Genetic Features Leading to Reduced Susceptibility to Aztreonam-Avibactam among Metallo-β-Lactamase-Producing *Escherichia coli* Isolates

Mustafa Sadek,[a] Mario Juhas,[a] Laurent Poirel,[a,b,c] Patrice Nordmann[a,b,c]

[a]Medical and Molecular Microbiology, Faculty of Science and Medicine, University of Fribourg, Fribourg, Switzerland
[b]Swiss National Reference Center for Emerging Antibiotic Resistance (NARA), University of Fribourg, Fribourg, Switzerland
[c]INSERM European Unit (IAME, France), University of Fribourg, Fribourg, Switzerland

Mustafa Sadek and Mario Juhas contributed equally. Author order was determined in order of increasing seniority.

ABSTRACT Metallo-β-lactamase (MBL)-producing *Escherichia coli* isolates resistant to the newly developed β-lactam/β-lactamase inhibitor drug combination aztreonam-avibactam (ATM-AVI) have been reported. Here, we analyzed a series of 118 clinical MBL-producing *E. coli* isolates of various geographical origins for susceptibility to ATM-AVI. The nature of the PBP3 protein sequence and the occurrence of bla_{CMY} genes for susceptibility to ATM-AVI were investigated. We showed here that elevated MICs of ATM-AVI among MBL-producing *E. coli* isolates resulted from a combination of different features, including modification of PBP3 protein sequence through specific amino acid insertions and production of CMY-type enzymes, particularly, CMY-42. We showed here that those insertions identified in the PBP3 sequence are not considered the unique basis of resistance to ATM-AVI, but they significantly contribute to it.

KEYWORDS *Escherichia coli*, aztreonam-avibactam, metallo-β-lactamase, PBP3, NDM, CMY-42

Among the most difficult-to-treat bacterial infections, those caused by carbapenemase-producing *Enterobacterales* (CPE) are considered in the top list. More specifically, there are extremely limited therapeutic options against enterobacterial isolates producing metallo-β-lactamases (MBL), being sources of nosocomial but also community-acquired infections worldwide. Among the most common MBL circulating, there are the New Delhi metallo-β-lactamase (NDM), the Verona-integron-mediated (VIM) enzyme, and imipenemase (IMP) that are all found in *Escherichia coli*, the most common human pathogen (1–4). MBLs hydrolyze penicillins, broad-spectrum cephalosporins, and carbapenems and are not inhibited by the β-lactamase inhibitors clavulanate and tazobactam. They use zinc ions for β-lactam hydrolysis and are inhibited neither by novel commercially used β-lactamase inhibitors such as diazabicyclooctanes avibactam (AVI) and relebactam nor by cyclic boronic acids such as vaborbactam (5). Aztreonam (ATM) is the only clinically used β-lactam antibiotic not degraded by MBLs. However, many MBL-producing bacteria coproduce extended-spectrum β-lactamases (ESBLs) or AmpC enzymes that hydrolyze ATM and thereby confer resistance to this β-lactam antibiotic (5). On the other hand, AVI is an excellent inhibitor of many non-MBL β-lactamases, including most ESBL and AmpC enzymes. Therefore, the recently developed ATM-AVI combination has been proposed for the treatment of MBL-producing *Enterobacterales* and is under commercial development (5–9). Considering that most MBL-producing *Enterobacterales* (including *E. coli*), and particularly those producing the NDM-type enzymes, are highly resistant to other

Address correspondence to Laurent Poirel, laurent.poirel@unifr.ch.

non-β-lactam antibiotics (aminoglycosides, trimethoprim-sulfamethoxazole, tetracycline, and fluoroquinolones), this ATM-AVI combination therapy is among the last-resort options against MBL-producing *E. coli*. However, MBL-producing *E. coli* isolates showing high MIC values for ATM-AVI were recently reported (1, 10). To date, breakpoints for determining susceptibility or resistance to ATM-AVI have still not been defined. Of note, the current EUCAST breakpoint for resistance to ATM in *Enterobacterales* is >4 μg/ml.

ATM has potent selective and specific activity toward the penicillin-binding protein 3 (PBP3) of *Enterobacterales* in contrast to that for other β-lactam molecules targeting other PBPs (10, 11). Due to its involvement in the peptidoglycan biosynthesis and cell division and its high conservation among Gram-negative bacteria, PBP3 is among the most widely used drug targets for developing novel antibiotics since the discovery of penicillin (12, 13). In *E. coli*, it was recently shown that the insertion of four specific amino acids in the PBP3 protein sequence (YRIN or YRIK) was a source of reduced susceptibility to ATM-AVI among NDM producers and, particularly, among isolates originating from India (1, 10).

Here, we report on the activity of ATM-AVI and other antibiotics against MBL-producing *E. coli* clinical isolates recovered from clinical samples of worldwide origin (Europe, Africa, Asia, and Australia). Furthermore, we investigated the impact of variable amino acid insertions within the PBP3 sequence in terms of ATM-AVI activity among NDM-producing *E. coli* isolates.

RESULTS

Activity of ATM-AVI against MBL-producing *E. coli* isolates. As expected for MBL-producing *E. coli*, all analyzed isolates were highly resistant to ceftazidime (CAZ) and CAZ-AVI. MICs of CAZ and CAZ-AVI were both at >256 μg/ml. MICs of imipenem (IMI) were also high, ranging from 32 μg/ml to 256 μg/ml (Table 1). Overall, results of susceptibility testing revealed a high rate of MBL-producing *E. coli* isolates with MICs of ATM-AVI superior or equal to 2 μg/ml (Table 1). A total of 47/118 (40%) of the isolates showed MICs of ATM-AVI ranging from 2 to 16 μg/ml. Overall, a total of 19 isolates was therefore considered "resistant" (MICs of >4 μg/ml) to ATM-AVI when considering the ATM resistance breakpoint (>4 μg/ml according to the EUCAST guidelines). Noticeably, all those resistant isolates produced an NDM-type MBL, namely, NDM-5 ($n = 15$), NDM-1 ($n = 2$), and NDM-4 ($n = 2$) (Table 1). In addition, a total of 28 MBL-producing isolates had MIC values of ATM-AVI ranging from 2 to 4 μg/ml, being further classified as "less susceptible" to ATM-AVI considering the ATM susceptibility breakpoint being at ≤1 μg/ml. Noteworthy, none of the VIM- or IMP-type MBL-producing *E. coli* isolates except one (strain R-74) showed reduced susceptibility to ATM-AVI (Table 1).

A four-amino-acid insertion in PBP3 identified among the NDM-producing *E. coli* isolates. To investigate the putative involvement of modifications in the PBP3 structure, to which ATM has unique selectivity, as a cause of reduced susceptibility to ATM-AVI, the PBP3-encoding genes of the 19 NDM-producing *E. coli* isolates exhibiting MICs of ATM-AVI of >4 μg/ml were sequenced. All those isolates had a four-amino-acid insertion into the PBP3 protein after residue 333 (Table 1). Two different amino acid sequences were identified for this insertion, namely, YRIN ($n = 16$) and YRIK ($n = 3$). Moreover, by analyzing the PBP3 sequences of 28 NDM-producing isolates identified as less susceptible to ATM-AVI, the same four-amino-acid insertions (YRIN or YRIK) were always identified.

Occurrence of the four-amino-acid insertions in PBP3 in susceptibility of *E. coli* to ATM-AVI. To investigate further the impact of such four-amino-acid insertions into the PBP3 sequence of *E. coli*, the corresponding genes were amplified and sequenced for all strains, including the ATM-AVI-susceptible MBL-producing *E. coli* isolates ($n = 71$) (with MICs of ATM-AVI of ≤1 μg/ml). As expected, the majority of those ATM-AVI-susceptible MBL-producing isolates (70%) did not display amino acid insertions in their PBP3 structure as previously reported. However, and surprisingly, almost 30% (21/71 isolates) of all the ATM-AVI-susceptible MBL-producing isolates investigated in this

TABLE 1 MICs and insertions in the PBP3 sequence of MBL-producing *E. coli* isolates

Strain	ST	Metallo-β-lactamase	Other β-lactamase(s)	Origin of isolation[a]	MIC (μg/ml)[b]					PBP3 insertion sequence[c]
					ATM	ATM-AVI	CAZ	CAZ-AVI	IMI	
R-3058	ND	NDM-5	CMY-42	Angola	32	16	>256	>256	32	YRIN
R-461	ND	NDM-1	CMY-42	France	32	16	>256	>256	64	YRIN
R-3038	ND	NDM-5	CMY-42	Angola	32	8	>256	>256	16	YRIN
R-3031	ND	NDM-5	CMY-42	Angola	128	8	>256	>256	32	YRIN
N-185	ND	NDM-5	CMY-42	Switzerland	32	8	256	256	16	YRIN
N-590	167	NDM-5	CMY-42	Switzerland	64	8	>256	>256	32	YRIN
N-1013	361	NDM-5	CMY-42	Switzerland	128	8	>256	>256	64	YRIN
N-1076	940	NDM-5	CMY-42, TEM-1B	Switzerland	64	8	>256	>256	32	YRIN
R-460	ND	NDM-1	CMY-42	France	>256	8	>256	>256	32	YRIN
R-3033	ND	NDM-5	CMY-42	Angola	64	8	>256	>256	16	YRIN
R-3040	ND	NDM-5	CMY-42	Angola	64	8	>256	>256	32	YRIN
R-3043	ND	NDM-5	CMY-42	Angola	64	8	>256	>256	32	YRIN
R-3029	ND	NDM-5	CMY-42, CTX-M group 1	Angola	32	8	>256	>256	32	YRIN
R-3039	ND	NDM-5	CMY-42	Angola	16	8	>256	>256	32	YRIN
R-3048	ND	NDM-5	CMY-42	Angola	16	8	>256	>256	32	YRIN
R-3054	ND	NDM-5	CMY-42	Angola	16	8	>256	>256	32	YRIN
N-57	ND	NDM-5	CMY-42	Switzerland	32	8	>256	256	32	YRIK
R-466	405	NDM-4	CMY-42, CTX-M-15, OXA-1	Cameroon	>256	8	>256	>256	16	YRIK
R-2222	ND	NDM-4	CMY-42	France	>256	8	>256	256	64	YRIK
R-474	ND	NDM-7	CMY-6	France	16	4	>256	>256	64	YRIN
N-6	ND	NDM-5	CMY-16	Switzerland	128	4	256	128	32	YRIN
N-204	ND	NDM-5	CTX-M-15	Switzerland	>256	4	>256	>256	64	YRIN
N-231	ND	NDM-5	CMY-156, CTX-M-15	Switzerland	>256	4	>256	>256	64	YRIN
N-640	167	NDM-5	CMY-142, TEM-1 B	Switzerland	64	4	>256	>256	32	YRIN
R-45	410	NDM-1	CMY-30, TEM-1, OXA-1	France	16	4	>256	>256	64	YRIN
R-467	ND	NDM-4	CMY-2, CTX-M-15, SHV-12, OXA-1, TEM-1	France	>256	4	>256	128	128	YRIN
R-3056	ND	NDM-5	CMY-156, CTX-M group 1	Angola	>128	4	>256	>256	64	YRIN
R-449	2527	NDM-1	CTX-M-15, TEM-1	Oman	>256	4	>256	256	64	YRIK
R-464	ND	NDM-4	OXA-1	France	>256	4	>256	256	32	YRIK
N-8	ND	NDM-5	CMY-16	Switzerland	>256	2	>256	>256	32	YRIN
N-665	167	NDM-5	CTX-M-15, OXA-1	Switzerland	≥256	2	>256	>256	64	YRIN
N-689	361	NDM-5	OXA-244	Switzerland	4	2	>256	>256	64	YRIN
R-74	ND	IMP-1	CMY-2	France	128	2	>256	>256	128	YRIN
R-456	ND	NDM-1	CMY, CTX-M-15, OXA-1, TEM-1	India	>256	2	>256	>256	16	YRIN
R-470	ND	NDM-5	CMY-6, CTX-M-15, TEM-1	UK	>256	2	>256	256	32	YRIN
R-2223	ND	NDM-4	CMY-2	France	>256	2	>256	>256	128	YRIN
R-2644	ND	NDM-1	CMY-4	France	32	2	>256	>256	32	YRIN
R-450	101	NDM-1	CMY-4, CTX-M-15, TEM-1	Australia	>256	2	>256	>256	64	YRIN
R-451	ND	NDM-1	CMY-2, CTX-M-15, OXA-1, OXA-2, TEM-1	India	>256	2	>256	>256	32	YRIN
R-462	ND	NDM-1		France	2	2	>256	256	32	YRIN
N-775	405	NDM-5	TEM-1B	Switzerland	16	2	>256	>256	128	YRIK
N-679	648	NDM-5		Switzerland	4	2	>256	>256	32	YRIK
N-1146	167	NDM-5	CMY-142, TEM-1	Switzerland	8	2	>256	>256	64	YRIN
N-935	648	NDM-5	TEM-1B	Switzerland	256	2	>256	>256	128	YRIK
R-468	ND	NDM-4	CMY-2, CTX-M-15, OXA-1, TEM-1	France	>256	2	>256	256	32	YRIK
R-469	ND	NDM-5	CMY-2	Netherlands	>256	2	>256	128	256	YRIK
N-901	354	NDM-5	CTX-M-24, TEM-1B	Switzerland	>256	2	>256	>256	128	YRIP
N-21	ND	NDM-5	CMY-2	Switzerland	>256	1	>256	>256	64	YRIN
R-2758	ND	NDM-1	CMY-2	France	>256	1	>256	>256	64	YRIN
R-475	ND	NDM-7	CMY-2	France	>256	1	>256	>256	64	YRIN
R-2225	ND	NDM-1	CMY-2	France	>256	1	>256	256	32	YRIN
R-3051	ND	NDM-5	CMY-2, CTX-M group 1	Angola	>256	1	>256	>256	32	YRIN
N-783	167	NDM-19		Switzerland	1	1	>256	>256	32	YRIN
N-1014	1588	NDM-5	CTX-M-15, SHV-1	Switzerland	>256	1	>256	>256	64	YRIN
R-2839	5079	NDM-1	CTX-M-15	Angola	>256	1	>256	>256	64	YRIN
R-472	ND	NDM-6	CTX-M-15, OXA-1	France	>256	1	>256	256	16	YRIN
N-489	405	NDM-5	CTX-M-15, TEM-1B	Switzerland	>256	1	>256	>256	64	YRIK
N-525	405	NDM-5	CTX-M-15	Switzerland	>256	1	>256	>256	64	YRIK
N-897	354	NDM-5	CTX-M-24, TEM-1 B	Switzerland	>256	1	256	256	128	YRIP
R-200	ND	VIM-4	CMY-2	France	8	1	>256	32	32	—
R-554	ND	VIM-4	CMY-4	Kuwait	8	1	>256	32	16	—
R-2646	ND	NDM-5	CMY-2	France	16	1	>256	>256	64	—
N-461	167	NDM-5	CTX-M-15, OXA-1	Switzerland	>256	0.5	>256	>256	32	YRIN
N-898	167	NDM-5	CTX-M-15, OXA-1	Switzerland	>256	0.5	>256	>256	128	YRIN

(Continued on next page)

TABLE 1 (Continued)

Strain	ST	Metallo-β-lactamase	Other β-lactamase(s)	Origin of isolation[a]	MIC (μg/ml)[b]					PBP3 insertion sequence[c]
					ATM	ATM-AVI	CAZ	CAZ-AVI	IMI	
N-568	167	NDM-5	CTX-M-15, OXA-1	Switzerland	>256	0.5	>256	>256	16	YRIN
R-3047	448	NDM-5	CTX-15, TEM-93, TEM-196	Angola	64	0.5	>256	≥256	32	YRIN
N-653	1284	NDM-5	CTX-M-15, TEM-1b, OXA-1	Switzerland	>256	0.5	>256	>256	16	YRIN
N-322	ND	NDM-5		Switzerland	1	0.5	>256	>256	32	YRIN
R-2219	ND	NDM-7		France	>256	0.5	>256	>256	32	YRIN
R-453	101	NDM-1	CTX-M-15, TEM-1, OXA-1, OXA-2	India	>256	0.5	>256	>256	8	YRIK
N-442	354	NDM-5	CTX-M-24, TEM-1	Switzerland	>256	0.5	>256	>256	64	YRIP
R-454	410	NDM-1	CMY-6	Norway	2	0.5	>256	256	8	—
R-458	ND	NDM-1	CMY-2, CTX-M-15, OXA-9, TEM-1	France	>256	0.5	>256	>256	128	—
R-2220	ND	NDM-6		France	>256	0.5	>256	>256	16	
R-455	10	NDM-1	CMY-16, OXA-1, OXA-10, TEM-1	India	16	0.5	>256	>256	32	—
R-457	ND	NDM-1	CMY-12, OXA-1	France	8	0. 5	>256	256	16	—
R-463	1706	NDM-1	CMY-6, CTX-M-15, TEM-1	France	64	0. 5	>256	>256	32	—
N-415	ND	NDM-5	CMY-4, CTX-M group 1	Switzerland	16	0.5	>256	>256	16	—
R-2224	ND	NDM-1		France	0.25	0.25	>256	256	16	—
R-2574	ND	VIM-1		France	0.25	0.25	0.125	0.063	0.25	—
R-549	ND	VIM-1	CMY-2	Spain	32	0.25	>256	32	8	—
N-292	ND	NDM-1	CMY-2	Switzerland	0.5	0.25	>256	256	1	—
N-1081	361	NDM-5		Switzerland	1	0.125	256	256	32	—
R-31	ND	NDM-5		France	8	<0.25	>256	>256	64	—
R-61	ND	VIM-1	CMY-2	France	>256	0.25	>256	8	4	—
R-62	ND	VIM-1	CMY-2-like	France	32	0.25	>256	>256	4	—
R-471	ND	NDM-5	TEM-1	France	16	<0.25	>256	256	64	—
R-178	ND	NDM-1	CMY-16	France	16	0.25	>256	>256	16	—
R-194	ND	IMP-8	CMY-2	France	≥256	0.25	>256	>256	16	—
N-1115	410	NDM-5	CMY-2, TEM-1, OXA-1	Switzerland	0.5	0.125	>256	>256	64	—
R-552	ND	VIM-1	TEM-1	France	>256	<0.25	>256	>256	64	—
R-452	131	NDM-1	OXA-1, TEM-1	India	0.06	0.06	>256	>256	32	—
N-401	ND	NDM-1	CMY-4, DHA-1	Switzerland	0.5	0.25	>256	256	16	—
N-426	ND	VIM-1	CTX-M group 1	Switzerland	32	<0.06	64	32	2	—
N-530	ND	NDM-1		Switzerland	1.	<0.06	>256	>256	16	—
N-632	ND	NDM-1		Switzerland	0.5	<0.06	>256	>256	16	—
N-737	540	NDM-1	OXA-10	Switzerland	2	<0.06	>256	>256	16	—
N-771	ND	VIM-1		Switzerland	0.125	<0.06	>256	>256	16	—
N-836	95	NDM-1	CTX-M-55, VEB-1, OXA-10	Switzerland	16	<0.06	>256	>256	16	—
N-1097	1431	NDM-5	TEM-1	Switzerland	0.5	<0.06	>256	>256	32	—
R-58	ND	VIM-19		France	4	<0.06	64	1	16	—
R-72	ND	IMP-1		France	0.125	<0.06	>256	128	4	—
R-167	ND	VIM-2		France	0.063	<0.06	128	64	2	—
R-168	ND	VIM-1		France	4	<0.06	0.25	0.125	2	—
R-174	ND	NDM-1		France	<0.016	<0.06	>256	128	16	—
R-177	ND	NDM-1	OXA-1	France	<0.016	<0.06	>256	>256	32	—
R-403	ND	IMP-8	SHV	Taiwan	64	<0.06	>256	>256	16	—
R-404	ND	IMP-1, TEM-1		France	0.125	<0.06	>256	>256	4	—
R-459	ND	NDM-1		France	<0.063	<0.06	>256	128	32	—
R-465	648	NDM-4	CTX-M-15	India	>256	<0.06	256	256	16	—
R-548	ND	VIM-1		Spain	0.094	<0.06	>256	256	16	—
R-550	23	VIM-1	SHV-12, TEM-1	Egypt	2	<0.06	0.5	0.063	2	—
R-551	ND	VIM-1		France	2	<0.06	>256	256	16	—
R-553	ND	VIM-2		France	0.06	<0.06	64	32	8	—
R-1688	ND	VIM-19		France	2	<0.06	>256	32	32	—
R-2221	ND	NDM-5		France	4	<0.06	>256	>256	32	—
R-2838	5079	NDM-1	CTX-M-15	Angola	>256	<0.06	>256	>256	32	—
R-2840	5693	NDM-1	CTX-M-15	Angola	0.125	<0.06	>256	256	16	—

[a]All NDM producers identified in Switzerland have a foreign origin.
[b]ATM, aztreonam; AVI, avibactam; CAZ, ceftazidime; IMI, imipenem.
[c]—, no insertion.

study harbored amino acid insertions in their PBP3 protein sequences, either corresponding to YRIN, YRIK, or YRIP quadruplets (Table 1).

Multilocus sequence typing (MLST) analyses performed for a selection of 41 isolates revealed a quite high genetic diversity. A total of 21 different STs were identified including ST167 ($n = 8$), ST405 ($n = 4$), ST354 ($n = 3$), ST361 ($n = 3$), ST410 ($n = 3$),

TABLE 2 MICs of β-lactams for various *E. coli* strains

Strain	MIC (μg/ml)[a]			
	Ceftazidime	Ceftazidime-AVI[b]	Aztreonam	Aztreonam-AVI[b]
R468 (NDM-4+CMY-2+YRIK)	>128	>128	>128	2
N590 (NDM-5+CMY-42+YRIN)	>128	>128	64	8
TOP10(pTOPO-CMY-2)	>128	0.5	16	0.25
TOP10(pTOPO-CMY-42)	>128	1	32	1
TOP10	0.25	<0.125	0.06	<0.06
N679 (NDM-5+YRIK)	>128	>128	4	2
N679 (NDM-5+YRIK/pTOPO-CMY-2)	>128	>128	16	4
N679 (NDM-5+YRIK/pTOPO-CMY-42)	>128	>128	32	16
N783 (NDM-19+YRIN)	>128	>128	1	1
N783 (NDM-19+YRIN/pTOPO-CMY-2)	>128	>128	8	2
N783 (NDM-19+YRIN/pTOPO-CMY-42)	>128	>128	32	8

[a]MIC data were determined by broth microdilution.
[b]Avibactam (AVI) was added at 4 μg/ml.

ST648 ($n = 3$), ST101 ($n = 2$), ST5079 ($n = 2$), and a single isolate for each the following STs: ST10, ST23, ST95, ST131, ST448, ST540, ST940, ST1284, ST1431, ST1588, ST1706, ST2527, and ST5693. Notably, all isolates belonging to either ST167, ST361, ST410, or ST5079 exhibited the same amino acid insertion (YRIN) in their respective PBP3 sequences. In contrast, isolates belonging to ST405 or ST648 possessed a YRIK insertion in the PBP3 sequence, and a YRIP insertion was identified in three NDM-producing isolates belonging to ST354 (Table 1), indicating a link between the nature of the PBP3 sequence and the strain background, regardless of their antibiotic susceptibility profile.

Occurrence of CMY-type enzymes among MBL-producing *E. coli* isolates, and their involvement in reduced susceptibility to ATM-AVI. To further decipher the genetic bases of reduced susceptibility to ATM-AVI, we aimed to evaluate whether additional β-lactamases might be involved, since the different insertions identified in PBP3 could not explain *per se* the AZT-AVI resistance. We focused on CMY-type AmpC β-lactamases, since whole-genome sequencing (WGS) data revealed their occurrence in many sequenced genomes. Hence, all studied isolates were tested for the bla_{CMY}-like genes by PCR. All the MBL-producing *E. coli* isolates categorized as resistant and most of the isolates identified as less susceptible carried a plasmid-borne bla_{CMY} β-lactamase gene (Table 1). Those data suggested a correlation between the occurrence of bla_{CMY} and the MIC level of ATM-AVI, the production of a CMY-type β-lactamase likely playing a significant role in the reduced susceptibility to ATM-AVI. When referring to the study by Alm et al. (10), it is important to highlight that the 14 NDM-producing *E. coli* isolates showing a decreased susceptibility to ATM-AVI in which amino acid insertions into the PBP3 sequence had been identified also carried a plasmid-borne bla_{CMY} gene, particularly, bla_{CMY-42}.

β-Lactamase CMY-42 is involved in resistance to ATM-AVI. Of note, all MBL-producing *E. coli* isolates categorized as resistant to ATM-AVI (MIC > 4 μg/ml) carried a plasmid-borne bla_{CMY-42} β-lactamase gene. Furthermore, since all the less-susceptible isolates also carried a bla_{CMY}-like gene, our hypothesis was that CMY-42 might play a significant role with respect to resistance to ATM-AVI, particularly, by comparison with CMY-2. To verify that hypothesis, both corresponding genes were cloned and expressed in the same *E. coli* background. Expression of the bla_{CMY-2} and bla_{CMY-42} genes in *E. coli* TOP10 conferred reduced susceptibility to ATM-AVI compared to that of the wild-type *E. coli* strain TOP10 (Table 2). Interestingly, the MIC of ATM-AVI was 4-fold higher for the CMY-42-producing than for the CMY-2-producing *E. coli* recombinant strain (1 versus 0.25 μg/ml, respectively), which is much higher than for the wild-type *E. coli* recipient strain TOP10 (<0.06 μg/ml).

To evaluate the impact of the bla_{CMY-42} and bla_{CMY-2} expression in clinical NDM-producing *E. coli* strains possessing either a wild-type or modified PBP3 protein sequence background, recombinant plasmids encoding CMY-2 and CMY-42 were introduced by transformation into clinical *E. coli* strain N679 (producing NDM-5 and

TABLE 3 Kinetic parameters of purified β-lactamases CMY-2 and CMY-42

	K_i (nM)	
β-Lactam	**CMY-2**	**CMY-42**
Avibactam	1.3	1.14
Aztreonam	1.6	0.47

possessing the YRIK quadruplet insertion) and clinical *E. coli* strain N783 (producing NDM-19 and possessing the YRIN quadruplet insertion). Upon production of those AmpC β-lactamases, MICs of ATM and ATM-AVI were further enhanced compared to that of a wild-type strain. MIC determination for ATM and ATM-AVI showed an 8-fold increase for the CMY-42-producing *E. coli* N679 (NDM-5 plus YRIK plus CMY-42) recombinant strain compared to that for the N679 (NDM-5 plus YRIK) isogenic counterpart (32 versus 4 μg/ml for ATM and 16 versus 2 μg/ml for ATM-AVI, respectively), confirming that the production of CMY-42 in a strain background possessing a modified PBP3 had a significant impact on susceptibility to not only ATM but also ATM-AVI (Table 2). Moreover, the impact on those respective MICs was more elevated for the CMY-42 producer than for the CMY-2 producer. Similar results were obtained when testing the CMY-42-producing N783 (NDM-19 plus YRIN plus CMY-42) *E. coli* recombinant strain.

Since our data showed that the impact of CMY-42 production on susceptibility to ATM and ATM-AVI was higher than that mediated by CMY-2, hydrolysis experiments were performed using purified extracts of both enzymes. Surprisingly, hydrolysis of ATM was not detected using ATM as the substrate and using both enzymatic extracts. It was therefore hypothesized that the discrepancy observed between the lack of evidence of ATM hydrolysis on one hand and the impact those enzymes may have on susceptibility to that antibiotic on the other hand could be explained by a very low initial rate of hydrolysis combined with an excellent affinity for the ATM substrate. Consequently, the K_i value of ATM was determined for both enzymes, considering that ATM could actually behave as an inhibitor rather than a substrate. Using various concentrations of ATM as inhibitor and cephalothin as reporter substrate, results of our kinetic determinations showed that CMY-42 exhibited a 4-fold higher affinity toward ATM (K_i = 0.47 nM) than CMY-2 (K_i = 1.6 nM), which may explain the discrepancies observed in terms of MICs (Table 3).

DISCUSSION

Our results showed that the ATM-AVI combination is highly effective against the majority of MBL-producing *E. coli* isolates of diverse geographic origins (Table 1). However, approximately 16% of the MBL-producing *E. coli* clinical isolates analyzed in this study showed MICs of ATM-AVI of >4 μg/ml if the EUCAST breakpoint for resistance to ATM is taken as the reference. Notably, all the isolates showing high MIC values for ATM-AVI identified produced an NDM-type MBL, while those producing IMP- and VIM-type MBLs exhibited low MIC levels (Table 1). Moreover, a total of 24% (28/118) of the MBL-producing *E. coli* clinical isolates showed a reduced susceptibility to ATM-AVI (MIC values of 2 to 4 μg/ml). Due to lack of the other alternatives, ATM in combination with CAZ-AVI is often used for the clinical treatment of NDM-producing *Enterobacterales*, which showed good results *in vitro* and *in vivo* (5, 14, 15).

Previous studies focusing on the mechanisms of decreased susceptibility of MBL-producing *Enterobacterales* to ATM-AVI highlight the role a four-amino-acid insertion in the PBP3 protein (1, 10). In our study, we identified a significant proportion of isolates possessing a four-amino-acid insertion of PBP3 protein, which is consistent with previously published studies (1, 10). All the MBL-producing isolates identified with an elevated MIC of ATM-AVI possessed a four-amino-acid insertion in the PBP3 protein, either YRIN or YRIK. Identical insertions had been found in non-NDM-producing *E. coli* showing reduced susceptibility to ATM-AVI (10). In our strain collection, the PBP3 insertion YRIN was observed more frequently than the YRIK quadruplet among the NDM-producing isolates. Periasamy et al. recently showed that elevated MICs of

ATM-AVI were also observed among isolates lacking any insertion within the PBP3 sequence (1). This suggested a possible role of an increased efflux, as previously described for ATM (16).

However, we identified identical four-amino-acid insertions within the PBP3 sequence among MBL-producing *E. coli* isolates with much lower MIC values of ATM-AVI, therefore showing that a PBP3-modified background, even if likely enhancing the occurrence of elevated MICs of ATM-AVI, is not sufficient to confer ATM-AVI resistance and that another mechanism(s) was likely involved in decreased susceptibility to that drug combination in *E. coli*. To sum up, all isolates with MICs of ATM-AVI of >4 μg/ml possessed CMY-42 and an insertion into PBP3, and all isolates with MICs of ATM-AVI of <0.5 μg/ml possessed a wild-type PBP3 sequence, regardless of the presence of a CMY-encoding gene.

In addition, we showed that the production of CMY-42 and, to a lesser extent, CMY-2 conferred significant increases in MICs of ATM and ATM-AVI when combined with the occurrence of PBP3 inserts. A previous study showed that a series of *E. coli* isolates exhibiting elevated MICs of ATM-AVI (8 μg/ml) actually possessed three serine-β-lactamase genes ($bla_{\text{CMY-42}}$, $bla_{\text{OXA-1/30}}$, and $bla_{\text{TEM-1}}$), membrane porin alterations, and a four-amino-acid insertion in PBP3 (7).

Finally, we showed here that elevated MICs of ATM-AVI among MBL-producing *E. coli* isolates results from a combination of different features, including modification of PBP3 protein sequence through specific amino acid insertions and production of CMY-type enzymes, particularly, CMY-42. We showed here that those insertions identified in the PBP3 sequence cannot be considered the unique basis of resistance to ATM-AVI, but they significantly contribute to it. Nevertheless, further work is required to precisely decipher the exact interplay between those different resistance mechanisms and the putative involvement of some other mechanisms, such as efflux overexpression, additional β-lactamases, or permeability defects.

To conclude, this study revealed a variety of *E. coli* clonal backgrounds exhibiting decreased susceptibility to ATM-AVI that have already disseminated worldwide. This may constitute a warning signal indicating that such drug combination which is under commercial development may not be the panacea for treating infections due to MBL producers. This result is of concern when considering that very few antibiotics may be still *in vitro* active against MBL producers (polymyxins, fosfomycin, and tigecycline).

MATERIALS AND METHODS

Bacterial isolates and antimicrobial agents. A total of 118 MBL-producing *E. coli* clinical isolates were included in this study. Isolates were obtained from various clinical sources (e.g., blood cultures, urine, and sputum) and from various continents (Europe, Asia, Africa, and Australia). All strains were previously characterized for their β-lactamase content by PCR, DNA sequencing, and, for some, whole-genome sequencing (WGS). The carbapenemase types were as follows: 96 NDM producers, including NDM-1 ($n = 31$), NDM-4 ($n = 7$), NDM-5 ($n = 52$), NDM-6 ($n = 2$), NDM-7 ($n = 3$), and NDM-19 ($n = 1$), 17 VIM producers, including VIM-1 ($n = 11$), VIM-2 ($n = 2$), VIM-4 ($n = 2$), and VIM-19 ($n = 2$), and 5 IMP producers, including IMP-1 ($n = 3$) and IMP-8 ($n = 2$). Antimicrobial agents were obtained from Sigma and Roche (Basel, Switzerland). Stock solutions were prepared according to CLSI guideline M07 (17).

Antimicrobial susceptibility testing. The MICs were determined using the broth microdilution in cation-adjusted Mueller-Hinton broth (Bio-Rad, Marnes-la-Coquette, France), and results were interpreted according to the latest EUCAST breakpoints (www.eucast.org/clinical_breakpoints) and Clinical and Laboratory Standards Institute (CLSI) (https://clsi.org/2018/) guidelines. ATM, ceftazidime (CAZ), imipenem (IMI), and AVI were obtained from Sigma-Aldrich (Buchs, Switzerland) and Roche (Basel, Switzerland). Stock solutions were prepared according to CLSI guideline M07 (17).

For the ATM-AVI and CAZ-AVI combinations, AVI was tested at a fixed concentration of 4 μg/ml. Since no breakpoint value for defining ATM-AVI resistance has yet been specified, that of ATM alone (>4 μg/ml; www.eucast.org/clinical_breakpoints) was arbitrarily chosen. Susceptibility testing was performed in duplicates on two different days. *E. coli* ATCC 25922 strain was used as quality control for all testing.

PBP3 gene amplification, sequencing, and analysis. Genomic DNA was extracted from *E. coli* isolates by boiling a single colony in 30 μl of sterile water at 95°C for 10 min. The *E. coli* PBP3-encoding gene was amplified by PCR using the FIREPol DNA polymerase (Solis BioDyne) and primers PBP3-Ec-For (5′-CTGCAAATGCAGCATGTTGATCCG-3′) and PBP3-Ec-Rev (5′-TCTCGCAGTGCTCGCGAAGGTGCG-3′) (10). PCR amplification products were visualized on an agarose gel and purified with the ExoSAP-IT PCR Product Cleanup reagent (Thermo Fisher). Sequencing was performed by Microsynth AG (Balgach,

Switzerland). Sequences were analyzed with Clustal Omega tool of the European Molecular Biology Laboratory of the European Bioinformatics Institute (https://www.ebi.ac.uk/Tools/msa/clustalo/). MLST of the analyzed isolates was performed according to EnteroBase (http://enterobase.warwick.ac.uk/species/ecoli/allele_st_search). MLST types were determined using the Center for Epidemiology tools (https://cge.cbs.dtu.dk/services/MLST/).

Cloning and antimicrobial susceptibility testing. The bla_{CMY-2} and bla_{CMY-42} genes were amplified from DNA of *E. coli* R468 and *E. coli* N590, respectively, using primers CMYF-cloning (5′-AACACACTGAT TGCGTCTGACG-3′) and CMYF-cloning (5′-GGCAAAATGCGCATGGGATT-3′). PCR products were cloned into pCR-BluntII-TOPO (Invitrogen, Thermo Fisher). Recombinant plasmids were further transformed into *E. coli* strain TOP10. Selection was based on plates containing kanamycin (50 μg/ml) and amoxicillin (30 μg/ml). Then, plasmids pTOPO-CMY-2 and pTOPO-CMY-42 were extracted and transformed into clinical *E. coli* strains N679 (NDM-5+YRIK) and N783 (NDM-19+YRIN). By using WGS, the β-lactamase content of those two isolates, namely, *E. coli* N679 and N783, was determined, confirming that they possessed only bla_{NDM-5} as single β-lactamase gene. Transformants were selected onto kanamycin (30 μg/ml)-containing Luria-Bertani agar plates. MICs for all obtained clones were determined by the broth microdilution method in Mueller-Hinton broth (Bio-Rad, Marnes-La-Coquette, France) for CAZ, CAZ-AVI, ATM, and ATM-AVI.

***β*-Lactamase purification.** Cultures of *E. coli* TOP10 harboring plasmid pTOPO-CMY-2 and pTOPO-CMY-42 were grown overnight at 37°C in 1 liter of Luria broth with kanamycin (30 μg/ml). The bacterial suspensions were pelleted, resuspended in 10 ml of 100 mM phosphate buffer (pH 7), disrupted by sonication (20 min for 30 s of sonication and 50 s of rest at 20 kHz with a Vibra Cell 75186), and centrifuged at 11,000 $\times$ *g* for 1 h at 4°C. This enzyme extract was dialyzed overnight against 20 mM Tris-HCl (pH 8) at 4°C and then was loaded onto a HiTrap Q HP column (GE Healthcare) preequilibrated with the same buffer. The resulting enzyme extract was recovered in the flowthrough and dialyzed against 20 mM piperazine sol (pH 10.2) overnight at 4°C. This extract was then loaded onto a preequilibrated (20 mM piperazine sol [pH 10.2]) HiTrap Q HP column and was then eluted with a linear NaCl gradient (0 to 1 M). The fractions showing the highest β-lactamase activity were pooled and dialyzed against 100 mM phosphate buffer (pH 7.0) prior to a 10-fold concentration with a Vivaspin 20 (GE Healthcare). The purified β-lactamase extract was immediately used for enzymatic determinations. The K_i value was determined by direct competition assays using 100 μM cephalothin. Inverse initial steady-state velocities (1/*V*0) were plotted against the inhibitor concentration ([I]) to obtain a straight line. The plots were linear and provided *y* intercept and slope values used for K_i determination. K_i was determined by dividing the value for the *y* intercept by the slope of the line and then corrected by taking into account the cephalothin affinity by the following equation: K_i (corrected) = K_i (observed)/(1 + [S]/K_m). Here, [S] is the concentration of cephalothin (100 μM) used in the assay and K_m is the Michaelis constant determined for cephalothin (18).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

ACKNOWLEDGMENTS

This work was financed by the University of Fribourg, Switzerland, and by the Swiss National Science Foundation (projects FNS-31003A_163432 and FNS-407240_177381).

L.P. and P.N. designed the study. M.S. and M.J. performed the experiments. All authors drafted the manuscript. L.P. and P.N. finalized the writing.

We declare no competing interests.

REFERENCES

1. Periasamy H, Joshi P, Palwe S, Shrivastava R, Bhagwat S, Patel M. 2020. High prevalence of *Escherichia coli* clinical isolates in India harbouring four amino acid inserts in PBP3 adversely impacting activity of aztreonam/avibactam. J Antimicrob Chemother 75:1650–1651. https://doi.org/10.1093/jac/dkaa021.
2. Berrazeg M, Diene SM, Medjahed L, Parola P, Drissi M, Raoult D, Rolain JM. 2014. New Delhi metallo-β-lactamase around the world: an eReview using Google Maps. Euro Surveill 19:20809. https://doi.org/10.2807/1560-7917.ES2014.19.20.20809.
3. Bushnell G, Mitrani-Gold F, Mundy LM. 2013. Emergence of New Delhi metallo-β-lactamase type 1-producing enterobacteriaceae and non-enterobacteriaceae: global case detection and bacterial surveillance. Int J Infect Dis 17:e325–e333. https://doi.org/10.1016/j.ijid.2012.11.025.
4. Rasheed JK, Kitchel B, Zhu W, Anderson KF, Clark NC, Ferraro MJ, Savard P, Humphries RM, Kallen AJ, Limbago BM. 2013. New Delhi metallo-β-lactamase-producing enterobacteriaceae, United States. Emerg Infect Dis 19:870–878. https://doi.org/10.3201/eid1906.121515.
5. Shields R, Doi Y. 2019. Aztreonam combination therapy: an answer to metallo-β-lactamase-producing Gram-negative bacteria? Clin Infect Dis 71:1159. https://doi.org/10.1093/cid/ciz1159.
6. Niu S, Wei J, Zou C, Chavda KD, Lv J, Zhang H, Du H, Tang Y-W, Pitout JDD, Bonomo RA, Kreiswirth BN, Chen L. 2020. *In vitro* selection of aztreonam/avibactam resistance in dual-carbapenemase-producing *Klebsiella pneumoniae*. J Antimicrob Chemother 75:559–565. https://doi.org/10.1093/jac/dkz468.
7. Sader HS, Mendes RE, Pfaller MA, Shortridge D, Flamm RK, Castanheira M. 2017. Antimicrobial activities of aztreonam-avibactam and comparator agents against contemporary (2016) clinical *Enterobacteriaceae* isolates. Antimicrob Agents Chemother 62:e01856-17. https://doi.org/10.1128/AAC.01856-17.
8. Chew KL, Tay MKL, Cheng B, Lin RTP, Octavia S, Teo JWP. 2018. Aztreonam-avibactam combination restores susceptibility of aztreonam in dual-carbapenemase-producing *Enterobacteriaceae*. Antimicrob Agents Chemother 62:e00414-18. https://doi.org/10.1128/AAC.00414-18.
9. Emeraud C, Escaut L, Boucly A, Fortineau N, Bonnin RA, Naas T, Dortet L.

2019. Aztreonam plus clavulanate, tazobactam, or avibactam for treatment of infections caused by metallo-β-lactamase-producing Gram-negative bacteria. Antimicrob Agents Chemother 63:e00010-19. https://doi.org/10.1128/AAC.00010-19.
10. Alm RA, Johnstone MR, Lahiri SD. 2015. Characterization of *Escherichia coli* NDM isolates with decreased susceptibility to aztreonam/avibactam: role of a novel insertion in PBP3. J Antimicrob Chemother 70:1420–1428. https://doi.org/10.1093/jac/dku568.
11. Davies TA, Page MG, Shang W, Andrew T, Kania M, Bush K. 2007. Binding of ceftobiprole and comparators to the penicillin-binding proteins of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*. Antimicrob Agents Chemother 51:2621–2624. https://doi.org/10.1128/AAC.00029-07.
12. Weiss DS, Pogliano K, Carson M, Guzman LM, Fraipont C, Nguyen-Distèche M, Losick R, Beckwith J. 1997. Localization of the *Escherichia coli* cell division protein FtsI (PBP3) to the division site and cell pole. Mol Microbiol 25:671–681. https://doi.org/10.1046/j.1365-2958.1997.5041869.x.
13. Weiss DS, Chen JC, Ghigo JM, Boyd D, Beckwith J. 1999. Localization of FtsI (PBP3) to the septal ring requires its membrane anchor, the Z ring, FtsA, FtsQ, and FtsL. J Bacteriol 181:508–520. https://doi.org/10.1128/JB.181.2.508-520.1999.
14. Shaw E, Rombauts A, Tubau F, Padullés A, Càmara J, Lozano T, Cobo-Sacristán S, Sabe N, Grau I, Rigo-Bonnin R, Dominguez MA, Carratalà J. 2018. Clinical outcomes after combination treatment with ceftazidime/avibactam and aztreonam for NDM-1/OXA-48/CTX-M-15-producing *Klebsiella pneumoniae* infection. J Antimicrob Chemother 73:1104–1106. https://doi.org/10.1093/jac/dkx496.
15. Jayol A, Nordmann P, Poirel L, Dubois V. 2018. Ceftazidime/avibactam alone or in combination with aztreonam against colistin-resistant and carbapenemase-producing *Klebsiella pneumoniae*. J Antimicrob Chemother 73:542–544. https://doi.org/10.1093/jac/dkx393.
16. Dean CR, Barkan DT, Bermingham A, Blais J, Casey F, Casarez A, Colvin R, Fuller J, Jones AK, Li C, Lopez S, Metzger LE, Mostafavi M, Prathapam R, Rasper D, Reck F, Ruzin A, Shaul J, Shen X, Simmons RL, Skewes-Cox P, Takeoka KT, Tamrakar P, Uehara T, Wei J-R. 2018. Mode of action of the monobactam LYS228 and mechanisms decreasing in-vitro susceptibility in *Escherichia coli* and *Klebsiella pneumoniae*. Antimicrob Agents Chemother 62:e01200-18. https://doi.org/10.1128/AAC.01200-18.
17. CLSI. 2015. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 10th ed: approved standard M07-A10. CLSI, Wayne, PA.
18. Poirel L, Ortiz de la Rosa JM, Kieffer N, Dubois V, Jayol A, Nordmann P. 2018. Acquisition of extended-spectrum β-lactamase GES-6 leading to resistance to ceftolozane-tazobactam combination in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 63:e01809-18. https://doi.org/10.1128/AAC.01809-18.