PLASMID COPY NUMBER AND *QNR* GENE EXPRESSION IN SELECTION OF FLUOROQUINOLONE-RESISTANT *ESCHERICHIA COLI*

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Fluoroquinolone resistance in Enterobacteriales is developed by chromosomal and plasmid-mediated mechanisms. Plasmids play an important role in dissemination of resistant genes and they carry genes that protect bacteria in different stress-induced situations. In this study, we studied *Escherichia coli* strains, each carried one plasmid-mediated quinolone resistance determinant namely, *qnrA1, qnrB1, qnrC1*, and *qnrD1*. We exposed 0.5 McFarland density of each strain to 0.5 mg/L ciprofloxacin from the period of 30, 60, 90, and 120 min over 24 h. All treated strains were further exposed to a constantly increasing 1, 2, 4, and 8 mg/L ciprofloxacin solution through 24, 48, and 120 h. In given timepoints, RNA was extracted from all treated strains. Expression of *qnrA1, qnrB1, qnrC1*, and *qnrD1* was investigated by quantitative PCR. Mutations in *gyrA* and *parC* genes were analyzed by PCR and nucleic acid sequencing. In this study, during 0.5 mg/L ciprofloxacin exposition, the following expression levels were detected: 1.2 for *qnrA1*, 1.47 for *qnrD1*, 12.44 for *qnrC1*, and 80.63 for *qnrB1*. In case of long-term study, we selected a resistant strain in *qnrB1*-positive *E. coli*, and its expression increased from 105.91 to 212.31. On the contrary, plasmid copy number increased in time from 1 to 4.13. No mutations in *gyrA* or in *parC* chromosomal genes of treated strains were detected. Our results show that *qnrB1*-positive *E. coli* strain was able to develop fluoroquinolone resistance by upregulated *qnrB1* expression that was linked to a minor increase in plasmid copy number but no mutations occurred in *gyrA* or *parC*.

Keywords: fluoroquinolone resistance, *qnr* determinants, *qnr* gene expression

Introduction

Fluoroquinolones are broad-spectrum antibiotics with bactericidal effect on Gram-negative and Gram-positive bacteria [1]. They bind to bacterial gyrase and

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topoisomerase IV enzymes, thereby inhibit DNA synthesis. Fluoroquinolones have a good penetration in various human tissues; therefore, they are used as first-line therapy of complicated urinary tract infections (UTIs) [2] and community-acquired pneumonia [3]. Furthermore, tuberculosis and infections of abdominal cavity, skin and soft tissue, bone, and joint can also be treated with fluoroquinolones [4].

Fluoroquinolone-resistant strains show a raising tendency worldwide and it causes new challenges for effective treatment. According to data of *Nature Reviews Urology*, global prevalence rate in Gram-negative bacteria demonstrated 75% in China, India, and Central America, whereas in Hungary, proportion of these resistant bacteria is about 15%–20% [5]. Based on ECDC database, fluoroquinolone resistance in *Escherichia coli* and *Klebsiella pneumoniae* is about 30%–40% [6].

Fluoroquinolone resistance is caused by chromosomal and plasmid-mediated mechanisms. High-level fluoroquinolone resistance with minimum inhibitory concentration (MIC) of 1 mg/L or higher values is developed by chromosomal mutations in quinolone-resistance determining regions (QRDRs), namely in gyrase enzyme coding *gyrA* and *gyrB* genes and in topoisomerase IV coding *parC* and *parE* genes. Plasmid-mediated quinolone resistance (PMQR) confers reduced susceptibility and low-level fluoroquinolone resistance [7, 8]. Plasmids are double-stranded, superhelical extrachromosomal DNA molecules with variable size from 1,000 to 100,000 base pairs. Plasmids are not essential elements for bacteria; therefore, microbes do not carry and express plasmid-coded genes under non-stressed conditions, because it would cause an unnecessary energy burden [9, 10]. Plasmids can replicate autonomously from the cell cycle's replication phase, but they consume ATP and ezymes of host cell. Plasmid copy number is an amount of plasmid DNA compared to that of housekeeping genes [10]. Plasmids carry various genes [11–13], and the most important of them are antibiotic resistance genes. Expression of these genes can protect bacteria in stress-induced conditions, e.g., in an environment containing antibiotic. PMQRs include three groups of determinants: Qnr-protective proteins, enzymatic modification of bifunctional aminoglycoside acetyltransferase-Ib-cr [ACC(6′)-Ib-cr], and efflux pumps (QepA and OqxAB) [14]. Each PMQR determinant is able to maintain 0.125–0.5 mg/L ciprofloxacin MIC value and facilitate selection of fluoroquinolone resistance, as frequency of chromosomal mutations will be increased [7, 8]. The first PMQR gene, *qnrA1*, was detected in 1998 in *K. pneumoniae* [7]. Since then, several determinants were described and have been identified worldwide in *Enterobacteriaceae*, mainly in *K. pneumoniae, Enterobacter* spp., *E. coli*, and *Salmonella enterica* both in community- and nosocomial-acquired infections [15]. PMQR genes are associated with other resistance determinants in

Enterobacteriaceae, often with extended-spectrum beta-lactamases (ESBLs) [16]. Conjugative plasmid that carry *qnrB19, bla*$_{KPC-3}$, *bla*$_{SHV-11}$, *bla*$_{TEM-1}$, and *aac(6′)-Ib* was detected in *K. pneumoniae* [17]. Furthermore, *armA, qnrS1, aac(6′)-Ib-cr, bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$, and *bla*$_{NDM-1}$ were also detected on transferable plasmid in *K. pneumoniae* [18]. In *E. coli, qepA, armA*, and *bla*$_{TEM-1}$ resistance determinants were found on a single conjugative plasmid [19].

The global prevalence of *qnr* determinants and *aac(6′)-Ib-cr* ranges from 0.2% to 94% depending on studied strains [19–21]. In Northwest Iran, prevalence of PMQRs in *Enterobacteriaceae* isolated from UTIs showed a very high prevalence about 89.1% and among them *aac(6′)-Ib-cr* was the most frequently detected [22]. In Hungary, prevalence of PMQR-positive *Enterobacteriaceae* isolated from urine clinical samples showed 17.7% [23]. In the past 8 years, incidence of PMQRs among ESBL-producing *E. coli* and *K. pneumoniae* from bloodstream infection increased in Hungary [24].

Materials and Methods

Strains

In this study, we included *E. coli* TG1 control strains, where each were transformed by plasmids carrying *qnrA1* (GenBank accession number: AY070235), *qnrB1* (GenBank accession number: DQ351241), *qnrC1* (GenBank accession number: EU917444), and *qnrD1* (GenBank accession number: FJ228229) [25]. The ciprofloxacin MIC of tested strains was 0.5 mg/L. Antimicrobial susceptibility testing was performed according to EUCAST 2016 protocol.

Ciprofloxacin exposure

In our investigation, we performed short-term study as we analyzed time dependence of fluoroquinolone resistance development. Each strain was adjusted to 0.5 McFarland density and was exposed to 0.5 mg/L ciprofloxacin from the period of 30, 60, 90, and 120 min over 24 h. After the strains adapted to ciprofloxacin, we performed long-term study to analyze concentration dependence of fluoroquinolone resistance. The 0.5 McFarland solutions of treated strains were exposed in constantly increasing 1, 2, 4, and 8 mg/L ciprofloxacin solutions through 24, 48, and 120 h. All investigations of ciprofloxacin exposition were performed in Mueller–Hinton broth.

Bacterial RNA extraction

At given timepoints, total RNA of treated strains was extracted by Qiagen RNeasy Mini Kit (Hilden, Germany). Briefly, samples were placed into an Eppendorf tube, and were centrifuged at $5,000 \times g$ over 10 min and supernatants were removed. Tris-EDTA pH 8 buffer containing 20 µl proteinase K and 200 µl lyzozime was added to the pellet of each strain. It was followed by incubation at 15–25 °C and samples were vortexed and RLT buffer was added. An amount of 700 µl from the solution was pipetted to RNeasy Mini spin columns and centrifuged at $8,000 \times g$ for 15 s. An amount of 700 µl RW1 buffer was added and centrifuged at $8,000 \times g$ through 15 s, 500 µl RPE buffer was added and centrifuged at $8,000 \times g$ over 15 s, and this step was repeated. An additional 500 µl RPE-buffer was added and centrifugation at $8,000 \times g$ for 2 min. After the last centrifugation step, the sample was transfered into a new tube, and eluation of RNA was performed into 50 µl RNase-free water by centrifugation at $8,000 \times g$ over 1 min.

RT-real-time PCR

Gene expressions of *qnrA1, qnrB1, qnrC1*, and *qnrD1* and plasmid copy numbers were quantified. Extracted RNA of each tested strain was applied in RT-real-time PCR in a Step One Real-Time PCR System (Applied BioSystems, Thermo Fisher Scientific, Foster City, CA, USA) using PCR protocol: 60 °C for 30 s, 50 °C for 5 min, 95 °C for 10 min, and (95 °C for 15 s and 60 °C for 1 min) $\times$ 40 cycles, and 60 °C for 30 s. Oligonucleotid primers and probes were designed by Primer Express 3.0 software (Applied BioSystems, Thermo Fisher Scientific, Foster City, CA, USA). Each *qnr* determinant and each plasmid backbone were targeted. Chromosomal housekeeping *icd* gene was used as internal control. We normalized each targeted sequence to *icd* gene according to C_T values with formula $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = (C_{Tgene \ of \ interest} - C_{Tinternal \ control})_{studied \ strain} - (C_{Tgene \ of \ interest} - C_{Tinternal \ control})_{control \ strain}$.

Mutations of gyrA *and* parC *genes*

Mutations in *gyrA* and *parC* genes were analyzed by PCR and nucleic acid sequencing. Each treated strain was suspended in 500 µl bidestillated water (Millipore Merk, Darmstadt, Germany) and each was incubated at 100 °C for 15 min, and centrifuged at 13,000 rpm for 10 min at 4 °C. Supernatant was applied as DNA template in PCR, and mixture contained 1 Unit DNA Taq polymerase (Sigma-Aldrich, St. Louise, MO, USA) and 10 pmol from each primer namely, gyrA F-5′-CAG CCC TTC AAT GCT GAT-3′ and gyrA R-5′-CGC TTT TAC

TCC TTT TCT GTT C-3′ and parC F-5′-CTC AAT CAG CGT AAT CGC C-3′ and parC R-5′-AAT CCT CAG CCG ATC TCA C-3′. Oligonucleotid primers of this study were designed by Eurofins Genomics online tools. PCR protocol was programmed as follows: 96 °C for 3 min, (95 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min) for 30 cycles, and it finished 72 and 4 °C for 5 min each as final step. PCR amplicons were purified by Qiagen PCR purification Kit (Hilden, Germany) and samples were sent to be sequenced (BIOMI Kft, Gödöllő). Obtained sequences were analyzed against NCBI GenBank database.

Results

In short-term study, during exposure to 0.5 mg/L ciprofloxacin solution from 30 min to 24 h, we detected the following results: *qnrA1* and *qnrD1* showed 1.2 and 1.47 level expressions, *qnrC1* was 12.44. Compared to these three studied *qnr* determinants, *qnrB1* demonstrated a 3.22–80.63 expression. We also studied copy numbers of plasmids carrying *qnrA1, qnrB1, qnrC1*, and *qnrD1* genes. In *qnrA1* and *qnrD1* plasmids, 1–1.4-folds were detected, in *qnrC1* plasmid, copy number changed from 3.1 to 4.42, while it reached fold change of 4.13 in *qnrB1* plasmid. Data are shown in Figure 1.
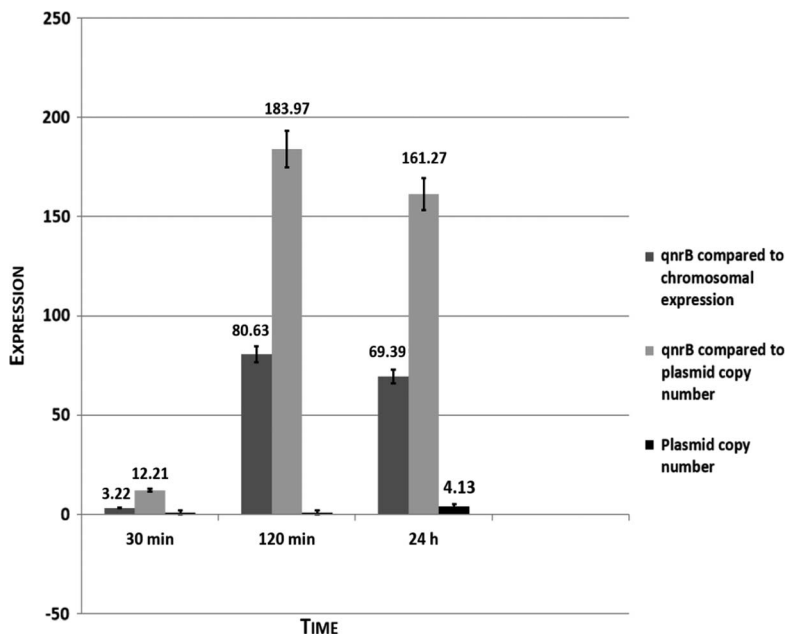

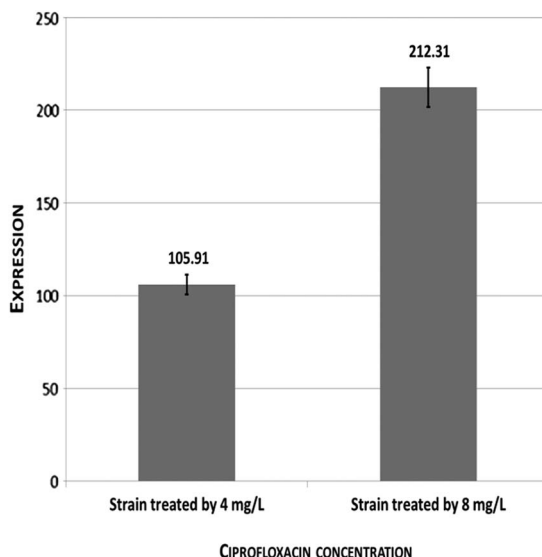**Figure 1.** Results of short-term study in case of *qnrB1*

Figure 2. Results of long-term study in case of *qnrB1*

We implicated the treated strains into long-term study. Treated strains were adjusted to 0.5 McFarland and were kept in constantly increasing 1, 2, 4, and 8 mg/L ciprofloxacin solutions. According to this model, resistant strain was selected in *qnrB1* carrier *E. coli*. In course of long-term study, 105.91 and 212.31 *qnrB1* level expressions were observed in 4 and 8 mg/L, respectively (Figure 2). On the other hand, *qnrB* plasmid copy number reached 4.13-fold increase. In this study, we did not find mutations in QRDRs.

Discussion

The selection of fluoroquinolone resistance in *E. coli* strains was investigated. In the case of *qnrB1*-positive *E. coli*, resistant strain was obtained by 0.5–8 mg/L ciprofloxacin exposure. Plasmid copy number change had minor role in this process, so our results show that selection of resistant strains in *qnrB1*-positive *E. coli* was mainly caused by increase of *qnrB* expression. Furthermore, in this study, no mutations in QRDR were detected. In the case of *qnrA*-, *qnrC*-, and *qnrD*-positive *E. coli*, in this study, these strains could adapt to 0.5 mg/L ciprofloxacin with *qnr* expressions ranging from 1.2 to 12.44, but further selection was not possible.

The importance of detected *qnrB* expression is that this PMQR determinant plays a role in SOS-response regulation system [26, 27]. This response is activated when bacteria gets into a stress-induced condition, such as a DNA damage by UV, oxidative stress, metabolic pH-change or when it senses antibiotic in its environment. Consequently, the SOS-response is triggered by a factor that can be the increasing ciprofloxacin concentration. Importance of SOS-response is to protect bacterial DNA from harmful effects by enhancing mutation frequence and by production of protective proteins. Two regulator proteins play key role in SOS-reponse, namely LexA transcription repressor and RecA coprotease. The interactions between these two proteins help bacteria to survive [28].

The function of these proteins is as follows. Promoter of *qnrB* gene includes CTGT binding site of LexA-protein. If bacteria do not sense any damaging effect, e.g., in ciprofloxacin-free condition, then LexA binds to CTGT region, so synthesis of RNA is blocked, and RecA protein is inactive and expression of *qnrB* takes a basic level (Figure 3). When antibiotic concentration shows an increase, it means a warning signal for bacteria, and RecA coprotease activates (with ssDNA arising) and aids autoproteolysis of LexA transcription repressor. Autoproteolized LexA leaves CTGT region, so RNA synthesis disengages from inhibition and synthesis of QnrB protein will be upregulated. QnrB is a pentapeptide-repeat protein that binds to gyrase through protein–protein interactions and protects it from ciprofloxacin. Consequently, MIC rises and susceptibility decreases (Figure 4) [26–28].
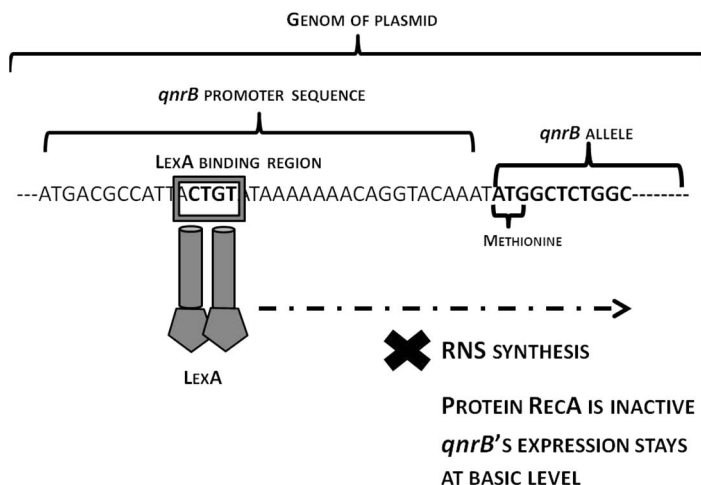

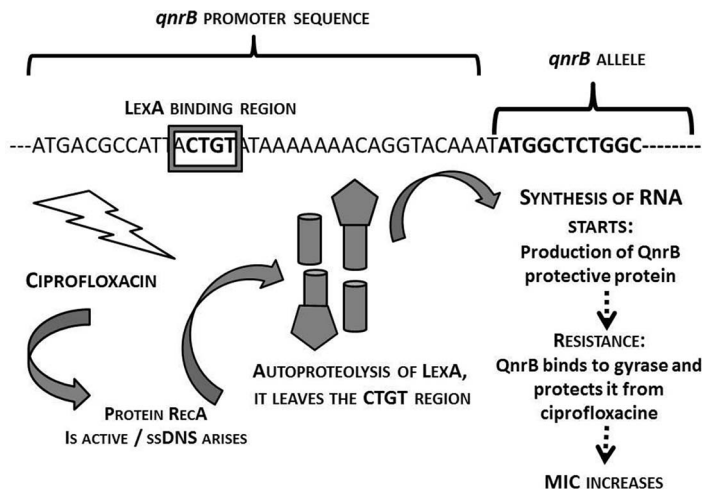**Figure 3.** SOS-response in ciprofloxacin-free environment

Figure 4. SOS-response in an environment with raising ciprofloxacin concentrations

Our studies were performed according to EUCAST protocol from 2016, but this was changed in January 2017. According to this new protocol, ciprofloxacin resistance breakpoint was revised from 1 to 0.5 mg/L [29]. Our results correlate with this change, as we demonstrated that each tested *E. coli* could survive in 0.5 mg/L ciprofloxacin concentration by an increased *qnr* expression.

The clinical relevance of our results is that ciprofloxacin is commonly subscribed to treat UTIs. It is estimated that around 150 million UTI cases are diagnosed each year and about 75% are caused by *E. coli* [22]. Ciprofloxacin concentrations used in this study demonstrate tissue concentrations during a per os therapy [30]. Thus, *E. coli* that carries a *qnr* determinant can develop resistance by increased *qnr* expression during a ciprofloxacin therapy that can lead to therapy failure.

Furthermore, the most common PMQR resistance gene is *qnrB* in the world and it has the highest number of variants (almost 100) among *qnr* determinants [25]. The reason of this abundance can have evolutionary advantage against other *qnr* determinants. This theory is supported by the fact that *qnrB* is often associated with other resistance genes, such as *bla*$_{CTX-M-15}$, *bla*$_{CTX-M-14}$, and *aac(6')-Ib-cr* [22], which can cause multiresistance and facilitate dissemination of resistant strains [9].

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control strains. This study was financially supported by OTKA Hungarian Scientific Fund, grant number 108481.

Conflict of Interest

There are no conflicts of interest.

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