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# Genotyping Investigation of Ciprofloxacin Resistance Genes (ParC and ParE) in Clinical Pseudomonas Aeruginosa Isolates

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**Abstract**
*Pseudomonas aeruginosa* appeared with burn patients is considered as one the most important opportunistic pathogen. This study aimed to determine the mutation within genes (*parC* and *parE*) encoded for topoisomerase IV which related to the Ciprofloxacin resistance. The distribution of *P. aeruginosa* among different types of specimens showed that highest percentage, 46 (66.7%) was for burn samples. Antibiotics susceptibility showed that 34 (73.9%), 36 (78.3%), 30 (65.2%) and 29 (63.04%) of *P. aeruginosa* isolates were resistant to ciprofloxacin, gentamicin, imipenem, and ceftazidime, respectively. The *gyrB* gene was detected in all 21 (100%) of *P. aeruginosa* isolates with product size (~510 bp). The *parC* and *parE* genes were detected in all the examined isolates. The presence of mutations in specific region (FRDR) in these genes were screened by sending a DNA extract for genetic sequencing. The mutations in the *parC* and *parE* genes play anessentil role in resistance to Ciprofloxacin in clinical isolates of *P.* aeruginosa.
**Keywords: Pseudomonas aeruginosa, Fluroquinolone, Ciprofloxacin resistance**

**الملخص**
ظهرت الزائفة الزنجارية مع مرضى الحروق وتعتبر من أهم مسببات الأمراض الانتهازية. هدفت هذه الدراسة إلى تحديد الطفرة داخل الجينات (parC و parE) المشفرة لـ topoisomerase IV والتي تتعلق بمقاومة السيبروفلوكساسين. أظهر توزيع الزائفة الزنجارية من العينات المختلفة أن أعلى نسبة كانت (66.7٪) لعينات الحروق. أظهرت الحساسية للمضادات الحيوية أن 34 عزلة (73.9٪) و36 (78.3٪) و30 (65.2٪) و29 (63.04٪) من عزلات الزائفة الزنجارية كانت مقاومة للسيبروفلوكساسين والجنتاميسين والإيميبينيم والسيفتازيديم على التوالي. تم الكشف عن جين gyrB في جميع عزلات الزائفة الزنجارية الـ 21 (100٪) مع حجم المنتج (حوالي 510 زوج قاعدي). تم الكشف عن جينات parC و parE في جميع العزلات المفحوصة. تم فحص وجود الطفرات في منطقة معينة (FRDR) في هذه الجينات عن طريق ارسال مستخلص الحمض النووي للتسلسل الجيني. تلعب الطفرات في جينات parC و parE دورًا أساسيًا في مقاومة السيبروفلوكساسين في العزلات السريرية للزائفة الزنجارية.

**الكلمات المفتاحية: الزائفة الزنجارية، الفلوروكوينون، مقاومة السيروفلوكسين**

## Introduction

*Pseudomonas aeruginosa* appeared with burn patients are considered as one the most important opportunistic pathogen, it causes an extensive range of acute and chronic nosocomial infections.[1] This kind of microorganism can be found in approximately 33% of all burn wounds while in 59% of extensive burns.[2] Due to *Pseudomonas aeruginosa's* low susceptibility to the development of antibiotic resistance and antimicrobial drugs, therapy and control of severe infections often result in recurrent difficulties. The Multidrug-Resistance (MDR) is defined as the bacterial isolate which is non-susceptible to at least one agent in greater than three antimicrobial categories, such as Aminoglycosides and Fluoroquinolones.[3] The production of *Pseudomonas aeruginosa* stains of virulence factors, including cytotoxic pigment pyocyanin, secreted elastase and rhamnolipids surfactants is strongly increased.[4] One of the major classes of antibiotics which are utilized in the treatment of infections is Fluoroquinolones (FQ), where the treatment of infections is caused by *Pseudomonas aeruginosa*. Moreover, an increasing rate of FQ resistance among clinical isolates of *Pseudomonas aeruginosa* has been reported by Large-scale surveillance studies.[5] Quinolone acts by restraining bacterial DNA replication by blocking DNA gyrase/topoisomerase IV enzyme activity. Mutation altering the drug targets (most common) and plasmids coding for defensive proteins can protect the bacteria from the deadly impacts of quinolones. The genes coding for DNA gyrase are *gyrA* and *gyrB* and topoisomerase IV are *parC* and *parE*.[6] Studies have linked to misusing the antibiotic and non-compliance to treatment as the major cause of antibiotic resistance. However, studies on the resistance design to quinolones in this locale are constrained.[7] This study detected the predominance of the Ciprofloxacin resistance design within the clinical isolates from this locale by phenotypic and molecular methods using Polymerase Chain Reaction (PCR) and Sanger's sequencing to screen Fluoroquinolones region determination resistance (FRDR).[8]

## Materials and method

Specimens collection and pathogens Isolation: One hundred and twenty samples were collected during November/2019 to March/2020 from different units of five hospitals in Baghdad. All swabs and

specimens were transported to the laboratory under suitable condition and cultured immediately on blood agar, MacConkey agar, nutrient agar and cetrimide agar selective media for Pseudomonas aeruginosa and incubated overnight at 37°C under aerobic conditions. P. aeruginosa was determined using various biochemical tests, including Oxidase test and Catalase tests. Furthermore, rapid identification method (VITEK 2 System) was used to confirm the identification, the identified isolates were stored at -20 °C after adding 20% glycerol. Antimicrobial Susceptibility: All 69 P.aeruginosa isolates were subjected to the antibiotic susceptibility test against specific antibiotics.

There are four categories used, including. Fluoroquinolones (Aminoglycoside(Gentamicin); Carbapenems (Imipenem) and Cephalosporins: (Ceftazidime).

**Molecular investigation:** In this study, 21 samples were selected for Molecular investigation. According to antibiotic profile 21 of multidrug resistance (MDR) *P. aeruginosa* isolates were chosen for Genomic DNA extraction from the culture by using a Genomic DNA kit (Geneaid, Korea) following the instruction of the manufacturing company. The extracted DNA has been subjected to detect the *gyrB* gene. The PCR technique has been used for molecular detection using master mix (GoTaq-Green) and specific primers for the *gyrB* gene with product size of 510bp: F-GCGGTGGAACAGGAGATGGGCAAGTAC; R-CTGGCGGAAGAAGAAGGTCAACA.

All 21 *P. aeruginosa* isolates that showed positive results for *gyrB* genes submitted to detect the presence of *parC* gene. PCR technique has been used for molecular detection using master mix with specific primers for the *parC* gene with product size of 357 bp: F-CGAGCAGGCCTATCTGAACTAT; R-AGCAGCACCTCGGAATAG. All isolates were submitted to detect the presence of *parE* gene using master mix with specific primers for the *parE* gene with product size of 592bp: F-CGGCGTTCGTCTCGGGCGTGGTGAAGGA; R-TCGAGGGCGTAGTAGATGTCCTTGCCG. All PCR reactions were done including 20 pmol for each primer, 12.5µl master mix, 3µl template DNA and complete the reaction with Nuclease free water up to the total volume 25µl for each reaction. PCR reaction tubes containing the mixture were placed in a thermo-cycler PCR instrument. DNA was amplified as the following conditions: Initial Denaturationat 95°Cfor 5 mins, 30 cycles including (Denaturation at95°C for30 secs. Annealingat 58°Cfor 30 secs and Extension at72for 40 secs). In addition, to one cycle of final Extension at 72 °C for 7 mins. Five microliters of the product were subjected to electrophoresis, while the remaining product was stored at -20°C. PCR products were sent for Sanger sequencing, automated DNA sequence, by micro gen corporation–Korea. DNA sequences were analyzed and similarity searches were carried out with the Basic Local Alignment Search Tool (BLAST). The statistical data analysis was performed with statistical Analysis System- SAS program and Chi-square test.

**Results and discussion**

The results showed that all of the 69 isolates were categorised into six groups of different types of specimens as summarized in table 1. The high prevalence percentage of P. aeruginosa in burn specimens 46 (66.7%) is supported by significant local studies.[2] According to our results, the distributions rates of P. aeruginosa isolates among other specimen types were 4 (5.8%) for each of the following specimens: wound, urine and sputum. For urine samples, the findings match the results of a study conducted by [9] in India, who reported 4.6% for the prevalence rate of P. aeruginosa isolates. The ear and ETT specimens showed 6 (8.7%) and 5 (7.2%), respectively. The results of the antibiotic susceptibility test of this study showed that 34 (73.9%), 36 (78.3%), 30 (65.2%) and 29 (63.04%) of P. aeruginosa isolates were resistant to Ciprofloxacin, Gentamicin, Imipenem, and Ceftazidime, respectively as showed in table 2.

In the current study, the resistance rate towards Aminoglycoside, including Gentamicin, was 36 (78.3 %), which is considered the highest percentage compared to other categories. Also, the resistance rate towards fluoroquinolones, including Ciprofloxacin, showed a high percentage 34 (73.9%). In contrast, the results of antibiotics resistance showed 30 (65.2%) and 29 (63.04%) towards Carbapenems (Imipenem) and Cephalosporins (Ceftazidime), respectively. The resistance rate against aminoglycosides (Gentamicin) (78.3%) to some extent were similar with local studies, which concluded that the highest resistance of P. aeruginosa examined isolates (burn samples) was against Gentamicin which reached 60%.[7] However, this finding was higher than a local study (46.96%) conducted by [10]. While this finding is slightly lower than the finding of the study achieved by[11]. In addition, the result of resistance rate towards Ciprofloxacin, the primary generation of fluoroquinolones category, showed a high percentage of 34 (73.9%). This finding was slightly similar to the number of local and international studies [9, 18] reported the resistance

rates Ciprofloxacin were 61.3%, 64% and 69.64%, respectively. On the contrary, current results were higher than previous local and international studies, which demonstrated the resistance rates of isolates to Ciprofloxacin ranged 28-35%.[4, 8, 12] The extracted DNA of 21 P. aeruginosa isolates has been subjected to detect the gyrB gene. The gyrB gene was detected in all 21 (100%) of P. aeruginosa isolates. In a successful PCR reaction, the gyrB gene product of ~510 bp molecular weight was observed in gel electrophoresis (Figure 1). This was considered a mandatory sign of a successful positive reaction. However, examined the negative control isolates (22C: klebsiella pneumoniae) showed negative PCR results which supports the accuracy and sensitivity of gyrB.

All 21 P. aeruginosa isolates that showed positive results for gyrB genes submitted to detect the presence of parC gene. The parC gene was detected in 21 (100%) of P. aeruginosa isolates with product of ~357 bp observed in gel electrophoresis as indicter of presence parC gene (Figure 2).

Aligning of the Obtained nine sequence with the reference strains (CP034435) in Gen Bank were analyzed for the presence of polymorphism of these genes and detection the mutations in the nucleotides. The results of the sequences alignment of the local isolates with the reference strain showed the presence of some mismatch in the nucleotides of our queries in the different positions including substitutions and deletions. In addition, it was found that the most of sequences had identical similarity (100%). According to the results shown in table 3, the isolates resistance towards Ciprofloxacin (P2, P3, P4 and P14) from burn and (P15 and P21) from sputum showed 100% identical to reference strains (CP034435). This could be mean absence of the different mutation in local isolates in comparison with reference P. aeruginosa strain. However, the sensitive isolates (P6, P11 and P13) towards Ciprofloxacin from burn, ear and urine respectively showed missense mutation (T/C: Leucine to Serine) in all of three isolates in addition to other missense mutation (A/G: Lysine to Serine) in isolate (P13) from urine. P: Pseudomonas aeruginosa R: Resistant S: Sensitive All 21 P. aeruginosa isolates that showed positive results for gyrB genes submitted to detect the presence of parE gene. The parE gene was detected in 21 (100%) of P. aeruginosa isolates with product size ~592 bp in gel electrophoresis as indicter of presence parE gene (Figure 3).

Aligning of the obtained sequences two with the reference strains (CP034435) in Gen Bank were analyzed for the presence of polymorphism of these genes and detection the mutations in the nucleotides. The results of the sequences alignment of the local isolates with the reference strain showed the presence of some mismatch in the nucleotides of our queries in the different positions including transition and transversion of the subject. According to the results shown in table 4, the isolates resistance towards Ciprofloxacin (P1 and P16) from burn in comparison with reference strains (CP034435) showed differences. The resistant isolate (P1) showed 9 mutations including 6 silent transition mutation (3 Glutamine to Glutamine; 1 Glycine to Glycine; 1 Lysine to Lysine and 1 Asparagine to Asparagine). While 3 transversion mutations were determined, two of them were silent mutation (1 Glycine to Glycine and 1 Arginine to Arginine) in addition to one missense mutation (1 Valine to Serine). While P16 showed one (Adenine) deletion causing frameshift mutation.

P: Pseudomonas aeruginosa R: resistance

P. aeruginosa isolates showed high resistance for most of the tested antibiotics. Bacteria can prevent the action of antibiotics through many mechanisms, including eliminating the accumulation of these antibiotics by efflux pump system.[12] Fluoroquinolones (such as Ciprofloxacin) are currently among the most commonly prescribed antibiotics worldwide due to their broad spectrum of activity.[13] The results revealed that 34 (74%) of P. aeruginosa isolates were commonly resistant to three of the tested antibiotics and above considered multidrug resistance (MDR). In comparison, 12 (26%) isolates were sensitive to more than one tested antibiotic. P. aeruginosa is capable of developing resistance to nearly all antibiotics categories through mutations in chromosomal genes in addition to plasmids.[14, 15, 16] Furthermore, gyrB gene is an essential gene in P. aeruginosa encoded for type II topoisomerase and plays an essential role in DNA replication. In the present study, the prevalence of this gene in target isolates is (100%). In current study, the specificity of gyrB gene P. aeruginosa in detection was examined through check subjected negative control, 22C (Klebsiella Pneumoniae) to PCR reaction and the result showed no amplified product as shown in figure 1. Thus, molecular techniques have essential role in species-specific detection of bacterial DNA and become a solution for detection issues. Presence of parC in all isolates was agreed with study showed 100% percentage of the parC gene in 350 P. aeruginosa bacteria.[15] Other study conducted by [16] showed that 115 isolates of

fluoroquinolones resistance P. aeruginosa possess parC gene.[17-23] reported that mutations in the parC gene play an essential role in resistance to Ciprofloxacin in clinical isolates of P. aeruginosa and PCR-sequencing was carried out to assess parC mutations in 30 drug-resistant isolates from different clinical sources. Thus, they reported that antibiotic resistance to Ciprofloxacin and mutations in parC gene in resistant isolates are significantly related to each other ($P<0.05$) (10). Many studies with clinical isolates have focused on ParC.

**Conclusion**

The following are the findings of the current study: Pseudomonas aeruginosa is more prevalent in burn samples than in other clinical samples. Misuse of these antibiotics may contribute to an increase in MDR Pseudomonas aeruginosa, particularly resistance to fluroquinolones (Ciprofloxacin). Therefore, applying molecular techniques in the identification of Pseudomonas aeruginosa using gyrB showed high accuracy and high sensitivity target gene. The resistance mechanisms of Pseudomonas aeruginosa to FQs are not related to the mutation in parC and parE only, but they are related to synergetic action with other type of mechanisms. Thus, mutations with targeted genes sequencing are reported in local isolates in comparison with reference strains. Increase the number of screened isolates is required for future study.

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**Table 1:** The distributions rates of P. aeruginosa isolates among the different spec imen types

| Type of Specimens | No. of *P. aeruginosa* isolates (%) |
|---|---|
| Burn | 46 (66.7) |
| Wound | 4(5.8) |
| Mid-stream urine | 4(5.8) |
| Sputum | 4(5.8) |
| Endotracheal tube | 5(7.2) |
| Ear swab | 6(8.7) |
| Total (%) | 69 (100) |
| Chi-Square ($\chi^2$) | 12.537 ** |
| P-value | 0.0001 |
| ** (P≤0.01). | |

**Table 2:** The percentage of sensitivity, intermediate resistant and resistance of P. aeruginosa according to categories of antipseudomonal antibiotics.

| Category of antipseudomonal antibiotics | Sensitive isolates | Intermediate resistant isolates | Resistant isolates | Total No. of Isolates | P-value |
|---|---|---|---|---|---|
| | No. (%) | No. (%) | No. (%) | No. (%) | |
| Fluoroquinolones:Ciprofloxacin<br>Aminoglycoside: Gentamicin | 9(19.6)<br>10(21.7) | 3(6.5)<br>0(0.0) | 34(73.9)<br>36(78.3) | 46(100)<br>46(100) | 0.0001**<br>0.0001** |
| Carbapenems: Imipenem | 16(34.8) | 0(0.0) | 30(65.2) | 46(100) | 0.0001** |
| Cephalosporins: Ceftazidime<br>Chi-Square($x^2$) | 13(28.26)<br>5.439* | 4(8.70)<br>1.877NS | 29(63.04)<br>5.317* | 46(100)<br>--- | 0.0001** |
| P-value | 0.0298 | 0.259 | 0.0207 | --- | |
| *(P≤0.05),**(P≤0.01). *Significant;**Highly Significant | | | | | |

**Table 3**: Mutations in the sequence of parC gene among local isolates in comparison with ATCC P. aeruginosa Strain (Accession number: CP034435).

| Sample | Wild type | Mutation | Position | Mutation type | Effect of mutation |
|---|---|---|---|---|---|
| P2-Burn-R | Identical to the reference | | | | |
| P3-Burn-R | Identical to the reference | | | | |
| P4-Burn-R | Identical to the reference | | | | |
| P14-Burn-R | Identical to the reference | | | | |
| P15-Sputum-R | Identical to the reference | | | | |
| P21-Sputum-R | Identical to the reference | | | | |
| P6-Burn-S | T | C | 513399 | Transition | Missense Mutations Leucine >Serine |
| P11-Ear-S | T | C | 513399 | Transition | Missense Mutations Leucine >Serine |
| P13-Urine-S | T | C | 513399 | Transition | Missense Mutations Leucine >Serine |
| | A | G | 513278 | Transition | Missense Mutations Lysine >Serine |

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**Table 4.** Mutations in the sequence of parE gene among local isolates in comparison with ATCC P. aeruginosa Strain (Accession number: CP034435).

| Sample | Wild type | Mutation | Position | Mutation type | Effect of mutation |
|---|---|---|---|---|---|
| | G | A | 510807 | Transition | Silent Mutations Glutamine >Glutamine |
| | T | C | 510832 | Transition | Silent Mutations Asparagine >Asparagine |
| | G | C | 510838 | Transversion | Silent Mutations Glycine >Glycine |
| | T | G | 510841 | Transversion | Silent Mutations Arginine >Arginine |
| P1-Burn-R | A | G | 510906 | Transition | Silent Mutations Lysine >Lysine |
| | A | G | 511006 | Transition | Silent Mutations Glutamine >Glutamine |
| | A | G | 511032 | Transition | Silent Mutations Glutamine >Glutamine |
| | A | G | 511058 | Transition | Silent Mutations Glycine >Glycine |
| | A | T | 511086 | Transversion | Missense Mutations Valine >Serine |
| P16-Burn-R | A | - | 5555278 | Deletion | framshift |

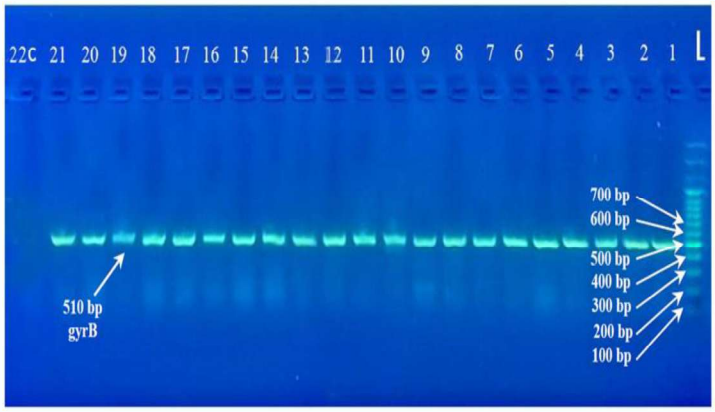

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**Figure 1:** Gel electrophoresis of PCR product of gyrB gene for Pseudomonas aeruginosa. Lane L: 100bp DNA ladder, lanes 1 21: examined isolates, lane 22C: klebsiella pneumoniae (1.6% agarose gel at 90vo lt for 40min 40 min.)

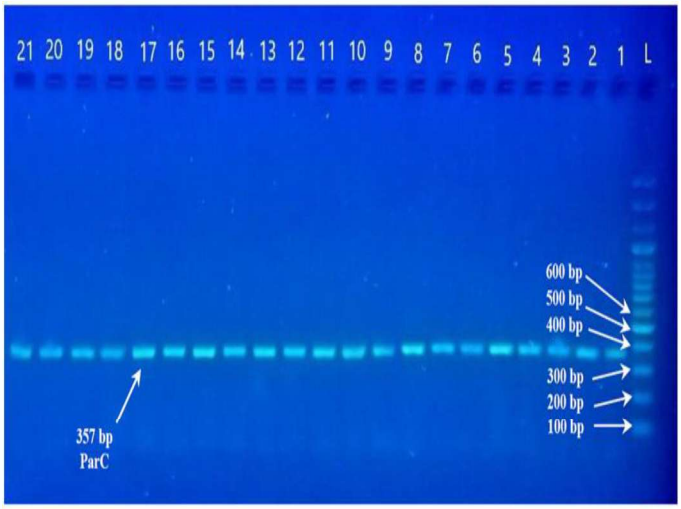


**Figure 2:** Gel electrophoresis of PCR product of ParC gene for Pseudomonas aeruginosa. Lane L: 100bp DNA ladder, lanes 1 21: examined isolates (1.6% agarose gel at 90volt for 40min).

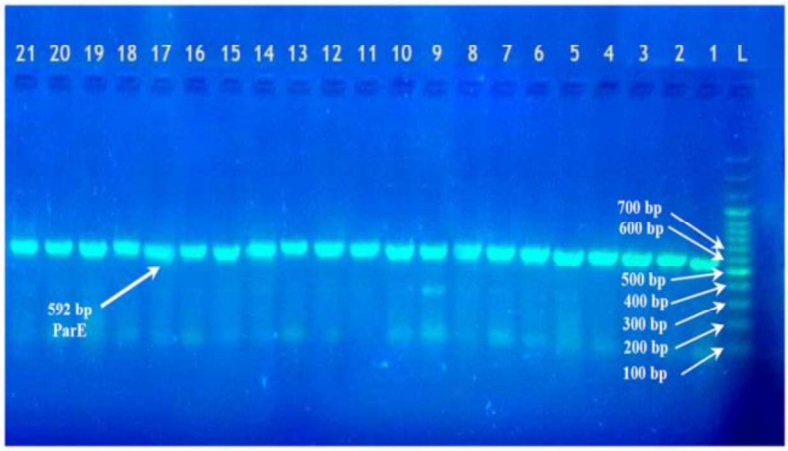


**Figure 3**: Gel electrophoresis of PCR product of ParE gene for Pseudomonas aeruginosa. Lane L: 100bp DNA ladder, lane s 1 21: examined isolates (1.6% agarose gel at 90volt for 40mi.