Closing report of K108481 ”Factors influencing the expression of plasmid-mediated quinolone determinants (PMQRs)”

I.Introduction

Fluoroquinolones are broad-spectrum antibiotics with bactericidal effect on both Gram-negative and Gram-positive bacteria. Fluoroquinolones bind to bacterial gyrase and 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 and community-acquired pneumonia. Furthermore, tuberculosis and infections of abdominal cavity, skin and soft tissue, bone and joint can also be treated with fluoroquinolones.

During the past years several attempts were followed to recognize antibacterial agents with new chemical structures, although numerous novel agents are derived from current antibiotic classes. The newly discovered fluoroquinolone agents should demonstrate more potent antimicrobial activity, efficacy against resistant pathogens, yield improved safety profiles and show enhanced pharmacokinetics. Based on pharmacological experience it is possible to design new molecules of ol antibiotic classes with improved pharmacokinetic and pharmacodynamic features in old antibiotic classes. The sophisticated crystallographic methods and structure–activity relationship studies play crucial role in drug discovery to detect multiple targets of a known antibiotic class. So far, novel quinolone agents have been developed namely, avarofloxacin, delafloxacin, finafloxacin, zabofloxacin and nemonoxacin. These agents demonstrate broad-spectrum antibacterial activity together with other beneficial features like intracellular accumulation, anti-inflammatory effect and inhibition of biofilm production. Moreover, these agents showed moderately severe or mild adverse events and demonstrated favorable tissue penetration. Among the novel quinolone agents zabofloxacin and delafloxacin have been introduced into clinical practice as treatment options in lower respiratory tract infections.

Fluoroquinolone resistance is caused by chromosomal and plasmid-mediated mechanisms. High-level fluoroquinolone resistance develops by chromosomal mutations in quinolone-resistance determining regions (QRDRs) namely, in gyrase enzyme coding *gyrA, gyrB* and in topoisomerase IV coding *parC* and *parE* genes. **Plasmid-mediated quinolone resistance (PMQR)** itself confers reduced susceptibility or low-level fluoroquinolone resistance. Expression of these genes can protect bacteria in stress-induced conditions for example 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). The first PMQR gene, *qnrA1*, was detected in 1998 in *K. pneumoniae*. Since then, many determinants were described and have been identified worldwide in Enterobacteriaceae, mainly in *Klebsiella pneumoniae*, *Enterobacter* spp., *Escherichia coli*, and *Salmonella enterica* both in community- and nosocomial acquired infections. PMQR genes are localized on mobile genetic elements - plasmids, transposons, integrons - and associated with other resistance determinants in Enterobacteriaceae, often with beta-lactamases: extended-spectrum beta-lactamases (ESBLs) and carbapenemases. Fluoroquinolone resistant strains show a raising tendency worldwide and it causes new challenges for effective treatment (https://ecdc.europa.eu/en/home).

Publications:

1. *Kocsis B, Szabo D. New treatment options for lower respiratory tract infections. Expert Opin Pharmacother. 2017 Sep;18(13):1345-1355. IF: 3.475*
2. *Kocsis B, Domokos J, Szabo D. Chemical structure and pharmacokinetics of novel quinolone agents represented by avarofloxacin, delafloxacin, finafloxacin, zabofloxacin and nemonoxacin. Ann Clin Microbiol Antimicrob. 2016 May 23;15(1):34. IF: 2.083*
3. *Kocsis B, Szabo D. Zabofloxacin for chronic bronchitis. Drugs Today (Barc). 2016 Sep;52(9):495-500. IF: 0.943*

II. Determination of the prevalence of plasmid-mediated quinolone resistance (PMQR) determinants in Enterobacteriaceae isolated from blood cultures and urine clinical samples

Plasmid-mediated quinolone resistance (PMQR) determinant genes including *qnrA, qnrB, qnrC, qnrD, qnrS, aac(6')-Ib-cr, oqxAB* and *qepA* were investigated in 103 extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae strains isolated from blood cultures and in 214 Enterobacteriaceae strains isolated from urine clinical samples at Semmelweis University. The isolates were screened for quinolone susceptibility by microdilution method and PMQR genes were detected by polymerase chain reaction (PCR). Altogether 40 ESBL-producing *E. coli* (39%) and 50 ESBL-producing *Klebsiella* spp. strains (48%) from blood cultures were resistant to ciprofloxacin based on their minimum inhibitory concentration (MIC) values according to the

EUCAST recommendations (www.eucast.org); 40 ESBL-producing *E. coli* (39%) and 47 ESBL-producing *Klebsiella* spp. strains (45%) were resistant to levofloxacin; and 88 strains including 40 ESBL-producing *E. coli* (39%) and 48 (47%) ESBL-producing *Klebsiella* spp. were resistant to moxifloxacin. Among the 103 ESBL-producing isolates, 77 (75%) isolates (30 *E. coli* and 47 *Klebsiella* spp.) harboured PMQR genes. From blood culture isolates the most commonly detected gene was the *aac(6')-Ib-cr* (65%) and the occurrence of *qnrS* gene was 6%. Out of 214 Enterobacteriaceae from urine samples, 38 yielded any PMQR determinant and altogether 45 genes were detected namely, six *qnrA*, one *qnrB*, two *qnrD* and eight *qnrS*, nine *aac(6')-Ib-cr* and 19 *oqxAB*. However, neither *qepA* nor *qnrC* were detected. Notably, from urine samples 18 *Klebsiella* spp., harbored *oqxAB*, nine *E. coli* were positive for *qnrS* and two *Morganella morganii* yielded *qnrD* resistance determinant. That was the first reported *qnrD*-resistance determinant in Hungary.

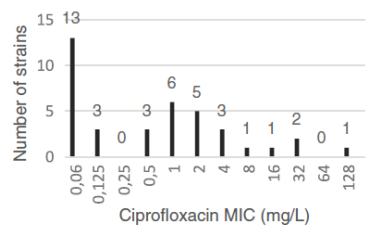
Table 1. Distribution of virulence determinant of the tested *E. coli* strains

Strain	afa	pap	pil	sfa/foc	kpsMT
E.coli 15	–	–	+	–	–
E.coli 38	–	+	+	+	+
E.coli 177	–	+	+	+	+
E.coli 178	–	–	+	–	+
E.coli 180	+	–	+	–	+
E.coli 184	–	–	+	+	+
E.coli 193	–	–	+	–	+
E.coli 199	–	+	+	+	+

Note: afa: afimbrial adhesions; pap: pili associated with pyelonephritis; pil: P fimbria sfa/foc: S and F1C fimbiriae; kpsMT: K-antigen.

The presence of virulence determinants, namely *afa*, *pap*, *pil*, *sfa/foc*, and *kpsMT* of eight *qnr* positive *E. coli* strains from urine samples were investigated and all eight investigated strains carried the *pil* gene, showing that P fimbria is a common virulence determinant among *qnr* positive *E. coli* from urine samples as seen on Table 1.

Figure 1. Ciprofloxacin MIC values of the 38 PMQR positive isolates from urine



Altogether, the PMQR-positive strains were susceptible or showed low-level resistant to ciprofloxacin with MIC between 0.06 and 1 mg/L, suggesting that prevalence of PMQR determinants is underestimated by phenotypic methods and screening among clinical isolates exhibiting reduced susceptibility is necessary as seen on Figure 1.

Publications:

4. *Domokos J, Kristof K, Szabo D.: Plasmid-mediated quinolone resistance among extended-spectrum beta-lactamase producing Enterobacteriaceae from bloodstream infections, Acta Microbiologica et Immunologica Hungarica 2016 Sep;63(3):313-323. 2016 IF:0,921*

5. *Szabó O, Gulyás D, Szabó N, Kristóf K, Kocsis B, Szabó D.: Plasmid-mediated quinolone resistance determinants in Enterobacteriaceae from urine clinical samples. Acta Microbiol Immunol Hung. 2018 Aug 1;65(3):255-265. IF:1,107*

III. The effect of ciprofloxacin exposure on different PMQR genes (*qnr*, *oqxAB*) in Gram-negative bacteria

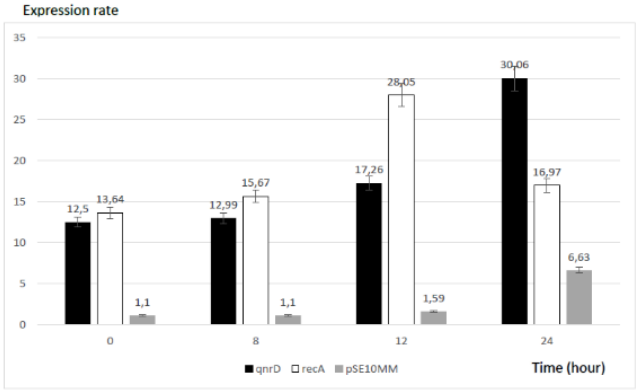
3.1. Ciprofloxacin promoted qnrD expression and its regulation in Morganella morganii

Morganella morganii SE10MM harboring *qnrD* quinolone resistance determinant was investigated in this study. An entirely sequenced novel 2,662 bp *qnrD*-plasmid pSE10MM was identified and deposited at **GenBank under accession number KU160530**. Promoter sequence of *qnrD* determinant contained LexA binding site. Nucleic acid sequence of pSE10MM showed 94-97% similarity to previously detected qnrD-plasmids of *Proteus mirabilis* strains. Phylogenetic analysis by Geneious 9.0.5 showed clusters of plasmids with possible common origin. Mutations in QRDRs were detected namely, *gyrA* S83I, *gyrB* S463A, and *parC* with S80I.

The role of *qnrD* expression, the plasmid copy number change during exposure to sub-MIC ciprofloxacin and the regulation of *qnrD* expression by SOS response was also investigated.

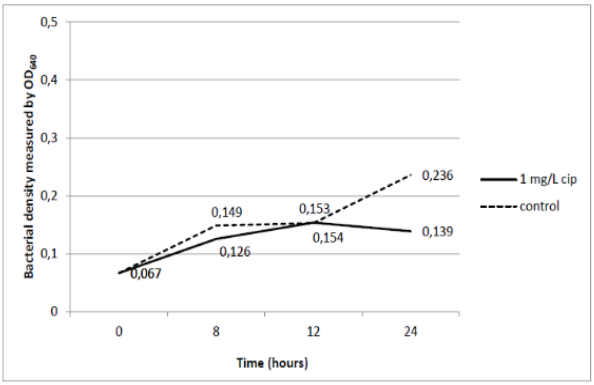
The *M. morganii* isolate exhibited resistance to nalidixic acid (MIC 128 mg/L), norfloxacin (MIC 2 mg/L), ofloxacin (MIC 2 mg/L), ciprofloxacin (MIC 2 mg/L) and was susceptible to all tested cephalosporins (cefotaxime, ceftazidime, ceftriaxone) each with 0.06 mg/L MIC. Based on these MIC values the *M. morganii* isolate was exposed to a sub-MIC (1 mg/L) ciprofloxacin for 24 hours and expression of the *qnrD*, the *recA* and the *rpoB* genes were detected by qPCR at different time points.

Figure 2. Expression rate differences of *qnrD*, *recA* genes and fold change of pSE10MM (*qnrD*-plasmid) normalized to *rpoB* ($2^{-\Delta Ct}$ values) after 0 hour, 8 hours, 12 hours and 24 hours exposure to 1 mg/L ciprofloxacin



The *qnrD* expression was upregulated within 24 hours and the

Figure 3. Growth rate of *M. morganii* SE10MM in presence and absence of 1 mg/L ciprofloxacin



qnrD-plasmid copy number was also increased in time from 1.1 to 6.63 (Figure 2). The *recA* expression (SOS response system regulator gene) correlated well with that of *qnrD* (Figure 2). There was no statistical differences in *qnrD* and *recA* expression (t-value = 0.76, *p*-value = 0.22). During exposure to sub-MIC ciprofloxacin the growth rate of tested strain was decreased at 24th hours (Figure 3) therefore the increased *qnrD* expression can be considered as protective factor under stressed condition even after multiple mutations in gyrase and topoisomerase enzymes. LexA binding site and expression level of *recA* that correlated with that of *qnrD* indicate SOS dependent regulation of *qnrD*. The *qnr* determinants role in the bacterial host cell is mainly dependent on promoter characterestics (eg.: SOS-dependent regulation) and on plasmid replicative capacity.

Publications:

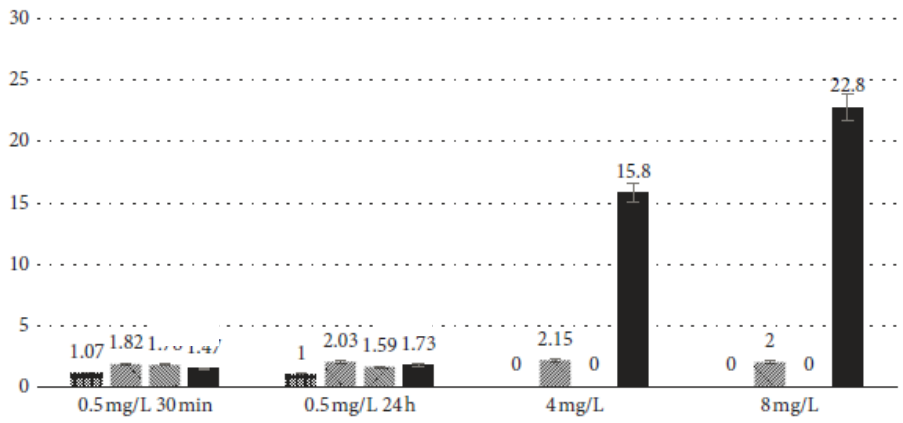
6. *Kocsis B, Szmolka A, Szabo O, Gulyas D, Kristóf K, Göcző I, Szabo D.: Ciprofloxacin Promoted qnrD Expression and Phylogenetic Analysis of qnrD Harboring Plasmids, Microb Drug Resist. 2018 Nov 21. doi: 10.1089/mdr.2018.0245. [Epub ahead of print], 2018 IF: 2.344*

3.2.Contribution of OqxAB efflux pump in selection of fluoroquinolone-resistant Klebsiella pneumoniae

The role of OqxAB efflux pump in *K. pneumoniae* was investigated in correlation with ciprofloxacin exposure. *K. pneumoniae* SE23 and *K. pneumoniae* SE191 isolates carried *oqxAB* resistance determinant and exhibited ciprofloxacin MIC of 0.06 and 0.5 mg/L, respectively. The strains were initially exposed to their ciprofloxacin MIC values for 24 hours. Later on, the ciprofloxacin exposition has been increased to a daily increasing 1, 2, 4, and to a final 8 mg/L. The

qPCR was performed and mutation of *gyrA* and *parC* genes was analyzed in each strain and multilocus sequence typing (MLST) was performed. Our study demonstrated role of OqxAB efflux pump in *K. pneumoniae* during exposure to increased ciprofloxacin concentrations. In our study *K. pneumoniae* SE23 and *K. pneumoniae* SE191 were included that belonged to different sequence types ST2567 and ST274, respectively, have been investigated. The *K. pneumoniae* SE23 and *K. pneumoniae* SE191 isolates showed varying ability to adapt to ciprofloxacin exposition, though both strains carried *oqxAB* efflux pump (Figure 4). 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 8 mg/L. This strain harboured already a Ser83Tyr amino acid substitution in gyrase A subunit, but no other mutations occurred as consequence to ciprofloxacin exposure. Ciprofloxacin exposure selected resistant strain from *K. pneumoniae* SE191; by contrast, *K. pneumoniae* SE23 was not adjustable to the increasing ciprofloxacin concentrations. An upregulation of *oqxB* 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 clone, as the *K. pneumoniae*

Figure 4. Expression rate ($2^{-\Delta Ct}$)of *oqxA* in *K. pneumoniae* SE23 and *K. pneumoniae* SE191 after exposure to different ciprofloxacin concentrations (mg/L)



ST274 clone has been identified as one of the disseminated clones in newborn and adult hospital wards in Hungary.

Publications:

7. *Szabo O, Kocsis B, Szabo N, Kristof K, Szabo D.: Contribution of OqxAB Efflux Pump in Selection of Fluoroquinolone-Resistant Klebsiella pneumoniae. Can J Infect Dis Med Microbiol. 2018 Sep 23;2018:4271638. doi: 10.1155/2018/4271638. eCollection 2018., IF: 1,271*

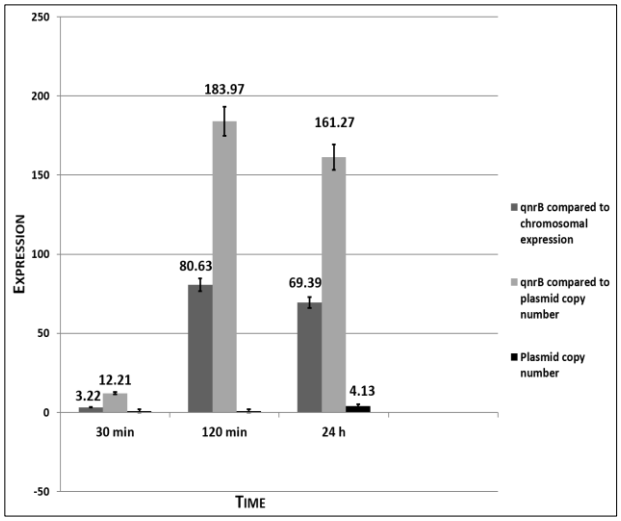
3.3 Plasmid copy number and qnr gene expression in selection of fluoroquinolone resistant Escherichia coli

In our research we performed short-term and long-term study to analyze the time-dependence in the development of fluoroquinolone resistance. During the study we used *E.coli* TG1 strains containing different PMQRs harboring plasmids – *qnrA1*, *qnrB1*, *qnrC1* and *qnrD1*. The ciprofloxacin MIC value was 0.5 mg/L for each strain.

During the study each strain was exposed to 0.5 mg/L ciprofloxacin from 30-60-90-120 minutes over 24 hours for the short term-study. After the strains adapted to ciprofloxacin, the treated strains were exposed in constantly increasing 1-2-4-8 mg/L ciprofloxacin solutions through 24-48-120 hours in order to analyze concentration-dependence of fluoroquinolone-resistance in the long-term study.

In the short term study, during exposure to 0.5 mg/L ciprofloxacin from 30 minutes to 24 hours the *qnrA1* and *qnrD1* showed 1.2 and 1.47 level expressions, the *qnrC1* was 12.44. Compared to these three studied *qnr* determinants, *qnrB1* demonstrated a 3.22 to 80.63 expression level. The copy numbers of plasmids carrying *qnrA1*, *qnrB1*, *qnrC1* and *qnrD1* genes were analyzed as well and the *qnrA1*- and *qnrD1*-harbouring plasmid copy number change was 1-1.4 fold, the *qnrC1*-harbouring plasmid copy number

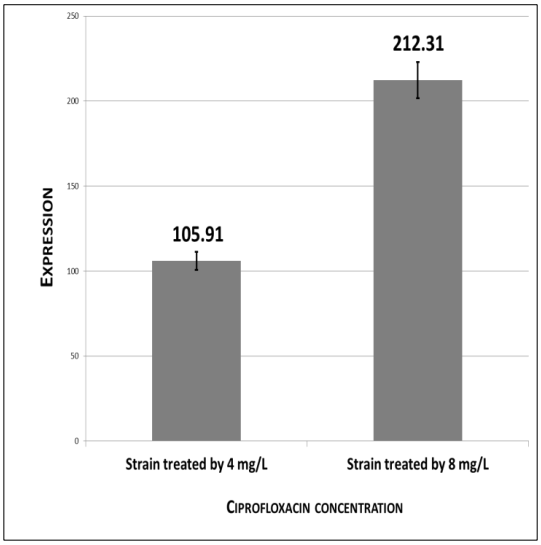
Figure 5. The results of the short-term study shows the different expression rate after 30 minutes, 120 minutes and 24 hours ciprofloxacin exposure



changed from 3.1 to 4.42, while in case of *qnrB1*-harbouring plasmid reached fold change of 4.13 Data are shown in Figure 5 .

We implicated the treated *E. coli* strains into the long term study and each were exposed to constantly increasing 1-2-4-8 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 mg/L and 8 mg/L ciprofloxacin concentration, respectively (Figure 6.). On the other hand the copy number of *qnrB*-harbouring plasmid reached 4.13 fold increase. In our study no mutation was found in the QRDRs.

Figure 6. The *qnrB1* expression rate in qnrB1-harbouring *E. coli* in different ciprofloxacin concentration



Our results showed that in the presence of 0.5-8 mg/L ciprofloxacin exposure the *qnrB1* positive *E. coli* developed quinolone resistance and in this process the plasmid copy number change had just a minor role, however resistance was maintained by increased expression of *qnrB1* gene. In the case of *qnrA*, *qnrC* and *qnrD* positive *E. coli*, these strains could adapt just to 0.5 mg/L ciprofloxacin with *qnr* expressions ranging from 1.2 to 12.44 but further selection was not possible.

The most common PMQR resistance gene is the *qnrB* in the world and it has the highest number of variants (almost 100) among the *qnr* determinants. The reason of its variability could 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 $bla_{CTX\text{-}M\text{-}15}$ and *aac(6')-Ib-cr* that can cause multiresistance and facilitate dissemination of resistant strains.

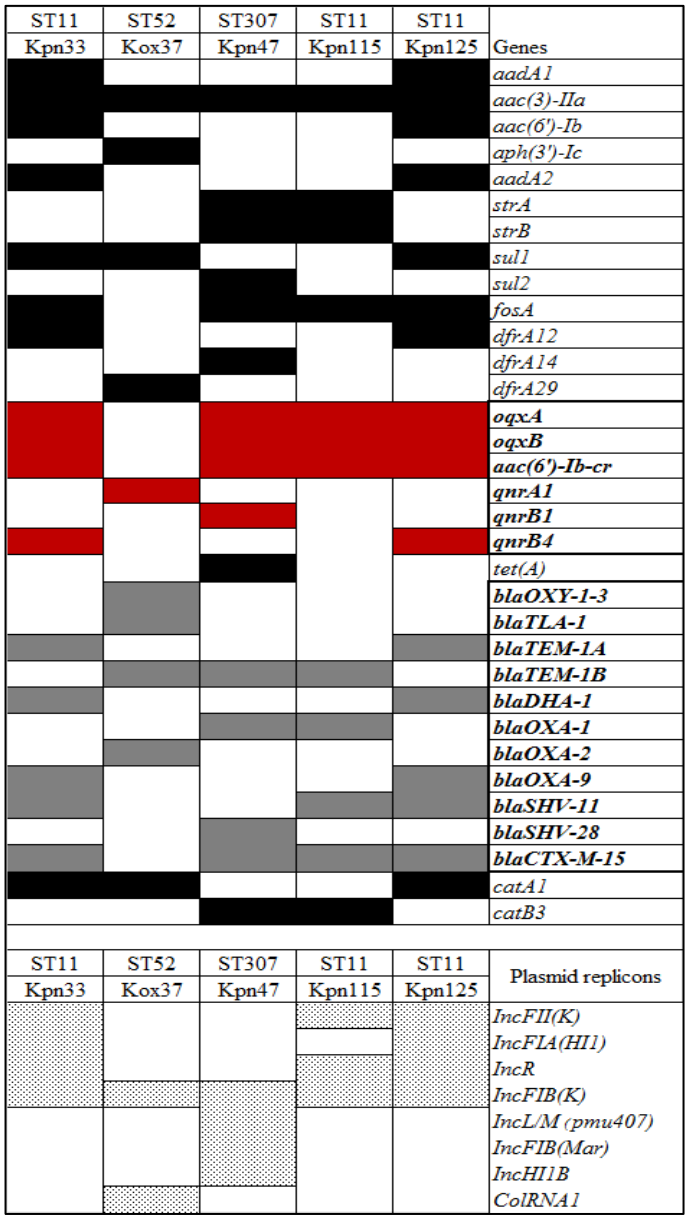
Publication:

8. *Gulyás D, Kocsis B, Szabó D.: Plasmid copy number and qnr gene expression in selection of fluoroquinolone-resistant Escherichia coli. Acta Microbiol Immunol Hung. 2018 Nov 22:1-10. doi: 10.1556/030.65.2018.049. [Epub ahead of print], 2018 IF: 1,107*

IV. Multiple benefits of PMQR determinants in *Klebsiella pneumoniae* ST11 and ST307 high-risk clone

The purpose of this study was the investigation of fluoroquinolone resistance in extended-spectrum beta-lactamase (ESBL)-producing strains, including four *K. pneumoniae* (Kpn33, Kpn47, Kpn115, Kpn125) and a single *Klebsiella oxytoca* (Kox37), isolated from bloodcultures in Hungary. Whole-genome sequencing and molecular typing including multilocus sequence typing (MLST) and pulsed-field gel electrophoresis were performed in selected strains. Expression of PMQR were investigated by qPCR. Three different STs were identified, including ST11 (Kpn33, Kpn115, Kpn125), ST307 (Kpn47) and ST52 (Kox37).

Table 2. Distribution of the different resistance genes and plasmid replicons of tested strains



Based on PFGE three pulsotypes (PT) were detected: KP053, S and KP197. Two isolates belonged to KP053 (Kpn33 and Kpn125) and one was detected as S PT (Kpn115). These strains belonged to the ST11 international high-risk clone. By contrast, Kp47 was classified as KP197 PT.

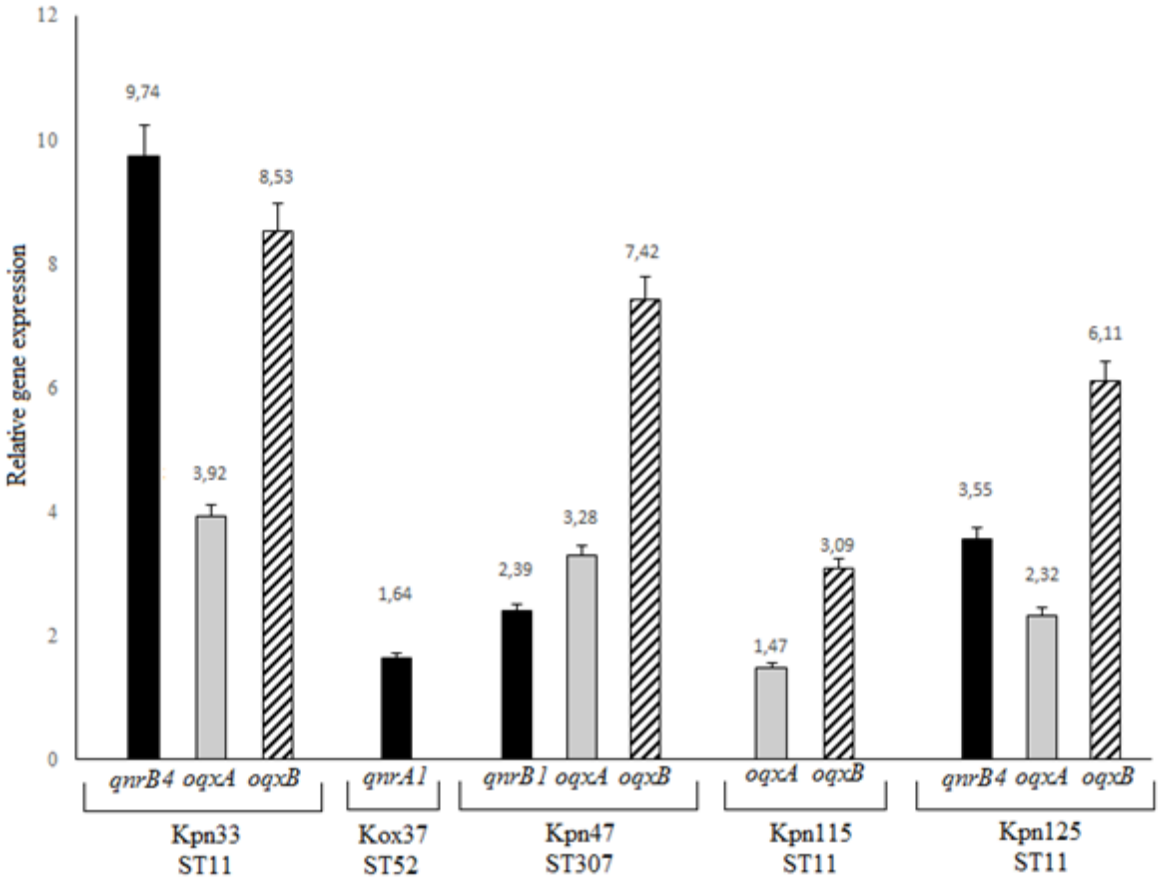
The initial assembled draft genome sequences were 5611026 bp

(Kpn33); 6370417 bp (Kox37); 5451744 bp, (Kpn47); 5450412 bp (Kpn115) and 5593358 bp, (Kpn125). Seventeen antibiotic resistance genes were found in two ST11 *K. pneumoniae* strains (Kpn33 and Kpn125), twelve were in the third ST11 strain (Kpn115), sixteen resistant genes were in ST307 strain (Kpn47) and ten resistance genes were observed in Kox37. Sequence analysis revealed, that the isolates harbored different β-lactamase genes, including *bla*$_{\text{DHA-1}}$, *bla*$_{\text{OXA-1}}$, *bla*$_{\text{OXA-2}}$, *bla*$_{\text{OXA-9}}$, *bla*$_{\text{SHV-11}}$, *bla*$_{\text{SHV-28}}$ and *bla*$_{\text{TEM-1A}}$, *bla*$_{\text{TEM-1B}}$, *bla*$_{\text{OXY-1-3}}$, *bla*$_{\text{TLA-1}}$; and all *K. pneumoniae* strains carried *bla*$_{\text{CTX-M-15}}$ genes. Among aminoglycoside resistance genes all isolates were positive for *aac(3)-II-a*. Only Kpn47 carried tetracyclin resistance (*tetA*) gene (Table 2). Except Kox37, all strains were identified positive for *fosA* gene nevertheless *sul1* or *sul2* and trimethoprim resistance (*dfrA12, dfrA14, dfrA29*) genes were detected in four strains too. PMQR genes were found in each tested strains namely, in Kpn33 *qnrB4,* in Kox37 *qnrA1,* in Kpn47 *qnrB1,* in Kpn125 *qnrB4.* All *K. pneumoniae* strains harbored *oqxAB* efflux pump and *aac(6')-Ib-cr,* but one of the ST11 strains (Kpn115) carried no *qnr* gene. Presence of phenicol resistance gene (*catA1* or *catB3*) was observed in all strains. Chromosomal mutations conferring fluoroquinolone resistance in *K. pneumoniae* strains were also detected, Ser83Phe and Asp87Ala substutions were in DNA gyrase subunit A of Kpn115 (ST11), but all other had only Ser83Ile in gyrase while on the other hand all *K. pneumoniae* had a Ser80Ile substitution in DNA topoisomerase IV. Based on the sequencing data *IncFIB, IncFII* and *IncR* replicons were uniformly present in all ST11 strains. In the case of ST307 *IncFIB, IncL/M, IncHI1B* were detected. The detected resistance genes and plasmid replicons are listed in Table 2.

Among *qnr* genes, *qnrB4* of two ST11 strains (Kpn33 and Kpn125) showed 9.74 and 3.55 fold expression, respectively. Interestingly, Kpn33 (ST11) was characterized approximately 3-fold higher expression, compared to the genetically similar Kpn125 (ST11). The lowest expression level (1.64) among *qnr* genes was detected in *K. oxytoca* that exhibited reduced susceptibility to ciprofloxacin. In the case of *qnrB1* in Kpn47 (ST307) it showed 2.39 fold expression (Figure 7).

Expression of *oqxA* ranged between 1.47 and 3.92 and that of *oqxB* from 3.09 to 8.53. The highest *oqxA* and *oqxB* expression were observed in Kpn33 (ST11) and Kpn47 (ST307). These were followed by Kpn125 (ST11) and Kpn115 (ST11). Interestingly, Kpn115 strain of ST11 high-risk clone carried no *qnr* gene, moreover it showed the lowest *oqxAB* expression. Conspicuous that in every *K. pneumoniae* strain the *oqxB* is expressed 2-3 fold higher than *oqxA*.

Figure 7. Level of *qnrB4* (Kpn33 and Kpn125), *qnrA1* (Kox37) and *qnrB1*, *oqxA* and *oqxB* relative gene expression. QRDRs: quinolone resistance determining regions. All MIC values are in mg/L.



	Kpn33	Kox37	Kpn47	Kpn115	Kpn125
MIC values					
NAL	>256	16	>256	>256	>256
CIP	128	0.25	128	128	128
LEV	128	0.25	32	8	64
MOX	128	0.5	128	8	64
QRDRs					
gyrA	Ser83Ile	-	Ser83Ile	Ser83Phe, Asp87Ala	Ser83Ile
parC	Ser80Ile	-	Ser80Ile, Asn304Ser	Ser80Ile	Ser80Ile

Acquisition of *qnr* determinants can have multiple advantages. In the case of *K. oxytoca* the presence and expression of *qnrA1* caused reduced susceptibility to quinolones (Figure 7). Further benefitial effect of Qnr proteins can be explained by the toxin-antitoxin effect. Qnr proteins are considers antitoxins, that protect gyrase and topoisomerase IV enzymes from naturally occurring toxins. It has been also established that the development of fluoroquinolone resistance is diverse among different clones and in the case of international high-risk *K. pneumoniae* clones the fluoroquinolone resistant strains retain fitness that facilitates their dissemination in hospital environment. Moreover, fluoroquinolone resistance played a key role in evolutionary success of *K. pneumoniae* clones. Emergence and possible dissemination of *K. pneumoniae* ST307 in hospital settings raises also public health concerns, therefore continuous monitoring of high-risk and potential high-risk clones is necessary.

Publication:

9. *Domokos J, Damjanova E, Kristóf K, Kocsis B, Szabo D. Multiple benefits of plasmid-mediated quinolone resistance determinants in Klebsiella pneumoniae ST11 high-risk clone and recently emerging ST307 clone. Front Microbiol. 2019 Feb 12;10:157. doi: 10.3389/fmicb.2019.00157. eCollection 2019. IF:4, 09*

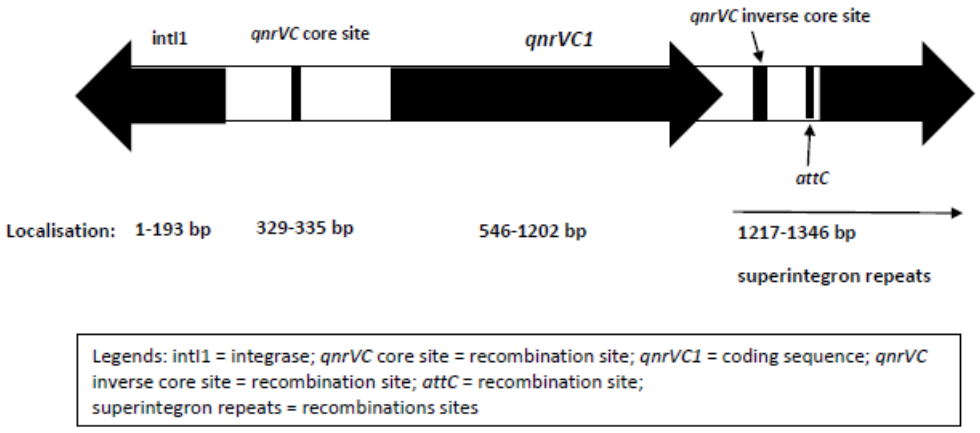
V. The expression and role of *qnrVC1* gene in an international high-risk *Pseudomonas aeruginosa* ST773 clone

In our study a multidrug-resistant *P. aeruginosa* PS1 isolated from urine clinical sample was investigated. *P. aeruginosa* PS1 exhibited resistance to piperacillin plus tazobactam (MIC 256 mg/L), ceftazidime (MIC 128 mg/L), imipenem (MIC 128 mg/L), meropenem (256 mg/L), ciprofloxacin (MIC 128 mg/L), levofloxacin (256 mg/L), tobramycin (256 mg/L), but it was susceptible to colistin (MIC 1 mg/L). The whole genome analysis detected 6815803 bp genome. The genome assembly of *P. aeruginosa* PS1 was deposited at **DDBJ/ENA/GenBank at RHDU00000000 accession number**. The sequence reads of *P. aeruginosa* PS1 were submitted to **Sequence Read Archive (SRA) at PRJNA503122**. Numerous resistance genes were identified: *aph(3')-IIb, rmtB, fosA, sul1, qnrVC1, tet(G), bla*$_{\text{OXA-50-like}}$, *bla*$_{\text{PAO}}$, *bla*$_{\text{NDM-1}}$ and *catB7*. Based on sequences of seven housekeeping genes namely, *acs*, *aro*, *gua*, *mut*, *nuo*, *pps*, *trp* the tested strain belonged to ST773. Nucleic acid sequence analysis done by NCBI Blast detected mutations in *gyrA*

and *parC* genes that correspond to aminoacid substitutions of Thr83Leu in *gyrA* and Ser87Leu in *parC*. In a 1346 bp class I integron incorporated *qnrVC1* was identified. The flanking region sequences including recombination sites of *qnrVC1* (**MH782277 accession number**) (Figure 8) were identical to that of found earlier in *V. cholerae* strain VC62. IS finder detected insertion sequences in flanking region of $bla_{\text{NDM-1}}$ namely, TnAs3 and ISAba125 (**MK109012 accession number**). The gene expression of *qnrVC1* and $bla_{\text{NDM-1}}$ was performed by qPCR and the expression of *qnrVC1* was 2-fold and $bla_{\text{NDM-1}}$ was 4-fold increased, compared to that of *rpsL* housekeeping gene.

Aquatic environment enables spread and exchange of resistance genes among waterborne bacterial species, notably several species within and out of Vibrionaceae were already found to carry *qnrVC* determinant on integrative elements. In our study a class I integron incorporated *qnrVC1* was detected in *P. aeruginosa* ST773 from a urine clinical sample. Integron of *qnrVC1* features *qnrVC* core and inverse core sites, *attC* site (Figure 8) that recombination sites are responsible for the cassette mobilization of chromosomal integrons moreover, *qnrVC1* carries its own functional promoter. In our study a 2-fold increased expression of *qnrVC1* was detected, that indicates role of this determinant even in presence of chromosomal mutations in gyrase and topoisomerase. A possible explanation of that could be the theory that Qnr proteins are antitoxins and protect DNA gyrase and topoisomerase IV from naturally occurring toxins. This protective effect of Qnr proteins can be an advantage in internationally disseminated high-risk clones.

Figure 8. Characteristics of qnrVC1 flanking regions in *Pseudomonas aeruginosa* ST773 NCBI Genbank accession number 782277



Mobile genetic elements play a key role in spread of resistance genes, and high-risk clones frequently integrate such determinants into their genomes. Emergence of *qnrVC1* and *bla*$_{\text{NDM-1}}$ resistance determinants in the clinically isolated high-risk *P. aeruginosa* clone indicates possible further dissemination of these genes among Gram-negative pathogens in hospital environments. The origin of *qnrVC1* gene in *P. aeruginosa* PS1 can be *V. cholera O1* that is explained by the identical sequences flanking *qnrVC1*. High-risk *P. aeruginosa* clones integrate various carbapenemase and other resistance determinants into their genomes that facilitates further dissemination of multiresistance among clinical isolate, and alerts public health issues. *P. aeruginosa* ST773 is an internationally disseminated clone, it was reported worldwide as multidrug resistant, VIM-2 producer, *bla*$_{\text{VEB-1}}$ positive. To the best of our knowledge this is the first report of *qnrVC1* and *bla*$_{\text{NDM-1}}$ in *P. aeruginosa* ST773.

Publication:

10. *Kocsis B, Toth A, Gulyas D, Ligeti B, Katalin K, Rokusz L, Szabo D. Acquired qnrVC1 and blaNDM-1 in international high-risk Pseudomonas aeruginosa ST773 clone. J. Med. Microbiol: 2019 Mar;68(3):336-338. doi: 10.1099/jmm.0.000927. IF:2,112*

VI. Investigation of *per os* antibiotics treatment for the gastrointestinal colonization with multiresistant *Klebsiella pneumoniae* strain harbouring- *aac6-Ib-cr*, $bla_{CXT\text{-}M\text{-}15}$ and $bla_{OXA\text{-}162}$ genes

Colonization resistance refers to the ability of the microbiota to prevent expansion and persistence of exogenously acquired bacterial species, a pivotal defense mechanism that can be impaired by antibiotic treatment. Changes in microbiota composition, mainly the elimination of specific groups of anaerobic bacteria in antibiotic treated mice, favoures colonization of multi-drug resistant bacteria in gastrointestinal tract in antibiotic-treated mice. Antibiotic-mediated depletion of commensals also decreases production of mucus and antimicrobial effector molecules, potentially increasing the risk for bacterial invasion of the intestinal epithelium.

The *aac(6')-Ib-cr* gene has spread rapidly among Enterobacteriaceae, and although only conferring a low-level resistance, it may create an environment facilitating the selection of more highly resistant determinants, especially those harbouring topoisomerase mutations. This fact is particularly worrisome at the nosocomial infections, where *aac(6')-Ib-cr* containing strains should be promptly detected and treated with non-hydrophilic fluoroquinolones, such as ciprofloxacin or other classes of antibiotics to prevent high-level resistance onset and spread.

In this study, our goal was to determine how the different antibiotic treatment would effect the rate of colonization and the resistance gene copy number in mice previously colonized by multiresustant *K. pneumoniae* strain 5825 producing an *aac(6')-Ib-cr* - a PMQR gene.

Materials and methods

Bacterial strains, conjugation

K. pneumoniae ST15 KP5825 clinical isolate produced an *aac(6')-Ib-cr* - a PMQR gene – an extended-spectrum beta-lactamase enzyme – CTX-M-15 – and a carbapenemase enzyme as well. The isolate was obtained from National Centre of Epidemiology.

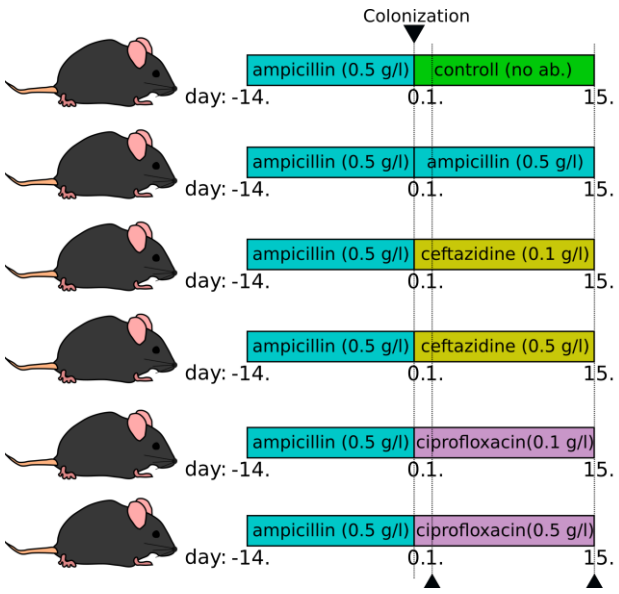
Conjugation experiments

The conjugation experiments were performed by using the CTX-M-15, OXA-162, *aac(6')-Ib-cr* -producing *Klebsiella pneumoniae* as a donor and *E. coli* J53 Azide^{R} as recipient. Each strain's inoculum reached density of 0.5 McFarland (that corresponds to 1.5 x 10^{8} bacterial cell/ml) when they were applied in reaction. Three different ratios of donor: recipient were applied 1:3 ratio 4 ml total volume of Luria-Bertani (LB) broth. The reaction tubes were incubated without shaking at 37 ºC for 5 hours and 100 µl reaction was plated on each selective LB agar medium containing combination of 100 mg/L sodium-azide and 2 mg/L imipenem 2 mg/L ceftazidime. After 24 hours incubation at 37 ºC agar plates were checked for transconjugants.

Intestinal colonization of mice

All experiments were carried out using 6–8 week-old C57BL/6 male mice purchased from Animalab and housed in sterile cages with irradiated food and acidified water. For experiments involving antibiotic treatment, 0.5g/L ampicillin was administered to animals in the drinking water for fourteen days and changed every day. For colonization experiments, 5x10^{6}CFU of *K. pneumoniae* KP5825 was administered by oral gavage in a 200µl volume on the fourteenth and fifteenth of ampicillin pretreatment. After the colonization 0.5g/L ampicillin, 0.1g/L ceftazidime, 0.5g/L ceftazidime, 0.1g/L ciprofloxacin and 0.5g/L ciprofloxacin was further administered to the animals in the drinking water for the two weeks and changed every day (Figure 9). Mice were single-housed at the time of colonization experiment. Animals were maintained in a specific pathogen-free facility at Institute of Medical Microbiology, Semmelweis University. All mouse handling, cage changes and fecal pellet collection were performed in a biosafety level 2 facility wearing sterile gowns, masks and gloves. Animals were maintained and handled in accordance with the recommendations of the Guidelines for the Care and Use of Laboratory Animals and the experiments were approved

Figure 9. The protocol of the colonization mice experiment



by the Animal Care Committee of Semmelweis University (permission no. PE/EA/60-8/2018, PE/EA/964-5/2018).

DNA extraction, V3-V4 16S rRNA gene amplification, multiparallel sequencing and sequence analysis

Fecal samples were frozen immediately after collection in a dry ice and stored at -80°C. DNA was extracted using QiaAmp Power fecal kit strictly as manufacture protocols described. The V3-V4 region of the 16S rRNA gene was amplified and sequenced with the Illumina Miseq platform (Illumina). The 16S rRNA amplicon data were analyzed using the QIIME2 microbiome bioinformatics platform. The raw sequence data was preprocessed using DADA2. The alpha and beta diversity values were computed using QIIME default parameters. The taxonomic classification was carried out using the SILVA 132 database and naive bayes classifier.

Determination of the antibiotic concentrations of the fecal samples in mice

The concentration of ampicillin, ceftazidime and ciprofloxacin level of the stool samples of each mice were determined by high pressure liquid chromatography (HPLC) at two different time points: first day and fifteenth day after colonization with KP5825

qPCR assay

DNA was extracted using QiaAmp Power faecal kit (Qiagen) after manufacturer's protocol. The rpoB (housekeeping gene) copy of *K. pneumoniae* ST15 KP5825 was detected by qPCR.

Results

The characterization of the *K. pneumoniae* strain KP5825

The whole genome of *K. pneumoniae* KP5825 was sequenced. There were many resistance genes detected in KP5825 as seen in Table 3. There were different plasmid types observed as well Table 4.

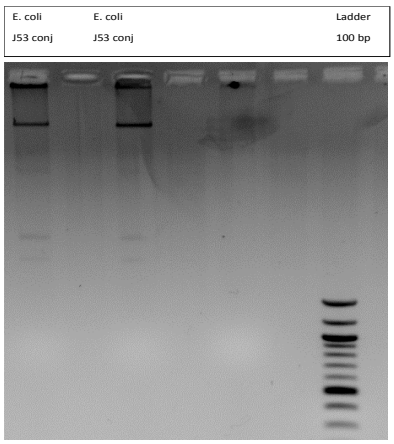
Table 3. The antibiotic resistance genes detected in the *K. pneumoniae* KP5825

Fluoroquinolone						
Resistance gene	**Identity**	**Query/HSP**	**Contig**	**Position in contig**	**Phenotype**	**Accession no.**
aac(6')-Ib-cr	**100.00**	**600/600**	**NODE_305_length_2280_cov_52.241226**	**1635..2234**	**Quinolone resistance**	DQ303918
oqxA	**99.23**	**1176/1176**	**NODE_40_length_190047_cov_23.768137**	**61474..62649**	**Quinolone resistance**	EU370913
oqxB	**98.86**	**3153/3153**	**NODE_40_length_190047_cov_23.768137**	**62673..65825**	**Quinolone resistance**	EU370913

Beta-lactam						
Resistance gene	**Identity**	**Query/HSP**	**Contig**	**Position in contig**	**Phenotype**	**Accession no.**
blaSHV-28	100.00	861/861	NODE_44_length_63538_cov_16.994854	12339..13199	Beta-lactam resistance	HM751101
blaOXA-162	100.00	798/798	NODE_186_length_2325_cov_67.126022	1503..2300	Beta-lactam resistance	GU197550
blaOXA-1	100.00	831/831	NODE_305_length_2280_cov_52.241226	674..1504	Beta-lactam resistance	J02967
blaCTX-M-15	100.00	876/876	NODE_239_length_10255_cov_45.244370	9174..10049	Beta-lactam resistance	DQ302097

Aminoglycoside						
Resistance gene	**Identity**	**Query/HSP**	**Contig**	**Position in contig**	**Phenotype**	**Accession no.**
aac(3)-IIa	100.00	861/861	NODE_108_length_2687_cov_38.806477	134..994	Aminoglycoside resistance	CP023555
aph(3')-Ia	100.00	816/816	NODE_306_length_1110_cov_58.953152	161..976	Aminoglycoside resistance	V00359
aac(6')Ib-cr	100.00	600/600	NODE_305_length_2280_cov_52.241226	1635..2234	Fluoroquinolone and aminoglycoside resistance	DQ303918

Figure 10. The plasmid isolation of the conjugated *E. coli* J53



The conjugation assay was performed successfully the *E. coli* J53 strain picked up one conjugative plasmid (Figure 10). The obtained transconjugant *E. coli* J53 that harboured the plasmid was resistant to cephalosporins, but remained susceptible to carbapenems. Based on the sequencing results the conjugative plasmid contained the CTX-M-15 ESBL gene, but did not contain the *aac(6')-Ib-cr* gene and the $bla_{\text{OXA-162}}$ gene. The plasmid isolation of the *E. coli* J53 after conjugation was performed in Figure 10.

Table 4. The plasmid types detected in the *K. pneumoniae* KP5825

Plasmid	%Identity	HSP length/Query	Contig	Position in contig
IncFII(K)	98.65	148 / 148	NODE_1_length_2347_cov_92.489990	1493..1640
IncL/M	100.00	661 / 661	NODE_169_length_17909_cov_65.945335	16227..16887
IncFIB	100.00	740 / 740	NODE_156_length_19663_cov_52.069775	2771..3510
ColpVC	98.45	193 / 193	NODE_24_length_2058_cov_601.307068	63..255

The antibiotic concentration in the stool samples

The ampicillin, ceftazidime and ciprofloxacin concentration were determined at two different time points. The first time point was one day after the beginning of per os antibiotic treatment and the second time was fifteen days after.

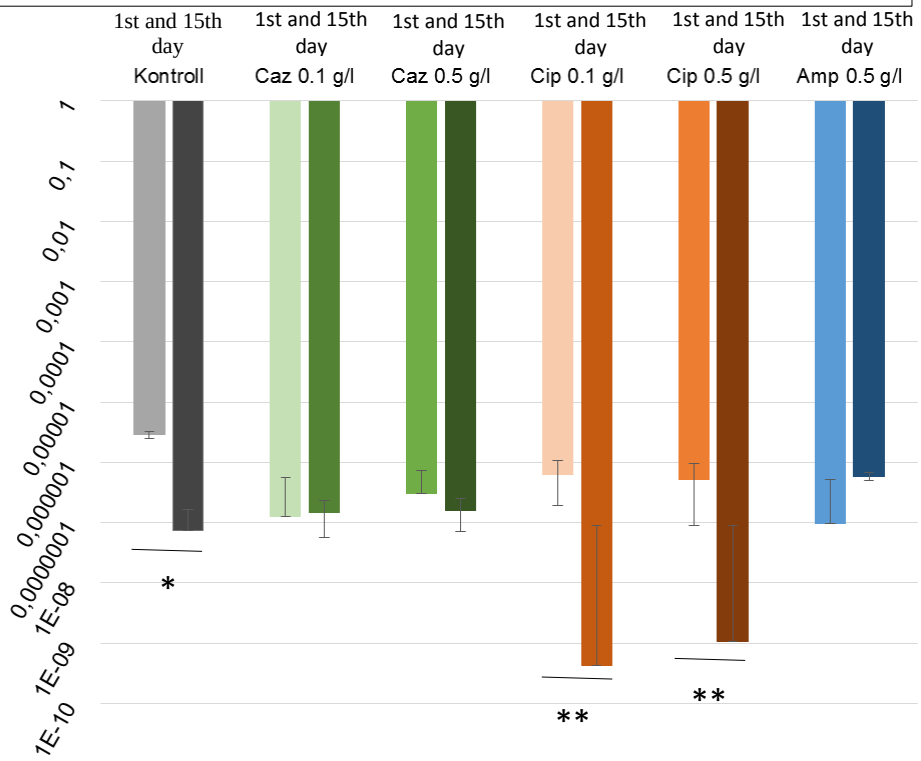
The average ampicillin concentration in the stool samples at the first time point in the group 0,5 g/L ampicillin treatment was 720,2 µg/g and the standard deviation was 247 µg/g. The average ampicillin concentration in the stool samples at the second time point in the group 0,5 g/L ampicillin treatment was 739,3 ug/g, the standard deviation was 219,4 µg/g. The average ciprofloxacin concentration in the stool samples at the first time point in the group 0,1 g/L ciprofloxacin treatment was 17,2 µg/g, the standard deviation was 5,96 µg/g. The average ciprofloxacin concentration in the stool samples at the first time point in the group 0,5 g/L ciprofloxacin treatment was 203,8 ug/g, the standard deviation was 46 µg/g. The average ciprofloxacin concentration in the stool samples at the second time point in the group 0,1 g/L ciprofloxacin treatment was 20,7 ug/g, the standard deviation was 4,97 µg/g. The average ciprofloxacin concentration in the stool samples at the second time point in the group 0,5 g/L ciprofloxacin treatment was 244,8 µg/g, the standard deviation was 61,9 µg/g. The ceftazidime concentration was unfortunately undetectable.

The effect of the different antibiotic on the gut microbiota colonized by *K. pneumoniae* K5825

Based on the C_T values of *rpoB1* the initial colonization with $5x10^6$ CFU *K. pneumoniae* K5825 after fourteen days the colonization rate (the K5825 amount in the feces) decreased significantly ($p<0,004$) in the control group with no antibiotic treatment and surprisingly also decreased

significant in the group with 0,1 g/L and 0,5g/L ciprofloxacin treatment (Figure 11). There was no remarkable differences detected between the first and 15th day of colonization in the ampicillin (0,5g/L) and in the ceftazidime (0,1 g/L and 0,5 g/L) treated groups, the amount of the K5825 isolates in feces did not change.

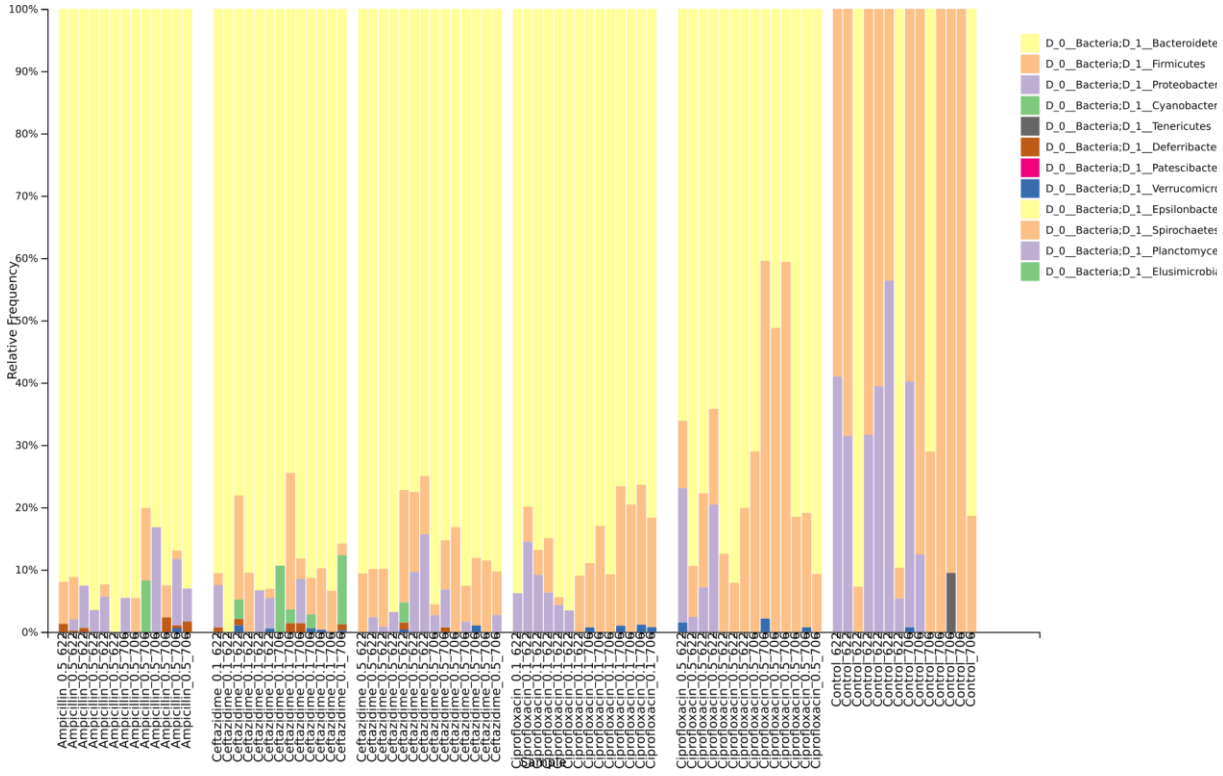
Figure 11. The change of the amount K5825 by qPCR in C57BL/6 mice fecal samples in the different treatment groups



The linearization of the data ($2^{(-Ct)}$) was followed by Mann-Whitney U-test. Comparing the data in the control group on the 15th day was 40 times less the amount of the K5825 strain than on the first day. In the ceftazidim groups – 0,1 g/l and 0,5 g/l –, and in the ampicillin group on the 1st and 15th day the amount of the the K5825 strain was identical from the stool samples. However, in case of 0,1 g/l ciprofloxacin group on the 15th day was 1400 times less the amount of the K5825 strain than on the first day, while in case of ceftazidime 0,5 g/l group 500 times decrease was observed.

After the colonization with K5825 strain the microbiota compositions was determined based on the 16S sequence in the gut of C57BL/6 mice on the first day of the antibiotic treatment (signed by

Figure 12 Antibiotic induced microbiota changes in the gut of C57BL/6 mice after colonization with *K. pneumoniae* K5825 strain



622) and on the 15th day (signed by 706) (Figure 12). The *Bacteroidetes/Firmicutes* ratio was higher in both time points in all the antibiotic treated group compared with control group. However, the ciprofloxacin treated groups were similar to the control groups. There were main differences among the groups based on which antibiotic treatment was used, however there were not so many differences among the groups with the different dosage within the same antibiotic treatment (ceftazidime, ciprofloxacin).

Proteobacteria was observed at the first time point observed in the highest ratios in control group and the high ratio was as observed in the ciprofloxacin groups as well. Despite of these observation at the second time point just in these groups (control, ciprofloxacin 0,1 g/L, ciprofloxacin 0,5 g/L) the Protebacteria decreased significantly, while in the other groups (ampicillin 0,5 g/L and ceftazidime 0,1 g/l and ceftazidime 0,5 g/L) it still existed or even increased the ratio.

Conclusion

Although intestinal colonization *K. pneumoniae* is asymptomatic, it increases the risk of extra-intestinal infection, including bacteremia. γ-Proteobacteria constitute a minor population within the human and murine microbiota and are, for the most part, harmless to the host unless intestinal homeostasis is perturbed. In clinical scenarios however, dense intestinal colonization with antibiotic-resistant bacteria is an important risk factor for systemic infection and for patient-to-patient spread.

It is a known fact that gastrointestinal colonization with multi-drug resistant pathogens can lead to serious clinical problems in the case of bacterial dissemination. In our experiment we aimed to find how the multidrug resistant *K. pneumoniae* gastrointestinal colonization is influenced by different doses of oral antibiotics with different structures. It is well known that antibiotics can induce and prolong cause of bacterial colonization. Surprisingly, in our studies, ciprofloxacin was a newcomer to the control both in terms of microbiology and carrier status.

Our new findings is that ciprofloxacin treatment can decrease the rate of the gastrointestinal colonization with multi-drug resistant *K. pneumoniae* strain harbouring *aac6'-1bcr* gene as well. Furthermore, the ciproflocaxin treatment induced microbiome change showed the less differences from normal flora.

Publication is in process.