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NOTES

Prospective Survey of β-Lactamases Produced by Ceftazidime-Resistant *Pseudomonas aeruginosa* Isolated in a French Hospital in 2000

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In 2000, at the Université d'Auvergne teaching hospital in Clermont-Ferrand, France, 44 (6.2%) strains of *Pseudomonas aeruginosa* were found to be resistant to ceftazidime. After genotyping, 34 strains were selected. Nine had an additional β-lactamase: OXA-21 ($n = 6$), PSE-1 (CARB-2) ($n = 2$), or PER-1 ($n = 1$). Ceftazidime resistance was related solely to the overproduction of the cephalosporinase in 30 strains. Sequencing of five bla_{AmpC} genes encoding cephalosporinases with different pIs showed 99% identity with the *ampC* gene of *P. aeruginosa* PAO1.

Resistance to extended-spectrum cephalosporins in *Pseudomonas aeruginosa* is associated in most cases with the overproduction of a naturally produced cephalosporinase (19). Derepression of this AmpC β-lactamase affects all β-lactams to various extents, which may lead to the misdetection of other acquired β-lactamases. Enzymes hydrolyzing extended-spectrum cephalosporins of Ambler classes A, B, and D have been reported in *P. aeruginosa* (19). Among the extended-spectrum β-lactamases (ESBLs) of class A, TEM-4 (22), TEM-24 (15), TEM-42 (16), PER-1 (18), SHV-2a (19), VEB-1 (21), and GES-2 (26) have been encountered.

Class B enzymes, IMP-1 (33), VIM-1 (10), VIM-2 (23), and VIM-related enzymes (11), hydrolyze carbapenems in addition to extended-spectrum cephalosporins.

The extended-spectrum class D enzymes are OXA-2 derivatives (OXA-15 [4] and OXA-32 [24]), OXA-10 derivatives (OXA-11 [7], OXA-14 [3], OXA-16 [5], OXA-17 [6], OXA-19 [17], and OXA-28 [25]), and OXA-18 (20).

Other mechanisms implicated in resistance to β-lactams in *P. aeruginosa* are impermeability of the outer membrane and increased efflux (12) The aim of this study was to characterize the β-lactamases produced by ceftazidime-resistant *P. aeruginosa* isolates.

Of the 713 nonduplicate *P. aeruginosa* strains isolated from the Université d'Auvergne teaching hospital in Clermont-Ferrand, France, between 1 January and 30 September 2000, 44 (6.2%) were resistant to ceftazidime, as determined by the disk diffusion method on Mueller-Hinton agar according to the recommendations of the Antibiogram Committee of the French Society for Microbiology (zone diameter, <15 mm; www.sfm.asso.fr/Sect4/atbuk.html). This prevalence of ceftazidime resistance was higher than the 4% prevalence in 1996 reported in a previous French national multicenter study (2). Isolates were identified with the ID 32 GN system (BioMérieux, Marcy l'Etoile, France). A duplicate isolate was defined as an isolate with the same antibiotic resistance phenotype as another isolate from the same patient during the same course of infection. The 44 strains were typed by random amplification of polymorphic DNA (RAPD) with two primers, ERIC-2 and 208, as described previously (Table 1) (14, 30). Isoelectric focusing analysis was performed with 6% polyacrylamide gels containing ampholines (Amersham Pharmacia Biotech, Uppsala, Sweden) with a pH range of 3.5 to 10, as reported previously (19). Among the 44 strains, 34 were selected on the basis of antibiotic resistance patterns, pIs, and patterns by RAPD analysis (Table 2). The chromosomal cephalosporinases focused at five pIs: 8.4 ($n = 13$), 8.0 ($n = 8$), 8.0 and 8.4 ($n = 7$), 7.8 ($n = 4$), and 8.6 ($n = 2$). Three additional β-lactamases were found at pIs 7.0 ($n = 6$), 5.7 ($n = 2$), and 5.4 ($n = 1$). The strains were distributed into 33 different RAPD profiles.

The MICs of drugs alone or in combination with a fixed concentration of clavulanate (2 μg/ml) and tazobactam (4 μg/ml) were determined by the dilution method in Mueller-Hinton agar with an inoculum of 10^4 CFU per spot, as described previously (20) (Table 2). The MICs of ceftazidime were in a narrow range from 32 to 128 μg/ml. The MICs of ticarcillin ranged from 64 to 2,048 μg/ml. The use of clavulanate did not restore susceptibility to ticarcillin. The MICs of piperacillin were between 16 and 256 μg/ml, and the MICs of aztreonam were between 16 and 128 μg/ml. The strains were

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TABLE 1. Oligonucleotides used as primers for amplification and/or sequencing

Primer	Sequence	Position[a]	Strand	Reference
ERIC-2	5′-AAGTAAGTGACTGGGGTGAGC-3′	NA[b]	NA	30
208	5′-ACGGCCGACC-3′	NA	NA	14
PER-A	5′-TGACGATCTGGAACCTTT-3′	1053	Forward	This study
PER-B	5′-AACTGCATAACCTACTCC-3′	204	Reverse	This study
OC1	5′-AATGGCAATCAGCGCTTC-3′	311	Forward	1
OC3	5′-GCGCGACTGTGATGTATA-3′	1009	Reverse	1
AMPC-PAC	5′-GGGGCGGTTTCTCATGCAGCCAACG-3′	510	Forward	This study
AMPC-PAD	5′-GAAGCGCTCATGGCACCATCATAGCC-3′	1822	Reverse	This study
AMPC-PAG	5′-CGACCTTGTAGTAACCGC-3′	1421	Reverse	This study
AMPC-28	5′-TCCTCATGCGCGATAC-3′	549	Forward	This study
AMPC-PAB1	5′-GTTGGAATAGAGGC-3′	1090	Reverse	This study
OXA-2,3	5′-GCCAAAGGCACGATAGTTGT-3′	239	Forward	This study
OXB-2,3	5′-GCGTCCGAGTTGACTGCCGG-3′	939	Reverse	This study

[a] Numbers correspond to the position of the first 5′ base of each oligonucleotide according to the numbering of the nucleotide sequences with GenBank accession nos. Z21957 (bla_{PER-1}), M69058 (bla_{PSE-1}), X03037 (bla_{OXA-2}), and X54719 (bla_{AmpC}).
[b] NA, not applicable.

moderately resistant to cefepime (MICs, 8 to 64 μg/ml). Fifteen strains were resistant to imipenem (MICs, >8 μg/ml), and of these, three were resistant to meropenem (MICs, >8 μg/ml).

Cephalosporinase inhibition, evaluated by the disk diffusion method on Mueller-Hinton agar containing cloxacillin at 250 or 500 μg/ml, was considered significant when the ceftazidime zone diameter increased by >10 mm. *P. aeruginosa* PAO1 was used as the reference strain (8). For 25 strains which did not have pI values that might correspond to those for an additional β-lactamase, ceftazidime activity was partially restored by cloxacillin at 250 μg/ml ($n = 15$) or only by cloxacillin at 500 μg/ml ($n = 10$). For these 25 strains without an additional β-lactamase, the level of production of the AmpC enzymes was measured by determination of their specific β-lactamase activities. Cultures were grown overnight at 37°C in 3 liters of brain heart infusion (Biokar Diagnostics, Beauvais, France). Bacterial suspensions were disrupted by sonication and centrifuged as described previously (19). The supernatants were dialyzed against 3 liters of 100 mM NaCl overnight at 4°C. Then, the specific β-lactamase activities of the crude extracts were determined by a computerized microacidimetric method described elsewhere (9) with 225 mM benzylpenicillin as the substrate. The total protein concentration in the enzyme preparation was estimated by a protein assay (Bio-Rad, Richmond, Calif.) with bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as the standard. One unit of specific β-lactamase activity was defined as the amount of enzyme which hydrolyzes 1 μmol of benzylpenicillin per min and per mg of protein at 37°C and at pH 7. For 21 strains the specific activity was 100- to 1,000-fold greater than that of PAO1, with values ranging from 124 to 15,373 mU mg^{-1} (Table 2). Variations in levels of resistance to ceftazidime were weakly related to different levels of enzyme overproduction (i.e., an MIC of 64 μg/ml corresponds to specific activities of 52 mU mg^{-1} for strain 33 and 15,373 mU mg^{-1} for strain 5).

The *ampC* genes of the strains that produced five cephalosporinases with different pI values were amplified with primers AMPC-PAC and AMPC-PAD (Table 1) under the following conditions: 94°C for 5 min and then 30 cycles of 94°C for 1 min, 50°C for 3 min, and 72°C for 3 min, with a single final extension at 72°C for 15 min. The PCR products were sequenced with the primers AMPC-PAD, AMPC-PAG, AMPC-28, and AMPC-PAB1 (Table 1). Sequence analysis showed a low level of sequence diversity (0.17 to 0.75%) and 99% nucleotide identity with the *ampC* gene of *P. aeruginosa* strain PAO1 (GenBank accession no. X54719). Compared to the PAO1 *ampC* gene sequence (8), all *amp*C genes encoding enzymes with pIs different from 8.0 harbored the mutation A866→G, which led to the substitution Thr105→Ala (Table 3).

Genes encoding enzymes of pI 8.0 + 8.4 and pI 8.4 harbored a mutation at position 1166 that led to the substitution Val205→Leu. The *ampC* gene encoding the enzyme of pI 7.8 harbored the mutation G789→A, which led to the substitution Arg79→Gln, and that encoding the enzyme of pI 8.6 harbored the mutation T1080→G, which led to the substitution Leu176→Arg. The Arg79→Gln substitution is associated with a change in charges and may have been responsible for the decrease in the pI value from 8.0 to 7.8, as reported previously (28). However, enzymes with identical pIs do not necessarily share identical amino acid sequences, and the different pIs suggested that some bands correspond to cleaved derivatives (isoenzymes) of the cephalosporinase, as reported previously (32).

For eight of the nine strains with an additional β-lactamase, ceftazidime activity was restored only by cloxacillin at 500 μg/ml (strains 18 to 25), and so ceftazidime resistance was very likely due to hydrolysis by the derepressed chromosomal cephalosporinase. For strain 4 the activity of ceftazidime was not restored by cloxacillin at 500 μg/ml. Strain 4 was the only one which had a positive modified disk synergy test result with ceftazidime and clavulanate (27). The genes of the six β-lactamases with a pI of 7.0 were amplified with OXA-2 primers (primers OXA-2,3 and OXB-2,3). Nucleotide sequence analysis of the bla_{OXA} PCR products revealed a bla_{OXA-21} sequence with only one silent mutation (T697→C) in the genes of four isolates compared to the sequence of bla_{OXA-21} previ-

TABLE 2. Characteristics of the 34 ceftazidime-resistant *P. aeruginosa* isolates

Strain[j]	pI	Cephalosporinase inhibition with CLO at[a]:		MIC (μl/ml)[b]									Sp act[c] (mU/mg) [mean ± SD])	βla[d]	Type[e]	Type by RAPD analysis	Site of recovery
		250 μg/ml	500 μg/ml	TIC	TCC	PIP	TZP	CAZ	FEP	ATM	IPM	MEM					
1	7.8	+	+	128	128	128	128	128	32	64	64	8	730 ± 89		NA[g]	1	Rectal swab
2	7.8	+	+	64	64	64	64	32	16	16	16	8	ND[h]		1	2	Wound
3	7.8	−	+	256	256	128	128	64	32	64	1	0.5	0.611 ± 20		6	3	Urines
4	7.8 + 5.4	−	−	256	64	16	16	128	32	128	16	16		PER-1	11	4	Rectal swab
5	8.0	−	+	256	256	256	256	64	32	64	1	0.5	15,373 ± 940		NA	5	Rectal swab
6	8.0	+	+	64	64	32	16	32	8	16	1	0.25	1,566 ± 22		6	6	BP[f]
7	8.0	−	+	128	128	128	128	32	32	16	16	16	1,602 ± 93		11	7	Wound
8	8.0	+	+	128	128	128	128	64	32	32	2	0.5	321 ± 1		3	8	Urine
9	8.0	+	+	64	64	64	32	32	16	16	32	8	231 ± 8		11	9	Urine
10[1]	8.0	−	+	128	128	128	128	64	16	32	1	0.5	1,679 ± 327		6	10	Rectal swab
11	8.0	+	+	128	128	128	128	128	32	128	16	8	124 ± 21		NA	11	Rectal swab
12	8.0	+	+	256	256	256	256	64	64	64	0.5	1	ND		11	12	Rectal swab
13	8.4	−	+	512	512	256	256	128	64	128	2	4	1,010 ± 24		NA	13	Blood
14	8.4	−	+	128	128	128	128	64	32	32	16	8	1,243 ± 52		6	14	BP
15	8.4	−	+	256	256	256	256	128	64	128	8	4	10,054 ± 30		6	15	BP
16	8.4	+	+	128	128	128	128	64	32	32	4	2	688 ± 28		1	16	BP
17	8.4	+	+	128	128	128	128	64	16	16	2	0.5	373 ± 12		NA	17	BP
18	8.4 + 5.7	−	+	2,048	1,024	256	256	64	32	32	32	16		PSE-1	12	18	Rectal swab
19	8.4 + 5.7	−	+	2,048	256	256	128	32	16	16	32	4		PSE-1	12	19	BP
20	8.4 + 7.0	−	+	256	256	64	32	64	32	16	2	0.25		OXA-21	3	20	PDF[k]
21	8.4 + 7.0	−	+	128	128	64	64	32	16	16	32	8		OXA-21	4	21	BP
22	8.4 + 7.0	−	+	256	256	64	64	32	32	32	32	8		OXA-21	3	22	BP
23	8.4 + 7.0	−	+	256	256	64	64	64	32	16	2	0.25		OXA-21	4	23	Urine
24	8.4 + 7.0	−	+	256	256	128	128	64	32	32	2	0.5		OXA-21	6	24	Rectal swab
25	8.4 + 7.0	−	+	256	256	64	64	64	32	16	2	0.25		OXA-21	3	25	Blood
26	8.0 + 8.4	+	+	128	128	128	128	64	32	32	16	8	894 ± 66		11	26	BP
27	8.0 + 8.4	+	+	128	128	128	128	64	32	32	0.5	1	680 ± 80		11	27	BP
28	8.0 + 8.4	+	+	128	128	64	64	32	16	16	16	8	693 ± 29		11	28	BP
29	8.0 + 8.4	−	+	256	256	128	128	64	32	32	1	0.5	678 ± 9		6	22	BP
30	8.0 + 8.4	+	+	128	128	64	64	128	32	128	16	8	177 ± 21		NA	29	Rectal swab
31	8.0 + 8.4	+	+	64	64	64	64	32	8	32	32	8	641 ± 10		11	30	Wound
32	8.0 + 8.4	+	+	64	64	64	32	32	8	16	2	0.12	1,761 ± 50		1	31	Rectal swab
33	8.6	+	+	128	128	64	32	64	16	64	1	0.25	52 ± 1		6	32	Rectal swab
34	8.6	−	+	256	256	128	128	64	64	64	0.5	0.5	ND		3	33	BP
PAO1	8.0	NAP[i]	NAP	≤16	≤16	≤4	≤4	≤2	2	4	1	0.25	<0.001		1		

[a] Cephalosporinase inhibition test on Mueller-Hinton agar with cloxacillin (CLO) at 250 or 500 μg/ml. +, ceftazidime zone diameter increase of >10 mm; −, ceftazidime zone diameter increase of ≤10 mm.

[b] Abbreviations: TIC, ticarcillin; TCC, ticarcillin-clavulanate (2 μg/ml); PIP, piperacillin; TZP, piperacillin-tazobactam (4 μg/ml); CAZ, ceftazidime; FEP, cefepime; ATM, aztreonam; IPM, imipenem; MEM, meropenem.

[c] AmpC activity was not determined for strains producing an additional β-lactamase.

[d] βla, additional β-lactamase.

[e] O serotype.

[f] BP, bronchopulmonary sample.

[g] NA, no agglutination.

[h] ND, not done.

[i] NAP, not applicable.

[j] The pairs of strains 10 and 24, 12 and 14, and 22 and 29 were each isolated from the same patient.

[k] PDF, peritoneal dialysis fluid.

ously reported for *Acinetobacter baumannii* (31) (EMBL database accession no. Y10693). The six strains that produced OXA-21 had different RAPD patterns (data not shown). To our knowledge, this is the first report of OXA-21 in *P. aeruginosa*.

The bla_{PSE-1} gene was detected in two strains by PCR with primers OC1 and OC3 (Table 1), and a protein with a pI of 5.7 conferred a very high level of resistance to ticarcillin (MICs, >2,048 μg/ml) and ticarcillin-clavulanate (MICs, 256/1,024 μg/ml).

The bla_{PER-1} gene of the strain producing the β-lactamase with a pI of 5.4 was identified by PCR with primers PER-A and PER-B (Table 1) and sequencing. Epidemics caused by strains with this enzyme, previously reported in Turkey (29) and Italy (13), are now observed in France.

The carbapenem resistance observed in 15 strains was

TABLE 3. Substitution in bla_{AmpC} genes according to the pI value for each cephalosporinase and comparison with the sequence of the bla_{AmpC} gene of *P. aeruginosa* strain PAO1 (pI 8.0)

Nucleotide (amino acid) position[a] in PAO1	Nucleotide (amino acid) substitution in cephalosporinase with accession no. (pI) of:				
	AY083593 (7.8)	AY083595 (8.0)	AY083592 (8.4)	AF490770 (8.0 + 8.4)	AY083594 (8.6)
G789 (Arg79)	A (Gln)				
A866 (Thr105)	G (Ala)		G (Ala)	G (Ala)	G (Ala)
C883 (Arg110)					T
A916 (Ala121)			G		
C994 (Phe147)		T			
C1060 (Tyr169)	T				
T1080 (Leu176)					G (Arg)
G1150 (Arg199)	A				
G1166 (Val205)			T (Leu)	C (Leu)	
C1204 (Asp217)	T				
T1243 (Tyr230)	C	C	C	C	C
C1399 (Thr282)	T				
G1519 (Pro322)	A			A	
T1585 (Gly344)	C			C	C
C1639 (Gly362)		A	A		
G1725 (Gly391)				C	

[a] Numbering is according to that for the bla_{AmpC} gene and that for the deduced amino acid sequence of *P. aeruginosa* PAO1 with GenBank accession no. X54719.

not related to enzymatic hydrolysis by a carbapenem-hydrolyzing β-lactamase. Although several ESBLs were reported in *P. aeruginosa*, their prevalences were very low in our study.

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