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Detection of the Novel Extended-Spectrum β-Lactamase OXA-161 from a Plasmid-Located Integron in *Pseudomonas aeruginosa* Clinical Isolates from Spain[▽]

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Two clonally related *Pseudomonas aeruginosa* isolates, recovered from two patients admitted to a pediatric intensive care unit, were found to harbor a new OXA-2 variant (Asn148Asp), designated OXA-161. The plasmid location of $bla_{OXA-161}$ was demonstrated through electroporation to PAO1, and its codification in a class I integron (together with *aacA4*) was demonstrated through PCR and sequencing. bla_{OXA-2} and $bla_{OXA-161}$ were cloned in parallel to demonstrate the extended-spectrum β-lactamase properties of OXA-161, conferring resistance to ceftazidime and reduced susceptibility to cefepime and aztreonam.

Pseudomonas aeruginosa is one of the most relevant nosocomial pathogens, particularly among patients admitted to the intensive care unit (ICU), where it is the main cause of ventilator-associated pneumonia (1). Furthermore, it is a major cause of chronic respiratory infections in patients with underlying diseases such as cystic fibrosis (11). In addition to its high intrinsic antibiotic resistance, *P. aeruginosa* may frequently acquire additional resistance by mutation and/or by horizontal transfer of resistance determinants such as β-lactamases, often carried within class 1 integrons, that can be mobilized by transposons and/or plasmids (10, 20). The OXA-type β-lactamases (belonging to Ambler's class D group) are often detected in *P. aeruginosa* but also in many other gram-negative microorganisms such as *Acinetobacter baumannii* (2, 13, 16). They were initially characterized by their high rates of hydrolysis of cloxacillin and oxacillin, although most of them did not significantly affect the extended-spectrum cephalosporins and carbapenems (12). Currently, over 125 OXA-type β-lactamases have been identified. Among them, single point mutations of OXA-2 and OXA-10 (also called PSE-2) provide extended-spectrum β-lactamase (ESBL) characteristics to these new derivatives, affecting ceftazidime (CAZ), aztreonam (ATM), cefotaxime, and ceftriaxone at different levels depending on the amino acid changes. They were first detected in Turkey and currently include several representatives: OXA-15 and -32 (deriving from OXA-2) and OXA-11, -14, -16, -17, -19, -28, and -35 (OXA-10 derivatives) (13). Their codification in plasmids and/or transposons may facilitate their horizontal diffusion, although many of them have been proved only to have a chromosomal location (12, 21).

In June 2008, a *P. aeruginosa* strain (PAjun08), displaying an unusual resistance phenotype, was isolated from a wound infection of a patient admitted to the Hospital Son Dureta pediatric ICU. One month later, a second *P. aeruginosa* isolate (PAjul08) with the same resistance phenotype was recovered from a lung biopsy specimen from a different patient admitted to the same ICU. MICs of piperacillin (PIP), PIP-tazobactam (PIP-TZ), CAZ, cefepime (FEP), ATM, imipenem (IMP), meropenem (MER), gentamicin (GEN), tobramycin (TOB), amikacin (AMK), ciprofloxacin, and colistin were determined by the Etest method (AB Biodisk, Solna, Sweden). Additionally, MICs of carbenicillin, CAZ, and CAZ plus 4 μg/ml clavulanate (CAZ-CLV) were determined by standard CLSI broth microdilution (3).

Both isolates were found to be resistant to all the tested aminoglycosides, CAZ, and IMP. They also showed reduced susceptibility to PIP, FEP, ATM, and MER but full susceptibility to PIP-TZ (Table 1). Furthermore, the double-disk synergy test using amoxicillin-CLV and CAZ disks (5-mm separation) yielded a slight synergy, suggesting the presence of an ESBL. Moreover, a slight synergy was also observed in a broth microdilution assay with CAZ-CLV (Table 1). To characterize the potential ESBL produced by the two *P. aeruginosa* isolates, PCRs using primers for the most common ESBL groups (OXA, PER, CTX-M, SHV, and TEM) were performed (4, 8). PCR using OXA-2 group primers provided positive results. Then, to amplify and sequence the complete bla_{OXA} gene, the external primers OXA-2-EXT-F (5′-ATGGCAATCCGAATC TTCGC-3′) and OXA-2-EXT-R (5′-TTATCGCGCAGCGTC CGAG-3′) were used. The obtained band was purified and sequenced using a BigDye Terminator kit (PE Applied Biosystems, Foster City, CA), and sequences were analyzed on an ABI Prism 3100 DNA sequencer (PE Applied Biosystems). In all cases, sequencing of two independent PCR products was performed to ensure the absence of errors occurring during amplification. The resulting sequences were then compared with those available at GenBank (www.ncbi.nih.gov/BLAST), revealing the presence of a not previously described polymorphism of OXA-2: Asn148Asp (amino acid numbering corresponding to the full-length precursor protein).The novel OXA-type variant was named OXA-161.

To find the epidemiological relatedness between the two

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TABLE 1. MICs for *P. aeruginosa* isolates and their transformants[a]

Strain and plasmid	MIC (μg/ml)[b]													
	PIP	PIP-TZ	CAR	CAZ	CAZ-CLV	FEP	ATM	IMP	MER	CIP	CST	GEN	TOB	AMK
PAjun08	24	6	128	>256	128	16	8	16	6	0.125	2	32	48	32
PAjul08	24	6	128	>256	128	12	8	32	8	0.25	2	64	64	64
PAO1	2	2	32	2	2	1	2	1.5	0.38	0.125	2	2	1	3
PAO1(pPAjun08)	16	4	128	128	64	8	8	1	0.75	0.125	2	12	12	12
PAO1(pPAjul08)	32	6	128	128	64	8	8	1	0.75	0.125	2	8	12	12
PAO1(pUCPOXA-2)	48	4	>256	8	4	2	2	1.5	0.38					
PAO1(pUCPOXA-161)	24	3	128	64	32	6	8	1.5	0.5					

[a] MICs for the two *P. aeruginosa* isolates producing the new $bla_{OXA-161}$ ESBL and their transformants in the reference strain, PAO1, harboring the plasmid DNA obtained from each strain, as well as the cloned (pUCP24) bla_{OXA-2} (pUCPOXA-2) and $bla_{OXA-161}$ (pUCPOXA-161).
[b] CAR, carbenicillin; CIP, ciprofloxacin; CST, colistin.

OXA-161-producing isolates, pulsed-field gel electrophoresis was performed using conventional protocols (9). Analysis of the results (following the criteria defined by Tenover et al. [19]) revealed that the two isolates belonged to the same clone. This finding was certainly not unexpected, given that overlapping of ICU admission periods was documented.

The possible location of $bla_{OXA-161}$ in a transferable plasmid was evaluated through transformation experiments. For this purpose, plasmid DNA (UltraClean plasmid prep kit; MO BIO, Carlsbad, CA) was introduced by electroporation into PAO1 as previously described (18). Transformants were selected in LB agar plates containing 20 μg/ml of CAZ. Transformants were checked by a double-disk synergy test and PCR. MICs of the obtained transformants are shown in Table 1. As can be observed, the resistance profile of the β-lactams (except carbapenems) and aminoglycosides could be horizontally transferred to PAO1, proving the location of aminoglycoside resistance determinants in the same plasmid as $bla_{OXA-161}$. Since carbapenem resistance was not transferred along with the $bla_{OXA-161}$ plasmid, additional mechanisms, most likely classical OprD inactivation, should be responsible for IMP resistance in the clinical strains. The integron potentially harboring the aminoglycoside-modifying-enzyme genes and $bla_{OXA-161}$ was characterized by PCR followed by DNA sequencing using previously described specific primers to amplify *intI1*, *qacEΔ1* (8), and the DNA regions located between *intI1* or *qacEΔ1* and $bla_{OXA-161}$. Reverse versions of cited primers OXA-2-EXT-F and OXA-2-EXT-R were used for this last purpose. The results showed the presence of $bla_{OXA-161}$ in a class 1 integron. No gene was detected between *intI1* and $bla_{OXA-161}$, but downstream, a 6′-*N*-aminoglycoside acetyltransferase gene (*aacA4*) was found. This Aac(6′)-Ib enzyme was identical to the one described by Galimand et al. (7) (GenBank number S67948), conferring resistance to AMK and TOB. Therefore, cotransferred resistance to GEN in PAO1 transformants might result from the presence of additional aminoglycoside-modifying enzymes in the plasmid.

To investigate the precise resistance profile conferred by OXA-161 in comparison with its ancestor oxacillinase, OXA-2, both genes were amplified and cloned into the pUCP24 vector. Primers OXAF-SmaI (TCCCCGGGATGGCAATCCGAATCTTCGC) and OXAR-BamHI (TCGGATCCTTATCGCGCAGCGTCCGAG) (the restriction targets are underlined) were used for cloning. The obtained products were checked by sequencing, and the resulting plasmids (pUCPOXA-2 and pUCPOXA-161) were transformed to *Escherichia coli* XL1-Blue as previously described (17). After the plasmids were extracted, they were electroporated into *P. aeruginosa* reference strain PAO1, and MICs of the antibiotics listed above were determined by Etest (Table 1). As can be observed, the resistance profile confirmed the predicted OXA-161 spectrum: high-level CAZ resistance and reduced susceptibility to PIP, FEP, and ATM. Two other OXA-2 ESBL derivatives, OXA-15 and OXA-32, have been previously described (5, 14). In both cases, the authors attributed the amplification of the hydrolytic spectrum (mainly directed to a greater cephalosporinase activity) to changes in conserved regions of the oxacillinase sequence. In this sense, OXA-161 has a Asn148Asp change, which affects the YGN conserved motif of class D β-lactamases, that is very close to a proposed β turn or loop (position 150, which in OXA-15 has a Asp150Gly substitution) involved in the hydrolysis of β-lactams (5). On the other hand, OXA-32 has a Leu-to-Ile change in the class D oxacillinase fourth conserved region (14). Moreover, as occurs with OXA-15 and OXA-32, the most affected cephalosporin by the novel OXA-161 is CAZ (conferring high-level resistance).

The presence of potent β-lactamases, such as metallo-β-lactamases or ESBLs, in plasmid-located integrons (harboring additional resistance determinants such as aminoglycoside-modifying enzymes) in *P. aeruginosa* is a growing threat with major clinical and epidemiological consequences. Although several outbreaks have been described worldwide (6, 15, 20), their overall prevalence seems to be still limited. For instance, this is the first report of OXA ESBLs in Spain. Nevertheless, it should be noted that the actual prevalence of ESBLs in *P. aeruginosa* is probably underestimated, since their detection in this species is made more difficult by the frequent chromosomal β-lactam resistance mechanisms such as the overexpression of AmpC and/or one of the several efflux pumps encoded in its genome. Therefore, active surveillance studies are urgently needed in order to determine the actual prevalence of these relevant resistance mechanisms and to establish directed measures for controlling their spread.

Nucleotide sequence accession numbers. The GenBank accession numbers for this study are as follows: $bla_{OXA-161}$, FJ617206; class 1 integron harboring $bla_{OXA-161}$, GQ202693.

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