

Molecular Characterization of Carbapenem-Nonsusceptible Enterobacterial Isolates Collected during a Prospective Interregional Survey in France and Susceptibility to the Novel Ceftazidime-Avibactam and Aztreonam-Avibactam Combinations

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An interregional surveillance program was conducted in the northwestern part of France to determine the prevalence of carbapenem-nonsusceptible *Enterobacteriaceae* (CNSE) isolates and their susceptibility to ceftazidime-avibactam and aztreonam-avibactam combinations. Nonduplicate CNSE clinical isolates were prospectively collected from six hospitals between June 2012 and November 2013. MICs of ceftazidime and aztreonam, alone or combined with a fixed concentration of avibactam (4 µg/ml), and those of carbapenems (comparator agents) were determined. MICs of ertapenem in combination with phenylalanine arginine-naphthylamide dihydrochloride (PAβN) were also determined to assess active efflux. Genes encoding carbapenemases, plasmid-mediated AmpC enzymes, extended-spectrum β-lactamases (ESBLs), and major outer membrane proteins (OMPs) were amplified and sequenced. OMPs were also extracted for SDS-PAGE analysis. Among the 139 CNSE isolates, mainly *Enterobacter* spp. and *Klebsiella pneumoniae*, 123 (88.4%) were ertapenem nonsusceptible, 12 (8.6%) exhibited reduced susceptibility to all carbapenems, and 4 *Proteaeae* isolates (2.9%) were resistant to imipenem. Carbapenemase production was detected in only two isolates (producing OXA-48 and IMI-3). In contrast, OMP deficiency, in association with AmpCs and/or ESBLs (mainly CTX-M-9, SHV-12, and CTX-M-15), was largely identified among CNSE isolates. The ceftazidime-avibactam and aztreonam-avibactam combinations exhibited potent activity against CNSE isolates (MIC₅₀/MIC₉₀, 1/1 µg/ml and 0.5/0.5 µg/ml, respectively) compared to that of ceftazidime and aztreonam alone (MIC₅₀/MIC₉₀, 512/512 µg/ml and 128/512 µg/ml, respectively). This study reveals the *in vitro* activity of ceftazidime-avibactam and aztreonam-avibactam combinations against a large collection of porin-deficient enterobacterial isolates that are representative of the CNSE recovered in the northern part of France.

Carbapenems are broad-spectrum antibiotics usually reserved for severe life-threatening infections. Some isolates of *Enterobacteriaceae* have developed carbapenem resistance, which results in limited options for the treatment of infections caused by these organisms. Except for the members of the *Proteaeae* tribe, carbapenem resistance in *Enterobacteriaceae* is almost always attributable to the production of β-lactamases, which can be distinguished according to their carbapenemase activity. “True” carbapenemases (e.g., KPC, OXA-48, and metallo-β-lactamases [MBLs]) confer resistance *per se* to carbapenems, whereas extended-spectrum β-lactamases (ESBLs) and AmpC-type enzymes require an additional mechanism of resistance, such as a decrease in the uptake of antibiotics by porin deficiency (1, 2) or efflux system overexpression (3), to be responsible for carbapenem resistance.

The rapid dissemination of carbapenemase-producing carbapenem-resistant *Enterobacteriaceae* (CP-CRE) that account for worldwide outbreaks (4) could be attributable to the location of the carbapenemase-encoding genes on mobile elements that facilitate transmission within and between isolates of *Enterobacteriaceae*. CP-CRE infections are associated with high rates of morbidity and mortality and occur most frequently among inpatients with a prolonged hospitalization stay and those who are critically ill or exposed to invasive devices (4). The antibiotic agents for treating carbapenem-nonsusceptible *Enterobacteriaceae* (CNSE)

infections, such as colistin, are extremely limited and are often associated with adverse reactions.

CP-CRE isolates emerged in France in the late 2000s, and their number has increased particularly in Paris and in the northern part of the country (5). The aim of this study was to conduct a prospective interregional surveillance program to assess the prevalence of CNSE clinical isolates at six health care facilities located in the North of France and to identify their mechanisms of resis-

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tance. We also tested the activity of ceftazidime-avibactam and aztreonam-avibactam against these strains to determine whether these novel combinations could constitute therapeutic alternatives in the future.

MATERIALS AND METHODS

Bacterial strains. Enterobacterial clinical isolates that were recovered from clinical samples and presented reduced susceptibility to at least one carbapenem, according to the CLSI criteria (6), were collected from six health care facilities (the teaching hospitals of Amiens, Lille, and Caen and the general hospitals of Abbeville, Compiègne, and Beauvais). The isolates were collected during a 1-year period, between June 2012 and November 2013, according to the hospital centers. Only one isolate per patient was included in the study. All isolates were sent to the laboratory of bacteriology of the teaching hospital of Amiens for reidentification by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), antimicrobial susceptibility testing, and characterization of resistance mechanisms.

Antimicrobial susceptibility testing. MIC measurements were performed by the reference broth microdilution method as described in CLSI document M07-A9 (6). MICs of ceftazidime and aztreonam alone or in combination with avibactam (at a fixed concentration of 4 µg/ml; AstraZeneca Pharmaceuticals) and MICs of ertapenem, imipenem, meropenem, and doripenem, which were used as comparators, were also measured. Moreover, MICs of carbapenems in combination with cloxacillin (at a fixed concentration of 250 µg/ml; bioMérieux, Marcy l'Etoile, France), which acts as an inhibitor of AmpC-type enzymes (7), were also determined. Interpretation was made according to the criteria in CLSI document M100-S23 (8). Quality control (QC) was performed using *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, and *Pseudomonas aeruginosa* ATCC 27853.

Efflux pump inhibitor tests. MICs of ertapenem in combination with phenylalanine arginine-naphthylamide dihydrochloride (PAβN; Sigma-Aldrich) (26.3 µg/ml), an inhibitor of RND pumps of enterobacteria (9), were determined. A 2-fold decrease in MIC after addition of PAβN was considered significant (9). The laboratory mutant *E. coli* AG102, which overexpresses the AcrAB transporter, was used as a positive control (10).

β-Lactamase detection. The double-disk synergy test was performed to screen for ESBLs as described by the CLSI (8). AmpC overproduction was investigated using agar plates containing cloxacillin (250 µg/ml) (7). Isolates were screened for class A, B, and D carbapenemases by using the modified Hodge test (MHT) as recommended by the CLSI (8) and the Carba NP test as described previously (11).

β-Lactamase identification. PCR was used to amplify carbapenemase genes (*bla*_{KPC}, *bla*_{NDM}, *bla*_{IMP}, *bla*_{VIM}, *bla*_{SIM}, *bla*_{SPM}, *bla*_{GIM}, *bla*_{IMI}, *bla*_{OXA-48-like}, *bla*_{OXA-23-like}, *bla*_{OXA-24-like}, *bla*_{OXA-58-like} and *bla*_{OXA-50-like}), ESBL-encoding genes (*bla*_{CTX-M}, *bla*_{TEM-like}, *bla*_{SHV-like}, *bla*_{PER}, *bla*_{VEB}, and *bla*_{GES}), and plasmid-mediated AmpC-encoding genes (*bla*_{CMY-2}, *bla*_{DHA}, *bla*_{ACC}, *bla*_{ECT}, *bla*_{MOX}, and *bla*_{FOX}) according to previously described procedures (12–15). All PCR products were sequenced by use of an ABI7570 sequencer (Applied Biosystems, Foster City, CA).

Examination of porin genes and porin expression. The sequences of the *ompK35* and *ompK36* genes from *K. pneumoniae*, the *ompC* and *ompF* genes from *Enterobacter cloacae* and *E. coli*, the *omp-35* and *omp-36* genes from *Enterobacter aerogenes*, and the *ompC*-like and *ompF*-like genes from *Citrobacter freundii* and *Serratia marcescens* were amplified and sequenced using the primers shown in Table S1 in the supplemental material. The amplification conditions used for the *ompK35*, *ompK36*, *ompC*, *ompF*, *omp-35*, and *omp-36* genes were identical to those previously described (16, 17). The *ompC*-like and *ompF*-like genes from *Citrobacter freundii* and *Serratia marcescens* were amplified using the following program: 95°C for 5 min; 35 cycles of 94°C for 1 min, 51°C for 1 min, and 72°C for 1 min; and a final elongation step of 72°C for 7 min. Strains used for the comparison of porin gene sequences were as follows: *K. pneumoniae* NTUH-

K2044, *E. cloacae* ATCC 13047, *E. aerogenes* ATCC 13048, *Citrobacter freundii* ATCC 8090, and *Serratia marcescens* ATCC 13880. Outer membrane proteins (OMPs) were isolated according to the rapid procedure of Carlone et al. (18) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (16).

Molecular typing. Relatedness among isolates was established by random amplified polymorphic DNA (RAPD) analysis and by enterobacterial repetitive intergenic consensus PCR (ERIC-PCR), using Ap12h and ERIC-2 primers, respectively, as previously described (19).

RESULTS

Diversity of CNSE isolates. A total of 139 CNSE isolates, including 104 *E. cloacae* (75%), 16 *K. pneumoniae* (11.5%), 5 *E. aerogenes* (3.5%), 5 *C. freundii* (3.5%), 3 *Morganella morganii* (2.1%), 3 *E. coli* (2.1%), 2 *S. marcescens* (1.6%), and 1 *Proteus mirabilis* (0.8%) isolate, were collected. Eighty-four (60.4%), 43 (31%), 9 (6.5%), 2 (1.4%), 1 (0.7%), and 0 (0%) isolates were collected from the hospitals of Amiens, Lille, Caen, Beauvais, Abbeville, and Compiègne, respectively. A total of 11,431, 12,338, 1,862, 2,186, 1,235, and 1,138 enterobacterial isolates were recovered in the respective hospital centers during the study period.

Genotyping analysis revealed that 36 *E. cloacae* and 4 *K. pneumoniae* isolates in the teaching hospital of Amiens displayed undistinguishable genotyping patterns (data not shown) (clones A and B, respectively). Moreover, 3 *E. cloacae* and 2 *K. pneumoniae* isolates were genotypically related in the teaching hospital of Lille (clones C and D, respectively). All other CNSE isolates were genotypically unrelated.

Characterization of carbapenem resistance. Only two strains were detected as CP-CRE (1.4%). One *K. pneumoniae* isolate and one *E. cloacae* strain, recovered in the hospital of Caen and in the hospital of Amiens, respectively, tested positive for carbapenemase production. The *K. pneumoniae* isolate expressed an OXA-48 enzyme (in association with an OXA-1 penicillinase), whereas the *E. cloacae* isolate produced an IMI-3 β-lactamase (in combination with the chromosomally encoded cephalosporinase) (Table 1).

The other 137 CNSE isolates (98.6%) did not produce true carbapenemases, as deduced from the Hodge test, PCR screening, and the Carba NP test. One hundred twenty-four strains (90.5%) among these 137 strains produced chromosomally encoded or plasmid-mediated AmpC-type enzymes, as deduced from the significant decreases in the MICs of cephalosporins with the use of cloxacillin-containing plates (Table 1). One hundred nineteen strains expressed their chromosomally encoded AmpC enzymes, whereas 4 *K. pneumoniae* strains, which were clonally related (clone B), and one *E. coli* isolate turned out to be positive for *bla*_{CMY-2} and *bla*_{ACC-1} genes, respectively.

Among the 139 CNSE isolates included in this study, 79 (57%) were detected as ESBL producers by using the CLSI screening criteria and the PCR screening method (Table 1) (8). They included 64 *E. cloacae* (62% of the overall samples for this species), 11 *K. pneumoniae* (69%), 2 *E. coli* (66%), 1 *E. aerogenes* (20%), and 1 *C. freundii* (20%) isolate. CTX-M-type β-lactamases were detected among 93% of ESBL-producing isolates. CTX-M-9 was the most prevalent ESBL detected in *E. cloacae* isolates (94% of ESBL-producing *E. cloacae* isolates), frequently in combination with the SHV-12 enzyme (66%). In contrast, CTX-M-15 was the most prevalent ESBL in *E. coli* and *K. pneumoniae* isolates (100% of ESBL producers).

PAβN failed to restore susceptibility to ertapenem in the 139

TABLE 1 β -Lactamase production and OMP profiles for the 139 CNSE isolates

OMP profile	Organism with β -lactamase production, β -lactamase(s) (no. of strains)				
	Carbapenemase	ESBL	AmpC	ESBL + AmpC	No β -lactamase
Wild-type outer membrane profile	<i>E. cloacae</i> , IMI-3 (1)		<i>M. morgani</i> (3)		<i>P. mirabilis</i> (1)
	<i>K. pneumoniae</i> , OXA-48 (1)				
Loss of one major outer membrane porin		<i>K. pneumoniae</i> , CTX-M-15 (10) <i>E. coli</i> , CTX-M-15 (2)	<i>E. aerogenes</i> (4) <i>E. cloacae</i> (39) <i>C. freundii</i> (4) <i>S. marcescens</i> (1) <i>E. coli</i> , ACC-1 (1)	<i>E. cloacae</i> , CTX-M-9/SHV-12 (36) <i>E. cloacae</i> , CTX-M-9 (20) <i>E. cloacae</i> , SHV-12 (4) <i>C. freundii</i> , CTX-M-9 (1)	
Loss of OmpC-like and OmpF-like porins		<i>K. pneumoniae</i> , CTX-M-15 (1)	<i>K. pneumoniae</i> , CMY-2 (4) <i>S. marcescens</i> (1)	<i>E. cloacae</i> , CTX-M-9/SHV-12 (4) <i>E. aerogenes</i> , TEM-24 (1)	

CNSE isolates included in this study, suggesting that efflux systems are unlikely to be involved. SDS-PAGE analysis of OMPs revealed a deficit of at least one major OMP for all CNSE isolates (Fig. 1), except the two CP-CRE strains and the four *Proteaceae* strains (Table 1). Sequence analysis of the *ompC*-like and *ompF*-like genes revealed that the porin deficiency was attributable, in 48% of cases, to disruption by insertion sequence, nonsense mutations leading to a premature stop codon, amino acid insertion in the L3 loop (20). Some *ompC*-like or *ompF*-like genes were not amplified, in agreement with complete gene deletion or a large insertion preventing amplification. Moreover, 52% of CNSE isolates with an altered OMP profile did not present mutations in the coding sequences of the *ompC*-analogue and *ompF*-analogue genes. Among the 133 porin-deficient CNSE isolates, 122 lacked only one major porin, and the other 11 had a simultaneous loss of both OMPs.

CNSE isolates fell into three groups according to their carbapenem MICs. The first one included 123 isolates (88% of the overall CNSE strains) that presented a reduced susceptibility to ertapenem, whereas they remained susceptible to imipenem, meropenem, and doripenem (Table 2). All these strains, except the OXA-48-producing isolate, were also resistant to extended-spectrum cephalosporins. The 12 isolates (9% of the overall CNSE strains) of the second group were resistant or had intermediate resistance to all carbapenems (Table 2). They included three clonally related SHV-12/CTX-M-9-producing *E. cloacae* strains, derived from clone A isolated in the city of Amiens, one clonally unrelated SHV-12/CTX-M-9-producing *E. cloacae* strain that was

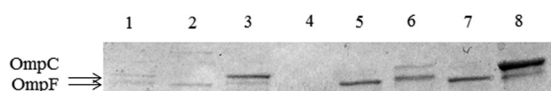


FIG 1 OMP profiles of representative CNSE isolates compared to wild-type reference strains. OMPs were profiled by SDS-PAGE. Lanes: 1, *E. coli* porin-deficient strain BH01043; 2, *E. coli* porin-deficient strain DP02127; 3, *E. coli* wild-type strain TOP10; 4, *E. cloacae* porin-deficient strain BH01124; 5, *E. cloacae* porin-deficient strain AJP01076; 6, *E. cloacae* wild-type strain ATCC 13047; 7, *K. pneumoniae* porin-deficient strain AG02123; 8, *K. pneumoniae* wild-type strain NTUH-K2044. The arrows indicate the positions of the OMPs OmpC and OmpF.

recovered in Caen, one IMI-3-producing *E. cloacae* isolate, four clonally related CMY-2-producing *K. pneumoniae* strains (clone B), one CTX-M-15-producing *K. pneumoniae* strain, one TEM-24-producing *E. aerogenes* strain, and one non-ESBL-producing *S. marcescens* strain. These strains were resistant to all β -lactams, except the IMI-producing *E. cloacae* isolate, which remained susceptible to cefepime. The four strains of the tribe *Proteaceae*, which were susceptible to all carbapenems but imipenem, were integrated in the third group (3% of the overall CNSE strains). They did not produce ESBL and carbapenemase and were susceptible to cefepime.

It is noteworthy that the level of resistance to carbapenems correlated with the number of porins expressed, except with regard to the two carbapenemase producers and the four *Proteaceae* strains. CNSE strains resistant only to ertapenem expressed one OMP, whereas those that were resistant or had intermediate resistance to all carbapenems produced neither OmpC nor OmpF analogues. Moreover, a ≥ 2 -fold decrease in the MIC of ertapenem in combination with the AmpC inhibitor cloxacillin was shown for all of the *E. cloacae*, *E. aerogenes*, *C. freundii*, and *S. marcescens* isolates, except the IMI-3-producing *E. cloacae* isolate, thus demonstrating the important contribution of class C β -lactamases to carbapenem resistance in our study.

To define the spectra of activity of ceftazidime-avibactam and aztreonam-avibactam against the CNSE isolates that were collected in our survey, we tested the susceptibilities of the 139 strains to these novel combinations. The ceftazidime-avibactam and aztreonam-avibactam combinations exhibited overall potent activities against CNSE isolates (MIC₅₀/MIC₉₀, 0.5/1 μ g/ml and 0.5/0.5 μ g/ml, respectively) compared to those with ceftazidime and aztreonam alone (MIC₅₀/MIC₉₀, 512/512 μ g/ml and 128/512 μ g/ml, respectively) or ertapenem and imipenem alone (MIC₅₀/MIC₉₀, 1/4 μ g/ml and 0.5/8 μ g/ml, respectively) (Table 2). The 123 ertapenem-nonsusceptible enterobacterial isolates exhibited ceftazidime-avibactam MICs and aztreonam-avibactam MICs of < 1 μ g/ml, whereas the 12 carbapenem-nonsusceptible enterobacterial strains exhibited ceftazidime-avibactam and aztreonam-avibactam MICs of ≤ 4 μ g/ml (Table 2). The three imipenem-nonsusceptible *M. morgani* isolates and the *P. mirabilis* isolate

TABLE 2 MIC distributions for the 139 carbapenem-nonsusceptible *Enterobacteriaceae* clinical isolates, with cumulative percentages

Organism (no. of strains)	Agent(s) ^a	MIC (μg/ml) (cumulative %)										MIC ₅₀	MIC ₉₀
		≤0.06	0.125	0.25	0.5	1	2	4	8	16	≥32		
<i>E. cloacae</i> (104)	CAZ								2 (2)	3 (5)	99 (100)	≥32	≥32
	CAZ-AVI		9 (9)	21 (29)	58 (85)	12 (96)	4 (100)					0.5	1
	ATM						2 (2)	3 (5)	14 (18)	85 (100)	≥32	≥32	
	ATM-AVI		14 (13)	23 (36)	63 (96)	4 (100)						0.5	0.5
	ERTA					82 (79)	12 (90)	5 (93)			5 (100)	1	2
	IMP		5 (5)	8 (13)	76 (86)	10 (95)			4 (99) ^b		1 (100) ^c	0.5	1
	MERO			11 (11)	79 (87)	9 (95)			4 (99) ^b		1 (100) ^c	0.5	1
	DORI		18 (17)	71 (86)	9 (94)	1 (95)		4 (99) ^b			1 (100) ^c	0.25	1
<i>K. pneumoniae</i> (16)	CAZ		1 (6)								15 (100)	≥32	≥32
	CAZ-AVI		3 (19)	8 (69)	1 (75)			4 (100)				0.25	4
	ATM	1 (6)									15 (100)	≥32	≥32
	ATM-AVI	1 (6)	9 (63)	2 (75)				4 (100)				0.125	4
	ERTA					7 (44)	3 (63)	2 (75)			5 (100) ^b	2	≥32
	IMP			2 (13)	8 (63)	1 (69)				1 (75) ^b	4 (100) ^b	0.5	≥32
	MERO				2 (13)	9 (69)				1 (75) ^b	4 (100) ^b	1	≥32
	DORI			2 (13)	7 (56)	2 (69)			1 (75) ^b	4 (100) ^b		0.5	16
<i>E. aerogenes</i> (5)	CAZ									1 (20)	4 (100)	≥32	≥32
	CAZ-AVI			2 (40)	2 (80)		1 (100)					0.5	2
	ATM							1 (20)	1 (40)	3 (100)	≥32	≥32	
	ATM-AVI		3 (60)	1 (80)		1 (100)					0.25	1	
	ERTA						4 (80)			1 (100) ^b	2	≥32	
	IMP					4 (80)			1 (100) ^b		1	8	
	MERO					4 (80%)			1 (100) ^b		1	8	
	DORI			1 (20%)	3 (80%)			1 (100%) ^b				0.5	4
<i>E. coli</i> (3)	CAZ						2 (67)				1 (100)	2	≥32
	CAZ-AVI		1 (33)	2 (100)								0.25	0.25
	ATM								2 (66)		1 (100)	8	≥32
	ATM-AVI	1 (33)	1 (67)	1 (100)								0.125	0.25
	ERTA			2 (67)	1 (100)							1	2
	IMP				3 (100)							0.25	0.25
	MERO				3 (100)							0.25	0.25
	DORI			1 (33)	2 (100)							0.25	0.25
<i>C. freundii</i> (5)	CAZ										5 (100)	≥32	≥32
	CAZ-AVI			2 (40)	3 (100)							0.5	0.5
	ATM									4 (80)	1 (100)	16	≥32
	ATM-AVI			4 (80)	1 (100)							0.25	0.5
	ERTA					1 (20)	3 (80)	1 (100)				2	4
	IMP			1 (20)	2 (60)	2 (100)						0.5	1
	MERO				3 (60)	2 (100)						0.5	1
	DORI			3 (60)	1 (80)	1 (100)						0.25	1
<i>S. marcescens</i> (2)	CAZ										2 (100)	≥32	≥32
	CAZ-AVI				1 (50)	1 (100)						0.5	1
	ATM										2 (100)	≥32	≥32
	ATM-AVI			1 (50)		1 (100)						0.25	1
	ERTA					1 (50)					1 (100) ^b	1	≥32
	IMP						1 (50)				1 (100) ^b	0.5	≥32
	MERO					1 (50)					1 (100) ^b	0.25	≥32
	DORI					1 (50)				1 (100) ^b		0.25	4
<i>P. mirabilis</i> (1)	CAZ	1 (100)										≤0.06	≤0.06
	CAZ-AVI	1 (100)										≤0.06	≤0.06
	ATM	1 (100)										≤0.06	≤0.06
	ATM-AVI	1 (100)										≤0.06	≤0.06
	ERTA	1 (100)										≤0.06	≤0.06
	IMP								1 (100)			8	8
	MERO	1 (100)										≤0.06	≤0.06
	DORI	1 (100)										≤0.06	≤0.06

(Continued on following page)

TABLE 2 (Continued)

Organism (no. of strains)	Agent(s) ^a	MIC ($\mu\text{g/ml}$) (cumulative %)										MIC ₅₀	MIC ₉₀
		≤ 0.06	0.125	0.25	0.5	1	2	4	8	16	≥ 32		
<i>M. morgani</i> (3)	CAZ	1 (33)							1 (67)		1 (100)	8	≥ 32
	CAZ-AVI	1 (33)	1 (67)	1 (100)								0.125	0.25
	ATM	1 (33)	1 (67)						1 (100)			0.125	1
	ATM-AVI	3 (100)										≤ 0.06	≤ 0.06
	ERTA	3 (100)										≤ 0.06	≤ 0.06
	IMP							2 (67)	1 (100)			8	16
	MERO	3 (100)										≤ 0.06	≤ 0.06
	DORI	3 (100)										≤ 0.06	≤ 0.06

^a CAZ, ceftazidime; CAZ-AVI, ceftazidime-avibactam; ATM, aztreonam; ATM-AVI, aztreonam-avibactam; ERTA, ertapenem; IMP, imipenem; MERO, meropenem; DORI, doripenem.

^b CNSE isolates with a complete loss of OmpC and OmpF porins.

^c True carbapenemase producers.

exhibited ceftazidime-avibactam and aztreonam-avibactam MICs of $\leq 2 \mu\text{g/ml}$ (Table 2).

DISCUSSION

This study was intended to determine the epidemiology of CNSE clinical isolates in the northern part of France and to assess the potency of the ceftazidime-avibactam and aztreonam-avibactam combinations against these isolates. A notable feature of our interregional study is that the CNSE isolates were prospectively and exhaustively collected during a 1-year period, thus providing an accurate picture of the epidemiology in the North of France. Most CNSE isolates in this study were collected in teaching hospitals (97% of the overall strains), whereas only a few CNSE strains were recovered in general hospitals (3%). Most of the CNSE isolates included were recovered in the teaching hospitals of Amiens and Lille, which could be attributable to the local spread of ESBL- and/or AmpC-producing epidemic strains. It is possible that carbapenem-containing regimens, which were used to treat infections due to ESBL producers, might have further selected for mutants lacking outer membrane permeability. By reducing the antibiotic concentration inside the periplasm, porin changes can amplify the β -lactamase effects of ESBLs and AmpC-type enzymes on weakly hydrolyzed substrates, such as carbapenems (1, 2). An additional study is ongoing to assess the contribution of carbapenem regimens to selection of porin-deficient mutants.

AmpC enzymes appeared to contribute greatly to carbapenem resistance in our collection. The carbapenem-hydrolyzing activity of class C β -lactamases has been reported previously (1, 7). This activity could be attributable, at least in part, to the asparagine residue at position 346, which plays a role in placing the acyl enzyme intermediate of extended-spectrum cephalosporins (ESCs) in a position that is more competent for hydrolytic attack (1, 21).

In contrast, the prevalence of isolates producing true carbapenemases was low in our study. Only 2 of 139 strains produced β -lactamases (IMI-3 and OXA-48). These results are in agreement with previous studies, carried out in Chile (22), Korea (23), China (24), United Kingdom (25), Belgium (26), and France (5, 27), which showed that the emergence of carbapenem resistance in *Enterobacteriaceae* could be supported mainly by the combination of ESBLs or AmpC β -lactamases with porin deficiency in certain areas. Recent reports highlighted the implication of carbapenem-nonsusceptible noncarbapenemase producers in nosocomial outbreaks (28) in different countries, mostly from *K. pneumoniae* or *Enterobacter* spp. (29–31). These isolates are commonly selected *in*

in vivo during the course of carbapenem therapy, as a consequence of a lack of membrane permeability.

In 48% of the overall porin-deficient CNSE isolates, loss of membrane permeability resulted from changes in OMP structure. Genes encoding major porins were affected by mutations causing protein structure changes in the L3 loop, by premature termination of translation, or by gene disruption. The L3 domain constitutes a pore constriction region that exhibits negative charges and plays a critical role in determining the characteristics of the pore (20). The two amino acid replacements identified in our study, Y214T and A141V, which were located in OmpC-like and OmpF-like porins of *E. cloacae* isolates, respectively, may perturb the electrostatic field acting in the eyelet and modify the physicochemical and biological channel properties. Most of these structural alterations occurred in only one of the two major OMPs, thus affecting the susceptibility to ertapenem (1). Reduced susceptibility to other carbapenems, such as imipenem and doripenem, was achieved once both major porins were altered. It is noteworthy that no significant changes in the *ompC*-like and *ompF*-like genes were identified in 52% of the porin-deficient CNSE isolates. We assume that a loss of permeability might result from decreased porin production. Further investigations into the nature of the purported regulatory system and its mutations are ongoing.

In addition, the peculiar resistance phenotype exhibited by the *M. morgani* and *P. mirabilis* isolates, which were fully susceptible to all carbapenems but imipenem, could be related to an alteration of penicillin-binding protein 2 (PBP2), as reported previously (32, 33).

The carbapenemase threat remains rare in French hospitals compared with the ESBL endemicity. In contrast, carbapenem resistance has arisen from local spread of *E. cloacae* and *K. pneumoniae* clones associated with the dissemination of ESBL (particularly CTX-M-9 and/or SHV-12 for *E. cloacae* and CTX-M-15 for *K. pneumoniae*) and AmpC production, leaving few therapeutic options available.

Several studies, conducted on a wide range of enterobacterial clinical isolates recovered throughout the world, have already demonstrated the overall activity of the ceftazidime-avibactam combination, including activity against ESBL, AmpC, and KPC producers (ceftazidime-avibactam MIC₅₀ of $\leq 0.5 \mu\text{g/ml}$ and ceftazidime-avibactam MIC₉₀ of $\leq 2 \mu\text{g/ml}$) (34–38). However, few data describe the *in vitro* activity of this combination against

porin-deficient enterobacterial isolates. To the best of our knowledge, only two articles showed the *in vitro* efficiency of this combination against well-characterized porin-deficient enterobacterial clinical isolates, namely, *Cedecea davisae* FUR and *K. pneumoniae* C2/pMG247 (7, 39). Livermore et al. also reported the activity of the ceftazidime-avibactam combination against a few ertapenem-resistant enterobacterial isolates, but these isolates were not characterized on the molecular level (40).

Our study shows the *in vitro* activity of the ceftazidime-avibactam and aztreonam-avibactam combinations against a representative collection of well-characterized CNSE clinical isolates lacking outer membrane permeability. It appears that porin deficiency does not impair the penetration of avibactam into the periplasmic space. The highest MIC value for the ceftazidime-avibactam and aztreonam-avibactam combinations against porin-deficient CNSE isolates was 4 µg/ml. According to the U.S. FDA ceftazidime-avibactam MIC breakpoints for *Enterobacteriaceae* (susceptible, ≤8 µg/ml; and resistant, ≥16 µg/ml) (41), which are based on pharmacokinetic/pharmacodynamic analyses (42), these porin-deficient CNSE isolates are susceptible. Additional studies are needed to establish what the potential roles of ceftazidime-avibactam and aztreonam-avibactam might be as substitutes for carbapenems to reduce the dissemination of CNSE in the future.

Concluding remarks. OMP deficiency in association with AmpCs and/or ESBLs was the main mechanism conferring carbapenem resistance in this survey. This study reveals the *in vitro* activity of the ceftazidime-avibactam and aztreonam-avibactam combinations against a representative collection of well-characterized CRE isolates with OMP deficiency.

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