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by Ahmed Majeed Abdzaid

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DNA Sequencing And Phylogenic Tree Analysis Of 16S Rrna And *Bla-Oxa-50* Genes Of Carbapenem Resistant *Pseudomonas Aeruginosa* In Diwanyah City/ Iraq

Ahmed Majeed Abdzaid[1]
[1]*Department of Pathological Analysis, College of Science, University of Al-Qadisiyah, Al-Qadisiyah Province, Iraq*

Ali Sabri Jabbar[2]
[2]*College of pharmacy, University of Al-Qadisiyah, Al-Qadisiyah Province, Iraq.*

Email: [1]ahmed.majeed@qu.edu.iq, [2]*ali.sabri@qu.edu.iq*

Abstract: *Pseudomonas aeruginosa is a major nosocomial pathogen known for its multidrug resistance, including carbapenems. This study aimed to investigate the prevalence of carbapenem resistanc P. aeruginosa of clinical sources and sequencing of 16S rRNA and blaOXA-50 genes Methods: A total of 53 P. aeruginosa isolates from burn, wound, urinary tract, and ear infections were analyzed. Antibiotic susceptibility testing identified carbapenem-resistant isolates. Polymerase Chain Reaction (PCR) detected 16S rRNA and blaOXA-50 genes, followed by DNA sequencing and phylogenetic analysis of 16S rRNA. Results: Among the isolates, 20 (37.73%) originated from wounds, 16 (30.19%) from burns, 10 (18.9%) from urinary tracts, and 7 (13.20%) from ears. Notably, 12 isolates (22.64%) exhibited carbapenem resistance. All carbapenem-resistant isolates harbored both 16S rRNA and blaOXA-50 genes. 16S rRNA sequencing demonstrated 99.66% homology to known P. aeruginosa strains, further confirmed by phylogenetic analysis. The blaOXA-50 gene displayed high sequence identity (98%-99%) with isolates from Russia, suggesting a potential clonal relationship. Conclusion: A moderate prevalence of carbapenem-resistant P. aeruginosa was revealed by this study and based on 16S rRNA and blaOXA-50 genes sequencing, these strains offered a high genetic similarity compared to the global strains. Further investigation is required to determine the role of blaOXA-50 carbapenem resistance in this bacterium.*

Keywords: *P. aeruginosa, carbapenem resistance, 16S rRNA, blaOXA-50, phylogenetic tree*

INTRODUCTION

Pseudomonas aeruginosa, is a gram-negative bacterium which is categorized as antibiotic resistant bacterium, known for its ability to acquire antimicrobial resistance through treatment making it of clinical concern significance (Girlich *et al*., 2004; Poole, 2011). This bacterium is known to be as multidrug-resistant (MDR) and responsible for a variety of infections particular in immunocompromised patients such as, pneumonia, urinary tract infections, and wound infections (Alhazmi, 2015). Identification of this bacterium can be done by determining phenotypic characteristics which consider popular identification methods, in another hand the 16S rRNA housekeeping gene sequencing is considered the standardized approach for diagnosis *P. aeruginosa* which is known to be highly precise compared to other methods (Drancourt *et al*., 2000). However, for effective antibiotic therapy, understanding the resistance profile is crucial. bla-OXA-50 gene is a

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**Ahmed Majeed Abdzaid, ahmed.majeed@qu.edu.iq*

mechanism of resistance encoding to an oxacillinase enzyme that inhibit beta-lactam antibiotics, a commonly used class to treat *P. aeruginosa* infections (Girlich *et al*., 2004). Recent studies have demonstrated the increasing dissemination of *bla-OXA-50*, particularly among MDR strains, posing a therapeutic challenge (Feng *et al*., 2017; Petrova *et al*., 2019; Stanton *et al*., 2022). Whole genome sequencing has become a potent method for thoroughly identifying P. aeruginosa antibiotic resistance genes, enabling a more focused therapeutic approach (Ahmed, 2022; Peykov & Strateva, 2023). The present study aimed to molecular identification of carbapenem resistant *P. aeruginosa* isolated from clinical sources using 16S rRNA sequencing and construction of phylogenetic tree along with *bla-OXA-50* sequencing in Al- Diwaniya city/ Iraq.

METHODOLOGY

Sample collection and identification

A total of 53 sample of *P. aeruginosa* of various clinical sources wound, urine and sputum recovered from patients attended to private clinics in Al- Diwaniya city/ Iraq during 2022. Samples were directly streaked onto MacConkey agar and incubated for 24 hours at 37°C, identification the isolates done by using VITEK 2 Compact system / Biomerieux – France by VITEK®2 GN cassette according to manufactures instruction.

Antimicrobial acceptability testing

Kirby-Bauer disc diffusion method was utilized for testing antimicrobial susceptibility (Andrews, 2009) using following antibiotics: cefoxitin (30µg), amikacin (30µg), ceftriaxone (10µg), cefixime (5µg), imipenem (10µg), Cefotaxime(30µg), Amoxicillin/ Clavulanic acid(30µg), gentamicin (10µg), ciprofloxacin (10µg), meropenem (10µg, ceftazidime (30µg), cefuroxime (30µg). Inhibition zone set based on Clinical and Laboratory Standards Institute (CLSI) 2020 (Institute, 2017)

Extraction of DNA from bacterial isolate

DNA was extracted from broth of bacterial growth by Genomic DNA Mini Kit's manufacturer's instructions (Geneaid/ Taiwan). The extracted DNA was evaluated with nanodrop to ensure validity concentration and purity of DNA sample. Finally, DNA contained microcentrifuge tubes were stored at -20°C in a deepfreeze for PCR.

PCR condition

Extracted DNA was set to PCR using forward and reverse primers (F and R) listed in (table 1) that Designed using Primer3 plus software.

Table 1. sequence of primers used in the study

Primers		Primer Sequences	Amplification	References
16s rRNA	F	TGGGAGGAAGGGCAGTAAAT	527 bp	Present study
	R	CGAATTAAACCACATGCTCCA		
Bla–OXA-50	F	TCGTCCTCTACGATGTGCAG	651 bp	Present study
	R	CCTTGAGACTGGCCTTGC		

A 25 µl reaction volumes prepared which contains: 12.5µl Go Taq® Green Master Mix X2, 2.5 µl(10 pico/ µl) forward primer, 2.5 µl(10 pico/ µl) reveres primer, 5 µl DNA template(250 ng), and2.5 µl nuclease-free water. PCR tubes that contain mixture above were placed in thermal cycler machine (BioRad, USA) to amplify target genes and programmed as in table (2).

Table 2. PCR thermocycling conditions of primers used in the present study

genes	Temperature (°C)/ Time						
	Initial denaturation	Cycling condition			Final extension		
		denaturation	annealing	extension			
16s rRNA	95/ 5min	94/ 30sec	50/ 30 sec	72/ 2min	72/ 7min	4 C	30
bla –OXA-50	95/ 5min	94/ 30sec	57/ 30 sec	72/ 2min	72/ 7min	4 C	30

1.5% Agarose gel was used for electrophoresis of the amplified products and stained with ethidium bromide (3µl) in a dark place. The electrode buffer used was Tris-borate-boric acid-EDTA (TBE). the first well of gel loaded with 100-2000bp ladder (Roche, New Jersey, USA) evaluate amplicon molecular weight. Electrophoresis device conducted in a constant voltage of 80 Volt for 1.5 hour. The DNA bands were visualizes using an UV light (UVItec, Paisley, UK).

DNA sequencing and genetic analysis of 16s rRNA and *bla –OXA-50*

The PCR products of 16s rRNA and *bla –OXA-50* genes amplicons were transported to Macrogen Company/ Korea by icebag by DHL for conducting DNA sequencing using AB DNA sequencing system. Molecular Evolutionary Genetics Analysis version 11 (MEGA 11) was utilized for analysis of phylogenetic tree. ClustalW was utilized for analysis of Multiple sequence alignment and Maximum Composite Likelihood method by (Kumar *et al*., 2018; Tamura & Nei, 1993) were used for evaluation of evolutionary distances. Genetic analysis of 16s rRNA and *bla –OXA-50* was performed using phylogenetic tree analysis between local *P*.

aeruginosa isolates and NCBI known isolates. Finally identified 16s rRNA and *bla –OXA-50* were submitted into NCBI-GenBank and got accession numbers.

RESULTS AND DISCUSSION

A Fifty-three sample of *P. aeruginosa* recovered from various clinical samples: Burn 20 (37.73%), wound 16(30.19%), urine 10 (18.9%) and ear 7(13.20%), figure (1). All of isolates diagnosed as a *P. aeruginosa* through VITEK 2 Compact system. These results are agreed with a study carried out in Al- Anbar province which stated that most of *P. aeruginosa* isolates recovered from burn infections (40%) followed by wound infections (16.3%) (Al-Mohammed & Mahmood, 2024). Present study also agrees with another local study which had the same findings (Al Fahadawi *et al*., 2019). This may be due to Compromised skin barrier functions in burn patients, coupled with frequent scrubbing and debridement procedures, creates a heightened risk of cross-contamination with MDR strains of Pseudomonas and colonization by these MDR bacterium (Zhapouni *et al*., 2009).

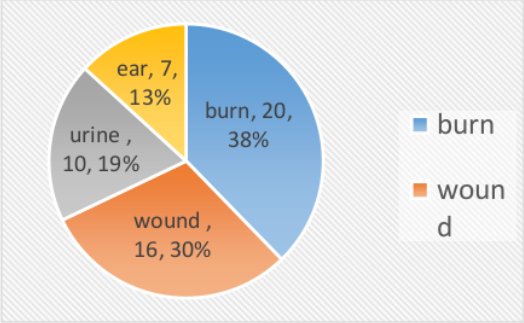


Figure 1. frequencies of *P. aeruginosa* sources of present study

Antimicrobial susceptibility testing of the present study to 53 sample of *P. aeruginosa* revealed that all of isolates were MDR, they were resistant at least three classes of antibiotics (14). However, isolates were highly resistant to amoxicillin/ clavulanic acid (86.79 %), Cefixime (83.02%), Cefotaxime (79.25 %) and Ceftriaxone (75.47%). Present results agree with local study in Hilla city which stated that *P. aeruginosa* isolates were highly resistant to treatment with these antibiotics (Al-Saffar & Jarallah, 2019) while disagree with a study conducted in Duhok city in resistant to Cefixime (39.4%) and Ceftriaxone (37%) but near to the results of the same study in resistant to Amikacin and Ciprofloxacin (Oumeri & Yassin, 2021). Antimicrobial susceptibility testing demonstrated moderate resistance rates for carbapenems in our study population of *P. aeruginosa* isolates. Imipenem resistance was noticed in 7.55% of isolates, while Meropenem resistance was present in 22.64% (Table 3).

Interestingly, only 4 isolates (33.3% of carbapenem-resistant isolates) exhibited resistance to both Imipenem and Meropenem. These findings contrast with several previous studies conducted in Iraq, which reported significantly higher carbapenem resistance rates in *P. aeruginosa* (Al-Saffar & Jarallah, 2019; Polse *et al*., 2024; Shilba *et al*., 2015). For instance, Polse *et al*. documented high resistance rates of 74.65% and 64.79% to Meropenem and Imipenem, respectively, in southern Iraq (Polse *et al*., 2024). Our results, however, align with a study from Al-Anbar province, which reported resistance rates to Meropenem and Imipenem of 20% and 5.5%, respectively (Al-Saffar & Jarallah, 2019).

Table 3. Antimicrobial Resistance Profile of 53 *P. aeruginosa*

Antibiotic	**No. of Resistant isolates (100%)**
Ceftriaxone	40 (75.47)
Cefotaxime	42(79.25)
Ceftazidime	34(64.15)
Cefoxitin	24(45.28)
Cefixime	44(83.02)
Amoxicillin/ Clavulanic acid	46(86.79)
Gentamicin	36(67.92)
Amikacin	22(41.51)
Ciprofloxacin	37(69.81)
Meropenem	12(22.64)
Imipenem	4(7.55)

Carbapenem resistance development in *P. aeruginosa* is a growing concern, particularly in regions with high antibiotic use, such as Iraq. Two primary mechanisms contribute to this phenomenon: Genetic acquisition of resistance including Plasmids or integrons harboring carbapenemase genes can be horizontally transferred between bacterial strains, leading to rapid dissemination of resistance. Secondly, Intrinsic mechanisms**:** Efflux pumps overexpression, decreased expression of outer membrane porins, and chromosomal cephalosporinase activity can all contribute to intrinsic carbapenem resistance in *P. aeruginosa* (Meletis *et al*., 2012) Further investigation into the specific mechanisms underlying carbapenem resistance in our isolates is warranted. This information is important for guiding appropriate antibiotic treatment strategies and using of an effective infection restriction measure to curb the dissemination of multidrug-resistant *P. aeruginosa*

All of 12 carbapenem resistant *P. aeruginosa* isolates that set for detection of 16s rRNA, agarose gel electrophoresis showed that all of isolates appeared to be positive to the gene in product size 527 bp, figure (2). 16S rRNA gene sequencing was employed for the definitive identification of the four selected isolates. The obtained sequences were submitted in GenBank under accession numbers OR394098.1, OR394099.1, OR394100.1, and OR394101.1. Subsequent BLAST analysis of the 16S rRNA gene sequences demonstrated a significant degree of homology (99.66%) with previously documented *P. aeruginosa* strains isolated in China and India (Table 3). This high level of sequence similarity strongly supports the identification of all four isolates as *P. aeruginosa*.

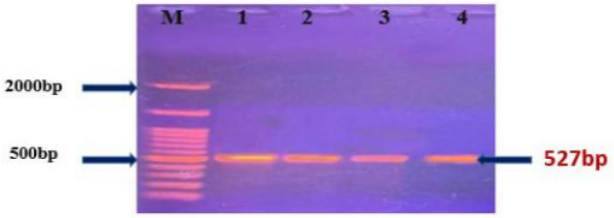


Figure 2. agarose gel electrophoresis showing positive results of 16s RNA (527 bp) of *P. aeruginosa*

Phylogenetic tree of the 16S rRNA gene sequences (Figure 3) further substantiated the identification. The analysis revealed that all four isolates clustered together within a clade containing established *P. aeruginosa* world strains. This clustering pattern suggests a close genetic relationship between the isolated strains and known *P. aeruginosa*.

The combined evidence from 16S rRNA gene sequencing, BLAST analysis, and phylogenetic tree construction provides compelling support for the definitive identification of all four isolates as *P. aeruginosa*.

Table 4. NCBI-BLAST Homology 16s rRNA sequence idenity beteween local *P. aeruginosa* isolates and NCBI-BLAST related *P. aeruginosa*

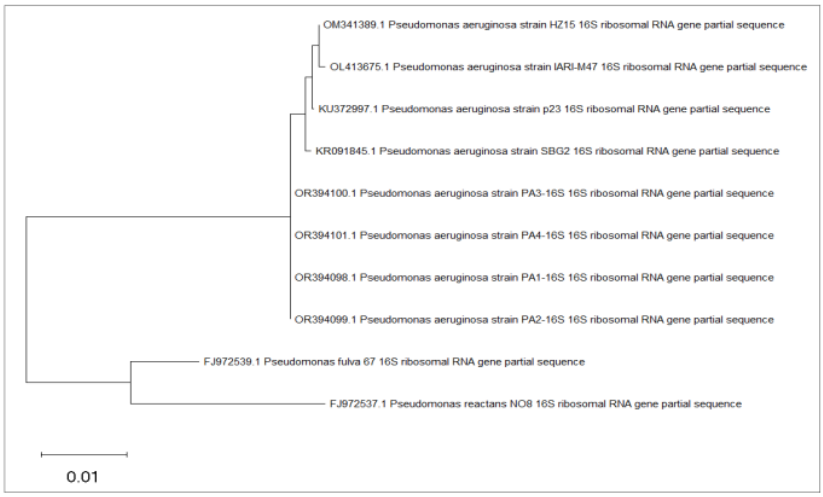
Local *P. aeruginosa*	Local isolates Accession number	NCBI-BLAST Homology sequence identity			
		Identical NCBI-BLAST *P. aeruginosa*	Accession number	Country	Idenitity (100%)
isolate No.1	OR394098.1	*P. aeruginosa* strain p23	KU372997.1	China	99.66%
isolate No.2	OR394099.1	*P. aeruginosa* strain SBG2	KR091845.1	India	99.66%
isolate No.3	OR394100.1	*P. aeruginosa* strain HZ15	OM341389.1	China	99.66%
isolate No.4	OR394101.1	*P. aeruginosa* strain IARI-M47	OL413675.1	India	99.66%

All *P. aeruginosa* isolates in this study that were specified as carbapenem-resistant harbored the blaOXA-50 gene. Gel electrophoresis confirmed the presence of this gene with

an expected amplicon size of 651 bp (Figure 3). This finding aligns with previous reports suggesting blaOXA-50 as a constituent gene in some carbapenem-resistant *P. aeruginosa* strains (Campana *et al*., 2017; Petrova *et al*., 2019). However, the role of blaOXA-50 in carbapenem resistance remains a subject of debate.

DNA sequencing of the *bla –OXA-50* gene of the four selected isolates of carbapenem resistant *P. aeruginosa* were submitted in GenBank under accession numbers: OR419505.1, OR419506.1, OR419507.1 and OR419507.1

BLAST analysis of sequencing of the gene revealed high sequence homology (98%-100%) with *blaOXA-50* sequences from isolates in Russia (Table 5). This suggests a potential clonal relationship or a common source of acquisition for this gene among these geographically distinct isolates.



Figur 3. Phylogenetic tree of *Pseudomonas aeruginosa* local strains 16s rRNA alignment with world strains.

Table 5. NCBI-BLAST Homology of *bla –OXA-50* sequence idenity beteween local *P. aeruginosa* isolates and NCBI-BLAST related *P. aeruginosa*.

Local *P. aeruginosa* isolate No.	Local isolates Accession number	NCBI-BLAST Homology Sequence Identity		
		Accession numbers	Countries	Identity (100%)
Isolate No.1	OR419505.1	CP051770.1	Russia	-100%
Isolate No.2	OR419506.1	CP051768.1	Russia	-100%
Isolate No.3	OR419507.1	CP051766.1	Russia	-100%
Isolate No.4	OR419508.1	CP051768.1	Russia	98%

The presence of *blaOXA-50* in all carbapenem-resistant isolates in this study warrants further investigation. While some studies have linked blaOXA-50 to carbapenem resistance, particularly when combined with other mechanisms, others suggest its intrinsic nature in P. aeruginosa with minimal impact on carbapenem susceptibility (5). The high sequence homology observed among isolates from Iraq and Russia highlights the potential for international spread of blaOXA-50-containing P. aeruginosa strains. Further studies are required to specify the specific contribution of *blaOXA-50* to carbapenem resistance in these isolates.

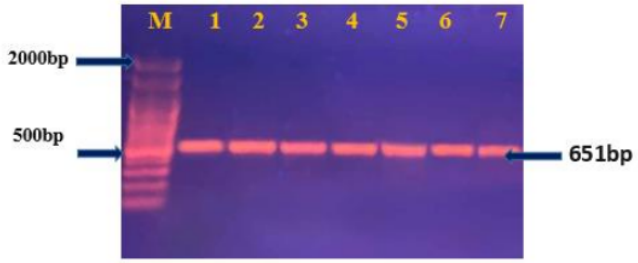


Figure 3. Agarose gel electrophoresis showing positive results of *bla –OXA-50* (651 bp) of *P. aeruginosa* that is carbapenem resistant

In conclusion, the study confirmed that carbapenem antibiotic is the most effective to treat *P. aeruginosa* infection, the study also revealed that 16S rRNA gene sequencing had high homology (>99.66%) to known *P. aeruginosa* strains. Phylogenetic analysis further supported this identification. Additionally, blaOXA-50 detection and sequencing revealed high identity (98%-100%) with isolates from Russia, suggesting a potential clonal relationship.

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