



Occurrence of Point Mutations in *gyrA* and *parC* Genes of Ciprofloxacin-Resistant *Pseudomonas aeruginosa* Isolated from Burn Infections

Hajer H. Abdulameer, Ghusoon A. Adulhassan*

Department of Biology, College of Science, University of Baghdad, Iraq

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Abstract

The spread of antibiotic resistant bacteria is a worldwide problem. Due to the importance of *P. aeruginosa* as a multidrug resistant bacterium, this study aimed, through molecular techniques, to detect point mutations in chromosomal genes responsible for the quinolones class of antibiotics resistance. A total of 52 isolates from burn infections were identified using specific primers for *P. aeruginosa* 16S rDNA. Ciprofloxacin minimum inhibitory concentrations (MIC) were estimated using the agar dilution assay. DNA sequences of the quinolone resistance-determining regions of *gyrA* and *parC* were determined for detecting the mutations found in these genes and the relations among the isolates by constructing phylogenetic trees. The results revealed that only 43 (82.7%) of isolates were *P. aeruginosa*, of which 31 (72.06%) were resistant to different concentrations of ciprofloxacin, ranging between 4 and >32 µg/ml. Twenty six isolates were selected for sequencing, including sensitive, intermediately resistant, and highly resistant to ciprofloxacin. The ciprofloxacin sensitive isolates did not exert any amino acid alterations in *gyrA* or *parC* genes; however, a single intermediately resistant isolate had a single mutation at each gene. Of the total resistant isolates (20), 6 isolates had no mutations at different MIC levels, while 14 isolates had Thr-83-Ile substitution in *gyrA* and Ser-87-Leu substitution in *parC*, only five isolates had a second mutation, namely Asp-87-Asn, in *gyrA*. The phylogenetic analysis of the studied groups showed divergence from the *P. aeruginosa* PAO1 and PAO1OR reference strains due to increased mutations and polymorphisms in studied isolates. In conclusion, *P. aeruginosa* occurrence was increased in burn infections and the fluoroquinolones in current use are not as effective as before; the main resistance mechanism in local clinical isolates of *P. aeruginosa* is mutations, where the main target of fluoroquinolones is *gyrA* gene.

Keyword: *P. aeruginosa*, *gyrA*, *parC*, Mutation, Phylogenetic tree.

حدوث طفرات نقطية في جينات *gyrA* و *parC* بين بكتريا *Pseudomonas aeruginosa*

المقاومة للسبروفلوكساسين معزولة من التهابات الحروق

هاجر هادي عبد الامير , غصون علي عبد الحسن*

قسم علوم الحياة، كلية العلوم، جامعة بغداد ، العراق.

الخلاصة

يمثل انتشار البكتيريا المقاومة للمضادات الحيوية مشكلة عالمية، وبسبب أهمية بكتريا *P. aeruginosa* باعتبارها بكتيريا مقاومة للأدوية المتعددة ، هدفت هذه الدراسة إلى اكتشاف الطفرات النقطية

*Email: ghusoon78@gmail.com

في جينات الكروموسومات المسؤولة عن صنف الكينولونات المقاومة للمضادات الحيوية ، من خلال التقنيات الجزيئية. تم الحصول على 52 عزلة من عدوى الحروق وكشف عنها باستخدام بواقي متخصصة لجين *16S rDNA* في بكتريا *P. aeruginosa* . تم تقييم التركيز المثبط الأدنى من سيبروفلوكساسين عن طريق فحص أجار التخفيف. تم تحديد تسلسل الحمض النووي لمنطقة الكينولونات المقاومة للكشف عن الطفرات النقطية الموجودة في جينات *gyrA* و *parC* وإيجاد العلاقة بين العزلات من خلال بناء أشجار النشوء والتطور. أظهرت النتائج أن 43 (82.7%) فقط من العزلات كانت تعود لبكتريا *P. aeruginosa* وكذلك 31 (72.06%) من هذه العزلات كانت مقاومة لتركيزات مختلفة من سيبروفلوكساسين تتراوح بين 4 إلى < 32 ميكروغرام/مل. اختيرت 26 عزلة بعضها حساسة ومتوسطة واغلبها مقاومة لمضاد السيبروفلوكساسين لمعرفة تسلسل القواعد النروجينية لها حيث لم تظهر أي من العزلات الحساسة للسيبروفلوكساسين تغيرات في الأحماض الأمينية في جينات *gyrA* أو *parC* ، لكن مع ذلك ظهرت طفرة واحدة في عزلة متوسطة المقاومة. من بين مجموع العزلات المقاومة (20)، لم يكن لدى 6 عزلات منها بمستويات MIC مختلفة أي طفرات، في حين أن 14 عزلة لديها طفرة استبدال Thr-83-Ile في *gyrA* و Ser-87-Leu في *parC*، 5 عزلات فقط لديها طفرة ثانية Asp-87-Asn في *gyrA*. أظهر تحليل الشجرة الجينية ان المجاميع المدروسة تتباعد عن السلالات القياسية المأخوذة من بنك الجينات والمستخدم في الدراسة وهي PAO1OR, PAO1 بسبب تزايد الطفرات وتعدد الأشكال في العزلات المدروسة. و قد تم الاستنتاج ان نسبة إصابة *P. aeruginosa* لخمج الجروح قد ازداد، و مضادات الكوينولات المستخدمة حالياً لم تعد فعالة كما كانت؛ الطفرة هي الآلية الأساسية لمقاومة الكوينولات بين العزلات المحلية لبكتريا *P. aeruginosa*، و ان *gyrA* هو الهدف الرئيسي للكوينولات.

Introduction

Burn injury is a worldwide public health issue due to increasing nosocomial infections [1,2]. Particularly in developing countries, *Pseudomonas aeruginosa* pathogen can cause severe nosocomial infections, especially in catheterized, immunocompromised, or burned patients [3, 4] resulting from its containment of intrinsic and acquired resistance to many antibiotic groups [5]. For effective oral treatment, fluoroquinolones are the only available antibiotics for infections caused by this organism [6]. In the treatment of *P. aeruginosa* infections, ciprofloxacin and levofloxacin are commonly used amongst the fluoroquinolones group [7]. None of the new fluoroquinolones after the third generation retains activity against ciprofloxacin-resistant isolates [8]. Fluoroquinolones work by inhibiting two enzymes involved in bacterial DNA synthesis, both of which are DNA topoisomerases that human cells lack and that are essential for bacterial DNA replication [9], thereby enabling these agents to be both specific and bactericidal [10]. A doubled increase in ciprofloxacin resistance was found among Gram -ve bacilli isolated from hospital's intensive care units in 1990s, because of raised (40%) fluoroquinolones consumption [11]. The main mechanisms of fluoroquinolones resistance in *P. aeruginosa* are resulting from mutations in DNA gyrase and topoisomerase IV [12], decreased permeability of the cell wall, multidrug efflux pump [13], and plasmid mediated resistance [14]. Most bacteria have both enzymes; however, gyrase is more sensitive to inhibition by quinolones than is topoisomerase IV in Gram -ve bacteria, whereas, in Gram +ve) bacteria, topoisomerase IV is usually the main target and gyrase is interestingly less susceptible [15]. Changes in topoisomerase IV in G-ve bacteria are alleged to have a secondary contribution in resistance development of *parC* or *parE* mutations, which confer resistance only in the existence of concomitant DNA gyrase mutations [16]. Resistance develops due to amino acid substitutions in regions in GyrA or ParC called the quinolone resistance determining regions (QRDRs) [17]. Mutations can enhance resistance to antibiotics by controlling enzyme expression or efficacy. Regulatory mutations provide limited resistance while higher resistance is provided by structural mutations via the improvement of the binding affinity between the resistance proteins and antibiotic [18]. The spread of antibiotic resistant bacteria is considered as a global problem. Due to the importance of *P. aeruginosa* as a multidrug resistant bacterium, this study aimed to detect mutations in chromosomal genes (*gyrA* and *parC*) responsible for resistance to the quinolones class of antibiotics. In addition, we aimed at constructing a phylogenetic tree for both genes to observe the effects of mutations on divergence between isolates.

Materials and Methods

Isolation of Bacteria

Fifty-two isolates from burn and wound infections were gathered from several hospitals in Baghdad, Iraq, which were, based on conventional (cultural and biochemical) tests considered to be *P. aeruginosa*.

16SrDNA for the Identification of *P. aeruginosa*

DNA was extracted from fifty-two *P. aeruginosa* isolates by utilizing a commercial kit (G-spin extraction kit, Intron, Korea), depending on the instructions of the production company. Specific primers were used for the identification of 16SrDNA from *P. aeruginosa* [19]. The PCR mixture consisted of premix (i-taq, Intron, Korea), 1 µl forward and reverse primers, and 3 µl DNA template. The volume was completed with 20 µl sterile distilled water. After optimization, the program was carried out with an initial denaturation at 94°C (2 min), 25 cycles of denaturation at 94°C (25 sec), annealing at 56.4°C (25 sec), extension at 72°C (40 sec), and final extension at 72°C (5 min).

Determination of Minimal Inhibitory Concentration

The agar dilution method was used to determine the minimal inhibitory concentration of ciprofloxacin (Hexal, Germany) against identified *P. aeruginosa*, as recommended by the Clinical and Laboratory Standards Institute CLSI [20]. The antibiotic dilution range was prepared to be between 0.0625 and 32 mg/L. The antibiotic solution was dispensed into universal containers containing 20 ml of Muller Hinton agar (Himedia, India) that have been allowed to equilibrate in a water bath to 45 to 50°C, then dispensed into petri dishes. The bacterial culture was set to 0.5 McFarland (1.5×10^8 CFU/ml), of which inoculums of 0.2 µL were applied to the agar surface with a micropipette. Plates were inverted and incubated at 37°C for 18-24 hr. The lowest concentration of antimicrobial agent that completely inhibits the growth was considered to be the minimal inhibitory concentration.

Amplification and sequencing of *gyrA* and *parC*

The two investigated genes, i.e. *gyrA* (417 bp) for detecting topoisomerase II subunit [21] and *parC* (303 bp) for detecting topoisomerase IV subunit of *P. aeruginosa* [17], were amplified. PCR mixture was composed of premix (i-taq, Intron, Korea), 1 µl of primers (for *gyrA*, F 5'-GACGGCCTGAAGC CGGTGCAC-3' and R 5'-GCCACGGCGATACCGCTGGA-3', while for *parC*, 1 µl of F 5'-CGAGCAGGCCTATCTGAACTAT-3' and R 5'-GAAGGACTTG GGATCGTCCGGA-3'), and 3 µl of DNA template, then the volume was completed with sterile D.W. After optimization, the program was carried out with initial denaturation at 94°C (3 min), 29 cycles of denaturation at 94°C (40 sec), annealing at 64.1°C (30 sec) for *gyrA* as well as 56.5°C at (30 sec) for *parC*, extension at 72°C (30 sec), and final extension at 72°C (5 min). Gel electrophoresis was employed using agarose gel (1.5%) with RedSafe dye for the confirmation of the presence and size of bands in gel under UV Transilluminator.

The conventional PCR products of 28 isolates for *gyrA* and *parC* were sent to Macrogen Company, USA, for sequencing by using forward primers of *gyrA* and *parC* on the genetic analyzer (Applied Biosystems) in addition to Sanger sequencing. Homology search and mutations detection were performed via the online Basic Local Alignment Search Tool (BLAST) program using blastn algorithms, which are available online at the National Center for Biotechnology Information (NCBI) and CDS feature.

The Bioedit program (V.7.2) was used for multiple sequence alignment in Clustal W, as well as to calculate the similarity matrix between bacterial isolate groups. Phylogenetic analysis was performed using MEGA7 software, employing the method of neighborhood-joining (NJ). For estimating the nod branching probability, 1000 bootstraps were used.

Statistical Analysis

Data were presented as percentage and/ or mean \pm standard error. The statistical analysis system IBM SPSS Statistics 23 (2015) was used to analyse the effects of different factors in this study. Chi-square (χ^2) test was employed to analyse the differences between study groups and assays. Levene's test was used to test homogeneity of variance and Spearman's correlation test was used to analyse other data. A p value of ≤ 0.05 was considered statistically significant.

Results and Discussion

Bacterial identification with 16S rDNA

DNA of fifty-two isolates was extracted and conventional PCR was performed to amplify the *PASS* gene. 43 (82.7%) isolates were identified as *P. aeruginosa*. These results corresponded with the result

of local studies [22, 23], where *P. aeruginosa* was isolated from different clinical specimens and identified using *16S rDNA*. This test gives 90% higher accuracy than other methods, allowing the differentiation of this species from other *Pseudomonas* species and other pathogens. The genotypic identification differs from the phenotypic one, being influenced by the presence or absence of non-conserved genes or characters expression variation; therefore, *16S rDNA* gene provides precise identification of isolates [24].

Minimal inhibitory concentration

Forty-three isolates of *P. aeruginosa* were tested to determine the minimal inhibitory concentration for ciprofloxacin by the agar dilution method. 31 (72.09%) isolates showed resistance to ciprofloxacin, 30.23% of which had resistance rate that ranged 4-32 $\mu\text{g/ml}$, while the rest (41.86%) were resistant at concentrations higher than 32 $\mu\text{g/ml}$. In addition, 8 (18.60%) isolates showed intermediate resistance at a concentration of 2 $\mu\text{g/ml}$, while 4 (9.30%) isolates were sensitive at 1 $\mu\text{g/ml}$. Significant differences were found among the different MIC concentrations [t_{obt} (199.56) = 16.16, $p \leq 0.05$].

The result of 72.09% resistance obtained here was higher than that of 9% reported by a previous local study in Baghdad [25] and that of 31% from different clinical sources [26]. However, non-local studies showed more similar results to ours. Thirty-eight clinical isolates of *P. aeruginosa* isolated from different clinical sources in north Lebanon showed 57.89% resistance to ciprofloxacin [17], whereas 64% of 100 isolates from different clinical specimens in Iran were resistant [12] and 100% (81 isolates) were resistant in Tunisia [27]. Differences in resistance rates could be attributed to isolation from different departments, patient populations, and surely the differences in the amount of this antibiotic used by different hospitals. However, the resistance rate is increasing year after year due to the misuse by both hospitals and community.

Amplification and sequencing of *gyrA* and *parC*

PCR was conducted over 43 isolates to amplify the constitutional genes *gyrA* and *parC*, where bands were confirmed with gel electrophoresis (Figure 1). All the isolates were positive for both the *gyrA* and *parC* genes, as these are essential genes that are constitutively expressed at a relatively constant level and their absence means cell death [28]. This result is consistent with that reported by a previous study [29].

The PCR products were sequenced for both genes from 26 isolates, some of which being sensitive, intermediate, or resistant. The results were analyzed and compared using BLAST with the reference strains available in the GenBank of the NCBI database. The sequencing results demonstrated 98-100% compatibility with the reference strains PAO1 (AE004091.2) and PAO1OR (LN871187.1).

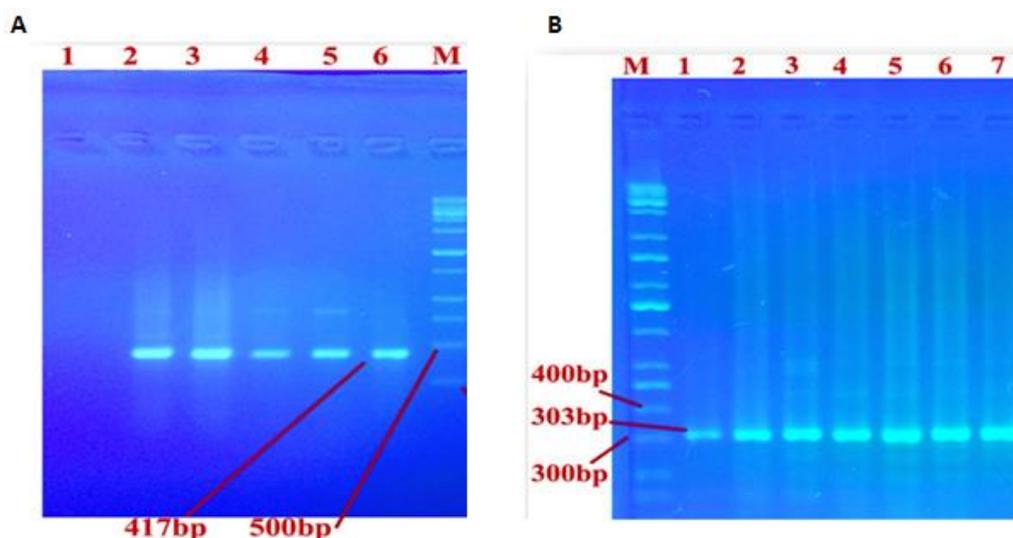


Figure 1- Gel electrophoresis of *P. aeruginosa* amplified genes; A: *gyrA* (417bp) and B: *parC* (303bp). Agarose gel (1.5%) stained with RedSafe stain, 80 V/cm for 1hrs and visualized by UV transilluminator. M: 1000 bp DNA ladder.

DNA sequencing of *gyrA* and *parC* was used to detect mutations related to the resistance of *P. aeruginosa* to the fluoroquinolone antibiotic (Figure 2). Transversion mutations showed a frequency of 19 for *gyrA* and *parC*, while transition mutations had a frequency of 83 in all the isolates. From 107 mutation, 71 were shown to be silent mutations in all the isolates while 36 were missense mutation.

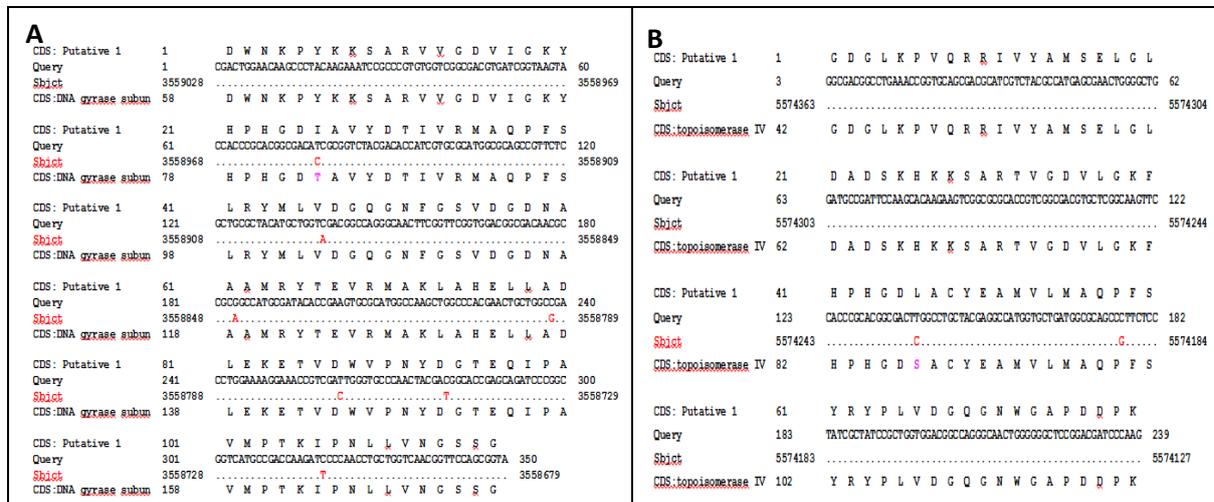


Figure 2- Sequence alignments and CDS subunits of *gyrA* (A) and *parC* (B) of *P. aeruginosa* local isolates with PAO1 NCBI reference strain AE004091.2. Black color indicates conserved bases and residues, red color indicates altered bases, pink color indicates amino acid alterations; Thr-83-Ile substitution in *gyrA* and Ser-87-Leu substitution in *parC*.

Three amino acid substitutions were found in this study; 15 (57.7%) and 5 (19.2%) isolates had Thr-83- Ile and Asp-87- Asn substitutions, respectively in *gyrA*, while 15 (57.7%) isolates had Ser-87- Leu substitution in *parC*. All isolates that showed Thr-83-Ile substitution in *gyrA* also had Ser-87-Leu substitution in *parC* (Table 1). The association of amino acid substitutions mentioned above with fluoroquinolone resistance in *P. aeruginosa* was reported in several investigations [30-32]. These amino acid substitutions were found as the main types of replacements [21, 33-35], thereby matching the results in this study. Furthermore, mutations that included Thr-83-Ile (92.44%) and Asn-87-Asp (0.44%) were present as single substitutions, whereas those of Thr-83-Ile and Asn-87-Asp (5.78%) were present as double substitutions [36]. Another study found that 100% of isolates resistant to ciprofloxacin had Thr-83-Ile substitution in *gyrA* and 68.75% had a further alteration in *parC* [12]. Amino acid alteration at positions Glu-91, Leu-95 [17], Pro-83, Pro-88, and Gly-85 [37] in *parC* were not detected in this study.

Relationship between Ciprofloxacin MIC and Mutations in QRDRs

Table 1 illustrates the Ciprofloxacin MIC for 26 isolates and their relationships to mutations in *gyrA* and *parC* genes. As observed, no mutations were found in the sensitive isolates. Isolates that had one *gyrA* (Thr-83-Ile) substitution and one *parC* substitution (Ser-87- Leu) revealed MIC values ranging 2 - >32 mg/mL. Moreover, five isolates with dual *gyrA* alterations (Thr-83-Ile and Asp-87-Asn) and one *parC* alteration (Ser-87-Leu) had MIC values ranging 4- >32 µg/ml, with most isolates having MIC >32 µg/ml.

Table 1- The MIC values of ciprofloxacin for 26 isolates and their relationships to mutations in *gyrA* and *parC* genes.

MIC value		*Isolate no.	Amino acid (codon) encoded by indicated gene at position:		
			<i>gyrA</i>		<i>parC</i>
			83(Thr)	87(Asp)	87(Ser)
Sensitive	1	B-PA22	----	----	----
		B-PA23	----	----	----
		B-PA26	----	----	----

Intermediate	2	B-PA6	----	----	----
		B-PA9	----	----	----
		B-PA10	Ile	----	Leu
Resistant	4	B-PA52	Ile	Asn	Leu
	8	B-PA4	----	----	----
	16	B-PA16	Ile	----	Leu
		B-PA19	Ile	----	Leu
		B-PA20	----	----	----
	32	B-PA21	----	----	----
		B-PA1	Ile	----	Leu
		B-PA2	----	----	----
		B-PA11	----	----	----
		B-PA12	----	----	----
		B-PA25	Ile	----	Leu
	>32	B-PA32	Ile	Asn	Leu
		B-PA13	Ile	----	Leu
		B-PA30	Ile	----	Leu
		B-PA31	Ile	Asn	Leu
		B-PA44	Ile	Asn	Leu
B-PA53		Ile	----	Leu	
B-PA54		Ile	Asn	Leu	
B-PA55		Ile	----	Leu	
B-PA56	Ile	----	Leu		

*B-PA: Burn-*Pseudomonas aeruginosa*

A high correlation was found between MIC values of 8->32 µg/ml and the presence of mutations [$r=0.672$, $P \leq 0.05$]. There was also a correlation between MIC value of 4 µg/ml and mutations [$r=0.454$, $P \leq 0.05$]. However, no correlation was found between MIC value of 2 µg/mL and mutations [$r=0.255$, $P > 0.05$]. The correlation results confirmed that substitution mutations in this study were generally responsible for ciprofloxacin resistance.

Based on the sequence analysis, the isolates with a mutation in *parC* hold one or double mutations in *gyrA*. This observation proved that gyrase was the main target for fluoroquinolone resistance in *P. aeruginosa* clinical isolates [38]. Yet, isolates possess two mutations in *gyrA* and one mutation in *parC* had elevated ciprofloxacin MICs compared with one mutation in *gyrA*. These findings suggest that the mutation in *parC* takes place after those occurring in *gyrA*, resulting in increased resistance to fluoroquinolone in *P. aeruginosa* [12].

Of twenty resistant isolates, 6 isolates had no mutations at different MIC levels, which suggests resistance by other mechanisms, such as efflux pumps overexpression, reduced membrane permeability, and *gyrB* and *parE* mutations, which were previously illustrated in *P. aeruginosa* fluoroquinolone resistance [17, 39].

Sequence similarity matrix and phylogenetic tree of for *gyrA* and *parC* in *P. aeruginosa*

For similarity matrix and phylogenetic tree construction, isolates were divided into groups depending on the similarity in their nucleotide sequences. Thus, the isolates divided into eight groups (A, B, C, D, E, F, G and H) for *gyrA* while for *parC* the isolates divided to five groups (1, 2, 3, 4 and 5). The sequence similarity matrix for *gyrA* and *parC* of 26 *P. aeruginosa* isolates were estimated by Bioedit program. The results showed that the studied groups (A-H) of *gyrA* have high sequence similarity, ranging between 97-99%, except for the group H which was observed to have 100% similarity with *P. aeruginosa* PAO1 strain. The sequence similarity for *parC* was also high when compared with *P. aeruginosa* PAO1 strain, ranging 89-99%, and reached to 100% in group 1.

The phylogenetic tree was constructed to determine the relationships among studied groups by comparing the *gyrA* and *parC* sequences obtained in this study with GenBank database sequences in NCBI. Figure 3 illustrates a neighbor-joining phylogenetic tree for *gyrA* sequences, which indicates one cluster that is divided into two subgroups. The first subgroup contained all the local isolates, except those isolates that belonged to groups A and C, which were placed in the second subgroup. Group G showed sister groups with PAO1 (USA) and PAO1OR (France) reference strains which were used for determining the mutations and polymorphisms in the local isolates of this study. Groups A

and C showed divergence with other groups, because these isolates had higher polymorphisms than those isolates of the remaining groups.

After phylogenetic analysis of *gyrA*, *P. aeruginosa* isolated from pet Chinese stripe-necked turtles was arranged in a distinct clade from clinical, soil and water isolates of *P. aeruginosa* clades, which means that the *gyrA* region is genetically more conserved [38].

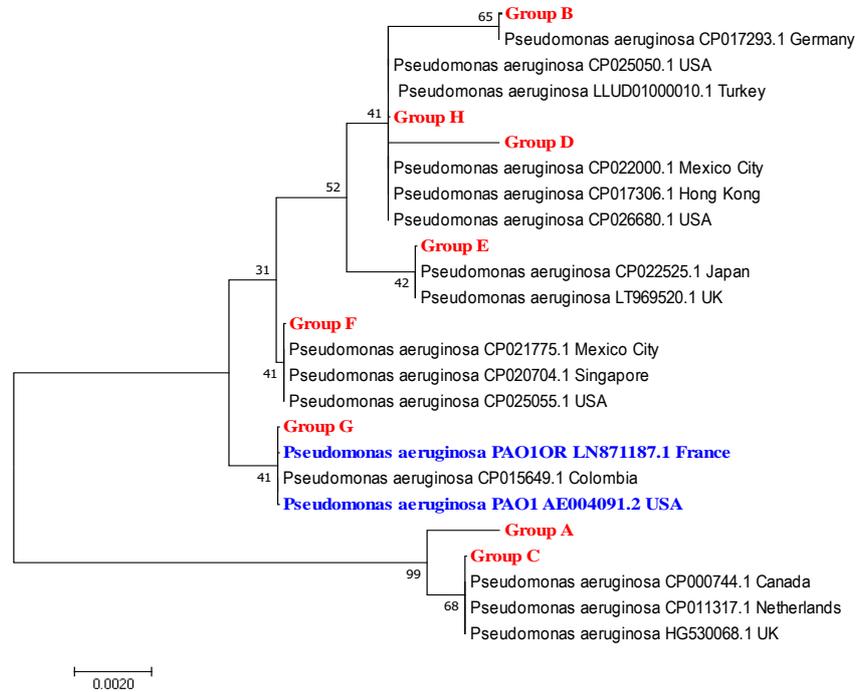


Figure 3- Neighbor-joining unrooted phylogenetic analysis of *gyrA* for 26 *P. aeruginosa* clinical local isolates arranged in eight groups (A, B, C, D, E, F, G and H). The analysis was performed using MEGA7.

Phylogenetic tree for *parC* sequences (Figure 4) indicated one cluster that was divided into two subgroups. Group 1 showed a sister group with PAO1 (USA) reference strain from GenBank. Local isolates in groups 3 and 5 revealed divergences, because they had polymorphisms not found in the strains of GenBank used for sequences comparison in this study.

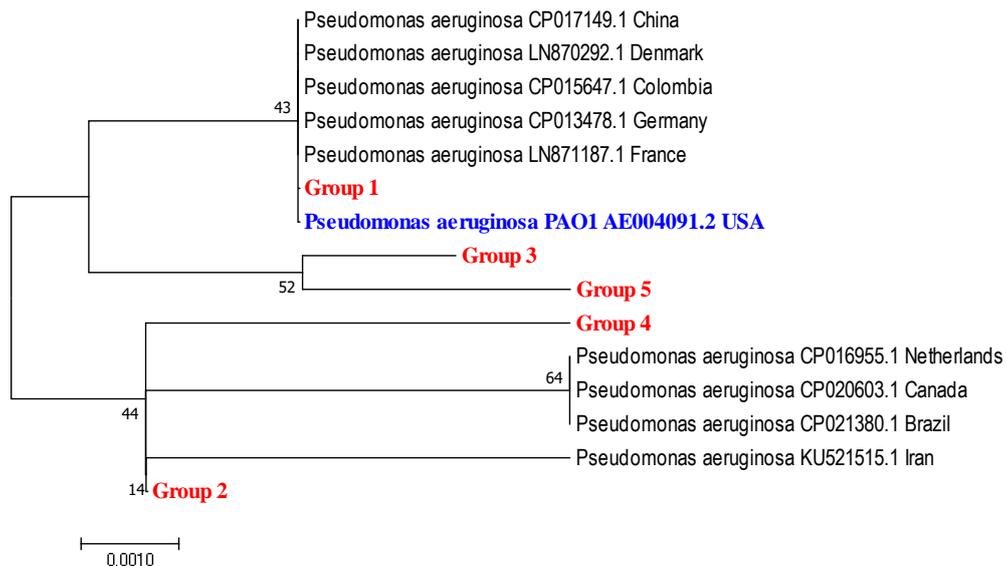


Figure 4- Neighbor-joining unrooted phylogenetic analysis of *parC* for 26 *P. aeruginosa* clinical local isolates showed an arrangement of five groups (1, 2, 3, 4 and 5). The analysis was performed using MEGA7.

In conclusion, *P. aeruginosa* occurrence was increased in burn infections. Also, the fluoroquinolones in current use are not as effective as before; the main resistance mechanism in local clinical isolates of *P. aeruginosa* is mutations, where the main target for fluoroquinolones is *gyrA* gene.

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