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Diversity of β-Lactamases Produced by Ceftazidime-Resistant *Pseudomonas aeruginosa* Isolates Causing Bloodstream Infections in Brazil[▽]

Renata C. Picão,[1,2] Laurent Poirel,[1] Ana C. Gales,[2] and Patrice Nordmann[1]*

Service de Bactériologie-Virologie, INSERM U914 Emerging Resistance to Antibiotics, Hôpital de Bicêtre, Assistance Publique/Hôpitaux de Paris, Faculté de Médecine Paris Sud, K.-Bicêtre, France,[1] and Laboratório ALERTA, Universidade Federal de São Paulo, São Paulo, Brazil[2]

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A retrospective survey was conducted to characterize β-lactamases in a collection of 43 ceftazidime-resistant *Pseudomonas aeruginosa* isolates recovered from patients with bloodstream infections hospitalized at a Brazilian teaching hospital between January and December 2005. Resistance rates for carbapenems, aminoglycosides, and quinolones were over 80%, with only colistin remaining active against all isolates. Pulsed-field gel electrophoresis analysis identified seven different genotypes. AmpC overproduction was found to be the sole β-lactamase-mediated mechanism responsible for ceftazidime resistance in four isolates (9.3%). Nine isolates (20.9%) produced an extended-spectrum β-lactamase (ESBL), either GES-1 ($n = 7$, 16.3%) or CTX-M-2 ($n = 2$, 4.6%). Carbapenemase activity was detected in 30 (70%) additional isolates. Among those isolates, two isolates (4.6%) produced the ESBL GES-5, possessing the ability to hydrolyze imipenem; a single isolate (2.3%) produced the metallo-β-lactamase (MBL) IMP-1; and 27 isolates produced the MBL SPM-1 (62.8%). None of the isolates coproduced both ESBL and MBL. Insertion sequence elements IS*CR4* and IS*CR1* were associated with $bla_{\text{SPM-1}}$ and $bla_{\text{CTX-M-2}}$ genes, respectively, whereas the $bla_{\text{GES-1}}$ and $bla_{\text{GES-5}}$ genes were part of class 1 integron structures. This study underlines the spread of MBL- and ESBL-producing *P. aeruginosa* isolates as an important source of ceftazidime resistance in Brazil.

Pseudomonas aeruginosa is a leading cause of hospital-acquired infections. Acquisition of β-lactamases, such as class A extended-spectrum β-lactamases (ESBLs) and class B metallo-β-lactamases (MBLs), by *P. aeruginosa* nosocomial isolates is detrimental to antimicrobial therapy in hospitalized patients (19).

The ESBLs reported for *P. aeruginosa* are SHV, TEM, PER, VEB, BEL, GES, and, more recently, CTX-M types (1, 7, 8, 16, 20, 23, 29). The GES-type enzymes are unusual since point amino acid changes in their active sites may extend their hydrolytic activity to carbapenems (31, 39, 40). ESBL production in *P. aeruginosa* has been documented in Brazil (2, 5, 21), but its prevalence remains unknown.

Five types of acquired MBLs have been identified in *P. aeruginosa*: IMP, VIM, SPM, GIM, and AIM (41, 42). In Brazil, IMP-, VIM-, and SPM-producing *P. aeruginosa* clinical isolates have been identified (35). In addition, SPM producers have been reported as endemic in Brazilian territory due to dissemination of a single clone (10).

The aim of this study was to investigate the diversity and frequency of both ESBL and MBL production and to characterize the genetic support of those acquired β-lactamase genes in a collection of ceftazidime-resistant *P. aeruginosa* clinical isolates from Brazil, taken as a model of a developing country.

MATERIALS AND METHODS

Bacterial strains. A total of 154 consecutive *P. aeruginosa* isolates were recovered from patients with bloodstream infections hospitalized at Hospital São Paulo between January and December 2005. A single isolate per patient was retained for this study. Among those isolates, 43 (28%) were ceftazidime resistant by the CLSI disk diffusion method (inhibition zone of ≤14 mm and MIC of ≥32 μg/ml) and thus were further characterized. *Escherichia coli* TOP10 was used as a recipient strain in cloning experiments (23). Transformation experiments were performed using both *E. coli* TOP10 and *P. aeruginosa* PAO1 as the recipients.

Clinical data. Clinical data including age, comorbidities, unit of the hospital, site of infection, therapeutic regimen, and final disposition (death or discharge) have been collected for each patient.

Susceptibility testing and screening for AmpC overproducers and/or ESBL production. Antibiotic susceptibility profiles of the 43 *P. aeruginosa* isolates were determined by the agar dilution method according to the CLSI guidelines (3). AmpC overproducers were identified by testing susceptibility to ceftazidime on Mueller-Hinton plates supplemented with 250 μg/ml cloxacillin (18, 33). Detection of ESBL production was carried out by a double disk synergy method testing ceftazidime, aztreonam, and cefepime at a distance of 15 mm from ticarcillin-clavulanic acid disks, on Mueller-Hinton plates supplemented or not with cloxacillin-containing plates (28).

Screening for carbapenemase activity. Hydrolysis of imipenem was assessed by UV spectrophotometry assays, as described previously (10, 11, 25). Briefly, 10 ml of an overnight broth culture was harvested and then disrupted by sonication. Whole-protein extracts were obtained after centrifugation. Hydrolytic activity of 20 μl of the crude extract was determined against 100 μM imipenem in 100 mM phosphate buffer (pH 7.0), and measurements were carried out at a wavelength of 297 nm.

PCR amplification for detection of ESBL and MBL genes; analysis of the genetic environment and sequencing. Specific primers were used under standard PCR conditions to detect ESBL- and MBL-encoding genes, namely, bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, bla_{GES}, bla_{PER}, bla_{VEB}, bla_{BEL}, bla_{KPC}, bla_{IMP}, bla_{VIM}, bla_{SPM}, bla_{GIM}, and bla_{AIM} (7, 12, 15, 16, 17, 20, 23, 26, 29, 30, 38, 42). The genetic environment of bla_{IMP} was determined by PCR using the previously published specific primers to anneal at the 5′ and 3′ conserved sequences (CSs) of class 1 integrons, followed by sequencing (24). The genetic environment of

* Corresponding author. Mailing address: Service de Bactériologie-Virologie, Hôpital de Bicêtre, 78 rue du Général Leclerc, 94275 Le Kremlin-Bicêtre cedex, France. Phone: 33-1-45-21-36-32. Fax: 33-1-45-21-63-40. E-mail: nordmann.patrice@bct.aphp.fr.

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$bla_{CTX\text{-}M\text{-}2}$ was determined by PCR and further sequencing using specific primers for the insertion sequence IS*CR1* located upstream and for the *qacEΔ1* and *sul1* tandem gene (17). The genetic context of $bla_{SPM\text{-}1}$ was determined by using primers hybridizing with IS*CR4*, as described previously (27). For direct DNA sequencing, PCR products were purified using PCR purification columns (Qiagen, Courtaboeuf, France). Sequencing reactions were performed using specific primers and an automated ABI 337 sequencer (Applied Biosystems, Foster City, CA). The nucleotide and deduced protein sequences were analyzed with software available over the Internet at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

PFGE analysis. Genetic relatedness among the ceftazidime-resistant *P. aeruginosa* isolates was evaluated by pulsed-field gel electrophoresis (PFGE) using restriction enzyme SpeI (GE Healthcare, Orsay, France) as described previously (27). Analysis of PFGE patterns was performed by visual inspection of photographs of ethidium bromide-stained gels. The isolates were classified according to the criteria described by Tenover et al. (36). The *P. aeruginosa* isolate 48-1997 (38), corresponding to the SPM-producing national clone, was included in PFGE experiments for direct comparison of genotypes.

Genetic support of β-lactamase-encoding genes. Plasmid extraction was performed by the Kieser technique (13). *E. coli* NCTC50192, harboring four plasmids of 154, 66, 38, and 7 kb, was used as a size marker for plasmids. Transformation assays were performed by electroporation with plasmid extracts from the identified positive isolates, and both *E. coli* TOP10 and *P. aeruginosa* PAO1 were used as recipient strains. Selection was performed on agar plates supplemented with 50 μg/ml amoxicillin (amoxicilline) and 50 μg/ml ticarcillin for *E. coli* and *P. aeruginosa*, respectively. DNA-DNA hybridization of plasmid extracts was performed with a Southern transfer onto a Hybond N^+ nylon membrane (GE Healthcare) as previously described. Labeling of the probe and signal detection were carried out using an enhanced chemiluminescence nonradioactive labeling and detection kit according to the manufacturer's instructions (GE Healthcare). The genetic localization of the β-lactamase-encoding genes was also attempted by using the endonuclease I-CeuI technique, as described previously (14).

Cloning experiments. Total DNA from *P. aeruginosa* 35 was HindIII restricted, ligated into the corresponding site of plasmid pBK-CMV, and then transformed in the *E. coli* TOP10 reference strain by electroporation as described previously (25). Recombinant plasmids were selected on Trypticase soy agar plates containing 1 μg/ml imipenem and 30 μg/ml kanamycin. The cloned DNA fragment of recombinant plasmid p35 was sequenced on both strands.

RESULTS

Clinical data, susceptibility testing, and screening for AmpC overproducers and ESBL producers. Forty-three out of the 154 *P. aeruginosa* isolates (28%) displayed ceftazidime resistance. Table 1 summarizes the clinical data, antimicrobial susceptibility profiles, and molecular typing of those 43 isolates. They were collected mainly from patients hospitalized in intensive care units (25 isolates), the emergency room (10 isolates), and the pediatric oncology unit (four isolates) (Table 1). In summary, the retrospective analysis of medical records showed that 24 patients (55.8%) had received adequate antimicrobial therapy, while empirical treatment was not optimal for 14 patients (32.5%). Additionally, the medical records of five patients (11.6%) were not available. Among the 24 patients who had received adequate empirical treatment or for whom antimicrobial therapy was correctly modified according to the results of susceptibility testing, 10 were discharged after 7 or more days of antimicrobial therapy, nine died after 7 or more days of treatment, and five died before 7 days of treatment. Among the 14 patients who had received inadequate empirical therapy, 10 died before results of antimicrobial susceptibility testing, whereas four patients were discharged. Notably, in quite a high number of cases, antibiotics such as carbapenems and the last-resort antibiotic polymyxin B had been used, although unsuccessfully for 20 out of 38 patients. Corresponding clinical data are presented in Table 1.

The 43 ceftazidime-resistant isolates were also resistant to ticarcillin, ticarcillin-clavulanic acid, cefpirome, and cefepime. The highest susceptibility rates were obtained for colistin (100%), followed by aztreonam (44.1%), piperacillin-tazobactam (39.5%), piperacillin (34.8%), imipenem (18.6%), and amikacin (18.6%). Only for piperacillin and piperacillin-tazobactam were susceptibility rates obtained following the breakpoints recommended by the European Committee on Antimicrobial Susceptibility Testing (an isolate is deemed susceptible when the MIC is ≤16 mg/liter). Overproduction of AmpC was identified to be likely the sole enzymatic mechanism responsible for ceftazidime resistance in four isolates (9.3%). Nine isolates were classified as ESBL producers.

Imipenem hydrolysis was observed for 30 isolates, all of them resistant to imipenem. A high imipenem hydrolysis rate was obtained for 28 isolates (average specific activity of 0.2 $U \cdot mg\ of\ protein^{-1}$), and a lower but significant rate was obtained for two isolates (average specific activity of 0.007 $U \cdot mg\ of\ protein^{-1}$).

Identification of acquired β-lactamase genes. The $bla_{GES\text{-}1}$ ($n = 7$) and $bla_{CTX\text{-}M\text{-}2}$ ($n = 2$) genes were identified in nine isolates exhibiting an ESBL phenotype. In addition, the $bla_{GES\text{-}5}$ gene, which encodes an unusual ESBL with carbapenemase activity, was identified in two isolates which did not display any ESBL phenotype but corresponded to the two isolates for which weak carbapenem hydrolysis was detected. The MBL-encoding genes $bla_{SPM\text{-}1}$ and $bla_{IMP\text{-}1}$ were identified in 27 isolates (62.8%) and one isolate (2.3%), respectively, corresponding to those isolates for which a high imipenem hydrolysis rate had been noted.

Clonal relationship. PFGE analysis performed with the 43 *P. aeruginosa* clinical isolates showed seven main genotypes. The 27 SPM-1-producing isolates belonged to a single genotype, A (corresponding to the genotype of the Brazilian epidemic clone), which can be divided into two subtypes, A1 and A2. GES-1-producing isolates belonged to either genotype B (six isolates) or genotype C1 (one isolate). The two GES-5-producing isolates belonged to genotype C2, which was closely related to genotype C1 (two-band difference). The four AmpC overproducers belonged to genotypes G (one isolate) and D (three isolates), the latter being subclassified into two subtypes, D1 (one isolate) and D2 (two isolates). Genotypes E and F corresponded to the two CTX-M-2-producing isolates and the single IMP-1-producing isolate, respectively (Table 1).

Genetics of β-lactamase-encoding genes. PCR mapping and sequencing revealed that the $bla_{CTX\text{-}M\text{-}2}$ gene was preceded by IS*CR1* and followed by the *qacEΔ1* and *sul1* tandem genes (Fig. 1), as previously reported for *Enterobacteriaceae* (34). IS*CR* elements are insertion sequences transposing in a particular way since they can mobilize adjacent sequences by rolling-circle transposition (37). Analysis of the SPM-1-producing strains showed that the $bla_{SPM\text{-}1}$ gene was preceded by IS*CR4* and followed by *groEL* and IS*CR4* (Fig. 1) with a perfect identity compared to the sequences previously reported from $bla_{SPM\text{-}1}$-producing *P. aeruginosa* isolates (27). Sequencing of the 5′ CS-3′ CS amplicon obtained from isolate 42 showed that the $bla_{IMP\text{-}1}$ gene was located at the first position of a class 1 integron possessing the strong promoter configuration with P_{ant} and P_2. Class 1 integrons are DNA structures that may integrate or excise antibiotic resistance genes as a form of gene cassettes (4). The $bla_{IMP\text{-}1}$ gene was associated with the *aadA1*

TABLE 1. Clinical features of the patients from whom ceftazidime-resistant *Pseudomonas aeruginosa* isolates were collected[a]

Isolate	Clone	Date of isolation (day/mo/yr)	Hospital unit	Antimicrobial susceptibility	β-Lactamase identified	Age (yr)	Underlying disease	Primary infection	Empirical treatment	Clinical outcome
1	A1	1/7/2005	ICU	CS	SPM-1	21	Acute lymphoblastic leukemia	Bloodstream	FEP, IMP, PMB	Death
2	A1	1/19/2005	ER	CS, PIP-TZP, ATM	SPM-1	61	None	Bloodstream	RIF, IMP, GM	Death
3	A1	1/19/2005	ICU	CS	SPM-1	17	Acute myeloid leukemia	Bloodstream	FEP	Death
4	A1	1/27/2005	ER	CS, ATM	SPM-1	62	Epilepsy	Pneumonia	FEP, IMP	Discharge
5	A1	1/27/2005	ER	CS, PIP-TZP	SPM-1	91	None	Urinary tract	FEP, IMP, TZP	Death
6	A1	2/10/2005	Pediatric oncology	CS, ATM	SPM-1	3	NA	NA	NA	NA
7	A1	3/1/2005	Bone marrow transplant	CS	SPM-1	53	Multiple myeloma	Bloodstream	IMP, PMB	Discharge
8	A1	4/12/2005	ICU	CS, PIP-TZP	SPM-1	48	Chronic pancreatitis	Bloodstream	IMP, PMB	Death
9	A1	5/3/2005	Pediatric oncology	CS, ATM	SPM-1	14	NA	NA	NA	NA
10	A1	5/22/2005	ICU	CS	SPM-1	60	Chronic pancreatitis	Bloodstream	CIP, IMP, PMB	Death
11	A1	5/31/2005	Hemodialysis	CS, ATM	SPM-1	1	NA	NA	NA	NA
12	A1	5/31/2005	Hemodialysis	CS, PIP-TZP, ATM	SPM-1	1	Chronic renal failure	Bloodstream	CIP, MEM, PMB	Discharge
13	A1	6/28/2005	ICU	CS	SPM-1	31	Acute myeloid leukemia	Bloodstream	FEP, IMP	Death
14	A1	7/7/2005	ICU	CS, ATM	SPM-1	37	HIV, HCV	Bloodstream	PMB	Discharge
15	A1	8/23/2005	Pediatric oncology	CS, PIP-TZP	SPM-1	10	NA	NA	NA	NA
16	A1	9/6/2005	ICU	CS, PIP-TZP	SPM-1	74	None	Intra-abdominal	IMP	Death
17	A1	9/20/2005	ICU	CS, ATM	SPM-1	70	Multiple myeloma	Pneumonia and urinary tract	FEP, IMP, PMB	Death
18	A1	10/27/2005	ICU	CS, PIP-TZP, ATM	SPM-1	70	Lymphoma	Pneumonia	PMB	Death
19	A1	11/10/2005	ICU	CS, PIP-TZP, ATM	SPM-1	38	Aortic paraganglioma	Pneumonia	IMP, TZP, PMB	Discharge
20	A1	12/15/2005	ICU	CS	SPM-1	76	None	Pneumonia	FEP, IMP, PMB	Death
21	A1	12/15/2005	ICU	CS, PIP-TZP, ATM	SPM-1	70	Chronic renal failure	Pneumonia	FEP, IMP, PMB	Discharge
22	A1	12/17/2005	ICU	CS, PIP-TZP, ATM	SPM-1	70	None	Pneumonia	PMB	Death
23	A1	12/20/2005	Pediatric oncology	CS	SPM-1	18	NA	NA	NA	NA
24	A2	1/27/2005	ICU	CS, PIP-TZP	SPM-1	29	Lymphoma	Intra-abdominal	IMP, PMB	Death
25	A2	7/15/2005	ICU	CS	SPM-1	78	Hepatitis C	Pneumonia	IMP	Death
26	A2	11/22/2005	ICU	CS, ATM, AMK	SPM-1	64	COPD	Intra-abdominal	FEP, IMP, PMB	Death
27	A2	11/29/2005	ER	CS, PIP-TZP, ATM, AMK	SPM-1	74	None	Bloodstream	CRO, NIT	Discharge
28	B	1/13/2005	ER	CS, PIP-TZP, IMP	GES-1	79	Wallenberg syndrome	Pneumonia	FEP	Death
29	B	1/19/2005	ER	CS, ATM, IMP, MEM, CIP, TM, AMK, GM, NET	GES-1	62	Chronic renal failure	Pneumonia	FEP, IMP, PMB, GM	Death
30	B	1/28/2005	ER	CS, PIP-TZP	GES-1	79	Chronic myeloid leukemia	Pneumonia	FEP, IMP, TZP	Discharge
31	B	8/2/2005	ER	CS, PIP-TZP, IMP	GES-1	49	Esophageal cancer	Pneumonia	PMB	Discharge
32	B	8/17/2005	ER	CS	GES-1	27	Mediastinal teratoma	Bloodstream	CIP, FEP	Death
33	B	9/20/2005	ICU	CS, IMP	GES-1	54	Bladder cancer	Pneumonia	FEP, IMP, PMB, TZP	Discharge

34	C1	2/1/2005	Surgery	CS	GES-5	60	Chronic obstructive pyelonephritis	Intra-abdominal	IMP	Death
35	C1	7/5/2005	ICU	CS, ATM	GES-5	44	None	Pneumonia	IMP, PMB	Discharge
36	C2	5/22/2005	ER	CS, PIP-TZP, IMP, MEM	GES-1	47	Bladder cancer	Urinary tract	FEP	Discharge
37	D1	1/19/2005	ICU	CS, CIP, TM, AMK, GM, NET	AmpC overproduction	66	None	Bloodstream	IMP, TZP	Death
38	D2	2/15/2005	ICU	CS, CIP, TM, AMK, GM, NET	AmpC overproduction	9 mo	Autoimmune endocrine disease	Pneumonia	CIP, MEM	Death
39	D2	4/12/2005	ICU	CS, IMP, CIP, TM, AMK, GM, NET	AmpC overproduction	79	None	Pneumonia	FEP, IMP, PMB	Death
40	E	3/22/2005	ICU	CS, ATM	CTX-M-2	46	HIV, HBV, HCV, pulmonary tuberculosis	Bloodstream	FEP, CIP	Discharge
41	E	3/30/2005	ICU	CS, ATM, IMP	CTX-M-2	36	HIV	Urinary tract	PMB	Discharge
42	F	5/31/2005	ICU	CS, PIP-TZP, ATM	IMP-1	63	Pancreatic cancer	Pneumonia	IMP, PMB	Death
43	G	8/12/2005	ICU	CS, TM, AMK, GM, NET	AmpC overproduction	39	Severe asthma	Bloodstream	CAZ, GM	Death

[a] Abbreviations: AMK, amikacin; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; CRO, ceftriaxone; CS, colistin; FEP, cefepime; GM, gentamicin; IMP, imipenem; MEM, meropenem; NET, netilmicin; NIT, nitrofurantoin; PMB, polymyxin B; TM, tobramycin; PIP, piperacillin; TZP, tazobactam; RIF, rifampin (rifampicin); COPD, chronic obstructive pulmonary disease; ER, emergency room; HBV, hepatitis B virus infection; HCV, hepatitis C virus infection; HIV, human immunodeficiency virus infection; ICU, intensive care unit; NA, not available.

gene cassette encoding resistance to aminoglycosides in that class 1 integron (Fig. 1).

Using the I-CeuI technique, no conclusive results regarding the genetic location of $bla_{\text{CTX-M-2}}$, $bla_{\text{SPM-1}}$, and $bla_{\text{IMP-1}}$ genes were obtained. No plasmid was identified in the two $bla_{\text{CTX-M-2}}$-positive isolates as well as in all $bla_{\text{SPM-1}}$-positive isolates, whereas a single plasmid of 100 kb was identified in the $bla_{\text{IMP-1}}$-positive isolate. Southern blot hybridization of plasmid DNA extracted from $bla_{\text{CTX-M-2}}$, $bla_{\text{SPM-1}}$, and $bla_{\text{IMP-1}}$-positive isolates using the corresponding probes did not give a positive signal. In addition, the transfer of these resistance determinants to both *E. coli* and *P. aeruginosa* PAO1 recipient strains remained unsuccessful.

Detailed analysis of bla_{GES}-positive isolates. In order to detail the environment of the bla_{GES} genes in *P. aeruginosa* isolates, a cloning step was necessary. Cloning of HindIII-digested total DNA from isolate 35 resulted in a $bla_{\text{GES-5}}$-positive recombinant plasmid containing an insert of approximately 10 kb. Sequencing of the insert revealed that the $bla_{\text{GES-5}}$ gene was located at the first position of a class 1 integron, followed by the *aacA7* and *aacA4* gene cassettes. The 59-base element (59-be) of the *aacA4* gene cassette was interrupted by a new insertion sequence element named IS*Pa29* belonging to the IS*1111* family, members of which have been shown to insert themselves into the recombination sites of gene cassettes by site-specific recombination (32). IS*Pa29* was 1,534 bp long, possessed 12-bp-long imperfect subterminal inverted repeats, and was not bracketed by any target site duplication as commonly observed for IS*1111* family members (Fig. 1) (32). Its transposase shared 63% identity with that of IS*1492* identified from *Pseudomonas putida*. The IS*Pa29* sequence has been deposited on the IS Biotoul website (http://www-is.biotoul.fr). This class 1 integron was bracketed by two copies of IS*26*, located in opposite orientations as drawn in Fig. 1, thus constituting a composite transposon structure, as already found for another β-lactamase gene ($bla_{\text{VEB-1}}$)-positive integron, In53 (22).

Further PCR mapping used to target IS*26* elements and the gene cassettes for the $bla_{\text{GES-5}}$- and $bla_{\text{GES-1}}$-positive isolates showed that they possessed the same class 1 integron structure. That integron possessed the promoter P_{ant}, whereas promoter P_2 was under an inactive form. As observed for the $bla_{\text{GES-1}}$-positive *Klebsiella pneumoniae* strain ORI-1 from French Guiana (26), the $bla_{\text{GES-1}}$ and $bla_{\text{GES-5}}$ genes identified in our isolates were purified as a form of gene cassette with a truncated 59-be of 19 bp. Again, the I-CeuI technique did not allow us to clarify whether those bla_{GES}-like genes were chromosomally or plasmid located. In addition, plasmid analysis followed by Southern blotting and hybridization with a bla_{GES}-specific probe performed with all bla_{GES}-positive isolates failed to identify any plasmid. In accordance with those latter negative results, repeated attempts to transfer the $bla_{\text{GES-1}}$ and $bla_{\text{GES-5}}$ genes by transformation into *E. coli* or *P. aeruginosa* recipient strains failed. Those results strongly suggested the chromosomal location of the bla_{GES}-like genes.

DISCUSSION

The rate of ceftazidime-resistant *P. aeruginosa* strains (28%) isolated from blood cultures was quite high in this study. A similarly high rate of resistance has been reported for many

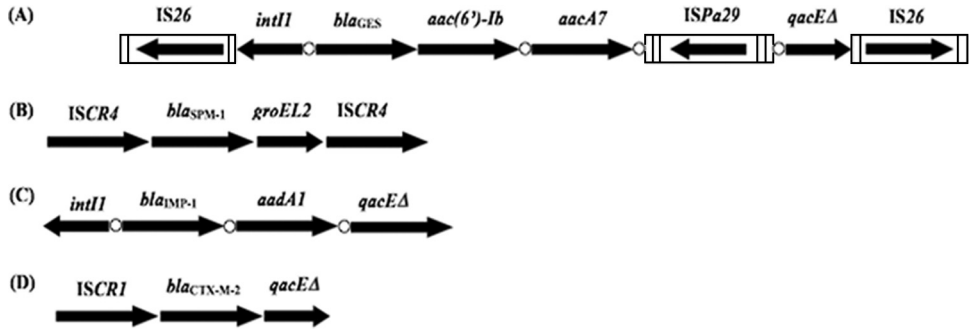

FIG. 1. Schematic representation of the genetic environment of β-lactamase-encoding genes identified in *P. aeruginosa* isolates. The coding genes are represented by arrows indicating their translation orientations, and 59-bes are indicated as white circles. (A) Genetic environment of bla_{GES-1} and bla_{GES-5}. (B) Genetic environment of bla_{SPM-1}. (C) Genetic environment of bla_{IMP-1}. (D) Genetic environment of $bla_{CTX-M-2}$.

developing countries worldwide (42). Here we showed that this high rate is mainly due to ESBL- and MBL-encoding genes, together with the clonal spread of several specific clones. Interestingly, AmpC overproduction as a single enzymatic mechanism for ceftazidime resistance was rarely observed; it seemed to be replaced by acquisition of β-lactamases with an expanded spectrum of activity. Worryingly, a very high proportion of those isolates were also resistant to imipenem (82%) and meropenem (95%), suggesting the presence of nonenzymatic mechanisms of carbapenem resistance, such as porin loss and/or overexpression of efflux pumps.

Our study showed that the bla_{GES}-type ESBL genes were identified in 13.9% of the ceftazidime-resistant *P. aeruginosa* isolates and were divided into two distinct clones. Since bla_{GES-1} and bla_{GES-5} genes were identified in the same *P. aeruginosa* clone, it may correspond to a local evolution of this clone. Considering that GES-5 possesses a wider spectrum of hydrolysis than that of GES-1, it might be speculated that this evolution could be due to a carbapenem-related selective pressure. Notably, those bla_{GES-1} and bla_{GES-5} gene cassettes had a truncated 59-be that was identical to that described in the bla_{GES-1}-positive *K. pneumoniae* ORI-I strain from French Guiana (34). Since French Guiana and Brazil are neighboring countries, those findings may suggest a spread of bla_{GES}-related structures in that part of the world.

The present study underlines the idea that the current spread of CTX-M enzymes may occur not only in the *Enterobacteriaceae* but also in *P. aeruginosa*. Interestingly, one recent study carried out in the same Brazilian hospital found that 44.8% of *K. pneumoniae* clinical isolates were CTX-M-2 producers (6). In addition, a CTX-M-2-producing *P. aeruginosa* strain that was susceptible to ceftazidime was recently identified in this hospital (22). Such a spread of CTX-Ms may be difficult to detect since many CTX-M variants confer a higher degree of resistance to cefotaxime than to ceftazidime (34). Therefore, since our selection criterion was based on ceftazidime resistance, the rate of CTX-M production among *P. aeruginosa* isolates identified here might be underestimated.

The genetic environments surrounding the bla_{CTX-M}- and bla_{GES}-type β-lactamase genes in *P. aeruginosa* were identical to those identified in the *Enterobacteriaceae*, underlining their common origin. It is likely that the location of the β-lactamase genes in *P. aeruginosa* results from their transfer from the *Enterobacteriaceae*.

Overall, the production of SPM-1 carbapenemase was the main source of resistance to ceftazidime, accounting for 62.8% of the isolates. SPM-1-producing *P. aeruginosa* isolates are widely disseminated in Brazil, with a single clone identified in different cities (10). This clone has been also recently identified in *P. aeruginosa* isolates recovered from Switzerland (9). We showed here that this SPM-1-producing clone had been responsible for a large outbreak causing septicemia in 27 hospitalized patients, leading to the death of at least 15 of them.

This study underlines the possibility that a variety of β-lactamases with wide spectra of activity may circulate in *P. aeruginosa* in Brazil, contributing to a multidrug resistance phenotype. Lack of their identification and consequently lack of isolation of carriers may lead to a further dissemination.

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REFERENCES

1. **Al Naiemi, N., B. Duim, and A. Bart.** 2006. A CTX-M extended-spectrum β-lactamase in *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*. J. Med. Microbiol. **55:**1607–1608.
2. **Castanheira, M., R. E. Mendes, T. R. Walsh, A. C. Gales, and R. N. Jones.** 2004. Emergence of the extended-spectrum β-lactamase GES-1 in a *Pseudomonas aeruginosa* strain from Brazil: report from the SENTRY antimicrobial surveillance program. Antimicrob. Agents Chemother. **48:**2344–2345.
3. **Clinical and Laboratory Standards Institute.** 2009. Performance standards for antimicrobial susceptibility testing. Nineteenth informational supplement. Clinical and Laboratory Standards Institute, Wayne, PA.
4. **Collis, C. M., and R. M. Hall.** 1995. Expression of antibiotic resistance genes in the integrated cassettes of integrons. Antimicrob. Agents Chemother. **39:**155–162.
5. **Da Fonseca, E. L., V. V. Vieira, R. Cipriano, and A. C. Vicente.** 2007. Emergence of bla_{GES-5} in clinical colistin-only-sensitive (COS) *Pseudomonas aeruginosa* strain in Brazil. J. Antimicrob. Chemother. **59:**576–577.
6. **Do Carmo Filho, J. R., R. M. Silva, M. Castanheira, M. C. Tognim, A. C. Gales, and H. S. Sader.** 2008. Prevalence and genetic characterization of bla_{CTX-M} among *Klebsiella pneumoniae* isolates collected in an intensive care unit in Brazil. J. Chemother. **20:**600–603.
7. **Dubois, V., C. Arpin, P. Noury, C. André, L. Coulange, and C. Quentin.** 2005. Prolonged outbreak of infection due to TEM-21-producing strains of *Pseudomonas aeruginosa* and enterobacteria in a nursing home. J. Clin. Microbiol. **43:**4129–4138.

8. **Dubois, V., L. Poirel, C. Marie, C. Arpin, P. Nordmann, and C. Quentin.** 2002. Molecular characterization of a novel class 1 integron containing bla_{GES-1} and a fused product of *aac3-Ib*/*aac6′-Ib′* gene cassettes in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **46:**638–645.
9. **El Salabi, A., M. A. Toleman, T. R. Walsh, R. Frei, and T. Bruderer.** 2008. The metallo-β-lactamase (MBL) SPM-1 arrives in Europe, abstr. C1-124, p. 92. Abstr. 48th Intersci. Conf. Antimicrob. Agents Chemother. (ICAAC)-Infect. Dis. Soc. Am. (IDSA) 46th Annu. Meet. American Society for Microbiology and Infectious Diseases Society of America, Washington, DC.
10. **Gales, A. C., L. C. Menezes, S. Silbert, and H. S. Sader.** 2003. Dissemination in distinct Brazilian regions of an epidemic carbapenem-resistant *Pseudomonas aeruginosa* producing SPM metallo-β-lactamase. J. Antimicrob. Chemother. **52:**699–702.
11. **Héritier, C., L. Poirel, D. Aubert, and P. Nordmann.** 2003. Genetic and functional analysis of the chromosome-encoded carbapenem-hydrolyzing oxacillinase OXA-40 of *Acinetobacter baumannii*. Antimicrob. Agents Chemother. **47:**268–273.
12. **Hirakata, Y., T. Yamaguchi, M. Nakano, K. Izumikawa, M. Mine, S. Aoki, A. Kondoh, J. Matsuda, M. Hirayama, K. Yanagihara, Y. Miyazaki, K. Tomono, Y. Yamada, S. Kamihira, and S. Kohno.** 2003. Clinical and bacteriological characteristics of IMP-type metallo-β-lactamase-producing *Pseudomonas aeruginosa*. Clin. Infect. Dis. **37:**26–32.
13. **Kieser, T.** 1984. Factors affecting the isolation of CCC DNA from *Streptomyces lividans* and *Escherichia coli*. Plasmid **12:**19–36.
14. **Liu, S. L., A. Hessel, and K. E. Sanderson.** 1993. Genomic mapping with I-Ceu I, an intron-encoded endonuclease specific for genes for ribosomal RNA, in *Salmonella spp.*, *Escherichia coli*, and other bacteria. Proc. Natl. Acad. Sci. USA **90:**6874–6878.
15. **Moland, E. S., N. D. Hanson, V. L. Herrera, J. A. Black, T. J. Lockhart, A. Hossain, J. A. Johnson, R. V. Goering, and K. S. Thomson.** 2003. Plasmid-mediated, carbapenem-hydrolysing β-lactamase, KPC-2, in *Klebsiella pneumoniae* isolates. J. Antimicrob. Chemother. **51:**711–714.
16. **Naas, T., L. Philippon, L. Poirel, E. Ronco, and P. Nordmann.** 1999. An SHV-derived extended-spectrum β-lactamase in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **43:**1281–1284.
17. **Nagano, N., Y. Nagano, C. Cordevant, N. Shibata, and Y. Arakawa.** 2004. Nosocomial transmission of CTX-M-2 β-lactamase-producing *Acinetobacter baumannii* in a neurosurgery ward. J. Clin. Microbiol. **42:**3978–3984.
18. **Nordmann, P., and H. Mammeri.** 2007. Extended-spectrum cephalosporinases: structure, detection and epidemiology. Future Microbiol. **2:**297–307.
19. **Nordmann, P., T. Naas, N. Fortineau, and L. Poirel.** 2007. Superbugs in the coming new decade; multidrug resistance and prospects for treatment of *Staphylococcus aureus*, *Enterococcus* spp. and *Pseudomonas aeruginosa* in 2010. Curr. Opin. Microbiol. **10:**436–440.
20. **Nordmann, P., E. Ronco, T. Naas, C. Duport, Y. Michel-Briand, and R. Labia.** 1993. Characterization of a novel extended-spectrum β-lactamase from *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **37:**962–969.
21. **Pellegrino, F. L., K. R. Netto-dos Santos, L. W. Riley, and B. M. Moreira.** 2006. bla_{GES} carrying *Pseudomonas aeruginosa* isolates from a public hospital in Rio de Janeiro, Brazil. Braz. J. Infect. Dis. **10:**251–253.
22. **Picão, R. C., L. Poirel, A. C. Gales, and P. Nordmann.** 2009. Further identification of CTX-M-2 extended-spectrum β-lactamase in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **53:**2225–2226.
23. **Poirel, L., L. Brinas, A. Verlinde, L. Ide, and P. Nordmann.** 2005. BEL-1, a novel clavulanic acid-inhibited extended-spectrum β-lactamase, and the class 1 integron In*120* in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **49:**3743–3748.
24. **Poirel, L., L. Cabanne, H. Vahaboglu, and P. Nordmann.** 2005. Genetic environment and expression of the extended-spectrum β-lactamase bla_{PER-1} gene in gram-negative bacteria. Antimicrob. Agents Chemother. **49:**1708–1713.
25. **Poirel, L., M. Guibert, D. Girlich, T. Naas, and P. Nordmann.** 1999. Cloning, sequence analyses, expression, and distribution of *ampC-ampR* from *Morganella morganii* clinical isolates. Antimicrob. Agents Chemother. **43:**769–776.
26. **Poirel, L., I. Le Thomas, T. Naas, A. Karim, and P. Nordmann.** 2000. Biochemical sequence analyses of GES-1, a novel class A extended-spectrum β-lactamase, and the class 1 integron In*52* from *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **44:**622–632.
27. **Poirel, L., M. Magalhaes, M. Lopes, and P. Nordmann.** 2004. Molecular analysis of metallo-β-lactamase gene bla_{SPM-1}-surrounding sequences from disseminated *Pseudomonas aeruginosa* isolates in Recife, Brazil. Antimicrob. Agents Chemother. **48:**1406–1409.
28. **Poirel, L., O. Menuteau, N. Agoli, C. Cattoen, and P. Nordmann.** 2003. Outbreak of extended-spectrum β-lactamase VEB-1-producing isolates of *Acinetobacter baumannii* in a French hospital. J. Clin. Microbiol. **41:**3542–3547.
29. **Poirel, L., T. Naas, M. Guibert, E. B. Chaibi, R. Labia, and P. Nordmann.** 1999. Molecular and biochemical characterization of VEB-1, a novel class A extended-spectrum β-lactamase encoded by an *Escherichia coli* integron gene. Antimicrob. Agents Chemother. **43:**573–581.
30. **Poirel, L., T. Naas, D. Nicolas, L. Collet, S. Bellais, J. D. Cavallo, and P. Nordmann.** 2000. Characterization of VIM-2, a carbapenem-hydrolyzing metallo-β-lactamase and its plasmid- and integron-borne gene from a *Pseudomonas aeruginosa* clinical isolate in France. Antimicrob. Agents Chemother. **44:**891–897.
31. **Poirel, L., G. F. Weldhagen, T. Naas, C. De Champs, M. G. Dove, and P. Nordmann.** 2001. GES-2, a class A β-lactamase from *Pseudomonas aeruginosa* with increased hydrolysis of imipenem. Antimicrob. Agents Chemother. **45:**2598–2603.
32. **Post, V., and R. M. Hall.** 2009. Insertion sequences in the IS*1111* family that target the *attC* recombination sites of integron-associated gene cassettes. FEMS Microbiol. Lett. **290:**182–187.
33. **Rodriguez-Martinez, J. M., L. Poirel, and P. Nordmann.** 2009. Extended-spectrum cephalosporinases in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **53:**1766–1771.
34. **Rossolini, G. M., M. M. D'Andrea, and C. Mugnaioli.** 2008. The spread of CTX-M-type extended-spectrum β-lactamases. Clin. Microbiol. Infect. **14**(Suppl. 1)**:**33–41.
35. **Sader, H. S., A. O. Reis, S. Silbert, and A. C. Gales.** 2005. IMPs, VIMs and SPMs: the diversity of metallo-β-lactamases produced by carbapenem-resistant *Pseudomonas aeruginosa* in a Brazilian hospital. Clin. Microbiol. Infect. **11:**73–76.
36. **Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. **33:**2233–2239.
37. **Toleman, M. A., P. M. Bennett, and T. R. Walsh.** 2006. IS*CR* elements: novel gene-capturing systems of the 21st century? Microbiol. Mol. Biol. Rev. **70:**296–316.
38. **Toleman, M. A., A. M. Simm, T. A. Murphy, A. C. Gales, D. J. Biedenbach, R. N. Jones, and T. R. Walsh.** 2002. Molecular characterization of SPM-1, a novel metallo-β-lactamase isolated in Latin America: report from the SENTRY antimicrobial surveillance programme. J. Antimicrob. Chemother. **50:**673–679.
39. **Vourli, S., P. Giakkoupi, V. Miriagou, E. Tzelepi, A. C. Vatopoulos, and L. S. Tzouvelekis.** 2004. Novel GES/IBC extended-spectrum β-lactamase variants with carbapenemase activity in clinical enterobacteria. FEMS Microbiol. Lett. **234:**209–213.
40. **Wachino, J., Y. Doi, K. Yamane, N. Shibata, T. Yagi, T. Kubota, and Y. Arakawa.** 2004. Molecular characterization of a cephamycin-hydrolyzing and inhibitor-resistant class A β-lactamase, GES-4, possessing a single G170S substitution in the omega-loop. Antimicrob. Agents Chemother. **48:**2905–2910.
41. **Walsh, T. R.** 2008. Clinically significant carbapenemases: an update. Curr. Opin. Infect. Dis. **21:**367–371.
42. **Yong, D., J. M. Bell, B. Ritchie, R. Pratt, M. A. Toleman, and T. R. Walsh.** 2007. A novel sub-group metallo-β-lactamase (MBL), AIM-1 emerges in *Pseudomonas aeruginosa* (PSA) from Australia, abstr. C1-593, p. 75. Abstr. 47th Intersci. Conf. Antimicrob. Agents Chemother.