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Original Article

Phenotypic and molecular characterization of fluoroquinolone resistant *Pseudomonas aeruginosa* isolates in Palestine

Caracterização fenotípica e molecular de isolados de *Pseudomonas aeruginosa* resistentes a fluoroquinolonas na Palestina

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Abstract

Fluoroquinolones are important antimicrobial agents for the treatment of *Pseudomonas* infections. A total of 11 isolates of *P. aeruginosa* were collected from different clinical samples from different medical centers in the North West Bank-Palestine during 2017. In this study, resistance to fluoroquinolones and secretions of β-lactamases were detected by phenotypic methods, while presence of β-lactamase gene sequences and other virulence factors were detected by PCR technique. PCR product for *gyrA*, *parC* and *parE* genes were sequenced for further analyses. The phylogenetic analyses, population diversity indices and haplotypes determination were conducted using computer programs MEGA version 6, DnaSP 5.1001 and median-joining algorithm in the program Network 5, respectively. Results of this study showed that the MIC for ciprofloxacin and norfloxacin had a range of 32-256 µg/ml. In addition, all isolates carried either *exoT* or *exoT* and *exoY* genes, different β-lactamase genes and 82% of these isolates harbored class 1 integrons. Analyses of the *gyrA*, *parC* and *parE* sequences were found to be polymorphic, had high haplotype diversity (0.945-0.982), low nucleotide diversity (0.01225-0.02001) and number of haplotypes were 9 for each *gyrA* and *parE* genes and 10 haplotypes for *parC* gene. The founder haplotypes being Hap-1 (18%), Hap-2 (27.3%) and Hap-6 (9.1%) for *gyrA*, *parC* and *parE* genes, respectively. Two of *ParE* haplotypes were detected as indel haplotypes. The Median-joining- (MJ) networks constructed from haplotypes of these genes showed a star-like expansion. The neutrality tests (Tajima's D test and Fu's Fs test) for these genes showed negative values. Palestinian fluoroquinolone resistant *P. aeruginosa* strains showed high MIC level for fluoroquinolones, β-lactamase producers, carried type III secretion exotoxin-encoding genes, most of them had integrase I gene and had high level of mutations in QRDR regions in *gyrA*, *parC* and *parE* genes. All these factors may play an important role in the invasiveness of these strains and make them difficult to treat. Isolation of these strains from different medical centers, indicate the need for a strict application of infection control measures in Medical centers in the North West Bank-Palestine that aim to reduce expense and damage caused by *P. aeruginosa* infections. Molecular analyses showed that Palestinian fluoroquinolone resistant *P. aeruginosa* haplotypes are not genetically differentiated; however, more mutations may exist in these strains.

Keywords:*P. aeruginosa*, *gyrA*, *parC*, *parE*, haplotypes.

Resumo

Fluoroquinolonas são agentes antimicrobianos importantes para o tratamento de infecções por Pseudomonas. Um total de 11 bacilos isolados de *P. aeruginosa* foram coletados de diferentes amostras clínicas provenientes de diferentes centros médicos na Cisjordânia-Palestina durante o ano de 2017. Neste estudo, resistência a fluoroquinolonas e secreções de β-lactamases foram detectadas por métodos fenotípicos, enquanto a presença de sequências do gene β-lactamase e outros fatores de virulência foram detectados pela técnica de PCR (Proteína C-reativa). O produto de PCR para os genes *gyrA*, *parC* e *parE* foram sequenciados para análises posteriores. As análises filogenéticas, os índices de diversidade populacional e a determinação de haplótipos foram realizados utilizando os *softwares MEGA* versão 6, *DnaSP* 5.1001 e o algoritmo de junção de mediana do programa *Network 5*, respectivamente. Os resultados deste estudo mostraram que a MIC para ciprofloxacina e norfloxacina tinha um intervalo de 32-256 µg/ml. Além disso, todos os bacilos isolados carregavam genes *exoT* ou *exoT* e *exoY,* genes de β-lactamase diferentes e 82% desses isolados continham integrons de classe 1. As análises das sequências *gyrA*, *parC* e *parE* foram consideradas polimórficas, com alta diversidade de haplótipos (0,945-0,982), baixa diversidade de nucleotídeos (0,01225-0,02001) e o número de haplótipos foi de 9 para cada gene de *gyrA* e *parE* e 10 haplótipos para o gene *parC*. Os haplótipos fundadores são Hap-1 (18%), Hap-2 (27,3%) e Hap-6 (9,1%) para os genes *gyrA*, *parC* e *parE*, respectivamente. Dois dos haplótipos *parE* foram detectados como haplótipos InDel. As redes *Median-joining* (MJ) construídas a partir de haplótipos desses genes mostraram uma expansão semelhante à de uma estrela. Os

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testes de neutralidade (teste D de Tajima e teste Fs de Fu) para esses genes apresentaram valores negativos. As cepas palestinas de *P. aeruginosa* resistentes a fluoroquinolonas mostraram alto nível de MIC para fluoroquinolonas, produtores de β-lactamase, genes codificadores de exotoxina de secreção tipo III, a maioria deles tinha o gene integrase I e tinha alto nível de mutações nas regiões QRDR nos genes *gyrA*, *parC* e *parE*. Todos esses fatores podem desempenhar um papel importante na invasão dessas cepas e torná-las difíceis de tratar. O isolamento dessas cepas em diferentes centros médicos, indica a necessidade de uma aplicação estrita de medidas de controle de infecção em centros médicos da Cisjordânia-Palestina que visam reduzir despesas e danos causados por infecções por *P. aeruginosa*. As análises moleculares mostraram que os haplótipos de *P. aeruginosa* resistentes à fluoroquinolona palestina não são geneticamente diferenciados; no entanto, mais mutações podem existir nessas cepas.

Palavras-chave:*P. aeruginosa*, *gyrA*, *parC*, *parE*, haplótipos.

1. Introduction

Pseudomonas aeruginosa is considered as one of the most important opportunistic Gram-negative pathogen that can cause many human infections including life-threatening (Lihua et al., 2013). Antibiotic resistance against this pathogen happens naturally as well as its ability to acquire resistance to a wide range of antimicrobial classes, make infections caused by this species of bacteria difficult to manage (Perez et al., 2014). The antipseudomonal agents are very limited and categorized into three major antimicrobial classes: lactams, aminoglycosides and fluoroquinolones (Giamarellou and Antoniadou, 2001). Fluoroquinolones such as levofloxacin and ciprofloxacin are considered as drugs of choice for treatment of *P. aeruginosa* infections (Llanes et al., 2011). These agents act by inhibiting the action of certain DNA replication enzymes such as DNA gyrase (type II topoisomerases) and topoisomerase IV (Dalhoff, 2012). DNA gyrase consists of an A2B2 heterotetramer encoded by the *gyrA* and *gyrB* genes, while topoisomerase IV exists as a C2E2 heterotetramer encoded by the *parC* and *parE* genes (Wydmuch et al., 2005).

It was reported that mutations in the DNA gyrase and topoisomerase IV (Agnello and Wong-Beringer, 2012; Kobayashi et al., 2013), efflux pump system overexpression and the innate impermeability of the membrane (Speciale et al., 2000; Teresa Tejedor et al., 2003) are mechanisms of fluoroquinolone resistance in *P. aeruginosa*. Sequence mutations in quinolone-resistance-determining region (QRDR) within DNA gyrase and topoisomerase IV are considered as major mechanism for fluoroquinolone resistance in *P. aeruginosa* (Nouri et al., 2016). Mutations in QRDR of DNA gyrase and topoisomerase IV, usually happen more frequently in *gyrA* and *parC* genes, respectively, and express the highest level of fluoroquinolone resistance. Mutation frequency in *gyrB* and *parE* genes is relatively low compared to that reported in *gyrA* and *parC* genes (Lee et al., 2005; Lister et al., 2009; Nouri et al., 2016).

This study aimed to determine the phenotypic and molecular characterization of clinical isolates of fluoroquinolone resistant *P. aeruginosa*. The current study designed to detect β-lactamases genes and their secretions, integrons 1, 2 and 3 and type III secretion system (T3SS) among clinical isolates of fluoroquinolone resistant *P. aeruginosa*. Additionally, It is designed to determine genetic diversity among these isolates depending on sequence analysis of *gyrA*, *parE* and *parC* genes. To our knowledge, this is the first study of its kind in Palestine and in many other countries around.

2. Materials and Methods

2.1. Bacterial strains collection and identification

A total of 11 isolates of *P. aeruginosa* were collected from different clinical samples during 2017. The samples included 2 urine, 6 wound swabs, 1 sputum trap and 2 ear swabs. The isolates were collected from different medical centers in the North West Bank-Palestine and identified by API 20NE system (Biomerieux) and identification was confirmed using conventional methods in microbiology research laboratory, at An-Najah National University. Replicate isolates from the same patient were excluded.

2.2. Antibiotic resistant test

Antimicrobial sensitivity testing was conducted according to instructions determined by the Clinical and Laboratory Standard Institute (CLSI, 2016) using the disk diffusion method. All *P. aeruginosa* isolates were examined using the following disks (Oxoid); Ciprofloxacin (CIP) 5 µg, Norfloxacin (NOR) 10 µg, Cefotaxime (CTX) 10 µg, Ceftazidime (CAZ) 30 µg, Imipenem (IPM) 10 µg and Meropenem (MEM) 10 µg. Mueller Hinton agar (MHA) (Oxoid) plates were seeded with McFarland 0.5 standard of *P. aeruginosa* strains, antibiotic disks were placed on the seeded plates. Then, the plates were incubated at 37 °C for 24 h. The inhibition zones were measured, and the isolates were classified as resistant or susceptible according to the criteria recommended by CLSI guidelines (CLSI, 2016). Isolates showed resistance to 3rd generation cephalosporins were tested for the production of ESBLs and AmpC β-lactamases, and those showed resistance to carbapenems were tested for the secretion of MBLs. The minimum inhibitory concentration (MIC) values for Ciprofloxacin and Norfloxacin were also determined by broth microdilution method following CLSI guidelines and breakpoints (CLSI, 2016). The reference strain of *P. aeruginosa* ATCC 27853 was used as a quality control in all of the experiments of antimicrobial susceptibility testing.

2.3. Detection of β-lactamases production by phenotypic tests

P. aeruginosa isolates that showed resistance to 3rd generation cephalosporins were tested for the production of extended spectrum β-lactamases (ESBLs) and AmpC β-lactamases by combination double disk test (CDDT) and combined disk method, respectively, as described previously (Ibrahim et al., 2013; Mansouri et al., 2014). Isolates showed resistance to carbapenems were tested

for the secretion of Metallo-β-lactamases (MBLs) using two different tests, combined disc diffusion test (CDDT) as described previously (Yong et al., 2002; Franklin et al., 2006) and double disc synergy test (DDST) as described previously by Lee et al. (2003)

2.4. DNA extraction

P. aeruginosa genome was prepared for PCR according to the method described previously (Adwan et al., 2013). Briefly, the cells were scraped off an overnight MHA plate, washed with 800 µL of 1X Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8]), centrifuged, and the pellet was resuspended in 400 µL of sterile double distilled H_2O, and boiled for 10-15 min. The cells were incubated on ice for ten minutes. The debris were pelleted by centrifugation at 11,500 X *g* for 5 min. The DNA concentration was determined using a nanodrop spectrophotometer (Genova Nano, Jenway). The DNA samples were stored at -20 ºC for further analyses.

2.5. Detection of antibiotic resistance genes

The presence of AmpC β-lactamase (Class C) genes, extended spectrum β-lactamases (Class A and D) genes and Metallo-β-lactamases (Class B) genes was investigated using multiplex PCR divided into pool 1, pool 2 and pool 3, respectively. The sequence of primers, amplicon sizes and annealing temperatures are listed in Table 1.

Table 1. Target genes for PCR amplification, fragment size and primer sequences that were used in this study.

Group	Targets	Primer sequence 5′→3′	Expected amplicon size (bp)	Annealing temperature (pool)	References
AmpC β-lactamase (Class C)	*MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11*	MOXM F GCT GCT CAA GGA GCA CAG GAT MOXM R CAC ATT GAC ATA GGT GTG GTG C	520	64 ºC (1)	Pérez-Pérez and Hanson (2002)
	LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1	CITM F TGG CCA GAA CTG ACA GGC AAA CITM R TTT CTC CTG AAC GTG GCT GGC	462	64 ºC (1)	Pérez-Pérez and Hanson (2002)
	DHA-1, DHA-2	DHAM F AAC TTT CAC AGG TGT GCT GGG T DHAM R CCG TAC GCA TAC TGG CTT TGC	405	64 ºC (1)	Pérez-Pérez and Hanson (2002)
	ACC	ACCM F AAC AGC CTC AGC AGC CGG TTA ACCM R TTC GCC GCA ATC ATC CCT AGC	346	64 ºC (1)	Pérez-Pérez and Hanson (2002)
	MIR-1T, ACT-1	EBCM F TCG GTA AAG CCG ATG TTG CGG EBCM R CTT CCA CTG CGG CTG CCA GTT	302	64 ºC (1)	Pérez-Pérez and Hanson (2002)
	FOX-1 to FOX-5b	FOXM F AAC ATG GGG TAT CAG GGA GAT G FOXM R CAA AGC GCG TAA CCG GAT TGG	190	64 ºC (1)	Pérez-Pérez and Hanson (2002)
extended spectrum β-lactamases (Class A)	*SHV*	SHV F ATG CGT TATATT CGC CTG TG SHV R TGC TTT GTT ATT CGG GCC AA	747	60 ºC (2)	Paterson et al. (2003)
	TEM	TEM F TCG CCG CAT ACA CTA TTC TCA GAA TGA TEM R ACG CTC ACC GGC TCC AGA TTT AT	445	60 ºC (2)	Monstein et al. (2007)
	CTX	CTX-M F ATG TGC AGY ACC AGT AAR GTK ATG GC CTX-M R TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	593	60 ºC (2)	Boyd et al. (2004)
	KPC	KPCM F CGTCTAGTTCTGCTGTCTTG KPCM R CTTGTCATCCTTGTTAGGCG	789	60 ºC (2)	Poirel et al. (2011)
extended spectrum β-lactamases (Class D)	*OXA*	OXA F 5-ATT ATC TAC AGC AGC GCC AGT G-3 OXA R 5-TGC ATC CAC GTC TTT GGT G-3	296	60 ºC (2)	Kim et al. (2009)

Table 1. Continued...

Group	Targets	Primer sequence 5′→3′	Expected amplicon size (bp)	Annealing temperature (pool)	References
Metallo-β-lactamases (Class B)	*Imp*	Imp-F 5-GGAATAGAGTGGCTTAAYTCTC-3 Imp-R 5-CCAAACYACTASGTTATCT-3	188bp	52 ºC (3)	Ellington et al. (2007)
	Vim	Vim-F 5-GATGGTGTTTGGTCGCATA-3 Vim-R 5-CGAATGCGCAGCACCAG-3	390bp	52 ºC (3)	Ellington et al. (2007)
	Gim	Gim-F 5-TCGACACACCTTGGTCTGAA-3 Gim-R 5-AACTTCCAACTTTGCCATGC-3	477bp	52 ºC (3)	Ellington et al. (2007)
	Spm	Spm-F 5-AAAATCTGGGTACGCAAACG-3 Spm-R 5-ACATTATCCGCTGGAACAGG-3	271bp	52 ºC (3)	Ellington et al. (2007)
	Sim	Sim-F 5-TACAAGGGATTCGGCATCG-3 Sim-R 5-TAATGGCCTGTTCCCATGTG-3	570bp	52 ºC (3)	Ellington et al. (2007)
Integrases	*intI1*	intI1 F 5-GCATCCTCGGTTTTCTGG-3 intI1 R 5-GGTGTGGCGGGCTTCGTG-3	457bp	58 ºC (4)	Shibata et al. (2003)
	intI2	intI2 F 5-CACGGATATGCGACAAAAAGG T-3 intI2 R 5-GTAGCAAACGAGTGACGAAATG-3	789bp	58 ºC (4)	Shibata et al. (2003)
	intI3	intI3 F 5-AT TGCCAAACCTGACTG-3 intI3 R 5-CGAATGCCCCAACAACTC-3	922bp	58 ºC (4)	Shibata et al. (2003)
type III secretion system (T3SS)	*exoS*	Exo S F: GCG AGG TCA GCA GAG TAT CG Exo S R: TTC GGC GTC ACT GTG GAT GC	118	56ºC (5)	Ajayi et al. (2003)
	exoT	Exo T F: AAT CGC CGT CCA ACT GCA TGC G Exo T R: TGT TCG CCG AGG TAC TGC TC	152	56ºC (5)	Ajayi et al. (2003)
	exoU	Exo U F: CCG TTG TGG TGC CGT TGA AG Exo U R: CCA GAT GTT CAC CGA CTC GC	134	56ºC (5)	Ajayi et al. (2003)
	exoY	Exo Y F: CGG ATT CTA TGG CAG GGA GG Exo Y R: GCC CTT GAT GCA CTC GAC CA	289	56ºC (5)	Ajayi et al. (2003)
Fluoroquinolone	*GyrA*	GyrA F: TTA TGC CAT GAG CGA GCT GGG CAA CGA CT GyrA R: AAC CGT TGA CCA GCA GGT TGG GAA TCT T	366	65ºC	Sekiguchi et al. (2005)
	ParC	ParC F: ATG AGC GAA CTG GGG CTG GAT ParC R: ATG GCG GCG AAG GAC TTG GGA	210	58ºC	Sekiguchi et al. (2005)
	ParE	ParE F: CGG CGT TCG TCT CGG GCG TGG TGA AGG A ParE R: TCG AGG GCG TAG TAG ATG TCC TTG CCG A	592	68ºC	Sekiguchi et al. (2005)

2.6. Detection of class 1, 2 and 3 integrons

The detection of integrons *intI1*, *intI2* and *intI3* was carried out using multiplex PCR (see Table 1, pool 4). Primer sequences, the size of amplicons and annealing temperature are presented in Table 1.

2.7. Detection of type III secretion exotoxin-encoding genes

The detection of type III secretion exotoxin-encoding genes (*exoS, exoT, exoU* and *exoY* genes) was performed using multiplex PCR (see Table 1, pool 5). The Primer

sequences, the size of amplicons and annealing temperature are shown in Table 1.

2.8. Detection of quinolone resistance-determining regions (QRDRs) and DNA Sequencing

The detection of quinolone resistance-determining regions (QRDRs) (*gyrA*, *parC*, and *parE*) of *P. aeruginosa* was performed using PCR. Primer sequences, the size of amplicons and annealing temperature are presented in Table 1. PCR products were analyzed by electrophoresis through 1.5% agarose gel. The amplified PCR products were purified by Wizard® SV Gel and PCR Clean-Up System (Promega) and sequenced using both forward and reverse primers by the dideoxy chain termination method (ABI PRISM sequencer, model 3130 Hitachi Ltd, Tokyo, Japan), at Bethlehem University, Bethlehem, Palestine. DNA sequence information was further submitted for accession numbers in GenBank.

2.9 Bioinformatics analyses

Continuous sequences were compared with previously available sequences of the *gyrA*, *parC*, and *parE* of *P. aeruginosa* in the National Center for Biotechnology Information (NCBI) using BLAST system. Multiple alignments were conducted using ClustalW of the computer program MEGA version 6 (Tamura et al., 2013). Phylogenetic analyses were based on alignments obtained from ClustalW of *gyrA* (365-bp), *parC* (209-bp) and *parE* (592-bp) sequences. Phylogenetic trees were constructed using the program Maximum Likelihood method in the same software. The robustness of the groupings in the Neighbor Joining analysis was assessed with 1000 bootstrap resamplings. The population diversity indices such as numbers of haplotype (h), haplotype diversity (Hd), nucleotide diversity (π), and the neutrality indices including (Tajima's D and Fu's Fs test) were calculated using DnaSP 5.1001 (Librado and Rozas 2009). Median-joining- (MJ) network of haplotypes of the *gyrA*, *parC*, and *parE* sequences of *P. aeruginosa* were analyzed using a median-joining algorithm in the program Network 5 (Bandelt et al. 1999). The *gyrA*, *parC*, and *parE* DNA sequences were numbered according to the reference sequences CP034434.1, CP025055.1 and CP021775.1, respectively, While the product of *gyrA*, *parC*, and *parE* sequences were numbered according to the reference sequences ANT75468.1, AUA79862.1 and ASA18488.1, respectively.

3. Results

3.1. Antibiotic resistance and other virulence genes

Results of the current study showed that all tested isolates were resistant to all tested antibiotics. The MIC for ciprofloxacin and norfloxacin had a range of 32-256 µg/mL. In addition, all of the investigated isolates carried either *exoT* or *exoT* and *exoY* genes. In addition, these isolates phenotypically were β-lactamase producers and all of them carried different β-lactamase genes. A total of 9 of *P. aeruginosa* isolates (82%) harbored class 1 integrons, while other classes were not detected (as shown in Table 2)

3.2. Bioinformatics and DNA analyses

The *gyrA*, *parC* and *parE* genes were amplified from all extracted DNA samples. The amplified products had 365-bp, 209-bp and 592-bp length for *gyrA*, *parC* and *parE,* respectively, were successfully sequenced and analyzed. The sequences were deposited in GenBank database under the accession numbers (MN064784-MN064794), and (MN067984- MN067994) and (MN067995-MN068005)

Table 2. Distribution of virulence genes and phenotypic characteristics among 11 fluoroquinolone resistant *P. aeruginosa* isolated in Palestine.

Isolate No.	Source	virulence genes**					Antibiotic resistance*			
		Integrons	ESBL	MBL	AmpC	Type III secretion exotoxin genes	CIP	NOR	MIC for CIP (µg/ml)	MIC for NOR (µg/ml)
1	Wound swab	Int1	N	P	P	*exoT*, *exoY*	R	R	32	64
2	Wound swab	Int1	P	P	N	*exoT*, *exoY*	R	R	64	64
3	Wound swab	Int1	N	P	N	*exoT*, *exoY*	R	R	32	128
4	Wound swab	Int1	N	P	P	*exoT*, *exoY*	R	R	32	128
5	Wound swab	Int1	N	P	P	*exoT*	R	R	64	32
6	Wound swab	Int1	P	P	P	*exoT*	R	R	128	128
7	Urine	N	N	P	P	*exoT*, *exoY*	R	R	128	64
8	urine	Int1	N	P	P	*exoT*, *exoY*	R	R	128	64
9	Ear swab	Int1	N	N	P	*exoT*, *exoY*	R	R	64	128
10	Ear swab	Int	N	P	N	*exoT*, *exoY*	R	R	128	64
11	Sputum trap	N	P	P	N	*exoT*, *exoY*	R	R	256	256

* CIP: Ciprofloxacin; NOR: Norfloxacin; R: Resistant; S: Sensitive; ** P: positive; N: Negative

for *gyrA*, *parC* and *parE* genes, respectively. Comparing the *gyrA*, *parE* and *parC* nucleotide sequences obtained from the *P. aeruginosa* strains isolated from Palestine with reference strains retrieved from GenBank confirmed that all these genes belonged to the species *P. aeruginosa* (as shown in Figure 1A, 1B and 1C).

To understand the genetic diversity of fluoroquinolone resistant *P. aeruginosa* in Palestine. Analyses of nucleotide sequences for *gyrA*, *parC* and *parE* genes were carried out using different softwares. Results of analyses showed that 9 haplotypes were detected for each *gyrA* and *parC* sequences, while 10 haplotyoes were detected for *parE* sequences. The founder haplotypes being Hap-1 (2 out of 11 isolates, 18%), Hap-2 (3 out 11, 27.3%) and Hap-6 (1 out 11, 9.1%) for *gyrA*, *parC* and *parE* genes, respectively. Two of *ParE* haplotypes (Hap-9 and Hap-10) were indel haplotypes (as shown in Figure 2A, 2B and 2C and Table 3). The black nodes (Hap) are the DNA sequences for these genes which represent the haplotypes, while light black nodes are median vectors (mv), which are considered the hypothesized (often ancestral) sequences for our sequences (Figure 2A, 2B and 2C). additionally, this study showed that number of parsimony informative sites were 8, 2 and 15, while number of singleton variable sites were 8, 16 and 7 for *gyrA*, *parC* and *parE* gene, respectively (as shown in Figure 3 and Table 4). Amino acid alteration in these genes are presented in Figure 4.

The haplotype diversity (Hd) was 0.964, 0.945 and 0.982, while the nucleotide diversity was 0.01225, 0.02001 and 0.01346 for *gyrA*, *parC* and *parE* genes, respectively (Table 4). The MJ networks constructed from haplotypes of *gyrA*, *parC* and *parE* sequences showed a star-like expansion with a major central Hap-1, Hap-2 and Hap-6 haplotype, respectively. The numbers of mutational steps between the major central haplotype and the others ranged from 1 to 7 for *GyrA* gene, 2 to 6 for *ParC* gene and 1 to 15 *ParE* gene (as shown in Figure 2). Low levels of polymorphism were detected in the both *GyrA* and *ParC* genes comparing to *parE* gene. Overall, there were 18, 22 and 31 point mutations between the major central haplotype and the other haplotypes for *GyrA*, *ParC* and *parE* genes (as shown in Figure 2). The neutrality tests (Tajima's D test and Fu's Fs test) for these genes showed negative values (see Table 4).

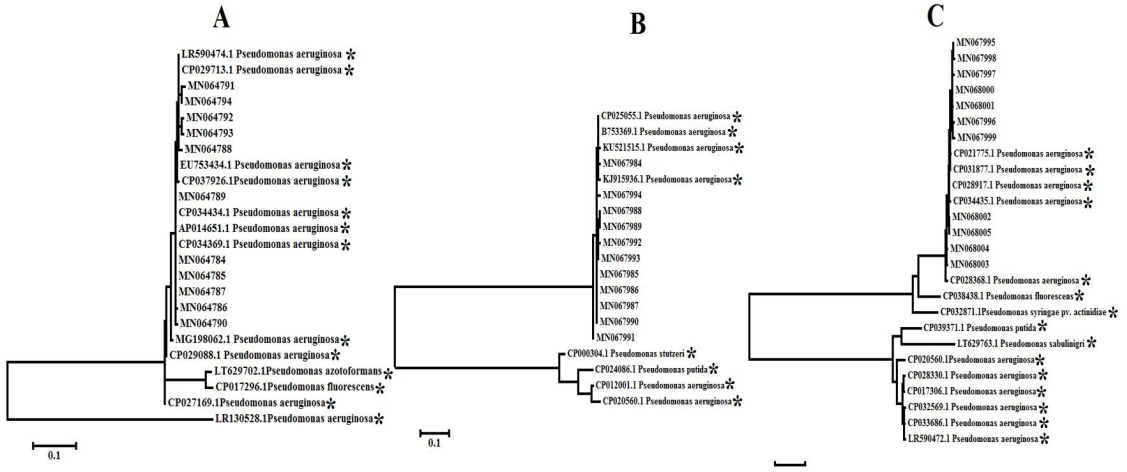

Figure 1. Molecular phylogenetic analysis by Maximum Likelihood method based on the GyrA (A), ParC (B) and ParE (C) sequence from fluoroquinolone resistant *P. aeruginosa* isolated in Palestine. Reference sequences retrieved from Genbank for the GyrA (A), ParC (B) and ParE (C) genes were denoted by asterisks (*). Sequences from Palestine and reference sequences were used to construct the phylogenetic tree. Evolutionary analyses were conducted in MEGA6.

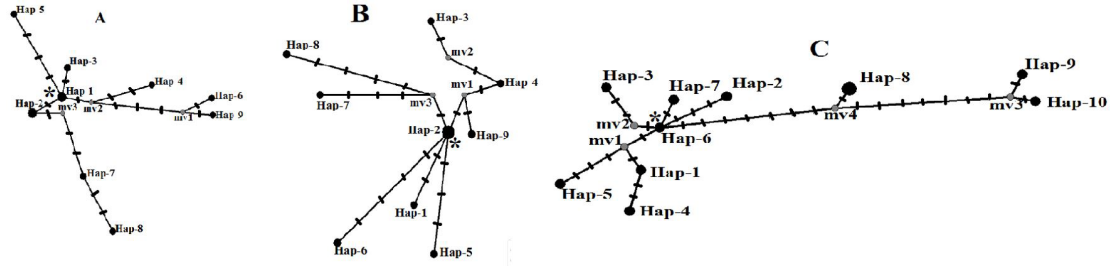

Figure 2. Median-joining network of *GyrA* (A), *ParC* (B) and *ParE* (C) of the haplotypes of fluoroquinolone resistant *P. aeruginosa* isolates. Each haplotype is represented by a circle. The asterisk (*) denotes the founder haplotype. The size of circle is relative to haplotype frequency. Bars indicate the number of nucleotide substitutions for *GyrA* (A), *ParC* (B) and *ParE* (C) sequences from fluoroquinolone resistant *P. aeruginosa* isolates recovered in Palestine.

Table 3. Haplotype Distribution for *gyrA*, *parC* and *parE* genes.

Haplotype	*GyrA* haplotype		*ParC* haplotype		*ParE* haplotype	
	No. of haplotypes	sequence	No. of haplotypes	sequence	No. of haplotypes	sequence
Hap-1	2	MN064784 MN064787	1	MN067984	1	MN067995
Hap-2	2	MN064785, MN064789	3	MN067985 MN067986 MN067987	1	MN067996
Hap-3	1	MN064786	1	MN067988	1	MN067997
Hap-4	1	MN064788	1	MN067989	1	MN067998
Hap-5	1	MN064790	1	MN067990	1	MN067999
Hap-6	1	MN064791	1	MN067991	1	MN068000
Hap-7	1	MN064792	1	MN067992	1	MN068001
Hap-8	1	MN064793	1	MN067993	2	MN068002 MN068005
Hap-9	1	MN064794	1	MN067994	1	MN068003 (indel haplotype)
Hap-10					1	MN068004 indel haplotype)

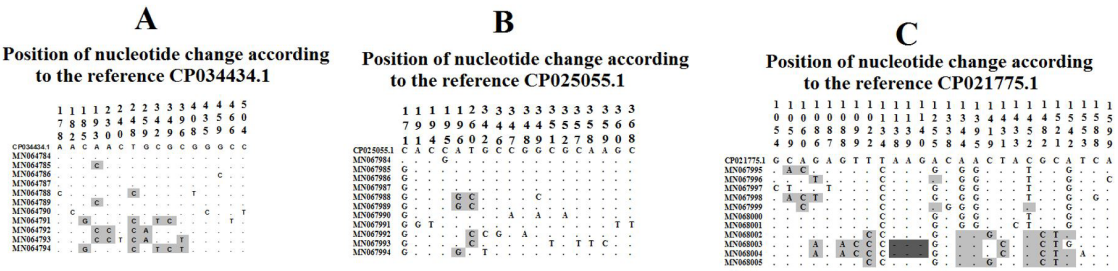

Figure 3. Nucleotide variation positions of *GyrA* (A), *ParC* (B) and *ParE* (C) genes among the studied fluoroquinolone resistant *P. aeruginosa* isolates according to the references from GenBank. Parsimony informative sites are shaded in light grey, while InDels are shaded in dark gray.

Table 4. Summary statistics for *gyrA*, *parC* and *parE* gene polymorphism in fluoroquinolone resistant *P. aeruginosa* isolates recovered in Palestine.

Nucleotide diversity indexes	Gene		
	GyrA	ParC	ParE
Number of sequences	11	11	11
Number of sites	365	209	592
Variable (polymorphic) sites	16	18	22
Total number of mutations	15	20	24
Singleton variable sites	8	16	7
Parsimony informative sites	8	2	15
Haplotype (gene) diversity (Hd)	0.964	0.945	0.982
Nucleotide diversity (per site), (π)	0.01225	0.02001	0.01346
Average number of nucleotide differences (k)	4.473	4.182	7.927
Tajima's D	-0.80774	-1.75596	-0.14914
Fu's Fs statistic	-3.154	-3.387	-2.906
Number of Haplotypes	9	9	10
Number of InDel Haplotypes	-	-	2
InDel Diversity per site	-	-	0.00055
InDel Haplotype Diversity	-	-	0.327
Tajima's D for InDel Haplotypes	-		-0.10001

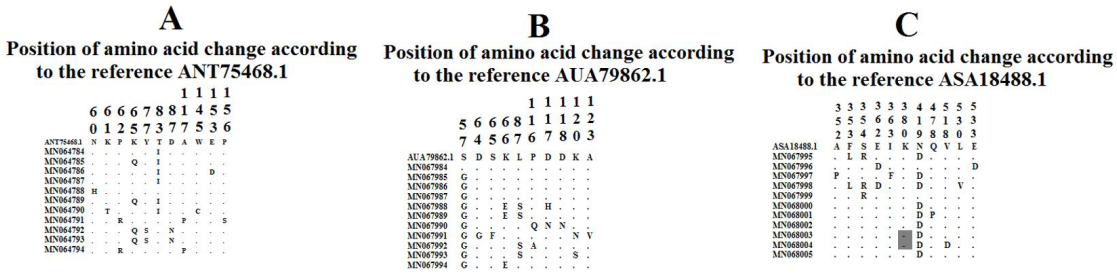

Figure 4. Amino acid variation positions of *GyrA* (A), *ParC* (B) and *ParE* (C) genes among the studied fluoroquinolone resistant *P. aeruginosa* isolates according to the references from GenBank.

4. Discussion

Fluoroquinolones are an important class of antimicrobial agents for the treatment of *P. aeruginosa* infections. The emergence of acquired resistance in *P. aeruginosa* is mainly due to mutations in the genes of DNA gyrase and topoisomerase IV. Several studies are suggested the correlation between the number of mutations in the genes of DNA gyrase and topoisomerase IV and the level of fluoroquinolone resistance (Lee et al., 2005; Nouri et al., 2016). The association was established between the wide use of fluoroquinolones in community practice and the frequency of bacteria resistance to these antimicrobial agents in hospitalized patients (MacDougall et al., 2005). This association between an inappropriate use of fluoroquinolones, the resistance selection to this class of antimicrobial agents, and the higher risk associated with treatment failure makes it mandatory to encourage a well-reasoned and appropriate use of these agents (Scheld, 2003).

This study highlights some of the phenotypic characteristics and genetic variations undergone by a fluoroquinolone resistant *P. aeruginosa* pathogen. These strains showed high MIC level for fluoroquinolones (ciprofloxacin and norfloxacin), β-lactamase producers, carried type III secretion exotoxin-encoding genes, most of them had integrase I gene and had high level of mutations in QRDR regions in *gyrA*, *parC* and *parE* genes. All these factors may play an important role in the invasiveness of these strains and make them difficult to treat. The identification of β-lactamase genes and other virulence genes and the dissemination of these strains in different Medical centers, indicate the need for a more strict application of infection control measures in Medical centers in the North West Bank-Palestine that aim to reduce expense and damage caused by *P. aeruginosa* infections.

Results of this study showed high level of fluoroquinolone resistance in these strains and this could be correlated with number of mutations in QRDR regions in *gyrA*, *parC* and *parE* sequences (Reinhardt et al., 2002; Lee et al., 2005; Nouri et al., 2016). Mechanisms of resistance to fluoroquinolones include two groups of mutation and acquisition of resistance-conferring genes. Resistance mutations in one or both of the two drug target enzymes, DNA gyrase and DNA topoisomerase IV, are commonly in a localized domain of the GyrA and ParE subunits of the respective enzymes and reduce drug binding to the enzyme-DNA complex. Other mechanism due to mutations occur in regulatory genes that control the expression of efflux pumps located in the bacterial cell membrane. Both types of these mutations can accumulate with selection pressure and produce highly resistant strains. The magnitude of resistance caused by single amino acid changes in the subunits of gyrase or topoisomerase IV varies by bacterial species and by quinolone (Hooper and Jacoby, 2015). Successive mutations in both gyrase and topoisomerase IV enzymes have been shown to provide rising levels of quinolone resistance. In many species, high-level quinolone resistance is often associated with mutations in both gyrase and topoisomerase IV (Schmitz et al., 1998).

The genetic variation of a species is distributed both within populations, expressed as differences between individuals and between populations, expressed as differences in the presence and frequency of alleles (Garg and Mishra, 2018). In this study, analyses of the *gyrA*, *parC* and *parE* sequences were found to be polymorphic with high haplotype diversity (Hd) (0.945-0.982) and low nucleotide diversity (0.01225-0.02001). The number of haplotypes were 9 for each *gyrA* and *parE* genes and 10 haplotypes for *parC* gene. The black nodes (Hap) are the DNA sequences for these genes which represent the haplotypes, while light black nodes are median vectors (mv), which are considered the hypothesized (often ancestral) sequences for our sequences. These are required to connect existing sequences within the network with maximum parsimony. Such gene flow in these Palestinian fluoroquinolone resistant *P. aeruginosa* haplotypes can potentially reduce genetic differentiation. The median joining network of *gyrA*, *parC* and *parE* sequences revealed "star-like" networks, suggesting population expansion haplotypes from a main founder haplotype. Along with the negative values of the neutrality tests Tajima's D and Fu's Fs statistic tests, further supported the hypothesis of population expansion of fluoroquinolone resistant *P. aeruginosa* in Palestine. The low nucleotide diversity is most likely a result due to different factors even had many haplotypes, this means that these haplotypes had very similarity between them. In addition, parsimony informative sites may play a role in low nucleotide diversity.

5. Conclusions

Fluoroquinolones used to treat infections due to first-step mutant strains may lead to the selection of

another mutation, which in turn could induce high-level of resistance to fluoroquinolones. Identifying first step mutants is important to adjust treatment and ensure the epidemiological monitoring of fluoroquinolone resistance.

Palestinian fluoroquinolone resistant *P. aeruginosa* strains showed high MIC level for fluoroquinolones, β-lactamase producers, carried type III secretion exotoxin-encoding genes, most of them had integrase I gene and had high level of mutations in QRDR regions in *gyrA*, *parC* and *parE* genes. All these factors may play an important role in the invasiveness of these strains and making the treatment of infections not easy. Identification and dissemination of these strains in different medical centers, indicate the need for adoption of a more strict application of infection control measures in Medical centers in the North West Bank-Palestine, that aim to reduce expense and damage caused by *P. aeruginosa* infections. Molecular analyses showed that Palestinian fluoroquinolone resistant *P. aeruginosa* haplotypes are not genetically differentiated and more mutations may exist in these strains. The tested number of isolates in the current study was very small, hence, future testing of a larger number of fluoroquinolone resistant *P. aeruginosa* isolates is required for a more clear picture about haplotype diversity, nucleotide diversity and genetic differentiation.

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