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Klebsiella pneumoniae mutants resistant to ceftazidime/avibactam plus aztreonam, imipenem/relebactam, meropenem/vaborbactam and cefepime/taniborbactam.

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Running Head: β-lactam/β-lactamase inhibitor resistance

ABSTRACT

We show that a previously described ceftazidime/avibactam plus meropenem/vaborbactam resistant *Klebsiella pneumoniae* variant having *ramR* plus *ompK36* mutation and producing the V239G variant KPC-3 (V240G *per* standard numbering system) confers resistance to ceftazidime/avibactam plus aztreonam and imipenem/relebactam but not cefepime/taniborbactam. The V239G variant does not generate collateral β-lactam susceptibility like many KPC-3 variants associated with ceftazidime/avibactam resistance. Additional mutation of *ompK35* and production of the OXA-48-like carbapenemase OXA-232 were required to confer cefepime/taniborbactam resistance.

TEXT

Aztreonam/avibactam (AZT/AVI) is a β-lactam/β-lactamase inhibitor combination currently in clinical trials, which has activity against Enterobacterales producing metallo-carbapenemases and those with AZT-hydrolysing enzymes such as plasmid-mediated AmpCs (pAmpCs), extended-spectrum β-lactamases (ESBLs) and the serine carbapenemase KPC. All these enzymes are increasingly carried in *Klebsiella pneumoniae*, and yet few studies have been performed to consider mechanisms of AZT/AVI resistance in this species. It was recently reported that among 8787 Enterobacterales isolates, 17 were AZT/AVI resistant. Of these, three *Klebsiella* spp. were identified. Production of the pAmpC, DHA-1 plus *acrA* efflux pump gene overexpression and mutation of *ompK35* or *ompK36* porins were identified in two resistant isolates. The other produced the ESBL PER-2 and carried an *ompK35* loss of function mutation (1). In one *in vitro* study, selecting AZT/AVI resistance identified mutations in the pAmpC, CMY-16 in a *K. pneumoniae* strain (2). AVI is currently in clinical use partnered by ceftazidime (CAZ/AVI) and here, mutations in KPC are known to confer resistance. However, such mutations tend to reduce hydrolytic activity to β-lactams other than CAZ, including carbapenems and AZT (3-6). Accordingly, it is conceivable that such mutant KPC enzymes might not confer AZT/AVI resistance.

Another recently licenced β-lactam/β-lactamase inhibitor combination is imipenem/relebactam (IMI/REL). Unlike AZT/AVI, this does not have efficacy against isolates producing metallo-carbapenemases, but is generally efficacious against Enterobacterales producing pAmpC, KPC and ESBLs (7). Again, analysis of clinical

isolates shows that IMI/REL resistance in *K. pneumoniae* is rare, but resistant isolates have mutations in or reduced expression of *ompK35* and/or *ompK36* porin genes and/or increased *acrA* efflux pump gene expression, alongside ESBL production (8). Similar impacts of porin and efflux pump production on IMI/REL susceptibility have been seen in *in vitro* studies using KPC-producing isolates (9).

Given seeming overlaps between AZT/AVI and IMI/REL resistance mechanisms in *K. pneumoniae*, we set out to dissect the mechanisms contributing to resistance to each in *K. pneumoniae* using a bank of clinical isolates and targeted recombinants having fully defined genotypes. **Table 1** reports MICs (determined using CLSI broth microdilution methodology [10,11]) of AZT/AVI and IMI/REL against a collection of clinical isolates, which have been previously described (12) and their β-lactam resistance genotypes characterised (13). All isolates, whether producing carbapenemases of classes A (KPC-3), B (NDM-1) or D (OXA-232) were AZT/AVI susceptible, but the NDM-1/OXA-232 producer KP4 was, as expected, IMI/REL resistant, as was the OXA-232 producer KP11, though with lower MICs (**Table 1**). Notably, KP4 and KP11 have *ramR* mutations (12), which lead to overproduction of AcrAB-TolC efflux pump, and reduced production of the OmpK35 porin in *K. pneumoniae* (14). Nonetheless, a *ramR* mutant clinical isolate producing KPC-3, KP30, was susceptible to both AZT/AVI and IMI/REL (**Table 1**) so we conclude that modulating production of these permeability-associated proteins is not sufficient to give resistance to either β-lactam/β-lactamase inhibitor combination in a KPC-3 positive background.

To investigate the effect of *bla*$_{KPC-3}$ mutations known to be associated with CAZ/AVI resistance (15) on AZT/AVI and IMI/REL susceptibility, we took *K. pneumoniae* clinical isolate KP21, which is a *ramR* mutant and fully susceptible to AZT and IMI (**Table 1**). We introduced *bla*$_{KPC-3}$ on a plasmid (pKPC-3), either wild-type or following site-directed mutagenesis to create the D178Y or V239G amino acid substitutions previously associated with CAZ/AVI resistance (15). Here we use numbering based on the KPC-3 amino acid sequence [16]; these substitutions are frequently referred to in the literature as D179Y and V240G using a standardised numbering system for class A β-lactamases (15). The construction of these variant KPC-3 plasmids has been reported previously (17). Reduced MICs of AZT and IMI were observed against KP21 carrying the D178Y variant, compared with KP21 carrying wild-type KPC-3 (**Table 1**). This phenomenon of reduced spectrum of β-lactamase activity has been described for other *bla*$_{KPC-3}$ mutants associated with CAZ/AVI resistance (3-6). However, in a KP21 background, this reduction in activity was seen to a lesser extent when the KPC-3 V239G variant was proesent (**Table 1**). This observation fits with previous reports that *K. pneumoniae* carrying the V239G mutant *bla*$_{KPC-3}$ remain meropenem resistant, while those carrying the D178Y mutant are meropenem susceptible (15, 17). However, AZT/AVI and IMI/REL MICs were not greatly elevated against KP21 carrying pKPC-3 V239G in comparison with KP21 carrying pKPC-3, and all these KP21 recombinants remained AZT/AVI and IMI/REL susceptible (**Table 1**). We conclude, therefore, that mutating *bla*$_{KPC-3}$ in a way that gives CAZ/AVI resistance is not sufficient to give AZT/AVI or IMI/REL resistance, even in a *ramR* mutant *K. pneumoniae* background.

Addition of an OXA-232 (class D carbapenemase) plasmid (pOXA-232, as described in our previous work [17]) to KP21 carrying pKPC-3 D178Y or pKPC-3 did not confer IMI/REL resistance (**Table 1**). This is because, despite the fact that REL does not notably inhibit class D β-lactamases (7), pOXA-232 does not confer IMI resistance, even in the absence of REL in KP21 (**Table 1**). Hence, if KPC-3 is inhibited by REL, OXA-232 cannot confer IMI resistance in KP21 alone (**Table 1**). However, importantly, adding pOXA-232 to the KP21 recombinant carrying pKPC-3 V239G conferred IMI/REL (but not AZT/AVI) resistance, showing that even the weakly expressed imipenemase OXA-232 can act in synergy with the partially inhibited KPC-3 V239G variant and together they can confer IMI/REL resistance.

Disruption of the *ompK36* porin gene in KP21 (as described previously[17]) conferred AZT/AVI and IMI/REL resistance when the recombinant was carrying pKPC-3 V239G, but not when it carried pKPC-3 D178Y or pKPC-3. Addition of pOXA-232 to the KP21 *ompK36* recombinants further raised IMI/REL MICs against the pKPC-3 V239G recombinant, and conferred IMI/REL resistance by acting in synergy with wild-type KPC-3 in the recombinant carrying pKPC-3, but not pKPC-3 D178Y (**Table 1**). Using the *ramR* wild-type isolate KP47 engineered to have an *ompK36* mutation we confirmed that *ramR* mutation is essential for the AZT/AVI (but not IMI/REL) resistance seen in KP21 *ompK36* pKPC-3 V239G (**Table 1**).

We therefore conclude that three steps: mutation of *ramR*, mutation of *ompK36* and carriage of the V239G variant of bla_{KPC-3} is sufficient for *K. pneumoniae* to become resistant to both AZT/AVI and IMI/REL. However, prior to clinical approval of AZT/AVI, this combination is usually created clinically by adding AZT to CAZ/AVI

therapy. A checkerboard assay confirmed that AZT/AVI and IMI/REL resistant derivative KP21[*ramR*] *ompK36* pKPC-3 V239G is also resistant to CAZ/AVI plus AZT, with MICs of CAZ (>32 μg.ml^{-1}) and AZT (16 μg.ml^{-1}) against this recombinant (**Figure 1**).

We have previously shown that this combination of *ramR* and *ompK36* mutation coupled with acquisition of pKPC-3 V239G also gives resistance to CAZ/AVI and another licenced β-lactam/β-lactamase inhibitor combination, meropenem/vaborbactam (17). Finally, therefore, we tested cefepime/taniborbactam a combination in late stage clinical trials (18). Notably, in the KP21[*ramR*] *ompK36* background, pKPC-3 D178Y supported lower cefepime MICs than pKPC-3 and pKPC-3 V239G (**Table 2**), as seen for the other β-lactams (**Table 1**) and this was also true for cefepime/taniborbactam MICs (**Table 2**). In contrast, pKPC-3 V239G supported the same cefepime/taniborbactam MIC as pKPC-3 in KP21[*ramR*] *ompK36*, being 8 μg.ml^{-1}, which is one doubling dilution below the cefepime resistance breakpoint (11) (**Table 2**). Further addition of pOXA-232 elevated cefepime MICs against the KP21[*ramR*] *ompK36* pKPC-3 D178Y recombinant (**Table 2**), as expected since OXA enzymes are known to hydrolyse cefepime (19). Even without OXA-232, cefepime MIC against KP21[*ramR*] *ompK36* pKPC-3 KPC-3 or pKPC-3 V239G were >256 μg.ml^{-1}, so any additional effect of OXA-232 could not be measured. Nonetheless, cefepime/taniborbactam MIC remained at ≤ 8 μg.ml^{-1} against all KP21[*ramR*] *ompK36* recombinants, indicating successful inhibition of OXA-232 (**Table 2**). However, additional insertional inactivation of the *ompK35* porin gene (performed as described previously [17]) pushed the cefepime/taniborbactam MIC against the KP21[*ramR*] *ompK36* recombinant carrying pOXA-232 and pKPC-3

V239G (but not pKPC-3 or pKPC-3 D178Y) to 16 μg.ml^{-1}, which is classed as cefepime resistant (**Table 2**).

We conclude, therefore, that whilst three events (*ramR*, *ompK36*, *bla*$_{KPC-3}$ V239G) are sufficient to cause CAZ/AVI/AZT, IMI/REL and, as previously shown, meropenem/vaborbactam resistance in *K. pneumoniae*, additional events are required to give cefepime/taniborbactam resistance. Furthermore, whilst many *bla*$_{KPC-3}$ mutations leading to CAZ/AVI resistance do come with the collateral effect of increased susceptibility to carbapenems, late generation cephalosporins and AZT, KPC-3 V239G does not suffer from this effect to the same degree. This explains why KPC-3 V239G, rather than KPC-3 D178Y, which does suffer from collateral increased susceptibility, is able to confer resistance to multiple β-lactam/β-lactamase inhibitor combinations, provided their accumulation is slowed. The biochemical basis of why KPC-3 V239G does not behave like KPC-3 D178Y, which as an activity biased towards ceftazidime (20) requires clarification. Nonetheless, the emergence of this *bla*$_{KPC-3}$ V239G variant should be watched with caution.

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Transparency declaration

The authors declare no conflict of interests.

Figure Legend

Figure 1. Checkerboard assays for CAZ and AZT in the presence of AVI against *K. pneumoniae* KP21[*ramR*] *ompK36* producing KPC-3 V239G.

The image represents duplicate assays for an 8×8 array of wells in a 96-well plate. All wells contained CA-MHB including avibactam (4 μg.ml^{-1}). A serial dilution of aztreonam (AZT, x-axis) and ceftazidime (CAZ, y-axis) was created from 32 μg.ml^{-1} in each plate as recorded. All wells were inoculated with a suspension of bacteria, made as per CLSI microtiter MIC guidelines (10), and the plate was incubated at 37°C for 20 h. Growth was recorded by measuring OD_{600} and growth above background (broth) is recorded as a yellow block; no growth is recorded as a white block. Growth in the red edged block indicates resistance to both AZT and CAZ.

Figure 1

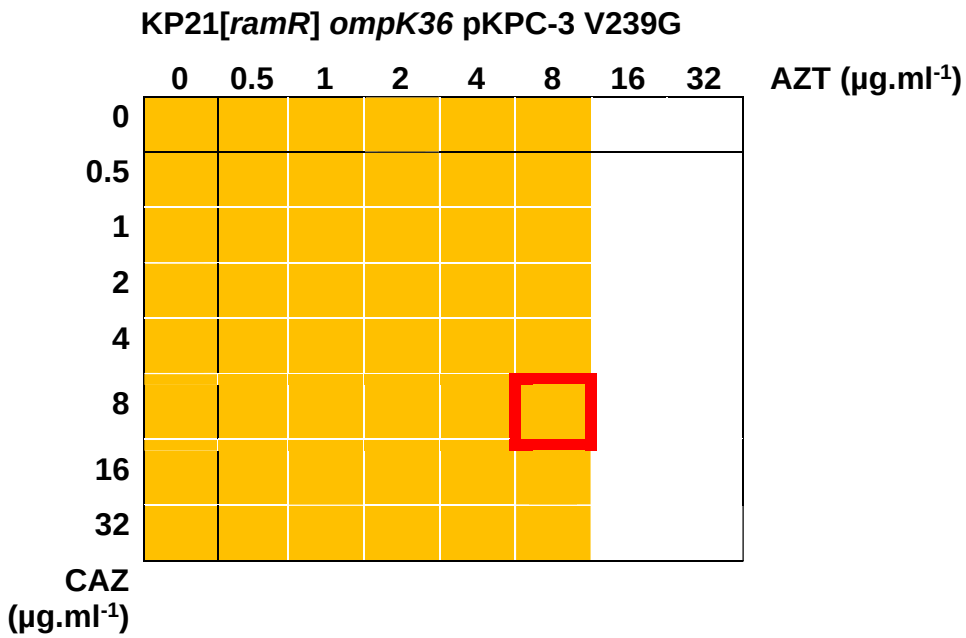
KP21[*ramR*] *ompK36* pKPC-3 V239G
0
0.5
1
2
4
8
16
32
AZT (µg.ml-1)
CAZ (µg.ml-1)

Table 1 MICs of aztreonam or imipenem with or without avibactam or relebactam against *K. pneumoniae* clinical isolates and derivatives of isolates KP21 and KP47

Isolate (relevant genotype	MIC (μg.ml^{-1})			
	AZT	**AZT/AVI**	**IMI**	**IMI/REL**
KP31 (wild-type)	≤0.5	≤0.5	≤0.5	≤0.5
KP21 (*ramR* TEM-1)	≤0.5	≤0.5	≤0.5	≤0.5
KP11 (*ramR* OXA-232 CTX-M-15 TEM-1)	>128	≤0.5	4	4
KP30 (*ramR ompK35* KPC-3 TEM-1)	>128	1	>128	1
KP4 (*ramR* NDM-1 OXA-232 CTX-M-15 TEM-1)	>128	≤0.5	64	16
KP21[*ramR*] pUBYT	0.5	≤0.5	0.5	≤0.5
KP21[*ramR*] pKPC-3	>128	1	64	2
KP21[*ramR*] pKPC-3 D178Y	1	≤0.5	1	0.5
KP21[*ramR*] pKPC-3 V239G	>128	2	32	2
KP21[*ramR*] pUBYT pOXA-232	0.5	≤0.5	2	1
KP21[*ramR*] pKPC-3 pOXA-232	>128	2	128	2
KP21[*ramR*] pKPC-3 D178Y pOXA-232	16	2	2	2
KP21[*ramR*] pKPC-3 V239G pOXA-232	>128	4	32	8
KP21[*ramR*] *ompK36* pUBYT	0.5	1	2	0.5
KP21[*ramR*] *ompK36* pKPC-3	>128	2	128	2
KP21[*ramR*] *ompK36* pKPC-3 D178Y	8	2	1	0.5
KP21[*ramR*] *ompK36* pKPC-3 V239G	>128	16	128	4
KP21[*ramR*] *ompK36* pUBYT pOXA-232	1	1	4	2
KP21[*ramR*] *ompK36* pKPC-3 pOXA-232	>128	2	>128	32
KP21[*ramR*] *ompK36* pKPC-3 D178Y pOXA-232	8	4	8	2
KP21[*ramR*] *ompK36* pKPC-3 V239G pOXA-232	>128	16	>128	32
KP47 *ompK36* pUBYT	0.5	≤0.5	0.5	0.5
KP47 *ompK36* pKPC-3	>128	1	>128	8
KP47 *ompK36* pKPC-3 D178Y	16	2	1	0.5
KP47 *ompK36* pKPC-3 V239G	>128	4	>128	16

Shading indicates resistance based on CLSI breakpoints (11)

Table 2 MICs of cefepime/taniborbactam against derivatives of *K. pneumoniae* clinical isolate KP21

Isolate	MIC (μg.ml^{-1})	
	Cefepime	**Cefepime/ Taniborbactam**
KP21[*ramR*] *ompK36* pUBYT	8	1
KP21[*ramR*] *ompK36* pKPC-3	>256	8
KP21[*ramR*] *ompK36* pKPC-3 D178Y	16	2
KP21[*ramR*] *ompK36* pKPC-3 V239G	>256	8
KP21[*ramR*] *ompK36* pUBYT pOXA-232	8	1
KP21[*ramR*] *ompK36* pKPC-3 pOXA-232	>256	8
KP21[*ramR*] *ompK36* pKPC-3 D178Y pOXA-232	64	1
KP21[*ramR*] *ompK36* pKPC-3 V239G pOXA-232	>256	8
KP21[*ramR*] *ompK36 ompK35* pUBYT	8	1
KP21[*ramR*] *ompK36 ompK35* pKPC-3	>256	8
KP21[*ramR*] *ompK36 ompK35* pKPC-3 D178Y	16	2
KP21[*ramR*] *ompK36 ompK35* pKPC-3 V239G	>256	8
KP21[*ramR*] *ompK36 ompK35* pUBYT pOXA-232	8	2
KP21[*ramR*] *ompK36 ompK35* pKPC-3 pOXA-232	>256	8
KP21[*ramR*] *ompK36 ompK35* pKPC-3 D178Y pOXA-232	64	2
KP21[*ramR*] *ompK36 ompK35* pKPC-3 V239G pOXA-232	>256	16

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