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In Vitro Development of Resistance to Subinhibitory Concentrations of Ciprofloxacin and Levofloxacin in Uropathogen *Escherichia coli*

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In this study we aimed to determine the development of resistance against subinhibitory concentrations of ciprofloxacin and levofloxacin of the *Escherichia coli* strains. Fifty *E.coli* strains that are susceptible to ciprofloxacin and levofloxacin have been included in this study. Minimal Inhibitory Concentration (MIC)s were detected by macro dilution method. In order to investigate the resistance development, the *E.coli* strains were left to incubate at 37°C for one night in subinhibitory concentrations (1/2 x MIC), then incubated in antibiotic free Mueller-Hinton broth for one night. In this way, after eight sequential passages, second MICs were detected. The third MICs were determined by repeating the same process based on the newly determined MIC values. Accordingly, as of the first MICs, after 16 successive passages an increase of MIC values against subinhibitory concentrations of ciprofloxacin was observed in all *E.coli* strains. This was the same for levofloxacin except for three strains; however, there were striking increases such as 64 or 128 times in the remaining strains. The use of antimicrobials for long treatment durations, taking care to monitor dose regulations and proper usage is recommended.

Key words: Resistance, *E.coli*, fluoroquinolones, Subinhibitory concentration.

Fluoroquinolones have been demonstrated to have high bacteriologic, clinical cure rates and effectiveness against most uropathogens. The use of broad spectrum antibiotics, insufficient treatment, usage of inappropriate antibiotics and usage of suboptimal concentrations of antibiotics are the reasons for the increase in resistant strains[1-3]. Bacterial resistance to fluoroquinolones usually results from mutations in the chromosomal genes which encode topoisomerases as well as the expression efflux pumps and loss of porines contributing to the development of fluoroquinolone resistance[4,5]. The aim of this study was to determine the development of resistance against subinhibitory concentrations of ciprofloxacin and levofloxacin of the *E.coli* strains.

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MATERIALS AND METHODS

Bacteria

In this study, *E.coli* strains isolated from urine samples were used. Recurrent isolates which were obtained in the same patient were excluded. Conventional bacteriologic methods were used for identification. Fifty *E.coli* strains which are susceptible to ciprofloxacin and levofloxacin have been included in this study. The MIC intervals for the strains were 0.25 - 0.0078 µg/ml for ciprofloxacin and 0.5-0.0078 µg/ml for levofloxacin.

Antimicrobials and Media

Ciprofloxacin (Toprak Drug Company, Turkey) and levofloxacin (Fako, Turkey) were used as antimicrobials. Mueller- Hinton broth (Merck,

Germany), Mueller-Hinton Agar (Merck, Germany) were used for the susceptibility test and serial passages in this study.

MIC determination

The determination of antibiotic susceptibility was performed by the broth macrodilution method with Mueller-Hinton broth according to the CLSI standards[6]. The susceptibility breakpoints were as follows; ciprofloxacin ≤1 µg/ml, levofloxacin ≤ 2 µg/ml.

Serial passages

Serial passage method in which bacteria are grown in the continuous presence of a drug concentration corresponding to half of the MIC was applied[7,8]. MICs were detected by the macro dilution method [6]. *E.coli* strains were grown in Mueller-Hinton broth at 37°C for 24 hours. Then, 5×10^7 cfu/ml of each strain was inoculated into eight series of tubes, containing 2 ml broth with different antibiotic concentrations for each of the two agents. After incubation, an inoculum from the tube nearest the MIC (1/2 x MIC), which had the same turbudity as the antibiotic free control was determined as the subinhibitory concentration. After defining the first MICs, each strain was incubated at 37°C for 24 hours in its own subinhibitory concentrations then, on the second day, transferred from the subinhibitory concentrations to antibiotic free Mueller Hinton broth and incubated at 37°C for one night. The strains were examined and their MICs were determined after every eight successive passages to the subinhibitory and antibiotic free Mueller-Hinton broth, respectively, for a change in MIC. In this manner, the second MICs of the strains were determined. The third MICs were determined by repeating the same process after a total of 16 passages (first and second round total). After each round, *E.coli* strains were inoculated in 15-20% glicerol and stored at -80°C. All strains were inoculated in tubes at the same time and under the same conditions in antibiotic free Mueller-Hinton broth as a control, and after 16 passages the MICs' of bacteria were determined. Assays were performed in replicates of three.

Statistical analyses

The Wilcoxon test was used to compare the observed difference in ciprofloxacin and levofloxacin resistance development ($p<0.05$ was considered statitically significant).

RESULTS

The MIC value increases of ciprofloxacin were observed in all *E.coli* strains that were exposed to subinhibitory concentrations of agents and antibiotic free Mueller-Hinton broth with 16 serial passages (rising from 0.25-0.0078 to 2 - 0.0312 µg/ml). For levofloxacin, in all except three *E.coli* strains, MIC values increased from 0.5-0.0078 to 4 - 0.0156 µg/ml after 16 serial passages. The second and third MICs and increase rates are summarized in Table 1. All strains were inoculated in tubes at the same time and under the same conditions in antibiotic free Mueller-Hinton broth as a control and the MIC values of bacteria had not changed.

Data were analyzed statistically by the Wilcoxon test. It was found that there was a statistically meaningful increase in second MIC values for ciprofloxacin and levofloxacin (p=0,026). However, from the second MIC to third MIC there was no statistically meaningful difference in the increase of MIC values between ciprofloxacin and levofloxacin (p=0.567). The increases of MIC values of ciprofloxacin, from the initial MICs to third MICs, were found to be higher than levofloxacin (p=0,024).

DISCUSSION

The infection location is important when selecting antibiotics, dosages and the necessary route of administration. Adequate concentrations of the drug should reach the infection location for effective antimicrobial treatment. In most cases, this means that the concentration of antibiotics in the related area equals or exceeds the MIC value (2-4 fold) [9]. The use of inadequate doses of antibiotics might cause them to be below the MIC in the infected area, which is important in ascertaining whether the bacteria developed resistance during the course of the treatment. In daily life, the reasons for low serum concentrations of antibiotics can be due to skipping doses, insufficient absorption of the drug from the intestine, ingestion of antibiotics found in animal meats, etc. In our study, *E.coli* strains were not constantly exposed to subinhibitory concentrations; the first day had subinhibitory concentrations and the second day had antibiotic free Mueller-Hinton broth, to represent this situation. In this way, with sixteen sequential

Table 1. Results for 50 *E.coli* strains with reduced susceptibility to ciprofloxacin and levofloxacin

S. No	Initial MIC value (µg/ml)		2nd MIc (µg/ml)		3rd MIC (µg/ml)		Total increase in folds	Total increase in folds
	CIP	LEV	CIP	LEV	CIP	LEV	CIP	LEV
1	0.0078	0.0156	0.0312	0.25	0.0625	1	8	64
2	0.0078	0.0156	0.0625	0.0312	0.25	0.5	32	32
3	0.0078	0.0156	0.0625	0.0312	0.25	0.125	32	8
4	0.0078	0.0156	0.0156	0.0625	0.0312	0.125	4	8
5	0.0156	0.0312	0.125	0.5	0.5	4	32	128
6	0.0078	0.0156	0.0625	0.0312	0.125	0.125	16	8
7	0.0156	0.0312	0.0625	0.125	0.5	0.5	32	16
8	0.0156	0.0625	0.0156	0125	0.125	0.5	8	8
9	0.0156	0.0156	0.0312	0.0312	0.125	0.0625	8	4
10	0.0078	0.0078	0.0078	0.0312	0.0312	0.125	4	16
11	0.0156	0.0312	0.125	0.25	0.25	2	16	64
12	0.0156	0.0312	0.5	0.25	1	1	64	32
13	0.0078	0.0156	0.0625	0.125	0.125	0.25	16	16
14	0.25	0.5	0.5	1	2	4	8	8
15	0.0078	0.0312	0.0312	0.0625	0.0625	0.125	8	4
16	0.0078	0.0312	0.0312	0.125	0.0625	0.25	8	8
17	0.0078	0.0156	0.0625	0.0312	0.25	0.0625	32	4
18	0.0039	0.0156	0.0078	0.125	0.0625	0.25	16	16
19	0.0625	0.125	0.125	0.25	0.125	0.5	2	4
20	0.0156	0.0312	0.125	0.125	0.25	0.25	16	8
21	0.0078	0.0156	0.0312	0.0312	0.0625	0.0625	8	4
22	0.0156	0.0312	0.0625	0.125	0.125	0.25	8	64
23	0.0156	0.125	0.125	0.25	0.5	0.5	32	4
24	0.0078	0.0312	0.0156	0.0625	0.0312	0.125	4	16
25	0.0039	0.0156	0.0312	0.125	0.125	0.5	32	16
26	0.0078	0.0312	0.0312	0.0625	0.125	0.125	16	4
27	0.0156	0.0156	0.0625	0.0625	0.25	0.125	16	8
28	0.0156	0.0312	0.0312	0.125	0.125	0.25	8	8
29	0.0039	0.0156	0.0078	0.0312	0.0625	0.125	16	8
30	0.25	0.5	0.25	1	0.5	2	2	4
31	0.0078	0.0156	0.0156	0.0156	0.125	0.0625	16	4
32	0.0078	0.0156	0.0312	0.0156	0.0312	0.0625	4	4
33	0.0078	0.0312	0.125	0.25	0.25	1	32	32
34	0.0078	0.0312	0.0312	0.25	0.0625	1	8	32
35	0.0078	0.0156	0.0625	0.0156	0.25	0.0625	32	4
36	0.0156	0.0312	0.0312	0.0625	0.125	0.125	8	4
37	0.0156	0.0312	0.25	0.125	0.25	0.25	16	8
38	0.0078	0.0312	0.0312	0.0625	0.0625	0.125	8	4
39	0.0078	0.0312	0.0625	0.0625	0.0625	0.125	8	4
40	0.125	0.25	0.125	0.5	0.25	1	2	4
41	0.0078	0.0156	0.0156	0.0625	0.0156	0.0625	2	4
42	0.25	0.5	0.25	1	0.5	1	2	2
43	0.0078	0.0156	0.0312	0.0625	0.0312	0.0625	4	4
44	0.0078	0.0156	0.0625	0.0156	0.125	0.125	16	8
45	0.0156	0.0312	0.125	0.125	0.5	0.25	32	8
46	0.0078	0.0156	0.0156	0.0156	0.0312	0.0156	4	0
47	0.0078	0.0156	0.0312	0.0156	0.0625	0.0156	8	0
48	0.0156	0.0156	0.0625	0.0312	0.0625	0.0625	4	4
49	0.0078	0.0156	0.0312	0.0156	0.125	0.0156	16	0
50	0.0156	0.0312	0.125	0.125	0.125	0.25	8	8

CIP: ciprofloxacin LEV: levofloxacin

passages, we investigated the development of resistance to subinhibitory concentrations of two agents.

In this study, we analyzed the resistance development of *E.coli* strains in subinhibitory concentrations. The MIC values of all *E.coli* isolates except three were increased. However, since the strains with MIC values of 0.5-0.0078 µg/ml were selected in our study, only two strains exceeded the resistance limit. We observed an 8 fold increase in the MIC value in one of these strains and 128 fold MIC value increase in the other. As for ciprofloxacin, a 64 fold increase was observed in one strain and a 32 fold increase was observed in ten strains. In addition, one strain became resistant by showing an eight fold increase in MIC value for both ciprofloxacin and levofloxacin. Based on these findings, it can be suggested that moderately sensitive and limitedly sensitive strains might develop resistance when they are exposed to lower concentrations than MIC.

Although there are studies in the literature investigating the effects of subinhibitory concentration of fluoroquinolones to some properties (hemagglutination, production of verotoxin) of *E.coli* strains[10,11], the literature contains only a limited number of studies releated with resistance in subinhibitory concentration of *E.coli* . This present study results are similar to results of the other studies. Both in this study and other studies on different microorganisms and methods revealed that subinhibitory concentrations of antibiotics caused increase of MIC values[1,12-15]. In a study[13] which used fluoroquinolones, five fluoroquinolones (ciprofloxacin, levofloxacin, grepafloxacin, sparfloxacin and travofloxacin) and amoxicillin clavulanate were used. Sequential passages were performed for ciprofloxacin with ten *Streptococcus pneumoniae* strains with a MIC value of 0.5–4 µg/ml in subinhibitory concentrations and the MIC values were observed to increase in all strains.

The study[14] which analyzed the development of resistance in methicillin-resistant *Staphylococcus aureus* strains in subinhibitory concentrations of fluoroquinolones (ciprofloxacin, gemifloxacin, sparfloxacin and travofloxacin) in a maximum of 50 sequential passages followed by a 10-day passage in antibiotic-free broth. The researchers reported that first MIC values of *S.aureus* strains were 0,016-0,063 mg/ml, however they increased to 2-64 mg/ml after 5-26 passages and that the strain developed resistance.

Similarly, in a study[15] that used fluoroquinolones, 10 sequential passages followed by 10-day passage in antibiotic-free broth were applied to *Streptococcus pneumoniae* strains with reduced sensitivity to ciprofloxacin. The results indicated that the MIC values were observed to increase.

As a result, the identification of varying degrees of decreasing sensitivity in different microorganisms which were exposed to subinhibitory concentrations of fluoroquinolones suggest that exposure to low concentrations in in-vivo conditions might result in the development of the loss of susceptibility or resistance and thus treatment failure especially in long term treatment as borne and joint infections. All related studies might be considered to indicate that for an effective use of antibiotics in the treatment of infectious diseases over a number of years, it is important to use them in appropriate doses.

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