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Strain-Tailored Double-Disk Synergy Test Detects Extended-Spectrum Oxacillinases in *Pseudomonas aeruginosa*▽

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The prevalence of class D extended-spectrum oxacillinases (ES-OXAs) in ceftazidime-resistant strains of *Pseudomonas aeruginosa* is often underestimated by double-disk synergy tests (DDST) using clavulanate. A DDST with a customized distance between a disk of ceftazidime or cefepime and inhibitors (clavulanate and imipenem) detected 14 out of 15 different ES-OXAs.

The development of enzymatic resistance to β-lactams in the opportunistic pathogen *Pseudomonas aeruginosa* results from mutational overproduction of the intrinsic cephalosporinase AmpC, from acquisition of transferable genes coding for a variety of secondary β-lactamases, or both (20). A growing number of Ambler class A extended-spectrum β-lactamases (ESBLs) such as TEM, SHV, PER, GES, VEB, BEL, KPC, and CTX-M, class B carbapenemases (metallo-β-lactamases [MBLs], such as IMP, VIM, SPM, and GIM), and 17 class D extended-spectrum oxacillinases (ES-OXAs) have been found in clinical strains of *P. aeruginosa* from various geographical origins (reviewed in references 10, 17, and 30 to 32). Inhibition of ESBL and MBL activities by clavulanate and cation chelators (EDTA, thiol compounds, and dipicolinic acid), respectively, has allowed the development of useful phenotypic screening assays to detect most of these enzymes in clinical strains (2, 22).

ES-OXA production confers a high-level resistance to antipseudomonal cephalosporins (10). Epidemiological studies usually neglect ES-OXAs. However, an unexpected proportion (33%) of ES-OXAs among β-lactamases with extended-spectrum produced has recently been found in clinical *P. aeruginosa* (15). In addition, new ES-OXAs have been found recently in several European countries (17, 30), as has as the spread of multidrug-resistant clones of *P. aeruginosa* producing ES-OXAs (5, 13a, 19). Most of the 17 ES-OXA genes identified so far in *P. aeruginosa* are located on plasmid-borne integrons, allowing their diffusion and spread (30). Altogether, these data suggest the emergence of ES-OXAs among clinical isolates of *P. aeruginosa* and support the need for a targeted screening test. A regular double-disk synergy test (DDST) using clavulanate usually failed to detect the ES-OXAs (with the exception of the clavulanate-inhibited OXA-18). ES-OXAs are characterized by an important genetic diversity: they derive from OXA-10 (OXA-11, -14, -16, -17, -142, -145, and -147), OXA-2 (OXA-15, -32, -144, and -161), OXA-1 (OXA-31), or OXA-13 (OXA-19, -28, and -183) or are more distant from these groups (OXA-18 and -45) (30). In addition, narrow-spectrum oxacillinase-encoding genes (e.g., $bla_{\text{OXA-10}}$) which are frequently expressed by clinical isolates (14, 15) differ from $bla_{\text{ES-OXA}}$ only by point mutations. This renders a PCR-based approach difficult for ES-OXA screening.

Although the inhibitory activity of clavulanate on ES-OXAs is poor, it has been repeatedly documented (5, 8, 12, 17, 24). Likewise, imipenem inhibits the activity of ES-OXAs derived from $bla_{\text{OXA-10}}$, as observed with members of the BEL, GES, and VEB groups (24). Jiang et al. have shown that reducing the distance between disks enhanced the performance of the DDST (16). Based on these data, we designed a strain-tailored DDST for the detection of *P. aeruginosa* producing ES-OXAs with a customized distance between the disks of substrates (ceftazidime and cefepime) and inhibitors (clavulanate and imipenem).

Strain-tailored double-disk synergy test. A routine disk susceptibility test was first performed to determine inhibition zones around disks containing separate compounds. Mueller-Hinton (MH) agar plates (Bio-Rad, Ivry-sur-Seine, France) were inoculated with a 1/100 dilution of a 0.1 McFarland suspension, and disks (Bio-Rad) containing clavulanate (10 μg in amoxicillin-clavulanate disk), ceftazidime (30 μg), cefepime (30 μg), and imipenem (10 μg) were tested (25). The day after and under identical conditions, the synergy between disks of substrates (ceftazidime and cefepime) and inhibitors (clavulanate and imipenem) was tested in duplicate. Distances between the disks were adapted to each strain based on inhibition zone diameters around disks containing each compound tested separately. Optimal detection of ES-OXAs was yielded, with a distance of 5 ± 1 mm between the edges of the two inhibition zones around the disks of considered agents (Fig. 1). A collection of clinical *P. aeruginosa* producing ES-OXAs (OXA-11, -14 to -19, -28, -32, -142, -144, -145, -147, -161, and -183) was assayed. We controlled the ability of that test to detect ESBL-producing (PER-1, VEB-1, GES-1, and BEL-1) and MBL-producing (VIM-1) isolates. In addition, *P. aeruginosa* isolates from our laboratory collection were tested (Table 1). The presence of the *bla* genes encoding ES-OXAs, ESBLs, and MBLs was assessed by sequencing experiments (see their origin in Table 1). As a negative control, we tested a reference strain (PAOΔ*dacB*) and a collection of 12 clinical isolates overproduc-

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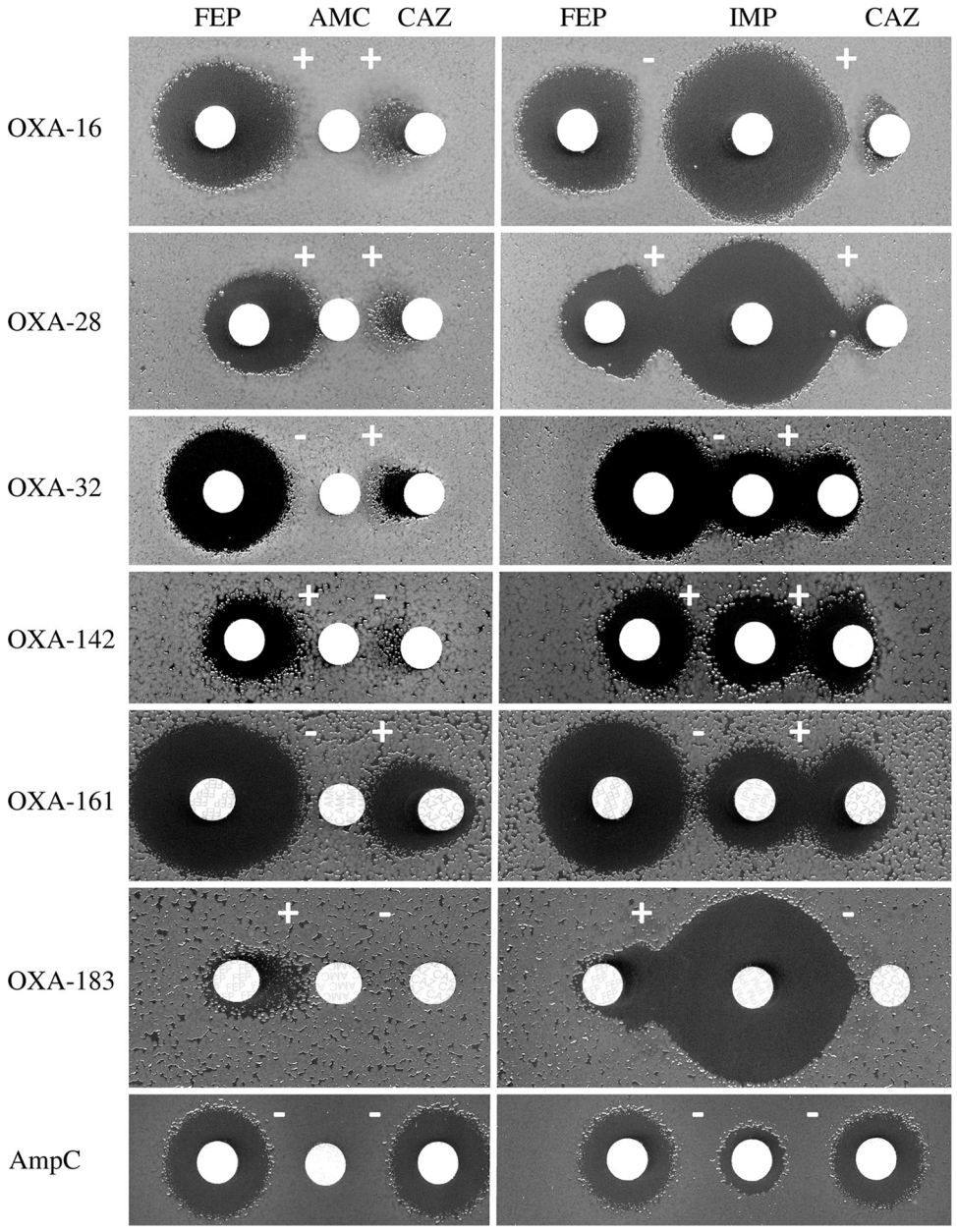

FIG. 1. Double-disk synergy test with *P. aeruginosa* isolates producing the extended-spectrum oxacillinases OXA-16 and OXA-142 (OXA-10 derived), OXA-28 and OXA-183 (OXA-13 derived), or OXA-32 and OXA-161 (OXA-2 derived) or overproducing the cephalosporinase AmpC (AmpC). Distances between the disks were adapted to each strain, based on the inhibition zone diameter around disks containing each compound tested separately. For instance, if no inhibition zone was noticed around clavulanate- and ceftazidime-containing disks, the distance between their two disks is 5 ± 1 mm. Abbreviations: FEP, cefepime (30 μg); AMC, amoxicillin-clavulanate (20/10 μg); CAZ, ceftazidime (30 μg); IMP, imipenem (10 μg). Interpretative results are given (see Table 1).

ing the chromosomally encoded cephalosporinase AmpC (3, 23). After 18 h of incubation, the presence of an enlarged zone (≥2 mm) or a synergy zone between any disks of antimicrobial agents and a disk of inhibitor was considered a positive result.

Strain-tailored DDST detects ES-OXAs (Fig. 1). Positive DDSTs were obtained with 13/15 and 14/15 reference strains producing different ES-OXAs by using clavulanate and imipenem, respectively. Only OXA-144 failed to be detected by both inhibitors (Table 1). Synergies were noticed with the 4 reference strains producing ESBLs but not with the MBL-producing strain. The reference strain overproducing AmpC was found to be negative with our DDST. All the additional clinical isolates taken from our collection behaved in the same way as the reference strains (Table 1).

The strain-tailored DDST thus appears to be a reproducible and cost-effective screening test for both ES-OXA- and ESBL-producing isolates in the medical laboratory. Since AmpC-overproducing mutants are very prevalent among β-lactam-resistant strains of *P. aeruginosa*, the DDST should optimize the use of molecular methods (i.e., PCR and DNA sequencing)

TABLE 1. Results of strain-tailored DDST applied to a collection of *P. aeruginosa* strains producing ES-OXAs, ESBLs, or MBLs or overproducing the AmpC cephalosporinase[a]

Enzyme produced	Reference strain		No. of additional clinical strains tested	Synergy between disks containing[b]:			
	Reference or accession no.	CAZ MIC (μg/ml)		CLA-FEP	CLA-CAZ	IMP-FEP	IMP-CAZ
OXA-11	13	512	0	+	+	+	+
OXA-14	7	512	1[c]	−	−	+	+
OXA-15	8	128	0	+	+	−	+
OXA-16	9	≥128	0	+	+	−	+
OXA-17	6	128	0	+	+	+	+
OXA-18	26	128	0	+	+	+	+
OXA-19	24	512	48[d]	+	+	+	+
OXA-28	29	256	13[e]	+	+	+	+
OXA-32	28	128	0	−	+	−	+
OXA-142	EU358785	128	0	+	−	+	+
OXA-144	18	>64	0	−	−	−	−
OXA-145	FJ790516	128	0	+	−	+	+
OXA-147	12	256	0	+	−	+	−
OXA-161	17	128	0	−	+	−	+
OXA-183	HQ111474	128	0	+	−	+	−
PER-1	21	512	3[f]	+	+	+	+
GES-1	11	32	0	+	+	+	+
VEB-1	1	>512	1[f]	+	+	+	+
BEL-1	27	32	0	+	+	−	+
VIM-1	1	>128	0	−	−	−	−
AmpC	23	32	12[g]	−	−	−	−

[a] CLA, clavulanate (10 μg); CAZ, ceftazidime (30 μg); FEP, cefepime (30 μg); IMP, imipenem (10 μg).
[b] +, enlarged zone of inhibition or synergy zone of inhibition; −, no synergy.
[c] Reference 4.
[d] Reference 5.
[e] Reference 13a.
[f] Reference 15.
[g] Reference 3.

by restricting the number of strains requiring complementary analyses. Production of ES-OXAs or ESBLs in *P. aeruginosa* typically leads to a high level of resistance to ceftazidime (10, 32). We therefore suggest testing of resistant isolates (as defined by the CLSI [25]) with a MIC of ≥32 μg/ml or with an inhibition zone of ≤15 mm around the ceftazidime-containing disk. It could be perfectly possible that rare ESBL- or ES-OXA-producing strains yield a MIC of <32 μg/ml, but a lower threshold could render this screening test hardly realistic and less cost-effective in the clinical laboratory. The test described here is now used routinely in our reference laboratory. It has allowed the detection of known ES-OXAs in clinical strains (4, 5, 15) (Table 1) and the discovery of new variants (OXA-145, OXA-147, and OXA-183) (12, 13a). However, it needs to be evaluated on a larger scale, but we believe that it may significantly improve the detection of ES-OXAs. In conjunction with an MBL-targeted screening test, it may help expedite implementation of control measures for preventing the spread of multidrug-resistant strains harboring emerging resistance mechanisms.

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