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ORIGINAL RESEARCH

bla$_{IMP-45}$ Amplification Facilitates Heterogeneous Resistance to Imipenem in *Pseudomonas aeruginosa*

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Objective: To investigate the contribution of *bla*$_{IMP-45}$ and its plasmid-borne genetic context in the development of imipenem heteroresistance in *Pseudomonas aeruginosa.*

Methods: Six clinical isolates of *P. aeruginosa* (HN41, HN66, HN67, HN125, HN148, and HN232) were analyzed. Broth microdilution confirmed imipenem(IMP) resistance in all the strains (MIC ≥8 mg/L). Whole-genome sequencing was performed to identify the presence of *bla*$_{IMP-45}$. E-test strips were used to indicate suspected heteroresistance phenotypes, while Population Analysis Profile (PAP) assays were conducted to definitively classify the strains. Complete plasmid sequencing of HN232 (IMP-HR) and HN41 (IMP-NHR) was performed to identify the genetic context of *bla*$_{IMP-45}$. Pre-exposure of IMP-HR strain HN232 to sub-inhibitory IMP (1, 4, and 32 mg/L) for 24h was conducted to assess regrowth frequency. Quantitative reverse-transcription PCR (qRT-PCR) and digital PCR (ddPCR) were used to measure *bla*$_{IMP-45}$ expression and gene copy number.

Results: Whole-genome sequencing revealed the absence of *bla*$_{IMP-45}$ in the HN67 and HN125 strains. E-test strips indicated suspected heteroresistance phenotypes, while Population Analysis Profile (PAP) assays definitively classified HN66, HN148, and HN232 as heteroresistant (heteroresistant subpopulation inhibitory concentration, HIC/HNIC ratio ≥8), with their maximum permissive growth concentrations (256 mg/L) 32-fold higher than non-heteroresistant strains HN67 and HN125 (8 mg/L), implicating *bla*$_{IMP-45}$ in driving phenotypic heterogeneity. Complete plasmid sequencing of HN232 (IMP-HR) and HN41 (IMP-NHR) identified *bla*$_{IMP-45}$ within the In786 integron, embedded in the Tn*7445* transposon (a Tn*1403* derivative) on IncP-2 type plasmids. Notably, the *bla*$_{IMP-45}$-flanking genetic environment was conserved between HR and NHR strains, excluding promoter region variations (eg, PcH2 and P2 promoters within In786) as drivers of differential expression. Pre-exposure of IMP-HR strain HN232 to sub-inhibitory IMP (1, 4, and 32 mg/L) for 24h induced dose-dependent increases in regrowth frequency at 4 and 8 mg/L IMP. Quantitative reverse-transcription PCR (qRT-PCR) and digital PCR (ddPCR) demonstrated that low-dose IMP exposure (1 mg/L, 24h) upregulated *bla*$_{IMP-45}$ expression and increased the gene copy number in HN232, whereas the NHR strain HN41 exhibited stringent plasmid regulation.

Conclusion: The study provided critical insights into *bla*$_{IMP-45}$-mediated IMP heteroresistance in *P. aeruginosa*, highlighting plasmid-encoded amplifiable resistance determinants as key modulators of phenotypic adaptability under antibiotic pressure.

Keywords: heteroresistance, carbapenem-resistant *Pseudomonas aeruginosa*, *bla*$_{IMP-45}$, IncP-2 plasmid

Introduction

Pseudomonas aeruginosa is ubiquitous in nature and a well-known opportunistic pathogen in hospitalized immunocompromised patients. *P. aeruginosa* can infect a range of systems and tissues, causing various types of inflammation and infections such as bacteremia, septicemia, septic pyemia, pneumonia, bronchitis, diarrhea, keratitis, and skin and wound infections.[1,2] Multidrug-resistant *P. aeruginosa* poses a serious clinical threat, and the development of carbapenem resistance is expected to exacerbate this situation.[3] Carbapenem-resistant *P. aeruginosa* (CRPA) has been categorized as a high-priority threat by the World Health Organization's updated bacterial priority pathogen List (BPPL) 2024.[4]

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Resistance to carbapenems in *P. aeruginosa* is generally due to a combination of mechanisms, including deficiency of the OprD porin, overexpression of efflux pumps, intrinsic chromosomally encoded AmpC β-lactamase, and production of carbapenemase.[2,5,6] The production, activity, transferability, and prevalence of metallo-β-lactamases (MBLs), particularly members of imipenem-type and Verona integron-encoded MBL families, are the most clinical significance.[7] A *P. aeruginosa* strain carrying the MBL-coding gene $bla_{\text{IMP-45}}$, which was first reported in 2014, was isolated from a canine in Beijing.[8] Recently, 23 $bla_{\text{IMP-45}}$-carrying conjugative IncP-2 megaplasmids, carried by *Pseudomonas spp*. from diverse genetic backgrounds, have been reported worldwide.[8–19] Among them, *P. aeruginosa* clone ST508 caused an outbreak of nosocomial infections at Hospital HS (Shanghai, China) from January to September 2015.[12]

Heteroresistance, which can generally be defined as the presence of subpopulations of cells within a culture of a single bacterial isolate with higher levels of antibiotic resistance, is a phenomenon that causes unreliability in antimicrobial susceptibility testing.[20] It is widely accepted that heterogeneous resistance arises from the unstable amplification of resistance genes, likely involving complex interactions between mobile genetic elements, including plasmids, resistance gene cassettes, transposons, and insertion sequences. Heteroresistance to carbapenem has been well-documented in current clinical isolates of *Escherichia coli, Klebsiella pneumoniae* and *Acinetobacter baumannii*.[21–23] Few studies have investigated the mechanisms of carbapenem-heteroresistant *P. aeruginosa*, which have shown the involvement of reduced OprD porin expression, efflux pump system overexpression, and biofilm formation.[24] The mechanisms by which MBLs and MBL-harboring plasmids mediate carbapenem-resistant *P. aeruginosa* remain unclear.

This study pioneers the exploration of imipenem resistance mechanisms in *P. aeruginosa* mediated by MBLs-encoding genes and their plasmid-borne transmission pathways.Heterogeneous resistant strains carrying the $bla_{\text{IMP-45}}$ gene showed a 32-fold increase in the maximum permissible growth concentration of 256 compared to non-heterogeneous resistant strains not carrying $bla_{\text{IMP-45}}$. The maximum allowable growth concentration of 256 was 32-fold higher in the former than in the latter and 2-fold higher in the latter than in the heterogeneously resistant strain carrying $bla_{\text{IMP-45}}$. Plasmid sequencing analysis showed that the genetic environment of $bla_{\text{IMP-45}}$ was consistent, and there was no variation in the promoter region. Pre-exposure of the strains to a low concentration of imipenem for 24h caused an increase in the expression and copy number of $bla_{\text{IMP-45}}$ in the heterogeneous resistant strains HN232 and HN41, except that the increase was more pronounced in the former.

Material and Methods

Bacteria Strains and Identification

The following non-duplicate clinical *P. aeruginosa* isolates were obtained from sputum or wound secretion samples collected mainly from two different surgical wards at the People's Hospital of Sanya City, Hainan Province, between 2013 and 2014: HN41, HN66, HN67, HN125, HN148, and HN232. Bacterial species were initially identified using matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF; Bruker,Germany). The criterion for strain inclusion was (IMP) readings of 1–3 mg/L (described below).

Genome Sequencing, Resistance Genes, Species Confirmation and MLST

Genomic DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) and sequenced on an advanced Illumina NovaSeq 6000 platform.A double-ended 150 bp (PE150) strategy was adopted. Average sequencing depth of andgt;100 × per sample, data volume of ≥6 GB, QC requirements: Q30 >85% (base-balanced library), and compliance with Illumina standard QRP39. Raw data was QC controlled by FastQC v0.12.1 and low-quality reads (Phred score andlt;20) and adapter sequences were filtered using Trimmomatic v0.39. The resistance gene profiles of the strains were analyzed using ResFinder (http://genepi.food.dtu.dk/resfinder) and multilocus sequence typing (MLST) was performed using the tool available at https://cge.food.dtu.dk/services/MLST/. Species identification was confirmed using SpeciesFinder at the Center for Genomic Epidemiology (http://genomicepidemiology.org/services/).

Antimicrobial Susceptibility Testing and Screening of HR Strains

Antimicrobial susceptibility testing was performed using the broth microdilution method with customized microtiter plates containing vacuum-dried antibiotics (BD,Phoenix NMIC-413, USA). The MIC values were interpreted according to the 2024 Clinical and Laboratory Standards Institute (CLSI) guidelines.[25] *E. coli* strain ATCC 25922 was used as a quality control. Initial screening of HR strains was conducted using E-test strips (BioMérieux, France). Suspicion of heterogeneous resistance was based on the presence of resistant clones inside the zone of inhibition or unclear boundaries.

Population Analysis Profile (PAP) Test

Bacterial suspensions containing 10^{-8}–10^{-4} colony-forming units (CFU)/mL were prepared from a 0.5 McFarland standard (~ 0.1×10^{8} CFU/mL). A 100-μL aliquot of each bacterial suspension was spotted in triplicate onto fresh gradient-diluted Mueller-Hinton agar (MHA) plates containing IMP at the following concentrations: 0, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, and 1024 mg/L. The plates were incubated at 37°C for 24h and the number of CFUs at each concentration was used to draw the PAP curves. The analysis was performed in triplicate with carbapenem-susceptible *P. aeruginosa* ATCC27853 as the control strain.[26]

Measurement of HN232 Resistance Frequency Under IMP Pre-Exposure

To investigate the effect of different IMP pre-exposure concentrations on the frequency of resistant clones, five different pre-exposure concentrations of IMP (0.5, 1, 4, 32, and 128 mg/L) were added to bacterial suspensions of HN232 activated for growth in Mueller-Hinton broth (MHB) and incubated for 24h at 37°C. Pre-exposure to 0 mg/L IMP served as a negative control. A 100 μL aliquot of each bacterial suspension was spotted in triplicate onto fresh gradient-diluted Mueller-Hinton agar (MHA) plates containing IMP at the following concentrations: 0, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, and 512 mg/L. The plates were incubated at 37°C for 24h and count the number of CFUs at each concentration (Figure 1). The analysis was performed in triplicate. The percentage of bacterial regrowth to IMP was calculated by dividing the number of CFUs on the IMP-containing MHA plates by the number of CFUs on the MHA plates without IMP.[27]

Determination of the bla_{IMP-45} Genetic Environment in Plasmids HN232 and HN41

The complete genomes of HN232 and HN41 were sequenced by combining the data obtained from a whole-genome shotgun strategy using the Ion Torrent Personal Genome Machine system (Life Technologies, USA) and

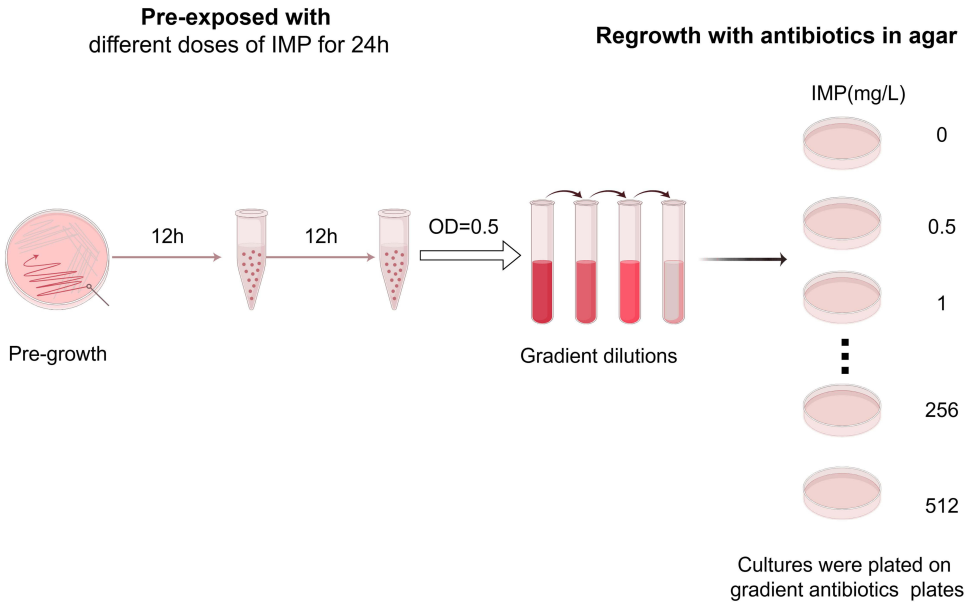

Figure 1 Pre-exposed with IMP to measure HN232 resistance frequency.

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Pacific Biosciences RSII DNA sequencing platform (PacBio, USA). The contigs were assembled using the Hierarchical Genome Assembly Process version 3.0. Sequence annotation, open reading frame prediction, and pseudogene identification of plasmids were performed using RAST 2.0 (https://rast.nmpdr.org/). Annotation of resistance genes, mobile elements, and other features was carried out using the following online databases: ResFinder (http://genepi.food.dtu.dk/resfinder), ISfinder (https://www-is.biotoul.fr/index.php), INTEGRALL (http://integrall.bio.ua.pt/?) and the Tn Number Registry (http://www.ucl.ac.uk/eastman/research/departments/microbial-diseases/tn). Gene organization diagrams were constructed using Inkscape 0.48.1 (http://inkscape.org).

qRT-PCR Determination of Gene Expression

To study the effect of local amplification of plasmid-derived $bla_{\text{IMP-45}}$ on the development of heterogeneous drug resistance, the IMP-HR strains HN232 and HN41 were treated with different pre-exposure concentrations of IMP. qRT-PCR was used to examine the expression of $bla_{\text{IMP-45}}$, replication initiation protein A (*repA*), and the chromosomal housekeeping gene *ecfX*. The mRNA levels of $bla_{\text{IMP-45}}$ and *repA* were expressed as fold-increases relative to that of *ecfX*, which served as the control.

For each strain, three independent cultures were exposed to a gradient of six IMP concentrations. After a 24h pre-exposure, the cultures were grown in MHB. The bacteria were collected and total RNA was extracted using an RNeasy R Mini Kit (Qiagen, Germany). The RNA sample concentrations were measured using a NanoDrop ND-1000 spectrophotometer. Reverse transcription of RNA was performed using the Prime Script RT Reagent Kit (Takara Bio, Japan) in accordance with the manufacturer's instructions. The incubation conditions for cDNA synthesis were 37°C for 15min, 85°C for 5min, and 4°C for 5min. Primers for the amplification of $bla_{\text{IMP-45}}$, plasmid-derived *repA* and *ecfX* were designed using the online tool (https://sg.idtdna.com/. All real-time PCRs were performed using a Bioer Quant Gene 9600 device. Each qRT-PCR reaction contained 5 μL 2 × SYBR Green (TIANGEN, China), 0.5 μL forward and reverse primers, 1 μL cDNA, and 3 μL RNase-free ddH_2O (Takara Bio, Japan). The cycling parameters were as follows: 95°C for 20s, followed by 40 cycles of 95°C for 10s, 55°C for 10s, and 72°C for 20s. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. At least three biological and technical replicates were performed for each set of experiments. The primers used are listed in Table S in supplementary material.

ddPCR Determination of Plasmid Copy Number

To study the copy numbers of $bla_{\text{IMP-45}}$ and *repA* in HN232 and HN41, ddPCR assays comprising of three reactions were performed. The reaction system comprised 10 μL of 2×ddPCR Supermix for Probes (no dUTP) (Bio-Rad, USA), 4 μL of template DNA, 0.4 μL of forward and reverse primers, 0.4 μL of probe, and 4.8 μL of ddH_2O, in a final reaction volume of 20 μL. Microdroplets of oil-encapsulated bacteria were generated using an automated microdroplet generator (Bio-Rad). Thermal cycling conditions using a Bio-Rad C1000 Touch™ Thermal Cycler were 95°C for 30s; 40 cycles at 95°C for 30s and 60°C for 30s; 98°C for 10min; and 4°C for 5min. The reaction data were examined using a QX200™ Droplet Analyzer and Bio-Rad Quanta Soft software for Poisson distribution analysis and calculation of absolute copy numbers. At least three biological and technical replicates were performed for each set of experiments. The primers used are listed in Supplementary material in Table S.

Nucleotide Sequence Accession Numbers

The complete sequences of HN41 chromosome, pHN41-MDR, HN232 chromosome, and pHN232-MDR were submitted to GenBank under accession numbers CP173219, CP173220, CP151543, and CP151544, respectively.

Results

Genomic Backgrounds and Antimicrobial Susceptibility Phenotypes and Genotypes of the CRPA Strains

Among the six CRPA isolates, two belonged to the same sequence type ST (ST446), whereas the remaining four had unique STs, namely ST110, ST111, ST2631, and ST693.

All six strains were susceptible to aztreonam and colistin and resistant to cephalosporins, including ceftazidime, cefepime, and others including piperacillin/tazobactam, and meropenem. However, these strains showed varying degrees of sensitivity and resistance to IMP, fluoroquinolones (ciprofloxacin and levofloxacin) and amikacin. Regarding the response to IMP, HN66 and HN67 exhibited sensitivity, HN125 and HN232 showed intermediate susceptibility, and HN41 and HN148 exhibited resistance. All six strains showed MICs of 1–3 mg/L IMP on E-test strip readings (Table 1), with either fuzzy borders or visible colonies scattered within the inhibition zone (Supplementary material: Figure S), and were suspected to be IMP-heterogeneous.

Regarding the antibiotic resistance genotype, strains HN67 and HN125 were negative for $bla_{\text{IMP-45}}$ and positive for the presence of five resistance genes: aminoglycoside-3'-phosphotransferase gene *aph (3')-IIb,* $bla_{\text{OXA-486/395/488/50/485}}$, $bla_{\text{PAO-1}}$, fosfomycin *fosA*, and *catB7*. These five genes were shared by all six isolates (excluding certain single-nucleotide polymorphisms within $bla_{\text{486/395/488/50}}$), suggesting that they were chromosomally located intrinsic resistance genes. Among the four $bla_{\text{IMP-45}}$-positive isolates, HN148 was found to carry a multidrug resistance operon and the following 14 resistance genes: rifampin gene *arr-2*; β-lactam genes $bla_{\text{OXA-485}}$, bla_{PAO}, $bla_{\text{IMP-45}}$ and $bla_{\text{OXA-1}}$; aminoglycoside genes *aph (3')-IIb* and *aac (6')-Ib3*, chloramphenicol genes *catB3* and *catB7*, fosfomycin gene (*fosA*), tetracycline gene cluster *oprJ*-t*mex*CD3, quinolone gene *qnrVC1*, trimethoprim gene *dfrA22*, and sulfonamide gene *sul1*. Compared with HN148, HN232 carries four additional resistance genes: aminoglycoside resistance gene *armA, aph (3')-Ia*, macrolide-specific efflux pump gene *msr(E)*, and macrolide phosphotransferase gene *mph(E)*. Compared to HN232, HN66 and HN41 carried *tet(C)* and ciprofloxacin resistance protein-encoding *crpP*.

Screening and Confirmation of IMP-HR and IMP-NHR *P. aeruginosa Strains*

The heteroresistance non-inhibitory concentration (HNIC), heteroresistance inhibitory concentration (HIC), HIC/HNIC ratio, and frequency of resistant clones at the HNIC of IMP for the six isolates were determined using the PAP test (Table 2). Based on the classical definition of heteroresistance (presence of a subpopulation of resistant bacteria at $\geq 10^{-7}$ frequency that can grow in an antibiotic concentration ≥ 8-fold higher than the highest concentration affecting the growth of the main population),[28,29] strains HN66, HN148, and HN232 were classified as IMP-HR, HN41, HN67and HN125 as IMP-NHR (Figure 2).

Notably, in strains HN67 and HN125, which did not carry $bla_{\text{IMP-45}}$, the IMP HICs were 8 mg/L. In contrast, four of the $bla_{\text{IMP-45}}$-positive strains, HN41, HN148, HN66, and HN232, exhibited HICs that were significantly elevated by at least 32-fold (≤ 256 mg/L) compared to HN67 and HN125.

Impact of IMP Pre-Exposure on IMP-HR Strain HN232

As shown in Figure 3, at pre-exposure IMP concentrations of 1, 4, and 32 mg/L, the regrowth percentage and frequency of HN232 on plates containing a range of IMP concentrations showed an overall trend of initially increasing and then decreasing; however, they remained higher than those of the controls. At lower (0.5 mg/L) and higher (128 mg/L) IMP concentrations, the effect of pre-exposure on regrowth was not significant. The HICs of IMP for HN232 after pre-exposure to 0.5, 1, 4, 32, and 128 mg/L were 256, 256, 256, 128, and 128 mg/L, respectively, consistent with the HIC observed in the PAP experiment without pre-exposure. These results indicated that pre-exposure treatment did not affect HIC, but selectively increased the aggregation of resistant subpopulations at specific concentrations of IMP.

At pre-exposure to 1 mg/L IMP, the frequencies of HN232 bacteria on regrowth plates (0.5, 1, 2, 4 and 8 mg/L IMP) were 7.3×10^{-6}, 1.03×10^{-7}, 9.2×10^{-6}, 8.6×10^{-6}, and 7.5×10^{-6}, respectively. The percentage of bacteria regrown on these plates was > 1. At pre-exposure to 4 mg/L IMP, the percentages of regrowth bacteria on plates containing 0.5, 1, 2 and 4 mg/L IMP were also > 1, with frequencies of regrowth bacteria of 7.19×10^{-6}, 7.79×10^{-6}, 7.42×10^{-6}, and 7.24×10^{-6}, respectively. However, this effect was not as evident at a pre-exposure IMP concentration of 1 mg/L, indicating that exposure to low IMP concentrations (1 and 4 mg/L) was sufficient to generate an IMP-resistant subpopulation that grew on plates containing ≥ 4 mg/L IMP (intermediate resistance) within a relatively short period. At pre-exposure to 32 mg/L IMP, the percentages of HN232 bacteria on regrowth plates at 0.5 and 1 mg/L IMP were also > 1, with frequencies of 7.1×10^{-6} and 7.35×10^{-6}, respectively. In contrast, at 128 mg/L pre-exposure, the percentage of regrowth bacteria across all plates was < 1, suggesting that high concentrations of IMP may exert bactericidal effects and cause toxicity, thereby inhibiting bacterial growth.

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Table 1 Characteristics of Six *P. Aeruginosa* Clinical Isolates Carrying bla_{IMP-45}

Clinical Isolates	Source	Sequence Type	E-test (IMP) mg/L	MICs (mg/L)										Resistance Gene
				TZP	CAZ	FEP	ATM	IMP	MEM	CIP	LEV	AMK	CT	
HN67	Sputum	ST110	1	>64/4	>64	>16	8	2	>8	<=0.5	<=2	<=8	2	*aph(3')-IIb,bla*OXA-486,*bla*PAO,*fosA,catB7*
HN125	Sputum	ST111	2	>64/4	>64	>16	8	4	>8	<=0.5	1	<=8	<=1	*aph(3')-IIb,bla*OXA-395,*bla*PAO,*fosA,catB7*
HN41	Wound secretion	ST446	2	>64/4	>64	>16	8	8	>8	16	8	>128	<=1	*aph(3')-IIb,armA,aac(6')-Ib3,aph(3')-Ia* *bla*IMP-45, *bla*OXA-1,*bla*OXA-395,*bla*PAO *fosA,msr(E),mph(E),catB7,catB3,arr-2,sul1* *crpP,qnrVC1,dfrA22,tet(C),oprJ-tmexCD3* *aph(3')-IIb,aac(6')-Ib3*
HN148	Sputum	ST2631	2	>64/4	>32	>16	8	>8	>8	4	64	>128	<=1	*bla*IMP-45,*bla*OXA-1,*bla*OXA-485,*bla*PAO *fosA,catB7,catB3,arr-2, sul1* *qnrVC1,dfrA22,oprJ-tmexCD3* *aph(3')-IIb,armA,aac(6')-Ib3,aph(3')-Ia*
HN66	Wound secretion	ST446	3	>64/4	>32	>16	8	<=0.25	>8	8	32	>128	2	*bla*IMP-45, *bla*OXA-1,*bla*OXA-395,*bla*PAO *fosA,msr(E),mph(E),catB7,catB3,arr-2,sul1* *crpP,qnrVC1,dfrA22,tet(C),oprJ-tmexCD3* *aph(3')-IIb,armA,aac(6')-Ib3,aph(3')-Ia*
HN232	Sputum	ST693	2	>64/4	>32	>16	8	4	>8	8	16	>128	2	*bla*IMP-45, *bla*OXA-1,*bla*OXA-50,*bla*PAO *fosA,msr(E),mph(E),catB7,catB3,arr-2,sul1* *qnrVC1,dfrA22,oprJ-tmexCD3*

Notes: E-test test strip method.

Abbreviations: MICs, minimum inhibitory concentrations; TZP, piperacillin/tazobactam; CAZ, ceftazidime; FEP, cefepime; ATM, aztreonam; IMP, imipenem; MEM, meropenem; CIP, ciprofloxacin; LEV, levofloxacin; AMK, amikacin; CT, colistin.

Table 2 Validation of the Heteroresistant Status of *P. Aeruginosa* Strains Using the PAP Method

Strains	bla_{IMP-45}	HIC	HNIC	FRC	HIC/HNIC Ratio	Heteroresistance
HN67	-	8	2	2.6×10^{-8}	4	-
HN125	-	8	1	8.5×10^{-8}	8	-
HN41	+	128	4	4.5×10^{-8}	32	-
HN148	+	256	0.5	1.6×10^{-7}	512	+
HN66	+	256	1	1.24×10^{-7}	256	+
HN232	+	256	0.5	1.25×10^{-7}	512	+

Abbreviations: HIC, heteroresistance inhibitory concentration; HNIC, heteroresistance non-inhibitory concentration; FRC, frequency of resistant clones at the HIC concentration.

Genetic Environment and bla_{IMP-45} Promoter in HN232 and HN41

Genomic sequencing and assembly revealed the presence of 433,528-kb and 449,083-kb closed circular DNAs carrying bla_{IMP-45} in HN232 and HN41 strains, which were hereafter designated as pHN232-MDR and pHN41-MDR, respectively. Plasmid pHN232-MDR encompassed 96% of the pHN41-MDR sequence and shared 100% nucleotide identity in the overlapping region. Both plasmids possessed the core characteristics of IncP-2 group megaplasmids (> 300 kb in size), including genes essential for replication (*repA2*, 1188 bp) and partition (*parB2-parAB*), and gene clusters coding for conjugation (*tra*), pilus assembly (*pil*), chemotaxis (*che*), and tellurium resistance (*ter*) functions in the plasmid backbone (data not shown).

The genetic environment adjacent to bla_{IMP-45} in both HN232 and HN41 was found to be associated with Tn*1403*-based Tn*6485*-like transposons (Figure 4). Tn*1403* is a Tn*3*-family transposon that was initially identified in a clinical *P. aeruginosa* isolate from the USA in the 1970s.[30] It has a core backbone composed of inverted repeat left (IRL), *tnpA* (transposase), *tnpR* (resolvase), *res* (resolution site), *sup* (sulfate permease), *uspA* (universal stress protein), *dksA* (RNA polymerase-binding transcription factor), *yjiK* (hypothetical protein), and inverted repeat right (IRR), with insertion sequence elements In28 and Tn*5393c* inserted into *res* and *dksA*, respectively. Tn*1403* and its close derivatives Tn*6060*,

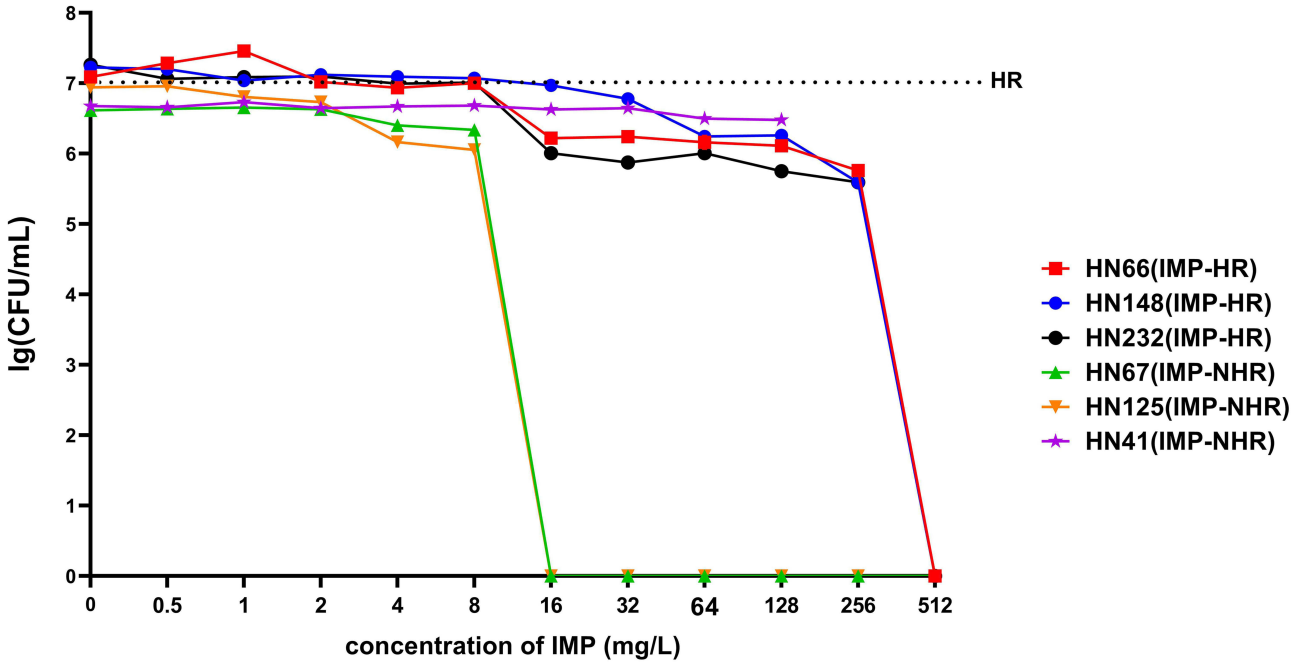

Figure 2 Population analysis profile (PAP) curves of six *P. aeruginosa* strains to IMP.
Abbreviations: CFU, colony-forming units; HR, heterogeneous resistance; NHR, non-heterogeneous resistance.

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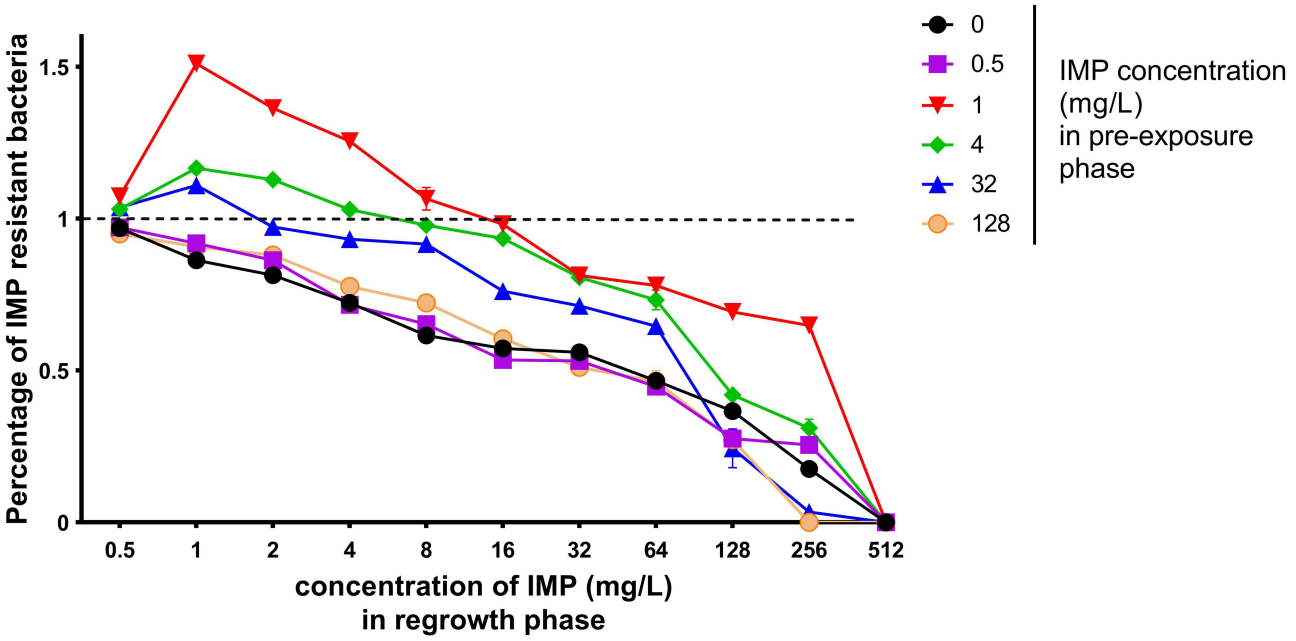

Figure 3 Impact of pre-exposure to IMP on the formation of IMP-resistant subpopulations. Schematic diagram illustrating the pre-exposure of HN232 to different doses of IMP-containing liquid medium for 24 h followed by regrowth on Mueller-Hinton agar plates containing a gradient of IMP concentrations (0–512 mg/L). The percentage of bacterial strains regrowing on IMP plates at different concentrations. Experiments were independently repeated three times. Error bars represent the standard error of the mean (n = 3).

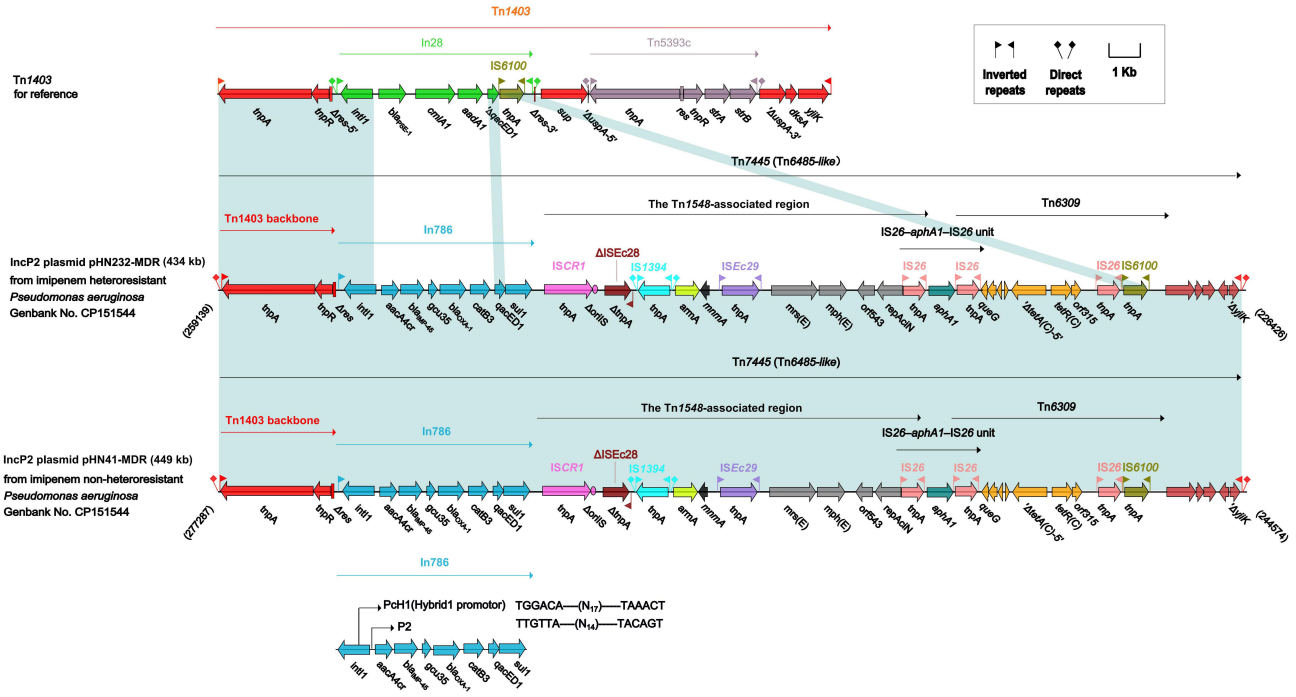

Figure 4 Genetic environment of bla_{IMP-45} in pHN232-MDR and pHN41-MDR, and comparison with Tn*1403*. Genes are denoted by arrows. Colors of genes, mobile elements and other features indicate their functional classification. Shading denotes regions of homology (> 95% nucleotide identity). Numbers in brackets indicate the nucleotide positions within the corresponding plasmids. Pchybrid 1 (PcH1) and P2 promotors within In*786*.

Tn*6061*, Tn*6217*, Tn*6249*, and Tn*6286*,[19,31–33] and the recently reported Tn*6485a-h* in *Pseudomonas*, often contain insertions of different foreign entities (eg, integrons and other transposons),[9,12,15,16] serving as important vehicles for the transmission of resistance genes.

Tn*6485*-like transposon Tn*7445*, found in both pHN232-MDR and pHN41-MDR, was 32,724 bp in length, with 38-bp IRLs and IRRs identical to those of Tn*1403* and bordered by the same 5-bp TCTCA direct repeat, that is, target site duplications that indicate acquisition of Tn*7445* by transposition. Compared to Tn*1403*, Tn*7445* undergoes several biological events.

1. In*28* was replaced with In*786*, which had a gene cassette array of *aacA4cr* (aminoglycoside and quinolone resistance)-*bla*$_{\text{IMP-45}}$-*gcu35* (unknown function)-*bla*$_{\text{OXA-1}}$ (β-lactamase resistance).
2. Evolution of In*786* into a complex class 1 integron via acquisition of the Tn*1548*-associated region extending from the IS*CR1* element to IS*26*, which harbors *armA* and the macrolide resistance operon *msr(E)–mph(E)*.
3. Accumulation of two entities, namely the IS*26*-*aphA1*-IS*26* aminoglycoside resistance unit and Tn*6309*, which carries *tetA(C)* downstream of the Tn*1548*-associated region. Furthermore, these three entities overlapped at one of the terminal IS*26* elements, strongly suggesting that they were acquired via IS*26*-mediated homologous recombination, rather than transposition.
4. Loss of most of the Tn*1403* backbone (Δ*res-sup-uspA-dksA-yjiK*) was replaced by five cryptic genes.

Relative Expression Levels and Copy Numbers of the *bla*$_{\text{IMP-45}}$ and *repA* Genes in HN232 and HN41

In the IMP-HR strain HN232, following pre-exposure to 1, 2, 8, 32, 128, and 512 mg/L IMP for 24h, the expression levels of *repA* relative to those of *ecfX* (1.36-, 1.23-, 1.17-, 0.96-, 0.73-, and 0.38-fold, respectively) suggested that plasmid replication was slightly induced at concentrations ≤ 8 mg/L, but was inhibited at concentrations ≥ 32 mg/L (Figure 5A). The expression levels of *bla*$_{\text{IMP-45}}$ relative to those of *ecfX* in the same range of IMP concentrations (20.00, 3.95, 1.59, 1.31, 1.03, and 0.53-fold, respectively) indicated that *bla*$_{\text{IMP-45}}$ expression might be induced by pre-exposure to ≤ 8 mg/L IMP, especially at 1 and 2 mg/L.

In IMP-NHR strain HN41, following exposure to 1, 2, 8, 32, 128, and 512 mg/L IMP, the expression levels of *repA* relative to those of *ecfX* (0.77-, 0.48-, 0.23-, 0.16-, 0.08-, and 0.02-fold) suggested that plasmid replication was inhibited

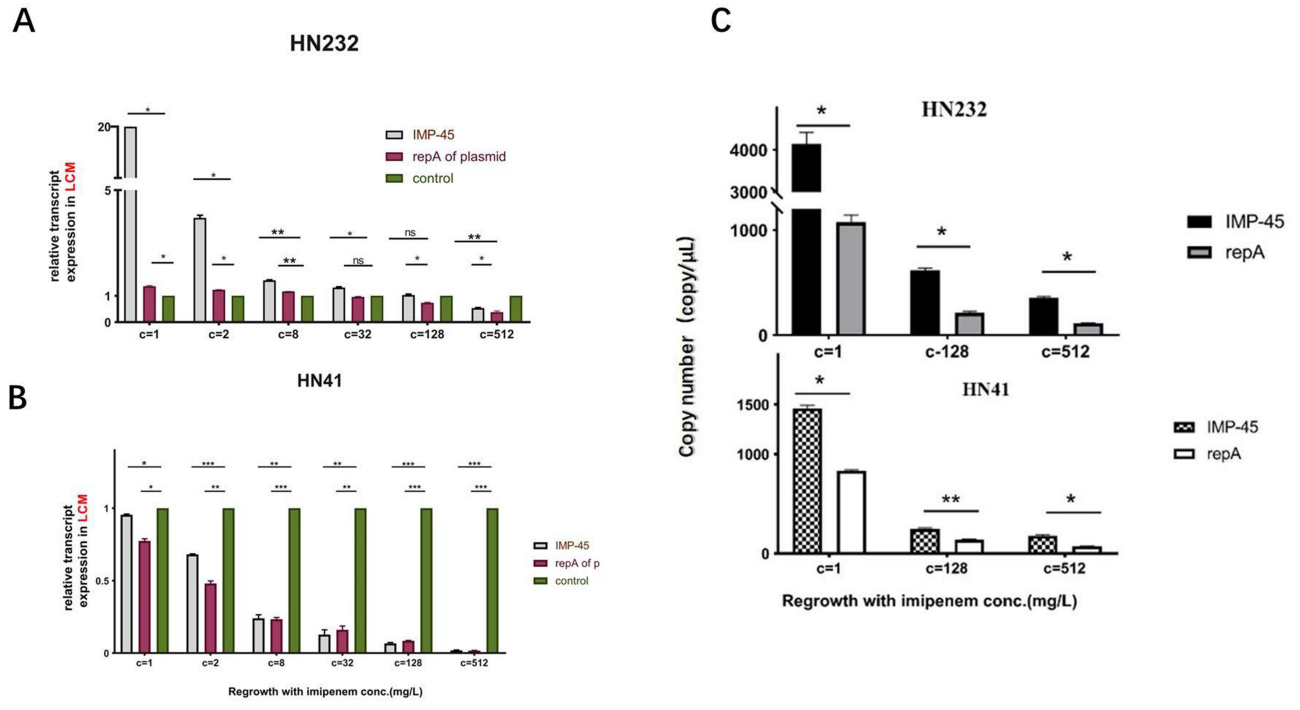

Figure 5 Quantitative real-time PCR and droplet digital PCR analyses of *bla*$_{\text{IMP-45}}$ in strains HN41 and HN232. (**A** and **B**) Expression of *bla*$_{\text{IMP-45}}$ and *repA* relative to *ecfX* in IMP-HR strain HN232 and IMP-NHR strain HN41 exposed to various concentrations of IMP. (**C**) Copy numbers of *bla*$_{\text{IMP-45}}$ and *repA* in HN232 and HN41 exposed to various concentrations of IMP. Experiments were independently repeated three times. Error bars represent the standard error of the mean (n = 3).

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at all concentrations, especially at ≥ 8 mg/L (Figure 5B). The expression levels of *bla*$_{IMP-45}$ relative to those of *ecfX* at the same range of IMP concentrations (0.95-, 0.6-, 0.2-, 0.1-, 0.07-, and 0.02-fold, respectively) suggested that pre-exposure to 1 and 2 mg/L slightly increased the expression of *bla*$_{IMP-45}$, all IMP concentrations inhibited the replication of the plasmid itself and the expression of *bla*$_{IMP-45}$, especially at concentrations ≥ 8 mg/L.

The absolute copy numbers of *bla*$_{IMP-45}$ and *repA* in HN232 and HN41 under pre-exposure IMP concentrations of 1 mg/L, 128 mg/L, and 512 mg/L were determined using ddPCR (Figure 5C). While the ratio of *bla*$_{IMP-45}$ to *repA* varied between 2.8–3.8, the greatest difference in absolute copy numbers was observed at 1 mg/mL IMP in HN232, in which the absolute copy number of *bla*$_{IMP-45}$ exceeded that of *repA* by > 3,000 copies, providing strong evidence for the independent replication of *bla*$_{IMP-45}$ resulting from unstable gene amplification. In HN41, the *bla*$_{IMP-45}$ copy number was also considerably higher than that of *repA*, but was not induced to the same extent as in HN232.

Discussion

The presence of carbapenemases often leads to an MDR phenotype in *P. aeruginosa*, with frequent reports of strains harboring the MBL gene *bla*$_{IMP-45}$ in recent years.[8,11,12,16,34,35] The STs of *P. aeruginosa* isolates include ST235, ST274, ST277, ST308, ST357, ST369, ST389, ST508, ST708, ST1420, and ST3014,[8,12,15,16] among these, ST508 and ST463 are high-risk clones involved in nosocomial infection outbreaks. To the best of our knowledge, ST693 and ST2631 identified in this study are novel *bla*$_{IMP-45}$-carrying STs in *P. aeruginosa*. Phenotypically, the MIC values of meropenem for the six *P. aeruginosa* strains in this study were all > 8 mg/L (Table 1), which is above the resistance breakpoint recommended by CLSI 2024.[25] CRPA isolates are generally defined by resistance to ≥ one carbapenems with antipseudomonal activity (doripenem, IMP, or meropenem);[36] therefore, all six of our strains were classified as CRPA. However, the four *bla*$_{IMP-45}$-carrying strains showed different MIC values (≤ 0.25 to > 8 mg/L) for IMP (Table 1). This may be attributable to the different pharmacokinetic traits of IMP and meropenem; the anti-bactericidal activity and killing speed of IMP against *P. aeruginosa* are significantly greater than those of meropenem.[37] Three of the *bla*$_{IMP-45}$-carrying isolates (HN66, HN148, and HN232) were confirmed to be IMP-HR; however, whether these strains are also HR to meropenem requires further investigation.In one study, rates of CRPA heteroresistance to meropenem and IMP have been reported 72.5% and 54.3%, respectively, indicating variability.[38]

The results of PAP testing and IMP pre-exposure experiments revealed that the HIC of *bla*$_{IMP-45}$-carrying IMP-HR isolates could be increased 32-fold (up to 256 mg/L) relative to that of *bla*$_{IMP-45}$-negative IMP-HR isolates. Additionally, pre-exposure of the strains to low concentrations of IMP (1 and 4 mg/L) led to an increased frequency of regrowth on plates containing IMP concentrations associated with intermediate susceptibility and resistance. Both factors could contribute to the difficulty in completely eradicating IMP-HR strains during clinical treatment, thereby increasing the likelihood of infections evolving into refractory infections and treatment failure.

Examination of the genetic environment of *bla*$_{IMP-45}$ in the CRPA isolates revealed that this gene is located in the second gene cassette of In*786* within the Tn*1403*-derived transposon, which resides in the Inc-P2 megaplasmid. The expression of *bla*$_{IMP-45}$ was governed by the same promoter in both IMP-HR HN232 and IMP-NHR HN41, excluding the possibility that the observed variation in gene expression levels was due to genetic and environmental differences. In contrast to the high genetic diversity of the genetic background of host strains, the genetic environment of *bla*$_{IMP-45}$ is relatively monotonous. Except for two cases of *bla*$_{IMP-45}$ found on the chromosomes of *P. aeruginosa* strains PA59 and M140A,[39] all the fully sequenced *bla*$_{IMP-45}$-carrying plasmids belonged to the IncP-2 group. Prior to 2013, the *bla*$_{IMP-45}$-encoding plasmid pOZ176 (500 kb) was the only fully sequenced IncP-2 plasmid with *bla*$_{IMP-45}$ residing in the second gene cassette located within In*244* (*aacA4'-bla*$_{IMP-9}$-*aacA4'*).[8] Subsequently, in 2017 and 2018, two IncP-2 plasmids, pSY153-MDR[18] and pBM413,[16] were reported in *Pseudomonas putida* and *P. aeruginosa*, respectively. Both were shown to carry *bla*$_{IMP-45}$ (the G214S variant of *bla*$_{IMP-9}$) in In*786*. To date, at least 23 *bla*$_{IMP-45}$ plasmids have been reported,[8–19] and phylogenetic analysis has revealed that most plasmids carrying *bla*$_{IMP-45}$ cluster into a distinct sublineage that also contains the full pOZ176 sequence, facilitating the dissemination of *bla*$_{IMP-45}$ among genetically diverse *P. aeruginosa* strains.Despite all carrying the identical bla$_{IMP-45}$ gene, the isolates displayed ≥ 32-fold variation in imipenem MICs (each exceeding the clinical resistance breakpoint).This divergence, in the absence of any promoter or cassette polymorphism, indicates that the variable resistance levels are not encoded by the carbapenemase itself but are driven by the

following mechanisms:inducible local amplification of the gene, differential plasmid replication control, and additional non-specific resistance traits such as efflux-pump overexpression.

In-depth mechanistic experiments indicated that plasmid replication in the IMP-NHR strain HN41 was suppressed at all IMP concentrations compared with chromosomal replication. In contrast, in the IMP-HR strain HN232, plasmid replication was inhibited at high concentrations of IMP but was roughly equivalent to chromosomal replication at concentrations between 1 and 32 mg/L. A common feature observed in both strains was the increased expression of $bla_{\text{IMP-45}}$ at IMP concentrations of 1 and 2 mg/L, with the fold increase being more pronounced in the HR strain. Absolute copy number quantification of $bla_{\text{IMP-45}}$ and *repA* using ddPCR further revealed that low concentrations of IMP induced an increase in $bla_{\text{IMP-45}}$ copy number in both HR and NHR isolates, albeit to a greater extent in the IMP-HR strain HN232. These findings support the hypothesis that $bla_{\text{IMP-45}}$ can undergo localized, unstable gene amplification independent of plasmid replication, leading to increased gene copy number and expression, which may play a major role in heteroresistance. Additionally, these findings suggest that plasmid replication and localized amplification of $bla_{\text{IMP-45}}$ are regulated by complex factors in both the strains. In comparison, plasmid replication and copy number may be more tightly controlled in IMP-NHR strain HN41.

Mobile genetic elements, such as IS*26* and transposons, have been reported to mediate the unstable amplification of adjacent antibiotic resistance genes. Wei et al reported that after pre-exposure to sublethal doses of meropenem or tobramycin, IS*26* mediated rapid and stable amplification of $bla_{\text{KPC-2}}$, leading to carbapenem resistance in clinical *E. coli* strains.[27] In *A. baumannii*, amplification of five resistance genes within the *aadB* region of plasmids has been reported to cause heteroresistance to tobramycin.[13] Similarly, in *Salmonella enterica serovar* Typhimurium, gene amplification at the chromosomal *pmrD* locus is associated with heteroresistance to colistin.[12] Whether the unstable downstream amplification of $bla_{\text{IMP-45}}$ observed in our study was driven by IS*26* or a Tn*1403*-like transposon remains to be confirmed.

While this study provides compelling evidence for the role of the $bla_{\text{IMP-45}}$-bearing plasmid and its inducible amplification in driving IMP heteroresistance in *P. aeruginosa*, it is important to acknowledge a potential limitation regarding the temporal scope of the isolates investigated. The clinical strains analyzed in this work were collected between 2013 and 2014. Antimicrobial resistance profiles and the prevalence of specific resistance mechanisms, including carbapenemases like IMP variants, are known to evolve dynamically over time in response to selective antibiotic pressure and infection control practices. Consequently, the specific resistance landscape and the relative abundance of $bla_{\text{IMP-45}}$ or similar determinants observed in this cohort may not fully represent the current clinical epidemiology of *P. aeruginosa* carbapenem resistance or the predominant mechanisms underpinning heteroresistance in contemporary settings. Future studies incorporating more recent clinical isolates would be valuable to confirm the ongoing relevance of the plasmid-mediated amplification mechanism described here and to assess potential shifts in the genetic drivers of IMP heteroresistance.

Conclusion

Carbapenem antibiotics remain the first-line treatment for *P. aeruginosa* infection. IncP-2 conjugative megaplasmids still serve as predominant vehicles for the dissemination of $bla_{\text{IMP-45}}$ among *P. aeruginosa* isolates. Our results indicate that, in IMP-HR $bla_{\text{IMP-45}}$-carrying *P. aeruginosa*, short-term (24 h) exposure to IMP concentrations as low as 1 mg/L can induce unstable localized gene amplification of plasmid-derived $bla_{\text{IMP-45}}$ in the frequency of occurrence of resistant sub populations. These findings emphasize the importance of rational antibiotic use in clinical treatment.

Ethics Statement

Human specimens were acquired with the patient's consent. The use of human specimens and all related experimental protocols was reviewed and approved by the Ethics Committee of the National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, in accordance with the medical research regulations of the Ministry of Health of China.The strain was routinely preserved in our laboratory repository, no patient interventions or additional sample collections were conducted. This study adhered to the guidelines outlined in the Declaration of Helsinki.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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