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Original Article

Analysis of Amino Acid Substitution Mutations of *gyrA* and *parC* Genes in Clonal Lineage of *Klebsiella pneumoniae* Conferring High-level Quinolone Resistance

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Introduction: The objective of this study was to determine the amino acid substitutions in gyrA and parC proteins in certain clonal lineages of *K. pneumoniae* conferring high-level quinolone resistance. **Methods:** One hundred and eleven *K. pneumoniae* isolates were recovered from clinical specimens in a teaching hospital in Kerman, Iran. The antibiotic susceptibility and MIC of quinolones were determined according to Clinical and Laboratory Standards Institute (CLSI) guidelines. Clonal lineages of the isolates were confirmed by enterobacterial repetitive intergenic consensus (ERIC)-PCR. Amino acid mutation profiles of *gyrA* and *parC* amplicons of 6 high quinolone-resistant isolates were also determined by DNA sequencing. **Results:** Twenty-two isolates were resistant to nalidixic acid (MIC, 256 µg/ml), ciprofloxacin (MIC, 32 µg/ml), levofloxacin (MIC, 32 µg/ml), and ofloxacin (MIC, 32 µg/ml). ERIC-PCR typing identified 4 clusters and 6 singleton, the largest belonged to cluster 3. Sequencing of *gyrA* gene showed 3 amino acid substitutions (Ser83→Ile, Lys154→Arg, and Ser171→Ala) in the strains 18, 20, 33; two substitutions (Lysine154→Arg; Ser171→Ala) in the strains 27 and 65 and 6 substitutions in the strain 66, of which, 3 (Ser83→Phe, Asp87→Ala, and Val190→Gly) were unique for this strain. Sequencing of *parC* gene revealed two substitutions (Ser129→Ala and Ala141→Val) in the strains 18, 27, 66, and 3 amino acid changes (Ser80→Ile, Ser129→Ala, and Ala141→Val) in the strains 20 and 33, respectively. Alignment and phylogenetic tree analysis of the *gyrA* from isolate 66 with homologous sequences obtained from the NCBI database, revealed 99.8% similarity to *gyrA* gene of *K. pneumoniae* ha10. **Conclusion:** The results suggest that acquisition of mutations in certain positions of *gyrA* and *parC* genes confers high-level resistance to quinolones. *J Med Microbiol Infec Dis, 2014, 2 (3): 109-117.*

Keywords: *Klebsiella pneumoniae*, Quinolone resistance gene, ERIC-PCR, Sequencing, Mutations.

INTRODUCTION

Klebsiella pneumoniae is considered as one of a group of pathogens responsible for most of the multidrug-resistant (MDR) nosocomial infections, in intensive care units (ICUs) of various hospitals [1]. *K. pneumoniae* is a major cause of nosocomial infections, primarily among immuno-compromised patients and those with long hospital stay. Emergence of MDR strains of *K. pneumoniae* has become a serious clinical concern for both patients' treatment (increase of mortality) and hospital management system. The most frequent resistances include resistance to aminoglycosides, fluoroquinolones (FQs), tetracyclines, chloramphenicol, and trimethoprim/sulfamethoxazole [2]. Reported carrier rates of multi-resistant strains in hospitalized patients are 77%, 19% and 42% in samples from stool, pharynx and patients' hands, respectively [3]. The high rate of nosocomial *K. pneumoniae* colonization appears to be associated with the use of antibiotics rather than hospital sanitation problems.

Ciprofloxacin is one of the treatment choices for infections caused by the Enterobacteriaceae family. This antibiotic acts by inhibiting bacterial DNA gyrase and topoisomerase IV, which are required for DNA replication [4]. Gyrase is more susceptible to inhibition by quinolones than topoisomerase IV, whereas, ciprofloxacin and levofloxacin are the two most frequently prescribed FQs. There are three mechanisms of resistance to quinolones: 1) mutations that alter the drug targets, 2) mutations that reduce drug accumulation, and 3) plasmids that protect cells from the lethal effects of quinolones [5].

Once a first-step mutation has reduced susceptibility of DNA gyrase in a Gram-negative organism, additional mutations in *gyrA* or mutations in *gyrB* or *parC* can further increase resistance to this class of antibiotics. Amino acid substitutions in a region of the gyrA or parC subunit, named "quinolone-resistance determining region" (QRDR), are involved in the resistance to quinolones. This region occurs on the DNA-binding surface of the enzyme [6].

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Resistance to FQ is now common in many extended spectrum beta-lactamase (ESBL)-producing bacteria including *K. pneumoniae* [7]. FQ resistance has been associated with specific amino acid substitutions in the QRDRs in gyrA and B subunits of DNA gyrase and parC and parE subunits of topoisomerase IV [8]. Typically, in both laboratory and clinical isolates, alterations in the serine residue comprise more than 90% of the mutant pool, with changes in the acidic residues comprising the bulk of the other mutations [9]. Mutations at Ser83 and Asp87 codons of gyrA subunit and Ser80 and Glu84 codons of parC subunit have been frequently reported in FQ-resistant *K. pneumoniae* isolates worldwide [9].

The objective of the present study is to determine the amino acid substitutions of gyrA and parC proteins in certain clonal lineages of the *K. pneumoniae* conferring high-level quinolone resistance.

MATERIAL AND METHODS

Specimen collection and bacterial identification. A total of 111 consecutive, isolates of *K. pneumoniae* were recovered from clinical specimens, such as urine, blood, bronchial aspirate, sputum, and patients' wounds of in surgical wards, burn units, and intensive care units (including NICU) of a teaching hospital, Kerman, Iran from June 2013 to March 2014. Samples taken by an expert laboratory technician were initially inoculated on MacConkey and bood agar (Merck, Darmstadt, Germany) plates and incubated at 37°C for 24 h. All isolates were confirmed as *K. pneumoniae* by conventional diagnostic tests for Enterobacteriaceae as described previously [10]. Demographic characteristics, including, sex, age, hospital unit, and length of hospitalization, were recorded. The individual colonies were inoculated onto sterile True North TM Cryogenic Vials (TNC) containing 1 ml of sterile tryptic soy broth (TSB) (BioMérieux, Marcy-I'Etoile, France) mixed with glycerol (40%) and stored at -70°C for further study.

Antimicrobial susceptibility testing. Antimicrobial susceptibility of all the hospital isolates of *K. pneumoniae* to four quinolones (nalidixic acid, ciprofloxacin, levofloxacin, and ofloxacin) was tested by the Kirby-Bauer agar disk diffusion breakpoint method [11]. The level of resistance was defined as: susceptible, intermediate, and resistant, based on the Clinical and Laboratory Standards Institute (CLSI) guidelines 2012 [12]. Oxoid antibiotic disks (Hi-Media, Mumbai, India) were used in the following concentrations (µg/ml): ciprofloxacin, 5; levofloxacin, 5; nalidixic acid, 30; ofloxacin, 5. Well-isolated colonies were inoculated into 5 ml TSB medium and incubated at 37°C for 24 h. The turbidity was then adjusted to 0.5 McFarland standard (CFU × 10^8). Two hundred microliters of the culture media were streaked on Muller-Hinton agar (Hi-Media, Mumbai, India) and the antibiotic disks were placed on the surface of agar plates. The plates were incubated at 37°C for 24 h and the inhibition zone around each disk was interpreted according to CLSI guidelines.

Minimum inhibitory concentrations (MICs) in quinolone resistant isolates were determined by E-test strips (Hi-Media, Mumbai, India) on Mueller-Hinton agar (MHA) according to the instructions of the manufacturer and CLSI guidelines. Resistance to nalidixic acid, ciprofloxacin, levofloxacin, and ofloxacin was defined as an MIC ≥4 µg/mL, and the susceptibility was defined as an MIC ≤1 µg/ml. *Escherichia coli* ATCC 25922 was used as quality control strain.

Molecular typing by ERIC-PCR. All quinolones resistant *K. pneumoniae* were typed by ERIC-PCR [13] to asses clonal relationships among the hospital isolates. Briefly, genomic DNA was extracted using a bacterial DNA extraction kit (Thermo Scientific, Vilnius, Lithuania) from a single colony grown on Luria- Bertani (LB) agar plates (BioMérieux, Marcy-l'Etoile, France) according to the manufacturer's instructions. The primers (Gene-Ray, Shanghai, China) used for ERIC-PCR typing were as follows; ERIC-1R (5'-AAGCTCCTGGGGATTCA-3') and ERIC-F (5'-AAGTAAGTGACTGGGGTGAGCG-3'). Each PCR amplification was performed on 50 µl of total volume containing 20 pM of target DNA, 0.2 mM of dNTPs, 50 pM of each primer, 5 µl of 10x PCR buffer solution (500 mM KCl, 100 mM Tris-HCl, pH 9.0), 2.5 mM $MgCl_2$, and 2 units of Taq DNA Polymerase (Ampliqon, Denmark). PCR conditions were as follows: denaturation at 95°C for 5 min, 40 cycles of denaturation at 92°C for 30 s, annealing at 59°C for 45 s, and extension at 72°C for 30 s. After reactions were completed, a final extension was performed at 72°C for 10 min [14]. The PCR products were separated by electrophoresis on 1.3% agarose gels (Merck, Darmstadt, Germany) and visualized using a gel documentation system (UVTEC, Cambridge). PCR profiles obtained by ERIC primers were used for dendrogram analysis and fingerprints by PyElph 1.4 software based on UPGMA (unweighted pair group method with arithmetic averages). The similarities between the fingerprints were calculated using the Pearson correlation test.

PCR amplification and sequencing of *gyrA* and *parC* genes. For PCR reactions, 22 quinolone resistant isolates of *K. pneumoniae* were grown on LB agar for 24 h at 37°C. Individual colonies were dissolved with 200 µl double distilled H20 and chromosomal DNA was extracted using a DNA extraction kit (Qiagen; Valencia, CA, USA). Oligonucleotide primers (Gene-Ray, Shanghai, China) used for amplification of the putative *gyr*A and *par*C genes, including QRDR were: *gyr*A-F (5′-CGCGTACTATACGCCATGAACGTA-3′); *gyr*A-R (5′-ACCGTTGATCACTTCGGTCAGG-3′); parC-F (5′-GCGTTGCCGTTTATTGGTGAT-3′); and *par*C-R (5′-GCAGGTTATGCGGTGGAAT-3′). PCR conditions for *gyr*A and *par*C amplifications consisted of an initial denaturation at 95°C for 30 s, followed by 30 cycles of denaturation at 95°C for 35 s, annealing at 62°C for 35 s for *gyr*A and at 59°C for 30 s for *par*C, extension at 72°C for 45 s, and a final extension at 72°C for 30 s. The PCR products were sent for sequencing (Bioneer, Seoul-Korea) in both directions with the same set of primers used for the PCR by Sanger dideoxy chain termination method using an Applied Biosystems 3730/3730Xl DNA Analyser (API, California, USA). Similarity searches for the nucleotide sequences were performed by BLAST algorithm software

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(http://www.ncbi.nlm.nih.gov/blast). Phylogenetic tree and evolutionary distance analysis of the *gyrA* gene of highly quinolone resistant isolate 66 were done by Maximum Likelihood method based on the Jones-Taylor-Thornton (JTT) matrix-based model and neighbour joining algorithms using MEGA6 software. Amino acid sequences of both gyrA and parC proteins were determined using UniProt database (http://www.uniprot.org) and compared for mutation analysis with reference sequences of Enterobacteriaceae *gyrA* and *parC* 281242.1 and NC_000913.3, respectively.

Statistical analysis. All numerical data were analysed using SPSS software for Windows (version 19; SPSS Inc., Chicago, IL, USA). Pearson's chi-square tests were used to evaluate the correlation between mutation and quinolone resistance. Statistical significance was defined as *p*-value less than 0.05.

RESULTS

Quinolone susceptibility. Out of a total number of 111 confirmed cases of *K. pneumoniae* infections in our hospital, 66% had signs of urinary tract infection (UTI), with the length of hospital stay of 15 to 20 days, 14% admitted with lower respiratory infections, such as chronic obstructive pulmonary disease (COPD) and ventilator associated pneumonia (VAP) with the highest length of stay in the ICU (20-25 days), 11% had blood infection, and 9% had wound infection and abscess with 13-15 days length of stay in hospital, respectively (Table 1). Among the infected patients, 53.2% were adult females, 32.4% were adult males, and 14.4% were infants. The majority (73%) of the patients aged from 46 to 62 years.

Antimicrobial susceptibility of all the isolates to four quinolone antibiotics are illustrated in Figure 1. Among the collected isolates, 22 (19%) were fully resistant to nalidixic acid and exhibited MIC ≥256 µg/ml, 77 (70%) were intermediate and showed MIC values of 0.5-1 µg/ml, 12 (11%) were susceptible with MIC ≤0.05 µg/ml. Similarly, resistance rates to ciprofloxacin, levofloxacin, and ofloxacin were, 19% (n=21), 19% (n=21), and 22.6% (n=25) with MIC ≥32 µg/ml, respectively (Figure1). Furthermore, 11% (n=12), 9% (n=10), %4 (n=4), and 2.7% (n=3) of the isolates were, susceptible to nalidixic acid, ciprofloxacin, levofloxacin, and ofloxacin with MIC ≤0.5 µg/ml.

ERIC-PCR Typing. Banding patterns of genomic DNA (range, 100 to 3500 bp) obtained by ERIC-PCR method are demonstrated in Figure 2a. The experiment was conducted twice, and reproducibility of the DNA patterns from the two gels was in the range of 98-100%. On the basis of the number and clarity of bands, their even distribution over the whole fingerprint, and discriminatory power, we identified 4 clusters (fingerprint) and 6 individual clones (singleton) (Figure 2b). The largest one belonged to cluster-3 (Cl-3 fingerprint) and consisted of 6 strains detected exclusively in UTI patients with length of hospital stay of 10-20 days. These strains showed high MIC (32 µg/ml) to all quinolones and had mutations in both *gyrA* and *parC* genes. Distinct ERIC-PCR banding patterns were obtained for cluster-1 (Cl-1 fingerprint) consisting of 4 isolates recovered from bronchial aspirate and sputum of COPD and VAP patients with length of hospital stay of 15 to 25 days and exhibited high MIC (MIC 32 µg/ml) to all the quinolones tested. The remaining clusters were isolated either from patients' blood or wound infections and abscesses with length of hospital stay of 15 to 25 days (Figure 2b).

Alignment and phylogenetic tree analysis of *gyrA* gene. Evolutionary distance analysis and phylogenetic tree of *gyrA* gene extracted from highly quinolone resistant *K. pneumoniae* isolate 66, in comparison with similar sequences in NCBI/BLAST search database, showed a high degree of similarity with *K. pneumoniae* strain ha10 (GenBank: JX123017.1) as illustrated in Figure 3. The alignment of the two sequences revealed 99.8% homology within 381 bp (Figure 3)

Table 1. Amino acid substitution mutations of gyrA and parC, clustral type, MIC, source of the samples, and length of hospitalization of 6 quinolone resistant isolates of *K. pneumoniae* recovered from Kerman hospital, Iran

Bacterial strain	Amino acid gyrA	Substitution parC	Cluster type	MIC (µg/ml)	Isolated from	Length of hospitalization (days)
18	S83→I K154→R S171→A	S129→A A141→V	Cl-3	32	Urine	15
20	S83→I K154→R S171→A	S80→I S129→A A141→V	Cl-3	32	Urine	18
27	K154→R S171→A	S129→A A141→V	Cl-3	32	Urine	17
33	S83→I K154→R S171→A	S80→I S129→A A141→V	Cl-1	32	Bronch	25
65	K154→R S171→A	ND	Cl-6	32	Wound	21
66	S83→F D87→A K154→R S171→A V190→G I191→F	S129→A A141→V	Cl-2	32	Sputum	20

ND= not detected, A= Alanine, R= Arginine, I= Isoleucine, K= Lysine, S= Serine, V= Valine, F= Phenylalanine, G= Glycine, D= Aspartic acid.

MIC was performed for nalidixic acid, ciprofloxacin, levofloxacin, and ofloxacin by E-test strip method. The inoculum concentration was CFU 1×10^8.

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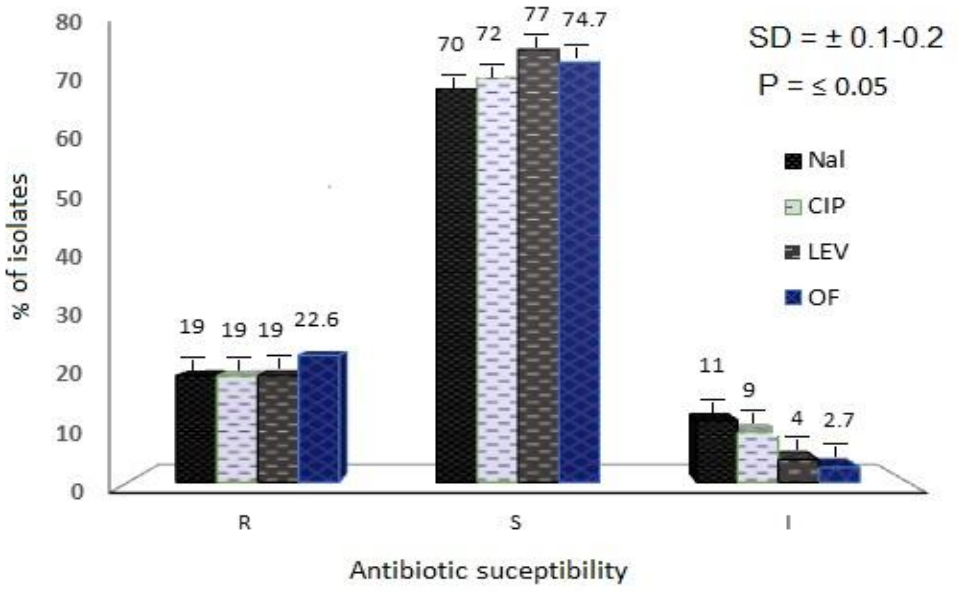


Fig. 1. Antibiotic susceptibility to four quinolones in *K. pneumoniae* strains isolated from a teaching hospital in Kerman, Iran. Nal, nalidixic acid; CIP, ciprofloxacin; LEV, levofloxacin; OF, ofloxacin. R= resistance, I= intermediate, S= sensitive. Breakpoint for each antibiotic was calculated according to the CLSI procedure

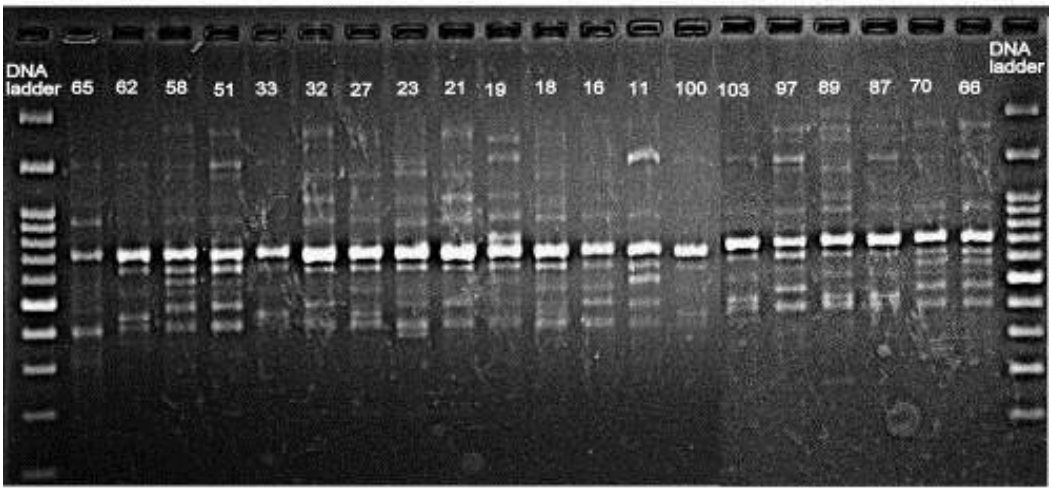


Fig. 2a. Agarose gel electrophoresis of ERIC-PCR of *K. pneumoniae* stains isolated from clinical samples in a teaching hospital in Kerman, Iran. The banding pattern showed 4 distinct clusters and 6 singleton.

Amino acid substitutions in gyrA and parC proteins. The PCR product of the *gyrA* and *parC* genes of the 6 *K. pneumoniae* isolates that showed high MIC to quinolones were sequenced, and amino acid sequences were evaluated for the presence of substitution mutation in certain positions. Six amino acid substitutions in gyrA and 3 amino acid substitutions in parC proteins were found in QR isolates (Figure 4a and 4b). In the case of gyrA protein, 3 point mutations and amino acid substitutions were detected at positions 83 (S → I), 154 (K → R), and 171 (S → A) in the strains 18, 20, and 33, respectively, while, double mutations were observed at positions 154 (K → R) and 171 (S → A) in the strains 27 and 65, respectively. Furthermore, strain 66 carried up to 6 mutations at positions 83 (S → F), 87 (D → A), 154 (K → R), 171 (S → A), 190 (V → G), and 191 (I → F), in which 3 amino acid changes (Valine → Glycine, Aspartic → Alanine, and Serine → Phenylalanine) were unique to this strain and reported for the first time. This strain exhibited a high MIC (64 µg/ml) to all quinolones ($p \leq 0.05$).

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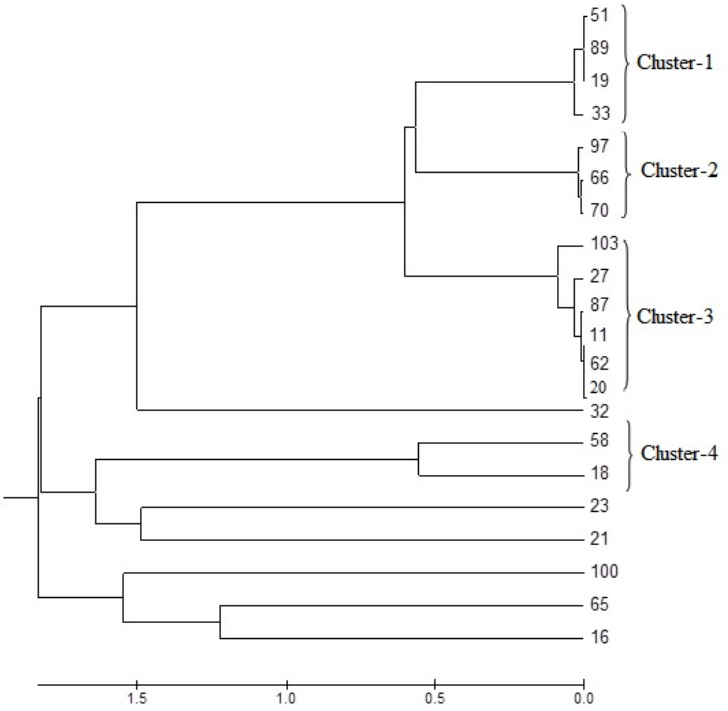


Fig. 2b. Dendrogram showing genetic relatedness of 21 quinolone resistant strains of *K. pneumoniae* was determined by (ERIC)-PCR fingerprint patterns.

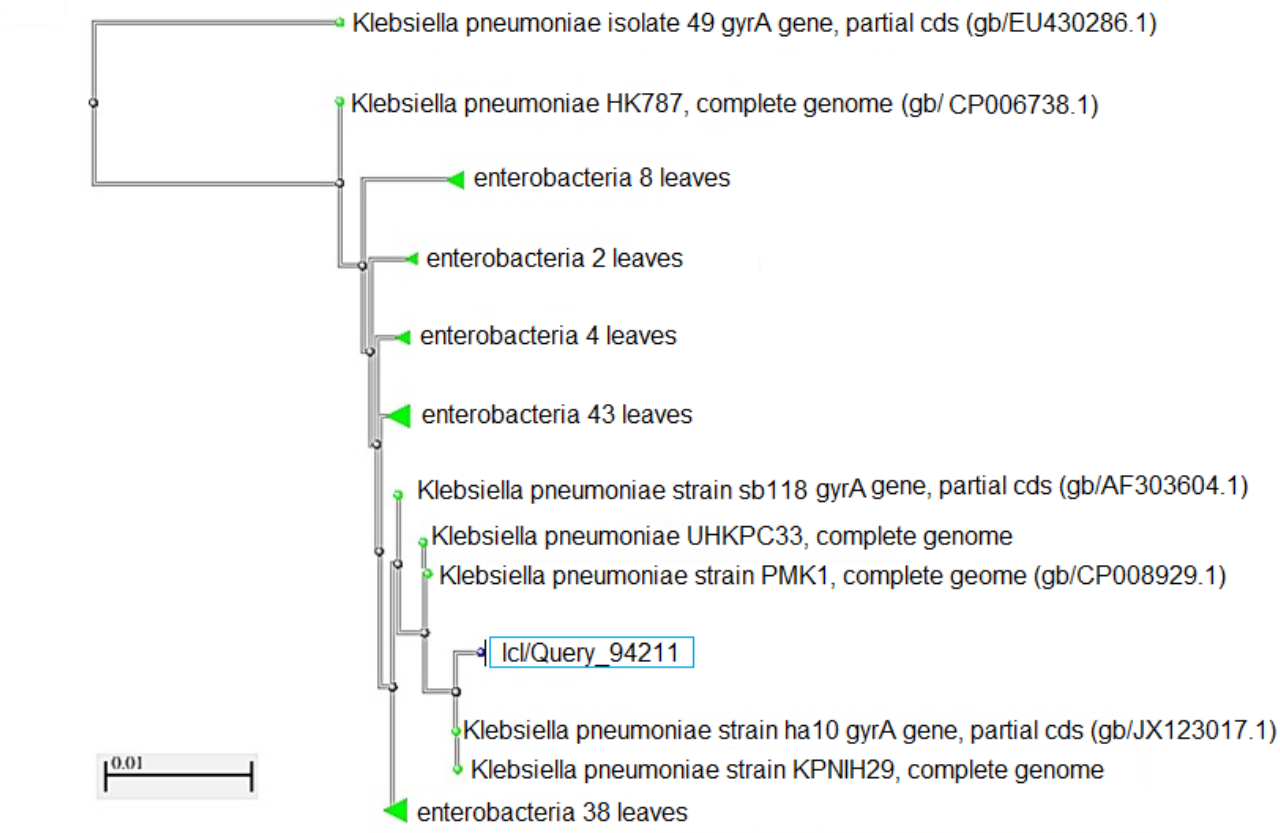
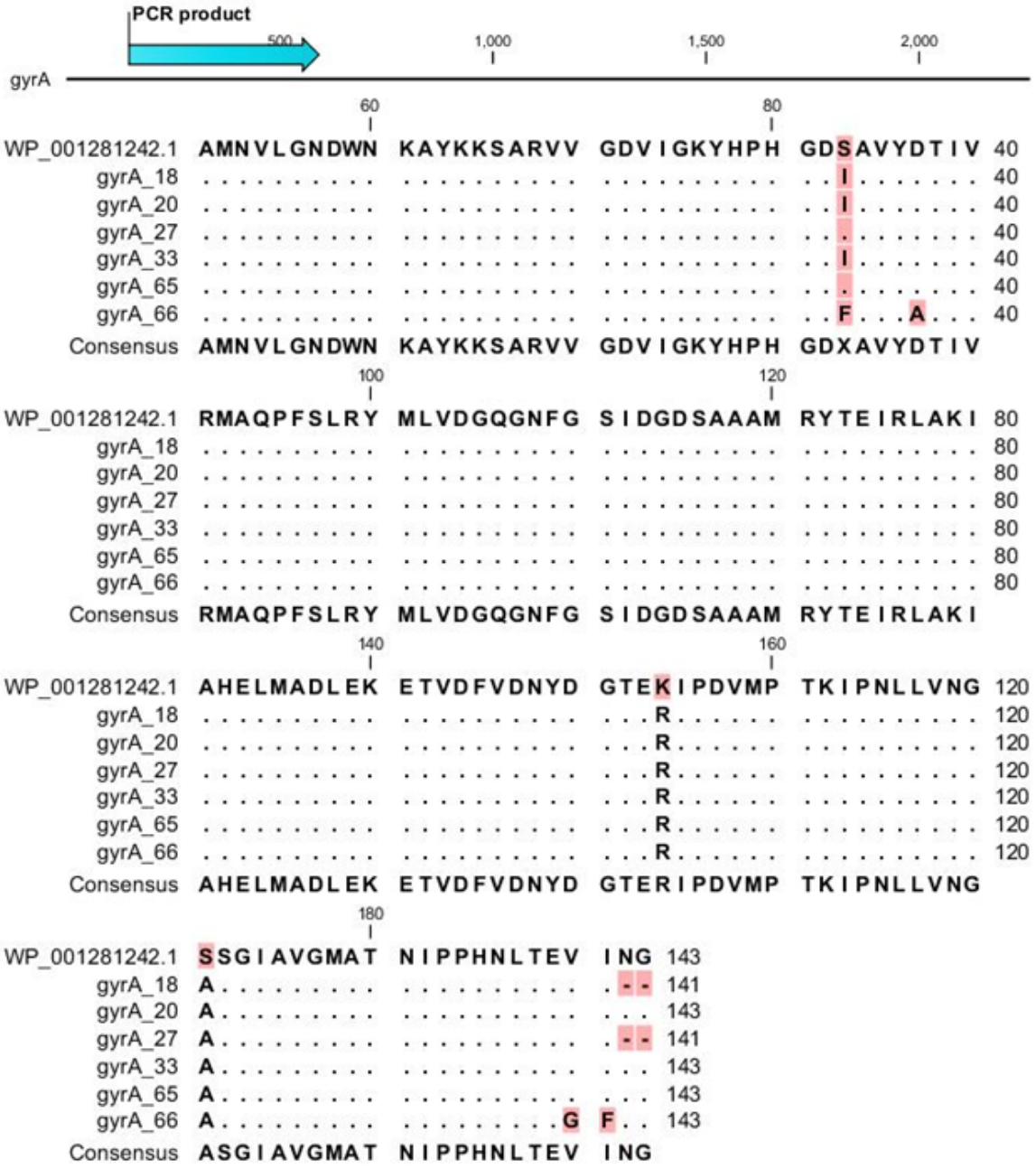


Fig. 3. A) Evolutionary distance analysis and phylogenetic tree of *gyrA* gene extracted from highly quinolone resistant isolate 66 with references sequences of *gyrA* in GenBank. BLAST computes a pairwise alignment between a query and the database sequences searched in NCBI. B) A schematic illustration of the aligned sequence of *gyrA* from *K. pneumoniae* stain 66 with *K. pneumoniae* strain ha10 (GenBank: JX123017.1).

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In the case of parC protein, 2 amino acid substitutions were located at codon positions 129 (S → A) and 141 (A → V) in the strains 18, 27, and 66. We also found 3 amino acid replacements at positions 80 (S → I), 129 (S → A), and 141 (A → V) in the strains 20 and 33 (Figure 4b). The most common mutations were (S → I), (K → R), and (S → A) ($p \leq 0.05$). It is interesting to note that the rates of resistance to ciprofloxacin, levofloxacin, and ofloxacin were significantly higher for those isolates that carried 3 amino acid substitutions in gyrA and parC proteins. We found single isolate with an identical mutation in *gyr*A to that of reference *K. pneumoniae* strain WP_001281242.1. Table 1 and Figure 5 shows amino acid substitution mutations in gyrA and parC, Cluster type, MIC, source of the samples, and length of hospitalization of the 6 strains of *K. pneumoniae* sequenced in this study.

a)



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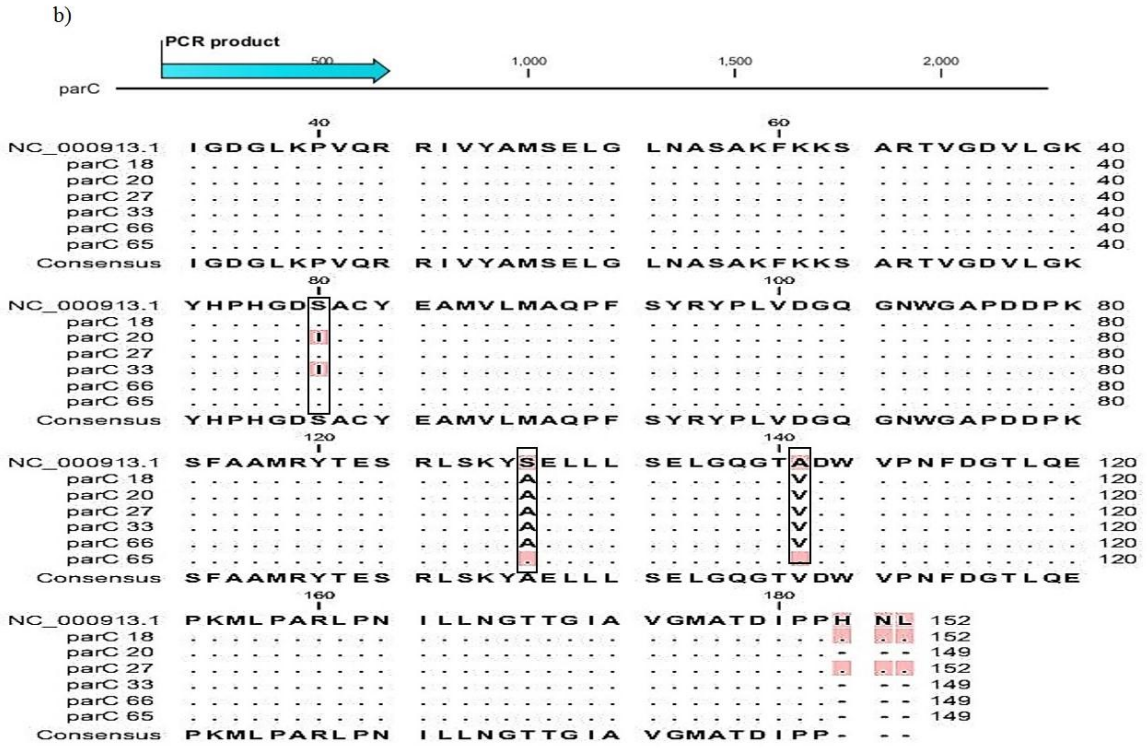


Fig. 4. Amino acid sequence of a) gyrA and b) parC of 6 *K. pneumoniae* strains exhibiting high MIC to quinolones. The amino acid substitution mutations were marked with color and showed in boxes. Sequences of both gyrA and parC proteins were characterized using UniProt database and compared with reference sequences of Enterobacteriaceae gyrA subunit A with accession number WP_001281242.1 and *E. coli* str. K-12 parC subunit A with accession number NC_000913.3. Dots indicate amino acids identical to the corresponding reference sequence.

DISCUSSION

Resistance to FQs in bacteria is mainly mediated by spontaneous mutations in the QRDR of *gyrA* and *parC* genes, either *gyrA* or *parC*, or both genes, especially at the highly conserved residues (Ser-83 and Asp-87) of gyrA [14]. In *K. pneumonia*, quinolone resistance is most commonly associated with specific mutations in gyrase and/or topoisomerase IV. Generally, mutation of type II enzyme confers ≤10-fold drug resistance. Selection for higher levels of resistance (~10–100-fold) usually yields strains with mutations in both enzymes [15].

A little information is available on *gyrA* and *parC* mutations among drug-resistant nosocomial bacteria in Iran. In a study conducted on 50 *A. baumannii* isolates in Tehran hospitals, 44 isolates were resistant to quinolones, and 42 isolates with double mutations in *gyrA* and *parC* genes showed a higher level of ciprofloxacin resistance compared to the 3 isolates with single mutations in *gyrA* or *parC* genes [16]. In another study, 23 out of 52 *K. pneumoniae* isolates were resistant to cephalosporins and/or quinolones; one isolate had the *qnrS*, and one harbored both *aac* (6')-Ib-cr and *qnrB* genes [17].

In the present study, we investigated the antimicrobial susceptibility of 111 *K. pneumoniae* isolates collected from clinical specimens of patients in a teaching hospitals in Kerman, Iran. We found that the rate of full and moderate resistance to quinolones was unexpectedly high, which may be due to the extensive administration of these drugs in hospitalized patients.

Analysis of the molecular nature of resistance suggests that MICs to quinolones in our isolates increased with the acquisition of additional mutations in *gyrA* and *parC* genes in the strains 18, 20, 27, 33, and 65. It seems that mutations in certain positions (83, 154, 171, and 191) of *gyrA* and *parC* genes resulted in high-level resistance to all quinolones. This was expected, as previous studies have shown that the progression from FQ susceptible towards resistant phenotype is a gradual process, starting from mutations in *gyrA*, the primary target of FQs, and followed by *parC* alterations that plays a complementary role in the development of higher levels of resistance [19]. This was confirmed by mutational analysis of strain 66, which had 6 base substitution mutations in *gyrA* (3 amino acid changes, *i.e.*, Valine → Glycine, Aspartic → Alanine, and Serine → Phenylalanine, were unique to this strain and reported for the first time) and 2 mutations in *parC* genes. The strain also showed the highest MIC (64 μg/ml) to ciprofloxacin, levofloxacin, and ofloxacin. To our knowledge, there have been no reports of such mutations. Protein modelling analysis of gyrA protein from the strains 18, 33, and 66 showed that its α/β structure is similar to those of other previously reported gyrA proteins, and the mutation may probably cause distortion of the helix. Dendrogram analysis by (ERIC)-PCR revealed 6 distinct fingerprints, the majority

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of which belonged to clusters 3 and cluster 1. Isolates with similarity >80% were considered to be in one group. Cluster 3 was more abundant among the isolates obtained from UTI patients. It is possible that this clone was spread among the UTI patients through contaminated catheters or contaminated urinary devices.

Effect of mutations on increased quinolone resistance due to mutations in gyrA and parC was also reported worldwide [20]. It has been shown that in *E. coli*, 3 or 4 mutations in both *gyrA* and *parC* genes are necessary to acquire high-level resistance to ciprofloxacin, while double mutations at positions 83 (Ser83) of gyrA and 80 (Ser80) of parC cause only moderate-level resistance [20]. Nakano *et al.* [21] reported that while a single amino acid change in gyrA, with or without a change in parC protein, was found in 14 isolates of *P. aeruginosa* with decreased susceptibility to FQs, 3 higher-level FQ-resistant isolates had a double amino acid change in gyrA and a single amino acid change in parC. Similarly, a Thr-86 to Ile mutation in *gyrA*, at a position equivalent to the *E. coli* Ser-83 to Ala mutation, has been reported to be responsible for high-level resistance to FQs in *Campylobacter jejuni in vivo* [22].

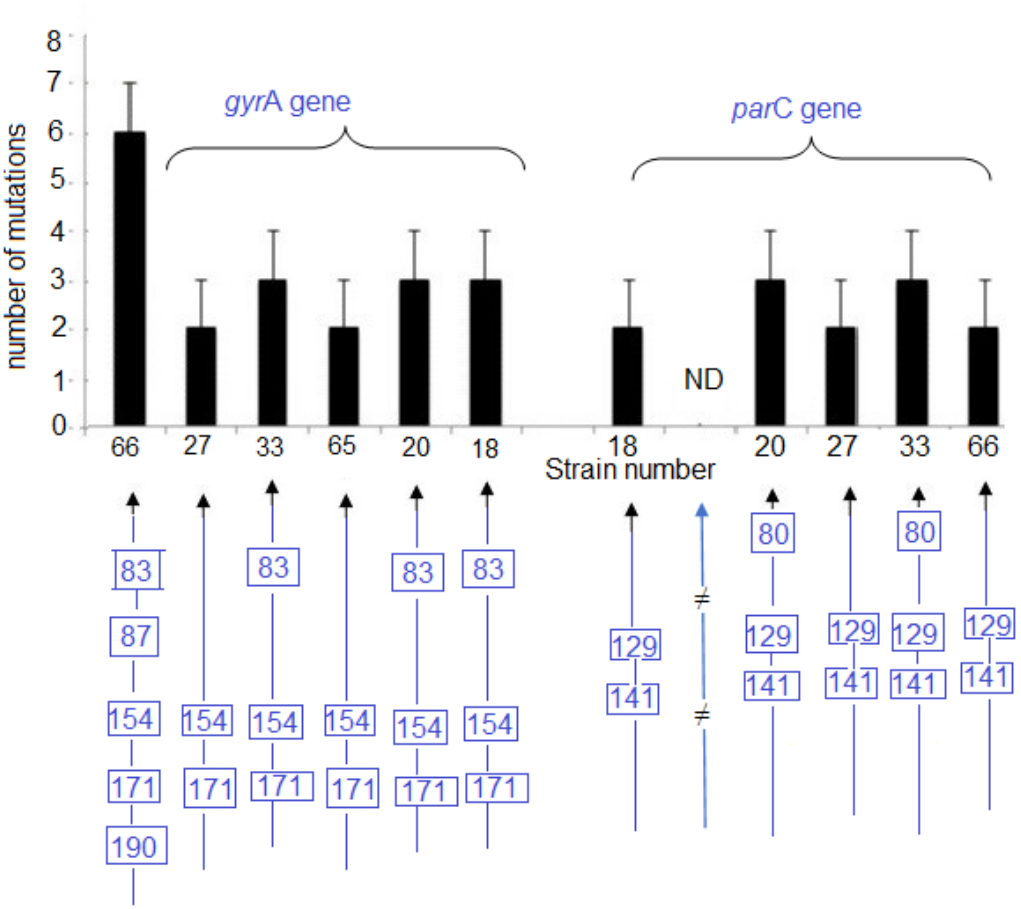


Fig. 5. Amino acid substitution mutations of gyrA and parC, Clustral type, and number of mutations in *gyrA* and *parC* amplicons.

In a study conducted on 61 ciprofloxacin-resistant isolates selected from 102 *K. pneumoniae* isolates, 4 major sequence types (STs) or clonal complexes (CCs), ST37, CC17 (consisting of ST17 and ST20), ST11, and CC528 (consisting of ST528 and ST1130), were found, and they accounted for 48.2% of the isolates tested. Amino acid substitutions in the QRDR and the presence of plasmid-mediated quinolone resistance (PMQR) genes, were detected in 20 (68.9%) and 18 (62.0%) isolates, respectively [23]. Another report revealed that 2 codons of Serine-83 and Aspartic acid-87 have the most frequent mutations, including Ser-83 → Phe (33%), Ser-83 → Tyr (20%), and Asp-87 → Asn (25%), Asp-87 → Ala (8%) that have high hydrophobicity compared to normal amino acids [24].

Weigel *et al.* [25] reported that low-level FQ resistance in *E. coli* was associated with single mutations in the gyrA protein, while high-level resistance required double mutations. They suggested with the exception of *Seratia marcescens*, that high-level resistance was associated primarily with alterations of the Serine or Threonine at position 83.

Overall, the results of our study suggest that acquisition of mutations in *gyrA* and *parC* genes plays a significant role in the development of high-level resistance to quinolones in certain clustral lineages of *K. pneumoniae*. To control the dissemination of clusters with certain mutations and high-level resistance in our hospital, excessive administration of quinolones must be discontinued.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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