



Molecular Detection of *gyrA* Mutation in Clinical Strains of *Klebsiella pneumoniae*

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Abstract

Background: Multi-drug resistant (MDR) *Klebsiella pneumoniae* strains cause the majority of community acquired and life-threatening infections. We aimed to detect the *gyrA* mutations in the clinical strains of nalidixic acid and ciprofloxacin resistant *K. pneumoniae* isolated from the patients with urinary tract infections.

Methods: Bacterial strains were isolated from the patients with urinary tract infections admitted to a major hospital in Tehran, Iran (2017-2018). Bacterial identification was done according to standard microbiological tests. Antimicrobial susceptibility testing for quinolones and fluoroquinolone antibiotics was done using both disc diffusion and minimal inhibitory concentrations (MICs) methods. PCR-RFLP was used to detect the probable mutation in the *gyrA* gene in nalidixic acid and ciprofloxacin resistant strains. Finally sequencing was performed to detect point mutations in isolated *K. pneumoniae* strains.

Results: One hundred *K. pneumoniae* isolates were recovered from the urine samples of the clinical cases. Antibiotic resistance testing showed that among all *K. pneumoniae* isolates, 26% and 19% of the strains were resistant to nalidixic acid and ciprofloxacin respectively. MIC value was ≥ 4 $\mu\text{g/ml}$ for ciprofloxacin resistant isolates. The results of RFLP on *gyrA* PCR amplicons using *HinfI* restriction enzyme showed point mutation in this gene in 46% of nalidixic acid and ciprofloxacin resistant *K. pneumoniae*. The data obtained from the sequencing confirmed the RFLP results and indicated the presence of point mutations in codons 83 and 87 in the *gyrA* gene which leads to the substitution of different amino acids in *gyrA* protein.

Conclusion: Our findings indicated a relative increased rate of resistance against quinolones and fluoroquinolone antibiotics that raised a concern about extensive dissemination of clinical strains of nalidixic acid and ciprofloxacin resistant *K. pneumoniae*. Point mutation of *gyrA* gene was responsible for the resistance in our strains however to gain more insight into the molecular characterization of quinolone-resistant isolates, other possible mechanisms of the resistance should also be investigated.

Keywords: *Klebsiella pneumoniae*; Antibiotic resistance; *gyrA* mutation; Sequencing

Introduction

Klebsiella pneumoniae is a gram negative and rod-shaped bacillus from the genus *Klebsiella* and

family Enterobacteriaceae. As a nosocomial pathogen, it causes several serious infections in-



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cluding pneumonia, bacteremia, hepatic abscesses and urinary tract infections particularly in the patients with underlying disease such as diabetes mellitus (1, 2).

Because of rapid emergence and spread of antibiotic resistance between the bacteria, there is an urgent need to monitor the use of antimicrobial agents and modify antibiotic therapies. Antibiotic resistance in bacteria is caused by several mechanisms including mutations in the genetic materials of bacterial cells. There are many reports through the world in which clinical isolates of *K. pneumoniae* have shown resistance to multiple antibiotic including quinolones and fluoroquinolones (3-5). The resistance against quinolones is often originated from chromosomes due to mutation of DNA gyrase gene. This enzyme consists of four subunits; a sub-unit is the main target of quinolone group antibiotics (3, 6). Unlike to abundance of global studies on quinolones and fluoroquinolones resistant *K. pneumoniae*, a few reports from Iran have shown the frequency of these resistance types and their genetic natures in clinical isolates of *K. pneumoniae* (6-10). Therefore, it is very important to continue studying the genes responsible for the resistance to different antibiotics in this pathogen particularly those are involved in quinolone resistance.

This study aimed to determine the frequency of resistance of clinical strains of *K. pneumoniae* toward quinolones and fluoroquinolones antibiotics. Moreover the genetic mutation in *gyrA* was also investigated.

Materials and Methods

Bacterial strains

In a cross-sectional study carried out in 2017 to 2018, *K. pneumoniae* isolates were recovered from the patients admitted to a major hospital in Tehran, Iran. The bacterial isolates were identified using conventional phenotypic and biochemical methods.

Antibiotics susceptibility testing

The antibiotic susceptibility testing of clinical *K. pneumoniae* isolates to fluoroquinolones (ciprofloxacin, 50 µg) and quinolone (nalidixic acid, 30 µg) was done using standard disk diffusion by culturing the isolates on the Mueller-Hinton agar medium and incubation for 18-24 h at 37 °C. The diameters of the antibiogram growth inhibition zones were measured according to the CLSI guidelines and the strains were classified based on how they reacted to each drug into susceptible and resistant (11).

The minimal inhibitory concentrations (MICs) was also carried out for ciprofloxacin by VITEK2 compact system (Biomérieux, Marcy, Petoile, France). Standard strain *K. pneumoniae* ATCC 12022 was used as a control.

Molecular detection of *gyrA* mutations

Total DNA was extracted by modified boiling method. The primers' *gyrAF* (CGCGTACTA-TACGCCATGAACGTA) and *gyrAR* (ACCGTTGATCACTTCGGTCAGG) were used for amplification of target gene of *gyrA* (12). Amplification reaction was performed according to the manufacturer's instructions, in an Eppendorf thermal cycler (Germany) in a reaction mixture (25µl) contained 10 µl Mastermix, 12 µl distilled water, 10 pmol of primer, and 1 µl DNA template. The PCR conditions for amplification of *gyrA* included an initial denaturation at 94-98 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, 1 min of annealing at 65 °C, and 2 min of extension at 72 °C, and final extension at 72 °C for 5 min. The PCR products were analyzed by electrophoresis on 1% gel agarose containing the safe stain.

RFLP analysis was used to determine the probable mutation in the *gyrA* gene of nalidixic acid and ciprofloxacin resistant strains. PCR products of *gyrA* gene was digested with *HinfI* enzyme. Briefly, 12 µl of PCR-amplified fragment was digested with 5 unit of the *HinfI* enzyme (MBI Fermentas) in 3 µl digestion buffer in final volume of 20 µl, and incubated at 37 °C for 16 h. Then, the restriction fragments were analyzed by electrophoresis on 2% agarose gel. Finally the sequencing was done to detect the point muta-

tions in *K. pneumoniae* strains. The obtained data were blasted in NCBI, and analyzed by Alignment Tools Pairwise software.

Ethical approval

This study was reviewed and approved by National Institute for Medical Research Development, Tehran, IRAN (ethic ID: IR.NIMAD 1398.172).

Results

Overall, 100 clinical isolates of *K. pneumoniae* were recovered from the patients under the study. Fifty seven patients were males and forty three were female. The age range of the patients was from 30 to 88 years. All bacterial strains were isolated from the urine specimens.

Antibiotic resistance testing showed that among all *K. pneumoniae* isolates, 26% and 19% of the strains were resistant to nalidixic acid and ciprofloxacin respectively. MIC value was ≥ 4 $\mu\text{g/ml}$ for ciprofloxacin resistant isolates. The most of the quinolone and fluoroquinolone resistant strains exhibited a low cip MIC value (4 $\mu\text{g/ml}$). The *gyrA* gene was amplified successfully in all nalidixic acid and ciprofloxacin resistant strains by PCR to produce amplicon products with expected size (Fig. 1). The results of RFLP using *HinfI* restriction enzyme showed point mutation in 46% of nalidixic acid and ciprofloxacin resistant *K. pneumoniae* isolates. As shown in Fig. 2, PCR-amplified *gyrA* fragments from non-mutant had three digested DNA bands however in mutant strains two fragments were produced.

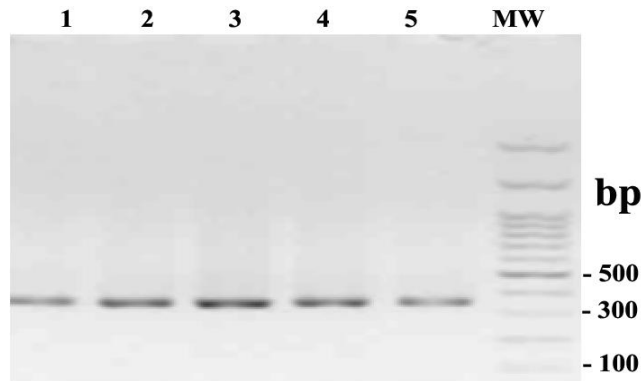


Fig. 1: PCR amplification of *gyrA* gene in *K. pneumoniae* strains

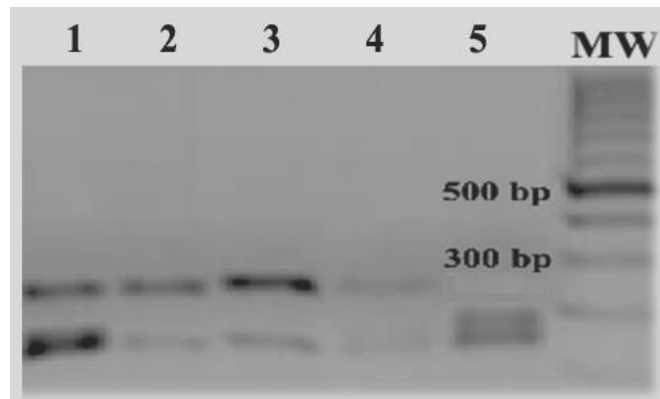


Fig. 2: Restriction fragment length polymorphism patterns of amplified *gyrA* gene in some representative strains of *K. pneumoniae* using *HinfI* restriction enzyme. Lanes 1-4 are mutant isolates with 2 fragments. Lane 5 is a non mutant isolate. MW is a 100bp DNA size marker

The sequencing of representative samples showed the presence of point mutations in codons 83 and 87 in the *gyrA* gene which leads to the substitution of different amino acids in *gyrA* protein. A double mutation in *gyrA* gene was identified in two strains.

Discussion

Many previous studies have reported that the *K. pneumoniae* strains were still susceptible to the fluoroquinolones indicated that these antibiotics might be considered as alternative and effective drug for treatment of the infections caused by this organism. However *K. pneumoniae* strains isolated in our study showed 26% and 19% resistance rates against nalidixic acid and ciprofloxacin respectively.

In our previous report with similar setting in 2016, 27% of the *K. pneumoniae* strains were resistant to these antibiotic classes (13). In Iran, low or moderate rates of resistance was indicated to quinolones in *K. pneumoniae* isolates. A very low frequency (6.8%) of the resistance reported to at least one of the quinolones among 220 *K. pneumoniae* isolates (7). Low frequency of resistance against nalidixic acid (29%) and ciprofloxacin (27%) in *K. pneumoniae* was also reported (9). Recently, among 100 isolates recovered in Borujerd hospitals, 38% of the *K. pneumoniae* strains were resistant to nalidixic acid and ciprofloxacin (6). Moreover, 39% and 27% of *K. pneumoniae* strains were resistant against nalidixic acid and ciprofloxacin respectively (10).

Comparison of the results obtained from previous studies in our country indicates that the resistance of *K. pneumoniae* strains toward quinolones and fluoroquinolone antibiotics is increasing. This alarming situation has been reported in other Asian countries. For example, the resistance rates for quinolones or fluoroquinolone antibiotics among *K. pneumoniae* strains have been reported 95% in Korea (14), 60%-80% in China (15,16) and 62.8% in Iraq (17).

By PCR, the desired *gyrA* DNA band was amplified in all resistant strains. RFLP was used to molecular analysis of quinolones or fluoroquinolone resistant *K. pneumoniae* strains. To detect the mutation in the *gyrA*, PCR products of *gyrA* were digested by *Hin*I restriction enzyme. Generally the target *gyrA* gene contains *Hin*I restriction sites that one of them is located at the codon 83 serine. RFLP with this enzyme will produce three fragments in non-mutant isolates. However, in mutant strains 2 fragments are expected to be produced due to loss of the restriction site at codon 83 serine. We found that 66% of nalidixic acid and ciprofloxacin resistant *K. pneumoniae* isolates had an altered restriction site at the codon 83 serine.

In the most reports, this mutation has been resulted in replacement of the amino acid of serine to phenylalanine (Ser83→Phe), serine to tyrosine (Ser83→Tyr), serine to leucine (Ser83→Leu) and other amino acid substitutions (3, 6, 7, 9).

The data obtained from the sequencing confirmed the RFLP results and indicated the presence of point mutations in codons 83 and 87 in the *gyrA* gene which leads to the substitution of different amino acids in *gyrA* protein.

In a recent study in Iran, the mutation rate of 40% in *gyrA* gene was in quinolone-resistant *K. pneumoniae* strains (6). Norouzi et al. reported substitution of Ser 83 →Ile and Ser 83 →Phe in *gyrA* gene among their *K. pneumoniae* strains (10). In Iraq, Ser83 → Leu was the most common mutation in *gyrA* that was observed in all quinolone resistant *K. pneumoniae* isolates, followed by Asp87→Asn (17). In China, more than 95% of ciprofloxacin resistant *Klebsiella* had point mutations in *gyrA* (16).

As a limitation, we didn't check other possible mechanisms of quinolone resistance including the mutation of *parC* gene and the presence of various *qnr* genes on the plasmids. As aforementioned, 46% of nalidixic acid and ciprofloxacin resistant *K. pneumoniae* isolates had point mutations in *gyrA* indicating other possible mechanisms may be involved to confer resistance toward quinolone resistance in our isolates including the

changes in the endocytic pathways, decreasing porin-expressions, increasing efflux pumps, and obtaining the qnr plasmids. Our findings showed a comparable rate of mutations in *gyrA* responsible for resistance against quinolones and fluoroquinolones in our *K. pneumoniae* isolates.

Here, we reported a relative increased rate of resistance against quinolone and fluoroquinolone antibiotics among *K. pneumoniae* strains. Acquisition of the virulence-associated genes encoding antibiotic resistance, biofilm formation and efflux pump by this organism can be considered as a major challenge for treatment of *K. pneumoniae* related infections and poses an important health issue in clinical settings (18).

Conclusion

Compared to previous studies, our findings indicated an increased rate of resistance against quinolones and fluoroquinolone antibiotics that raises a concern about extensive dissemination of clinical strains of nalidixic acid and ciprofloxacin resistant *K. pneumoniae*. Point mutation of *gyrA* gene was responsible for the resistance in our strains however to gain more insight into the molecular characterization of quinolone-resistant isolates, other possible mechanisms of resistance should also be investigated.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interest.

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