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In Vivo Emergence of Resistance to Novel Cephalosporin–β-Lactamase Inhibitor Combinations through the Duplication of Amino Acid D149 from OXA-2 β-Lactamase (OXA-539) in Sequence Type 235 *Pseudomonas aeruginosa*

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ABSTRACT Resistance development to novel cephalosporin–β-lactamase inhibitor combinations during ceftazidime treatment of a surgical infection by *Pseudomonas aeruginosa* was investigated. Both initial (97C2) and final (98G1) isolates belonged to the high-risk clone sequence type (ST) 235 and were resistant to carbapenems (*oprD*), fluoroquinolones (GyrA-T83I, ParC-S87L), and aminoglycosides (*aacA7/aacA8/aadA6*). 98G1 also showed resistance to ceftazidime, ceftazidime-avibactam, and ceftolozane-tazobactam. Sequencing identified bla_{OXA-2} in 97C2, but 98G1 contained a 3-bp insertion leading to the duplication of the key residue D149 (designated OXA-539). Evaluation of PAO1 transformants producing cloned OXA-2 or OXA-539 confirmed that D149 duplication was the cause of resistance. Active surveillance of the emergence of resistance to these new valuable agents is warranted.

KEYWORDS extended-spectrum OXA, *Pseudomonas aeruginosa*, multidrug resistance, ceftolozane-tazobactam, ceftazidime-avibactam

The increasing prevalence of nosocomial infections produced by multidrug-resistant (MDR) or extensively drug-resistant (XDR) *Pseudomonas aeruginosa* strains severely compromises the selection of appropriate treatments and is therefore associated with significant morbidity and mortality (1–3). This growing threat results from the extraordinary capacity of this pathogen to develop resistance to nearly all available antibiotics by the selection of mutations in chromosomal genes and from the increasing prevalence of transferable resistance determinants, particularly those encoding class B carbapenemases (metallo-β-lactamases [MBLs]) or extended-spectrum β-lactamases, frequently cotransferred with genes encoding aminoglycoside-modifying enzymes (4, 5). The emergence of MDR/XDR global clones, deemed high-risk clones, disseminated in several hospitals worldwide adds further concern (6, 7).

The recent introduction of novel β-lactam–β-lactamase inhibitor combinations, namely, ceftolozane-tazobactam and ceftazidime-avibactam, which are stable against most mutational resistance mechanisms, including the overexpression of the chromosomal cephalosporinase AmpC, partially alleviates the urgent clinical need for new agents that combat infections by MDR/XDR *P. aeruginosa* (8–10). Thus, the potential emergence of resistance to these antibiotics is of particular concern. Therefore, we report here the characterization of the resistance mechanisms of a *P. aeruginosa* clinical

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TABLE 1 Antimicrobial susceptibility profiles of studied clinical isolates

	97C2		98G1	
Antibiotic[a]	MIC (μg/ml)	CLSI interpretation[b]	MIC (μg/ml)	CLSI interpretation[b]
TIC	256	R	256	R
PTZ	16/4	S	16/4	S
AZT	8	S	8	S
FEP	8	S	32	R
CAZ	4	S	>64	R
TOL-TAZ	1/4	S	>32/4	R
CAZ-AVI	2/4	S	>32/4	R
IMP	16	R	16	R
MER	16	R	16	R
TOB	>32	R	>32	R
AMK	64	R	64	R
CIP	>16	R	>16	R
COL	4	S	4	S

[a]TIC, ticarcillin; PTZ, piperacillin-tazobactam; AZT, aztreonam; FEP, cefepime; CAZ, ceftazidime; TOL-TAZ, ceftolozane-tazobactam; CAZ-AVI, ceftazidime-avibactam; IMP, imipenem; MER, meropenem; TOB, tobramycin; AMK, amikacin; CIP, ciprofloxacin; COL, colistin.
[b]R, resistant; S, susceptible.

isolate recovered from a surgical wound in a patient with colon cancer that was documented to develop resistance to ceftazidime, ceftazidime-avibactam, and ceftolozane-tazobactam during ceftazidime therapy.

Isolates 97C2 and 98G1, obtained with a 30-day interval from a surgical wound from a patient treated with ceftazidime, were found to belong to the same clone through pulsed-field gel electrophoresis (PFGE) assays using SpeI as the restriction enzyme (11). Moreover, multilocus sequence typing (MLST), following stablished schemes (http://pubmlst.org/paeruginosa), identified both isolates as sequence type (ST) 235 international high-risk clones (7).

Table 1 shows the comparative broth microdilution susceptibility profiles of the two isolates, according to CLSI guidelines and breakpoints (12). As shown, although both isolates were resistant to carbapenems, fluoroquinolones, and aminoglycosides, the second isolate also developed resistance to cefepime, ceftazidime, ceftazidime-avibactam, and ceftolozane-tazobactam. However, both isolates remained within the susceptibility breakpoints for piperacillin-tazobactam (16/4 μg/ml) and aztreonam (8 μg/ml).

To decipher the involved resistance mechanisms, the presence of horizontally acquired β-lactamases was investigated through previously established phenotypic and molecular (PCR) methods (11). Moreover, sequence variation on 146 chromosomal genes related to antimicrobial resistance was evaluated as previously described (13). Briefly, indexed paired-end libraries were generated from genomic DNA using a commercial library preparation kit (Nextera XT, Illumina, USA) and sequenced on an Illumina MiSeq benchtop sequencer with a MiSeq reagent kit (version 2). Obtained paired-ended reads were aligned to the *P. aeruginosa* PAO1 reference genome, and sequence variation was further analyzed for the 146 chromosomal genes related to antimicrobial resistance (13). The presence of horizontally acquired resistance determinants was further explored using online databases (https://cge.cbs.dtu.dk//services/ResFinder/).

Both isolates showed the same *oprD* inactivating mutation [insertion of 1 pb (C) in nucleotide 1205] responsible for the carbapenem-resistant phenotype and the GyrA T83I and ParC S87L mutations responsible for fluoroquinolone resistance. However, differential mutations between the strains for the 146 genes studied were not detected, including classic ceftazidime resistance mutations in AmpC regulators (e.g., *ampD*, *dacB*, or *ampR*) or MexAB-OprM efflux pump regulators (e.g., *mexB*, *nalC*, or *nalD*), suggesting that chromosomal mutations were not the cause of the developed resistance phenotype. Moreover, PCR assays for acquired β-lactamases yielded a single positive result for OXA-2-type β-lactamases in both isolates. However, sequencing of the amplicons showed that, although $bla_{\text{OXA-2}}$ was identified in 97C2, isolate 98G1 harbored

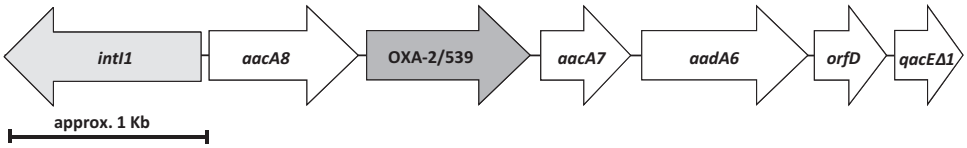

FIG 1 Structure of the In78 integron harboring OXA-2/539.

a previously undescribed 3-bp insertion leading to the duplication of the key residue D149 (https://www.lahey.org/studies/webt.asp#OXA; D151 according to the full-length precursor OXA-2 protein). This novel OXA-2-derived enzyme was designated OXA-539 (GenBank accession no. KY094077). Remarkably, the substitution of this key aspartate D149 residue has been described to confer an extended-spectrum phenotype, such as that reported for OXA-15 (14) or OXA-36 (15), in which it is replaced by glycine and tyrosine, respectively.

Both OXA-2 and OXA-539 were documented to be located in an In78 class 1 integron (16), together with several aminoglycoside resistance determinants responsible for documented tobramycin and amikacin resistance (Fig. 1). Southern blot hybridization of the I-CeuI nuclease-digested genomes, following described protocols (17), suggested chromosomal location of OXA-2/OXA-539 genes, given that the OXA gene probes hybridized with bands that also hybridized with the rRNA gene probes (not shown). Moreover, all attempts to transfer OXA-2/OXA-539 through electroporation and conjugation consistently yielded negative results, supporting the chromosomal location of the β-lactamases (17).

To evaluate the impact of the D149 duplication on β-lactam resistance, $bla_{\text{OXA-2}}$ and $bla_{\text{OXA-539}}$ PCR products were cloned into pUCP24, transformed into *Escherichia coli* XL1-Blue, and finally electroporated into *P. aeruginosa* PAO1, following previously described protocols (18). Table 2 shows the β-lactam MICs of PAO1 derivatives harboring the cloning vector or the cloned OXA-2 or OXA-539 β-lactamases. As shown, OXA-539 notably increased cefepime, ceftazidime, ceftazidime-avibactam, and ceftolozane-tazobactam MICs compared with OXA-2. In contrast, meropenem MICs were less affected by the expression of OXA-539 than by that of OXA-2.

To our knowledge, this is the first report documenting the *in vivo* emergence of an extended-spectrum OXA β-lactamase during treatment of human infection with broad-spectrum cephalosporins. Likewise, this is the first report in which an extended spectrum is generated from narrow-spectrum OXA β-lactamases through the duplication of a key amino acid. The fact that this resistance mechanism emerged in the ST235 high-risk clone, characterized by its worldwide dissemination, its extraordinary association with transferable resistance, and its higher virulence, associated with the production of the ExoU cytotoxin, adds further concern to our findings (7, 19–22). Moreover, according to a recent review, OXA-2-producing ST235 has been reported in at least 14 countries (7). However, of possible greater concern is the development of cross-

TABLE 2 MICs for the PAO1 transformants producing OXA-2 or OXA-539 β-lactamases

Antibiotic[a]	MIC (μg/ml)		
	PAO1 (pUCP24)	PAO1 (pUCP-OXA-2)	PAO1 (pUCP-OXA-539)
TIC	16	256	128
PTZ	≤4/4	≤4/4	≤4/4
AZT	4	4	4
CAZ	≤1	4	>64
TOL-TAZ	≤0.5/4	≤0.5/4	16/4
CAZ-AVI	1/4	1/4	32/4
FEP	≤1	2	8
IMP	2	2	2
MER	≤0.5	8	1

[a]TIC, ticarcillin; PTZ, piperacillin-tazobactam; AZT, aztreonam; CAZ, ceftazidime; TOL-TAZ, ceftolozane/tazobactam; CAZ-AVI, ceftazidime/avibactam; FEP, cefepime; IMP, imipenem; MER, meropenem.

resistance to ceftolozane-tazobactam and ceftazidime-avibactam, because they are particularly useful for combating infections by MDR/XDR *P. aeruginosa*. The prevalence of primary resistance to these agents depends on the geographic location, but it is still globally low and mostly linked to the production of horizontally acquired MBLs (23–26). The main advantage of ceftolozane-tazobactam and ceftazidime-avibactam over other antipseudomonal β-lactams is their stability against most mutation-driven resistance mechanisms, including the overexpression of the chromosomal cephalosporinase AmpC. *In vitro* studies revealed that the emergence of resistance occurs at lower frequency than with other agents, and it is linked to specific mutations leading to the structural modification of AmpC- or efflux-related mechanisms in the case of ceftazidime-avibactam (9, 27–30). In this work, we describe one further mechanism for development of resistance to these new antibiotics, through the selection of extended-spectrum mutations from narrow-spectrum OXA β-lactamases, such as OXA-2, which is relatively frequent among *P. aeruginosa* isolates worldwide (31). Altogether, these findings argue for the need to maintain an active surveillance of the mechanisms involved in the emergence of resistance to these new valuable agents.

Accession number(s). The novel OXA-2-derived enzyme OXA-539 was deposited in GenBank under the accession no. KY094077.

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