International Journal of Microbiological Research 3 (2): 82-86, 2012
ISSN 2079-2093

DOI: 10.5829/idosi.ijmr.2012.3.2.635

# *gyrA* and *parC* Mutations in Quinolone-Resistant Clinical Isolates of *Pseudomonas aeruginosa* from Nini Hospital in North Lebanon

[1,2]Rayan M Salma, [1,2]Fouad A Dabboussi, [1]Imad O Kassaa, [1,3]Rami H Khudary and [1,2]Monzer M Hamze

[1]Medical Microbiology Laboratory, Azm Center for Research in Biotechnology and its Applications, Doctoral School of Sciences and Technology, Lebanese University
[2]Faculty of public health, Lebanese University, Tripoli, Lebanon
[3]American University of Middle East, Kuwait

**Abstract:** Excessive use of broad-spectrum antibiotics in hospitals has led to the emergence of highly resistant strains of *Pseudomonas aeruginosa*. The major mechanism of resistance of this bacterium to fluoroquinolones is the modification of type II topoisomerases (DNA gyrase and topoisomerase IV). In this study, we examined the mutations in quinolones resistance-determining regions (QRDR) of *gyrA* and *parC* genes of 38 clinical isolates using the technique of DNA pyrosequencing. The most common origin of isolates was sputum (44.7%), followed by wounds (11%) and urine (5%) & ear discharge (5%). Serotypes O:11 (21%), O:2 (18.4%) and O:6 (7.8%), were the most predominant. Among these 38 clinical isolates of *P. aeruginosa*, 11 were susceptible, 22 were resistant and 5 were intermediate to ciprofloxacin. We found that 19 (50%) of these strains have a mutation in the *gyrA* gene (Threonine 83 Isoleucine), one of them presents a new mutation (Histidine 80 Arginine). 8 (21.05%) strains have an additional mutation in the *parC* gene (Serine 80 Leucine), one of these strains have two new mutations not previously reported (Glutamine 84 Aspartic acid, Alanine 85 Glycine). Fluoroquinolone-Sensitive strains had no mutations in the sequence area examined. 81.8% of isolates that were resistant to the ciprofloxacin had a mutation in the *gyrA*. Some strains also had a mutation in the *parC* gene. Results of this study suggest pyrosequencing as a rapid and reliable technique for the determination of antibiotic resistance pattern of a given bacterial strain.

**Key words:** *P. aeruginosa* · Fluoroquinolones · Pyrosequencing · GyrA · ParC

## INTRODUCTION

*Pseudomonas aeruginosa* is an important opportunistic pathogen which plays an important role in hospital intensive care units, causing a wide spectrum of nosocomial infections. The spread of this organism in health care settings is often difficult to control, due to the presence of multiple intrinsic and acquired mechanisms of resistance to a wide variety of antibiotics [1,2]. Furthermore, One of the important characteristics of *P. aeruginosa* is biofilm formation that helps them to survive different antibiotic therapy [3].

Fluoroquinolones are bactericidal, rapidly acting antimicrobial drugs with wide spectrums. They are very effective against many Gram negative bacterial pathogens *in vitro* [4]. Their effect against Gram negative bacilli, including *P. aeruginosa*, is one of their most important features. However, fluoroquinolones resistance among *P. aeruginosa* isolates has increased at an alarming rate due to its extensive use, which severely limits their usefulness [5-7]. Fluoroquinolones resistance can lead to treatment failure in *P. aeruginosa* [6]. The main mechanisms of resistance are mutations in the target genes, those encoding DNA gyrase (*gyrA*) and topoisomerase IV (*parC*) [6, 8-10] and in regulatory genes for drug efflux pumps. [11-13]. The aim of our study was to examine for the occurrence of mutations related to fluoroquinolones resistance (*gyrA* and *parC* genes) using the pyrosequencing technique of 38 clinical strains of *P. aeruginosa*, isolated from Nini hospital in north Lebanon.

**Corresponding Author:** Fouad Dabboussi, Faculty of public health, Lebanese University, Tripoli, Lebanon.
Tel.: +961 (6) 213 252 - Mob: +961 (3) 228 881 - Tele/Fax: +961 (6) 213 383. Tripoli, Lebanon.

Table 1: Primers used for *gyrA* and *parC* genes amplification and the sequencing primers used in this study.

| Primer | Comments | Sequence (5' to 3') | Reference |
|---|---|---|---|
| *gyrA* $F_b$ | *gyrA* Forward amplification Primer, biotinylated | GTGTGCTTTATGCCATGAG | [4] |
| *gyrA* R | *gyrA* Reverse amplification Primer | GGTTTCCTTTTCCAGGTC | [4] |
| *parC* $F_b$ | *parC* forward amplification Primer, biotinylated | CATCGTCTACGCCATGAG | [4] |
| *parC* R | *parC* Reverse amplification primer | AGCAGCACCTCGGAATAG | [4] |
| Seq *gyrA* | *gyrA* Sequencing primer | TCT ACC AGC ATG TAG CG | this study |
| Seq *parC* | *parC* Sequencing primer | CAC CAG CGG ATA GCG A | this study |

## MATERIALS AND METHODS

**Clinical Isolates:** 38 clinical isolates of *Pseudomonas aeruginosa* with a resistant profile to fluoroquinolones were obtained from Microbiology Laboratory at NINI hospital between 2005 and 2011 and kept on deep agar in Azm center. The isolates were considered to be sensitive, intermediately resistant or resistant to fluoroquinolones after testing by the disk diffusion method in Muller Hinton agar according to the recommendations of 'Comité de l'antibigramme de la Société Française de Microbiologie' (CA-SFM) [14].

The isolates were obtained following conventional bacteriological culture techniques from a range of specimens, The most common origin of isolates was sputum (44.7%), followed by wounds (11%), urine (5%) and ear discharge (5%) and were initially identified with the API 20 NE system (Biomerieux, Marcy l'Etoile, France). *P. aeruginosa* strain ATCC10145 was used as a control strain; this strain is sensitive to ciprofloxacin but resistant to pefloxacin and ofloxacin.

**Typing:** The determination of the O serotype was made by slide agglutination [15] with a set of four pools (OMA, OMC, OME, OMF) and 16 monovalent antisera numbered Ol to O16 (Biorad, France).

**DNA Extraction:** The DNA of all isolates of *P. aeruginosa* was extracted using the Qiagen Kit (QIAamp® DNA Mini Kit, Germany) according to the manufacturer's instructions and were used as template for PCR reactions.

**PCR Reactions:** Primers *gyrA*-$F_b$ and *gyrA*-R were used to amplify 287 bp of the fluoroquinolones resistance-determining region of the *gyrA* gene [6]. Primers *parC*-$F_b$ and *parC*-R were used to amplify 267 bp of the fluoroquinolones resistance-determining region of *parC* [6] (Table 1). PCR amplification was performed in a 50µL mixture containing 1× Buffer (Sigma-Aldrich, St Louis, MO), 3 mM MgCl2, 0.2 mM mix of deoxynucleotide triphosphates dNTPs (Sigma-Aldrich), 1.5 U of DNA *Taq* Polymerase (Sigma-Aldrich), 10 pmol of each primer and 50 ng of the DNA template. Amplification of the target regions was performed in the thermal cycler MyCycler C1000 (Bio-Rad), in 35 cycles consisting of initial heat activation at 95°C for 7 min, denaturation at 95 °C for 45 s, annealing at 52°C for 30 s and elongation at 72°C for 45 s, with a final elongation at 72°C for 7 min [6]. The efficacy of the amplification was determined by gel electrophoresis [6]. The PCR products obtained from this step were used for pyrosequencing.

**Pyrosequencing:** Sample preparation for pyrosequencing was performed according to the manufacturer's instructions and as described previously [16]. Single-stranded DNA amplicons were prepared semi-automatically using a Vacuum Prep Tool and Vacuum Prep Worktable (Biotage, Uppsala, Sweden). A 20µl of biotinylated PCR products was immobilized onto 4µL of streptavidin-coated Sepharose™ High Performance Beads (Amersham Biosciences, Piscataway, NJ) with 26µl of Binding Buffer – pH 7.6 (10 mM Tris- HCl 2 M NaCl; 1 mM EDTA; 0.1% Tween 20) and incubated at 65 °C under agitation at 1400 rpm for at least 5 min. Double-stranded DNA immobilized on Sepharose beads was washed with 70% ethanol and denatured with 0.2 M NaOH. Unbound single-stranded DNA was washed with 0.1 M Wash buffer [0.1 M Tris-HCl (pH 7.6)]. All the steps were performed according to the manufacturer's instructions for the Vacuum Prep Station. The beads carrying single-stranded DNA amplicons were suspended in 38.4µl of annealing buffer – pH 7.6 (20 mM Tris- Acetate; 5 mM Mg-acetate) containing 1.6µl of sequencing primers (Table 1). The single-stranded DNA was annealed to the sequencing primer at 80 °C for 2 min followed by incubation for 2 min at room temperature. Single stranded PCR products were sequenced using the PyroMark™Q96 ID System (Biotage). Sequencing was performed according to the manufacturer's instructions. To identify point mutations, sequences from clinical isolates were compared with that of wild-type *P. aeruginosa* PAO1.

## RESULTS AND DISCUSSION

The mutations found in *gyrA* and *parC* genes of 38 *P. aeruginosa* strains were described in the Tables 2 and 3. The results showed that among the 38 clinical isolates of *P. aeruginosa*, 19 (50%) had mutations in the *gyrA* gene while only 8 strains (21.05%) present mutations in the *parC* gene. It is notable that all of the isolates with *parC* alterations had an alteration in *gyrA*. Our results showed the absence of mutations in the *parC* gene alone.

To assess the correlation between mutations and resistance to fluoroquinolones, results from the molecular analysis were compared with the antibiotic susceptibility profile of bacterial isolates (Table 2).

Among the 22 fluoroquinolones-resistant isolates (ciprofloxacin, pefloxacin, ofloxacin resistant), 10 had a single mutation in the *gyrA* gene, 1 had a double mutation in this gene and 8 had a mutation in both *gyrA* and *parC* genes. On the basis of biochemical, genetic and epidemiological studies, DNA gyrase is known to be the primary target enzyme for fluoroquinolones and topoisomerase IV is known to be the secondary target in *P. aeruginosa* [17, 18]. Thus, the alteration in *parC* occurs after *gyrA* alteration and is associated with the development of higher-level fluoroquinolones resistance [17, 19].

Among the 22 strains resistant to all test fluoroquinolones, 4 had no mutations on *gyrA* and *parC*. This results show that detection of *gyrA* and *parC* mutations does not always imply resistance to fluoroquinolones, but that acquiring these mutations increases the likelihood of resistance. These discrepancies could suggest the existence of other additional molecular mechanisms for fluoroquinolones resistance such as the mutation in the regulatory gene *mexR* leading to hyper expression of the efflux pump *MexAB-OprM* [20]. Finally, The 11 strains, those were sensitive to ciprofloxacin, in addition to the reference strain *P. aeruginosa* ATCC 10145 (included as control) had no mutations in the sequence areas examined.

Common mutations in fluoroquinolones-resistant strains occurred in codon 83 of the *gyrA* gene (Fig.1 and Table 3). The nucleic acid alterations that occurred in this codon changed the amino acid profile from Threonine to Isoleucine, consistent with previous reports [6, 7, 18, 21, 22]. It was the principal replacement (19 of 38 isolates; 50%). In addition, a novel mutation related to fluoroquinolones resistance in codon 80 of the *gyrA* gene, changing amino acid Histidine to Arginine, was detected.

Common mutations in fluoroquinolones-resistant strains occurred in codon 80 of the *parC* gene (Fig. 2 and Table 3). The amino acid sequences in the QRDR of *parC* showed a replacement of Serine-80 to Leucine consistent with previous reports [7, 17, 19]. The 2 novel mutations detected were existing at the codon 84 (Glutamine 84 Aspartic acid) and the codon 85 (Alanine 85 Glycine).

Table 2: Correlation between susceptibility to ciprofloxacin, pefloxacin and ofloxacin of the 38 clinical isolates of *P. aeruginosa* and mutations in *gyrA* and *parC* genes.

| Antibiogram | | | | | | | |
|---|---|---|---|---|---|---|---|
| Ciprofloxacin | Pefloxacin | Ofloxacin | No. of strains | Mutation in *gyrA* only | Mutation in *parC* only | Mutation in both *gyrA* and *parC* | No mutations in *gyrA* or *parC* |
| R[a] | R | R | 22 | 10 | 0 | 8 | 4 |
| I[b] | R | R | 5 | 1 | 0 | 0 | 4 |
| S[c] | I | I | 2 | 0 | 0 | 0 | 2 |
| S | S | I | 2 | 0 | 0 | 0 | 2 |
| S | R | R | 7 | 0 | 0 | 0 | 7 |
| Total | | | 38 | 11 | 0 | 8 | 19 |

[a], Resistant. [b], Intermediate. [c], susceptible.

Table 3: Type of point mutations in *gyrA* and *parC* genes of *P. aeruginosa* strains.

| Genes | No. of strains | Codon | Mutations | Amino acid change |
|---|---|---|---|---|
| *GyrA* | 18 | 83 | ACC→ATC | Thr→Ile |
| | 1 | 83 | ACC→ATC | Thr→Ile |
| | | 80 | CAC→CGC | His→Arg |
| *ParC* | 7 | 80 | TCG→TTG | Ser→Leu |
| | 1 | 80 | TCG→TTG | Ser→Leu |
| | | 84 | GAG→GAC | Glu→Asp |
| | | 85 | GCC→GGC | Ala→Gly |

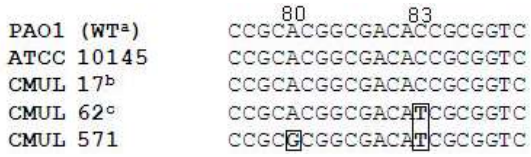


Fig. 1: Pyrosequencing showing point mutations in *gyrA* gene. *gyrA*. [a], wild type; [b], CMUL 17, 37, 45, 68, 107, 110, 115, 118, 127, 318, 345, 364, 426, 460, 481, 502, 516, 562, 574; [c], CMUL 62, 116, 117, 120, 122, 123, 125, 126, 229, 317, 324, 341, 348, 443, 495, 520, 541, 499

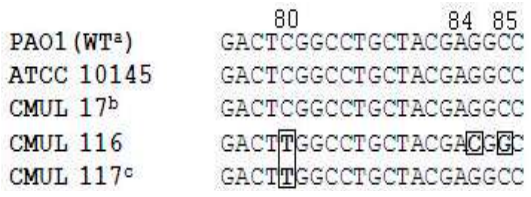


Fig. 2: Pyrosequencing showing point mutations in *parC* gene. *parC*. [a], wild type; [b], CMUL 17, 37, 45, 62, 68, 107, 110, 115, 118, 123, 125, 126, 127, 318, 324, 341, 345, 364, 426, 443, 460, 481, 495, 502, 516, 541, 562, 571, 574, 499; [c], CMUL 117, 120, 122, 229, 317, 348, 520.

The serotyping of *P. aeruginosa* showed that serotypes O:11 (21%), O:2 (18.4%) and O:6 (7.8%), were the most predominant. It was determined whether different O serotypes were associated with particular mutations on *gyrA* or *parC* among the *P. aeruginosa* isolates. No direct relation between the presence of mutations and serologic results could be proved.

Pyrosequencing is a real-time DNA sequencing method that, on average, analyses 50–60 bases by detecting the release of pyrophosphate. It is faster, less expensive and easier to perform than conventional sequencing [23]. This technology has been used previously for the detection of other genes responsible for antibiotic resistance, e.g. fluoroquinolones resistance in *Neisseria gonorrhoeae*, rifampicin resistance in *Mycobacterium tuberculosis*, macrolide resistance in *Streptococcus pneumoniae* and *Streptococcus pyogenes* and linezolid resistance in enterococci [24, 25].

In conclusion, point mutations in clinical isolates associated with antibiotic resistance were rapidly and reliably detected by DNA sequencing using pyrosequencing. No direct relation between the presence of mutations and serologic results could be proved.

## REFERENCES

1. Landman, D., J.M. Quale, D. Mayorga, A. Adedeji, K. Vangala, J. Ravishankar, C. Flores and S. Brooks, 2002. Citywide Clonal Outbreak of Multiresistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* in Brooklyn, NY: The Preantibiotic Era Has Returned. Arch. Intern. Med., 162: 1515-1520.
2. Murugan, S., R. Bakkiya Lakshmi, P. Uma Devi, K.R. Mani, 2010. Prevalence and antimicrobial susceptibility pattern of metallo β lactamase producing *Pseudomonas aeruginosa* in diabetic foot infection. Int. J. Microbiol. Res., 1: 123-128.
3. Chakraborty, D., S. Basu, P. Chatterjee, S.K. Dey and S. Das, 2011. Concurrent Determination of Collagenase and Biofilm Formation Activities in Metallo-Beta-Lactamase Producing *Pseudomonas aeruginosa*. Int. J. Microbiol. Res., 2: 208-212.
4. Swiatlo, E., E. Moore, J. Watt and L.S. Mc Daniel, 2000. In vitro activity of four floroquinolones against clinical isolates of *Pseudomonas aeruginosa* determined by the E test. Int. J. Antimicrob. Agents. 15: 73-76.
5. Gasink, L.B., N.O. Fishman, M.G. Weiner, I. Nachamkin, W.B. Bilker and E. Lautenbach, 2006. Fluoroquinolone-resistant *Pseudomonas aeruginosa*: assessment of risk factors and clinical impact. Am. J. Med., 119: 19-25.
6. Jalal, S., O. Ciofu, N. Hoiby, N. Gotoh and B. Wretlind, 2000. Molecular mechanisms of fluoroquinolone resistance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. Antimicrob. Agents. Chemother. 44: 710-712.
7. Yonezawa, M., M. Takahata, N. Matsubara, Y. Watanabe and H. Narita, 1995. DNA gyrase gyrA mutations in quinolone-resistant clinical isolates of *Pseudomonas aeruginosa*. Antimicrob. Agents. Chemother. 39: 1970-1972.
8. Hancock, R.E., 1998. Resistance mechanisms in *Pseudomonas aeruginosa* and other nonfermentative Gram-negative bacteria. Clin. Infect. Dis., 27: 93-99.
9. Ruiz, J., 2003. Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection. J. Antimicrob. Chemother. 51: 1109-1117.

10. Ghosh, U.K. and A. Hore, 2011. Isolation and Characterization of Nalidixic Acid Resistant Mutant of a Thermophilic Actinomycete. Int. J. Microbiol. Res., 2: 120-123.
11. Aeschlimann, J.R., 2003. The role of multidrug efflux pumps in the antibiotic resistance of *Pseudomonas aeruginosa* and other Gram-negative bacteria. Insights from the Society of Infectious Diseases Pharmacists. Pharmacotherapy. 23: 916-924.
12. Masuda, N., E. Sakagawa, S. Ohya, N. Gotoh, H. Tsujimoto and T. Nishino, 2000. Substrate specificities of MexAB-OprM, MexCD-OprJ and MexXY-oprM efflux pumps in *Pseudomonas aeruginosa*. Antimicrob. Agents. Chemother. 44: 3322-3327.
13. Ziha-Zarifi, I., C. Llanes, T. Kohler, J.C. Pechere and P. Plesiat, 1999. In vivo emergence of multidrug-resistant mutants of *Pseudomonas aeruginosa* overexpressing the active efflux system MexA-MexB-OprM. Antimicrob. Agents. Chemother. 43: 287-291.
14. Bonnet, R., J.D. Cavallo, H. Chardon, C. Chidiac, P. Courvalin, H. Dabernat, H. Drugeon, L. Dubreuil, B. Guery, V. Jarlier, F. Jehl, T. Lambert, R. Leclercq, M.H. Nicolas-Chanoine, P. Plesiat, C. Quentin, B. Rouveix, C.J. Soussy, E. Varon and P. Weber, 2010. Communiqué 2010, Comité de l'antibiogramme de la Société française de microbiologie, Édition de janvier.
15. Kusama, H., 1978. Serological classification of *Pseudomonas aeruginosa* by a slide agglutination test. J. Clin. Microbiol., 8: 181.
16. Gorgani, N., S. Ahlbrand, A. Patterson and N. Pourmand, 2009. Detection of point mutations associated with antibiotic resistance in *Pseudomonas aeruginosa*. Int. J. Antimicrob. Agents. 34: 414-418.
17. Mouneimne, H., J. Robert, V. Jarlier and E. Cambau, 1999. Type II topoisomerase mutations in ciprofloxacin-resistant strains of *Pseudomonas aeruginosa*. Antimicrob. Agents. Chemother. 43: 62-66.
18. Nakano, M., T. Deguchi, T. Kawamura, M. Yasuda, M. Kimura, Y. Okano and Y. Kawada, 1997. Mutations in the gyrA and parC genes in fluoroquinolone-resistant clinical isolates of *Pseudomonas aeruginosa*. Antimicrob. Agents. Chemother. 41: 2289-2291.
19. Akasaka, T., M. Tanaka, A. Yamaguchi and K. Sato, 2001. Type II topoisomerase mutations in fluoroquinolone-resistant clinical strains of *Pseudomonas aeruginosa* isolated in 1998 and 1999: role of target enzyme in mechanism of fluoroquinolone resistance. Antimicrob. Agents. Chemother. 45: 2263-2268.
20. Livermore, D.M., 2002. Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa*: our worst nightmare?. Clin. Infect. Dis., 34: 634-640.
21. Lee, J.K., Y.S. Lee, Y.K. Park and B.S. Kim, 2005. Alterations in the gyrA and gyrB subunits of topoisomerase II and the parC and parE subunits of topoisomerase IV in ciprofloxacin-resistant clinical isolates of *Pseudomonas aeruginosa*. Int. J. Antimicrob. Agents. 25: 290-295.
22. Wydmuch, Z., O. Skowronek-Ciolek, K. Cholewa, U. Mazurek, J. Pacha, M. Kepa, D. Idzik and R.D. Wojtyczka, 2005. gyrA mutations in ciprofloxacin-resistant clinical isolates of *Pseudomonas aeruginosa* in a Silesian Hospital in Poland. Pol. J. Microbiol., 54: 201-206.
23. Ronaghi, M. and E. Elahi, 2002. Pyrosequencing for microbial typing. J. Chromatogr. B. Anal. Technol. Biomed. Life. Sci., 782: 67-72.
24. Haanpera, M., P. Huovinen and J. Jalava, 2005. Detection and quantification of macrolide resistance mutations at positions 2058 and 2059 of the 23S rRNA gene by pyrosequencing. Antimicrob. Agents. Chemother. 49: 457-460.
25. Sinclair, A., C. Arnold and N. Woodford, 2003. Rapid detection and estimation by pyrosequencing of 23S rRNA genes with a single nucleotide polymorphism conferring linezolid resistance in enterococci. Antimicrob. Agents. Chemother. 47: 3620-3622.