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Investigation of mechanisms responsible for decreased susceptibility of aztreonam/avibactam activity in clinical isolates of Enterobacterales collected in Europe, Asia and Latin America in 2019

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Background: The combination aztreonam/avibactam is currently under Phase 3 trials for the treatment of serious infections caused by Gram-negative bacteria including those with MBLs.

Objectives: To investigate the resistance mechanisms in Enterobacterales exhibiting aztreonam/avibactam MICs of ≥4 mg/L.

Methods: Among 8787 Enterobacterales, 17 (0.2%) isolates exhibited an aztreonam/avibactam MIC of ≥4 mg/L. Isolates were sequenced and screened for β-lactamases. Sequences of porins, penicillin-binding protein 3 (PBP3) and expression levels of AmpC and AcrA were evaluated.

Results: Eleven (11/4154 isolates; 0.26%) *Escherichia coli*, three (3/1981; 0.15%) *Klebsiella pneumoniae* and three (3/628; 0.5%) *Enterobacter cloacae* were identified. All *E. coli* showed either an 'YRIK' or 'YRIN' insertion in PBP3. In general, these isolates carried bla_{CMY} and/or $bla_{CTX\text{-}M}$ variants, except for one isolate from Korea that also produced NDM-5 and one isolate from Turkey that produced OXA-48. Two DHA-1-producing *K. pneumoniae* overexpressed *acrA* and had a premature stop codon in either OmpK35 or OmpK36, whereas a third *K. pneumoniae* carried $bla_{PER\text{-}2}$ and had a premature stop codon in OmpK35. All three *E. cloacae* expressed AmpC at levels ≥570-fold, but sequence analysis did not reveal known amino acid alterations associated with decreased avibactam binding or increased hydrolysis of β-lactams. Minor amino acid polymorphisms within OmpC, OmpF and PBP3 were noted among the *E. cloacae*.

Conclusions: A small number of isolates (0.2%) met the inclusion criteria. *E. coli* showed altered PBP3 as the most relevant resistance mechanism, whereas *K. pneumoniae* had multiple resistance mechanisms. Further investigations are needed to clarify resistance in *E. cloacae*.

Introduction

Antimicrobial resistance remains a great concern worldwide, especially among Gram-negative bacteria. The latest report from the US CDC estimated 197 400 cases and 9100 deaths caused by Enterobacterales resistant to expanded-spectrum cephalosporins (ESC), and 13 100 cases and 1100 deaths caused by Enterobacterales resistant to carbapenems (CRE).[1] In Europe, 31.7% of *Klebsiella pneumoniae* were reported as resistant to ESC and 7.5% of *K. pneumoniae* were reported as resistant to carbapenems in 2018. Resistance rates varied greatly (0%–78%) among the 30 European countries, but most countries (18) reported resistance rates for ESC higher than 20%. Moreover, carbapenem resistance among *K. pneumoniae* remained below 4% in most countries, but occurrences between 8% and 30% were reported in seven countries, and a rate as high as 64% was reported in Greece.[2]

The occurrences of resistance phenotypes to ESC and carbapenems are considered serious and urgent threats, respectively.[1] These threats prompted the development of new therapeutic options and/or strategies as part of a global action plan against antimicrobial resistance.[3] Aztreonam/avibactam, a monobactam/β-lactamase inhibitor (BLI) combination, is undergoing Phase 3 clinical trials for treating infections caused by Gram-negative organisms including those producing MBLs.[4] In contrast to most β-lactams, monobactams are not substrates for MBLs, whereas avibactam reversely inactivates most Class A and C and some D β-

lactamase enzymes.[5] Thus, this combination mitigates resistance caused by most ESBL, including carbapenemases.[6]

The *in vitro* activity of aztreonam/avibactam was assessed against a large collection of contemporary (2019) clinical Enterobacterales recovered from patients hospitalized in medical centres located in Europe, Latin America and the Asia-Pacific region.[7] In this previous study, a total of 18 Enterobacterales displayed an aztreonam/avibactam MIC of ≥4 mg/L. These isolates were selected for molecular characterization to investigate the resistance mechanisms associated with this phenotype. This study expands on the previous publication[7] to report on the epidemiological typing and resistance mechanisms observed among these select pathogens.

Materials and methods

The original study included 8787 Enterobacterales collected consecutively in 2019 from 64 medical centres in Europe, Russia and Turkey ($n = 6170$); the Asia-Pacific region ($n = 1456$); and Latin America ($n = 1161$). Information related to these organisms can be obtained in Sader *et al.* (2021).[7] Within this collection, 18 (0.2%) isolates exhibited an aztreonam/avibactam MIC of ≥4 mg/L (Table 1): 11 *Escherichia coli*, 3 *K. pneumoniae*, 3 *Enterobacter cloacae* species complex, and 1 *Proteus vulgaris*. The *P. vulgaris* was later found to be non-viable and was therefore excluded from further characterization (Table 1). Susceptibility testing was performed by reference broth microdilution according to CLSI.[8,9]

DNA extraction was performed with the ThermoScientific™ KingFisher™ Flex Magnetic Particle Processor (Cleveland, OH, USA) and used as input material for library construction. Libraries were normalized using the bead-based normalization procedure (Illumina) and then sequenced on MiSeq (Reagent Kit v2; 2 × 250 paired reads; 500 cycles). *De novo* assembled FASTQ files were screened for β-lactamases, as previously described.[10] Gene sequences encoding for penicillin-binding protein 3 (PBP3), OmpC/OmpK36 and OmpF/Ompk35 were investigated. Sequence analysis comparison was performed using sequences from a control isolate belonging to the same MLST as the query sequence. Isolates were subjected to the quantification of AmpC (except for *K. pneumoniae*) and AcrA (AcrAB-TolC) expression.[11]

Results

Eleven (11/4154 surveillance isolates; 0.26%) *E. coli* had elevated aztreonam/avibactam MICs (4–16 mg/L). Ceftazidime/avibactam MICs of 1–8 mg/L were obtained against these isolates, except for one *E. coli* from Korea that carried bla_{NDM-5} and $bla_{OXA-181}$ (MIC, >32 mg/L) (Tables 1 and 2). Elevated MIC results for aztreonam and ESC (≥8 mg/L) were obtained against *E. coli*, whereas low MIC values were noted for meropenem (0.03–0.12 mg/L) and imipenem (≤0.12–1 mg/L), except against the isolate from Korea that carried bla_{NDM-5} and $bla_{OXA-181}$ (imipenem and meropenem MIC, >8 mg/L) and one isolate from Turkey with a bla_{OXA-48} (imipenem MIC, 4 mg/L) (Tables 1 and 2).

These 11 *E. coli* isolates carried multiple ESBL and plasmid AmpC-encoding genes, mostly consisting of CTX-M and CMY variants (Table 2). Four ST types were observed, with five isolates from two sites in Turkey belonging to ST410. Additionally, the NDM-5-producing *E. coli* strain from Korea belonged to ST410 (Table 2). All *E. coli* showed amino acid alterations in the PBP3 sequence either as an 'YRIK' or 'YRIN' insertion after amino acid 333. Overexpression of either the intrinsic *ampC* (≤1.2-fold) or *acrA* (≤4.5-fold) gene was not detected in any *E. coli*.

Aztreonam/avibactam MICs of 8 mg/L or >16 mg/L and ceftazidime/avibactam MICs of 4 mg/L or 16 mg/L were observed in three *K. pneumoniae* among a collection of 1981 (0.15%) isolates (Table 1). In general, these isolates had elevated MICs for β-lactams and β-lactam/BLI combinations; however, isolate 1122568 had a lower MIC for aztreonam/clavulanate (0.25 mg/L) and ceftazidime/clavulanate (1 mg/L). These *K. pneumoniae* remained susceptible to carbapenems (MIC, 0.5–1 mg/L), except for one strain from Bangkok, which displayed an imipenem and meropenem MIC of 4 mg/L (Table 1). All three isolates had elevated MICs for ertapenem.

The *K. pneumoniae* isolate 1116221 carried DHA-1 and had a premature stop codon at position 43 of OmpK36, whereas WT sequences were observed for OmpK35 and PBP3 (Table 2). This isolate showed expression of *acrA* 6.2-fold higher than the control strain (Table 2). bla_{PER-2} and bla_{DHA-1} were detected in isolates 1122568 and 1125511, respectively, and displayed premature stop codons in OmpK35 as well as amino acid alterations in PBP3 (Table 2). Expression of *acrA* in isolate 1125511 was 5.5-fold higher than the control strain.

Three (3/628; 0.5%) isolates identified as *E. cloacae* species complex displayed aztreonam/avibactam MICs of 4–16 mg/L and ceftazidime/avibactam MICs of 1–4 mg/L. These isolates exhibited elevated MICs to other β-lactams and β-lactam/BLI combinations, but remained susceptible to carbapenems, with the exception of isolate 1108008 (MIC, ≥2 mg/L) and isolate 1118254 (ertapenem MIC, 2 mg/L). All three isolates demonstrated a high-level expression of AmpC (≥570-fold). Additionally, isolates 1108008 and 1118254 carried $bla_{CTX-M-15}$ and bla_{SHV-12}, respectively (Table 2). In general, the *E. cloacae* complex isolates showed minor amino acid polymorphisms within OmpC and OmpF, except for isolate 1102685, which had multiple alterations within OmpF (Table 2). No amino acid alterations within the AmpC enzyme were noted (Figure S1, available as Supplementary data at *JAC* Online), but isolates 1102685 and 1118254 showed within PBP3, respectively, a G306V and a glutamic acid insertion at position 259 (Table 2).

Discussion

A total of 11 *E. coli* isolates were selected for this study; of these isolates, 9 isolates had the 'YRIK' insertion and 2 isolates had the 'YRIN' insertion after position 333 of PBP3. These insertions were previously described by Alm *et al.*[12] to cause decreased aztreonam binding at the target site and were further evaluated by Sadek *et al.* (2020).[13] Isolates possessing an altered PBP3 and bla_{NDM} would be refractory to aztreonam/avibactam and any clinically available β-lactams and β-lactam/BLI combinations. Recent studies reported a high prevalence of NDM-producing *E. coli* with PBP3 insertions, which seem to be more prevalent in India.[13,14] However, other surveillance studies reported a low proportion (≤0.3%) of Enterobacterales with aztreonam/avibactam MICs of ≥4 mg/L; these isolates tended to be carbapenem susceptible.[15] A narrow aztreonam/avibactam MIC range (4–16 mg/L) was obtained against *E. coli* as well as for ceftazidime/avibactam (1–8 mg/L), except against the NDM-5-producing *E. coli* (>32 mg/L). These results indicate that the PBP3 mutations are essentially driving the higher aztreonam/avibactam MICs and the MIC variation (4–16 mg/L) may be caused by the β-lactamase background, as demonstrated previously.[13]

The three *K. pneumoniae* had aztreonam/avibactam MICs of ≥8 mg/L and ceftazidime/avibactam MICs of 4–16 mg/L. The

Table 1. Isolates exhibiting aztreonam/avibactam MIC results ≥4 mg/L selected for further characterization of resistance mechanisms

					MIC (mg/L)													
Collection number	Site code	Country	City	Organism	ATM	ATM/AVI	ATM/CLA	CAZ	CAZ/AVI	CAZ/CLA	COZ/TZB	CRO	FEP	SAM	TZP	MEM	IPM	ETP
1108470	68	Turkey	Ankara	*E. coli*	>64	8	32	>128	2	>128	>16	>8	>256	>64	>128	0.03	0.25	0.12
1108523	68	Turkey	Ankara	*E. coli*	32	8	32	>128	2	>128	>16	>8	32	>64	>128	0.03	0.25	0.12
1108694	68	Turkey	Ankara	*E. coli*	64	8	64	>128	2	>128	>16	>8	8	64	128	0.06	0.5	0.12
1114251	69	Turkey	Istanbul	*E. coli*	>64	16	32	>128	8	>128	>16	>8	>256	>64	>128	0.06	1	0.5
1114255	69	Turkey	Istanbul	*E. coli*	64	8	64	>128	8	>128	>16	>8	128	>64	>128	1	4	>2
1118669	606	Korea	Kangwondo	*E. coli*	>64	4	32	>128	>32	>128	>16	>8	>256	>64	>128	32	>8	>2
1116284	603	Thailand	Bangkok	*E. coli*	>64	8	16	>128	4	16	>16	>8	>32	64	>128	0.03	≤0.12	0.5
1128667	380	France	Rennes Cedex	*E. coli*	>64	4	8	>128	1	8	>16	>8	>256	64	64	0.03	≤0.12	0.25
1130864	86	Italy	Rome	*E. coli*	>64	8	16	>128	8	16	>16	>8	>256	>64	>128	0.12	0.25	2
1116957	263	Australia	Sydney	*E. coli*	16	8	32	>128	2	>128	>16	>8	64	>64	>128	0.03	0.25	0.25
1126350	283	Vietnam	Hanoi	*E. coli*	>64	16	64	>128	4	>128	>16	>8	>256	>64	>128	0.12	0.25	>2
1122568	40	Argentina	Buenos Aires	*K. pneumoniae*	>64	8	0.25	>128	16	1	>16	>8	32	>64	>128	1	0.5	>2
1125511	215	Taiwan	Taipei	*K. pneumoniae*	>64	8	>64	>128	4	>128	>16	>8	8	>64	>128	0.5	1	>2
1116221	603	Thailand	Bangkok	*K. pneumoniae*	>64	>16	>64	>128	16	>128	>16	>8	16	>64	>128	4	4	>2
1102685	614	Australia	Melbourne	*E. cloacae*	64	4	>64	>128	1	>128	>16	>8	32	16	128	0.03	≤0.12	0.03
1108008	81	Poland	Warsaw	*E. cloacae*	64	4	64	>128	4	128	>16	>8	128	>64	>128	2	2	>2
1118254	81	Poland	Warsaw	*E. cloacae*	>64	16	>64	>128	2	>128	>16	>8	32	>64	>128	0.12	0.5	2

ATM, aztreonam; ATM/AVI, aztreonam/avibactam (at fixed concentration of 4 mg/L); ATM/CLA, aztreonam/clavulanate (at fixed concentration of 4 mg/L); CAZ, ceftazidime; CAZ/AVI, ceftazidime/avibactam; CAZ/CLA, ceftazidime/clavulanate; COZ/TZB, ceftolozane/tazobactam (at fixed concentration of 4 mg/L); CRO, ceftriaxone; FEP, cefepime; SAM, ampicillin/sulbactam; TZP, piperacillin/tazobactam; MEM, meropenem; IMP, imipenem; ETP, ertapenem.

Table 2. Isolates exhibiting ATM/AVI MIC results ≥4 mg/L selected for further characterization and main resistance mechanisms documented

		MIC (mg/L)			β-lactamase genes	mRNA expression[a]		Amino acid alterations		
Collection number	Organism	ATM/AVI	CAZ/AVI	MLST		AcrA	AmpC	OmpF/OmpK35	OmpC/OmpK36	PBP3
1108470	*E. coli*	8	2	410	CMY-42, CTX-M-15, OXA-1	3.6	1.2	WT	WT	R333insYRIK
1108523	*E. coli*	8	2	410	CMY-42, OXA-1	3.8	<1	WT	L14Q	R333insYRIK
1108694	*E. coli*	8	2	410	CMY-141	2.7	<1	WT	G137D	R333insYRIK
1114251	*E. coli*	16	8	410	CMY-42, CTX-M-15, OXA-1, TEM-1	3.5	<1	WT	WT	R333insYRIK
1114255	*E. coli*	8	8	410	OXA-48, CMY-42, CTX-M-14, TEM-190	2.3	<1	WT	WT	R333insYRIK
1118669	*E. coli*	4	>32	410	NDM-5, OXA-181, CMY-2, CTX-M-15, OXA-1, TEM-1	2.4	<1	WT	R199L	R333insYRIN
1116284	*E. coli*	8	4	405	CTX-M-15, OXA-1	<1	<1	N258X	WT	R333insYRIK
1128667	*E. coli*	4	1	405	CTX-M-55, OXA-1	<1	<1	N258X	WT	R333insYRIK
1130864	*E. coli*	8	8	405	CTX-M-15, OXA-1	<1	<1	N258X	WT	R333insYRIK
1116957	*E. coli*	8	2	38	CMY-42, OXA-1	<1	<1	WT	WT	R333insYRIK
1126350	*E. coli*	16	4	617	CMY-42, CTX-M-27	4.5	<1	WT	WT	R333insYRIN
1122568	*K. pneumoniae*[b]	8	16	872	PER-2, SHV-11	1.1	NA	Y286X	WT	M6T, A33V, V41I, L370I, Q374K, H396R, E434A, I447M, N455S, L577Q, A578G
1125511	*K. pneumoniae*[b]	8	4	15	DHA-1, SHV-28	5.5	NA	A119X	WT	Y432C
1116221	*K. pneumoniae*[b]	>16	16	273	DHA-1, LAP-2, SHV-11, TEM-1	6.2	NA	WT	Y43X	WT
1102685	*E. cloacae*	4	1	350	ACT-27	<1	3667	A19S, S159L, Y199F, E200D, Y208L, E224K, G225A, G234E, L235M, Y236H, T243K, N276A, Q276A, F277H, D278_F279insENT	P177A	G306V
1108008	*E. cloacae*	4	4	121	ACT-25, CTX-M-15, OXA-1, TEM-1	<1	3060	WT	WT	WT
1118254	*E. cloacae*	16	2	78	ACT-24, SHV-12, TEM-1	<1	570	WT	P177A, D188E	E258_S259insE

ATM/AVI, aztreonam/avibactam; CAZ/AVI, ceftazidime/avibactam.
[a] Reported expression results are relative to a control isolate.
[b] *K. pneumoniae* isolates had WT sequences of OmpK37.

aztreonam/ and ceftazidime/clavulanate MICs (0.25–1 mg/L) were 16- to 32-fold lower than when these drugs were combined with avibactam against a PER-2 producer (1122568). Avibactam seems to inhibit PER-2 to a lesser extent than other ESBLs, which can partially explain the elevated MICs.[16] Notably, clavulanate did not bring the aztreonam (0.25 mg/L) and ceftazidime (1 mg/L) MICs down to WT levels (modal MIC, 0.03 mg/L and 0.12 mg/L, respectively; data not shown). The absence of OmpK35 or OmpK36 does not significantly affect susceptibility to ceftazidime.[17] However, the absence of both porins or absence of any porin and the presence of an ESBL increases the ceftazidime MIC around 4-fold, which seems to fit the results observed for 1122568.[17]

The remaining *K. pneumoniae* isolates 1116221 and 1125511 produced DHA-1. The former isolate had a premature stop codon within OmpK36, whereas the latter isolate had a premature stop codon within OmpK35. Both isolates expressed moderate levels of AcrAB-TolC. Nicolas-Chanoine *et al.*[18] demonstrated that a DHA-1-producing *K. pneumoniae* strain exhibited a ceftazidime/avibactam MIC of 2 mg/L, and isogenic strains expressing DHA-1 and additional resistance mechanisms associated with drug influx or efflux had MICs of 4–16 mg/L. These results are consistent with those obtained here (MIC, 4–16 mg/L) and suggest that the aztreonam/avibactam and ceftazidime/avibactam MICs obtained against isolates 1116221 and 1125511 were likely due to the production of DHA-1 in combination with drug efflux and porin deficiencies.[5]

One possible hypothesis for the elevated aztreonam/avibactam MICs in isolates 1102685 and 1108008 (MIC, 4 mg/L) would be the similar elevated expression of AmpC. It is tempting to speculate that the amount of enzyme produced could overcome the *in vitro* inhibitory capability of avibactam used at 4 mg/L. However, while isolate 1108008 had a WT PBP3 sequence, isolate 1102685 showed a G306V mutation. This glycine is located within the η3 loop region. Although it is considered a conserved amino acid, it is situated at the opposite side of the active β-lactam binding site and may not affect enzyme–substrate affinities, unless G306V causes conformational changes in the PBP3 structure that affect the active site. *E. cloacae* 1118254 had a higher aztreonam/avibactam MIC (16 mg/L), but a much lower expression of AmpC compared with isolates 1102685 and 1108008. However, isolate 1118254 had a glutamic acid insertion in the transpeptidase domain (amino acid 237–577) of PBP3. This insertion was previously reported in an *E. cloacae* that displayed an aztreonam/avibactam MIC of >8 mg/L, [19] and it is located adjacent to the conserved alanine at position 257 at the end of the α8 loop, which adjoins the active binding site.[20]

This study further analysed 17 (17/8787; 0.2%) Enterobacterales isolates that showed a decreased susceptibility to aztreonam/avibactam to discern their associated resistance mechanisms. In summary, *E. coli* tended to be carbapenem susceptible and produce an altered PBP3, likely as a relevant aztreonam/avibactam resistance mechanism acting in conjunction with the β-lactamase background.[13] The *K. pneumoniae* showed multiple mechanisms, whereas the *E. cloacae* did not show clear evidence to explain their elevated MICs, other than an overexpression of AmpC.

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Transparency declarations

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Supplementary data

Figure S1 is available as Supplementary data at *JAC* Online.

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