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Carbapenem Resistance Related with Biofilm Formation and Pilin Genes in Clinical *Pseudomonas aeruginosa* Isolates

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Abstract

Pseudomonas aeruginosa is a common cause of nosocomial infections worldwide, and infections caused by this bacterium are difficult to eradicate because it is intrinsically resistant or less susceptible to several antimicrobial agents. The study aimed to identify the genotype distribution or frequency of virulence factors genes (*algD, pilA, and pilB*) involved in alginate and the type 3 system of carbapenem-resistant *P. aeruginosa* local isolates. The genotype distribution of 25 carbapenem-resistant *P. aeruginosa* involving the alginate was noted with the highest frequency (100%), the genes that encoded for pilin structural subunits were noted with the lowest frequency (4%) for *pil B* gene, and *pil A* gene showed (92%). The results of the PCR detection revealed a high spread for the *alg D* gene, all resistant isolates contained this gene at the same time these isolates were 100%, there is a correlation between the alginate production and *P. aeruginosa* ability to form biofilm. chi-square showed a significant association between the biofilm density and carbapenem resistance ($X^2 = 14.62$, $P < 0.023$).

Keywords: Carbapenem Resistance, Genotype distribution, virulence factors, *Pseudomonas aeruginosa.*

مقاومة الكاربابينيم المرتبطة بتكوين الغشاء الحيوي وجينات البيلين في عزلات سريرية لبكتريا الزوائف الزنجارية المعزولة محليا

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الخلاصة

تعتبر بكتريا الزوائف الزنجارية هي من المسببات الشائعة لعدوى المستشفيات في جميع أنحاء العالم ، ومن الصعب القضاء على الالتهابات التي تسببها هذه البكتيريا لأنها تمتلك صفة المقاومة أو أقل حساسية لتأثير العديد من العوامل المضادة للميكروبات. هدفت الدراسة إلى تحديد توزيع التركيب الجيني أو تواتر جينات عوامل الضراوة في العزلات السريرية حيث درست توزيع النمط الوراثي لـخمس وعشرون عزلة من بكتريا المقاومة للكاربابينيم .حيث كانت جينات الألجينات بتكرار عالي (١٠٠٪) ، وقد لوحظت ان جينات البيليين بأقل تردد (٤٪) لجين بيل **A** ، في حين أظهر الجين بيل **B** تردد (٩٢٪). أظهرت نتائج الكشف عن تفاعل البلمرة المتسلسل انتشارًا عاليًا لجين الالجينات **D** ، حيث احتوت جميع العزلات المقاومة على هذا الجين في نفس الوقت الذي كانت فيه هذه العزلات تنتج غشاء حيوي بنسبة ١٠٠٪ ، هناك علاقة بين إنتاج الألجينات وقدرة الزوائف الزنجارية على تكوين الاغشية الحيوية . أظهرتحليل مربع كاي ارتباطًا معنويًا بين كثافة الأغشية الحيوية والمقاومة **($X^2 = 14.62$ ، $P < 0.023$)**.

الكلمات المفتاحية: المقاومة للكاربابينيم ، توزيع النمط الجيني،عوامل الضراوة ، بكتريا الزوائف الزنجارية .

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that is one of the five most important nosocomial microbes in the world (Al-Azzawi, 2018). It is a heterotrophic, rod-shaped bacterium that is 1-5mm long and 0.5-1.0mm wide (Diggle & Whiteley, 2020). Non spores forming and it is motile by one or two polar flagella **(**Haiko *et al*., 2013**;** AL-Essawi *et al*.,2021**)**. This pathogen can cause both acute and chronic infections in humans but it can cause opportunistic infections in people with weakened immune systems, it is also a major contributor to antibiotic resistance and hospital-acquired infections (Al-Wrafy *et al*., 2017; Khudair & Mahmood, 2021; Mahmood, et al., 2020). Pathogenesis of *P. aeruginosa* is caused by the development of a variety of extracellular and cellular virulence factors such as adhesion factor type IV pili and alginate (Shugang *et al*., 2022).

These virulence factors play a significant role in pathogenicity by supporting bacterial surface colonization, survival, and invasion of host tissues (Hogardt & Heesemann, 2011; Al-Ani *et al.*, 2020). The biofilm is composed of many microcolonies involved in an Exopolysaccharide called Alginate produced by *P. aeruginosa* (Al-Azzawi*l*., 2018). The development of *P. aeruginosa* mucoid colonies formed of alginates and involving the *algD* gene, and the pathogen resistance to the host immune response and antimicrobials (Ra'oof, 2011; AlRawi *et al*.,2022). Carbapenem antibiotics are widely used to treat multidrug-resistant Gram-negative pathogenic bacteria due to their broad antibacterial spectrum (Meletis, 2016).

P. aeruginosa with multidrug resistance (MDR) and extended drug resistance (XDR) had clinically emerged as a result of the widespread use and excessive of carbapenem antibiotics, posing a significant burden and challenge to clinical anti-

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infection treatment (Kunz Coyne *et al*., 2022). Carbapenem resistance mechanisms can occur due to a variety of reasons, such as decreased outer membrane permeability, downregulation in outer membrane porins such as OprD, production of beta-lactamase enzymes, and overexpression of pump efflux systems (Qader *et al*., 2022). The study aimed to identify the frequency of virulence factors genes (*algD*, *pilA*, and *pilB*) involved in alginate and the type III secretion system toxins of carbapenem-resistant *P. aeruginosa* local isolates.

Methods

Sample collection protocol

Clinical samples were taken from wounds, burns, urine, nose, and ear swabs, and 110 isolates of *Pseudomonas aeruginosa* were obtained from two hundred ten samples gathered from affected patients from the National Center for Educational Laboratories Specialized Burns Hospital and Al-Ramadi Teaching Hospital, the study period from November 2021 to February 2022.

***Pseudomonas aeruginosa* identification protocol**

After collecting the samples, they were quickly transported to the laboratory, where they were isolated using MacConkey agar (Neogen UK) and aerobically at 37°C for 24 hrs. *Pseudomonas aeruginosa* colonies in MacConkey agar are colorless due to a lack of lactose fermentation, which is essential in distinguishing *P. aeruginosa* from other bacteria in the sample after that re-cultured on nutrient agar (Himedia, India) at 37°C for 24 hrs. These samples sub-culture on a selective medium Cetrimide agar (Himedia Indian) and incubated at 42°C for 24 hrs. to clarify and confirm while permitting the growth of *Pseudomonas* species, mainly *P. aeruginosa*. After sub culturing on MacConkey and Cetrimide, they were tested for identification depending on colony morphology, Gram-negative rod, growth at 42°C, and other tests confirmed that it was *P. aeruginosa* by using an automated Vitek-2 compact system and molecular diagnosis.

Antimicrobial susceptibility test:

The discs diffusion method was used to assess the resistance of bacterial isolates based on CLSI-2021 according to (Matuschek *et al*., 2014): A few colonies (2-4) were added to (2 ml) brain-heart broth (Himedia, India)and cultured at 37°C for 18 hrs to obtain a bacterial suspension with a McFarland turbidity of (0.5) McFarland corresponding to (1.5×10^8 CFU/ml), a sterile cotton swab was used to inoculate the bacterial suspension into Muller Hinton agar plates (Himedia, India), the plates were then set aside to dry, antimicrobial discs were placed on the surface of the medium, and the plates were incubated for 24 hrs at 37°C, the diameter of each antibiotic discs inhibitory zone was measured, then, these results were compared with the standard inhibition zone of the CLSI-2021.

Biofilm formation detection

Biofilm formation was detected by using the microtiter plate method which is the quantitative detection of biofilm by a microplate reader (Kırmusaoğlu, 2019): Bacterial suspensions equivalent to the McFarland No (0.50) turbidity standard were inoculated in the nutrient broth (Hitachi Japan) and incubated at 37ºCfor 18-24 hours. Then was distributed 20 µl of bacterial suspension into the microplate in three replicates per isolate, with 180 µl sterile broth added to three wells as a negative control, the plate was covered with a lid and parafilm, then the plate is incubated at 37 °C for 18–24 hour. After incubation, bacterial cultures were removed from each well, and the plate was washed twice with distilled water to eliminate non-adherent bacterium cells, the plate then was dried at room temperature.

After that 200ul of methanol was added to fix the adhesive cells for 10 minutes, then 200ul of 0.1% crystal violet dye was added for each well for 15 minutes, and then the plate was washed twice with distilled water to remove excess dye. Then it was added 200ul of 95% ethanol for 10 minutes before reading it, the results were read to detect the biofilm with optical density (OD 630), and the biofilm formation was calculated using three replicates for each isolate by regarding to the cutoff value where it was classified into biofilm productivity as no, weak and strong as follows:

- $OD \leq ODC^*$ Non-biofilm
- $ODC < OD \leq 2\ ODC$ Weak
- $2ODC < OD \leq 4\ ODC$ Moderate
- $4ODC < OD$ Strong

ODC* **:**optical density of control.

Detection of PCR products by agarose gel electrophoresis

Molecular detection of (*alg D, pil B*, and *pil A*) genes by using Pioneer kit (Korea) PCR.

Table 1. Sequence of PCR primers, molecular size of PCR products:

Gene	Primer	bp	Reference
alg D	F: ATGCGAATCAGCATCTTTGGT	1310	(Bogiel et.,al .,2021)
	R: CTACCAGCAGATGCCCTCGGC		
Pil A	F: ACAGCATCCAACTGAGCG	1675	(Bogiel *et al*., 2021)
	R: TTGACTTCCTCCAGGCTG		
Pil B	F: TCGAACTGATGATCGTGG	408	(Bogiel *et al*., 2021)
	R: CTTTCGGAGTGAACATCG		

Table 2. Conditions of polymerase chain reactions for *alg D* gene.

Gene	Step	Temp. (°C)	Time	No. of cycle
alg D	Initial denaturation	95	5 min.	1
	Denaturation	95	30 s.	
	Annealing	63.1	1 min.	30
	Extension	72	30 s	
	Final extension	72	7 min.	1
	Hold temperature	4	4 min.	

Table 3. Conditions of polymerase chain reactions for *pil A* gene.

Gene	Step	Temperature(°C)	Time	No. of cycle
pil A	Initial denaturation	95	5 min	1
	Denaturation	95	30 s.	
	Annealing	63	30 s.	
	Extension	72	1 min.	30
	Final extension	72	7 min	1
	Hold temperature	4	4 min.	

Table 4. Conditions of polymerase chain reactions for *pil B* gene.

Gene	Step	Temperature(°C)	Time	No. of cycle
pil B	Initial denaturation	95	5 min.	1
	Denaturation	95	30 s.	
	Annealing	63.1	1min.	
	Extension	72	30 s	30
	Final extension	72	7 min.	1
	Hold temperature	4	4 min.	

Statistical analysis

The Chi-square test was used to determine the relationship between carbapenem resistance and biofilm intensity using the statistical package for social sciences (SPSS) program at the probability value below ($P< 0.05$).

Result and Discussion

The current study includes 110 confirmed as *P. aeruginosa* out of a total of 210 specimens (burn, wound, ear, and UTI). Most of the isolates (40%) were found in burn samples, while isolates from wounds samples were the lowest (16.3%), these results agreed with (Al. Fahadawi *et al*., 2019), which showed that *P.aeruginosa* from burn samples contained most proportion of isolates (46.8%), while the wound samples contained the greatest proportion of isolates (31.6%). Whereas the proportion of other infections is (20-23.7)% from the ear and urine swabs, Farhan found the proportion was (20-68.4)% of urine, and ear swabs, and in contrast to Umar *et al*, 2016 detected that (23.2%) of samples of otitis media were positive for *P. aeruginosa*, but Farhan's results revealed a high incidence (68.4%) of *P. aeruginosa* among samples collected from patients suffering from otitis media (Farhan *et al*., 2019), Figure (1).

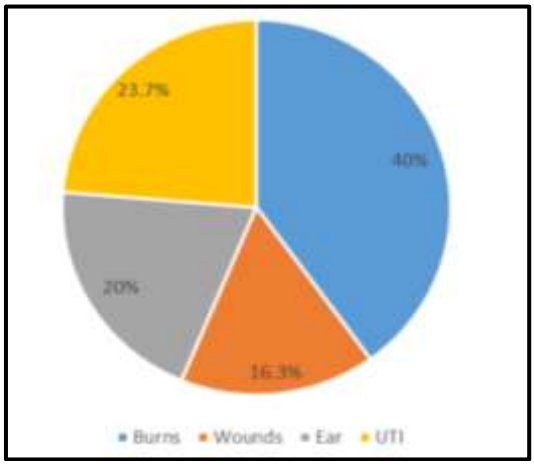

Figure 1. Prevalence of *Pseudomonas aeruginosa* local isolates in clinical specimens.

The variations in the percentage of infections among the current results and those of other studies may be due to the percentage of the spread of isolates, which differed depending on the location of clinical samples collection, or the number, type of samples, environmental elements, virulence factors, nutrient requirements (Bunyan *et al*., 2018). In the current study, a large percentage of *P. aeruginosa* isolates exhibited high levels of resistance to meropenem (20%), and the lowest percentage to imipenem (5.5%), Figures (2 & 3). These varying values are in agreement with the study by Gupta that showed the meropenem resistance was higher (22.16%) than imipenem (17.32%) and the imipenem was more active than meropenem (Gupta *et al*., 2006).

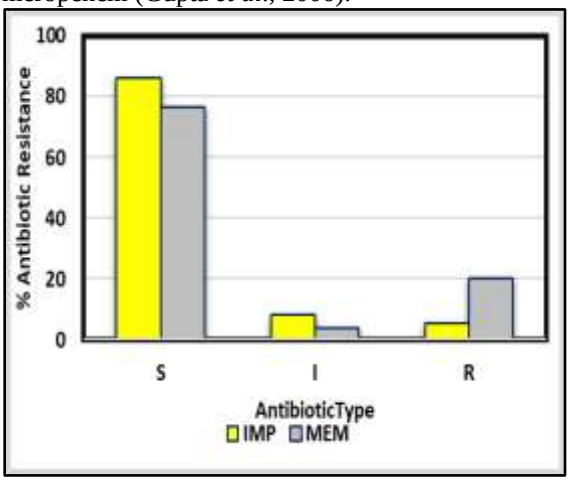

Figure 2. Antibiotic resistance percentage among *P. aeruginosa* local isolates, (IPM): Imipenem, (MEM): Meropenem, S: Sensitive, I: Intermediate, R: Resistant

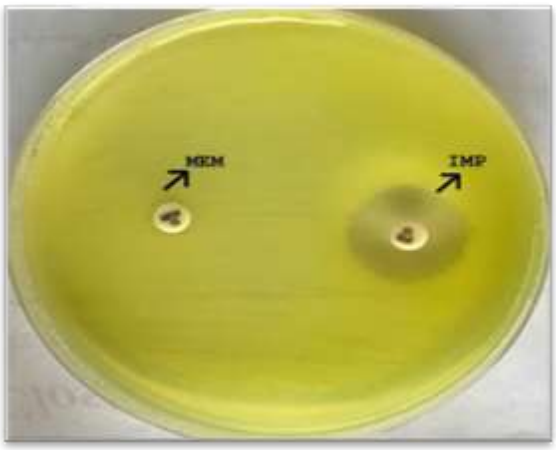

Figure 3. Well diffusion test for local *P. aeruginosa* isolates on Mueller Hinton agar at 37°C for 18 hrs.

The carbapenems are a class of β-lactam antibiotics that have excellent antimicrobial activity against *P. aeruginosa*, but the emergence of acquired carbapenem resistance in this species has impacted therapeutic and control efforts (Riera *et al*., 2011).

Quantitative biofilm analysis using the microtiter test revealed that 100% of isolates produced biofilm, the isolates can form biofilm ranging in intensity from strong and moderate to weak (Table 5), and the prevalence was (52,32and16) % of isolates (strong, moderate, and weak) producing biofilm respectively, these results in agreement with other studies such as Hadadi-Fishani et al., 2020 showed the prevalence was (47.7,30.2 and 27.4)% of isolates (strong, moderate, weak) producing biofilm respectively (Hadadi-Fishani *et al*., 2020), Other studies described by Rossi that all strains tested were strong biofilm producers that patients with bacteremia caused by carbapenem-resistant *P. aeruginosa* strains which had a higher mortality rate this demonstrates the significance of this organism as a major source of infection in hospitals (Rossi Gonçalves *et al*., 2017).

Table 5. Relationship between carbapenem resistance and biofilm strength (Strong, moderate, and weak producer).

Biofilm density	No. of isolates	Resistance to MEM,10)	Resistance to (IPM,10)	Resistance to both antibiotics
Strong	13	12	1	0
Moderate	8	4	1	3
Weak	4	3	0	1

Chi-square showed a significant association between the biofilm density and carbapenem resistance ($X^2 = 14.62$, $P < 0.023$). El-Mahdy indicated that biofilm formation was consistently correlated with carbapenem-resistant *P. aeruginosa*, biofilm formation was 65.2% and 94.1% in carbapenem-sensitive and carbapenem-resistant, respectively (El-Mahdy & El-Kannishy, 2019). The polymerase chain reaction used for the detection of 25 DNA samples of carbapenem resistance *P. aeruginosa* isolates, the results of the reaction and gel electrophoresis revealed the *alg D* gene percentage was 100% with bands appearing within the estimated size of the gene (1310 bp.) that showed in the Figure (4).

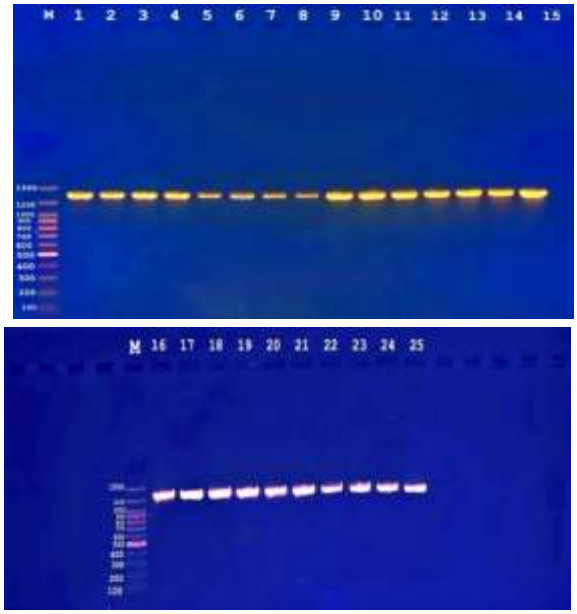

Figure 4. Gel electrophoresis of *alg D* gene (1310bp), (1 % agarose), 70 V/cm^2 for 90 min), lane M represents (100_ 1500bp) DNA ladder, lanes 1-25 represent *alg D* bands of carbapenem resistance *P. aeruginosa* isolates.

This result is consistent with Ellappan *et al* 2018 who indicated that *alg D* was 93% of carbapenem resistance isolates they indicated that the coexistence of different antimicrobial resistance mechanisms together with virulence genes in carbapenem-resistant isolates has become an alarming increasing threat, constantly monitoring of multidrug-resistant pathogens is critical for clinicians to identify treatment options against such infections (Ellappan *et al*., 2018). The genes that encode for pilin structural subunits (*pil A* and *pil B*) were also detected, the results of the molecular analysis of 25 isolates showed that the percentage of the *pil B* gene was (4%), and only one isolate was positive for this gene, the result is inconsistent with the result of Bogiel who showed that the lowest frequency of *pil B* gene was ranged (4.2_4.7)% (Bogiel *et al*., 2021, 2022), Figure (5).

Figure 5. Gel electrophoresis of *pil B gene* (408bp),(agarose1.4 %, 70 V/cm^2 for 90 min.). Lane M represents (100_ 1500bp) DNA ladder, and lanes 1- 25 represent bands of carbapenem resistance *P. aeruginosa* isolates.

While the result of gene *pil A* was (92%), these results disagreed with the results obtained by (Bogiel et al., 2021, 2022) who showed the frequency of *pil A* gene ranged from (19.7-to 15.9) %, this finding is very interesting because it suggests that the presence of this specific gene is essential for the colonization or infection of the human gastrointestinal tract, therefore, it might be a correlation between the presence of the chosen virulence genes and *P. aeruginosa* ability to form biofilm or colonize a specific location, but more studies are required to confirm this result (Bogiel et al., 2021), Figure (6)..

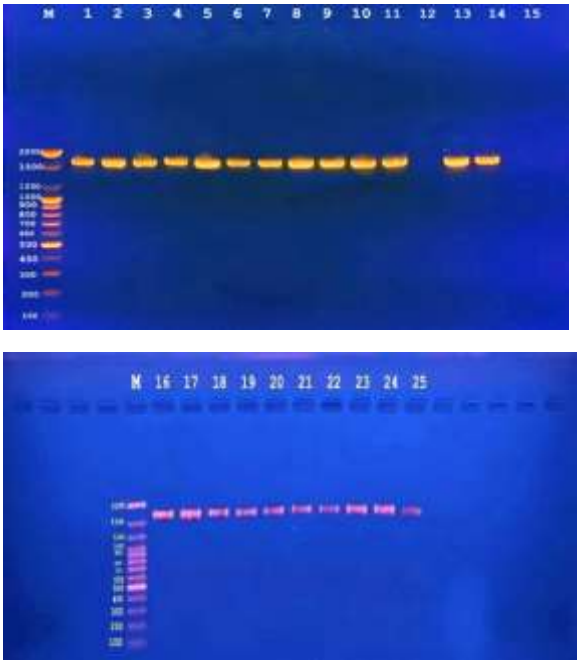

Figure 6. Gel electrophoresis of pil A gene (1675bp), (1.5 % agarose, 70 V/cm2 for 90 min), lane M represents (100-2000 bp.) DNA ladder, lanes 1-25 represent pil A bands of carbapenem resistance P. aeruginosa isolates

Conclusions

A large percentage of *P. aeruginosa* isolates exhibited high levels of resistance to meropenem 20% and the lowest percentage to Imipenem 5.5%. Most isolates with carbapenem resistance of *P. aeruginosa* were 100% produced biofilm, the highest percentage of strong biofilm production was observed in meropenem-resistant isolates, chi-square showed a significant association between the biofilm density and carbapenem resistance (X^2 = 14.62, P < 0.023). The results showed that most isolates have *alg D* genes with the lowest frequency of the pilin gene (*Pil B*).

Conflict of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Author Contribution

I want to declare that the contribution of authors: Tiba A. Al-Mohammed: execute the lab. work and collect the results .Huda M. Mahmood: is furnished the idea of the research, analyzed the data, and wrote the manuscript.

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