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 (X² = 14.62, P < 0.023).

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

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). No 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-Wrify 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 pilin 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., 2018). The development of P. aeruginosa mucoid colonies formed of alginites 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-
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 (1.5×10⁶ 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 (Kuruusahaan, 2019): Bacterial suspensions equivalent to the McFarland No (0.50) turbidity standard were inoculated in the nutrient broth (Hitachi Japan ) and incubated at 37°C for 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≤ ODC* : Non-biofilm
- ODC < OD ≤ 2 ODC : Weak
- 2ODC < OD ≤ 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>
<thead>
<tr>
<th>Table 1. Sequence of PCR primers, molecular size of PCR products:</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gene</strong></td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td><em>alg D</em></td>
</tr>
<tr>
<td><em>Pil A</em></td>
</tr>
<tr>
<td><em>Pil B</em></td>
</tr>
</tbody>
</table>
Table 2. Conditions of polymerase chain reactions for alg \( D \) gene.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Step</th>
<th>Temp. (°C)</th>
<th>Time</th>
<th>No. of cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>alg ( D )</td>
<td>Initial denaturation</td>
<td>95</td>
<td>5 min.</td>
<td>1</td>
</tr>
<tr>
<td>alg ( D )</td>
<td>Denaturation</td>
<td>95</td>
<td>30 s.</td>
<td></td>
</tr>
<tr>
<td>alg ( D )</td>
<td>Annealing</td>
<td>63.1</td>
<td>1 min.</td>
<td>30</td>
</tr>
<tr>
<td>alg ( D )</td>
<td>Extension</td>
<td>72</td>
<td>30 s.</td>
<td></td>
</tr>
<tr>
<td>alg ( D )</td>
<td>Final extension</td>
<td>72</td>
<td>7 min.</td>
<td>1</td>
</tr>
<tr>
<td>alg ( D )</td>
<td>Hold temperature</td>
<td>4</td>
<td>4 min.</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Conditions of polymerase chain reactions for pil \( A \) gene.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Step</th>
<th>Temperature(°C)</th>
<th>Time</th>
<th>No. of cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>pil ( A )</td>
<td>Initial denaturation</td>
<td>95</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>pil ( A )</td>
<td>Denaturation</td>
<td>95</td>
<td>30 s.</td>
<td></td>
</tr>
<tr>
<td>pil ( A )</td>
<td>Annealing</td>
<td>63</td>
<td>30 s.</td>
<td>30</td>
</tr>
<tr>
<td>pil ( A )</td>
<td>Extension</td>
<td>72</td>
<td>1 min.</td>
<td>1</td>
</tr>
<tr>
<td>pil ( A )</td>
<td>Final extension</td>
<td>72</td>
<td>7 min.</td>
<td></td>
</tr>
<tr>
<td>pil ( A )</td>
<td>Hold temperature</td>
<td>4</td>
<td>4 min.</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Conditions of polymerase chain reactions for pil \( B \) gene.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Step</th>
<th>Temperature(°C)</th>
<th>Time</th>
<th>No. of cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>pil ( B )</td>
<td>Initial denaturation</td>
<td>95</td>
<td>5 min.</td>
<td>1</td>
</tr>
<tr>
<td>pil ( B )</td>
<td>Denaturation</td>
<td>95</td>
<td>30 s.</td>
<td></td>
</tr>
<tr>
<td>pil ( B )</td>
<td>Annealing</td>
<td>63.1</td>
<td>1 min.</td>
<td>30</td>
</tr>
<tr>
<td>pil ( B )</td>
<td>Extension</td>
<td>72</td>
<td>30 s.</td>
<td>1</td>
</tr>
<tr>
<td>pil ( B )</td>
<td>Final extension</td>
<td>72</td>
<td>7 min.</td>
<td></td>
</tr>
<tr>
<td>pil ( B )</td>
<td>Hold temperature</td>
<td>4</td>
<td>4 min.</td>
<td></td>
</tr>
</tbody>
</table>

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

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).

Chi-square showed a significant association between the biofilm density and carbapenem resistance (X² = 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).

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

<table>
<thead>
<tr>
<th>Biofilm density</th>
<th>No. of isolates</th>
<th>Resistance to MEM,10</th>
<th>Resistance to (IPM,10)</th>
<th>Resistance to both antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strong</td>
<td>13</td>
<td>12</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Moderate</td>
<td>8</td>
<td>4</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Weak</td>
<td>4</td>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

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

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.

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).

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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This study carried out under self-funding.

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.

References

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