

ORIGINAL ARTICLE

Involvement of Mutation in Serine 83 of Quinolone Resistance-Determining Region of gyrA Gene in Resistance to Ciprofloxacin in *Escherichia Coli*

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ABSTRACT

Appearance of bacteria resistant to antibacterial agents puts physicians in trouble and threatens the health of the world. The rapid development of bacterial resistance in *Escherichia coli* to ciprofloxacin makes difficult the treatment of infectious diseases. So, detection of the locations of possible mutations in gyrase A gene (*gyrA*) in these mutants is very important to determine the mechanism of this resistance. In the present study, ciprofloxacin-resistant mutants were isolated from medium containing ciprofloxacin. Polymerase chain reaction (PCR) was used to amplify the *gyrA* gene in these mutants and DNA sequencing was used to determine the location of mutation in this gene. Results showed that the most of ciprofloxacin-resistant mutants in quinolone resistance- determining region (QRDR) of A subunit of DNA gyrase and specially at serine 83. However, mutations outside of this region were also found at tyrosine 50 and alanine 119. In conclusion, this study confirms that mutation in *E. coli*.

Keywords: Escherichia coli, Ciprofloxacin, DNA gyrase, Quinolone resistance, Mutation

Escherichia coli is one of the most important members of Enterobacteriaceae family. This bacterium is the most common cause of some infections such as bacterial urinary tract infections, enteric infections and diarrhea in travelers [1-2]. Due to frequent and sometimes inappropriate use of antibiotics to treat a wide range of different infections caused by this bacterium, resistance to these antibiotics is gradually spreading around the world [3-4]. Up to now, two major mechanisms to explain the resistance to guinolones have been discussed [5-6]. The mode of action of quinolones is inhibiting the activity of topoisomerase II (DNA gyrase) and topoisomerase IV [7]. The DNA gyrase enzyme breaks both strands of DNA, reseals the broken area, and leads to formation of negative supercoils in DNA. This enzyme is a tetramer and consists of two A subunits and two B subunits [8]. A and B subunits are coded by gyrA and gyrB genes, which are located at 48 and 83 min, respectively, on the E. coli genome [9]. In most cases, resistant mutants in gram-negative bacteria such as E.coli are associated with changes in gyrA and gyrB genes [10]. Studies have shown that a small area in the N-terminal of gyrA from amino acids 67 to 106 causes resistance to these drugs. This area is called quinolone resistance-determining region (QRDR) [11-13]. Most of E. coli mutations occurred in the QRDR of gyrA, are in nucleotide 248 and 620, lead to changes in amino acids aspartic acid 83 and serine 87 [14]. Quinolones targets in gram -negative and gram -positive microorganisms are different . DNA gyrase and topoisomerase IV are targets for gram-positives and gram-negatives, respectively [15-18]. In clinical isolated strains, those with multiple mutations in codon 83 and 87, have higher amounts of minimum inhibitory concentration (MIC) [19]. Mutations in codon 426 (aspartic acid to asparagine) and codon 447 (lysine to

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glutamic acid) in *gyrB* gene can cause resistance to quinolones [20]. Reported mutations in codon 67, 81, 82, 83, 84, 87, and 106 of *gyrA* are related to the increase of *E. coli* resistance to quinolones [13, 21-22]. Although QRDR of protein GyrA has been known as the catalytic site of this protein, mutations outside the QRDR (codon 51) might also result in resistance to ciprofloxacin [14]. This issue implicates the necessity of further investigations on mutants resistant to ciprofloxacin. However the study of *E. coli* genetic structure is a major guide in the treatment of infections caused by this bacterium. Polymerase chain reaction (PCR) is a very effective, rapid, and specific method for detection of many pathogens and resistance to

antibiotics. The aim of this study was to isolate mutants resistant to ciprofloxacin and to detect the location of the mutation on the *gyrA* gene by PCR and sequencing methods.

MATERIALS AND METHODS

The present experimental study was carried out during 12 months (2014-2015) in Tehran University of Medical Sciences, Tehran, Iran.

Materials

Ciprofloxacin powder was purchased from Bayer, Germany. E. coli ATCC 25922 strain was purchased from the collection of fungi and bacteria of scientific and industrial research organization of Iran (IROST). E. coli MG1655 was a gift from Professor Lloyd (institute of genetics, University of Nottingham, UK). Mueller-Hinton broth (MHB) and Mueller-Hinton agar (MHA) were purchased from Merck, Germany. PCR buffer, Taq polymerase, $MgCl_2$ and deoxynucleotide triphosphates (dNTPs) were purchased from Cinnagen, Iran. Primers were prepared by Gen Fanavaran, Iran, and DNA ladder (1 kb DNA) was purchased from Fermentas, Canada.

Treatment with antibiotic and MIC determination

We used sterilized glassware and solutions. MIC is the lowest concentration of antibiotic that inhibits the growth of microorganisms. Ciprofloxacin MIC was determined by the standard agar dilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [23-25]. For this purpose, we made 24-hour culture of bacteria, then in test tubes containing cation-adjusted MHB medium, serial concentrations of the ciprofloxacin (0.9, 1.8, 3.7, 7.5, 15, 23.1, 56.2, 125, 250, 500, and 1000 µg/ml) were added, and finally 0.1 ml of bacterial suspension containing 10⁶ microorganisms per ml was subjoined. We considered two MHB tubes as controls: the first one only containing MHB and the other containing the bacteria and antibiotic. These tubes incubated at 37°C for 24 hours, and then we examined them in term of growth of microorganisms [23-25].

Viable microbial count

We prepared serial dilutions of the bacteria and then we spread 1 ml of these dilutions on cation-adjusted MHA medium incubated at 37° C for 24 hours. Then, we counted the colonies grown on plates. We considered plates with 30-300 colonies for counting.

Isolation of mutants resistant to antibiotics

After MIC determination for *E. coli* MG1655 and ATCC 25922 control bacteria, we selected the appropriate concentrations of ciprofloxacin to add to the MHA medium. For isolation of resistant mutants, we took 0.1 ml liquid of the MHB medium, then spread on palates that the desired antibiotic concentrations had already been added to them. Then, we placed them in incubator (Memmert, Germany) at 37° C for 3 days and harvested resistant colonies daily.

Mutation frequency

To determine the mutation frequency, the ratio of mutant to total bacteria on each day was obtained. For this purpose, we added 0.1 ml of 24-hour bacterial culture medium to MHA containing antibiotics and incubated it at 37 °C. The number of mutant bacteria appeared per day at the plate was counted. To obtain the total number of bacteria, we used cultured bacteria for preparation of consecutive dilution in normal saline , then one ml of desired dilution was added to antibiotics-free MHA and incubated at 37 °C, then we counted the number of colonies per day.

MIC determination for mutants

We isolated ciprofloxacin-resistant mutants from ciprofloxacin-containing MHA plate, which used for determining the frequency of mutations. Five colonies were randomly selected from each plate and a total of 100 ciprofloxacin-resistant colonies of the plates were separated for later tests. We used higher concentrations of ciprofloxacin for MIC determination for antibioticresistant mutants.

Polymerase chain reaction

We used fresh 24-hour cultures of resistant mutant bacteria for DNA extraction. Then, we solved one colony in 50 μ l of sterile water by a toothpick and put it in a water bath (Memmert, Germany) at 95 °C for two minutes, then cooled it on the ice. The primers were designed as below:

Forward primer: 5'-GCCATGAACGTACTAGGC-3'

Reverse primer: 5'-GGATATACACCTTGCCGC-3'

The amplification procedure was performed in a final volume of 100 μ l of the reaction mixture contained 10 mM tris hydrochloride (pH=8.3), 50 mM KCl, 2 mM MgCl₂, 0.001% gelatin, 200 μ M each of the deoxyribonucleotides, 300 ng each of the oligonucleotides, 2.5 units of *Taq* polymerase, and 20 picomol primer. The mixtures were processed in a programmable DNA thermal cycler (Techne-PCR, UK),

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 Table 1. MICs for ciprofloxacin-resistant colonies

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| Strain | MIC (ng/ml) | Strain | MIC (ng/ml) |
|------------|-------------|------------|-------------|
| MG1655 | 35 | ATTC 25922 | 9 |
| MW1- MW22 | 62.5 | MP1- MP32 | 18 |
| MW23- MW43 | 75 | MP33- MP43 | 75 |
| MW44- MW46 | 125 | MP44- MP46 | 312 |
| MW47- MW48 | 312 | MP47- MP48 | 625 |
| MW49- MW50 | 625 | MP49- MP50 | 1250 |

Table 2. Changes in amino acid sequences in different colonies

| Colony name | Mutation | Colony name | Mutation |
|-------------|---|-------------|----------------------------|
| MG1655 | Wild type | ATTC 25922 | Wild type |
| MW1-MW4 | Serine 83 to leucine (TTG) | MP1 | Wild type |
| MW14 | Tyrosine 50 (TAC) to phenylalanine (TTT) | MP2-MP11 | Serine 83 to leucine (TTG) |
| MW23-MW26 | Wild type | MP33-MP42 | Serine 83 to leucine (TTG) |
| MW27 | Tyrosine 50 (TAC) to phenylalanine (TTT) | MP44 | Serine 83 to leucine (TTG) |
| MW28 | Serine 83 deletion | MP47-MP49 | Serine 83 to leucine (TTG) |
| MW29-MW38 | Serine 83 to leucine (TTG) | MP50 | Serine 83 to leucine (TTG) |
| MW43 | Alanine 119 (GCA) to glutamic acid (GAA) | | |
| MW44 | Serine 83 to leucine (TTG) | | |
| MW47 | Serine 83 to leucine (TTG) | | |
| MW49 | Serine 83 to leucine (TTG) | | |

and subjected to 30 cycles of amplification. Each cycle consisted of 15 seconds at 95 °C, 25 seconds at 50 °C, and one minute at 72 °C. Electrophoresis was carried out on 10 μ l of amplified sample in 2% agarose gel with a 1 kb DNA ladder as a molecular mass marker. We immersed the gel in 100 ml of distilled water, and then added 0.1 μ l of ethidium bromide (0.5 μ g/ml) to it and shook it for 5 to 10 minutes slowly. The image of the gel was captured by a UV gel documentation system (Topac Inc., USA).

Sequencing

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We sent the PCR products and both primers to the company (Bionid, Korea) for sequence determination.

RESULTS

Colony counting

The total number of bacteria was 11×10^8 for *E. coli* MG1655 control and 22×10^7 for ATCC 25922 control. Mutation frequency (the ratio of the number of bacteria *Published online: July 12, 2015*

grown in medium containing ciprofloxacin to ciprofloxacin-free medium) for *E. coli* ATCC 25922 and MG1655 *E. coli* were 10×10^{-6} and 40×10^{-7} per day, respectively. These numbers were the averages of 10 independent counting.

MIC determination

The MICs of ciprofloxacin for *E. coli* MG1655 and ATCC 25922 were determined as 35 ng/ml and 9 ng/ml, respectively, while MICs for most resistant colonies that obtained from strains MG 1655 and ATCC 25922 were 75 ng/ml and 18 ng/ml, respectively. MICs for ciprofloxacin-resistant colonies are shown in Table 1.

PCR analysis

Fig 1 shows the gel electrophoresis of PCR product. Comparison of sequences of the gyrA gene of ciprofloxacin-resistant colonies with sequences of controls revealed the changes in nucleotides (Fig 2). Alterations in amino acid sequences in different colonies are demonstrated in Table 2.



Fig 1. Gel electrophoresis for PCR products. Column 1: DNA marker, columns 2-8: ciprofloxacin-resistant mutants.

DISCUSSION

The emergence of antimicrobial-resistant bacteria is considered as a serious threat against world health. Repeated and improper use of antibiotics causes bacterial resistance in the period of time . Extensive use of antibiotics has led to the emergence of resistant

pathogens and the problem of infectious diseases is now more complicated [25-26]. In this study, ciprofloxacin, a member of the fluoroquinolone family that is widely used to treat infections caused by gram-negative bacteria especially E. coli, was studied. Improper use of antibiotics (high doses or unnecessary administration) in the emergence and has an important role development of resistant bacteria. Chromosomal mechanisms of resistance to quinolones include changes in the genes related to A subunit of DNA gyrase as well as alterations in genes involved in permeability and transmission of materials outside of the cells . DNA gyrase is a member of the class II topoisomerase enzymes. This enzyme causes the formation of negative supercoils in bacterial DNA. It possesses two A and two B subunits that are encoded by gyrA and gyrB genes, respectively. DNA gyrase and especially GyrA are targets for quinolones in gram-negative bacteria. In this research, we studied E. coli, an important member of the Enterobacteriaceae family in term of resistance to ciprofloxacin. Nucleotide variations such as substitution, deletion or addition can lead to mutations and the emergence of fluoroquinolone-resistant E. coli. We showed that 45 colonies of 50 analyzed colonies had a mutation in the gyrA gene, and the rest probably had mutations in other ciprofloxacin targets, such as parC, gyrB, or parE. Our research revealed that there were 41 cases of mutation of serine 83 to leucine in 45 colonies with mutations in gyrA gene. This event has been reported previously [16-17]. In a research conducted by Drlica et al. on the protein GyrA in E. coli, mutations in two amino acids including serine 83 and aspartic acid 87 were the main causes of resistance

to ciprofloxacin [14]. It is also reported that mutations at serine 83 typically have more impact on resistance than mutations in aspartic acid 87 [14, 19]. Unlike the previous studies, we did not observe any mutations in codon 87. In this study, we showed codon changes including removal and alteration of the amino acid serine 83 to leucine in pathogenic and non-pathogenic strains. In addition, in the previous studies, other mutations including substitutions in the QRDR were reported [12,27]. We observed deletion of amino acid serine 83 in the colonies contained mutation at gyrA. This implicates the importance of this area in resistance to ciprofloxacin. We also showed substitution of tyrosine 50 by phenylalanine in two other colonies. Mutations outside of the QRDR had been reported, but these mutations were seen in codon 51 [27-28]. We found a substitution of alanine 119 by glutamic acid, and this mutation has not been reported yet. Since in this study the majority of ciprofloxacin-resistant colonies had mutations in gyrA, the primary assumption of the current study that was the direct relationship between resistance to ciprofloxacin in E. coli and changes in the gyrA gene, was approved. In the present study, all of mutants isolated from pathogenic parent strain had mutations in this region, while isolated mutants from the laboratory parent strain contained mutations outside of this region. This issue eases the design of new antibiotics to treat infections caused by pathogenic E. coli strains. To ensure the prevention of rapid resistance, it is better to consider the importance of the locations 50 and 119 that might possess a dramatic role in the reaction between enzyme and antibiotic, especially with codon 119 that is close to the position tyrosine 122 (GyrA active site). This active site is near the QRDR and this area possesses an important role in binding to DNA [29]. These mutations also approved the importance of amino acids around the enzyme active site (tyrosine 122) in reaction between enzyme and antibiotics.

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Fig 2. Nucleotide sequences of PCR products from ciprofloxacin-resistant mutants

CONCLUSION

In is concluded that mutation in serine 83 of QRDR in A subunit of DNA gyrase is the main cause of resistance to ciprofloxacin in E. coli. Other mechanisms implicated in ciprofloxacin resistance, which might be responsible for the differences in MIC values for ciprofloxacin were not considered in this study. Changes in active efflux pump and permeability are another mechanisms that cannot be excluded and may enhance development of antimicrobial resistance and contribute to the selection of fluoroquinolone-resistant isolates in the course of treatments with these antibiotics, especially in hospital environment. Studies to determine the further locations of mutation, which cause resistance to ciprofloxacin provide useful information about the interaction between DNA gyrase and antibiotics. In the other words, mechanisms of action of antibiotics and these outcomes can be used to design and synthesize new fluoroquinolones. It should be also noted that in many cases antibiotic resistance is transmitted to humans, hospitalized patients, and hospital environment through other sources including food plants, animals, fish, poultries, and other Published online: July 12, 2015

industries, in which antibiotics are used for different purposes and may lead to emerging resistant strains [30-31]. Isolation, identification and antimicrobial susceptibility of pathogens can be helpful in optimizing the antimicrobial use.

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