Contribution of OqxAB Efflux Pump in Selection of Fluoroquinolone-Resistant Klebsiella pneumoniae

Orsolya Szabo,1 Bela Kocsis,1 Nikolett Szabo,1 Katalin Kristof,2 and Dora Szabo1

1Institute of Medical Microbiology, Semmelweis University, Budapest 1082, Hungary
2Institute of Laboratory Medicine, Clinical Microbiology Laboratory, Semmelweis University, Budapest 1089, Hungary

Correspondence should be addressed to Bela Kocsis; kocsis.bela@med.semmelweis-univ.hu

Received 4 April 2018; Accepted 7 August 2018; Published 23 September 2018

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The role of OqxAB efflux pump in Klebsiella pneumoniae was investigated in correlation with ciprofloxacin exposure. K. pneumoniae SE23 and K. pneumoniae SE191 were isolated from urinary tract infections and were analyzed in this study. Each carried oqxAB resistance determinant and exhibited ciprofloxacin MIC of 0.06 and 0.5 mg/L, respectively. Tested strains were initially exposed to their ciprofloxacin MIC values for 24 hours. Later on, the ciprofloxacin exposition has been increased to a daily 1, 2, 4, and to a final 8 mg/L. Total cellular RNA was extracted at 30, 60, 90, and 120 minutes of initial exposure and after every 24 hours. Quantitative reverse-transcriptase PCR was performed from each RNA sample. Mutation in gyrA and parC genes was analyzed in each strain and multilocus sequence typing (MLST) was performed. Ciprofloxacin exposure selected resistant strain from K. pneumoniae SE191; by contrast, K. pneumoniae SE23 was not adjustable to the increasing ciprofloxacin concentrations. During initial exposure, both oqxA and oqxB expression remained low ($2^{-\Delta Ct}$ = 1.2-2.9). However, increasing ciprofloxacin promoted oqxB expression as it reached fold increase of 15.8-22.8, while oqxA expression was maintained ($2^{-\Delta Ct}$ = 2-2.15). An amino acid substitution Ser83Tyr in gyrA was detected in K. pneumoniae SE191, but no additional mutations occurred as consequence to ciprofloxacin exposure. MLST identified K. pneumoniae SE191 as ST274, while K. pneumoniae SE23 belonged to the novel ST2567. Ciprofloxacin concentration-dependent upregulation of oqxAB efflux pump in K. pneumoniae is clonally related and contributes to selection for higher level of fluoroquinolone resistance.

1. Introduction

Klebsiella pneumoniae is one of the most widespread nosocomial pathogen and responsible for various infectious diseases, including urinary tract, bloodstream, and respiratory tract infections. It is capable of developing resistance to cephalosporins, carbapenems, fluoroquinolones, aminoglycosides, and recently to polymyxins [1–3]. Fluoroquinolone resistance in K. pneumoniae is explained by mutations in gyrase and topoisomerase IV enzymes; however, reduced susceptibility to fluoroquinolones and low-level resistance is maintained by plasmid-mediated quinolone resistance (PMQR) determinants, namely, Qnr determinants, QepA and OqxAB efflux pumps, and aminoglycoside-acetyltransferase Ib-cr enzyme [4].

OqxAB efflux pump belongs to the resistance nodulation division family and it is constructed by two main domains, namely, OqxA, a periplasmic part, and OqxB, a transmembrane protein. Its efflux pump activity is described in a TolC-independent way; however, presence of an outer membrane protein enhances its effect [5]. OqxAB has been commonly found chromosomally in K. pneumoniae and usually plasmid located in other Enterobacteriaceae species. This resistance determinant is responsible to develop reduced susceptibility and even resistance to olaquindox and other fluoroquinolone agents such as ciprofloxacin, norfloxacin, and flumequine [5, 6]. High prevalence of OqxAB has been described in K. pneumoniae on both chromosome and plasmids representing a potential reservoir of this resistance determinant [7–10]. In K. pneumoniae, upregulation of oqxAB efflux pump is rarA mediated; by contrast, oqXR is capable of down-regulating it [11–13]. It has been recently reported that AcrAB associated with overexpression of OqxAB is required for high virulence potential in K. pneumoniae [14].
Internationally successful high-risk clones of K. pneumoniae have disseminated in Hungary. These clones were fluoroquinolone resistant and harboured extended-spectrum beta-lactamases (blaCTX-M-15) or carbapenemases (blaVIM-4, blaKPC); however, they retained fitness and these combined features contributed to the widespread dissemination of ST11, ST15, ST147, and ST258 in hospital settings and made them responsible for the majority of healthcare-associated infections [15–17]. Recently, ST274 has emerged in adult and newborn hospital wards and has been reported in several nosocomial infections [17]. The aim of this study was to analyze role of oqxAB efflux pump in selection of fluoroquinolone resistance in different K. pneumoniae clones.

2. Materials and Methods

2.1. Bacterial Strains. Two K. pneumoniae strains (K. pneumoniae SE23 and K. pneumoniae SE191) have been included in this study. Both have been previously found positive to oqxAB resistance determinant by PCR and nucleic acid sequencing and lacked all other PMQRs. Antimicrobial susceptibility testing was performed by microdilution method based on documents of the European Committee on Antimicrobial Susceptibility Testing (EUCAST), and both K. pneumoniae SE23 and K. pneumoniae SE191 were found susceptible to ciprofloxacin with MIC 0.06 and 0.5 mg/L, respectively, based on EUCAST breakpoints issued in the year 2016. However, ciprofloxacin resistance breakpoint of Enterobacteriaceae has been revised by EUCAST in 2017, indicating 0.5 mg/L value as non-susceptible to fluoroquinolones.

2.2. Exposure to Ciprofloxacin. Each tested strain has been prepared in 0.5 McFarland density in Mueller-Hinton broth and has been initially exposed to their ciprofloxacin MIC values of 0.06 and 0.5 mg/L for 24 hours at 37°C. RNA extraction has been carried out at 30, 60, 90, and 120 minutes and at 24 hours of ciprofloxacin exposition. Later on, after the initial incubation, each tested strain has been adjusted to 0.5 McFarland density and has been exposed to an increased ciprofloxacin concentration. In the case of K. pneumoniae SE23, the peak ciprofloxacin exposition was 0.5 mg/L. On the other hand, K. pneumoniae SE191 was capable of adapting to a daily increasing 1, 2, 4, and 8 mg/L ciprofloxacin exposition, where each incubation at 37°C lasted for 24 hours. After each daily exposure, RNA extraction has been performed from each sample to analyze expression of oqxAB efflux pump.

2.3. Quantitative Reverse-Transcriptase PCR Analysis for Expression of oqxAB. Total cellular RNA was extracted from both tested strains at given time point of incubation by RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions. The quantitative RT-PCR was carried out in a Step One Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific) with following thermal profile: 60°C for 30 sec; 50°C for 5 min and 95°C for 10 min; 40 cycles of 95°C for 15 sec and 60°C for 1 min; and 60°C for 30 sec.

To test separate expression of the oqxA and oqxB gene, the internal fragments were used to design set of primers and 6-FAM- or VIC-labelled probes. Chromosomal rpoB was chosen as housekeeping gene. All oligonucleotide primers and probes for quantitative RT-PCR were designed by Primer Express 3.0 software and are listed in Table 1. Each sample was tested in triplicate. The Ct values of genes of interest were normalized (ΔCt) to the Ct values of housekeeping gene rpoB, and the relative expression of each gene of interest was calculated as 2−ΔΔCt.

2.4. Analysis of Mutations in gyrA and parC Genes. Mutations in gyrA and parC genes have been investigated by PCR and nucleic acid sequencing. Set of primers was designed by online tools of MWG Eurofins Operon and synthesised by IDT Bioscience. PCR thermal profile was as follows: initial denaturation at 95°C for 3 min, 30 cycles of 95°C for 1 min, 52°C for 1 min, 72°C for 1 min, and an additional extension at 72°C for 5 min.

2.5. Multilocus Sequence Typing (MLST) of K. pneumoniae. Each tested strain has been analyzed by multilocus sequence typing based on sequences of seven housekeeping genes, namely, gapA, infB, mdh, pgi, phoE, rpoB, and tonB. Oligonucleotide primers and PCR thermal profiles were applied after Diancourt et al. article [18] and as it is highlighted on MLST database website http://pubmlst.org/klebsiella/primers_used.html. Amplicons of MLST, gyrA and parC PCR were analyzed by electrophoresis in a 1.5% agarose gel (Sigma-Aldrich) at 120 V for 20 min in 1xTAE (40 mM Tris-HCl (pH 8.3), 2 mM acetate, and 1 mM EDTA), and gel was stained with 0.05 mg/L GelRed dye (Biotium) and visualised on a UV transilluminator. PCR-positive amplicons were purified by QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany), and nucleic acid sequencing was performed by BIOMI Kft. Gödöllő, Hungary.

2.6. Statistical Analysis. Expression level differences were analyzed by t-test for 2 independent means.

3. Results

K. pneumoniae SE23 and K. pneumoniae SE191 strains were positive to oqxAB by PCR and were both initially exposed to ciprofloxacin concentration of their MIC values 0.06 and 0.5 mg/L, respectively. However, selection of resistant strains was successful only by K. pneumoniae SE191 and it was found to be adjustable for further ciprofloxacin exposition. Hence, multiple conditions K. pneumoniae SE23 was not capable of adapting to increased ciprofloxacin concentrations. The highest ciprofloxacin value was 0.5 mg/L, where RNA could have been extracted from K. pneumoniae SE23, but no further exposition was possible.
Quantitative RT-PCR was performed on RNA samples taken at given time points of ciprofloxacin exposure to investigate expression level differences of oqxAB efflux pump. Expression in SE191 to 0.5mg/L for 30 minutes was 1.82 and no relevant expression change to 4 and 8mg/L as 2−ΔCt values were 2.15 and 2.00. In the case of SE23 expression, 2−ΔCt values were 1.07 and 1 at maximum 0.5mg/L (Figure 1).

In the case of OqxAB, expression level values calculated as 2−ΔCT were 1.47 and 1.73 for SE191 while in SE23 values were 1.78 and 1.59. The change in expression of SE191 to 4 and 8 mg/L concentrations was in time 15.8 and 22.8 (Figure 1). Expression analysis by t-test showed t value = 1.88 and p value = 0.04.

In the case of SE191, a Ser83Tyr mutation in gyrA subunit has been detected. This mutation presented the 0.5mg/L MIC value. In SE23, amino acid substitutions have not been at 0.5mg/L exposition.

MLST has been conducted on both strains. K. pneumoniae SE191 was identified as ST274, while K. pneumoniae SE23 presented the novel ST2567. This novel sequence type incorporated a new tonB variant 371, which had two mutations at C48A and G61C compared to its closest match allele 87. Other alleles of K. pneumoniae SE23 yielded gapA 4, infB 31, mdh 13, pgi 1, phoE 1, and rpoB 1. The nucleic acid sequence of novel tonB allele 371 and the MLST profile of ST2567 have been submitted to K. pneumoniae database (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html).

4. Discussion

Our study demonstrated role of OqxAB efflux pump in K. pneumoniae during exposure to increased ciprofloxacin concentrations. K. pneumoniae SE23 and K. pneumoniae SE191 belonging to different sequence types ST2567 and ST274, respectively, have been investigated. They showed varying ability to adapt to ciprofloxacin exposition, though both strains carried oqxAB efflux pump. The two tested strains exhibited different ciprofloxacin MIC values 0.06 and 0.5 mg/L, respectively, and both were susceptible based on the EUCAST 2016 documents (valid at the time when this experiment was started). The initial exposure selected resistant strains only in the ST274 clone and it was further exposed to higher ciprofloxacin concentrations until 8mg/L. This strain harboured already a Ser83Tyr amino acid substitution in gyrA subunit, but no other mutations occurred as consequence to ciprofloxacin exposure. An
upregulation of oqxAB has been detected with a fold change of 22.8 times increase in expression during the ciprofloxacin exposure from 0.5 till 8 mg/L. This upregulation and selection of resistant strains seem clonally dependent as K. pneumoniae SE23, a strain of ST2567, could not increase the expression level of oqxAB and failed to adapt to an increasing ciprofloxacin concentration. This upregulation of efflux pump can contribute to the fitness of ST274, as it has been identified as one of the disseminated clones in newborn and adult hospital wards in Hungary [17].

Results of our study are in accordance with recent ciprofloxacin breakpoint modification of EUCAST. Our study had begun in 2016; therefore, antimicrobial susceptibility testing and interpretation of results were based on EUCAST 2016 documents where 0.5 mg/L ciprofloxacin value was in susceptible range. The resistance breakpoint of Enterobacteriacea has been revised in 2017 and set 0.5 mg/L in nonsusceptible range. K. pneumoniae SE191 exhibiting 0.5 mg/L ciprofloxacin MIC was capable of developing further fluoroquinolone resistance by upregulation of oqxAB efflux pump due to ciprofloxacin exposure. By contrast, K. pneumoniae SE23 exhibiting 0.06 mg/L ciprofloxacin MIC and lacking increased oqxAB expression during exposure to ciprofloxacin was not able to adapt to fluoroquinolone exposition and could not develop resistance.

In our study, a novel K. pneumoniae sequence type has been detected, namely, ST2567 that was distinguished by two mutations in tonB allele from its closest sequence type 2387. The new K. pneumoniae ST profile has been deposited to MLST database (http://bigdb.pasteur.fr/klebsiella/klebsiella.html).

Earlier studies demonstrated OqxAB efflux pump in extended-spectrum beta-lactamase-producing and fluoroquinolone-resistant K. pneumoniae high-risk clones [19–21]. In Hungary, K. pneumoniae with oqxAB resistance determinant was reported in bloodstream infections [22]. In all these reported studies, tested strains were resistant to fluoroquinolones. In our study, we demonstrated the role of oqxAB efflux pump in K. pneumoniae in selection of resistance to fluoroquinolones. Our two tested strains were susceptible to fluoroquinolones, but after exposure to ciprofloxacin, the one with gyrA mutation could be selected to higher levels of fluoroquinolone resistance. Additional chromosomal mutations of gyrA and parC genes in tested strain did not occur, suggesting that the development of resistance during exposure to 0.5–8 mg/L ciprofloxacin was mainly caused by the increased expression of the oqxAB efflux pump gene. Strains were exposed to the maximum of 8 mg/L ciprofloxacin concentration, and in human tissues, ciprofloxacin is unable to reach higher concentrations during a per os dosing; thus, the concentrations used in our study can represent a fluoroquinolone exposure during per os therapy.

Data Availability

Novel K. pneumoniae MLST profile of ST2567 and new tonB variant 371 sequence data have been submitted to MLST database (http://bigdb.pasteur.fr/)

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

We thank the team of curators of the Institut Pasteur MLST and whole-genome MLST databases for curating the data of K. pneumoniae ST2567 and making them publicly available at http://bigdb.pasteur.fr/. This study has been financially supported by OTKA Hungarian Research Fund, Grant Number 108481.

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