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High Prevalence of Metallo-β-Lactamase and 16S rRNA Methylase Coproduction among Imipenem-Resistant *Pseudomonas aeruginosa* Isolates in Brazil[▽]

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Rates of metallo-β-lactamase and 16S rRNA methylase production were investigated in 51 imipenem-resistant *Pseudomonas aeruginosa* clinical isolates collected from hospitals in São Paulo, Brazil. Of them, 57% and 75% produced SPM-1 and RmtD, respectively. Of note, 51% produced both enzymes, suggesting that their coproduction is already common in this geographic area.

Treatment of infections due to *Pseudomonas aeruginosa* is becoming increasingly complicated by its tendency to acquire resistance to multiple classes of antimicrobials (12). The agents typically used to treat these infections include antipseudomonal penicillins, cephalosporins, carbapenems, fluoroquinolones, and aminoglycosides. The production of metallo-β-lactamases (MBLs) contributes substantially to panresistant phenotypes in *P. aeruginosa* because they confer resistance to all classes of β-lactam antimicrobials except aztreonam (10). Various MBLs including IMP, VIM, SPM, and GIM types have been reported for *P. aeruginosa* (12). Of note, SPM-type MBLs have been found only in Brazil thus far (13).

Methylation of 16S rRNA has emerged as a mechanism of high-level aminoglycoside resistance among gram-negative pathogens in recent years (3). Five such methylases, ArmA and RmtA through RmtD, have been reported to date. The most recently identified methylase is RmtD, which we reported previously for a panresistant *P. aeruginosa* strain isolated near São Paulo, Brazil (4). This enzyme confers high-level resistance to most aminoglycosides in clinical use. In this particular strain, the coproduction of RmtD and SPM-1 played a substantial role in the panresistant phenotype. The present study was conducted to determine the prevalence of coproduction of these enzymes among *P. aeruginosa* clinical isolates in Brazil.

Nonrepetitive imipenem-resistant *P. aeruginosa* isolates from a total of 49 patients hospitalized at seven hospitals in the São Paulo area in 2005 and 2006 were collected. One patient had two isolates with different pulsed-field gel electrophoresis (PFGE) patterns, and another patient had two isolates with identical PFGE patterns but markedly different susceptibility patterns. Thus, 51 imipenem-resistant *P. aeruginosa* isolates were included in the study. Sites of the specimens were as follows: 28 from urine, 8 from blood, 7 secretions from various sites, 3 from cerebrospinal fluid, 2 from catheter tip, 1 from bronchoalveolar lavage fluid, 1 from ascitic fluid, and 1 from nasal swab.

The MICs of imipenem, meropenem, aztreonam, amikacin, gentamicin, arbekacin, ciprofloxacin, and colistin were obtained using either the standard agar dilution method or Etest (AB Biodisk, Solna, Sweden) (2). The MICs of imipenem were 32 μg/ml or higher for all the isolates, verifying the high degree of resistance to this agent in this collection. For meropenem, MICs were 8 μg/ml or higher. When the phenotypic test using sodium mercaptoacetic acid was performed to detect MBL production (1), 29 of 51 isolates (57%) yielded a positive result.

PFGE was performed with SpeI (New England Biolabs, Beverly, MA) as the restriction enzyme for the genomic DNA. Electrophoresis was performed with the CHEF III DR system (Bio-Rad, Hercules, CA). The pulses were increased linearly from 5.3 to 34.9 s for 24 h at 14°C. Seventeen pulsotypes were observed among the 51 isolates by PFGE. Eight of the pulsotypes comprising 32 isolates from six hospitals were possibly related to each other ("pulsotype A," consisting of subtypes A1 through A8), whereas the other nine pulsotypes were unrelated (pulsotypes B through J) according to criteria described previously by Tenover et al. (Table 1 and Fig. 1) (14). Pulsotype A was thus the most prevalent genotype in hospitals in this area.

PCR was conducted to detect the MBL gene bla_{SPM} (6) and the 16S rRNA methylase gene *rmtD*. PCR for bla_{IMP-1}, bla_{IMP-2}, and bla_{VIM-2} was performed for all the bla_{SPM}-negative isolates (11). For *rmtD*, the following primers were used to produce a 401-bp amplicon: rmtD-F (5′-CGGCACGCGATT GGGAAGC-3′) and rmtD-R (5′-CGGAAACGATGCGACG AT-3′). The thermal cycle conditions included an initial denaturation step at 96°C for 5 min followed by 30 cycles at 96°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final extension step at 72°C for 5 min. All the 29 isolates that produced a positive phenotypic test for MBL production yielded an amplicon consistent with bla_{SPM}. When representative amplicons from different PFGE pulsotypes were sequenced, their deduced amino acid sequences matched that of SPM-1 within the amplicons (15). PCR results for bla_{IMP-1}, bla_{IMP-2}, and

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TABLE 1. Characteristics of study isolates

Pulsotype and subtype	Total no. of isolates	Hospital(s)	No. of isolates (%)	
			bla$_{SPM}$ positive	*rmtD* positive
A	32	b, c, d, e, f, g	29 (91)	29 (91)
1	12	b, c, e, f, g	11	12
2	10	b, d, g	9	8
3	1	g	1	1
4	3	b	2	3
5	3	b	3	3
6	1	b	1	1
7	1	b	1	1
8	1	b	1	0
B	1	a	0 (0)	0 (0)
C	5	b	0 (0)	0 (0)
D	1	a	0 (0)	0 (0)
E	7	b	0 (0)	7 (100)
F	1	g	0 (0)	0 (0)
G	1	b	0 (0)	0 (0)
H	1	d	0 (0)	1 (100)
I	1	a	0 (0)	0 (0)
J	1	b	0 (0)	1 (100)

TABLE 2. MICs of imipenem, meropenem, aztreonam, amikacin, ciprofloxacin, and colistin in the presence or absence of *bla*$_{SPM}$

bla$_{SPM}$ status (no. of isolates)	Antibiotic	No. of isolates with MICs (μg/ml) of:									
		>256	256	128	64	32	16	8	4	2	≤1
Positive (29)	Imipenem	21	6			2					
	Meropenem	17	10				1	1			
	Aztreonam					5	19	5			
	Amikacin	26	3								
	Ciprofloxacin	1	1	8	15	2	1				1
	Colistin							12	8	7	2
Negative (22)	Imipenem				17	5					
	Meropenem				2	10	9	1			
	Aztreonam	1	2	3	4	7	3		2		
	Amikacin	12	4		1		4	1			
	Ciprofloxacin		4	3	3	7	1		1		3
	Colistin						2	3	11	5	1

bla$_{VIM-2}$ were all negative. These results suggested that SPM-1 is indeed the most predominant MBL among *P. aeruginosa* isolates in this geographic area. The median MIC of imipenem was greater than 256 μg/ml for *bla*$_{SPM}$-positive isolates and 64 mg/ml for *bla*$_{SPM}$-negative isolates (Table 2). For *rmtD*, 38 isolates (75%) yielded an amplicon. Deduced amino acid sequences of representative amplicons from different PFGE profiles were identical to that of RmtD within the amplicons. The MICs of arbekacin, amikacin, and gentamicin for the *rmtD*-positive isolates were always greater than 256 μg/ml. Of the 29 *bla*$_{SPM}$-positive isolates, 26 were positive for *rmtD* as well. Thus, the prevalence of coproduction of SPM-1 and RmtD in this collection of imipenem-resistant *P. aeruginosa* isolates was 51% (26/51), and that among the SPM-1-producing isolates reached 90% (26/29). All of the *bla*$_{SPM}$-positive isolates belonged to pulsotype A. Most of the *rmtD*-positive strains were also observed within pulsotype A, whereas some belonged to pulsotypes E, H, and J. These results suggest that the *rmtD* and *bla*$_{SPM}$ genes are spreading in São Paulo hospitals mostly by means of interhospital transmission of strains belonging to pulsotype A.

When the MICs of aminoglycosides were stratified according to the PCR results for *rmtD*, the sensitivities of arbekacin, amikacin, and gentamicin MICs greater than 256 μg/ml in predicting the presence of *rmtD* were all 100% (Table 3). On the other hand, the specificities of their MICs equal to or less than 256 μg/ml were 77%, 100%, and 54%, respectively. Therefore, amikacin was the best single agent to predict aminoglycoside resistance mediated by the production of RmtD. This result is in contrast with a previous finding with a collection of *Acinetobacter* sp. strains, where arbekacin displayed better specificity than amikacin (8). Although arbekacin generally retains better activity than amikacin in the presence of an aminoglycoside-modifying enzyme among gram-negative bacteria, this advantage may be lost when multiple modifying enzymes with different substrate specificities are produced simultaneously in a bacterium (5). Both agents are also known to

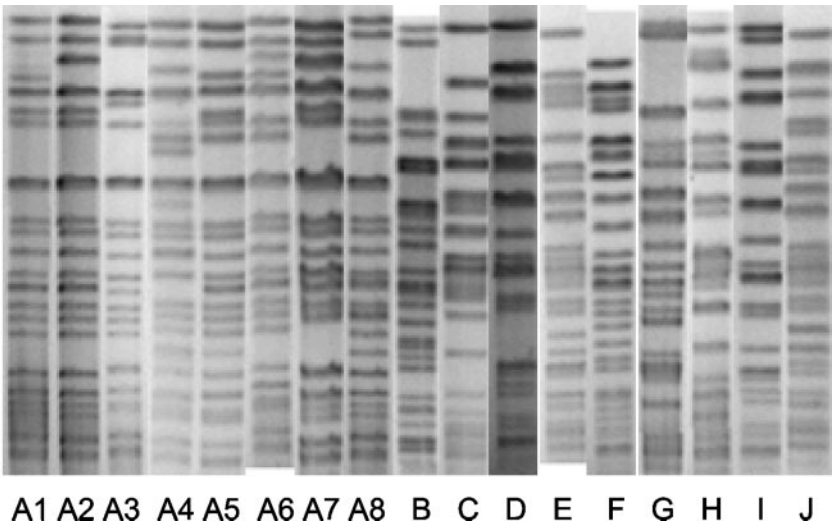

FIG. 1. PFGE patterns of the study isolates. The pulsotypes were aligned using Bionumerics software version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium).

TABLE 3. MICs of aminoglycosides in the presence or absence of *rmtD*

rmtD status (no. of isolates)	Aminoglycoside	No. of isolates with MIC (μg/ml) of:							
		>256	256	128	64	32	16	8	4
Positive (38)	Arbekacin	38							
	Amikacin	38							
	Gentamicin	38							
Negative (13)	Arbekacin	3	2	1				6	1
	Amikacin		4		1		7	1	
	Gentamicin	6		1				3	3

be substrates of efflux pumps in *P. aeruginosa* (9). A combination of these resistance mechanisms may thus lead to high aminoglycoside MICs even in the absence of 16S rRNA methylase. The MICs of imipenem, meropenem, aztreonam, amikacin, ciprofloxacin, and colistin were then stratified depending on the PCR results for bla_{SPM} (Table 2). Higher MICs of the carbapenems and lower MICs of aztreonam were observed in bla_{SPM}-positive isolates, as expected, reflecting the substrate specificities of the MBL (10). High-level amikacin resistance was more frequent in bla_{SPM}-positive isolates due to the frequent coproduction of RmtD. MICs of ciprofloxacin were variable regardless of the presence or absence of bla_{SPM}. Some isolates with elevated colistin MICs (8 to 16 μg/ml) were observed in both groups, which differed from a previous study that reported 100% susceptibility to this agent for imipenem-resistant *P. aeruginosa* isolates in Brazil (6).

In summary, our study demonstrates that the coproduction of SPM-1 and RmtD is a common phenomenon observed in half of the imipenem-resistant *P. aeruginosa* isolates in hospitals in São Paulo, Brazil. A recent nationwide surveillance study from Brazil reported that as many as 37% of *P. aeruginosa* isolates recovered in Brazilian hospitals were resistant to imipenem (7). Furthermore, it was also reported that MIC_{90}s of amikacin, tobramycin, and gentamicin against *P. aeruginosa* were all greater than 256 μg/ml. Our findings, coupled with those surveillance data, suggest that high-level aminoglycoside resistance mediated by the production of 16S rRNA methylase may already be disseminated in Brazilian hospitals.

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