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Bacteriological and Molecular Study of Fluoroquinolones Resistance in *Pseudomonas aeruginosa* Isolated From Different Clinical Sources

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Abstract

The present study was conducted to investigate the resistance of fluoroquinolones (FQs) and the effects of mutations in the resistance gene in clinical isolates of *P. aeruginosa* isolated from different sources in Al-Hussein Hospital, Al-Samawah city, Iraq. The basic mechanism of the resistant of fluoroquinolones in *P. aeruginosa* is via mutations occurring in the basic bacterial *gyrA* gene encoding-subunit A of DNA gyrase . Forty clinical isolates from various sourced (burn 7 (17.5 %), wound 7 (17.5 %), ear 2 (5 %), operation room 12 (30 %), urine 3 (7.5 %), and industrial dialysis center 9 (22.5 %)) were isolated based on bacteriological methods confirmed by *16s rRNA* gene using PCR technique. A sensitivity test was conducted to all isolates by Kirby-Pour method using 7 antibiotics of fluoroquinolones. Amongst the 40 clinical isolates, 10 were resistant and 3 were sensitive to all tested antibiotics, while 27 were intermediate, resistant and sensitive to two or more of tested antibiotics, with the resistance being confirmed by the minimum inhibitor concentration (MIC) test. The ten resistant isolates were used to examine the mutations in *gyrA* gene. A direct sequence method was used and revealed eight mutations in *gyrA* gene at different positions. In addition, we found that fluoroquinolone activity in the sensitive isolates, after sequencing for these isolates, is a bacteriostatic activity. The results of this study showed the *gyrA* mutations resulting from the excessive use of antibiotics are one of the mechanisms may be that leading to fluoroquinolone resistance.

Keywords: Fluoroquinolone, *gyrA*, direct sequencing, *P. aeruginosa*, *16s rRNA*, antibiotic resistance.

دراسة بكتريولوجية و جزيئية لبكتريا الزوائف الزنجارية المقاومة للفلوروكوينالون و المعزولة من مصادر سريرية مختلفة

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الخلاصـــة

أجريت هذه الدراسة للتحقق من مقاومة الفلوروكينولونات (FQs) في العزلات السريرية لبكتريا الزوائف الزنجارية المعزولة من مصادر مختلفة من مستشفى الحسين في مدينة السماوة مع دراسة تأثير الطفرات في موروث المقاومة. الآلية الأساسية لمقاومة الفلوروكينولونات في بكتريا الزوائف الزنجارية هي طفرات تحدث في مورث البكتيريا الاساسي التي يشفر للوحدة الفرعية A من الـ DNA gyrase بواسطة مورث الـ *gyrA* . في

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هذه الدراسة, شخصت اربعين عزلة سريرية (الحروق 7 (17.5 ٪)، الجروح 7 (17.5 ٪)، الأذن 2 (5 ٪)، غرفة العمليات 12 (30 ٪)، الأدرار 3 (7.5 ٪) و 9 (22.5 ٪) مركز غسيل الكلى الصناعي) بانها بكتريا الزوائف الزنجارية بناءً على طرق البكتريولوجية مع تأكيد بواسطة موروث الـ *16s rRNA* بأستخدام تقنية تفاعل البلمرة المتسلسل. تم إجراء اختبار الحساسية لجميع العزلات بواسطة اختبار Kirby–Pour باستعمال سبعة مضادات حيوية من الفلوروكينولونات. من بين اربعين عزلة سريرية، عشرة عزلات كانت مقاومة و ثلاثة حساسة أتجاه جميع المضادات الحيوية المختبرة، و سبعة وعشرون عزلة كانت ما بين متوسطة، مقاومة وحساسة لاثنين أو أكثر من المضادات الحيوية المختبرة مع تأكيد مقاومة العزلات بواسطة اختبار التركيز الحد الأدنى المثبط. استعملت طريقة التحليل المباشر للتتابعات لعشرة عزلات مقاومة لمضادات الفلوروكينولونات، حددت ثمانية طفرات في مورث الـ *gyrA* في مواقع مختلفة. بالإضافة إلى ذلك حددت فعالية الفلوروكينولون في العزلات الحساسة كفعالية مثبطة للجراثيم بعد تحديد التتابعات في هذه العزلات. أظهرت نتائج هذه الدراسة أن طفرات الـ *gyrA* الناتجة عن الاستخدام المفرط للمضادات الحيوية هي احدى الآليات التي قد تؤدي الى مقاومة الفلوروكينولونات.

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is an ubiquitous bacterium known as a pathogenic microbe responsible for serious opportunistic infections, especially in the immunocompromised subjects [1]. The spread of this organism in health institutions is very dangerous, with infiltrates, via any defect at the basic defense line of the human host, enter the body through the skin, especially in the hospital intensive care units (ICUs) leading to nosocomial infections [1, 2]. *P.aeruginosa* pathogenesis is multifactorial, due to the existence of multiple mechanisms of veritable resistance to most antibiotics, resulting in the production of a variable set of cellular structures and extracellular molecules which play a significant role in increasing pathogenicity [3]. In general, antibiotics are natural products inhibiting the microbes in either bactericidal or bacteriostatic pattern [4]. Fluoroquinolones (FQs) are synthetic antibiotics with bactericidal activity against many species of Gram negative bacterium [4]. Fluoroquinolones are used basically in the treatment of infections generated by *P. aeruginosa*, but in recent years an increase of fluoroquinolones resistance was observed between clinical isolates at high levels, leading to the failure of the treatment of *P. aeruginosa* [5]. The basic mechanism of the resistant of fluoroquinolones in *P. aeruginosa* is mutations occurring in the bacterial *gyrA* gene encoding subunit A of DNA gyrase [2], thus leading to inhibiting the transcription and blocking DNA replication [6]. The aim of the current study was to detect the occurrence of mutations associated with fluoroquinolones resistance (*gyrA* gene) using partial fragments from 40 clinical isolates of *P. aeruginosa* using direct sequencing technique.

Materials and methods

Collection of clinical samples

A total of 258 clinical samples were collected from varied clinical sources of patients and teaching laboratories of Al-Hussein Hospital in Al-Samawah city during the period from November 2018 to February 2019. These samples were taken accurately from each source using a sterile swab, which was then placed in a plane tube containing BHI broth and transferred to public health laboratory (bacteriological unit) in Al-Samawah city, where they were incubated at 37 ˚C for 24hr. The main sources of the samples were Burn, Wound, Ear, Operation Room, Urine and Industrial Dialysis Center.

Identification of bacterial isolates

As cultural method, all samples were grown on blood and macconkey agars and incubated-at 37 ˚C for 24hr as an initial diagnostic approach. Additionally, sub-cultures onto macconkey agar were performed to remove bacterial mixed growth and obtain pure colonies of each isolate [7]. For purification, *P. aeruginosa* was isolated using selective media (Pseudomonas and Cetrimide agars) to confirm the inhibition of other bacterial growth and the production of Pigments [8, 9, 10]. For microscopic examination, Gram stain was used [11]. Diagnostic tests were performed using a variety set of tests (oxidase, catalase, blood hemolysis, urease, motility, indole, citrate, gelatin, and KIA) [12] with confirmed by $VITEK_2$ Compact was achieved using Gram-negative card kits (GN ID) through the colorimetric technique [7]. Then, molecular detection of all isolates was performed by the *16s rRNA* gene using PCR technique [13].

Antibiotic sensitivity test

Antibiotic sensitivity testing (AST) was performed by Kirby-Bauer test using Agar Disc Diffusion Method in Mueller-Hinton medium, following recommendations of the Clinical Laboratory Standards Institute (CLSI, 2018, 28th) for Antibiotic sensitivity testing in the medical laboratories. The following 7 antibiotics of fluoroquinolones were used: Nalidixic Acid (30µg), Ciprofloxacin (10µg), Norfloxacin (10µg), Ofloxacin (5µg), Lomafloxacin (10µg), Levofloxacin (5µg), and Trovafloxacin (10µg), which were all obtained from BioAnalyse, Turkey [14].

Minimum inhibitor concentration test

The MICs were performed by agar well dilution method (AWDM) in Mueller- Hinton medium, with following the CLSI 2018 recommendations for 'MIC breakpoints' and using 7 antibiotics of fluoroquinolones (NA, CIP, NOR, OFX, LOM, LEV, and TRV) [15].

DNA extraction

Genomic DNA was extracted from all isolates using a specific Kit (GenomicDNA Mini Kit, FavorGEN, Korea) in accordance with the user manual of the manufacturer company (specific protocol for gram negative-bacterium). In addition, concentration and purity of DNA for each sample were estimated by a NanoDrop device with a range of 147.8 µg/ml within 1.7 purity to 1236.8 µg/ml within 1.9 purity.

PCR reaction

The PCR technique was used to amplify *16s rRNA* and *gyrA* genes, using specific primers (table 1) [16, 17]. A volume of 20 µL was used for amplification in accordance with the user manual of the manufacturer company, which included 10µL of master mix, 1µL of each primer, 3 µL of the DNA template, and 5 µL of nuclease free water. The proper program for this experiment is presented in table 2. Several trails were conducted to determine the optimum conditions to amplify *16s rRNA* and *gyrA* genes in a thermal-cycler device depending on different primers and the DNA template. Afterward, 5 µL was analyzed to detect *16s rRNA* and *gyrA* genes of each PCR product through electrophoresis on 1% agarose gel containing 3 µL of ethidium bromide, and then viewed on UV transilluminator.

Table 1-Nucleotides sequences for primers (acc.no.NC_002516.2)

ID	Gene	PCR Primers 5'…….. 3'		X (bp)	Ref.
1.	*16s rRNA*	F	*TCAACCTGGGAACTGCATCC*	468	16
		R	*ACATCTCACGACACGAGCTG*		
2.	*gyrA*	F	*GTGTGCTTTATGCCATGAG*	287	17
		R	*GGTTTCCTTTTCCAGGTC*		

Note: X= PCR product length, Ref= Reference, F= Forward primer, R= Reverse primer.

Table 2-Basic PCR conditions in this experiment

ID	Steps	Temp (°C)	Time	Cycle
1.	Initial Denaturation	95	5 min	1
2.	Denature	95	40 sec	25
3.	Anneal	X	45 sec	
4.	Extend	72	1 min	
5.	Final Extension	72	5 min	1

X= 58.8 °C for *16s rRNA*, X= 51.6 °C for *gyrA*.

DNA sequencing

In the current study, PCR products of the 15 isolates (13 for *gyrA* and 2 for *16s rRNA*) were sent to the MacroGenecompany, South Korea, to define the basic nucleotides sequences for the genes. The results of sequencing were then analyzed by BioEdit and MEGA7 software with the NCBI engine.

Results

Amongst the 258 clinical samples, forty *P.aeruginosa* isolates from different sources were obtained. The isolates of *P.aeruginosa* distributed among clinical samples based on their source are listed in Table-3. All clinical isolates were identified as *P.aeruginosa* by a set of a variety of techniques (biochemical tests, cultural, pigment production and $VITEK_2$ Compact). The results are demonstrated in Table-4.

Table 3-Distribution of *P.aeruginosa* isolates.

ID	Source of Samples	No. of Samples	Isolates	
			No.	%
1.	Burn	58	7	17.5
2.	Wound	50	7	17.5
3.	Ear	20	2	5
4.	Operation Room	54	12	30
5.	Urine	34	3	7.5
6.	Industrial Dialysis Center	42	9	22.5
7.	Total	258	40	100

Table 4-Biochemical tests for the identification of *P.aeruginosa*.

Test	Gram stain	Oxidase	Catalase	Hemolysis	Urease	Motility	Indole	Citrate	Gelatin	Ferment	Cetrimide	Pseudomonas
Result	-	+	+	+	-	+	-	+	+	-	+	+

(+)= Positive, (-)= Negative.

Antibiotic sensitivity test

Amongst the 40 clinical isolates, 10 were resistant and 3 were sensitive for all tested antibiotics, whereas 27 were intermediate, resistant and sensitive for two or more of the tested antibiotics (Table-5), These results agree with those of previous reports on clinical isolates of *P.aeruginosa* [16, 17, 18]. This study showed (Fig. 1) that most of *P.aeruginosa* isolates showed the highest level of resistance against the tested antibiotics in accordance with the percentage for each antibiotic; Nalidixic Acid 34 (85%), Trovafloxacin 25 (62.5%), Lomefloxacin 18(45%), Norfloxacin 13(32.5%), Ciprofloxacin 12 (30%), Levofloxacin 12 (30%), and Ofloxacin 11 (27.5%).

Table 5-Results of the antibiotic sensitivity test for *P.areuginosa* isolates.

ID	Antibiotics	Resistant No.(%)	Sensitive No.(%)	Intermediate No.(%)
1.	Nalidixic Acid	34 (85 %)	3 (7.5 %)	3 (7.5 %)
2.	Ciprofloxacin	12 (30 %)	26 (65 %)	2 (5 %)
3.	Norfloxacin	13 (32.5 %)	27 (67.5 %)	0 (0 %)
4.	Ofloxacin	11 (27.5 %)	28 (70 %)	1 (2.5 %)
5.	Lomafloxacin	18 (45 %)	16 (40 %)	6 (15 %)
6.	Levofloxacin	12 (30 %)	28 (70 %)	0 (0 %)
7.	Trovafloxacin	25 (62.5 %)	14 (35 %)	1 (2.5 %)

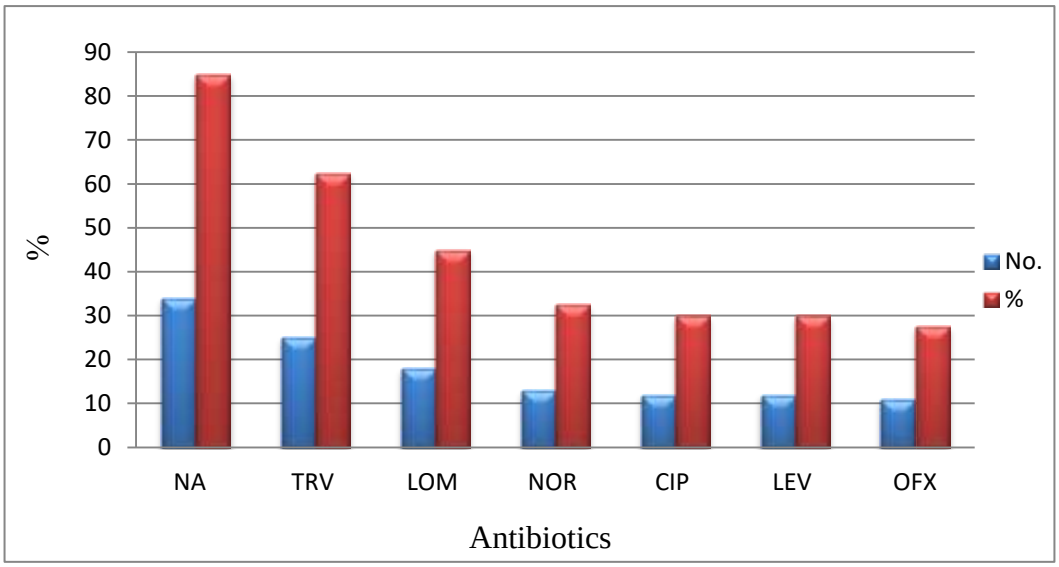


Figure 1-The number and percentage of resistant isolates of *P.aeruginosa*.

MIC Test

Amongst the 40 clinical isolates, 10 isolates that were resistant to all tested antibiotics were selected for the MIC test to confirm the resistance of isolates in accordance with the antibiotic sensitivity test, based on recommendations of the CLSI, 2018. The results are listed in the Table-6.

Table 6-The results of resistant isolates in actual MIC range

Id	Resistance Isolates		Actual MIC range (mg/ml)						
	Source	No.	NA	CIP	NOR	OFX	LOM	LEV	TRV
1.	Burn	Mu 3	> 128	32	32	4	4	32	> 0.016
2.	Wound	Mu 10		128	128	16			0.016
3.		Mu 12							
4.		Mu 14		8	8	4		16	> 0.016
5.	Operation Room	Mu 17		128	128	16		32	
6.		Mu 21	128	1	1	0.25			
7.		Mu 27	> 128	128	128	16		16	
8.	Urine	Mu 30				8		32	
9.		Mu 31							
10.	IDC	Mu 32		8	8	4			

Note: Mu= No. of resistance isolates (pink color), IDC= Industrial Dialysis Center, NA= Nalidixic Acid, CIP= Ciprofloxacin, NOR= Norfloxacin, OFX= Ofloxacin, LOM= Lomefloxacin, LEV= Levofloxacin, TRV= Trovafloxacin.

Molecular detection of *16s rRNA* and *gyrA*

The *16s rRNA* was used as a molecular marker for the diagnosis of *P. aeruginosa*. The results of the current study showed that forty isolates of *P. aeruginosa* contained the *16s rRNA* gene, which is considered as the diagnostic gene for this bacteria. Specific primers were used for the amplification of *16s rRNA*, with 468 bp by conventional PCR based on 58.8 °C annealing temperature (Figure-2). Whereas, the *gyrA* gene in the current study was detected by PCR technique in *P. aeruginosa* isolates associated with fluoroquinolones resistance. Specific primers were used to amplify 287 bp of *gyrA* gene based on 51.6 °C annealing temperature (Figure-3).

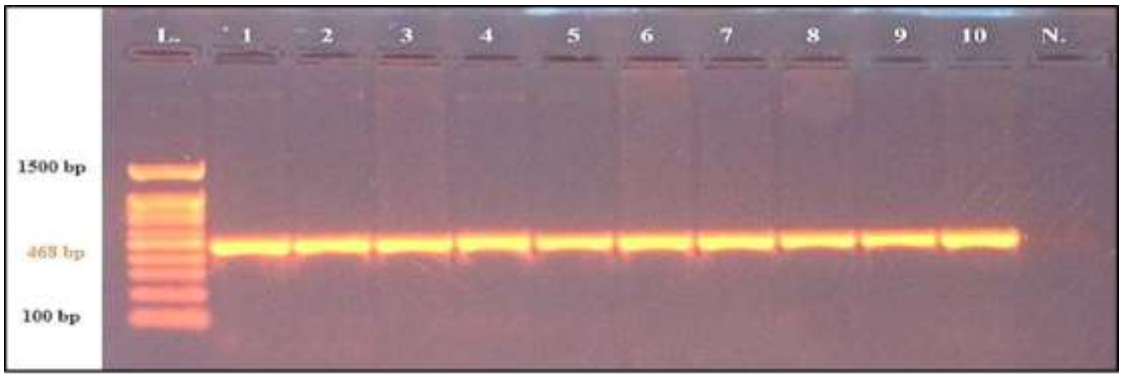


Figure 2-Ethidium-Bromide stained agarose gel (1% agarose) showing the PCR amplification products with *16s rRNA* gene (468 bp) primers for *P.aeruginosa* extracted DNA. The electrophoresis was conducted at 70 volts for 70 min. Lane (L) is DNA molecular size marker (100→1500 bp ladder). Lanes (1→10) represent bacterial isolates. Lane (N) represents negative control.

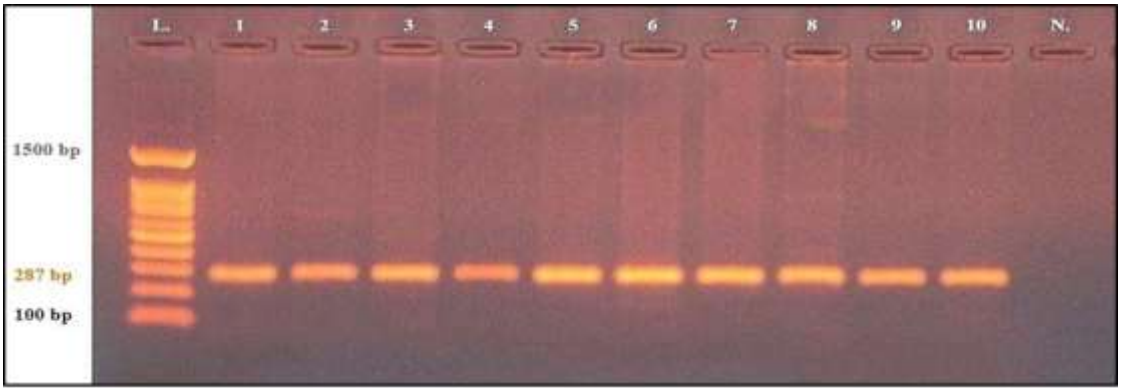


Figure 3-Ethidium-Bromide stained agarose gel (1% agarose) showing the PCR amplification products with *gyrA* gene (287 bp) primers for *P.aeruginosa* extracted DNA. The electrophoresis was conducted at 70 volts for 70 min. Lane (L) is DNA molecular size marker (100→1500 bp ladder). Lanes (1→10) represent bacterial isolates. Lane (N) represents negative control.

Data analysis using direct sequencing

1. Phylogenetic tree analysis

Phylogenetic analysis was conducted through following up the alignment using the $BLAST_n$ program for *16s rRNA* sequences obtained from the current isolates by Sanger sequencing after trimming by MEGA7 software [19]. The phylogenetic tree (dendrogram) was created by MEGA7 software using the neighbor-joining method as shown in the figure 4 which illustrates the relationships amongst the local isolate of *16s rRNA* with the reference strains in GenBank.

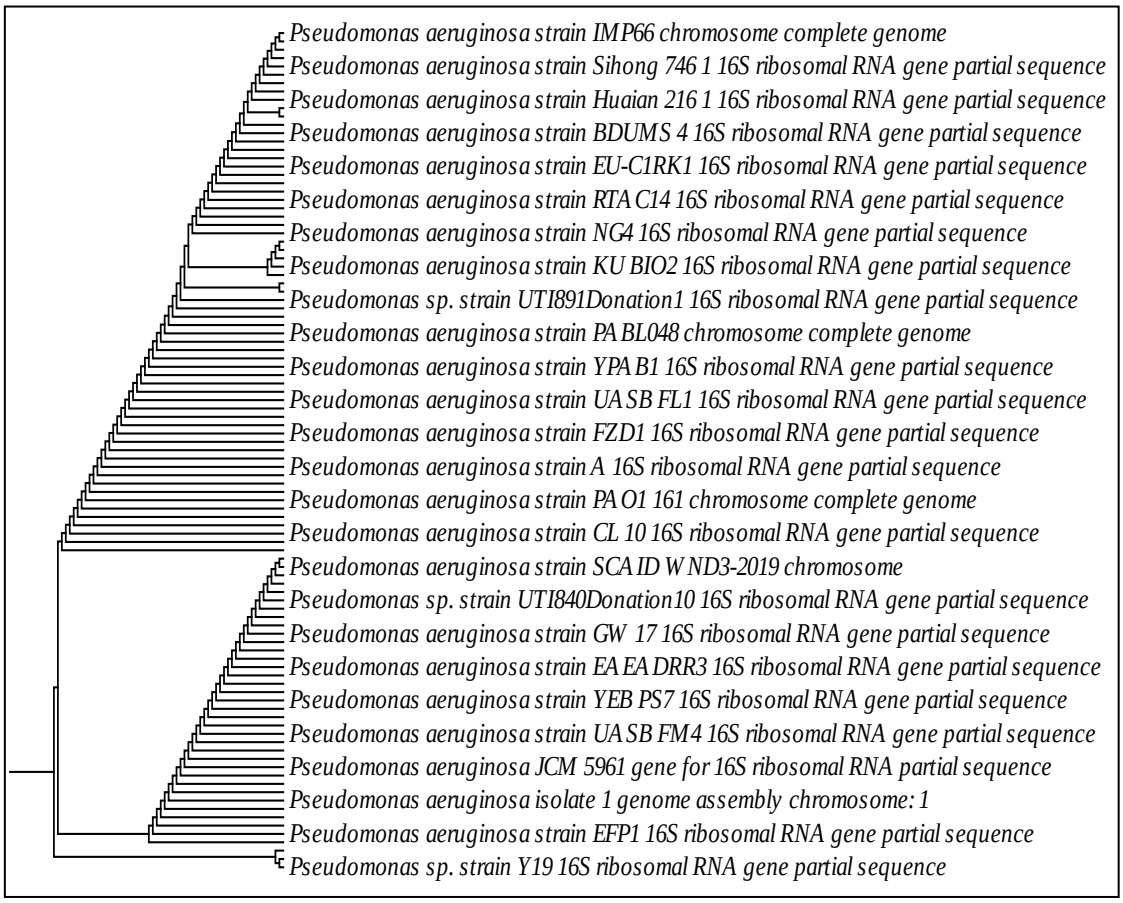
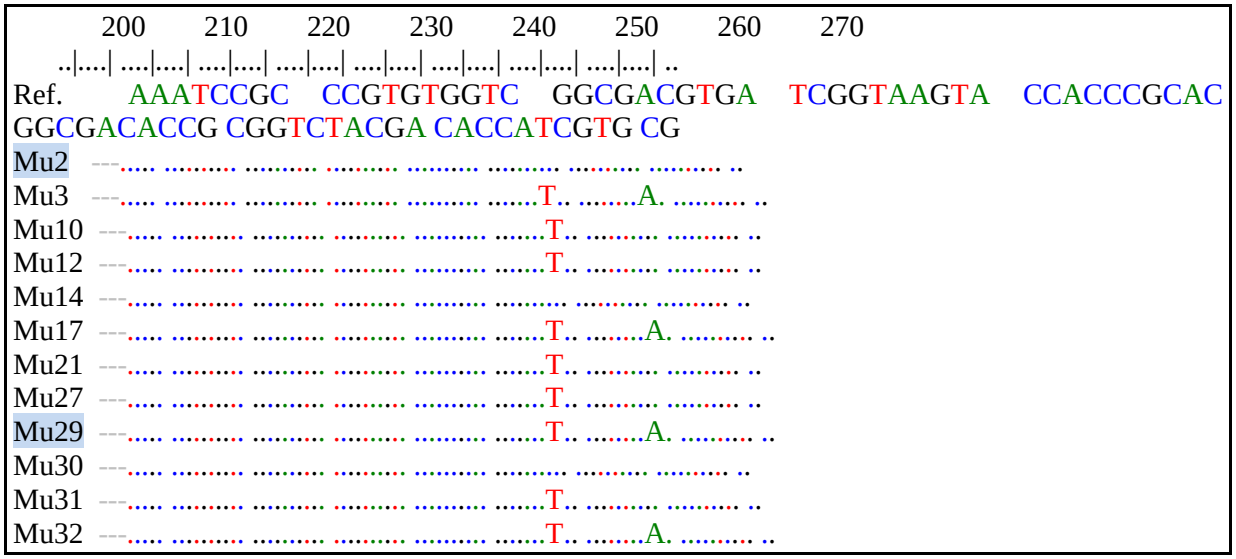


Figure 4-Phylogenetic tree by MEGA7 software, showing the classification of the isolates used in this study and nearest strains of *P.aeruginosa*. Note, strain PAO1 is target strain in this study (shown in green color).

2. *GyrA* gene

The *gyrA* sequences were translated by BioEdit software through comparing the reference sequence (acc.no.NC_002516.2) with local isolates, using option toggle translation from the alignment menu after the sequences alignment (Figure-5), to detect the alterations in amino acids in each isolate (Figure-6). In addition, the *gyrA* sequences were treated by MEGA7 software before starting the sequences alignment, to remove the trimming from the ends. All amino acids and nucleotides with alterations or substitution are listed in the Table-7.



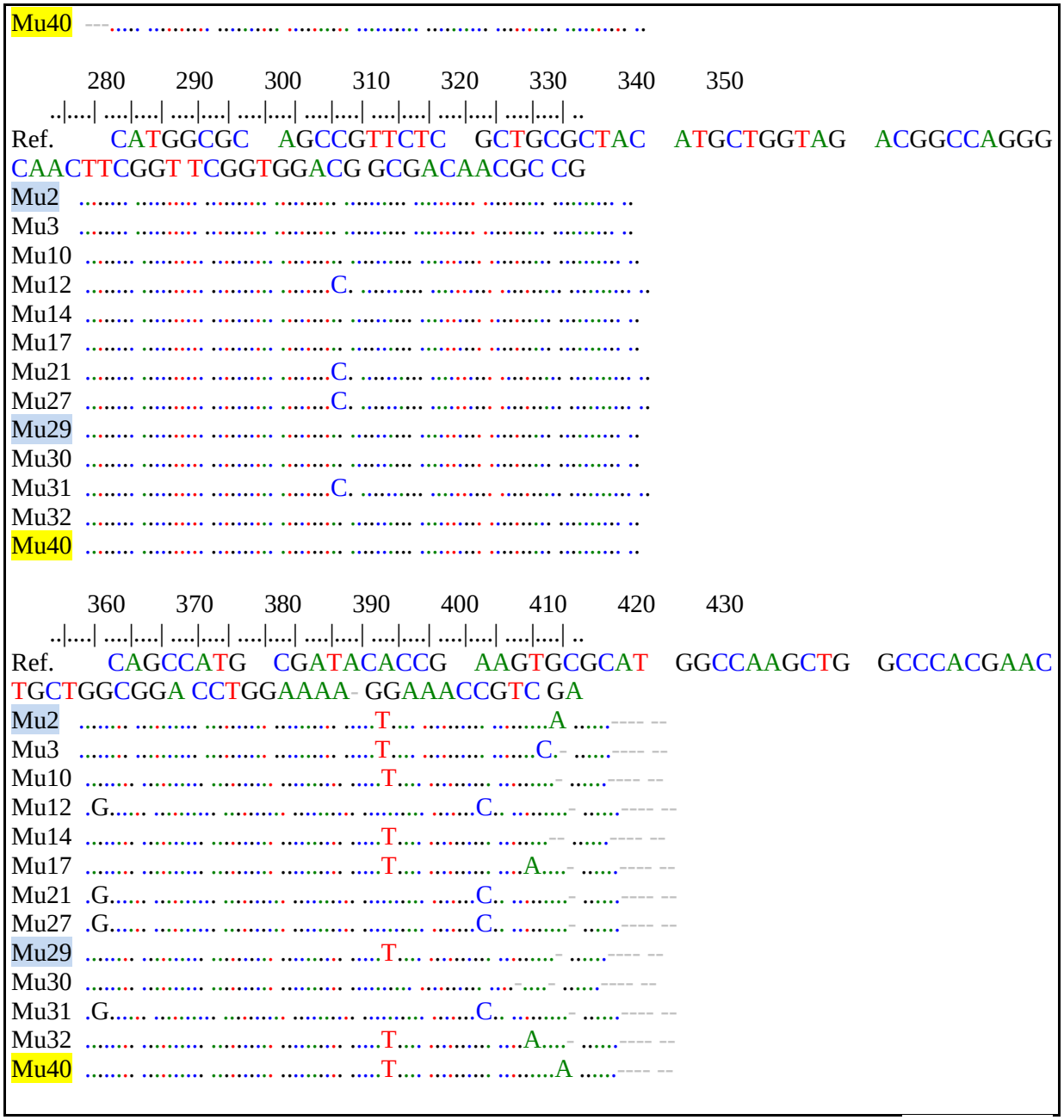


Figure 5-DNA sequences alignment of local isolates with reference sequence (acc.no.NC_002516.2) of *gyrA* gene by BioEdit software, with alterations in each isolate (table 7). The symbol "Mu" in black color indicates resistant isolates, in blue sensitive isolates, in yellow intermediate isolate.

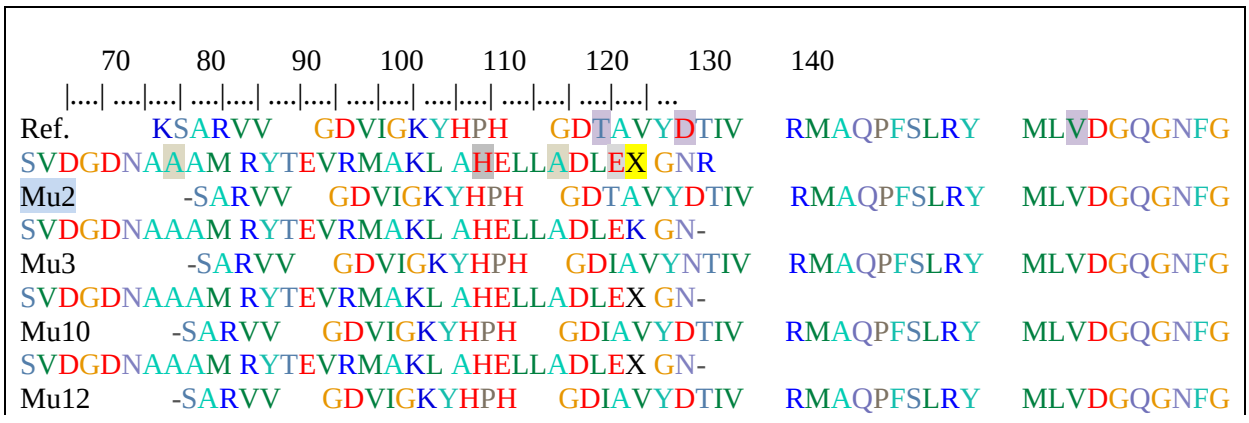


Figure 6-Amino acid alignment of the *gyrA* sequences by BioEdit software version (7.1) with alterations in each isolate (Table-7).

Table 7-All amino acids and nucleotides with alterations in the *gyrA* gene

ID	Mu	Nucleotides			Amino Acids		Type of Mutation
		Position		Change codons	Position	Change	
		X	Y				
1.	3,10,12,17,21,27,29,31,32	248	53	ACC→ATC	83	T→I	Missense
2.	3,17,29,32	259	64	GAC→AAC	87	D→N	Missense
3.	12,21,27,31	309	114	GTA→GTC	103	V→V	Silent
4.	12,21,27,31	354	159	GCA→GCG	118	A→A	Silent
5.	2,3,10,14,17,29,32,40	396	201	CAC→CAT	132	H→H	Silent
6.	12,21,27,31	408	213	GCG→GCC	136	A→A	Silent
7.	30	415	220	GAA	139	E→X	Deletion
8.	17, 32	415	220	GAA→AAA	139	E→K	Missense
9.	2, 40	420	225	AAA	140	X→K	Insertion

Mu_ No. of mutant isolates, Position X_= nucleotides position in reference gene, Position Y_= nucleotides position in the amplicon, X_ any acid of the twenty standard amino acids, basic nucleotides in red _= the locations of the alterations.

In our study, after the sequencing of 10 isolates resistant to all tested antibiotics of fluoroquinolones, we identified 8 mutations in *gyrA* gene at different positions, of which 3 (37.5 %) were missense mutations, 4 (50 %) were silent mutations, and 1 (12.5 %) was a deletion mutation. Whereas when sequencing for the 3 control isolates (2 sensitive and 1 intermediate), we identified 4 mutations in *gyrA* gene at different positions, of which 2 (50 %) were missense mutations, 1 (25 %)

was a silent mutation, and 1 (25 %) was insertion mutation. Based on this, we identified the fluoroquinolone activity in the sensitive isolates as a bacteriostatic activity, in accordance with the basic tests (antibiotic sensitivity, MIC and sequencing), due to the presence of several mutations which are limiting the use of this class of antibiotics in the future. Nevertheless, all the mutations diagnosed in the *gyrA* gene were related with all antibiotics (NA, CIP, NOR, OFX, LOM, LEV, and TRV) in accordance with the 10 isolates of *P.aeruginosa* that were resistant for all tested antibiotics of fluoroquinolones class.

Discussion

Fluoroquinolones are synthetic antibiotics with bactericidal-activity against many species of Gram negative bacterium [20]. In most hospitals, excessive use of a wide spectrum of antibiotics led to the appearance of highly resistant isolates of *P. aeruginosa* [17]. Based on biochemical, epidemiological, and molecular previous studies, the first target for FQs in *P.aeruginosa* is DNA gyrase [18]. In the current study, the mutations in *gyrA* gene of the 13 isolates of *P. aeruginosa* (10 resistant, 2 sensitive, and 1 intermediate) obtained from the 40 clinical isolates are demonstrated in Table-7) above. To reveal the correlation amongst mutations and isolates associated with fluoroquinolones resistance, a comparison was made for the molecular analysis results of the target gene with MIC test in accordance with the antibiotic sensitivity test. The results in our study showed that, amongst the 40 clinical isolates, 10 were resistant and 3 were sensitive for all tested antibiotics, while 27 were intermediate, resistant and sensitive for two or more of the tested antibiotics. In addition, amongst the 10 isolates of *P.aeruginosa* resistant to all tested antibiotics of fluoroquinolones, 2 had a single mutation, 1 had a double mutation, and 7 had several mutations. While for the 2 sensitive isolates to fluoroquinolones, 1 had several mutations and 1 had a double mutation, with 1 intermediate isolate that had a double mutation in *gyrA* gene. These results agree with those of a previous molecular study [17]. However, we identified 9 mutations in *gyrA* gene in the 13 clinical isolates (10 associated with fluoroquinolones resistant, 2 sensitive, and 1 intermediate) that occurred at different codons (83, 87, 103, 118, 132, 136, 139, and 140). The alterations of nucleic acid in these codons were detected through the change of the amino acid Threonine in the codon 83 to Isoleucine (Thr-83→Ile), which was found in 9 isolates. Additionally, Aspartate in the codon 87 was changed to Asparagine (Asp-87→Asn) in 4 isolates. These results agree with those of a previous reports on clinical isolates of *P.aeruginosa* [6, 17, 18, 21]. The present analysis also revealed changes of Valine in the codon 103 to Valine (Val-103→Val) in 4 isolates, Alanine in the codon 118 to Alanine (Ala-118→Ala) in 4 isolates, Histidine in the codon 132 to Histidine (His-132→His) in 8 isolates, and Alanine in the codon 136 to Alanine (Ala-136→Ala) inn 4 isolates. These results agree with those of a previous molecular study [2]. In addition, we found changes of Glutamate in the codon 139 to Lysine (Glu-139→Lys) in 2 isolates, Glutamate in the codon 139 to X (deletion G) (Glu-139→X) in 1 isolate, and insertion A in the codon 140 to Lysine (X-139→Lys) in 1 isolate. These results are in disagreement with the results of previous molecular studies [2, 17]. In this study, the highest levels of resistant were to Nalidixic Acid (85%), Trovafloxacin (62.5%), Lomefloxacin (45%), Norfloxacin (32.5%), Ciprofloxacin (30%), Levofloxacin (30%), and Ofloxacin (27.5%). The importance of this resistance in *P. aeruginosa* isolates is that it gradually leads to increased secretion of the virulence factors [2]. Moreover, the highest levels of resistant are usually associated with the existence of *gyrA* mutations and lead to the failure of the treatment of *P. aeruginosa*, thus limiting the use of this class of antibiotics in the future.

Conclusions

In conclusion, the results of the present study, in agreement with the previous reports, showed the *gyrA* mutations resulting from the excessive use of antibiotics are one of the mechanisms may be that leading to fluoroquinolone resistance in *P. aeruginosa* clinical isolates, where we proved that DNA gyrase encoding *gyrA* gene is considered as the basic target for fluoroquinolones.

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