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Simple Microdilution Test for Detection of Metallo-β-Lactamase Production in *Pseudomonas aeruginosa*

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A microdilution test measuring imipenem MICs in the presence or absence of a mixture of EDTA plus 1,10-phenanthroline was developed and tested on 190 *Pseudomonas aeruginosa* isolates, including 18 VIM- and 4 IMP-type metallo-β-lactamase (MBL) producers. The chelator mixture reduced by fourfold or more the imipenem MICs for MBL producers, while a lower effect or no effect was usually observed with MBL nonproducers.

Acquired metallo-β-lactamases (MBLs) of the IMP or VIM type, encoded by integron-borne mobile gene cassettes, have started spreading worldwide as a cause of broad-spectrum β-lactam resistance in *Pseudomonas aeruginosa* and other gram-negative nosocomial pathogens (2, 10). Resistance mediated by MBLs is not overcome by conventional β-lactamase inhibitors (2, 10). For this reason, MBLs are presently included among the resistance determinants of increasing clinical importance (2, 10) and their monitoring has become an important issue in clinical microbiology. Although certain resistance phenotypes might be suggestive of acquired MBL production in isolates of naturally susceptible species, production of these enzymes is not readily detectable by conventional susceptibility testing and must be confirmed by enzyme assays and molecular detection of the corresponding genes. To facilitate the screening for MBL producers in the clinical microbiology laboratory, phenotypic tests based on the principle of disk diffusion for rapid detection of MBL-producing isolates have recently been proposed (1, 7).

In this work, we developed a new test, based on a simple microdilution technique, for phenotypic detection of MBL-producing *P. aeruginosa*. The test was named the EPI (after EDTA-phenanthroline-imipenem) microdilution test.

Bacterial strains. The strains used in this study included 188 nonreplicate clinical isolates of *P. aeruginosa* and the reference *P. aeruginosa* strains PAO1 (12) and ATCC 27853. Clinical isolates were from various geographic areas and hospitals and included 22 MBL producers (producing either IMP- or VIM-type enzymes) and 166 MBL nonproducers. MBL producers included some strains that have already been described, namely, 101/1477 (5), VR-143/97 (6), VR-193/98 (15), VA-182/00 (3), and NTU-26/99 (17), and additional clinical isolates in which MBL production was confirmed by enzyme assays (6), and the nature of the MBL determinant was identified by PCR analysis. Detection and identification of bla_{VIM} genes were carried out as described previously (3). Detection and identification of bla_{IMP} genes were carried out by amplification of a 361-bp internal region of bla_{IMP} genes (from nucleotides 2168 to 2528; GenBank accession no. AF416297) and analysis of the *Alu*I restriction pattern of the amplicon. PCR amplification was carried out, using the IMP-DIA primers (IMP-DIA/f, 5′-ggAATAgAgTggCTTAATTCTC; IMP-DIA/r, 5′-gTgATgCg TCYCCAAYTTCACT), in a 50-μl volume containing 50 pmol of each primer, 200 μM deoxynucleoside triphosphates, 10 ng of genomic DNA of the test strain, and 3.5 U of the Expand high fidelity PCR system (Roche Biochemicals, Mannheim, Germany), in the reaction buffer provided by the manufacturer, under the following cycling parameter conditions: initial denaturation at 94°C for 240 s; denaturation at 94°C for 60 s, annealing at 52°C for 60 s, and extension at 72°C for 90 s, repeated for 25 cycles; and final extension at 72°C for 600 s. All of the MBL nonproducers were confirmed to lack bla_{IMP}- or bla_{VIM}-related sequences by colony blot hybridization using a bla_{IMP-1} probe and a bla_{VIM-1} probe (16). All the MBL producers and most of the nonproducers were resistant to imipenem, ceftazidime and cefepime. Twelve of the MBL producers were known to produce the PER-1 extended-spectrum β-lactamase (11; L. Pagani, unpublished results). Twenty-six of the imipenem-resistant isolates not producing MBL activity were known to be deficient in the OprD protein, as detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane protein extracts (9). Thirty-four of the imipenem-resistant isolates not producing MBL activity were known to constitutively produce the AmpC enzyme, as revealed by isoelectric focusing analysis of crude extracts (9). OprD loss, constitutive AmpC production, and PER-1 production were investigated in some but not all of the isolates.

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TABLE 1. Results of the EPI test carried out with 190 *P. aeruginosa* strains

MBL production (no. of strains)	MBL type (no. of strains)	Phenotype (no. of strains)[c]	MIC (μg/ml) for[d]:		No. of strains showing a MIC reduction of:								
			IMP[c]	EPI	>128-fold	128-fold	64-fold	32-fold	16-fold	8-fold	4-fold	2-fold	0
Negative (168)		Imp^{s} Caz^{s} (2)[a]	2	1								2	
		Imp^{s} Caz^{r} (5)	2–4	1–4								1	4
		$Imp^{r\text{-}i}$ $Caz^{r\text{-}i}$ (161)	8–64	2–32						1	4	103	53
Positive (22)[b]	VIM-1 (7)	Imp^{r} Caz^{r}	128–256	0.5–16	1	1		5					
	VIM-2 (10)	Imp^{r} Caz^{r}	32–512	1–8	2	1	4	2		1			
	VIM-3 (1)	Imp^{r} Caz^{r}	64	2				1					
	IMP-1 (1)	Imp^{r} Caz^{r}	512	16				1					
	IMP-2 (3)	Imp^{r} Caz^{r}	128–256	8–32				1		1	1		

[a] Including the *P. aeruginosa* reference strains PAO-1 and ATCC27853.
[b] Including strains from references 3, 5, 6, and 15 to 17.
[c] Phenotypes listed indicate levels of susceptibility to the drugs imipenem (Imp) and ceftazidime (Caz). The superscript letters indicate the following: s, sensitivity, r, resistance; and r-i, resistance to intermediate susceptibility.
[d] Where a single value is not listed, the MIC range is given. IMP, imipenem; EPI, EDTA-phenanthroline-imipenem.

Identification of isolates was carried out according to standard procedures (4). Susceptibility testing was carried out by disk diffusion and interpreted according to the guidelines of the National Committee for Clinical Laboratory Standards (14).

EPI microdilution test. The EPI microdilution test was carried out as follows. Imipenem MICs were determined with a standard microdilution assay (13) in 96-well microtiter plates (Greiner Labortechnik GmbH, Kremsmünster, Austria), using Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) and a bacterial inoculum of 5×10^4 CFU per well, in a final volume of 100 μl. Imipenem concentrations in the range of 512 to 0.25 μg/ml were tested. The MICs were determined either in the absence or in the presence of a mixture of EDTA plus 1,10-phenanthroline. The final concentrations of the chelators to be used in the test were selected on the basis of the results of preliminary experiments in which the inhibition of MBL activity and the toxicity of variable concentrations of the chelators were tested with a subset of 20 randomly selected isolates, including 10 MBL producers (2 producing IMP-type and 8 producing VIM-type enzymes) and 10 MBL nonproducers (randomly selected from imipenem-resistant isolates). These experiments revealed the following: (i) the growth of the tested strains was not significantly inhibited by a final EDTA concentration of up to 0.4 mM; (ii) at a fixed EDTA concentration of 0.4 mM, the growth of the tested strains was not significantly inhibited by a final 1,10-phenanthroline concentration of up to 0.04 mM, while at a 0.2 mM 1,10-phenanthroline concentration, toxicity was observed with some strains; and (iii) the best results in terms of comparative MIC reduction were observed using final concentrations of EDTA and 1,10-phenanthroline of 0.4 and 0.04 mM, respectively, and these final concentrations were used in the test. The mixture of divalent ion chelators was added (at a 2× final concentration) to the bacterial suspension just before inoculation of the wells. A growth control in the presence of the chelators mixture was always included. Results were recorded by visual inspection of microtiter plates after 18 h of incubation at 37°C. The tests were conducted in a blinded study by a person who did not know which were the MBL producers. All tests were performed in triplicate, with highly reproducible results. Within each series, the imipenem and the imipenem-plus-chelators MICs were identical or differed by 1 twofold dilution at most. In different experiments, the MIC ratio reduction caused by the presence of the chelators remained constant or differed for a few MBL nonproducers by twofold at most. In the latter cases, the highest ratios were used for comparisons. Chemicals were from Sigma Chemical Co. (St. Louis, Mo.). Media and chemicals of the same lot number were used.

Detection of MBL producers by the EPI microdilution test. Using the EPI microdilution test, all the *P. aeruginosa* isolates which produced an acquired MBL exhibited a notable reduction of imipenem MICs in the presence of the mixture of chelators. The magnitude of reduction ranged from 4- to 512-fold (median, 32-fold). In 19 of 22 (86%) cases, the reduction was equal to or higher than 32-fold. The lowest reductions were observed with two of the IMP-2 producers and with one of the VIM-2 producers (Table 1). Under the same conditions, isolates that did not produce acquired MBLs usually exhibited no reduction or a twofold reduction of imipenem MICs, while only a minority of them showed a fourfold reduction or, in one case, an eightfold reduction (Table 1). In particular, imipenem resistance sustained by mechanisms other than MBL production was not significantly influenced by the presence of chelators.

With a minimum fourfold imipenem MIC reduction designated as the cutoff value for detection of MBL producers, the EPI test could detect all the isolates which produced an acquired MBL (95% confidence interval [CI], 81.5 to 100%), with 3% of the results false positives (CI, 1.1 to 7.2%). With a minimum eightfold imipenem MIC reduction designated as the cutoff value, the test detected 95% of the MBL producers (CI, 75.1 to 99.8%), with 0.6% false positives (CI, 0 to 3.8%). Finally, with a minimum 16-fold imipenem MIC reduction designated as the cutoff value, the test detected 86% of the MBL producers (CI, 64 to 96.4%), with no false positives (CI, 0 to 2.8%). It should be noted that the only MBL producer to exhibit a fourfold MIC reduction exhibited an imipenem MIC (128 μg/ml) greater than those observed with nonproducers showing the same MIC reduction (64 to 16 μg/ml).

Concluding remarks. The development of simple screening tests that are designed for the detection of acquired MBL production and that are suitable for routine use in the clinical microbiology laboratory is a critical step toward large-scale monitoring of these emerging resistance determinants in vari-

ous clinical settings. Such tests will eventually be useful for the design of containment measures and for verification of their efficacy. A similar approach would be particularly useful for *P. aeruginosa*, in which carbapenem resistance is often caused by mechanisms other than acquired MBLs, such as decreased permeability of the outer membrane and/or active efflux, possibly associated to overproduction of the endogenous class C β-lactamase (8). Although phenotypic tests based on disk diffusion have already been described for that purpose (1, 7), they are not suitable for automation and must rely upon analogical interpretation. The EPI microdilution test described in this paper appears to be a simple, specific, and sensitive method for the screening of acquired MBL production in *P. aeruginosa.* Compared with the tests based on disk diffusion, in which identification of MBL producers must rely upon the evaluation of changes in the appearance of growth inhibitory zones in proximity to a disk containing an inhibitor (1, 7), the EPI microdilution test should allow better standardization in recording results and might also be amenable to automation.

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