

Original Research

Prevalence of AmpC β -Lactamase and MexAB-OprM Efflux Pump Genes Among Multidrug-Resistant *Pseudomonas aeruginosa* Clinical Isolates from Babylon Province, Iraq

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Abstract

Multidrug-resistant *Pseudomonas aeruginosa* is a major cause of hospital-acquired infections and is responsible for high mortality rates due to its intrinsic and acquired resistance mechanisms to various antibiotics. This study aimed to investigate the prevalence of the AmpC beta-lactamase enzyme and the MexAB-OprM efflux pump genes, as well as the effect of the inhibitors cloxacillin and Phe-Arg β -naphthylamide dihydrochloride (PA β N) on its antibiotic resistance. The current cross-sectional study included 227 *P. aeruginosa* isolates collected from patients hospitalized at AL-Hillah Teaching Hospital, Babil Teaching Hospital, Marjan Medical City, and associated medical microbiology laboratories in Babylon province. The antibiotic resistance pattern, beta-lactamase production, and the effect of inhibitors on these isolates were determined phenotypically, while the prevalence of beta-lactamase and efflux pump genes was investigated using the PCR technique. Of these isolates, 114 (50.2%) were multidrug-resistant. These multidrug-resistant isolates exhibited complete resistance (100%) to aztreonam, cefepime, and ceftazidime-clavulanic acid, but were most susceptible to piperacillin-tazobactam (59.6%). Antibiotic resistance in the multidrug-resistant isolates decreased after adding cloxacillin and PA β N inhibitors, with



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resistance to several antibiotics markedly reduced (e.g., ceftazidime from 84% to 42% and meropenem from 97% to 48%). Among the genes encoding the beta-lactamase AmpC, the *bla*_{DHA} was the most prevalent, detected in 55% (17/31) of the isolates, followed by *bla*_{FOX} (39% (12/31)). The *bla*_{ACT-1/MIR-1} and *bla*_{MOX} were each detected in 7% (2/31) of the isolates, while the *bla*_{LAT} was detected in only one isolate (3%). Among the MexAB-OprM efflux pump systems, the *mexB* was the most prevalent (48.4% (15/31)) compared to the *mexA* (16.1% (5/31)), while the *oprM* was identified in 12.9% (4/31) of isolates. High resistance has been detected in *P. aeruginosa* isolates, where the use of cloxacillin and PAβN plays a role in decreasing antibiotic resistance. This high resistance is concerning and warrants stricter antibiotic prescribing policies.

Keywords

Pseudomonas aeruginosa; antibiotic resistance; PAβN; cloxacillin; β-lactamase genes; AmpC β-lactamase

1. Introduction

Pseudomonas aeruginosa is an important cause of hospital-acquired infections, particularly in immunocompromised patients such as those with neutropenia, burn injuries, and cystic fibrosis [1]. Recent studies from Iraq have reported a high prevalence of multidrug-resistant *Pseudomonas aeruginosa* isolates in clinical settings, emphasizing that antimicrobial resistance has become a major therapeutic challenge and underscoring the need for continuous surveillance of resistance mechanisms [2]. *Pseudomonas aeruginosa* is an important opportunistic pathogen responsible for a wide range of healthcare-associated infections. It is commonly isolated from respiratory tract infections, urinary tract infections, burn and wound infections, bloodstream infections, and other invasive infections, particularly among hospitalized and immunocompromised patients. The diversity of clinical specimens reflects the organism's ability to colonize different body sites and cause severe infections associated with high morbidity and limited therapeutic options due to antimicrobial resistance [3].

The antibiotic resistance in *P. aeruginosa* has become a world-concerning problem, which results in reduced and ineffective therapy efficacy. Besides intrinsically resistant to various antibiotics due to low permeability of the outer membrane, production of chromosomal β-lactamases, and the presence of efflux pump systems [4]. Infections caused by *P. aeruginosa* isolates with multidrug resistance increase mortality, hospitalization, and treatment costs, especially in intensive care units. Mortality decreases significantly if early and appropriate antipseudomonal treatment is initiated [5]. The rate of multidrug-resistant *P. aeruginosa* (MDR) has increased significantly. It has become a major challenge to global public health, which is linked to high morbidity and mortality because of its ability to acquire resistance to the most effective antibiotics [2]. Production of β-lactamase enzymes is a major mechanism of intrinsic resistance in *P. aeruginosa*. It can be categorized into 4 categories, including class A extended-spectrum beta-lactamases (ESBLs), class B metallo-beta-lactamases (MBLs), class C AmpC beta-lactamases (AmpC), and class D oxacillinases (OXA type), based on their amino acid sequences; these enzymes

hydrolyze β -lactams but differ in their active sites. A, C, and D classes possess serine in their active site, whereas class B β -lactamases are metallo-enzymes that need zinc ions for β -lactam hydrolysis [6]. In *P. aeruginosa*, the overexpression of the naturally occurring AmpC is associated with decreased susceptibility or resistance to expanded-spectrum cephalosporins, such as ceftazidime [7]. Bacteria that produce ampC β -lactamase genes are clinically important because they are often resistant to most β -lactams other than 4th-generation cephalosporins and carbapenems [8]. The class C AmpC β -lactamases are the predominant cephalosporinases encoded on both chromosomes and plasmids. The major families of AmpC β -lactamases include the CMY, FOX, MIR, ACT, and DHA families, which are widely distributed in multiple Gram-negative bacteria. AmpC enzymes are inducible, and overexpression confers resistance to broad-spectrum cephalosporins such as cefotaxime, ceftazidime, and ceftriaxone, as well as to penicillin, aztreonam, and cephamycin. Penicillin, aztreonam, and cephamycins. The majority of AmpC β -lactamases have no carbapenemase activity. However, mutated AmpC β -lactamases do have resistance to carbapenem antibiotics [9]. *P. aeruginosa* possesses at least 12 structural genes for multidrug efflux pumps belonging to the resistance-nodulation-cell division (RND) family where MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM efflux pumps have shown to be of clinical relevance system [10]. The MexAB-OprM efflux system contributes to its intrinsic resistance to β -lactams, quinolones, tetracycline, chloramphenicol, novobiocin, and macrolides, and its overexpression confers cross-resistance or reduced susceptibility to several antibiotics [11].

The inhibitors have been used to stop bacterial efflux pumps from working, where Phe-Arg β -naphthylamide dihydrochloride (PA β N) is the first characterized inhibitor of multiple resistance-RND transporters from *P. aeruginosa*. Phe-Arg β -naphthylamide dihydrochloride potentiated the activities of the antibiotics that were substrates for the efflux pump (MexAB-OprM), which included fluoroquinolones, tetracyclines, β -lactams, and rifampin, but did not potentiate the activities of antibiotics that were not substrates for MexAB-OprM. PA β N showed no significant activity against strains lacking known efflux pumps. Cloxacillin is another inhibitor used to inhibit bacterial AmpC enzyme activity [12]. Multidrug resistance in *Pseudomonas aeruginosa* is usually mediated by the combined action of multiple resistance mechanisms, particularly AmpC β -lactamase overproduction and overexpression of the Mex efflux pumps. These mechanisms act synergistically to reduce susceptibility to several antimicrobial agents, making their combined investigation important for understanding the molecular basis of multidrug resistance [13].

This study aimed to investigate the prevalence of Ampc β -lactamase and MexAB-OprM efflux pumps genes and the effects of cloxacillin and Phe-Arg β -naphthylamide dihydrochloride (PA β N) inhibitors on the antibiotic resistance in *P. aeruginosa* isolates.

2. Material and Methods

2.1 Study Design and Sample Collection

We conducted a cross-sectional study between April and September 2025. A total of 227 *Pseudomonas aeruginosa* isolates were collected from patients hospitalized at AL-Hillah Teaching Hospital, Babil Teaching Hospital, Marjan Medical City, and associated medical microbiology laboratories in Babylon province. We collected clinical specimens from various infection sites, including diabetic foot infections (n = 87), burns (n = 46), pus (n = 38), sputum (n = 35), ear swabs (n = 17), and urine (n = 4). Standard procedures were followed for sample collection: burn and pus

swabs were taken from the depth of the wound; sputum specimens were collected in sterile containers before antibiotic therapy; ear swabs were inserted to collect middle ear exudate while avoiding external canal contamination. All samples were transported to the laboratory immediately for processing, and Figure 1 shows all experimental steps.

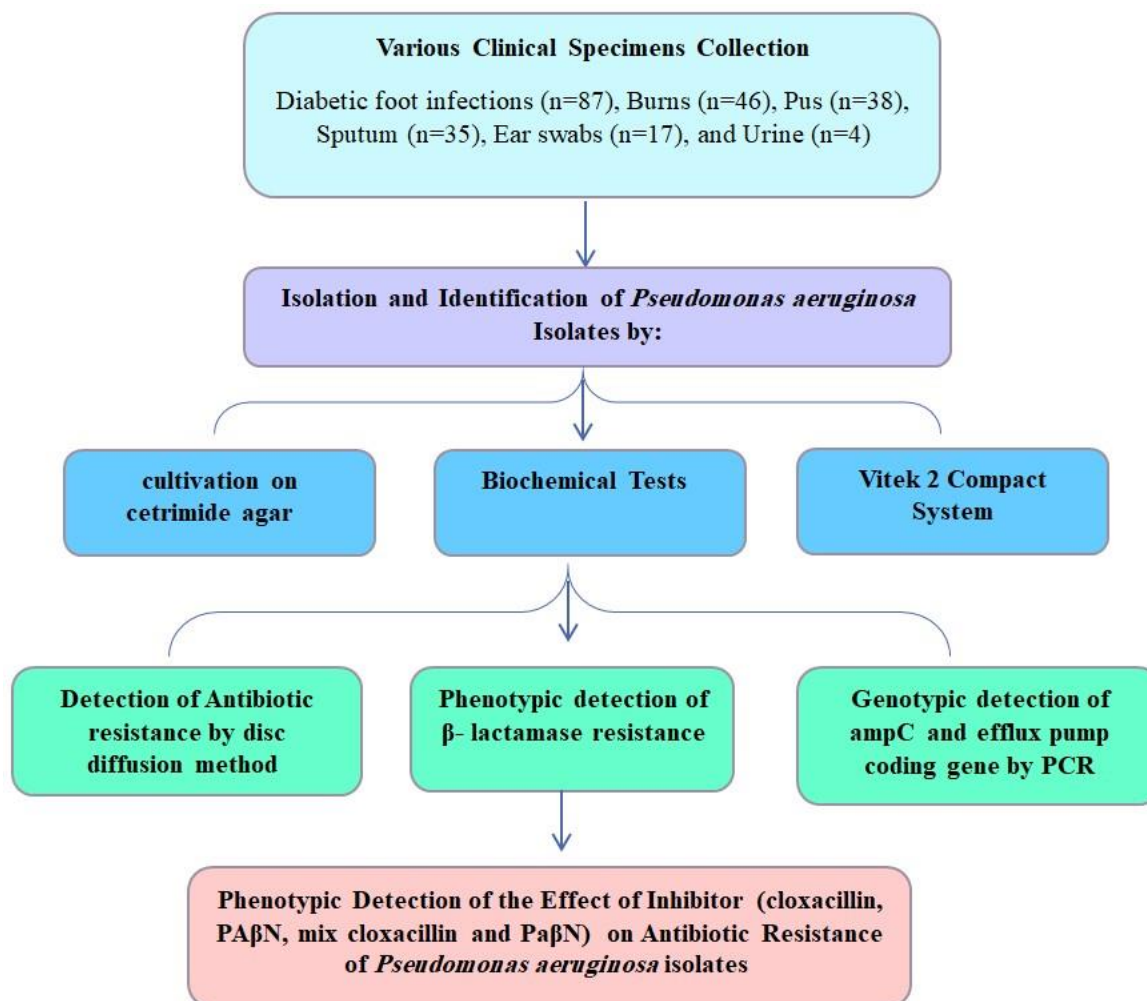


Figure 1 Study design showing all steps of the experiment.

2.2 Isolation and Identification of *P. aeruginosa*

We identified the collected isolates using conventional microbiological techniques. This included cultivation on cetrimide agar (Himedia, India) and a series of biochemical tests. Confirmatory identification was performed using the Vitek 2 Compact system (bioMérieux, France).

2.3 Antimicrobial Susceptibility Test to β-Lactams

Antimicrobial susceptibility of the isolates was determined following the standard Kirby-Bauer disc diffusion technique on Mueller-Hinton agar (Himedia, India) [14] using the following antibiotic discs (Himedia, India): Piperacillin (PI, 100 µg), Piperacillin-Tazobactam (PIT, 100/10 µg), Ceftazidime (CAZ, 30 µg), Ceftazidime-clavulanate (CAC, 30/10 µg), Cefotaxime (CTX, 30 µg), Cefotaxime-clavulanate (CEC, 30/10 µg), Cefepime (CPM, 30 µg), Cefoxitin (CX, 30 µg), Aztreonam (AT, 30 µg), Imipenem (IPM, 10 µg), Meropenem (MRP, 10 µg), Polymyxin B (PB, 300 units),

Gentamicin (GEN, 10 µg), Amikacin (AK, 30 µg), Ciprofloxacin (CIP, 5 µg), and Levofloxacin (LEV, 5 µg). We interpreted the results according to [15]. Multidrug resistance (MDR) was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories [16]. We detected ESBL production using combined disk tests and DDSTs. We inferred ESBL production when the cephalosporin zone expanded with clavulanate. Combined disk tests were also performed by placing disks of ceftazidime, cefotaxime, ceftazidime-clavulanic acid, and cefotaxime-clavulanic acid on MH plates at a distance of 30 mm (center to center) from each other. ESBL production was inferred if the zones produced by the disks with clavulanate were 5 mm larger than those without the inhibitor. To conveniently perform these two tests and observe the results, we integrated the DDSTs and combined disk tests into one plate by placing disks of ceftazidime, cefotaxime, aztreonam, cefepime (30mg each), ceftazidime-clavulanic acid, and cefotaxime-clavulanic acid on MH plates [12].

2.4 Determination of the Effect of Inhibitor on Antibiotic Resistance

DDSTs and combined disk tests were also performed on cloxacillin inhibitor alone to inhibit the activities of the AmpC enzyme (200 µg/ml)-containing plates and MC-207,110 (20 µg/ml)-containing plates alone to inhibit the activities of efflux pumps, and plates that contained both cloxacillin (200 µg/ml) and MC-207,110 (20 µg/ml) [12].

2.5 DNA Extraction and Detection of the β -Lactamase Encoding Genes

A total of 227 *Pseudomonas aeruginosa* isolates were included in this study, of which 114 isolates were identified as multidrug-resistant (MDR). Among these MDR isolates, the remaining 113 isolates were non-MDR and were excluded from further molecular analysis because they did not show resistance to multiple antimicrobial categories. Thirty-one isolates exhibiting the highest resistance profiles against the tested antimicrobial agents were selected based on their phenotypic response after the addition of inhibitors, including (cloxacillin and PA β N). These selected isolates were further subjected to molecular analysis to detect AmpC β -lactamase-associated genes and efflux pump genes.

We extracted total genomic DNA from the MDR isolates using the EasyPure Genomic DNA Kit (TransGen Biotech, China) according to the manufacturer's instructions. The extracted DNA was stored at -20°C until PCR amplification, following the manufacturer's instructions. We used the DNA as a template for polymerase chain reaction (PCR) analysis. Multiplex-PCR was subsequently performed using a set of primers (Table 1) for AmpC genes (*bla*_{DHA}, *bla*_{ACT}, *bla*_{MOX}, *bla*_{LAT}, and *bla*_{FOX}), and efflux pumps (*mexA*, *mexB*, *oprM*). The total reaction mixture (20 µL) consisted of 8 µL of master mix, 7.5 µL of MgCl₂, 0.5 µL of sterile nuclease-free water, 1 µL each of forward and reverse primers, and 2 µL of DNA template. Table 1 lists the thermocycling conditions.

Table 1 Sequences of primers used for this study (Macrogen, South Korea).

Genes	Primer Sequence (5'-3')	PCR Conditions	Amplicon Size (bp)	Reference
<i>bla</i> _{ACT1}	TCGGTAAAGCCGATGTTGCGG CTTCCACTGCGGCTGCCAGTT	Initial denaturation at 94°C for 3 min, 25 cycles, denaturation at 94°C for 30 s, annealing temperature 64°C for 30 s, extension at 72°C for 60 s, with final extension at 72°C for 7 min.	302	[1]
<i>bla</i> _{FOX}	AACATGGGGTATCAGGGAGATG CAAAGCGCGTAACCGGATTGG		190	
<i>bla</i> _{MOX}	GCTGCTCAAGGAGCACAGGAT		520	
<i>bla</i> _{LAT}	TGGCCAGAACTGACAGGCAAA TTTCTCCTGAACGTGGCTGGC		462	
<i>bla</i> _{DHA}	AACCTTTCACAGGTGTGCTGGGT CCGTACGCATACTGGCTTTGC		405	
<i>mexA</i>	CTACGAGGCCGACTACCAGA TGCAGGCCTTCGGTAATGAT	Initial denaturation at 94°C for 5 min, 35 cycles, denaturation at 94°C for 30 s, annealing temperature 59°C for 30 s, extension at 72°C for 1.5 min, and final extension at 72°C for 7 min.	772	[17]
<i>mexB</i>	CCGTGAATCCCGACCTGATG TGACATGATGGCTTCCGCAT		255	
<i>opr-M</i>	TACCAGAAGAGTTTCGACCTGAC CATGTGTCAAACAGTCACCTCC		812	

3. Results

3.1 Distribution of Isolates and Prevalence of MDR

A total of 227 *P. aeruginosa* isolates were collected from various clinical specimens. The primary sources were diabetic foot infections (n = 87, 38.3%), burns (n = 46, 20.3%), sputum (n = 35, 15.4%), ear swabs (n = 17, 7.5%), wound (n = 19, 8.4%), pus (n = 19, 8.4%), and urine (n = 4, 1.8%) (Figure 2). Overall, 114 of the 227 isolates (50.2%) met the criteria for multidrug resistance (MDR). The distribution of MDR isolates by specimen type was as follows: diabetic foot infections (n = 55, 48.3% of MDR isolates), burns (n = 21, 18.4%), sputum (n = 14, 12.3%), ear swabs (n = 10, 8.8%), wound (n = 8, 7.0%) and pus (n = 6, 5.3%) (Figure 3). Notably, all four urine isolates were susceptible to the tested antibiotics.

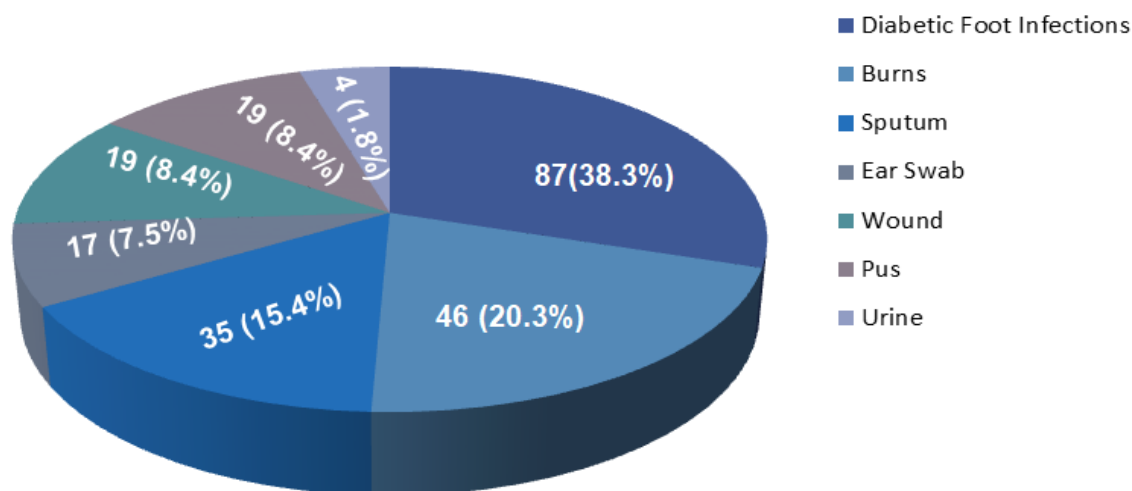


Figure 2 The percentage of clinical *Pseudomonas aeruginosa* isolated from different source.

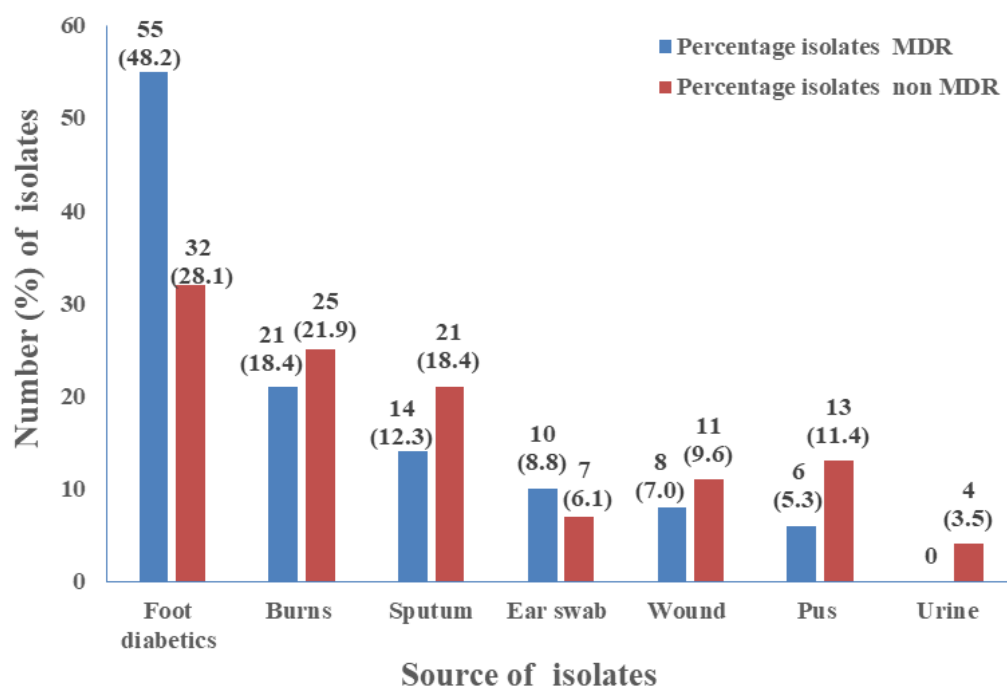


Figure 3 The percentage of multidrug resistance *Pseudomonas aeruginosa* isolated from different source.

3.2 Antibiotic Resistance Profiles of MDR Isolates

The 114 MDR isolates exhibited high resistance rates to most tested antibiotics (Figure 4). All isolates showed resistance (100%) to Piperacillin (PI), Cefepime (CPM), Ceftazidime-Clavulanic acid (CAC), Cefotaxime-clavulanic acid (CEC), Aztreonam (ATM), Polymyxin B (PB), and Gentamicin (GEN). Resistance to other critical agents was also very high, including Cefotaxime (CTX, 92.9%), Meropenem (MRP, 97.3%), Amikacin (AK, 96.4%), and Ciprofloxacin (CIP, 90.3%). Imipenem (IMP, 78.9%) and Levofloxacin (LEV, 88.5%) had the lowest resistance rates. Piperacillin-Tazobactam (PIT) was the most effective agent, with 59.6% of MDR isolates remaining susceptible. Although all

isolates showed resistance to piperacillin (PI), the addition of tazobactam resulted in a different susceptibility pattern for piperacillin-tazobactam (PIT), reflecting the inhibitory effect of tazobactam against some β -lactamase-mediated resistance mechanisms.

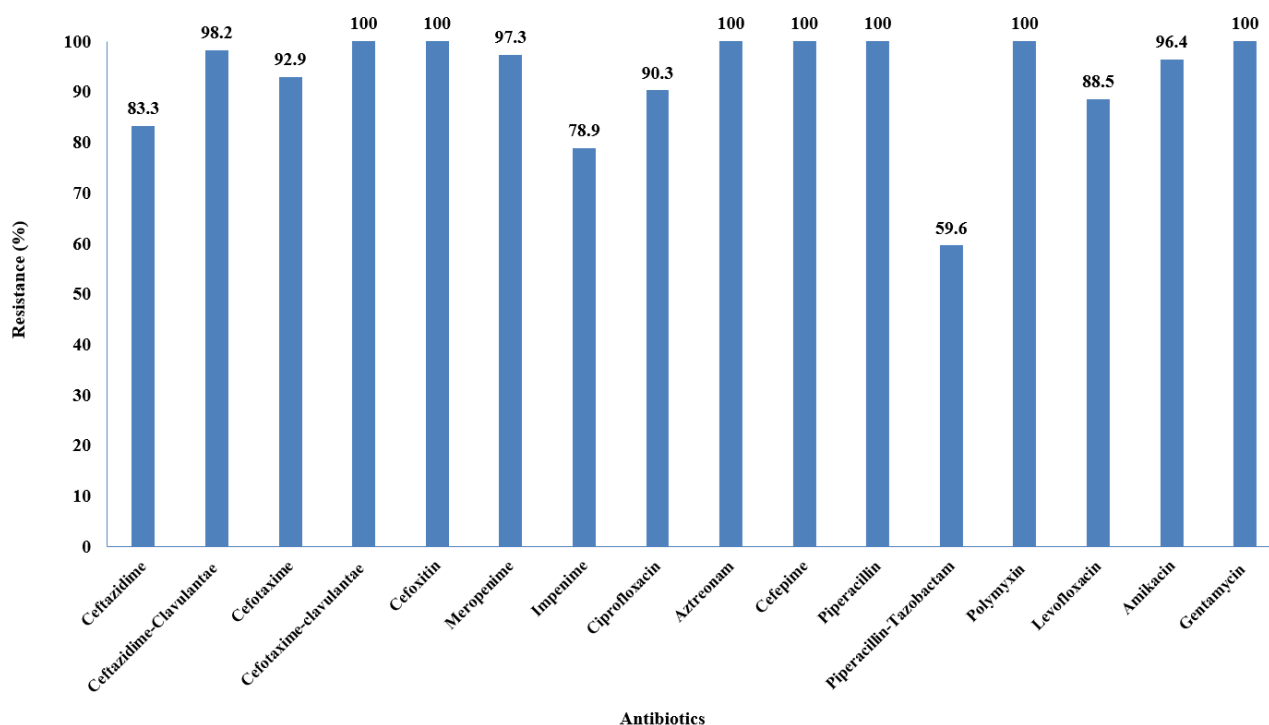


Figure 4 Prevalence of resistance to various antimicrobial agents among MDR *Pseudomonas aeruginosa* isolates.

3.3 Selection of Highly Resistant MDR Isolates and Effect of Inhibitors

Among the 114 multidrug-resistant (MDR) isolates identified in this study, 31 isolates demonstrating the highest levels of antimicrobial resistance were selected for further examination before and after the addition of cloxacillin, Phe-Arg β -naphthylamide dihydrochloride (PA β N), and their combination. We compared the proportion of resistant isolates (R) for each tested antibiotic (Table 2). The results showed noticeable variations in resistance rates after addition of inhibitors. For instance, resistance to ceftazidime (CAZ) decreased from 84% before inhibitor addition to 68% after cloxacillin and 65% after PA β N, and 42% after the combination of both inhibitors. Resistance to cefotaxime (CTX) was 94% before inhibitor addition, which decreased to 48% after cloxacillin, 55% after PA β N, and 71% after the combined inhibitors. Resistance to meropenem (MRP) was 97% before inhibitor addition, which decreased to 68% after cloxacillin, remained 68% after PA β N, and further decreased to 48% after the combined inhibitors. For imipenem (IMP), resistance was 77% before inhibitor addition, which decreased to 42% after cloxacillin, increased to 71% after PA β N, and decreased again to 42% after the combined inhibitors. Resistance to cefepime (CPM) was 100% before inhibitor addition, which decreased to 55% after cloxacillin, and increased to 77% after both PA β N and the combined inhibitors. Resistance to piperacillin (PI) was 100% before inhibitor addition, which slightly decreased to 97% after cloxacillin, 88% after PA β N, and 81% after the combined inhibitors. Resistance to piperacillin-tazobactam (PIT) was 55% before inhibitor addition, which decreased to 52% after cloxacillin, 42% after PA β N, and 39% after the combined inhibitors.

Table 2 The percentage of antibiotic resistance before and after adding Inhibitors (cloxacillin, PA β N, mix of cloxacillin and PA β N).

Antibiotics	The percentage of antibiotic resistance:			
	Before adding inhibitors	After adding cloxacillin	After adding PA β N	After adding mix inhibitors
CAZ	84	68	65	42
CAC	100	100	100	100
CTX	94	48	55	71
CEC	100	100	100	100
CX	100	100	100	100
MRP	97	68	68	48
IMP	77	42	71	42
CIP	84	94	77	77
ATM	100	55	59	71
CPM	100	55	77	77
PI	100	97	88	81
PIT	55	52	42	39
PB	100	100	100	100
LEV	81	81	81	71
AK	97	55	55	65
GEN	100	97	84	81

CAZ, Ceftazidime; CAC, Ceftazidime-clavulanate; CTX, Cefotaxime; CEC, Cefotaxime-clavulanate; CX, Cefoxitin; MRP, Meropenem; IMP, Imipenem; CIP, Ciprofloxacin; ATM, Aztreonam; CPM, Cefepime; PI, Piperacillin; PIT, Piperacillin-tazobactam; PB, Polymyxin B; LEV, Levofloxacin; AK, Amikacin; GEN, Gentamicin.

Resistance to levofloxacin (LEV) was 81% before inhibitor addition, remained 81% after both cloxacillin and PA β N, and decreased to 71% after the combined inhibitor. Resistance to amikacin (AK) was 97% before inhibitor addition, which decreased to 55% after cloxacillin, remained 55% after PA β N, and increased to 65% after the combined inhibitors. Resistance to gentamicin (GEN) was 100% before inhibitor addition, which decreased to 97% after cloxacillin, 84% after PA β N, and 81% after the combined inhibitors. Universal resistance (100%) was observed against Ceftazidime-Clavulanic acid (CAC), Cefotaxime-clavulanic acid (CEC), and Cefoxitin (CX); Polymyxin B (PB) remained at 100% under all tested conditions (Table 2). Resistance percentages were calculated based on the total number of tested isolates (n = 114).

3.4 Molecular Detection of AmpC and Efflux Pump Genes

A total of 31 MDR isolates were subjected to multiplex PCR analysis to detect plasmid-mediated AmpC β -lactamase genes. The results revealed that *bla*_{DHA} was the most prevalent, detected in 55% (17/31) of the isolates, followed by *bla*_{FOX}, which was identified in 39% (12/31) of isolates. Both *bla*_{ACT-1/MIR-1} and *bla*_{MOX} were detected in 7% (2/31) of the isolates each, whereas *bla*_{LAT} was detected in only one isolate (3%). The distribution of AmpC genes among the studied isolates is summarized in Table 3, whereas the results of agarose gel electrophoresis are shown in Figure 5.

Table 3 Prevalence of AmpC β -lactamase genes among 31 multidrug-resistant *Pseudomonas aeruginosa* isolates.

Ampc genes	Positive isolates	Percentage
<i>bla</i> _{DHA}	17	55
<i>bla</i> _{FOX}	12	39
<i>bla</i> _{ACT-1/MIR-1}	2	7
<i>bla</i> _{MOX}	2	7
<i>bla</i> _{LAT}	1	3

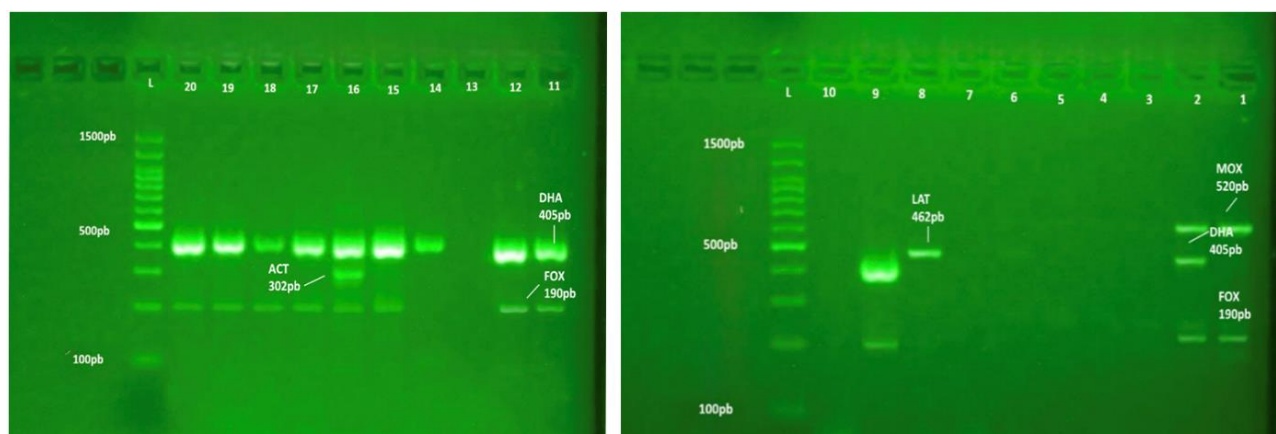
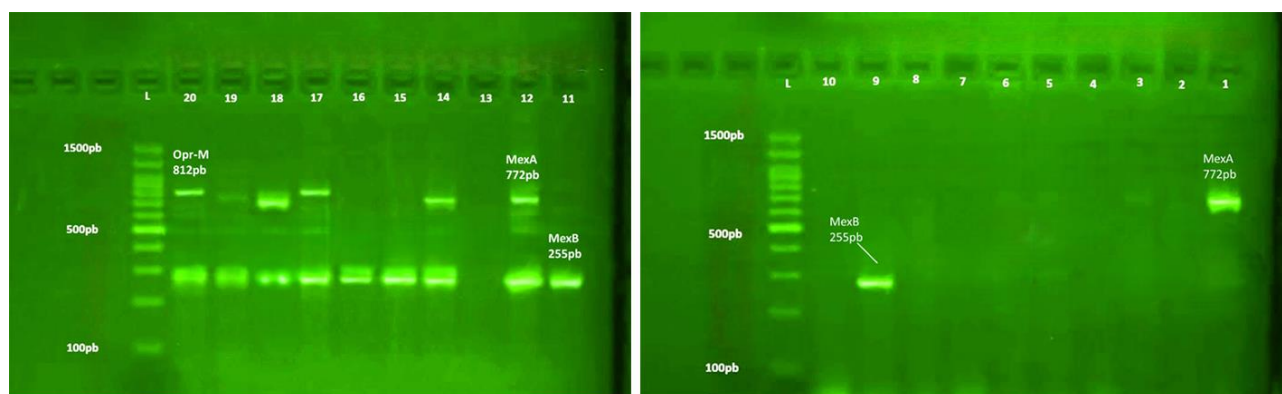


Figure 5 Agarose gel electrophoresis (2% agarose gel for 1 hour) of *bla*_{DHA} (405 bp), *bla*_{LAT} (462 bp), *bla*_{FOX} (190 bp), *bla*_{ACT} (302 bp), and *bla*_{MOX} (520 bp) in *Pseudomonas aeruginosa* isolates. Lane L: 1kb DNA ladder, lanes 1-20: amplified *Pseudomonas aeruginosa* PCR products.

The results of 31 MDR isolates were examined for the presence of efflux pump-associated genes the targeted genes included *mexA*, *mexB*, and, which are components of the MexAB-OprM efflux pump system. It showed that *mexB* was the most prevalent, detected in 48.4% (15/31) of the isolates and *mexA* was detected in 16.1% (5/31) of the isolates, whereas *oprM* was identified in 12.9% (4/31) of the isolates. The distribution of efflux pump genes among the studied isolates is summarized in Table 4. The PCR amplification products of the detected genes were visualized by agarose gel electrophoresis, as shown in Figure 6.

Table 4 Distribution of efflux pump genes among 31 multidrug-resistant *Pseudomonas aeruginosa* isolates.

Efflux pumps genes	Positive isolates	Percentage
<i>mexA</i>	5	16
<i>mexB</i>	15	48
<i>opr-M</i>	4	13

**Figure 6** Agarose gel electrophoresis (2% agarose gel for 1 hour) of Efflux pump genes *mexA* (772 bp), *mexB* (255 bp), *opr-M* (812 bp) in *Pseudomonas aeruginosa* isolates. Line L: 1 kb DNA ladder, lines 1-20: amplified *Pseudomonas aeruginosa* PCR product.

3.5 Correlation Between Phenotypic Inhibitor Assays and Molecular Detection of AmpC and Efflux Pump Genes

To evaluate the association between the phenotypic effect of cloxacillin inhibition and the presence of AmpC β -lactamase genes, the results showed that 12 isolates carrying AmpC genes exhibited a reduction in resistance after the addition of cloxacillin, whereas 8 AmpC-positive isolates did not show any effect; among the isolates lacking AmpC genes, 9 isolates demonstrated a reduction in resistance following cloxacillin treatment, while 2 isolates showed no change. Table 5 presents the distribution of isolates by AmpC gene presence and the phenotypic effect of cloxacillin. Statistical analysis using the Chi-square test showed no significant association between the presence of AmpC genes and the phenotypic effect of cloxacillin ($p = 0.214$).

Table 5 Correlation between AmpC gene detection and phenotypic effect of cloxacillin among 31 MDR of *P. aeruginosa* isolates.

Ampc genes	Cloxacillin effect	Number
Positive	yes	12
Positive	no	8
Negative	yes	9
Negative	no	2
P-value	>0.05	0.214

The phenotypic effect of the PA β N inhibitor was compared with the presence of efflux pump genes. The results showed that 12 isolates carrying efflux pump genes exhibited a reduction in resistance after the addition of PA β N, whereas 6 efflux-positive isolates did not show any effect. Among the isolates lacking efflux pump genes, 7 isolates demonstrated a reduction in resistance following PA β N treatment, while 6 isolates showed no change. The distribution of isolates according to the presence of efflux pump genes and the phenotypic effect of PA β N is presented in Table 6. Statistical analysis using the Chi-square test showed no significant association between the presence of efflux pump genes and the phenotypic effect of PA β N (p value = 0.47).

Table 6 Correlation between Efflux pump gene detection and the phenotypic effect of PA β N among MDR *Pseudomonas aeruginosa*.

Efflux pump genes	PA β N effect	Number
Positive	yes	12
Positive	no	6
Negative	yes	7
Negative	no	6
P-value	>0.05	0.470

The phenotypic effect of mix inhibitors (cloxacillin, PA β N) was compared with the presence of AmpC β -lactamase and efflux pump genes. The results showed that 15 isolates carrying resistance genes exhibited a reduction in resistance after the addition of the inhibitor combination (cloxacillin + PA β N), whereas 6 isolates did not show any effect. Among isolates lacking resistance genes, 9 showed reduced resistance after treatment with the inhibitor mixture, while only one showed no change. The distribution of isolates according to the presence of resistance genes and the phenotypic effect of the inhibitor combination is presented in Table 7. Statistical analysis using the Chi-square test showed no significant association between the presence of resistance genes and the phenotypic effect of the inhibitor combination ($\chi^2 = 1.336$, p = 0.248).

Table 7 Correlation between resistance gene detection and the phenotypic effect of the inhibitor combination (cloxacillin + PA β N) among MDR isolates (n = 31).

Resistance genes	Mix effect	Number
Positive	yes	15
Positive	no	6
Negative	yes	9
Negative	no	1
P-value	>0.05	0.248

4. Discussion

Our findings reveal a concerning epidemiological situation in Babylon province, characterized by a high prevalence of MDR (50.2%). This high MDR rate aligns with several studies from the region, including reports from Basra (66.3%) and Erbil (66%), and exceeds rates previously documented in other Iraqi cities like Al-Hilla (20%) and Al-Najaf (12.4%) [18-22]. This variability

underscores the localized nature of antibiotic resistance, likely driven by differences in antibiotic prescription policies and infection control practices. The high prevalence of resistance in our setting may reflect empirical and often indiscriminate use of broad-spectrum antibiotics, underscoring the need for stricter stewardship programs [19].

A critical finding of our study is the alarming level of resistance to last-resort antibiotics. The observed resistance to carbapenems (Meropenem 97.3%, Imipenem 78.9%) is substantially higher than rates reported in neighboring countries like Jordan (21%) and Saudi Arabia (30%), and even exceeds a previous report from Basra (68.4%) [20, 23]. Similarly, the near-universal resistance to aminoglycosides and quinolones, coupled with 100% resistance to Polymyxin B. The 100% resistance rate observed to Polymyxin B is clinically concerning because polymyxins are considered last-line therapeutic options for multidrug-resistant Gram-negative infections. However, this finding should be interpreted cautiously due to the limitations of the susceptibility testing method used. Further confirmation using reference methods is recommended to accurately determine polymyxin resistance severely limits therapeutic options. This finding is particularly disturbing as it suggests a potential loss of efficacy for drugs considered the final line of defense against MDR Gram-negative infections. The most effective agent in our study, Piperacillin-tazobactam (59.6% susceptibility), may soon become compromised if current trends continue.

In our study, the results after the addition of inhibitors (cloxacillin, PA β N) revealed variable effects on antimicrobial resistance patterns and noticeable reductions in resistance rates for several antibiotics, indicating the possible contribution of different resistance mechanisms in the studied isolates. Our findings appeared after the addition of PA β N to tested antibiotic was appeared moderate changes in resistance levels for some antibiotics this similar to a study conducted in California by [24] finding the resistance of MDR *Pseudomonas aeruginosa* isolates reduced after the addition of PA β N inhibitors. Another study in Canada by [25] appeared the PA β N significantly reduced MICs for piperacillin, cefotaxime, ceftazidime, and ciprofloxacin. In this study a considerable reduction in resistance was observed after the addition of cloxacillin for several β -lactam antibiotics. This result aligns with a study in France by [26], which demonstrated that inhibition of AmpC β -lactamase using cloxacillin can reduce resistance to extended-spectrum cephalosporins in *Pseudomonas aeruginosa*. Similar results were reported in a study conducted in China by [12], which demonstrated that the use of cloxacillin and the efflux pump inhibitor PA β N significantly improved the detection of ESBL-producing *Pseudomonas aeruginosa*. The sensitivity of the screening tests increased from about 29% with conventional methods to approximately 94-97% when both inhibitors were used together. The study used several β -lactam antibiotics, including ceftazidime, cefotaxime, aztreonam, and cefepime, in double-disk synergy and combined disk tests. In this study PCR testing showed that 31 MDR isolates revealed the presence of several plasmid-mediated AmpC β -lactamase genes, Similar results were reported in a study conducted in Al-Najaf city by [27] demonstrated that the *bla*_{DHA} family was the most prevalent (37.5%) followed by the *bla*_{FOX}, *bla*_{MOX}. Another study in Al-Najaf city conducted by [28] revealed high rate and frequency of *bla*_{DHA} 30 (93.75%), while *bla*_{MOX}, *bla*_{FOX} not detected in this study. while a study done in Iran by [29] they recorded different rate among AmpC-family genes in *P. aeruginosa* isolates included 22.1%, 36.6% for *bla*_{FOX}, *bla*_{MOX} genes respectively, and 2.1% for *bla*_{DHA} genes compared to our study. However, in Iran a study conducted by [1] showed the *bla*_{LAT} and *bla*_{FOX} were found both as low as 1.95% and *bla*_{DHA}, *bla*_{ACT-MIR1}, *bla*_{MOX} genes were not found. In this study PCR testing showed that 31 MDR isolates revealed the presence of efflux pump-associated genes similar to a

study in Diyala city by [30] demonstrated that the (94.7%) of resistant *P. aeruginosa* isolates have *mexB*, this rate is higher than in our study. In Pakistan a study conducted by [31] appeared that *mexB* was identified in 69% resistant isolates and also found that it was a major factor in antibiotic resistance. Whereas a research in Al-Hilla city performed by [32] they noted that *mexA* was present (100%), *mexB* was found in (93.84%), and the *oprM* was present in (97.69%). In contrast, another study conducted by [33] in Iran reported that *mexA* (55.5%), *mexB* (53.3%) and *oprM* (35.5%). In Egypt a study performed by [34] appeared that *mexA* (63.8%), *mexB* (75.9%) and *oprM* (34.5%). This is following [35] who found that 83.54%, 63.29%, and 48.1% of MDR *P. aeruginosa* isolates were positive for *mexA*, *mexB*, and *oprM*, respectively. Differences between the present findings and previous studies may be attributed to geographic variation, differences in antimicrobial usage patterns, Alteration in mechanism of antibiotic resistance such as using alternative pathway for antibiotic resistance, patient populations, and study design.

5. Conclusion

The resistance rates in *P. aeruginosa* were higher than global values; this resistance was attributed to several mechanisms. This high resistance is alarming and necessitates applying strict antibiotic prescription policies.

5.1 Limitation of the Study

Several limitations should be considered in this study. Quality control testing using reference strains, such as *Pseudomonas aeruginosa* ATCC 27853, was not performed, which limit the antimicrobial susceptibility testing. In addition, polymyxin B resistance was determined by the disc diffusion method, and further confirmation using reference methods such as broth microdilution is recommended. Molecular analysis was performed on a limited number of selected MDR isolates (31 isolates), which may reduce the statistical power of correlation analyses between phenotypic and genotypic findings. We did not sequence PCR products to confirm amplicon identity; future studies should include sequencing. Other important resistance mechanisms, including carbapenemase production and 16S rRNA methylase-mediated resistance, were not investigated and should be evaluated in future studies. Furthermore, clinical outcome data were not available, limiting the assessment of the clinical impact of the detected resistance mechanisms.

Author Contributions

Investigation, methodology, resources, and experimental validation: Fatima Abbas Nasser Albawi; supervision, data curation, formal analysis, project administration: Mourouge Saadi Abbas Alwash; software, manuscript drafting: Liqaa Yehya Mohsen; manuscript review and editing: Fatima Abbas Nasser Albawi, Mourouge Saadi Abbas Alwash, and Liqaa Yehya Mohsen. All authors reviewed and approved the final manuscript.

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Competing Interests

The authors declare no competing financial interests or personal relationships that could have influenced the work reported in this paper.

Data Availability Statement

The datasets generated and/or analyzed in this study are available from the corresponding author upon reasonable request.

AI-Assisted Technologies Statement

Artificial intelligence (AI) tools were used solely for basic grammar correction and language refinement in the preparation of this manuscript. Specifically, OpenAI's ChatGPT was employed to improve the readability and linguistic clarity of the English text. All scientific content, data interpretation, and conclusions were developed independently by the author. The authors have thoroughly reviewed and edited the AI-assisted text to ensure its accuracy and accept full responsibility for the content of the manuscript.

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