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MOLECULAR INVESTIGATIONS OF FLUOROQUINOLONE RESISTANCE IN *ESCHERICHIA COLI* AND *PSEUDOMONAS AERUGINOSA*

PhD thesis

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LIST OF ABBREVIATIONS

aac(6')-Ib-cr	aminoglycoside 6′-N-acetyltransferase type Ib variant cr
ABSSSI	acute bacterial skin and skin-structure infections
AG	aminoglycoside
AME	aminoglycoside modifying enzyme
AmpC	ampicillinase beta-lactamase
AMR	antimicrobial resistance
BL	beta-lactam
bp	base pair
CAP	community-acquired pneumonia
CC	clonal complex
COVID-19	Coronavirus Disease 2019
CREC	carbapenem-resistant *E. coli*
CTX-M	cefotaxime-hydrolyzing beta-lactamase
DPMT	degenerate primer MOB („mobility gene") typing
ECDC	European Center for Diseases Prevention and Control
EHEC	enterohemorrhagic *E. coli*
EMA	European Medicines Agency
ESBL	extended-spectrum beta-lactamase
EU / EEA	European Union / European Economic Area
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ExPEC	extraintestinal pathogenic *E. coli*
FDA	U.S. Food and Drug Administration
F-factor	fertility factor
FQ	fluoroquinolone

GES	Guiana extended-spectrum beta-lactamase
HAI	healthcare-associated infection
HGT	horizontal gene transfer
HUS	hemolytic uremic syndrome
ICE	integrative conjugative element
IMP	imipenemase metallo-beta-lactamase
In	integron
Inc	plasmid incompatibility group
IPEC	intestinal pathogenic *E.coli*
IS	insertion sequence
KPC	*Klebsiella pneumoniae* carbapenemase
MALDI-TOF MS	matrix-assisted laser desorption ionization time-of-flight mass spectrometry
MBL	metallo-beta-lactamase
mcr	mobilized colistin resistance gene
MDR	multidrug-resistance
MDRPAE	multidrug-resistant *P. aeruginosa*
MfpA	*Mycobacterium* fluoroquinolone resistance protein A
MIC	minimum inhibitory concentration
MLST	multilocus sequence typing
mob	mobility gene
MPF complex	membrane-associated mating pair formation-complex
NDM	New Delhi metallo-beta-lactamase
NMEC	neonatal meningitis causing *E. coli*
oqxAB	olaquindox resistant efflux pump
OXA	oxacillinase beta-lactamase

PBP	penicillin-binding protein
PBRT	PCR-based replicon typing
PER	*Pseudomonas* extended resistant beta-lactamase
pMLST	plasmid multilocus sequence typing
PMQR	plasmid-mediated quinolone resistance
pmrA*, *pmrB	phosphoetanolamin transferase coding genes
PRP	Pentapeptide repeat proteins
qepA	quinolon resistance efflux pump
qnr	quinolone resistance gene
qPCR	quantitative polymerase chain reaction
QRDR	quinolone-resistance determining region
SNP	single nucleotid polymorphismus
SPM	Sao Paulo metallo-beta-lactamase
SRA	sequence read archive
ST	sequence type
T4SS	type 4 secretion system
Tn	transposon
UPEC	uropathogenic *E. coli*
UTI	urinary tract infection
VAP	ventillator-associated pneumonia
VEB	Vietnam extended-spectrum beta-lactamase
VIM	Verona integron-encoded metallo-beta-lactamase
WGS	Whole genome sequencing
WHO	World Health Organization
XDR	extensively drug-resistant

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1. INTRODUCTION

1.1 . Medical relevance of antibiotic resistance - The „silent" pandemic

Antibiotics are one of the most remarkable discoveries in medical history, as they presented a novel opportunity to treat infections and to decrease mortality rates **(1)**. Based on Darwinian evolution theory, antimicrobial resistance (AMR) occurs when certain microorganisms, such as bacteria cope with antimicrobial selection pressure in order to survive by uptaking and expressing resistance genes. These acquired genes are transmitted later to other bacteria that results in dissemination of antibiotic resistance **(2)**. The most important drivers of this process are the misuse and abuse of antimicrobial agents however, other factors are also important, these are shown on **Figure 1.** **(3)**

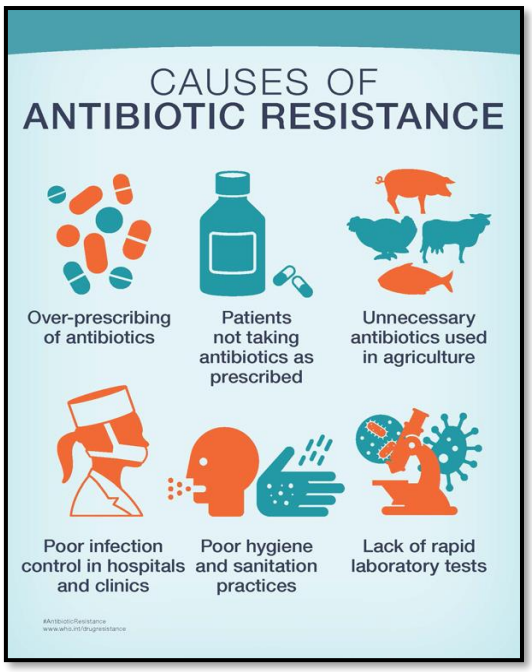

Figure 1. Main causes of antibiotic resistance development **(3)**

The problem of AMR had been already forseen by Sir Alexander Fleming, Nobel prize laureate for the discovery of penicillin in 1945, however, realization of this urgent issue is only reaching a larger audience nowadays **(2, 4)**. Currently, emergence and dissemination of multidrug-resistant (MDR) and extensively drug-resistant (XDR) bacteria pose a great challenge for modern medicine **(5, 6)**. In the shadow of COVID-19, local and global circulation of MDR bacteria and their resistance genes among various

reservoire organisms (humans, animals, plants) and shared environment (soil, agri- and aquaculture) resulted in a paralel, „silent" pandemic **(6-8)**. This situation is of great concern, it is characterized by a multisectoral and transdisciplinary theory, called as „One health" triad, as we have one common life to save (Figure 2.) **(2, 9-10)**.

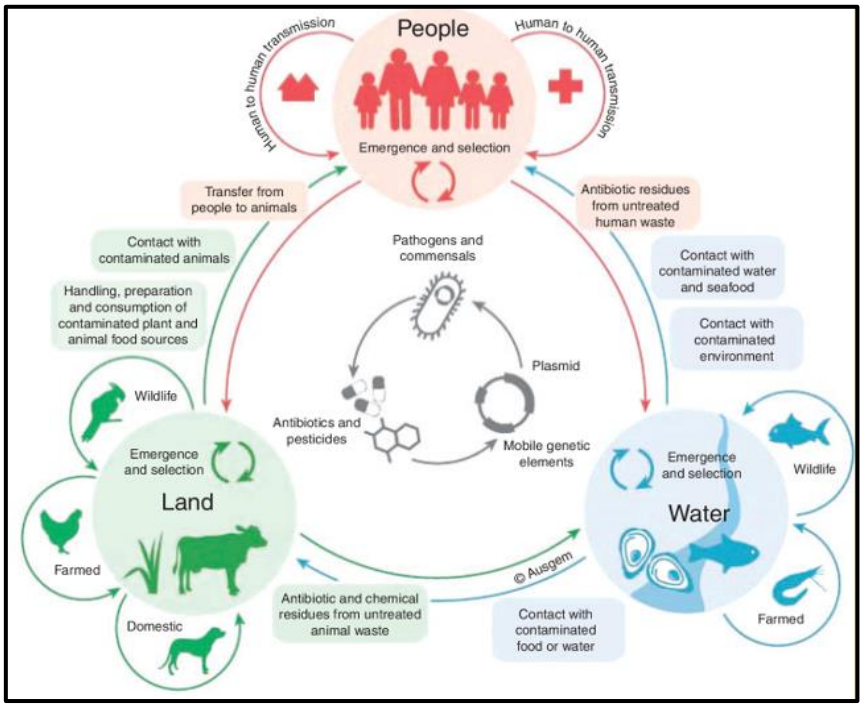

Figure 2. Aspects of „One Health" Approach **(10)**

This pandemic is recently considered as one of the biggest threats to public health worldwide. Due to limited therapeutic options in infections caused by antibiotic resistant bacteria, AMR is linked to significantly high mortality and morbidity rates **(11)**. According to data of European Center for Diseases Prevention and Control (ECDC), 33.000 people die every year in Europe as a direct consequence of an infection induced by bacteria resistant to antibiotics **(12)**. This number reached 35.000 in the United States of America in 2019 **(13)**. As a global summary, a recent study reported that MDR bacteria demanded the lives of altogether 1.27 million people in a single year worldwide and further 5 million deaths were also connected to AMR, meaning that these microbes kill more people than HIV/AIDS or Malaria **(14-16)**. If this process continues at this rate, it may become the most frequent cause of death with an estimated 10.000 000 deaths by 2050, instead of the currently leading cancer-associated lethal cases (ca. 8 million) (Figure 3.) **(16)**.

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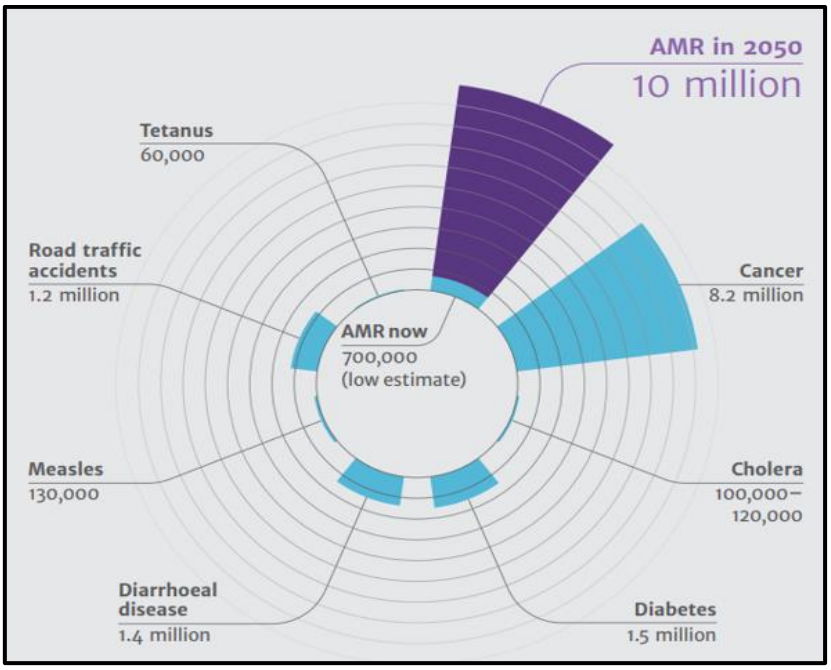

Figure 3. Estimated number of AMR-attributed deaths every year compared to other major causes of death **(16)**

In view of this urgent situation, the World Health Organization (WHO) released a priority list about the most common threatening causative agents of difficult-to-treat infections in 2018 **(17)**. Accordingly, in the last few years huge efforts have been done in order to find novel antimicrobial agents to combat MDR bacteria. Several new antibiotics with different chemical structures have already been marketed and recommended when the standard drugs cannot be administered. Among others next-generation aminoglycosides (AGs), like the crystallic plazomicin against difficult-to-treat urinary tract infections (UTIs) **(18-19);** cefiderocol, a siderophore cefalosporin recommended for UTIs, healthcare-associated infections (HAIs) and ventillator-associated pneumonia (VAP) as well as beta-lactams (BLs) combined with beta-lactamase-inhibitors were added to the antibiotic pipeline **(20-22)**. Finally, the fifth generation of fluoroquinolones (FQs), namely, zabofloxacin, finafloxacin and delafloxacin with different chemical structures were also developed in order to treat MDR bacterial infections **(23)**.

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1.2. Role of mobile genetic elements in antimicrobial resistance

1.2.1. Concept of antibiotic resistome

Following the rising tendency of MDR pathogens, theory of antibiotic resistome was introduced in 2006 and it was later implemented into „One Health" Approach. Currently, it is defined as a special collection of all types of antibiotic resistance genes, that often circulate in nature. It contains already known vertically and / or horizontally acquired and intrinsic (natural) resistance mechanisms and silent (cryptic)- as well as proto-resistance (precursor form). The novel (anthropogenic) resistance genes are emerging from precursors, that show a susceptible phenotype and a low or the lack of activity until development of mutations. As a prequel form of proto-resistance, cryptic genes are functional, but not expressed genes with phenotypic sensitivity **(24-25)**.

All of these genetic materials belong to the microbial ecological system, called as microbiome. The relatively stable part of microbiome is the so-called core resistome and the highly variable and flexible moiety is known as mobile resistome (mobilome). Furthermore, recent studies sketch a third set named as accessory resistome. (**Figure 4.**) Mobilome is genetically connected to mobile genetic elements, namely, plasmids, insertion sequences (ISs), transposons (Tn), integrons (In) and integrative conjugative elements (ICEs) **(24-26)**. This genetic process is followed by clonal expansion of MDR bacteria **(27)**.

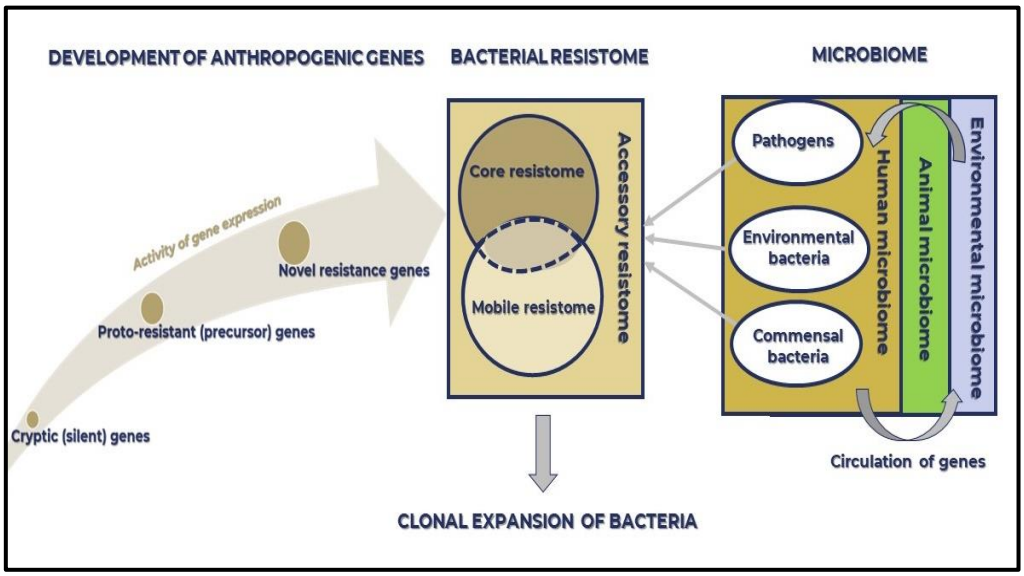

Figure 4. Ecological genesis of antibiotic resistance based on „One Health" Approach (own figure)

1.2.2. Importance of plasmids

1.2.2.1. General physiology and structure of plasmids

As one of the clinically most relevant part of mobile resistome, notion of plasmids was introduced by Joshua Lederberg more than 70 years ago **(28)**. Plasmids are defined as double-stranded, superhelical extrachromosomal DNA molecules with variable size ranging from 1.000 to 100.000 base pairs (bp). Although they are non-essential components of microbes, approximately 50% of bacteria, including MDR superbugs like Enterobacteriales or *Pseudomonas aeruginosa* and Gram-positive bacteria, such as *Staphylococcus aureus* and *Bacillus anthracis* carry plasmids **(29)**. Generally, microbes do not carry and express plasmid-encoded genes under non-stressed circumstances, because it would generate an unnecessary energy burden **(30)**.

Plasmids are characterized by self-replication, so they can be replicated independently from the cell cycle's replication process, although their function is linked to the host cell's ATP pool and enzymes (mainly DNA-polymerases) **(31)**. In order to detect their quantity, plasmid copy number is used. It means the quantity of plasmid DNA compared to that of chromosomal housekeeping genes **(32)**.

Regarding the structure of plasmids, a distinction is made between coding and non-coding sections, the latter is called as plasmid backbone. Coding regions are divided to accessory or additional genes and the so-called core genes, that encode central functions for plasmid physiology, including DNA replication and plasmid transfer from one host to an other **(33)**. Furthermore, accessory genes give an advantage for the bacterial host cell by acquisition of plasmids, as they carry functions that are selected by evolutionary effects. Of them, cargo genes are mainly responsible for spread of antibiotic resistance, production of antimicrobial agents, tolerance against heavy-metal and disassembly of xenobiotics and toxins. Further sequences encode virulome, a group of genes, that contribute to virulence **(33-34)**. Consequently, plasmids are significant gene reservoirs in bacterial genetics and play a key role in life and evolution of microbial environments as well **(33-35)**. „One Health" Approach also emphasizes their importance, as plasmid metagenome (plasmidome) refers to complete genetic content of present plasmids in a given environment **(36)**.

1.2.2.2. Aspects of plasmid mobility

Plasmids and their resistance genes are generally transfered among bacteria during horizontal gene transfer (HGT), namely, conjugation, a contact-dependent intercellular unidirectional transmission. Based on this process, conjugative and non-conjugative plasmids are defined. In case of *Enterobacteriaceae* a bridge-like structure, named as sex pilus („mating pore") or fertility factor (F-factor) is required for conjugation; it was first described in *Escherichia coli* strains. Plasmid-donor bacteria are F-factor positive microbes, marked as F+, non-carriers and recipients as F— **(37)**. With the presence of F-factor the given bacterium acquires a donor property, so if energetic level of a carrier cell and genetic markers of the plasmid are appropriate, the plasmid is transferable /mobilizable.

As a criteria, conjugative plasmids need to express essential sequences, including DNA-transfer-duplication inducer mobility (*mob*) gene variants and the *membrane-associated mating pair formation (MPF)-complex*, that builds up type 4 secretion system (T4SS), a key part of conjugation bridge (Figure 5.) **(38-39)**. Developed from the double DNA chain of plasmid genetic material, only a linear molecule will be passed through the conjugation apparatus, the missing complementer part will be synthetized in donor and recipent cells as well. It is the so-called „rolling circle replication", that plays a potential role in dissemination of AMR as it results in two copies of the same plasmid's genetic material **(31)**.

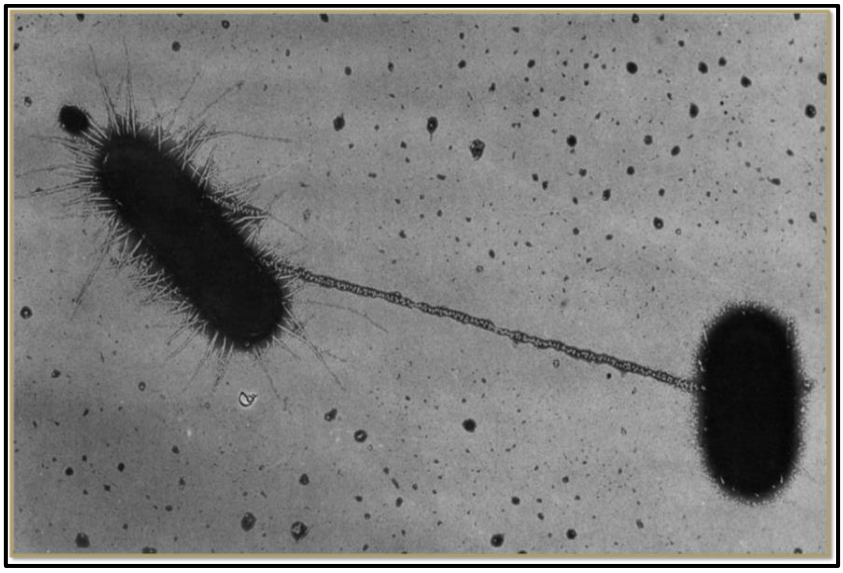

Figure 5. Conjugation between plasmids via sex pilus **(39)**

1.2.2.3. Evolution dynamic of plasmids: regulation of plasmid copy number

In general, bacterial cells are able to carry more plasmids, it is also described as „plasmid co-infection". Uptake of more than one plasmid is not a random event, it is strictly regulated by their selective pressure on plasmid backbone-encoded proteins. As vehicles of HGT, plasmid evolution is regulated by interplays not only between plasmids and their host cells but also between more plasmid subtypes. This phenomenon is also known as plasmid replication incompatibility. It influences population dynamics of AMR, hence a resident plasmid determines uptake of a novel genetic element from other bacteria. These interactions may be beneficial or they can perform an evolutionary disadvantage that leads to loss of these elements. Most of these changes are linked to alterations of plasmid copy number control, that is dependent from replication origo, number and size of plasmids **(34, 40-41)**.

Incompatibility is strongly associated to plasmid instability. Also known as plasmid paradox, it means that these genetic materials are able to circulate long-lasting among bacteria, despite of the extra metabolic burden they cause through their copy number and gene expression (**42-43)**. Thus, it is more beneficial for bacteria, if co-expression of multiple resistance phenotypes is associated to the same operon in the same MDR plasmid.

Based on their medical importance in AMR, identification of plasmids is a relevant part of bacterial typing. Formerly, members of Plasmid Section of the National Collection of Type Cultures established 27 groups within *Enterobacteriales.* Since this classification is based on incompatibility properties, these are named as plasmid incompatibility (Inc) groups. Currently, the most widely performed methods to detect plasmid incompatibility groups are PCR-based replicon typing (PBRT) and degenerate primer MOB („mobility") typing (DPMT). PBRT is available since 2005, it targets replicons of major plasmid families of Enterobacteriales (namely, HI2, HI1, I1-γ, X, L/M, N, FIA, FIB, FIC, W, Y, P, A/C, T, K, B/O) **(30, 44)**. On the other hand, DPMT detects Gammaproteobacteria relaxase genes, that are common sequences in transferable plasmids **(45)**. Due to limitations of these methods (e.g., diagnostic difficulties of rare or novel plasmid variants) plasmid multilocus sequence typing (pMLST) with bioinformatic tools is recommended for analysis of plamids' phylogenetic profile. In the course of pMLST 400-500 bp amplified plasmid alleles are sequenced and compared to GenBank data from the website

https://pubmlst.org. Then novel or already isolated sequence types (STs) and their variants are differentiated **(44, 46)**.

1.2.3. Transposable elements, genomic islands, integrons and ICEs

„Jumping genes" or transposable elements, like ISs (e.g., IS26) and Tns (e.g., Tn3 family) are separate DNA segments that are able to transpose themselves (and carried resistance genes) by their transposase enzyme almost fortuitously to novel intracellular positions in the same or even to different DNA molecules, like bacterial chromosome or plasmids. This moving effect occurs in most common cases as a „cut and paste" (whole IS will insert into new site) or „copy and paste" (half of replicated IS will integrate), eventually „copy out and paste in" (whole duplicated IS will incorporate) mechanism. They generally own a strong promoter that aims expression of given resistance gene frequently against BLs, AGs, FQs and tetracyclines. Moreover, many MDR pathogens, such as *Acinetobacter baumannii* is able to build genomic islands or gene cassettes, that are commonly composed of transposable elements **(47-48)**.

Gene cassettes are short mobile sequences (ca. 0.5 to 1 bp) composed of a single or sometimes two genes, but generally they do not have a promoter or an *attC* recombination site. These missing parts, so correct tools for expression of resistance genes, are provided by Ins, into which the gene cassettes are inserted. In other words, Ins are special DNA molecules that work as genetic assembly platforms, that convert uptaken open reading frames into functional genes. Correspondingly, they are incorporated in chromosomal material of many bacteria. Multiple gene cassettes can be obtained by the same In, these variants are the so-called superintegrons (formerly gene cassette arrays) **(47-50)**. Of them, class-1 integrons are the most frequently detected ones in MDR pathogens. They are characterized by *int1* gene, that contains the promoter sequence **(47)**.

Finally, ICEs make a diverse group of mobile components, those are frequently found in both Gram-negative and Gram-positive bacteria. Interestingly, ICEs are also self-transferable by using conjugation bridge like plasmids. In comparison, in most common cases ICEs are integrated into host bacterial cell's chromosome and join to its replication as an original part of it. They are built up by a backbone, into which resistance genes are acquired **(47, 51)**. As a current threat, mobilization of carbapenemase enzymes is prevalently linked to these elements **(52)**.

As a summary, evolutionary changes of these mobile genetic materials play a determining role in dissemination of MDR, especially in emergence of the so-called international high-risk clones of ESKAPE pathogens.

1.3. ESKAPE-pathogens

1.3.1. Clonal expansion of resistant bacteria

The most frequently detected MDR bacteria belong to the group of ESKAPE-pathogens, namely, *Enterococcus faecium*, *S. aureus, Klebsiella pneumoniae, A. baumannii*, *P. aeruginosa* and further members of Enterobacteriales, such as *Enterobacter* spp. or *E. coli*. Additional bacteria, like *Stenotrophomonas maltophilia* and *Clostridioides difficile* are also added to this group according to their healthcare burden **(53-54)**. These pathogens easily develop resistance against numerous antibiotics, and are prevalent causative agents of difficult-to-treat nosocomial infections, including sepsis, UTIs, wound- and skin infections and VAP **(13, 55)**.

Furthermore, ESKAPE-pathogens are also highlighted as "critical" and "high" priority microbes on WHO priority list, therefore development of novel effective antibiotics is urgently needed worldwide against these pathogens. In „critical" category carbapenem-resistant *P. aeruginosa* and carbapenem- and third-generation cefalosporin-resistant Enterobacteriales are also listed **(17)**.

International high-risk clones of these superbugs are defined as being prevalent across the globe, able to colonise and persist for more than 6 months in different hosts and later these can be successfully passed on. They also have augmented pathogenicity and fitness associated to multiple resistance determinants and they induce severe and/or recurrent infections **(56)**. Based on their genetic markers, MLST determined phylogroups, STs, clonal complexes (CCs), clades and subclades can be classified. Furthermore, these lineages often demonstrate different geographic distributions, therefore their evolution and dissemination can be tracked during surveillance programs and epidemiological studies **(56-57)**. Accordingly, early diagnosis and genome analysis are key points in understanding their evolutionary puzzle **(58)**.

Emergence of MDR high-risk clones follows the so-called "genetic capitalism principle", meaning that the more resistant clones with the highest fitness-associated

resistance carrier genetic elements are selected out and then they are able to disseminate **(27)**. Genomes of these bacteria share a remarkable plasticity and complexity. Thus, they are able to acquire and express multiple virulence and resistance markers at the same time, including the most frequently reported extended-spectrum beta-lactamases (ESBLs), carbapenemases (e.g., metallo-beta-lactamases (MBLs)), mutations of quinolone-resistance determining regions (QRDR), plasmid-mediated quinolone resistance markers (PMQRs), aminoglycoside modifying enzymes (AMEs), sulfonamide-trimethoprim, macrolide or tetracycline resistance mechanisms **(57)**. They obtain this arsenal of resistance genes through various ways (**Figure 6.**), namely, transformation, transduction, conjugation, and through uptake of mobile genetic elements **(57)**.

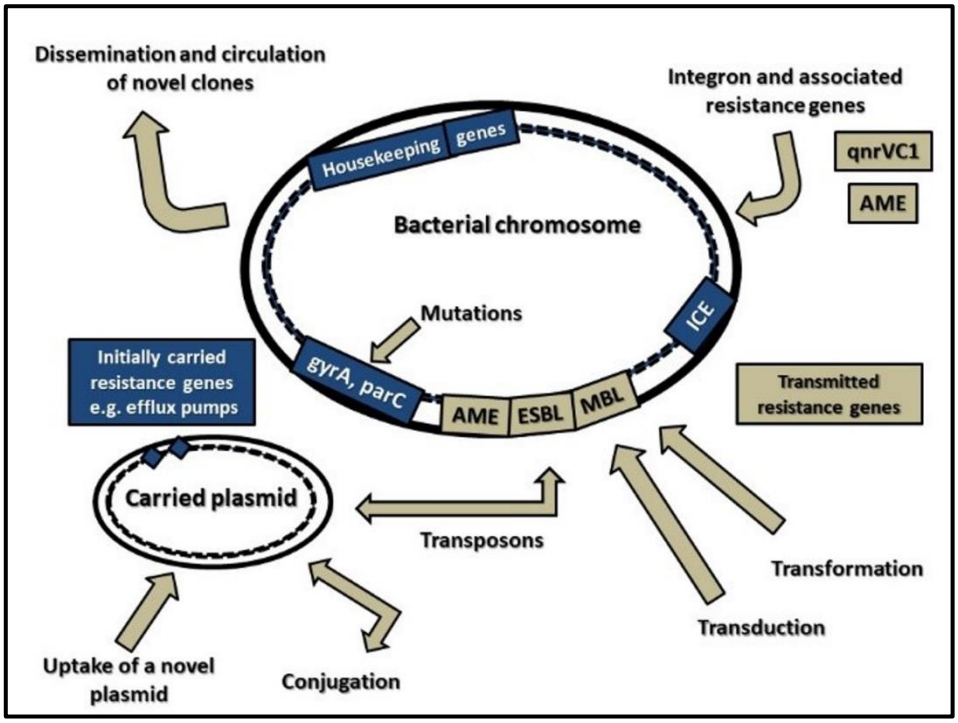

Figure 6. Uptake of resistance mechanisms in case of a MDR international high-risk clone (own figure)

As a common feature, FQ resistance generally plays a central role in emergence of high-risk clones **(59-60)**. In case of *E.coli* and *P.aeruginosa* clones it has been already described, that major clones suffer lower cost for maintaining their favourable fitness after evolvement of mutational FQ resistance. Consequently, it promotes their stabilisation, survival acquisition of further resistance genes and transmission **(60)**. Moreover, according to "perfect storm" theory, sequentional uptake of virulence factors also

remarkably contributes to clonal expansion of MDR bacteria, because obtainment of these genes is linked to elevated antibiotic resistance rates **(59)**.

1.3.2. *E. coli* high-risk clones

E. coli belongs to Enterobacteriales, and it is a Gram-negative rod-shaped commensal bacterium in human gastrointestinal tract and it is also responsible for several infections. Intestinal pathogenic *E.coli* (IPEC) strains are capable of taking up toxin genes and virulence factors, those are associated to variable severity diarrhoea with possible complications, such as hemolytic uremic syndrome (HUS) in case of Enterohemorrhagic *E.coli* (EHEC) **(61)**.

On the other hand, extraintestinal pathogenic *E. coli* (ExPEC) is a well-known causative agent of severe community-associated and nosocomial infections. The predominant types of ExpEC are uropathogenic *E.coli* (UPEC), neonatal meningitis causing *E.coli* (NMEC), and further *E. coli* strains cause peritonitis and bloodstream infections **(62-65)**. Additionally, diseases related to MDR ExPEC are linked to high number of hospital outbreaks worldwide associated to longer hospital stays, increased health care costs and mortality rates **(11, 66-67)**. Typical colonies of *E.coli* on Eosin-methylene blue agar are presented on **Figure 7.**

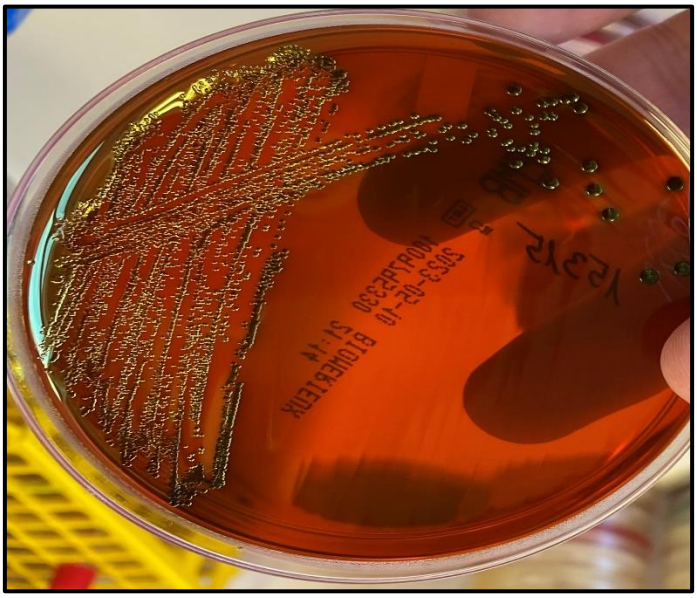

Figure 7. Colonies of *E.coli* (own picture)

E. coli strains are clustered into eight phylogroups (A, B1, B2, C, D, E, F and G) according to Achtman scheme (PCR-based MLST) and complete genome sequences **(68)**. This phylogenetic classification is applicable for comparing serogroups, virulome and resistance patterns, plasmids and evolution lines of ExPEC **(67-70)**. The most successful subclone at the moment is clade C of ST131, however, further frequently reported lineages are ST69, CC10, ST405 and CC38 **(56, 70)**. Additionally, novel emerging subtypes, for instance sister clones of ST131, ST43, ST457 and ST73 are also reported **(71-73)**. These strains are equally reported from human isolates, soil samples and zoonotic sources, including marine mammals and birds as well **(68-73)**.

Alarmingly, high prevalence of ESBL-producing and an increasing number of carbapenem-resistant *E. coli* (CREC) are detected worldwide **(17)**. The most commonly reported ESBLs are CTX-M-type (cefotaxime-hydrolyzing beta-lactamase) enzymes, namely, $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-27}}$ **(74)**. MBLs and other carbapenemases are rapidly transfered by different mobile genetic elements. The most frequently reported carbapenemases in *E. coli* are NDM (New Delhi metallo-beta-lactamase), VIM (Verona integron-encoded metallo-beta-lactamase), IMP (imipenemase), KPC (*K. pneumoniae* carbapenemase), OXA-48 (oxacillinase) and as a novel pan-European risk, OXA-244. **(75-77)** A recent study showed that prevalence of MDR ExPEC reached nearly 60% among $bla_{\text{NDM-5}}$ positive isolates in Europe. In this case the most frequent clinical sample was urine and relatively few MDR clones were found in hemoculture. Moreover, resistome of these strains are characterised by harbouring other resistance markers against FQs, AGs, fosfomycin and nitrofurantoin resistance genes **(78-80)**.

Of great concern, due to the limited number of available effective antibiotics, colistin is one of the few remaining last resort antibiotics against CREC **(67)**. Worryingly, MDR ExPEC clones increasingly contain colistin resistance genes, namely, plasmid-mediated *mcr* (mobile-colistin resistance gene) variants or chromosomal mutations of *pmrA* or *pmrB* (phosphoetanolamin transferase coding genes) **(70, 81)**. Moreover, high variability of ExPEC genome is seen through exchange of resistance genes between different strains of Enterobacteriales (e.g.: *K. pneumoniae)* or even non-fermentative pathogens, such as *P. aeruginosa* **(82)**.

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1.3.3. *P. aeruginosa* high-risk clones

P. aeruginosa is a Gram-negative rod-shaped bacterium. It is an ubiquitous bacterium, therefore, it can be present in different niches including water, soil, and environment. In addition, a massive biofilm-producing capacity aids its long-lasting survival in special circumstances including nosocomial environment. Based on these features, *P. aeruginosa* is one of the most important opportunistic human pathogen, that is responsible for a wide range of severe diseases, such as VAP, catheter-associated infection and burn wound infections, bacteriaemia, sepsis, as well as contact lense-related keratitis, and infections of cystic fibrosis patients **(83)**. It is able to produce a variety of virulence factors, that gives the bacterium a diverse macroscopic appearance. (**Figure 8.**)

Figure 8. Unique colonies of *P.aeruginosa* on blood agar (own picture)

Furthermore, this pathogen has intrinsic resistance connected to its extraordinary low outer-membrane permeability and upregulation of efflux pumps **(84)**, and it can also use acquired resistance mechanisms against a variety of antimicrobial drugs **(85)**. These special properties resulted in selection and clonal expansion of MDR and XDR *P.aeruginosa*. Additonally, evolvement of these strains is strongly dependent on the given geographic region, particularly local antibiotic use, circulation of transferable genes among these pathogens, travelling trends and earlier hospitalization events of patients **(86-88)**.

Recently, the following MDR *P. aeuginosa* (MDRPAE) international high-risk clones are commonly detected by MLST from HAIs: the predominant ST235 and ST111, ST175, ST773, ST233, ST357, ST654 as well as ST277 **(89-91)**.

They are also characterised by a resistome that harbour a colourful collection of beta-lactamases, AMEs, mutations of QRDR determinants, PMQRs and colistin resistance determinants **(85)**. The most prevalent BL-hydrolyzing enzymes are frequently associated to each other in MDRPAE clones. They are ampicillinases (AmpC), ESBLs like *Pseudomonas* extended resistant beta-lactamases (PER), Guiana extended-spectrum beta-lactamases (GES) as well as Vietnam Extended Spectrum Beta-lactamase (VEB), just as ICE-mediated OXA-type beta-lactamases and MBLs, such as IMP, VIM, NDM types **(92)**. Among carbapenemases the $bla_{\text{NDM-1}}$ was earlier the most frequent one in the predominant ST235 clone **(93)**, but nowadays its novel variants are increasingly detected in other MDRPAE high-risk clones as well **(94)**. Of great concern, a novel oxacillinase variant, called as OXA-681 was also described; it easily clefts ceftolozane and facilitates cross-resistance to new synergistic antibiotic combinations **(93)**.

Interestingly, as an exception among MDRPAE high-risk clones, ST277 is localised mainly in South-America (Brazil) as an endemic clone. Its resistome is built up by well-defined genomic islands, CRISPR-Cas system type I-C and numerous single nucleotid polymorphismus (SNP) linked to a certain type of carbapenemases, called as Sao Paulo metallo-beta-lactamase ($bla_{\text{SPM-1}}$). Furthermore, this clone is curiously susceptible to colistin **(95-96)**. Based on „One Health" Approach, zoonotic spread of ST277 was also investigated. The results showed that migratory birds played a relevant role in transmission of ST277 in Brazilian region **(97)**.

Moreover, other studies confirmed a zooanthroponotic transmission way of VIM-2-producer ST233 clone as well. In this case this pathogen was able to colonize in a 50-year old man during a hospital stay. Based on anamnestic data, he suffered from a brain infection originated from a complicated traumatic brain injury by a traffic accident, thus, he stayed 1 month long in intensive care unit and then 4 other months in the clinic. After a few weeks arriving home from the hospital, his dog had a massive ear canal infection with a great amount of smelly secretion. Causative agent was the same ST233 clone that was isolated from the patient after hospitalization and also from household environment of his house, including surface of his sofa **(98)**.

1.4. Fluoroquinolones

1.4.1. General features: generations, clinical indication, side-effects

FQs are nucleic-acid synthesis inhibitor broad-spectrum bactericidal antibiotics. Their main targets are ATP-dependent subunit A (GyrA) of DNA gyrase (topoisomerase II) and subunit A (ParC) of topoisomerase IV enzymes. Energetic background is covered by subunit B (GyrB or ParE) of their tetrameric structure. These enzymes create a negative supercoil in bacterial DNA; in this way they uncoil the helical structure and initiate duplication that is necessary for cell division **(99-100)**. Based on the relative binding affinity on topoisomerases, its linkage to their antimicrobial spectrum, chemical structure and pharmacokinetic properties FQs are divided into different classes (**Table 1.**) **(101-107)** .

One of the first zwitterionic quinolones, nalidixic acid, that lacked a fluorine atom was developed in 1960s. It was recommended for treatment of UTIs, however as a narrow-spectrum drug nowadays it is not used for therapy. On the other hand, its chemical structure was modified multiple times in the last decades by supplementing different side-chains to its bicyclic quinolone ring in order to reach a higher tissue penetration and enhanced antibacterial activity **(23, 108)**.

The clinically most relevant substituents of the basic two-part quinolone ring are a fluorine atom in position C6, cyclopropyl or difluorophenyl at C1, a piperazine ring at C7 and a halogenic part, a methoxy-derivate or a fused third ring at C8 (**Figure 9.**) **(23, 108-109)**. These structural changes lead to the synthesis of classic FQs from 1970s, such as norfloxacin, ofloxacin, ciprofloxacin, that are predominantly active against Gram-negative bacteria or moxifloxacin, that is mainly active against Gram-positive bacteria. As an important agent of this group, levofloxacin (a stereoisomeric molecule of ofloxacin) shows a remarkable activity against both Gram-negatives and Gram-positives **(23, 108)**. Their concentration- and time-dependent broad-spectrum bactericidal effect develops fast in the presence of Mg^{2+} as they reach a therapeutical concentration in various tissues. Due to these favourable clinical features they were earlier worldwide used to treat various infections like UTIs, community-acquired pneumonia (CAP), enteric-, skin-, soft tissue-, bone- and joint infections. Consequently, widespread resistance developed, that resulted in the currently restricted clinical indication of FQs **(101-102, 109-110)**.

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Table 1. Generations and pharmacological features of FQs **(101-107, 111, 112)**

*ABSSSI refers to acute bacterial skin and skin-structure infections

Generation		Agent	Antibacterial spectrum	Indication
Quinolone		**nalidixic acid**	Enterobacterales	Currently not used
Classic FQs	**1.**	**norfloxacin**	Enterobacterales	UTI, gastrointestinal infections
	2.	**ciprofloxacin, ofloxacin**	mainly Gram-negative bacteria	UTI, osteomyelitis, skin- and soft tissue infections, gastrointestinal infections
	3.	**levofloxacin**	Gram-negative, Gram-positive and intracellular bacteria	systemic and respiratory tract infections
	4.	**moxifloxacin**	Gram-negative, but mainly Gram-positive bacteria and anaerobes	systemic and respiratory tract infections
Next-generation (5.) (dual-targeting) FQs		**finafloxacin**	Gram-negative and Gram-positive bacteria	otitis media caused by *S.aureus* and *P.aeruginosa*
		zabofloxacin	Gram-negative and Gram-positive bacteria, lack of potency against nosocomial MDR bacteria	acute bacterial exacerbation of COPD
		delafloxacin	Gram-negative, Gram-positive, anaerobic, intra- and epicellular bacteria	ABSSSI*, CAP, sepsis and intraabdominal infections

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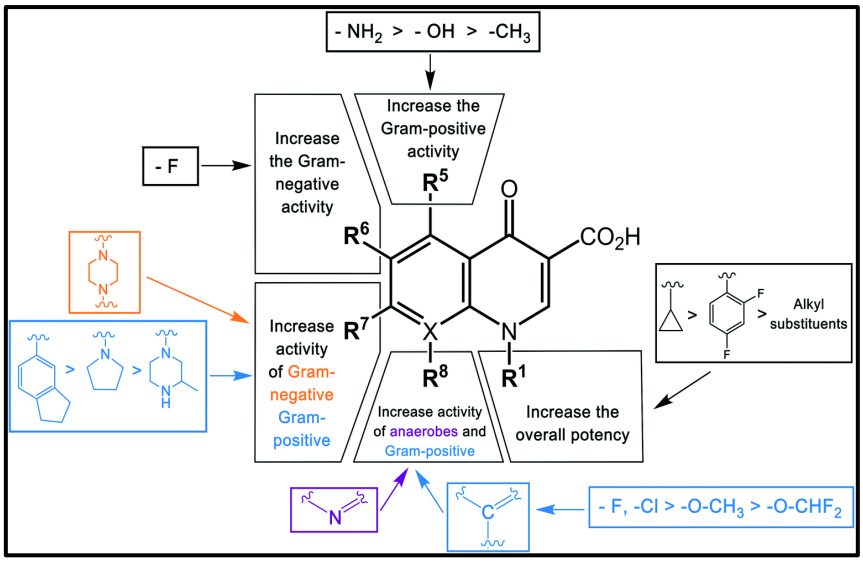

Figure 9. Basic structure and clinical relevance of substituents of FQs **(109)**

In addition, all known FQs induce several possible side-effects, such as gastrointestinal symptoms (nausea, diarrhoea), photosensitivity (dermatitis), neurological problems (e.g.: convulsion, peripheral neuropathy), spontaneous tendon rupture, exacerbations of myasthenia gravis, bone development disorders, hypersensitivity, hyerglycaemia and renal or hepatic impairment as well as cardiovascular complications such as long QT-syndrome. They may also provoke dysbacteriosis, that is significantly associated to *C. difficile*-infections. Based on these adverse effects, FQs are contraindicated during pregnancy and for pediatric use **(101-102, 109)**.

Recently marketed next-generation FQ agents, like delafloxacin or zabofloxacin represent favourable side effect profiles, as unlike earlier generations, they lack pharmacological interactions with commonly used medicines and arrhythmia **(103-105, 111)**. However, finafloxacin is contraindicated for ophtalmic use as teratogenic complications were reported in animal studies **(106)**. These novel agents are also known as dual-targeting FQs as they bind to topoisomerases of both Gram-positives and Gram-negatives with equal intensity **(103-107)**. They have a special chemical structure that contains a chiral cyano-substituent and pyrrolo-oxazinyl element in case of finafloxacin and associated to the original quinolone ring, zabofloxacin carries methoxyimino and naphthyridine side chains **(106-107)**. (**Figure 10.**) In regard to the high number of MDR bacteria, these new drugs are approved only in well-described clinical cases, such as ABSSSI of adults, CAP or otitis externa (**Table 1.**) **(101-107)**.

1.4.2. Delafloxacin, a unique next-generation fluoroquinolone

Delafloxacin is recently the only anionic (non-zwitterionic) FQ, that has a special chemical structure described as 1-(6-amino-3,5-difluoro-2-pyridinyl)-8-chloro-6-fluoro-7-(3-hydroxy-1-azetidinyl)-4-oxo-1,4-dihydro-3-quinolinecarboxylate **(108)**. (**Figure 10.**) This composition provides a weak acidity (uncharged form by acidic pH), that contributes in its transmembrane uptake. Thus, delafloxacin will be accumulated intracellularly (neutral pH), where it develops its anionic (deprotonated) form **(105, 112)**. Anionic appearence displays a wide-spectrum concentration-dependent bactericidal effect by dual inhibition of bacterial gyrase and topoisomerase IV enzymes of both Gram-positives (e.g., Staphylococci, Enterococci) and Gram-negatives (e.g., ESBL-producer *E.coli*, *K.pneumoniae*, *P.aeruginosa*, *Neisseria gonorrhoeae*) **(103-105, 112)**. This effect is prevailed under acidic circumstances, including abscesses, infected regions of human skin and soft tissue and inside of phagolysosomes **(108)**.

Chlorine atom of delafloxacin aids inhibition of DNA-synthesis of anaerobic bacteria (e.g.: *Bacteroides fragilis*) and it also targets intra- and epicellular microbes causing atypical pneumonia (e.g.: *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*) **(103-105, 112)**. Therefore, the current guidelines recommend delafloxacin as a drug of choice for ABSSSI and CAP **(103-105)**. Furthermore, according to its augmented bactericidal activity in acidic environment it is also a promising novel antibiotic against *Helicobacter pylori* **(113)**.

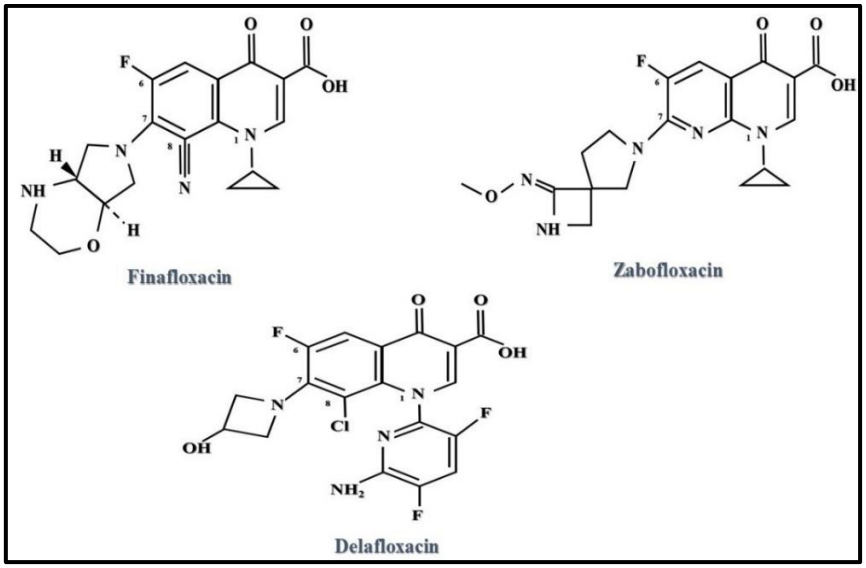

Figure 10. Chemical structures of next-generation FQs (own figure)

Moreover, delafloxacin has a heteroaromatic ring connected to nitrogen atom of quinolone ring, that lends a larger molecular surface. It is one of the most important components, as it provides an extended antibacterial efficacy against certain bacteria that are non-susceptible to classic FQs, because delafloxacin binds to more sites of the main target enzymes **(108, 112)**.

FDA and EMA approved delafloxacin for oral and intravenous use as well as switch dosing. Based on these protocols, per os a 450 mg dose and as an infusion a 300 mg dose is necessary to be given to the patients to reach the adequate concentration-time profile. Compared to earlier generations, delafloxacin does not bind to P450 isoenzymes, so in most common cases it does not induce pharmacological interactions, it is not influenced by even other antibiotics or hepatic impairment. Rarely, it may show a chelation interplay with multivalent metal cation (e.g., Al3+, Mg2+, Fe2+, Zn2+) containing drugs that results in a decreased oral bioavailability (it is ca. 58,8% without any effects) **(103-105)**.

Besides, similarly to zabofloxacin, delafloxacin performs frequently occuring well-tolerated adverse effects, namely, diarrhoea, vomiting, infusion site extravasation and other uncommon complications, containing higher blood sugar level and elevated liver enzymes. Interestingly, the special anionic structure is also linked to the lack of some well-described FQ-related side effects, like photosensitivity and QT interval prolongation **(103-105, 108, 112)**. Other classic secondary effects, like *C.difficile*-associated diarrhoea or hypersensitivity were described as less serious and dose-dependent symptoms **(112)**. Teratogenic features were not reported during animal studies, but currently delafloxacin is not recommended for pregnants or children **(103-105, 108)**.

1.4.3. Resistance mechanisms

1.4.3.1. Mutations of QRDR determinants

FQ resistance is explained by mutations in gyrase and topoisomerase IV enzymes encoded by QRDRs in *gyrA*, *gyrB*, *parC* and *parE* chromosomal genes **(100)**. This mechanism is also known as high-level FQ resistance because ciprofloxacin minimum inhibitory concentration (MIC) can reach 1 mg/L or even higher values **(114-115)**. In case of Gram-negative bacteria gyrase displays a higher susceptibility to FQs than that of topoisomerase IV. Correspondingly, the first aminoacid alteration of the DNA-binding surface ordinarily develops in GyrA and it decreases affinity and/or function of gyrase to

antibiotics **(116)**. Generally, further mutations are accumulated later in *parC*, *gyrB* and sometimes *parE* (in descending order of frequency), because a single change of GyrA provides resistance only against nalidixic acid, for higher level FQ resistance additional mutations are needed. For instance, a second mutation of GyrA or double mutations of GyrA-ParC / GyrA-ParE as well as triple mutations of GyrA-ParC-ParE were described in FQ-resistant Gram-negative bacteria **(100, 117-118)**. On the other hand, co-expression of a complementary gene (e.g., plasmid- or ICE-carried resistance mechanisms like efflux pumps) together with a single *gyrA* aminoacid substitution may also result in a clinically resistant phenotype **(117-120)**. To solve this clinical problem, delafloxacin was synthesized in order to reduce this mutational process by targeting more sites of these enzymes with equal affinity **(108, 112)**.

In general, the higher the FQ MIC of a given strain (probability of developing resistance), the more mutations of *gyrA*, *gyrB*, *parC* and *parE* are present **(121-122)**. The clinically most relevant bacteria are able to control their mutation frequence within certain limits. It is a key-issue for surviving, because if the mutational rate is too high it may lead to accumulation of harmful mutations and cell death **(123)**. Furthermore, FQs are also able to activate SOS-response system **(124)**, which induces an increased mutation frequence that promotes the survival and selection of resistant bacteria.

Basically, FQs bind to polar aminoacids (such as Ser or Thr, that own polar hydroxyl groups) with a remarkable high affinity. In case of Gram-negatives, for instance gyrase of *E.coli* owns Ser at position 83 and its topoisomerase IV carries Ser at position 80. When these aminoacids are substituated by apolar aminoacids, like Leu, Arg, Val or Lys binding capacity of FQs is significantly decreased **(122)**.

On the other hand, mutational resistance linked to QRDRs are also commonly detected among MDR international high-risk clones of ESKAPE pathogens **(59-60, 78, 85)**. Double Ser alterations of topoisomerase sequences are generally carried by high-risk clones, which are connected to a beneficial fitness, that contributes to survival and acquisition of further resistance genes **(60)**. Moreover, QRDR mutation rate can be elevated up to a hundredfold by PMQRs, namely, quinolone resistance genes (*qnr*), quinolone resistance- (*qepA*) and olaquindox resistant efflux pump (*oqxAB*) and the bifunctional cr variant of aminoglycoside 6′-N-acetyltransferase type Ib enzyme (*aac(6')-Ib-cr*) (**Figure 11.**) **(100, 114-115)**.

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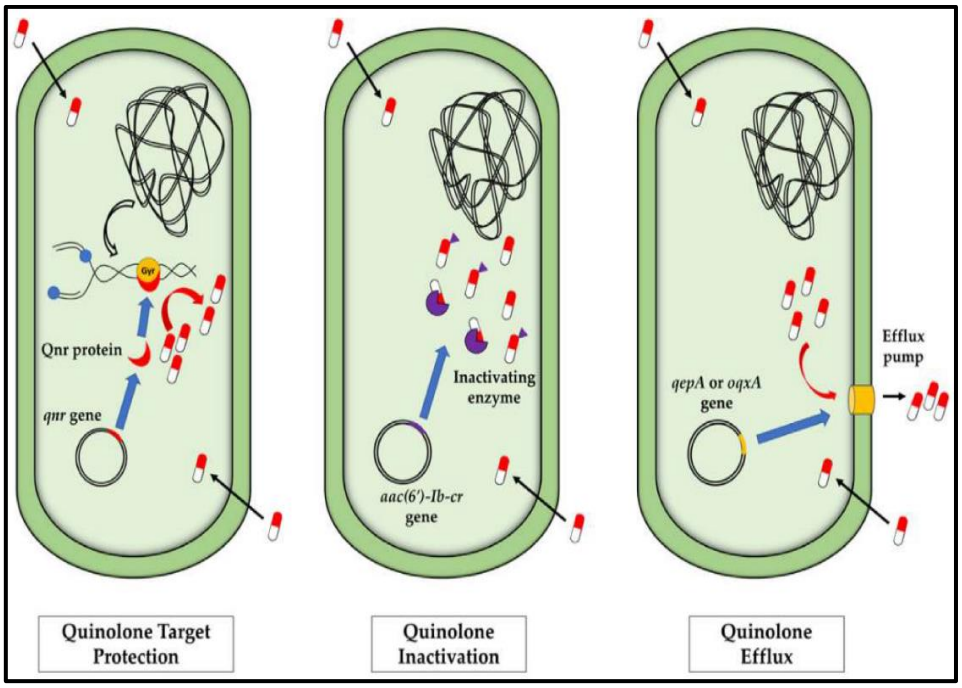

Figure 11. PMQR determinants **(100)**

1.4.3.2. Plasmid-mediated quinolone resistance (PMQR)

Plasmid-associated FQ resistance was described as low level resistance as they can maintain 0.125–0.5 mg/L ciprofloxacin MIC value **(114-115)**. It was first reported in 1998 in ciprofloxacin-resistant *K. pneumoniae* **(114)**. This resistance mechanism is generally associated to other antibotic resistance genes on different MDR plasmids with variable size and Inc groups. This high heterogeneity may explain diversity of bacterial host cells and the widespread FQ resistance **(30)**.

As one of the main PMQR determinant (**Figure 11.**) **(100)**, Qnr protective proteins were first described as chromosomal sequences of Gram-negatives, but as an evidence for their transfer, currently they are predominantly identified on mobile genetic elements, like plasmids and ICEs **(125)**. The main function of Qnrs is to bind to gyrase and topoisomerase enzymes in order to prevent the effects of FQs. Based on their chemical structure they belong to the family of Pentapeptide repeat proteins (PRP), that includes more than 500 members of prokaryotes and eukaryotes. PRPs consist of or contain domains built up by a duplicate of tandemly repeated five amino acids according to a consensus sequence of (A,C)(D,N)(L,F)XX indicating a special motif of [Ser, Thr, Ala, or Val][Asp or Asn][Leu or Phe][Ser, Thr or Arg][Gly] **(126)**. Their 3D structure was

first observed in case of *Mycobacterium* fluoroquinolone resistance protein A (MfpA), that was isolated from ciprofloxacin-resistant *Mycobacterium tuberculosis*. Interestingly, PRPs create a quadrilateral beta-conformation after dimerisation, that mimics the composition of bacterial DNA **(127)**. In Gram-negatives all of their monomers own a smaller (A) and a larger (B) loop; that contain 8 and 12 aminoacids. Qnrs perform a conserved aminoacid sequence exclusively by loop B, that plays an essential role in emergence of FQ resistance **(128-129)**.

PRPs are classified by their „topoisomerase poison resistance factor" activity, that is encoded by chromosomal genes (here we find MfpA) or it can be mediated by plasmids, like Qnrs. **(128)** Compared to each other, these PRPs differ 40% or more in their aminoacid sequences and inside of a subfamily they are identical by 90% (**Figure 12.**) **(130-131)**. According to sequence analysis and phylogeny, these protective proteins have a wide spectrum of subfamilies, such as *qnrA* (A1–A8), *qnrB* (B1–B97), *qnrS* (S1–S13)**,** *qnrC1*, *qnrD* (D1-D3), *qnrVC* (VC1-12)**,** *qnrE* (E1-E4) and an additonal group of *smqnr* (SmQnr 1-58) (**Table 2.**) **(100, 132-137)**. In 2010s the highest number of variants belonged to QnrB group and its alleles, like *qnrB1*, *qnrB2*, *qnrB4*, *qnrB6*, *qnrB10* and *qnrB19* were the most commonly detected ones across the globe **(30, 125)**. Currently, QnrB is still one of the most successful subfamily, however, a noteworthy prevalence of QnrS (close to 30%) is recently detected in Egypt, Romania and Iran **(138-140)**.

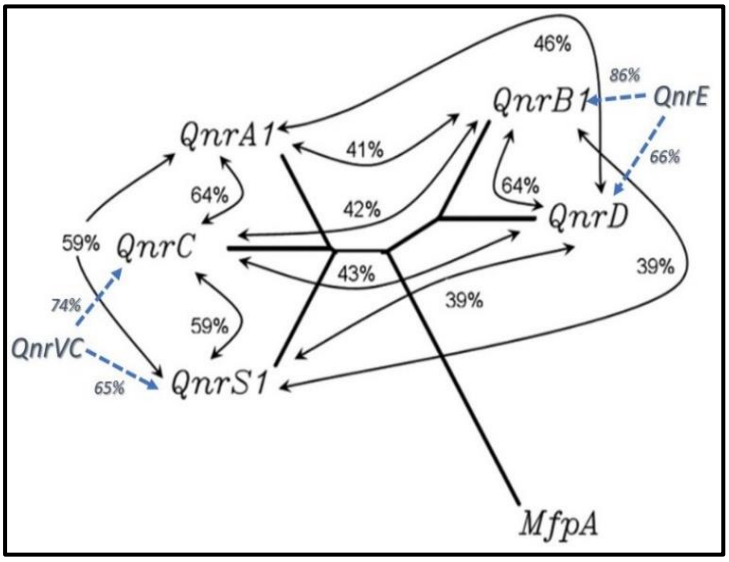

Figure 12. Phylogeny of classic Qnr protective proteins. The original figure was created by **Wang et al, 2009 (131)**, novel determinants highlighted in blue were added to the figure.

Table 2. General features of Qnr protective proteins **(100, 132-137)**

Qnr determinants	Currently reported variants	Number of aminoacids	First description
QnrA	8	218	*K. pneumoniae* (1998)
QnrB	97	214 (216)	*K. pneumoniae* (2006)
QnrC	1	221	*Proteus mirabilis* (2009)
QnrD	3	214	*Salmonella enterica* (2009)
QnrS	13	218	*Shigella flexneri* (2003)
QnrVC	12	218	*Vibrio cholerae* (2008)
QnrE	4	214	*K.pneumoniae* (2016)
SmQnr	58	219	*S.maltophilia* (2008)

Beside Qnr protective proteins, other PMQRs, like the worldwide reported QepA and OqxAB efflux pumps also contribute in FQ resistance (**Figure 11.**)**(100)**. Their mechanism of action is basically to remove toxic substances from cytoplasm or periplasmic space to the extracellular space through their transport canals. Belonging to the so-called Major facilitator superfamily of efflux pumps, QepA1 and its variants, QepA2, A3 and A4 are composed of 14 transmembrane transporter proteins. Each of them is able to cause non-susceptibility to ciprofloxacin, norfloxacin and nalidixic acid **(141-142)**. Compared to QepA, OqxAB and its subtypes (e.g.: OqxA2, B2 and B3) have a wider activity against antibiotics, including ciprofloxacin, norfloxacin, olaquindox, chloramphenicol and sulfonamides as well. This MDR efflux pump is a member of a Resistance-nodulation-division family, it has a large periplasmatic part (is called as OqxA), a transmembrane canal protein and a periplasmatic adapter domain (named as OqxB). It was first isolated in 2003 from *E.coli*, then many members of Enterobacterales showed positivity **(143-144)**.

Finally, FQs are also inactivated by enzymatic modification (**Figure 11.**) **(100)**. In this group of PMQRs we find AAC(6')-Ib-cr, that neutralizes certain AGs (e.g.: amikacin, tobramycin, kanamycin) and FQs, that contain a nitrogen atom associated to their piperazinyl ring, like ciprofloxacin and norfloxacin **(145)**. Based on these features

this enzyme is also known as a bifunctional FQ-modifying protein. Compared to other AG-inactivating enyzmes, it has alterations of two codons (namely, Trp102Arg and Asp179Tyr), which are responsible for this special function **(125)**. Presence of *aac(6')-Ib*-cr was isolated worldwide in *Enterobacteriales* and *P. aeruginosa*, but in total it is the most frequent in *E. coli.* Interestingly, it is more commonly reported than Qnr proteins in case of *E. coli* **(146)**.

1.4.3.3. Epidemiology of fluoroquinolone resistance

Recently, the Antibiotic resistance surveillance in Europe 2023 survey reported *E. coli* as the most common species (39.4% of samples) and FQ resistance as the second most frequent resistance mechanism of *E. coli* invasive strains from 45 countries of European Union / European Economic Area (EU/EEA) as it presented ca. 22% population-weighted mean resistance percentage. In addition, it was the most significant in Southern- and Eastern-Europe as 25% or higher FQ resistance percentage was identified in 17 countries and further 4 countries presented 50% or even higher rate. More than half of *E. coli* isolates were resistant to aminopenicillins, ca. 14% showed non-susceptibility to third-generation cefalosporins and ca. 10% developed resistance against AGs as well. As a summary, more than half of the samples exhibited resistance to at least one antibiotic group under surveillance. These rates are lower compared to the period 2016-2020, however, a novel challenge, carbapenem-resistant *E.coli* was also found **(75, 78)**.

On the other hand, in case of *K. pneumoniae* FQ resistance reached almost 34% and carbapenem resistance showed around 12% prevalence. Alarmingly, *P.aeruginosa* showed the highest EU/EEA population-weighted mean resistance percentages regarding FQs and piperacillin-tazobactam (18.7%, respectively) followed by carbapenems (18.1%) in this survey. In addition, resistance to multiple antibiotic groups was also frequent in these bacteria, it was detected close to 18% of *P.aeruginosa* and it ranged from 3 to 10% of *E.coli* strains in all tested clinical specimens. Data and geographic distribution are summarized on **Figure 13.** **(75)**.

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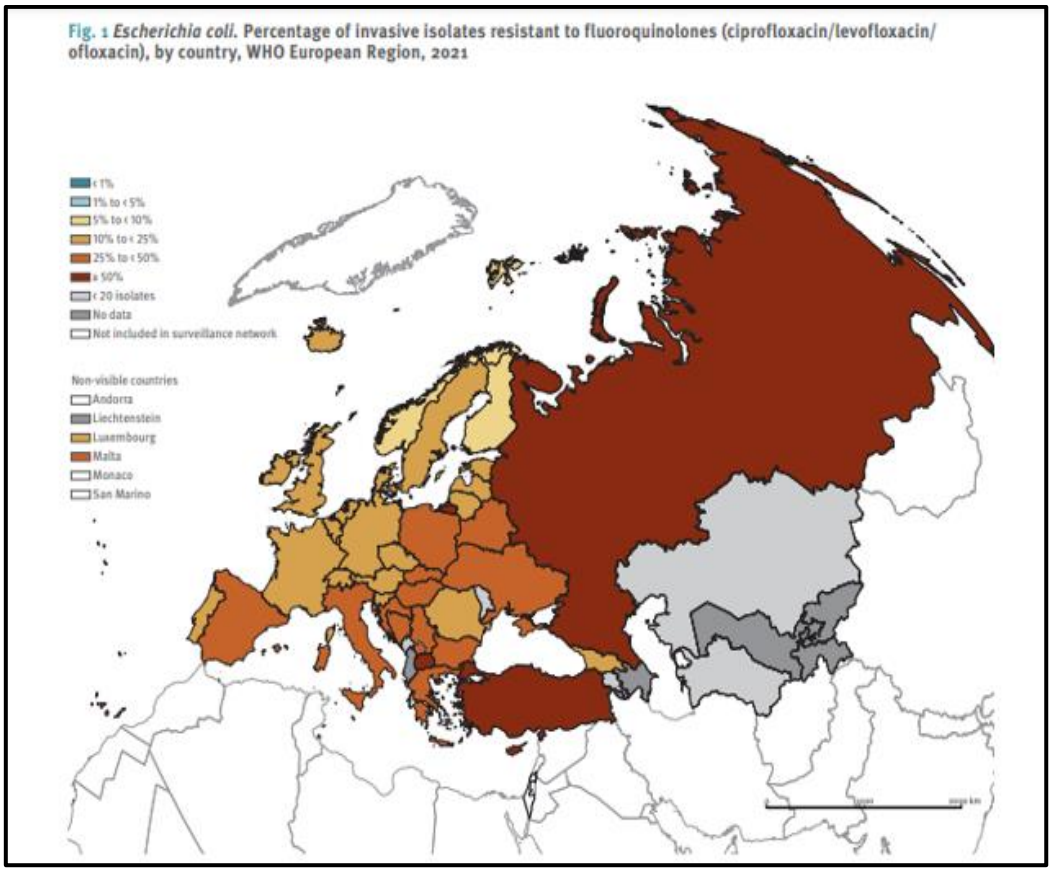

Figure 13. Geographic distribution of FQ-resistant *E.coli* in WHO European Region (Data summary from 2021) **(75)**

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2. OBJECTIVES

The main objectives of this PhD thesis are detection and analysis of FQ resistance in *E. coli* and *P. aeruginosa* strains. This scientific work has three parts.

2.1. Analysis of fluoroquinolone resistance in plasmid-carrier *E.coli* control strains

- Investigation of time- and concentration-dependence in FQ resistance mediated by chromosomal and plasmid-encoded determinants: The aim of our first study was to analyze time- and concentration-dependence of ciprofloxacin resistance based on *qnr* gene expression levels in PMQR-carrier *E. coli* TG1 control strains.
- Role of plasmids, PMQRs and chromosomal mutations after FQ exposition: During our first work we also compared the possible roles of *qnr* gene expression, plasmid copy number and mutations of QRDR after adaptation to ciprofloxacin.

2.2. Detection of *qnrVC1* and of $bla_{\text{NDM-1}}$ in MDR *P.aeruginosa*

The second study analyzed contribution of *qnrVC1*, a PMQR determinant as well as $bla_{\text{NDM-1}}$ in MDR *P. aeruginosa* PS1 strain isolated from urine clinical sample.

- Resistance profiling: Our first aim was to observe antibiotic susceptibility to clinically approved antimicrobial agents, such as BLs, AGs, FQs and colistin.
- Molecular characterization: Other purpose was determination of the strain's ST and resistance determinants based on whole genome sequencing (WGS). We also aimed to analyze *qnrVC1* and $bla_{\text{NDM-1}}$ gene expression by qPCR.

2.3. Investigation of delafloxacin resistance among *E.coli* clinical strains

- Determination of prevalence of delafloxacin resistance: During the third part of our work we studied 47 *E. coli* strains from various clinical samples, including hemoculture and urine.
- Molecular characterization of two delafloxacin-resistant MDR *E.coli* strains by WGS in order to detect their resistance pattern
- Comparison of antibacterial efficacy of delafloxacin and earlier FQs

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3. METHODS

3.1. Investigations of plasmid copy number and gene expression of *qnr* determinants

3.1.1. Strains of our model

In the course of our work, we used artificially modified *E. coli* TG1 control strains (presented by **Figure 14.**), that were provided by Prof. Giuseppe Cornaglia (Università degli Studi di Verona, Verona, Italy). These strains were transformed by plasmids, which separately carried different *qnr* determinants namely, *qnrA1*, *qnrB1*, *qnrC1* or *qnrD1*. GenBank accession numbers of these determinants are shown in **Table 3**. We performed broth microdilution method to determine ciprofloxacin MIC values based on the latest European Committee on Antimicrobial Susceptibility Testing (EUCAST) protocol (ISO 20776-2) **(147)**. Each strain displayed 0.5 mg/L MIC value for ciprofloxacin.

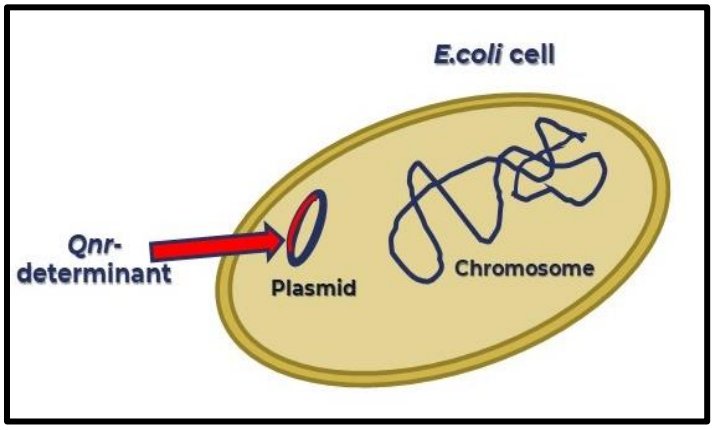

Figure 14. *E. coli* TG1 control strains carried different *qnr* determinants in our model (own figure)

Table 3. Genbank accession numbers of carried *qnr*-determinants **(132)**

Qnr-determinants	GenBank Accession Numbers
qnrA1	*AY070235*
qnrB1	*DQ351241*
qnrC1	*EU917444*
qnrD1	*FJ228229*

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3.1.2. Adaptation to ciprofloxacin exposition and selection

At first we performed short-term study as we detected time-dependence of FQ resistance development. 0.5 McFarland solutions of each *qnr*-carrier strain was exposed to 0.5 mg/L ciprofloxacin in the period from 30 minutes to 24 hours. We cultured these treated strains and at given timepoints (30, 60, 90 and 120 minutes as well as 24 hours) samples were taken for RNA extraction.

After the strains adapted to ciprofloxacin, we accomplished long-term study in order to observe concentration-dependence of plasmid copy number and Qnr protective protein expression. We adjusted the treated strains from short-term study to 0.5 McFarland and 1 mg/L ciprofloxacin was added. Bacterial growth was detected through observation of turbidity by naked eye. Then we isolated the given strain and a 0.5 McFarland bacterial solution of that, was exposed to the next ciprofloxacin concentration (2 mg/L). Then we repeated these steps coherently with 4 and 8 mg/L ciprofloxacin concentration. Each step was conducted in 24 hours and they were performed in Mueller-Hinton broth. Similarly to short term-study, in given timepoints (24, 48, 72, 96 and 120 hours) samples were taken for RNA extraction. Methodical background is presented on **Figure 15.**

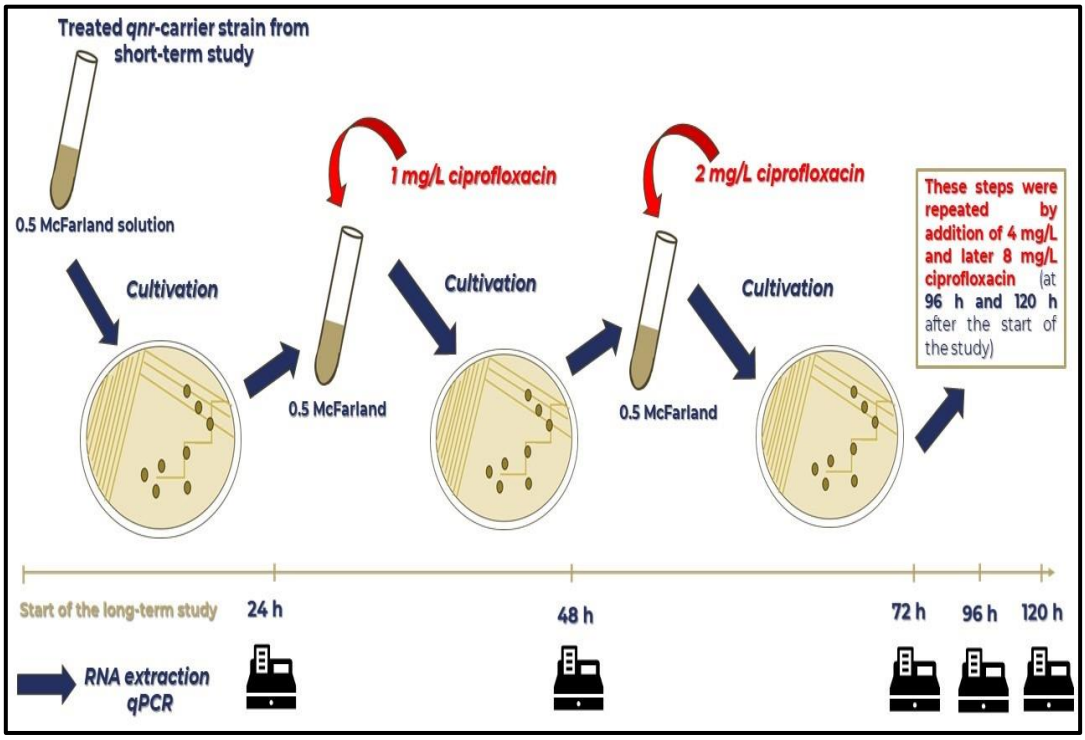

Figure 15. Principles of long-term study (own figure)

3.1.3. Bacterial RNA extraction

At given timepoints total RNA of exposed strains was isolated by Qiagen RNeasy Mini Kit (*Hilden, Germany*). Accordingly, we performed the following steps:

- We put our samples into Eppendorf tubes, and we centrifuged them at 5,000 × g over 10 min and then the supernatants were removed.
- Tris-EDTA pH 8 buffer including 20 µl proteinase K and 200 µl lyzozime was added to the pellet of each strain. Then they were incubated at 15–25°C and vortexed and RLT buffer was added as well.
- 700 µl from each solution was pipetted to RNeasy Mini spin columns and centrifugation was performed at 8,000 × g for 15 s.
- 700 µl RW1 and 500 µl RPE buffers were added separately. These steps were followed by centrifugation at 8,000 × g over 15 s and this phase was repeated.
- Additional 500 µl RPE-buffer was added and it was centrifugated at 8,000 × g for 2 min.
- Finally, the sample was placed into a new tube and eluation of RNA was performed into 50 µl RNase-free water by centrifugation at 8,000 × g over 1 min.

3.1.4. qPCR

We quantified expression rates of *qnrA1*, *qnrB1*, *qnrC1*, *qnrD1* genes and the carrier plasmids. Extracted RNA of each tested strain was investigated by quantitative PCR (qPCR) in a Step One Real-Time PCR System (*Applied BioSystems, Thermo Fisher Scientific, Foster City, CA, USA*).

Here we used the following 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) × 40 cycles, and 60 °C for 30 s. Oligonucleotid primers and probes were composed by Primer Express 3.0 software (*Applied BioSystems, Thermo Fisher Scientific, Foster City, CA, USA*) in order to target each *qnr* determinant and plasmid backbone sequences.

We normalized each targeted sequence to *icd* according to CT values with formula $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = (CT_{\text{gene of interest}} - CT_{\text{internal control}})_{\text{studied strain}} - (CT_{\text{gene of interest}} - CT_{\text{internal control}})_{\text{control strain.}}$ We chose chromosomal housekeeping *icd* gene as internal control and the control strains were the non-treated strains. The designed primers and probes are shown in **Table 4.** and **Table 5.**

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Table 4. Primers and probes for gene expression analysis

Target	Forward primer sequences (5' to 3')	Reverse primer sequences (5' to 3')	Probe sequences (5' to 3')
qnrA1	*TTG AGT GAC AGC CGT TTT CG*	*GCA GCTG ACA GTG GCT GAA G*	*FAM-CTGCCGCTTTTATC-MGB*
qnrB1	*GTG CGC TGG GCA TTG AA*	*CGG AAA TCT GCG CCT TGT*	*FAM-TTCGCCACTGCCGC-MGB*
qnrC1	*CGA TAA GCA ATG CCT TGA AAA G*	*GGA ATA ACA ATC ACC CCC AAC*	*FAM-TCTCACTCATTCAGAATTAAATGGC TTAGAACCTCGTA-MGB*
qnrD1	*AAA GTG CGA ACT GTG GGA AAA*	*ACA CGG CGC CAG TTA TCAC*	*FAM-CGCTGGAATGGCA-MGB*
icd	*GTT CGT GCA GCG ATC GAA*	*GCA CCA GGG TCA CAG AGT CA*	*VIC-ACGCAATTGCTAACGAT-MGB*

Table 5. Primers and probes for plasmid copy number analysis

Target	Forward primer sequences (5' to 3')	Reverse primer sequences (5' to 3')	Probe sequences (5' to 3')
plasmid qnrA1	*ATG AGG AGC AAA AAG GTG GTT TAT*	*CGC GCC TTC CCA ACA AC*	*FAM-CTTCCTATACCCCTGCAAA-MGB*
plasmid qnrB1	*GCT CCC TGC CCT TAT ACA AT AGT G*	*TGC GTC GAA ACG TG TGA CTT*	*FAM-CCGCCCTTGCCTTA-MGB*
plasmid qnrC1	*AAA ATT TTT TCT AGT TTG CCG GAT AG*	*GGC ACC TAA CAG CCC CTT AAA*	*FAM-TTGATCGCATTTCTC-MGB*
plasmid qnrD1	*TTC GAT ACC GCA GCG TGA T*	*AAC GTA CCG GAA AAC GAA ATT C*	*FAM-CGGAGGCAGAGCC-MGB*
icd	*GTT CGT GCA GCG ATC GAA*	*GCA CCA GGG TCA CAG AGT CA*	*VIC-ACGCAATTGCTAACGAT-MGB*

3.1.5. Detection of QRDR mutations

The role of mutations in QRDRs, namely, *gyrA* and *parC* chromosomally genes in our model was investigated by PCR and nucleic acid sequencing. In the course of mutational resistance analysis, we performed these steps:

- Each treated strain was suspended in 500 µl bidestillated water *(Millipore Merk, Darmstadt, Germany*) and they were incubated at 100 °C for 15 min.
- It was followed by centrifugation at 13,000 rpm for 10 min at 4 °C. Supernatant was applied as DNA template in PCR, and the mixture contained 1 Unit DNA Taq polymerase (*Sigma-Aldrich, St. Louise, MO, USA*) and 10 pmol of each oligonucleotid primer. (**Table 6.**)
- Oligonucleotid primers of this study were designed by Eurofins Genomics online tools.
- We performed the following PCR protocol: 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 as final step 72 and 4 °C for 5 min.
- PCR amplicons were purified by Qiagen PCR purification Kit (*Hilden, Germany*) and we sent our samples to be sequenced by *BIOMI Kft, Gödöllő*. The detected sequences were studied according to NCBI GenBank database.

Table 6. Oligonucleotid primers designed for detection of mutational FQ resistance

Target	Oligonucleotid primer sequences *(5' to 3')*	
	Forward	Reverse
gyrA	*CAG CCC TTC AAT GCT GAT*	*CGC TTT TAC TCC TTT TCT GTT C*
parC	*CTC AAT CAG CGT AAT CGC C*	*AAT CCT CAG CCG ATC TCA C*

3.2. Analysis of *qnrVC1* and *bla*$_{NDM-1}$ in MDR *P. aeruginosa*

3.2.1. Studied strain

In this work a MDR *P. aeruginosa* PS1 strain, that was isolated from urine clinical sample was studied.

3.2.2. Antibacterial susceptibility testing

Antimicrobial susceptibility testing was performed by microdilution method in Mueller–Hinton broth in 96-well microplates. We determined MIC vaules for piperacillin-tazobactam, ceftazidime, imipenem, meropenem, ciprofloxacin, levofloxacin, tobramycin and colistin. These results were interpreted based on the latest EUCAST documents **(147)**. We used *P.aeruginosa* ATCC27853 as control strain.

3.2.3. Whole genome sequence analysis

Whole genome sequence of PS1 strain was investigated by the Illumina MiSeq system that uses MiSeq reagent kit v2 generating 250 bp paired-end reads in order to analyze its resistome. Then following methods were performed:

- DNA was extracted by UltraClean Microbial DNA Isolation Kit (*Qiagen GmbH, Hilden, Germany*). Libraries were composed by SureSelect QXT Library Prep Kit (*Agilent Technologies, Santa Clara, USA*).
- De novo genome assembly was performed with SPAdes Genome Assembler 3.9.0.
- Assembled sequences were uploaded to the online bioinformatics tool ResFinder (*Center for Genomic Epidemiology, Technical University of Denmark, Lyngby, Denmark*) to assay resistance markers of PS1 strain **(149)**.

3.2.4. Quantification of gene expression

During WGS we identified *qnrVC1* determinant and *bla*$_{NDM-1}$ metallo-beta-lactamase in *P. aeruginosa* PS1 strain. Their gene expression rates were investigated by qPCR. Total bacterial RNA of the tested strain was extracted by Qiagen RNeasy Mini Kit (*Hilden, Germany*) based on the manufacturer's instructions. The qPCR was applied in a Step One

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Real-Time PCR System (*Applied BioSystems, Thermo Fisher Scientific, Foster City, CA, USA).*

Separate expression of *qnrVC1* and $bla_{\text{NDM-1}}$ was detected whereas chromosomal *rpsL* was chosen as internal control. The qPCR was carried out with default setting and CT values of these two genes were normalized to that of *rpsL* gene. We calculated expression rates with the above detailed schema: $2^{-\Delta CT}$, where $\Delta CT = (CT_{\text{gene of interest}} - CT_{\text{internal control}})$. The designed primers are presented in **Table 7.**

Table 7. Primers and probes used for gene expression analysis

	Forward primer sequences *(5' to 3')*	Reverse primer sequences *(5' to 3')*	Probe sequences *(5' to 3')*
qnrVC1	AAA CCT CCG AGA TAC ACA GTT CAT TA	ATC GCA CCC TTC CAA TGC	CTG TAC TTT CAT AGA GCA GG
$bla_{\text{NDM-1}}$	TGC ATG CCC GGT GAA ATC	GTC GCC AGT TTC CAT TTG CT	CCC GAC GAT TGG C
rpsL	CAA GCG CAT GGT CGA CAA	TTG CGG GCA GTT TTG CA	AGC GAC GTG CCT GC

DOI:10.14753/SE.2024.3011

3.3. Determination of delafloxacin resistance

3.3.1. Strain collection

A total of 47 non-repetitive *E. coli* strains were collected between September and December 2022 at South-Pest Central Hospital, National Institute of Hematology and Infectious Diseases, from diverse clinical samples, such as hemoculture and urine. All isolates were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI *Biotyper, Bruker, Bremen, Germany*). The inclusion criteria of *E. coli* strains in our study were non-susceptibility to ciprofloxacin and/or resistance to third-generation cefalosporins or ESBL-production, that was confirmed by double-disk synergy test. (**Figure 16.**)

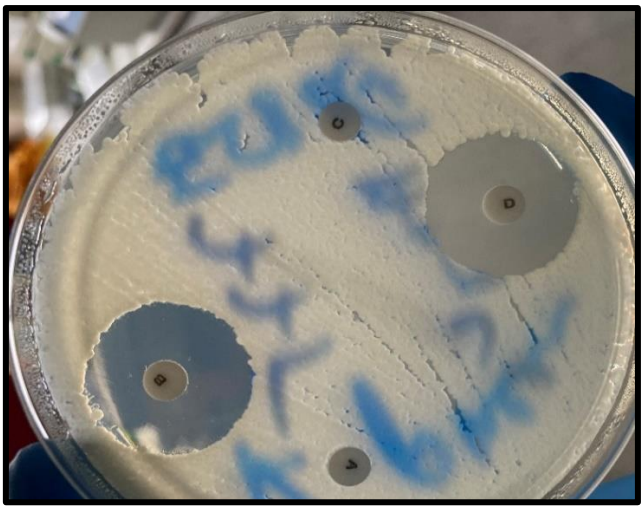

Figure 16. Confirmation of ESBL-phenotype with double disc synergy test on Mueller-Hinton agar. (Biomérieux Hungária Kft.) (own picture) Third-generation cefalosporins (disc A-*cefotaxime* and disc C-*ceftazidime*) are ineffective against ESBL enzymes, but the combination of the given beta-lactam and clavulanic acid (disc B and D) results in wide inhibition zones.

3.3.2. Determination of MIC

Antibiotic susceptibility testing was carried out for delafloxacin, ciprofloxacin, levofloxacin, moxifloxacin, ceftazidime, cefotaxime, and imipenem. MIC values were determined by broth microdilution method in Muller–Hinton broth in 96-well microplates. The MIC results were interpreted according to the latest EUCAST protocol, accessed on 10 January 2023 **(147)**. We used *E. coli* ATCC 25922 as control strain.

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3.3.3. Selection of strains and whole genome sequencing

Whole genome sequence analysis was performed on two selected *E. coli* strains (ECO-SEOMI-LKH 920/1 and 951/2) as they exhibited the highest FQ MIC values in our collection. (**Figure 17.**) Besides, our selection criteria for WGS also included ciprofloxacin and delafloxacin resistance together with ESBL production.

WGS was performed by Illumina MiSeq system in Eurofins BIOMI Kft (*Gödöllő, Hungary*). Genomic DNA was extracted by the NucleoSpin Microbial DNA Mini kit (*Macherey-Nagel, Düren, Germany*). The quantity of isolated DNA was measured by Qubit fluorometer, and the quality of DNA was tested by microcapillary electrophoresis (*Tape Station 4150, Agilent, Waldbronn, Germany*). Libraries were prepared by Illumina DNA Prep kit, according to the manufacturer's instruction.

Sequencing was performed on an Illumina Miseq system using MiSeq Reagent Kit v2 generating 250 bp paired-end reads. Genome assembly was applied with the SPAdes Genome assembler algorithm v3.15.3. Resistance determinants were identified in the assembled sequences by Bionumerics v8.1 software.

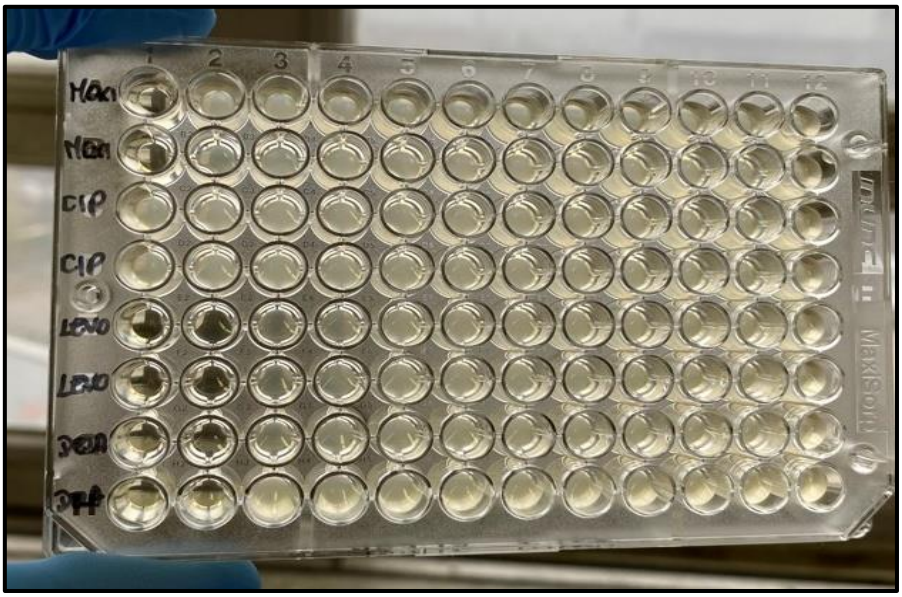

Figure 17. FQ MIC values of 951/2 strain (own picture)

Lines on this plate refer to the given FQ, namely, Moxi: moxifloxacin, Cip: ciprofloxacin, Levo: levofloxacin and Dela: delafloxacin. (Two lines belong to the same FQ.)

Columns indicate the bisecting dilution of antibiotic concentrations between 128 mg/L (column one on the left side) and 0,125 mg/L > (last column on the right side).

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4. RESULTS

4.1. Role of plasmid copy number and *qnr* gene expression of *E. coli* strains

During short-term study we detected the following results. In the period from 30 minutes to 24 h *qnrA1* and *qnrD1* demonstrated similar, 1.0-1.2 and 1.2-1.47 level expressions and *qnrC1* displayed an almost 12.5-fold rise. As a comparison, in the same period *qnrB1* performed 3.22–80.63 expression. In case of plasmid copy numbers we detected analogue results, as by *qnrA1*- and *qnrD1*-carrier plasmids 1.0–1.4-folds were found and a 4-fold value was exhibited in case of *qnrB1* plasmid. In short, the highest plasmid copy number was performed by *qnrC1*-positive plasmid from period 30 min to 120 min, although this result was similar to *qnrB1*-positive plasmid as it stayed relatively low in the last period of time (nearly 4.5). Results of time-dependence study are detailed on **Table 8.**

Table 8. Results of short-term study. The highest *qnr* expression and plasmid copy number are indicated in red in each time period.

Tested plasmid (strains)	*qnr* expression 30 min - 120 min	*qnr* expression 120 min - 24 h	Plasmid copy number 30 min- 120 min	Plasmid copy number 120 min-24 h
qnrA1	***1.0***	***1.2***	***1.0***	***1.47***
qnrB1	***3.22***	***80.63***	***1.0***	***4.13***
qnrC1	***3.82***	***12.44***	***3.0***	***4.42***
qnrD1	***1.2***	***1.47***	***1.0***	***1.40***

All in all, the most significant increase of gene expression was introduced by *qnrB1*, data are shown by **Figure 18.** Brown colour represent *qnrB1* expression compared to plasmid expression. In addition, grey indicates plasmid copy number (plasmid expression compared to chromosomal expression). Here we detected that the remarkable gene

expression rate was not associated to a relevant plasmid expression level, it reached altogether 4.13-fold value. Nevertheless, the detected expression rates are lower after 24 h than that of at 120 min, but they remain still remarkable. Interestingly, expression of *qnrC1* also showed an increasing tendency during short-term study, but it could not reach the constantly high rates of *qnrB*-variant. (**Table 8**.)

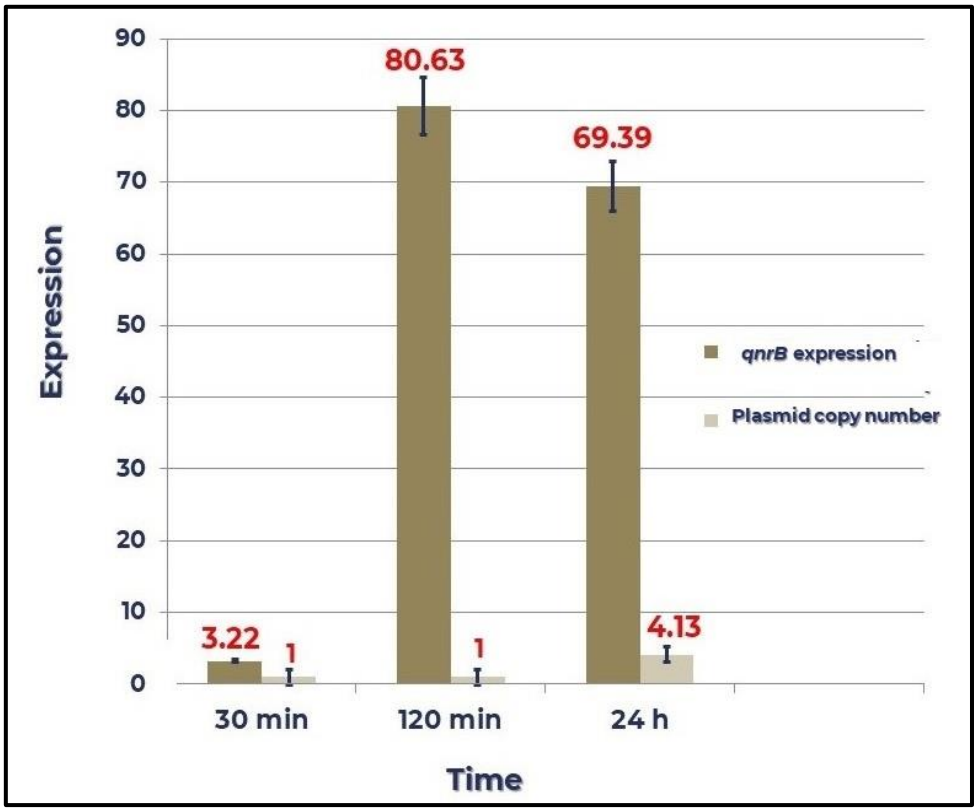

Figure 18. Results of short-term study in case of *qnrB1*. Results are normalised to untreated control strains. Diagram shows the rates of *qnr* gene expression and plasmid copy number after ciprofloxacin exposition.

After the strains adapted to ciprofloxacin, we accomplished long-term study in order to observe concentration-dependence of FQ resistance. In this phase we performed the same steps as it was mentioned by short-term study to quantify gene expression. According to our model, a resistant strain was selected only in *qnrB1* carrier *E. coli*. The *qnrA1*, *qnrC1* and *qnrD1*-carrier strains died after short-term study, we could not perform further selection.

In long-term study nearly 106- and 212-fold *qnrB1* expression levels were detected by 4 and 8 mg/L ciprofloxacin-concentrations, respectively. Data are shown on **Figure**

19. In contrast, plasmid copy number of *qnrB1*-carrier plasmid reached only 4-fold increase. Results are summarized by Table 9.

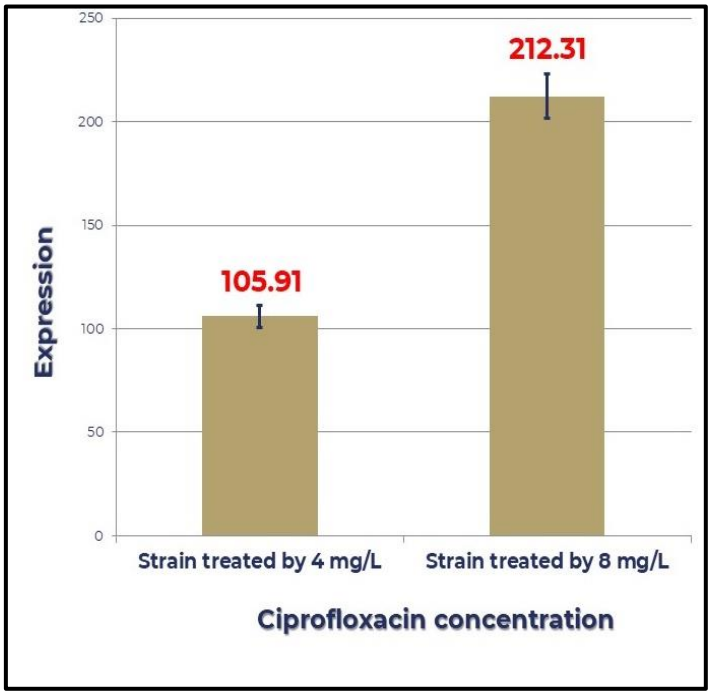

Figure 19. Results of long-term study in case of *qnrB1*

Table 9. Plasmid copy number changes of *qnrB1*-carrier plasmid in course of increasing ciprofloxacin-concentrations

Tested plasmid (strain)	Plasmid Copy Number by 1-2 mg/L ciprofloxacin	Plasmid Copy Number by 4-8 mg/L ciprofloxacin
***qnrB1*-plasmid**	***1***	***4.13***

Finally, besides the role of PMQRs in selection of resistant bacteria we also analysed the presence of chromosomal mutations in QRDRs, namely, *gyrA* and *parC*. In the course of our study no mutations in these genes were identified.

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4.2. Contribution of *qnrVC1* and *bla*$_{NDM-1}$ in *P. aeruginosa*

In our work a MDR *P. aeruginosa* strain, referred to PS1, isolated from urine clinical sample was investigated. In the course of antimicrobial susceptibility testing we detected resistance to piperacillin-tazobactam, ceftazidime, imipenem, meropenem, ciprofloxacin, levofloxacin and tobramycin, but it was sensitive to colistin. MIC-values and the latest EUCAST-breakpoints are summarized on Table 10. **(147)**.

Table 10. MIC-results of PS1 strain and EUCAST breakpoints **(147)**.
Resistance values are highlighted in red.

Antibiotic	MIC (mg/L)	EUCAST MIC breakpoints (mg/L)	Result of susceptibility
Ciprofloxacin	128	0.5	R
Levofloxacin	256	2	R
Meropenem	256	8	R
Imipenem	128	4	R
Ceftazidime	128	8	R
Piperacillin-tazobactam	256	16	R
Tobramycin	256	2	R
Colistin	1	2	S

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During WGS analysis of the tested strain a 6815803 bp genome was identified. According to its characteristic housekeeping genes (*acs*, *aro*, *gua*, *mut*, *nuo*, *pps* and *trp*) **(148)**, PS1 was identified as MDR *P.aeruginosa* ST773 international high-risk clone. It was built up by 148 contigs harbouring several different resistance determinants, including *aph(3')-IIb*, *rmtB*, *fosA*, *sul1*, *qnrVC1*, tet(G), $bla_{\text{OXA-50-like}}$, bla_{PAO}, $bla_{\text{NDM-1}}$ and *catB7*. (**Table 11.**) We deposited sequence data of our strain in NCBI database under accession number *RHDU00000000*.

Table 11. Resistance markers of PS1 strain.

The two genes of interest are highlighted in red.

Resistance markers	Resistance phenotypes
aph(3')-Iib	aminoglycoside resistance
rmtB	aminoglycoside resistance
fosA	fosfomycin resistence
sul1	sulfonamide resistance
qnrVC1	**fluoroquinolone resistance**
tet(G)	tetracycline resistance
$\boldsymbol{bla}_{\text{OXA-50-like}}$	beta-lactam resistance
$\boldsymbol{bla}_{\text{PAO}}$	beta-lactam resistance
$\boldsymbol{bla}_{\text{NDM-1}}$	**beta-lactam resistance**
catB7	phenicol resistance

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Based on the high-level FQ resistance, we performed nucleic acid sequence analysis by NCBI Blast, that identified aminoacid alterations by certain positions of QRDRs. Results are shown on **Table 12.**

Table 12. Mutations of QRDR determinants in PS1 strain

Position	Aminoacid substitutions
Subunit A of gyrase (*gyrA*)	Thr83Leu
Subunit A of topoisomerase (*parC*)	Ser87Leu

Nevertheless, we determined separate gene expressions of *qnrVC1* and $bla_{\text{NDM-1}}$ with qPCR. Results are shown on **Figure 20.**

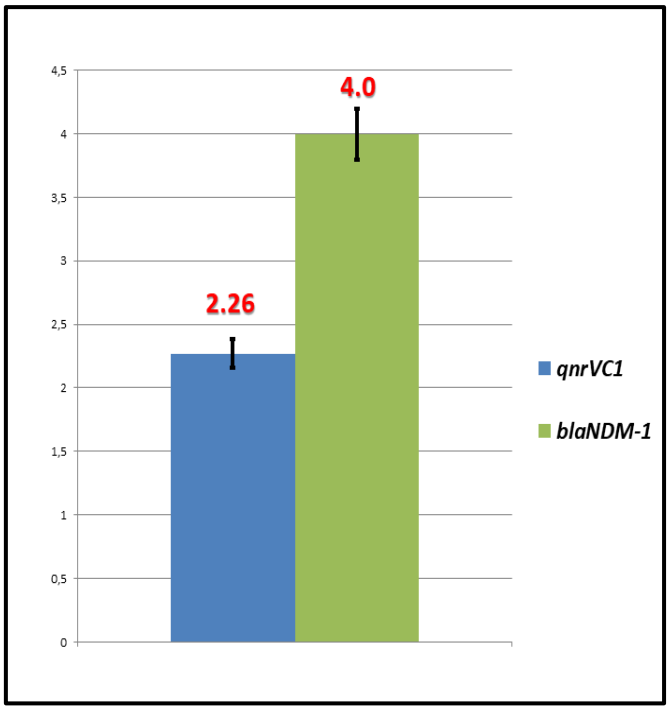

Figure 20. Expression rates of *qnrVC1* and $bla_{\text{NDM-1}}$

Based on sequence analysis, *qnrVC1* was incorporated in a class I superintegron with 1346 bp, that carried gene cassette mobilization elements, namely *qnrVC* core, inverse core and *aatc* site. Associated to *V. parahaemolyticus* repeat (VPR) site, coding region of this Qnr protective protein had its own functional promoter sequence as well. Compared to available data, the flanking region including recombination sites of *qnrVC1* was identical to that of were found in *V. cholerae* O1 strain in Brazil **(150)**. We uploaded sequences of *qnrVC1*-integron in NCBI Genbank under accession number *MH782277*. Additionally, with IS finder we analysed the flanking region of $bla_{\text{NDM-1}}$ as well. In this case further resistance markers were identified, such as microsomal dipeptidase and bleomycin resistance gene referred to ble_{MBL} (accession number: *MK109012*). Structure of *qnrVC1* gene cassette and genetic environment of $bla_{\text{NDM-1}}$ is presented by **Figure 21.**

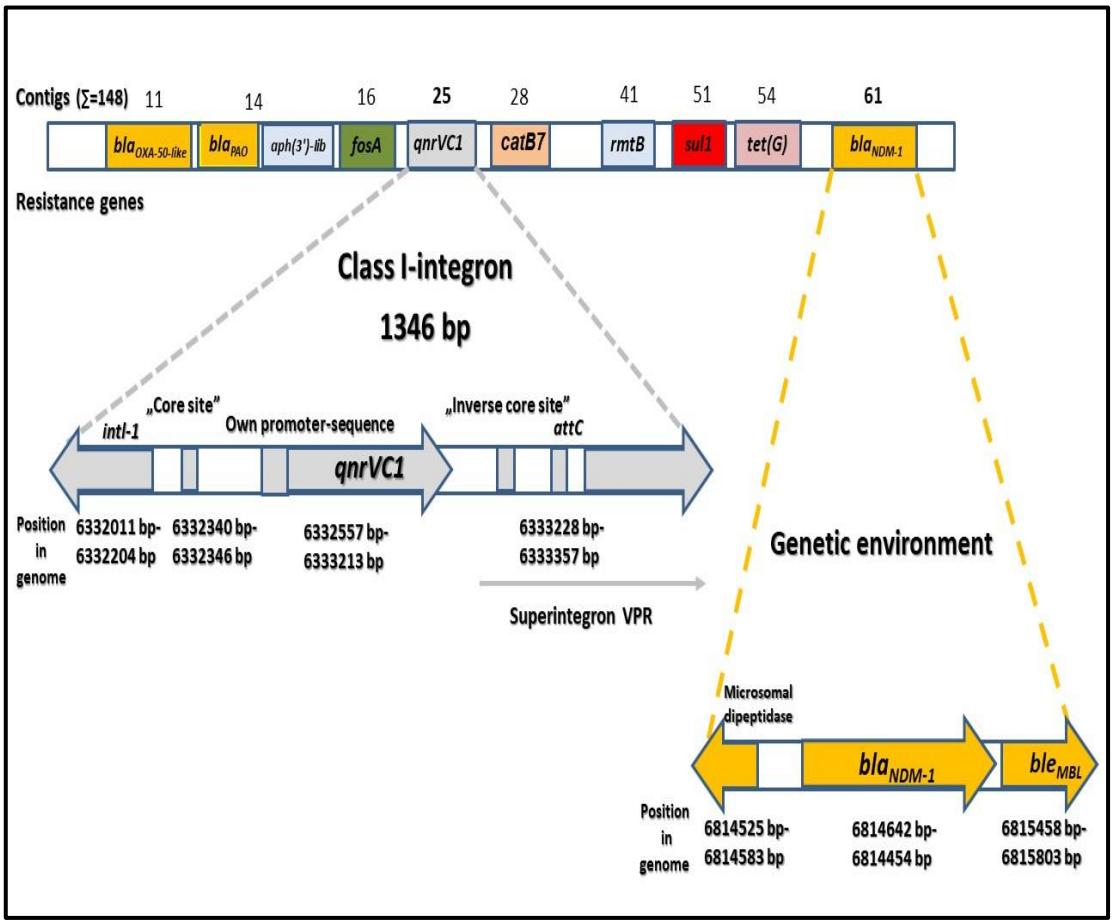

Figure 21. Flanking regions of *qnrVC1* and $bla_{\text{NDM-1}}$

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4.3. Delafloxacin-resistant *E.coli* high-risk clones

The investigated 47 *E. coli* clinical strains displayed a wide range of FQ MIC distribution. Altogether, 20 of them were susceptible and further 18 showed resistance to all tested FQs. (**Figure 22.**) Furthermore, in our collection ceftazidime MIC values demonstrated a range of 0.5–128 mg/L and cefotaxime MIC values were in the range of 0.125–128 mg/L. Altogether, 46 *E. coli* strains were verified as ESBL-producer. Among the studied strains, 43 out of 47 *E. coli* showed imipenem MIC values between 1 and 4 µg/mL. (**Figure 23.**)

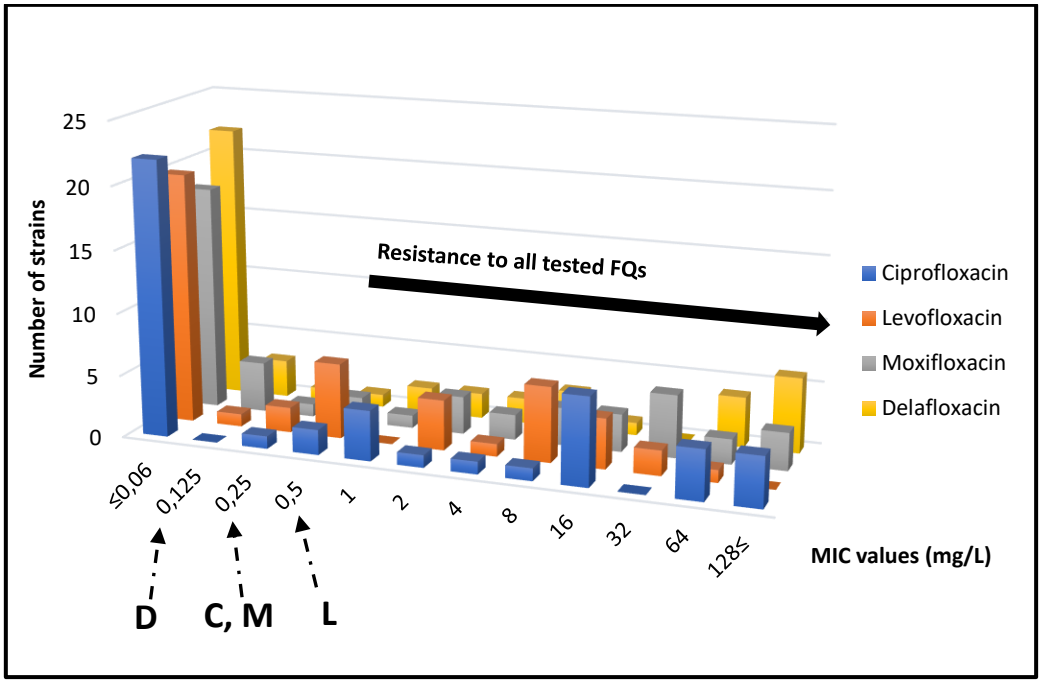
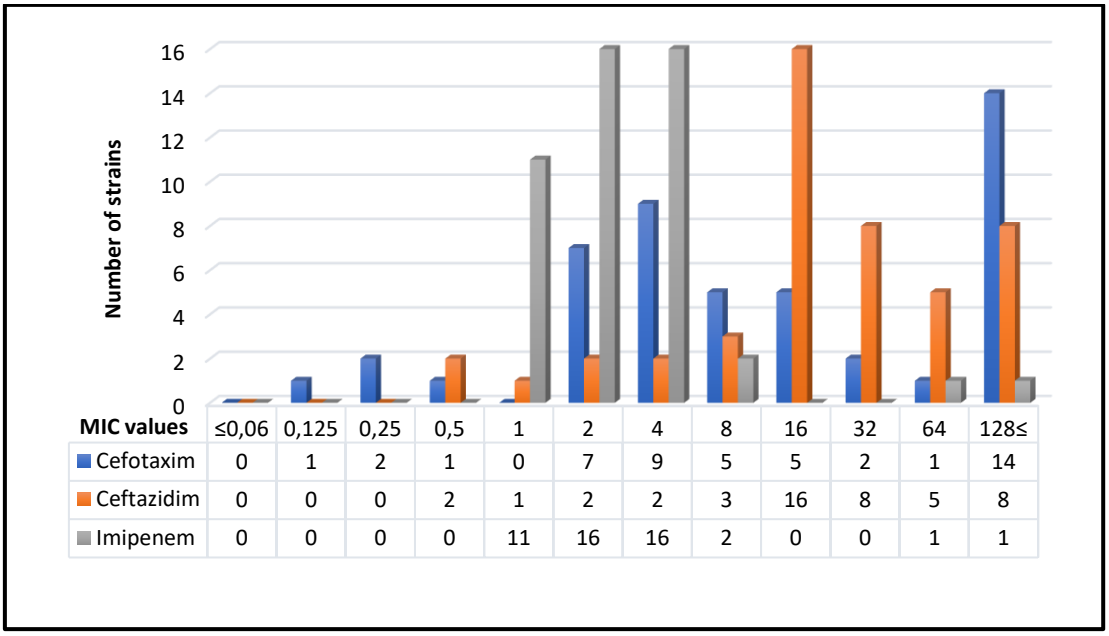


Figure 22. Distribution of FQ MIC values of the 47 *E. coli* strains in our study. Arrows indicate EUCAST breakpoints for each FQ, namely, D: delafloxacin, C: ciprofloxacin, M: moxifloxacin, and L: levofloxacin **(147)**.

DOI:10.14753/SE.2024.3011



MIC values	≤0,06	0,125	0,25	0,5	1	2	4	8	16	32	64	128≤
Cefotaxim	0	1	2	1	0	7	9	5	5	2	1	14
Ceftazidim	0	0	0	2	1	2	2	3	16	8	5	8
Imipenem	0	0	0	0	11	16	16	2	0	0	1	1

Figure 23. Distribution of BL MIC values of the 47 *E. coli* strains in our study.

(All values are in mg/L.)

Altogether, 25 out of 47 *E. coli* strains showed susceptiblity to delafloxacin, and a single strain was sensitive only to delafloxacin but developed resistance to all other FQs. Delafloxacin resistance rate was 47% (22/47), ciprofloxacin resistance was 51% (24/47), moxifloxacin resistance was 51% (24/47), and levofloxacin resistance was 38% (18/47). We also determined MIC50 and MIC90 values in the course of our work. They indicate the MIC value of 50% and 90% of the tested strains, respectively.

In our study, MIC50 value was 0.125 mg/L for delafloxacin and all other FQs had 0.25 mg/L. MIC90 values for delafloxacin, ciprofloxacin, moxifloxacin, and levofloxacin were 64 mg/L, 64 mg/L, 32 mg/L, and 16 mg/L, respectively. 20 *E. coli* strains of our collection were delafloxacin-susceptible however they performed ciprofloxacin resistance and an ESBL phenotype. The strains, which displayed ciprofloxacin MIC value above 1 mg/L were resistant to delafloxacin. Moreover, the strains exhibiting 4 and 8 mg/L delafloxacin MIC values were moxifloxacin-resistant.

On the other hand, we also found unusual phenotypes as levofloxacin MIC values did not significantly correlate with the other FQs. Two *E. coli* strains were levofloxacin-susceptible but resistant to all other tested FQs and further two strains showed sensitivity to levofloxacin and delafloxacin, but were resistant to other FQs. A single *E. coli* strain was levofloxacin- and moxifloxacin-susceptible, but resistant to other observed FQs.

Moreover, an another strain was ciprofloxacin- and levofloxacin-susceptible, but resistant to other FQs. Finally, two strains were resistant only to ciprofloxacin, but were susceptible to all other FQs. Resistance phenotypes are summarized on **Figure 24.**

Number of strains (∑=47)	Ciprofloxacin	Levofloxacin	Moxifloxacin	Delafloxacin
18	R	R	R	R
20	S	S	S	S
2	R	S	R	R
1	R	S	S	R
2	R	S	R	S
1	S	S	R	R
1	R	R	R	S
2	R	S	S	S

Figure 24. FQ resistance patterns of the 47 *E. coli* strains.

R refers to resistance and S indicates susceptibility.

Based on our results of antibiotic susceptibility testing, we chose two MDR *E. coli* strains, namely, ECO-SEOMI-LKH 920/1 and 951/2 for WGS analysis. MIC results of theirs are presented on **Table 13.**

Table 13. MIC values of the two *E. coli* strains selected for whole-genome sequencing.

Antibacterial agents	Ciprofloxacin	Levofloxacin	Moxifloxacin	Delafloxacin	Imipenem	Ceftazidime	Cefotaxime
920/1	128	16	64	4	4	128	128<
951/2	128<	64	128	64	8	128<	128<

Both strains belonged to *E. coli* ST43 international high-risk clone and both of them carried varied resistance genes, including ESBLs, namely, $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-15}}$. In case of 920/1 strain, five aminoacid alterations were identified in multiple positions of QRDR, namely, *gyrA* S83L, D87N, *parC* S80I, E84V and *parE* I529. *E. coli* 951/2 was also

marked by similar multiple mutations of these chromosomal sequences; such as *gyrA* S83L, D87N and *parC* S80I,E84V. Moreover, in this strain a PMQR determinant, *aac(6')-Ib-cr* was detected as well. Further resistance mechanisms were also identified, they are presented on **Table 14.**

The assembled genomes of the two MDR strains were submitted to NCBI Genbank at Bioproject PRJNA971108; sequence read archive (SRA) identifiers: SAMN35019574 (ECO-SEOMI-LKH 920/1 strain) and SAMN35019575 (ECO-SEOMI-LKH 951/2 strain).

Table 14. Results of WGS of the two selected *E. coli* strains (920/1, 951/2).
n.d. refers to not detected

Strain	920/1	951/2
Sequence type (ST)	ST43	ST43
Serotype	*H4-O25*	*H4-O25*
Plasmid groups	IncFII, IncFIA, IncFIB, IncX1	IncFII, IncFIA, IncFIB, col156,
Beta-lactamases	$bla_{CTX\text{-}M\text{-}1}$	$bla_{CTX\text{-}M\text{-}15}$, $bla_{OXA\text{-}1}$
Macrolide resistance genes	*mph*(A)	*mph*(A)
Tetracycline resistance genes	*tet(A)*	*tet(A)*
Sulfonamide resistance genes	*sul1, dfrA17*	*sul1, dfrA17*
PMQR	n.d.	*aac(6')-Ib-cr*
QRDR	*gyrA*: S83L, D87N *parC*: S80I, E84V *parE*: I529L	*gyrA*: S83L, D87N *parC*: S80I, E84V

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5. DISCUSSION

5.1. Selection of *qnrB*-carrier *E.coli*

In the first part of our studies we investigated time and concentration-dependence of FQ resistance established by Qnr protective proteins. We selected a FQ-resistant strain from *qnr*-positive *E. coli*. In case of *qnrB1*-carrier *E. coli*, a resistant strain developed during 0.5–8 mg/L ciprofloxacin exposure. Our results demonstrate that selection of resistant *qnrB1*-positive *E. coli* was particularly induced by the elevated expression of QnrB and copy number change of *qnrB1*-bearing plasmid played only a minor role in this process. Moreover, in this study we also detected the lack of mutations in QRDRs. Although the other Qnr-variant strains, namely *qnrA*-, *qnrC*-, and *qnrD*-bearing *E. coli* were also able to adapt to 0.5 mg/L ciprofloxacin concentration with *qnr* expressions ranging from 1.2 to 12.44, further selection was not possible.

Importance of this *qnrB* expression is that this PMQR marker takes part in bacterial SOS-response regulation system **(151-152)**. Briefly, this response reaction is triggered if the bacteria gets into stressful circumstances, including DNA damage by UV, oxidative stress, metabolic pH-change as well as presence or increasing concentration of antibiotics like ciprofloxacin in its environment. The evolutional purpose of SOS-response is providing protection for bacterial DNA-synthesis against harmful effects by intensified mutation rate and via production of Qnr protective proteins.

Two regulator molecules have key function in SOS-reponse, they are LexA transcription repressor („master regulator") and RecA coprotease. The interplays between these two proteins help bacteria to survive **(153)**. Promoter of *qnrB* sequence contains CTGT binding site of LexA-protein. If bacteria do not sense any damaging influences, e.g., FQ-free environment, LexA binds to CTGT region, so RNA synthesis is inhibited, RecA protein remains inactive and *qnrB* expression performs a basic level. (**Figure 25/a.**) When antibiotic concentration displays an increasing tendency, it is a warning signal for bacteria. Consequently, RecA coprotease will be activated (with ssDNA arising) that leads to autoproteolysis of LexA transcription repressor. Autoproteolized LexA leaves its binding region, so synthesis of RNA will be released from hindrance and production of QnrB PRP will be upregulated. QnrB protects gyrase through protein–protein interactions

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from antibacterial effects of ciprofloxacin. This process will result in elevated MIC values and decreased FQ susceptibility **(151-153)**. (Figure 25/b.)

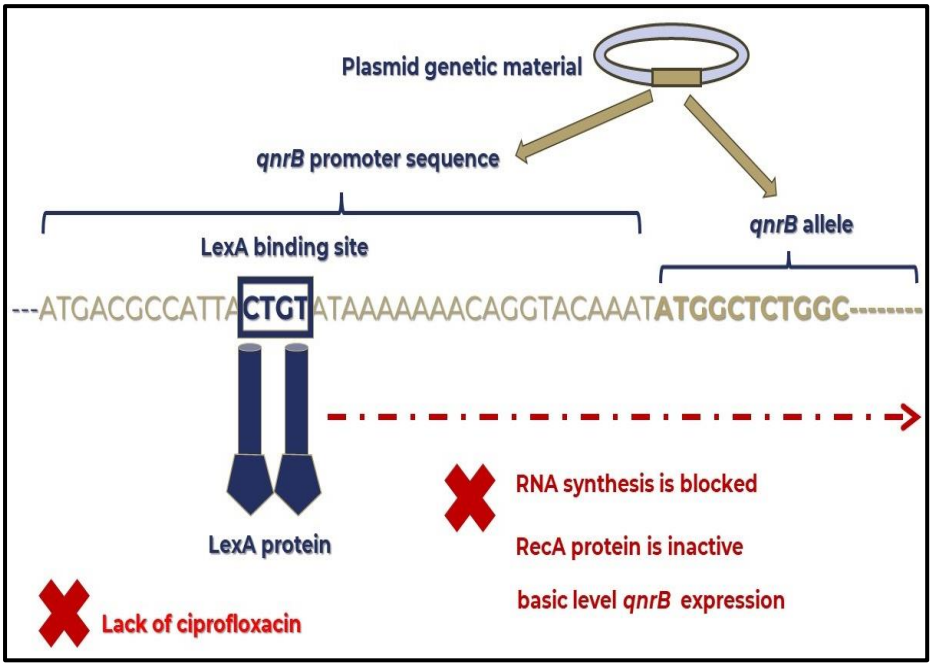

Figure 25/a. SOS-response in ciprofloxacin-free environment (own figure)

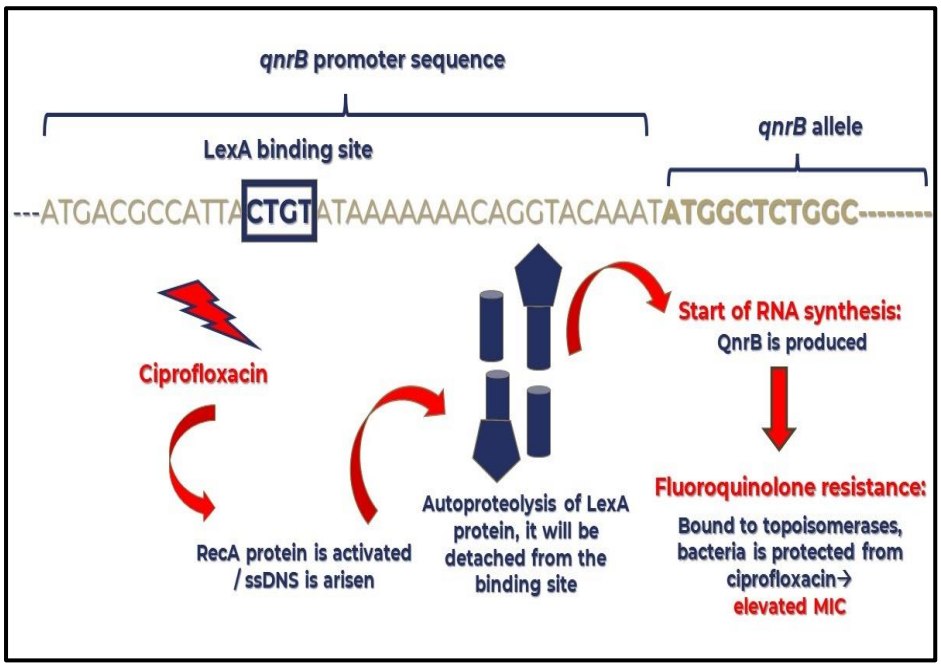

Figure 25/b. SOS-response in an environment with increasing ciprofloxacin concentration (own figure)

We accomplished antibiotic susceptibility testing according to EUCAST protocol from 2016, but this was changed in January 2017. According to the current recommendation, ciprofloxacin resistance breakpoint was modified from 1 to 0.5 mg/L **(147)**. Our results correlate with this revision, because we found that all of the tested *E. coli* strains were able to survive in 0.5 mg/L ciprofloxacin concentration linked to an intensified *qnr* expression. Medical relevance of our results is that ciprofloxacin was the first-line treatment for UTIs in the past years. It was also estimated that around 150 million UTI cases are reported per year and approximately 75% are caused by *E. coli* **(154)**.

Furthermore, we used ciprofloxacin concentrations in our work that correlate well with tissue concentrations during a per os therapy **(155)**. Since, a *qnr*-carrier *E. coli* is able to exhibit resistance by an increased *qnr* expression in the course of FQ therapy, this can lead to treatment failure. Additionally, activated by the SOS-system, *qnrB* is currently one of the most commonly detected PMQR determinants in the world and it has the biggest number of variants (almost 100) among Qnr determinants **(132)**. The explanation for this remarkably high prevalence is probably the evolutionary advantage compared to other *qnr* subfamilies. This theory is supported by the fact that *qnrB* is often carried by MDR plasmids, that also bear further associated resistance genes, like $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-14}}$, and *aac(6')-Ib-cr* **(154)**.

5.2. Role of integron-associated *qnrVC1* and *bla*$_{NDM-1}$ in MDR *P. aeruginosa* ST773

In the second phase of our work we analysed the resistance pattern of MDR *P. aeruginosa* ST773 hig-risk clone, that was isolated from urine sample. In this study we identified the co-existence of a transferable quinolone resistance determinant, namely *qnrVC1* and *bla*$_{NDM-1}$ carbapenemase. The *qnrVC1* determinant was carrying its own promoter sequence, it was harboured by a class I superintegron. This sequence contained core and inverse core sites of *qnrVC, intI1* as well as *attC* recombination sites, which are responsible for genetic transfer and insertion of gene cassettes in chromosomal integrons. Furthermore, activity of *intI1* sequence is also regulated by LexA, that plays an important role in the above detailed SOS response process. When the adaptation is needed for the bacteria against novel challenges, SOS response mechanism is triggered, so the uptaken mobilised cassettes are more likely could be incorporated **(49)**.

Aquatic environment promotes dissemination and exchange of resistance genes among waterborne pathogens **(156-157)**, thus Vibrionaceae family is hypothesized as the origin and reservoirs of Qnr determinants **(156, 158)**. Accordingly, *qnrVC1* allele was first described in the same class 1 integron of *Vibrio cholerae* O1 from Brazil **(150)**, and during their quite fast evolution other eleven *qnrVC* variants were also reported from integrons and plasmids **(137, 156)**.

In case of our *P. aeruginosa* strain, *qnrVC1* performed an active, more than twofold expression, indicating that it owns a relevant role even in the presence of QRDR mutations namely, Thr83Leu in GyrA and Ser87Leu in ParC. However, the reason for obtaining and expressing a Qnr determinant carried by transferable genetic elements that is associated to low-level FQ resistance in an isolate exhibiting high-level FQ resistance is not completely clear. A possible reason of this could be the theory that Qnr proteins have an „antitoxin" function and they provide an additional protection for DNA gyrase and topoisomerase IV from toxins in the environment, including FQs **(128)**. Similarly to the succesful contribution of QnrB in the selection of resistant strains, this protective effect of Qnr determinants may give an evolutionary advantage to worldwide reported high-risk clones. Development of FQ resistance by our *P. aeruginosa* strain is detailed on **Figure 26/a** and **26/b.**

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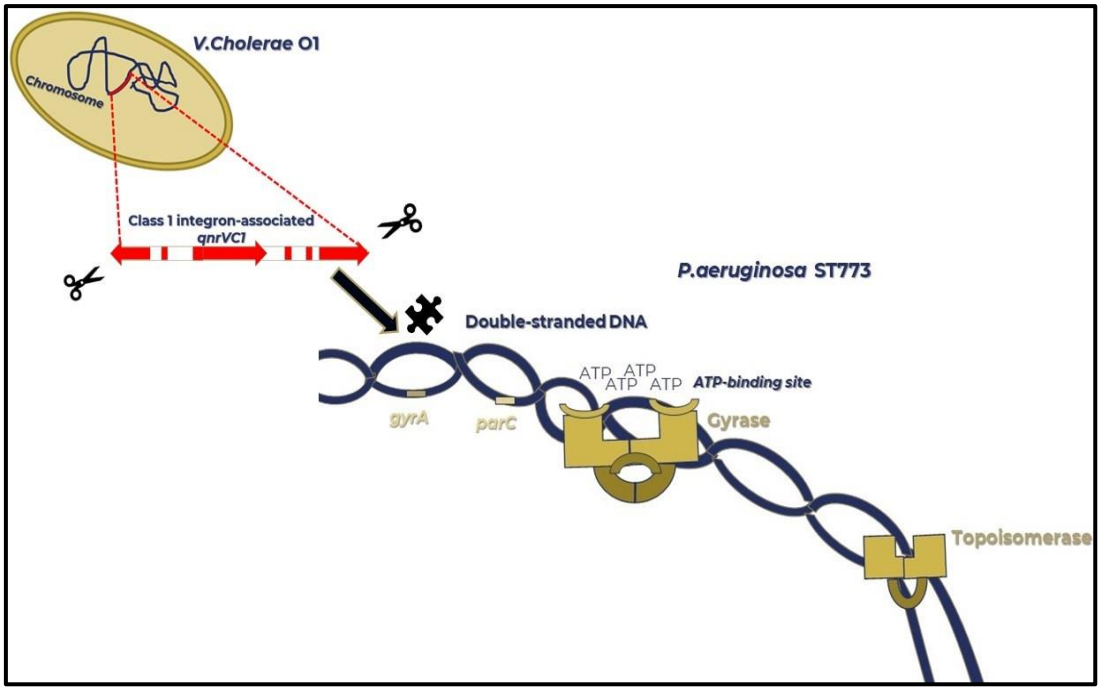

Figure 26/a. Acquisition of class 1 integron encompassing *qnrVC1* in our strain (own figure)

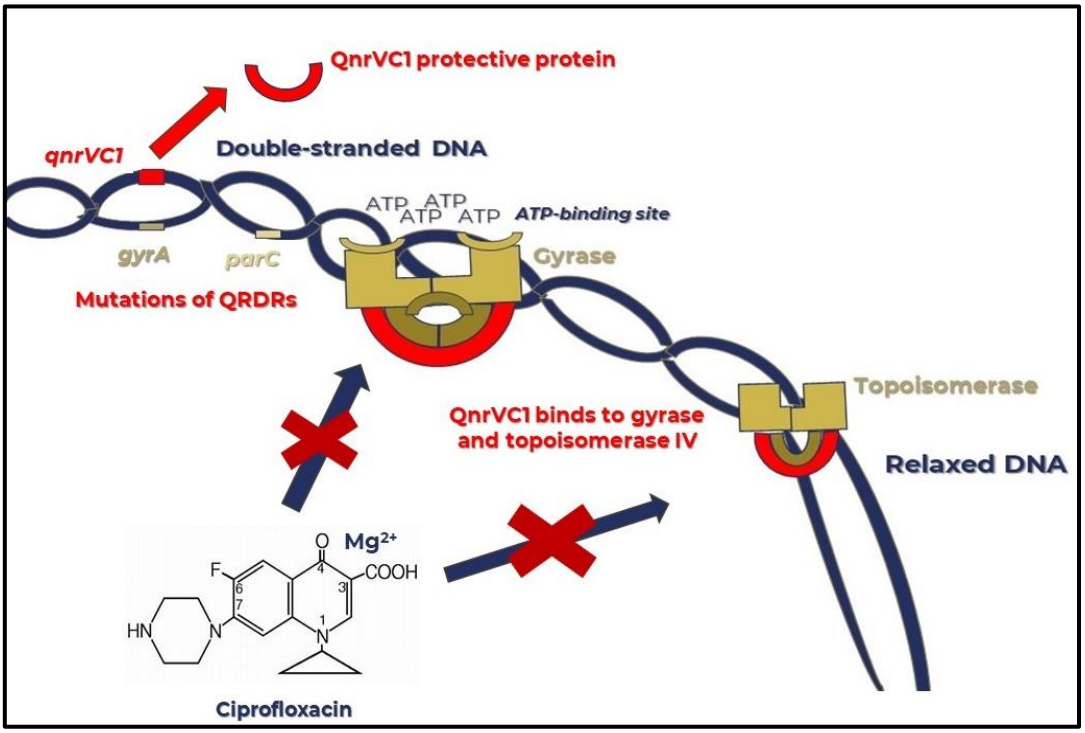

Figure 26/b. Development of FQ resistance in case of our strain (own figure)

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Based onWGS analysis, the studied strain in our work belonged to *P. aeruginosa* ST773. It is a MDR high-risk clone, that is identified across the globe as a *bla*$_{VIM-2}$ and *bla*$_{VEB-1}$-producer **(159-161)**, but instead of these determinants we detected NDM-1, that performed a fourfold activity compared to expression of *rpsl* housekeeping gene. Phylogenetic relationships among MDRPAE clones is summarized on **Figure 27.**

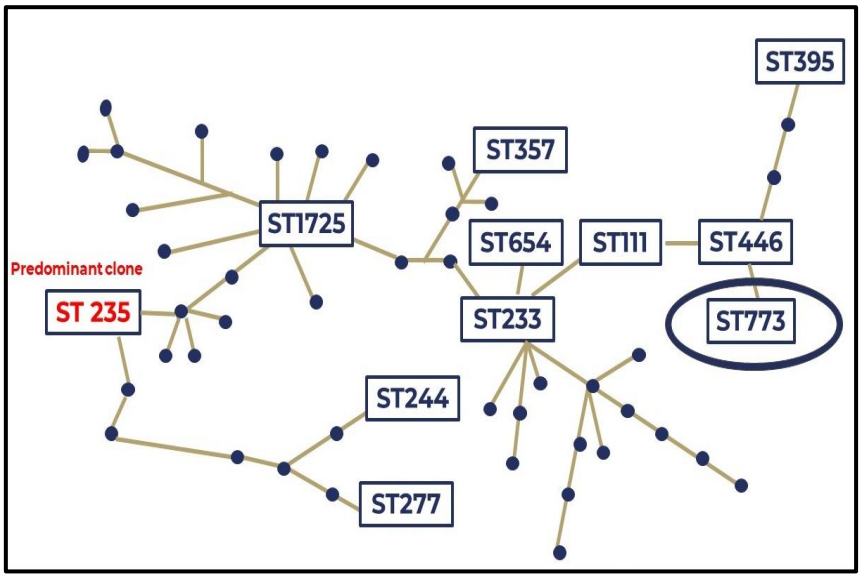

Figure 27. Schematic summary of MDRPAE clones' phylogeny based on the available data of NCBI. Red colour indicates the currently predominant ST235 clone and the relative position of ST773 is circled in blue on the phylogenetic tree. (own figure)

Of great concern, high-risk clones can persist in a longer period of time in hospital environment, and a horizontal gene transfer of different resistance genes to other bacterial pathogens is also possible. Therefore, our results indicate possibility of further spread of these genes among Gram-negative bacteria in hospitals **(137, 156)**. Our results underline the medical relevance of the mobile genetic elements, as they play a major role in the circulation of resistance genes, furthermore high-risk clones commonly acquire and integrate such determinants into their genomes **(24-26)**. (**Figure 6**.) Accordingly, an earlier study has already found *qnrVC1* in *P. aeruginosa*, although in that case a distinct integron structure involved this determinant **(162)**. Other studies reported various resistance mechanisms that were transferred between different bacterial species through mobile genetic elements, such as *bla*VIM-4, that was encompassed by an identical integron stucture in species like *A. hydrophila* and *K. pneumoniae* **(163-164)**.

5.3. Analysis of delafloxacin resistance and detection of ST43 *E.coli*

In this study, we investigated the delafloxacin resistance rate in 47 *E. coli* strains which were isolated from clinical samples (e.g., hemoculture and urine). Delafloxacin is a novel FQ agent, which was approved for clinical application in recent years. Our results demonstrate that 47% (22/47) of the *E. coli* strains developed resistance to delafloxacin. In comparison, ciprofloxacin and moxifloxacin resistance performed 51% (24/47), respectively and 38% (18/47) of the tested strains showed levofloxacin resistance. The MIC50 value of delafloxacin was 0.125 mg/L, while all other observed FQs had a 0.25 mg/L MIC50 value. Despite of the fact that delafloxacin is not available for clinical use in Hungary, it can be clearly seen that *E. coli* develops resistance against this new antibacterial agent.

Moreover, we also detected that ciprofloxacin and moxifloxacin resistance are frequently (20/47) linked to delafloxacin resistance. Notably, *E. coli* strains exhibiting ciprofloxacin MIC above 1 mg/L were already resistant to delafloxacin. This phenotype can be used as a marker to indicate delafloxacin resistance. Our results also present possible clinical indications for delafloxacin, as 20 *E. coli* strains were delafloxacin-susceptible, but they exhibited an ESBL phenotype and ciprofloxacin resistance. On the other hand, 22 delafloxacin-resistant strains were also detected and they produced ESBL as well.

Interestingly, levofloxacin MIC values did not strongly correlate to other FQs in our work. Two *E. coli* strains demonstrated delafloxacin resistance, but one strain was susceptible against ciprofloxacin and levofloxacin, while the other was sensitive to levofloxacin and moxifloxacin.

In our study, we selected two MDR *E. coli* strains for WGS analysis. Both strains belonged to ST43 *E. coli* international high-risk clone based on Institut Pasteur's MLST database. Interestingly, *E. coli* ST43 in Pasteur's MLST database corresponds to ST131, the worldwide predominant *E. coli* clone in the Achtman MLST scheme **(165-167)**. Belonging to phylogroup B2, the vast majority of ST131 *E. coli* is characterised by resistance to several antibiotics, including cefalosporins (based on CTX-M-type ESBL-production), carbapenems, AGs, FQs (mainly due to mutations of QRDRs and additional PMQRs), sulfonamides, nitrofurantoin, and tetracycline **(168)**. As MDR ST131 high-risk clone is the main source of resistance determinants and further emerging sister clones,

like ST1193 **(67)**, its phylogeny has been clustered into three major clades based on resistance traits and population genetics, namely clade A (H41), B(H22), and C(H30S). Worryingly, although subclones A and B are smaller groups, colistin-reistant strains of clade A are commonly isolated from environmental samples and foodborne pathogens from clade B are also often reported from urine and blood samples. In contrast, the biggest medical challenge is posed by clade C, that has two further subclones, namely C1/H30R and C2/H30Rx. Originated from clade B, clade C *E. coli* strains usually express bla_{TEM}; subclade C1 usually carry $bla_{\text{CTX-M-14}}$ or $bla_{\text{CTX-M-27}}$ ESBLs, and in contrast, subclade C2 is mostly linked to $bla_{\text{CTX-M-15}}$ **(70-71, 168)**. Phylogenetic tree of *E. coli* international high-risk clones is presented by **Figure 28.**

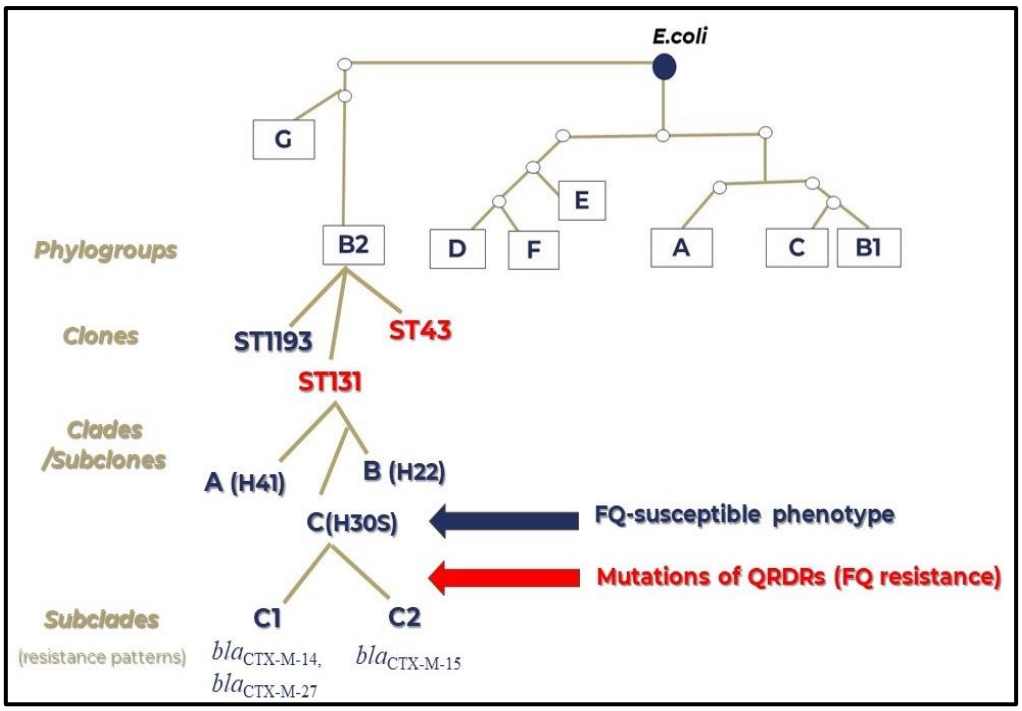

Figure 28. Phylogenetic tree of *E. coli* high-risk clones. (based on Pasteur's MLST schema) (own figure)

Additionally, *E. coli* ST43 was isolated from clinical samples in a few countries. In Panama, Central America, it was detected as CTX-M-15-positive and ciprofloxacin-resistant **(165)**. In Italy, it performed KPC-3-positivity and FQ resistance **(169)**. On the other hand, ST43 *E. coli* from USA was reported as FQ-resistant due to multiple QRDR mutations, but it showed sensitivity to third-generation cefalosporins and carbapenems

(170). In our study, the two strains of *E. coli* ST43 were ESBL-producers, bearing *bla*$_{CTX-M-1}$ and *bla*$_{CTX-M-15}$, and both strains were resistant to all tested FQ agents, including delafloxacin. The genetic background of FQ resistance in these two strains demonstrated the accumulation of multiple QRDR mutations. In case of strain 920/1, five mutations were present: GyrA S83L, D87N, ParC S80I, E84V and ParE I529L. Meanwhile, 951/2 established four mutations, namely GyrA S83L, D87N and ParC S80I, E84V and a PMQR determinant, *aac(6')-Ib-cr* was also detected (**Figure 29**).

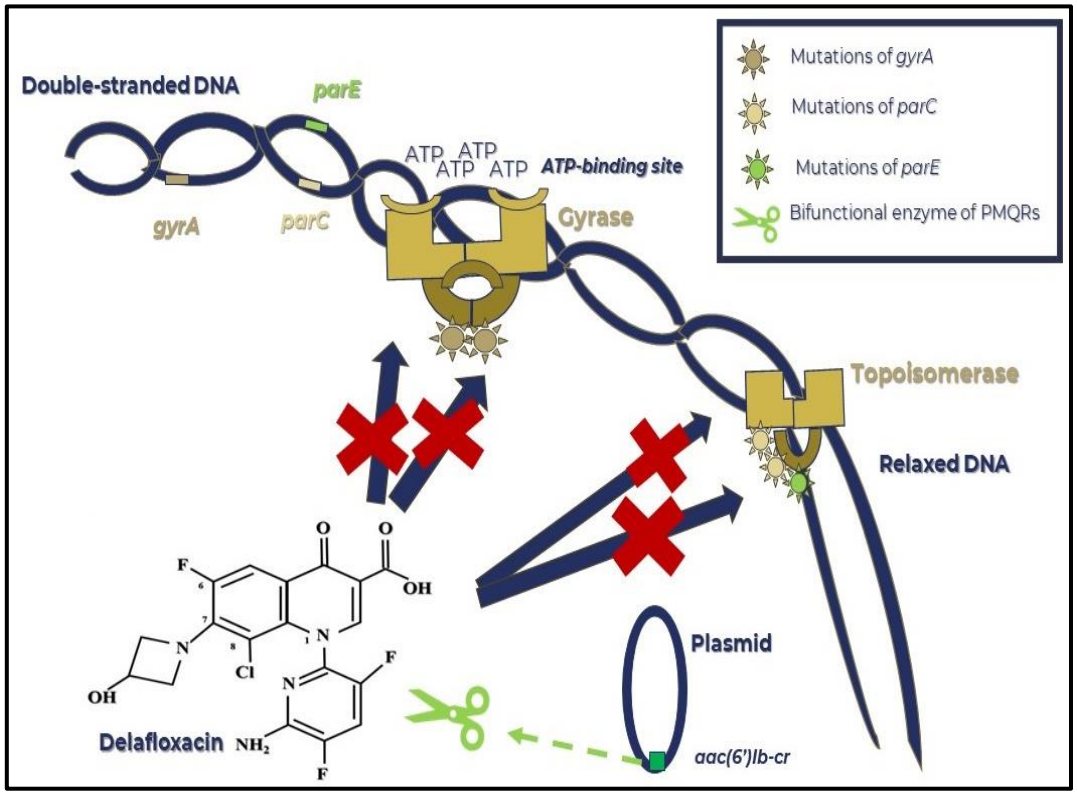

Figure 29. Development of delafloxacin resistance in case of 920/1 and 951/2 strains (own figure)

FQ resistance is commonly identified in Enterobacterales, especially in MDR international high-risk clones, including *E. coli* ST131, ST1193, ST69 and CC10 strains **(71, 163, 171-172)**, as well as *K. pneumoniae* ST11, ST15, ST101, ST147 and ST307 **(173-178)**. High-risk clones suffer a lower fitness cost and retain a favorable fitness after the development of FQ resistance, which facilitate their survival and further circulation

in hospital settings **(175, 179-180)**. It is an important feature, because they are able to persist and disseminate for a long period of time causing severe infections and meanwhile they can exchange their resistance determinants. This favourable fitness is promoted by double Ser mutations in QRDR sequences **(60)**. Our results of the third study phase correlate well to these earlier data as we detected delafloxacin resistance in MDR *E. coli* ST43 international high-risk clone carrying double Ser mutations in *gyrA* and *parC* genes. To date, delafloxacin resistance has been scarcely reported among bacterial isolates, e.g., in *S. aureus*, *N. gonorrhoeae*, and non-tuberculous Mycobacteria **(181-183)**.

In summary, in the third work we analyzed the efficacy of delafloxacin in a collection of *E. coli* strains. A limitation of our study is that we analysed only 47 strains; however, these early results are useful to gain insights into the further possible clinical use of delafloxacin. We detected a remarkably high prevalence (47%) of delafloxacin resistance among MDR *E. coli* strains. This report can be considered as a baseline result, since delafloxacin is not yet available for clinical application in Hungary. We also reported the detection of *E. coli* ST43 high-risk international clone in Hungary. This clone has been found in different geographic regions with various resistance patterns, in our study both *E. coli* ST43 strains established ciprofloxacin and delafloxacin resistance together with CTX-M-1- and CTX-M-15-type ESBL production.

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6. CONCLUSIONS

The main aspects of my PhD thesis were detection and analysis of FQ resistance development in *E. coli* and *P. aeruginosa* strains. This scientific work was divided into three sections.

In our first study model FQ-resistant *qnrB*-carrier *E.coli* was selected out after ciprofloxacin exposition. We detected a significantly high expression rate in case of QnrB1 (183-fold during short-term study and 212-fold in the course of long-term study), but it was not linked to chromosomal mutations of QRDR. During time-dependence analysis we found rising dynamics of *qnrC1* expression as well, but apart from *qnrB1*-bearer *E.coli* all of the challenged strains died after short-term study, thus further selection was not feasible. Furthermore, QnrA1, QnrB1, QnrC1 and QnrD1 expression rates in *E. coli* were not connected to a relevant plasmid copy number change (it ranged from 1.4 to 4.43-fold expression rate).

In the course of the second part we identified a MDR *P. aeruginosa* international high-risk clone, namely ST773 by WGS in a urine clinical sample. We deposited WGS data of this strain in NCBI database under accession number *RHDU00000000*. We also reported the co-existence of QnrVC1 and NDM-1, both of them were detected for the first time in *P. aeruginosa* ST773 clone. Sequences of *qnrVC1*-integron and flanking region of $bla_{\text{NDM-1}}$ were also uploaded in NCBI Genbank under accession numbers *MH782277* and *MK109012*, respectively.

Flanking region of *qnrVC1* was identical to that of described in *V.cholerae* from Brazil. In addition, following the earlier published data of *P.aeruginosa* high-risk clones, appearence of these novel resistance genes indicates the potential evolutional changes and further possible dissemination between even different genera in waterborne and nosocomial environments. Our results also display that class 1-integron harboured *qnrVC1* determinant played an additional protective role even after alterations of GyrA and ParC in MDR *P. aeruginosa*.

Despite of the fact, that delafloxacin is not yet available in our country, in the third part of our scientific work we reported a significant 47% prevalence of delafloxacin resistance among 47 *E.coli* clinical isolates, that originated from hemoculture and urine. Our results show that delafloxacin resistance is caused by multiple mutations of QRDR

in *E. coli*. To the best of our knowledge, our study was the first observation of delafloxacin resistance in Enterobacterales.

Furthermore, based on MIC distribution we found a correlation between the different generations of FQs as *E.coli* strains with ciprofloxacin MIC values above 1 mg/L were resistant to delafloxacin and the strains exhibiting 4 and 8 mg/L delafloxacin MIC values were moxifloxacin-resistant. Interestingly, levofloxacin displayed an unusual resistance phenotype, as it seemed independent from delafloxacin during our work.

Two MDR *E.coli* strains of our collection (ECO-SEOMI-LKH 920/1 and 951/2) performed the highest FQ MIC values combined with ESBL-production, thus they were chosen for WGS. Both of them were identified as ST43 international high-risk clones. Their assembled genomes were uploaded to NCBI Genbank, sequence read archive (SRA) identifiers: SAMN35019574 (ECO-SEOMI-LKH 920/1 strain) and SAMN35019575 (ECO-SEOMI-LKH 951/2 strain). In these two strains of ST43 clone double Ser alterations of QRDR determinants were detected as main causes of FQ resistance and for stabilisation during evolutional changes. Furthermore, $bla_{\text{CTX-M-1}}$ in *E.coli* ST43 clone was reported for the first time. Finally, we also detected an association between CTX-M-1, CTX-M-15 ESBL production and delafloxacin resistance.

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7. SUMMARY

Antibiotic resistance turned into a „silent" pandemic in the wake of COVID-19 as it is reponsible for the death of 1.27 million patients per year. As a multifaced threat (including circulation of resistance genes linked to mobile genetic elements and clonal expansion of nosocomial pathogens) it is currently one of the most urgent clinical challenges. Accordingly, huge efforts were made in order to find novel therapeutic agents, such as new fluoroquinolone agents (e.g., delafloxacin) to combat infections caused by antibiotic resistant bacteria. The earlier generations of FQs have been widely used in the last decades, due to their favourable features like broad antibacterial spectrum as well as excellent pharmacokinetics. Their frequent consumption led to the escalation of mutational and plasmid-mediated quinolone resistance (PMQR), those are connected to the evolution of multidrug-resistant (MDR) bacteria. In the first stage of our studies, we investigated *qnr*-carrier *E. coli* control strains in order the analyse the role of plasmid copy number and Qnr protective proteins after exposure to ciprofloxacin. Here we detected the selection of ciprofloxacin-resistant QnrB-positive *E.coli,* that was able to establish FQ resistance with increased *qnrB* gene expression. In the second phase of our work we identified a MDR *P. aeruginosa* ST773 by whole-genome sequencing. This strain expressed a class 1-integron-harboured *qnrVC1* determinant. An upregulation of QnrVC1 indicated a protective role against FQs even after development of chromosomal mutations namely, Thr83Leu of GyrA and Ser87Leu of ParC. In ST773 clone we also detected NDM-1. In the third part of our studies, our aim was to analyze resistance to delafloxacin in a collection of 47 *E.coli* isolated from hemoculture and urine samples. Despite of the fact, that delafloxacin is not yet available for therapy in Hungary, we detected a remarkable high prevalence (47%) of delafloxacin resistance. We investigated delafloxacin resistance mechanisms in two *E. coli* strains by WGS, and our results demonstrated that multiple mutations of *gyrA* and *parC* mediate delafloxacin resistance. Moreover, delafloxacin resistance correlated with ciprofloxacin and moxifloxacin resistance. In this study we identified MDR *E.coli* ST43 clone that was associated with CTX-M-1, CTX-M-15 ESBL production as well as with ciprofloxacin and delafloxacin resistance.

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9. BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

9.1. Publications related to the topic of the thesis

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9.2. Further Publications

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