

Characterization of antimicrobial resistance mechanisms in carbapenem-resistant *Pseudomonas aeruginosa* carrying IMP variants recovered from a Mexican Hospital

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Purpose: *Pseudomonas aeruginosa* infections in hospitals constitute an important problem due to the increasing multidrug resistance (MDR) and carbapenems resistance. The knowledge of resistance mechanisms in *Pseudomonas* strains is an important issue for an adequate antimicrobial treatment. Therefore, the objective was to investigate other antimicrobial resistance mechanisms in MDR *P. aeruginosa* strains carrying bla_{IMP} make a partial plasmids characterization, and determine if modifications in *oprD* gene affect the expression of the OprD protein.

Methodology: Susceptibility testing was performed by Kirby-Bauer and by Minimum Inhibitory Concentration (presence/absence of efflux pump inhibitor); molecular typing by Pulsed-field gel electrophoresis (PFGE), resistance genotyping and integrons by PCR and sequencing; OprD expression by Western blot; plasmid characterization by MOB Typing Technique, molecular size by PFGE-S1; and bla_{IMP} location by Southern blot.

Results: Among the 59 studied *P. aeruginosa* isolates, 41 multidrug resistance and carbapenems resistance isolates were detected and classified in 38 different PFGE patterns. Thirteen strains carried bla_{IMP} , 16 bla_{GES} and four carried both genes. This study centered on the 17 strains harboring bla_{IMP} . New variants of β -lactamases were identified (bla_{GES-32} , bla_{IMP-56} , bla_{IMP-62}) inside of new arrangements of class 1 integrons. The presence of bla_{IMP} gene was detected in two plasmids in the same strain. The participation of the OprD protein and efflux pumps in the resistance to carbapenems and quinolones is shown. No expression of the porin OprD due to stop codon or IS in the gene was found.

Conclusions: This study shows the participation of different resistance mechanisms, which are reflected in the levels of MIC to carbapenems. This is the first report of the presence of three new variants of β -lactamases inside of new arrangements of class 1 integrons, as well as the presence of two plasmids carrying bla_{IMP} in the same *P. aeruginosa* strain isolated in a Mexican hospital.

Keywords: *Pseudomonas aeruginosa*, resistance mechanisms, plasmid, integrons

Introduction

Pseudomonas aeruginosa is a well-known cause of severe and potentially life-threatening nosocomial infections, which include pulmonary disease, urinary tract infections and bacteremia. *P. aeruginosa* has an extraordinary capacity to develop antimicrobial resistance due to chromosomal mutations and the acquisition of resistance genes encoding β -lactamases, particularly carbapenemases, and combined with the presence of other resistance mechanisms, has generated an increase of multidrug resistance (MDR) strains.^{1,2} Previous studies have demonstrated that metallo- β -lactamases (MBLs)

represent an important antimicrobial resistance determinant among *P. aeruginosa* strains causing nosocomial infections.³ The first MBL, variant *bla*_{IMP} was described in Japan,⁴ and to date 64 variants of *bla*_{IMP}³ have been reported which are available in GenBank. The *bla*_{IMP} genes are usually located in integrons, which are prevalent in gram-negative bacteria, and can be located in plasmids and transposons, facilitating their dissemination.⁵ *P. aeruginosa* strains harboring integrons with *bla*_{IMP} genes cassettes have been reported in Europe, Asia, and South America.^{6–8} However, its presence has been recently found in Mexico, reports include the presence of carbapenem-resistant *P. aeruginosa* strains containing either *bla*_{IMP-15} or *bla*_{IMP-18} genes, both inside integrons class 1.^{9–11}

In addition, a number of studies describe the presence of *bla*_{IMP-17}, *bla*_{IMP-9}, *bla*_{IMP-15}, *bla*_{IMP-29}, *bla*_{IMP-45}, in conjugative plasmids of narrow and broad-host range belonging to incompatibility groups IncP-2 and IncP-9 in some cases.^{4,10,12–14} In *P. aeruginosa*, at least 13 incompatibility groups have been identified: IncP-1 to IncP-7 and IncP-9 to IncP-14. The plasmids belonging to the groups IncP-2, IncP-5, IncP-7, IncP-10, IncP-12 and IncP-13 are defined as plasmids of host narrow range. The plasmids with IncP-1, IncP-4 and IncP-6 are broad-range host plasmids.^{13,15,16} Due to this diversity in the Inc groups, Alvarado et al¹⁷ designed degenerate oligonucleotides that allow the identification of transmissible plasmids based on the sequence of relaxases, which are multidomain proteins present in mobilizable and conjugative plasmids.

Other mechanisms such as alterations or lack of porin OprD, and overexpression of efflux pumps have been associated with resistance to drugs in strains isolated from hospitalized patients. Loss of porin OprD decreases the susceptibility to carbapenems.¹⁸ Previous studies have shown that mutations in *oprD* gene results in the absence corresponding to the OprD porin band in SDS-PAGE gels.^{19,20}

On the other hand, in *P. aeruginosa*, four clinically relevant RND efflux pumps: MexAB-OprM,²¹ MexCD-OprJ²² MexEF-OprN²³ and MexXY-OprM²⁴ have been identified, and the overexpression of these pumps, has been associated with increases of β -lactam, aminoglycosides and fluoroquinolone resistance.²⁵ Also, it has been reported that the pump inhibitor phenylalanine arginyl β -naphthylamide (EPI), allows the participation of these pumps in the resistance to meropenem and fluoroquinolones, through a mechanism of competitive inhibition, this is because the pumps recognize EPI as a substrate instead of the target antibiotics.^{25,26}

According to the information about the participation of different resistant mechanisms developed by *P. aeruginosa*,

this work was carried out to investigate other antimicrobial resistance mechanisms in MDR *P. aeruginosa* strains carrying *bla*_{IMP} as well as to make a partial characterization of plasmids and determine if mutations in the *oprD* gene could have an effect on the expression of the OprD protein.

Material and methods

Bacterial strains and culture conditions

Fifty-nine *P. aeruginosa* clinical isolates were obtained from 57 patients with nosocomial infection (two patients with two different strains) hospitalized in Hospital Regional del ISSSTE of Puebla, Mexico, during the period April 2013 to July 2015. The isolates were identified by conventional biochemical tests and VITEK-2 system.

Antimicrobial susceptibility testing

Susceptibility to 13 anti-pseudomonal agents (piperacillin, ticarcillin, piperacillin/tazobactam, ceftazidime, cefepime, aztreonam, imipenem, meropenem, gentamicin, tobramycin, amikacin, ciprofloxacin, and norfloxacin) was performed by the disc-diffusion method. Minimum Inhibitory Concentration (MIC) of CAZ, FEP, IPM, MEM, CIP, AN, and GM was determined in the strains carrying *bla*_{IMP} by agar dilution method using the breakpoints recommended by the Clinical and Laboratory Standard Institute.²⁷ The MDR strains and carbapenem resistance (RC) were selected to continue the study. MDR was defined according to Magiorakos' proposal as: non-susceptible to ≥ 1 agent in ≥ 3 antimicrobial categories.²⁸

Molecular typing of *P. aeruginosa* isolates

The clonal relationship was performed by Pulsed-field gel electrophoresis (PFGE). Genomic DNA was digested using SpeI restriction enzyme (Thermo Fisher Scientific, Waltham, MA, USA) and macrorestriction fragments were separated as previously described²⁹ on a CHEF-DR II (BioRad Laboratories Inc., Hercules, CA, USA). Banding patterns were visualized by ethidium bromide staining and photographed. Dendrogram and cluster analysis were performed with NTSYSpc2.2. software, using the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) and based on the criteria of Tenover et al.³⁰ The sequence type (ST) was determined in the three strains with new variants of β -lactamases by the multilocus sequence typing (MLST) technique as previously described by Curran et al³¹ Allelic profile and a sequence type (ST) were assigned using the *Pseudomonas aeruginosa* MLST website.³²

Detection of resistance genes

The presence of different resistance genes was studied by PCR using specific primers for resistance to: β -lactams (*bla*_{IMP}, *bla*_{VIM}, *bla*_{GIM}, *bla*_{SPM}, *bla*_{NDM}, *bla*_{GES}, *bla*_{KPC}, *bla*_{VEB}, *bla*_{PSE}, *bla*_{CMY}, *bla*_{PER}, *bla*_{OXA-2}, *bla*_{OXA-40}, *bla*_{OXA-50}), aminoglycosides (*aac(3)-IIa*, *aacA4*), quinolones (*qnrA*, *qnrB*, *qnrS*, *aac(6')Ib-cr*) and colistin (*mcr-1*). Primers used for amplification and for sequencing are listed in [Table S1](#). The amplicons obtained were sequenced. The nucleotide and deduced protein sequences were analyzed and compared with those available at GenBank. The strains carrying *bla*_{IMP} were selected and used for the assays that are described below.

Detection and characterization of the genetic structures of integrons

The presence of type 1 and 2 integrases genes (*int11* and *int12*), as well as the 3'-conserved segment of class 1 integrons (*qacEΔ1+sul1*) were studied by PCR in all *bla*_{IMP}-harboring strains. The characterization of class 1 integron variable regions was determined by PCR and sequencing ([Table S1](#)).³³

Mechanisms of resistance to quinolones

Amino acid changes in GyrA and ParC proteins were studied in the *P. aeruginosa* strains. For this purpose, *gyrA* and *parC* genes were amplified by PCR, amplified fragments were purified and sequenced.³⁴ Deduced amino acid sequences were compared with those previously reported for GyrA and ParC in *P. aeruginosa* PAO1 (GenBank accession number for GyrA/ParC: NP_251858.1 and NP_253651.1 respectively).

Modifications of porin OprD

The *oprD* gene was amplified by PCR and sequenced in all *bla*_{IMP}-harboring strains. The mutations were determined by comparison with the sequence of the *P. aeruginosa* PAO1 strain (GeneID: 881970).^{35,36}

Expression of protein OprD

The strains were cultured in BHI medium for 20 hours; the cells were collected and lysed with RIPA buffer (Sigma Aldrich, St. Louis, MO, USA). The total protein extract was precipitated with acetone, recovered by centrifugation and proteins were separated by means of 12% SDS-PAGE gel. Proteins were separate in duplicate to obtain two gels, one was stained with Coomassie brilliant blue²⁰ and the other was used for Western blot.

Western blot

Gel destined for Western blot was transferred to a nitrocellulose membrane (Hybond-ECL; Amersham Pharmacia Biotech, Amersham, UK) for detection of OprD protein. The membrane was blocked using 5% non-fat skim milk overnight at 4°C. Then was washed with PBS and incubated with rabbit polyclonal antisera (1:10000) against OprD (obtained in this laboratory) followed by incubation with a second antibody phosphatase alkaline-conjugated goat anti-mouse IgG (1:5000) (Zymed Laboratories, Thermo Fisher Scientific, Waltham, MA, USA) and visualized with 5-bromo-4-chloro-3-indolyl phosphate/p-nitro blue tetrazolium system (BCIP/NBT) (Sigma Aldrich). The total extract protein of *P. aeruginosa* PAO1 and the OprD recombinant protein (obtained in this laboratory) were used as positive controls.

Influence of efflux pumps inhibitor on MIC to meropenem and ciprofloxacin

The efflux pumps inhibitor EPI (L-phenylalanine-arginine-naphthylamide, PAβN, Sigma-Aldrich) was used to determine the involvement of the RND pumps in the resistance to MEM and CIP. The MIC of MEM and CIP was performed by the agar diffusion method without and with EPI at 25 mg/L. A decrease of two-fold or higher in MIC values in presence of EPI was considered as a participation of RND-type efflux pumps in the resistance to these two antibiotics.³⁷

Extraction and size determination of plasmids

Plasmid extraction was performed using the Quick Prep method,³⁸ and the size and number of plasmids were determined by PFGE-S1. For this method, the bacterial DNA was embedded in agarose plugs as described by Kaufmann.³⁹ Plasmid digestions were done with nuclease S1 (8 U per plug) (New England Biolabs, Ipswich, MA, USA). The PFGE conditions were used as follows: pulse time ranging from 1 s to 30 s for 22 hours, the gel was run at 6 V cm and at 14°C. Two strains with plasmids of known size were used for size determination: *E. coli* NCTC 50192 with four plasmids (154, 66, 48 and 7 kb) and *Rhizobium etli* CFN42Δe with six plasmids (624, 371, 250, 194, 184 and 60.5 kb).

Plasmid typing

The types of relaxases were determined in the strains carrying plasmids by the Degenerate Primer MOB Typing (DPMT) method,¹⁷ and the incompatibility groups as was reported by Caratolli et al.⁴⁰

Location of *bla*_{IMP} gene in plasmids by southern blot

The PFGE conditions used were as follows: pulse time ranging from 5 s to 45 s for 20 hours at 6 V/cm and at 14°C after nuclease S1 (180 U/μL, Takara Bio Inc., Kusatsu, Shiga Prefecture, Japan) digestion. Southern blot was performed using the DIG HIGH Prime DNA Labeling and Detection Starter Kit II. The hybridization was performed overnight at 40°C with the *bla*_{IMP} gene as probe; detection was made as is described by the manufacturer (Hoffmann-La Roche, Basel, Switzerland).

Ethics

All isolates were collected during routine sampling, and patient's data were maintained under anonymity. The protocol for this study was approved by the Ethical Committee of Hospital Regional ISSSTE, Puebla (Registry number 188.2018)

Results

The 59 isolates were recovered from 57 patients hospitalized in different units (Internal Medicine, 51%; Intensive Care Unit, 17%; Urology, 7%; Surgery, 9%; and other units, 16%) and from different types of samples (bronchial secretions, 36%; urine culture, 32%; wounds, 8%; blood culture, 8%; catheter, 7%; and others, 9%).

Antimicrobial susceptibility

Forty-one of the 59 *P. aeruginosa* isolates showed MDR-RC (69%). The following percentages of resistance were detected: ticarcillin 74%, imipenem 69%, ciprofloxacin 69%, norfloxacin 69%, meropenem 68%, gentamicin 68%, tobramycin 66%, amikacin 63%, cefepime 61%, ceftazidime 59%, piperacillin 47%, aztreonam 41% and piperacillin/tazobactam 24%. All isolates were susceptible to colistin.

Clonal relationship of the isolates

A total of 38 different PFGE patterns were detected among the 41 MDR-RC *P. aeruginosa* isolates. Dendrogram shows that six strains were grouped into three clusters (A to C). Cluster A included strains PE09 and PE16, with an 84% of similarity, these strains were collected from the same service (Internal Medicine); cluster B included the strains PE54 and PE55 presenting a similarity of 95%, these strains were obtained from different services (Urology and Internal Medicine, respectively); and cluster C included strains PE57 and PE58 with 87% of similarity, these strains were also obtained from different services (Neurology and Internal Medicine, respectively). The strains from the same patient

PE06 and PE52 (isolated from urine) and PE26 and PE30 (from expectoration and urine respectively) were non-related (Figure S1). MLST was performed on strains PE06, PE21, and PE52 that carried new genetic variants; results showed that these belong to lineages ST308, ST167, and ST385, respectively.

Detection of resistance genes

Among the 41 MDR *P. aeruginosa* strains, the following resistance genes were detected (% strains) to β-lactams: *bla*_{IMP} (41%), *bla*_{GES} (49%), *bla*_{OXA-2} (85%) and *bla*_{OXA-50} (100%); and to aminoglycosides: *aacA4* (70%) and *aac(3')-IIa* (7%). None of the strains harbored the genes: *bla*_{VIM}, *bla*_{GIM}, *bla*_{SPM}, *bla*_{NDM}, *bla*_{VEB}, *bla*_{PSE}, *bla*_{KPC}, *bla*_{CMY}, *bla*_{PER}, *bla*_{OXA-40}, *qnrA*, *qnrB*, *qnrS* or *aac(6')Ib-cr*

The following variants were detected among the 16 *bla*_{GES}-positive strains: *bla*_{GES-2} (n=6 strains), *bla*_{GES-19} (n=4), *bla*_{GES-20} (n=1) and *bla*_{GES-26} (n=5); and the variants among the 17 *bla*_{IMP}-positive strains were as follows: *bla*_{IMP-18} (n=10 strains), *bla*_{IMP-18}+*bla*_{IMP-56} (n=2), *bla*_{IMP-56} (n=1), and *bla*_{IMP-62} (n=4). The two new variants, *bla*_{IMP-56} and *bla*_{IMP-62}, were deposited in the GenBank database with the accession number KU351745.1 for *bla*_{IMP-56} sequence, and accession number KX753224.1 for *bla*_{IMP-62} sequence (Table 1). Association of *bla*_{IMP} and *bla*_{GES} genes was detected in four isolates: *bla*_{IMP-62}+*bla*_{GES32} in PE21; *bla*_{IMP-62}+*bla*_{GES-2} in PE23; *bla*_{IMP-18}+*bla*_{GES-1} in PE63; and *bla*_{IMP-62}+*bla*_{GES-26} in PE83 (Table 1). The sequence of the new variant *bla*_{GES-32} was deposited in the GenBank database (accession number KX753225).

Nine *bla*_{IMP}-positive strains also carried the *aacA4* gene (amikacin MIC 64 to >512 μg/mL), and two strains contained *aac(3')-IIa*+*aacA4* (amikacin MIC 128 to >512 μg/mL and gentamicin MIC >512 μg/mL). None of the acetylases studied were amplified in three strains resistant to amikacin MIC >512 μg/mL and gentamicin MIC 16–32 μg/mL (Table 1).

Detection and characterization of the genetic structures of integrons

To determine the genetic context of *bla*_{IMP} class 1 integrons were detected in all strains, but not class 2 integrons. Two new class 1 integron arrangements were identified in two strains obtained from the same patient (PE06 and PE52): *bla*_{IMP-18}+*aadA1*+*bla*_{OXA-2} and *bla*_{IMP-56}+*aadA1*+*bla*_{OXA-2}. These new arrangements were deposited in Integrall and GenBank database with the accession numbers KY646160 and KY646161 for the In1215/In1343 sequence of PE52 respectively.

The strains PE21 and PE83 showed an identical class 1 integron array without 3'-CS: *aacA7*+*bla*_{IMP-62}+*qacH*

Table 1 Phenotype and genotype of resistance of *P. aeruginosa* clinical isolates carrying *bla*_{IMP} gene

Strain	PFGE type	Phenotype of resistance										Genotype										Amino acid changes	
		CAZ	FEP	MEM (+ ^b EPI)	IMP	CIP (+ ^b EPI)	AN	GM	CAZ	FEP	MEM (+ ^b EPI)	IMP	CIP (+ ^b EPI)	AN	GM	GyrA	ParC						
PE06	15	PIP, TIC, TZR, CAZ, FEP, ATM, IPM, MEM, GM, NN, AN, CIP, NOR	>512	>512	>512	>512	>512	>512	>512	>512	>512	32	<i>bla</i> _{IMP-18} , <i>bla</i> _{IMP-56} , <i>bla</i> _{OXA-2} , <i>bla</i> _{OXA-50} , <i>aacA1</i> , <i>aacA6</i>			T831	S87L						
PE09	6a	TIC, CAZ, FEP, IPM, MEM, GM, NN, AN, CIP, NOR	>512	512	>512	64	64	64	64	256	>512	>512	256	>512	>512	T831	S87L						
PE10	8	PIP, TIC, CAZ, FEP, ATM, IPM, MEM, GM, NN, AN, CIP, NOR	512	256	64	64	64	64	64	32	>512	>512	64	>512	>512	T831	S87L						
PE15	7	TIC, CAZ, FEP, IPM, MEM, GM, NN, AN, CIP, NOR	512	256	64	64	64	64	64	32	>512	>512	64	>512	>512	T831	S87L						
PE16	6b	TIC, CAZ, FEP, IPM, MEM, GM, NN, AN, CIP, NOR	512	256	64	64	64	64	64	32	>512	>512	64	>512	>512	T831	S87L						
PE20	1	PIP, TIC, CAZ, FEP, IPM, MEM, GM, NN, AN, CIP, NOR	>512	>512	>512	>512	>512	>512	>512	128	>512	>512	>512	>512	>512	T831	S87L						
PE21	28	TIC, CAZ, FEP, IPM, MEM, GM, NN, AN, CIP, NOR	>512	512	>512	128	128	128	128	64	>512	>512	>512	>512	>512	T831	S87L						
PE22	12	TIC, CAZ, FEP, IPM, MEM, GM, NN, AN, CIP, NOR	512	256	16	64	64	64	64	128	>512	>512	128	>512	>512	T831	S87L						
PE23	10	TIC, FEP, IPM, MEM, GM, NN, AN, CIP, NOR	16	32	32	64	64	64	64	32	>512	>512	64	>512	>512	T831	S87L						
PE27	25	TIC, CAZ, FEP, IPM, MEM, GM, NN, AN, CIP, NOR	512	256	64	>512	>512	64	64	64	>512	>512	128	512	>512	T831	S87L						
PE46	32	TIC, CAZ, FEP, IPM, MEM, GM, NN, AN, CIP, NOR	>512	512	>512	64	64	64	64	64	>512	>512	512	>512	>512	T831	S87L						
PE52	16	PIP, TIC, TZR, CAZ, FEP, ATM, IPM, MEM, GM, AN, CIP, NOR	>512	>512	>512	>512	>512	>512	>512	>512	>512	32	<i>bla</i> _{IMP-18} , <i>bla</i> _{IMP-56} , <i>bla</i> _{OXA-2} , <i>bla</i> _{OXA-50} , <i>aacA1</i> , <i>aacA6</i>		T831	wild type							
PE62	20	PIP, TIC, CAZ, FEP, ATM, IPM, MEM, GM, NN, AN, CIP, NOR	>512	>512	>512	>512	>512	>512	>512	>512	>512	16	<i>bla</i> _{IMP-18} , <i>bla</i> _{OXA-2} , <i>bla</i> _{OXA-50}		T831	S87L							
PE63	38	PIP, TIC, TZR, CAZ, FEP, ATM, IPM, MEM, GM, NN, AN, CIP, NOR	256	232	512	512	512	512	512	128	512	>512	512	>512	wild type	P105T							
PE64	21	PIP, TIC, CAZ, FEP, ATM, IPM, MEM, GM, NN, AN, CIP, NOR	256	512	>512	>512	>512	>512	>512	512	>512	32	<i>bla</i> _{IMP-18} , <i>bla</i> _{OXA-2} , <i>bla</i> _{OXA-50}		T831	S87L							
PE73	26	PIP, TIC, CAZ, FEP, ATM, IPM, MEM, GM, NN, AN, CIP, NOR	>512	>512	>512	>512	>512	>512	>512	>512	>512	32	<i>bla</i> _{IMP-56} , <i>bla</i> _{OXA-2} , <i>bla</i> _{OXA-50}		T831	S87L							
PE83	13	TIC, CAZ, FEP, IPM, MEM, GM, NN, AN, CIP, NOR	>512	>512	>512	64	64	256	128	>512	>512	>512	>512	>512	T831	S87L							

Notes: Minimum Inhibitory Concentration; ^bPAβN (L-phenylalanine-arginine-β-naphthylamide).

Abbreviations: PFGE, pulsed-field gel electrophoresis; PIP, piperacillin; TIC, ticarcillin; TZR, tazobactam; CAZ, ceftazidime; FEP, cefepime; IMP, imipenem; MER, meropenem; GM, gentamicin; NN, tobramycin; AN, amikacin; CIP, ciprofloxacin; NOR, norfloxacin; ATM, aztreonam.

+ *aacA4*+ *aadA1*+ *bla*_{OXA-2} (Figure 1). These strains were unrelated according to PFGE and they were obtained from different patients. By analyzing the variable regions of class I integrons, we identified some other resistance genes in these strains, such as *aacA33*, *aadA1*, *aadA6* and *aacA7*.

Modifications and expression of porin OprD

The *oprD* gene was analyzed in the 17 *bla*_{IMP}-carrying strains (Table 2). In two strains the *oprD* gene was not amplified and by immunoblot the protein could not be detected. Four strains showed deletions of two amino acids: 378 and 379 (called loop L7-short), which generated an OprD protein of 441 amino acids, and the protein expression was confirmed by immunoblot, showing a positive signal of approximately 48 kDa. The OprD porin was not detected by immunoblot in nine strains with premature stop codon (amino acid 295, 417, 422 and 426), and in the PE20 strain with an insertion sequence (IS1394) in the amino acid 232. The PE63 strain showed the *oprD* gene wild type and the expression of protein was detected (Table 2, Figure 2).

Influence of efflux pumps inhibitor on MIC to meropenem and ciprofloxacin

In order to investigate if RND-superfamily efflux pumps were involved in resistance to meropenem and ciprofloxacin, strains carrying *bla*_{IMP} were exposed with EPI. In the strains PE09 and PE46 there was a decrease in meropenem MIC value ≥ 16 -fold (≥ 512 – 32 $\mu\text{g/mL}$), in the strain PE20 there

was a 4-fold decrease (>512 – 128 $\mu\text{g/mL}$) and in the strain PE64 there was a decrease of ≥ 2 fold (>512 – 512 $\mu\text{g/mL}$). In the other strains, no change was detected in meropenem MIC value by EPI presence. In the 17 strains, the ciprofloxacin MIC value without EPI (32 to >512 $\mu\text{g/mL}$) showed a decrease in the order of ≥ 2 fold (16– 512 $\mu\text{g/mL}$) (Table 1). In the case of the strains with ciprofloxacin or meropenem MIC values of >512 $\mu\text{g/mL}$ and that decreased to 512 $\mu\text{g/mL}$ in presence of EPI, additional experiments were performed using concentrations of 1024 $\mu\text{g/mL}$ of the antibiotic to corroborate this decrease (data not showed).

Mechanisms of resistance to quinolones

The changes in the QRDR region of the GyrA and ParC proteins were determined. A single amino acid substitution in GyrA protein (Thr-83→Ile) was detected in all the strains. In ParC protein, 15 strains showed the substitution Ser-87→Leu and one strain (PE63) the change Pro-105→Thr. ParC protein in the strain PE52 was wild type. The ciprofloxacin MIC showed different values in the 17 strains: four showed 32 $\mu\text{g/mL}$, three strains 64 $\mu\text{g/mL}$, four strains 128 $\mu\text{g/mL}$ and six strains 256– >512 $\mu\text{g/mL}$ (Table 1).

Partial characterization of plasmids

Eight strains showed plasmids with different size as was determined by PFGE-S1 method. We confirm the presence of plasmids by Quick Prep method, however, in the strain PE63 was not possible to detect any plasmid (Figure S2). The relaxase MOB P11 was detected in the strains PE06, PE21,

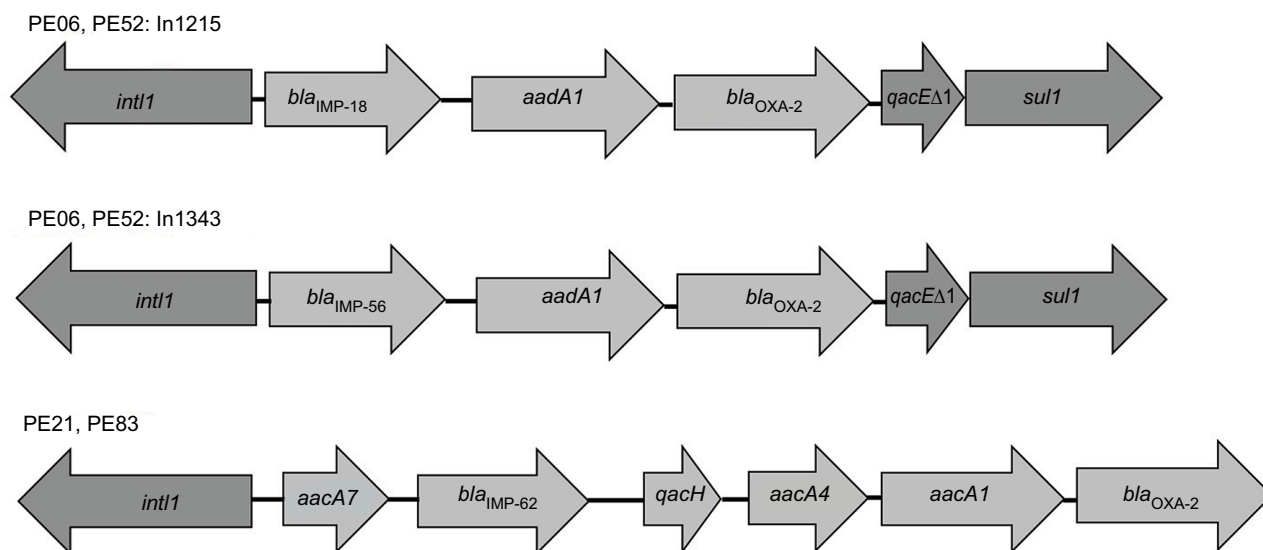


Figure 1 Diversity of class I integron arrangements detected in *bla*_{IMP}-carrying *P. aeruginosa* clinical isolates.

Notes: The In1215 containing *bla*_{IMP-18} with the accession number KY646160 and the In1343 containing *bla*_{IMP-56} with the accession number KY646161. Arrows indicate the open reading frames.

Table 2 Changes in OprD porin in clinical isolates of *P. aeruginosa* carrying *bla*_{IMP}

Strain	^a MIC (µg/mL)		OprD size (Number of amino acids)	Amino acid changes in OprD sequence	Insertion/deletion
	IMP	MEM			
PE06	>512	>512	426	D43N, S57E, S59R, E202Q, I210A, E230K, S240T, N262T, A267S, A281G, K296Q, Q301E, R310G, V359L, M372V, S373D, D374S, N375S, N376S, V377S, Δ378, Δ379, K380Y, N381A, Y382G, G383L E426STOP	Stop codon
PE09	64	>512	441	D43N, S57E, S59R, E202Q, I210A, E230K, S240T, A267S, K296Q, Q301E, R310G, V359L, M372V, S373D, D374S, N375S, N376S, V377S, Δ378, Δ379, K380Y, N381A, Y382G, G383L	Loop L7-short
PE10	64	64	441	D43N, S57E, S59R, E202Q, I210A, E230K, S240T, N262T, A267S, A281G, K296Q, Q301E, R310G, V359L, M372V, S373D, D374S, N375S, N376S, V377S, Δ378, Δ379, K380Y, N381A, Y382G, G383L	Loop L7-short
PE15	>512	64	^b NA	^b NA	^b NA
PE16	>512	64	422	D43N, S57E, S59R, E202Q, I210A, E230K, S240T, N262T, A267S, A281G, K296Q, Q301E, R310G, V359L, M372V, S373D, D374S, N375S, N376S, V377S, Δ378, Δ379, K380Y, N381A, Y382G, G383L A422STOP	Stop codon
PE20	>512	>512	232	D43N, S57E, S59R, E202Q, I210A, E230K, I232M, Y233A, R234A, IS1394	Truncated by IS1394
PE21	128	>512	417	D43N, S57E, S59R, E202Q, I210A, E230K, S240T, N262T, A267S A281G, K296Q, Q301E R310G V359L, M372V, S373D, D374S, N375S, N376S, V377S, Δ378, Δ379, K380Y, N381A, Y382G, G383L W417STOP	Stop codon
PE22	64	16	441	D43N, S57E, S59R, E202Q, I210A, L224F, E230K, S240T, N262T, A267S A281G, K296Q, Q301E, R310G, V359L, M372V, S373D, D374S, N375S, N376S, V377S, Δ378, Δ379, K380Y, N381A, Y382G, G383L	Loop L7-short
PE23	64	32	417	D43N, S57E, S59R, E202Q, I210A, E230K, S240T, N262T, A267S A281G, K296Q, Q301E, L307M, R310G, A316G, M372V, S373D, D374S, N375S, N376S, V377S, Δ378, Δ379, K380Y, N381A, Y382G, G383L W417STOP	Stop codon
PE27	>512	64	^b NA	D43N, S57E, S59R, E202Q, I210A, E230K, S240T, N262T, A267S	^b NA
PE46	64	>512	441	D43N, S57E, S59R, E202Q, I210A, E230K, S240T, N262T, A267S, W277R, A281G, A282G, Y283S, T284G, K296Q, Q301E, R310G, V359L, M372V, S373D, D374S, N375S, N376S, V377S, Δ378, Δ379, K380Y, N381A, Y382G, G383L	Loop L7-short
PE52	>512	>512	426	D43N, S57E, S59R, E202Q, I210A, E230K, S240T, N262T, A267S, A281G, K296Q, Q301E, R310G, V359L, M372V, S373D, D374S, N375S, N376S, V377S, Δ378, Δ379, K380Y, N381A, Y382G, G383L, E426STOP	Stop codon
PE62	>512	>512	426	D43N, S57E, S59R, E202Q, I210A, E230K, S240T, N262T, A267S, A280G, A281G, Y283S, T284A, K296Q, Q301E, R310G, V359L, M372V, S373D, D374S, N375S, N376S, V377S, Δ378, Δ379 K380Y, N381A, Y382G, G383L, E426STOP	Stop codon
PE63	512	512	443	Wild type	-
PE64	>512	>512	295	D43N, S57E, S59R E202Q, I210A, E230K, S240T, N262T, A267S, A281G, K295STOP	Stop codon
PE73	>512	>512	426	D43N, S57E, S59R, E202Q, I210A, E230K, S240T, N262T, A267S, A281G, K296Q, Q301E, R310G, V359L, M372V, S373D, D374S, N375S, N376S, V377S, Δ378, Δ379, K380Y, N381A, Y382G, G383L, E426STOP	Stop codon
PE83	64	>512	417	D43N, S57E, S59R, E202Q, I210A, E230K, S240T, N262T, A267S A281G, K296Q, Q301E, R310G, V359L, M372V, S373D, D374S, N375S, N376S, V377S, Δ378, Δ379, K380Y, N381A, Y382G, G383L W417STOP	Stop codon

Notes: ^aMinimum Inhibitory Concentration; ^bNot amplified or partially amplified; IMP, imipenem; MEM, meropenem.

PE52, PE62, PE64, PE73 and PE83, and a second relaxase MOBH2 was amplified in the PE21 and PE83 strains. The relaxase MOBP14 was detected in the strain PE63 (Table 3, Figure 3).

Location of the *bla*_{IMP} gene by southern blot

Results obtained showed that *bla*_{IMP} gene was detected in only four of the eight strains carrying plasmids. The PE06

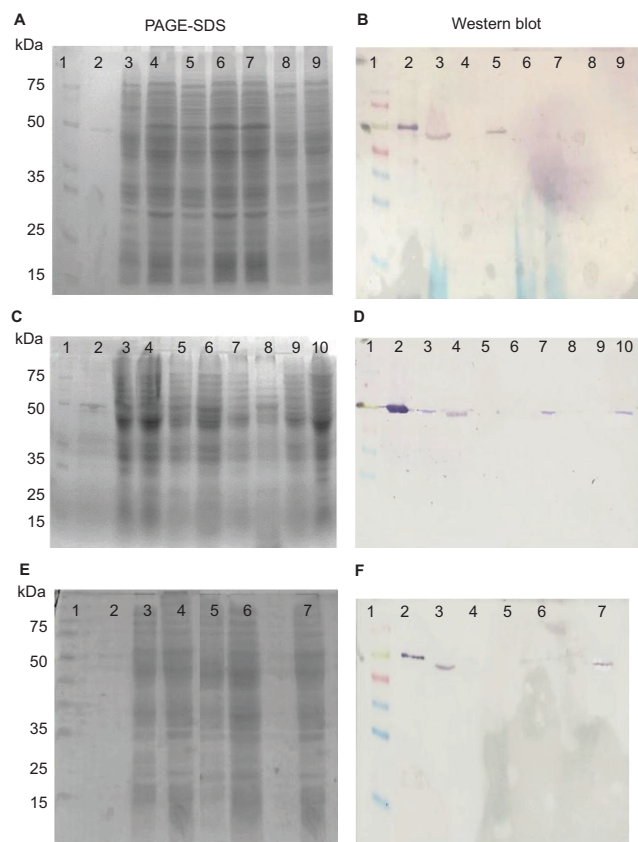


Figure 2 Detection of the OprD protein in 17 clinical *bla_{IMP}*-carrying *P. aeruginosa*. (A, C, E) PAGE-SDS of extract total protein, (B, D, F) Western blot of extract total protein with polyclonal antisera anti-oprD.

Notes: For each lane, 30 µg of proteins were loaded. *P. aeruginosa* PAO1 and OprD protein recombinant were used as positive controls. (A, B) Line 1: Molecular weight marker (Spectra™); Line 2: recombinant OprD protein; Line 3: *P. aeruginosa* PAO1; Line 4: PE06 (stop codon); Line 5: PE09 (L7-short); Line 6: PE15 (not amplified); Line 7: PE16 (stop codon); Line 8: PE20 (IS1394); Line 9: PE21 (stop codon). (C, D) Line 1: Size marker; Line 2: recombinant OprD protein; Line 3: *P. aeruginosa* PAO1; Line 4: PE22 (L7-short); Line 5: PE23 (stop codon); Line 6: PE27 (partially amplified); Line 7: PE46 (L7-short); Line 8: PE52 (stop codon); Line 9: PE62 (stop codon); Line 10: PE63 (wild type). (E, F) Line 1: Size marker; Line 2: recombinant OprD protein; Line 3: *P. aeruginosa* PAO1; Line 4: PE64 (stop codon); Line 5: PE73 (stop codon); Line 6: PE83 (stop codon) and Line 7: PE10 (L7-short). The experiment was made in duplicate.

and PE52 strains, that carried two *bla_{IMP}* variants (*bla_{IMP-18}* and *bla_{IMP-56}*), and the PE62 and PE63 strains that carried *bla_{IMP-18}*, each showed two positive hybridization signals in two plasmids (Figure 4). No hybridization signals were detected in the remaining strains (data not shown)

Discussion

It has been reported that most of the nosocomial infections caused by *P. aeruginosa* most frequently affect patients with pneumonia associated with mechanical ventilation and this is the main cause of chronic respiratory infection in patients with cystic fibrosis, bronchiectasis or COPD.⁴¹ In the present

Table 3 Plasmid characterization of 17 clinical *bla_{IMP}*-carrying *P. aeruginosa* isolates

Strain	β-lactamase gene	Plasmids size (kb)	*Relaxase MOB subfamily
PE06	<i>bla_{IMP-18}</i> , <i>bla_{IMP-56}</i>	2(approx 224, approx 188)	MOB _{P11}
PE09	<i>bla_{IMP-18}</i>	Absent	Absent
PE10	<i>bla_{IMP-18}</i>	Absent	Absent
PE15	<i>bla_{IMP-18}</i>	Absent	Absent
PE16	<i>bla_{IMP-18}</i>	Absent	Absent
PE20	<i>bla_{IMP-18}</i>	Absent	Absent
PE21	<i>bla_{IMP-62}</i> , <i>bla_{GES-32}</i>	1 (approx 368)	MOB _{P11} MOB _{H2}
PE22	<i>bla_{IMP-18}</i>	Absent	Absent
PE23	<i>bla_{IMP-62}</i> , <i>bla_{GES-2}</i>	Absent	Absent
PE27	<i>bla_{IMP-62}</i>	Absent	Absent
PE46	<i>bla_{IMP-18}</i>	Absent	Absent
PE52	<i>bla_{IMP-18}</i> , <i>bla_{IMP-56}</i>	2(approx 242, approx 194)	MOB _{P11}
PE62	<i>bla_{IMP-18}</i>	2(approx 248, approx 200)	MOB _{P11}
PE63	<i>bla_{IMP-18}</i> , <i>bla_{GES-1}</i>	3(approx 230, approx 200, approx 182)	MOB _{P14}
PE64	<i>bla_{IMP-18}</i>	1 (approx 146)	MOB _{P11}
PE73	<i>bla_{IMP-56}</i>	1 (approx 266)	MOB _{P11}
PE83	<i>bla_{IMP-62}</i> , <i>bla_{GES-26}</i>	1 (approx 350)	MOB _{P11} MOB _{H2}

Notes: *Detection of relaxases was performed by Degenerate Primer MOB Typing (DPMT).¹⁷

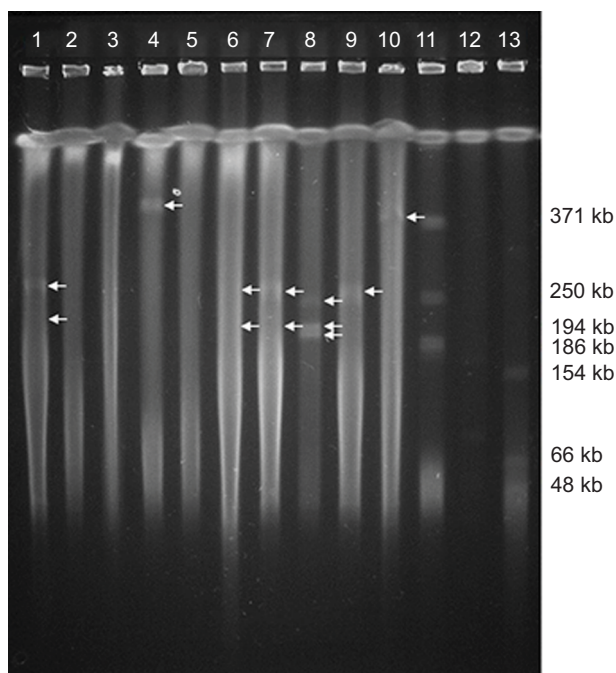


Figure 3 PFGE-SI plasmids of 10 clinical isolates of *P. aeruginosa* carrying *bla_{IMP}*. **Notes:** The arrows indicate the plasmids. Lane 1: PE06, Lane 2: PE09, Lane 3: PE20, Lane 4: PE21, Lane 5: PE46, Lane 6: PE52, Lane 7: PE62, Lane 8: PE63, Lane 9: PE73, Lane 10: PE83, Lane 11: Control strain *Rhizobium etli* CFN42 Δe, Lane 12: Negative control PAO1, Lane 13: Control strain *E. coli* NCTC 50192 with four plasmids: 154 kb, 66 kb, 48 kb and 7 kb. The experiment was performed twice. **Abbreviation:** PFGE-SI, Pulsed-field gel electrophoresis with nuclease S1.

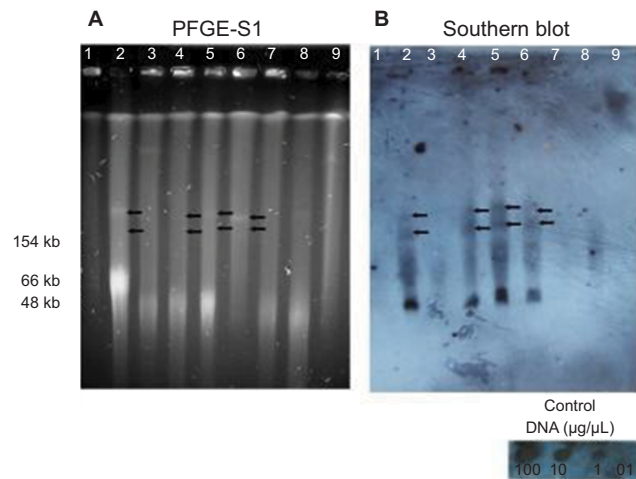


Figure 4 Detection of *bla*_{IMP} in plasmids of eight *P. aeruginosa* strains by Southern blot.

Notes: (A) PFGE after digestion with nuclease S1 (180 U/µL, Takara Bio Inc.), (B) Southern hybridization of plasmids (DIG HIGH Prime DNA Labeling and Detection Starter Kit II) using *bla*_{IMP} as a probe. Line 1: Control strain *E. coli* NCTC 50192 with four plasmids: 154 Kb, 66 Kb, 48 Kb and 7 Kb, Line 2: PE06; Line 3: PE21; Line 4: PE52; Line 5: PE62; Line 6: PE63; Line 7: PE64; Line 8: PE73 and Line 9: PE83. The black arrows indicate the positive signal corresponding to the hybridization with *bla*_{IMP} in plasmids. The experiment was done three times.

Abbreviation: PFGE-S1, Pulsed-field gel electrophoresis with nuclease S1.

study, isolates were obtained from bronchial secretions (36%). On the other hand, in the last few years an increase of strains with carbapenems' resistance has been reported all over the world.^{3,25} In this study we detected that 69% of the strains were resistant to carbapenems, and resistant to nine or more of the 13 antibiotics tested, indicating the dissemination of MDR and CR strains, and an emerging problem in this hospital.

Of 41 MDR-CR strains, 24 were harboring β-lactamase with carbapenemase activity; six strains were carrying *bla*_{GES-2*}, one strain had *bla*_{GES-20} and 17 strains were carrying different variants of *bla*_{IMP}. Twelve strains were carrying *bla*_{IMP-18*}, which is one of the most frequent variants detected in Mexico,^{9,11} USA⁴² and Latin America.⁸ Two new variants, *bla*_{IMP-56} and *bla*_{IMP-62} were detected in seven strains, alone or with *bla*_{IMP-18*}, these new variants derived from *bla*_{IMP-18} and *bla*_{IMP-15*}, respectively and showed the same change (S214G) in the amino acid sequence. Coexistence of *bla*_{IMP} and *bla*_{GES} genes in the same strain has not been reported in Mexico, and there is only one report of *P. aeruginosa* carrying *bla*_{GES-5} and *bla*_{VIM}.⁴³ In this study we found a new variant *bla*_{GES-32}, this variant had 96% of similarity with *bla*_{GES-1}, *bla*_{GES-5*}, *bla*_{GES-6} and *bla*_{GES-7*}.

On the other hand, 17 MDR-RC strains did not carry any β-lactamase genes related with carbapenems' resistance.

Previous studies have reported the absence of these genes in CR isolates, suggesting the existence of other mechanisms responsible for the resistance.^{19,25}

Integrations are ubiquitous platforms of great importance that participate in the capture of resistance genes, which are carried by mobile elements, plasmids, and transposons. These elements allow bacteria to acquire the capacity to adapt to environmental changes. Class 1 integrations are the most frequently reported in *P. aeruginosa* related to resistance to β-lactams and aminoglycosides.⁴⁴ In this study, the PE06 and PE52 strains harbored two new class 1 integrations: *In1245* and *In1343*, and the strains PE21 and PE83 carried an integration with the identical partial arrangement in the variable region: *aacA7+* *bla*_{IMP-62}+*qacH*+*aacA4+* *aadA1*+*bla*_{OXA-2}. These results suggest the existence of a dissemination of integrations in unrelated strains. Other strains with *bla*_{IMP} were found in class 1 integrations, but the genetic array in the variable region could not be determined.

Three main high-risk clones ST111, ST175 and ST235 have been reported.⁴⁵ In our study, strain PE21 corresponds to a ST167; when performing the analysis of eBURST_{V3}, we find that it derives from group O where high-risk ST111 is found, but they only share two alleles, so we think that they are not related. The other two, ST308 and ST385, were not found to be related to the high risk clones reported. In the case of high risk clones, only ST235 has been reported in Mexico.⁴⁵ It is important to note that our strains carry resistance mechanisms different from those reported in high risk clones.¹⁹ The ST found in this study has already been reported in different parts of the world⁴⁵ and the ST308 in Mexico.⁴⁶ So this is the first report that shows the presence of ST167 and ST385 in Mexico.

Since this study was focused on the strains carrying *bla*_{IMP} and they showed different levels of resistance to carbapenems, it was interesting to analyze other mechanisms that could be contributing to the resistance such as mutations of the *oprD* gene and its expression, and the participation of the efflux pumps.

Four groups were formed according to MIC values for carbapenems. In the first group, eight strains were grouped with high MIC values for imipenem and meropenem (128 to >512 µg/mL), in all of them, we detected one or two carbapenemases and they presented a premature stop codon or SI in the *oprD* gene. Previous studies have shown that strains with a premature stop codon in the middle of the protein OprD sequence exhibited a loss in protein expression^{19,47}; in the analyzed strains, the premature stop codon was located at the

end of the sequence, however, the protein was not expressed. The insertion sequence (IS1394) found in the PE20 strain, correspond to a 1,100 bp sequence, coding for a transposase. The first report of an inactivation of OprD porin in clinical isolates by insertion sequences that lead to carbapenems resistance in *P. aeruginosa* occurred in 2004.³⁶ At present there are several reports that show the inactivation of OprD porin by insertion sequences.^{18,19,48} In regard to IS1394 inserted into *oprD* gene in *P. aeruginosa*, the first report appears in Puerto Rico.⁴⁹ It is important to mention that this is the first report that describes the presence of IS1394 in a strain isolated in Mexico. We suggest that the OprD porin, and the presence of MBL contributed synergistically to the acquisition of a higher level of MIC to carbapenems, except for the PE63 strain, which showed a wild type porin. Another mechanism that may be contributing to the bacteria resistance is the efflux pumps, as is suggested by the results observed in the strains PE20 and PE64, which showed a decrease of CMI for MEM in presence of EPI, as has been reported in other studies.²⁶ These results also suggest that other resistance mechanisms, eg, the role of AmpC,⁵⁰ could be involved.

The second group included three strains (PE15, PE16 and PE27), these strains showed high MIC values for imipenem (>512 µg/mL) and low levels for meropenem (64 µg/mL). These strains exhibited a premature stop codon and did not express OprD porin. In these strains we did not observe the contribution of efflux pumps suggesting that another mechanism might participate in carbapenem resistance, as has been reported in other works.^{1,2}

The third group with three strains, showed low MIC values for IMP (64 µg/mL) and high values for meropenem (>512 µg/mL). Two of these strains (PE09, PE46) showed a Loop L7-short; therefore, the porin OprD probably is not participating in the carbapenems' resistance. It is important to highlight that the strains under study showed a high polymorphism in the *oprD* gene, which may be involved in the variable susceptibility presented.¹⁹ These strains in the presence of EPI, showed a decrease of MIC value for meropenem, suggesting that efflux pump is participating in the meropenem resistance. In PE83 strain, a premature stop codon was detected, and the porin was not expressed, suggesting that the OprD porin and the two new *bla*_{IMP-62} and *bla*_{GES-26} variants detected in this isolate, could be related in the resistance to carbapenems.

The fourth group involved three strains with low MIC values for IMP (64 µg/mL) and low values for MEM (16–64 µg/mL). Imipenem resistance in PE10 y PE22 strains is probably due only to the presence of *bla*_{IMP-18}. These strains showed

a Loop L7-short, so that we suggested that porin is perhaps involved with the low resistance to meropenem.⁵¹ Results obtained in the PE23 strain suggest that the presence of the two carbapenemases, and the porin *oprD* are participating in carbapenems' resistance, because EPI did not modify MIC values for meropenem.

The presence of aminoglycoside-modifying enzymes was detected in 14 strains, all of them showed high MIC values for aminoglycosides (gentamicin and amikacin). Therefore, there is no doubt that in addition to the participation of these enzymes, there are other aminoglycoside resistance mechanisms involved.⁵²

The mutations detected in *gyrA* and *parC* genes are not enough to explain the high ciprofloxacin MIC values shown by 10 strains (128 to >512 µg/mL), therefore, it was necessary to identify other mechanisms involved. The 17 strains showed a decrease of ciprofloxacin MIC value in the presence of EPI, suggesting that efflux pumps also are participating in the resistance to ciprofloxacin since it has been reported that PAβN is a broad-spectrum efflux pump inhibitor (EPI), which is capable of significantly reducing fluoroquinolone resistance in *P. aeruginosa*.⁵³

In eight strains carrying *bla*_{IMP} we detected 1–3 plasmids of different sizes, but only the *bla*_{IMP} gene was detected in two plasmids of four strains. Strain PE06 and PE52 (*bla*_{IMP-18} and *bla*_{IMP-56}) have a relaxase MOB_{P11}, and this relaxase has been found in conjugative plasmids of a broad host range belonging to the complex IncP1.

On the other hand, *bla*_{IMP-18} was detected in PE62 and PE63 strains, in two plasmids in each strain. It is important to mention that *bla*_{IMP-18} has not been reported in plasmids,^{8,9,11} therefore, in Mexico this is the first report of plasmids that carry *bla*_{IMP-18}. In the PE62 strain the relaxase MOB_{P11} was detected and in the strain PE63 the relaxase MOB_{P14} was detected, the latter one is present in mobilizable plasmids with a broad range belonging to the incompatibility groups IncP6 and IncQ2.^{17,54}

It has been reported that IncP1 in *Pseudomonas* corresponds to the IncP of *E. coli*, a complex formed by six subgroups (α, β, γ, ε, δ and ζ), with the difference between them the protein TrfA, a protein that initiates the replication of plasmids (determined by phylogenetic studies and amino acid sequencing).⁵⁵ In these strains we performed a search for IncP1, but it was not possible to amplify them. Probably other types of Inc belonging to the IncP complex could be present.⁵⁶

Results obtained in this work suggest the existence of horizontal gene transference between bacteria within this

hospital. It is important to underline that the knowledge of the mechanisms, mainly of carbapenems' resistance in clinical isolates of *P. aeruginosa* will help to decide the best therapeutic strategy. This information is of great value in taking measures to avoid the dissemination of emergent mechanisms of resistance in the hospital setting.

Conclusion

This study highlights the participation of various resistance mechanisms that are reflected in the levels of MIC to carbapenems shown by the studied strains. This is the first report in Mexico that describes the presence of three new variants of β -lactamases (bla_{GES-32} , bla_{IMP-56} , bla_{IMP-62}) inside of new arrangements of class 1 integrons and the presence of two plasmids carrying bla_{IMP} in the same strain isolated in a Mexican hospital. Non-expression of the OprD protein is shown when it presents premature stop codons at the end of the sequence.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

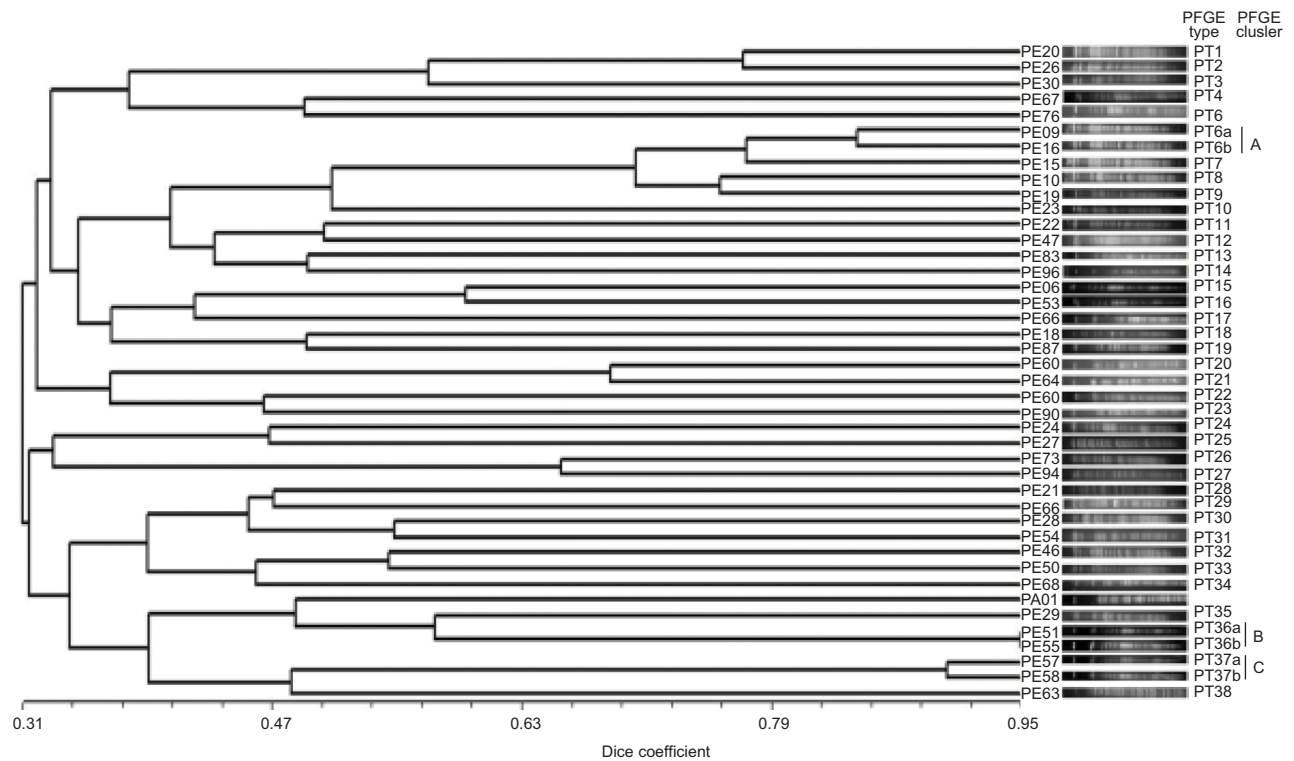


Figure S1 Dendrogram of 41 multidrug-resistant *P. aeruginosa* clinical isolates obtained in ISSSTE Regional Hospital of Puebla, Mexico.
Notes: The isolates showing a Dice coefficient $\geq 80\%$ were considered as genetically related for this study. PFGE type (PT), PFGE clusters (A, B and C).
Abbreviation: PFGE, pulsed-field gel electrophoresis.

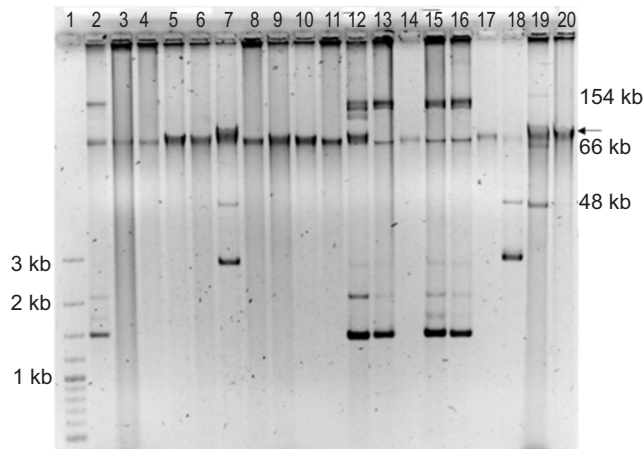


Figure S2 Plasmid extraction by the Quick Prep method of 17 clinical *bla*_{IMP}-carrying *P. aeruginosa* isolates.
Notes: The position of chromosomal DNA is indicated by an arrow at the right.
 Line 1: Size marker; Line 2: PE06; Line 3: PE09; Line 4: PE15; Line 5: PE16; Line 6: PE20; Line 7: PE21; Line 8: PE22; Line 9: PE23; Line 10: PE27; Line 11: PE46; Line 12: PE52; Line 13: PE62; Line 14: PE63; Line 15: PE64; Line 16: PE73; Line 17: PE10; Line 18: PE83; Line 19: Control *E. coli* NCTC 50192; Line 20: *P. aeruginosa* PAO1.

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