Medical Microbiology

The role of gyrA and parC mutations in fluoroquinolones-resistant Pseudomonas aeruginosa isolates from Iran

Roghayeh Nouri\textsuperscript{a,b,c}, Mohammad Ahangarzadeh Rezaee\textsuperscript{a,b,*}, Alka Hasani\textsuperscript{a,b}, Mohammad Aghazadeh\textsuperscript{b}, Mohammad Asgharzadeh\textsuperscript{d}

\textsuperscript{a} Tabriz University of Medical Sciences, Infectious and Tropical Diseases Research Center, Tabriz, Iran
\textsuperscript{b} Tabriz University of Medical Sciences, Faculty of Medicine, Department of Microbiology, Tabriz, Iran
\textsuperscript{c} Tabriz University of Medical Sciences, Student Research Committee, Tabriz, Iran
\textsuperscript{d} Tabriz University of Medical Sciences, Biotechnology Research Center, Tabriz, Iran

\textbf{ARTICLE INFO}

Article history:
Received 15 July 2015
Accepted 25 March 2016
Available online 26 July 2016
Associate Editor: Alfonso Luis Barth

Keywords:
Pseudomonas aeruginosa
Fluoroquinolone resistance
\textit{gyrA}
\textit{parC}

\textbf{ABSTRACT}

The aim of this study was to examine mutations in the quinolone-resistance-determining region (QRDR) of gyrA and parC genes in \textit{Pseudomonas aeruginosa} isolates. A total of 100 clinical \textit{P. aeruginosa} isolates were collected from different university-affiliated hospitals in Tabriz, Iran. Minimum inhibitory concentrations (MICs) of ciprofloxacin and levofloxacin were evaluated by agar dilution assay. DNA sequences of the QRDR of gyrA and parC were determined by the dideoxy chain termination method. Of the total 100 isolates, 64 were resistant to ciprofloxacin. No amino acid alterations were detected in gyrA or parC genes of the ciprofloxacin susceptible or ciprofloxacin intermediate isolates. Thr-83 \textarrow{\textrightarrow} Ile substitution in gyrA was found in all 64 ciprofloxacin resistant isolates. Forty-four (68.75\%) of them had additional substitution in parC. A correlation was found between the number of the amino acid alterations in the QRDR of gyrA and parC and the level of ciprofloxacin and levofloxacin resistance of the \textit{P. aeruginosa} isolates. Ala-88 \textarrow{\textrightarrow} Pro alteration in parC was generally found in high level ciprofloxacin resistant isolates, which were suggested to be responsible for fluoroquinolone resistance. These findings showed that in \textit{P. aeruginosa}, gyrA was the primary target for fluoroquinolone and additional mutation in parC lead to highly resistant isolates.

© 2016 Sociedade Brasileira de Microbiologia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

* Corresponding author at: Infectious and Tropical Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, Iran. E-mail: rezaeetb@tbzmed.ac.ir (M. Ahangarzadeh Rezaee).
Introduction

_Pseudomonas aeruginosa_ is an important opportunistic pathogen leading to life-threatening infections, especially in immunocompromised patients. Often these infections are difficult to treat due to the intrinsic resistance of the species as well as its remarkable ability to acquire resistance to a wide range of antimicrobial agents. Fluoroquinolones are the only accessible antibiotics for effective oral treatment of infections caused by this organism. Among fluoroquinolones, ciprofloxacin and levofloxacin are widely used in the treatment of _P. aeruginosa_ infections.

Fluoroquinolones act by inhibiting the action of target enzymes, DNA gyrase and topoisomerase IV, with both enzymes playing a principal role in DNA replication. DNA gyrase and topoisomerase IV are heterotetrameric enzymes that are composed of two subunits encoded by the _gyrA_, _gyrB_ and _parC_, _parE_, respectively. The _gyrA_ and _gyrB_ genes are homologs to _parC_ and _parE_, respectively. The mechanisms of fluoroquinolone resistance in _P. aeruginosa_ include mutations in the DNA gyrase and topoisomerase IV, overexpression of efflux pump system and the innate impermeability of the membrane. Alterations in the so-called quinolone-resistance-determining region (QRDR) within DNA gyrase and topoisomerase IV are the major mechanisms for fluoroquinolone resistance in _P. aeruginosa_.

Moreover, isolates with mutations in QRDR of _gyrA_ and _parC_ show the highest levels of fluoroquinolone resistance. Although amino acid alterations in the _gyrB_ and _parE_ genes have been described, the frequency of these mutations is low, with only a complementary role in fluoroquinolone resistance.

Several studies from Iran have reported the high prevalence of MDR strains among Iranian hospitals and strains isolated from hospitalized patients; especially, burn patients have show high level resistance to most available antibiotics.

The prevalence of mutations in DNA gyrase and topoisomerase IV has not been well studied in Iran. This is the largest analysis of the QRDR of _gyrA_ and _parC_ in the clinical isolates of _P. aeruginosa_ from Iran. The aim of this study was to examine the mutations in _gyrA_ and _parC_ genes and characterize their correlation with ciprofloxacin and levofloxacin resistance, as well as the evaluation of their effect on ciprofloxacin and levofloxacin Minimum inhibitory concentrations (MICs) among the clinical isolates of _P. aeruginosa_.

### Materials and methods

#### Bacterial isolates

In a prospective study, a total of 100 non-repetitive clinical isolates of _P. aeruginosa_ were collected, between December 2013, and July 2014, from four Educational-Health Care Centers of Tabriz University of Medical Sciences in Northwest Iran. The bacterial isolates were recovered from different clinical specimens such as urine (29%), wound discharge (25%), tracheal aspirates (21%), blood (8%) and other clinical specimens (Table 1). All isolates were identified by standard conventional biochemical tests.

#### Antimicrobial susceptibility testing

Antimicrobial susceptibility to ticarcillin (75 µg), piperacillin-tazobactam (100/10 µg), ceftazidime (30 µg), cefepime (30 µg), aztreonam (30 µg), imipenem (10 µg), meropenem (10 µg), gentamicin (10 µg), amikacin (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg) and ofloxacin (5 µg) (Mast, UK) was performed by employing the standard disk diffusion method. MICs of ciprofloxacin and levofloxacin (Sigma-Aldrich) were determined by the standard agar dilution assay. The results of disk diffusion assay as well as MIC were interpreted according to the clinical and laboratory standard institute (CLSI) guidelines.

_Pseudomonas aeruginosa_ ATCC 27853 was used as the control strain for susceptibility testing.

---

### Table 1 – Distribution of _Pseudomonas aeruginosa_ isolates by the site of isolation and hospital origin.

| Source of isolates | Imam Reza | Sina | Koodakan | Shahid Madani | Total no. of isolates |
|--------------------|------------|------|----------|---------------|-----------------------|
| Tracheal aspirate   | 7          | 4    | 6        | 4             | 21                    |
| Wound discharge     | 7          | 8    | 4        | 6             | 25                    |
| Urine               | 7          | 6    | 9        | 7             | 29                    |
| Blood               | 1          | 4    | 1        | 2             | 8                     |
| Throat culture      | 1          | 0    | 3        | 0             | 4                     |
| Catheter            | 0          | 0    | 2        | 2             | 4                     |
| Sputum              | 0          | 1    | 1        | 0             | 2                     |
| Bronchial washing   | 0          | 0    | 0        | 2             | 2                     |
| Fleural fluid culture| 1        | 0    | 0        | 0             | 1                     |
| Urethral discharge  | 1          | 0    | 0        | 0             | 1                     |
| Peritoneal fluid    | 1          | 0    | 0        | 0             | 1                     |
| Ear discharge       | 0          | 1    | 0        | 0             | 1                     |
| Stool               | 0          | 1    | 0        | 0             | 1                     |
| Total               | 27         | 24   | 26       | 23            | 100                   |
PCR amplification and DNA sequencing

Chromosomal DNA from the isolates of P. aeruginosa was extracted by DNA extraction kit (Yekta Tajhiz Azma, Iran) according to the manufacturer’s instructions. The PCR reaction was performed in a 50 µL mixture containing 1.5 mM MgCl2, 0.5 pmol of each primer, 0.2 mM dNTPs (Yekta Tajhiz Azma, Iran), 1U of Pfu DNA polymerase (Yekta Tajhiz Azma, Iran), 1X Pfu DNA polymerase buffer and 10–100 ng of the template DNA.

The amplification of gyrA and parC genes was carried out using the polymerase chain reaction and specific primer sets as described previously.26 Purified PCR products were sequenced using the Applied Biosystems 3730/3730xl DNA analyzers sequencing (ABI) system, (Bioneer Co., Korea).

Statistical analysis

Categorical variables were compared by the Chi-square test or Fisher’s exact test using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL). A statistically significant difference was considered as a P-value < 0.05.

Results

Antimicrobial susceptibility testing

Of the 100 P. aeruginosa clinical isolates tested for antimicrobial susceptibility, 71 of them were found to show multidrug resistance (MDR). MDR was defined as resistance to three or more unrelated antibiotics.27 The highest rate of resistance was observed against ticarcillin (84%) and ofloxacin (82%). Moreover, the highest susceptibility rate was obtained against ceftazidime (45%), followed by gentamicin (44%). Of the total isolates, 64 (64%) were resistant, 2 (2%) were intermediate and 34 (34%) were observed to be susceptible to ciprofloxacin. Moreover, 63% of isolates were resistant, 1% were intermediate, and 36% were susceptible to levofloxacin.

DNA sequences analysis

DNA sequences of all P. aeruginosa isolates were compared with the corresponding sequences of P. aeruginosa PA01 (Accession: NC_002516.2 GI: 110645304). Aside from ciprofloxacin susceptible, levofloxacin susceptible and ciprofloxacin intermediate isolates which had no amino acid alterations in their gyrA or parC genes, the amino acid alterations were recognized in gyrA and parC QRDR of 64 ciprofloxacin resistant, 63 levofloxacin resistant and 1 levofloxacin intermediate isolates, as described in Table 2. The total mutations found in these isolates were classified into 4 distinct groups according to the pattern of amino acid alteration. Group I: isolates contained single mutation Thr-83 → Ile in gyrA. Group II: isolates contained one mutation Thr-83 → Ile in gyrA and one mutation Ala-88 → Pro in parC. Group III: isolates contained one mutation Thr-83 → Ile in gyrA and one mutation Ser-87 → Leu in parC. Group IV: isolates contained two mutations Thr-83 → Ile and Asp-87 → Asn in gyrA and one mutation Ser-87 → Leu in parC.

DNA sequences of QRDR gyrA showed Thr-83 → Ile substitution for all 64 ciprofloxacin resistant isolates. Of 64 isolates, 20 (31.25%) had a mutation (Thr-83 → Ile) in gyrA alone (group I). A double mutation in gyrA (Thr-83 → Ile and Asp-87 → Asn) was detected in 5 of 64 isolates. Amino acid alteration in the QRDR parC was observed in 44 (68.75%) of 64 isolates. All of these isolates possessed additional mutations in gyrA. No double mutations in parC were found. The Ser-87 → Leu substitution was found in 31 (48.4%) of 64 isolates. Moreover, the substitution of Pro for Ala-88 was observed in 8 (12.5%) of 64 isolates.

Correlation between fluoroquinolones MIC and QRDRs mutations

The MIC values of ciprofloxacin and levofloxacin for 64 resistant isolates and their correlation with different types of mutations in gyrA and parC genes are shown in Table 3. As shown, ciprofloxacin MIC for isolates with a single gyrA substitution (Thr-83 → Ile) ranged from 4–64 µg/mL and for levofloxacin, the 4–32 µg/mL range was observed. Isolates with a single gyrA (Thr-83 → Ile) substitution and a single parC substitution (Ala-88 → Pro or Ser-87 → Leu) had ciprofloxacin MICs ranging from 8 to 128 or 16 to 256 µg/mL and levofloxacin MICs varied from 8 to 64 or 8 to 256 µg/mL. Moreover, the isolates with double gyrA substitutions (Thr-83 → Ile and Asp-87 → Asn) and a single parC substitution (Ser-87 → Leu) had ciprofloxacin and levofloxacin MICs ranging from 32 to 256 µg/mL. Our results showed that the two concurrent mutations in gyrA and parC genes were associated with a higher level of ciprofloxacin and levofloxacin MICs, as compared to a single mutation in gyrA. (Geometric mean MICs of ciprofloxacin, 29.34 (group 2) and 32 (group 3) versus 16.56 (group 1) µg/mL, p < 0.05; geometric mean MICs of levofloxacin, 24.67 (group 2) and 28.6 (group 3) versus 14.42 (group 1) µg/mL p < 0.05). Moreover, three concurrent mutations in gyrA and parC genes were associated with a higher level of ciprofloxacin and levofloxacin MICs, as compared to
two concurrent mutations in gyrA and parC genes (Geometric mean MICs of ciprofloxacin, 73.5 (group 4) versus 29.3 (group 2) μg/mL, and 32 (group 3) μg/mL p < 0.05; geometric mean MICs of levofloxacin, 64 (group 4) versus 24.6 (group 2) and 28.61 (group 3) μg/mL p < 0.05) or single mutation in gyrA. (Geometric mean MICs of ciprofloxacin, 73.51 (group 4) versus 16.56 (group 1) μg/mL, p < 0.05; geometric mean MICs of levofloxacin, 64 (group 4) versus 14.42 (group 1) μg/mL p < 0.05).

Discussion

Fluoroquinolones such as ciprofloxacin and levofloxacin are an important class of antibiotics for the treatment of P. aeruginosa infections. However, P. aeruginosa rapidly becomes resistant to these drugs during antibiotic therapy. The principle mechanism of fluoroquinolones resistance in P. aeruginosa involves mutations in the genes of DNA gyrase and topoisomerase IV.

In the present study, the alteration of Thr-83 to Ile in gyrA was found in all ciprofloxacin and levofloxacin resistant P. aeruginosa isolates. Moreover, a double concomitant mutation in gyrA (Thr-83 → Ile and Asp-87 → Asn) was observed in five ciprofloxacin and levofloxacin resistant isolates. More noticeably, the MIC values of tested fluoroquinolones among these isolates were significantly higher. However, no amino acid change was detected in ciprofloxacin susceptible, levofloxacin susceptible and ciprofloxacin intermediate isolates. So Thr-83 → Ile was shown to be the chief mechanism of fluoroquinolones resistance. This was consistent with the results of other studies.

Furthermore, the amino acid sequences analysis in the QRDR of parC showed that more than half of ciprofloxacin and levofloxacin resistant isolates had an alteration in parC and the Ser-87 → Leu substitution was the predominant amino acid change (48.4%). Lee et al. and Mouneime et al. have previously reported this amino acid change in 35.9% and 25% of the ciprofloxacin resistant isolates, respectively. Also, another alteration in parC, Ala-88 → Pro substitution, was highly frequent in our isolates, in comparison to the results of Akasaka et al. and Sekiguchi et al. They found this amino acid change only in one of all studied isolates. More importantly, this substitution was observed generally among high level ciprofloxacin resistant isolates suggested to be responsible for fluoroquinolone resistance. However, we did not detect amino acid alteration at positions Pro-83, Gly-85, Glu-91 and Leu-95 in parC, as reported in the previous studies.

Based on the analysis of sequencing results, all of the isolates with parC mutation had one or two mutations in gyrA. This observation confirmed that the DNA gyrase was the primary target for fluoroquinolone resistance in the clinical isolates of P. aeruginosa. However, isolates that had double mutation in gyrA and parC had higher ciprofloxacin and levofloxacin MICs than those with a single mutation in gyrA, thereby suggesting that alteration in parC occurred after gyrA, leading to higher level fluoroquinolone resistance in P. aeruginosa. Moreover, the addition of a second gyrA alteration to gyrA and parC mutations had a significant effect on ciprofloxacin and levofloxacin MICs. Our results suggested that there could be a correlation between the number of gyrA and parC alterations and the level of fluoroquinolone resistance. This has been reported by Lee et al. for the clinical isolates of P. aeruginosa. Differences in the MICs of ciprofloxacin and levofloxacin for isolates had a single alteration in QRDR of gyrA with or without an alteration in QRDR of parC, revealing that other resistance factors were involved in fluoroquinolone resistance. Mechanisms such as overexpression of efflux pumps (MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM) and mutation in gyrB and parC have been described for fluoroquinolone resistance in P. aeruginosa.

However, the impact of these mechanisms on MICs of ciprofloxacin and levofloxacin can be clarified by further studies.

To conclude, mechanisms other than mutations in gyrA and parC (such as active efflux pumps, alterations in gyrB, parE or innate impermeability of the membrane) may contribute to the level of fluoroquinolone resistance in the clinical isolates of P. aeruginosa, but a single amino acid alteration, Thr-83 → Ile, in gyrA, is sufficient to cause clinically important levels of resistance to fluoroquinolones, and the simultaneous presence of mutation in parC (Ser-87 → Leu or Ala-88 → Pro) mediates significantly higher level fluoroquinolone resistance.

Finally, our results revealed that the mutations in gyrA and parC were the main mechanism of fluoroquinolone resistance among the clinical isolates of P. aeruginosa in Tabriz, Iran.
the QRDR of gyrA and parC in the clinical isolates of *P. aeruginosa* from Iran.

**Conflicts of interest**

The authors declare no conflicts of interest.

**Acknowledgment**

This work was fully supported by Infectious and Tropical Diseases Research Center (grant No. 93-02), Tabriz University of Medical Sciences. It is also a report originating from a database developed for the thesis of first author registered in Infectious and Tropical Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, Iran. The authors also thank Dr. Hossein Samadi Kafli for his kind help in analyzing sequencing results, and Mrs. Maryam Rasoli and Mr. Jaber Kamran for collecting the clinical isolates.

**References**

1. Ahangarzadeh Rezaee M, Behzadiannezhad Q, Najjar-Piraye S, Oulia P. In vitro activity of imipenem and ceftazidime against mucoid and non-mucoid strains of *Pseudomonas aeruginosa* isolated from patients in Iran. Arch Iran Med. 2002;5:251–254.
2. Lihua L, Jianhui W, Jialin Y, Yayan L, Guanxin L. Effects of allicin on the formation of *Pseudomonas aeruginosa* biofilm and the production of quorum-sensing controlled virulence factors. Pol J Microbiol. 2013;62:243–251.
3. Wolka K, Szewda P. Genetic features of clinical *Pseudomonas aeruginosa* strains. Pol J Microbiol. 2009;58:255–260.
4. Ahangarzadeh Rezaee M, Behzadiannezhad Q, Najjar PS, Oulia P. Higher aminoglycoside resistance in mucoid *Pseudomonas aeruginosa* than in non-mucoid strains. Arch Iranian Med. 2002;5:108–110.
5. Oliveira ACd, Maluta RP, Stella AE, Rigobelo EC, Marin JM, Ávila FAd. Isolation of *Pseudomonas aeruginosa* strains from dental office environments and units in Barretos, state of São Paulo, Brazil, and analysis of their susceptibility to antimicrobial drugs. Braz J Microbiol. 2008;39:579–584.
6. Wolka K, Kot B, Jakubczak A. Phenotypic and genotypic diversity of *Pseudomonas aeruginosa* strains isolated from hospitals in Siedlice (Poland). Braz J Microbiol. 2012;43:274–282.
7. Perez LRR, Limberger MF, Costi R, Dias CAG, Barth AL. Evaluation of tests to predict metallo-β-lactamase in cystic fibrosis (CF) and non-CF *Pseudomonas*. Braz J Microbiol. 2014;45:835–839.
8. Straeva T, Yordanov D. *Pseudomonas aeruginosa*—a phenomenon of bacterial resistance. J Med Microbiol. 2009;58:1133–1148.
9. Kugelberg E, Löfmark S, Wretlind B, Andersson DI. Reduction of the fitness burden of quinolone resistance in *Pseudomonas aeruginosa*. J Antimicrob Chemother. 2005;55:22–30.
10. Llanes C, Köhler T, Patry I, Deheq B, Van Delden C, Pléiat P. Role of the efflux system MexEF-OprN in low level resistance of *Pseudomonas aeruginosa* to ciprofloxacin. Antimicrob Agents Chemother. 2011;55:5676–5684.
11. Dalhoff A. Global fluoroquinolone resistance epidemiology and implications for clinical use. Interdisc Perspect Infect Dis. 2012;1:37.
12. Wydmuch Z, Skowronek-Ciolek O, Cholewa K, Mazurek U, Pacha J, Kepa M. gyrA mutations in ciprofloxacin-resistant clinical isolates of *Pseudomonas aeruginosa* in a Silesian Hospital in Poland. Pol J Microbiol. 2005;54:201–206.
13. Akasaka T, Onodera Y, Tanaka M, Sato K. Cloning, expression, and enzymatic characterization of *Pseudomonas Aeruginosa* topoisomerase IV. Antimicrob Agents Chemother. 1999;43:530–536.
14. Agnello M, Wong-Beringer A. Differentiation in quinolone resistance by virulence genotype in *Pseudomonas aeruginosa*. PLoS ONE. 2012;7:e42973.
15. Jalah S, Ciofu O, Høiby N, Gotoh N, Wretlind B. Molecular mechanisms of fluoroquinolone resistance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. Antimicrob Agents Chemother. 2000;44:710–712.
16. Mouneimné H, Robert J, Jarlier V, Cambau E. Type II topoisomerase mutations in ciprofloxacin-resistant strains of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 1999;43:62–66.
17. Akasaka T, Tanaka M, Yamaguchi A, Sato K. 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. 2001;45:2263–2268.
18. Lee JK, Lee YS, Park YK, Kim BS. 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. 2005;29:290–295.
19. Nakano M, Deguchi T, Kawamura T, Yasuda M, Kimura M, Okano Y. Mutations in the gyrA and parc genes in fluoroquinolone-resistant clinical isolates of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 1997;41:2289–2291.
20. Salma R, Daboussi F, Kassaa I, Khudary R, Hamze M. gyrA and parc mutations in quinolone-resistant clinical isolates of *Pseudomonas aeruginosa* from Nini Hospital in north Lebanon. J Infect Chemother. 2013;19:77–81.
21. Lister PD, Wolter DJ, Hanson ND. Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. Clin Microbiol Rev. 2009;22:582–610.
22. Japoni A, Farshad S, Alborzi A. *Pseudomonas aeruginosa*: burn infection, treatment and antibacterial resistance. Iran Red Crescent Med J. 2009;2009:244–253.
23. Ranjarb R, Owlia P, Saderi H, Mansouri S, Jonaidi-Jafari N, Izadi M. Characterization of *Pseudomonas aeruginosa* strains isolated from burned patients hospitalized in a major burn center in Tehran, Iran. Acta Med Iran. 2011;49:675–679.
24. Hall GS. Nonfermenting and miscellaneous gram-negative bacilli. In: Mahon CR, Leman DG, Manyeslis G, eds. *Textbook of Diagnostic Microbiology*. 3rd ed. Ohio: Saunders-Elsevier; 2007:564–585.
25. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing. Document Approved Standard M100-S20. PA, USA: Wayne; 2010.
26. Gorgani N, Ahlbrand S, Patterson A, Pourmand N. Detection of point mutations associated with antibiotic resistance in *Pseudomonas aeruginosa*. Int J Antimicrob Agents. 2009;34:414–418.
27. Ahangarzadeh Rezaee M, Sheikhalizadeh V, Hasani A. Detection of integrons among multi-drug resistant (MDR) Escherichia coli strains isolated from clinical specimens in northern west of Iran. Braz J Microbiol. 2011;42:1308–1313.
28. Higgins P, Fluit A, Milatovic D, Verhoef J, Schmitz F-J. Mutations in GyrA, ParC, MexR and NfxB in clinical isolates of *Pseudomonas aeruginosa*. Int J Antimicrob Agents. 2003;21:409–413.
29. Kureishi A, Diver JM, Beckthold B, Schollaardt T, Bryan LE. Cloning and nucleotide sequence of *Pseudomonas aeruginosa* DNA gyrase gyrA gene from strain PA01 and
quinolone-resistant clinical isolates. Antimicrob Agents Chemother. 1994;38:1944–1952.

30. Takenouchi T, Sakagawa E, Sugawara M. Detection of gyrA mutations among 335 Pseudomonas aeruginosa strains isolated in Japan and their susceptibilities to fluoroquinolones. Antimicrob Agents Chemother. 1999;43:406–409.

31. Sekiguchi J-I, Asagi T, Miyoshi-Akiyama T, et al. Outbreaks of multidrug-resistant Pseudomonas aeruginosa in community hospitals in Japan. J Clin Microbiol. 2007;45:979–989.

32. Lambert P. Mechanisms of antibiotic resistance in Pseudomonas aeruginosa. J R Soc Med. 2002;95:22–26.

33. Poole K. Efflux-mediated resistance to fluoroquinolones in gram-negative bacteria. Antimicrob Agents Chemother. 2000;44:2233–2241.

34. Van Bambeke F, Glupczynski Y, Plesiat P, Pecher J, Tulkens PM. Antibiotic efflux pumps in prokaryotic cells: occurrence, impact on resistance and strategies for the future of antimicrobial therapy. J Antimicrob Chemother. 2003;51:1055–1065.