Feno- and genotypic examination of fluoroquinolone resistance in *Escherichia coli* and *Klebsiella* species isolated from hemoculture

Ph.D. thesis

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1. Introduction

Fluoroquinolones possess an excellent activity against Gram-negative bacteria especially against *Enterobacteriaceae* and have been extensively used for treatment of infections caused by these pathogens since their introduction in the 1980s. The mode of action of fluoroquinolones is the inhibition of type II DNA topoisomerases, namely, gyrase and topoisomearse IV. DNA gyrase is the primary target for quinolones in Gram-negative bacteria. Resistance to fluoroquinolones was explained by accumulation of mutations in the chromosomal genes (*gyrA*, *gyrB*, *parC*, and *parE*), encoding DNA gyrase and topoisomerase IV enzymes. Further resistance mechanisms are the decreased intracellular drug accumulation by upregulation of native chromosomally encoded efflux pump (e.g. AcrAB) or decreased expression of outer membrane porins (e.g. OmpF in *Escherichia coli*). Plasmid-mediated quinolone resistance (PMQR) determinants – such as *qnr* genes (*qnrA*, *qnrB*, *qnrS*, *qnrC*, and *qnrD*), aminoglycoside acetyltransferase *(6')-Ib-cr* variant, and specific efflux pump genes (*qepA* and *oqxAB*) – were discovered as new ways of fluoroquinolone resistance. PMQR genes are frequently associated with various β-lactamase genes, including extended-spectrum β-lactamases (ESBLs) and narrow spectrum β- lactamases, as these resistance determinants are located often on same plasmids. Resistance plasmids are usually transferable by conjugation that facilitates their dissemination between different *Enterobacteriaceae* species.

International high-risk clones of *Klebsiella pneumoniae* are among the most common Gram-negative pathogens. Multi-drug resistant (MDR) *K. pneumoniae* clones emerged and dramatically increased prevalence of nosocomial infections while *K. oxytoca* has been isolated in hospital infections with less frequency. Multi-drug resistant *K. pneumoniae* acquires various resistance mechanisms that confer resistance to commonly used antibiotics. Among the most frequent resistance mechanisms are ESBLs, plasmid-mediated AmpC enzymes (pAmpCs), carbapenemases, PMQR genes, aminoglycoside-modifying enzymes (AMEs), as well as exogenously acquired 16S rRNA methyltransferase that have been detected in clinical isolates. Presence of PMQR genes confer reduced susceptibility to fluoroquinolones and facilitate selection of fluoroquinolone resistant strains in Enterobacterales. High-risk *K. pneumoniae* clones have acquired these antibiotic resistance determinants, that enabled them to increase their pathogenicity and survival skills. Consequently, increased diversity of plasmid-encoded

antimicrobial resistance genes facilitates spread of these clones, causing significant therapeutic difficulties. Multi-drug resistant *K. pneumoniae* strains mainly belong to ST11, ST14, ST15, ST37, ST101, ST147, ST258, ST336, ST340, and ST874. These represent high-risk international clones that played major role in dissemination in hospital settings and increased frequency in nosocomial infections. International high-risk *K. pneumoniae* ST11 has been frequently detected worldwide as a successful pathogen being associated with important co-resistance and virulence factors. However, in recent years, new drug-resistant lineages have emerged internationally and among them, KPC-producing *K. pneumoniae* ST307 has been recognized in the United States which was initially associated with production of CTXM- 15. Later on, this clone has been reported in several countries including Italy, United Kingdom, Columbia, Pakistan, Morocco, Korea, Tunisia, China as well as Serbia. Recent studies related to dissemination and antibiotic resistance of *K. pneumoniae* clones clearly showed that "fitness cost advantage" associated with high-level resistance to fluoroquinolones contributed to emergence of international high-risk *K. pneumoniae* clones. Emergence and dissemination of *K. pneumoniae* ST307 in hospital settings raises public health concerns, therefore continous monitoring of high-risk and potential highrisk clones is necessary.

2. Objectives

In our study, 103 ESBL-producing *Enterobacteriaceae* isolates from bloodstream infections (49 *E. coli* and 54 *Klebsiella spp*.) of patients treated at intensive care units of Semmelweis University between 2010 and 2014 were collected. Our work aimed at investigating the pheno- and genotypic characteristics in these strains by focusing on:

- Determination of the antibiotic susceptibility (fluoroquinolone MIC values).
- Detection of the PMQR determinants.

Based on preliminary data five strains were selected.
Selection of strains was done based on the following criteria:

I. Presence of *qnr* gene and non-wild type fluoroquinolone MIC values: Kox37,
II. Presence of *qnr* gene and high fluoroquinolone MIC values: Kpn47, Kpn115, Kpn125,
III. Multiple PMQR gene carriage together with high fluoroquinolone MIC values: Kpn33.

- Identification of sequence type (ST) of all selected strains by multilocus sequence typing (MLST), determination of pulsotype (PT) of *K. pneumoniae* strains by pulsed-field gelelectrophoresis (PFGE) as well as implementation of whole genome sequence (WGS) analysis of selected strains.
- Investigation of relative gene expression of PMQR genes by quantitative polymerase chain reaction (qPCR).
- To compare our results to the international data. Exploring correlations.

3. Methods

Identification of bacterial strains

Identification of 103 ESBL-producing *Enterobacteriaceae* isolates was done by MALDI-TOF/MS (Bruker Daltonik GmbH, Bremen, Germany).

Phenotypic detection of ESBL production

Double-disk synergy tests were performed by placing disks of ceftazidime, cefotaxime, and cefepime at a distance of 20 and 30 mm (center to center) from a disk containing amoxicillin plus clavulanic acid (20/10 μg). A "keyhole" phenomenon was regarded as positive for ESBL production.

Examination of antibiotic susceptibility

MIC values of ciprofloxacin, levofloxacin, and moxifloxacin were determined by broth microdilution methods. Results were interpreted in accordance with established EUCAST breakpoint criteria (www.eucast.org). *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were reference strains for antimicrobial susceptibility testing.

Detection of PMQR genes by polymerase chain reaction

Detection of genes encoding PMQR – *qnrA, qnrB, qnrC, qnrD, qnrS, aac(6')-Ib-cr*, *qepA* and *oqxAB* – was performed by PCR on all isolates. PCRs were performed with RedTaq DNA polymerase (Sigma-Aldrich, St. Louis, MO, USA), according to manufacturer's instructions in a total reaction volume of 30 μl. Amplicons were analyzed by gelelectrophoresis in 0.8% agarose gel (Sigma-Aldrich) with 120 V for 30 min in electrophoresis system (Bio-Rad, Hercules, CA, USA).

Determination of STs by multilocus sequence typing

Genotype of each strain was determined by MLST. The sequences of seven housekeeping genes namely, *gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB* were amplified and directly sequenced. Alleles and sequence types were assigned by using the MLST database (http://www.pasteur.fr/mlst/Kpneumoniae.html).

Bacterial whole genome sequence typing database (BacWGSTdb)

The distance based relationship between the strains was investigated by BacWGST using both the whole-genome MLST and SNP (sequenced based) strategies. Multiple genome analysis was carried out using all the draft genomes of this study and the HS11286_CP003200_ST11 as a reference genome.

Determination of PTs by pulsed-field gelelectrophoresis

Clonal relatedness of the four *K. pneumoniae* strains was analyzed by PFGE according to CDC (2000) protocol. Prepared genomic DNA of each strain was digested by XbaI restriction endonuclease (Fermentas, ABI, Germany), and DNA fragments were separated in a PFGE CHEF-DR II system (Bio-Rad Laboratories, Hercules, CA, United States). Banding patterns were analyzed by Fingerprinting II Informatix Software (Bio-Rad). *Salmonella enterica* serotype Braenderup H9812 was used as a size marker.

Whole genome sequencing

DNA of each strain was extracted by UltraClean Microbial DNA Isolation Kit (Qiagen GmbH, Hilden, Germany). Libraries were prepared using SureSelect QXT Library Prep Kit (Agilent Technologies, Santa Clara, United States). Sequencing was performed on an Illumina MiSeq system using the MiSeq reagent kit v2 generating 250-bp pairedend reads. Trimmomatic was used for preprocessing the WGS data. Only the reads longer than 50 nucleotides were used for subsequent analysis. De novo genome assembly was performed with SPAdes Genome Assembler 3.13.0. Each assembled genome was accepted for further analysis if it met all of the following quality criteria: (i) average coverage > 30 times, (ii) N50 > 15,000 bases, (iii) maximum contig length > 50,000 bases, and (iv) assembled genome size between 5,000,000 and 6,500,000 bases.

Assembled genomes were uploaded to the online bioinformatics tools ResFinder, PlasmidFinder (Center for Genomic Epidemiology, Technical University of Denmark, Lyngby, Denmark) to analyse resistome and plasmid replicon types of the isolates.

Quantitative polymerase chain reaction

Total RNA of tested strains was isolated by RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The qPCR was carried out in a Step One Real-Time PCR System (Applied BioSystems, Thermo Fisher Scientific). Separate expression of *qnrA1*, *qnrB1*, *qnrB4*, *oqxA* and *oqxB* genes were investigated whereas chromosomal *rpoB* was chosen as housekeeping gene. Set of primers and 6-FAM or VIC labeled probes were designed by Primer Express 3.0 software. The CT values of genes of interest were normalized to the CT values of housekeeping gene *rpoB.*

4. Results

Results of antibiotic susceptibility testing

About 40 ESBL-producing *E. coli* (39%) and 50 ESBL-producing *Klebsiella spp*. strains (48%) were resistant to ciprofloxacin; 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. The distribution of MIC values of ESBL-producing *E. coli* and *Klebsiella spp.* is shown in Figures 1 and 2.

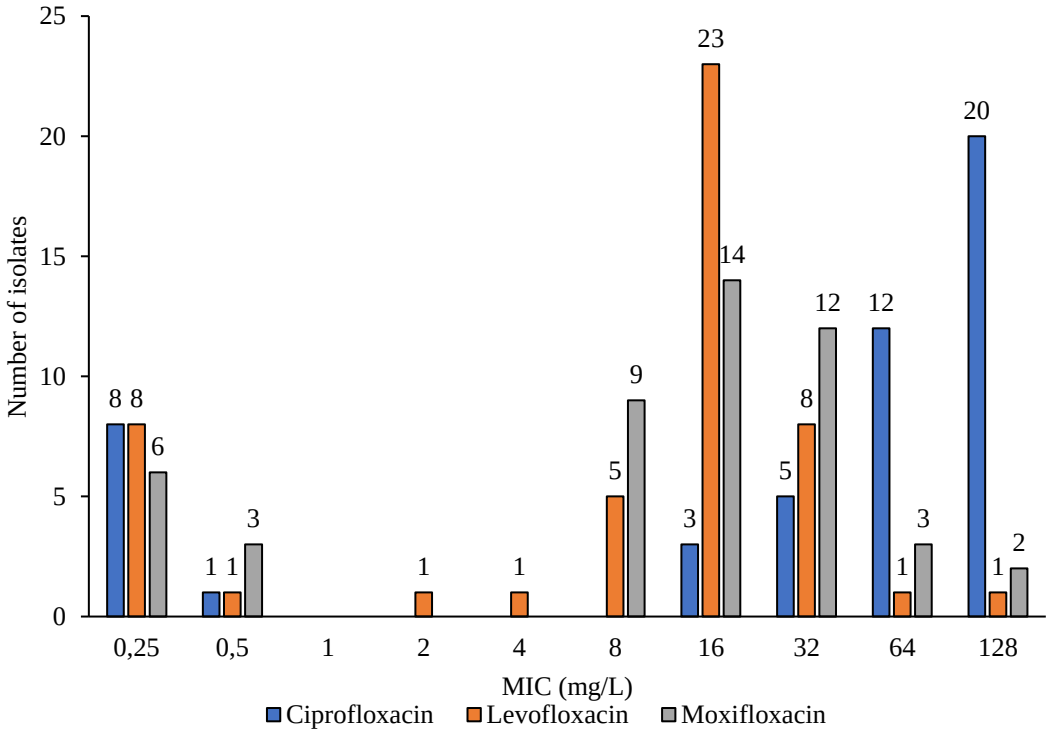


Figure 1. The MIC values (mg/L) of ESBL-producing *E. coli* isolates

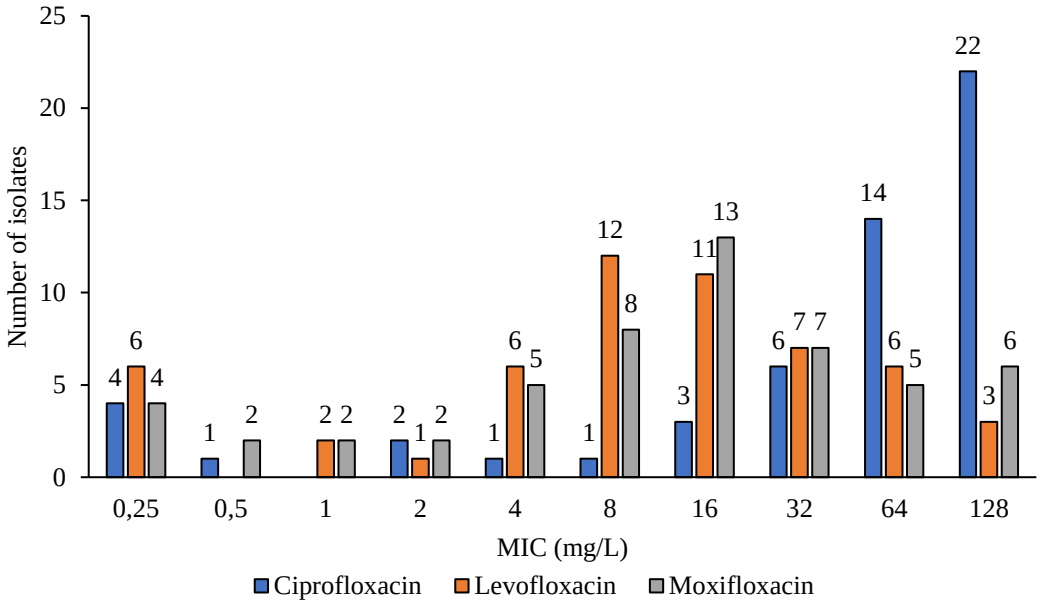


Figure 2. The MIC (mg/L) values of ESBL-producing *Klebsiella spp.*

Results of polymerase chain reaction

Among 103 ESBL-producing isolates, 77 (75%) harbored PMQR genes, and 30 *E. coli* and 47 *Klebsiella spp.* were detected. The most commonly detected gene was *aac(6')-Ib-cr* (65%). The occurrence of *qnrS* gene was 6% (1 *E. coli*, 4 *K. pneumoniae*, 1 *K. oxytoca*). Interestingly, *qnrA*, *qnrB*, *qnrC*, *qnrD*, and *qepA* were not found in any isolates. 26 *K. pneumoniae* isolates were positive for *oqxA* and 22 for *oqxB* genes. Among 77 PMQR-positive isolates, 27 (35.1%) and 1 (1.3%) carried two and three different PMQR genes, respectively. Only *Klebsiella spp*. harbored more than one gene. There is no correlation between ciprofloxacin MIC values and number of the harbored PMQR genes, as ciprofloxacin MIC values of these strains ranged between 0.25 and 128 mg/L.

Results of multilocus sequence typing

In our study, four *K. pneumoniae* and a single *K. oxytoca* were investigated by MLST and PFGE. Three different STs were identified, including ST11 (Kpn33, Kpn115, Kpn125), ST307 (Kpn47), and ST52 (Kox37).

Results of pulsed-field sequence typing and bacterial whole genome sequence typing

PFGE analysis detected three pulsotypes (PT) among *K. pneumoniae* strains, namely, 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, Kpn47 was classified as KP197 PT (**Figure 2**).

Results of whole genome sequencing analysis

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 detected in Kox37. Sequence analysis revealed that the isolates harbored different β-lactamase genes. All *K. pneumoniae* strains carried $bla_{\text{CTX-M-15}}$. Among aminoglycoside resistance genes all isolates were positive for *aac(3)-IIa*. PMQR genes were found in each tested strain 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. Chromosomal mutations conferring fluoroquinolone resistance in *K. pneumoniae* strains were also detected. Based on the sequencing data, IncFIB(K) was uniformly present in all strains. The detected resistance genes and plasmid replicons are listed in **Table 1**.

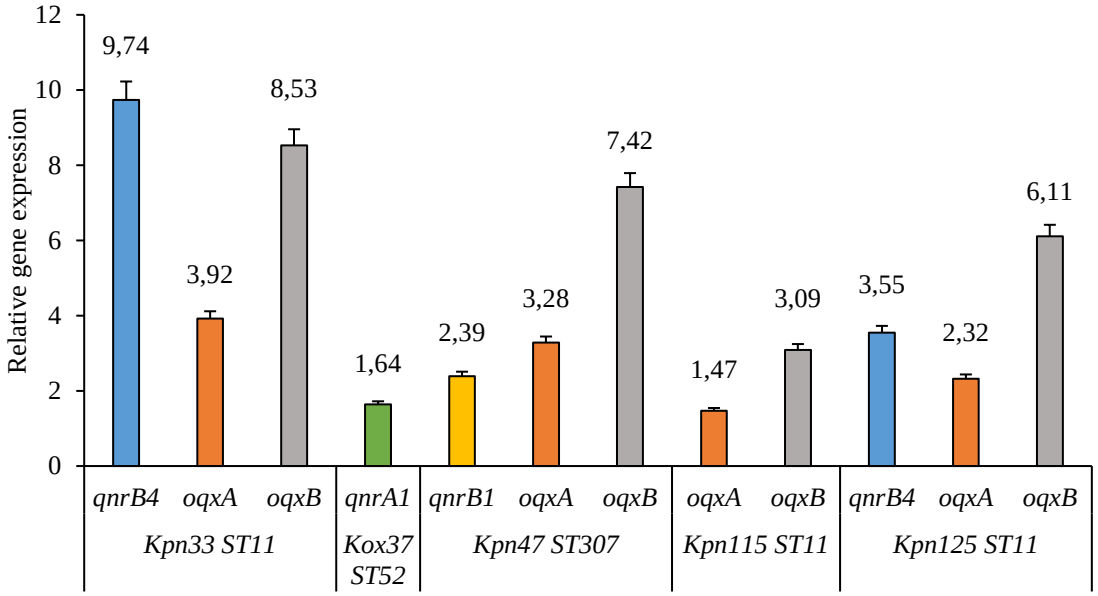
Results of quantitative polymerase chain reaction

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. 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* expressions were observed in Kpn33 (ST11) and Kpn47 (ST307). These were followed by Kpn125 (ST11) and Kpn115 (ST11). Interestingly, Kpn115 a strain of ST11 high-risk clone carried no

qnr gene moreover, it showed the lowest *oqxAB* expression. It is conspicuous that in every *K. pneumoniae* strain the *oqxB* is expressed 2–3 fold higher than *oqxA*.

Table 1. Distribution of the different resistance genes and plasmid replicons of tested strains.

ST11 Kpn33	ST52 Kox37	ST307 Kpn47	ST11 Kpn115	ST11 Kpn125	Genes
					aadA1
					aac(3)-IIa
					aac(6')-Ib
					aph(3')-Ic
					aadA2
					strA
					strB
					sul1
					sul2
					fosA
					dfrA12
					dfrA14
					dfrA29
					oqxA
					oqxB
					aac(6')-Ib-cr
					qnrA1
					qnrB1
					qnrB4
					tet(A)
					blaOXY-1-3
					blaTLA-1
					blaTEM-1A
					blaTEM-1B
					blaDHA-1
					blaOXA-1
					blaOXA-2
					blaOXA-9
					blaSHV-11
					blaSHV-28
					blaCTX-M-15
					catA1
					catB3
ST11 Kpn33	ST52 Kox37	ST307 Kpn47	ST11 Kpn115	ST11 Kpn125	Plasmid replicons
					IncFII(K)
					IncFIA(HI1)
					IncR
					IncFIB(K)
					IncL/M (pmu407)
					IncFIB(Mar)
					IncHI1B
					ColRNA1



	Kpn33	Kox37	Kpn47	Kpn115	Kpn125
MIC (mg/L)					
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
Detected mutations in QRDRs					
gyrA	Ser83Ile	-	Ser83Ile	Ser83Phe Asp87Ala	Ser83Ile
parC	Ser80Ile	-	Ser80Ile Asn304Ser	Ser80Ile	Ser80Ile

Figure 3. Level of *qnrB4* (Kpn33 and Kpn125), *qnrA1* (Kox37), and *qnrB1*, *oqxA*, and *oqxB* relative gene expression as well as the detected mutations in QRDRs. QRDRs: quinolone resistance determining regions. All MIC values are in mg/L.

5. Conclusions

We have reached the following conclusions and findings in our studies:

- The ESKAPE pathogens including *K. pneumoniae* are the leading cause of nosocomial infections (e.g. bloodstream infection) throughout the world.
- Based on our results the prevalence of *aac(6')-Ib-cr* increased dramatically from 26.6% to 68,8%, since the first detection in Hungary
- In this study, there was no correlation between fluoroquinolone MIC values and harbored PMQR determinants
- Our data also prove the spread of KP053/ST11 clone in our country.
- To the best of our knowledge, our study is the first description of ST307 in Hungary that is has been reported as a potential high-risk clone.
- This is the first report of the *qnr* gene in *K. oxytoca* ST52.
- In our study, all strains of ST11 international highrisk clone carried $bla_{\text{CTX-M-15}}$ ESBL that correlates well with earlier studies as the most common global ESBLs are the CTX-M type beta-lactamases in Enterobacterales.
- Regarding plasmid replicon types, the most common replicon was IncFIB, that was present in all strains, which confirms earlier studies.
- In every *K. pneumoniae* strain the *oqxB* is expressed 2–3 fold higher than *oqxA* which correlates well with earlier studies.
- Assembled genomes of all investigated strains were deposited in NCBI Genbank for epidemiological and phylogenetic studies.

6. List of own publications

Bibliography of the candidate's publications

[1] **Domokos J**, Kristof K, Szabo D. (2016) Plasmid-mediated quinolone resistance among extended-spectrum beta-lactamase producing *Enterobacteriaceae* from bloodstream infections. Acta Microbiol Immunol Hung, 63(3): 313-323. IF: 0,921

[2] **Domokos J**, Damjanova I, Kristof K, Ligeti B, Kocsis B, Szabo D. (2019) Multiple Benefits of Plasmid-Mediated Quinolone Resistance Determinants in *Klebsiella pneumoniae* ST11 High-Risk Clone and Recently Emerging ST307 Clone. Front Microbiol, 10: 157. IF: 4,236

Candidate's publications not related to the PhD thesis

[1] Kocsis B, **Domokos J**, Szabo D. (2016) Chemical structure and pharmacokinetics of novel quinolone agents represented by avarofloxacin, delafloxacin, finafloxacin, zabofloxacin and nemonoxacin. Ann Clin Microbiol Antimicrob, 15(1): 34. IF: 2,376

[2] Khayer B, **Domokos J**, Magyar T, Wehmann E. (2015) Antibiotic susceptibility of Hungarian *Bordetella bronchiseptica* strains isolated from pigs. Acta Microbiol Immunol Hung, 62 (Suppl): 44-45.