

Molecular Characterization of Siderophore-Encoding Genes in Multi-Drug Resistant *Pseudomonas Aeruginosa* from Wound Infections

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ABSTRACT

Background: *Pseudomonas aeruginosa* is a gram-negative bacillus commonly associated with wound infections. This pathogen has demonstrated a remarkable ability to acquire antibiotic resistance genes, including those conferring resistance to carbapenems. **Objective:** This study aimed to identify and characterize siderophore genes in carbapenem-resistant *P. aeruginosa* isolates from wound infections. **Materials and Methods:** Bacterial isolates were obtained from swab samples of patients with complicated wound infections treated at three hospitals in Baghdad, Iraq: Medical City Baghdad (Al-Burns Specialist Hospital), Al-Yarmouk Teaching Hospital, and Al-Kindi Teaching Hospital. Samples were collected between November 2023 and February 2024, totaling 200 swabs from patients displaying signs of wound infection. The majority of patients were female (59%), with males comprising 41%. Samples were cultured on nutrient agar, blood agar, MacConkey agar, and Cetrimide agar, followed by incubation at 37°C for 24 hours. **Results:** From the 200 clinical samples, 59 isolates (29.5%) were identified as *P. aeruginosa*. Molecular analysis revealed that all isolates (100%) tested positive for the 16S rRNA housekeeping gene, while 80% of isolates harbored the *plcN* gene, and 80% tested positive for the *exoS* gene. These findings indicate a strong association between the presence of *plcN* and *exoS* genes and the virulence of multidrug-resistant *P. aeruginosa*. **Conclusion:** The high prevalence of the *plcN* and *exoS* virulence genes in multidrug-resistant *P. aeruginosa* isolates underscores their potential role in enhancing the pathogen's virulence and resistance. These findings emphasize the need for targeted strategies to mitigate the impact of these highly virulent strains in clinical settings.

Keywords: Multidrug Resistant, *Pseudomonas Aeruginosa*, *PlcN*, *ExoS*

INTRODUCTION

A prevalent opportunistic pathogen linked to infections acquired in healthcare settings is the gram-negative bacteria *P. aeruginosa* [1]. It is recognized bacterial pathogen that has the potential to cause severe infections in humans. *Pseudomonas aeruginosa* exhibits both inherent and acquired resistance to a broad range of antibiotics, which significantly contributes to the incidence of health- multidrug resistant (MDR) strains in both community and healthcare settings [2]. The number of *Pseudomonas aeruginosa* infections has surged in recent years, affecting public health globally and leading to illnesses that are challenging to treat [3]. The World Health Organization (WHO) has consequently classified *P. aeruginosa* as one of the bacteria that is most resistant to drugs, requiring immediate study and the creation of potent treatment approaches [4]. *Pseudomonas aeruginosa* is known to cause an extraordinary range of human diseases. The pathogenesis of wound infection by *P. aeruginosa* is attributed to its ability to produce multiple virulence factors (VFs) [5]. It has different virulence factors, such as flagella, pili and lipopolysaccharide which assist the bacteria attach or /colonize the host, it also has secretion systems that carry effectors and toxins to the host, proteases that cause tissue damage, biofilm and quorum sensing which allow the bacteria to communicate and promote drug resistance [6]. Numerous bacteria produce Siderophores, which are low molecular weight iron chelators (less than 10 KD), under iron-limited conditions, large-scale complexes with free iron, which it then transfers into the cell via membrane receptor molecules generated by five genes in an operon that is deactivated once the cell has detected adequate iron [7]. Almost all kinds of bacteria require Iron as a

nutrition. as a host innate immunological response to invasive infections, iron regulation serves human [8]. Nosocomial infections, often called hospital-acquired infections (HAIs), are a worldwide epidemic that especially impacts hospitals' surgical, burn, and critical care units (ICUs). Hospital stays, patient quality of life, treatment expenses, and mortality are all negatively impacted by nosocomial infection complications, which in turn worsen the underlying disease prognosis [9]. *Pseudomonas aeruginosa* responsible for (10 to 11) percent of HAI cases [10].

This disease has gained resistance to a range of antibacterial medications and disinfectants, resulting in its high incidence in hospitals. *Pseudomonas aeruginosa* causes respiratory, urinary tract, and wound infections in hospitals and can induce bacteremia, especially in patients in intensive care units and on mechanical ventilators [11, 12]. The MDR *Pseudomonas aeruginosa* phenotype is defined as a bacterium that is resistant to at least three classes of antibiotics (penicillins / cephalosporins, carbapenems, fluoroquinolones and aminoglycosides) by using specific enzymes that make carbapenems and beta-lactams deficient, such as metallo β -lactamases (MBLs) and extended- spectrum beta lactamases (ESBLs) [13].

MATERIALS AND METHODS

Collection of Samples

Two hundred wound swab samples from patients with wound infections of all ages and genders were collected from the Al - Kindi

Teaching Hospital, Al-yarmok Teaching Hospital and Medical City Baghdad, Iraq, (Al- Burns Specialist Hospital). from early November 2023 to late February 2024. Identification of *P. aeruginosa* manual biochemical tests used and the VITEK2 compact system.

Antimicrobial Susceptibility

In according with (Institute 2017) this experiment was carried out [13]. For each isolate, a 0.5. McFarland standard was determined. Mueller-Hinton. agar plates have been covered with the standard suspension, which has been let dry for five minutes to test for antibiotic sensitivity.

The *P. aeruginosa* isolates (59) have been examined against nine antibiotics Azetreonom (AT), Amikacin (AK), Ceftazidime (CAZ), Ciprofloxacin (CIP), Gentamicin (GM), Imipenem (IPM), Levofloxacin (LE), Meropenem (MEM), Tobramycin (TOB), using the disc diffusion technique. After that, the plates were turned over and incubated at 37°C for 24 hours. a metric ruler was used to measure the inhibitory zones that the discs produced in millimeters

(mm), as reported through the Laboratory and Clinical. Standards Institute [14]. The isolates were compared to. Typical inhibitory zones in order to determine if they were intermediate, sensitive, or resistant to the antibiotic [15].

DNA Extraction and its Quantified Concentration and Purity

Using an extraction kit manufactured by BIOMERIEUX (France), DNA was extracted from the *P. aeruginosa* isolates, detected by the Vitek-2 system. the extraction operation went smoothly [16].

Detection of the *Pseudomonas Aeruginosa*

Extraction of DNA

The genes encoding the siderophore genes resistance determinants, *plcN* and *exoS*, were analyzed by using the specific primers listed in Table (1). in a 25 µL PCR amplifying procedure, each primer experienced a five-minute initial denaturation step at 94°C, which was followed by 38 cycles of 95°C for 30 seconds, 54°C for 45seconds, and 72°C for seven minutes, as indicated in Table (2).

Table 1: Primers used in PCR to Detect *plcN*, *exoS*, and 16srRNA Genes of *P. aeruginosa*

Gene	Sequence of Oligonucleotide Primers: 5' To 3'	Size of Pcr Product (Bp)	Reference
Exos	F: CTGGAAGGGACTCGACAAGG R: TTCAGGTCCGCGTAGTGAAT	504	[16]
Plcn	F: GTTATCGCAACAGCCCTAC R: AGGTCGAACACCTGGAACAC	466	[6]
16s Rma	F: GGGGGATCTTCGGACCTCA R: TCCTTAGAGTGCCACCCG	956	[17]

Table 2: Constituent of PCR Master Mix Reaction. The cycle parameters for amplifying the 16srRNA gene of *Pseudomonas aeruginosa*.

Components for the PCR Master Mix Reaction			Volume
One Master Mix of Taq (NEB®)			12.5µl
Template of the DNA			3µl
Primers	Forward		1µL
	Reverse		1µL
Nuclease-free H ₂ O			7.5µL
Total Volume			25Ml

Polymerase Chain Reaction (PCR)

This step was carried out by adding (12.5 µl) from One Taq (NEB®) master mix, (3 µl) of DNA sample, (1 µl 10) pmol/µl from each primer and (7.5 µl) of free-nuclease water. The reaction done under the optimal PCR conditions for gene. add nuclease-free water to get a final volume of (25µl).

The PCR premix, primers, and extracted DNA were thawed at 4°C, vortexed, then brief centrifugation to concentrate the contents. to the

bottoms of the tubes. Following a series of experiments, the PCR was fine-tuned to achieve optimal performance.

The negative control consisted of all components, except for DNA. instead of using template DNA, nuclease-free water was added. PCR programs were executed on the Thermal-cycler manufactured by Applied Biosystems in the United States. the PCR Thermocycler quantities were determined using the Conventional PCR Thermocycler, as specified in Table (3) and (4).

Table 3: PCR Thermo-Cycle Conditions for 16S rRNA Genes

Loop's steps	Temp. (C°)	Time,	Number of cycles
Initial Denaturation.	94	5 minutes	1
Denaturation.	94	30 second	38
Annealing.	49	45 second	
Extension	72	45 second	
Final Extension	72	7 minutes	1

Table 4: PCR Thermo- Cycle Conditions for the *plcN* and *exoS* Genes

Loop's steps.	Temp (C°)	Time.	Number of cycles
Initial Denaturation.	95	5 minutes	1
Denaturation.	95	30 second	38
Annealing	52	45 second	
Extension	72	45 second	
Final Extension	72	5 minutes	1

Results and Discussion

Isolating and Identifying of *P. Aeruginosa*

By using sterile cotton swabs, samples were collected and then transported to laboratory immediately. The swabs were streaked on nutrient, MacConkey, blood, and on the selective medium *Pseudomonas* cetrimide agar (PCA) plates and then they were kept overnight at 37°C to observe colony morphology. Colonies that are suspected as *P. aeruginosa*, were processed further for conventional biochemical tests: indole, cytochrome oxidase, catalase, motility, and citrate utilization tests. VITEK 2 system and molecular markers based on the amplification of 16S rRNA were used to characterize and confirm the identification of presumptive *P. aeruginosa* isolates. Morphological characterization of the *P. aeruginosa*. These 59 isolates were capable of growing on cetrimide agar as a selective medium and appeared as smooth and greenish-yellow colony as well as it appeared as lactose-non-fermenting mucoid colonies on MacConkey agar; beta-hemolytic brownish gray colonies on blood agar; and smooth greenish blue colonies on nutrient agar with grape like odor. They showed negative result for indole test and positive results for

cytochrome oxidase, catalase, mannitol motility, and citrate utilization tests [18, 19]. Cetrimide agar as a selective medium highly selective and sensitive media for *P. aeruginosa* (smooth greenish). Approximately 59 isolates of the *P. aeruginosa* were verified on the cetrimide agar, a diagnostic and differential medium used to distinguish between different isolates obtained from wound infections samples [20]. these isolates were previously grown on the conventional culture media and then identified and diagnosed [21].

Analysis of bacterial isolates obtained from wound infections.

Table (5) showed, 156 (78%) of the specimens exhibited growth, while 44 (22%) had no growth, *P. aeruginosa* had the highest percentage, representing (29.5%), followed by *E. coli* with (10.5%), followed by *Staphylococcus aureus* (9.5%), and *Klebsiella pneumonia* (8.5%), *Proteus mirabilis* (7.5%), *Acinetobacter* spp (6.5%), *Strep. Pyogenes* (4.5%) and *Enterococcus* spp (1.5%). This similar with local study Hateet [22], Revealed that *P. aeruginosa* was the predominant pathogen (20%) followed by *Enterobacter* spp. (16.19%), *S. aureus* (17.14%), *Proteus vulgaris* (13.33%), *Klebsiella pneumoniae* (6.6%), *E. coli* (7.6%), and *Staphylococcus lentus* (4.76%), *P. mirabilis* (10.47%).

Table 5: Numbers and Percentage of all Specimens (N=200)

Bacteria	Number	Percentage %
<i>Pseudomonas Aeruginosa</i>	59	29.5 %
<i>E.Coli</i>	21	10.5 %
<i>Staphylococcus Aureus</i>	19	9.5 %
<i>Klebsiella Pneumonia</i>	17	8.5 %
<i>Proteus Mirabilis</i>	15	7.5 %
<i>Acinetobacter Spp</i>	13	6.5 %
<i>Strep. Pyogenes</i>	9	4.5 %
<i>Enterococcus Spp</i>	3	1.5%
No Growth	44	22 %
Total	200	100%
Chi Square		114.651
P –Value		* (P≤ 0.05)

As for age, the age groups between (21-30) years had the greatest infection rate 24%, followed by the age groups between (11-20) were 21%. the rates for the groups (1-10) were 18%, while the group (31-40) were 16%, and (41-50) were rate 14%, The lowest rates were 2.5% for the age (61-80) and 4.5% for the group (51-60). This study is comparable to Rao et al.[5], Among the 50 samples collected from

patients with burns, 18 (36%) were from males and 32 (64%) were from females. The largest proportion of patients fell within the 21-30 age range (32%), followed by the 0-10 age range (28%), 11-20 age range (18%), 31-40 age range (8%), 41-50 age range (8%), and 51-60 age range (4%), with the 61-70 age range having the smallest number of patients (2%).

Table 6: Rates of Infection by Age Categories

Age	No. Infection	Percentage%
1 - 10 years	36	18%
11 - 20 years	42	21%
21 - 30 years	48	24%
31 - 40 years	32	16%
41 - 50 years	28	14%
51 - 60 years	9	4.5%
61 - 80 years	5	.25%
Total	200	100%
Chi Square		41.334
P –value		* (P≤ 0.05)

In this cross-sectional study, two hundred *P.aeruginosa* strains were isolated from various clinical wound infections samples. The following is a full account of the hospital wards, samples and patients: of the strains of *P. aeruginosa*, 41% (n = 82) came from males and 59 % (n = 118) from females.

This is shown in the Table (7) where it was found that the rate of infections in female highest than male [23, 24]. This study is similar

to Bazghandi et al.[25], In this cross-sectional study, 84 *P. aeruginosa* strains were isolated from various clinical samples. The following is a full description of the hospital wards, specimens and patients: were as follows: The *P. aeruginosa* strains, 52.4% (n = 44) came from females and 47.6% (n = 40) from males. Also local study by Raheem and Abdalshheed [12], that found male (28%) and female (72%).

Table 7: Percentages of Infections by Gender

Gender	Number	Percentage %
Female	118	59%
Male	82	41%
Total	200	100 %
Chi Square		6.48
P –value		* (P≤ 0.05)

The antibiotic susceptibility test for *P. aeruginosa* was done using the Kirby Bauer disk diffusion method on a Mueller-Hinton agar, as indicated in Table (8). all detected *P. aeruginosa* strains were tested with antimicrobial agents. The results showed that all strains of *P. aeruginosa* showed highest resistance to Tobramycin, with a prevalence of (71.18%). This corresponds to Shatti et al. [13], were the highest rate of resistance against tobramycin (75%). and resistance to Aztreonam at (66.10%), Ceftazidime (42.3%), Gentamycin (35.5%), and Amikacin (30.5%), lower than local study Alkhulaifi and

Mohammed [19], with resistance rate Aztreonam (82.11%), Ceftazidime (98.95%), Gentamycin (68.40%), Amikacin (69.48%) and the lowest resistance was Imipenem (25.4%) Meropenem (28.8%). Similar to study done by AL-Ameen and Ghareeb. (28), were demonstrated Carbapenems class Imipenem (21.7 %) and Meropenem (23.9%). Quinolones resistance of Ciprofloxacin (33.8%), Levofloxacin (32.2), similar to local study done by Kazem and Muhammad [26], were recorded Ciprofloxacin (38.2% Levofloxacin (41.2 %).

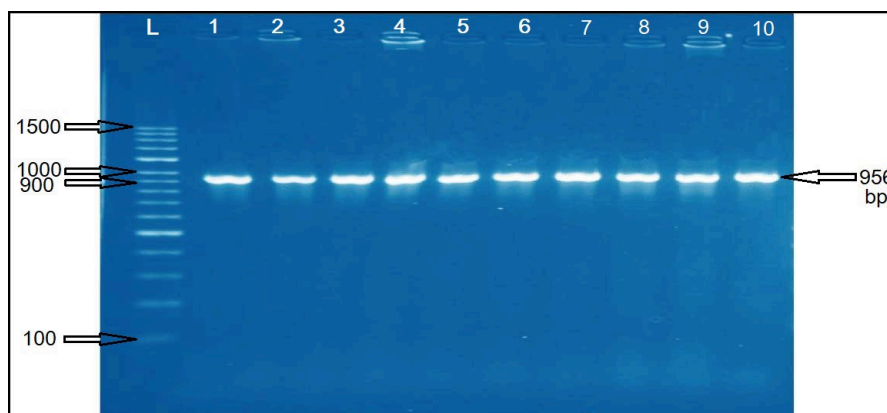
Table 8: Antimicrobial Susceptibility Test of 59 *P. aeruginosa* Isolates Against Nine Antimicrobial Agents

Antibiotics	Disc content (µg)	No. isolated	R	No. isolated	I	No. isolated	S
Aztreonam (AT)	30	39	66.10%	0	0%	20	33.8%
Amikacin (AK)	30	18	30.5%	5	8.4%	38	64.4%
Ceftazidime (CAZ)	30	25	42.3%	10	16.9%	23	38.9%
Ciprofloxacin (CIP)	5	20	33.8%	8	13.5%	23	38.9%
Gentamycin (GEN)	10	21	35.5%	0	0%	36	61%
Imipenem (IPM)	10	15	25.4%	4	6.7%	30	50.8%
Levofloxacin (LE)	5	19	32.2%	12	20.3%	27	45.7%
Meropenem (ME)	10	17	28.8%	2	3.3%	33	55.9%
Tobramycin (TOB)	10	42	71.18%	0	0%	17	28.8%

Identification of *P. aeruginosa* by Molecular Detection of the 16SrRNA Gene.

The study examined a total of n=10 isolates that were resistant to mutidrugs. The identified strains were discovered in samples taken from clinical wound infections [27, 28]. Employing the specific initiator of the *16S rRNA* gene for identification purposes to confirm the diagnosis made by PCR. all of the bacterial isolates were identified

as forming the species *Pseudomonas aeruginosa*. The positive isolates identified by using the Vitek2 system the assay yielded results that were positive when the 16SrRNA gene was identified using polymerase chain reaction amplification. The PCR results are verified through the analysis of bands using gel electrophoresis comparing their molecular size with a 1500 bp DNA Ladder. The PCR Figure (1) displays the outcomes of the reaction for the *16S rRNA* gene [29].

**Figure 1:** The PCR Product of 16SrRNA gene (956 bp) of *P. aeruginosa* was Electrophoresed on a 1500bp DNA ladder in gel. (1% Agarose, 90 V for 70 minutes).

Identification of *Pseudomonas aeruginosa* *plcN* and *exoS* Genes.

To identify the existence of the *P. aeruginosa* phospholipase C (*plcN*) and exoenzyme S (*exoS*) genes. The PCR results were verified through the analysis of bands using gel electrophoresis and by comparing their molecular size with a 1500 bp DNA Ladder. Among of the total isolates, n=8 (80 %) was positive for the *plcN* gene, as shown

in figure (2). And n= 8 (80%) isolates were positive for *exoS*, as shown in figure (3). And consistent with Ali, Al-Kenanei, and Bdaiwi. [28], Conventional PCR was used to detect the genotypes of many virulence factors associated with *P. aeruginosa*. The genes that were included include *exoS*, which codes for exoenzyme S, and *plcN*, which codes for phospholipases C.

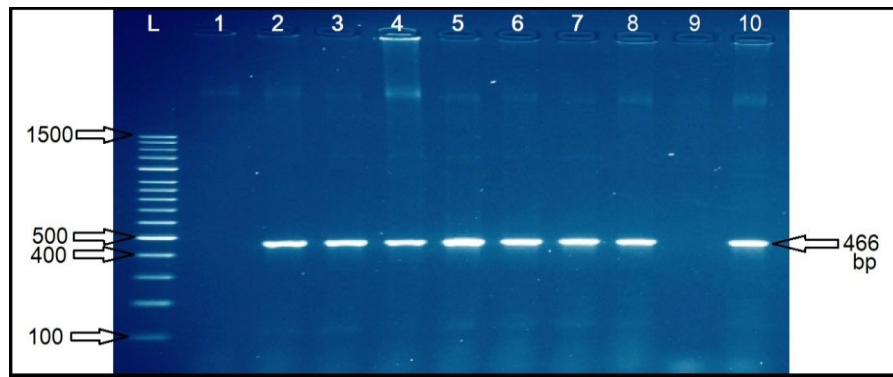


FIGURE 2: The PCR product of *plcN* Gene (466 bp) of *P. aeruginosa*, was Electrophoresed on a 1500bp DNA ladder in gel. (1% Agarose, 90 V for 70 minutes)

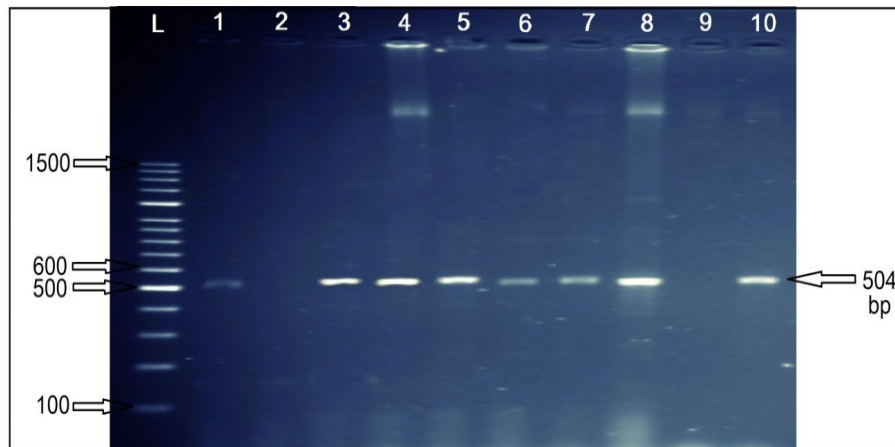


FIGURE 3: The PCR product of *exoS* gene (504 bp) of *P. aeruginosa*, was Electrophoresed on a 1500bp DNA ladder in gel. (1% Agarose, 90 V for 70 minutes)

Conclusion

The study found that *P. aeruginosa* pathogenesis were common and several isolates showed a high level of the multidrug resistant, which

are considered the one of last options for treating infections. Also found that the genes *plcN* and *exoS* are essential in the creation of siderophores, which are associated with a high rate of detection and multidrug resistance in the *P. aeruginosa*.

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