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Dissemination of High-Risk Clones of Extensively Drug-Resistant *Pseudomonas aeruginosa* in Colombia

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The ability of *Pseudomonas aeruginosa* to develop resistance to most antimicrobials represents an important clinical threat worldwide. We report the dissemination in several Colombian hospitals of two predominant lineages of extensively drug-resistant (XDR) carbapenemase-producing *P. aeruginosa* strains. These lineages belong to the high-risk clones sequence type 111 (ST111) and ST235 and harbor $bla_{\text{VIM-2}}$ on a class 1 integron and $bla_{\text{KPC-2}}$ on a Tn*4401* transposon, respectively. Additionally, *P. aeruginosa* ST1492, a novel single-locus variant of ST111, was identified. Clonal dissemination and the presence of mobile genetic elements likely explain the successful spread of XDR *P. aeruginosa* strains in Colombia.

Pseudomonas aeruginosa is an opportunistic pathogen associated with a variety of hospital-associated infections, often in critically ill patients. The treatment of infections caused by *P. aeruginosa* is challenging due to the expression of metallo-β-lactamases (e.g., VIM and IMP) and serine enzymes (e.g., *Klebsiella pneumoniae* carbapenemase [KPC]) that confer resistance to most commercially available β-lactams. The acquisition of carbapenemase-encoding genes, combined with the presence of mechanisms of resistance to multiple other antimicrobials, has led to the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) *P. aeruginosa* (1). Clinicians are often left with very limited options to treat infections caused by MDR and XDR *P. aeruginosa* strains, which are associated with increased morbidity, mortality, and health care costs (2, 3).

Studies on the molecular epidemiology and population structure of Gram-negative bacteria have identified MDR strains that successfully disseminate across diverse geographic locations and patient populations and are therefore known as high-risk clones. Genetic fingerprinting with multilocus sequence typing (MLST) has identified sequence type 111 (ST111), ST175, and ST235 as high-risk clones that are prevalent among carbapenemase-producing *P. aeruginosa* strains from Europe and Asia (4–7). Carbapenem-resistant *P. aeruginosa*, mainly mediated by KPC- and VIM-type enzymes, is endemic in Colombia (8, 9). Although initial reports suggested that high-risk clones circulate in Colombia (10, 11), data on the molecular epidemiology of carbapenemase-producing *P. aeruginosa* isolates are limited. In this study, we characterize XDR *P. aeruginosa* isolates from seven cities in Colombia, focusing on the identification of high-risk clones and of genetic elements associated with the dissemination of carbapenemase genes.

Single-patient isolates of XDR *P. aeruginosa* (defined as resistant to antipseudomonal carbapenems, cephalosporins, penicillins, fluoroquinolones, and aminoglycosides [1]) were selected from the Colombian Bacterial Resistance Surveillance Network strain collection (2008 and 2010) at the Centro Internacional de Entrenamiento e Investigaciones Médicas (CIDEIM). A total of 161 isolates were recovered from 16 tertiary care hospitals in seven Colombian cities. The majority of the isolates (77%) were from intensive care units, and the most frequent sample sources were urine ($n = 40$), blood ($n = 36$), respiratory secretions ($n = 34$), and skin or soft tissue ($n = 17$). The study was approved by the ethics committee of CIDEIM.

An in-house multiplex quantitative PCR (qPCR) designed to detect bla_{KPC}, bla_{VIM}, bla_{IMP}, bla_{NDM}, bla_{TEM}, bla_{SHV}, and $bla_{\text{CTX-M}}$ was performed using a CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). The probes (dual-labeled black hole quencher [BHQ] probes) and primers (Table 1) were designed using Beacon Designer 8.0 (Premier Biosoft International). Total DNA (100 ng) was used as the template for the reaction. The thermal cycling conditions for the multiplex qPCR were 50°C for 2 min, 95°C for 2 min, followed by 40 cycles at 95°C (15 s) and 60°C (1 min). The samples with a threshold cycle (C_T) value of <35 were considered positive. Additionally, conventional PCR was used to screen for the presence of bla_{PER} and bla_{GES} (12). We performed phenotypic tests for carbapenemase production using the Carba NP test and a combined-disk method (CDM) using imipenem and cloxacillin in isolates that tested negative for the presence of carbapenemase genes (13, 14). The sequencing of

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TABLE 1 Primers and probes designed for the detection of *bla* genes among XDR *P. aeruginosa* from Colombia

Target gene	5′–3′ sequence for[a]:		
	Forward primer	Reverse primer	Probe
bla_{KPC}	GGACACACCCATCCGTTA	GCGGGCGTTATCACTGTATTG	FAM-TCCGCCACCGTCATGCCTGTTG-BHQ1[b]
bla_{VIM}	GCTTCGGTCCAGTAGAACTC	AGACGTGCGTGACAACTC	CR610-AATCGCACAACCACCATAGAGCACACT-BHQ2
bla_{IMP}	GCGGCTATAAAATAAAAGGCAGTA	GATGCATACGTGGGGATAGA	CY5.5-CACATTTCCATAGCGACAGCACGGGC-3BHQ3
bla_{NDM}	CAACGGTTTGGCGATCTG	DGCCATCCCTGACGATCAA	GOLD540-CGCACCGAATGTCTGGCAGCACA-BHQ1
bla_{CTX-M}	ATGTGCAGYACCAGTAARGTKATGGC	ATCACKCGGRTCGCCXGGRAT	CR610-CCCGACAGCTGGGAGACGAAACGT-BHQ2
bla_{TEM}	TGGCATGACAGTAAGAGAATTATG	CAAGGCGAGTTACATGATCC	CG540-AAGCGGTTAGCTCCTTCGGTCCTCC-BHQ1
bla_{SHV}	CAGGATCTGGTGGACTACTC	CGCTGTTATCGCTCATGG	Q670-CGCAGAGTTCGCCGACCGTCA-BHQ2

[a] The final primer and probe concentrations in the multiplex reaction were 0.2 μM each. Additional volumes of magnesium and deoxynucleoside triphosphates (dNTPs) were used in the multiplex reaction.
[b] FAM, 6-carboxyfluorescein.

PCR products was performed in order to confirm the identities of the detected genes. Pulsed-field gel electrophoresis (PFGE) using XbaI was performed in the 161 isolates to assess their genetic relatedness, as previously described by Gautom (15). Two or more strains were considered genetically related if the Dice coefficient was >75%. MLST was performed in at least one isolate from each PFGE type, as described previously (15). STs were assigned using the available Web-based scheme (*Pseudomonas aeruginosa* MLST database [http://pubmlst.org/paeruginosa]). The location of carbapenemase genes within the bacterial chromosome was investigated with S1 nuclease/I-CeuI probe hybridization using previously described protocols (16, 17), and the genetic environment surrounding bla_{VIM} or bla_{KPC} was further analyzed by PCR and sequencing (11, 18).

PCR amplification and sequencing confirmed the presence of bla_{VIM-2} and bla_{KPC-2} in 128 out of 161 isolates that fit the definition of XDR *P. aeruginosa* (1). Of note, isolate 3386 was found to harbor both bla_{VIM-2} and bla_{KPC-2}, as previously described in detail (10). All isolates that tested negative for the presence of carbapenemase genes by PCR were also negative for carbapenemase production, according to phenotypic tests (Carba NP and CDM). We did not characterize these isolates further, but we suspect that their phenotype can be explained by the hyperexpression of the MexAB-OprM efflux pump, modification of the OprD2 porin, and cephalosporinase hyperproduction, as described elsewhere (14, 19).

PFGE revealed 18 different types, seven of which were represented by single isolates. Seven PFGE types contained isolates with bla_{VIM-2}, and six contained isolates with bla_{KPC-2}. As mentioned above, one isolate harbored both bla_{VIM-2} and bla_{KPC-2}, but other isolates from the same PFGE type (PTG2) harbored bla_{VIM-2} only. Among 12 isolates in PFGE type PTG1, only one contained bla_{VIM-2}, whereas carbapenemase-encoding genes were not found in the remaining isolates. We were not able to detect carbapenemase genes in the isolates belonging to four PFGE types (Table 2).

We selected 32 isolates (at least one from each PFGE type) for further typing with MLST; 13 of these isolates harbored bla_{VIM-2} only, 10 isolates harbored bla_{KPC-2} only, and one isolate harbored both bla_{VIM-2} and bla_{KPC-2}. Eight isolates were negative for all the carbapenemases tested. MLST indicated that 86% of the *P. aeruginosa* strains harboring bla_{VIM-2} belonged to ST111 ($n = 11$), while all *P. aeruginosa* strains carrying bla_{KPC-2} belonged to ST235. Interestingly, a novel single-locus variant of ST111 (designated ST1492) was identified in one isolate. Carbapenem-resistant *P. aeruginosa* isolates that did not carry any of the target genes belonged to ST111, ST235, ST481, and ST227 (Table 2). All these sequence types have >3 allele differences and are considered unrelated.

Using an S1/I-CeuI hybridization protocol, we were able to determine that bla_{VIM-2} and bla_{KPC-2} were located in the chromosome in the majority of the isolates (70%). In the remaining isolates, these genes were located in plasmids of variable length (80 to 190 kb). In the 12 isolates belonging to *P. aeruginosa* ST111, bla_{VIM-2} was found in a class 1 integron with *aacA29a* in the upstream region and *aacA29b* and *qacEΔ1sul*-1 in the downstream region; this genetic structure was previously designated In59 (10, 20). The transposon Tn*4401* was found in 8 isolates carrying bla_{KPC-2}; in the two remaining isolates, bla_{KPC-2} did not appear to be associated with this genetic structure (Table 2).

The emergence of MDR and XDR bacteria causes alarm and is deemed a global public health crisis. Colombia is a particular hotspot for antibiotic resistance, where the acquisition of genes coding for KPC and VIM enzymes among *Enterobacteriaceae* and *P. aeruginosa* is of great concern (8–11, 18, 21–23). Previous reports (24, 25) described the predominance of certain genetic lineages of *P. aeruginosa* in various clinical settings and geographic locations; *P. aeruginosa* ST111 and ST235, mostly harboring bla_{VIM} and other metallo-β-lactamases, have been identified as being among these high-risk clones (4, 26–30). Similar to findings elsewhere, our survey of *P. aeruginosa* from Colombian hospitals found that ST111 is a common host of bla_{VIM-2}. In contrast, we found that *P. aeruginosa* ST235 is most commonly associated with the dissemination of bla_{KPC-2} and is present in hospitals from 6 out of the 7 cities included in our study (see Fig. S1 in the supplemental material). Although we had previously identified other *P. aeruginosa* STs (ST308, ST1006, and ST1060) associated with the dissemination of bla_{KPC-2}, this more comprehensive survey revealed that ST235 is the predominant carbapenemase-producing *P. aeruginosa* strain type in Colombia. *P. aeruginosa* ST235 harboring other carbapenemase-encoding genes, such as bla_{VIM}, bla_{IMP}, and bla_{GES}, has been reported in other countries (8, 30). Of note, the set of XDR *P. aeruginosa* strains analyzed in this survey displayed consistent susceptibility to polymyxin B (except for isolate 3386 that harbors both bla_{VIM-2} and bla_{KPC-2}), indicating that polymyxins remain one of the few options for the treatment of infections caused by XDR *P. aeruginosa* strains in Colombia.

In summary, VIM-2 and KPC-2 carbapenemases are the main contributors to β-lactam resistance among XDR *P. aeruginosa* strains found in Colombian hospitals. Almost all *P. aeruginosa* strains harboring bla_{VIM-2} belong to ST111, while a single se-

TABLE 2 Characterization of representative pulsed-field gel electrophoresis types of XDR *P. aeruginosa* from Colombia

ID[a]	PFGE type[b]	MLST[c]	Carbapenemase gene	MIC (μg/ml) for antibiotic[d]:									Carba NP result	CDM result[e]	Genetic environment of bla_{VIM-2} and bla_{KPC-2}
				IPM	MEM	AMK	ATM	FEP	CAZ	CIP	TZP	PMB			
90	PTG1	ST481	NEG[f]	64	8	64	64	32	128	>8	256/4	2	NEG	NEG	Negative for carbapenemases
127	PTG1	ST481	NEG	32	16	<8	32	32	64	>8	256/4	2	NEG	NEG	Negative for carbapenemases
93	PTG1	ST481	bla_{VIM-2}	>128	64	64	<8	16	32	>8	32/4	1	ND[g]	ND	*intI1-aac(6′)29a-* bla_{VIM-2}*-aac(6′)29b-qacEΔ1sul-1*
142	PTG2	ST111	bla_{VIM-2}	>128	128	64	32	16	32	>8	32/4	2	ND	ND	*intI1-aac(6′)29a-* bla_{VIM-2}*-aac(6′)29b-qacEΔ1sul-1*
2146	PTG2	ST111	bla_{VIM-2}	128	128	128	32	16	32	>8	32/4	2	ND	ND	*intI1-aac(6′)29a-* bla_{VIM-2}*-aac(6′)29b-qacEΔ1sul-1*
2369	PTG2	ST111	bla_{VIM-2}	128	128	64	32	16	16	>8	32/4	1	ND	ND	*intI1-aac(6′)29a-* bla_{VIM-2}*-aac(6′)29b-qacEΔ1sul-1*
3386	PTG2	ST111	bla_{VIM-2} and bla_{KPC-2}	128	128	128	64	128	64	>8	>128/4	64	ND	ND	*intI1-aac(6′)29a-* bla_{VIM-2}*-aac(6′)29b-qacEΔ1sul-1 tnpR-tnpB-istB-*bla_{KPC-2}*-tnpA*
120	PTG3	ST111	bla_{VIM-2}	128	128	128	32	32	64	>8	>256/4	2	ND	ND	*intI1-aac(6′)29a-* bla_{VIM-2}*-aac(6′)29b-qacEΔ1sul-1*
2826	PTG3	ST111	bla_{VIM-2}	128	128	128	16	32	64	>8	64/4	2	ND	ND	*intI1-aac(6′)29a-* bla_{VIM-2}*-aac(6′)29b-qacEΔ1sul-1*
2961	PTG3	ST111	bla_{VIM-2}	128	128	64	32	128	64	>8	64/4	2	ND	ND	*intI1-aac(6′)29a-* bla_{VIM-2}*-aac(6′)29b-qacEΔ1sul-1*
712	PTG9	ST111	bla_{VIM-2}	>128	64	32	16	16	16	>8	32/4	2	ND	ND	*intI1-aac(6′)29a-* bla_{VIM-2}*-aac(6′)29b-qacEΔ1sul-1*
1275	PTG9	ST111	bla_{VIM-2}	64	16	32	16	16	32	>8	64/4	1	ND	ND	*intI1-aac(6′)29a-* bla_{VIM-2}*-aac(6′)29b-qacEΔ1sul-1*
275	PTG10	ST1492	bla_{VIM-2}	32	16	128	<8	32	64	<1	64/4	2	ND	ND	*intI1-aac(6′)29a-* bla_{VIM-2}*-aac(6′)29b*
192	PTG4	ST235	bla_{KPC-2}	128	>128	64	32	128	256	>8	128/4	2	ND	ND	*tnpR-tnpB-istB-*bla_{KPC-2}*-tnpA*
1332	PTG4	ST235	bla_{KPC-2}	128	>128	32	64	128	128	>8	128/4	2	ND	ND	*tnpR-tnpB-istB-*bla_{KPC-2}*-tnpA*
343	PTG4	ST235	NEG	32	32	<8	64	16	64	>8	256/4	2	NEG	NEG	Negative for carbapenemases
3144	PTG5	ST235	bla_{KPC-2}	128	128	128	64	128	64	>8	>128/4	2	ND	ND	bla_{KPC-2}
2651	PTG5	ST235	bla_{KPC-2}	32	32	64	64	128	64	>8	>128/4	1	ND	ND	*tnpR-tnpB-istB-*bla_{KPC-2}*-tnpA*
1986	PTG5	ST235	bla_{KPC-2}	128	128	64	>16	128	64	>8	>128/4	2	ND	ND	*tnpR-tnpB-istB-*bla_{KPC-2}*-tnpA*
1602	PTG5	ST235	bla_{KPC-2}	>128	>128	32	64	128	128	>8	128/4	2	ND	ND	*tnpR-tnpB-istB-*bla_{KPC-2}*-tnpA*
542	PTG6	ST235	bla_{KPC-2}	>128	>128	64	64	128	128	>8	256/4	2	ND	ND	*tnpR-tnpB-istB-*bla_{KPC-2}*-tnpA*
1669	PTG7	ST235	bla_{KPC-2}	16	64	64	64	128	32	>8	>256/4	1	ND	ND	bla_{KPC-2}
2537	PTG7	ST235	NEG	16	32	<8	32	16	8	>8	32/4	1	NEG	NEG	Negative for carbapenemases
2902	PTG8	ST235	bla_{KPC-2}	32	64	64	64	128	64	>8	>128/4	2	ND	ND	*tnpR-tnpB-istB-*bla_{KPC-2}*-tnpA*
1516	PTG11	ST235	bla_{KPC-2}	>128	>128	32	64	32	256	>8	256/4	2	ND	ND	*tnpR-tnpB-istB-*bla_{KPC-2}*-tnpA*
2697	POLI1	ST298	bla_{VIM-2}	32	16	32	<8	64	64	4	128/4	2	ND	ND	*intI1-aac(6′)29a-* bla_{VIM-2}*-aac(6′)29b*
185	POLI2	ST111	bla_{VIM-2}	>128	128	64	32	32	32	>8	32/4	2	ND	ND	*intI1-aac(6′)29a-* bla_{VIM-2}*-aac(6′)29b-qacEΔ1sul-1*
209	POLI3	ST111	bla_{VIM-2}	>128	>128	64	32	32	32	>8	32/4	2	ND	ND	*intI1-aac(6′)29a-* bla_{VIM-2}*-aac(6′)29b-qacEΔ1sul-1*
154	POLI4	ST111	NEG	>128	128	64	32	32	16	>8	32/4	2	NEG	NEG	Negative for carbapenemases
1499	POLI5	ST235	NEG	4	16	64	64	32	>256	>8	128/4	2	NEG	NEG	Negative for carbapenemases
406	POLI6	ST481	NEG	16	4	32	64	32	64	>8	>256/4	2	NEG	NEG	Negative for carbapenemases
646	POLI7	227	NEG	16	16	<8	64	8	128	>8	>256/4	4	NEG	NEG	Negative for carbapenemases

[a] ID, identification.
[b] PFGE, pulsed-field gel electrophoresis; PTG, PFGE type with >1 isolate; POLI, PFGE type with a single isolate.
[c] MLST, multilocus sequence typing; ST, sequence type.
[d] IPM, imipenem; MEM, meropenem; AMK; amikacin; ATM, aztreonam; FEP, cefepime; CIP, ciprofloxacin; TZP, piperacillin-tazobactam; PMB, polymyxin B.
[e] CDM, combined disk method.
[f] NEG, negative.
[g] ND, not determined.

quence type, ST235, is associated with *P. aeruginosa* strains harboring bla_{KPC-2}. These XDR high-risk clones mainly rely on class 1 integrons and the well-known transposable element Tn*4401* as the principal structures for gene mobilization. The coexistence of these lineages of XDR *P. aeruginosa* in this South American country suggests complex transmission dynamics that need to be explored further. Our findings indicate that the spread of XDR *P. aeruginosa* high-risk clones is a real threat in Colombian hospitals; this knowledge should serve as the basis for nationwide strategies to improve infection prevention and control efforts.

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