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Plasmid-mediated fluoroquinolone resistance in Enterobacteriaceae

S.S.D. MED07

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1. Introduction

Fluoroquinolone resistance is emerging in Gram-negative pathogens, as reported in 2009 by the European Antimicrobial Resistance Surveillance Network that highlighted over 20% of invasive *Escherichia coli* isolates were resistant to fluoroquinolones in Europe. In Italy, Spain, Portugal, Hungary, Bulgaria and Cyprus the resistance rates exceeded 25% (1). In 2003, a survey of enteric bacteria in US intensive care units found more than 10% of these organisms to be resistant to ciprofloxacin (2). In 1999, a study found about 25% of healthy individuals living in Barcelona to be intestinally colonised with quinolone-resistant *Escherichia coli* (3). Levels of quinolone resistance in clinical *E. coli* isolates have been reported at 40% in Hong Kong in 2006 (4). These increasing numbers of resistant Enterobacteriaceae isolates worldwide are hardly explained only by chromosomal mutations, the traditional way of developing resistance to fluoroquinolones. The recent discovery of plasmid-mediated horizontally transferable genes encoding quinolone resistance might shed light on these phenomena (5).

Quinolones were introduced into clinical use in 1962 in the form of nalidixic acid, a total syntethic agent. Pharmacological improvements of the basic quinolone molecule resulted in fluoroquinolones (**Figure 1**), which became clinically available in the 1980s (6). Nowadays, several members of this antimicrobial agent group are used worldwide, since they possess excellent activity against Enterobacteriaceae and are the recommended therapy according to both CLSI and EUCAST (7, 8).

Over the decades, Enterobacteriaceae developed resistance to these agents, resulting in clinical therapy failure. Resistance to quinolones and fluoroquinolones is generally due to chromosomal mutations in genes coding for the target molecules of these agents, namely the DNA gyrase and topoisomerase IV enzymes. Both have characteristic sequences in their genes, called quinolone resistance determining regions (QRDR), where mutations occur. Interpretation of a pathogen as 'resistant' depends on the internationally approved breakpoints: the EUCAST ciprofloxacin breakpoint for Enterobacteriaceae is 1µg/ml (8, 9). However, low-level resistance corresponding to minimal inhibitory concentration (MIC) values exceeding those of wild-type *E. coli* but still below the resistance breakpoint (**Figure 3**), not necessarily entail mutations in the target molecules (9). Spontaneus chromosomal mutations occur in one out of 10^8 - 10^9 cells, and double mutations required for clinically important ciprofloxacin resistance are even rarer events,

one from 10^{14} (10, 11). Mutation frequency is 100 times higher in bacterial cells in low-level resistance, facilitating the selection to higher resistance levels (12). Permeability of Gram-negative cell wall to fluoroquinolones was described; notably, in rough lipopolysaccharide (LPS) mutants of *Salmonella typhimurium* MICs to hydrophobic quinolones like nalidixic acid are 2-4 folds lower than the smooth LPS-variant, but MICs to hydrophilic ones (e.g: ciprofloxacin and norfloxacin) are not affected (13). In the case of OmpF mutants of *E. coli* the MIC of nalidixic acid, norfloxacin and ciprofloxacin had a two-fold increase, i.e. in the range of low-level resistance (13, 14). Active efflux systems (acrAB-TolC) resulting in decreased intracellular accumulation of fluoroquinolones in *Escherichia coli* and *Klebsiella pneumoniae* were detected (15, 16, 17, 18, 19).

In 1998, the first plasmid-mediated quinolone resistance (PMQR) determinant, the *qnr* gene, was discovered on a transferable plasmid of a *K. pneumoniae* human clinical isolate, after conjugation causing low-level resistance in recipient strains (12). Later on, other plasmidic genes were described conferring low-level fluoroquinolone resistance, such as ciprofloxacin modifying enzyme, the cr variant of aminoglycoside-acetyltransferase(6')-Ib in 2006 and fluoroquinolone specific efflux pumps, *qepA* being the first detected in 2007 followed in 2009 by *oqxAB* (20, 21, 22).

Plasmids, defined as bacterial extrachromosomal, doublestranded, circular DNA molecules capable of autonomous replication have crucial role in the dissemination of resistance genes like extended-spectrum beta-lactamase (ESBL) and PMQR genes (23). Nevertheless, association between these two resistance mechanisms in Enterobacteriaceae has been detected worldwide (5, 24, 25, 26). These findings are alarming, since ESBLs confer resistance to third generation cefalosporins, while PMQR causes low-level fluoroquinolone resistance. These antimicrobial agents are in the first line of option for several infectious diseases (e.g. urinary tract infections and pneumonia). If they are found to be ineffective because of resistance, clinicians will face worrisome situations.

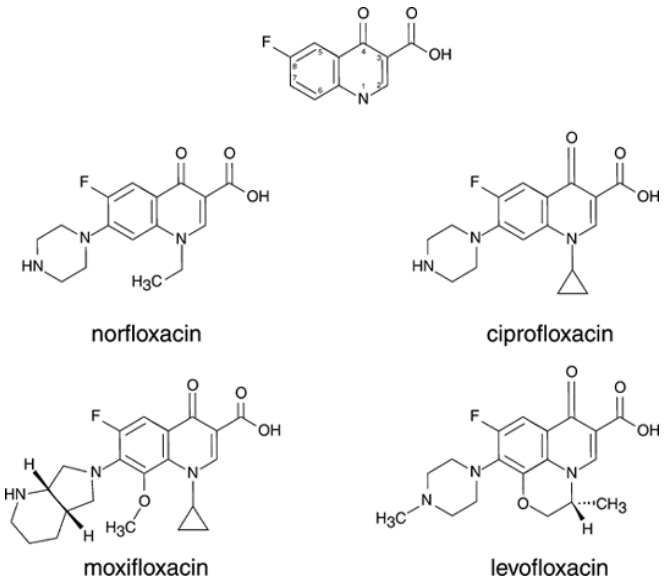
1.1 Quinolone and fluoroquinolone antimicrobial agents

Quinolones and fluoroquinolones are effective antimicrobial agents showing different efficacy against infections. Nalidixic acid is a quinolone agent effective against Enterobacteriaceae in the site of urinary tract, since it reaches therapeutically active concentrations only there (27).

Pharmacological improvements on the basic quinolone ring with the addition of fluorin at position C6 and piperazynil or related rings at position C7 yielded the fluoroquinolones (**Figure 1**). Several agents are used in clinical practice such as norfloxacin, ciprofloxacin, levofloxacin and moxifloxacin. They achieve higher serum levels leading to better tissue penetration and more potency against Enterobacteriaceae and other species (6, 27).

Norfloxacin and ciprofloxacin are active against several Gram-negative bacteria (besides Enterobacteriaceae they can be used successfully also against other pathogens like *Neisseria gonorrhoeae*), generally used in urinary tract infections (e.g.: cystitis, urethritis). Ciprofloxacin is active against *Pseudomonas aeruginosa* and also used against enteric infections of *Salmonella* spp. and *Shigella* spp. Levofloxacin and moxifloxacin have therapeutically active concentration at the respiratory tract and show therapeutic activity against Gram-positive bacteria as well (e.g.: *Staphylococcus aureus, Streptococcus pneumoniae*), these features make them useful in lower respiratory tract infections such as pneumonia. Antimicrobial spectrum of moxifloxacin includes anaerobic bacteria too (e.g.: *Bacteroides* spp. and *Clostridium* spp.) (27).

Adverse effects of fluoroquinolone therapy are rare, including fotosensitivity and with allergic reactions developing only in less than 0.5% of the population. Joint cartilage formation was damaged when ciprofloxacin was experimentally administered in animals, therefore treatment with fluoroquinolones is not recommended for pregnant, breastfeeding women and children under age sixteen (27).



Basic quinolone chemical structure, with fluorin at position C6.

Addition of piperazynil ring at position C7. Active in urinary tract infections against Gram-negative bacteria.

Addition of substituents at position C7. Active in respiratory tract infections mainly against Gram-positive bacteria.

Figure 1. Overview of fluoroquinolones frequently used in clinical practice (5).

1.2 Mechanism of action of fluoroquinolones is the inhibition of type II topoisomerase enymes, namely gyrase and topoisomerase IV. Both of them are involved in the bacterial DNA replication (**Figure 2**); gyrase introduces negative supercoils (or relaxes positives) into DNA by looping, topoisomerase IV is responsible for unlinking DNA following replication. Fluoroquinolone-gyrase-DNA and fluoroquinolone-topoisomerase IV-DNA lethal complexes are formed and the DNA replication is blocked. This leads to bactericid effect therefore bacterial cells die (27, 29).

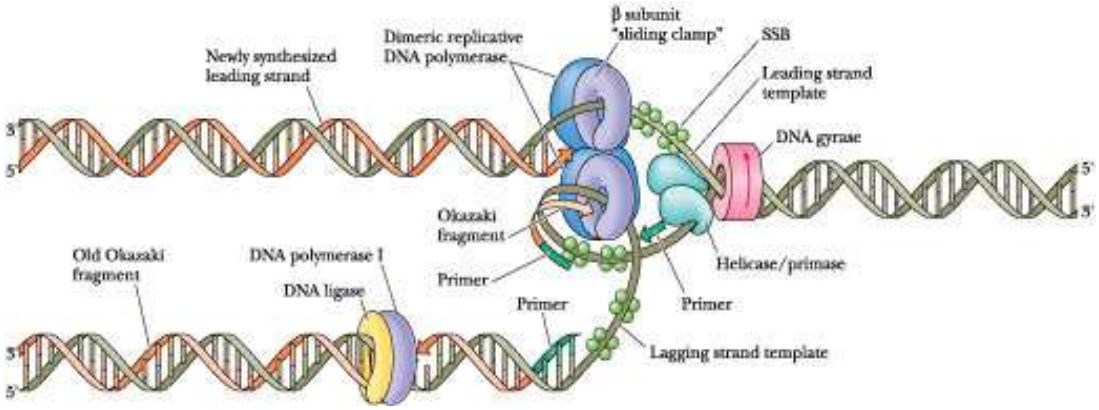


Figure 2. DNA replication.
http://web.virginia.edu/Heidi/chapter30/Images/8883n30_15.jpg

1.3 Mechanisms of resistance against fluoroquinolones

Until recently, two mechanisms had been found to determine resistance to fluoroquinolones (and quinolones, since in almost all cases organisms resistant to fluoroquinolones are resistant to nalidixic acid as well) (5). The most important of these mechanisms in Enterobacteriaceae is the accumulation of mutations in the bacterial enzymes targeted by fluoroquinolones: DNA gyrase and DNA topoisomerase IV (28). Quinolones and fluoroquinolones bind to these enzymes and stabilise a drug-enzyme-cleaved DNA complex allowing lethal double-stranded DNA breaks to accumulate unrepaired (29). Each target enzyme's coding gene (namely *gyrA*, *gyrB* for DNA gyrase and *parC*, *parE* for DNA topoisomerase IV) has quinolone-resistance determining regions (QRDRs), i.e. portions of the DNA-binding surface of the enzyme (30) at which aminoacid substitutions can diminish quinolone binding. Generally, multiple mutations are required to achieve clinically important resistance in Enterobacteriaceae; when such organisms are quinolone resistant they are nearly always found to have one or more QRDR mutations (**Table 1**). Another well-described resistance mechanism is the decreased intracellular drug accumulation by upregulation of native efflux pumps (31) (e.g. AcrAB-TolC in *E. coli*) and/or decreased

expression of outer membrane porins (32). Several Enterobacteriaceae species own a chromosomal native AcrAB-TolC efflux pump belonging to the resistance-nodulation division (RND) family. AcrA is a membrane fusion protein, AcrB is an inner-membrane pump and TolC is an outer-membrane protein and they build up an efflux pump whose overexpression leads to high-level fluoroquinolone resistance observed in *E. coli* and *Klebsiella* spp. (15, 16, 31).

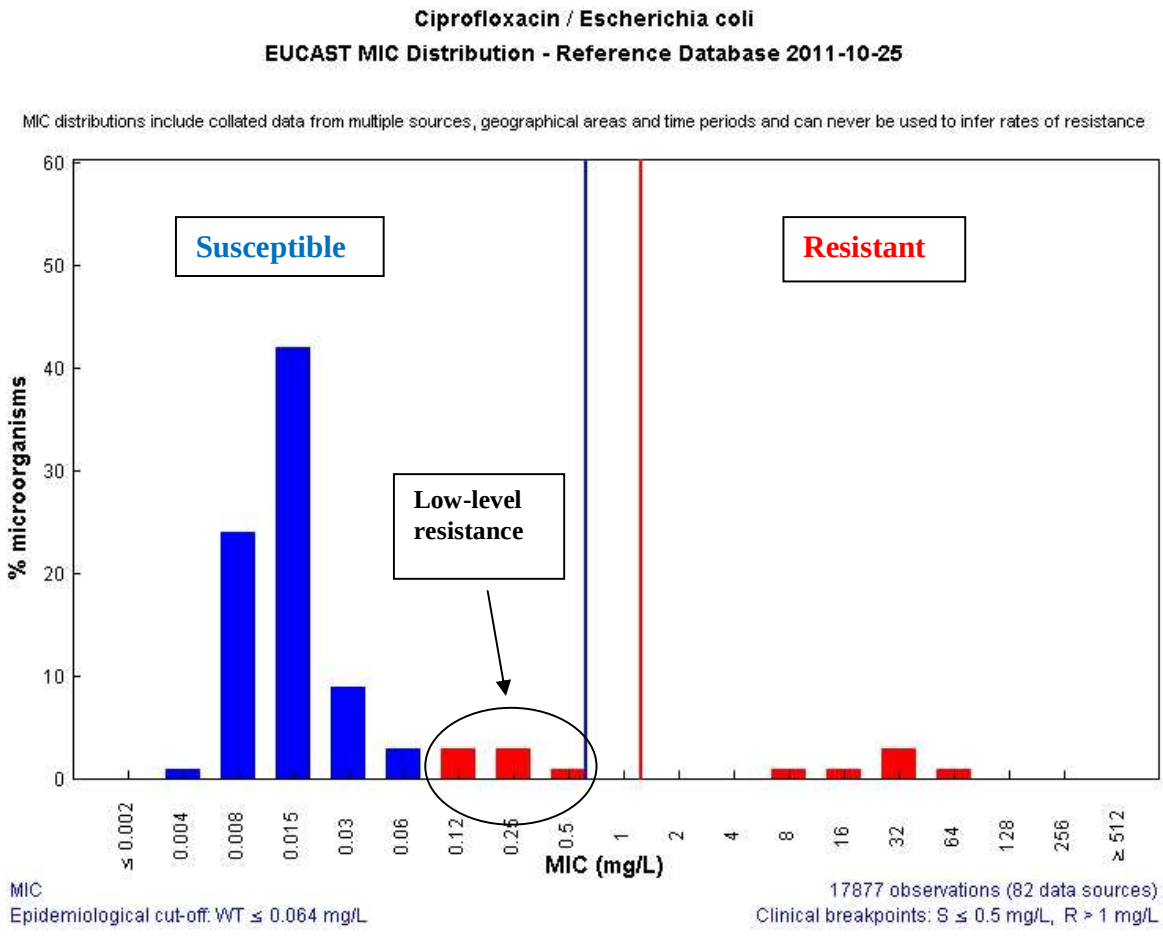
Permeability of Gram-negative cell wall to fluoroquinolones was also described, notably rough LPS-mutants of *Salmonella typhimurium* have an MIC 2-4 folds decrement to hydrophobic agents like nalidixic acid compared to the smooth LPS-variant, but MICs to hydrophilic (e.g: ciprofloxacin and norfloxacin) are not affected (13). In the case of OmpF mutants in *E. coli* a two-fold increase in the MIC of nalidixic acid, norfloxacin and ciprofloxacin was observed in the range of low-level resistance (13, 14).

These mechanisms of resistance are mutational, arising in an individual organism and then passing vertically to its surviving progeny. Neither of the above mentioned mechanism seems to transfer effectively on mobile genetic elements (5).

Table 1. MIC values with aminoacid substitutions due to mutations in *gyrA*, *gyrB* and *parC*.

Strain	**Ciprofloxacin MIC (µg/ml)**	***gyrA***	***gyrB***	***parC***
C20	0.007	Ser83… Asp87	Lys447	Ser80…Glu84
C1	0.06	Ser83… Asp87	Lys447	Ser80…Glu84
C8	0.125	Ser83… Asp87	Lys447	Ser80…Glu84
C5	0.25	Ser83… Asp87	Lys447	Ser80…Glu84
C4	0.25	Leu83…Asp87	Lys447	Ser80…Glu84
C10	1	Leu83…Asp87	Lys447	Arg80…Glu84
1327	2	Leu83…Asp87	Lys447	Ile80…Val84
1289	4	Leu83…Asp87	Lys447	Arg80…Glu84
1363	4	Leu83…Asp87	Glu447	Ser80…Lys84
1273	8	Leu83…Tyr87	Lys447	Ser80…Lys84
1331	8	Leu83…Asn87	Lys447	Ser80…Lys84
1574	8	Leu83…Asn87	Lys447	Ile80…Glu84
1283	16	Leu83…Asn87	Lys447	Arg80…Glu84
1334	16	Leu83…Asn87	Lys447	Ile80…Glu84
1360	32	Leu83…Tyr87	Lys447	Ser80…Lys84
1416	32	Leu83…Asn87	Lys447	Ile80…Glu84
1323	64	Leu83…Asn87	Lys447	Ile80…Glu84
1319	64	Leu83…Asn87	Lys447	Ile80…Val84
1383	128	Leu83…Tyr87	Lys447	Ile80…Lys84

(9)



(8)

Figure 3. Distribution of *E. coli* clinical isolates in susceptible and resistant MIC values.

Internationally-approved resistance breakpoints are required to determine which organism is susceptible or resistant. **Figure 3** shows the EUCAST distribution in *E. coli* of different ciprofloxacin MIC values, and low-level resistance (from 0.06 µg/ml to 0.5 µg/ml) are also highlighted (8). This unique status is below the resistance breakpoint and referres to a heterogenous population which does not necessarily have aminoacid substitution in gyrase or topoisomerase IV (9) because plasmid-mediated quinolone resistance determinants can maintain this phenotype. These determinants increase also the mutation frequency and in case of exposure to fluoroquinolones will facilitate the selection of high-level resistance (12).

1.4 Plasmid-mediated quinolone resistance determinants

Three different mechanisms were detected, namely Qnr determinants (12), quinolone specific efflux pumps (20, 21) and the aminoglycoside acetyltranferase(6')-Ib-cr variant enzyme (22). The genes of these determinants are plasmid localized and individually cause low-level resistance.

1.4.1 Qnr determinants

The Qnr proteins belong to the pentapeptide-repeat family, which is defined by a tandem five aminoacid repeat with the recurrent motif [Ser, Thr, Ala or Val] [Asp or Asn] [Leu or Phe] [Ser, Thr or Arg] [Gly] (33). More than 500 proteins are known to contain such pentapeptide-repeat motifs, but the function of nearly all of these proteins is unknown (5).

So far several members of Qnr determinants were identified and labeled as Qnr A, B, S, C and D while aminoacid variations are indicated in numbering (**Figure 4**) (34). Their genes were described in human clinical bacterial isolates worldwide (5, 24, 25, 26, 35). The first *qnr*, now named *qnrA1* was detected in 1998 from *K. pneumoniae* (12), and to date 7 different variants of *qnrA* are known (36). In the case of *qnrS*, the first was found in 2005, in *Shigella flexneri* 2b (37), and until today 5 variants are recognised (36). The most heterogenous cluster of the *qnr* gene family is *qnrB*, having 47 different alleles (36, 38), the first of them detected in 2006 in *K. pneumoniae* (39). Two additional members of the *qnr* gene family were detected in 2009, namely *qnrC* in *Proteus mirabilis* and *qnrD* in *Salmonella enterica* Serovar Kentucky and Bovismorbificans. Only one allele is known for each of these last two determinants (36, 40, 41).

Chromosomal *qnr* genes were also discovered and named after the specific organism where it was found and shortened like Vv*qnr* from *Vibrio vulnificans* or *Efqnr* from *Enterococcus faecalis* (34). The progenitor of *qnrA* gene was discovered in an environmental bacterium *Shewanella algae*, while the source of *qnrB* determinants was found to be *Citrobacter* spp. (34, 38). All *qnr* determinants are listed in a homepage: www.lahey.org/qnrStudies and updated regulary after the new publications (36).

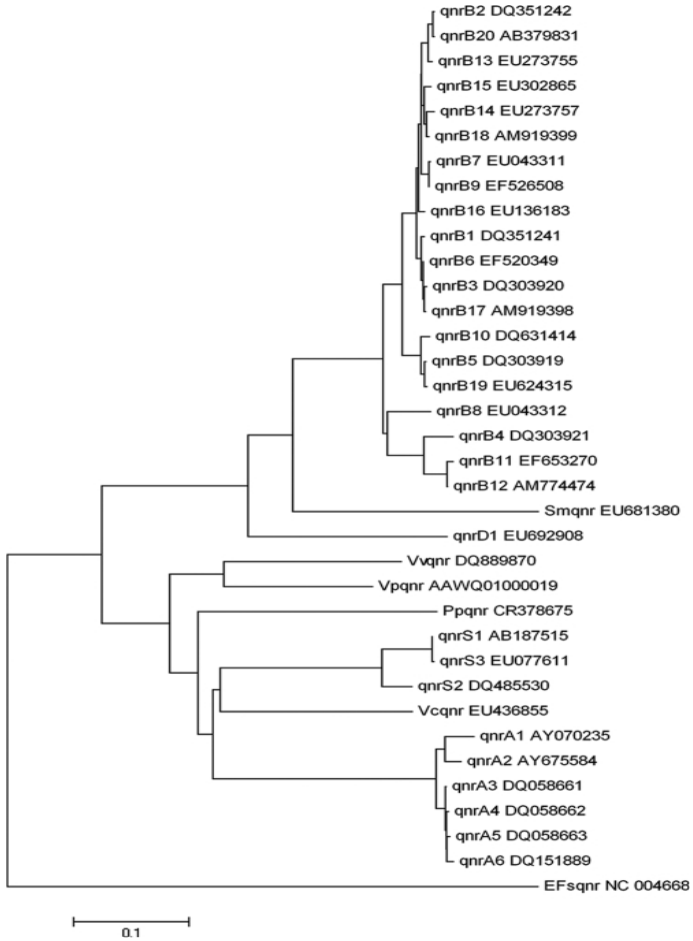


Figure 4. Family of the *qnr* determinants with Genbank accession numbers (40).

The direct effect of Qnr A is the reverse of the inhibition of gyrase-mediated DNA supercoiling caused by ciprofloxacin. Qnr A binding to gyrase or topoisomerase IV inhibits the gyrase-DNA interaction, and could account at least in part for the protection against quinolones by minimising opportunities for these agents to stabilise the lethal gyrase-DNA-quinolone complex (5). The first *qnr*-harbouring plasmid (pMG252) had a broad host range feature and could be transferred by conjugation from *E. coli* to *C. freundii*, *S. typhimurium* and *P. aeruginosa* (12). Quinolone resistance was expressed in all transconjugant strains with ciprofloxacin MICs showing 2-5 times increment at the range of low-level resistance (0.125 μg/mL in *C. freundii*, 0.25 μg/mL in *S. typhimurium,* 0.5 μg/mL in *P. aeruginosa*). In all genera, the highest resistance was seen with nalidixic acid and the lowest with MICs of clinafloxacin. Mutation frequency increased 100 times (from $1.6x10^{-8}$ to $3.5x10^{-6}$) in J53 *E. coli* with pMG252 comparing to the one without the plasmid (12).

The clinical importance and the selection of *qnr* positive cells to high-level fluoroquinolone resistance can be demonstrated by mutant preventive concentration (MPC), the lowest concentration at which no mutants can be obtained. **Figure 5** shows how an inoculum of 10^{10} *E. coli* J53 carrying *qnr*-harbouring plasmid (pMG252) has 1.25 µg/ml MPC value, compared to the wild-type *E. coli* J53 without *qnr*-harbouring plasmid (pMG252) having less than 0.2 µg/ml MPC value. This data explains the mutation window, from 0.2 µg/ml to 1.25 µg/ml, at which during ciprofloxacin therapy novel mutations can be promoted. Since with *per os* administered ciprofloxacin the maximum serum concentration reaches 2.9 µg/ml and 35% bounds to serum proteins, the remaining ~1.8 µg/ml is free. This free ciprofloxacin will not be able to penetrate equally in each tissue, and bacterial cells will be exposed to lower concentrations, giving opportunity for *qnr* positive cells to develop mutation.

Figure 6 gives similar data comparing an inoculum of 10^{10} *E. coli* J53 with one *gyrA* mutation has also 1.25 µg/ml MPC value, compared with *E. coli* J53 lacking *gyrA* mutation has less than 0.2 µg/ml. The two graphs together show that *qnr* positive and 1-time *gyrA* mutant bacterial cells behave similary after exposure to ciprofloxacin, both can develop new mutation (42).

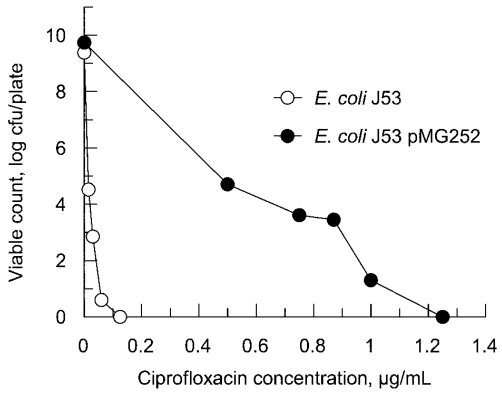


Figure 5. (42)
E. coli J53 with and without pMG252 (*qnrA1*-harbouring plasmid)

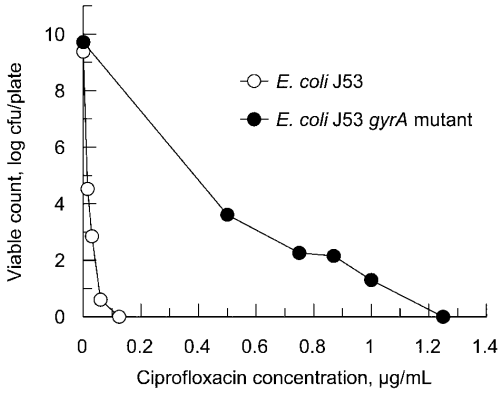


Figure 6. (42)
E. coli J53 with and without *gyrA* mutation (Ser83Leu)

Further explanation of acquiring chromosomal mutations by *qnr* determinants is based on the induction of SOS response of bacterial cells when DNA damage occurs. LexA is the central regulator of the SOS response system, which represses *qnrB* expression (45). All known *qnrB* determinants have a conservated region in its promoter sequence with a LexA-binding site allowing controll (**Figure 7**). Ciprofloxacin, a known inducer of SOS response, upregulates LexA which deliberates *qnrB* from the repression and enhances its RNA and protein synthesis (**Figure 8**). The regulation of other *qnr* determinants is not known (38, 44, 45).

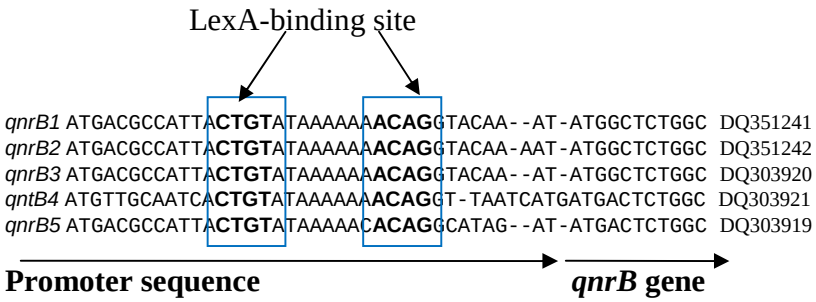


Figure 7. Promoter region of each *qnrB* determinant starts with ATG triplet and has conservative motifs CTGT and ACAG acting as LexA binding site. This part explains the role of *qnrB* determinants in SOS response. The *qnrB* gene coding sequence starts at the second ATG triplet, encoding for methionin (44).

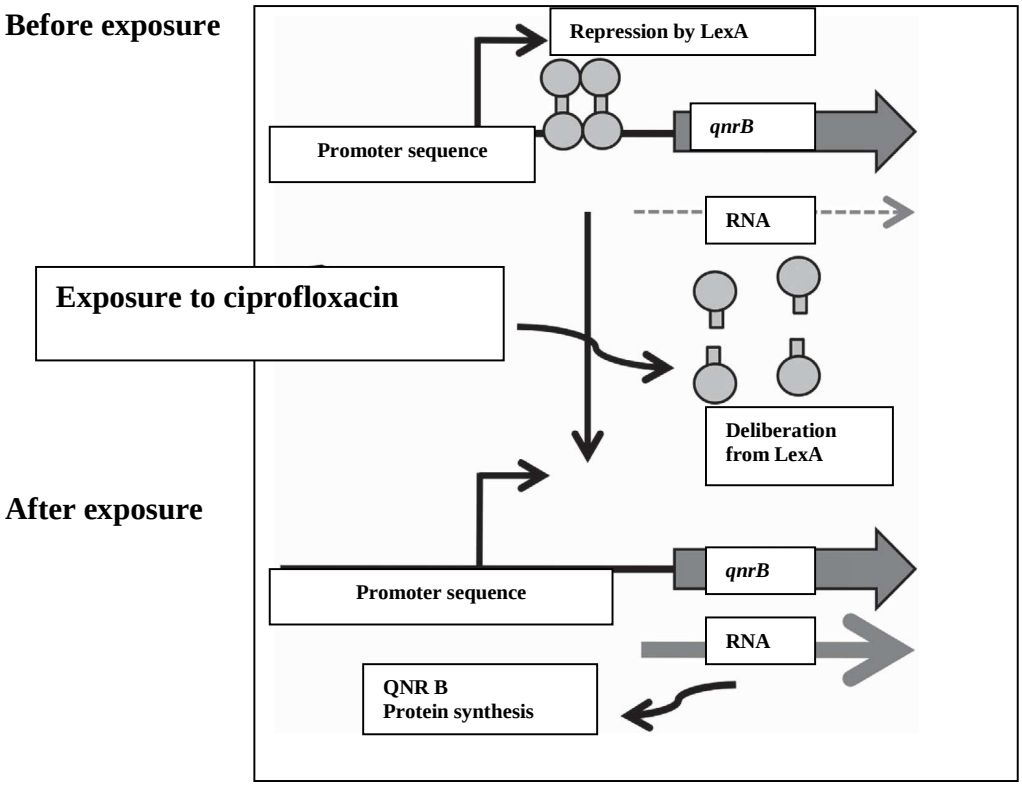


Figure 8. SOS response is activated after DNA damage. Exposure to ciprofloxacin can induce SOS response leading to the deliberation of *qnrB* gene expression from LexA repression. This promotes RNA and protein synthesis of Qnr B determinant (45).

1.4.2 Fluoroquinolone specific efflux pumps

QepA and QepA2 are 511-aminoacid proteins related to the major facilitator superfamily (MFS) of 14-transmembrane segment efflux pumps (**Figure 9**). Both cause a moderate increase (five or more times) in the range of low-level resistance to norfloxacin and ciprofloxacin, but do not significantly affect activity of less hydrophilic (levofloxacin, moxifloxacin, pefloxacin, gatifloxacin, sparfloxacin) or hydrophobic (nalidixic acid) derivatives. Its function is an active efflux, the energy is provided by a proton motive force and inhibited by carbonyl cyanide m-chlorophenylhydrazone (20, 46, 47). It was first described in Japan, in 2007 on a plasmid of a clinical urine isolate of *E. coli*. Other surveys from Belgium, France and China reported this determinant (20, 24, 35, 47, 48).

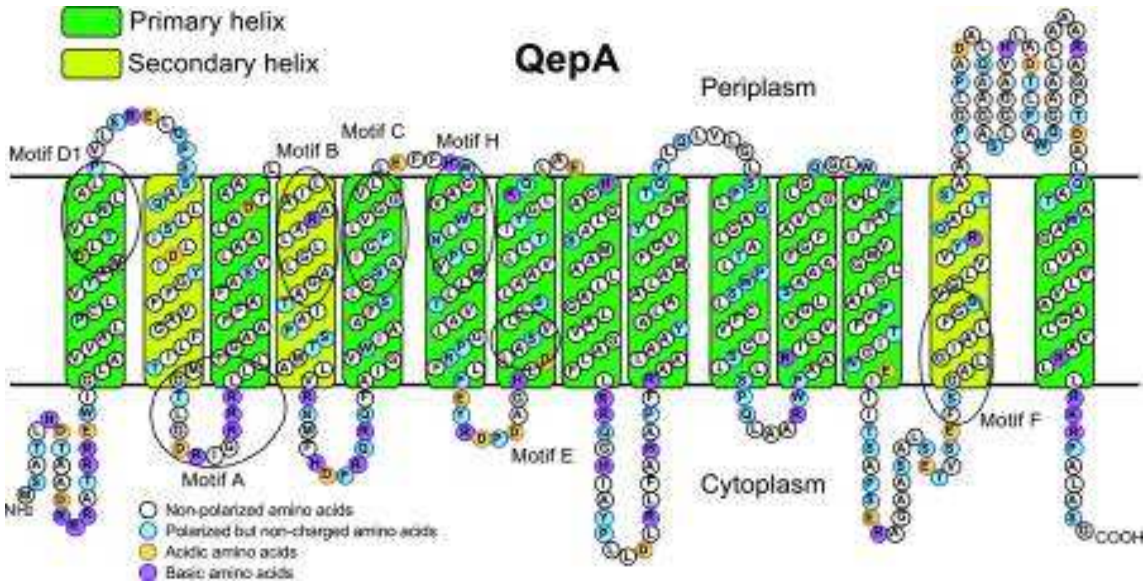


Figure 9. The secondary transmembrane structure of QepA (511-aminoacid protein) (20).

The OqxAB (**Figure 10**) belongs to the resistance-nodulation division (RND) family, being one of the first plasmid-borne efflux pumps described. The *oqxA* gene codes for the OqxA membrane fusion protein while the *oqxB* codes for the OqxB protein containing 12-transmemrbane α-helices for the inner-membrane pump. This system requires TolC outer-membrane protein to function fully (21). This is the first identified genetic resistance mechanism towards olaquindox, an agent used as growth enhancer in pigs (21). Later on other substrates of this pump were detected such as: chloramphenicol (21), nalidix acid, norfloxacin and ciprofloxacin (49). The *oqxAB* was first detected in 2004 in porcine isolates in Denmark and Sweden, on a plasmid of *E. coli* (21). In 2009 a report from the Republic of Korea detected in human clinical isolates, located on a plasmid of *E. coli* and in the chromosome of *K. pneumoniae.* In 2010 in a survey of farmworkers in China revealed this dererminant in *E. coli.* (35, 50). Inhibitor of this efflux pump carbonyl cyanide m-chlorophenylhydrazone (21).

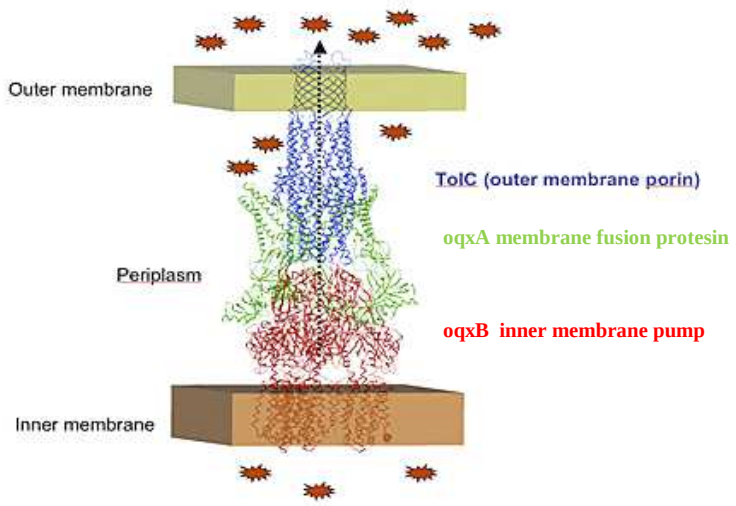
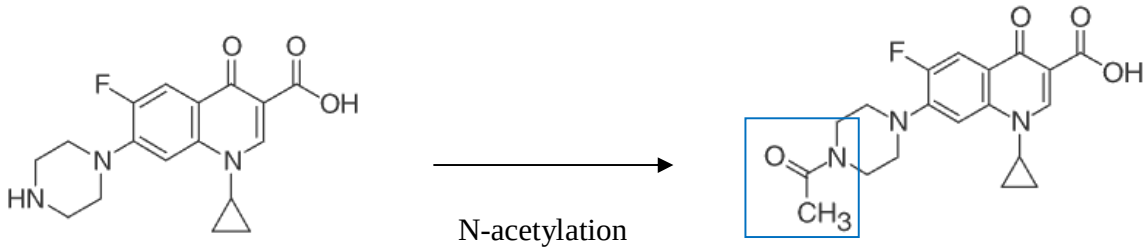


Figure 10. Structure of OqxAB efflux pump as a RND type efflux pump.
http://www.mpexpharma.com/images/illustration.gif

1.4.3 Aminoglycoside acetyltranferase(6')-Ib-cr variant

Approximately 30 variants of aminoglycoside acetyltransferase(6')-Ib have already been described since 1986. Two codon changes, namely Trp102Arg and Asp179Tyr, are found to be necessary, sufficient and unique for the cr-variant (ciprofloxacin resistance phenotype) comparing to the wild-type aminoglycoside acetyltransferase(6')-Ib (22). The function of the enzyme is to N-acetylate ciprofloxacin at the amino nitrogen on its piperazinyl substituent (**Figure 11**) (22). Norfloxacin has also free amino nitrogen acting as substrate of enzyme, while other fluoroquinolones lacking an unsubstituted piperazinyl nitrogen, like levofloxacin and moxiflxacin are unaffected (5). The effectiveness of this enzyme is modest, leading to an increase in the MIC of ciprofloxacin and norfloxacin three-fold to four-fold in the range of low-level resistance (22).



ciprofloxacin N-acetyl ciprofloxacin

Figure 11. Action of aac(6')-Ib-cr variant enzyme on ciprofloxacin: N-acetylation of amino nitrogen on the piperazinyl substituent (5).

Although the activity of the aac(6')-Ib-cr variant enzyme is modest, it increases the mutant preventive concentration (MPC) of the host cell to 3.2 µg/mL thus facilitating the selection of high-level fluoroquinolone resistance. **Figure 12** shows the ciprofloxacin values where *aac(6')-Ib-cr* positive isolates have opportunity to develop new mutations in quinolone-resistance determining region (QRDR). During *per os* ciprofloxacin therapy the peak free serum ciprofloxacin concentration is approximately 1.8 µg/mL, it will not reach MPC value. Furthermore, the *qnr* and *aac(6')-Ib-cr* variant work additively, since these two determinants have different mechanism of action and they together enhance the development of fluoroquinolone resistance (22).

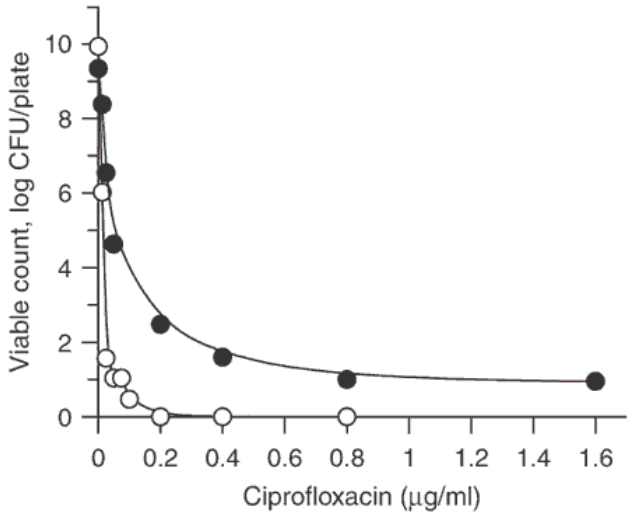


Figure 12. Mutant preventive concentration (MPC) assay for comparing wild-type *E. coli* J53 ○ with *E. coli* J53 carring pBCSK-*aac(6')-Ib*-cr ●. The graph shows that, at 1.6 µg/ml the peak serum ciprofloxacin concentration surviving mutants could be still recovered. The presence of *aac(6')-Ib*-cr increased the mutant preventive concentration from 0.2 µg/ml to 3.2 µg/ml (22).

2. Aim of the thesis

The aim of this thesis was to investigate the plasmid-mediated quinolone resistance determinants. For this purpose we collected clinical Enterobactericaeae isolates from microbiological diagnostic laboratories of North-East Italy. We focused on low-level and high-level fluoroquinolone resistant strains, plus those exhibiting beta-lactam-resistant phenotypes.

Our objectives were:

1) to screen for genes of plasmid-mediated quinolone resistance (PMQR) determinants by polymerase chain reaction (PCR) and sequencing, and to detect their prevalence and distribution in the collection

2) to assess the ciprofloxacin and levofloxacin MIC distributions among PMQR-positive strains

3) to check PMQR-positive strains for beta-lactamase genes by PCR and sequencing

4) to investigate the PMQR-harbouring plasmids by Southern blot to identify the plasmidic localization of PMQR-genes or by conjugation to analyse the transferability of plasmids or by PCR-based replicon typing to detect the genes of autonomus plasmid replication or by plasmid DNA sequencing

5) to characterize the clonal relationship of PMQR-positive strains by pulsed-field gelelectrophoresis or random-amplified polymorphic DNA or multilocus sequence typing

3. Materials and methods

3.1 Strain collection: 756 Enterobacteriaceae strains were collected from the Microbiology diagnostic laboratory of Bolzano Central Hospital, Ospedale „Santa Maria del Carmine" of Rovereto, Ospedale „Santa S. Chiara" of Trento, Policlinico GB Rossi of Verona and Ospedale „S. Bortolo" of Vicenza. All the isolates included in the study were from specimens from inpatients admitted at hospitals or long-term care facilities, and from outpatients. Collection of nonreplicate Enterobacteriaceae isolates was carried out during three days a week, for a total period of six months, according to the following criteria: i) all isolates exhibiting an intermediate or resistance phenotype to ciprofloxacin and/or levofloxacin; ii) all isolates suspected/confirmed for extended-spectrum beta-lactamases (ESBLs) production; iii) all isolates, regardless of their resistance phenotype (during one of the three days of collection).

3.2 Minimal Inhibitory Concentration (MIC) for ciprofloxacin and levofloxacin by broth microdilution method.

Ciprofloxacin and levofloxacin MICs were measured in microdilution plates with a final volume of 200 µl/well. 100 µl Mueller Hinton broth was put in each well adding an extra 74.4 µl only in the first well and 25.6 µl ciprofloxacin from a 2 mg/ml mother solution. Serial dilution was carried out and 100 µl of bacterial inoculum with 10^5 cfu/ml was added in each well, obtaining ciprofloxacin concentration of 128 µg/ml in the first well and 0.06 µg/ml in the last. In case of the levofloxacin 10.24 µl was added in the first well from a 5 mg/ml mother solution. After serial dilution and the addition of bacterial inoculum with 10^5 cfu/ml in each well levofloxacin concentrations of 128 µg/ml in the first and 0.06 µg/ml in the last were obtained.

3.3 Screening by PCR for *qnr*, *aac(6')-Ib-cr* variant, *qepA*, *oqxAB* and beta-lactamase genes

Colonies of each Enterobacteriaceae strain were transferred to 500 µl distilled water in Eppendorf tubes, boiled at 100°C for 15 minutes and centrifuged at 13000 rpm for 10 minutes. 3 µl of the supernatant was used to prepare 200 ng of DNA templates, 1.25 U Taq DNA polymerase, 0.5 µM of each primer, 0.2 mM dNTP mix, 2.5 mM Buffer with Mg^{2+} filled up with distillated water for a total volume of 50 µl PCR reaction. Positive (containing strains with known *qnr* genes) and negative (without DNA template) controls were included in each run. DNA fragments were

analyzed by electrophoresis in a 0.8% agarose gel at 100 V for 45 min in 1x TAE [40 mM Tris–HCl (pH 8.3), 2 mM acetate and 1 mM EDTA] and stained with 0.05 mg/L ethidium bromide solution (51).

All the primers used in the screen are listed in **Table 2**. Multiplex PCR of *qnrA*, *qnrB* and *qnrS* genes was performed with qnrA Fwd and qnrA Rev to give a 516 bp product; with qnrB Fwd and qnrB Rev to give a 469 bp product; with qnrS Fwd and qnrS Rev to give a 417 bp product. The PCR conditions were 94°C for 45 s, 53°C for 45 s, and 72°C for 60 s for 32 cycles and a final elongation at 72°C for 10 min (52). Screening was carried out by PCR for *qnrC* gene using primers qnrC Fwd and qnrC Rev with PCR conditions 94°C for 30 s, 50°C for 30 s, 72°C for 30 s for 30 cycles and a final extension at 72°C for 10 minutes to obtain a 447 bp product (41).

Screening for *qnrD* gene was performed with primers qnrD Fwd and qnrD Rev with PCR conditions 94°C for 1 min, 50 °C for 1 min for 72°C for 1 min for 30 cycles and a final extension at 72°C for 10 min for 644 bp PCR product (40).

A*ac(6')-Ib* was amplified by PCR with primers aac(6')-Ib Fwd and aac(6')-Ib Rev to produce a 482 bp product. Primers were chosen to amplify all known aac(6')-Ib variants. PCR conditions were 94°C for 45 s, 55°C for 45 s, and 72°C for 45 s for 34 cycles and a final elongation at 72°C for 10 min (53).

A 199 bp fragment of *qepA* gene was amplified by PCR with primers qepA Fwd and qepA Rev. The PCR conditions were: denaturation at 96°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min for 30 cycles a final step was at 72°C for 5 min (54). Screening by PCR for OqxAB efflux pump was performed, to amplify *oqxA* gene of membrane fusion protein with primers oqxA Fwd and oqxA Rev with thermal profile for 34 cycles of 94^{o}C for 45 s, 57^{o}C for 45 s, 68^{o}C for 60 s was used to obtain 392 bp PCR product. To amplify *oqxB* gene of inner-membrane pump, oqxB Fwd and oqxB Rev set of primer was used with PCR conditions as follows: 94^{o}C for 45 s, 64^{o}C for 45 s and 72^{o}C for 60 s for 32 cycles to obtain 512 bp PCR product (50).

PMQR positive strains were screened for genes of SHV, TEM, CTX-M, VIM, IMP, OXA and LAP beta-lactamases with primers listed in **Table 2**. SHV PCR reaction was performed with 1 min denaturation at 94°C, 1 min annealing at 56 °C 1 min elongation at 72°C for 30 cycles and a final elongation at 72°C for 10 min, obtaining a PCR product of 930 bp (55).

TEM PCR was performed for product of 867 bp as follows: 94°C for 1 min, 58°C for 1 min,

72°C for 1 min for 30 cycles and a final elongation at 72°C for 10 min (55).

CTX-M-1 group PCR was carried out with CTX-M-1 Fwd and CTX-M-1 Rev primers denaturation at 94°C for 1 min, annealing at 62°C for 1 min, elongation at 72°C for 1 min for 35 cycles, with a final elongation 72°C for 10 min for a 688 bp PCR product (56).

CTX-M-9 group PCR was carried out with CTX-M-9 Fwd and CTX-M-9 Rev primers denaturation at 94°C for 1 min, annealing at 62°C for 1 min, elongation at 72°C for 1 min for 35 cycles, with a final elongation at 72°C for 10 min 561 bp PCR product (56).

VIM PCR was carried out with VIM Fwd and VIM Rev primers, initial denaturation at 94°C for 10 min and 30 cyles of 94°C for 40 s, 55°C for 40 s and 72°C for 1 min and one cycle of final elongation at 72°C for 10 min obtaining 390 bp product (56).

IMP PCR was performed with IMP Fwd and IMP Rev set of primers with initial denaturation at 94°C for 10 min and 30 cyles of 94°C for 40 s, 55°C for 40 s and 72°C for 1 min and one cycle of final elongation at 72°C for 10 min obtaining 139 bp PCR product (56).

OXA beta-lactamase genes were screened with OXA Fwd and Rev set of primers with a thermal profile of initial denaturation at 94°C for 10 min and 30 cyles of 94°C for 40 s, 60°C for 40 s and 72°C for 1 min and one cycle of final elongation at 72°C for 10 min to obtain 564 bp PCR product (56). LAP Fwd and LAP Rev set of primers were used to amplify bla_{LAP} gene with a thermal profile of 3 min at 93°C and 40 cycles of 1 min at 93°C, 1 min at 55°C, and 1 min at 72°C and, finally, 7 min at 72°C for 858 bp PCR product (57).

3.4 Sequencing of PCR products and analysis by NCBI Genebank database

Positive PCR products were purified by QIAquick PCR Purification Kit (Qiagen) and sent to be sequenced on both strands to Eurofins MWG Operon, Germany. Sequence analysis was performed with Blast program of NCBI Genebank database http://blast.ncbi.nlm.nih.gov/ .

3.5 Cloning of *qnr* and *aac(6')-Ib-cr* genes in plasmid vector

Positive PCR products of *qnr* and *aac(6')-Ib-cr* genes were purified by QIAquick PCR Purification Kit (Qiagen) and ligated in plasmid vector from Perfect PCR cloing kit (5Prime). Ligation reaction was performed at 4°C for 1 hour in a total volume of 10 µl, including 500 ng pPrime cloning vector, 100 ng of insertion PCR product, 1x Ligation mastermix and RNase free water. After the reaction, ligation enzyme was inactivated at 70°C for 10 min.

3.6 Transformation of DH5 alpha *E. coli* cells by heat shock

100 µl DH5 alpha cells and 10 µl ligation reaction were transfered to a falcon tube and kept on ice for 30 min. Heat pulse was performed at 42°C for not more the 40 sec. The shocked cells were incubated in SOC medium (triptone 2% w/v, yeast extract 0.5% w/v and salt solution 10% v/v [6 g NaCl, 1.87 g KCl, 20.33 g $MgCl_2$, 24.65 g $MgSO_4$ and 36 g glucose for 1 liter]) for 5 hours at 37°C. 80 µl of inoculums was plated on 20 ml Luria-Bertani agar (yeast extract 0.5% w/v, triptone 1% w/v, NaCl 1% w/v, 1.5% w/v agar) plates containing 100 µg/ml ampicillin for an overnight incubation at 37°C.

3.7 Incompatibility grouping of plasmids by PCR-based replicon typing

PCR-based replicon typing (PBRT) was performed in 50 µl of PCR reaction, using specific primers to detect inc types of plasmids. Five multiplex PCR reactions were applied, 1. multiplex PCR: HI 1 Fwd and Rev, HI2 Fwd and Rev, I1 Fwd and Rev, 2. multiplex PCR: X Fwd and Rev, L/M Fwd and Rev, N Fwd and Rev, 3. multiplex PCR: FIA Fwd and Rev, FIB Fwd and Rev, W Fwd and Rev, 4. multiplex PCR: Y Fwd and Rev, P Fwd and Rev, FIC Fwd and Rev, 5. muliplex PCR: A/C Fwd and Rev, T Fwd and Rev, FII_S Fwd and Rev. Seven simplex PCR reactions were included namely: 1. F_{repB} Fwd and Rev, 2. K/B Fwd and K Rev, 3. K/B Fwd and B Rev, 4. Q Fwd and Rev, 5. U Fwd and Rev, 6. R Fwd and Rev, 7. ColE Fwd and Rev (**Table 2**). For each reaction 1.25 U Taq DNA polymerase, 0.5 µM of each primer, 0.2 mM dNTP mix, 2.5 mM Buffer with Mg^{2+} 200 ng DNA prepared from each *qnr* and *aac(6')-Ib-cr* positive strain, filled up with distilled water for a total volume of 50 µl PCR reaction. Thermal profile of replicon typing for all the multiplex PCR reactions and simplex PCR 2, 3, 5, 6, 7 was as follows: initial denaturation at 94°C for 5 min, and 30 cycles of 94°C for 1 min, 60°C for 30 sec, 72°C for 1 min and final elongation at 72°C for 5 min. Simplex PCR 1. was used with annealing at 52 °C, and Simplex PCR 4. with annealing at 62 °C (58, 59, 60).

3.8 Conjugation of *qnr* positive plasmids was performed. Each *qnr* positive strain was plasmid donor and J53 $Azid^R$ *E.coli* was used as recipient cell. Each strain's inoculum reached 0.6 $OD_{640,}$ measured by spectrophotometer (T60 UV-Visible Spectrophotometer, PG Instruments) when they were applied in reaction (500 µl of donor inoculum, 500 µl of recipient inoculum in total of 5 ml Luria-Bertani broth). The reaction tubes were incubated at 37 ºC for 5 hours.

Transconjugant cells were selected on Luria-Bertani agar plates containing 100 µg/ml sodium-azid combined with either 16 µg/ml nalidixic acid or 10 µg/ml ceftazidime. Transconjugant cells were analyzed by PCR for the presence of the *qnr* genes and MIC values of the transconjugant cells were measured by microdilution method for nalidixic acid, ciprofloxacin and levofloxacin.

3.9 Plasmid extraction and detection: plasmid extraction was carried out by commercial kit (Qiagen) from an overnight incubation of 5 ml Luria-Bertani broth of each *qnr* positive strain. The plasmid extractions were loaded in a 0.4% (w/v) agargel and runned at 60 V for 60 min. The gel was stained with 1x Gel Red solution (Biotum).

3.10 Southern blot

Plasmid extractions from each *qnr* positive strain were treated with Plasmid-safe ATP-dependent DNase (Epicenter). Both untreated and treated plasmids were loaded on a 0.4% (w/v) agargel and separated by electrophoretion on 60 V for 60 min. The plasmids in the gel were denaturated for 30 min in denaturation buffer [0.5 M NaOH, 1.5 M NaCl] and neutralized in neutralizing buffer [pH 7, 0.5 M Tris-HCl, 1.5 M NaCl], transferred onto the surface of a nylon membrane by overnight blotting in transfer buffer 20xSSC (pH 7, 3 M NaCl, 0.3 M $Na_3C_6H_5O_7$ x $2H_2O$). The denaturated transferred plasmids were dried in the membrane by heating on 80^{o}C for 2 hours. Specific *qnr* probes incorporating digoxigenin-11-dUTP (Roche, Mannheim, Germany) were performed by PCR. After denaturating the probe on 100 ^{o}C for 15 min, hybridization was carried out on 44.6 ^{o}C for 16 hours. Detection was carried out by developing chemiluminescens detection film (Roche, Mannheim, Germany) in development and fixation buffers (Sigma).

3.11 Inverse PCR of *qnrD*-harbouring plasmids

PCR reaction was carried out on the plasmid extraction of *qnrD* positive strains. The complementar antiparalelle strand of qnrD Fwd and qnrD Rev primers named as INVDF/Rev and INVDR/Fwd, respectively (listed in **Table 2**) were used with the following thermal profile: initial denaturation at 95^{o}C for 2 min, 30 cycles of 94 ^{o}C for 30 sec, 53 ^{o}C for 30 sec, 72 ^{o}C for 3 min with a final elongation of 72 ^{o}C for 10 min to obtain 2200 bp PCR product. Sequencing of positive PCR product was carried out (Eurofins MWG Operon, Germany) and based on the DNA sequence four additional primers were designed, namely Pl 1 Rev, Pl 2 Fwd, Pl 3 Fwd and Pl 4

Rev (**Table 2**, **Figure 16**) by tools of Eurofins MWG Operon, Germany. These primers were used with the same thermal profile to amplify 994 bp PCR product with Pl1 Fwd and Pl2 Rev, while 710 bp with Pl3 Fwd and qnrD Rev and 972 bp with qnrD Fwd and Pl4 Rev. Positive PCR products were purified by QIAquick PCR Purification Kit (Qiagen) and sequenced by Eurofins MWG Operon, Germany.

3.12 PCR and sequencing of quinolone-resistance determining regions of *qnrD* positive isolates

Quinolone-resistance determining regions of four *qnrD* positive *Proteus mirabilis* strains were amplified in PCR reactions, with 1 unit Taq polymerase, 45 mM KCl, 2.5 mM Mg^{++}, 200 µM each dNTPs, 0.5 µM each primer, 3 µl DNA from boiled colony. 538 bp PCR product with gyrA Pro Fwd and gyrA Pro Rev, 535 bp with gyrB Pro Fwd and gyrB Pro Rev, 351 bp parC Pro Fwd and parC Pro Rev, 460 bp with parE Pro Fwd and parE Pro Rev were obtained (Primers listed in **Table 2**) The PCR reaction was performed for *gyrA* with initial denaturation at 94^{o}C for 5 min, followed by 35 cycles of denaturation at 95^{o}C for 45s, annealing at 52^{o}C for 20 s, elongation at 72^{o}C for 30s and final elongation at 72^{o}C for 10 min. The same PCR reaction was used with annealing at 54^{o}C for *gyrB,* at 52^{o}C for *parC* and at 56^{o}C for *parE* (61). Positive PCR products were sequenced by Eurofins MWG Operon and analyzed by Blast program of NCBI Genbank.

3.13 Pulsed-field gelelectrophoresis (PFGE)

5 ml Luria-Bertani broth overnight incubation of each *qnrS* positive strain. 500 microliter inoculum to spin down at 8000 rpm for 5 min. Pellet resuspended in 500 microliter TE buffer (10 mM Tris-HCl pH 7.5, 1 mM Edta pH 8). Mixed with 500 microliter 2 % (w/v) low-melting agarose (BioRad). Pipet the plugs in frame (BioRad). Solidified at room temperature for 15 min. Plugs washed in 5 ml of lysis buffer [6 mM Tris-HCl pH 8, 100 mM Edta pH 8, 1 M NaCl, 1% Brij, 0.4 % deoxycolat, 1 % sarkozyl, 0.1 % w/v lyzozim] at 37 ^{o}C overnight. Plugs washed in 5 ml of proteinase K buffer [0.5 M Edta pH 8, 1 % w/v sarkozyl, 0.005 % w/v proteinase K] at 50 ^{o}C overnight. Plugs washed in 20 ml of TE buffer [Tris-HCl 10 mM pH 7.5, EDTA 1 mM pH 8] 5 times at room temperature. 30 U Xba I digestion overnight and run in CHEF DR™ Electrophoresis carried out with BioRad 6V/cm, 120^{o} angel, initial switch time 2 s and final switch time 30 s for 21 hours run.

3.14 Multilocus sequence typing (MLST) for *K. pneumoniae*

Seven housekeeping genes *rpoB, gapA, mdh, pgi, phoE, infB, tonB* were amplified with specific primers (listed in **Table 2**). PCR reaction in total volume of 50 µl including 1 unit Taq polymerase, 45 mM KCl, 2.5 mM Mg^{++}, 200 µM each dNTPs, 0.5 µM each primer, 3 µl DNA from boiled colony. 501 bp PCR product with rpoB Fwd and Rev, 450 bp with gapA Fwd and Rev, 477 bp with mdh Fwd and Rev, 432 bp pgi Fwd and Rev, 420 bp pho Fwd and Rev, 318 bp infB Fwd and Rev, 414 bp tonb Fwd and Rev were amplified. PCR thermal profile was used with initial denaturation at 95^{o}C for 5 min, and 35 cycles of 95^{o}C for 1 min, 50^{o}C for 1 min and 72^{o}C for 1 min with final elongation 72^{o}C for 10 min. Exception was gapA used with 60^{o}C and tonB with 45^{o}C of annealing. Positive PCR products were sequenced and allelic numbers were obtained after the alignament of sequences of these seven housekeeping genes at the following homepage http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae (62).

3.15 Random amplified polymorhic DNA (RAPD)

Three single primers were used in separate PCR reations, namely OPA 11, OPX13 and OPZ08 (**Table 2**) to obtain polymorphic bands for the four *qnrD* positive *Proteus mirabilis* strains. PCR reaction was performed in total volume of 50 µl including 1 unit Taq polymeras, 45 mM KCl, 2.5 mM Mg^{++}, 200 µM, each dNTPs, 4 µM primer, 3 µl DNA from boiled colony. PCR cycling thermal profile was 92^{o}C for 4 min, 40 cycles of denaturation at 94^{o}C for 1 min, annealing at 40^{o}C for 1 min and elonagation at 72 ^{o}C for 5 min, and a final elongation at 72 ^{o}C for 5 min. The obtained PCR products were run in 1.5% agarose gel and stained with 1x GelRed (Biotum) (63).

Table 2. Primers used in the study

Primers for screening of PMQR and beta-lactamase genes			Primers for plasmid replicon typing		
Primer name	**Ref.**	**Primer sequence**	**Primer name**	**Ref.**	**Primer sequence**
qnrA Fwd	51	5-ATTTCTCACGCCAGGATTTG-3	**HI 1 Fwd**	58	5-ggagcgatggattacttcagtac-3
qnrA Rev	51	5-GATCGGCAAAGGTTAGGTCA-3	**HI 1 Rev**	58	5-tgccgtttcacctcgtgagta-3
qnrB Fwd	51	5-GATCGTGAAAGCCAGAAAGG-3	**HI 2 Fwd**	58	5-tttctcctgagtcacctgttaacac-3
qnrB Rev	51	5-ACGATGCCTGGTAGTTGTCC-3	**HI 2 Rev**	58	5-ggctcactaccgttgtcatcct-3
qnrB FQ 1	30	5-ATGACGCCATTACTGTATAA-3	**I1 Fwd**	58	5-cgaaagccggacggcagaa-3
qnrB FQ 2	30	5-GATCGCAATGTGTGAAGTTT-3	**I1 Rev**	58	5-tcgtcgttccgccaagttcgt-3
qnrS Fwd	51	5-ACGACATTCGTCAACTGCAA-3	**X Fwd**	58	5-aaccttagaggctatttaagttgctgat-3
qnrS Rev	51	5-TAAATTGGCACCCTGTAGGC-3	**X Rev**	58	5-tgagagtcaatttttatctcatgttttagc-3
qnrC Fwd	41	5-GGGTTGTACATTTATTGAATC-3	**L/M Fwd**	58	5-ggatgaaaactatcagcatctgaag-3
qnrC Rev	41	5-TCCACTTTACGAGGTTCT-3	**L/M Rev**	58	5-ctgcaggggcgattctttagg-3
qnrD Fwd	40	5-CGAGATCAATTTACGGGGAATA-3	**N Fwd**	58	5-gtctaacgagcttaccgaag-3
qnrD Rev	40	5-AACAAGCTGAAGCGCCTG-3	**N Rev**	58	5-gtttcaactctgccaagttc-3
aac(6')-Ib Fwd	53	5-TTGCGATGCTCTATGAGTGGCTA-3	**FIA Fwd**	58	5-ccatgctggttctagagaaggtg-3
aac(6')-Ib Rev	53	5-CTCGAATGCCTGGCGTGTTT-3	**FIA Rev**	58	5-gtatatccttactggcttccgcag-3
qepA Fwd	54	5-GCAGGTCCAGCAGCGGGTAG-3	**FIB Fwd**	58	5-ggagttctgacacacgattttctg-3
qepA Rev	54	5-CTTCCTGCCCGAGTATCGTG-3	**FIB Rev**	58	5-ctcccgtcgcttcagggcatt-3
oqxA Fwd	50	5-CTCGGCGCGATGATGCT-3	**W Fwd**	58	5-cctaagaacaacaaagccccg-3
oqxA Rev	50	5-CCACTCTTCACGGGAGACGA-3	**W Rev**	58	5-ggtgcgcggcatagaaccgt-3
oqxB Fwd	50	5- TTCTCCCCCGGCGGGAAGTAC-3	**Y Fwd**	58	5-aattcaaacaacactgtgcagcctg-3
oqxB Rev	50	5- CTCGGCCATTTTGGCGCGTA-3	**Y Rev**	58	5-gcgagaatggacgattacaaaacttt-3
SHV Fwd	55	5-GGGTTATTCTTATTTGTCGC-3	**P Fwd**	58	5-ctatggccctgcaaacgcgccagaaa-3
SHV Rev	55	5-TTAGCGTTGCCAGTGGTC-3	**P Rev**	58	5-tcacgcgccagggcgcagcc-3
TEM Fwd	55	5-ATGAGTATTCAACATTTCCG-3	**FIC Fwd**	58	5-gtgaactggcagatgaggaagg-3
TEM Rev	55	5-CTGACAGTTACCAATGCTTA-3	**FIC Rev**	58	5-ttctcctcgtcgccaaactagat-3
CTX-M-1 Fwd	56	5-TCCCGACGGCTTTCCGCCTT-3	**A/C Fwd**	58	5-gagaaccaaagacaaagacctgga-3
CTX-M-1 Rev	56	5-ATGGTTAAAAAATCACTGCGCC-3	**A/C Rev**	58	5-acgacaaacctgaattgcctcctt-3
CTX-M-9 Fwd	56	5-GTGACAAAGAGAGTGCAACGG-3	**T Fwd**	58	5-ttggcctgtttgtgcctaaaccat-3
CTX-M-9 Rev	56	5-ATGATTCTCGCCGCTGAAGCC-3	**T Rev**	58	5-cgttgattacacttagctttggac-3
VIM Fwd	56	5-GATGGTGTTTGGTCGCATA-3	**FII_S Fwd**	58	5-ctgtcgtaagctgatggc-3
VIM Rev	56	5-CGAATGCGCAGCACCAG-3	**FII_S Rev**	58	5-ctctgccacaaacttcagc-3
IMP Fwd	56	5-TTGACACTCCATTTACDG-3	**F_{repB} Fwd**	58	5-tgatcgtttaaggaattttg-3
IMP Rev	56	5-GATYGAGAATTAAGCCACYCT-3	**F_{repB} Rev**	58	5-gaagatcagtcacaccatcc-3
OXA Fwd	56	5-ggcaccagattcaactttcaag-3	**K/B Fwd**	58	5-gcggtccggaaagccagaaaac-3
OXA Rev	56	5-gaccccaagtttcctgtaagtg-3	**K Rev**	58	5-tctttcacgagcccgccaaa-3
LAP Fwd	57	5-CAATACAAAGCACAGAAGACC-3	**B Rev**	58	5-tctgcgttccgccaagttcga-3
LAP Rev	57	5-CCGATCCCTGCAATATGCTC-3	**Q Fwd**	59	5-tcgtggtcgcgttcaagg tacg-3
Primers to amplify QRDR of *Proteus mirabilis*			**Q Rev**	59	5-ctg taa gtc gat gat ctg ggcgtt-3
gyrA Pro Fwd	This study	5-TGCCAGAGAAATCACACCAG-3	**R Fwd**	60	5-tcg ctt cat tcc tgc ttc agc-3
gyrA Pro Rev	This study	5-TTTCCAAATCCGCCAGCAG-3	**R Rev**	60	5-gtg tgc tgt ggt tat gcc tca -3
gyrB Pro Fwd	61	5- TGA(C/T)GATGC(G/C/A)CG(T/C)GAAGG-3	**U Fwd**	60	5-tca cga cac aag cgc aag gg-3
gyrB Pro Rev	61	5- CGTACG(A/G)ATGTG(C/A)GA(G/A)CC-3	**U Rev**	60	5- tca tgg tac atc tgg gcgc-3
parC Pro Fwd	61	5- TTGCC(A/T)TTTAT(C/T)GG(G/T)GATGG-3	**Col E Fwd**	60	5-gtt cgt gca tac agt cca-3
parC Pro Rev	61	5- CGCGC(A/T)GGCAGCATTTT(A/T)GG-3	**Col E Rev**	60	5-ggc gaa acc cga cag gac t-3
parE Pro Fwd	61	5- GCA(G/A)GA(T/G)(C/G)CGCA(G/A)TT(T/C)G-3			
parE Pro Rev	61	5-ATC(A/C/G)GAGTC(C/T/G)GCATCCG-3			
Primers for multilocus sequence typing			**Primers for random amplified polymoprhic DNA**		
Primer name	**Ref.**	**Primer sequence**	**Primer name**	**Ref.**	**Primer sequence**
rpoB Fwd	62	5-GGCGAAATGGCWGAGAACCA-3	**OPA 11**	63	5-caatcgccgt-3
rpoB Rev	62	5-GAGTCTTCGAAGTTGTAACC-3	**OPX 13**	63	5-acgggagcaa-3
gapA Fwd	62	5-TGAAATATGACTCCACTCACGG-3	**OPZ 08**	63	5-gggtgggtaa-3
gapA Rev	62	5-CTTCAGAAGCGGCTTTGATGGCTT-3	**Primers for *qnrD*-plasmid sequencing**		
mdh Fwd	62	5-CCCAACTCGCTTCAGGTTCAG-3	**INVDR/Fwd**	This study	5-CAGGCGCTTCAGCTTGTT-3
mdh Rev	62	5-CCG TTTTTCCCCAGCAGCAG-3	**INVDF/Rev**	This study	5-TATTCCCCGTAAATTGATCTCG-3
pgi Fwd	62	5-GAGAAAAACCTGCCTGTACTGCTGGC-3	**Pl1 Rev**	This study	5-GCCGAAGAAAATAGCCAGAG-3
pgi Rev	62	5-CGCGCCACGCTTTATAGCGGTTAAT-3	**Pl2 Fwd**	This study	5- TGCTAAGATTGGATTGCGAC-3
phoE Fwd	62	5-ACCTACCGCAACACCGACTTCTTCGG-3	**Pl3 Fwd**	This study	5-TAATGGGGATGAACGGGAG-3
phoE Rev	62	5-TGATCAGAACTGGTAGGTGAT-3	**Pl4 Rev**	This study	5-TCGCAATCCAATCTTAGCAAC-3
infB Fwd	62	5-CTCGCTGCTGGACTATATTCG-3			
infB Rev	62	5-CGCTTTCAGCTCAAGAACTTC-3	**Primers used to amplify *qnrS1* and bla_{LAP} genetic context**		
tonB Fwd	62	5-CTTTATACCTCGGTACATCAGGTT-3	**Ins LAP Fwd**	This study	5-TGGATGATAGCGGATAAAACAG-3
tonB Rev	62	5-ATTCGCCGGCTGRGCRGAGAG-3	**Ins LAP Rev**	This study	5- GCCAATGCAAGAGCGATAG-3

4. Results

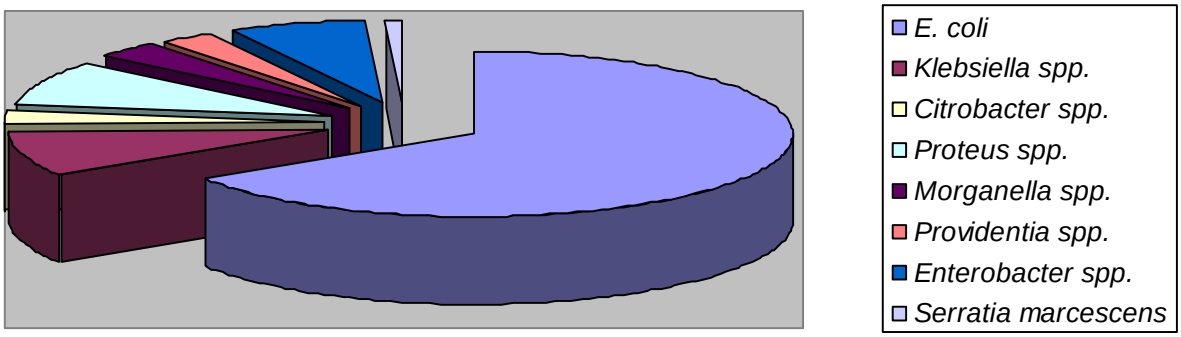
4.1 Results of strain collection and MIC distribution

Altogether 756 Enterobacteriaceae clinical isolates were collected from the microbiological diagnostic departments: 75 from Bolzano, 31 from Rovereto, 94 from Trento, 101 from Vicenza and 455 from Verona. The largest part were *E. coli*, but many Enterobacteriaceae species such as *Klebsiella* spp., *Proteus* spp., *Citrobacter* spp. were also in the collection (**Table 3, Graph 1**).

Table 3. Number of strains collected in each center by species

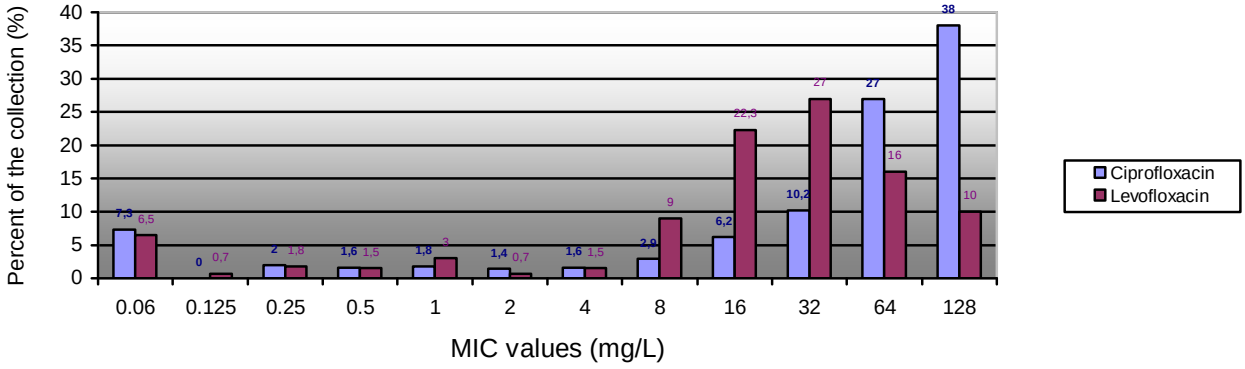
	Bolzano	**Rovereto**	**Trento**	**Vicenza**	**Verona**	**Total**
Escherichia coli	51	23	72	80	271	**497**
***Klebsiella* spp.**	12	1	3	6	46	**68**
***Citrobacter* spp.**	1	1	3	1	12	**18**
***Proteus* spp.**	6	4	9	5	45	**69**
***Morganella* spp.**	1	2	2	2	17	**24**
***Providentia* spp.**	3	0	1	6	11	**21**
***Enterobacter* spp.**	1	0	3	1	47	**52**
Serratia marcescens	0	0	1	0	6	**7**
Total	75	31	94	101	455	**756**

Graph 1. Distribution of the 756 Enterobacteriaceae isolates



MICs of ciprofloxacin and levofloxacin were measured for all strains. We could demonstrate a range of 0.06-128 mg/L. **Graph 2** shows the distribution of the MIC values in the collection. In total sixty-five percent of the strains in the collection had 64 or 128 mg/L MICs for ciprofloxacin while for levofloxacin 65.3% had 16, 32 or 64 mg/L. Low-level resistant strains (0.06 - 0.5 mg/L) were found to represent 10.5% of the collection.

Graph 2. MIC distribution of ciprofloxacin and levofloxacin of 756 Enterobacteriaceae strains



4.2 Results of screening for *qnr, aac(6')-Ib-cr* variant, *qepA* and *oqxAB* genes

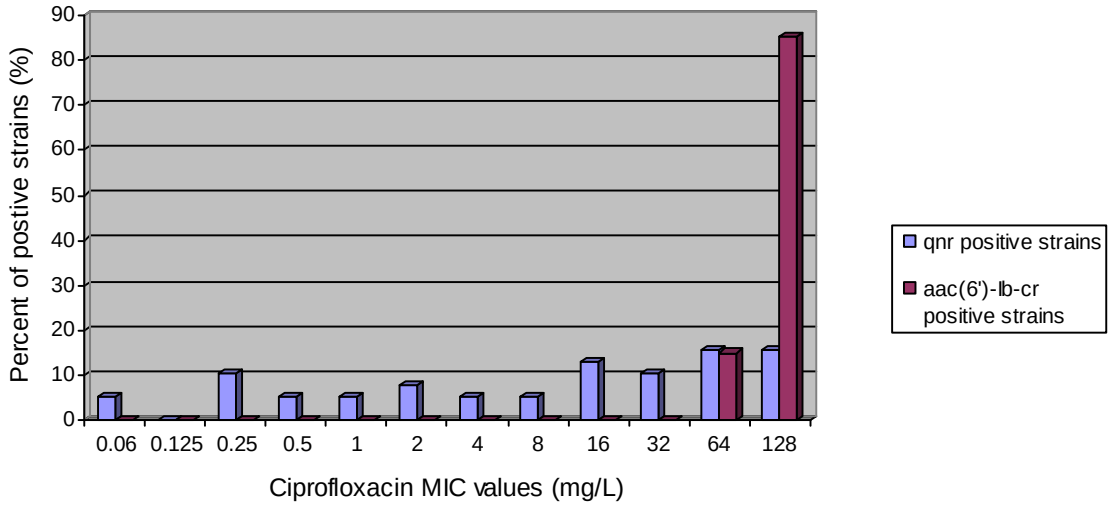
Screening by PCR for PMQR genes found 104 isolates positive for any of the tested PMQR genes, showing their 13.3% prevalence in the collection. 108 PMQR genes were found since four *E. coli* strains carried a *qnrS1* and an *aac(6')-Ib-cr* too. 38 out of the 756 strains were positive for any of the tested *qnr* determinants, showing a 5.02% prevalence. 7 *qnrB*, 26 *qnrS* and 5 *qnrD* were found and further investigated by DNA sequencing and analysis verified: 2 *qnrB2*, 2 *qnrB6*, 2 *qnrB8*, 1 *qnrB19*, 26 *qnrS1* and 5 *qnrD*. Screening by PCR for *aac(6')-Ib-cr* genes proved 70 positives and verified by DNA sequencing showing 9.2% prevalence. Among the positives 60 were *E. coli*, 8 *Klebsiella* spp., 1 *P. mirabilis* and 1 *M. morganii*. Neither *qnrA*, *qnrC* or *qepA*, *oqxAB* were found in the collection (**Table 4**).

The overview of the positive strains shows the highest prevalence in *Klebsiella* spp. and *Citrobacter* spp. with 33.8% and 27.7%, respectively. *Klebsiella* spp. had 15 *qnrS1* and 8 *aac(6')-Ib-cr*. Noteworthy, *E. coli* accounted for 65.7% (497/756) of the collection, only 14.1% carried a PMQR gene, 10 *qnrS1* and 60 *aac(6')-Ib-cr* genes were detected. Interestingly, *Proteus* spp. carried three different types of PMQR genes (1 *qnrS1*, 4 *qnrD* and 1 *aac(6')-Ib-cr*), though these 3 determinants were detected only in 8.7% of this genus. Among *Morganella morganii* 1 *qnrD* and 1 *aac(6')-Ib-cr* positive strains were identified.

Table 4. Isolates found positive for PMQR genes in the collection

	qnrA	*qnrB*	*qnrS*	*qnrC*	*qnrD*	*aac(6')-Ib-cr*	*qnrS1+ aac(6')-Ib-cr*	*qepA*	*oqxAB*	**Prevalence**
Escherichia coli	0	0	**6**	0	0	**56**	**4**	0	0	**66/497 = 13.3%**
***Klebsiella* spp.**	0	0	**15**	0	0	**8**	0	0	0	**23/68 = 33.8%**
***Citrobacter* spp.**	0	**5**	0	0	0	0	0	0	0	**5/18 = 27.7 %**
***Proteus* spp.**	0	0	**1**	0	**4**	**1**	0	0	0	**6/69 =8.7 %**
***Morganella* spp.**	0	0	0	0	**1**	**1**	0	0	0	**2/24 = 8.3 %**
***Enterobacter* spp**	0	**2**	0	0	0	0	0	0	0	**2/52 = 3.8 %**
***Providentia* spp.**	0	0	0	0	0	0	0	0	0	**0/21 = 0%**
S. marcescens	0	0	0	0	0	0	0	0	0	**0/7 = 0%**
Total	**0**	**7**	**22**	**0**	**5**	**66**	**4**	**0**	**0**	**104/756 = 13.7%**

MIC values of *qnr* and *aac(6´)-Ib-cr* positive strains are listed in **Table 5, 6** and **7**. **Graph 3** shows the distribution of the positive strains ranging over 11 different ciprofloxacin MIC values from susceptible to resistant. In the case of *aac(6')-Ib-cr* positives, they were found only in the resistance range (64 and 128 mg/L). Three *qnrB* positive *Citrobacter* spp. and two *qnrS* positive *Klebsiella pneumoniae* were found at low-level resistance range (0.06 - 0.5 mg/L). These five isolates account for 13.1% (5/38).



Graph 3. Comparison of ciprofloxacin MIC values of *qnr* positive and *aac(6')-Ib-cr* positive strains

Table 5. MIC (μg/ml) values of *aac(6')-Ib-cr* variant postive *K.pneumoniae, P.mirabilis and M. morganii*

Strain	PMQR	cip	lev	ceftazidime	cefotaxim	cefepime	aztreonam	imipenem
***K.pneumoniae* VR C191**	***aac(6')-Ib-cr***	>128	32	128	64	16	128	<0.0625
***K.pneumoniae* VR E102**	***aac(6')-Ib-cr***	128	8	>128	>128	>128	>128	<0.0625
***K.pneumoniae* VR E154**	***aac(6')-Ib-cr***	128	8	>128	>128	>128	>128	0.125
***K.pneumoniae* VR E157**	***aac(6')-Ib-cr***	>128	16	>128	>128	>128	>128	<0.0625
***K.pneumoniae* VR E165**	***aac(6')-Ib-cr***	>128	16	>128	>128	64	>128	1
***K.pneumoniae* VR E176**	***aac(6')-Ib-cr***	>128	32	128	>128	>128	>128	<0.0625
***K.pneumoniae* V29**	***aac(6')-Ib-cr***	128	16	>128	>128	>128	>128	0.5
***K.pneumoniae* V91**	***aac(6')-Ib-cr***	128	16	>128	>128	>128	>128	0.5
***P.mirabilis* T40**	***aac(6')-Ib-cr***	128	16	>128	>128	>128	>128	0.0625
***M. morganii* T53**	***aac(6')-Ib-cr***	64	8	16	128	32	64	1

Table 6. MIC (μg/ml) values of *aac(6')-Ib-cr* variant postive *E. coli* strains

Strain	PMQR	cip	lev	ceftazidime	cefotaxim	cefepime	aztreonam	imipenem
***E. coli* T6**	***aac(6')-Ib-cr***	128	16	128	>128	32	>128	0.125
***E. coli* T10**	***aac(6')-Ib-cr***	>128	128	>128	>128	>128	>128	0.0625
***E. coli* T27**	***aac(6')-Ib-cr***	>128	32	128	>128	>128	>128	0.0625
***E. coli* T30**	***aac(6')-Ib-cr***	>128	128	1	1	1	1	0.25
***E. coli* T39**	***aac(6')-Ib-cr***	>128	64	>128	>128	>128	>128	0.125
***E. coli* T47**	***aac(6')-Ib-cr***	128	32	>128	>128	>128	>128	0.125
***E. coli* T87**	***aac(6')-Ib-cr***	64	16	>128	>128	>128	>128	0.0625
***E. coli* V18**	***aac(6')-Ib-cr***	>128	16	64	>128	32	>128	0.125
***E. coli* V21**	***aac(6')-Ib-cr***	>128	16	32	>128	16	128	0.0625
***E. coli* V25**	***aac(6')-Ib-cr***	128	16	1	1	1	2	<0.0625
***E. coli* V27**	***aac(6')-Ib-cr***	64	32	>128	>128	>128	>128	<0.0625
***E. coli* V32**	***aac(6')-Ib-cr***	128	16	1	1	2	0.5	0.0625
***E. coli* V41**	***aac(6')-Ib-cr***	64	16	0.5	0.125	0.25	1	0.125
***E. coli* V47**	***aac(6')-Ib-cr***	>128	128	32	>128	1	128	<0.0625
***E. coli* V55**	***aac(6')-Ib-cr***	128	16	16	>128	8	64	<0.0625
***E. coli* V65**	***aac(6')-Ib-cr***	128	64	1	2	2	0.5	0.25
***E. coli* V85**	***aac(6')-Ib-cr***	128	64	1	0.25	0.5	1	0.0625
***E. coli* B33**	***aac(6')-Ib-cr***	128	64	2	0.125	0.25	0.25	0.0625
***E. coli* B38**	***aac(6')-Ib-cr***	>128	32	>128	>128	128	>128	0.0625
***E. coli* B44**	***aac(6')-Ib-cr***	>128	16	128	>128	64	>128	<0.0625
***E. coli* B65**	***qnrS1, aac(6')-Ib-cr***	>128	128	>128	>128	128	>128	0.0625
***E. coli* R25**	***aac(6')-Ib-cr***	>128	64	128	>128	128	>128	0.0625
***E. coli* VR B18**	***aac(6')-Ib-cr***	64	16	>128	>128	>128	>128	<0.0625
***E. coli* VR B88**	***aac(6')-Ib-cr***	64	16	64	>128	64	>128	<0.0625
***E. coli* VR B127**	***aac(6')-Ib-cr***	64	8	>128	>128	>128	>128	<0.0625
***E. coli* VR B227**	***aac(6')-Ib-cr***	128	16	>128	128	>128	>128	<0.0625
***E. coli* VR B232**	***aac(6')-Ib-cr***	64	8	>128	>128	128	>128	<0.0625
***E. coli* VR B248**	***aac(6')-Ib-cr***	>128	16	0.25	0.5	4	<0.0625	<0.0625
***E. coli* VR B262**	***aac(6')-Ib-cr***	>128	16	0.125	2	16	0.5	<0.0625
***E. coli* VR B269**	***aac(6')-Ib-cr***	128	8	128	>128	128	>128	<0.0625
***E. coli* VR B292**	***aac(6')-Ib-cr***	64	8	0.125	<0.0625	<0.0625	<0.0625	<0.0625
***E. coli* VR B649**	***aac(6')-Ib-cr***	64	8	0.5	0.25	0.5	0.125	<0.0625
***E. coli* VR B656**	***aac(6')-Ib-cr***	64	8	64	>128	64	>128	<0.0625
***E. coli* VR B715**	***aac(6')-Ib-cr***	64	2	64	>128	128	>128	<0.0625
***E. coli* VR B 717**	***aac(6')-Ib-cr***	128	16	128	>128	>128	>128	<0.0625
***E. coli* VR B754**	***aac(6')-Ib-cr***	128	16	0.5	0.25	1	0.5	<0.0625
***E. coli* VR B770**	***qnrS1, aac(6')-Ib-cr***	64	16	128	>128	64	>128	<0.0625
***E. coli* VR B775**	***qnrS1, aac(6')-Ib-cr***	64	16	128	>128	64	>128	<0.0625
***E. coli* VR B780**	***aac(6')-Ib-cr***	128	16	>128	>128	>128	>128	<0.0625
***E. coli* VR B784**	***aac(6')-Ib-cr***	>128	16	128	128	128	128	<0.0625
***E. coli* VR B786**	***aac(6')-Ib-cr***	128	32	0.125	0.125	<0.0625	<0.0625	<0.0625
***E. coli* VR B787**	***aac(6')-Ib-cr***	64	8	128	128	128	>128	<0.0625
***E. coli* VR B793**	***aac(6')-Ib-cr***	>128	16	8	16	8	32	<0.0625
***E. coli* VR B808**	***aac(6')-Ib-cr***	>128	8	64	128	64	128	<0.0625
***E. coli* VR B814**	***qnrS1, aac(6')-Ib-cr***	128	16	>128	>128	128	>128	<0.0625
***E. coli* VR C17**	***aac(6')-Ib-cr***	64	8	0.25	0.125	0.5	0.25	<0.0625
***E. coli* VR C18**	***aac(6')-Ib-cr***	128	8	>128	>128	>128	>128	<0.0625
***E. coli* VR C19**	***aac(6')-Ib-cr***	128	8	>128	>128	128	>128	<0.0625
***E. coli* VR C24**	***aac(6')-Ib-cr***	64	4	>128	>128	128	>128	<0.0625
***E. coli* VR C66**	***aac(6')-Ib-cr***	64	4	64	>128	128	>128	<0.0625
***E. coli* VR C93**	***aac(6')-Ib-cr***	64	4	0.5	<0.0625	0.5	0.25	<0.0625
***E. coli* VR C105**	***aac(6')-Ib-cr***	64	4	64	>128	128	>128	<0.0625
***E. coli* VR C109**	***aac(6')-Ib-cr***	64	16	64	128	64	>128	<0.0625
***E. coli* VR E13**	***aac(6')-Ib-cr***	64	8	64	128	128	64	<0.0625
***E. coli* VR E25**	***aac(6')-Ib-cr***	64	8	64	128	128	>128	<0.0625
***E. coli* VR E39**	***aac(6')-Ib-cr***	64	8	>128	>128	128	>128	<0.0625
***E. coli* VR E47**	***aac(6')-Ib-cr***	64	8	8	1	2	>128	<0.0625
***E. coli* VR E64**	***aac(6')-Ib-cr***	128	16	32	>128	>128	>128	<0.0625
***E. coli* VR E98**	***aac(6')-Ib-cr***	64	8	64	>128	>128	>128	<0.0625
***E. coli* VR E108**	***aac(6')-Ib-cr***	128	8	64	>128	>128	>128	<0.0625

Table 7. MIC (μg/ml) values of *qnr* positive isolates

Strain	PMQR	cip	lev	ceftazidime	cefotaxim	cefepime	aztreonam	imipenem
***E. coli* B24**	***qnrS1***	>128	>128	128	16	2	0.125	<0.0625
***E. coli* B61**	***qnrS1***	64	16	0.5	0.125	<0.0625	<0.0625	<0.0625
***E. coli* B65**	***qnrS1,aac(6')-Ib-cr***	>128	128	>128	>128	128	>128	<0.0625
***E. coli* V38**	***qnrS1***	64	32	0.5	<0.06	<0.0625	<0.0625	<0.0625
***E. coli* T57**	***qnrS1***	128	64	1	0.25	<0.0625	0.125	<0.0625
***E. coli* VR301**	***qnrS1***	32	16	>128	>128	128	>128	<0.0625
***E. coli* VR770**	***qnrS1,aac(6')-Ib-cr***	64	16	128	>128	64	>128	<0.0625
***E. coli* VR775**	***qnrS1,aac(6')-Ib-cr***	64	16	128	>128	64	>128	<0.0625
***E. coli* VR805**	***qnrS1***	8	8	0.25	<0.0625	<0.0625	0.125	<0.0625
***E. coli* VR814**	***qnrS1,aac(6')-Ib-cr***	128	16	>128	>128	128	>128	<0.0625
***K.pneumoniae* B49**	***qnrS1***	32	16	>128	64	16	>128	0.0625
***K.pneumoniae* B56**	***qnrS1***	0.25	0.25	>128	128	16	>128	0.0625
***K.oxytoca* B67**	***qnrS1***	1	1	>128	64	16	0.125	1
***K.oxytoca* B68**	***qnrS1***	64	16	>128	64	16	0.5	0.5
***K.pneumoniae* B69**	***qnrS1***	8	8	>128	64	8	>128	0.25
***K.pneumoniae* T52**	***qnrS1***	16	64	>128	128	2	128	<0.0625
***K.pneumoniae* VR586**	***qnrS1***	16	8	>128	>128	128	>128	<0.0625
***K.pneumoniae* VR782**	***qnrS1***	16	16	>128	>128	32	>128	<0.0625
***K.pneumoniae* VR818**	***qnrS1***	32	16	0.25	<0.0625	<0.0625	0.125	<0.0625
***K.pneumoniae* VR824**	***qnrS1***	>128	128	2	0.125	0.125	0.5	<0.0625
***K.pneumoniae* VRC160**	***qnrS1***	0.25	0.25	>128	128	16	>128	<0.0625
***K.pneumoniae* VRE73**	***qnrS1***	16	2	>128	64	8	>128	<0.0625
***K.pneumoniae* VRE94**	***qnrS1***	16	8	0.5	<0.0625	<0.0625	0.5	<0.0625
***K.pneumoniae* VRE185**	***qnrS1***	32	16	64	128	32	>128	<0.0625
***K.pneumoniae* VRE196**	***qnrS1***	16	8	128	>128	64	>128	<0.0625
***P. mirabilis* T18**	***qnrS1***	64	32	2	4	0.25	<0.0625	0.0625
***P. mirabilis* VR67**	***qnrD***	4	8	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625
***P. mirabilis* VR758**	***qnrD***	1	2	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625
***P. mirabilis* VR1048**	***qnrD***	4	4	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625
***P. mirabilis* T80**	***qnrD***	2	2	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625
***M. morganii* VR831**	***qnrD***	2	4	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625
***C. freundii* R11**	***qnrB6***	128	64	2	0.5	<0.0625	1	<0.0625
***C. freundii* T35**	***qnrB6***	<0.0625	<0.0625	>128	128	2	128	<0.0625
***C. koserii* V96**	***qnrB19***	0.25	0.5	128	16	1	>128	<0.0625
***C. freundii* VR669**	***qnrB8***	32	0.0625	>128	64	1	128	<0.0625
***C. freundii* VRE14**	***qnrB8***	0.25	0.25	>128	32	0.25	32	<0.0625
***E. cloacae* VRD25**	***qnrB2***	16	2	>128	>128	64	>128	<0.0625
***E. cloacae* VR739**	***qnrB2***	256	64	>128	>128	32	>128	<0.0625

4.3 Characterization of *qnr* positive isolates

All thirty-eight *qnr* positive strains were further screened for beta-lactamase genes such as: bla_{SHV}, bla_{TEM}, bla_{CTX-M}, bla_{OXA}, $bla_{LAP,}$ bla_{VIM} and bla_{IMP} and plasmid incompatibility groups (inc type) were also determined (**Table 8, Graph 4**). Ten *qnrS1* positive *E. coli* isolates were found to be positive for a variety of beta-lactamase genes. The most prevalent was bla_{TEM-1} (7/10), but others such as $bla_{CTX-M-15}$ (4/10), bla_{OXA-1} (4/10), bla_{SHV-12} (1/10) and bla_{VIM-1} (1/10) were also identified. Their plasmid incompatiblity types were also recognized, whereas incN (7/10) and incF (9/10) replicons were predominant, and others like incFia (7/10), incFib (3/10) and incColE (2/10) were also detected.

The four *E. coli* isolates carrying both *qnrS1* and *aac(6')-Ib-cr* variant were all found to be positive for $bla_{CTX-M-15,}$ bla_{OXA-1} and bla_{TEM-1}, and even their plasmid incompatibility types showed similarity, since incN, incFia and incF were common, and only one had incColE.

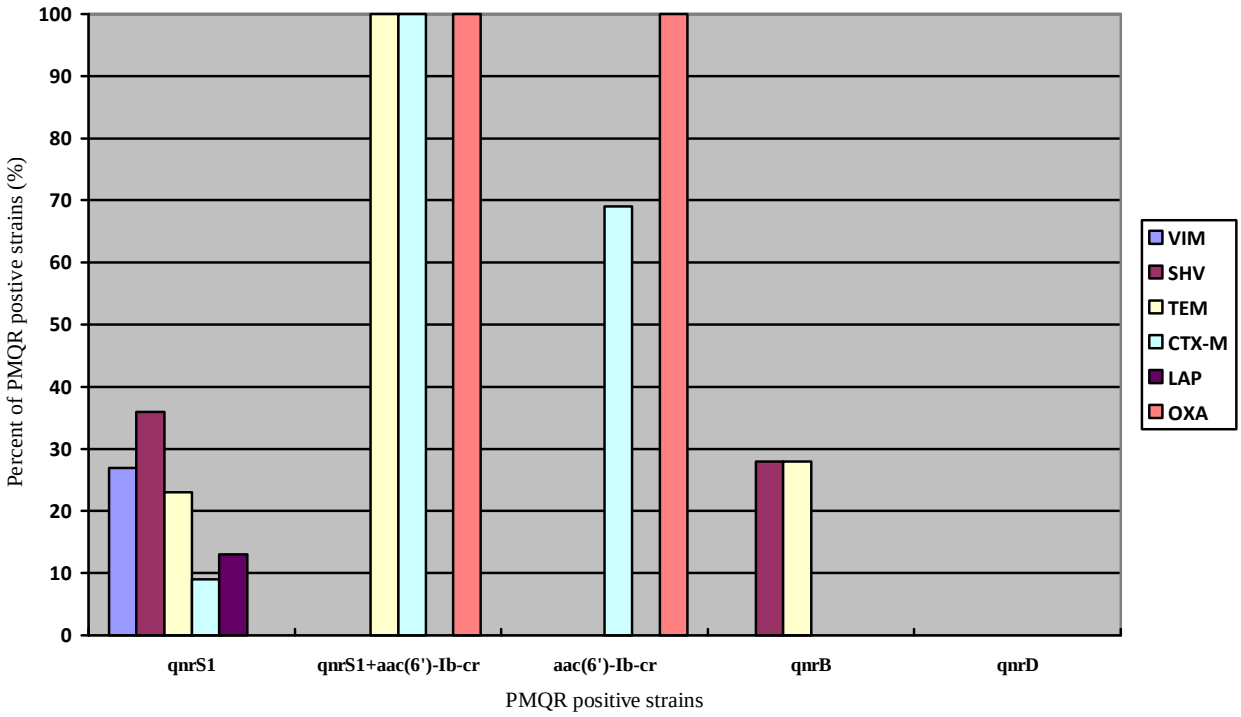
Fifteen *Klebsiella* spp. carring *qnrS1* determinant were detected and found positive for bla_{VIM-1} (5/15), bla_{SHV-12} (6/15), bla_{SHV-28} (1/15), bla_{TEM-3} (1/15), $bla_{CTX-M-15}$ (2/15) and bla_{LAP-2} (3/15). In case of their incompatibility groups, incN (12/15) and incColE (13/15) were the dominant ones, while incR (3/15) and incL/M (2/15) were also identified.

One *qnrS1* positive *P. mirabilis* carried TEM-52 and harboured incN plasmid. Four *qnrD* positive *P. mirabilis* and one *M. morganii* were detected and all of them were found negative for the tested beta-lactamases and were untypeable for the incompatibility groups.

Among *qnrB* positive isolates, five *Citrobacter* spp. and two *Enterobacter cloacae* were detected. Associations with beta-lactamases were rare, only one bla_{SHV-12} and one bla_{TEM-1} positive *Citrobacter* spp. were detected, while one *E. cloacae* was found bla_{SHV-12} and bla_{TEM-1} positive. Common incompatibility group, incHI2 was found for *E. cloacae* but all the *Citrobacter* isolates were untypeable.

Table 8. *Qnr* positive strains and their association with beta-lactamse genes and inc types

	inc type	*PMQR*	*VIM*	*SHV*	*TEM*	*CTX-M*	*OXA*	*LAP*
***E. coli* B24**	**N, Fia, Fib, F**	***qnrS1***	***VIM-1***	neg	neg	neg	neg	neg
***E. coli* B61**	**N, Fia, Fib**	***qnrS1***	neg	neg	neg	neg	neg	neg
***E. coli* V38**	**F, ColE**	***qnrS1***	neg	neg	***TEM-1***	neg	neg	neg
***E. coli* T57**	**Fib, F**	***qnrS1***	neg	neg	***TEM-1***	neg	neg	neg
***E. coli* B65**	**N, Fia, F, ColE**	***qnrS1, aac(6')-Ib-cr***	neg	neg	***TEM-1***	***CTX-M-15***	***OXA-1***	neg
***E. coli* VR 770**	**N, Fia, F**	***qnrS1, aac(6')-Ib-cr***	neg	neg	***TEM-1***	***CTX-M-15***	***OXA-1***	neg
***E. coli* VR 775**	**N, Fia, F**	***qnrS1, aac(6')-Ib-cr***	neg	neg	***TEM-1***	***CTX-M-15***	***OXA-1***	neg
***E. coli* VR 814**	**N, Fia, F**	***qnrS1, aac(6')-Ib-cr***	neg	neg	***TEM-1***	***CTX-M-15***	***OXA-1***	neg
***E. coli* VR 805**	**F**	***qnrS1***	neg	neg	***TEM-1***	neg	neg	neg
***E. coli* VR 301**	**N, Fia, F**	***qnrS1***	neg	***SHV-12***	neg	neg	neg	neg
***K. pneumoniae* B49**	**N, ColE**	***qnrS1***	***VIM-1***	***SHV-12***	neg	neg	neg	neg
***K. pneumoniae* B56**	**N, ColE**	***qnrS1***	***VIM-1***	***SHV-12***	neg	neg	neg	neg
***K. pneumoniae* B69**	**N, ColE**	***qnrS1***	***VIM-1***	***SHV-12***	neg	neg	neg	neg
***K. pneumoniae* T52**	**N, ColE**	***qnrS1***	neg	***SHV-28***	***TEM-3***	neg	neg	neg
***K. oxytoca* B67**	**N**	***qnrS1***	***VIM-1***	neg	neg	neg	neg	neg
***K. oxytoca* B68**	**N**	***qnrS1***	***VIM-1***	neg	neg	neg	neg	neg
***K.pneumoniae* VR 586**	**ColE**	***qnrS1***	neg	***SHV-12***	neg	neg	neg	***LAP-2***
***K.pneumoniae*VRE185**	**ColE**	***qnrS1***	neg	neg	neg	***CTX-M-15***	neg	***LAP-2***
***K.pneumoniae*VRE196**	**ColE**	***qnrS1***	neg	neg	neg	***CTX-M-15***	neg	***LAP-2***
***K.pneumoniae* VR782**	**N, R, ColE**	***qnrS1***	neg	***SHV-12***	neg	neg	neg	neg
***K.pneumoniae* VR818**	**N, R, ColE**	***qnrS1***	neg	neg	neg	neg	neg	neg
***K.pneumoniae* VR824**	**N, R, ColE**	***qnrS1***	neg	neg	neg	neg	neg	neg
***K.pneumoniae*VRC160**	**N, L/M, ColE**	***qnrS1***	neg	neg	neg	neg	neg	neg
***K.pneumoniae*VRE73**	**N, L/M, ColE**	***qnrS1***	neg	***SHV-12***	neg	neg	neg	neg
***K.pneumoniae*VRE94**	**N,ColE**	***qnrS1***	neg	neg	neg	neg	neg	neg
***P. mirabilis* T18**	**N**	***qnrS1***	neg	neg	***TEM-52***	neg	neg	neg
***P. mirabilis* T80**	Untypeable	***qnrD***	neg	neg	neg	neg	neg	neg
***P. mirabilis* VR 67**	Untypeable	***qnrD***	neg	neg	neg	neg	neg	neg
***P. mirabilis* VR 758**	Untypeable	***qnrD***	neg	neg	neg	neg	neg	neg
***P. mirabilis* VR1048**	Untypeable	***qnrD***	neg	neg	neg	neg	neg	neg
***M.morganii* VR 831**	Untypeable	***qnrD***	neg	neg	neg	neg	neg	neg
***C. freundii* R11**	Untypeable	***qnr B6***	neg	neg	***TEM-1***	neg	neg	neg
***C. freundii* T35**	Untypeable	***qnr B6***	neg	neg	neg	neg	neg	neg
***C. koserii* V96**	Untypeable	***qnr B19***	neg	***SHV-12***	neg	neg	neg	neg
***C.freundii* VR 669**	Untypeable	***qnrB8***	neg	neg	neg	neg	neg	neg
***C.freundii*VR E14**	Untypeable	***qnrB8***	neg	neg	neg	neg	neg	neg
***E. cloacae* VR 739**	**HI2, ColE**	***qnrB2***	neg	neg	neg	neg	neg	neg
***E. cloacae* VR D25**	**HI2**	***qnrB2***	neg	***SHV-12***	***TEM-1***	neg	neg	neg



Graph 4. Association of PMQR-positive isolates with beta-lactamase genes

Graph 4 shows that *qnrS1* positive strains were found to be positive for several beta-lactamase genes, namely, 27% VIM, 36% SHV, 23% TEM, 9% CTX-M and 13% LAP. All the strains co-harbouring *qnrS1* with *aac(6')-Ib-cr* were carrying genes of TEM, CTX-M and OXA. The *aac(6')-Ib-cr* positive strains had a high correlation with OXA beta-lactamase genes (96%) and with CTX-M beta-lactamase genes (69%). The *qnrB* positive isolates were 28.6% SHV and CTX-M postive. All the *qnrD* strains were negative for the tested beta-lactamase genes.

Transformation of DH5 alpha *E. coli* cells with recombinant vectors containing *qnr* genes

The *qnr* genes found in our survey were analyzed in isogenic *E. coli* DH5 alpha cells after transformation with recombinant vectors ligated with *qnrB*, *qnrS* and *qnD* genes. After transformation, MIC increments for nalidixic acid, ciprofloxacin and levofloxacin were measuerd by broth microdilution method.

The transformed DH5 alpha cells showed 1-5 fold MIC increment to nalidixic acid (from 32 µg/ml to 64, 128 and 1024). In case of ciprofloxacin and levofloxacin no increment was measured (**Table 9**).

Table 9. MIC values of DH5 alpha *E. coli* and its transformant (Tn) harbouring different *qnr* genes.

Antimicrobial	**MIC** (µg/ml)				
	DH5 apha *E. coli* Recipient cell	**Tn DH5 alpha *E. coli* R11-QNR B6**	**Tn DH5 alpha *E. coli* T35-QNR B6**	**Tn DH5 alpha *E. coli* V38-QNR S1**	**Tn DH5 alpha *E. coli* T80-QNR D**
nalidixic acid	32	128	1024	64	1024
ciprofloxacin	0.016	0.016	0.016	0.016	0.016
levofloxacin	0.032	0.032	0.032	0.032	0.032

Transformation of DH5 alpha *E. coli* cells with recombinant vectors containing *aac(6')-Ib-cr* variant gene

The *aac(6')-Ib-cr* variant genes found in our survey, were analyzed in isogenic *E. coli* DH5 alpha cells after transformation with recombinant vectors ligated with *aac(6')-Ib-cr* genes. After transformation, MIC increments for kanamycin, gentamicin, amikacin, ciprofloxacin and levofloxacin were measured by broth microdilution method.

The transformed DH5 alpha cells showed 2-3 fold MIC increment to gentamicin (from 0.125 µg/ml to 0.5, 1 and 2), 7-9 fold to kanamycin (from 0.5 µg/ml to 64 and 256), 1-3 fold to amikacin (from 0.125 µg/ml to 0.25, 0.5 and 1). In case of ciprofloxacin and levofloxacin no increment was measured (**Table 10**).

Table 10. MIC values of DH5 alpha *E. coli* and its transformant (Tn) counterparts harbouring *aac(6')-Ib-cr* genes.

Antimicrobial	**MIC (µg/ml)**				
	DH5 alpha *E. coli*	**Tn DH5 alpha *E. coli* V25-aac(6')-Ib-cr**	**Tn DH5 alpha *E.coli* V27-aac(6')-Ib-cr**	**Tn DH5 alpha *E. coli* V41-aac(6')-Ib-cr**	**Tn DH5 alpha *E. coli* T87-aac(6')-Ib-cr**
gentamicin	0.125	1	0.5	0.125	2
kanamycin	0.5	256	256	256	64
amikacin	0.125	0.5	0.25	0.125	1
ciprofloxacin	0.016	0.016	0.016	0.016	0.016
levofloxacin	0.032	0.032	0.032	0.032	0.032

4.4 Characterization of *qnrD* positive isolates

Four *P. mirabilis* (labelled: VR67, VR758, VR1048, T80) and one *M. morganii* (labelled: VR831) isolates were found to be positive by PCR for the *qnrD* determinant. Sequencing confirmed all to belong to the same allel. PCR-based replicon typing found all isolates untypeable and conjugation experiment did not find transconjugable plasmids. The strains were susceptibile to third and fourth generation cefalosporins, aztreonam and carbapenem but resistant to fluoroquinolones.

Plasmid characterization

Linearization of plasmids by digestion with *Nhe* I and *Xba* I restriction enzymes

Plasmid extractions of all *qnrD* positive isolates were treated with restriction enzymes having recognition site in the *qnrD* gene. **Figure 13** shows the agarose gelelectrophoresis of *qnrD*-harbouring plasmids after digestion with *Nhe* I and *Xba* I, approximately 2800 bp plasmid is common to all the isolates.

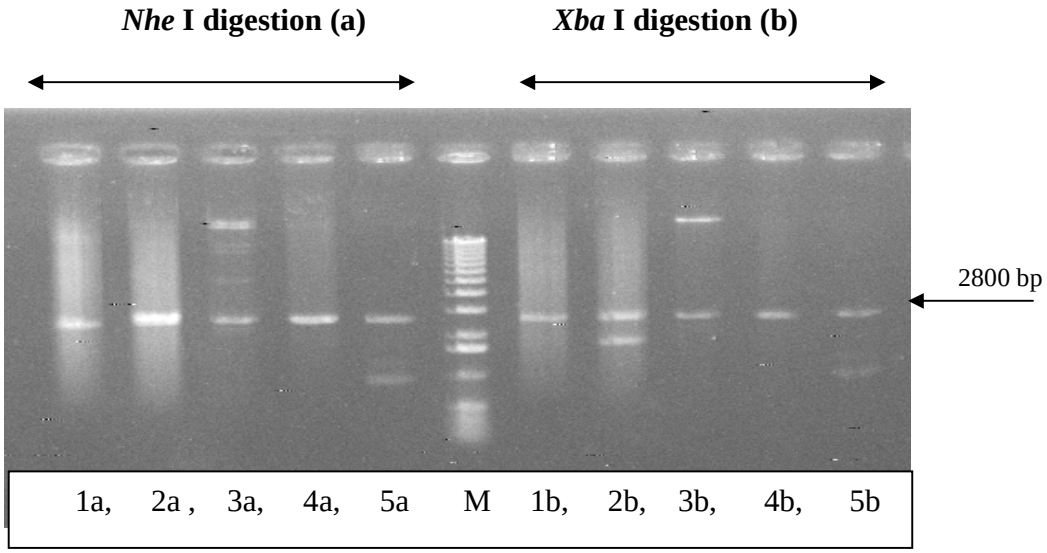


Figure 13. Plasmid profile of *qnrD* positive isolates digested with *Nhe* I (a) and *Xba* I (b) restriction enzymes: 1: VR67, 2: VR758, 3: VR831, 4: VR1048, 5: T80, M: 1kb ladder (Invitrogen)

Southern blot of *qnrD*-harbouring plasmids

Plasmid extractions of *qnrD* positive isolates were treated with ATP-dependent DNase, to eliminate chromosomal DNA. Plasmid extractions before and after treatment were loaded onto 0.6% (w/v) agarose gel which showed approximately 2800 bp common plasmid. After denaturation of plasmid DNA and overnight blot on positively-charged nylon membrane, hybridization was carried out at 44.6^{o}C with digoxigenin-labeled *qnrD* probe. After developing chemiluminescens detection film positive bands verified *qnrD* gene to be localized on the common plasmid, since both plasmid extractions without (a) and with (b) chromosomal treatment had given positive signal (**Figure 14**).

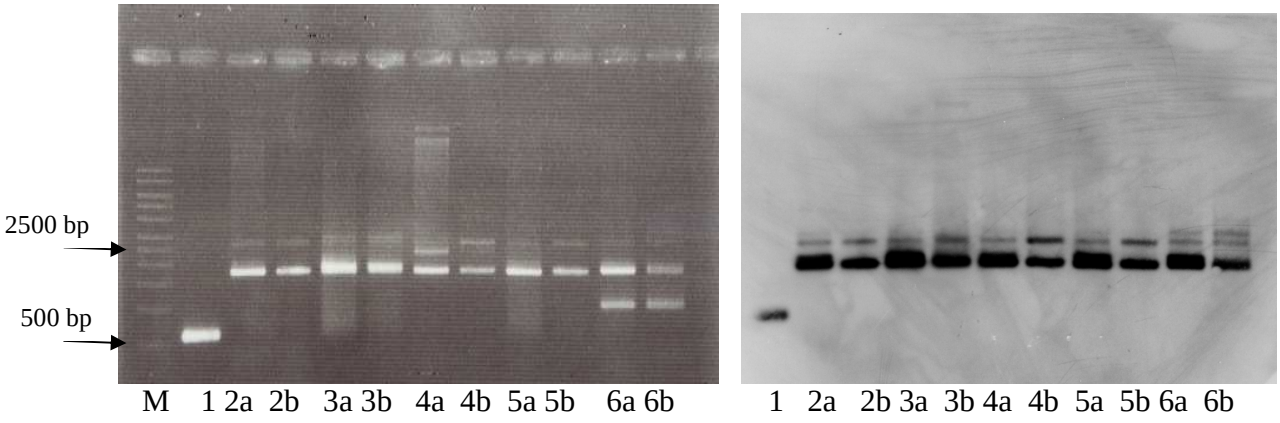


Figure 14. Plasmid profiles (left) and corresponding Southern blot (right) of 5 *qnrD* positive strains. Each strain was running as untreated (a) and treated (b) to remove any trace of chromosome DNA. M: 1kb (Sigma); 1: *qnrD* gene; 2a-b: strain VR67; 3a-b: strain VR758; 4a-b: strain VR831; 5a-b: strain VR1048; 6a-b: strain T80

Results of inverse PCR on *qnrD*-harbouring plasmids

Inverse PCR was performed by complementar and antiparallel oligonucleotids of qnrD Fwd and qnrD Rev primers labeled INVDF/Rev and INVDR/Fwd (**Table 2, Figure 16**), to amplify the plasmid DNA located upstream and downstream to the *qnrD* gene. Thermal profile was used as follows: 94^{o}C for 1 min, 53.1^{o}C for 1 min and elongation at 72^{o}C for 3 min for 30 cycles, resulted in 2200 bp products (**Figure 15**). Sequencing was carried out and two additional set of primers were designed Pl 1 Rev with Pl 2 Fwd and Pl 3 Fwd with Pl 4 Rev (**Table 2, Figure 16**) to obtain overlapping regions of the *qnrD*-plasmid. PCR was performed with these set of primers, with thermal profile as follows: 30 cycles of 94^{o}C for 1 min, 53.1^{o}C for 1 min and 72^{o}C for 3 min and after sequencing the positive PCR products overlapping regions were obtained. The whole sequences of *qnrD*-plasmid were 2687 bp for the four *P. mirabilis* while 2684 bp for the *M. morganii*.

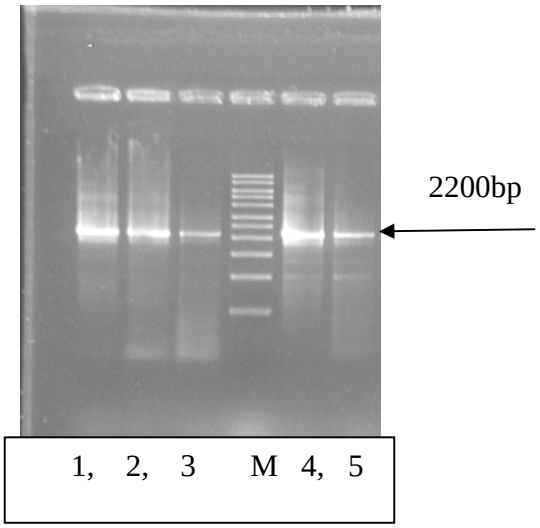


Figure 15. Inverse PCR on *qnrD*-harbouring plasmids 1: VR67, 2: VR758, 3: VR831, M: 1kb (Sigma), 4: VR1048, 5: VRT80

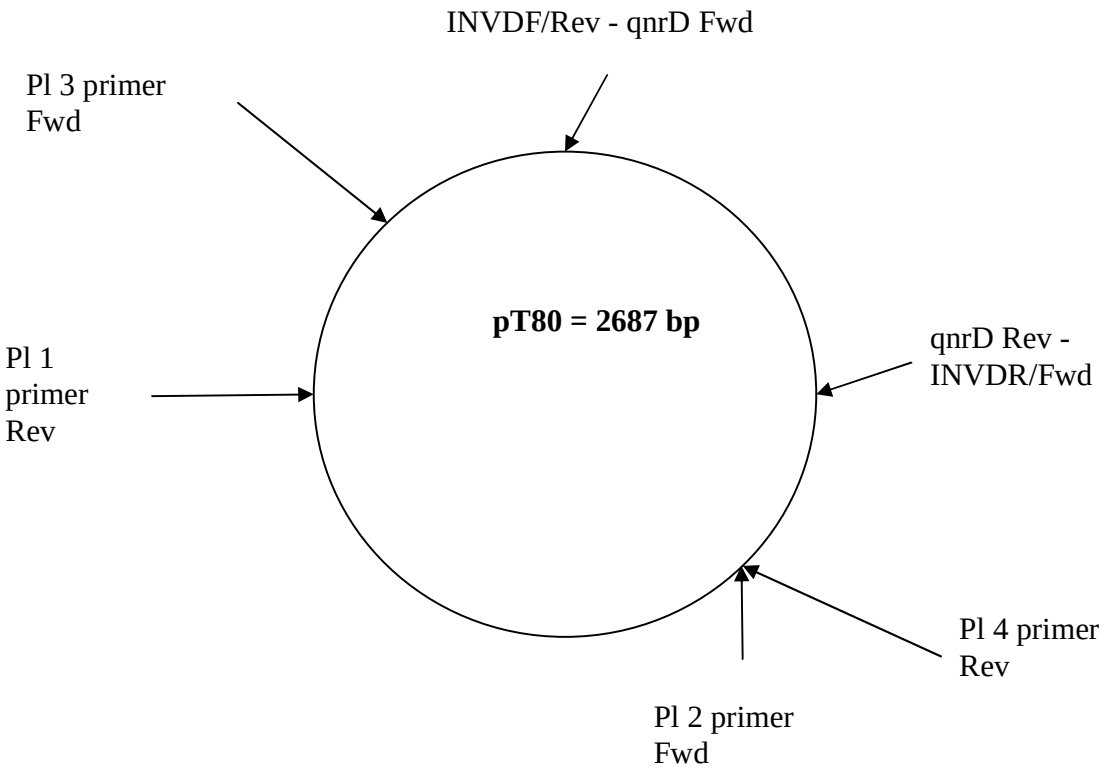


Figure 16. Primers highlighted on the schematic figure of pT80.

Sequence analysis

The *qnrD*-harbouring plasmid sequence analysis found 100% similarity with the 1450 bp (54%) of the first described *qnrD* plasmid. Besides the *qnrD* gene the upstream region included a promoter sequence and the downstream region included an Orf2 gene, without known function. The remaining 1237 bp sequence of the plasmid was unknown.

Deposition of plasmid DNA sequence in Genbank was done, with accession number JN183060 and JN183061 for *P. mirabilis* and *M. morganii*, respectively.

Table 11. MIC of the *qnrD* postive strains for fluoroquinolones and beta-lactam antibiotics

Strains	**MIC (µg/ml)**						
	cip	lev	ceftazidime	cefotaxime	cefepime	aztreonam	imipenem
P.mirabilis VR67	4	8	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625
P.mirabilis VR758	1	2	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625
P.mirabilis VR1048	4	4	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625
P. mirabilis T80	2	2	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625
M. morganii VR831	2	4	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625

Table 11 shows all the *qnrD* positve strains were resistant to the tested fluoroquinolones (cip : ciprofloxacin, lev : levofloxacin) and susceptible to the tested beta-lactam antibiotics.

Table 12. QRDR aminoacid substitutions in *qnrD* positive strains

Strains	***QRDR***					
	gyrA		*gyrB*		*parC*	
P. mirabilis ATCC 29906	83 S	87 E	464 S	466 E	78 G	80 S
P. mirabilis VR67	**S 83 I**	E	**S**	**E**	**G**	**S 80 I**
P. mirabilis VR758	**S 83 I**	E	**S**	**E**	**G**	**S 80 R**
P. mirabilis VR1048	**S 83 I**	E	**S**	**E**	**G**	**S 80 I**
P. mirabilis T80	**S 83 I**	E	**S**	**E**	**G**	**S 80 I**
M. morganii VR831	n.d.	n.d.	**S**	**E**	n.d.	n.d.

(n.d.: not detected)

Table 12 shows the QRDR regions of the *qnrD* postive strains. The detected aminoacid changes can explain the fluoroquinolone resistant phenotype. In case of *M. morganii* we could not obtain PCR product for *gyrA* and *parC*.

Clonality analysis

Random Amplified Polymorphic DNA (RAPD) was performed with OPX13 primer. **Figure 17** shows that each strain has a different RAPD pattern, thus explaining the clonal unrelatedness

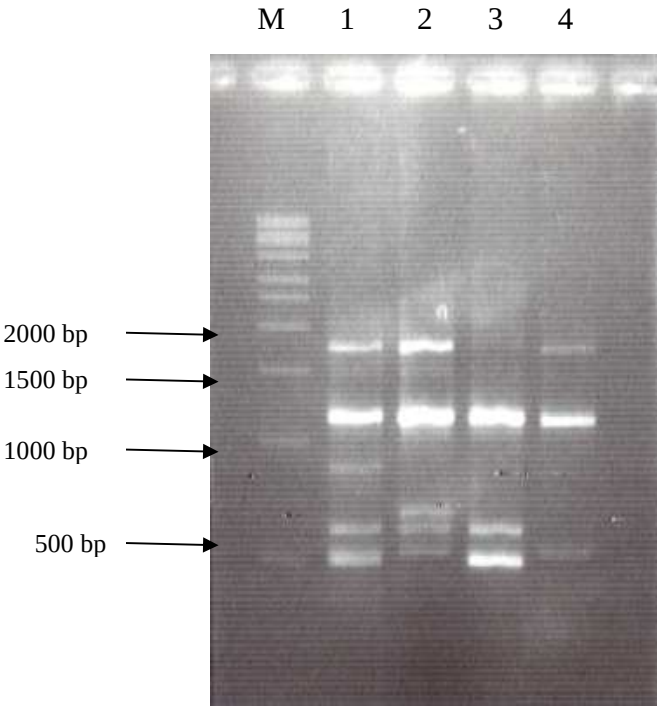


Figure 17. RAPD profile of four *qnrD* positive *P. mirabilis* with primer Opx13.

Legend : M: 1kb (Sigma); 1:strain T80; 2: strain VR67; 3: strain VR758; 4: strain VR1048.

4.5 Characterization of *qnrS1* postive isolates

Our survey found twenty-six *qnrS1* positive isolates namely, 10 *E.coli*, 15 *Klebsiella* spp. and 1 *P. mirabilis*. Four *E. coli* strains besides *qnrS1* were positive for the aac(6)-Ib-cr variant too. High correlation with beta-lactamases was detected. The 22 *qnrS1* positive strains were found to be positive for several beta-lactamase genes too, namely 27% bla_{VIM}, 36% bla_{SHV}, 23% bla_{TEM}, 9% $bla_{\text{CTX-M.}}$ All the four *qnrS1* and aac(6)-Ib-cr variant positive strains were $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1}}$ and $bla_{\text{TEM-1}}$ positive (**Table 8**).

Characterization of *qnrS1* postive plasmids

Characterization of *qnrS1* positive strains was performed by PCR-based replicon typing where incN plasmid type was the predominant, but incColE, incFia, incFib, incF, incL/M, and incR were also identified (**Table 8**). **Figure 18** shows the *qnrS*1-harbouring plasmids, identified as incN type. Conjugation experiment found incN, approximately 100 kb plasmids harbouring *qnrS1* and $bla_{\text{VIM-1}}$ genes to be transferable from two *Klebsiella oxytoxa* and one *Klebsiella pneumoniae* strains to *E. coli* J53 Azid^{R}. The increase of MIC values in transconjugants were checked by microdilution method and showed these plasmids to be able to raise the MIC in host cells up to the low-level resistance (0.06-0.5μg/ml). The MIC increased by 1-2 fold in the case of nalidixic acid, 2-4 fold in the case of ciprofloxacin, and 2-3 fold in the case of levofloxacin (**Table 13**).

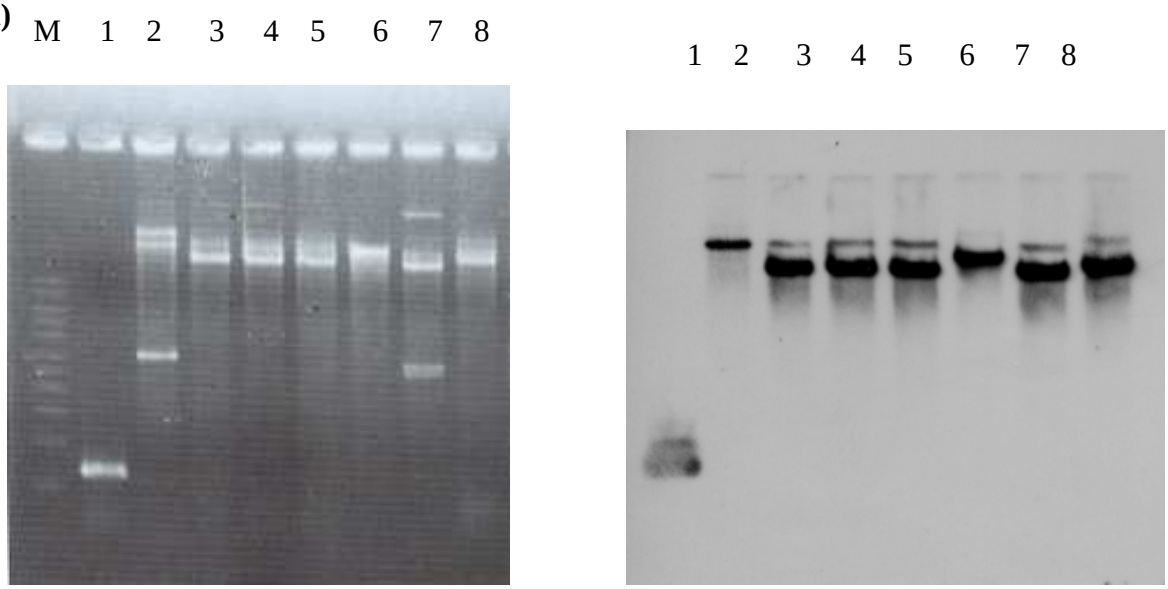
Table 13. MIC increment in the transconjugants carrying *qnrS1*-harbouring plasmid comparing to the recipient J53 *E. coli*

Antimicrobial	**MIC (μg/ml)**			
	J53	**J53-pB49**	**J53-pB67**	**J53-pB68**
nalidixic acid	4	8	16	16
ciprofloxacin	0.03	0.125	0.5	0.25
levofloxacin	0.06	0.25	0.5	0.5

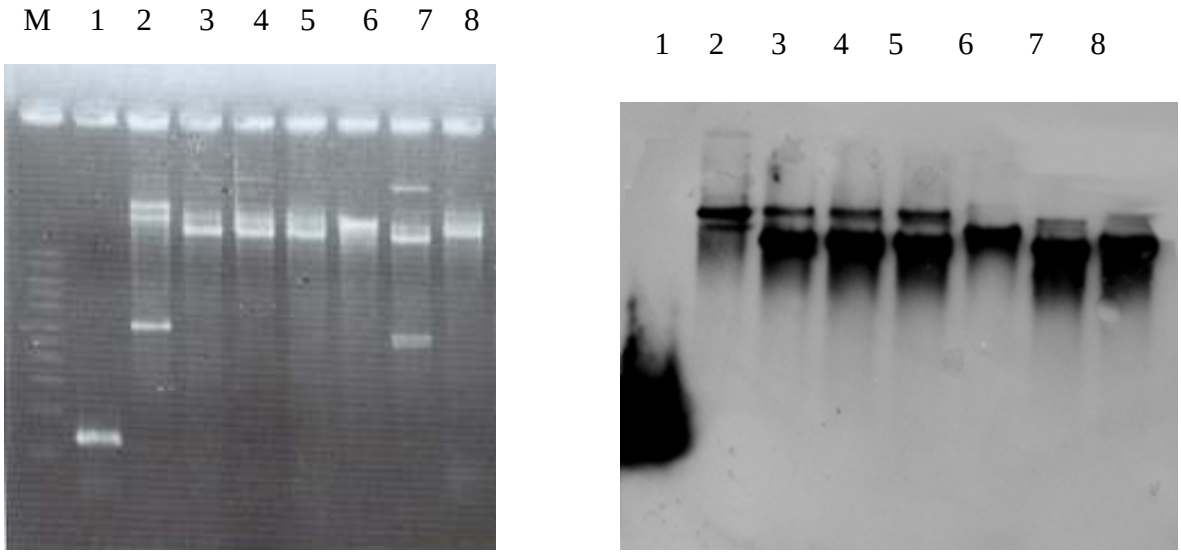
Figure 18. Southern blot analysis of *qnrS1* positive *E. coli* strains:
A) Plasmid profile (left) and Southern blot (right) with *qnrS1* probe
B) Plasmid profile (left) and Southern blot (right) with incN probe

Figure 18.

(A)



(B)



M: 1kb (Sigma); 1 (A) *qnrS1* PCR product; 1 (B) *incN* PCR product; 2: pB65; 3; pVR770; 4: pVR775; 5: pVR814; 6: pB24; 7: pB61; 8: pVR301

Results of pulsed-field gelelectrophoresis (PFGE)

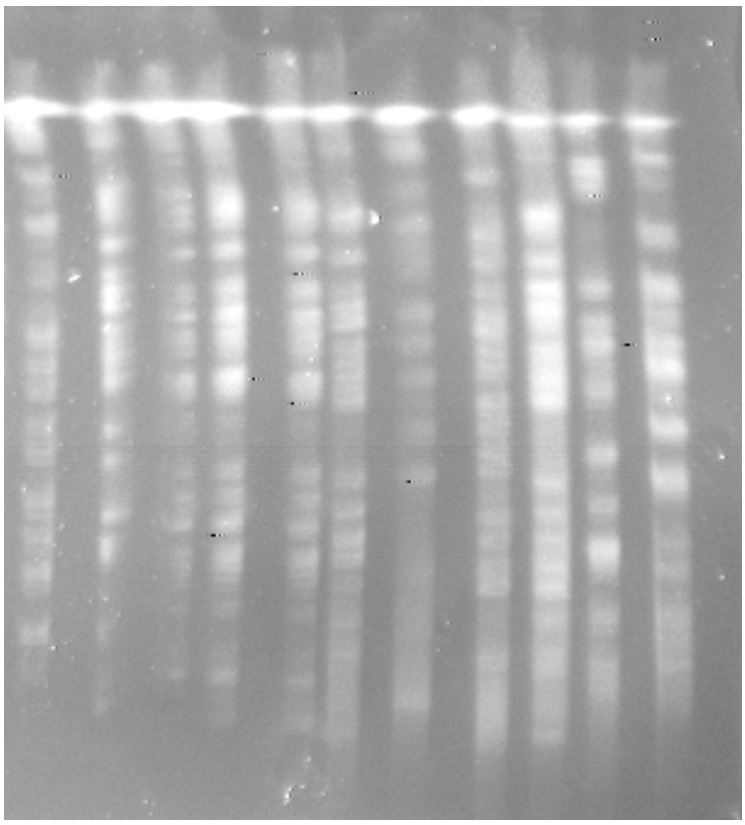
PFGE figure of *qnrS1* positive *E. coli* strains after digestion of 30 units *XbaI* overnight at 37 ^{o}C, run at 14 ^{o}C for 21hours, 6V/cm, initial switch time 2sec, final switch time 30sec.

Seven pulso-types (A-B-C-D-E-F-G) can be distinguished among the ten *qnrS1* positive *E. coli* isolates and the R25 *E. coli* used as an indifferent strain (**Figure 19**).

Figure 19. PFGE of the *qnrS1* positive *E. coli:*

1: VR805; 2: VR301, 3: VR770, 4: VR775, 5: VR814, 6: R25, 7: B24, 8: B61, 9: B65, 10: T57, 11: V38

1:A 2:B 3:B 4:B 5:B 6:B 7: C 8:D 9:E 10:F 11:G



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4.6 Characterization of *qnrS1* and *bla*$_{\text{LAP-2}}$ positive *K. pneumoniae* strains

Among twenty-six *qnrS1* positive isolates, three *K. pneumoniae* strains were found positive by PCR for *bla*$_{\text{LAP}}$ using the LAP Fwd and LAP Rev set of primer. Sequencing verified them as LAP-2.

The genetic context incorporating *bla*$_{\text{LAP-2}}$ and *qnrS1* genes was mapped by PCR using LAP Fwd and qnrS Rev primers, resulting in 2600 bp product. After sequencing of this PCR product additional primers namely, Ins lap Fwd and Ins lap Rev were designed by tools of Eurofins MWG Operon Germany (**Table 2**). This new set of primer was used in amplification of the internal part of the complex genetic material in a PCR reaction as follows: 40 cycles of 1 min at 93°C, 1 min at 55°C, and 3 min at 72°C and finally, 72^{o}C for 7 min. We obtained 800 bp PCR products, and after sequencing we found overlapping regions with the sequence of the 2600 bp PCR product. The sequence of the 800 bp PCR product was identified as IS*Ecl2*, which separates *bla*$_{\text{LAP-2}}$ and *qnrS1* determinants. IS*Ecl2* is built up by orfA and orfB (**Figure 20**).

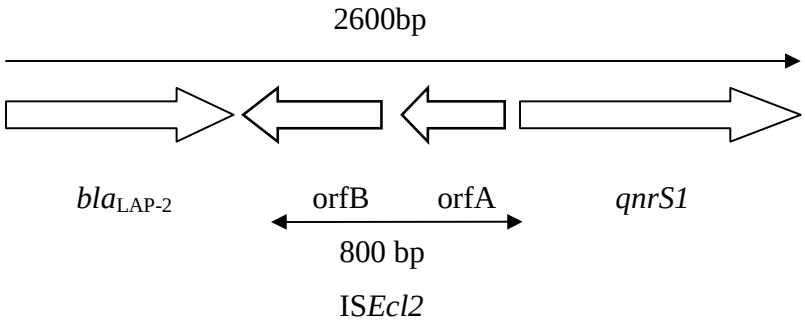

Figure 20. Genetic scheme of *bla*$_{\text{LAP-2}}$ and *qnrS1* genes.

The localization of this complex genetic material was further investigated by Southern blot. Plasmid extractions of each strain before and after treatment of ATP-dependent DNase were run in a 0.6% (w/v) agarose gel, denatureated and blotted on a positively charged nylone membrane. Hybridization at 47^{o}C with digoxigenin-labeled LAP-probe was done. A chemiluminescens detection film was developed in dark chamber, where positive bands verified the localization on approximately 100 kb plasmid (**Figure 21**).

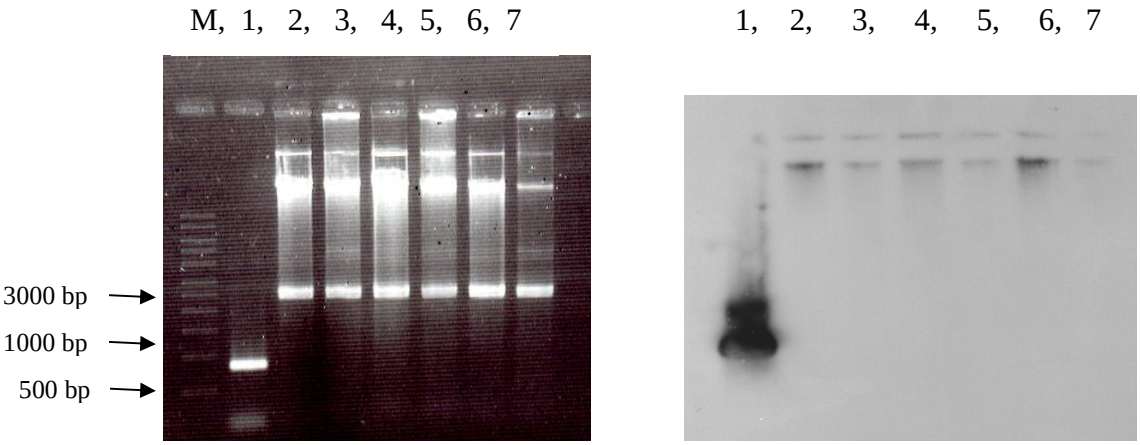

Figure 21. Plasmid extraction (left) and Southern-blot (right) of *bla*$_{LAP-2}$ and *qnrS1* positive strains. Legend: M: 1kb (Sigma), 1: *bla*$_{LAP}$ PCR product, 2: pVR185 without DNase treatment, 3: pVR185 after DNase treatment, 4: pVR196 without DNase treatment, 5: pVR196 after DNase treatment, 6: pVR586 without DNase treatment 7: pVR586 after DNase treatment.

Transferability of *qnrS1* and *bla*$_{LAP-2}$ positive plasmids was also checked by conjugation. No transconjugants were selected on Luria-Bertani agar plates containing 100 μg/ml sodium-Azid and 16 μg/ml nalidixic acid by using J53 Azid^{R} *E. coli* as recipient cells.

PCR–based replicon typing was also performed and all three *Klebsiella pneumoniae* strains were found positive only for incColE. Southern blot was carried out with digoxigenin labeled colE-probes and clarified the 3 kb plasmid to belong to this type.

Clonality analysis of the three *Klebsiella pneumoniae* strains was performed by pulsed-field gelelectrophoresis, 30 U *Xba* I digestion overnight and run in CHEF DR^{TM} Electrophoresis (BioRad) 6V/cm, 120^{o} angel, initial switch time 2s and final switch time 35s for 21h run. An identical pulso-type was detected for all the three *Klebsiella pneumoniae* isolates.

Strain genotyping was performed by Multilocus Sequence typing (MLST) to determine the sequence type (ST) of the isolates. Allelic numbers were obtained on the basis of sequences of 7 housekeeping genes at the http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae . MLST identified the strains as a ST 147.

Table 14. MIC (µg/ml) of *K. pneumoniae* strains harbouring bla_{LAP2} and *qnrS1* genes

Strain	PMQR	ciprofloxacin	levofloxacin	ceftazidime	cefotaxime	cefepime	aztreonam	imipenem
K.pneumoniae VRE185	***qnrS1***	32	16	64	128	32	>128	<0.0625
K.pneumoniae VRE196	***qnrS1***	16	8	128	>128	64	>128	<0.0625
K.pneumoniae VR 586	***qnrS1***	16	8	>128	>128	128	>128	<0.0625

Tabel 14 shows MIC values of the three *qnrS1* and LAP-2 positive *K.pneumoniae* strains. All of them were resistant to fluoroquinolones and to third- and fourth-generation cephalosporins, but susceptible to imipenem.

Table 15. Genetic determinants of *K. pneumoniae* strains harbouring bla_{LAP2} and *qnrS1* genes

Strain	inc type	PMQR	VIM	SHV	TEM	CTX-M	OXA	LAP
K.pneumoniae VRE185	**ColE**	***qnrS1***	neg	neg	neg	***CTX-M-15***	neg	***LAP-2***
K.pneumoniae VRE196	**ColE**	***qnrS1***	neg	neg	neg	***CTX-M-15***	neg	***LAP-2***
K.pneumoniae VR 586	**ColE**	***qnrS1***	neg	***SHV-12***	neg	neg	neg	***LAP-2***

Tabel 15 shows the incompatibility types and the tested beta-lactamase genes of the three *qnrS1* and LAP-2 positive *K. pneumoniae* strains. All of them were carrying incColE type plasmid, two were found positive for CTX-M-15 and one was SHV-12 positive.

4.7 Characterization of *aac(6')-Ib-cr* variant positive isolates

Altogether 70 from the 756 isolates tested were found positive for this plasmid-mediated quinolone resistance determinant showing 9.25% prevalence. Among them were 60 *E. coli*, 8 *Klebsiella* spp, one *P. mirabilis* and *M. morganii*.

All the positive strains were found resistant to fluoroquinolones and 78% (55/70) were resistant to third generation cefalosporins too (**Table 5**, **Table 6**). High correlation of CTX-M-15 and OXA-1 beta-lactamase was remarkable in the case of *E. coli*, (42/60), all the *Klebsiella* spp., (8/8) and *M. morganii*. Exception was *P.mirabilis* where TEM-52 and OXA-1 was detected.

PCR-based replicon (inc) typing found incFia, incFib, incF and incColE for *E. coli*, while for *Klebsiella* spp. incColE was the predominant. In the case of *P. mirabilis* incFia and incF types were detected, but *M. morganii* was untypeable (**Table 16, Table 17**).

Four *E. coli* strains were positive for *qnrS1* determinant too, and shared an identical plasmid replicon profile incN, incFIA, incF. The two determinants [*qnrS1* and *aac(6')-Ib-cr*] have additive effect on developing fluoroquinolone resistance in Enterobacteriaceae, but this phenomenon was detected in 0.5% (4/756) of the isolates in our collection.

Table 16. The *aac(6')-Ib-cr* variant positive *K. pneumoniae, P. mirabilis, M. morganii* with their beta-lactamase genes and incompatibility types

Strain	**PMQR**	**inc type**	***SHV***	***TEM***	***CTX-M***	***OXA***
K.pnenumoniae VR C191	***aac(6')-Ib-cr***	**col E**	neg	neg	***CTX-M-15***	***OXA-1***
K.pnenumoniae VR E102	***aac(6')-Ib-cr***	**col E, F**	***SHV-11***	***TEM-1***	***CTX-M-15***	***OXA-1***
K.pnenumoniae VR E154	***aac(6')-Ib-cr***	**col E, F**	neg	***TEM-1***	***CTX-M-15***	***OXA-1***
K.pnenumoniae VR E157	***aac(6')-Ib-cr***	**col E, F**	***SHV-11***	***TEM-1***	***CTX-M-15***	***OXA-1***
K.pnenumoniae VR E165	***aac(6')-Ib-cr***	**col E, F**	neg	neg	***CTX-M-15***	***OXA-1***
K.pnenumoniae VR E176	***aac(6')-Ib-cr***	**col E**	neg	neg	***CTX-M-15***	***OXA-1***
K.pnenumoniae V29	***aac(6')-Ib-cr***	**col E**	***SHV-11***	***TEM-1***	***CTX-M-15***	***OXA-1***
K.pnenumoniae V91	***aac(6')-Ib-cr***	**col E**	***SHV-11***	***TEM-1***	***CTX-M-15***	***OXA-1***
P.mirabilis T40	***aac(6')-Ib-cr***	**Fia, F**	neg	***TEM-52***	neg	***OXA-1***
M.morganii T53	***aac(6')-Ib-cr***	Untypeable	neg	neg	***CTX-M-15***	***OXA-1***

Table 17. The *aac(6')-Ib-cr* variant positive *E. coli* strains their beta-lactamase genes and incompatibility groups

Strain	PMQR	inc type	*SHV*	*TEM*	*CTX-M*	*OXA*
E. coli T6	***aac(6')-Ib-cr***	**Fia, Fib**	neg	neg	***CTX-M-15***	***OXA-1***
E. coli T10	***aac(6')-Ib-cr***	**Fia**	neg	neg	***CTX-M-15***	***OXA-1***
E. coli T27	***aac(6')-Ib-cr***	**Fia**	neg	***TEM-1***	***CTX-M-15***	***OXA-1***
E. coli T30	***aac(6')-Ib-cr***	**Fia, col E**	neg	neg	neg	***OXA-1***
E. coli T39	***aac(6')-Ib-cr***	**Fia**	neg	***TEM-1***	***CTX-M-15***	***OXA-1***
E. coli T47	***aac(6')-Ib-cr***	**Fia, F**	neg	***TEM-1***	***CTX-M-15***	***OXA-1***
E. coli T87	***aac(6')-Ib-cr***	**Fia, Fib, F, col E**	neg	***TEM-1***	***CTX-M-15***	***OXA-1***
E. coli V18	***aac(6')-Ib-cr***	**Fia, F**	neg	neg	***CTX-M-15***	***OXA-1***
E. coli V21	***aac(6')-Ib-cr***	**Fia, F, col E**	neg	neg	***CTX-M-15***	***OXA-1***
E. coli V25	***aac(6')-Ib-cr***	**Fia, F**	neg	neg	neg	***OXA-1***
E. coli V27	***aac(6')-Ib-cr***	**Fia, Fib, F, col E**	neg	neg	***CTX-M-15***	***OXA-1***
E. coli V32	***aac(6')-Ib-cr***	**Fia, F**	neg	neg	neg	***OXA-1***
E. coli V41	***aac(6')-Ib-cr***	**Fia, F**	neg	***TEM-1***	neg	***OXA-1***
E. coli V47	***aac(6')-Ib-cr***	**Fia, F**	neg	neg	***CTX-M-15***	***OXA-1***
E. coli V55	***aac(6')-Ib-cr***	**Fia, F**	neg	neg	***CTX-M-15***	***OXA-1***
E. coli V65	***aac(6')-Ib-cr***	**Fia, F**	neg	neg	neg	***OXA-1***
E. coli V85	***aac(6')-Ib-cr***	**Fia, F**	neg	neg	neg	***OXA-1***
E. coli B33	***aac(6')-Ib-cr***	**Fia, Fib, P, Fic**	neg	neg	neg	***OXA-1***
E. coli B38	***aac(6')-Ib-cr***	**Fia, col E, P**	neg	neg	***CTX-M-15***	***OXA-1***
E. coli B44	***aac(6')-Ib-cr***	**Fia, Fib**	neg	neg	***CTX-M-15***	***OXA-1***
E. coli B65	***qnrS1, aac(6')-Ib-cr***	**N, Fia, F, colE**	neg	***TEM-1***	***CTX-M-15***	***OXA-1***
E. coli R25	***aac(6')-Ib-cr***	**Fia, F**	neg	***TEM-1***	***CTX-M-15***	***OXA-1***
E. coli VR B18	***aac(6')-Ib-cr***	**Fia, F**	neg	***TEM-1***	***CTX-M-15***	***OXA-1***
E. coli VR B88	***aac(6')-Ib-cr***	**Fia, Fib, F**	neg	***TEM-1***	***CTX-M-15***	***OXA-1***
E. coli VR B127	***aac(6')-Ib-cr***	**col E, F**	neg	***TEM-1***	neg	***OXA-1***
E. coli VR 227	***aac(6')-Ib-cr***	**Fia, F**	neg	***TEM-1***	***CTX-M-15***	***OXA-1***
E. coli VR 232	***aac(6')-Ib-cr***	**Fia, F**	neg	neg	***CTX-M-15***	***OXA-1***
E. coli VR B248	***aac(6')-Ib-cr***	**Fia, F**	neg	***TEM-1***	neg	***OXA-1***
E. coli VR B262	***aac(6')-Ib-cr***	**Fia, F**	neg	***TEM-1***	neg	***OXA-1***
E. coli VR B269	***aac(6')-Ib-cr***	**Fia, F**	neg	***TEM-1***	***CTX-M-15***	***OXA-1***
E. coli VR B292	***aac(6')-Ib-cr***	**Fia, Fib, F**	neg	***TEM-1***	neg	***OXA-1***
E. coli VR B649	***aac(6')-Ib-cr***	**Fia, Fib, F**	neg	neg	neg	***OXA-1***
E. coli VR B656	***aac(6')-Ib-cr***	**Fia, F**	neg	***TEM-1***	***CTX-M-15***	***OXA-1***
E. coli VR B715	***aac(6')-Ib-cr***	**Fia, F**	neg	***TEM-1***	***CTX-M-15***	***OXA-1***
E. coli VR B 717	***aac(6')-Ib-cr***	**Fia, F**	neg	neg	***CTX-M-15***	***OXA-1***
E. coli VR B754	***aac(6')-Ib-cr***	**Fia, F**	neg	neg	neg	***OXA-1***
E. coli VR B770	***qnrS1, aac(6')-Ib-cr***	**N, FIA, F**	neg	***TEM-1***	***CTX-M-15***	***OXA-1***
E. coli VR B775	***qnrS1, aac(6')-Ib-cr***	**N, FIA, F**	neg	***TEM-1***	***CTX-M-15***	***OXA-1***
E. coli VR B780	***aac(6')-Ib-cr***	**Fia, Fib**	neg	***TEM-1***	***CTX-M-15***	***OXA-1***
E. coli VR B784	***aac(6')-Ib-cr***	**Fia, F**	neg	***TEM-1***	neg	***OXA-1***
E. coli VR B786	***aac(6')-Ib-cr***	**Fia, F**	neg	***TEM-1***	neg	neg
E. coli VR B787	***aac(6')-Ib-cr***	**Fia, F**	neg	***TEM-1***	***CTX-M-15***	***OXA-1***
E. coli VR B793	***aac(6')-Ib-cr***	**Fia, Fib, F**	neg	neg	***CTX-M-15***	***OXA-1***
E. coli VR B808	***aac(6')-Ib-cr***	**Fia, col E, F**	neg	***TEM-1***	***CTX-M-15***	***OXA-1***
E. coli VR B814	***qnrS1, aac(6')-Ib-cr***	**N, FIA, F**	neg	***TEM-1***	***CTX-M-15***	***OXA-1***
E. coli VR C17	***aac(6')-Ib-cr***	**Fia, Fib, F**	neg	neg	neg	***OXA-1***
E. coli VR C18	***aac(6')-Ib-cr***	**Fia, Fib**	neg	***TEM-1***	***CTX-M-15***	***OXA-1***
E. coli VR C19	***aac(6')-Ib-cr***	**Fia, Fib, F**	neg	***TEM-1***	***CTX-M-15***	***OXA-1***
E. coli VR C24	***aac(6')-Ib-cr***	**Fia, F**	neg	***TEM-1***	***CTX-M-15***	***OXA-1***
E. coli VR C66	***aac(6')-Ib-cr***	**Fia, Fib, F**	neg	neg	***CTX-M-15***	***OXA-1***
E. coli VR C93	***aac(6')-Ib-cr***	**Fia, F**	neg	neg	neg	***OXA-1***
E. coli VR C105	***aac(6')-Ib-cr***	**Fia, Fib, F**	neg	neg	***CTX-M-15***	***OXA-1***
E. coli VR C109	***aac(6')-Ib-cr***	**Fia, Fib, F**	neg	neg	***CTX-M-15***	***OXA-1***
E. coli VR E13	***aac(6')-Ib-cr***	**Fia, Fib, F**	neg	***TEM-1***	***CTX-M-15***	***OXA-1***
E. coli VR E25	***aac(6')-Ib-cr***	**Fia, F**	neg	***TEM-1***	***CTX-M-15***	***OXA-1***
E. coli VR E39	***aac(6')-Ib-cr***	**Fia, Fib, F**	neg	***TEM-1***	***CTX-M-15***	***OXA-1***
E. coli VR E47	***aac(6')-Ib-cr***	**Fia, Fib, F**	neg	neg	neg	***OXA-1***
E. coli VR E64	***aac(6')-Ib-cr***	**F**	neg	neg	***CTX-M-15***	***OXA-1***
E. coli VR E98	***aac(6')-Ib-cr***	**Fia, Fib, F**	neg	***TEM-1***	***CTX-M-15***	***OXA-1***
E. coli VR E108	***aac(6')-Ib-cr***	**Fia, F**	neg	***TEM-1***	***CTX-M-15***	***OXA-1***

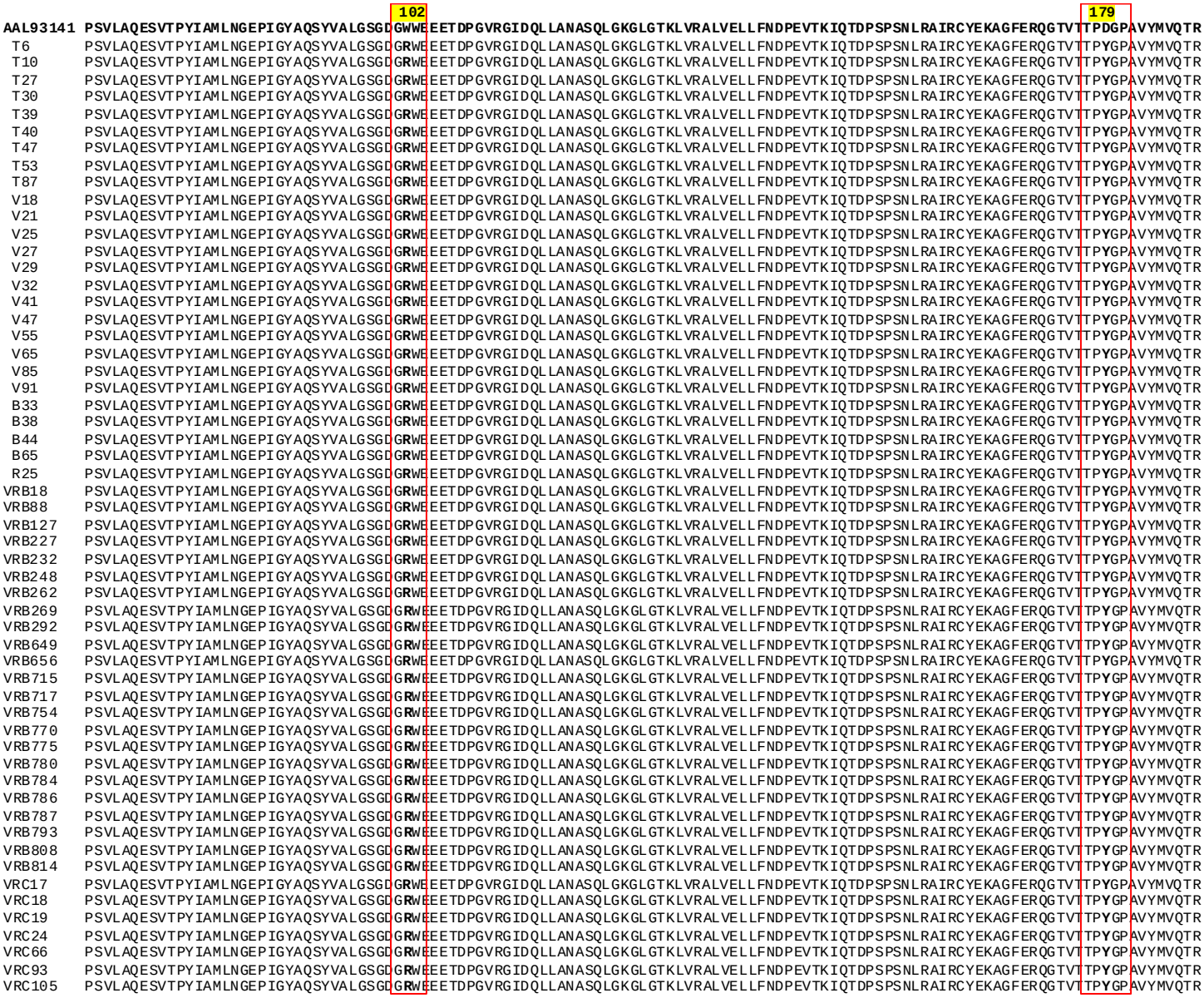

Figure 22. Aminoacid sequence alignament of aac(6')-Ib-cr variant enzymes in our collection. Characteristic aminoacid substitutions at Trp102Arg and Asp179Tyr are highlighted and compared to the AAL93141 wild-type aac(6')-Ib.

5. Conclusion and discussion

The survey for plasmid-mediated quinolone resistance determinants demonstrates a 14.3% (108/756) prevelance in our collection of clinical Enterobacteriaceae isolates from North-East Italy. These determinants were found in 104 isolates, since four *E. coli* strains carried *qnrS1* and *aac(6')-Ib-cr* variant too. Among the *qnr* genes 5.02 % (38/756) were positive; *qnrS, qnrB* and *qnrD* were detected, neither *qnrA* nor *qnrC* were found.

Four *P. mirabilis* and one *M. morganii* were positive for *qnrD*. These are the first *qnrD* genes in Europe, and the first ever *qnr* determinant found in *M. morgani.* Earlier this determinant was reported only from China, in four *Salmonella enterica* Serovar Bovismorbificans and Kentucky clinical isolates (40). The sequences of these *qnrD* genes were found to be 100% the same as in the first described allel. The sequence analysis of the five *qnrD*-harbouring plasmids showed 1450 bp of 2687 bp (54%) to be same as the first described plasmid, where the *qnrD* gene together with its upstream localized promoter region tends to be conserved. In the downstream sequence Orf2 was localized on each of the plasmids, without additional information about its function. The remaining sequences (1237 bp, i.e. 46% of the plasmid) was unknown. All *qnrD* plasmids lacked replication gene, suggesting chromosal support for the replication, moreover genes for horizontal transfer were also not identified.

Among all isolates, 26 *qnrS1* positive were identified, namely fifteen *Klebsiella* spp, ten *E.coli* and one *P. mirabilis.* Their plasmid profile was analyzed and incN replicon type, which carried the *qnrS1* gene, was the dominant. Association of *qnrS1* and $bla_{\text{VIM-1}}$ metallo beta-lactamase is remarkable, and it was found in one *E. coli* and five *Klebsiella* spp. Among them, three incN plasmids carrying both $bla_{\text{VIM-1}}$ and *qnrS1* detected in three *K. pneumoniae* isolates were found to be conjugable. This is an alarmig phenomenon, since these plasmids are capable to spread between different species.

Our survey found the first LAP narrow-spectrum beta-lactamase in Italy, in three *qnrS1* positive *K. pneumoniae* ST147 strains. The $bla_{\text{LAP-2}}$ gene was incorporated with *qnrS1* in genetic context named, ISE*cl2*. LAP beta-lactamase was first identified in six *Enterobacter cloacae* clinical isolates from France and Vietnam (64). The complex genetic material, ISE*cl2* together with *qnrS1* and $bla_{\text{LAP-2}}$ can explain of co-selection towards resistance of narrow-spectrum beta-lactam and fluoroquinolone antibiotics.

In the case of *qnrB*, *Citrobacter freundii* carries *qnrB6*, while *Citrobacter koseri* carries *qnrB19*, and 2 *Enterobacter* spp. were positive for *qnrB2*. The *qnrB* carriage in *Citrobacter* spp. is common (38).

Screening for *aac(6')-Ib-cr* shows high prevalence in *E. coli*, 60 from the 70 positive isolates (85.7%) belong to this species. Interestingly, four *E. coli* strain harboured two PMQR genes, *qnrS1* and *aac(6')-Ib-cr* simultaneously.

None of the quinolone specific efflux pumps (qepA and oqxA) were found in our collection. This is not surprising since these determinants are rarely found worldwide. QepA was detected first in Japan and later in isolates from France and Belgium (20, 46, 47). The oqxAB was detected only in one clinical *E. coli* and *K. pneumonaie* in South-Korea and in environmental isolates from China (35, 50).

The ciprofloxacin MIC distribution showed the *qnr* positive isolates to coincide with both susceptible and resistant values, while *aac(6')-Ib-cr* variant positive strains could only be found among highly resistant phenotype. This data show that *qnr* positive strains do not have a common phenotype and, their prevalence is underestimeted when resistance breakpoints are used as markers.

In our survey, the detected plasmids harbouring quinolone-resistance determinants were analyzed by replicon typing and it showed that these plasmids are able to replicate in Enterobactericeae. PMQR positive strains are clonaly unrelated as we detected by means of PFGE for *qnrS1* positive *E.coli* isolates and RAPD technique for *qnrD* positive *P. mirabilis* confirmed.

The landscape of fluoroquinolone resistance is getting diverse. Besides the chromosomal mutations leading to high-level fluroquinolone resistance, we can point out the low-level resistance as well. This latter phenotype is maintained by plasmid-coded quinolone resistance determinants and play a role in selection of higher-level resistance. MIC values alone will not give enough infomation about resistance mechanisms. Molecular screening of at-risk isolates seems mandatory.

6. Abstract (English)

Clinical isolates of fluoroquinolone-resistant Enterobacteriaceae are emerging worldwide. The traditional resistance mechanism is the accumulation of mutations in the chromosome coding for the target molecules of fluoroquinolone. All known plasmid-mediated quinolone resistance determinants - namely, Qnr determinants, aac(6')-Ib-cr enzyme, qepA and oqxAB efflux pumps - can individually confer low-level resistance. In this condition, bacterial cells have increased mutation frequency, making it easier the selection of higher-level fluoroquinolone resistance.

Our aims were to survey for plasmid-mediated quinolone resistance determinants a collection of Enterobacteriaceae clinical isolates originating from clinical microbiological laboratories of North-East Italy, and to characterize both the resistant strains and the plasmids harbouring quinolone resistance determinants and beta-lactamase genes.

Altogether, 756 Enterobacteriaceae clinical isolates were collected: 497 *Escherichia coli*, 68 *Klebsiella* spp., 18 *Citrobacter* spp., 69 *Proteus* spp., 24 *Morganella* spp., 21 *Providencia* spp., 52 *Enterobacter* spp. and 7 *Serratia marcescens*. MIC values were determined by microdilution for ciprofloxacin and levofloxacin, showing values between 0.06 and 128 µg/ml. Screening by PCR for plasmid-mediated quinolone resistance determinants yielded 108 positives [38 *qnr* and 70 *aac(6')-Ib-cr*] out of 104 isolates. Sequencing and analysis of PCR-positive products verified: 26 *qnrS1*, 2 *qnrB2*, 2 *qnrB6*, 2 *qnrB8*, 1 *qnrB19* and 5 *qnrD*. Four *Proteus mirabilis* and one *Morganella morganii* isolates were *qnrD* positive. Plasmid extraction was performed, and Southern blot analysis verified the plasmid localization of the *qnrD* determinants. Inverse PCR-based sequencing of *qnrD*-harbouring plasmids, resulted in a 2687 bp and 2684 bp plasmid DNA for *Proteus mirabilis* and *Morganella morganii*, respectively. Both plasmid sequences are deposited at Genbank with accession numbers JN183060 and JN183061. Among the *qnrS1* positive isolates, 10 *E. coli*, 15 *Klebsiella* spp. and one *Proteus mirabilis* were detected. Detection of beta-lactamase genes by PCR and sequencing yielded: 6 VIM-1, 7 SHV-12 and 3 LAP-2 positives. All four *qnrS1 E. coli* with the *aac(6')-Ib-cr* variant were CTX-M-15, OXA-1 and TEM-1 positive. PCR-based replicon typing found incN type plasmid to be the most prevalent. Conjugation experiment found 3 *qnrS1* and *VIM-1* harbouring conjugable plasmids. We could also demonstrate in three *Klebsiella pneumoniae* ST147 strains that *qnrS1* and $bla_{\text{LAP-2}}$ are integrated in the same IS*Ecl2* genetic context. Southern blot verified that the *qnrS1* gene is

localized on incN or untypeable plasmids. Altogether 70 *aac(6')-Ib-cr* variant positive isolates were found: 60 *E. coli*, 8 *Klebsiella* spp., one *P.mirabilis* and one *M.morganii*. Replicon typing of plasmids found incFia and incF types to be common for *E. coli* and *P. mirabilis* while incColE type to be predominant in *Klebsiella* spp., whilst *M.morganii* was untypeable. A high correlation of the *aac(6)-Ib-cr* variant with OXA-1 and CTX-M-15 beta-lactamases was found in *E. coli* (42/60) and in *Klebsiella* spp. (8/8), whilst in *P. mirabilis* the *aac(6)-Ib-cr* variant was associated with TEM-52 and OXA-1.

Our survey for plasmid-mediated quinolone resistance determinants found a 13.7% prevalence (104/756). MIC values for ciprofloxacin and levofloxacin showed uneven resistance levels among the *qnr*-positive isolates, with only 13.1% of strains (5/38) in the range of low-level resistance, while in case of the *aac(6')-Ib-cr* variant all positive isolates were resistant to fluoroquinolones. Our survey found the first *qnrD* determinants in Europe, and we could also demonstrate the first LAP-2 beta-lactamase in Italy.

7. Abstract (Italian)

In tutto il mondo si sta assistendo ad un aumento dell'incidenza di isolati clinici di Enterobacteriaceae resistenti ai fluorochinoloni.

La resistenza può essere dovuta ad un accumulo di mutazioni cromosomiche a livello dei geni che codificano le topoisomerasi, bersaglio d'azione dei fluorochinoloni; mediata da determinanti plasmidici, in particolare quelli che codificano per proteine di protezione Qnr, per l'enzima di acetilazione aac(6')-Ib-cr e per le pompe di efflusso QepA e OqxAB. Tutti questi determinanti da soli conferiscono al batterio bassi livelli di resistenza ma consentono un aumento della frequenza di mutazione,facilitando la selezione di alti livelli di resistenza.

Lo scopo di questo lavoro è stato quello di valutare l'incidenza della presenza di determinanti plasmidici che mediano la resistenza ai fluorochinoloni (PMQR), in una collezione di isolati clinici di Enterobacteriaceae provenienti da vari laboratori del nord-est Italia. Tutti i ceppi risultati positivi per la presenza di questi determinanti sono stati caratterizzati dal punto di vista plasmidico e correlati alla presenza di geni che codificano per beta-lattamasi.

Sono stati raccolti 756 isolati clinici di enterobatteri: 497 *Escherichia coli*, 68 *Klebsiella* spp., 18 *Citrobacter* spp., 69 *Proteus* spp., 24 *Morganella* spp., 21 *Providencia* spp., 52 *Enterobacter* spp. and 7 *Serratia marcescens*.

Per tutti i ceppi sono stati eseguiti i saggi di sensibilità con il metodo della microdiluizione a ciprofloxacina e levofloxacina, ottenendo valori di MIC variabili da 0.06 a 128 µg/ml.

Lo screening dei PMQR mediante PCR ha evidenziato 108 positivi (38 *qnr* e 70 *aac(6')-Ib-cr*) su 104 isolati. L'analisi delle sequenze degli ampliconi ha permesso di classificare le varianti qnr in 26 *qnrS1*, 2 *qnrB2*, 2 *qnrB6*, 2 *qnrB8*, 1 *qnrB19* e 5 *qnrD*. Per quest'ultimo determinante sono risultati positivi 4 *P. mirabilis* e una *M. morganii* verificandone la localizzazione plasmidica con Southern blot e ottenendo la sequenza completa mediante PCR inversa (2687 bp per *P. mirabilis* e 2684 bp per *M. morganii* depositate in Genbank con i numeri di accesso JN183060 and JN183061).

I ceppi positivi per *qnrS1* erano suddivisi in 10 *E. coli*, 15 *Klebsiella* spp. e un *P. mirabilis*. In questi ceppi *qnrS1* era accompagnato da VIM-1, SHV-12 e LAP-2. I 4 *E. coli* che ospitavano sia *qnrS1* che *aac(6')-Ib-cr* ospitavano anche CTX-M-15, OXA-1 and TEM-1.

I plasmidi dei positivi sono stati caratterizzati con il metodo dell'origine della replicazione. I ceppi *qnrS1* ospitano questo gene su plasmidi di tipo incN. Con esperimenti di coniugazione si è visto che il plasmide con *qnrS1* e VIM-1 è coniugabile. Inoltre, nei tre ceppi di *K. pneumoniae* ST147 con *qnrS1* e $bla_{\text{LAP-2}}$ si è dimostrato che questi sono integrati nello stesso contesto genetico di IS*Ecl2*.

I 70 ceppi che presentano la variante *aac(6')-Ib-cr* erano suddivisi in 60 *E. coli*, 8 *Klebsiella* spp., 1 *P. mirabilis* e 1 *M. morganii*. La tipizzazione dei plasmidi ha messo in evidenza i tipi incFia e incF sia in *E. coli* che *P. mirabilis*, mentre incColE era predominante in *Klebsiella* spp. e *M. morganii* era caratterizzata da un plasmide non tipizzabile. Si è inoltre evidenziata una alta correlazione fra la variante *aac(6)-Ib-cr* e le beta-lattamasi OXA-1 e CTX-M-15 sia in *E. coli* (42/60), che in *Klebsiella* spp. (8/8), con l'eccezione di *P. mirabilis* dove invece con OXA-1 è presente TEM-52.

Dalla nostra indagine è emersa una prevalenza di determinanti plasmidici che mediano la resistenza ai fluorochinoloni del 13.7% (104/756). Solo il 13.1% (5/38) degli isolati positivi per il qnr risultano in un range di bassa resistenza, mentre i ceppi positivi per la variante *aac(6')-Ib-cr* presentavano tutti alti livelli di resistenza.

La nostra indagine ha consentito di descrivere il primo determinante *qnrD* isolato in Europa e la prima beta-lattamasi LAP-2 isolata in Italia.

8. References

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