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Original article

DETECTION OF THE EFFLUX PUMP-MEDIATED QUINOLONE RESISTANCE IN ESBL POSITIVE *ESCHERICHIA COLI* AND *KLEBSIELLA PNEUMONIAE* ISOLATES BY PHE-ARG-BETA-NAPHTHYLAMIDE

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Abstract

In Extended Spectrum Beta-Lactamase (ESBL) producing Escherichia coli and Klebsiella pneumoniae strains, fluoroquinolone resistance is acquired mostly by target mutations in topoisomerase genes and increased expression of efflux pumps. ESBL positive 65 E. coli and 48 K. pneumoniae strains isolated in Department of Microbiology and Clinical Microbiology, Faculty of Medicine, Ege University were investigated in this study. 58 E. coli and 21 K. pneumoniae isolates displayed reduced susceptibility (intermediately-resistant or resistant) to ciprofloxacin or nalidixic acid by disc diffussion method. Ciprofloxacin, Phenyl-arginine-beta-naphthylamide (Phe-Arg-β-naphthylamide-PβNA) and nalidixic acid MIC values of these isolates were determined. The changes in nalidixic acid and ciprofloxacin MIC values in the presence of fixed concentration of PβNA (20 µg/mL) were investigated. Among 21 K.pneumoniae isolates, at least four fold reductions were observed in MIC values of ciprofloxacin, nalidixic acid and both for four, five and two isolates respectively. Among 58 E. coli isolates, at least four fold reductions were observed in MIC values of ciprofloxacin, nalidixic acid and both for eight, four and three isolates respectively. This result was evaluated to be indicative for presence of efflux pump mediated resistance in these isolates.

Key words: *Phe-Arg-β-naphthylamide-PβNA, Efflux, Quinolone-resistance, E.coli, K.pneumoniae.*

GSBL Pozitif *Escherichia coli* ve *Klebsiella pneumoniae* İzolatlarında Efluks Pompası Aracılığı ile Gelişen Florokinolon Direncinin Fenil Arginin Beta Naftilamit ile Araştırılması

Genişlemiş Spektrumlu Beta-Laktamaz (GSBL) sentezleyen Escherichia coli ve Klebsiella pneumoniae izolatlarında florokinolon direnci genellikle topoizomeraz genlerindeki nokta mutasyonlar ve efluks pompalarının artan ekspresyonu sonucu ortaya çıkar. Bu çalışmada Ege Üniversitesi Tıp Fakültesi, Mikrobiyoloji ve Klinik Mikrobiyoloji Anabilim Dalı'nda izole edilen GSBL pozitif 65 E.coli ile 48 K. pneumoniae izolatı incelendi. 58 E. coli ve 21 K. pneumoniae izolatının siprofloksasin veya nalidiksik aside azalmış duyarlılık sergilediği (dirençli veya orta duyarlı) disk difüzyon yöntemi ile saptandı. Bu izolatların siprofloksasin, nalidiksik asit ve Phenyl-arginine-beta-naphthylamide (Phe-Arg-β-naphthylamide-PβNA) MİK değerleri belirlendi. Sabit konsantrasyondaki (20 µg/mL) PβNA'nın, siprofloksasin ve nalidiksik asit MİK değerlerinde oluşturduğu değişim araştırıldı. 21 K. pneumoniae izolatının 4 tanesinde sadece siprofloksasin, 5 tanesinde sadece nalidiksik asit, 2 tanesinde hem nalidiksik asit hem de siprofloksasin; 58 E. coli izolatının 8 tanesinde sadece siprofloksasin, 4 tanesinde sadece nalidiksik asit, 3 tanesinde hem nalidiksik asit hem de siprofloksasin MİK değerlerinde en az 4 katlık azalma gözlendi. Bu sonuçlar, sözü edilen izolatlarda efluks pompası aracılığı ile gelişen direncin göstergesi olarak değerlendirildi.

Anahtar kelimeler: *Fenil Arginin Beta Naftilamit-PβNA, Efluks, Kinolon direnci, E.coli, K.pneumoniae.*

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INTRODUCTION

Gram-negative bacteria are one of the important causes of both hospital and community acquired infections. Extended-Spectrum Beta-Lactamase (ESBL) producing *Escherichia coli* and *Klebsiella pneumoniae* strains are among the most commonly isolated pathogens from hospital acquired infections. The main feature of ESBLs is that they inactivate narrow and broad spectrum cephalosporins, penicillins and monobactams. The first ESBLs are mutant enzymes derived from narrow spectrum SHV-1 and TEM-1 beta-lactamases. The fact that the genes encoding ESBLs can be mobilized increases their prevalence among Gram-negative pathogens (1, 2).

Following carbapenems, fluoroquinolones are the second choice of treatment in infections originating from ESBL-producing Gram-negative bacilli (3). However, the prevalence of quinolone resistance tends to increase in ESBL-producing strains. The rate of quinolone resistance changes between 10 and 40% in these strains (4-6).

The resistance against the quinolone group of antibiotics complicates the treatment of infections originating from ESBL producing *Enterobacteriaceae* strains. Thus, it is important to elucidate the mechanisms of the resistance that developed against quinolone antibiotics. Chromosomal mutations and efflux pump over-expression are mechanisms that frequently encountered in quinolone resistance. Plasmids can also carry genes that play role in quinolone resistance. Efflux pumps have a significant role in the multipl antibiotic resistance of microorganisms. The excessive production of RND (resistance-nodulation-division) type of efflux pump systems causes quinolone resistance, along with beta-lactam, tetracycline, chloramphenicol and trimethoprim resistance (7-9). Additionally, high resistance to fluoroquinolones in *E. coli* isolates involves excessively increased production of the AcrAB membrane efflux pump.

In Gram-negative bacteria, the efflux pump inhibitors act through inhibiting main pump systems such as AcrAB-TolC and MexAB-OprM which cause multi-drug resistance. In case of bacteria with multi-drug resistance involving efflux pump systems, efflux pump inhibitors themselves do not exhibit an antibiotic effect at concentrations that alter antibiotic effectiveness. Phenyl-arginine-beta-naphthylamide (Phe-Arg-β-naphthylamide -PβNA), which inhibits the extrusion of the fluoroquinolones via efflux pump systems in Gram-negative bacteria, is one of the first peptidomimetics. PβNA is a substrat of efflux pump systems of *Pseudomonas aeruginosa, E. coli, Enterobacter aerogenes, K. pneumoniae, Acinetobacter baumannii* and *Camphylobacter jejuni.* As a result of the competition between PβNA and the antibiotic, PβNA is extruded outside by the efflux pump and the antibiotic could reach effective concentration inside the cell (10).

In the present study, it was aimed to investigate the efflux pump-mediated quinolone resistance of ESBL-positive *E. coli* and *K. pneumoniae* strains isolated from various clinical samples.

EXPERIMENTAL

ESBL-positive 65 *E. coli* and 48 *K. pneumoniae* strains isolated from various clinical samples in Bacteriology Laboratory of Department of Microbiology and Clinical Microbiology, Faculty of Medicine, Ege University, were included in the study. Detection of ESBL producing isolates had been done by Vitek-2 (Biomerieux). *E. coli* ATCC 25922 was used as the reference strain for all tests.

Investigation of ciprofloxacin and nalidixic acid resistance by the disc diffusion method

The bacteria stored at -80^{0}C in Brain Heart Infusion broths containing 10% glycerin were regenareted and the sensitivities of the isolates against ciprofloxacin (Oxoid) and nalidixic acid

(Oxoid) were determined according to Kirby-Bauer disc diffusion method Following the overnight incubation at 35^{0}C, antibiotic susceptibilities of the isolates were evaluated according to the Clinical and Laboratory Standards Institute (CLSI) criteria (11).

Determination of the MIC values of ciprofloxacin, nalidixic acid and PβNA by the microdilution method

The minimum inhibitory concentrations (MICs) of 58 *E. coli* and 21 *K. pneumoniae* isolates, which were found to be resistant or moderately sensitive to ciprofloxacin and nalidixic acid, were determined by the microdilution method, according to the CLSI (formerly NCCLS) recommendations (12). The final concentrations in the wells were adjusted between 1024-0.5 µg/mL for ciprofloxacin (Koçak) and PβNA (Sigma) and between 4096-2 µg/mL for nalidixic acid (Sigma). The lowest antibiotic concentration that inhibits the growth considered as the MIC value.

Detection of the efflux pump-mediated quinolone resistance

50 µL of Mueller Hinton broth was added to the wells of a sterile microdilution plate. By adding 50 µL of suitable consantrations of ciprofloxacin and nalidixic acid to the first line of wells, serial dilutions were performed. 20 µg/mL constant PβNA concentration was obtained by adding 40 µL of the bacterial suspension and 10 µL of the stock PβNA (200 µg/ml) solution to each well. At least four fold decrease in the MIC values of ciprofloxacin and nalidixic acid was evaluated as the presence of the efflux pump.

RESULTS

It was determined that 58 (89.23%) *E. coli* and 21 (43.75%) *K. pneumoniae* isolates were resistant or intermediate susceptible to ciprofloxacin and/or nalidixic acid (Table 1).

Table 1. Antibiotic resistance rates of *E. coli* and *K. pneumoniae* isolates by disc diffusion method.

Strians	Nalidixic acid			Ciprofloxacin		
	R[a] (%)	I[b] (%)	S[c] (%)	R (%)	I (%)	S (%)
E. coli	89.23	0	10.77	84.62	0	15.38
K. pneumoniae	41.67	2.08	56.25	29.17	4.17	66.66

[a]R: Resistant, [b]I: Intermediate Susceptible, [c]S: Susceptible

In these isolates, the effect of the 20 µg/mL constant concentration of PβNA on the MIC values of ciprofloxacin and nalidixic acid was investigated. Reduction of ciprofloxacin and/or nalidixic acid MIC values of 52 (89,7 %) out of 58 *E. coli* isolates was observed in the presence of PBNA. Of the 58 *E. coli* isolates included in the study, at least four-fold decrease in MIC values was observed in eight, four and three isolates for ciprofloxacin only, nalidixic acid only and both antibiotics, respectively (totally, 15 isolates). Six out of 58 *E. coli* isolates showed no change in ciprofloxacin and/or nalidixic acid MIC values in the presence of PBNA (Table 2).

Table 2. Effect of PβNA on ciprofloxacin and nalidixic acid MIC values of *E.coli* isolates.

			MIC(µg/ml)		
Strain No	[a]PβNA	[b]CIP	CIP+ PβNA	[c]NA	NA+ PβNA
1	512	64	64	4096	2048
2	512	256	256	4096	2048
3	256	128	128	4096	2048
4	256	128	128	4096	2048
5	512	128	128	4096	2048
6#	256	256	256	2048	2048
7	512	256	128	4096	2048
8•	1024	64	2	2048	16
9*	1024	1024	256	2048	2048
10°	1024	512	256	4096	128
11°	1024	256	128	4096	512
12•	512	512	128	4096	1024
13	512	256	128	4096	2048
14	512	128	128	4096	2048
15	512	128	128	4096	2048
16	512	128	128	4096	2048
17°	512	<0.5	<0.5	2048	32
18	512	128	128	4096	2048
19*	512	512	128	4096	2048
20#	512	256	256	4096	4096
21*	512	256	64	4096	4096
22*	>1024	256	32	4096	4096
23	512	1024	512	4096	2048
24	512	256	128	4096	2048
25	512	256	128	4096	2048
26	512	16	16	4096	2048
27	512	128	128	4096	2048
28	256	128	128	4096	2048
29	512	32	32	4096	2048
30*	1024	512	128	2048	2048
31*	512	>1024	128	4096	2048
32	512	128	128	4096	2048
33*	1024	512	128	2048	2048
34	1024	256	128	4096	2048
35	1024	128	128	4096	2048
36	512	128	128	4096	2048
37	512	8	8	4096	2048
38	512	256	128	4096	2048
39#	512	256	256	2048	2048
40	>1024	64	64	4096	2048
41	>1024	128	128	4096	2048
42°	>1024	1	0.5	4096	16
43	>1024	512	512	4096	2048
44#	512	64	64	2048	2048
45	512	128	128	4096	2048
46	512	128	128	4096	2048
47	1024	256	128	4096	2048
48	1024	256	128	4096	2048
49	1024	128	128	4096	2048
50	512	128	128	4096	2048
51	512	128	256	4096	2048
52	256	128	64	4096	2048
53*	128	256	64	4096	2048
54	128	16	16	4096	2048
55#	128	128	128	2048	2048
56	128	128	128	4096	2048
57#	128	256	256	<2	<2
58•	512	256	64	2048	256

[a] PβNA: Phe-Arg-β-naphthylamide, [b] CIP: Ciprofloxacin, [c] NA: Nalidixic acid
* Isolates that have 4-fold or more reduction in the CIP MIC values
° Isolates that have 4-fold or more reduction in the NA MIC values
• Isolates that have 4-fold or more reduction in the CIP and NA MIC values
Isolates that show no change in the CIP and NA MIC values

On the other hand, reduction of ciprofloxacin and/or nalidixic acid MIC values of 16 (76,2 %) out of 21 *K.pneumoniae* isolates was observed in the presence of PBNA. Of the 21 *K. pneumoniae* isolates included in the study, an at least four-fold decrease in MIC values was observed in four, five and two isolates for ciprofloxacin only, nalidixic acid only and both ciprofloxacin and nalidixic acid, respectively (totally, 11 isolates). Five out of 21 *K.pneumoniae* isolates showed no change in ciprofloxacin and/or nalidixic acid MIC values in the presence of PBNA (Table 3).

Table 3. Effect of PβNA on ciprofloxacin and nalidixic acid MIC values of *K.pneumoniae* isolates.

			MIC(µg/mL)		
Strain No	PβNA	CIP	CIP+ PβNA	NA	NA+ PβNA
1#	1024	512	512	2048	2048
2°	>1024	8	4	256	8
3	>1024	512	512	4096	2048
4	1024	256	128	4096	2048
5•	>1024	8	2	4096	32
6°	>1024	2	2	2048	32
7#	>1024	32	32	4096	4096
8*	1024	4	1	4096	4096
9°	>1024	<0.5	<0.5	4096	64
10	256	64	32	4096	2048
11•	>1024	8	2	2048	256
12#	1024	128	128	4096	4096
13	>1024	256	128	4096	4096
14°	512	512	256	4096	1024
15°	>1024	1	<0.5	1024	128
16*	>1024	8	2	2048	2048
17#	1024	<0.5	<0.5	1024	1024
18#	1024	128	128	2048	2048
19*	>1024	1024	128	4096	2048
20*	1024	16	0.5	2048	2048
21	>1024	128	64	4096	2048

[a] PβNA: Phe-Arg-β-naphthylamide, [b] CIP: Ciprofloxacin, [c] NA: Nalidixic acid
* Isolates that have 4-fold or more reduction in the CIP MIC values
° Isolates that have 4-fold or more reduction in the NA MIC values
• Isolates that have 4-fold or more reduction in the CIP and NA MIC values
Isolates that show no change in the CIP and NA MIC values

DISCUSSION

Quinolone resistance mediated by the efflux pump is one of the most frequently encountered resistance mechanisms. The efflux pump system causes multi-antibiotic resistance in *Enterobacteriaceae* members. Although it has been determined that the resistance due to efflux pump systems are regulated by 38 genes or operons in bacteria, only 19 of them could be shown to be directly involved with the development of antibiotic resistance (13, 14). It has been shown in the previous studies that the efflux-mediated resistance of the bacteria is activated more efficiently after the exposure of the bacteria to antibiotics. The increase in the penetration of the fluoroquinolone into the bacterial cell may result in over-expression of the efflux pump. The developing resistance is low, however, one mutation can trigger another and as a result, after the regulatory gene mutation, the level of resistance may be increased exponentially by other mutations such as changes in target molecule and decreases in permeability (15). There are

many studies showing via phenotypic and genotypic methods that quinolone resistance is mediated by the efflux pump. One of the most widely used phenotypic methods to show the presence of efflux pumps is evaluation of the changes in the MIC levels of antibiotics by using the molecules known to inhibit the efflux pumps.

Escherichia coli AcrAB operon encode a stress-induced efflux system. In the clinical isolates of *E. coli* that demonstrate high resistance to fluoroquinolones, in addition to mutations in *gyrA* and *parC* genes, overproduction of AcrAB is also observed. In a study by Mazzariol et al. (16), it was determined that a high level of AcrAB was synthesized in 9 of the 10 *E. coli* strains with high ciprofloxacin resistance (MIC ≥32 μg/ml) but it was not synthesized at all in the 15 *E. coli* strains with low ciprofloxacin resistance (MIC <1 μg/ml). In this study, changes in ciprofloxacin MIC values were determined in the presence of PβNA, an AcrAB inhibitor.

In our study, the effect of the 20 μg/mLconstant concentration of PβNA on ciprofloxacin and nalidixic acid MIC values was investigated in 58 *E. coli* and 21 *K. pneumoniae* strains resistant to ciprofloxacin and/or nalidixic acid. An at least four-fold decrease was observed in 11 of the 21 *K. pneumoniae* and 15 of the 58 *E. coli* strains. Six out of 58 *E. coli* isolates and five out of 21 *K.pneumoniae* isolates showed no change in ciprofloxacin and/or nalidixic acid MIC values in the presence of PBNA. Target enzyme mutations and plasmid-mediated quinolone resistance mechanisms could have played role in these isolates.

In a study conducted by Saenz et al. (17) the effect of different concentrations of PβNA (20, 40, 80 or 160 μg/ml) on nalidixic acid, ciprofloxacin and norfloxacin MICs was studied in 25 *E. coli* isolates. They determined that the influence of PβNA was homogeneous in the range of 20 to 160 μg/ml in all the isolates tested. In our country, few studies concerning this subject have been performed. In a study of Coban et al. in 2004, MIC values of ciprofloxacin were decreased two folds in six isolates and four folds in two isolates out of 14 clinical *E. coli* isolates, in the presence of 20 μg/ml PβNA (18). In light of these findings, PβNA was used at a concentration of 20 μg/mLin the present study.

In the study of Hasdemir et al. (19) conducted in 2004, active efflux and porin changes were investigated in *K. pneumoniae* strains demonstrating multi-antibiotic resistance and presence of PβNA sensitive efflux pump was determined in 39% of the strains that most of them were ESBL-positive. In the study, PβNA was determined to reduce the MIC values of quinolone, chloramphenicol and tetracycline by fivefold for at least one of these antibiotic classes. In our study, ESBL-producing strains were included due to their high efflux-pump mediated resistance and this resistance was detected in 22.9% of the *K. pneumoniae* strains.

Saenz et al. (17) investigated the effect of PβNA on the MIC values of nalidixic acid, fluoroquinolone (norfloxacin, ciprofloxacin), tetracycline and chloramphenicol. They determined a decrease in nalidixic acid MIC values of all *E. coli* isolates sensitive and resistant to nalidixic acid in the presence of PβNA and varying levels of effect in fluoroquinolone MIC values. Authors were concluded that this inhibitory molecule could have affected two or more efflux systems in the study strains. As for our study, in the presence of PβNA, a decrease was detected in the nalidixic acid and ciprofloxacin MIC values of the majority of the *E. coli* strains, which were found to be intermediate susceptible and resistant to nalidixic acid.

Tohidpour et al. (20) investigated the prevalence of efflux pump over-expression in *P. aeruginosa* isolates and showed that PβNA causes a decrease in ciprofloxacin MIC values. Efflux pump over-expression was detected in 35% of the strains demonstrating only ciprofloxacin resistance whereas this rate tended to decrease in case of cross resistance to antibiotics such as gentamicin (31%), ceftazidime (29%) and imipenem (18%). This result indicated that PβNA is an effective agent to ensure fluoroquinolone susceptibility and the authors concluded that the increase in the use of ciprofloxacin can affect the sensitivity of other antipseudomonal antibiotics.

CONCLUSION

The mechanisms causing multi-drug resistance in Gram negatives are intensively investigated through out the world and in our country. The presence of quinolone resistance in addition to ESBL-production in Gram-negative bacteria complicates the treatment of infections caused by these strains. Besides to their role in quinolone resistance, efflux pumps have a crucial role in the development of multi-antibiotic resistance. In the present study, the presence of the efflux pump-mediated resistance to the quinolone group of antibiotics was detected phenotypically in a considerable number of the ESBL-positive *E. coli* and *K. pneumoniae* strains isolated from various clinical samples. Determining the role of efflux pumps in antibiotic resistance will greatly contribute to limiting the resistance development. In addition to be an indicator of efflux pumps, the molecules that inhibit many efflux pump systems, such as PβNA, could be an important step for the production of antibiotic/efflux pump inhibitor combinations similar to beta-lactam/beta-lactamase combinations.

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