	**CTX-M-14. TEM-1**		**32**	**0.5**	**0.25**	**4**	**0.5**	**128**	**0.12**	**8**	**0.25**	**>4.0**	**>128**	**0.06**	**4**	**128**	**2**
PF_19-17m (2x MIC@1)	CTX-M-14	BaeS Q163H	32	1	0.5	4	1	128	0.12	8	0.12	>4.0	>128	0.06	8	128	4
***K. pneumoniae* PF_19-21**	**SHV-5, SHV-1**		**>128**	**0.5**	**0.12**	**>128**	**0.5**	**8**	**0.06**	**8**	**0.25**	**>4.0**	**128**	**0.12**	**8**	**1**	**4**
PF_19-21c (2x MIC@4)			>128	8	1	>128	4	32	0.06	8	0.12	>4.0	>128	0.12	>128	1	2
***K. pneumoniae* PF_19-22**	**SHV-2**		**16**	**0.06**	**0.12**	**64**	**0.5**	**32**	**0.06**	**8**	**0.12**	**>4.0**	**>128**	**0.12**	**>128**	**0.06**	**4**
PF_19-22g (2x MIC@1)		EnvZ F284V	>128	1	0.25	>128	2	>128	0.06	32	0.12	>4.0	>128	0.12	>128	0.06	2
***E. coli* PF_19-23**	**TEM-10**		**128**	**0.25**	**0.12**	**>128**	**0.5**	**4**	**≤0.03**	**4**	**0.06**	**>4.0**	**4**	**≤0.03**	**8**	**≤0.03**	**2**
PF_19-23d (2x MIC@4)		EnvZ L23P	>128	2	1	>128	8	64	1	8	1	>4.0	16	0.25	64	≤0.03	2

[a] For mutant notation and abbreviations see Table 2. Parent strains are shown in bold font

589 **Table 6.** Mutations selected with aztreonam/avibactam in Isolates with AmpC β-lactamases
590

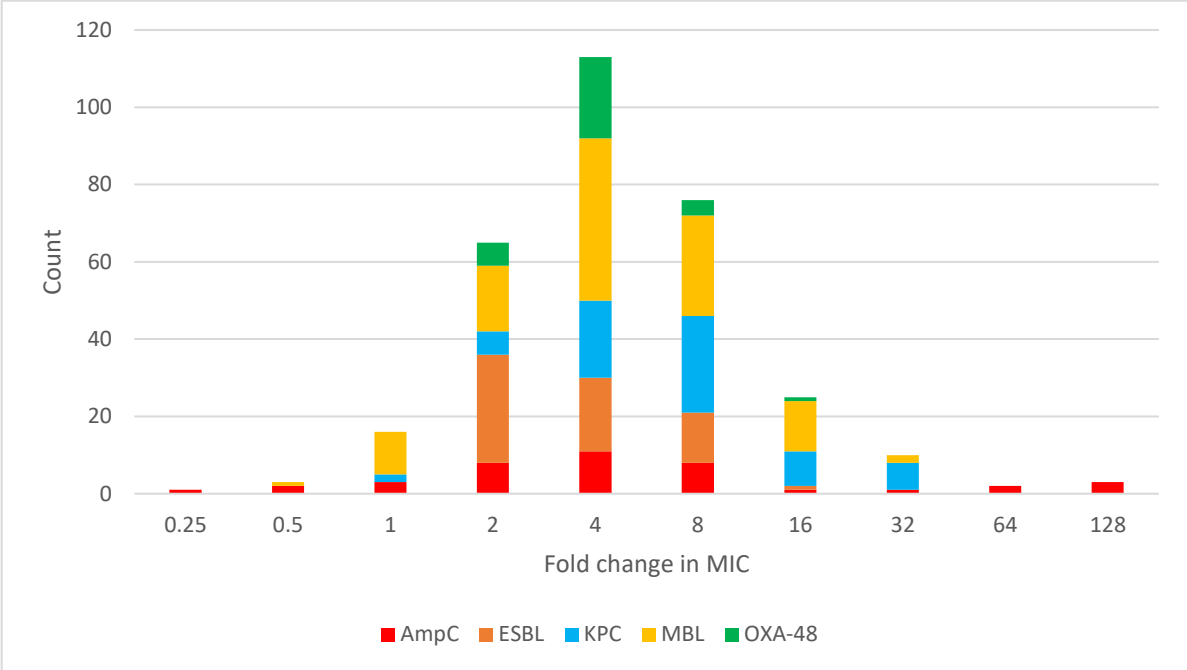
			MIC (mg/L)													
Strain	β-Lactamase(s)	Mutations found	ATM	ATM AVI1	ATM AVI4	CAZ	CAZ AVI4	FEP	FEP AVI4	CPT	CPT AVI4	AVI	CTX	MEM	TZP	CIP	AMK
***E. coli* PF_19-25**	**CMY-2; OXA-1; TEM-1**		**8**	**0.25**	**0.12**	**32**	**0.25**	**1**	**0.06**	**4**	**0.12**	**>4.0**	**8**	**≤0.03**	**64**	**128**	**16**
PF_19-25c (2x MIC@1)[a]		None found	>128	8	1	>128	1	8	0.12	32	0.25	>4.0	128	0.25	>128	128	16
***E. coli* PF_19-26**	**CMY-44[v]; TEM-1**		**16**	**0.5**	**0.25**	**64**	**0.5**	**0.5**	**0.06**	**8**	**0.06**	**>4.0**	**16**	**≤0.03**	**8**	**8**	**4**
PF_19-26a (4x MIC@4)		CMY-44 Y150C	64	64	16	4	0.5	0.25	0.06	2	0.25	>4.0	2	≤0.03	16	8	4
PF_19-26l (2x MIC@1)		repZ silent	32	32	8	>128	4	2	0.06	32	2	>4.0	64	0.06	128	8	8
***E. cloacae* PF_19-27**	**AmpC**		**64**	**2**	**0.5**	**128**	**1**	**1**	**0.12**	**16**	**1**	**>4.0**	**>128**	**0.12**	**128**	**≤0.03**	**2**
PF_19-27s (2x MIC@1)		UgpE I211L	>128	128	4	>128	8	16	0.25	>128	4	>4.0	>128	8	>128	≤0.03	1
***E. cloacae* PF_19-28**	**AmpC**		**32**	**2**	**0.5**	**>128**	**0.5**	**2**	**0.12**	**16**	**0.5**	**>4.0**	**>128**	**0.06**	**128**	**≤0.03**	**2**
PF_19-28b (4x MIC@4)		PBP3 A498T	64	16	4	>128	8	32	2	32	2	>4.0	>128	0.12	>128	≤0.03	2
PF_19-28r (2x MIC@1)		None found	128	16	2	>128	2	32	0.5	64	2	>4.0	>128	0.25	>128	≤0.03	2

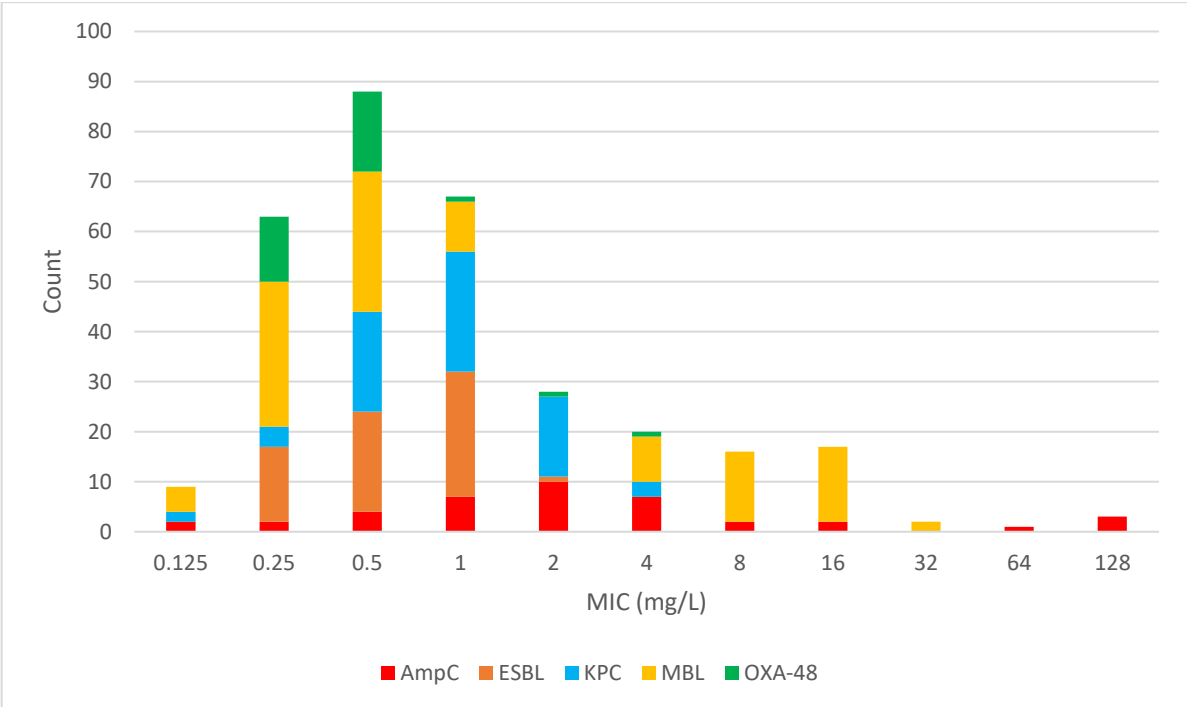
***E. cloacae* PF_19-29**	**AmpC**		**64**	**2**	**0.5**	**128**	**1**	**2**	**0.25**	**32**	**0.5**	**>4.0**	**>128**	**0.25**	**128**	**0.06**	**4**
PF_19-29f (2x MIC@4)		RpoC G82V; LptD G389V	>128	16	4	>128	4	16	1	32	2	>4.0	>128	0.25	>128	0.06	2
PF_19-29t (2x MIC@1)		LptD G389V	128	16	1	128	1	8	0.12	64	0.5	>4.0	>128	4	>128	≤0.03	2
***E. cloacae* PF_19-30**	**AmpC**		**64**	**4**	**1**	**>128**	**1**	**4**	**0.12**	**16**	**1**	**>4.0**	**>128**	**0.12**	**>128**	**≤0.03**	**2**
PF_19-30a (8x MIC@4)		AmpC Y150C	>128	>128	128	8	1	0.5	0.25	16	4	>4.0	8	0.06	128	0.06	2
PF_19-30h (2x MIC@4)		AmpC N346Y	64	32	16	>128	128	8	2	128	64	>4.0	128	≤0.03	128	≤0.03	2
PF_19-30n (4x MIC@4)		GalU R21W	128	16	4	>128	1	16	0.12	64	1	>4.0	>128	4	>128	0.06	2

[a] For mutant notation and abbreviations see Table 2

Parent strains are shown in bold font

Figure 1





MIC shifts for mutants selected with aztreonam/avibactam: panel A, distribution of fold changes of aztreonam/avibactam 4 mg/L; panel B, distribution of MICs of aztreonam/avibactam 4 mg/L