ISSN 0891-4168, Molecular Genetics, Microbiology and Virology, 2014, Vol. 29, No. 4, pp. 211–215. © Allerton Press, Inc., 2014.

EXPERIMENTAL WORKS

Molecular Genetic Analysis of Quinolone Resistance-Determining Region of DNA Gyrase-A in Fluoroquinolones Resistant *Klebsiella pneumoniae* Based on GenBank Data and Reported Studies[1]

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Abstract—Infectious diseases caused by fluoroquinolone-resistant strains of *Klebsiella pneumoniae* is a considerable challenge in healthcare issues. Mutations in *gyrA* gene nucleotide sequences are the most important subject in the field of bacterial antibiotic-resistance mechanisms. In this study 200 cases of *Klebsiella pneumoniae* have been studied. For this purpose, 80 partial sequences of *gyrA* belonging to fluoroquinolone-resistant strains of *Klebsiella pneumoniae* obtained from the National Center for Biotechnology Information (NCBI) were analyzed via bioinformatic software and 120 ready cases from several reported studies directly imported into this investigation. Results showed that, two 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 in comparison with normal amino acids. According to the results of this investigation, the high distribution of hot spot mutations in *gyrA* gene sequences may lead to critical problems in the field of antibiotic therapy. Thus, the use of bioinformatics and statistical analyses are prompt approaches to perceive and control the probable drug-resistant bacterial strains.

Keywords: fluoroqinolone-resistant, *Klebsiella pneumoniae*, *gyrA* gene, mutation

DOI: 10.3103/S0891416814040041

INTRODUCTION

Klebsiella as an important member of the *Enterobacteriaceae*, is an encapsulated, Gram-negative and a rod shaped bacterium with no motility. Despite the presence of *Klebsiella* in human alimentary tract as a normal flora, some species like *K. pneumoniae* can cause different infectious diseases in open wounds, lung or urinary tract infections (UTIs) [1–6]. Depending on the anatomic position of bacterial infections, different types of antibiotics including tetracycline, aminoglycosides, fluoroquinolones are used in pharmaceutical therapy [7, 8].

Quinolones and fluoroquinolones inhibit the bacterial DNA gyrase enzyme. These are synthetic wide spectrum antibiotics, which affect on DNA gyrase enzyme as the target molecule and used in various bacterial infections caused by both Gram-negative and Gram-positive bacteria. This enzyme plays an important role in DNA replication and transcription. DNA gyrase introduces negative supercoils, which prevents over winding or excessive positive supercoiling of DNA in front of the replication fork. The inhibition of DNA gyrase by quinolones and fluoroquinolones leads to the cell death. Mammalian cells contain topoisomerase II instead of DNA gyrase to remove positive supercoils during DNA replication. Concentrations more than about hundred times of quinolones and fluoroquinolones are needed to inhibit topoisomerase II that demonstrates their selectivity of bacterial cells. Nalidixic acid introduced by George Lesheris is known as the first generation of quinolones, which was applied to treat UTIs during past years. Nowadays, other generations of fluoroquinolone especially Ciprofloxacin (second-generation) are widely used to treat infections caused by susceptible bacteria. The ligase domain of DNA gyrase is targeted by quinolones and leads to DNA fragmentation [9–11]. Up to now, three different types of quinolones resistance mechanisms are determined including the reduction of antibiotic concentration through the activity of some efflux pumps, the inhibition of quinolones activity through plasmid sourced protecting protein which binds to DNA gyrase, and the reduction of topoisomerase II and IV affinity to antibiotics via

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Table 1. The frequency of mutations in 83 and 87 codon positions in all resistant mutant strains of *Klebsiella pneumoniae*

Common codons of mutation	Percent of substitutions	Number of substitutions	Mutation type, codon no.	
TCC → TTC	33%	42	S → F	83
TCC → TAC	20%	25	S → Y	
TCC → ATC TCC → ATA	16%	20	S → I	
TCC → CTT TCC → CTC TCC → CTA	10%	13	S → L	
TCC → ACT TCC → ACC	1%	2	S → T	
GAC → AAC GAC → AAT	25%	31	D → N	87
GAC → GCC GAC → GCT	8%	10	D → A	
GAC → GGC GAC → GGT	4%	8	D → G	
GAC → TAC	4%	5	D → Y	
GAC → CAC	2%	3	D → H	

F: Phenylalanine, Y: tyrosine, I: isoleucine, L: leucine, T: threonine, N: asparagine, A: alanine, G: glycine, H: histidine, S: serine, D: aspartic acid.

point mutations [12]. According to different studies, specific point mutations in quinolone resistance-determining region (QRDR) of *gyrA* gene have a significant role in bacterial resistance [13, 14]. This study is mainly aimed to determine the types and the frequencies of point mutations occurred in the key position of *gyrA* of fluoroquinolone resistant *K. pneumoniae* which are recorded in GenBank and related studies.

MATERIALS AND METHODS

Data Collection Among Different Sources

First, the search was done on the NCBI nucleotide database to find complete or partial coding sequences of DNA gyrase subunit A belonging to fluoroquinolone-resistant strains of *K. pneumoniae*. Furthermore, several valuable papers were reviewed and ready analyzed data extracted and imported into the study [15–19].

Screening of Sequences

Second, the selected sequences, recorded in the NCBI nucleotide database were aligned together by Muscle (a multiple alignment algorithm) [20] via MEGA5.05 software [21] and compared with *K. pneumoniae* DNA gyrase subunit A reference sequence of NC_012731. Then, the phylogenetic tree of sequences was traced and redundant or similar sequences were omitted. Shared data between articles and GenBank were removed.

Sequence Alignment

Third, the remained mutant sequences and the RefSeq were aligned by the Muscle (a multiple alignment algorithm). Furthermore, the DNA sequences were translated into amino acid sequences in the correct reading frame and were aligned together via Muscle. Then the changes in specific points were investigated. Finally, the type and frequency of mutations were determined in both nucleotide and amino acid sequences. Data found in previous studies were collected and merged with other data.

RESULTS

In this study, 200 filtered cases of *K. pneumoniae* were selected from different sources including NCBI nucleotide database (80 partial sequences of *gyrA*) and several articles (120 reported cases). In 124 resistant strains, a single or double mutation was detected in codons of 83 and 87 but in 54 resistant strains, no mutation was found in mentioned positions. On the other hand, 22 cases were antibiotic-susceptible in spite of having a mutation in these positions. Our study revealed that among all resistant mutant cases, 57% are single codon mutant (83 or 87). So that 68% and 32% of mutations are occurring respectively in codons of 83 and 87 in QRDR. In 43% of antibiotic-resistant mutant strains, double mutations were observed in two mentioned codons (Table 1 and 2). The predominant mutations in codon 83 were respectively "Ser → Phe, Ser → Tyr, Ser → Ile, Ser → Leu, Ser → Thr" and in codon 87 were "Asp → Asn, Asp → Ala, Asp → Gly, Asp → Tyr, Asp → His" (Table 1). Besides, in this investigation between sequences recorded in the NCBI nucleotide database, 3 and 1% of mutations respectively for Serine and Aspartic acid lead to silent mutations, which have no effect on protein sequence. Among the cases extracted from previous studies, 22 antibiotic-susceptible cases had just a single mutation in 83 or 87 codon positions in the *gyrA* gene (Table 3).

DISCUSSION

Klebsiella pneumoniae is one of the most important bacterial causes of nosocomial infections. Multi-drug resistance (MDR) strains including Extended-Spectrum Beta-Lactamase (ESBL) and fluoroquinolones resistant strains are enhancing rapidly around the world and the drug therapy is going to be limited in a vast range [22–27]. In this study we investigated and analyzed the mutation types and frequency in DNA gyrase subunit A. As the results showed the mentioned subunit contains a specific region called quinolone resistance-determining region (QRDR) has a high

Table 2. The frequency of double mutations in resistant mutant strains of *Klebsiella pneumoniae*

Codons	Double mutation type	Number of mutations	Percent of mutations	Common codons of mutation
83 87	S → I D → G	3	3%	TCC → ATC GAC → GGC GAC → GGT
83 87	S → F D → G	3	3%	TCC → TTC GAC → GGC GAC → GGT
83 87	S → F D → A	7	6%	TCC → TTC GAC → GCC GAC → GCT
83 87	S → F D → H	2	2%	TCC → TTC GAC → CAC
83 87	S → L D → N	12	10%	TCC → CTT GAC → AAC
83 87	S → F D → N	22	17%	TCC → TTC GAC → AAC GAC → AAT
83 87	S → Y D → Y	4	3%	TCC → TAC GAC → TAC
83 87	S → Y D → A	3	3%	TCC → CTC GAC → GCC GAC → GCT
83 87	S → Y D → H	1	1%	TCC → TAC

degree of affinity which enables it to bind to fluoroquinolone antibiotics [28]. Studies have shown that any mutation either in *gyrA* or *gyrB* can cause resistance to fluoroquinolones but mutations in *gyrA* leads to a greater resistance compared with *gyrB* mutations. It seems that the quinolone resistance-determining region in *gyrA* is proximal to the enzyme active site while mutation sites in *gyrB* are distal to the active site [29].

According to our studies, about 70% of *K. pneumoniae* resistant strains showed single or double mutations in the codons 83 and 87 of *gyrA*. In the codon 83, mutations lead to the substitution of phenylalanine, tyrosine, isoleucine, leucine and threonine amino acids instead of serine as a polar-uncharged amino acid. Phenylalanine, tyrosine, isoleucine and leucine are non-polar amino acids with hydrophobic property whereas threonine is a polar-uncharged amino acid similar to Serine. As the results show, about 99% of the mutations in codon 83 have changed the class of amino acids from polar-uncharged to non-polar property.

Also mutation in the codon 87 that normally is translated to aspartic acid with polar-negative charged property, leads to the substitution of asparagine, alanine, glycine, tyrosine and histidine. Similar to the codon 83, non-polar amino acids (Glycine, alanine and tyrosine) are significant substituted amino acids with about 41% frequency in mutant codon 87. Also aspargine as a polar-negative charged amino acid has a remarkable frequency in a mutant form of gyrase A (54%), this amino acid has a lesser negative charge in comparison with aspartic acid. Histidine is another amino acid that is substituted in this position with the lowest frequency (5%) as a polar-positively charged amino acid and low hydrophilic property (Table 4).

Table 3. The frequency of codon alteration in DNA gyrase subunit A in 22 antibiotic-susceptible *Klebsiella pneumoniae*

Percent of substitutions	Number of substitutions	Mutation type	Codon no.
4%	1	S → I	83
14%	3	S → F	
41%	9	S → Y	
14%	3	S → T	
4%	1	S → L	
9%	2	S → A	
14%	3	D → N	87

Table 4. The hydrophobicity of amino acids that are substituted with normal amino acids in the codon of 83 and 87 of *Klebsiella pneumoniae* DNA gyrase subunit A sequences

Codon 83		Codon 87	
normal amino acid	hydrophobicity index	normal amino acid	hydrophobicity index
Serine	−5	*Aspartic Acid*	−55
mutant amino acid	hydrophobicity index	mutant amino acid	hydrophobicity index
Phenylalanine	100	*Asparagine*	−28
Isoleucine	99	*Glycine*	0
Leucine	97	*Tyrosine*	63
Tyrosine	63	*Alanine*	41
Alanine	41	*Histidine*	8
Threonine	13	–	–

The hydrophobicity index is a measure of relative hydrophobicity, or solubility of an amino acid is in water. The values of hydrophobicity index in the table are normalized in comparison with glycine (hydrophobicity index value = 0), so that the most values of hydrophobic and hydrophilic amino acids are 100 and −100 respectively.

All of amino acids that have been replaced with serine and aspartic acid are more hydrophobic so that in serine position more than 70% substitutions are amino acids with complete hydrophobicity while serine is almost a neutral amino acid, also for 87 codon, aspargin is twice more hydrophobic than aspartic acid (Table 4) [30]. Also in similar to single codon mutations, double codon mutations (in both 83 and 87 codon positions) lead to substitution of phenylalanine, leucine, isoleucine, tyrosine with serine and replacement of aspargin, alanine, glycine, tyrosine and histidine with aspartic acid that are more hydrophobic compared to normal amino acids. Studies show that the mutation in serine 83 to a hydrophobic amino acid generally confers more resistance rather than mutation in position 87. When both sites undergo mutation, levels of resistance can be two- to three-fold higher than the single mutation [31].

In other quinolone-resistant bacteria, spontaneous mutations in QRDR of *gyrA* at Ser 83 and Asp 87 have been indicated. Numerous studies have revealed that these residues are crucial for binding of quinolone to GyrA. Although the molecular interaction mechanism is not known, some ligand docking studies on *gyrA* mutations in *Escherichia coli* and *Salmonella enterica* predicted the role of these mutant positions in altering of antibiotic-enzyme interactions. According to these studies, reducing the hydrogen bonds between enzyme and antibiotic, and the change of amino acid hydrophobicity, which leads to the alteration of active site configuration, are the main factors that can decrease the enzyme binding affinity to quinolones and therefore decrease the drug's effectiveness [29].

The *gyrA* gene of *Klebsiella pneumoniae* is similar to its *E. coli* homolog with about 85% at the nucleotide level, 90% at the amino acid level and 100% in QRDR [32], on the other hand, the type of amino acid substitutions in *Klebsiella pneumoniae* mutants are similar to *E. coli*, so these parameters can be effective for quinolone resistance in these bacteria.

Finally, this study revealed that it is possible to identify the best antibiotic candidate against resistance bacteria in antibiotic-enzyme interaction models by determining the type and frequency of amino acids in enzyme hot spot mutation regions that leads to antibiotic resistance and in silico modeling of selective antibiotics with high frequency enzyme mutants. Also these analyses help us to design a new class of drugs to prevent the onset of pathogenic resistant bacteria [33, 34].

ACKNOWLEDGMENT

Authors are particularly grateful for the assistance provided by Ms Samaneh Khodi for her helpful and constructive comments during the revising of this research work.

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